
# The role of dietary lipids and essential fatty acids in the processes of skeletogenesis and pigmentation in Senegalese sole (*Solea senegalensis)* larvae

## Efecto de los lípidos y ácidos grasos esenciales de la dieta sobre el desarrollo esquelético y la pigmentación en larvas del lenguado senegalés (*Solea senegalensis)*

Anaïs Boglino

**DOCTORAL THESIS**

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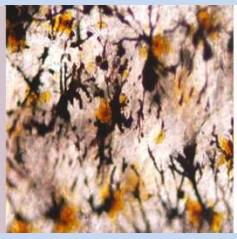
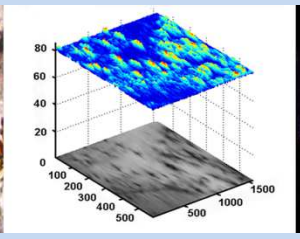


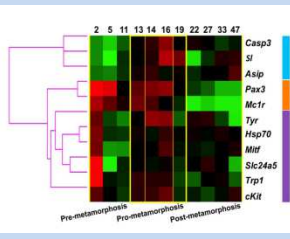


**ANAÏS BOGLINO**

**Barcelona, 2013**

UNIVERSITAT DE BARCELONA
FACULTAD DE BIOLOGÍA
DEPARTAMENTO DE FISIOLOGIA

IRTA
RECERCA I TECNOLOGIA AGROALIMENTARIES
CENTRE DE SANT CARLES DE LA RÁPITA
UNITAT DE CULTIUS EXPERIMENTALS

**Tesis Doctoral**

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Memoria presentada por

**Anaïs Boglino**

para optar al grado de

**Doctor por la Universitat de Barcelona**

Tesis realizada bajo la dirección de

Dr. Enric Gisbert Casas del IRTA-Sant Carles dela Rápita (España) y

Dra. Maria José Darias Caceres del IRD-UMR 226 ISE-M, Université Montpellier II (Francia)

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Dr. Enric Gisbert  Dr. Maria J. Darias  Dra. Isabel Navarro  Anaïs Boglino

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# INDEX

# SUMMARY

**The role of dietary lipids and essential fatty acids in the processes of skeletogenesis and pigmentation in Senegalese sole (*Solea senegalensis*) larvae**

Skeletal deformities and pigmentary disorders are two of the most important problems currently affecting the intensive production of flatfish juveniles. These anomalies, occurring from the early larval stages, affect the external appearance of fish, downgrade the market value of the final product and represent an important bottleneck for flatfish aquaculture. Among the factors involved in skeletal and pigmentary disorders, lipid nutrition at first feeding is one of the key parameters known to influence skeletogenesis and pigmentation processes during larval development. Since dietary lipids represent the main energy source in developing larvae and a source of essential fatty acids (EFA) required for normal larval growth, proper bone formation and pigmentation, unbalanced levels of lipids or EFA or their inappropriate form of supply in the diet may lead to skeletal deformities and pigmentary disorders. In this sense, the research work of this thesis aimed to study the effect of dietary lipids and EFA, especially arachidonic acid (ARA), on skeletogenesis and pigmentation and their role in the incidence of skeletal and pigmentary disorders in Senegalese sole (*Solea senegalensis*, Kaup 1858), a flatfish of high market value currently produced in the Iberian Peninsula. Thus, this thesis was divided in two sections, a first one devoted to evaluate the effects of EFA on skeletogenesis and a second one focused on the impact of nutrition on pigmentation disorders. In a first part, the effect of different commercial enriching products for *Artemia* metanauplii, presenting distinct fatty acid (FA) profiles, was evaluated on survival rate, growth, digestive system maturation, lipid deposition in target tissues, ossification, incidence of skeletal deformities in Senegalese sole early juveniles, and molecular regulation of these processes during the morphogenesis. Better larval growth performance, development and ossification degree were found for fish fed *Artemia* enriched with Aquagrow Gold© (ABN, AGG), so that their FA profile was considered as the most balanced for Senegalese sole among all tested diets, and this product was considered as a reference for enriching live prey for the rest of trials conducted in this thesis, while fish fed *Artemia* enriched with Easy Selco© (INVE, ES) showed the lowest performance. The incidence of skeletal deformities was high in all the dietary treatments and not significantly affected by the dietary FA composition. On the contrary, the patterns of fat deposition in the intestine, liver and vascular system were affected among dietary treatments, and *Artemia* enriched with AGG was considered as the most appropriate for proper growth and development of fish. The absolute levels of DHA, EPA and ARA of 9.5, 3.1 and 0.7% TFA, respectively, and (n−3)/(n−6) PUFA, DHA/EPA, ARA/ DHA and OA/PUFA ratios of 5.2, 3.0, 0.1, 0.5, respectively, of the AGG diet were recommended as more suitable for Senegalese sole larval development. A variation, even slight, of these proportions might have modified the metabolism of lipids and disrupted the profile of lipid accumulation in the target tissues, leading to severe intestinal and hepatic steatosis in fish fed the other diets and reduced larval growth performance and development. The expression of most of the genes involved in vitamin A and lipid absorption and metabolism (*igf1, igf2, igfr, crabp1, crbp2α, rbp, rarα1, rxrα, pparβ, i-fabp* y *l-fabp*) was invariant between fish presenting the higher and the lowest growth performance and development (AGG and ES); thus, not sensitive to the dietary FA composition. However, the lower osteocalcin (*oc*) expression in fish fed the ES diet, related to lower larval growth and bone mineralization, compared to those fed the AGG diet, was associated with a deficiency in ARA. The effects of dietary oxidized lipids were also evaluated on larval survival, growth, activity of antioxidant enzymes, intestinal lipid deposition, ossification and incidence of skeletal deformities in Senegalese sole. Fish appeared to be sensitive to the increasing amounts of dietary peroxidized lipids (34.5 to 78.8 nmol MDA $g^{-1}$ w.w.), although no remarkable physiological alterations were detected, since larvae were similar in terms of their FA profile, oxidative stress status, survival, growth performance or metamorphosis. Senegalese sole seemed to activate antioxidant defense mechanisms in response to the dietary oxidative stress, through the consumption of the dietary vitamin E provided and the activation of antioxidant enzymes. Feeding fish with highly oxidized diets caused an increase of accumulation of oxidized fat in the intestinal enterocytes and a reduction in bone mineralization. The vitamin E was suggested to interact with ossifying cartilage, with an excess in vitamin E in highly oxidized diets affecting the structures formed by endochondral ossification (haemal arches).

However, the duration of feeding of diets that promote high oxidative stress seemed to have reduced the activity of antioxidant enzymes, possibly resulting of an adaptive process. The effects of dietary ARA on larval survival, growth, ossification, incidence of skeletal malformations and pigmentary disorders were evaluated on 50 dph-aged Senegalese sole. The amount of 4.5% TFA of ARA fed to larvae during the *Artemia* feeding period was found as the optimal for proper growth and ossification, while 1% TFA of ARA was insufficient to meet the initial larval requirements and 7% TFA of ARA led to imbalanced ARA/EPA and n-6/n-3 ratios that, in both cases, was suspected to disrupt the relative proportions of prostaglandins PGE2 and PGE3, and significantly delayed somatic growth and skeletal ossification. None of the dietary ARA contents influenced differentially the incidence of skeletal deformities in the vertebral and caudal regions, but high levels of dietary ARA (10.2% TFA in rotifer and 7.1% in *Artemia* metanauplii) induced an impaired eye migration in larvae, altered their head shape and cranial bone remodeling. Feeding larvae with these high levels of dietary ARA in enriched live prey during the pre-, pro- and/or post-metamorphosis enhanced survival, without affecting growth and eye migration process, and induced malpigmentations. The ontogeny of the pigmentation of the ocular side skin at both morphological and molecular levels was studied in Senegalese sole larvae in order to detect possible alterations of a normal pigmentation developmental pattern, and the appearance of the ARA-induced pseudo-albino phenotype was characterized. A high “sensitivity window” to ARA-induced malpigmentation was identified during pre- and pro-metamorphosis stages, resulting in pseudo-albino specimens that presented reduced density of melanophores in their dorsal skin, which lost their ability to change the shape from round to dendritic and to disperse melanin compared to normally pigmented individuals. Supplying high ARA amounts in the live prey changed the dietary ARA/EPA ratio, which in turn altered the relative concentrations of prostaglandins of the 2 and 3-series, causing pigmentary disorders. The pseudo-albino phenotype was characterized by the absence of differentiation of post-metamorphic populations of chromatophores, the progressive disintegration of the larval melanophores and xanthophores, and the inhibition of the melanogenesis, reflected by the disrupted expression of genes involved in melanophore differentiation and melanin synthesis (*asip, pax3, cKit, mitf, tyr, trp1 and slc24a5).* Altogether, these studies provide helpful knowledge to understand how dietary lipids and essential fatty acids affect the processes of skeletogenesis and pigmentation, in order to improve actual Senegalese sole larval rearing and ultimately the quality of the juveniles.

**Keywords:** *Solea senegalensis*, metamorphosis, larvae, bone, ossification, skeletogenesis, pigmentation, pseudo-albinism, nutrition, lipids, fatty acids.

# RESUMEN

**Efecto de los lípidos y ácidos grasos esenciales de la dieta sobre el desarrollo esquelético y la pigmentación en larvas del lenguado senegalés (*Solea senegalensis*)**

Las malformaciones esqueléticas y problemas pigmentarios (pseudo-albinismo) son dos de los problemas más importantes que afectan actualmente a la producción masiva de juveniles de peces planos. Estas anomalías, que ocurren desde las etapas iniciales del desarrollo larvario, afectan su apariencia externa, disminuyen su precio final en el mercado y representan un importante cuello de botella para la acuicultura. Entre los factores implicados en la aparición de los citados desórdenes del desarrollo, la nutrición lipídica es uno de los parámetros más importantes detectados hasta la fecha, pues los lípidos afectan a los procesos de esqueletogénesis y pigmentación durante el desarrollo larvario. Los lípidos representan la principal fuente de energía en larvas de peces y una fuente de ácidos grasos esenciales (AGE) necesarios para un crecimiento larvario normal y por la formación correcta de los huesos y el desarrollo de células pigmentarias, mientras que un desequilibrio en los niveles de lípidos y/o puede conducir a un desarrollo anormal del esquelético y despigmentación. En este sentido, la presente tesis doctoral tuvo como objetivo estudiar el efecto de los lípidos y AGE (particularmente el ácido araquidónico, ARA) administrados en la dieta, sobre la esqueletogénesis y pigmentación en larvas del lenguado senegalés (*Solea senegalensis*, Kaup 1858); especie de pez plano con alto precio en el mercado y que actualmente es producido en la península Ibérica. Por consiguiente, esta tesis fue dividida en dos secciones, la primera dedicada a la evaluación de los efectos de los AGE sobre la esqueletogénesis y la segunda enfocada sobre el impacto de la nutrición sobre la pigmentación. En la primera parte, el efecto de diferentes productos comerciales con distintos perfiles de ácidos grasos (AG) usados comúnmente para enriquecer *Artemia*, fue evaluado sobre la tasa de supervivencia, el crecimiento, la maduración del sistema digestivo, la deposición de lípidos en diversos tejidos objetivos, la osificación y la incidencia de deformaciones esqueléticas en juveniles de lenguado senegalés, trabajos que fueron completados con el estudio de la regulación molecular de dichos procesos durante el proceso de metamorfosis. Los resultados mostraron que los peces alimentados con *Artemia* enriquecida con Aquagrow Gold© (ABN, AGG) presentaron un mejor crecimiento, desarrollo y grado de osificación, considerándose así el perfil de AG de esta dieta como el más equilibrado entre todas las dietas probadas. Así mismo, y este producto para enriquecer la presa viva fue considerado como referencia. Por el contrario, las larvas que fueron alimentadas con para el resto de *Artemia* enriquecida con Easy Selco© (INVE, ES) mostraron el peor crecimiento y calidad en relación al resto de dietas analizadas. La incidencia de deformaciones esqueléticas fue alta en todos los tratamientos; no obstante, no se vio significativamente afectada por la composición en AG de la dieta. Por el contrario, los perfiles de deposición de grasa en el intestino, el hígado y el sistema vascular sí se vieron afectados por la dieta. Los niveles recomendados para las larvas de lenguado para los distintos AG son los siguientes: DHA (9.5%), EPA (3.1%) y ARA (0.7%) en base al porcentaje total de AG (AGT) y las relaciones de AG Polinsaturados (AGPI; n-3/n-6), DHA/EPA, ARA/DHA y AO/AGPI de 5.2, 3.0, 0.1, 0.5, respectivamente. Una variación, aun ligera, de las proporciones anteriormente indicadas, es capaz de modificar el metabolismo de los lípidos y alterar el perfil de acumulación de lípidos en los tejidos objeto de estudio, resultando en una severa esteatosis intestinal y hepática en los peces alimentados con otras dietas que no fuera AGG, así como también un menor crecimiento y desarrollo. La expresión de la mayoría de los genes analizados implicados en la absorción de la vitamina A y de los lípidos (*igf1, igf2, igfr, crabp1, crbp2α, rbp, rarα1, rxrα, pparβ, i-fabp* y *l-fabp*) no se vio alterada entre los peces que mostraron un mejor y pero rendimiento en cuanto a crecimiento y grado de desarrollo (grupos AGG y ES, respectivamente); por consiguiente, estos genes no fueron sensibles a la composición en AG de la dieta. No obstante, la menor expresión de la osteocalcina en los peces alimentados con la dieta ES, los cuales mostraron una menor tasa de crecimiento y nivel de mineralización de sus esqueleto en comparación a los juveniles alimentados con la dieta AGG, fue asociada a una deficiencia en ARA en la dieta. Los efectos de los lípidos oxidados en la dieta también fueron evaluados sobre la supervivencia larvaria, el crecimiento, la actividad de enzimas antioxidantes, la deposición intestinal de lípidos, la osificación y la incidencia de deformidades esqueléticas en el lenguado senegalés. Los peces parecieron ser sensibles al incrementar la cantidad de lípidos peroxidados en la dieta (34.5 to 78.8 nmol MDA $g^{-1}$ P.S.), no obstante, ninguna alteración fisiológica remarcable fue detectada debido a que las larvas mostraron similitudes en sus perfiles de AG, niveles de estrés oxidativo,

supervivencia, crecimiento y/o metamorfosis. Estos resultados vendrían explicados porque las larvas de lenguado serían capaces de activar los mecanismos de defensa antioxidante en respuesta al estrés oxidativo en la dieta, por el consumo de la vitamina E (proveída en la dieta) y la activación de enzimas antioxidantes. Alimentar los peces con dietas altamente oxidadas causó un aumento de la acumulación de grasa en los enterocitos intestinales y una reducción de la mineralización ósea. Se sugiere que la vitamina E interactúa con el cartílago y que un exceso de vitamina E en la dieta altamente oxidada afectó las estructuras formadas por osificación endocondral (arcos haemales). No obstante, al mantener dietas que promueven el estrés oxidativo por un largo periodo de tiempo parece haber reducido la actividades de las enzimas antioxidantes, posiblemente resultado de un proceso adaptativo. Los efectos del ARA en la dieta sobre la supervivencia larvaria, el crecimiento, la osificación, la incidencia de malformaciones esqueléticas y los desórdenes pigmentarios fueron evaluados a los 50 días post-eclosión. La cantidad de 4.5% AGT de ARA suministrada a las larvas durante el periodo de alimentación con *Artemia* resultó ser la más óptima en cuanto a un crecimiento y osificación correctos, mientras que 1% y 7% AGT de ARA condujeron a desequilibrios en las relaciones ARA/EPA y n-3/n-6 AGPI. A su vez, dichos cambios en los niveles de ARA en la dieta resultaron en alteraciones de las proporciones relativas de prostaglandinas PGE2 y PGE3, retrasando significativamente el crecimiento somático y el grado de osificación esquelética. Ninguno de los contenidos de ARA en la dieta influyó la incidencia de deformidades esqueléticas en las regiones vertebral y caudal, pero altos niveles de ARA en la dieta (10.2% AGT en los rotíferos y 7.1% AGT en los metanauplios de Artemia) indujeron una migración del ojo defectuosa en las larvas, unas alteraciones de la forma de su cabeza y de la remodelación de los huesos craneales. Al alimentar los juveniles con presas vivas con estos altos niveles de ARA durante la pre-, pro- y /o post-metamorfosis, mejoró la supervivencia, no afectó al crecimiento ni al proceso de migración del ojo, e indujo desórdenes pigmentarios (pseudo-albinismo). La ontogenia de la pigmentación de la piel del lado ocular fue estudiada a nivel morfológico y molecular con el fin de detectar posibles alteraciones de un perfil normal de desarrollo de la pigmentación, mientras que la aparición del fenotipo pseudo-albino inducido nutricionalmente por un exceso de ARA fue también caracterizada. Las larvas de lenguado presentan una ventana durante su desarrollo (etapas pre- y pro-metamórficas de su desarrollo) en las que son altamente sensibles a niveles elevados de ARA y responsables de afectar el desarrollo normal de las células pigmentarias. Así y como norma general, los ejemplares pseudo-albinos presentaron una menor densidad de melanóforos en su piel dorsal, que perdieron la capacidad de cambio de forma de redundo a dendrítico y de dispersar la melanina, en comparación a los individuos normalmente pigmentados. El suministro de altas proporciones de ARA en la presa viva cambió el ratio alimenticio de ARA/EPA, a su vez, alteró a las concentraciones relativas de prostaglandinas de las series 2 y 3, causando desordenes pigmentarios. El fenotipo pseudo-albino fue caracterizado por la ausencia de diferenciación de las poblaciones post-metamórficas de cromatóforos, la progresiva desintegración de los melanóforos y xantóforos larvarios, y la inhibición de la melanogenesis, reflejado por la alteración de la expresión de genes involucrados en la diferenciación de los melanóforos y la síntesis de melanina (*asip, pax3, cKit, mitf, tyr, trp1 and slc24a5).* El conjunto de estos estudios proveen un conocimiento útil para entender como los lípidos y ácidos grasos esenciales de la dieta influencian los procesos de esqueletogénesis y de pigmentación, con el objetivo de mejorar el cultivo larvario del lenguado senegalés y la calidad de los juveniles.

**Palabras llaves:** *Solea senegalensis*, metamorfosis, larvas, esqueletogénesis, pigmentación, pseudo-albinismo, nutrición, lípidos, ácidos grasos.

# INTRODUCTION

## 1. World Aquaculture and the issue of species diversification.

According to the Food and Agriculture Organization (FAO) of the United Nations, aquaculture is defined as “the farming of aquatic organisms in inland and coastal areas, involving intervention in the rearing process to enhance production and the individual or corporate ownership of the stock being cultivated”.

Aquaculture is a very old activity, which more ancient tracks are natural ponds of freshwater for maintenance of carps (*Cyprinus carpio*) in China dating from five millenaries and for rearing of Tilapia (*Tilapia sp.*) in Egypt for more than four centuries. However, fish aquaculture production has considerably increased from the last 60 years with the fast world demographical expansion, the consequent augmentation of the request for seafood and the overexploitation and exhaustion of marine fisheries resources. With an average annual growth rate of 8.7% worldwide, aquaculture account for around 40% of world fish food supply (Figure 1).

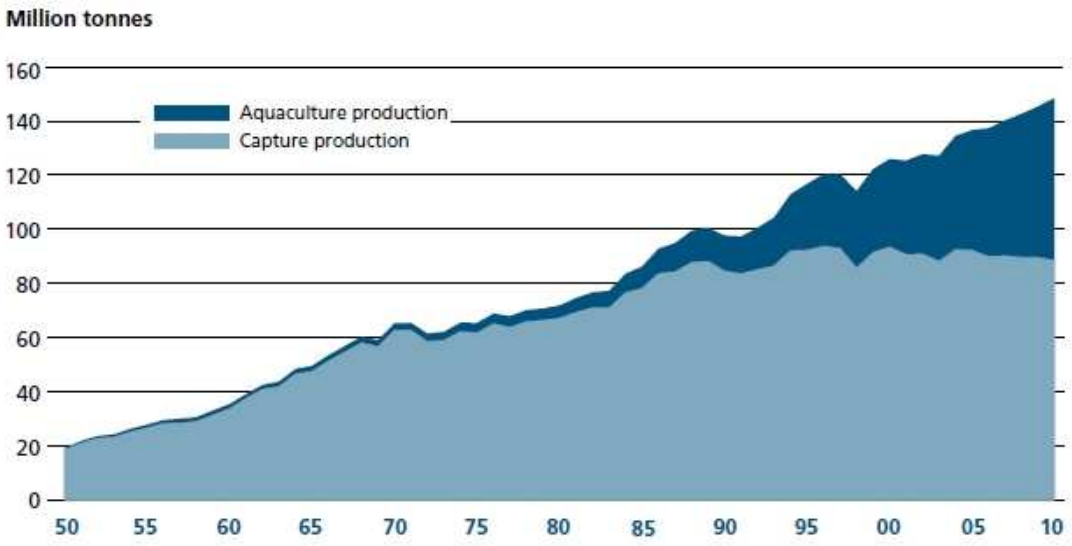


**Figure 1.** World capture fisheries production and aquaculture in million of tones during the last decades (FAO, 2012).

Asia, in particular China, is actually the world highest generator of products from aquaculture, with 53.3 million of tones declared in 2010 (FAO, 2012), representing the 90% of world aquaculture activities (Figure 2). The European aquaculture sector, with an average production of 2.5 million of tones in 2010 (FAO, 2012), is a modern industry employing around 190,000 people (directly or indirectly) with a €7 billion ex-farm value (EATIP, 2012).

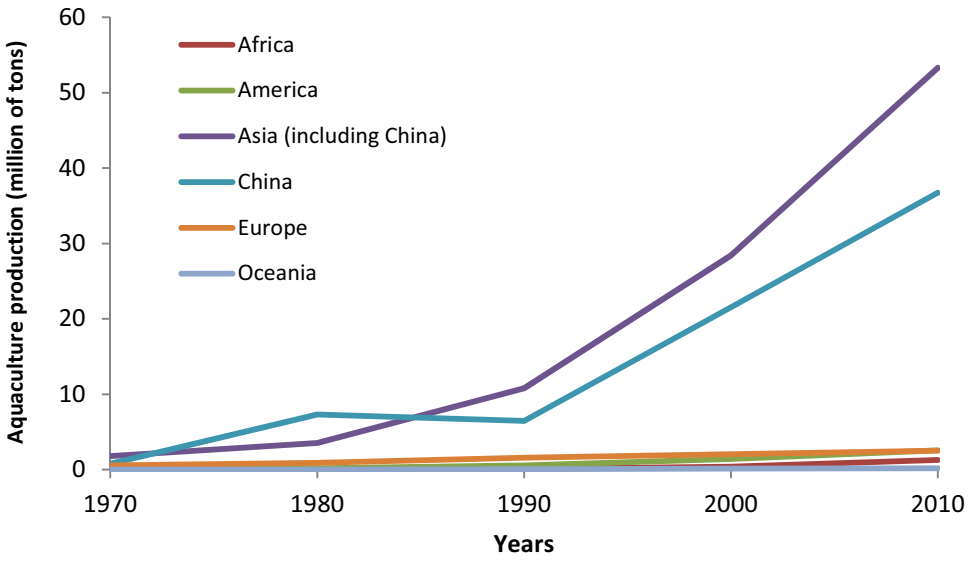


**Figure 2.** Aquaculture production by region in million of tones, from 1970 to 2010 (FAO, 2012).

Many world-class researchers and facilities exist in specialized research centers and universities throughout Europe, while the private sector employs highly skilled and educated personnel, with modern production facilities. Compared to the rest of the world, the EU has high sustainability and food safety demands with respect to aquaculture products and production methods. In order to meet these requirements, EU food safety regulations for wild and aquaculture fish are becoming increasingly stringent. This sector is well situated to be among the world leaders in the efficient and sustainable production of safe seafood of the highest quality and nutritional value, taking into account consumer preferences and lifestyles, and the immense diversity of aquatic products to which the consumer is accustomed to (EATIP, 2012).

Even though some 35 aquatic species are cultured in Europe, finfish aquaculture production is dominated both in volume and value by a handful of species, such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*), common carp (*Cyprinus carpio*) and European sea bass (*Dicentrarchus labrax*), what limits the variety of farmed fish available in the market (Table 1). Besides, the aquaculture industry for these species has experienced periods of market value problems when production has surpassed demand, resulting in ex-farm price that often decreases to prices close to or below cost of production. This, combined with less expensive imports, has slowed-down the growth rate of the aquaculture production in the European Economic Area and production has leveled off in the EU. An efficient, competitive, sustainable and market-oriented expansion of the EU aquaculture sector based on new species and products will reduce the dependence of the EU consumer on imports from countries of questionable production, health, environmental and social standards, and the pressure on over-exploited fisheries in the EU.

**Table 1.** Main species produced in aquaculture, in 2010, in the European Union, in tones (FAO, 2012).

| **Species** | **tones** |
|---|---|
| Rainbow trout (*Onchorynchus mykiss*) | 192,856 |
| Blue mussel (*Mytilus edulis*) | 368,207 |
| Atlantic salmon (*Salmo salar*) | 171,215 |
| Japanese oyster (*Crassostrea gigas*) | 104,102 |
| Mediterranean mussel (*Mytilus galloprovincialis*) | 102,532 |
| Gilthead sea bream (*Sparus aurata*) | 87,596 |
| Common carp (*Cyprinus carpio*) | 65,965 |
| European sea bass (*Dicentrarchus labrax*) | 53,833 |
| Japanese carpet shell (*Ruditapes philippinarum*) | 37,544 |
| Turbot (*Psetta maxima*) | 8,549 |
| Eel (*Anguilla anguilla*) | 6,819 |
| African sharptooth catfish (*Clarias gariepinus*) | 5,308 |
| Brown trout (*Salmo trutta*) | 4,502 |
| Silver carp (*Hypophthalmichthys molitrix*) | 3,329 |
| Meagre (*Argyrosomus regius*) | 3,937 |
| Other species | 45,422 |
| **Total European Aquaculture** | 1,261,716 |

During the last decades, several species belonging to the *Sparidae*, *Scianidae* and *Soleidae* families have been seriously considered for diversifying the European marine aquaculture. Among them, the Senegalese sole (*Solea senegalensis,* Kaup, 1858) has been suggested since the late eighties as an adequate candidate for aquaculture in intensive open flow and recirculating water systems (Dinis *et al.*, 1987; Dinis, 1992; Vázquez *et al.*, 1994; Marín-Magán *et al.*, 1995, Anguis and Cañavate, 2005; Garcia and Garcia, 2006; Gavaia *et al.*, 2009; Padrós *et al.*, 2011, among others). This species presents several advantages, such as i) the high market price (*ca.* 11 €/kg; APROMAR, 2012) and wide acceptance by the consumer, ii) the natural spawning of wild broodstock held in captivity, iii) the massive egg production, iv) the high survival and fast development of eggs and larvae and v) the high growth rate of juveniles. Moreover, Senegalese sole is tolerant to variations of environmental rearing conditions (oxygen, salinity, temperature…) and presents a better growth performance than common sole (*Solea solea*, Linnaeus 1758) (Howell *et al.*, 2005). The scientific effort coupled with the increasing economic interest for this species have led to the development of the zootechnical knowledge necessary to successfully produce individuals until their commercial size in conditions of intensive industrial production (Imsland, 2003; Cañavate, 2005).

## 2. Taxonomy, geographical distribution, external morphology and general biology of *Senegalese sole*.

Senegalese sole belongs to the *Soleidae* family (order Pleuronectiformes), a group constituted by fish referred as “flatfishes” that is characterized by a flat and ovoid shape, presenting an altered bilateral symmetry.

In the Mediterranean, there are many species of this family. Senegalese sole is an inshore species, mainly allocated from the Bay of Biscay to the coasts of Senegal, particularly along the Atlantic coasts of Spain and Portugal, until the Mediterranean coasts of Spain, Morocco and Algeria (Figure 3).

**Figure 3.** Senegalese sole coastal distribution (source: Fishbase, 2013)

Senegalese sole presents both eyes on the right side that is named the ocular side. The dorsal fin starts just before the location of both eyes. Dorsal and anal fins do not contain spines but soft rays and they are straggling along mostly all the periphery of the body until the caudal fin (Figure 4). The pectoral fin on the ocular side has a black colored interradial membrane, which distinguished it from common sole (*S. solea*) presenting a large black spot on the posterior part of this membrane. The supratemporal branch of the lateral line forms an arch. The small and semi-circular mouth is located under the inferior edge of the left eye. The coloration of the ocular side is usually brown, with wide variations from dark to light and the skin has high capacity to adapt to the environment by mimicry (Healey, 1999).

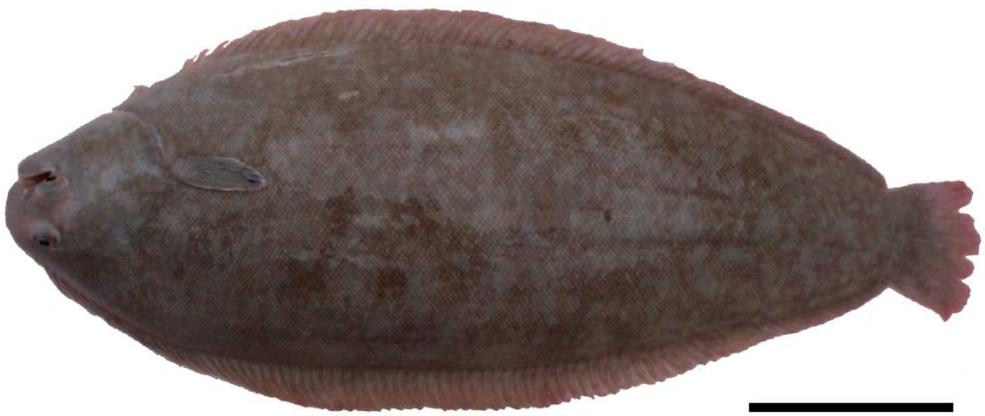

**Figure 4.** Adult specimen of Senegalese sole. Scale bar represents 10 cm.

Senegalese sole is a benthonic species living in sandy or muddy bottoms of coastal areas, up to 100 m depth. It feeds on benthonic invertebrates, as polychetes larvae (mostly *Hediste diversicolor*), bivalve mollusks (mainly *Scrobicularia plana*) and small crustacean (isopods, amphipods and decapods) (Arias and Drake, 1990; Abellan and Basurco, 1999; Cabral, 2000).

An adult specimen of Senegalese sole generally measures 45 cm in average and occasionally it may reach 60 cm (Abellan and Basurco, 1999). This species is gonochoric with no external dimorphism. Females mature during their third year of life, when their length approximately reaches 32 cm and they have an average fecundity of 500,000 eggs/kg. The spawning period takes place between March and June (Andrade, 1990; Dinis *et al.*, 1999; Anguis and Cañavate, 2005). For specimens in captivity, spawnings are also obtained in autumn, although the number and quality of eggs is lower than in spring (Anguis and Cañavate, 2005). In both seasons, most of the spawning episodes (65–73%) occur after temperature has increased up to 2.5°C within 3 days (Anguis and Cañavate, 2005). Normally, a good spawning contains ca. 100,000 to 150,000 of floating eggs per kg of female, with a diameter that varies between 929.6 ± 0.01 µm and 960.6 ± 0.03 µm, which tends to decrease along the spawning period (Dinis *et al.*, 1999, Anguis and Cañavate, 2005). Egg incubation depends on rearing temperature, ranging from 36h at 20°C to 48h at 17°C (Dinis and Reis, 1995; Cañavate and Fernández-Díaz, 1999; Dinis *et al.*, 1999). After the incubation phase, eggs hatch and liberate planktonic larvae with a bilateral symmetry that measure ca. 2.4 ± 0.1 mm, depending on the egg size and quality (Dinis *et al.*, 1999), and feed on zooplankton, mainly copepods. There is some variability in the relationship between individual egg size and individual larval size at hatching although, in average, larger eggs produce larger larvae (Geffen *et al.*, 2007).

Around 15 days after hatching (300 degree days), larvae progressively lose their bilateral symmetry, their body starts to flatten and the left eye begins its migration into the ocular side (Fernández-Díaz *et al.*, 2001). This process, called “metamorphosis” (Figure 5) lasts around one week, although its length depends on water temperature. The process of metamorphosis is often

associated with settlement, when larvae migrate towards the bottom, change their feeding habits, behavior and ecological niche. Metamorphosis involves important morphological and physiological changes associated with eye migration, a 90° rotation in posture and asymmetrical pigmentation. These processes are often assumed to be critical in determining recruitment in flatfish, through their impact on feeding, growth and survival (see review in Geffen *et al.*, 2007). It has been shown that feeding continues during metamorphosis, but not as effectively, and prey ingestion and daily ration decline at early metamorphosis (Yúfera *et al.*, 1999; Fernández-Díaz *et al.*, 2001; Cañavate *et al.*, 2006). Moreover, the stomach and the gut are not fully functional until after metamorphosis (Ribeiro *et al.*, 1999), making digestion and assimilation less efficient. In this sense, pre-metamorphic flatfish larvae increase their storage of lipids in liver, obtained from planktonic preys, to utilize these reserves as a source of energy during metamorphosis, when feeding abilities are reduced (Brewster, 1987; Yúfera *et al.*, 1999). Development may be delayed until the larvae had accumulated sufficient reserves (Brewster, 1987). In agreement with this assumption, Fernández-Díaz *et al.* (2001) observed a decrease in the C:N ratio in metamorphosing Senegalese sole, indicating the utilization of carbohydrate and lipid resources. Metamorphosis and settlement are energically demanding because of the high requirements for physical remodeling through an asymmetrical flat morphology and a benthic behavior. Moreover, developmental changes associated with metamorphosis and asymmetry acquirement take place at the cost of somatic growth, which significantly decline during this transition period (Cañavate *et al.*, 2006). Larval size, energy reserves and the feeding capacity during metamorphosis determine the flexibility in completing metamorphosis and settlement (Geffen *et al.*, 2007).

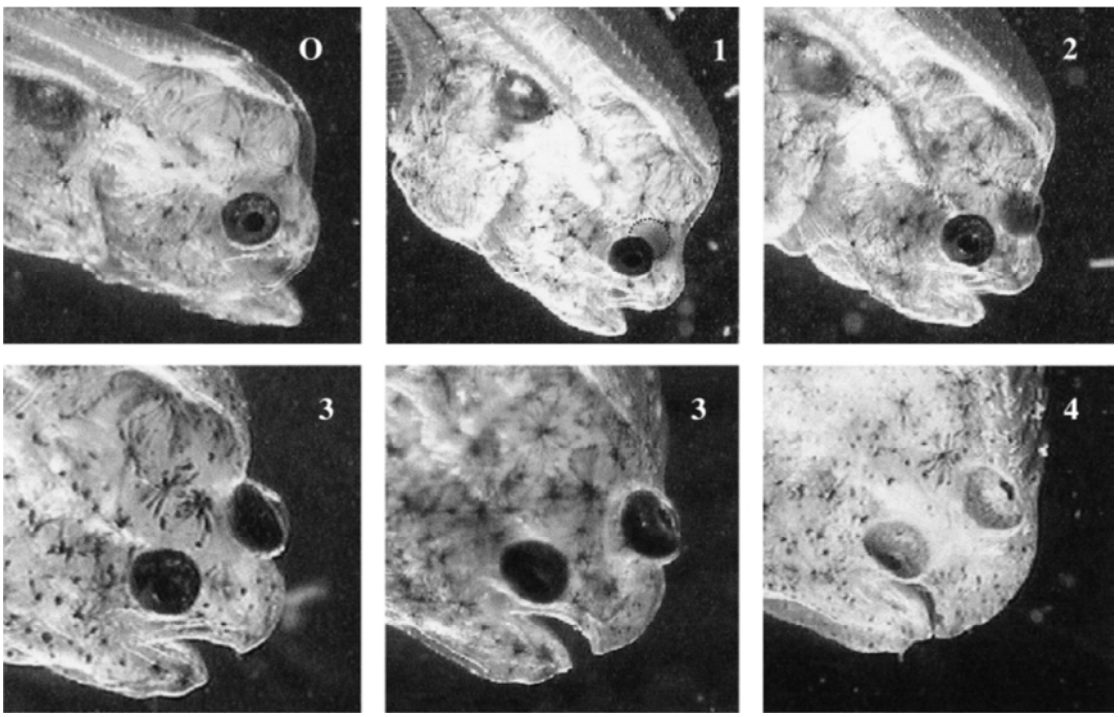


**Figure 5.** Eye position at different stages of metamorphosis in Senegalese sole larvae (Fernández-Díaz *et al.*, 2001).

### 3. The industrial culture of Senegalese sole

Senegalese sole is mainly cultured in European and Asian countries, where its global production has dramatically increased from 26.310 to 148.807 tons between 2000 and 2008, representing a growth rate of 565% in production and 50% in value (FAO, 2010). In Europe, Senegalese sole production is mainly located in Spain (autonomous communities of Galicia, Andalucía and Canary Islands) with 204 tons produced in 2010 and 110 tons in 2011 (APROMAR, 2012). The decline in production observed during these last years may be attributed to a lack of fry for on-growing purposes (Figure 6).

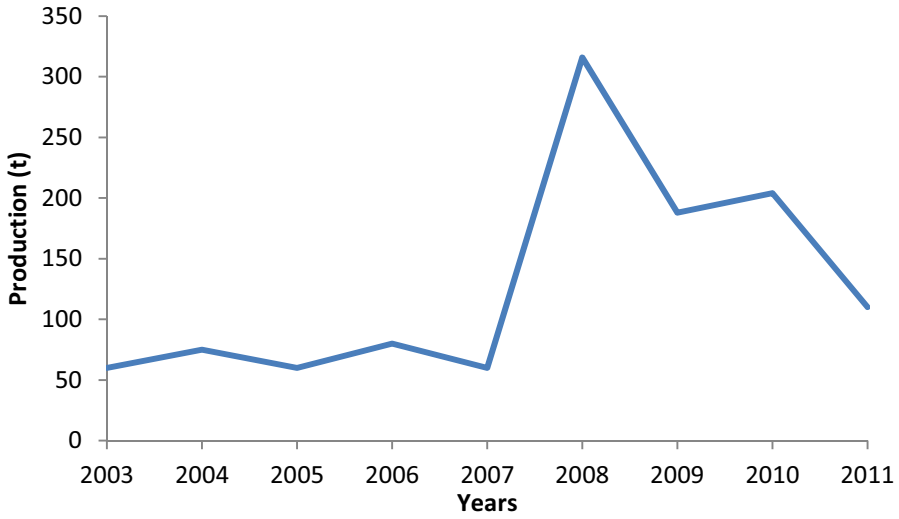


**Figure 6.** Production of Senegalese sole in Spain in tones from 2003 to 2011 (APROMAR, 2012).

Although Senegalese sole is a species of high commercial interest, its intensive rearing presents some factors that still limit the economical development and profitability of this activity, which are mainly related to the reproduction of the F1 specimens, the incidence of pathologies and, larval weaning and quality (Dinis *et al.*, 1999; Padrós *et al.*, 2003; Toranzo *et al.*, 2003; Fernández and Gisbert, 2011).

Getting **regular spawns** from broodstock kept in captivity has been limiting the development of Senegalese sole farming for a long time. Although spawning of wild Senegalese sole in captivity provides large quantities of eggs, the reproduction in captivity of this species is not totally controlled, since the reproduction of individuals from the first rearing generation (F1) or even from the F2 is still limited, resulting in a strong dependency on wild broodstocks (Cañavate, 2005). Evidence suggests that the poor spawning in G1 broodstock cannot be attributed to any hormonal dysfunction (Bertotto *et al.*, 2006; Agulleiro, 2007; Guzmán *et al.*, 2008) and a failure of G1 males to display normal courtship behavior has been demonstrated (Carazo *et al.*, 2009; 2011), while G1 females were shown to be able to produce fertilized eggs from natural spawning as long as they were accompanied by wild males (Mañanos, 2011). The reason for the failure of courtship and spawning remains unclear and,

consequently, the reproduction of cultured Senegalese sole remains a bottleneck to establish a sustainable industrial production of the species (Howell *et al.*, 2011). Several factors are suspected to be at the origin of this reproductive problem of G1 broodstock: the culture environment during early developmental stages, the effects of broodstock selection on the genetic composition of the broodstock, and/or the broodstock nutrition (Bromage, 1995, Mañanos *et al.*, b, 2008, Howell *et al.*, 2011; Norambuena, 2012).

Another critical period in the lifecycle of Senegalese sole that represents an important bottleneck for the development of the industrial production is the **weaning** period. This stage is characterized by the transition from feeding on live prey to inert compound diets. During metamorphosis, Senegalese sole larvae settle at the bottom of the tanks and change its feeding behavior, passing from an active pelagic live prey hunter to a benthonic feeder, grazing continuously on dead *Artemia* on the bottom of the tanks (Dinis *et al.*, 2000). This feeding behavior complicates the weaning of Senegalese sole onto inert diets, and weaning has traditionally been limiting Senegalese sole farming (Howell 1997; Dinis *et al.*, 1999). Early work suggested that lower growth and higher mortality in fish larvae fed inert diets was related to low acceptance and attractiveness of inert diets, combined with poor ingestion, digestion and assimilation (Koven *et al.*, 2001a), leading to poor quality of juveniles. In this sense, a period of co-feeding with live prey and inert diet was introduced in feeding protocols. Dinis (1992) was able to wean Senegalese sole until 30 days post hatching (dph) with 35.8% of survival rate, with a co-feeding regime using an inert diet including attractants, although one of the major problems was the large variation in survival and growth dispersion between batches. Various studies have been undertaken to determine feeding strategies at weaning (Engrola *et al.*, 2005; 2007, 2009a, b; 2010) and co-feeding larvae with inert diets from mouth opening produced larger and better quality post-larvae at the end of the weaning (Engrola *et al.*, 2009a). Engrola *et al.* (2007) determined that the choice of the feeding strategy to adopt at weaning should be based on the post-larvae weight, which indicates the developmental stage and the physiological status of the post-metamorphic larvae.

Opportunistic **pathologies** regularly occurring during Senegalese sole rearing are one of the main factors that currently limit the on-growing and the commercial development of this species (Padrós *et al.*, 2003; Toranzo *et al.*, 2003). Major pathologic problems are related to bacterial diseases, such as (i) Pasteurelosis, which are septicemic infections due to *Photobacterium damsela* subs. *piscicida,* particularly affecting Senegalese sole when rearing temperatures exceed 18°C, leading to high mortality rates, and (ii) Flexibacteriosis, which are cutaneous ulcers due to *Flexibacter maritimus* associated with vibriosis infections by *Vibrio harveyi, Vibrio alginolyticus* or *Vibrio pelagius.* Cutaneous ulcers are highly frequent in Senegalese sole rearing, at both juvenile and fattening stages, and are characterized by progressive ulcerative lesions on the skin and the fins, leading to cause septicemias and massive mortalities (Padrós *et al.*, 2003). The incidence of bacterial pathologies is particularly high in hatcheries when starting the weaning phase and during the pre-fattening phase, associated with episodes of high mortalities and severe growth decreases. In order to reach a more

reliable production of Senegalese sole, the possible future solutions to these pathologic problems will necessary begin with (i) the improvement in rearing and production systems; (ii) a better knowledge of its nutritional requirements to formulate more adequate diets during the weaning and the pre-fattening phases; (iii) an improvement in the application of sanitary measures; (iv) the development of specific vaccines and (v) the utilization of immunostimulants.

Finally, **larval quality** in hatchery-reared Senegalese sole is a major concern to get adult fish with the adequate standard commercial size and shape and optimal market value. In reared fish, the presence of morpho-anatomical abnormalities, defined as deviations of external morphology associated with defects of internal anatomy, is a frequent and important issue in fish aquaculture with high economic consequences (reviewed by Koumoundouros, 2010). These morpho-anatomical abnormalities downgrade both the image of the product and the biological performances (e.g. growth rate, mortality, susceptibility to diseases) of reared fish, with direct negative effects on the market value and the production costs, as well as in fish welfare (Divanach *et al.*, 1996; Koumoundouros *et al.*, 1997; Boglione *et al.*, 2001; Gavaia *et al.*, 2002; Cahu *et al.*, 2003), and their incidence in fish commercial farms represents one of the main bottlenecks to improve profit margins of industrial aquaculture. Morpho-anatomical abnormalities include two main categories affecting fish phenotype: pigmentary disorders and skeletal deformities. In the aquaculture industry, losses due to deformities mostly occur at two different levels. At hatcheries, reducing survival rate and growth efficiency of deformed fish; and during on-growing rearing period, where deformed fish have to be discarded or sold at lower market prices. Moreover, that lower larval survival rate and the deletion of deformed fish imply an over-dimension of the production facilities that also increases the production costs. While the incidence of skeletal deformities in gilthead sea bream and European sea bass are around 30% (Villeneuve *et al.*, 2005a), in Senegalese sole it could affect up to the 80% of the fish produced under standard protocols (Gavaia *et al.*, 2002). Moreover, pigmentation abnormalities, mainly including pseudo-albinism and ambicoloration, affect the external aspect of the hatchery-reared flatfish species, downgrading their market values. The incidence of malpigmented flatfish could reach the 61% of the overall fish production (Estévez *et al.*, 1995, 1999; Copeman *et al.*, 2002; Villalta *et al.*, 2005a). During the last decade, the productive sector together with research groups have put interest in developing investigation studies (as the one presented here) aiming to improve the quality of reared fish by reducing the incidence of skeletal deformities and pigmentation abnormalities in this flatfish species.

## 4. The problem of larval quality in intensive aquaculture: pigmentation abnormalities and skeletal deformities.

Two general types of morpho-anatomical abnormalities affecting fish normal phenotype may be considered: **pigmentary disorders** and **skeletal deformities**. As Prentiscola *et al.* (2013) recently reviewed, the presence of skeletal and pigmentary anomalies in reared fish is generically attributable to a general lowering of individual homeostasis, that is the tendency of a biological system to resist to

changes and to maintain itself in a state of stable equilibrium (i.e. the capacity to buffer environmental and genetic variations through canalization and developmental stability). These alterations allow the expression of deviated ontogenetic and growth processes, such as anatomical anomalies, fluctuating asymmetry, altered meristic counts and anomalous pigmentation. Accordingly, all these anomalies may be considered as developmental disturbances, indicative of the presence of inappropriate rearing conditions or genetic impairment. The aim of this section is not to present a deep review on this topic; thus, readers are encouraged to consult the reviews of Lall and Lewis-McCrea (2007) and Boglione *et al.* (2013a, b) for a thoroughly revision on the typology of this abnormalities and the causative factors responsible for their development.

The presence of skeletal anomalies in farmed teleosts is a constant worldwide problem in aquaculture and it entails economical, biological and welfare issues. Deformed fishes have to be manually and repeatedly culled out from production or products from these fish are downgraded to filets or fish meal (flour) with loss of profit. Even filet processing is impaired by the presence of skeletal (particularly vertebral) deformations as machines are designed for normal shaped fish and more manual processing and extra trimming are necessary. The prevalence of skeletal deformities in farmed fish suggests that we still need to improve our knowledge about genetic and epigenetic factors in rearing conditions that can provoke such malformations (see review in Boglione *et al.*, 2013a, b). According to Hough (2009), the minimum estimate of the annual loss due to deformed fish is more than 50,000,000 €/year for European aquaculture, and a reduction of 50% in deformed fish rate could save *ca.* 25,000,000 €/year, increase production and profitability and enhance aquaculture reputation.

In spite of the improvement in rearing techniques, fish quality remains a significant issue in finfish mariculture. The incidence of morpho-anatomical abnormalities damage the biological performances of the fish (*e.g.* growth rate, survival, resistance to diseases) and the external appearance and quality of the products, affect the market value and the production costs and lead to remarkable negative economic consequences for the industry (Koumoundouros, 2010).

**Pigmentation abnormalities** in flatfish cover a broad range of different conditions ranging from pseudo-albinism (on the ocular side), ambicoloration (pigmentation on both ocular and blind sides), hyperpigmentation and mosaicism (Bolker and Hill, 2000; Tagawa and Aritaki, 2005). They affect the external aspect of reared flatfish species, as well as their survival, growth and development, reducing the market value of the final product, although their relative impact may depend on the way in which flatfish are commercialized, *i.e.*, intact fish or fillets (Power *et al.*, 2008). The high incidence of malpigmented fish in intensive rearing conditions represents a current major bottleneck in flatfish farming and limits the development of the production sector (Power *et al.*, 2008; Boglione *et al.*, 2013a, b). Skin pigmentation in fish is the result of the amount and spatial combination of several types of chromatophores, such as melanophores (black), xanthophores (yellow-orange), erythrophores (red), iridophores (iridescent), leucophores (white) and cyanophores (blue), that produce a huge variety of pigment patterns contributing to sex recognition, camouflage and predator

avoidance (Fujii, 2000; Couldridge and Alexander, 2002; Puebla *et al.*, 2007). Because of their remarkable capacity to change skin color to mimic texture and color of the background (Healey, 1999), skin morphology in flatfish and the chromatic biology and physiology of chromatophores (types, location in tissues) associated with color pattern changes has been extensively studied (Cunnunghanm and McMunn, 1893; Burton *et al.*, 2010). Fish color changes can be divided into two main categories: (i) physiological changes involved in quick modification caused by the immediate aggregation or dispersion of pigmentary organelles within chromatophores in response to environmental signals and (ii) morphological changes, long-term modifications resulting from the gradual decrease or increase in the number and the size of chromatophores and found after background adaptation (Izquierdo and Koven, 2011). The mechanisms regulating these changes are hormonally (melanocyte-stimulating hormone, MSH and melanin-concentrating hormone, MCH) and neutrally (sympathetic innervation) controlled (see review in Fujii, 2000). Pigmentation development in pleuronectids occurs at the same time as asymmetry acquisition during metamorphosis, through the differentiation of the different chromatophores, leading to the adult pattern of pigmentation. In Japanese flounder, the pattern of development is accompanied by changes in skin histology, enzymatic activity, and pigment cell distribution (Seikai *et al.*, 1987). On the ocular side of flatfish, adult small-sized melanophores appear in cluster around degenerating larval chromatophores (Seikai *et al.*, 1993), which may result either from the migration of differentiated cells or from the differentiation of existant precursors (Bolker and Hill, 2000). A proper adult pigmentation pattern is based on the appropriate differentiation of chromatophores, as well as their correct organization trough interactions among them, at precise time and location of their differentiation (Izquierdo and Koven, 2011). Once metamorphosis successfully completed, the blind side of the fish is mainly constituted by iridophores, while the ocular side is formed by a mosaic of melanophores and iridophores, aggregated in melanosomes, which give the final color of the skin (Burton, 2010). Pigmentary disorders seem to rely on a disruption of the differentiation of melanophores during the process of metamorphosis and alterations in melanogenesis (Bolker and Hill, 2000; Nakamura *et al.*, 2010), as a result of a dietary nutritional imbalances in fatty acids or vitamin A, among other factors (Kanazawa, 1993; Estévez and Kanazawa, 1995; Matsumoto and Seikai, 1992; Villalta *et al.*, 2005a; Hamre *et al.*, 2005, 2007). It has been demonstrated that the process of metamorphosis and pigmentation in fish is mirrored in the molecular features (Fu *et al.*, 2011) and the evolution in gene expression patterns during metamorphosis reflect the transition from the larval to the adult phenotype. The molecular and physiological processes underlying malpigmentations in juvenile flatfish may have their origin in earlier larval stages, before being manifested in the external morphology. Characterizing the molecular ontogeny of pigmentation underlying skin morphological features, by studying the expression pattern of pigmentation-related genes during the development of flatfish is a reliable tool for elucidating the formation mechanisms of the adult pigmentation pattern and understanding the origins of pigmentary disorders. The identification of molecular markers in differentiating pre-metamorphosis larvae will be helpful to differentiate normally and abnormally pigmentation in developing larvae before morphological differences become evident (Power *et al.*, 2008).

**Table 2.** Deformities affecting different European flatfish species.

| Species | | Deformities % | Origin Rearing system | Most affected area | Ref. |
|---|---|---|---|---|---|
| *Hippoglossus hippoglossus* |  | 1% (jaw)<br>41 - 89%<br>83 - 91% | Norway / Intensive<br>Canada / Intensive<br>Canada / Intensive | Jaw<br>Eye migration<br>Pre-haemal vertebrae | 1<br>2<br>3 |
| *Scophthalmus maximus* |  | 6% (0 – 29.3%)<br>< 20% | Turkey / Intensive<br>France - Intensive | Eye migration<br>Skeleton | 4<br>5 |
| *Solea senegalensis* |  | 44 – 85%<br>69%<br>41 – 57% | Portugal / Intensive<br>Portugal / Intensive<br>Portugal / Extensive | Caudal/Haemal vertebrae<br>Caudal complex | 6<br>7<br>8 |
| *Solea solea* |  | 17 – 48% | France / Intensive | Jaw, cranium, caudal vertebrae | 9 |
| *Dicologlossa cuneata* |  | nd | Spain / Intensive | nd | - |

References: 1: Ottesen and Babiak, 2007; 2: Lewis and Lall, 2006; 3: Lewis *et al.*, 2004; 4: Üstündağ *et al.*, 2002; 5: Person-Le Ruyet, 2002; 6: Gavaia *et al.*, 2002; 7: Dâmaso-Rodrigues *et al.*, 2010; 8: Gavaia *et al.*, 2009; 9: Lagardère *et al.*, 1993). Abbreviations: nd, no data available; ref, bibliographic references.

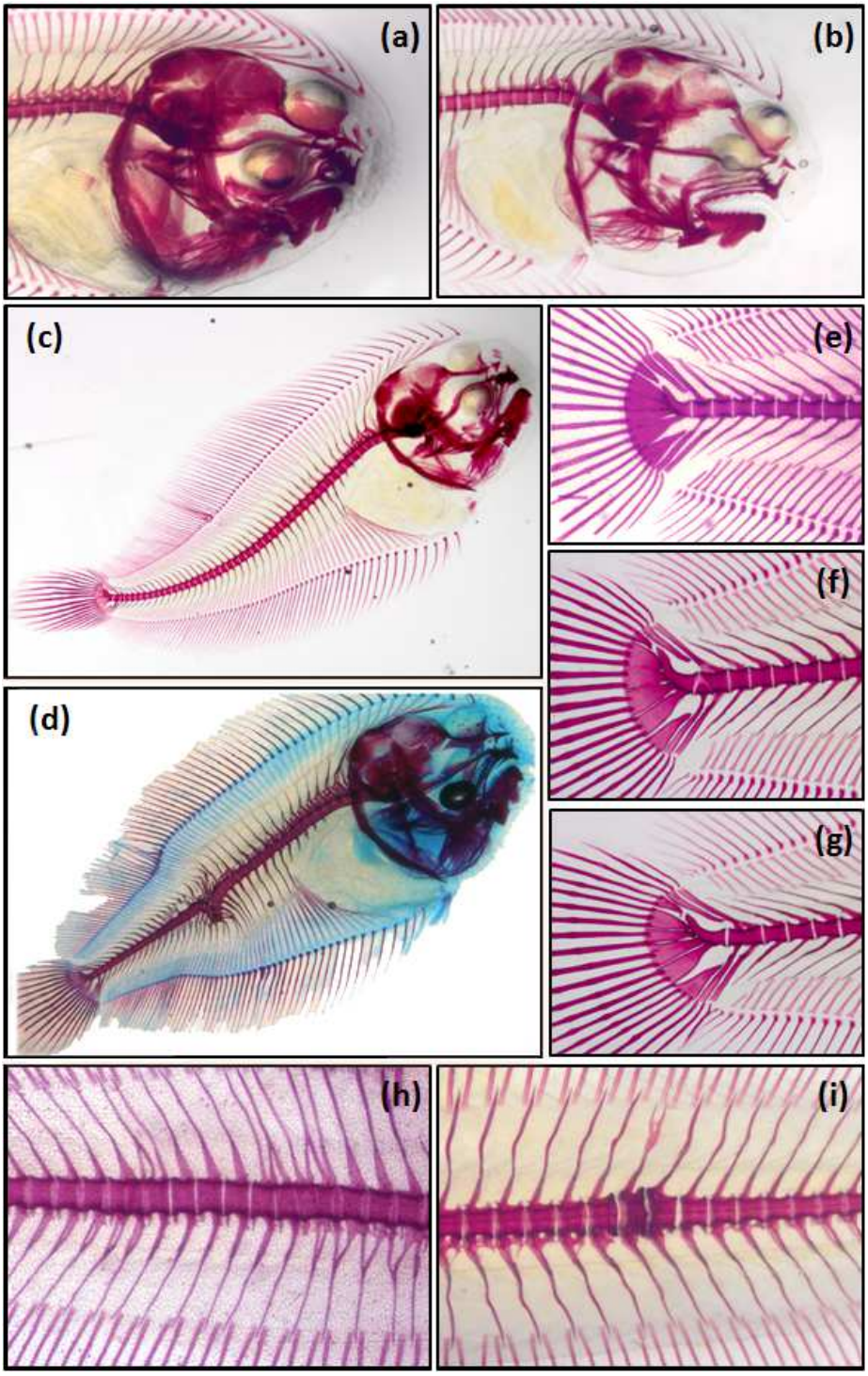


**Figure 7.** Different types of skeletal deformities identified in skull, vertebral and caudal fin regions in Senegalese sole larvae stained with alizarin red and alcian blue. Head with normal splanchnocranium structures (a); Head with parasphenoid deformity and eye migration anomaly (b); Scoliotic fish (c); Fish presenting a severe deformity in the vertebral region (d); Normal developed caudal fin complex (e); Supernumerary vertebrae before the urostyle (f); Fusion of the last two vertebrae before the urostyle (g); Severe vertebral fusions with fusions of neural and haemal arches (h); Compression of vertebral bodies (i).

**Skeletal deformities** can affect all body regions, from the skull to the vertebral column and the fins, consisting in multiplication, lack, shape and position modifications of the different skeletal structures. These malformations mostly occur during early developmental stages in hatcheries, they reduce fish survival rate and growth performance and alter development and external morphology (Divanach *et al.*, 1996; Koumoundouros *et al.*, 1997; Boglione *et al.*, 2001, 2013a, b). Existing data about the incidence of anomalies are collected using different diagnostic tools observations, X-rays, palpation, whole mount staining, computed tomography, histology, histopathology, histochemistry and immunohistochemistry) that provide different levels of accuracy (varying according to the life stage considered) (Boglione *et al.*, 2013b). While the incidence of skeletal deformities in hatchery-reared gilthead sea bream and European sea bass is around 15-50% (Villeneuve *et al.*, 2005a; Darias *et al.*, 2010, 2011a; Prentiscola *et al.*, 2013), in flatfish species there exists some variability depending on the species and rearing system (Table 2). It was observed that in the post-larval and juvenile stages of Senegalese sole, produced under intensive rearing conditions, the incidence of individuals affected by vertebral anomalies can reach levels ranging from 44% to 80% (Gavaia *et al.* 2002, 2009; Engrola *et al.*, 2009a; Fernández and Gisbert, 2011), while wild captured larvae display a low incidence of deformed fish (Gavaia *et al.,* 2009).

Different types of skeletal deformities have been identified and classified by Koumoundouros (2010) in three categories depending on the affected body region: cranial deformities (splanchnocranium and neurocranium), vertebral column deformities (vertebral bodies and their neural or haemal spines) and fin deformities (Figure 7).

**Cranial deformities**, which mainly affect the splanchnocranium, are evident at gross examination and mainly affect dentary, pre- and maxillary, glossohyal and opercular plate. They appear during larval development, when the implicated skeletal structures are ossifying, and are mainly sublethal anomalies (Boglione *et al.*, 2013b). Affected fish generally show difficulty in efficiently nourishing, so they grow slower and are weaker than unaffected fish. Opercular plate or gill-cover anomalies are anatomically attributed to inside or outside folding, shortening or abnormal positioning of the opercular and subopercular bones, leaving the branchial arches more exposed to injuries or parasites, then affecting the health status of affected fish (Boglione *et al.*, 2013a, b)**.** Gill-cover anomalies appear early in development during the pre-flexion and flexion stage (Koumoundouros 2010), mostly in fish reared under intensive conditions (Prestinicola, 2012). Beraldo and Canavese (2011) recently showed that the monolateral inside folding of the gill-cover presents a partial recovery during the on-growing phase of fish in sea cages, but only when the degree of anomaly is low. Pugheadness, cross-bite and lower jaw reduction or elongation are the main types of jaw anomalies that can affect the finfish reared in Mediterranean aquaculture (Barahona-Fernandes 1982; Koumoundouros *et al.*, 2004; Abdel *et al.* 2005). In pugheadness, the ethmoid region and upper jaws are antero-posterior compressed. In cross-bite, the dentary appears affected by asymmetric growth: the result is that the dentary tip appears skewed off-center so it is not oriented parallel to the upper jaw. Finally, the reduction of the lower jaw is almost identical to the intense cross-bite and attributed to

the ventro-lateral distortion of the jaw elements (Hickey *et al.*, 1977; Cobcroft *et al.*, 2001). Prognathism, which is a prolongation of the dentary, is also frequently observed in intensively reared European sea bass and gilthead sea bream (Boglione *et al.*, 2013b). These jaw anomalies develop mainly during the early larval stages (Koumoundouros 2010, Darias *et al.*, 2010, 2011a). Hyobranchial anomalies are related to the ventral projection of glossohyal, or basihyal or hypohyal elements, often associated with bending and/or dislocations of ceratohyals. In flatfish species, some cranial deformities are related to the eye migration and head remodeling occurring during metamorphosis and the achievement of the asymmetric shape and benthic lifestyle. Senegalese sole displayed up to 5% of specimens with ocular migration related abnormalities (Gavaia *et al.*, 2009), which have been associated with the presence of a deformed pseudomesial bar in other flatfish species (Okada *et al.*, 2003). The importance of the cephalic region in visual, sensorial, feeding and respiratory functions makes fish affected by these types of deformities very susceptible to disease outbreaks and reduction of survival, biological performance and market value (Koumoundouros, 2010).

**Vertebral column** anomalies have been documented in all the reared fish species, in the form of curvature, dislocation, shortening and twisting. In severe cases, anomalies of vertebrae centra and/or arches are associated with macroscopic deviations of the vertebral axis. These latter involve lordosis (V-shaped dorsal–ventral curvature), kyphosis (Λ-shaped dorsal–ventral curvature) and scoliosis (lateral curvature). The angle formed by axis bending must be of a certain magnitude in order to identify the anomaly under gross external examination, but it has to be considered that many fish may present this problem to a lesser severe degree (Koumoundouros, 2010; Boglione *et al.*, 2013a, b). Vertebral anomalies occur later than skull deformities, mainly during notochord segmentation and the differentiation of vertebral bodies. Given the essential role of skeletal axis in supporting the musculature for the swimming activity, deformities in this region have a strong repercussion on growth, survival, external appearance and thus final market price (Koumoundouros, 2010). Existing literature indicates that finfish species of European aquaculture could be divided into two different groups according to the type, severity and frequency of some vertebral anomalies. The first group includes gilthead sea bream, European seabass, flatfish and most of the candidate new species, whereas the second group includes the reared salmonids. In the non-salmonid group, vertebral axis anomalies are quite frequent in the same lot or species, and concern almost all the recorded types to date. In salmonid species, vertebral axis deviations appear dramatically only after smoltification, being rarely observed in early juveniles, and they mainly concern the compression of the vertebral axis due to fused and compressed vertebral bodies. Furthermore, it must be taken into account that belonging to the same family should not justify the transfer of knowledge from one species to the other, considering that the farming environment for one species to another can be very diverse (Boglione *et al.*, 2013b). Main vertebral deformities observed in reared fish are lordosis (pre-haemal and haemal), kyphosis and scoliosis, and are often but not always associated with non-inflation of the swim bladder (Chatain, 1994; Andrades *et al.*, 1996). Haemal lordosis has been proposed also to be linked with inappropriate tank hydrodynamism and forced swimming although lordotic fish have been found also in gentle water flow tanks (Chatain, 1994; Andrades *et al.*, 1996; Divanach *et al.*, 1997). In red porgy, haemal lordosis

has been considered the consequence of coupling insufficient feeding (causing skeleton weakness) and excessive swimming activity for feeding (Izquierdo *et al.*, 2010). Lordosis has been associated with fused vertebrae and a reduced or excessive number of vertebrae in red porgy and red sea bream (Izquierdo *et al.*, 2010; Hattori *et al.*, 2003; Matsuoka, 2003), their localization along the column being affected by culture intensity. The incidence of pre-haemal kyphosis has been associated with haemal lordosis and abnormalities of the branchiostegal rays (Boglione *et al.*, 1995; Koumoundouros *et al.*, 2002). Scoliosis is the most easily identifiable anomaly in live fish, with the best detection being from the dorsal or ventral side of the whole fish. In the most severe cases, various combinations of these three anomalies occur, as in the LSK syndrome (a consecutive repetition of lordosis–scoliosis–kyphosis, from the head to the caudal fin) described in gilthead sea bream by Afonso *et al.* (2009) and attributed in red porgy to a very poor nutritional quality of live prey during the very early feeding (Izquierdo *et al.*, 2010). In Atlantic halibut juveniles, (i) low phosphorus diets enhanced scoliosis in the cephalic and haemal regions of the vertebral column; (ii) diets without ascorbic acid supplement caused scoliosis and lordosis in the haemal vertebrae; (iii) high levels of vitamin A in the diet caused scoliosis, spanning the cephalic/pre-haemal and anterior haemal vertebrae; (iv) oxidized oil diet induced scoliosis, spanning the cephalic/pre-haemal/anterior haemal regions (Lewis-McCrea and Lall, 2010). The pattern and type of abnormalities observed in fish fed these experimental diets were similar to those observed in a commercial halibut hatchery.

In addition to changes in curvature or twisting of the vertebral column axis, fish may also display vertebrae anomalies. They include: (i) dislocation, fusion, shortening, deformation or lack of the vertebra centra; (ii) dislocation, compression, deformation, lack or extra formation of the haemal and neural arches and apophysis, and (iii) dislocation, shortening, deformation, lack or separation of the ribs (see review in Boglione *et al.*, 2013b). Compressed vertebrae with intervertebral spaces are generally classified as platyspondyly, and compressed vertebrae without intervertebral spaces are classified as vertebral ankylosis and platyspondyly. The most severe vertebrae anomalies are those affecting the vertebral body such as fusion, compression and modified shape because, if including many vertebrae, the fish length could be reduced. In contrast, these anomalies altering vertebral arches and ribs are considered slight, as they do not affect the external shape of the fish (Koumoundouros, 2010; Boglione *et al.*, 2013a, b). However, their presence is a physiological sign of altered skeletogenic processes. Severe anomalies affecting many neural arches and spines, which protect the spinal cord and furnish the insertion for dorsal musculature, could significantly affect fish performance (Hall, 2005). Severe and extended deformities of haemal arches, which protect the arteria and venae caudalis, could interfere with blood flow in the organism and oxygen levels, influencing chondrogenesis (Hall, 2005). Compressed and fused vertebrae are considered as true deformations, as it has been reported that vertebral bodies often develop normally up to a certain point (Witten *et al.*, 2009), then the tissue of the intervertebral space (notochord tissue) is replaced by cartilage and fibrocartilage and, concurrently, cells of the vertebral growth zone change their character from osteoblastic to chondroblastic (Witten *et al.*, 2009). In Senegalese sole, this has been considered a pathological response to a compressive mechanical environment (Cardeira *et al.*, 2012). Sufficient

dietary mineral content for promoting proper mineralization is a factor that favors containment and prevents aggravation of a vertebral anomaly. Besides, under and over-mineralized vertebrae have also been considered to be anomalies, as they can evolve into anomalous vertebral bodies (Darias *et al.*, 2010, 2011a). Senegalese sole is mostly affected by vertebrae fusion and anomalies, and abnormalities of the vertebral arches (Engrola *et al.*, 2009a; Fernández *et al.*, 2009; Cardeira *et al.*, 2012), with a special incidence on the preural vertebrae and caudal fin regions, in both reared and wild specimens (Gavaia *et al.* 2002, 2009; Engrola *et al.*, 2009; Fernández *et al.*, 2009; Fernández and Gisbert, 2010). Cardeira *et al.* (2012) described the microanatomical changes that occur at tissue and cellular levels in lordo-kyphotic Senegalese sole. In affected individuals, ectopic cartilage-like tissue within bone matrix was found at the growth regions (contact area between two vertebrae) of affected vertebrae. The authors hypothesized that the increase in strain supported by deformed vertebrae may trigger the onset of metaplastic tissue formation through an osteochondroprogenitor precursor from the condensation of mesenchymal cells or by trans-differentiation of (pre-) osteoblastic cells to a chondrocytic lineage. In addition, a change in the phenotypes observed from juveniles to adults suggests that the response to altered loads will vary according to fish size or to the mineralization state of the vertebrae. Cartilaginous vertebrae have been also reported in European sea bass fed low dietary ascorbic acid levels (Darias *et al.*, 2011a).

Depending on their level of severity, **fin deformities** can affect the appearance and the overall body shape. Fins anomalies are frequently observed in reared fish, but the frequency, the affected fin and the severity of anomaly largely vary according to the species, the rearing methodology and the tank features. In intensive conditions, fin erosions or bitten caudal fins are quite frequent due to crowding effects: these are not to be considered as skeletal anomalies. However, the poor state of bitten or eroded fins prevents the monitoring of true fin anomalies. In general, the most severe observed fin anomalies are the complete lack of a fin, the presence of a supernumerary fin or fin dislocation, but they are very rare or limited to some peculiar lot/farm or observed in the experimental rearing of a new candidate species (Noble *et al.*, 2012; Boglione *et al.*, 2013a, b). An incomplete development of dorsal (saddleback syndrome, depression in the anterior dorsal profile) or anal fins, the lateral bending, duplication, partial lack and stricture of the caudal fin are ones of the most severe osteological fin deformities. Caudal fin complex deformities appear during the development of posterior notochord or even earlier (Koumoundouros, 2010). Because of the involvement of the caudal fin in swimming, caudal fin deformities can induce malformations on the adjacent vertebrae, such as kyphosis, lordosis or scoliosis. Lighter fin deformities consisting in fusions, lacks, extra formation or displacements of bony structures supporting the fin (epural, hypurals, parahypural, urostyle and modified neural and haemal spines) and in alterations of the rays and spines have negligible impact on the external morphology (Boglione *et al.*, 2013b). In Senegalese sole, a significant number of fusions or malformations of hypurals have been observed in the caudal fin and of anomalies in pterygiophores in the dorsal and anal fins (Gavaia *et al.*, 2002; Engrola *et al.*, 2009a; Fernández *et al.*, 2009; Fernández and Gisbert, 2010).

## 5. Causative factors responsible for the development of skeletal deformities and pigmentary disorders

Several studies have suggested that most of the skeletal deformities in fish are induced during embryonic and larval stages, where many developmental processes (*e.g.* organogenesis, morphogenesis and metamorphosis) take place in a short period of time, and attempted to understand the causes and the mechanisms responsible for these deformations (Daoulas *et al.*, 1991; Koumoundouros *et al.*, 1997; Cahu *et al.*, 2003; Lall and Lewis, 2007; Boglione *et al.*, 2013a, b). According to the available literature, each environmental (biotic and abiotic) factor/parameter (oxygen, temperature, pH, stocking density, water flow, $CO_2$, rearing volumes, inappropriate alimentation (i.e. unbalanced feeds), heavy metals, bacteria, parasites, toxicants, etc), if at a non-optimal level for the reared species or life stage, can cause skeletal anomalies in reared fishes. Thus, the development of skeletal abnormalities is linked to a poorly understood relationship between nutrition, environment and genetic factors (see reviews in Cahu *et al.*, 2003; Lall and Lewis-McCrea, 2007; Boglione *et al.*, 2013b).

The larval stage is a very sensitive period influenced by many factors: **abiotic** (*i.e.* temperature, salinity, pH, $CO_2$ and $O_2$ water concentration, light intensity and photoperiod, radiation, flow rate and tank shape, volume and color) (Doroshev and Aronovich, 1974; Bengtsson *et al.*, 1988; Divanach *et al.*, 1996; Villamizar *et al.*, 2011); **biotic** (*i.e.* stocking density, parasites, handling, mechanical shock, pathological infections) (Pommeranz, 1974; Lom *et al.*, 1991; Divanach *et al.*, 1996; Prentiscola *et al.*, 2013); **physiological** (*i.e.* stress, infectious diseases, mechanical lesions, etc); **xenobiotic** (*i.e.* pollutants, algaecides, insecticides, heavy metals, pesticides); **genetic** (*i.e.* mutations, hybridization, inbreeding) (Madsen and Dalsgaard, 1999; Saddler *et al.*, 2001) and **nutritional** (deficiencies or excesses in macro- and micronutrients) (Cahu *et al.*, 2003; Darias *et al.*, 2011b, Boglione *et al.*, 2013a, b). However, some skeletal deformities may appear at later stages of development, *i.e.* during the on-growing period, and they are indicative of non-optimal rearing and nutritional conditions (Boglione *et al.*, 2013a, b; Prentiscola *et al.*, 2013). The effects of dietary factors and nutrients on skeletal development are better defined in humans and terrestrial animals (Beattie and Avenell, 1992; Wallach, 2002). A high level of fiber impairs calcium (Ca), lipid and fat-soluble nutrient absorption and isoflavonoids decrease bone resorption through estrogen like properties. Certain vitamins (A, C, E and K) and minerals (calcium, phosphorus, boron, zinc, copper, silicon, vanadium, selenium, manganese, strontium and fluoride) either promote or delay bone formation or mineralization (Darias *et al.*, 2010, 2011b; Fernández *et al.*, 2009, among others), in function of their quantity in the diet. Excessive intake of vitamin A and some minerals (strontium, lithium, aluminum, iron, cadmium, tin and lead) has deleterious effects on skeletal tissue metabolism (Lall and Lewis-McCrea, 2007). Finally, imbalances in dietary contents of lipids, HUFA and fatty acids are known to be causative agents of skeletal abnormalities in farmed fish (Lall and Lewis-McCrea, 2007, see section 6 from the Introduction).

Imbalances in dietary vitamins levels have been shown to affect ossification and skeletogenesis processes in various marine fish larvae. In Senegalese sole larvae, vertebral centrums were ones of the most affected structures by an increase of dietary VA (Fernandez *et al.*, 2009). The incidence of a supernumerary vertebra in the haemal region of the vertebral column under increased dietary VA levels in Senegalese sole and gilthead sea bream (Fernández *et al.*, 2008, 2009) has also been observed is in Japanese flounder (Haga *et al.*, 2002), European sea bass (Villeneuve *et al.*, 2006) and red sea bream (Hernández *et al.*, 2007). In gilthead sea bream, an increasing incidence of specimens with lordosis, kyphosis and/or scoliosis was noticed when fish fed high dietary VA levels (Fernández *et al.*, 2008), as observed in European sea bass larvae (Villeneuve *et al.*, 2005a), generally originating from skeletal compression and fusion of one or two vertebral centrums in gilthead sea bream (Fernández *et al.*, 2008), Japanese flounder (Dedi *et al.*, 1995) and red sea bream (Hernández *et al.*, 2006). In Senegalese sole, the body shape was not affected by high VA doses (Fernández *et al.*, 2009), although vertebral compression and fusion was observed (Fernández *et al.*, 2009; Fernández and Gisbert, 2010a). Higher incidence of vertebral deformities in the cephalic vertebrae (the first developed and less exposed to VA-induced imbalanced) was found in gilthead sea bream and Senegalese sole fishes exposed to the highest VA level; whereas fishes fed with all supplemental VA diets exhibited increasing frequencies of deformities in haemal and caudal vertebrae (the latest developed and most exposed to en excess of VA) (Fernández *et al.*, 2008, 2009; Fernández and Gisbert, 2010). Therefore, the dose of VA and the timing of morphogenesis directly affect the incidence of skeletal disorders (Villeneuve *et al.*, 2006; Fernández *et al.*, 2008, 2009; Mazurais *et al.*, 2008; Fernández and Gisbert, 2010). Fernández and Gisbert (2010) have shown that high dietary VA levels disrupted the skeletogenesis in Senegalese sole post-metamorphosed larvae by increasing the incidence of skeletal deformities in the axial skeleton and caudal fin complex, which were dependent on both bone morphogenesis and ossification processes. Depending on the ossification process from which different skeletal structures are derived, bones might be differentially affected by high dietary VA content, those directly originated from the connective tissue with a preliminary cartilage stage being more sensitive to dietary VA excess than those formed by intramembranous ossification.

The effect of imbalanced dietary levels of vitamin C (VC) and D (VD) on mineralization and incidence of skeletal deformities have been investigated in European sea bass (Darias *et al.*, 2009, 2011b). Insufficient VC levels (0-30 mg VC/kg diet) conducted to cartilage damages with poor mineralization, the absence of formation of the haemal arches, the loss of one vertebra and deformities in the caudal complex, while excessive VC levels 400 mg VC/kg diet) led to poor mineralization, supernumerary vertebrae, and deformities in the cranial and fins region (Darias *et al.*, 2009, 2011a, b). VC is a co-substrate for hydrolase and oxygenase enzymes involved in the biosynthesis of pro-collagen and extreme dietary concentrations in VC might disrupt the proper skeletogenesis process in European sea bass. VD is known to protect the skeletal integrity. Insufficient levels of $VD_3$ in the diet (11.2 IU $VD_3$/g diet) led to poor mineralization, vertebral deformities such as kyphosis and scoliosis, deformities of the caudal fin and in the cranial region,

whereas an excess of $VD_3$ (42-120IU $VD_3$/g diet) might delay the mineralization and induced vertebral deformities (Darias *et al.*, 2010). Excess and lack of essential vitamins both damaged the processes of mineralization and bone formation and dietary balance in these vitamins is crucial for proper skeletogenesis.

Pigmentation in fish is generally dependent on bilaterally symmetric embryonic chromatoblasts, which either differentiate into larval pigment cells or remain undifferentiated, leading to the adult pigmentation morphological pattern (Power *et al.*, 2008). Although some gene mutations have been identified to be at the origin of the albinos phenotype, defects in chromatophores differentiation in flatfish, leading to the incidence of pigmentary disorders, seems to be mainly controlled by environment, nutrition and by the development of appropriate innervation (Dickey-Collas, 1993; Estévez and Kanazawa, 1995; Rainuzzo *et al.*, 1997; Bolker and Hill, 2000; Bell and Sargent, 2003; Hamre *et al.*, 2005; Villalta *et al.*, 2005a; Lund *et al.*, 2007, among others). Inappropriate lighting has been suspected to be responsible for the prevalence of pigmentary abnormalities in hatchery-reared flatfish, flatfish larvae hatched in total darkness being predisposed to pigment abnormalities later in life. In partially pseudo-albinos southern flounder *Paralichthys lethostigma* reared until 37 dph under low light conditions, the incidence of normally pigmented specimens increased once light intensity was increased (Denson and Smith, 1997). However, too strong light intensity at metamorphosis may also lead to pseudo-albinism and ambicoloration (Von Ubisch, 1951). In other research works, Sugiyama *et al.* (1985), Fukusho *et al.* (1986) and Yamamoto *et al.* (1992) studied the effect of abiotic factors on the pigmentation of Japanese flounder (i.e. the shape of the tank, the aeration and the light, respectively), but they were not able to correlate the rate of pigmentary disorders to any of the above-mentioned parameters. In summer flounder reared under intensive conditions, the use of a sandy substrate has been shown to help preventing the apparition of ambicoloration (Stickney and White, 1975). Besides, larval nutrition until metamorphosis is thought to be critical for normal pigmentation, some nutrients probably arresting the development of pigment cells at a specific stage in larval development or avoiding melanin synthesis (Seikai, 1985; Fukusho *et al.*, 1987; Yamamoto *et al.*, 1992; Kanazawa, 1993; Reitan *et al.*, 1994; Guo *et al.*, 2003). High levels of vitamin A (VA) in live prey or in the rearing water enhance pigmentation of Japanese flounder, by stimulating the development of adult-type chromatophores, but this also induced cranial and spinal deformities (Seikai, 1985; Takeuchi *et al.*, 1995; Dedi *et al.*, 1997; Haga *et al.*, 2002). Moreover, dietary absolute and relative contents of some essential fatty acids, such as EPA (20:5n-3) and DHA (22:6n-3) have been found related with normal pigmentation in turbot (Reitan *et al.*, 1994). A reduction of the incidence of pigmentation anomalies in several flatfish has been attributed to increased dietary DHA levels in larval feeds (Kanazawa, 1993; Reitan *et al.*, 1994; McEvoy *et al.*, 1998a), although it was not identified if malpigmentations originated from a deficiency in DHA or from an excess in EPA and the resulting imbalance of the DHA/EPA ratio (Devresse *et al.*, 1994; Rainuzzo *et al.*, 1994; Sargent *et al.*, 1997, 1999b; Bell and Sargent, 2003). Moreover, high dietary contents of ARA (20:4n-6) have been associated to increasing incidence of pigmentary disorders in turbot and halibut (McEvoy *et al.*, 1998a, b; Estévez *et al.*, 1999), yellowtail flounder (Copeman *et al.*, 2002), Japanese flounder

(Estévez *et al.*, 2001) and Senegalese sole (Villalta *et al.*, 2005a), and the addition of EPA to the diet reversed the effect of high dietary ARA levels and improved pigmentation. Hence, the ratio EPA/ARA seems to play an important role in the pigmentation pattern of flatfish species. Indeed, ARA and EPA are both precursors for prostaglandins synthesis, directly competing for cyclooxygenase and lipooxygenase enzymes, to give rise to the prostaglandins of the 2-series ($PGE_2$) and 3-series ($PGE_3$), respectively (Bell *et al.*, 1995). Prostaglandins enhance the immune system and the resistance to stress (Bell and Sargent, 2003), and their relative proportions ($PGE_2/PGE_3$) are suspected to be involved in the proper pigmentation in flatfish, although their concrete effect on pigmentation patterns has not been demonstrated yet (Izquierdo and Koven, 2011). High amounts of dietary ARA, resulting in imbalances in the relative content of EPA and DHA (Moren *et al.*, 2011), and therefore in the relative proportions of $PGE_2$ and $PGE_3$ (Bell and Sargent, 2003), has been suggested to cause biochemical stress and developmental disorders related to pigmentation patterns and delayed eye migration in several flatfish species (McEvoy *et al.*, 1998a, b; Estévez *et al.*, 1999; 2001; Copeman *et al.*, 2002; Villalta *et al.*, 2005a, 2008; Lund *et al.*, 2008; Hamre and Harboe, 2008). More work is needed to determine the precise role of the dietary ARA and EPA contents on prostaglandin formation and their intervention in the pigmentation of flatfish.

## 6. The importance of lipid nutrition in Senegalese sole larval development and quality

Among all the factors influencing the incidence of skeletal deformities and pigmentary disorders (*i.e.*, pseudo-albinism), larval nutrition at first feeding has been recognized by many studies as one of the key parameters that affect skeletogenesis and pigmentation during early development (Cahu *et al.*, 2003; Lall and Lewis-McCrea, 2007; Boglione *et al.*, 2013a, b). Several studies have demonstrated that different nutrients (*i.e.* lipids, amino acids, vitamins and minerals) are responsible for the appearance of skeletal anomalies when their level and/or form of supply in the diet are inappropriate or unbalanced (Cahu *et al.* 2003; Lall and Lewis-McCrea 2007; Darias *et al.*, 2011b; Fernández and Gisbert, 2011; Boglione *et al.*, 2013a, b). Among them, dietary lipids have been shown to be particularly important for early development of marine finfish larvae (Watanabe, 1982; Izquierdo *et al.*, 2000, 2003; Sargent *et al.*, 1999b) because they represent the main energy source and a source of HUFA and EFA required for normal formation of new cell and tissue membranes, organ development, larval growth, morphogenesis and bone formation (Izquierdo *et al.*, 2000, 2003; Cahu *et al.*, 2003; Pousão-Ferreira *et al.*, 2003; Lall and Lewis-McCrea, 2007). However, they remain one of the least understood and enigmatic nutrients in aquaculture nutrition (Glencross, 2009). This might be due in part to the relatively complex chemistry and the varied functional roles of lipids (for review see Sargent *et al.*, 2002).

All fatty acids can serve as an energy source, but some specific long-chain polyunsaturated fatty acids (PUFA) also have a number of essential roles in metabolism and especially in bone metabolism, as demonstrated in mammals, and are highly vulnerable to lipid peroxidation. Lipid

requirements of marine fish larvae have been extensively studied during the past two decades and particular attention has been paid to PUFA and phospholipids (Cahu *et al.*, 2009; Boglione *et al.*, 2013b). In this context, several studies have highlighted the negative effects of diets deficient in EFA and phospholipids on flatfish larval growth performance, metamorphosis and survival (Dickey-Collas and Geffen, 1992; Izquierdo *et al.*, 1992; Morais *et al.*, 2004; Tzoumas, 1988; Villalta *et al.*, 2005b), incidence of skeletal deformities (Dâmaso-Rodrigues *et al.*, 2010) and pigmentary disorders (Estévez and Kanazawa, 1995; Reitan *et al.*, 1994; Villalta *et al.*, 2005a; Vizcaíno-Ochoa *et al.*, 2010). In addition, previous studies on lipid nutrition of Senegalese sole larvae have shown that this species has very specific requirements in EFA compared to other marine fish species (Morais *et al.*, 2004 and Villalta *et al.*, 2005a, b). This fact compromises the direct transfer of the existing knowledge on the impact of lipids in fish larval development and quality (i.e. European sea bass, gilthead sea bream) (Cahu *et al.*, 2003; Robin and Peron, 2004; Fountoulaki *et al.*, 2009; Atalah *et al.*, 2011a, b) and indicates that specific nutritional studies dealing with this group of nutrients are needed for Senegalese sole.

### *6.1. Function and structure of lipids*

As first constituents of cellular biomembranes, dietary lipids (mainly phospholipids) play a vital role in maintaining cell structures (*i.e.* viscosity, elasticity, resistance) and represent highly energetic metabolic substrates and provide essential nutritive elements for the organism to function. Dietary lipids also serve as carriers for absorption of other nutrients, including the fat-soluble vitamins and pigments. Lipids are components of hormones and precursors for synthesis of intra- and extra-cellular messengers, such as prostaglandins and other eicosanoids (Izquierdo *et al.*, 2000; Sargent *et al.*, 2002).

Lipids are constituted of fatty acids (FA), molecules with a long aliphatic chain from four to twenty-eight carbons, including no (saturated FA, SFA), one (monounsaturated FA, MUFA) or several (polyunsaturated FA, PUFA) double bounds. FA structure and function vary according to the degree of unsaturation (the number of double bounds) and the position of the double bound on the carbon chain. In this sense, lipids present varied structures and biosynthesis pathways, but two main categories can be identified within this group of compounds: polar lipids (*i.e.* phospholipids, including phosphoglycerids and sphingolipids) and neutral lipids (*i.e.* acylglycerols, sterols like cholesterol and wax esters). Any lipid class containing fatty acids can act as a source of energy, which is released through β-oxidation of the acyl chains producing acetyl-CoA and NADH that are further metabolized via the tricarboxylic acid cycle and oxidative phosphorylation, respectively. Triacylglycerols are the primary class for lipid storage and energy provision, but phospholipids can serve as a source of energy in fish in certain circumstances, such as embryonic and early larval development (Tocher *et al.*, 2008). The beneficial effects of dietary phospholipids on growth and survival have been demonstrated in larvae and juvenile stages of various marine and freshwater species. However, the growth-promoting effects of dietary phospholipids diminish with age and it appears to have little or no effect in

adult fish (Izquierdo and Koven, 2011). Some authors have highlighted that marine finfish larvae have more efficient capacity to utilize dietary polar lipids than neutral lipids; thus, improving their growth, survival and development (Kanazawa *et al.*, 1983a, 1983b; Koven *et al.*, 1993; Geurden *et al.*, 1997; Cahu *et al.*, 2003; Morais *et al.*, 2007). This seems to be due to the pattern of expression and regulation of the two main digestive enzymes involved in lipid digestion, lipase and phospholipase A2 (PLA2), during larval development. Lipase expression and activities were not proportionally increased with an augmentation in the neutral lipid (NL) concentration in the diet in European sea bass (Cahu *et al.*, 2003; Morais *et al.*, 2004), while PLA2 activity and expression appeared to be accurately correlated with the dietary PL content in 40 dph European sea bass fed increasing PL levels from first feeding (Cahu *et al.* 2003) and in 24 dph red drum *Scianops ocellatus* (Buchet *et al.*, 2000). This accurate regulation of PLA2 in fish larvae may explain the more effective utilization of dietary PL than NL for growth and development of marine fish larvae (Cahu *et al.*, 2009).

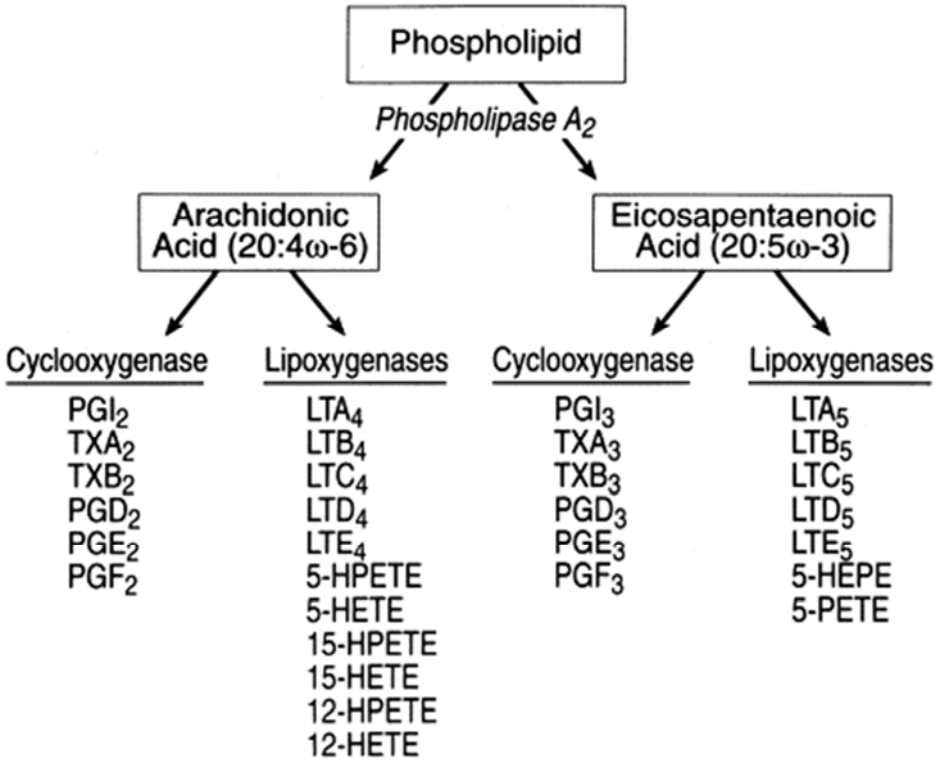


**Figure 8.** Biosynthesis of eicosanoids from ARA and EPA precursors (from Teitelbaum and Allan, 2001). PG: prostaglandin; TX: thromboxanes; LT: leukotrienes; HPETE: hydroperoxy eicosatetraenoic acids; HETE: hydroxyl eicosatetraenoic acids.

In addition, phospholipids are the source of the substrate fatty acids for the formation of eicosanoids, a range of highly bioactive derivatives of, in particular C20, highly unsaturated fatty acids (HUFA), especially arachidonic acid (ARA, 20:4n-6) and eicosapentaenoic acid (EPA, 20:5n-3) (Figure 8). Fatty acids released from membrane phospholipids by the action of phospholipase A2 are converted by either cyclooxygenase enzymes, which produces cyclic oxygenated derivatives, collectively called

prostanoids, including prostaglandins, prostacyclins and thromboxanes, or lipoxygenase enzymes, which produce linear oxygenated derivatives including hydroperoxy- and hydroxy fatty acids, leukotrienes and lipoxins. Eicosanoids are produced by many tissues in response to various extracellular stimuli and are autocrines, hormone-like compounds of short half-life that act in the immediate vicinity of their production. They are implicated in many physiological processes including blood clotting and cardiovascular tone, immune and inflammatory responses, reproduction, and renal and neural functions. The distribution and production of eicosanoids in fish species and tissues and their possible roles have been reviewed previously (Tocher *et al.*, 2008).

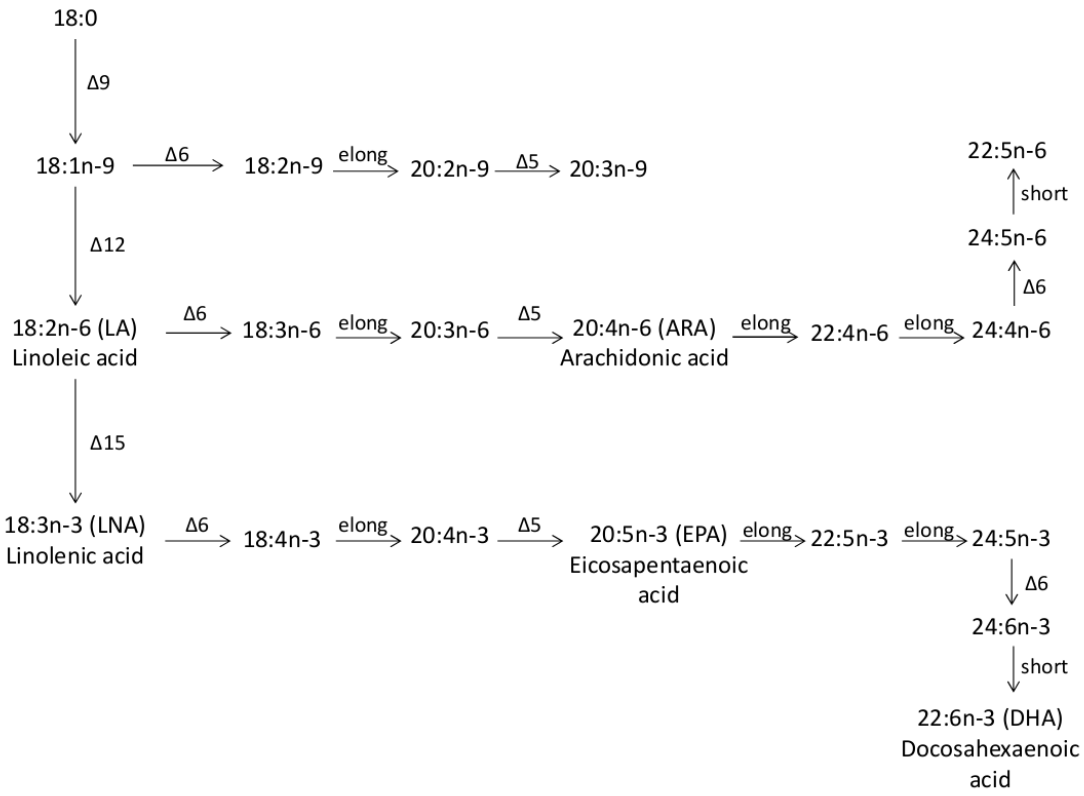


**Figure 9.** Biosynthesis pathways of essential PUFA (modified from Tocher, 2003).
Enzymes: elong: elongase; Δ5: Δ5-desaturase; Δ6: Δ6-desaturase; short: chain cortening.

Marine fish lipids are rich in a great variety of saturated and monounsaturated fatty acids, which are "de novo" synthesized, whereas polyunsaturated fatty acids must be provided in the diet (Figure 9). Three very long chain polyunsaturated fatty acids (PUFA), namely docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have a variety of very important functions in fish species, as in most vertebrates. Freshwater fish seem to have sufficient Δ5 and Δ6 desaturase and elongase activities to produce ARA, EPA and DHA if their precursors, linoleic (18:2n-6) and linolenic (18:3n-3) acids, are present in the diet, those five fatty acids being considered essential for freshwater species. However, such enzymatic activity is very restricted

in marine fish and, as a consequence, DHA, EPA and ARA have to be included in the diet and are considered essential. Besides of their high requirements in EFA from the n-3 series (EPA and DHA) (Watanabe, 1982; Kanazawa, 1985), marine fish have also slightly lower needs for the n-6 series (ARA) (Sargent *et al.*, 1999a).

### *6.2. Function of essential fatty acids*

EPA, DHA and ARA are essential PUFA (Figure 10), indispensable for physiological functions in organisms, such as the maintenance of the structure and functioning of cell biomembranes and the biosynthesis of physiologically active substances, that could not be covered by others fatty acids.

A number of studies have shown that DHA promotes growth more efficiently than EPA and ARA in marine fish larvae. The contribution of DHA to weight gain lies in its structural function in the phospholipids bilayer of the cellular membrane and its influence on membrane fluidity (Izquierdo and Koven, 2011). Additionally, particularly abundant in fish, DHA also plays an important role in larval development, especially in neural tissues, such as retina and brain. Larval eyes are particularly rich in DHA, and this fatty acid is the main one in photoreceptors, cones and bones, of larval fish retina, which hints broadly at the role of DHA in vision (Izquierdo *et al.*, 1992; Devresse *et al.*, 1994; Bell *et al.*, 1995; Kanazawa, 1995; Estévez and Kanazawa, 1995; Rainuzzo *et al.*, 1997; Sargent *et al.*, 1997, 1999b). In this sense, a number of authors have reported that increased dietary DHA or n-3 HUFAs levels improve feeding behavior in the larval stages of several marine fish species. Nevertheless, other essential fatty acids may play essential roles in neural tissues. For instance, EPA presence in live prey enhances development of the brain, whereas ARA functions as a retrograde signal to regulate the dynamic growth of the retina (Izquierdo and Koven, 2011).

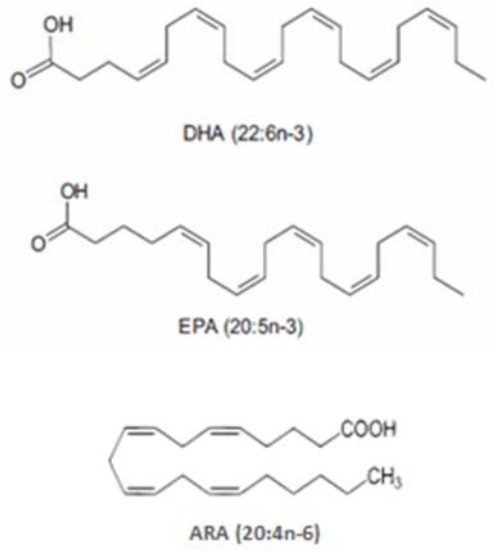


**Figure 10.** Chemical structures of DHA, EPA and ARA

Arachidonic acid is the main precursor of prostaglandins (PG), highly bioactive molecules playing a primordial role in reproduction function, immune and inflammatory responses, osmoregulation processes or in the control of stress response (Lands, 1993; Sargent *et al.*, 1999a; Koven *et al.*, 2001a; Tocher, 2003). EPA, another 20-carbon fatty acid, is also a competitive precursor with ARA for eicosanoid synthesis, since both fatty acids compete for lipoxygenase and cyclo-oxygenase enzymes that convert them into eicosanoids (Tocher, 2003). However, prostaglandins derived from EPA (series 3, $PGE_3$) are significantly less bioactive than ARA derivatives (series 2, $PGE_2$) (Sargent *et al.*, 1999a, b). Thus, ARA is preferentially used as a substrate for eicosanoids biosynthesis, but EPA, being more abundant than ARA in fish tissue, modulates eicosanoid production (Sargent *et al.*, 1999b). However, EPA can be an important source of eicosanoids in marine fish, particularly in an environment where ARA is not very abundant (Izquierdo and Koven, 2011).

Competitive metabolic interactions between n-3 and n-6 HUFA, specifically between DHA, EPA and ARA for their biosynthesis and function in the organism lead to consider relative proportions of these FA as well as their absolute amounts. The **ratio of n-3 to n-6 HUFA** plays a role as important as absolute quantities of each HUFA series, above all during larval development when high requirements are needed to build new cells and for proper bone formation and correct pigmentation. Dietary ARA is more efficiently incorporated into larval tissues than EPA, leading to EPA/ARA ratios lower in larval tissues than in diets, and probably reflecting a higher affinity of this EFA by enzymes from the 3-glycerol-3-phosphate pathways that synthesize TAG and PL (Caballero *et al.*, 2006). However, competition for the incorporation into the different lipid classes differ among tissues since fatty acid composition of each lipid class markedly differs among cellular types (Lie *et al.*, 1992). Nevertheless, the higher mitochondrial β-oxidation of EPA than ARA (Froyland *et al.*, 1997) could also be responsible for the more efficient incorporation and retention of dietary ARA. The optimum EPA/ARA ratio described in the literature as adequate for flatfish pigmentation ranged from 3.5 to 5, being different among fish species. In turbot and halibut, the optimum EPA/ARA ratios for proper pigmentation were 4 and 3.5, respectively (McEvoy *et al.*, 1998a; Hamre and Harboe, 2008). In European sea bass, Atalah *et al.* (2011b) found that, when DHA requirements were satisfied, a significant improvement in growth and survival is only observed when increased EPA and ARA maintain a ratio ranging from 3.3 to 4.0. The dietary DHA/EPA ratio also have been shown to be important for proper pigmentation, as increasing the total n-3 PUFA content from 6 to 8.8% DW and the DHA/EPA ratio from 0.6 to 1.82 in the diet significantly improved the pigmentation of turbot (Reitan *et al.*, 1994). Increased dietary EPA contents have been found to reduce dietary DHA incorporation into larval PL, and increased dietary DHA amounts notably decreased dietary EPA incorporation in phosphatidylethanolamine (Izquierdo *et al.*, 2000), suggesting that suitable DHA/EPA ratios are required for optimizing the dietary utilization of both fatty acids. The DHA/EPA ratio of marine fish eggs is generally close to 2 in several species, which has been suggested as a general value for first-feeding marine larvae (Sargent *et al.*, 1997). More detailed studies about species-specific requirements revealed that optimum dietary DHA/EPA ranged from around 1.2 for gilthead sea bream

(Rodríguez *et al.*, 1997, 1998) and 2 for red drum (Brinkmeyer and Holt, 1998), to 8 for yellowtail flounder (Copeman *et al.*, 2002). Finally, the **ratio of dietary DHA:EPA:ARA** is of high importance to evaluate the requirements in EFA of fish larvae, since modifying DHA levels alters EPA levels, as well as the relation EPA to ARA and can affect the quantity and way of interaction of eicosanoids (Tocher, 2003). Consequently, these molecular imbalances can lead to a defective osteogenesis with the emergence of skeletal deformities or a chemical stress leading to the apparition of abnormal pigmentation (Sargent *et al.*, 1999b).

### *6.3. Lipid and fatty acid requirements of Senegalese sole larvae in hatchery*

In marine environment, copepods represent a major part of natural alimentation of Senegalese sole larvae and constitute a substantial source of essential fatty acids (EFA). Rich in polar lipids, they contain high quantities of EPA and DHA, and lower but significant amounts of ARA (Tocher and Sargent, 1984; Mourente andTocher, 1992) and they meet the high nutritional requirements of developing larvae. However, in intensive production, the live preys the most commonly used for practical reasons, rotifer (*Brachionus plicatilis*) and nauplii of *Artemia sp.*, are deficient in these EFA, and it is thus necessary to enrich them with commercial emulsions, based on fish oils rich in EFA, before to supply them to fish larvae. Suboptimal levels of these EFA in larval feeds may give raise to several behavioral and morphological alterations such as reduced feeding and swimming activity, poor growth and increasing mortality, fatty livers, hydropesia, deficient swim bladder inflation, abnormal pigmentation, disintegration of gill epithelia, immune deficiency, elevated basal cortisol levels and skeletal deformities (Izquierdo and Koven, 2011). However, the process of live prey enrichment in polar lipids and essential HUFA encounter its own limits in the instable nature of the enriching solutions (autoxidation) and the own metabolism of live prey: utilization of the absorbed lipids for their own metabolism, conversion of the absorbed phospholipids in triglycerides, less energetic during storage, and retroconversion of DHA into EPA (Navarro *et al.*, 1999). Due to these processes, the FA composition of the enriching emulsion is never strictly totally similar to the FA profile in the enriched live prey supplied to fish larvae, which should be taken into consideration to meet EFA requirements of fish larvae.

Moreover, the efficiency of assimilation of HUFA and their effect on fish larval performance also depend on the dietary lipid class that they originate from, NL or PL. Marine fish larvae have higher requirements for dietary PL than NL (Cahu *et al.*, 2009) as they use dietary n−3 PUFA contained in the PL fraction more efficiently than those from the NL fraction (Salhi *et al.*, 1999; Gisbert *et al.*, 2005). The addition of PL in live-prey enrichment emulsions has resulted in improving larval growth, survival, maturation of the digestive function, stress tolerance and larval quality (Coutteau *et al.*, 1997; Izquierdo *et al.*, 2000; Sargent *et al.*, 2002; Bell and Sargent, 2003; Cahu *et al.*, 2003; Gisbert *et al.*, 2005). However, in aquaculture industry, dietary EFA are currently provided to fish larvae by fish oils, mainly composed of NL (Izquierdo *et al.*, 2000; Cahu *et al.*, 2003) and even in enriching products designed to contain higher PL levels, *Artemia* usually converts the absorbed PL in

less energetic triglycerides (NL) during storage. This is another point to take into consideration when investigating marine fish lipid requirements.

As previously outlined, requirements of Senegalese sole larvae regarding EFA have been previously demonstrated to be very specific and lower compared to other marine fish species (Morais *et al.*, 2004; Villalta *et al.*, 2005a). Regarding EPA, Senegalese sole larvae have low requirements for this FA when DHA is present in the diet and increasing amounts of dietary EPA appeared to have a detrimental effect on morphogenesis instead of enhancing it (Villalta *et al.*, 2008). Contrary to various others marine organisms, for which the range of EPA levels suitable for larval growth and survival has been estimated between 3 and 4% total fatty acids (TFA) (Léger *et al.*, 1986; Izquierdo *et al.*, 2000), Senegalese sole larvae are able to grow and survive on *Artemia* nauplii and metanauplii containing negligible EPA contents (0.4% of TFA) up to 40 dph, even showing a higher growth than when fed *Artemia* with 10.7 and 20.3% EPA of TFA (Villalta *et al.*, 2008).

Concerning DHA requirements, in spite of the high importance paid to this FA for larval development (Sargent *et al.*, 2002), Senegalese sole larvae has been shown to have low and almost negligible needs for this FA, being able to survive, grow and metamorphose on *Artemia* practically devoid of DHA, but containing other sources of n−3 PUFA (Morais *et al.*, 2004; Villalta *et al.*, 2005a; Dâmaso-Rodrigues *et al.*, 2010). Regarding the relative proportions of EPA and DHA, Morais *et al.* (2004) reported that Senegalese sole have a high dietary DHA requirement relative to EPA. While Sargent *et al.* (1997) have determined an optimal dietary DHA/EPA ratio of 2:1 for newly hatched larvae, data that was estimated from the lipid and fatty acid composition of the yolk sac of most of marine fish species, eggs from Senegalese sole contained a significantly higher DHA/EPA ratios (4.3:1), which is indicative of the species-specific nutritional requirements in this flatfish species.

The interest for the role of n-3 PUFA and the preponderance in fish tissues of DHA and EPA compared to ARA have resulted in neglecting for a long time the importance of this EFA in marine fish lipid nutrition. ARA is a ubiquitous constituent of fish tissue, albeit at low concentrations, usually around 1% of TFA. Although dietary levels of ARA have been shown to not affect growth and survival, even at high levels (8% of TFA) in various flatfish species (McEvoy *et al.*, 1998a; Estévez *et al.*, 1999; Copeman *et al.*, 2002; Willey *et al.*, 2003), including Senegalese sole (Villalta *et al.*, 2005a), increasing dietary ARA levels have been found to slow the metamorphic events during flatfish metamorphosis (Villalta *et al.*, 2005a) and to induce pigmentation anomalies of various flatfish species (McEvoy *et al.*, 1998a; Estévez *et al.*, 1999; Copeman *et al.*, 2002; Bell and Sargent, 2003; Willey *et al.*, 2003). Actually, many nutritional experiments on larvae of various flatfish species supported the idea that, beyond the absolute requirements in dietary ARA, the dietary imbalances in the relative high proportions of ARA to EPA and ARA to DHA are related to developmental and pigmentary disorders (Seikai *et al.*, 1987; Yamamoto *et al.*, 1992; Dickey-Collas and Geffen, 1992; Estévez and Kanazawa, 1995; Naess and Lie, 1998; McEvoy *et al.*, 1998b; Sargent *et al.*, 1999a, b; Estévez *et al.*, 1999; Shields *et al.*, 1999; Copeman *et al.*, 2002; Villalta *et al.*, 2005a). The imbalance in eicosanoids

production resulting from the deregulation of dietary ARA and EPA relative proportions is one of the possible causes for the apparition of pigmentary disorders (Sargent *et al.*, 1997). In available commercial products for live prey enrichment, ARA is included in low but significant amounts (around 1% TFA), but there is no evidence if this amount is sufficient for Senegalese sole larvae, nor if the relative proportion of ARA to DHA and EPA contained in these enrichers is the optimal for this flatfish species.

Due to the impact of the interaction between DHA, EPA and ARA on fish physiology, the requirements for all three HUFA have to be considered simultaneously (Sargent *et al.*, 1999a). The ideal balance between n-3 and n-6 PUFA series for marine fish larvae has been the subject of several reviews (Izquierdo, 1996; Sargent *et al.*, 1999a), highlighting evidences for higher requirements in n−3 EFA than in n−6 EFA. In vertebrate, higher dietary ratios of (n−3)/(n−6) PUFA are associated with beneficial effects on bone health (Maggio *et al.*, 2009).

However, HUFA and EFA requirements cannot meaningfully be considered without at the same time considering SFA and MUFA, as major energy yielding nutrients in fish, since the balance between energy and essentiality is crucial for proper larval development of Senegalese sole, and marine fish in general. Although the nutritional requirements of Senegalese sole have been investigated during the last years (Morais *et al.*, 2004, 2007; Villalta *et al.*, 2005a, b, 2008; Silva *et al.*, 2010; Dâmaso-Rodrigues *et al.*, 2010; Padrós *et al.*, 2011), there still exists some gaps in the knowledge of the effects of different absolute and relative quantifications of EFA such as DHA, ARA and EPA, as well as SFA and MUFA on the growth performance and developmental processes in Senegalese sole larvae, and consequently, this thesis attempted to complete the knowledge about these mechanisms.

### *6.4. Lipid peroxidation and fish defense against oxidative stress*

Although PUFA are essential for optimal growth and development in marine fish larvae, they also impose a significant peroxidation burden (Mourente *et al.*, 2000). Actually, PUFA are highly susceptible to damages by oxygen derivatives and free radicals, such as reactive oxygen species (ROS), produced during normal cellular functioning (Matés, 2000). Lipid peroxidation of these PUFA is a chain reaction that results in highly deleterious deterioration of cellular biomembranes with potential pathological effects on cells, tissues and morphogenesis (Fontagné *et al.*, 2006). Besides, marine fish tissues contain particularly high amounts of n-3 PUFA (Sargent *et al.*, 1999a, b), that make them more at risk from peroxidative damages than mammals.

Under a normal physiological status, these harmful effects of oxidative stress on cellular membrane lipids are neutralized by effective antioxidant defense systems operating in fish (Jacob, 1995). According to Halliwell and Gutteridge (2006), an antioxidant molecule is a substance that, present at low concentrations, significantly delays, prevents or removes oxidative damage of that target substrate. Antioxidant systems involve many molecules differing in the way that they interact

with oxidant components to reduce oxidative stress in organisms. Among them, “sacrificial agents” are molecules preferentially oxidized by ROS to preserve more important biomolecules, such as glutathione (GSH), NADH/NADPH, protein with sulfhydryl (-SH) groups, uric acid, among others, and dietary antioxidant micronutrients such as α-tocopherol (vitamin E, VE), ascorbic acid (vitamin C) and carotenoids. Most of them are not synthesized by animals and must be obtained from the diet (Hess, 1993). The ingestion of very low quantities of antioxidant molecules is sufficient to effectively inhibit lipid peroxidation by scavenging lipid peroxyl radical much faster than these radicals can react with adjacent fatty acid (FA) side chains or with membrane proteins (Halliwell and Gutteridge, 2006; Halver, 2002). Increasing the quantities of unsaturated oxidized fat in fish diets has been shown to increase the dietary requirements for α-tocopherol and other antioxidants in many freshwater and marine fish species (Tocher *et al.*, 2003; Puangkaew *et al.*, 2005; Peng and Gatlin, 2009; Betancor *et al.*, 2011; Lebold *et al.*, 2011, Abdel-Hameid *et al.*, 2012, among others).

Antioxidant defenses also include agents that catalytically remove ROS and thus disrupt lipid peroxidation chain reaction, such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione S-transferase (GST) among others (Halliwell and Gutteridge, 2006). CAT and SOD are scavengers of ROS eliminating hydrogen peroxide ($H_2O_2$) and superoxide anion radical ($O_2^{\cdot-}$), respectively (Miller *et al.*, 1993). GR maintains the level of reduced glutathione (GSH) by means of the reduction of glutathione disulfide (GSSG) and the oxidation of NADH and some glutathione S-transferase (GST) isoforms have the capacity to detoxify peroxidized compounds (Halliwell and Gutteridge, 2006). Numerous studies have shown an increase in the activity of these antioxidant enzymes when fish were fed oxidized diets, like in Adriatic sturgeon *Acipenser naccarii* (Trenzado *et al.*, 2006), Siberian sturgeon *A. baerii* (Fontagné *et al.*, 2006), rainbow trout (Puangkaew *et al.*, 2005; Trenzado *et al.*, 2006; Fontagné *et al.*, 2008; Zambrano and Landines, 2011), gilthead sea bream (Mourente *et al.*, 2002; Tocher *et al.*, 2003) and turbot (Tocher *et al.*, 2003). However, there is a lack of information about the potential effects of oxidized lipids in early development and metamorphosis process in Senegalese sole. This information may be of practical interest due to the high levels of PUFAs that are normally incorporated in tailor-made and commercial emulsions or enrichers used for improving the nutritional quality of live feed.

# OBJECTIVES

**The general objective of this Thesis is to analyze the effect of lipids and essential fatty acids on skeletogenesis and pigmentation processes in Senegalese sole (*Solea senegalensis*) larvae.**

This objective may be subdivided in several specific objectives as follows:

1. Evaluating the effect of essential fatty acids and oxidized lipid on larval performance and development and on the incidence of skeletal deformities and pigmentary anomalies.
2. Analyzing the gene expression of several potential molecular players involved in proper morphogenesis, skeletogenesis and pigmentation and understanding the molecular mechanisms leading to skeletal and pigmentary disorders.

In order to reach the above-mentioned specific objectives, the present research work is organized in two main sections, the first being centered on the skeletogenesis process and the second on the pigmentation process:

**<u>Section 1:</u> Effects of lipid and essential fatty acids on the skeletogenesis in Senegalese sole:**

- Evaluation of the effects of several commercial products for *Artemia* enrichment on larval performance in Senegalese sole larvae, by means of survival rate, growth, digestive system maturation, lipid deposition in target tissues, ossification, incidence of skeletal deformities and molecular regulation of these processes during the morphogenesis.
- Evaluation of the effects of dietary oxidized lipids on larval survival, growth, activity of antioxidant enzymes, intestinal lipid deposition, ossification and incidence of skeletal deformities in Senegalese sole larvae by a dose-response experimental approach.
- Evaluation of the effects of dietary arachidonic acid on larval survival, growth, ossification, incidence of skeletal malformations, eye migration process and cranial bones remodeling during the metamorphosis in Senegalese sole by a dose-response experimental approach.

**<u>Section 2:</u> Effects of lipid and essential fatty acids on the pigmentation in Senegalese sole:**

- Molecular and morphological characterization of the ontogeny of the pigmentation in Senegalese sole larvae.
- Determination of a sensitive developmental period to high dietary arachidonic acid levels for the incidence of pigmentary disorders in Senegalese sole larvae.
- Molecular and morphological characterization of the pseudo-albinos phenotype in Senegalese sole larvae.

# INFORME DEL FACTOR DE IMPACTO

El Dr. Enric Gisbert Casas y la Dra. María José Darias Cáceres, respectivamente como director y co-directora de la tesis titulada “Efecto de lípidos y ácidos grasos esenciales de la dieta en el desarrollo del esqueleto y de la pigmentación en larvas de lenguado senegalés” realizada por Anaïs Boglino, manifiestan la veracidad del factor de impacto y la implicación de la doctoranda en cada artículo científico realizado en coautoría que se presenta en esta Tesis, y que ninguno de los artículos ha sido utilizado para la elaboración de alguna otra tesis doctoral, como a continuación se expone:

*1. AUTORES/AS (p.o. de firma): Boglino A., Darias M.J., Ortiz-Delgado J.B., Özcan F., Estévez A., Andree K.B., Hontoria F., Sarasquete C., Gisbert E.*


*TITULO:* **Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae**

REF. REVISTA/LIBRO: *Aquaculture 324-325: 290–302 (2012)*

FI: 2.041 (Q1 – FISHERIES)

Anaïs Boglino colaboró en la preparación del diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado y análisis de las muestras biológicas (bioquímica, crecimiento, supervivencia, calidad larvaria, funcionalidad del sistema digestivo), interpretación de los datos y redacción final del artículo científico junto con el resto de coautores del trabajo.

*2. AUTORES/AS (p.o. de firma): Boglino A., Gisbert E., Darias M.J., Estévez A., Andree K.B., Sarasquete C., Ortiz-Delgado J.B.*


*TITULO:* **Isolipidic diets differing in their essential fatty acid profiles affect the deposition of unsaturated neutral lipids in the intestine, liver and vascular system of Senegalese sole (*Solea senegalensis* Kaup 1858) larvae and early juveniles**

REF. REVISTA/LIBRO: *Comparative Biochemistry and Physiology, Part A 162: 59–70 (2012)*

FI: 2.235 (Q1 – ZOOLOGY, Q2- PHYSIOLOGY)

Anaïs Boglino realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, interpretación de los datos y participó en la redacción final del artículo científico junto con el resto de coautores del trabajo.

*3. AUTORES/AS (p.o. de firma): Darias M.J., Boglino A., Manchado M., Ortiz-Delgado J.B., Estévez A., Andree K.B., Gisbert E.*


*TITULO:* **Molecular regulation of both dietary vitamin A and fatty acid absorption and metabolism associated with larval morphogenesis of Senegalese sole (*Solea senegalensis* Kaup 1858)**

REF. REVISTA/LIBRO: *Comparative Biochemistry and Physiology, Part A 161: 130–139 (2012)*

FI: 2.235 (Q1 – ZOOLOGY, Q2- PHYSIOLOGY)

Anaïs Boglino realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado y colaboró en el análisis de las muestras biológicas (expresión génica), interpretación de los datos y redacción final del artículo científico junto con el resto de coautores del trabajo.

*4. AUTORES/AS (p.o. de firma): Boglino A., Darias M.J., Estévez A., Andree K.B., Sarasquete C., Solé M., Gisbert E.*


*TITULO:* **The effect of dietary oxidized lipid levels on growth performance, antioxidant enzyme activities, intestinal lipid deposition and skeletogenesis in Senegalese sole (*Solea senegalensis* Kaup 1858) larvae**

REF. REVISTA/LIBRO: *Aquaculture Nutrition en revisión (2013)*

IF: 1.203 (Q1 – FISHERIES)

Anaïs Boglino colaboró en la preparación del diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado y análisis de las muestras biológicas (bioquímica, crecimiento, supervivencia, calidad larvaria, niveles de oxidación y actividad de las enzimas antioxidantes), interpretación de los datos y redacción final del artículo científico junto con el resto de coautores del trabajo.

*5. AUTORES/AS (p.o. de firma): Boglino A., Darias M.J., Estévez A., Andree K.B., Gisbert E.*


*TITULO:* **The effect of dietary ARA during the *Artemia* feeding period on larval growth and skeletogenesis in Senegalese sole (*Solea senegalensis)***

REF. REVISTA/LIBRO: *Journal of Applied Ichthyology 28: 411-418 (2012)*

IF: 0.869 (Q3 – FISHERIES)

Anaïs Boglino colaboró en la preparación del diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado y análisis de las muestras biológicas (bioquímica, crecimiento, supervivencia, calidad larvaria), interpretación de los datos y redacción final del artículo científico junto con el resto de coautores del trabajo.

*6. AUTORES/AS (p.o. de firma): Boglino A., Wishkerman A., Darias M.J., Andree K.B., De la Iglesia P., Estévez A., Gisbert E.*


*TITULO:* **High dietary ARA levels affect the process of eye migration and head shape in pseudo-albino Senegalese sole (*Solea senegalensis,* Kaup 1858) early juveniles**

REF. REVISTA/LIBRO: *Journal of Fish Biology, en prensa (2013)*

IF: 1.685 (Q2 – FISHERIES)

Anaïs Boglino elaboró el diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado y análisis de las muestras biológicas (bioquímica, crecimiento, supervivencia, calidad larvaria, fotos) y colaboró a la interpretación de los datos y participó en la redacción del artículo científico junto con el resto de coautores del trabajo.

*7. AUTORES/AS (p.o. de firma): Darias M.J., Andree K.B., Boglino A., Fernández I., Estévez A., Gisbert E.*

*TITULO:* **Coordinated regulation of chromatophore differentiation and melanogenesis during the ontogeny of skin pigmentation of Senegalese sole (*Solea senegalensis)***

REF. REVISTA/LIBRO: *PLoS ONE 8(5), e63005 (2013)*

IF: 4.09 (Q1 – BIOLOGY)

Anaïs Boglino colaboró en la preparación del diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado de las muestras biológicas (fotos) y participó en la redacción final del artículo científico junto con el resto de coautores del trabajo.

*8. AUTORES/AS (p.o. de firma): Boglino A., Wishkerman A., Darias M.J., Estévez A., De la Iglesia P., Andree K.B., Gisbert E.*

*TITULO:* **Senegalese sole (*Solea senegalensis)* metamorphic larvae are more sensitive to pseudo-albinism induced by high dietary ARA levels than post-metamorphic larvae**


REF. REVISTA/LIBRO: *British Journal of Nutrition, en revisión (2013)*

IF: 3.9 (Q2 - NUTRITION & DIETETICS)

Anaïs Boglino elaboró el diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva, muestreos, colaboró al procesado y análisis de las muestras biológicas (bioquímica, crecimiento, calidad larvaria) y participó en la interpretación de los datos y en la redacción final del artículo científico junto con el resto de coautores del trabajo.

*9. AUTORES/AS (p.o. de firma): Darias M.J., Andree K.B., Boglino A., Rotllant J., Cerda-Reverter J.M., Estévez A., Gisbert E.*

*TITULO:* **Morphological and molecular characterization of dietary-induced pseudo-albinism during post-embryonic development of Senegalese sole (*Solea senegalensis)***

REF. REVISTA/LIBRO: *PLOS ONE, en prensa (2013)*

IF: 4.09 (Q1 – BIOLOGY)

Anaïs Boglino colaboró en la preparación del diseño experimental, realizó todo el cultivo larvario de lenguado senegalés, producción y enriquecimiento de la presa viva (rotíferos y *Artemia*), muestreos, procesado de las muestras biológicas (fotos) y participó en la redacción final del artículo científico junto con el resto de coautores del trabajo.

Enric Gisbert Maria J. Darias

# SECTION 1

## Effects of lipids and essential fatty acids on the skeletogenesis in Senegalese sole

# 1

# Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae

Anaïs Boglino[1]
Maria J. Darias[1]
Juan B. Ortiz-Delgado[2]
Filiz Özcan[1]
Alicia Estévez[1]
Karl B. Andree[1]
Francisco Hontoria[3]
Carmen Sarasquete[2]
Enric Gisbert[1]

Published in Aquaculture 324-325 (2012), 290-302

[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] Instituto de Ciencias Marinas de Andalucia (CSIC), Campus Universitario Río San Pedro, Apdo Oficial, 11510 Puerto Real, Cádiz, Spain

[3] Instituto de Acuicultura de Torre de la Sal (CSIC), 12595 Torre de la Sal, Castellón, Spain

**Diferentes productos comerciales para el enriquecimiento de *Artemia* afectan al crecimiento, la maduración del sistema digestivo, la osificación y la incidencia de deformaciones esqueléticas en larvas de lenguado Senegalés (*Solea senegalensis*)**

**Resumen**

Una adecuada nutrición inicial en larvas de peces marinos es un factor importante para lograr éxito en su cultivo. No obstante, las presas vivas que sirven de alimento carecen de ciertos ácidos grasos esenciales, lo que conlleva a los productores comerciales a enriquecer sus presas vivas para proveer una dieta balanceada. Actualmente, muchos productos comerciales disponibles para enriquecer presas vivas presentan considerables diferencias en su formulación y composición de nutrientes esenciales. Asimismo, se encuentra bien especificado que los requerimientos nutricionales de cada especie varían, por lo cual, resulta necesario definir los enriquecedores más apropiados para cada especia en términos de calidad y nutrición para inducir un buen desarrollo. El objetivo del presente estudio fue evaluar seis diferentes enriquecedores en términos del crecimiento, desarrollo del sistema digestivo y la esqueletogénesis en larvas del lenguado Senegalés y determinar si algunos de estos enriquecedores promueve un mejor desarrollo durante el cultivo de larvario de la especie. Larvas de 8 a 37 días post-eclosión (dpe) fueron alimentadas con metanauplios de *Artemia* previamente enriquecidos con: Easy Selco© (INVE, ES), Easy Selco© (INVE) diluido a la mitad con aceite de oliva (ES/2), Multigain© (BioMar, MG), RedPepper© (Bernaqua, RP), Aquagrow Gold© (ABN, AGG), Aquagrow DHA (ABN, AGD), estos dos últimos diluidos a un tercio con aceite de olivo. Los resultados muestran que los tratamientos afectaron significativamente al crecimiento y el desarrollo de las larvas; las larvas alimentadas con la dieta AGG tuvieron un mayor peso seco final, mayor crecimiento y un desarrollo intestinal más avanzado al compararlas con las larvas alimentadas con la dieta ES, que fueron significativamente más pequeñas y menos desarrolladas. De igual forma, la dieta AGG promovió una metamorfosis más acelerada, lo mismo que en el proceso de osificación, en tanto que las larvas alimentadas con ES/2, RP, AGD y MG presentaron valores inferiores en estos dos parámetros. No se detectaron diferencias significativas en cuanto a la sobrevivencia final y la incidencia de deformidades esqueléticas con ninguna dieta. Se concluye que de los resultados analizados, el enriquecedor a base de AGG es el más apropiado para larvas de lenguado Senegalés.

**Palabras claves:** Lenguado senegalés, enriquecimiento, nauplios de *Artemia*, ácidos grasos, morfogénesis, esqueletogénesis.

**Abstract**

Proper nutrition at first feeding in marine fish larvae is an important factor for successful larval rearing. However, live prey used to feed marine finfish larvae lack essential fatty acids requiring commercial hatcheries to enrich live prey to provide a balanced diet. Currently, the many commercially available enrichments for live prey present considerable differences in their formulas, and compositions of essential nutrients. Since nutritional requirements are species-specific it is necessary to define those enrichments most appropriate for each species in terms of larval development and quality. This study aimed to evaluate the effect of six enrichments on Senegalese sole larval performance including growth, digestive system development and skeletogenesis, to determine whether any of these products are more suitable for Senegalese sole larval rearing. From 8 to 37 days post hatching (dph), larvae were fed *Artemia* nauplii previously enriched with six different formulae: Easy Selco© (INVE, ES), Easy Selco© (INVE) half diluted with olive oil (ES/2), Multigain© (BioMar, MG), Red Pepper© (Bernaqua, RP), Aquagrow Gold© (ABN, AGG) and Aquagrow DHA© (ABN, AGD), with the last two being diluted by a third with olive oil. The dietary treatments significantly affected larval growth and performance; larvae fed *Artemia* enriched with AGG presented significantly higher final dry weight (2.0±0.4 mg), growth and intestinal maturation than larvae fed ES-enriched *Artemia* (final dry weight: 1.1±0.3 mg). Larvae fed the AGG treatment also were those most advanced in metamorphosis and ossification processes. Larvae fed ES/2, RP, AGD and MG treatments presented intermediary values for all these parameters. No significant effect of the dietary treatments was detected in final survival and incidence of skeletal deformities. We conclude that, among the enrichments tested, AGG is the most appropriate for larvae of Senegalese sole.

**Keywords:** Senegalese sole, enrichment, *Artemia* nauplii, fatty acid, morphogenesis, skeletogenesis

**List of abbreviations**: AGD, Aquagrow DHA© treatment; AGG, Aquagrow gold© treat-ment; AP, alkaline phosphatase; ARA, arachidonic acid, 20:4n-6; DHA, docosahexaenoic acid, 22:6n-3; DPA, docosapentaenoic acid, 22:5n-3; dph, days after hatching; DW, dry weight; EA, eicosanoic acid, 20:1n-9; EFA, essential fatty acids; EPA, eicosapentaenoic acid 20:5n-3; ES, Easy Selco© treatment; ES/2 Easy Selco©, diluted by a half with olive oil treatment; FA, fatty acids; HUFA, highly unsaturated fatty acids; IEM, eye migration index; LA, linoleic acid, 18:2n-6; LAP, leucine–alanine peptidase; LNA, linolenic acid, 18:3n-3; MG, Multigain© treatment; MUFA, monounsaturated fatty acids; OA, oleic acid, 18:1n-9; PCNA, proliferating cell nuclear antigen; PUFA, polyunsaturated fatty acids; RP, Red Pepper© treatment; SD, standard deviation; S.E. M., standard error of the mean; SFA, saturated fatty acids; SGR, specific growth rate; SL, standard length; TFA, total fatty acids; VA, vitamin A.

**Introduction**

A current major bottleneck in fish farming is the natural deficiency in essential fatty acids (EFA) of live prey, such as rotifers and *Artemia* nauplii, commonly used in marine fish hatcheries to feed larvae (Conceição *et al.*, 2010). Enrichments have to be performed to at-tempt to provide live prey with the adequate nutritional value for the larvae (McEvoy *et al.*, 1998). Many commercial enrichment products are formulated to contain high amounts of essential nutrients. However, among all these products there are large differences in their physical forms (emulsions, pastes, and spray-dried powders), ingredients (fish oils, vegetal oils, single-cell algal and fungal hetero-trophic or phototrophic organisms, *e.g. Crypthecodinium cohni* or *Schizochytrium sp.*) and composition of their primary nutrients, such as lipids, fatty acids, amino acids, minerals and vitamins. Although these enriching products are currently used in most commercial hatcheries to improve the nutritional value of live prey for feeding marine fish larvae, no comparative study has been conducted up to now to determine whether these different commercial enriching products significantly affect larval performance and quality.

Senegalese sole (*Solea senegalensis* Kaup 1858) is a high-valueflat-fish, commonly reared in intensive aquaculture production systems in Spain and Portugal. Although enriched *Artemia* is commonly used in Senegalese sole larval rearing, high incidence of skeletal deformities and pigmentary disorders during larval stages were noticed (Fernández *et al.*, 2009; Gavaia *et al.*, 2002; Soares *et al.*, 2001). These alterations to their normal appearance are a cause of reduced commercial value and limit the development of the fish production sector for this species.

Among other factors, nutritional imbalances are known to play a key role in morphogenesis and skeletogenesis at early stages and several dietary components have been identified that affect correct larval development (Cahu *et al.*, 2003; Lall and Lewis-McCrea, 2007). Among them, dietary lipids have been shown to be particularly important for early development of marine finfish larvae (Sargent *et al.*, 2002), because they represent the main energy source for larvae and a source of highly unsaturated fatty acids (HUFA) and EFA needed for the new cellular structures and required for normal larval growth, morphogenesis and bone formation (Cahu *et al.*, 2003; Izquierdo *et al.*, 2000; Lall and Lewis-McCrea, 2007). Moreover, the ingestion of HUFA from the diet is the only way for marine fish to get EFA from the n-3 series, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), as well as from the n-6 series, such as arachidonic acid (ARA, 20:4n-6), since they are unable to synthesize these fatty acids from their precursors 18:3n-3 and 18:2n-6 (Sargent *et al.*, 2002). Previous studies have highlighted the negative effect on flatfish larval growth performance, metamorphosis and survival (Dickey-Collas and Geffen, 1992; Izquierdo *et al.*, 1992; Morais *et al.*, 2004; Tzoumas, 1988; Villalta *et al.*, 2005a), skeletal disorders (Dâmaso-Rodrigues *et al.*, 2010) and in the incidence of pigmentary disorders (Estevez and Kanazawa, 1995; Reitan *et al.*, 1994; Villalta *et al.*, 2005b; Vizcaíno-Ochoa *et al.*, 2010), when these EFA are not provided in sufficient amount or in adequate form in the diet. Besides, previous studies on lipid nutrition of Senegalese sole larvae have shown that this species has very specific requirements regarding EFA compared to other marine fish species (Morais *et al.*, 2004 and Villalta *et al.*, 2005a).

Among all enriching products commercially available, it is unknown which is the most appropriate for Senegalese sole larval rearing.

The objective of this study was to compare the effect of six different enriching products for live prey, commonly used in Senegalese sole hatcheries, with graded levels of n-3 polyunsaturated fatty acids (PUFA) and graded DHA/EPA ratios on larval performance and skeletogenesis. Particular focus was made on the differential dietary content of two types of nutrients important in morphogenesis: fatty acids, because they are the main source of energy during the larval period, and vitamin A (VA) which plays an important role at early stages of larval development (Cahu *et al.*, 2003; Fernández and Gisbert, 2011). Further, because of the strong interactions existing be-tween EFA (*e.g.* the competitiveness for the biosynthesis and molecular signaling pathways) this study set the focus not only on the effect of each nutrient in particular on larval performance, but in a more global and integrative approach considering the whole diet and its entire fatty acid profile. The results of this work provide practical guidelines to the industrial sector to improve both the current knowledge on Senegalese sole larval feedings, and the production of high quality animals.

**Materials and methods**

*Larval rearing and feeding protocol*

Two-day-old Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, A Coruña, Spain) and acclimated at the IRTA-SCR facilities. Larvae were reared in 18 cylindrical tanks of 60 l (initial density 80 larvae.l$^{-1}$) connected to a water recirculation unit IRTA-mar$^{TM}$. Water conditions were as follows: temperature 20.0±2.8 °C, salinity 35‰, pH 8.0±0.2, dissolved oxygen 7.5±1.3 ppm and 50% daily water renewal in the recirculation system with gentle aeration in each tank. Photoperiod was 16 L: 8D, and light intensity was 500 lx at the water surface. Larvae were fed twice a day, from 2 dph to 10 dph, with rotifers (*Brachionus plicatilis*) enriched with microalgae (*Tetraselmis suecica*), at a density of 10 rotifers.ml$^{-1}$ from 2 to 7 dph and of 5 rotifers.ml$^{-1}$ from 8 to 10 dph. Enriched *Artemia* metanauplii were supplied to larvae from 8 to 38 dph twice a day, at increasing density from 0.5 to 12 metanauplii.ml$^{-1}$ , adjusted based upon the increase of weight of the larvae and to the daily food ration calculated as described by Cañavate *et al.* (2006).

*Experimental diets and live prey enrichment*

Several commercially available enriching products were used to enrich *Artemia*: Easy Selco© (INVE, Belgium,fish oil based-emulsion), Red Pepper© (Bernaqua, Belgium, encapsulated fish oil-based emulsion), Aquagrow Gold© (Advanced Bio Nutrition, ABN, USA, *Schizochytrium sp*. based spray dried product), Aquagrow DHA© (ABN, USA, *Crypthecodinium cohnii*-based spray dried product) and Multigain© (Bio-Mar, Denmark, single cell marine organism-based spray dried product). Currently, AquagrowGold© and AquagrowDHA© products are not manufactured anymore by ABN, but the composition of *Schizochytrium sp*. based spray dried Algamac 3050© (Bio-marine Inc., Aquafauna, USA) is the closest to the one of Aquagrow Gold©. It is worth noting that the formulation and

biochemical composition of each commercial emulsion did not change from one batch to another, as should be expected, in order to maintain quality and the consumer's trust. *Artemia* was enriched with these products in order to obtain five dietary treatments with graded levels of n-3 PUFA and graded DHA/EPA ratios: Easy Selco© (ES), Red Pepper©(RP), Multigain©(MG), AquagrowGold©(AGG) and Aquagrow DHA© (AGD). Aquagrow Gold© and Aquagrow DHA© were diluted by a third with olive oil in order to adjust the increasing gradient of n-3 PUFA in enriched *Artemia*. Olive oil contains around 80% of oleic acid, so it is an appropriate solvent to dilute PUFA in saturated fatty acids (SFA). Moreover, a sixth dietary treatment containing a level of n-3 PUFA and a DHA/EPA ratio significantly was designed by enriching *Artemia* with Easy Selco© diluted by a half with olive oil and used as a negative control.

**Table 1.** Total lipid and total fatty acids contents, fatty acid composition (in % TFA) and retinoid content in *Artemia* nauplii enriched with the six enriching products (mean ± SD; n = 3). Totals include some minor components not shown. Superscripts letters denote significant differences among diets ($P < 0.05$).

| | **Dietary treatments** | | | | | |
|---|---|---|---|---|---|---|
| | **ES** | **ES/2** | **RP** | **AGG** | **AGD** | **MG** |
| Total lipids (mg·g$^{-1}$ DW) | 219.8±17.4 | 175.3±44.6 | 174.7±17.3 | 184.4±17.0 | 202.9±1.3 | 159.6±8.3 |
| Total FA (mg·g$^{-1}$ DW) | 155.9±54.8 | 112.2±20.0 | 104.9±15.6 | 124.0±43.0 | 106.3±6.0 | 116.1±24.1 |
| 14:0 | 0.7±0.1 | 0.3±0.1 | 0.7±0.5 | 0.8±0.4 | 0.6±0.4 | 0.6±0.3 |
| 16:0 | 9.9±0.4 | 8.3±0.9 | 12.2±1.2 | 10.3±1.7 | 9.5±3.6 | 10.7±0.4 |
| 18:0 | 4.2±0.2 | 4.3±0.4 | 4.7±0.8 | 4.5±0.2 | 4.2±1.5 | 3.8±0.7 |
| Total saturated | 14.9±0.3 | 13.0±1.2 | 17.8±1.2 | 15.9±2.6 | 14.6±5.4 | 15.1±0.7 |
| 16:1n-7 | 2.1±0.6 | 1.1±0.1 | 1.0±0.9 | 1.1±0.3 | 1.6±0.6 | 1.1±0.2 |
| 18:1n-9 | 33.3±2.6$^{ab}$ | 50.8±2.0$^{a}$ | 20.2±7.0$^{b}$ | 26.2±10.1$^{ab}$ | 27.7±19.0$^{ab}$ | 17.8±6.1$^{b}$ |
| 18:1n-7 | 0.0±0.0 | 0.0±0.0 | 3.6±6.2 | 4.1±7.0 | 0.0±0.0 | 3.1±5.4 |
| 20:1n-9 | 2.5±0.2$^{a}$ | 1.7±0.2$^{ab}$ | 1.2±0.1$^{b}$ | 1.1±0.2$^{b}$ | 1.5±0.7$^{ab}$ | 1.2±0.2$^{b}$ |
| Total monounsaturated | 38.8±3.7$^{ab}$ | 54.1±1.6$^{a}$ | 26.5±1.9$^{b}$ | 32.7±3.2$^{ab}$ | 30.9±17.7$^{b}$ | 23.5±2.4$^{b}$ |
| 18:2n-6 | 8.28±0.04$^{a}$ | 6.3±0.5$^{ab}$ | 7.6±0.4$^{ab}$ | 5.2±1.0$^{b}$ | 6.9±1.9$^{ab}$ | 5.5±0.5$^{ab}$ |
| 18:3n-6 | 0.56±0.03 | 0.3±0.2 | 0.4±0.2 | 0.5±0.2 | 0.6±0.5 | 0.4±0.1 |
| 20:4n-6 | 0.5±0.1$^{cd}$ | 0.3±0.1$^{cd}$ | 1.4±0.3$^{a}$ | 0.7±0.1$^{bc}$ | 0.2±0.2$^{d}$ | 1.1±0.2$^{ab}$ |
| 22:5n-6 | 0.1±0.07 | 0.0±0.0 | 2.8±2.5 | 2.2±1.9 | 0.04±0.04 | 3.7±3.2 |
| Total n-6 PUFA | 9.5±0.2 | 7.0±0.4 | 12.3±2.3 | 8.6±2.3 | 7.8±2.6 | 10.8±3.7 |
| 18:3n-3 | 18.9±1.8 | 17.4±2.3 | 20.5±3.4 | 24.1±6.1 | 25.1±5.5 | 21.4±2.1 |
| 18:4n-3 | 2.3±0.3 | 1.92±0.02 | 2.4±0.3 | 2.9±0.6 | 3.4±1.0 | 2.9±0.4 |
| 20:4n-3 | 0.4±0.6 | 0.3±0.3 | 0.5±0.4 | 0.6±0.5 | 0.4±0.4 | 0.8±0.1 |
| 20:5n-3 | 7.6±0.6$^{a}$ | 3.1±0.9$^{c}$ | 5.1±0.6$^{b}$ | 3.2±0.4$^{bc}$ | 3.7±1.2$^{bc}$ | 4.9±0.4$^{bc}$ |
| 22:5n-3 | 1.4±0.3 | 1.6±1.8 | 1.8±2.6 | 1.2±2.0 | 1.9±2.7 | 1.8±2.4 |
| 22:6n-3 | 5.5±1.5$^{cd}$ | 1.3±0.5$^{d}$ | 11.5±1.0$^{b}$ | 9.5±0.9$^{bc}$ | 11.8±2.7$^{b}$ | 16.9±2.0$^{a}$ |
| Total n-3 PUFA | 36.5±3.5$^{ab}$ | 25.7±3.2$^{b}$ | 42.9±1.6$^{a}$ | 42.5±4.6$^{a}$ | 46.4±9.5$^{a}$ | 50.4±2.3$^{a}$ |
| Total PUFA | 46.0±3.7$^{ab}$ | 32.7±2.9$^{b}$ | 55.3±2.8$^{a}$ | 51.1±5.9$^{a}$ | 54.1±12.0$^{a}$ | 61.1±2.3$^{a}$ |
| (n-3)/(n-6) | 3.9±0.3 | 3.7±0.6 | 3.6±0.7 | 5.2±1.5 | 6.2±0.9 | 5.2±2.4 |
| DHA/EPA | 0.7±0.1$^{c}$ | 0.4±0.1$^{c}$ | 2.3±0.3$^{b}$ | 3.0±0.1$^{a}$ | 3.3±0.3$^{a}$ | 3.5±0.3$^{a}$ |
| ARA/DHA | 0.1±0.0$^{bc}$ | 0.22±0.04$^{a}$ | 0.12±0.01$^{b}$ | 0.08±0.01$^{bc}$ | 0.01±0.01$^{d}$ | 0.07±0.01$^{cd}$ |
| ARA/EPA | 0.07±0.01$^{b}$ | 0.09±0.02$^{b}$ | 0.3±0.1$^{a}$ | 0.23±0.02$^{a}$ | 0.04±0.03$^{b}$ | 0.23±0.03$^{a}$ |
| OA/PUFA | 0.73±0.11$^{b}$ | 1.57±0.20$^{a}$ | 0.37±0.14$^{b}$ | 0.53±0.24$^{b}$ | 0.58±0.43$^{b}$ | 0.29±0.10$^{b}$ |
| Retinoids (µg·g DW$^{-1}$) | 24.8±9.6$^{a}$ | 13.2±7.4$^{ab}$ | 0.5±0.3$^{b}$ | 0.2±0.2$^{b}$ | 0.4±0.6$^{b}$ | 0.1±0.2$^{b}$ |

One-day-old *Artemia* nauplii (EG strain, INVE) were enriched in 100 l containers at 300 nauplii.ml$^{-1}$ for 16 h at 28 °C with 0.6 g.l$^{-1}$ of ES, AGG or AGD, 1.5 g.l$^{-1}$ of RP and 0.5 g.l$^{-1}$ of MG, following manufacturer's instructions. The biochemical analysis of lipids and fatty acid com-position of enriched *Artemia* is shown in Table 1. After enrichment, *Artemia* metanauplii were washed with UV-treated, filtered seawater and freshwater to reduce the bacterial load and remove residues of the enrichment emulsions, and kept at 4 °C in UV-treated, filtered seawater with aeration until administered to larvae. From 20 dph onwards, when larvae began to settle to the bottom of the tank, enriched *Artemia* were supplied frozen to the larvae. The effect of the different dietary treatments on Senegalese sole skeletogenesis was only evaluated during the *Artemia* feeding period to avoid introducing variability due to the different pattern of fatty acid accumulation between both types of live preys (rotifers and *Artemia*) (Giménez *et al.*, 2006).

*Lipid, fatty acid and retinoid analysis*

Enriched live prey and larvae were sampled at 2 dph (endogenous feeding period before mouth opening), 8 dph (end of the rotifer feed-ing period and start of the *Artemia* feeding phase) and 38 dph, then washed with distilled water to remove salt and bacteria, and frozen at -80 °C until analysis. Total lipids were extracted in chloroform:methanol (2:1, v:v) using the method of Folch *et al.* (1957) and quantified gravimetrically after evaporation of the solvent under a nitro-gen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1, 20 mg.ml$^{-1}$) containing 0.01% butylated hydroxytoluene (BHT) at-20 °C prior to analysis.

Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane:diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo ElectronTraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m×0.25 mm id; SGE, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C.min$^{-1}$ and holding at 250 °C after ramping at 2 °C.min$^{-1}$ helium (1.2 ml.min$^{-1}$ constant flow rate) as the carrier gas and on-column injection and flame ionization detection at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chromcard for Windows (TraceGC, Thermo Finnigan, Italy). Results of fatty acid content are expressed as a percentage of total fatty acids (TFA).

Retinoids in enriching emulsions and live prey were analyzed by HPLC using a modification of the method proposed by Takeuchi *et al.* (1998). Extracted lipids were evaporated and redissolved on methanol:acetone (1:1 v:v) prior to their HPLC analysis. The HPLC system (Thermo Separation Products, San Jose, CA, USA) was equipped with a Lichrospher C-18 reverse phase column (Merck, Darmstadt, Germany) and a UV–visible detector set at a wavelength of 325 nm. The con-centration of each retinoid was calculated from the calibration curves constructed with the peak area ratios of their

external standards and an internal standard of retinol acetate added to the samples. All the reference retinoids were purchased from Sigma-Aldrich (Spain).

*Larval performance*

*Growth and survival*

Standard length (SL) and dry weight (DW) of Senegalese sole larvae were measured at 2, 4, 8, 15, 22, 31 and 38 dph. Thirty larvae from each tank were randomly sampled and euthanized with an overdose of tricaine methane sulphonate (MS-222, Sigma). SL was measured with a digital camera connected to a microscope (Nikon SMZ 800) and an image analysis system (AnalySIS, Soft Imaging Systems, GmbH). DW determination was performed by rinsing larvae with dis-tilled water to remove salt and then drying them at 60 °C for 24 h. Samples were weighed with an analytic microbalance (Sartorius BP211D). Specific growth rate (SGR, in $\%d^{-1}$) was calculated as $(\ln DW_f - \ln DW_i)/(t_f - t_i)*100$; where $DW_f$, $DW_i$, $t_f$ and $t_i$ represented final and initial DW and time of the experiment, respectively. Final survival was evaluated by counting the animals surviving at the end of the experiment and calculated according to Buckley *et al.* (1984), which considers the number of sampled individuals during the experiment.

*Metamorphosis*

Eye migration in Senegalese sole larvae, used as a measure of the progress of the metamorphosis process, was assessed for 30 individuals per tank at 10, 15, 22, 31 and 38 dph, according to Villalta *et al.* (2005b). Data are presented as the relative amount of larvae in each stage of development at the same age. Eye migration index ($I_{EM}=\Sigma$ (%fish in each stage $*$ stage)/100) was calculated according to Solbakken *et al.* (1999).

*Organization and functionality of the digestive tract*

*Digestive enzyme activity*

The specific enzyme activity of one intestinal cytosolic enzyme (leucine-alanine peptidase: leu-ala peptidase), two intestinal brush border enzymes (alkaline phosphatase and N-aminopeptidase) and three pancreatic enzymes (amylase, lipase and trypsin) were used to assess the degree of development and maturation in the digestive system of Senegalese sole larvae fed the six dietary treatments. Enzyme activity was measured at 38 dph on 30 individuals per tank and each sample was assayed in triplicate.

Sampled fish were washed with distilled water and stored at -80 °C until enzyme activity analysis. Fish heads at 38 dph were re-moved to reduce the protein content. Samples were homogenized (Ultra-Turrax D25 basic, IKA©-Werke) in thirty volumes (v/w) of Tris–Mannitol (50 mM, HCl 2 mM, pH = 7.5) and CaCl2 (0.1 M) for 5 min and subjected to sonication (Vibra-Cell©, Sonics) for 1.5 min, maintaining them in ice to restrict enzymatic activity. An aliquot was taken for pancreatic enzyme quantification and stored at -80 °C. Intestinal brush border membranes for determination of intestinal enzymes were purified according to Crane *et al.* (1979). The remaining fraction was

centrifuged at 9000×g (10 min) at 4 °C, and then the supernatant was collected and centrifuged at 14,000×g (30 min) at 4 °C. The pellet containing the intestinal brush border was recovered, diluted in a KCl, DTT, Hepes buffer (pH = 7.5) and kept at -80 °C.

Trypsin (E.C. 3.4.21.4) activity was assayed at 25 °C and 407 nm, using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide, Sigma) as substrate (Holm *et al.*, 1988). Amylase (E.C. 3.2.1.1) activity was measured at 580 nm, using soluble starch (0.3%) dissolved en $Na_2HPO_4$ buffer (pH = 7.4) as substrate (Métais and Bieth, 1968). Alkaline phosphatase (E.C.3.1.3.1) was quantified at 37 °C and 407 nm, using PNPP (4-nitrophenyl phosphate, Sigma) as substrate (Bessey *et al.*, 1946). N-aminopeptidase (E.C.3.4.11.2) activity was determined at 25 °C and 410 nm, according to Maroux *et al.* (1973) using sodium phosphate buffer 80 mM (pH = 7.0) and L-leucine p-nitroanilide as substrate (Sigma, in 0.1 mM DMSO). Leu-ala peptidase (E.C.3.4.11) activity was quantified at 37 °C and 530 nm, using leu-ala (Sigma) as substrate (Maroux *et al.*, 1973). Lipase (E.C.3.1.1) activity was measured at 30 °C and 405 nm, using p-nitrophenyl myristate (Sigma) as substrate (Iijima *et al.*, 1998). Enzymatic activities were read using a spectrophotometer (Synergy HT, Bio-Tech) and expressed as specific enzyme activity, in units per milligram of protein ($U.mg^{-1}$ protein). Soluble protein of crude enzyme extracts was quantified by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

*Enterocyte height and cell proliferation capacity in the intestinal mucosa.*

For morphometric and cell proliferation capacity studies, ten larvae from each experimental tank at 38 dph were randomly sampled, euthanized with an overdose of MS-222 and fixed in 4% formalin buffered to pH = 7.0 with 0.1 M phosphate buffer during 24 h. Samples were routinely dehydrated, embedded in paraffin wax, sectioned using a rotary microtome and placed on microscope slides precoated with poly-L-Lysine (Sigma-Aldrich).

The height of the mucosal epithelial cells, defined as the distance between the basal lamina and the extremity of the brush border was measured for 10 enterocytes located in the mucosal folds of the intestine in three larvae per treatment.

To detect the proliferation capacity of the intestinal mucosa, sections were stained immune-histochemically using a monoclonal anti-body (PC10, Santa Cruz Biotechnology Inc., Heidelberg, Germany) against proliferating cell nuclear antigen (anti-PCNA), following the method of Piñuela *et al.* (2004). Mounted tissues were dewaxed, hydrated in ddH2O and then placed in 3% triton in phosphate buffer saline (PBS-T). Endogenous peroxidase activity was blocked by incubation in a solution of 3% H2O2in methanol. Background was reduced by incubation in a solution of 3% BSA in triton/PBS with 1% di-methyl sulfoxide (DMSO) for 30 min at RT. Sections were incubated with anti-PCNA IgG2a mouse monoclonal antibody (PC10, Santa Cruz Biotechnology) 1:500 overnight in a humid chamber at RT. Samples were then incubated with a secondary antibody (biotinylated anti-mouse IgG from horse, Vector antibodies) for 1 h at RT. Before rinsing in PBS-T during 10 min, the slides were incubated with horse-radish peroxidase-conjugated avidin-biotin complex (ABC complex; Vectastain, Vector Laboratories Inc., USA) for 1 h at RT. The colorimetric reaction was developed by exposing the

slides to 0.04% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.015% $H_2O_2$ in Trizma base (TB, 100 mM, pH=7.6) for about 10 min. Sections were then dehydrated, mounted and counterstained with eosin.

Intestinal cell proliferation was calculated following the quantification criteria described by Sanden *et al.* (2005). For that purpose, five intestinal folds were examined randomly in two sections per fish, in three larvae from each dietary group. For each intestinal fold measured, the thickness of 10 cells was selected above the basal area and positive PCNA and negative PCNA cells were counted. Cell proliferation was expressed as the percentage of PCNA positive cells/non-PCNA cells.

Measurement of enterocyte height and proliferation capacity was realized by using a digital camera connected to a microscope (Leica DM 2000, Leica Microsistemas S.L.U, Barcelona, Spain) and an image analysis system (Image J, National Institute of Health, Bethesda, USA).

*Ossification degree and skeletal deformities*

To evaluate the impact of different dietary treatments on the degree of ossification of the larval skeleton, identify different typologies of skeletal deformities, and quantify their incidence in larvae, 40 larvae per tank were randomly sampled at the end of the experiment, fixed in 4% formalin buffered to pH = 7.0 with 0.1 M phosphate buffer and stored until double staining. Animals were stained with alcian blue and alizarin red to detect cartilaginous and bony tissues, respectively (Darias *et al.*, 2010a). Quantification of the degree of ossification was performed in 38 dph Senegalese sole larvae according to Darias *et al.* (2010a). Briefly, stained fish were placed on their blind (left) side and scanned using a digital scanner (HP Scanjet G3010) to create 1200 dpi images for subsequent analysis. A computerized image analysis package (IMAQ Vision Builder©, National Instruments) was used to determine the number of red pixels of each stained larvae which corresponded to the ossified structures.

Skeletal structures were identified and named according to Wagemans and Vandewalle (2001) and Gavaia *et al.* (2002). The incidence of skeletal abnormalities was determined in the cranium, vertebral column and caudal fin complex. Special attention was given to vertebral deformities, which were divided in two categories: severe (fusion, compression, deformed vertebral centrums, torsion and scoliosis) and light (haemal spines and neural spines) deformities and caudal fin complex deformities (modified haemal and neural spines). Fusion of the hypurals, parahypural and/or epural was not considered as a skeletal deformity, but rather the normal development of the caudal complex occurring in juveniles (Barrington, 1937).

*Statistics*

Results were expressed as means ± SD (n = 3). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). A nested-ANOVA was performed with "treatment" as the fixed-effects factor nested within the random-effects factor "tank" (triplicates per treatment for final SL, final DW and ossification variables measured on 30–40 larvae per replica) or "larvae"(triplicates per treatment for enterocyte height or PCNA variables, measured on 10 cells or 10

intestinal folds per larvae) (Zar, 1974). Post-hoc Bonferroni's test was performed when significant differences were found at Pb0.05. However, for variables such as FA composition, SGR, survival, $I_{em}$, enzymatic activities and incidence of skeletal deformities, measurements were performed on a pool of in-dividuals per replicate but with just one value of these variables per replicate, a nested ANOVA cannot be carried out. For these variables, a one-way ANOVA was performed among treatments and the post-hoc Tukey's test was performed when significant differences were found (Pb0.05). A correlation test of Pearson was performed between alkaline phosphatase specific activity and cell proliferation data. All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA).

**Results**

*Lipid, fatty acid composition and retinoid content of live prey and fish larvae*

*Live prey*

No significant differences were detected in the total lipid and total fatty acid contents among the *Artemia* nauplii enriched with the different commercial enrichments (Table 1, $P < 0.05$).*Artemia* enriched with ES/2 exhibited significantly higher total monounsaturated fatty acids (MUFA) levels than *Artemia* groups enriched with AGD, RP or MG. Significant differences were found in the proportions of 18:1n-9 (oleic acid, OA) and 20:1n-9 (eicosanoic acid, EA) be-tween dietary treatments. *Artemia* enriched with ES/2 presented significantly higher amounts of OA than those enriched with RP or MG. *Artemia* enriched with ES showed higher levels of EA than groups enriched with AGG, RP or MG. In relation to n-6 PUFA, significantly different concentrations among dietary treatments were noticed for 18:2n-6 (linoleic acid, LA). Incorporation of LA was higher in the ES group and lower in the AGG group. For 20:4n-6 (ARA), the higher incorporation was observed in the RP group and the lower in the ES, ES/2 and AGD groups; with AGG having intermediate values. Concerning the n-3 PUFA, only EPA and DHA contents were significantly different between enrichment treatments. In the case of EPA, *Artemia* enriched with ES showed the highest content, those enriched with RP, MG, AGD and AGG intermediate values and ES/2 the lowest. Regarding DHA, *Artemia* enriched with MG exhibited the highest content and ES/2 enrichment the lowest. The rest of the treatment groups presented intermediate DHA values. In terms of total PUFA content, *Artemia* enriched with ES showed an intermediate value between the lowest (the ES/2 group) and the highest (the MG, RP, AGD, and AGG groups).*Artemia* enriched with MG, AGD, RP and AGG presented significantly higher amounts of total n-3 PUFA than ES/2 but total n--6 PUFA were similar in all dietary groups, and the n-3/n-6 ratio was not significantly different among the nauplii. *Artemia* enriched with MG, AGG and AGD showed the highest DHA/EPA ratios followed by the RP group, while the lowest DHA/EPA ratios were observed in *Artemia* enriched with ES and ES/2.*Artemia*enriched with ES/2 presented a significantly higher ARA/DHA ratio as compared with those enriched with RP, ES, AGG, MG and AGD. *Artemia* enriched with RP, AGG and MG showed significantly higher ARA/EPA ratios than those enriched with ES/2, ES

and AGD. *Artemia* enriched with ES showed the highest retinoid content followed by ES/2 and the rest of the dietary treatments.

**Table 2.** Total lipid and total fatty acids contents (in mg.g$^{-1}$ DW), fatty acid composition (in % TFA) and retinoid content (in µg·g$^{-1}$ DW) in newly hatched Senegalese sole larvae at 2 dph and 38 dph fed *Artemia* enriched with the six commercial enrichments (mean ± SD, n = 3). Totals include some minor components not shown. Superscripts denote significant differences among diets ($P < 0.05$).

| | | | Dietary treatments (38 dph) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | (2dph) | (8dph) | ES | ES/2 | RP | AGG | AGD | MG |
| Total lipids | 43.4±8.4 | 49.9±10.1 | 67.3±17.0 | 88.3±11.3 | 79.0±8.6 | 97.9±5.8 | 93.4±11.1 | 86.6±7.5 |
| Total FA | 26.2±7.8 | 25.7±7.3 | 34.7±10.0 | 53.5±10.3 | 36.9±9.6 | 41.6±3.0 | 49.0±9.7 | 41.9±2.3 |
| 14:0 | 1.0±0.5 | 0.5±0.1 | 0.4±0.1$^{bc}$ | 0.3±0.0$^{c}$ | 0.6±0.1$^{ab}$ | 0.6±0.1$^{a}$ | 0.5±0.0$^{ab}$ | 0.6±0.0$^{a}$ |
| 16:0 | 20.0±2.6 | 12.8±1.5 | 10.4±1.1$^{bc}$ | 8.6±0.1$^{c}$ | 12.8±1.1$^{a}$ | 11.4±0.4$^{ab}$ | 10.0±1.1$^{bc}$ | 11.5±0.6$^{ab}$ |
| 18:0 | 7.3±0.4 | 10.2±2.0 | 5.8±0.5$^{bc}$ | 4.7±0.4$^{d}$ | 7.3±0.4$^{a}$ | 6.4±0.2$^{ab}$ | 5.1±0.4$^{cd}$ | 6.2±0.1$^{b}$ |
| Total saturated | 29.1±3.4 | 23.6±3.5 | 16.9±1.7$^{bc}$ | 13.6±0.3$^{c}$ | 20.7±1.6$^{a}$ | 18.5±0.6$^{ab}$ | 16.1±2.0$^{bc}$ | 18.4±0.5$^{ab}$ |
| 16:1n-7 | 3.7±0.9 | 1.0±0.1 | 1.0±0.1 | 1.3±0.8 | 0.7±0.0 | 0.7±0.2 | 0.7±0.1 | 0.6±0.1 |
| 18:1n-9 | 12.9±5.8 | 23.9±3.9 | 32.1±4.9$^{bc}$ | 42.4±4.9$^{a}$ | 24.5±0.7$^{cd}$ | 30.3±1.2$^{bc}$ | 34.8±1.2$^{ab}$ | 21.5±1.6$^{d}$ |
| 20:1n-9 | 1.1±1.1 | 2.6±0.3 | 2.8±0.3$^{a}$ | 2.7±0.3$^{a}$ | 1.8±0.4$^{ab}$ | 2.0±0.1$^{ab}$ | 2.1±0.3$^{ab}$ | 1.6±0.7$^{b}$ |
| Total | 21.3±0.8 | 27.5±4.0 | 35.9±5.1$^{b}$ | 46.4±4.3$^{a}$ | 27.1±0.6$^{cd}$ | 33.0±1.2$^{bc}$ | 37.6±1.5$^{b}$ | 23.7±1.5$^{d}$ |
| 18:2n-6 | 1.9±1.4 | 5.2±0.5 | 7.9±1.0$^{ab}$ | 9.2±0.1$^{a}$ | 7.9±0.4$^{ab}$ | 5.8±0.8$^{c}$ | 6.8±0.4$^{bc}$ | 5.4±0.4$^{c}$ |
| 18:3n-6 | 0.17±0.03 | 0.3±0.1 | 0.6±0.1$^{a}$ | 0.4±0.0$^{b}$ | 0.5±0.1$^{a}$ | 0.6±0.0$^{a}$ | 0.6±0.1$^{a}$ | 0.5±0.0$^{ab}$ |
| 20:4n-6 | 2.9±0.1 | 2.3±1.3 | 1.1±0.2$^{c}$ | 0.9±0.2$^{cd}$ | 3.3±0.1$^{a}$ | 2.1±0.1$^{b}$ | 0.5±0.1$^{d}$ | 3.0±0.1$^{a}$ |
| 22:5n-6 | 0.66±0.03 | 0.9±0.5 | 0.3±0.2$^{c}$ | 0.1±0.1$^{c}$ | 4.6±0.6$^{b}$ | 3.8±0.1$^{b}$ | 0.1±0.2$^{c}$ | 5.6±0.4$^{a}$ |
| Total n-6 PUFA | 6.2±1.4 | 9.2±2.0 | 10.2±1.3$^{cd}$ | 10.8±0.3$^{bc}$ | 16.6±1.0$^{a}$ | 12.5±0.6$^{b}$ | 8.1±0.7$^{d}$ | 14.9±0.6$^{a}$ |
| 18:3n-3 | 0.4±0.1 | 9.7±5.0 | 14.3±1.6 | 15.5±1.1 | 12.6±1.2 | 15.5±0.8 | 15.4±1.1 | 14.5±0.6 |
| 18:4n-3 | 0.15±0.02 | 1.1±0.7 | 1.6±0.3$^{ab}$ | 1.3±0.2$^{ab}$ | 1.1±0.2$^{b}$ | 1.7±0.2$^{ab}$ | 1.8±0.1$^{a}$ | 1.6±0.2$^{ab}$ |
| 20:4n-3 | 0.23±0.05 | 1.0±0.1 | 0.8±0.1$^{a}$ | 0.7±0.1$^{ab}$ | 0.5±0.1$^{b}$ | 0.8±0.1$^{a}$ | 0.7±0.0$^{ab}$ | 0.7±0.0$^{ab}$ |
| 20:5n-3 | 4.4±0.7 | 3.9±0.7 | 5.1±0.7$^{a}$ | 3.4±0.5$^{b}$ | 3.0±0.2$^{b}$ | 2.3±0.2$^{b}$ | 2.6±0.4$^{b}$ | 3.2±0.3$^{b}$ |
| 22:5n-3 | 4.1±0.1 | 2.0±0.3 | 4.0±0.7$^{a}$ | 2.1±0.2$^{b}$ | 2.0±0.4$^{b}$ | 1.7±0.3$^{b}$ | 1.5±0.1$^{b}$ | 1.9±0.1$^{b}$ |
| 22:6n-3 | 28.6±6.3 | 19.8±4.1 | 10.4±1.9$^{c}$ | 5.6±1.4$^{d}$ | 15.3±0.7$^{b}$ | 12.9±1.5$^{bc}$ | 15.1±1.7$^{b}$ | 20.0±1.6$^{a}$ |
| Total n-3 PUFA | 38.1±7.2 | 37.6±4.3 | 36.2±4.8$^{ab}$ | 28.6±3.5$^{b}$ | 34.6±2.1$^{ab}$ | 34.9±2.2$^{ab}$ | 37.2±3.1$^{ab}$ | 42.0±2.1$^{a}$ |
| Total PUFA | 44.2±8.6 | 46.8±5.3 | 46.4±6.1$^{b}$ | 39.4±3.8$^{b}$ | 51.3±2.3$^{ab}$ | 47.5±1.6$^{ab}$ | 45.2±3.7$^{b}$ | 56.9±2.0$^{a}$ |
| (n-3)/(n-6) | 6.2±0.3 | 4.3±1.1 | 3.6±0.0$^{b}$ | 2.7±0.3$^{cd}$ | 2.1±0.2$^{d}$ | 2.8±0.3$^{c}$ | 4.6±0.3$^{a}$ | 2.8±0.2$^{c}$ |
| DHA/EPA | 6.5±0.4 | 5.2±1.6 | 2.0±0.1$^{c}$ | 1.7±0.2$^{c}$ | 5.1±0.4$^{b}$ | 5.6±0.2$^{ab}$ | 5.8±0.6$^{ab}$ | 6.2±0.5$^{a}$ |
| ARA/DHA | 0.1±0.0 | 0.1±0.1 | 0.1±0.0$^{c}$ | 0.2±0.0$^{b}$ | 0.2±0.0$^{a}$ | 0.2±0.0$^{b}$ | 0.03±0.0$^{d}$ | 0.2±0.0$^{b}$ |
| ARA/EPA | 0.7±0.1 | 0.6±0.4 | 0.2±0.0$^{c}$ | 0.3±0.0$^{c}$ | 1.1±0.1$^{a}$ | 0.9±0.1$^{b}$ | 0.2±0.0$^{c}$ | 0.9±0.1$^{b}$ |
| OA/PUFA | 0.3±0.1 | 0.5±0.1 | 0.7±0.2$^{b}$ | 1.1±0.2$^{a}$ | 0.5±0.0$^{b}$ | 0.6±0.1$^{b}$ | 0.8±0.1$^{ab}$ | 0.4±0.0$^{b}$ |
| Retinoids | | | 3.2±0.5$^{b}$ | 4.6±0.4$^{ab}$ | 6.3±1.1$^{a}$ | 4.1±1.1$^{ab}$ | 4.4±1.6$^{ab}$ | 6.5±0.9$^{a}$ |

*Fish larvae*

In order of relative abundance, 2 dph-old larvae contained particularly high proportions of DHA, 16:0, OA, 18:0, EPA and 22:5n-3 (docosapentaenoic acid, DPA) (Table 2), resulting in high n-3/n-6 and DHA/EPA ratios. At the end of the rotifer feeding period, 8 dph-old larvae contained equivalent levels of total lipids, total FA, total SFA and PUFA and equivalent EFA ratios as 2 dph-old larvae, but lower levels of 14:0, 16:0, 16:1n-7 and higher amounts of OA, EA, LA, LNA and 20:4n-3. At 38 dph, the fatty acid composition of the larvae reflected the composition of the diets (Tables 1 and 2). Significantly higher levels of MUFA, particularly OA and EA were identified in larvae fed *Artemia* enriched with ES/2. Larvae from the RP and MG dietary treatments contained significantly more n-6 PUFA than the rest of the groups, principally LA, 18:3n-6 (Gamma Linoleic Acid, GLA) and ARA for larvae from the RP group, and GLA, ARAand22:5n-6 for larvae of the MG group. Larvae from the ES/2 and ES groups also contained high LA levels. Larvae from the AGD, ES/2 and ES groups exhibited particularly significant low levels of ARA. Larvae from the MG group contained significantly higher amounts of total n-3 PUFA and DHA than the other groups; whereas EPA and DPA were significantly more abundant in larvae fed *Artemia* enriched with ES. Reflecting the *Artemia* profile, total PUFA amounts were higher in larvae from the MG group; intermediate in larvae from RP and AGG groups; and lower in the AGD, ES and ES/2 groups. The higher n-3/n-6 PUFA ratio was noted in larvae fed *Artemia* enriched with AGD, followed by ES, AGG, MG, ES/2 and RP. The higher DHA/EPA ratio was obtained from larvae of the MG group (6.2 ± 0.5), and those from the ES and ES/2 groups the lowest, with the AGD, AGG and RP larvae showing intermediate values. Values of ARA/DHA and ARA/EPA ratios followed the same de-creasing trend among larvae from the RP, AGG, MG, ES/2, ES and AGD groups. Larvae fed *Artemia* enriched with MG and RP contained significantly higher amounts of retinoid, while the ES group had lower values, and ES/2, AGD and AGG intermediate values in retinoid content.

*Larval performance*

*Larval growth and survival*

Senegalese sole larvae from the six dietary treatments did not show significant differences in SL and DW until 22 dph (metamorphosis period) (Table 3), the age at which larvae fed AGG-enriched *Artemia* appeared to be significantly longer in SL (except from larvae fed ES/2-enriched *Artemia*) and heavier in DW than those of the other groups (data not shown, $P = 0.007$ and $P = 0.004$, respectively). At the end of the experiment, larvae fed AGG-enriched *Artemia* presented significantly higher SL ($F_{10, 578}=6.90$; $P \leq 0.001$), DW ($F_{10, 134} = 6.79$; $P \leq 0.001$) and SGR values ($P = 0.022$) during the whole larval rearing period than larvae fed ES-enriched *Artemia*, with the other groups showing intermediate values (Table 3). The SGR of larvae fed AGG-enriched *Artemia* was significantly higher than the others particularly during pre-metamorphic and metamorphic stages (2–22 dph, $P = 0.006$). Senegalese sole larvae final survival was not affected by any diet (Table 3, $P = 0.328$), reaching a mean of 95.7 ± 1.0%.

**Table 3.** Final larval size in standard length (SL, in mm) and dry weight (DW, in mg), specific growth rate (SGR, in %.$d^{-1}$) during the whole period of rearing (2–38), the pre-metamorphic and metamorphic periods (2–22) and the post-metamorphic period (22–38), and survival rate (in %) of Senegalese sole larvae fed *Artemia* nauplii enriched the different commercial products. Initial standard length and dry weight of larvae were 2.90 ± 0.28 mm and 16.5 ± 0.0µg, respectively. Values are expressed as mean ± SD (n = 3). Different letters within the same column show significant differences ($P < 0.05$).

| | Final SL | Final DW | SGR (2-38) | SGR (2-22) | SGR (22-38) | Survival rate |
|---|---|---|---|---|---|---|
| **ES** | 7,21±1,14$^{e}$ | 1,07±0,25$^{c}$ | 0,115±0,007$^{b}$ | 0,191±0,004$^{b}$ | 0,044±0,010$^{b}$ | 94,1±2,3 |
| **ES/2** | 7,76±1,17$^{cd}$ | 1,33±0,26$^{c}$ | 0,122±0,002$^{ab}$ | 0,196±0,004$^{ab}$ | 0,054±0,001$^{ab}$ | 96,3±0,2 |
| **RP** | 7,59±1,39$^{de}$ | 1,25±0,41$^{c}$ | 0,119±0,008$^{ab}$ | 0,191±0,004$^{b}$ | 0,054±0,015$^{ab}$ | 96,4±0,4 |
| **AGG** | 8,93±1,29$^{a}$ | 2,01±0,42$^{a}$ | 0,133±0,004$^{a}$ | 0,203±0,004$^{a}$ | 0,072±0,013$^{ab}$ | 94,8±2,6 |
| **AGD** | 8,42±1,47$^{ab}$ | 1,68±0,45$^{b}$ | 0,129±0,004$^{ab}$ | 0,189±0,004$^{b}$ | 0,077±0,005$^{a}$ | 96,2±0,4 |
| **MG** | 8,20±1,41$^{bc}$ | 1,63±0,47$^{b}$ | 0,127±0,006$^{ab}$ | 0,189±0,002$^{b}$ | 0,073±0,013$^{ab}$ | 96,2±0,8 |

*Metamorphosis*

The process of eye migration in Senegalese sole larvae was not significantly affected by any dietary treatment (Table 4). During the metamorphosis process, no differences in $I_{EM}$ were detected between the six experimental groups ($P > 0.05$). However, only in the AGG group was the eye migration process completed (stage 5) at the end of the experiment. No malpigmented fish were recorded at the end of the experiment in any of the six groups.

**Table 4.** Eye migration index ($I_{EM}$) of Senegalese sole larvae fed *Artemia* nauplii enriched the different commercial products at 10, 15, 22, 31 and 38 dph. Values are expressed as mean ± SD (n = 3). No significant differences ($P > 0.05$) were detected among dietary treatments for any sampled day.

| | Age (days post hatching) | | | | |
|---|---|---|---|---|---|
| | **10** | **15** | **22** | **31** | **38** |
| **ES** | 0,96±0,10 | 1,25±0,23 | 2,82±0,23 | 4,17±0,03 | 4,77±0,29 |
| **ES/2** | 0,97±0,08 | 1,25±0,14 | 2,93±0,07 | 4,21±0,54 | 4,84±0,14 |
| **RP** | 0,93±0,09 | 1,50±0,17 | 2,99±0,45 | 4,17±0,62 | 4,78±0,16 |
| **AGG** | 0,96±0,07 | 1,29±0,13 | 3,13±0,10 | 4,26±0,49 | 5,00±0,00 |
| **AGD** | 0,93±0,03 | 1,12±0,16 | 2,99±0,23 | 4,50±0,30 | 4,95±0,07 |
| **MG** | 0,98±0,04 | 1,36±0,08 | 2,77±0,06 | 4,04±0,57 | 4,90±0,07 |

*Organization and functionality of the digestive tract*

*Digestive enzyme activity.*

The specific activities of selected intestinal and pancreatic enzymes from fish fed the six different diets at 38 dph are represented in Figure 1. Intestinal maturation in Senegalese sole larvae was measured as the ratio of specific activities of brush border membrane enzymes/cytosolic enzymes: alkaline

phosphatase/leu-ala peptidase and N-aminopeptidase/leu-ala peptidase. These ratios were both significantly higher ($P$ = 0.001 and $P$ = 0.006) in larvae fed AGG-enriched *Artemia* (0.96 ± 0.04 and 17.01 ± 1.90 respectively), intermediate in larvae fed *Artemia* enriched with AGD (0.50 ± 0.12 and 10.83 ± 0.96) and lower in larvae fed MG (0.49 ± 0.15 and 8.51 ± 3.32), RP (0.34 ± 0.11 and 8.52 ± 3.46), ES (0.25 ± 0.01 and 8.09 ± 1.66) and ES/2-enriched *Artemia* (0.09 ± 0.01 and 6.04 ± 3.78).

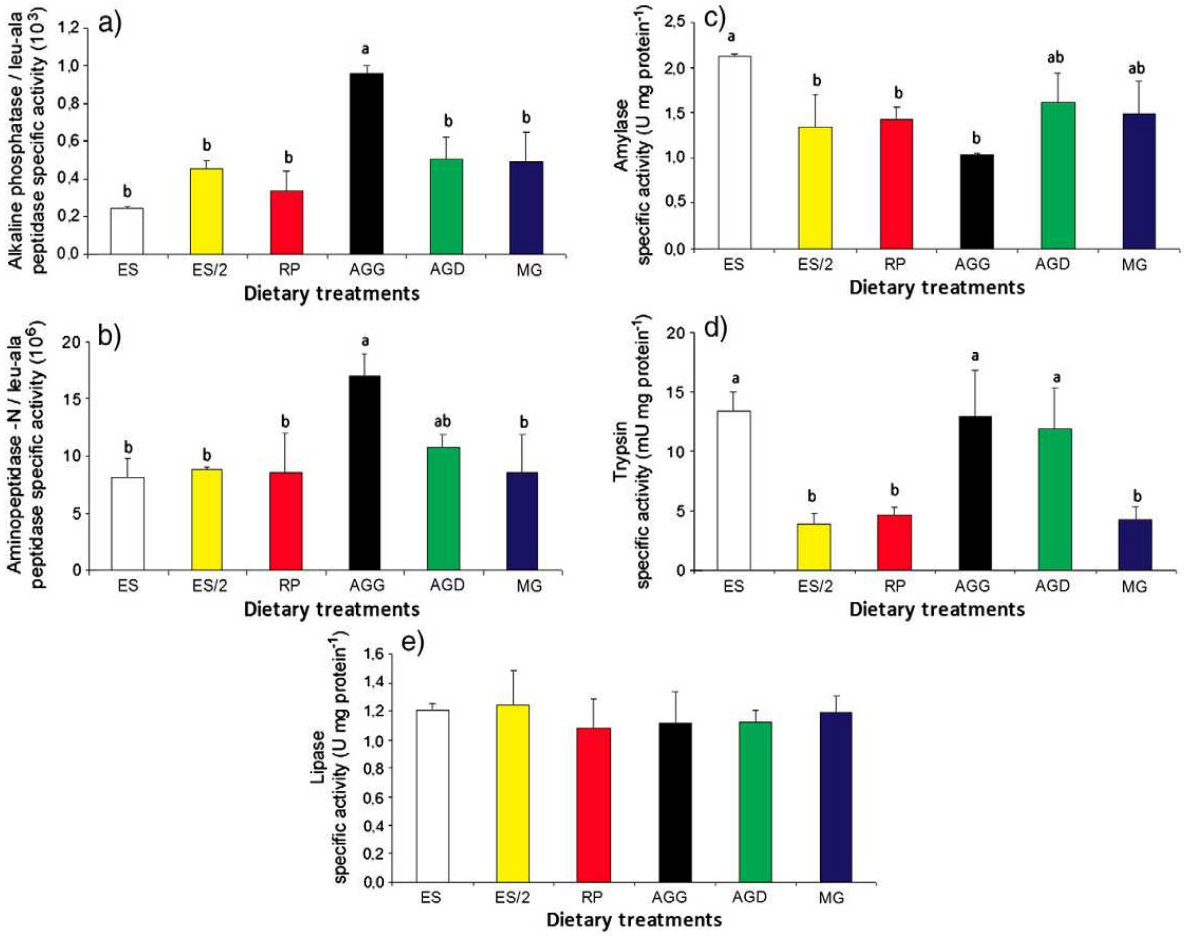


**Figure 1.** Intestinal maturation measured as the ratio of specific activity of brush border membrane enzymes/cytosolic enzymes: alkaline phosphatase/leucine–alanine peptidase (a), N-aminopeptidase/leu-ala peptidase (b), and specific activity of pancreatic enzymes: amylase (c), trypsine (d) and lipase (e) in 38 dph-Senegalese sole larvae fed the six different dietary treatments. Data are expressed as the mean ± D.E. (n = 3). Different letters show significant differences between the six groups (ANOVA, $P$< 0.05).

At 38 dph, amylase specific activity was significantly ($P$ = 0.005) higher in larvae from the ES group (2.11±0.03 U.mg protein$^{-1}$) than in those from RP, ES/2 and AGG groups (1.42 ± 0.15, 1.34 ± 0.36 and 1.04 ± 0.01 U mg.protein$^{-1}$, respectively) ($P$ < 0,001), being intermediate in larvae from the AGD and MG groups (1.62 ± 0.32 and 1.50 ± 0.36 U.mg protein$^{-1}$, respectively). Trypsin specific activity in 38

dph-larvae fed *Artemia* enriched with ES, AGG or AGD (13.42 ± 1.56, 12.92 ± 3.94 and 11.94 ± 3.47 U.mg protein$^{-1}$, respectively) was significantly ($P < 0.001$) higher than that of larvae from the RP, MG and ES/2 groups (4.64 ± 0.63, 4.26 ± 1.04 and 3.87 ± 0.89 U.mg protein$^{-1}$, respectively). Lipase specific activity at 38 dph was not affected by the dietary treatments ($P = 0.816$).

*Enterocyte height and cell proliferation capacity in the intestinal mucosa.*

The mean heights of enterocytes from fish fed the six different diets are represented in Figure 2a. There were significant differences in enterocyte heights between dietary treatments ($F_{10, 162} = 6.24$; $P \leq 0.001$) being higher in larvae fed *Artemia* enriched with ES, lower in larvae fed *Artemia* enriched with RP, AGG and MG and intermediate in larvae fed ES/2- and AGD-enriched *Artemia*. The rate of cell proliferation was significantly different among the dietary groups, being higher in those exposed to AGG, AGD and MG with respect to those fed with ES-, ES/2- and RP-enriched *Artemia* (Figure 2b, $F_{10, 162} = 3.98$; $P \leq 0.001$). Alkaline phosphatase specific activity was significantly and positively correlated with enterocyte proliferation ($R^2 = 0.83$; $P = 0.04$).

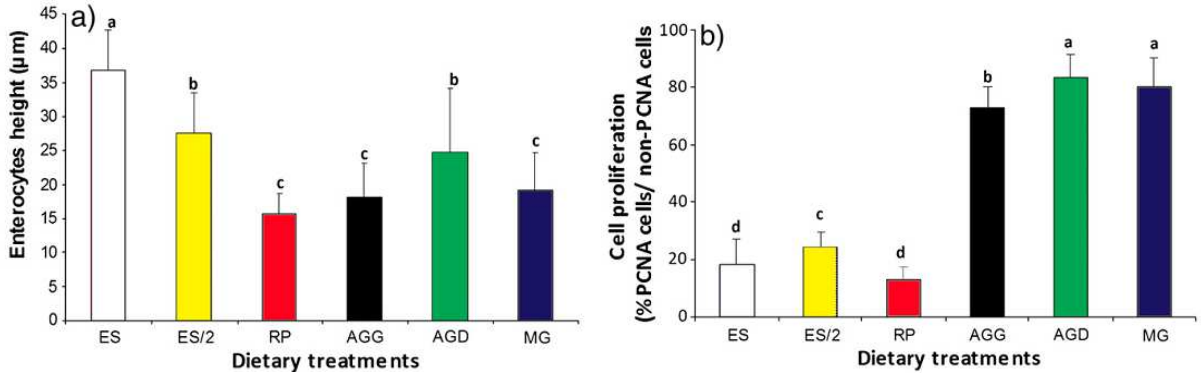


**Figure 2.** Effect of the six different dietary treatments on the intestinal maturation at 38 dph in Senegalese sole larvae fed the six different dietary treatments, measured as the enterocyte height (a) and cell proliferation capacity in mid intestine (b). Data are expressed as the mean ± S.E.M. (n = 30). Letters denote significant differences between groups (ANOVA, $P < 0.05$).

*Ossification degree and skeletal deformities*

At 38 dph, the ossification degree in Senegalese sole larvae was significantly affected by dietary treatments (Figure 3, $F_{10, 213} = 4.61$; $P \leq 0.001$). Larvae fed *Artemia* enriched with ES/2, AGG or AGD exhibited a significantly higher ossification degree than those from the ES group, with the larvae from the RP and MG groups showing intermediate ossification.

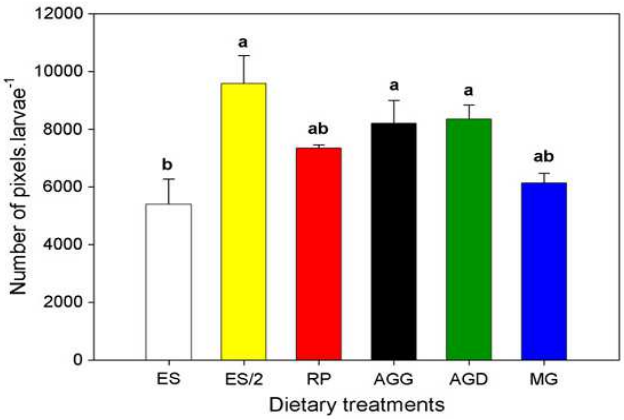


**Figure 3.** Degree of ossification of Senegalese sole larvae fed *Artemia* enriched with the six different enrichments at 38 dph. Data are expressed as the mean ± S.E.M. (n = 3). Indexed letters indicate significant differences among treatments (ANOVA, $P < 0.05$).

**Table 5.** Incidence of skeletal deformities (in %) in Senegalese sole fed with the six different dietary treatments, considering the number of abnormal skeletal elements per fish (mean ± SD). Indexed letters show significant differences among treatments (ANOVA.Pb0.05). Mns, modified neural spine; Mhs, modified haemal spines; HYP, hypurals; PHY, parahypural; EP, epural.

| | **Dietary treatments** | | | | | |
|---|---|---|---|---|---|---|
| | **ES** | **ES/2** | **RP** | **AGG** | **AGD** | **MG** |
| **Total deformities** | 71.9±9.9 | 74.2±10.2 | 74.5±2.0 | 79.2±3.4 | 77.9±5.3 | 79.3±3.9 |
| **Vertebral abnormalities** | 68.0±13.6 | 60.4±11.4 | 65.2±5.1 | 69.7±4.6 | 66.3±9.4 | 67.3±7.1 |
| *Severe* | 46.7±15.0 | 40.0±5.7 | 37.7±7.0 | 39.6±1.6 | 50.8±10.2 | 36.7±4.3 |
| Fusion | 18.7±6.9$^{ab}$ | 20.0±0.6$^{ab}$ | 17.2±5.7$^{ab}$ | 22.5±4.4$^{ab}$ | 30.6±7.1$^{a}$ | 15.7±2.4$^{b}$ |
| Compression | 13.1±12.6 | 10.4±6.4 | 13.4±3.6 | 18.9±3.6 | 16.8±7.1 | 15.0±4.9 |
| Vertebral centrums | 16.4±4.0 | 9.4±4.0 | 14.8±3.2 | 7.0±4.2 | 15.2±6.7 | 8.0±3.1 |
| Torsion | 8.5±7.2 | 6.8±5.4 | 10.7±7.6 | 3.4±3.8 | 10.9±10.5 | 6.9±3.9 |
| Scoliosis | 7.5±7.9 | 2.8±0.4 | 4.4±1.4 | 6.8±3.8 | 6.5±1.4 | 2.5±2.4 |
| *Light* | 56.5±9.3 | 57.6±8.9 | 62.6±4.9 | 61.3±6.9 | 47.1±2.6 | 62.8±5.8 |
| Haemal spines | 40.3±11.3 | 33.2±7.2 | 35.5±8.2 | 31.8±2.8 | 30.0±7.2 | 36.1±12.2 |
| Neural spines | 50.6±11.2 | 56.8±10.3 | 59.0±4.6 | 58.7±6.9 | 42.3±2.0 | 57.6±8.4 |
| **Caudal abnormalities** | 40.8±1.9$^{b}$ | 54.2±6.5$^{a}$ | 47.4±2.8$^{ab}$ | 55.9±4.4$^{a}$ | 44.7±4.4$^{ab}$ | 49.3±6.7$^{ab}$ |

Dietary treatments did not affect the incidence of total skeletal deformities in Senegalese sole (Table 5). The frequency of deformed fish was similar between the six groups ($P = 0.668$), ranging from 70 to 80%. No cranial deformities were observed in any larvae of the different dietary treatments. In all experimental groups, skeletal abnormalities affected mainly vertebral and caudal fin complex regions, particularly the pre-haemal vertebral region (Figure 4b), haemal vertebral region in the middle of the column (Figure 4c, d) and the last two vertebrae before the urostile (Figure 4e, f). The main vertebral column in Senegalese sole is composed of 45 vertebrae, divided in 8 pre-haemal and 37

haemal vertebrae (including the urostile). No significant differences were detected in the mean percentage of fish with 43 (13.6%), 44 (39.0%) or 45 (47.3%) vertebrae among the six experimental groups (P>0.05). The frequency of skeletal abnormalities in the vertebral column (pre-haemal and haemal regions) was similar among the six dietary treatments (P=0.751). However, when exam-ining each kind of vertebral deformity, the incidence of vertebral fusion was affected by the dietary treatment ($P$ = 0.042), with larvae from the AGD group presenting double the number of individuals (30.6 ± 7.1%) with total or partial vertebral fusion as compared to those from the MG group (15.7 ± 2.4%). No significant differences were detected in the remaining severe deformities (compression, torsion, scoliosis, vertebral centrums) nor in the minor ones (haemal or neural spines) among the dietary groups ($P$ > 0.05). Skeletal structures composing the caudal fin complex were also affected by dietary treatments, associated with a total or partial fusion of the last two vertebrae, modified neural and haemal spines presenting deformities or defects in ossification. The incidence of deformities in these structures significantly decreased ($P$ = 0.017) from larvae fed *Artemia* enriched with AGG (55.9%) and ES/2 (54.2%) to larvae fed *Artemia* enriched with ES (40.8%), whereas those fed *Artemia* enriched with MG, RP and AGD showed intermediate values relating to the incidence of abnormal modified neural and haemal spines (49.3, 47.4 and 44.7%, respectively).

**Discussion**

*Effect of the dietary treatments on the fatty acid profiles in live prey and on fish larvae*

Enriched *Artemia* nauplii from the six dietary treatments were isolipidic and contained similar content of total fatty acids. The fatty acid profile, particularly the profile of total (n-3) and total (n-6) HUFA, EPA, DHA, ARA, DHA/EPA ratio and the retinoid content of enriched *Artemia* nauplii varied among dietary treatments, in accordance to the composition of commercial emulsions (data not shown). However, *Artemia* are not passive carriers of fatty acids, but have specific physiological needs that can alter metabolically the original composition of the diet by retroconverting DHA into EPA, and by redistributing the incorporated fatty acids among lipid classes with high unpredictability (Navarro *et al.*, 1999). The metabolism inherent to *Artemia* nauplii cause them to accumulate EPA at higher levels than DHA, and consequently decreased the DHA/EPA ratio from the enriching product, as it had been previously described by Izquierdo (1988) and McEvoy *et al.* (1995).

In addition, the diverse forms in which enriching products are commercialized could be influencing the enrichment efficiency of *Artemia*. Indeed, during the enriching period, the digestive tract of *Artemia* nauplii were differentiating (Navarro *et al.*, 1999) and the differences in the type of enriching compounds could likely be influencing their onto-genesis and, hence, their absorption efficiency and the nutritional value of this type of live prey. All the above suggests that a combination of the initial lipid composition of the commercial enrichment, its physical form of presentation, and the lipid metabolic pathways of *Artemia* could be responsible for the final lipid profile of the *Artemia*.

The fatty acid profile of Senegalese sole larvae at 38 dph closely reflected the composition of their diet, as observed in striped trumpeter *Latris lineata* (Bransden *et al.*, 2004), turbot *Scophthalmus*

*maximus* (Estévez *et al.*, 1999), sea bream *Sparus aurata* (Koven *et al.*, 2001), white bass *Morone chrysops* (Harel *et al.*, 2000) or Senegalese sole larvae (Villalta *et al.*, 2005a, b), among others. In agreement to Villalta *et al.* (2005b), diets with medium and high amounts of DHA (MG, AGD, RP and AGG) contained low levels of OA and MUFA, which were normally used as energy for larval growth and development, and this trend was reflected in fish larvae fed these diets. The DHA content in larvae reflected the DHA amounts of the diet. This level of DHA was maintained in larvae fed the MG treatment com-pared to 8 dph-old larvae, whereas DHA content decreased from 1.3 to 3.5-fold in larvae from the others. Among all dietary treatments, ES-enriched *Artemia* presented a significantly higher EPA content than the other enriched *Artemia* which might have induced the particular fatty acid profile observed in larvae from this treatment, with high retention of EPA and DPA in larval tissues, whereas larvae from the other treatments seemed to have used these fatty acids during their development. The provision of dietary ARA in RP- and MG-enriched *Artemia* allowed the larvae to maintain the initial ARA con-tent at 38 dph, but larvae fed ES-, ES/2-, AGG and AGD diets significantly depleted their ARA stock during development, with larvae from the AGD group presenting the lowest ARA content at the end of the experiment. Curiously, the retinoid content in larvae did not follow the profile found in the enriched *Artemia*. Amounts of VA in *Artemia* enriched with ES was between 50 and 205-times higher than in *Artemia* enriched with RP, AGD, AGG or MG, and twice as high as the ES/2-enriched*Artemia*, reflecting the composition of commercial emulsions. However, this trend was completely normalized in larvae, probably revealing a capacity of elimination and detoxification of the dietary VA surplus (Fernández and Gisbert, 2010).

*Effect of the dietary treatments on the larval developmental performance*

At 38 dph, Senegalese sole larvae fed AGG-enriched *Artemia* presented a significantly improved larval performance and development than fish fed ES-enriched *Artemia*, whereas larvae from the ES/2, RP, AGD and MG groups showed an intermediate profile. In this sense, larvae from the AGG group grew faster than the others, particularly during the pre-metamorphic phase, showing higher final DW and SL values than larvae fed ES-enriched *Artemia*. Larvae fed AGD, MG, ES/2 and RP showed intermediate growth values, but larvae from the AGD and MG groups tended to grow slightly faster during the post-metamorphic phase than larvae from ES/2 and RP groups. However, in this study, 38 dph-Senegalese sole larvae were smaller than those of Villalta *et al.* (2005a, b) and Morais *et al.* (2004) at the same developmental stage, far from the suitable window of 5-10 mg required to start weaning (Conceição *et al.*, 2007). Such variations might be related to differences in the initial larval size among trials and rearing conditions.

Senegalese sole larvae were able to complete metamorphosis in all dietary treatments, but no significant effect of the different dietary treatments was observed in the process of eye migration. However, larvae from AGG, AGD and MG groups showed a trend toward a faster metamorphosis compared to larvae from the ES, ES/2 and RP groups. Larvae that metamorphose faster seemed to grow faster too, as the relative profiles for the metamorphosis process among dietary treatments were

in agreement with the trends for larval growth, as observed in Atlantic halibut *Hippoglossus hippoglossus* (Naess and Lie, 1998) and other flatfishes (Geffen *et al.*, 2007).

Dietary treatments did not affect significantly the survival rate of Senegalese sole larvae. Survival at 38 dph was higher than that reported in other studies where it ranged between 15 and 90% (Cañavate and Fernández-Díaz, 1999; Dâmaso-Rodrigues *et al.*, 2010; Dinis, 1992; Dinis *et al.*, 1999; Fernández *et al.*, 2008; Morais *et al.*, 2004; Villalta *et al.*, 2005a, b), but equivalent to the survival rate in Senegalese sole larvae fed *Artemia* HUFA-deficient during their post-metamorphic stage (>90%, Morais *et al.*, 2005a, b).

The activity of the pancreatic and intestinal enzymes provides a reliable marker for assessing the development of the digestive function in fish larvae (Zambonino-Infante *et al.*, 2008). During development, the specific activity of the brush border enzymes (alkaline phosphatase and N-aminopeptidase) increased with a parallel decrease in the activity of leu-ala peptidase (Ribeiro *et al.*, 1999), increasing the ratios between the brush border and the cytosolic enzymes, which provide an indication of the degree of maturation of the digestive system. Significantly higher values for both ratios observed in larvae fed AGG-enriched *Artemia* than in larvae fed other diets indicated a more physiologically advanced digestive system of larvae fed the AGG diet than those from the other dietary treatments, which coincided with the results of growth and metamorphosis for this treatment. Larvae from the AGD group showed intermediate values of the N-aminopeptidase/leu-ala peptidase ratio in concordance with their intermediate growth profiles. Amylase and trypsin activities are markers of the maturation of the digestive tract (Ribeiro *et al.*, 1999; Zambonino-Infante and Cahu, 2007). These pancreatic enzymes play an extremely important role during early stages and their specific activities normally decrease during larval development with the maturation of the stomach and the intestine. At the end of the experiment, larvae fed AGG-enriched *Artemia* presented the lowest amylase specific activity values, whereas larvae fed ES-enriched *Artemia* showed the highest, suggesting a more advanced maturation of the digestive system in larvae from the AGG group than those from the ES group. Larvae fed ES/2, RP, AGD or MG diets showed low or intermediate amylase specific activity values. Lipase specific activity was not significantly different among larvae fed *Artemia* enriched with the different products. Lipase secretion is regulated by lipid concentration in the diet (Martínez *et al.*, 1999) and this observation probably reflected the fact that the treatments were isolipidic (Morais *et al.*, 2006).

Parallel to these physiological changes in enzyme specific activities in Senegalese sole larvae during development, the intestinal mucosa matured as evidenced by structural changes. Enterocyte proliferation was significantly higher in 38 dph-larvae fed AGG-, AGD-, MG-enriched *Artemia* than in those fed ES-, ES/2- and RP-enriched *Artemia*. Apart from that, enterocyte height was lower in larvae fed AGG-, RP-and MG-enriched *Artemia* as compared to larvae from the ES fed group and intermediate in larvae from the ES/2 and AGD fed groups. These results are inversely correlated to

results of cell proliferation, since cells allocating energy in intensive mitotic divisions would spend less energy in cell growth (Lodish *et al.*, 2000). Moreover, the significant and positive correlation between the specific activity of alkaline phosphatase and enterocyte proliferation indicated that both parameters are useful biomarkers providing complementary biochemical and histochemical information for the evaluation of gut maturation. These results supported the hypothesis of a more advanced degree of maturation of the digestive system for larvae from the AGG fed group, followed by those from the AGD and MG fed groups, after which by those from the RP and ES/2 fed groups, and finally by larvae fed ES-enriched *Artemia*, which is in agreement with the results of growth and eye migration.

Dietary treatments also affected the degree of ossification of post-metamorphic Senegalese sole larvae. Larvae from the AGG group showed most of their skeleton ossified at 38 dph, whereas larvae from the ES dietary treatments were significantly less ossified, and larvae from the other groups showed intermediate ossification. Al-though there was no clear correlation with the trend observed in growth and intestinal maturation, it can be noticed that larvae fed ES-enriched *Artemia* showed the lowest growth, intestinal maturation and ossification values; whereas larvae fed AGG-enriched *Artemia* showed the opposite pattern. A correlation between a delay in growth and ossification had been previously highlighted in Atlantic cod (Kjørsvik *et al.*, 2009), gilthead sea bream (Fernández *et al.*, 2008) and European sea bass (Darias *et al.*, 2010b) larvae fed different diets. The former studies reported that animals with lower growth values were also delayed in digestive system maturation and skeletogenesis, resulting in the incidence of skeletal disorders.

However, in the current study, no correlation between the degree of ossification and the incidence of skeletal deformities could be established; although all experimental groups showed a large incidence of skeletal deformities (76% in average), as previously observed in Senegalese sole larvae, particularly during early stages of development (Engrola *et al.*, 2009; Fernández *et al.*, 2009; Gavaia *et al.*, 2002). No significant effect from the dietary treatment on the incidence of total abnormalities, nor malformations of the vertebral column were detected. Neither were there observed significant differences among treatments in types of skeletal deformities in the vertebral region, apart from the larvae fed AGD-enriched *Artemia* which were more affected by fusion of vertebral centrums than larvae fed MG-enriched *Artemia*. In the caudal fin complex, significant differences were detected, with larvae fed AGG-and ES/2-enriched*Artemia*presenting higher frequency of individuals with malformations of modified neural and haemal spines than larvae fed ES-enriched *Artemia*. However, these variations among treatments are too minor and therefore fail to indicate which dietary treatment is responsible for a higher frequency of skeletal deformities.

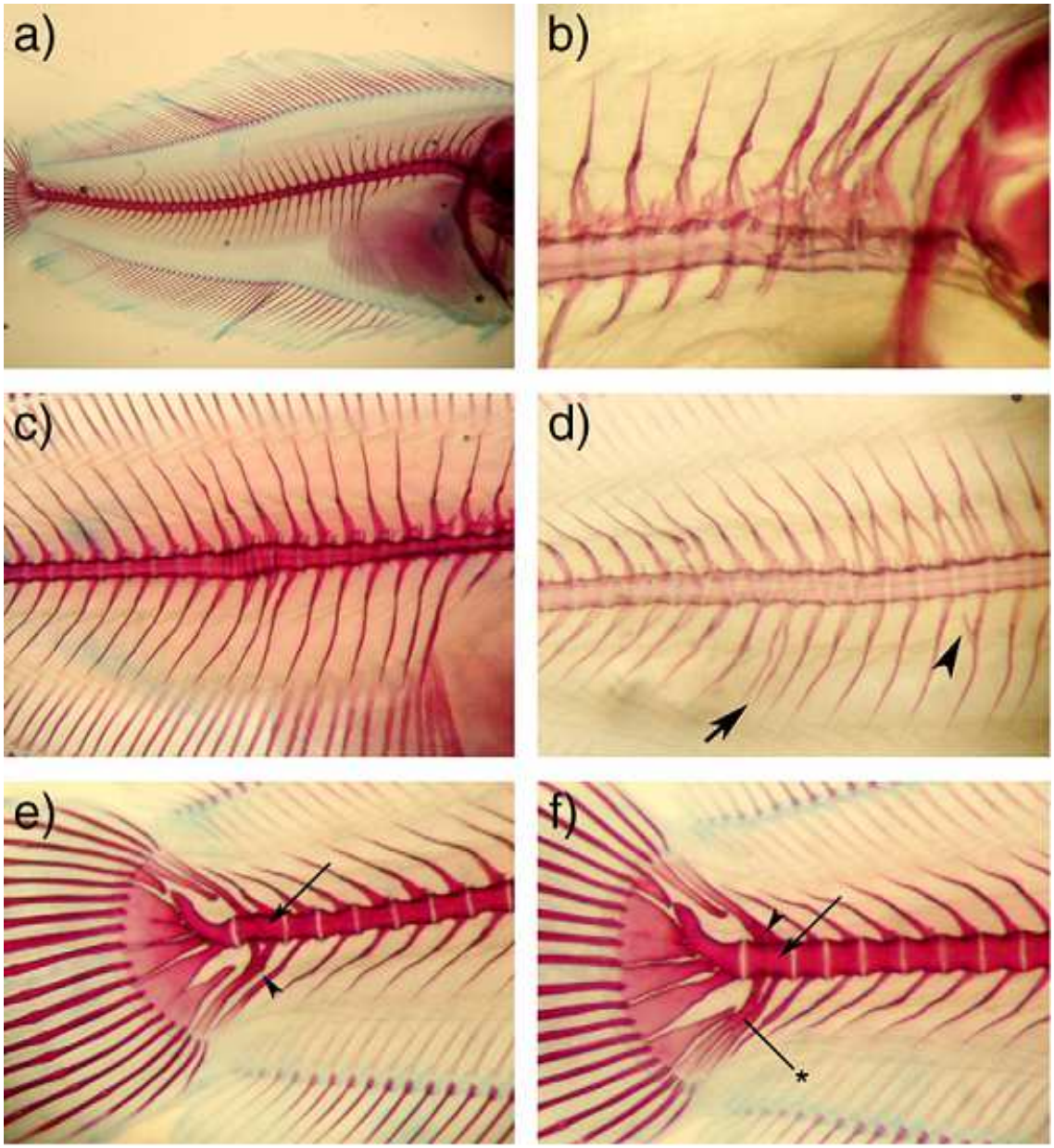


**Figure 4.** Examples of different typologies of skeletal deformities found in 38-day-old Senegalese sole. (a) View of the vertebral region of a larva presenting scoliosis. (b) Strong compression of the first five pre-haemal vertebrae resulting in abnormal neural spines. (c) Compression in the haemal region, resulting in a thickened area, raising the height of the vertebrae compared to the vertebral axis, and a defect in the haemal and neural spines parallelism. (d) Torsion in the haemal region, resulting in a deviation of the vertebral axis and of the apparent shape of the vertebral centrums (which seem inclined). Note the visible space of the neural and haemal arches as an indicator of the vertebral torsion and the absence of fusion of two haemal spines (arrow). (e) Partial fusion of the last two vertebrae before the urostile (the intervertebral space is still visible) and fusion of the modified haemal spines. (f) Total fusion of the last two vertebrae before the urostile and fusion of the modified neural spines.

*Effect of the dietary fatty acid profile on the larval performance in Senegalese sole larvae*

The nutritional causes affecting larval developmental performance cannot be evaluated by taking into account the individual effect of each fatty acid alone due to the complexity of the interactions between the fatty acids and the nutrients composing the diet. Therefore it is necessary to examine the relative proportions of dietary fatty acids, especially the ratios between n-3andn-6 HUFA and between EPA, DHA and ARA. Results of growth, eye migration, intestinal maturation, and dietary amounts of the main essential nutrients were gathered in Figure 5 in order to integrate and better visualize the differences in dietary fatty acid composition and larval performance between dietary treatments.

In this study, the AGG diet was associated with the best overall larval performance and quality among all dietary treatments tested. The fatty acid profile of the AGG diet presented intermediate values of total MUFA, ARA, EPA, DHA, total PUFA, DHA/EPA and n-3/n-6PUFA ratios (3.0:1 and 5.2:1, respectively). This subtle balance between all the components in the AGG diet certainly helps to explain the improved results observed in Senegalese sole larvae in the present study, since all the other diets that induced less efficient larval development had one or several compounds in a different proportion as com-pared to the composition of the AGG formulae.

The ES diet contained significantly higher EPA levels than AGG. This excessive dietary EPA accumulated in larval tissues in the form of EPA or DPA, probably because Senegalese sole larvae, as with many marine fish larvae, are unable to elongate and desaturate them into DHA at a significant rate (Morais *et al.*, 2004). A surplus of EPA in larval tissues could have a detrimental effect on larval development. Previous studies (Izquierdo *et al.*, 2000; Léger *et al.*, 1986) estimated between 3 and 4% TFA as the range of EPA levels, suitable for larval growth and survival of various marine organisms. Moreover, Villalta *et al.* (2008) proved that Senegalese sole larvae were able to grow and survive on *Artemia* nauplii with negligible EPA content (0.4% of TFA) up to 40 dph, even showing a superior growth than when fed *Artemia* with 10.7 and 20.3% EPA of TFA. Senegalese sole larvae have low requirements for EPA when DHA is present in the diet and increasing EPA amounts in the diet rather than enhance growth and development had a detrimental effect on morphogenesis, as demonstrated for the RP, AGD and MG diets.

The ES/2 diet was significantly poorer in DHA in comparison to the AGG diet, but both contained the same amounts of EPA; in spite of this, the ES/2 diet resulted in less developed larvae. Although previous studies have demonstrated that Senegalese sole larvae have low or negligible requirements for DHA, being able to survive, grow and metamorphose on *Artemia* practically devoid of DHA, but containing other n-3 PUFA (Dâmaso-Rodrigues *et al.*, 2010; Morais *et al.*, 2004; Villalta *et al.*, 2005a), the dietary DHA minimum requirement is an important nutritional parameter that should not be neglected even if it is “just a drop in the ocean” relative to the other nutritional factors affecting larval physiology. In this sense, an increase from 9.5 to 11.5–11.8% TFA in the RP and AGD diets did not significantly improve larval development; and in the case of MG, DHA content was too excessive

to be efficiently used by sole larvae and accumulated in larval tissue (Boglino, unpublished data) which may have been detrimental for proper development (Roo *et al.*, 2009). Such an in-crease in DHA was compensated by a diminution in MUFA, and particularly in OA; fatty acids that are more easily catabolized by fish larvae to produce energy for growth and development. The same trend was observed in the RP diet where higher n-6 PUFA levels than in the AGG diet were offset by a lower MUFA content. As shown by Villalta *et al.* (2008), disrupting the balance between energy (saturated FA and MUFA) and essentiality (HUFA) had a detrimental effect on Senegalese sole larvae.

An optimal dietary DHA/EPA ratio of 2:1 for newly hatched larvae has been previously determined by Sargent *et al.* (1997) from the lipid composition of the yolk sac in marine fish eggs. According to these authors, a DHA/EPA ratio inferior or equal to 1 corresponds to a sub-optimal diet by providing to larvae insufficient amounts of DHA or an excess of EPA, and could be deleterious in larval fish feeds, as in the ES and ES/2 diets. The DHA/EPA ratio was largely reduced in ES, ES/2 and RP diets compared to the AGG diet. Morais *et al.* (2004)evaluated that Senegalese sole have a high dietary DHA requirement relative to EPA, with eggs of these species containing a DHA/EPA ratio (4.3:1) higher than that found in most marine species eggs (2:1–3:1); although no correlation was found between the prey's DHA/EPA ratio and Senegalese sole larval growth and survival. Brinkmeyer and Holt (1998) found that the optimal DHA/EPA ratio for red drum larvae was greater than 2.5:1, and further, a ratio of 3.78:1 in the diet produced larvae more resistant to stress. Other authors (Harel *et al.*, 2002; Rodriguez *et al.*, 1998; Watanabe *et al.*, 1989) have demonstrated the wide superiority of DHA to EPA to im-prove growth and survival in other marine fish larvae, such as red sea bream (DHA/EPA = 1.1:1), gilthead sea bream (DHA/EPA = 1.3:1), striped bass, European sea bass, Atlantic halibut (DHA/EPA = 2:1-2.5:1); and to ameliorate survival and vitality in blackspot sea bream *Pagellus bogaraveo* (DHA/EPA = 3.8:1). In this study, a DHA/EPA ratio of 3.0:1 provided the best results in terms of larval performance, whereas diets with diverging ratios were less efficient.

The decrease in n-3 PUFA content in the ES and ES/2 diets and the increase of n-6 PUFA content in the RP diet compared to the AGG diet also disrupted the balance between n-3 and n-6 PUFA, since a ratio of 5.2:1 seemed to produce better growth and development than a ratio of 3.9:1, 3.7:1 or 3.6:1 (ES, ES/2 and RP diets, respectively). The optimal balance between these two series of PUFA has been the subject of several reviews (Izquierdo, 1996; Sargent *et al.*, 1999). A higher dietary n-3/n-6 PUFA ratio is associated with beneficial effects on bone health (Maggio *et al.*, 2009) and some evidence exists for higher requirements in n-3 EFA than in n-6 EFA in marine fish larvae. Sargent *et al.* (1999) showed that an excess of ARA to EPA impaired pigmentation and metamorphosis in turbot larvae. The dietary ARA/EPA ratio plays an important role, be-cause ARA is the major eicosanoid precursor in fish and an excess of EPA can be harmful by reducing the desirable ARA/EPA ratio in live feeds (Sargent *et al.*, 1997).

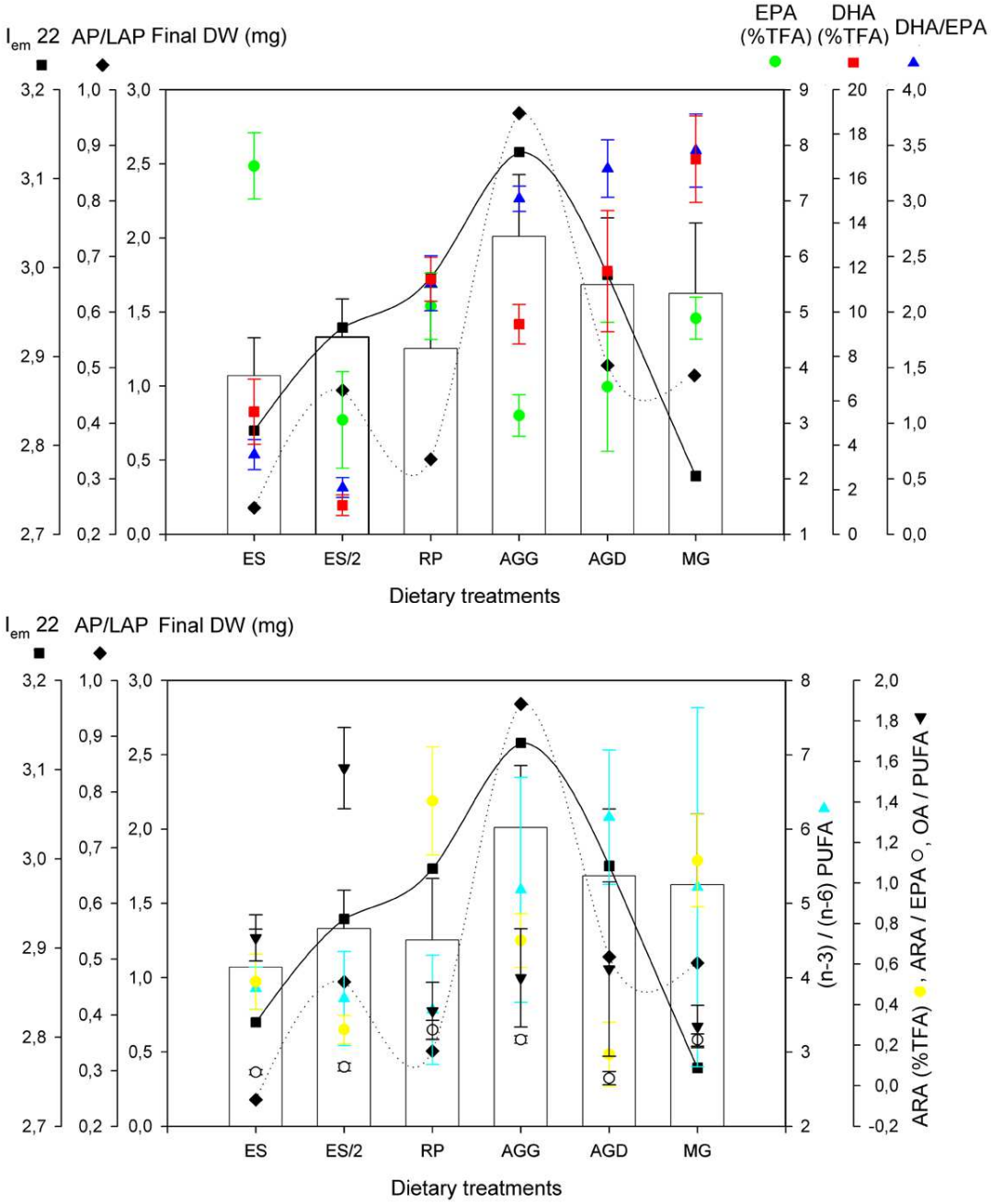


**Figure 5.** Final DW (bars, in mg), $I_{EM}$ at 22 dph (squares and solid line) and Alkaline phosphatase/leu-ala peptidase ratio at 22 dph (AP/LAP), diamonds and dotted line) in Senegalese sole larvae, DHA (red squares), EPA (green circles), ARA (yellow circles) contents and n-3/n-6 PUFA (light blue triangles), DHA/EPA (dark blue triangles), ARA/EPA (white circles), OA/PUFA (black inversed triangles) ratios in *Artemia* enriched the six different dietary treatments. Data are expressed as mean ± D.E. (n=3). The highest final DW and the most advanced metamorphosis and maturation of the digestive system were obtained for larvae fed AGG-enriched *Artemia*, which diet presented intermediate values of DHA, EPA and ARA amounts (9.5, 3.1, 0.7% TFA) and intermediate values of n-3/n-6 PUFA, DHA/EPA, ARA/DHA and OA/PUFA ratios (5.2, 3.0, 0.1 and 0.5, respectively).

Although ARA is included in enrichments in low but significant amounts (usually about 1% TFA), there is no evidence if this amount is sufficient, nor if the relative proportion of ARA to DHA and EPA is optimal for marine fish larval feeds. In common sole *Solea solea*, neither standard growth nor larval survival was significantly affected by different dietary absolute concentrations of ARA, EPA and DHA, or their ratios (Lund *et al.*, 2007). In this study, a 2-fold increase in the amount of ARA in the RP diet (1.4% TFA) or a deficiency in ARA for the AGD, ES and ES/2 diets (0.2, 0.5 and 0.3% TFA, respectively) in comparison to the AGG diet (0.7% TFA), together with an excess of EPA in the ES diet, contributed to a disruption in the balance between EFAs, that might have had consequences on the overall developmental performance of Senegalese sole.

The effect of the origin of the dietary HUFA should not be neglected, since their efficiency of assimilation and their effect on larval performance depends on the dietary lipid class [neutral lipids (NL) or phospholipids (PL)]. EFA are generally provided in the diet by fish oils, which are mainly composed of NL (Cahu *et al.*, 2003; Izquierdo *et al.*, 2000). However, fish larvae have high requirements for dietary PL (Cahu *et al.*, 2009). Adding PL to live-prey enrichment emulsions results in beneficial effects on the larval growth, survival, maturation of the digestive function, stress tolerance and larval quality (Bell and Sargent, 2003; Cahu *et al.*, 2003; Coutteau *et al.*, 1997; Gisbert *et al.*, 2005; Izquierdo *et al.*, 2000; Sargent *et al.*, 2002) as marine fish larvae use dietary n-3 PUFA contained in the PL fraction more efficiently than those from the NL fraction (Gisbert *et al.*, 2005; Salhi *et al.*, 1999). Although the different origin of the ingredients composing the enriching products, under the present experimental conditions, enriched *Artemia* from the different dietary treatments showed similar total lipid content, as well as similar values of polar and neutral lipids (Boglino, unpublished data). As was found by Harel *et al.* (2002), this study demonstrated the potential of single cell heterotrophs (AGG, AGD, MG) as replacements for fish-based emulsions (ES, ES/2, RP) in larval diets, producing at least as good results in larval growth and development. However, further work is needed to examine possible effects of the different products on lipid absorption, transport and metabolism. Optimizing larval diets requires a careful balance of all the component nutrients and there is a need to consider the interactions between all of these components to create the most benefit for growth and development.

**Conclusion**

Under current experimental conditions, none of the tested enriching products were inappropriate for proper larval performance and quality, although they differentially affected Senegalese sole larval growth and development. Each dietary treatment presented its own specific accumulation pattern for each fatty acid, none of which were optimal for proper larval performance and survival. Larvae fed the AGG diet had the best larval performance so that their FA profile can be considered as the most balanced for Senegalese sole larvae among all tested diets. Given the fatty acid profile of the AGG diet and our results from this study we recommend absolute levels of DHA, EPA and ARA of 9.5, 3.1

and 0.7% TFA, respectively, and n-3/n-6 PUFA, DHA/EPA, ARA/DHA and OA/PUFA ratios of 5.2, 3.0, 0.1, 0.5, respectively, as more suit-able for Senegalese sole larval development.

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# 2

# Isolipidic diets differing in their essential fatty acid profiles affect the deposition of unsaturated neutral lipids in the intestine, liver and vascular system of Senegalese sole larvae and early juveniles

Anaïs Boglino[1]
Enric Gisbert[1]
Maria J. Darias[2]
Alicia Estévez[1]
Karl B. Andree[1]
Carmen Sarasquete[3]
Juan B. Ortiz-Delgado[3]

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[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

[3] Instituto de Ciencias Marinas de Andalucia (CSIC), Campus Universitario Rio San Pedro, Apdo Oficial, 11510 Puerto Real, Cádiz, Spain

**Unas dietas isolipídicas con diferentes perfiles de ácidos grasos esenciales afectan a la deposición de lípidos neutrales insaturados en el intestino, el hígado y el sistema vascular de larvas y juveniles de lenguado Senegalés.**

**Resumen**

La manera como el contenido y la composición lipídica de una dieta es utilizada por diversos órganos y tejidos en los peces se refleja en su estructura; iniciando por el intestino por donde los lípidos administrados en la dieta serán digeridos y absorbidos, el sistema vascular que está involucrado en su transporte, y el hígado, por donde los lípidos serán almacenados y movilizados. No obstante, a la fecha actual no se han realizado estudios sobre el efecto de diferentes niveles de ácidos grasos poliinsaturados (AGPI) y ácidos grasos esenciales (AGE) en dietas de peces marinos sobre la deposición en diversos órganos en larvas y juveniles de peces. Por tanto, los efectos de seis dietas isolipídicas (*Artemia* salina enriquecida) fueron evaluadas, con distintos perfiles de ácidos grasos, sobre los patrones de acumulación lipídica en tres tejidos objetivos (intestino, hígado y sistema vascular) en larvas y juveniles de lenguado Senegalés (*Solea senegalensis*). Los resultados mostraron que el perfil de acumulación de lípidos en estos tres tejidos se vieron significativamente afectados por los diferentes tratamientos, el estadio de desarrollo del pez (pre-, pro- o post-metamorfosis), tanto como por las interacciones entre estos dos factores que fueron responsable de los cambios en la organización histológica de los tejidos. Los resultados histológicos revelaron que al variar ligeramente los niveles de AGE (e.g. EPA, DHA o ARA) o sus ratios (EPA/DHA, ARA/EPA, ARA/DHA, n-3/n-6 PUFA, OA/PUFA) el metabolismo de los lípidos fue modificado y el patrón de acumulación de lípidos en los tejidos objetivos fue afectado, lo que condujo a la esteatosis intestinal y hepática en larvas y juveniles del lenguado Senegalés.

**Palabras claves**: *Solea senegalensis*, larvas, lípidos, ácidos grasos, acumulación de grasa, esteatosis.

**Abstract**

How lipid content and composition in the diet is utilized by the various organs and tissues offish is reflected in their structure, such as the intestine through which dietary lipids are digested and absorbed, the vascular system which is involved in their transport, and the liver where lipids are stored and metabolized. However, no study has been conducted to compare the effect of different diets containing different levels of highly unsaturated fatty acids (HUFA) and essential fatty acids (EFA) on lipid deposition in fish larvae and early juveniles. Thus, we evaluated the effects of six isolipidic diets (enriched *Artemia salina*), differing in their fatty acid profile, on the lipid accumulation patterns in selected target tissues (intestine, liver and vascular system) in Senegalese sole (*Solea senegalensis*) larvae and early juveniles. Results showed that the profile of fat accumulation in these three tissues was significantly affected by the dietary treatments, the developmental stage of the fish (pre-metamorphosis, metamorphosis or post-metamorphosis), as well as by the interaction between these two factors that were responsible for changes in the histological organization of the tissues. Histological results revealed that a slight variation in the EFA levels (e.g. EPA, DHA or ARA) or in their ratios (EPA/DHA, ARA/EPA, ARA/DHA, (n-3)/(n-6) HUFA, OA/PUFA) modified the metabolism of lipids and disrupted the pattern of lipid accumulation in the target tissues, leading to intestinal and hepatic steatosis.

**Keywords:** *Solea senegalensis*, larvae, lipid, fatty acid, fat accumulation, steatosis

**Introduction**

Dietary lipids are particularly important for normal growth and development, since they represent the main energy source in developing fish larvae and a valuable source of essential fatty acids (EFA) needed for the synthesis of new cellular structures and organs during larval morphogenesis (Sargent *et al.*, 1999). Lipid requirements of marine fish larvae have been studied during the last two decades, and particularly highly unsaturated fatty acids (HUFA) and EFA, such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA), since marine fish are unable to synthesize these fatty acids (FA) from their precursors 18:3n-3 and 18:2n-6 (Sargent *et al.*, 2002). The important morphological and physiological changes occurring during larval development affect dietary lipid utilization and make larvae very susceptible to changes in dietary lipid composition, classes (phospholipids and neutral lipids) and levels. Thus, when lipids and HUFA, particularly EFA, are not provided to the fish larvae in sufficient amount or adequate form in the diet, they can be responsible for delayed growth, increased mortality, reduced resistance to stress, appearance of skeletal deformations, physiological or anatomical alterations and pigmentary disorders (see reviews in Izquierdo *et al.*, 2000; Tocher *et al.*, 2008; Cahu *et al.*, 2009).

Since dietary requirements offish larvae and early juveniles are different from those of adults, larval nutrition should be always considered along the organization and functionality of the digestive sys-tem, and nutritional needs at different stages of development (Lazo *et al.*, 2011). How lipid content and composition (*e.g.* HUFA, phospholipids and neutral lipid levels) in the diet is utilized by the various organs and tissues offish is reflected in their structure, such as the intestine through which dietary lipids are digested and absorbed, the vascular system which is involved in their transport in the form of very low-density lipoproteins (VLDL) or chylomicrons, and the liver where lipids are stored and metabolized (see review in Tso, 1994). As the above-mentioned tissues employed different cellular mechanisms in response to qualitative and quantitative changes of

the diet, their use as nutritional and physiological biomarkers is well established in fish larvae (see review in Gisbert *et al.*, 2008; Cahu *et al.*, 2009). In this sense, changes in the histological organization of the liver, the exocrine pancreas, the intestine and the muscular fibers have been used on a regular basis as histological targets to analyze the nutritional condition offish larvae and elucidate the effects of different dietary regimes or nutrients on larval physiology, nutrition and early development. These tissues and organs are especially sensitive to non-optimal feeding conditions or nutritional stress during larval development, because they are under progressive and intensive morphogenesis, and consequently, they respond rapidly and sensitively to nutritional disorders (Theilacker and Porter, 1995; Catalán and Olivar, 2002; Caballero *et al.*, 2003, 2004 among others). For instance, the size of lipid vacuoles in enterocytes and hepatocytes depends on the dietary fat content and the degree of unsaturation of the lipids ingested, which gives an idea if the dietary fat supply man-ages to balance the lipid metabolic rate (Sheridan, 1988).

Senegalese sole (*Solea senegalensis*, Kaup 1858) is a high-value flatfish, reared in intensive production systems in Iberian Peninsula. However, larvae from this species have specific lipid and fatty acids requirements (Morais *et al.*, 2004; Villalta *et al.*, 2005) and among all commercial enriching

products for live prey used nowadays in flatfish larval production, the most appropriate for Senegalese sole larval rearing and correct development remains unknown. Within this context, this work is a part of a larger study that aimed to evaluate by simulating dietary conditions in commercial fish hatcheries, the effects of six different isolipidic diets (*Artemia* nauplii enriched with different commercial emulsions/enrichers resulting in graded levels of EFA) on Senegalese sole larval performance, in which a wide range of biological and molecular markers, like growth, digestive system development, lipid and fatty acid accumulation in fish larval tissues, skeletal development and gene expression associated to lipid transport, bone mineralization and retinoid metabolism, were evaluated in order to assess the impact of the diet on larval morphogenesis (Boglino *et al.*, 2012; Darias *et al.*, 2012). Although this approach could have been con-ducted using enrichment emulsions formulated with different fatty acid profiles (Villalta *et al.*, 2005, among others) differing in one or several fatty acids, we decided to choose different commercial products as this approach is closer to real practices in commercial fish hatcheries.

This study aimed to describe by means of histological procedures how different isolipidic diets with similar levels of polar and neutral lipids but differing in their FA profile affect lipid metabolism and utilization in Senegalese sole. In this sense, the levels of accumulated neutral lipids in the intestine, liver and vascular sys-tem were evaluated by histological means for a better understanding of lipid assimilation and deposition processes in early life stages of this flatfish species.

## Materials and methods

*Larval rearing and experimental diets*

Senegalese sole larvae (2 days post hatch, dph) were obtained from Stolt Sea Farm SA (Cambre, La Coruña, Spain) and reared for 38 days in eighteen cylindrical 60 L-tanks connected to an IRTAMAR® water recirculation unit. The system was housed in a constant environment room maintained at 16 h light: 8 h dark photoperiod and 20.0 ± 2.8 °C water temperature. Water quality in terms of dissolved oxygen, pH and salinity were 7.5 ± 1.3 ppm, 8.0 ± 0.2 and 35‰, respectively. The feeding sequence for Senegalese sole larvae used was already described in Boglino *et al.* (2012). In brief, larvae were fed twice a day with rotifers (*Brachionus plicatilis*) enriched with microalgae (*Tetraselmis suecica*) from 2 to 10 dph, and with enriched *Artemia salina* metanauplii from 8 to the end of the study at 38 dph.

In order to provide insight on how different levels of dietary HUFA affected the levels of neutral lipids deposition in the intestine, vascular system and liver, *Artemia* were enriched with various commercially enrichers, differing in their FA composition: Aquagrow Gold© (ABN, USA), Aquagrow DHA© (ABN, USA), Easy Selco© (INVE, Belgium), Red Pepper© (Bernaqua, Belgium), and Multigain© (BioMar, Denmark). By enriching *Artemia* with these products, five dietary treatments with graded levels of n-3 PUFA and graded DHA/EPA ratios was obtained: Aquagrow Gold© (AGG), Aquagrow DHA© (AGD), Easy Selco© (ES), Red Pepper© (RP), and Multigain© (MG). Aquagrow Gold© and Aquagrow DHA© were diluted by a third with olive oil in order to adjust the increasing gradient of n-3 PUFA in enriched *Artemia*. Moreover, a sixth dietary treatment containing a level of n-3 PUFA and a

DHA/EPA ratio significantly lower was designed by enriching *Artemia* with Easy Selco© diluted by a half with olive oil (ES/2) and used as a negative control. *Artemia* nauplii were enriched as described in Boglino *et al.* (2012). Lipid and total fatty acid contents and the fatty acid composition of *Artemia* metanauplii enriched the six products are shown in Table 1.

**Table 1.** Total lipid and fatty acid content (in mg.g$^{-1}$ DW), lipid classes (in %; phospholipids, PL and neutral lipids, NL) and fatty acid composition (% TFA) in *Artemia* nauplii (mean ± SD; n = 3) enriched with Aquagrow Gold© (AGG), Aquagrow DHA© (AGD), Easy Selco© (ES), Easy Selco© diluted by a half with olive oil (ES/2), Red Pepper© (RP) and Multigain© (MG). Totals include some minor components not shown. Superscript letters denote significant differences among diets ($P < 0.05$).

| | | | | | | |
|---|---|---|---|---|---|---|
| Total lipids | 184.4±17.0 | 202.9±1.3 | 219.8±17.4 | 175.3±44.6 | 174.7±17.3 | 159.6±8.3 |
| Total PL | 13.9±0.7 | 14.1±1.1 | 15.6±2.5 | 22.0±2.3 | 17.0±2.2 | 16.4±4.0 |
| Total NL | 86.2±1.3 | 85.5±2.1 | 84.4±2.1 | 77.9±8.9 | 82.9±6.2 | 83.6±3.9 |
| Total FA | 124.0±43.0 | 106.3±6.0 | 155.9±54.8 | 112.2±20.0 | 104.9±15.6 | 116.1±24.1 |
| Total saturated | 15.9±2.6 | 14.6±5.4 | 14.9±0.3 | 13.0±1.2 | 17.8±1.2 | 15.1±0.7 |
| 18:1n-9 | 26.2±10.1$^{ab}$ | 27.7±19.0$^{ab}$ | 33.3±2.6$^{ab}$ | 50.8±2.0$^{a}$ | 20.2±7.0$^{b}$ | 17.8±6.1$^{b}$ |
| Total monounsaturated | 32.7±3.2$^{ab}$ | 30.9±17.7$^{b}$ | 38.8±3.7$^{ab}$ | 54.1±1.6$^{a}$ | 26.5±1.9$^{b}$ | 23.5±2.4$^{b}$ |
| 18:2n-6 | 5.2±1.0$^{b}$ | 6.9±1.9$^{ab}$ | 8.28±0.04$^{a}$ | 6.3±0.5$^{ab}$ | 7.6±0.4$^{ab}$ | 5.5±0.5$^{ab}$ |
| 20:4n-6 | 0.7±0.1$^{bc}$ | 0.2±0.2$^{d}$ | 0.5±0.1$^{cd}$ | 0.3±0.1$^{cd}$ | 1.4±0.3$^{a}$ | 1.1±0.2$^{ab}$ |
| Total (n-6) PUFA | 8.6±2.3 | 7.8±2.6 | 9.5±0.2 | 7.0±0.4 | 12.3±2.3 | 10.8±3.7 |
| 18:3n-3 | 24.1±6.1 | 25.1±5.5 | 18.9±1.8 | 17.4±2.3 | 20.5±3.4 | 21.4±2.1 |
| 20:5n-3 | 3.1±0.4$^{cb}$ | 3.7±1.2$^{cb}$ | 7.6±0.6$^{a}$ | 3.1±0.9$^{c}$ | 5.1±0.6$^{b}$ | 4.9±0.4$^{cb}$ |
| 22:6n-3 | 9.5±0.9$^{bc}$ | 11.8±2.7$^{b}$ | 5.5±1.5$^{cd}$ | 1.3±0.5$^{d}$ | 11.5±1.0$^{b}$ | 16.9±2.0$^{a}$ |
| Total (n-3) PUFA | 42.5±4.6$^{a}$ | 46.4±9.5$^{a}$ | 36.5±3.5$^{ab}$ | 25.7±3.2$^{b}$ | 42.9±1.6$^{a}$ | 50.4±2.3$^{a}$ |
| Total PUFA | 51.1±5.9$^{a}$ | 54.1±12.0$^{a}$ | 46.0±3.7$^{ab}$ | 32.7±2.9$^{b}$ | 55.3±2.8$^{a}$ | 61.1±2.3$^{a}$ |
| (n-3)/(n-6) | 5.2±1.5 | 6.2±0.9 | 3.9±0.3 | 3.7±0.6 | 3.6±0.7 | 5.2±2.4 |
| DHA/EPA | 3.0±0.1$^{a}$ | 3.3±0.3$^{a}$ | 0.7±0.1$^{c}$ | 0.4±0.1$^{c}$ | 2.3±0.3$^{b}$ | 3.5±0.3$^{a}$ |
| ARA/DHA | 0.08±0.01$^{bc}$ | 0.01±0.01$^{d}$ | 0.1±0.0$^{bc}$ | 0.22±0.04$^{a}$ | 0.12±0.01$^{b}$ | 0.07±0.01$^{cd}$ |
| ARA/EPA | 0.23±0.02$^{a}$ | 0.04±0.03$^{b}$ | 0.07±0.01$^{b}$ | 0.09±0.02$^{b}$ | 0.3±0.1$^{a}$ | 0.23±0.03$^{a}$ |
| OA/PUFA | 0.53±0.24$^{ab}$ | 0.58±0.43$^{ab}$ | 0.73±0.11$^{ab}$ | 1.57±0.20$^{a}$ | 0.37±0.14$^{b}$ | 0.29±0.10$^{b}$ |

*Larval growth and biochemical analysis*

The effects of the dietary treatments on larval performance were presented in Table 2, in terms of dry weight (DW) in Senegalese sole at 15, 22, 31 and 38 dph. Other parameters of larval performance (growth in terms of standard length and specific growth rate, survival and maturation of the digestive system) have already been described in depth in Boglino *et al.* (2012). Larvae fed *Artemia* enriched with AGG presented significant higher DW at 22 and 38 dph (Table 2) and better overall performance at the end of the study (Boglino *et al.*, 2012) than larvae from the other dietary treatments, thus fish from the AGG diet were considered as a reference group for comparative purposes among different dietary treatments.

Total lipids from diets and larvae at 38 dph were extracted according to the method of Folch *et al.* (1957) and fatty acid compositions were determined as described in detail in Boglino *et al.* (2012), in triplicate for each experimental condition. Lipid class composition of enriched *Artemia* was determined by high-performance thin layer chromatography (HPTLC) (Olsen and Henderson, 1989). HPTLC plates (TLCP) were previously washed using hexane:diethyl ether (1:1), dried in an oven at 100 °C for 30 min and loaded with 1µL of total lipid solution (10 mg/mL), run in polar solvent (5 mL methyl acetate; 5 mL chloroform; 2 mL methanol and 1.8 mL 0.25% KCl) to two-thirds distance and desiccated for 30 min, then fully run in neutral solvent (16 mL hexane; 4 mL diethyl ether and 0.4 mL acetic acid glacial), desiccated for 30 min, sprayed with Fewster's reagent (3% (w/v) cupric acetate in 8% (v/v) aqueous orthophosphoric acid) and finally charred in an oven at 100 °C for at least 1 h to visualize lipid spots. Identification of lipid spots on the TLCPs was done using polar and neutral lipid standards (Sigma, catalog number P3817 and 1781, respectively). The concentration of each lipid class on TLC plates was determined by scanning the plates with a GS-800 Calibrated densitometer followed by image densitometry analysis (Quantity One, Bio-Rad, Inc, USA). Total lipid content, lipid class and fatty acid com-positions for enriched *Artemia* and Senegalese sole larvae at 38 dph are shown in Tables 1 and 2, respectively. The different enriching products did not affect significantly the lipid classes in enriched *Artemia*, in terms of phospho- and neutral lipids contents. At the end of the trial, no differences in total lipid content were observed among the fish fed different diets, although the fatty acids composition of the diet significantly affected the fatty acids profile in the whole fish.

*Sampling and histological analyses*

For histological purposes, five larvae from each experimental tank (n = 15 per dietary treatment) and different ages (15, 22, 31 and 38 dph) were randomly sampled before the first feeding of the day (around 15 h after the last feeding of the anterior day), euthanized with an overdose of tricaine methanesulphonate (MS-222, Sigma-Aldrich), fixed in 4% formalin buffered to pH7.0 with 0.1 M phosphate buffer for 24 h and preserved in 70% ethanol. Afterwards, samples were post-fixed in 1% osmium tetraoxide and 2.5% potassium dichromate for 8 h, washed in running tap water and dehydrated before the wax embedding procedure (Ortiz-Delgado *et al.*, 2008). Serial sections (6–7µm thick) were cut and mounted in gelatinized slides. In order to identify lipid inclusions, slides were cleared and mounted in Eukitt©. The areas of tissue sections showing fat inclusions stained in black (insolubilized neutral lipids by osmium tetraoxide) were measured and compared to the total areas of those sections. Histological images were obtained by light microscopy (Leica DM 2000™coupled to a dig-ital camera Leica DFC 420C™) and analyzed using the digital image analysis software ImageJ (U.S. National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/index.html). The quantification of the areas occupied by fat was conducted as described by Caballero *et al.* (2003) and Hulver *et al.* (2003). Briefly, fat deposits were assumed to be homogeneous in each specific tissue (intestine, liver and trunk musculature) and the area of fat deposits was based on the analysis of six randomly chosen fields for each target tissue. Fat deposition ($F_d$) in each target tissue was determined using the following formula: $F_d$ (%) = ($\Sigma$ $ARF_d$/$\Sigma$ $ART_t$) * 100, where $ARF_d$ is the area of black stained

fat deposits and $ART_t$ the total area of the target tissue in the optical field. In this study, steatosis was described as a non-pathological accumulation of lipids largely above the average in larval tissues from the different dietary treatments, due to an unbalanced dietary intake of lipids which saturated the physiological capability of the intestine, liver and vascular system to handle them, leading to lipid droplet accumulation (Spisni *et al.*, 1998).

**Table 2.** Dry weight (DW, in mg) at 15, 22, 31 and 38 dph (mean ± SD, n = 3) and total lipid (in $mg.g^{-1}$DW) and total fatty acids composition (in% TFA) at 38 dph (mean ± SD, n = 3) in Senegalese sole fed *Artemia* enriched with Aquagrow Gold© (AGG), Aquagrow DHA© (AGD), Easy Selco© (ES), Easy Selco© diluted by a half with olive oil (ES/2), Red Pepper© (RP) and Multigain© (MG). Totals include some minor components not shown. Superscripts denote significant differences among diets ($P$<0.05).

| | Dietary treatments | | | | | |
|---|---|---|---|---|---|---|
| | **AGG** | **AGD** | **ES** | **ES/2** | **RP** | **MG** |
| $DW_{15dph}$ | 0.13±0.02 | 0.15±0.02 | 0.09±0.01 | 0.15±0.03 | 0.13±0.02 | 0.10±0.02 |
| $DW_{22dph}$ | 0.64±0.09$^{a}$ | 0.50±0.05$^{b}$ | 0.52±0.06$^{b}$ | 0.56±0.06$^{b}$ | 0.51±0.04$^{b}$ | 0.50±0.04$^{b}$ |
| $DW_{31dph}$ | 0.99±0.47 | 1.07±0.35 | 0.71±0.22 | 0.83±0.37 | 0.85±0.40 | 0.91±0.44 |
| $DW_{38dph}$ | 2.01±0.42$^{a}$ | 1.68±0.45$^{b}$ | 1.07±0.25$^{c}$ | 1.33±0.26$^{c}$ | 1.25±0.41$^{c}$ | 1.63±0.47$^{b}$ |
| Total lipids ($mg \cdot g^{-1}$ DW) | 97.9±5.8 | 93.4±11.1 | 67.3±17.0 | 88.3±11.3 | 79.0±8.6 | 86.6±7.5 |
| Total FA ($mg \cdot g^{-1}$ DW) | 41.6±3.0$^{b}$ | 49.0±9.7$^{b}$ | 34.7±10.0$^{b}$ | 53.5±10.3$^{b}$ | 36.9±9.6$^{b}$ | 41.9±2.3$^{b}$ |
| Total saturated | 18.5±0.6$^{ab}$ | 16.1±2.0$^{bc}$ | 16.9±1.7$^{bc}$ | 13.6±0.3$^{c}$ | 20.7±1.6$^{a}$ | 18.4±0.5$^{ab}$ |
| 18:1n-9 | 30.3±1.2$^{bc}$ | 34.8±1.2$^{ab}$ | 32.1±4.9$^{bc}$ | 42.4±4.9$^{a}$ | 24.5±0.7$^{cd}$ | 21.5±1.6$^{d}$ |
| Total monounsaturated | 33.0±1.2$^{bc}$ | 37.6±1.5$^{b}$ | 35.9±5.1$^{b}$ | 46.4±4.3$^{a}$ | 27.1±0.6$^{cd}$ | 23.7±1.5$^{d}$ |
| 18:2n-6 | 5.8±0.8$^{c}$ | 6.8±0.4$^{bc}$ | 7.9±1.0$^{ab}$ | 9.2±0.1$^{a}$ | 7.9±0.4$^{ab}$ | 5.4±0.4$^{c}$ |
| 20:4n-6 | 2.1±0.1$^{b}$ | 0.5±0.1$^{d}$ | 1.1±0.2$^{c}$ | 0.9±0.2$^{cd}$ | 3.3±0.1$^{a}$ | 3.0±0.1$^{a}$ |
| Total n-6 PUFA | 12.5±0.6$^{b}$ | 8.1±0.7$^{d}$ | 10.2±1.3$^{cd}$ | 10.8±0.3$^{bc}$ | 16.6±1.0$^{a}$ | 14.9±0.6$^{a}$ |
| 18:3n-3 | 15.5±0.8 | 15.4±1.1 | 14.3±1.6 | 15.5±1.1 | 12.6±1.2 | 14.5±0.6 |
| 20:5n-3 | 2.3±0.2$^{b}$ | 2.6±0.4$^{b}$ | 5.1±0.7$^{a}$ | 3.4±0.5$^{b}$ | 3.0±0.2$^{b}$ | 3.2±0.3$^{b}$ |
| 22:6n-3 | 12.9±1.5$^{bc}$ | 15.1±1.7$^{b}$ | 10.4±1.9$^{c}$ | 5.6±1.4$^{d}$ | 15.3±0.7$^{b}$ | 20.0±1.6$^{a}$ |
| Total n-3 PUFA | 34.9±2.2$^{ab}$ | 37.2±3.1$^{ab}$ | 36.2±4.8$^{ab}$ | 28.6±3.5$^{b}$ | 34.6±2.1$^{ab}$ | 42.0±2.1$^{a}$ |
| Total PUFA | 47.5±1.6$^{ab}$ | 45.2±3.7$^{b}$ | 46.4±6.1$^{b}$ | 39.4±3.8$^{b}$ | 51.3±2.3$^{ab}$ | 56.9±2.0$^{a}$ |
| (n-3)/(n-6) | 2.8±0.3$^{c}$ | 4.6±0.3$^{a}$ | 3.6±0.0$^{b}$ | 2.7±0.3$^{cd}$ | 2.1±0.2$^{d}$ | 2.8±0.2$^{c}$ |
| DHA/EPA | 5.6±0.2$^{ab}$ | 5.8±0.6$^{ab}$ | 2.0±0.1$^{c}$ | 1.7±0.2$^{c}$ | 5.1±0.4$^{b}$ | 6.2±0.5$^{a}$ |
| ARA/DHA | 0.2±0.0$^{b}$ | 0.03±0.0$^{d}$ | 0.1±0.0$^{c}$ | 0.2±0.0$^{b}$ | 0.2±0.0$^{a}$ | 0.2±0.0$^{b}$ |
| ARA/EPA | 0.9±0.1$^{b}$ | 0.2±0.0$^{c}$ | 0.2±0.0$^{c}$ | 0.3±0.0$^{c}$ | 1.1±0.1$^{a}$ | 0.9±0.1$^{b}$ |
| OA/PUFA | 0.6±0.1$^{b}$ | 0.8±0.1$^{ab}$ | 0.7±0.2$^{b}$ | 1.1±0.2$^{a}$ | 0.5±0.0$^{b}$ | 0.4±0.0$^{b}$ |

*Statistics*

A nested-ANOVA was performed with “treatment” as the fixed-effects factor nested within the random-effects factor “tank”(triplicates per treatment for final DW variable, measured on 30–40 larvae per replicate) (Zar, 1999). Post-hoc Bonferroni's test was performed when significant differences were found at $P < 0.05$. However, for fatty acids quantification, the analysis was performed on a pool of individuals per replicate (just one value of this variable per replicate), thus a nested ANOVA cannot be carried out and instead was performed a one-way ANOVA among treatments, with a post-hoc Tukey's test when significant differences were found ($P < 0.05$).

Data in terms of differences in fat accumulation in target tissues along larval development and among different dietary treatments were expressed as means ± SD and compared by Two-Way ANOVA (arc-sine square root transformed data), considering diets and larval ages as factors. The Pearson product moment correlation test was used to measure the correlation of lipid accumulation among different target tissues. When a statistical correlation was detected be-tween these two variables, data were analyzed by means of linear regression and regression lines compared by ANCOVA (Zar, 1999). Data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test). The similarity among dietary treatments with regards to lipid accumulation in target tissues was evaluated by means of a cluster analysis. Average linkage was selected as the amalgamation rule, and Euclidean distances were used for computing distances between objects in the multidimensional space. Statistical analyses were conducted with IBM® SPSS® Statistics v18 (Somers, NY, USA).

**Results**

The main histological developmental events of the liver, intestine and vascular system observed in the present study between 15 and 38 dph were similar to those already described by Ribeiro *et al.* (1999) and Padrós *et al.* (2011). Figures 1 and 2 summarize the most relevant histological changes in the lipid deposition in the target tissues at 31 and 38 dph, showing differences in the size, distribution and localization of lipid deposits in those tissues among the dietary treatments.

At 31 dph (Figure 1), lipid distribution in the tissues varied depending on the dietary treatments. Larvae from all the dietary treatments showed a differential lipid distribution in the mid-intestine, in the supranuclear or the infranuclear portion of enterocytes from the intestinal mucosal or in the lamina propria-submucosal layer. Moreover, the lipid droplets detected in the enterocytes varied in size ranging from small spots to clear big deposits (diameter higher than 5µm). Sections in the mid-intestine of larvae from the AGG group (Figure 1k) showed large lipid deposits at the base of the folds, close to the lamina propria-submucosa and in the adjacent vasculature, but no lipid deposits were observed in the cytoplasm of the enterocytes. On the contrary, fat deposits in the mid-intestine of larvae from the ES/2 and AGD groups (Figures 1e, n) occupied more than 50% of the intestinal folds. The whole cytoplasm of enterocytes from both apical and basal parts of the intestinal folds was completely filled with small to medium size lipid deposits. Lipid deposits were larger in larvae from the AGD group than in larvae from the ES/2 group, measured from 5 to 15µm and were mostly observed in the supranuclear cytoplasm of the absorbing cells, but also in adjacent layers (in the lamina propria-submucosa and occasionally in the muscularis) in larvae from the AGD group (Figure 1n). Mid-intestine of larvae from the ES and RP groups (Figure 1b, h) showed high columnar enterocytes with small central nuclei. A small amount of fat appeared in scarce and small lipid globules in supranuclear position in enterocytes from the apical folds. Fat deposits were also detected in the adjacent submucosa and in the vasculature. Larvae from the MG group showed scarce medium-size lipid

deposits in enterocytes (Figure 1q), more abundant and larger than those observed in larvae from the ES and RP groups.

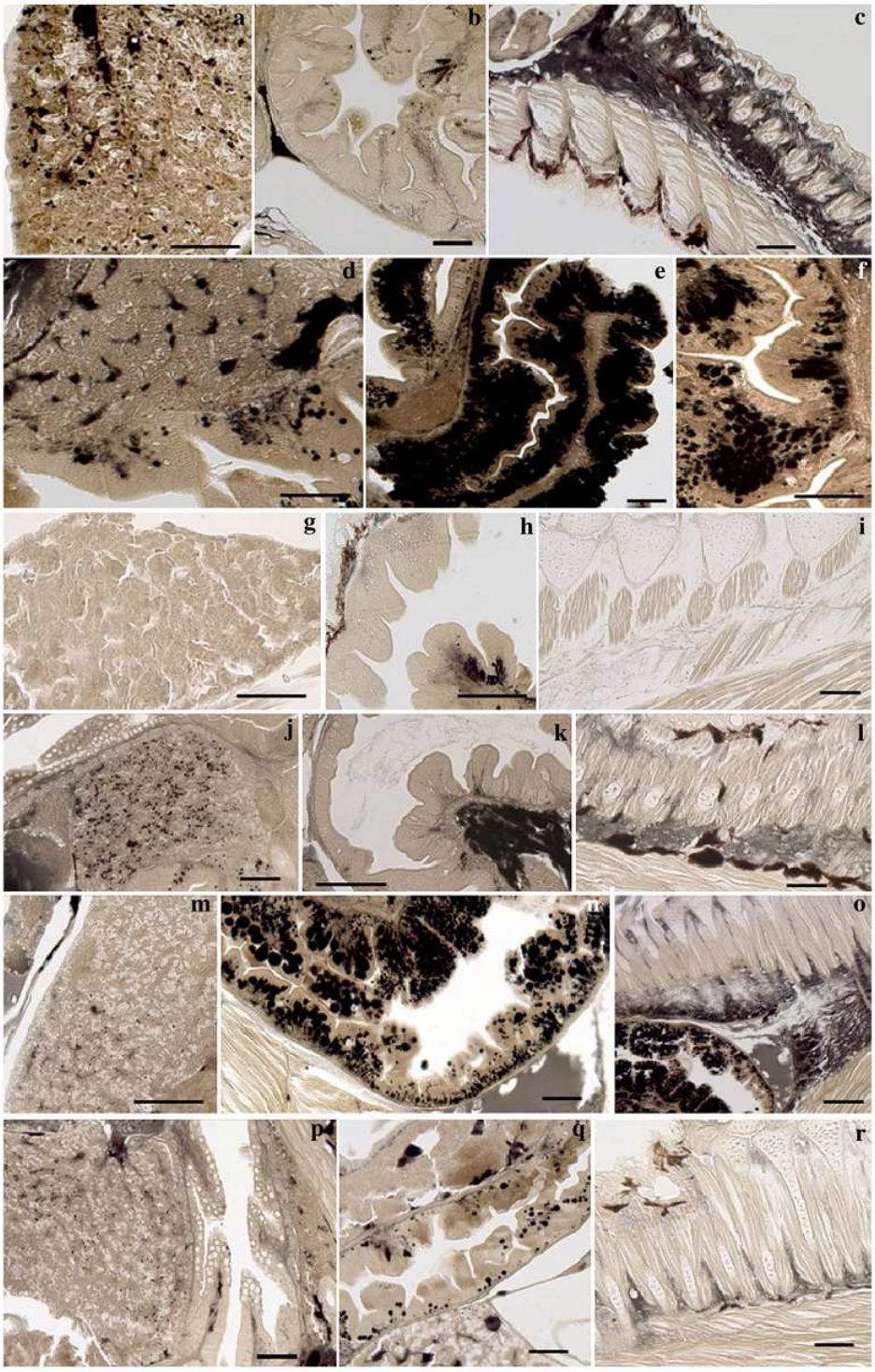


**Figure 1.** Longitudinal paraffin sections of the liver (left side), mid-intestine (middle) and vascular system (right side) of Senegalese sole larvae at the end of metamorphosis (31dph) fed the six dietary treatments (ES: a to c; ES/2: d to f; RP: g to i; AGG: j to l; AGD: m to o and MG: p to r) showing different levels of neutral lipid accumulation (in black). Scale bar represents 50µm.

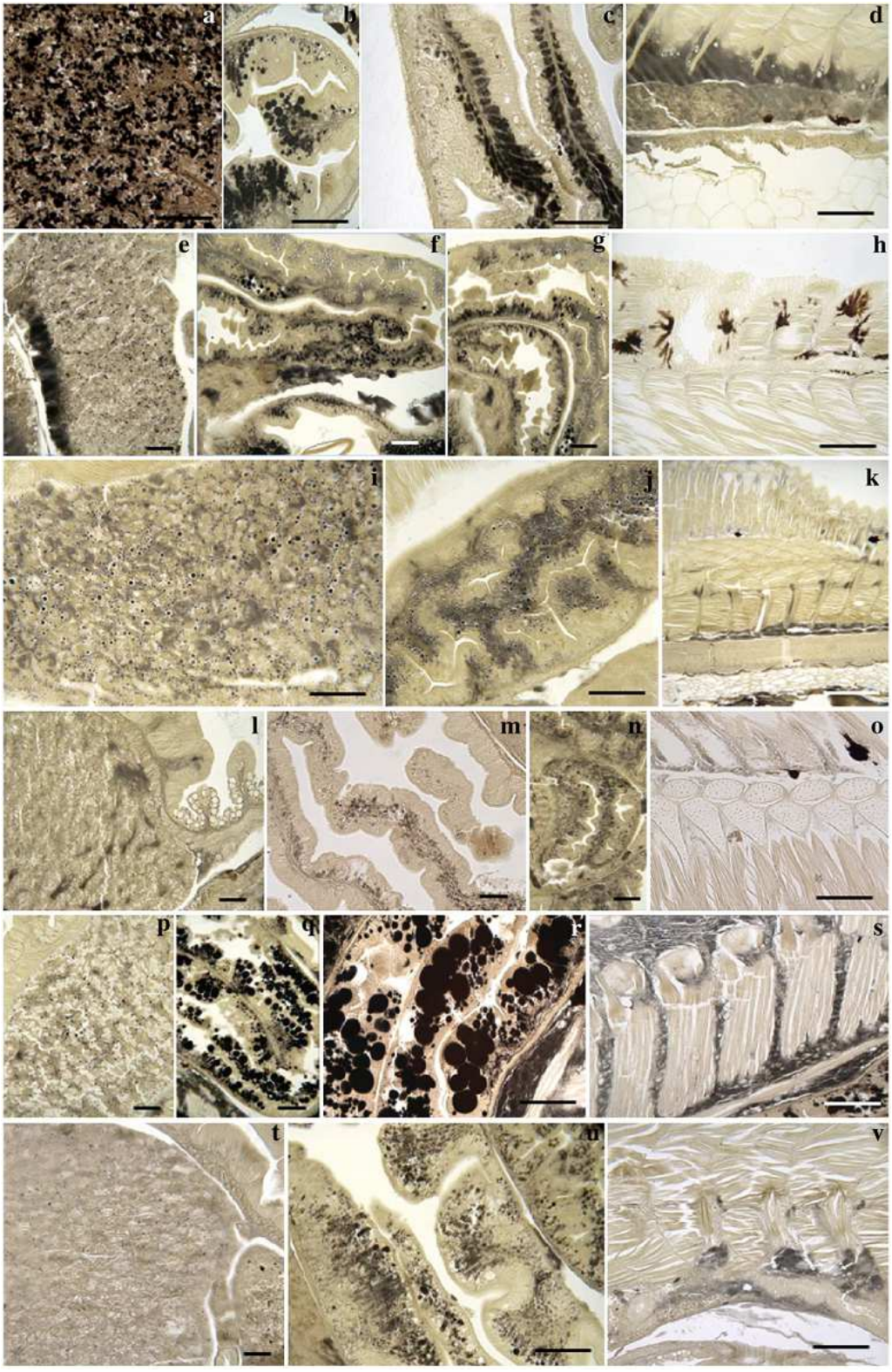


**Figure 2.** Longitudinal paraffin sections of the liver (left side), mid intestine (middle) and vascular system (right side) of Senegalese sole early juveniles at the post-metamorphic stage (38 dph) fed the six dietary treatments (ES: a to d; ES/2: e to h; RP: I to k; AGG: l to o; AGD: p to s and MG: t to v) showing different levels of neutral lipid accumulation (in black). Scale bar represents 50µm.

Liver of larvae from the AGG diet (Figure 1j) showed regular and homogeneous shaped hepatocytes with large nuclei. The cell of the hepatic parenchyma presented a uniform distribution of small lipid globules detected as small black spots spreading along the entire parenchyma. The size of lipid droplets in larvae from the ES group (Figure 1a) varied in the row of hepatocytes preferentially adjoining the vasculature. Lipid deposition was also detected in the plasma filling the vascular lumen. Similar low accumulation of lipids was detected in liver of larvae from the AGD and MG groups (Figures 1m, p). In larvae fed the ES/2 diet (Figure 1e), lipid deposits were preferentially detected in the vascular lumen with a clear dilation of the lipid-filled vessels without fat accumulation in hepatocytes. Finally, larvae from the RP group did not show any lipid deposition in the hepatocytes nor in the vasculature (Figure 1g).

Fat deposits in vascular system sections of 31 dph-larvae were preferentially located within the collagen myoseptum spaces. The largest deposits were detected in larvae from the ES and AGD groups (Figures 1c, o), being intermediate in larvae from the ES/2 and AGG groups (Figures 1f and l) and minor in larvae from the RP and MG groups (Figures 1i, r).

At 38 dph (Figure 2), fat lipid deposits in the target tissues presented a similar histological profile than at 31 dph. Deposition of lipids in the intestine offish from the AGG group was localized at the infranuclear position in the form of small globules (Figures 2m, n). Similar profiles of lipid accumulation were detected in the intestine of fish fed the RP diet (Figure 2j), but with more lipid deposits than in fish from the AGG group. Fish from the ES/2 and AGD groups showed the highest intestinal fat deposition (Figures 2f, g, q, r), mostly in the form of big lipid de-posits from 5 to 15µm at the supranuclear position probably formed as a coalescence of smaller ones and also with smaller lipid globules at the infranuclear position. Fat deposits were detected both in the adjacent layers and in the vasculature. To a lesser extent, fish fed the ES and MG diets also presented large lipid deposits at the supra-nuclear position and smaller ones at the infranuclear position (Figure 2b, c, u), but with more small lipid globules in enterocytes of juveniles from the MG group than in those offish from the ES group.

Despite the presence of some lipid deposits in the parenchymal sinusoids, no lipid accumulation was detected in hepatocytes of fish fed the AGG diet (Figure 2l). The highest hepatic fat distribution was detected in fish fed the ES diet (Figure 2a), followed by fish fed the ES/2 and RP groups (Figures 2e, i). Fat deposits in the liver of juveniles from the ES group occupied the whole cytoplasm of hepatocytes, in the form of lipid deposits larger than 5µm, and displaced the nucleus to the periphery of the cell (Figure 2a). The fat deposits were homogeneously distributed along the whole hepatic parenchyma. Similar profiles of fat distribution were detected in the livers of both fish from the ES/2 and RP groups (Figures 2e and j), but the lipid deposits were smaller in fish fed the ES/2 diet than in fish fed the ES diet. Hepatic lipid deposits in fish fed the AGD diet consisted of small lipid

globules homogeneously distributed along the parenchyma (Figure 2p). No accumulation of lipids was detected in liver offish from the MG group, nor in hepatocytes or the vascular system (Figure 2t).

The fat accumulation profile in the vascular system of 38 dph-juveniles was similar to the profile observed at 31 dph, with fish from the ES and AGD groups presenting the highest intermyotomal fat deposition (Figures 2d and s) and fish from the ES/2, RP, AGG and MG groups showing lower lipid deposition in the vascular system (Figures 2h, k, o, v).

Profiles of fat accumulation ($F_d$) in the intestine, the liver and the vascular system along larval development at 15, 22, 31 and 38 dph for the six dietary treatments are shown in Figure 3. Larvae from the ES/2 and AGD groups presented a severe intestinal steatosis at 31 dph ($F_d$ = 71.0 ± 2.0% and 77.7 ± 0.8% respectively vs. 47.5 ± 11.3% in average) and 38 dph ($F_d$ = 62.0 ± 2.2% and 53.8 ± 1.6% respectively vs. 34.6 ± 8.1% in average). Larvae from the AGG and ES/2 groups showed moderate hepatic steatosis in liver at 31 dph ($F_d$ = 33.4 ± 1.0% and 25.5 ± 1.1% respectively vs. 16.9 ± 5.2% in average), whereas fish from the ES group showed severe hepatic steatosis at 38 dph ($F_d$ = 40.5 ± 1.0% vs. 17.8 ± 5.5% in average). Finally, larvae from the ES and AGD groups presented a severe accumulation of neutral lipids in the vascular system at 31 ($F_d$ = 14.4 ± 0.7% and 14.6 ± 0.6% respectively vs. 6.4 ± 2.6% in average) and 38 dph ($F_d$ = 35.8 ± 1.0% and 31.2 ± 1.2% respectively vs. 18.6 ± 5.4% in average).

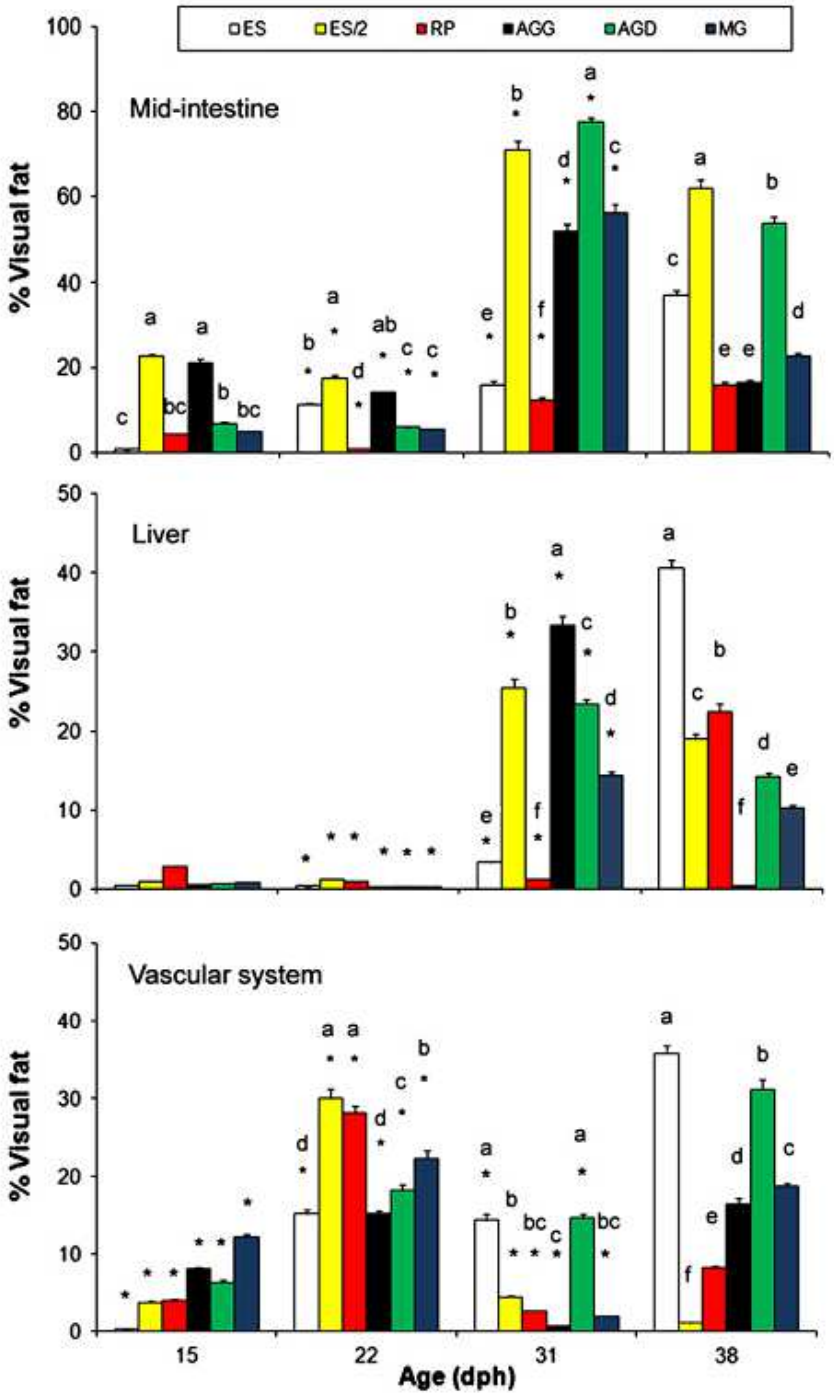


**Figure 3.** Morphometrical quantitative estimates of accumulated neutral lipids in the mid intestine, liver and vascular system of Senegalese sole larvae fed the six dietary treatments at 15, 22, 31 and 38 dph. Each bar represents mean values ± S.E.M (n = 18). Different letters indicate significant differences (two-way ANOVA, $P < 0.05$) among groups within the same age. Asterisks represent significant differences between one develop-mental stage and the next one, within the same dietary treatment (for instance, an asterisk put above ES bar at 22 dph for the mid-intestine means that this value was significantly different from the one of fat deposit in the mid-intestine for larvae from ES group at 31 dph).

**Table 3.** Results of the two-way ANOVA analysis comparing values of the morpho-metrical quantitative estimation of accumulated lipids in each target tissue (mid-intestine, liver and vascular system) in Senegalese sole larvae fed different diets, considering the larval age and the diet as factors for the analysis.

| | Age | | Diet | | Age x diet | |
|---|---|---|---|---|---|---|
| **Tissues** | *F* | *P* | *F* | *P* | *F* | *P* |
| Intestine | 1274.63 | <0.001 | 391.48 | <0.001 | 123.65 | <0.001 |
| Liver | 1426.98 | <0.001 | 49.74 | <0.001 | 285.17 | <0.001 |
| Vascular system | 638.03 | <0.001 | 72.79 | <0.001 | 122.21 | <0.001 |

Results of the Two-Way ANOVA indicated that the level of lipid accumulation in the target tissues depended (1) on the larval stage of development, (2) the diet and (3) the interaction between these two factors (Table 3). Independently of the dietary group considered (factor = age), the level of lipid accumulation in the liver and intestine followed the same pattern (Figures 3a, b) with significantly similar lipid deposits from the onset of larval metamorphosis at 15 dph until its climax at 22 dph ($P > 0.05$), a significant increase from 22 dph to the end of metamorphosis at 31 dph ($P < 0.001$) and a significant decrease from 31 dph to the post-metamorphic stage at 38 dph ($P < 0.001$). In contrast, the changes in fat accumulation in the vascular system followed a different pattern (Figure 3c). Thus, lipid deposits significantly increased between 15 and 22 dph ($P < 0.001$), were significantly reduced until 31 dph ($P < 0.001$) to levels significantly similar to those recorded at the onset of metamorphosis at 15 dph ($P > 0.05$) and significantly increased between 31 dph and the post-metamorphic stage at 38 dph ($P < 0.001$). The level of lipid accumulation in target tissues was also affected by the dietary treatments (factor = diet, Table 3). Intestinal lipid accumulation (Figure 3a) was significantly different depending on the experimental group ($P < 0.001$). In the liver (Figure 3b), only fish from the ES and ES/2 groups, and from the RP and MG groups showed similar fat accumulation patterns ($P > 0.05$), whereas it was significantly different for the rest of the groups ($P < 0.001$). The degree of lipid accumulation in the vascular system (Figure 3c) was similar among fish from the AGG, RP and ES/2 groups ($P > 0.05$), while the rest of dietary groups were significantly different ($P < 0.001$). Considering the interaction between both factors (age × diet), significant differences exist in the level of lipid deposition during larval ontogeny depending on the tar-get tissue and diet (Table 3). Larvae from the ES and RP groups showed similar patterns of lipid accumulation in the intestinal mucosa (Figure 3a), with similar amounts between the onset of metamorphosis at 15 dph to its climax at 22 dph ($P > 0.05$), then increasing until the post-metamorphic stage ($P < 0.001$). In the case of the fish from the ES/2, AGG, AGD and MG groups, intestinal lipid deposits increased until the end of metamorphosis at 31 dph and then significantly de-creased at 38 dph ($P < 0.001$). In all the treatments, the hepatic accumulation of lipids (Figure 3b) remained constant from 15 to 22 dph ($P > 0.05$) and then increased significantly until the end of the study in fish fed ES and RP, but not in the rest of the groups. In fish fed ES/2, AGG, AGD and MG diets, liver deposits increased until 31 dph and then significantly decreased at the post-metamorphic stage ($P < 0.001$), in a particularly strong way for fish fed AGG

diet. Lipid deposits in the vascular system (Figure 3c) increased from 15 to 38 dph in all dietary groups ($P < 0.001$), with the exception of ES/2 which decreased between 31 and 38 dph.

Figure 4 integrates the results of the hierarchical cluster analysis of the lipid deposition in the intestine, liver and vascular system of larvae from the six dietary treatments at 15, 22, 31 and 38 dph. Larvae from each experimental tank were gathered according to the similarity in their fat accumulation profile, indicating tissue- and diet-specific differences in lipid accumulation as detailed in Figure 4and Table 3. Three primary clades can be distinguished, with one clade (ES–RP) being a sister clade of the other major clade, which is com-posed of two subclades (MG–AGG and ES/2–AGD, respectively). All the experimental replicates fell into their own respective subclades for each dietary treatment.

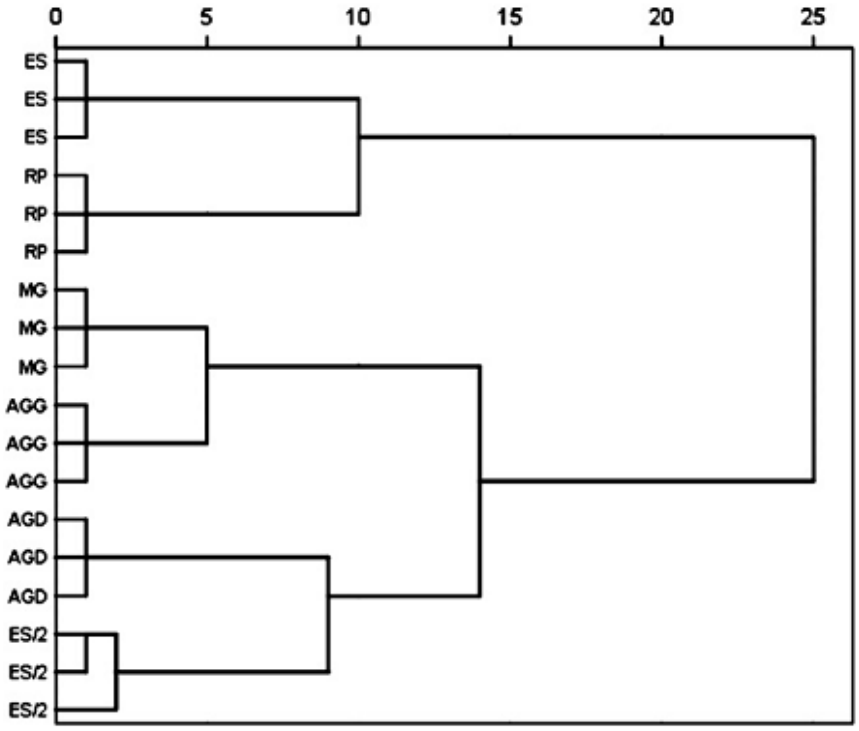


**Figure 4.** Result of the cluster multivariate analysis including tissue and developmental stage data of fat accumulation in Senegalese sole larvae fed the six dietary treatments. Dietary treatments and replicates are represented on the X-axis and the percentage of similarity among fish from the different tanks on the Y-axis.

Independently of the dietary group and larval age considered, there was a positive and significant correlation between the levels of intestinal and liver fat accumulation ($F_d$; n = 432, r = 0.696; $P < 0.001$, Figure 5). No significant correlation was found between the degree of lipid accumulation ($F_d$) in the vascular system and liver ($P > 0.05$), whereas a negative and weak significant correlation was found between the levels of fat in the vascular system and the intestinal mucosa ($F_d$; n = 432, r = -0.104, $P = 0.031$). When the levels of lipid deposition were analyzed considering dietary treatments,

lipid accumulation in the intestine and liver varied depending on the experimental group, as indicated by the slope values (b) of the regression lines (Figure 5). In this sense, larvae fed *Artemia* enriched with AGD, MG and ES/2 tended to accumulate higher levels of lipids in the intestinal mucosa rather than in the liver (1.9 < b < 3.0). In contrast, fish from the ES and RP groups followed the opposite trend (b < 0.7), whereas larvae from the AGG group showed relatively similar levels of lipid accumulation in the intestine than in the liver (b = 1.0).

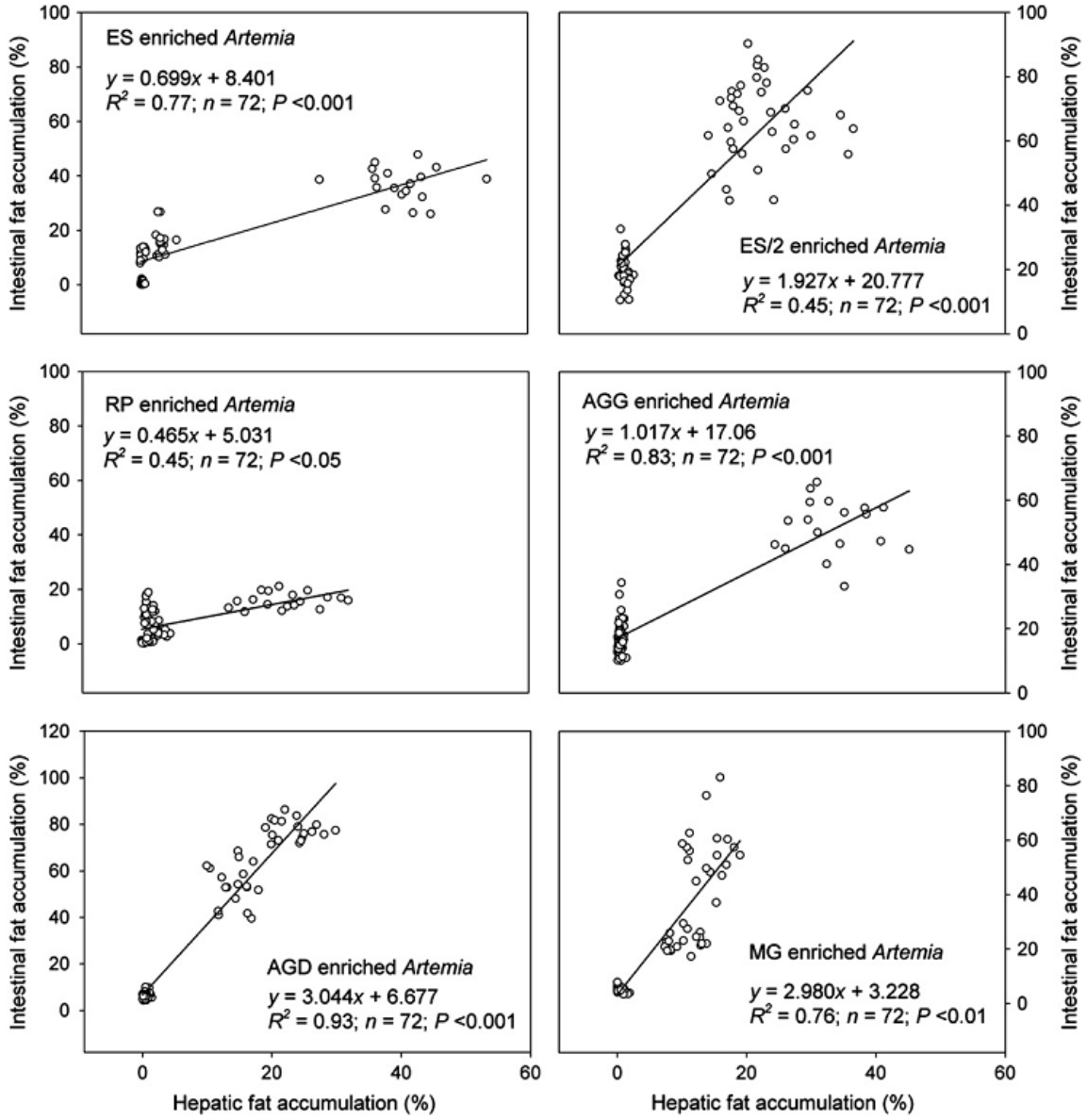


**Figure 5.** Linear regression between the level of fat accumulation in the mid-intestine and liver in Senegalese sole larvae fed the six dietary treatments. Plotted data of fat accumulation correspond to values recorded at different sampling times (15, 22, 31 and 38 dph).

**Discussion**

In this study, we evaluated the effects of different diets (enriched *Artemia*) differing in their fatty acid profile on the lipid accumulation in selected target tissues (intestine, liver and vascular system) of Senegalese sole. Results showed that the profile of fat accumulation in these three tissues was significantly affected by the diet (fatty acid profile), the developmental stage of the fish (pre-metamorphosis, metamorphosis or post-metamorphosis), as well as by the interaction between these two factors that were responsible for changes in the pattern of fat deposition in the above-mentioned tissues.

*Changes in fat deposition during Senegalese sole larval development*

In all dietary groups, fat accumulation in the intestine and liver remained low during pre-metamorphosis and metamorphosis (15–22 dph), progressively increased until the end of the metamorphosis (31 dph) and slightly decreased at post-metamorphosis (38 dph), with the exception of fish fed *Artemia* enriched with ES. This trend is consistent with existing data on the process of maturation of the digestive system occurring in this species at these stages of development (Ribeiro *et al.*, 1999). During the process of metamorphosis, the digestive tract of Senegalese sole elongates, with a clear increase in the intestinal absorptive surface and the liver increases in size (Sarasquete *et al.*, 1996). These histomorphological changes are coupled with the maturation of enterocytes and the acquisition of an adult mode of digestion at the end of metamorphosis (Ribeiro *et al.*, 1999). Thus, the accumulation of lipids in the intestinal mucosa, considered as an indicator of luminal absorption and temporal storage of lipids (Sarasquete *et al.*, 1995; Gisbert *et al.*, 2004), reflected the morpho-physiological development of the intestine and was positively correlated with changes in the degree of lipid deposits in the liver at pre- and metamorphic stages in Senegalese sole, confirming that this organ responds sensitively to changes in the diet (Hoehne-Reitan and Kjørsvik, 2004).

Metamorphosis is a key process occurring at early stages of flatfish larvae, including many physiological and anatomical changes. Thus, metamorphosis and settlement are extremely energetically demand-ing processes due to the extra requirements of physical remodeling (body asymmetry), hormone production and energy acquisition for growth (see review in Geffen *et al.*, 2007). In this study, most of the larvae from all dietary treatments started to metamorphose around 15 dph, reaching the climax of metamorphosis at ca. 22 dph, and completing it at 31 dph (Boglino *et al.*, 2012). Similar to other studies, during metamorphosis, food ingestion and growth were significantly reduced in Senegalese sole larvae (Parra *et al.*, 1999; Cañavate *et al.*, 2006), which might explain the low lipid accumulation levels observed in the intestine and liver between 15 and 22 dph. During this pre-metamorphic stage, the energy stored in the liver might be mobilized to complete larval metamorphosis (Brewster, 1987). Once the metamorphosis was accomplished atca.31 dph, Senegalese sole juveniles resumed their feeding behavior as seen by the increase and higher accumulation of lipids in the intestine with regards to the liver, indicating that at that developmental stage the intestinal mucosa acted as a temporary site for lipid storage. High feeding rates observed

during this period (Cañavate *et al.*, 2006) would result in the capacity of the intestine to transport the absorbed lipids to the liver being surpassed (Segner *et al.*, 1994; Gisbert *et al.*, 2005, 2008). After metamorphosis, dietary lipids would be mainly used as a source of energy for tissue differentiation and somatic growth in early juveniles (Brewster, 1987), which would have resulted in a reduction of lipid deposits in all the experimental groups, with the exception of fish fed *Artemia* enriched with ES, where fat deposits still increased. This differential trend in fat accumulation at post-metamorphic stages in fish fed ES-enriched *Artemia* is discussed in the following section.

In this study, fat deposits in the vascular system were interpreted as transported lipids in the form of chylomicrons to the liver where they are going to be metabolized and stored, after being absorbed by the intestine. The size of the chylomicrons depends on the concentration of dietary unsaturated fatty acids, the degree of unsaturation and the metabolic pathway through which the fatty acids are esterified (Sire *et al.*, 1981). In this study, fat accumulation in the vascular system during larval development presented a different pattern than that of the intestine and liver for all dietary treatments. Low lipid deposition in the vascular system at 15 dph reflected the low absorption and accumulation of fat in the intestine. Although lipid deposition was similar at 15 and 22 dph in the intestine and liver, it significantly increased in the vascular system at the climax of metamorphosis, suggesting that lipid exportation to developing tissues could be enhanced during this critical phase of the flatfish development. The resumption of a higher ingestion rate at the end of the metamorphosis (31 dph) resulted in an increase of fat accumulation in the intestine, and a decrease of lipid deposits in the vascular sys-tem, except in larvae from the ES and AGD treatments. Histological observations suggested that lipids were nevertheless transported through the circulatory system towards the liver, as shown by the in-crease of hepatic fat deposits at this developmental stage. Finally, fat deposition in the vascular system significantly increased between 31 and 38 dph in fish from all dietary treatments, particularly in the ES and AGD groups. An adaptation period seems to be necessary for the vascular system to recover the capacity to transport lipids absorbed in the intestine towards the liver. The particular profile of fat accumulation in the vascular system of fish fed ES- and AGD-enriched *Artemia* are discussed in the next section.

*Effects of dietary fatty acid profiles on fat deposition in Senegalese sole larvae and early juveniles*

The intestine and the liver are considered reliable nutritional and physiological biomarkers because their histological organization is very sensitive to dietary changes. Thus, the size and type of lipid inclusions in the enterocytes is dependent on the diet and the size of lipoprotein particles increasing with the fat content of the feed and the degree of unsaturation of the lipids ingested (see reviews in Hoehne-Reitan and Kjørsvik, 2004; Gisbert *et al.*, 2008). The histological organization of the liver, notably the fat deposition in hepatocytes, accurately reflects any physiological disorder originated from a nutritionally unbalanced diet or unsuitable feeding conditions (Segner *et al.*, 1994; Mobin *et al.*, 2000, 2001; Gisbert *et al.*, 2008).

Dietary phospholipids have been found to greatly affect lipid digestion, absorption and transport in cultured fish, as it is generally accepted that they have a beneficial effect on lipid emulsification in the intestinal lumen (Tso, 1994), whereas their absence in the diet may lead to morphological alterations in the epithelial gut (Liu *et al.*, 2002). Under the present experimental conditions, enriched *Artemia* from different nutritional groups showed similar total lipid content, as well as similar values of polar and neutral lipids; thus differences in fat deposition and steatosis among dietary groups were not linked to their different total lipid levels and/or class lipid content, as several studies have reported in common carp (Fontagné *et al.*, 1998), gilthead sea bream (Salhi *et al.*, 1999), European sea bass (Gisbert *et al.*, 2005), Atlantic cod (Wold *et al.*, 2007), Atlantic herring and Senegalese sole (Morais *et al.*, 2006). Consequently, in this study the pattern of fat deposition in the target tissues has been investigated considering the different fatty acid profile of different diets.

Under this context and for comparative purposes among dietary treatments, the pattern of fat deposition in target tissues offish fed AGG-enriched *Artemia* was considered as a reference, since those fish showed the best somatic growth, maturation of the digestive system and level of skeletal ossification (Boglino *et al.*, 2012). An im-balance in the composition of the diet can modify the larval capacity to absorb and export lipids through the circulatory system towards the liver to be stored and mobilized for larval development when needed (Tso, 1994). In this study, larvae fed ES- and RP-enriched *Artemia* tended to accumulate lower levels of fat in the intestine and liver than larvae from the AGG treatment at 31 dph. Such differences might be explained by the delayed maturation of the digestive tract in these larvae (Boglino *et al.*, 2012), which affected the capacity of the intestinal mucosa to absorb and mobilize lipids to the liver. In addition, post-metamorphic fish fed *Artemia* enriched with ES and RP tended to accumulate higher levels of dietary lipids in the liver than in the intestine, contrary to the larvae from the AGG group that accumulated similar amounts of lipids in both tissues, indicating a more balanced metabolism (Tso, 1994; Tocher, 2003). Early juveniles fed AGG-enriched *Artemia* seem to have an optimal mobilization of dietary lipid energy from the target tissues and especially from the liver to promote growth and achieve metamorphosis, although the decrease in hepatic fat observed between 31 and 38 dph might be a consequence of a higher growth and a more advanced development of these fish compared to those from other treatments. The high degree of fat deposition in all target tissues observed in fish fed ES-enriched *Artemia* compared to the AGG control group has produced a physiological hepatic steatosis, not affecting the survival (Spisni *et al.*, 1998). Similarly, an increase in lipid deposition in the liver of post-metamorphic larvae from the RP group together with lower lipid mobilization for growth might explain the lower somatic growth and underdevelopment of these fish. Considering the former authors, a problem in the regulation of fat storage due to an imbalance in the fatty acid profile of the *Artemia* enriched with ES probably existed. Thus, an excess in EPA (145% higher) and a deficiency in DHA (42% lower) in the ES diet compared to the AGG diet, combined with the well known incapacity of marine fish larvae to convert EPA into DHA by desaturation and elongation (Sargent *et al.*, 2002) may have imbalanced the DHA/EPA ratio (77% lower than in AGG diet) on one hand, and may also have disrupted the ARA/EPA ratio (70%

lower than in AGG diet), essential in eicosanoids synthesis (Sargent *et al.*, 2002). These nutritional imbalances provide a possible explanation for the high fat deposition observed in the liver of fish fed ES-enriched *Artemia* even leading to saturation in the capacity of their vascular system to transport lipids. In addition, *Artemia* enriched with ES contained more MUFA, particularly in the form of oleic acid (OA) and less PUFA that the AGG-enriched *Artemia*. Considering that OA is generally used for energetic purposes, whereas PUFA are mainly used for biochemical, cellular and physiological functions (see reviews in Sargent *et al.*, 1999; Tocher, 2003), such an unbalance in the OA/PUFA ratio (38% higher in the ES than in the AGG diet) would have disrupted the balance between energy and essentiality, leading to an excessive fat accumulation in the liver and vascular sys-tem in fish fed the ES diet. Spisni *et al.* (1998) observed that teleost liver can tolerate moderate variations in the n-3/n-6 PUFA and MUFA/PUFA ratios, thus, an increase in dietary MUFA would have affected DHA esterification and induce hepatic steatosis. Similar observations were reported in fish fed RP-enriched *Artemia*, although the degree of fat accumulation in the target tissues was lower than in fish from the ES group. In both cases, larval growth performance and development were similarly affected by the diet (Boglino *et al.*, 2012), but the level of lipid deposition in the intestine, liver and vascular system was less modified in fish fed the RP diet, as a result of a more balanced fatty acid profile than in ES diet compared to the AGG diet. *Artemia* enriched with RP showed 23 and 30% lower DHA/EPA and OA/PUFA ratios, and 65 and 30% higher EPA levels and ARA/EPA ratio than the AGG diet.

Regarding fish fed *Artemia* enriched with ES/2 and AGD, animals of both treatments showed the highest levels of intestinal fat deposits and moderate hepatic steatosis at metamorphic and post-metamorphic stages in comparison to the AGG group and the rest of dietary treatments; although the fatty acid profile of these two diets were quite different. In this sense, the ES/2 diet contained ca. 65% more MUFA, especially OA, than the AGG diet and 40% lower amounts of PUFA, including 57 and 86% less ARA and DHA, respectively. This FA profile resulted in different OA/PUFA and ARA/DHA ratios (196% and 175% higher than the AGG diet, respectively), and also in the n-3/n-6 HUFA, DHA/EPA and ARA/EPA ratios, which were 29, 87 and 61% lower than in the AGG diet, respectively. The higher proportion of OA with regards to the PUFA content in *Artemia* enriched with ES/2 have inversed the OA/PUFA ratio to a value higher than 1, that might have affected the absorption, transport, storage and mobilization of dietary lipids. Although MUFA are considered to be used for energy more easily than PUFA (Tocher, 2003), this diet clearly presented an excess of OA that might have impaired lipid transport from the intestinal mucosa to the liver, resulting in intestinal steatosis. Thus, partial replacement of fish oil by olive oil rich in OA in the ES/2diet has affected lipid intestinal absorption leading to intestinal steatosis, and should be performed with caution, as suggested by Sargent *et al.* (2002) about vegetal oil in diets for marine fish larvae. In addition, fish fed ES/2 and AGD diets showed moderate levels of hepatic steatosis. The inclusion of vegetal oils in the diet has been associated with hepatic steatosis in gilthead sea bream juveniles (Alexis, 1997; Caballero *et al.*, 2003, 2004). As previously reported for the ES group, high levels of dietary OA might be responsible for hepatic steatosis; in this case, not only high OA levels but also the disruption of the OA/DHA ratio

might have been responsible for the moderate hepatic steatosis found in fish fed *Artemia* enriched with ES/2, as supported by the results of Spisni *et al.* (1998). These authors, as well as Yang *et al.* (1990) and Braunbeck *et al.* (1990), pointed out that a high dietary OA/DHA ratio coupled with a lack of adaptative peroxisomal proliferation in teleost fish was the primary cause of lipid droplets formation leading to hepatic steatosis. In addition, the imbalance in the OA/MUFA ratio coupled with the decrease in n-3 and n-6 HUFA, particularly in DHA and ARA, might also account for the reduced mobilization of lipid reserves in the liver offish from the ES/2 diet after metamorphosis, leading to lower growth and development (Boglino *et al.*, 2012), as observed in gilthead sea bream juveniles fed an EFA deficient diet (Montero *et al.*, 2001). Although fish fed the AGD diet showed comparable severe intestinal steatosis and moderate hepatic steatosis as fish fed the ES/2 diet and similar severe steatosis in the vascular system as fish fed the ES diet, the differences in the fatty acid profile of AGD diet compared to the AGG diet were less important than those in ES and ES/2 diets. The AGD diet has the same OA/PUFA ratio than the AGG diet, but a large deficiency in ARA (71% less than the AGG diet) and subsequently lower ARA/DHA and ARA/EPA ratios (88% and 83% lower, respectively) that might have been responsible for the higher accumulation of lipids in the intestine and the vascular system. These results are in agreement with those by Fountoulaki *et al.* (2003) who observed an increasing trend of lipid deposition in the muscle of gilthead sea bream juveniles with decreasing dietary ARA levels. Light modifications in the amounts of just one EFA seem to be enough to disrupt the profile of the diet, imbalance the accumulation of lipids in larval tissues and change their mobilization for proper growth and development.

Finally, fish fed MG-enriched *Artemia* presented a similar pattern of fat accumulation in the intestine and liver as fish from the AGG diet, except for a relatively lower mobilization of hepatic lipids be-tween 31 and 38 dph. Although the MG diet had 28% less MUFA and 20% more PUFA (mainly due to 32% less OA and 79% more DHA) as the AGG diet, the n-3/n-6PUFA, DHA/EPA, ARA/EPA and ARA/DHA ratios were similar and only the OA/PUFA ratio was 51% lower than in the AGG diet. In comparison to fish oil-based emulsions (ES, ES/2 and RP), both AGG and MG diets, which are products including spray-dried single cell organisms for enriching live prey, showed the best results in terms of lipid absorption, storage and mobilization, as indicated by the histological organization of target tissues. In this sense, products based on single-cell heterotrophs have been shown to meet larval requirements in terms of EFA profile and ratios and show potential for the substitution or replacement offish-based ingredients in larval diets (Harel *et al.*, 2002). Although AGD is also an enriching product made of dried single-cell heterotrophic organisms as is AGG and MG, fish fed AGD-enriched *Artemia* had a completely different pattern of fat deposition, with large amounts of lipids in the intestine and vascular system at metamorphic and post-metamorphic stages. These results indicate that rather than the raw material and/or the lipid source used for enrichment (single-cell organisms in the case of AGG and AGD products, or a mixture of fish oil and single-cell organisms like MG), it is the fatty acid profile and the EFA balance of the product that matters for proper lipid metabolism, at least in the case of Senegalese sole larvae and early juveniles.

## Conclusion

Senegalese sole larvae and early juveniles fed *Artemia* enriched with different products showed significantly different patterns of fat (neutral lipids) accumulation in their intestine, liver and vascular sys-tem during their development. As fish fed the AGG diet showed the best larval performance, the pattern of fat deposition in larval tissues was considered as the most appropriate for proper growth and development and the FA profile of AGG diet was considered as the most balanced for Senegalese sole larvae among all tested diets. Optimizing larval diets requires a careful balance of all the component nutrients. A variation, even slight, in the EPA, DHA or ARA levels or in the EPA/DHA, ARA/EPA, ARA/DHA, (n-3)/(n-6) HUFA ratios and particularly in the OA/PUFA ratio might have modified the metabolism of lipids and disrupted the profile of lipid accumulation in the target tissues, leading to intestinal and hepatic steatosis. The steatosis observed in the intestine of larvae fed the ES/2 and AGD diets, in the liver of fish fed the ES diet and in the vascular system of fish fed the ES and AGD diets could not be considered as pathological because it did not affect the survival of the fish; nonetheless, this elevated deposition of lipids in the target tissues negatively affected larval growth and development.

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# 3

# Molecular regulation of both dietary vitamin A and fatty acid absorption and metabolism associated with larval morphogenesis of Senegalese sole (*Solea senegalensis*)

Maria J. Darias[1]
Anaïs Boglino[1]
Manuel Manchado[2]
Juan B. Ortiz-Delgado Juan[3]
Alicia Estévez[1]
Karl B. Andree[1]
Enric Gisbert[1]

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[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain
[2] IFAPA Centro El Toruño, Junta de Andalucía, Camino Tiro de pichón s/n, 11500 El Puerto de Santa María, Cádiz, Spain
[3] Instituto de Ciencias Marinas de Andalucia (CSIC), Campus Universitario Río San Pedro, Apdo Oficial, 11510 Puerto Real, Cádiz, Spain

**Regulación molecular de la absorción de vitamina A y de ácidos grasos de la dieta y del metabolismo asociado con la morfogénesis larvaria del lenguado Senegalés (*Solea senegalensis*)**

**Resumen**

El presente estudio tiene como objetivo ampliar el conocimiento de los mecanismos moleculares que rigen la absorción y el metabolismo de ciertos nutrientes que influyen sobre el crecimiento y el desarrollo larvario del lenguado senegalés. El uso de *Artemia* enriquecida con Easy Selco© (ES, INVE) y Aquagrow Gold© (AGG, ABN) con diferentes niveles de vitamina A (VA) y distintas composiciones de ácidos grasos fueron probado en larvas de 22, 30 y 38 días post-eclosión (dpe) y el efecto de estos enriquecedores fue analizado en su expresión de genes involucrados en el metabolismo de la VA (*crbp2, rbp, crabp1*), transporte lipídico (*i-fabp, l-fabp*), receptores nucleares a la VA y ácidos grasos (*rarα1, rxrα, pparβ*), crecimiento (*igf1, igf2* y su receptor *igf1r*) y desarrollo de esqueleto (*bgp*). Los resultados sugieren que la absorción de la VA está controlada a nivel intestinal por CRBP2 en ambos grupos, asimismo, se encontró que esta molécula previene la acumulación excesiva de VA en otros tejidos específicos. La expresión del gen *i-fabp* en larvas del grupo ES se observó que es estable con la edad y que también puede inducir a una acumulación excesiva de grasa en el intestino, que a su vez, induce la esteatosis en el hígado y en el sistema vascular de las larvas. La regulación de la expresión de los genes *rbp* y *l-fabp* en el hígado reflejaron el estado de las funciones fisiológicas demandantes de VA y lípidos en las larvas. Estos resultados revelaron que la composición de la dieta indujo a diferentes estrategias para la absorción y el metabolismo de la VA y de lípidos, que a su vez, afecto el crecimiento, el desarrollo y la salud de las larvas.

**Palabras claves:** *Solea senegalensis*, larvas, osificación, expresión génica, nutrición, vitamina A, ácidos grasos, enriquecimiento.

**Abstract**

The present study aimed to deepen the understanding of molecular mechanisms governing the absorption and metabolism of some nutrients, growth and development in larvae of Senegalese sole (*Solea senegalensis*) fed with *Artemia* enriched with Easy Selco© (ES, INVE) or Aquagrow Gold© (AGG, ABN), which mainly differed in their vitamin A (VA) content and fatty acid composition. The expression profile of genes involved in VA metabolism (*crbp2, rbp, crabp1*), lipid transport (*i-fabp, l-fabp*), nuclear receptors for VA and fatty acids (*rarα1, rxrα, pparβ*), growth (*igf1, igf2* and their receptor *igf1r*) and development (*bgp*) was analyzed at 22, 30 and 38 days post hatching. The main results suggested that the amount of VA absorbed by larvae is controlled at the intestinal level by *crbp2* in both groups, preventing excessive accumulation of this vitamin in the target tissues. The stable expression of *i-fabp* in the ES group with age could cause an excessive fat accumulation in the intestine inducing, in turn, the steatosis found in the liver and vascular system of these specimens. In liver, the regulation of *rbp* and *fabp* expression reflected the status of the physiological functions demanding VA and lipids. The findings revealed that dietary composition induced different strategies for VA and lipid absorption and metabolism affecting, in turn, larval development, growth and health.

**Keywords:** *Solea senegalensis*, larvae, ossification, gene expression, nutrition, vitamin A, fatty acids, enrichment

**Introduction**

Marine teleosts represent a suitable biological model for studying larval ontogenesis as it takes place within a short period of time. During that process, when major physiological and morphological changes occur (Darias *et al.*, 2008), nutrition is one of the key parameters affecting morphogenesis. This is especially true at the very early stages, where lipids are the main energy source, and other nutrients such as proteins, fatty acids or vitamins are needed for body patterning, building new cellular structures, developing tissues and organ systems, and affecting ultimately, growth (Lall and Lewis-McCrea, 2007). Several studies have demonstrated that some nutrients are responsible for the appearance of developmental disorders when their type and/or amounts are inappropriate or unbalanced (Lall and Lewis-McCrea, 2007; Darias *et al.*, 2011a; Fernández and Gisbert, 2011). Indeed, there is still a lack of knowledge about the nutritional requirements of marine fish larvae that in most cases prevents their feeding with inert diets. In-stead, rotifers and *Artemia* nauplii are used although it is well known that these live preys are deficient in polar lipids and essential fatty acids in comparison to natural zooplankton (Sargent *et al.*, 1999). There-fore, the improvement of their nutritional value to meet fish larval requirements is achieved by enrichment. Many enrichment products containing diverse fatty acid profile and vitamin A content exist in the market. This represents a good research opportunity for comparing the influence of such variety of regimes on fish larval morphogenesis, the molecular processes influenced by nutrition and the synergistic effects influenced by different groups of nutrients.

Senegalese sole (*Solea senegalensis*) is a commercially valuable flatfish for the Atlantic and Mediterranean populations. Given the dramatic morphologic remodeling that takes place with flatfish, and the fact that this species is already under cultivation with husbandry practices in place to enable careful reproducible results to be obtained from its study, the species provides a good model for studying the effects of nutrition on growth and development. This is the third part of a study that evaluated the effects of five different commercial enrichments on Senegalese sole larval performance, including a wide range of biological, physiological and molecular parameters, like growth, digestive system development, lipid accumulation in tissues, skeletal development and gene expression associated to lipid transport, bone mineralization and vitamin A metabolism, among others (Boglino *et al.*, 2012a, b). Previous studies have given clues about fish nutritional demands and behavior, which could be quite different among marine fish. For instance, compared to other teleosts, it has been shown that Senegalese sole larvae were able to grow, survive and metamorphose with negligible amounts of dietary docosahexaenoic acid (DHA) when eicosapentaenoic acid (EPA) was present (Morais *et al.*, 2004; Villalta *et al.*, 2005). Recent results showed that Senegalese sole larvae require lower amounts of DHA than those currently employed in other marine fish species (Dâmaso-Rodríguez *et al.*, 2010).Boglino *et al.* (2012a) found that EPA/DHA ratio had greater influence on the physiology of Senegalese sole than the DHA content alone. Moreover, the authors reported negative effects of the Easy Selco (ES) enriching product on larval growth, intestinal maturation and degree of ossification in this species, whereas the AquaGrow Gold (AGG) enriching product gave the best results in terms of morphogenesis among the five products tested (Boglino *et al.*, 2012a). Lipid

absorption and accumulation evolved differently among these dietary treatments and throughout their larval development being associated with the amount and dietary composition of fatty acids. In some cases, differences in the fatty acid profile disrupted the absorption and mobilization of dietary lipids, resulting in hepatic steatosis and the presence of large lipid droplets in the vascular system of the larvae especially if fed *Artemia* enriched with ES (Boglino *et al.*, 2012a).

Considering the findings observed for growth, lipid accumulation in tissues, intestinal maturation and skeletal ossification (Boglino *et al.*, 2012a, b) we decided to analyze the expression of some genes involved in diverse biological processes in larvae from the dietary treatments that showed significantly different results (extreme groups, ES and AGG), with the aim of better under-standing the mechanisms underlying the physiological and morphological changes observed in Senegalese sole larvae fed these commercial enrichments. For this purpose, a set of genes involved in morphogenesis, and directly or indirectly related with skeletal development, has been chosen in order to find out whether the nutritional composition of the enriching products tested could influence intestinal uptake of fatty acids and skeletogenesis in Senegalese sole: insulin growth factors 1and 2 (*igf1* and *igf2*) and insulin growth factor receptor (*igf1r*), which positively regulates cell proliferation; fatty acid binding protein in the intestine (*i-fabp*) and the liver (*l-fabp*), responsible for lipid transport; and osteocalcin (*bgp*) implicated in osteoblast mineralization. Due to the important variability in VA content between enrichments, and the shared molecular pathways between VA and fatty acid metabolism, some key genes involved in the cellular metabolism of VA were also analyzed to determine to what extent dietary VA content could modulate the expression pattern of these genes: retinoic acid receptor (*rarα*), retinoic X receptor (*rxrα*), cellular retinoic acid binding protein I (*crabp1*), cellular retinol binding protein II α (*crbp2α*), and retinol binding protein (*rbp*), which are genes involved in the metabolism and transport of VA; peroxisome proliferator-activated receptor (*pparβ*), which is a shared target gene for fatty acids and VA and is highly expressed in adipocyte tissues.

The knowledge of the molecular mechanisms underlying the metabolic pathway of a certain nutrient (*e.g.* vitamin A), its synergistic effects with others (e.g. fatty acids) and their implications on the overall larval physiology, digestive function and skeletogenesis of fish larvae is of great interest for fish physiologists, as well as for aquaculturists, since these nutrients have a great impact on fish development and quality.

**Material and methods**

*Larval rearing and feeding protocol*

Senegalese sole larvae were obtained from Stolt Sea Farm SA (Cambre, La Coruña, Spain), acclimated at the IRTA-SCR facilities and incubated at 20.0±2.8 °C and 35 of salinity in cylindrical tanks of 60 L (initial density: 80 larvae.$l^{-1}$) connected to a recirculation unit IRTAmar™. Water was daily renewed (50%) with gentle aeration in each tank, pH and dissolved oxygen being 8.0 ± 0.2 and 7.5 ± 1.3 ppm, respectively. Photoperiod was 16 L: 8D, and light intensity was 500 lx at the water surface. Larvae were fed twice a day, from 2 days post hatching (dph) to10 dph, with rotifers

(*Brachionus plicatilis*) enriched with microalgae (*Tetraselmis suecica*), at a density of 10 rotifers.ml$^{-1}$ from 2 to 7 dph and of 5 rotifers.ml$^{-1}$ from 8 to 10 dph. Enriched *Artemia* metanauplii were supplied to larvae from 8 to 38 dph twice a day, at increasing density from 0.5 to 12 metanauplii.ml$^{-1}$, adjusted according to the increase of weight of the larvae and to the daily food ration calculated as described in Boglino *et al.* (2012a).

*Experimental diets, live prey enrichment and sampling*

Two commercial enriching products Easy Selco©(ES, INVE, Belgium) and Aquagrow Gold© (AGG, ABN, USA) were used to enrich *Artemia*. Enrichment was carried out following the protocol described in Boglino *et al.*, 2012a). Senegalese sole larvae were fed with enriched *Artemia* metanauplii after being washed with UV filtered seawater and fresh-water to reduce the bacterial load and remove enrichment residue. Larval rearing was performed in triplicate for each dietary treatment. From 20 dph onwards, when larvae began to settle, enriched *Artemia* were supplied frozen to the larvae most of which were lying on the bottom of the tanks. The fatty acid profile of enriched *Artemia* with ES and AGG, and larvae at 38 dph, is shown in Table 1.

**Table 1.** Total lipid and total fatty acid contents, fatty acid composition (in % TFA) and retinoid content in *Artemia* nauplii enriched with ES and AGG and in 38 day-old Senegalese sole larvae fed these diets (mean ± SD; n = 3). Totals include some minor components not shown. Superscripts letters denote significant differences among diets ($P < 0.05$).

| | **Artemia** | | **Larvae** | |
|---|---|---|---|---|
| | **ES** | **AGG** | **ES** | **AGG** |
| Total lipids (mg·g$^{-1}$ DW) | 219.8±17.4 | 184.4±17.0 | 67.3±17.0 | 97.9±5.8 |
| Total FA (mg·g$^{-1}$ DW) | 155.9±54.8 | 124.0±43.0 | 34.7±10.0 | 41.6±3.0 |
| Total saturated | 14.9±0.3 | 15.9±2.6 | 16.9±1.7 | 18.5±0.6 |
| Total monounsaturated | 38.8±3.7 | 32.7±3.2 | 35.9±5.1 | 33.0±1.2 |
| 18:2n-6 | 8.28±0.04$^{a}$ | 5.2±1.0$^{b}$ | 7.9±1.0$^{a}$ | 5.8±0.8$^{b}$ |
| 20:4n-6 | 0.5±0.1$^{b}$ | 0.7±0.1$^{a}$ | 1.1±0.2$^{b}$ | 2.1±0.1$^{a}$ |
| 22:5n-6 | 0.1±0.07 | 2.2±1.9 | 0.3±0.2$^{b}$ | 3.8±0.1$^{a}$ |
| Total n-6 PUFA | 9.5±0.2 | 8.6±2.3 | 10.2±1.3$^{b}$ | 12.5±0.6$^{a}$ |
| 20:5n-3 | 7.6±0.6$^{a}$ | 3.1±0.4$^{b}$ | 5.1±0.7$^{a}$ | 2.3±0.2$^{b}$ |
| 22:5n-3 | 1.4±0.3 | 1.2±2.0 | 4.0±0.7$^{a}$ | 1.7±0.3$^{b}$ |
| Total n-3 PUFA | 36.5±3.5 | 42.5±4.6 | 36.2±4.8 | 34.9±2.2 |
| Total PUFA | 46.0±3.7 | 51.1±5.9 | 46.4±6.1 | 47.5±1.6 |
| (n-3)/(n-6) | 3.9±0.3 | 5.2±1.5 | 3.6±0.0$^{a}$ | 2.8±0.3$^{b}$ |
| DHA/EPA | 0.7±0.1$^{b}$ | 3.0±0.1$^{a}$ | 2.0±0.1$^{b}$ | 5.6±0.2$^{a}$ |
| ARA/DHA | 0.1±0.0 | 0.08±0.01 | 0.1±0.0$^{b}$ | 0.2±0.0$^{a}$ |
| ARA/EPA | 0.07±0.01$^{b}$ | 0.23±0.02$^{a}$ | 0.2±0.0$^{b}$ | 0.9±0.1$^{a}$ |
| Vitamin A (µg·g DW$^{-1}$) | 24.8±9.6$^{a}$ | 0.2±0.2$^{b}$ | 3.15±0.5 | 4.09±1.1 |

For gene expression analyses, 200 mg wet weight larvae were sampled at 22, 30 and 38 dph from both the ES and AGG groups, sacrificed with an overdose of anesthetic (Tricaine methanesulfonate, MS-222, Sigma), rinsed in distilled water and preserved in RNA later© (Ambion) at -80 °C for further analyses. These sampling points were chosen based on the main changes observed in morphogenesis and lipid accumulation during development (Boglino *et al.*, 2012a, b) and to cover metamorphosis and post metamorphosis periods, as they constitute the most important stages in flatfish development.

*Gene expression*

Several genes involved in diverse biological processes were chosen as markers for Senegalese sole morphogenesis (Table 2). Expression of the following genes was analyzed at 22, 30 and 38 dph in larvae from the ES and AGG groups: insulin growth factors I and II (*igf1* and *igf2*) and insulin growth factor receptor (*igf1r*), which positively regulates cell proliferation; retinoic acid receptor (*rarα*), retinoic X receptor (*rxrα*), cellular retinoic acid binding protein I (*crabp1*), cellular retinol binding protein IIα(*crbp2α*), and retinol binding protein (*rbp*),which are genes involved in the metabolism and transport of VA; peroxisome proliferator-activated receptor (*pparβ*), which is a shared target gene for fatty acids and VA and is highly expressed in adipocyte tissues; fatty acid binding protein in the intestine (*i-fabp*) and the liver (*l-fabp*), responsible for lipid transport; and osteocalcin (*oc*) implicated in osteoblast mineralization.

Total RNA of a pool of larvae (80 to 3 individuals per sample time and tank, depending on fish size) was extracted using TRIzol™ (Invitrogen). The quantity of RNA isolated was determined using a Gene-Quant spectrophotometer (Amersham Biosciences), measuring optical density at 260 nm and its purity was established by the absorbance ratio 260/280 nm (1.7–2.0). The quality of the RNA was examined using 1.2% agarose gel electrophoresis. Equal quantities of total RNA (1µg) from each sample were reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen®, GmbH, Germany). Real-time PCR analysis was performed using an ABI PRISM 7300 (Applied Bio-systems). For each gene, a species-specific Taqman assay was designed (Applied Biosystems) from available sequences in the Gen-Bank database (Table 2). The efficiency of the Taqman assay for each gene was previously evaluated to assure that it was close to 100%. Amplification reactions were performed in 96 well plates in triplicate in a total volume of 20µl containing 1µl of cDNA (dilution, 1/15), 1µl of Taqman probe, 10µl of Taqman mix and 8µl of sterile water. The reference gene $ef_{1\text{-}3}$ was chosen as a reference since it did not exhibit any significant variation in expression among the samples. The amplification conditions were 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

**Table 2.** Accession numbers and main biological processes of the analyzed genes.

| Gene name | GenBank accession no. | Biological process | Amplicon size | Hydrolysis probes | 5' to 3' sequence |
|---|---|---|---|---|---|
| *ef1-3* | AB326304 | Translational elongation | 86 | Forward | GAAGAAAACCCCAAAAGTGTCAAGT |
| | | | | Reverse | GCAAACGCCTCAACACACA |
| | | | | FAM probe | CATGGCTGCATCTCCA |
| *igf1* | AB248825 | Cell proliferation | 73 | Forward | AGAACTCAAGTCGAGGCAACAC |
| | | | | Reverse | TGGGCGTTTGTCCATCCA |
| | | | | FAM probe | CCGTCCCTACATTCTG |
| *Igf2* | AB248826 | Cell proliferation | 63 | Forward | CATGATGCCCGCAATAAAGCA |
| | | | | Reverse | TGCCACACCTCGTATTTGGAAT |
| | | | | FAM probe | CTTCCTCGGGACTTCC |
| *igfr* | FJ515914 | Cell proliferation | 75 | Forward | TGGCTGAGGACTTTGCTGTTAAAAT |
| | | | | Reverse | TGCGGTAGTAATCCGTCTCATAGAT |
| | | | | FAM probe | CATGCCGAAATCTCCT |
| *crbp2α* | FF290878 | Vitamin A metabolism | 69 | Forward | CTGGACGATGATTTTGGTCTGATG |
| | | | | Reverse | GCCCTTGACATCGACTTTGC |
| | | | | FAM probe | ATGGACAGCAATCTTT |
| *rbp* | FF290795 | Vitamin A metabolism | 76 | Forward | CCAGTAAGCAGATCTCCCTCTTCT |
| | | | | Reverse | TCCCGTCATATCACTGGTCTGA |
| | | | | FAM probe | CCATCGCCTGGTCCTC |
| *crabp1* | FF287712 | Vitamin A metabolism | 66 | Forward | ACAGAGTGTGGTGGGTTGTG |
| | | | | Reverse | GGGAATGACACACCTGGATCAG |
| | | | | FAM probe | CCAACTGGGTTTCTGC |
| *rarα1* | AB668026 | TR, morphogenesis | 77 | Forward | GAAGAAGAAGGACGAGAAGAAGCA |
| | | | | Reverse | TGTCTATCATCTGCTCCGTGTCT |
| | | | | FAM probe | CAGGACGTAGCTCTCC |
| *rxrα* | AB668024 | Transcription regulation (TR) | 68 | Forward | CTCATCGTTCCATAGCCGTTAAAGA |
| | | | | Reverse | GCTGTTGCGGTGAACGT |
| | | | | FAM probe | TCGCCAACAGAATCC |
| *pparβ* | GU219855 | Fatty acid metabolism | 75 | Forward | GCTCTGGAGCTGGATGATAGTG |
| | | | | Reverse | CAGCCCGGGACGATCTC |
| | | | | FAM probe | ATGGCAGCAACAAACA |
| *l-fabp* | FF284817 | Lipid transport | 81 | Forward | TGTGACGGTCACCTTGAAGTG |
| | | | | Reverse | GAGCTCATCCAGAAAGGCAAAGATA |
| | | | | FAM probe | CCGTCTCCTCAATCTC |
| *i-fabp* | FF287004 | Lipid transport | 73 | Forward | GCGAGGCTGTACTCAAAAGTGA |
| | | | | Reverse | TCAAGGAGACCAGCGCTTT |
| | | | | FAM probe | CCGCACCCTGGAATTA |
| *oc* | AY823525.1 | Bone mineralization | 56 | Forward | CGAGCACATGATGGACACTGA |
| | | | | Reverse | GTCCGTAGTAGGCCGTGTAG |
| | | | | FAM probe | CAGCGATGATTCCC |

Real-time PCR efficiencies (E) were determined for each gene from the slopes obtained with Applied Biosystems software, applying the equation E=10[−1/slope], where E is PCR efficiency. The

relative gene expression ratio (R) for each gene was based on the E and the quantification cycle (Cq) of a sample compared with the control, and expressed in comparison to the reference gene, according to Pfaffl's formula: $R = (E_{target\ gene})^{\Delta Cq\ target\ gene\ (mean\ sample - mean\ ref\ sample)} / (E_{ef1\text{-}3})^{\Delta Cqef1\text{-}3(mean\ sample - mean\ ref\ sample)}$, where $ef_{1\text{-}3}$ is the reference gene, mean sample corresponds to triplicate average, and the initial time point from the AGG group was chosen as the reference group.

*Statistics*

Results were expressed as mean ± SD (n = 3). All data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett's test) and transformed when necessary. Data were log transformed when required. Two-Way ANOVA was per-formed to analyze differences during larval development and be-tween dietary treatments. When significant differences were found at $P < 0.05$. All Pairwise Multiple Comparisons were performed using the Holm-Sidak method. All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA).

**Results**

The average efficiency of amplification for all assays was 98%. Expression patterns of *igf1, -2* and *igf1r* did not show significant differences with age nor among dietary treatments ($P > 0.05$; Figure 1). *Crbp2α* expression decreased from 22 to 30 dph ($P < 0.05$) in larvae from the ES remained constant in larvae fed with the AGG diet ($P > 0.05$; Figure 1). At 38 dph, the expression level of *crbp2α* was higher in AGG than in ES group ($P = 0.05$).

Also, there was a statistically significant interaction between age and dietary treatment ($P = 0.05$).The gene expression of *rbp* presented a V-shape from 22 to 38 dph in ES group (Figure 1), whereas it remained constant from 22 to 30 dph and increased from 30 to 38 dph in AGG group ($P = 0.017$; Figure 1). No differences in *rbp* gene expression were found between dietary treatments (Figure 1). *Crabp1* (Figure 1) did not show significant differences in gene expression throughout development ($P = 0.467$) or among dietary treatments ($P = 0.393$). The expression of the nuclear receptors *rarα1, rxrα* and *pparβ* remained invariable from 22 to 38 dph in both dietary groups ($P > 0.05$; Figure 2). *l-fabp* gene expression decreased from 22 to 30 dph in both dietary groups ($P < 0.05$; Figure 2) and remained constant thereafter ($P > 0.05$). No differences in *l-fabp* expression were observed among dietary treatments ($P = 0.263$). *i-fabp* expression remained constant with age in ES group ($P = 0.05$) whereas it de-creased from 22 to 30 dph in the AGG group ($P = 0.017$; Figure 2). How-ever, no differences in gene expression were observed among dietary treatments ($P = 0.435$).

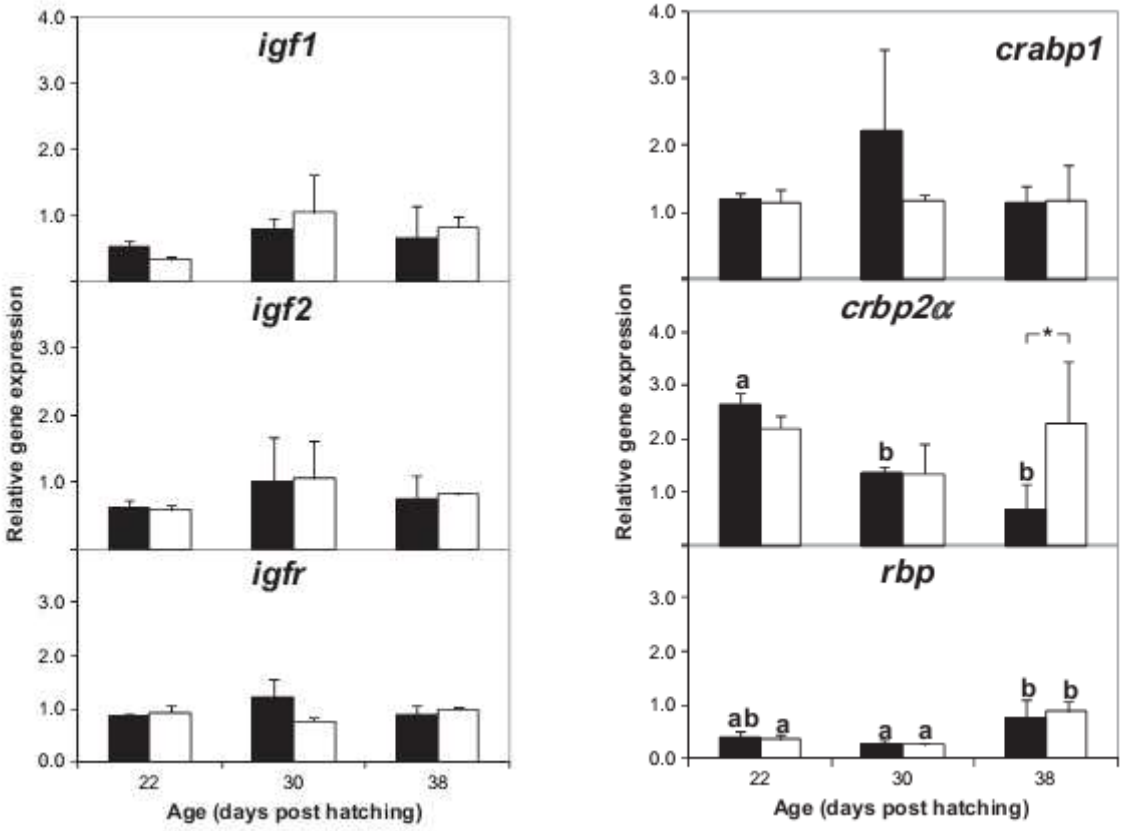


**Figure 1.** Relative gene expression of *igf1l, igf12ll, igf1r crabp1, crbp2α* and *rbp* with respect to the reference gene $ef_{1\text{-}3}$ in Senegalese sole larvae fed with *Artemia* enriched with ES (black bars) or AGG (white bars) at 22, 30 and 38 dph. Different superscripts denote significant differences between days of the same dietary treatments and asterisk shows differences between dietary treatments of the same age (Two way ANOVA, $P < 0.05$, n = 3).

Finally, the expression of *oc* was constant in larvae fed with the ES diet ($P = 0.05$), while it sharply in-creased in larvae fed with AGG from 22 to 30 dph ($P = 0.017$; Figure 3) to remain stable thereafter ($P > 0.05$; Figure 3). At 30 and 38 dph, the expression levels of *oc* were significantly different between ES and AGG groups ($P = 0.04$; Figure 3). There was a statistically significant interaction between age and dietary treatment in *oc* expression ($P = 0.043$).

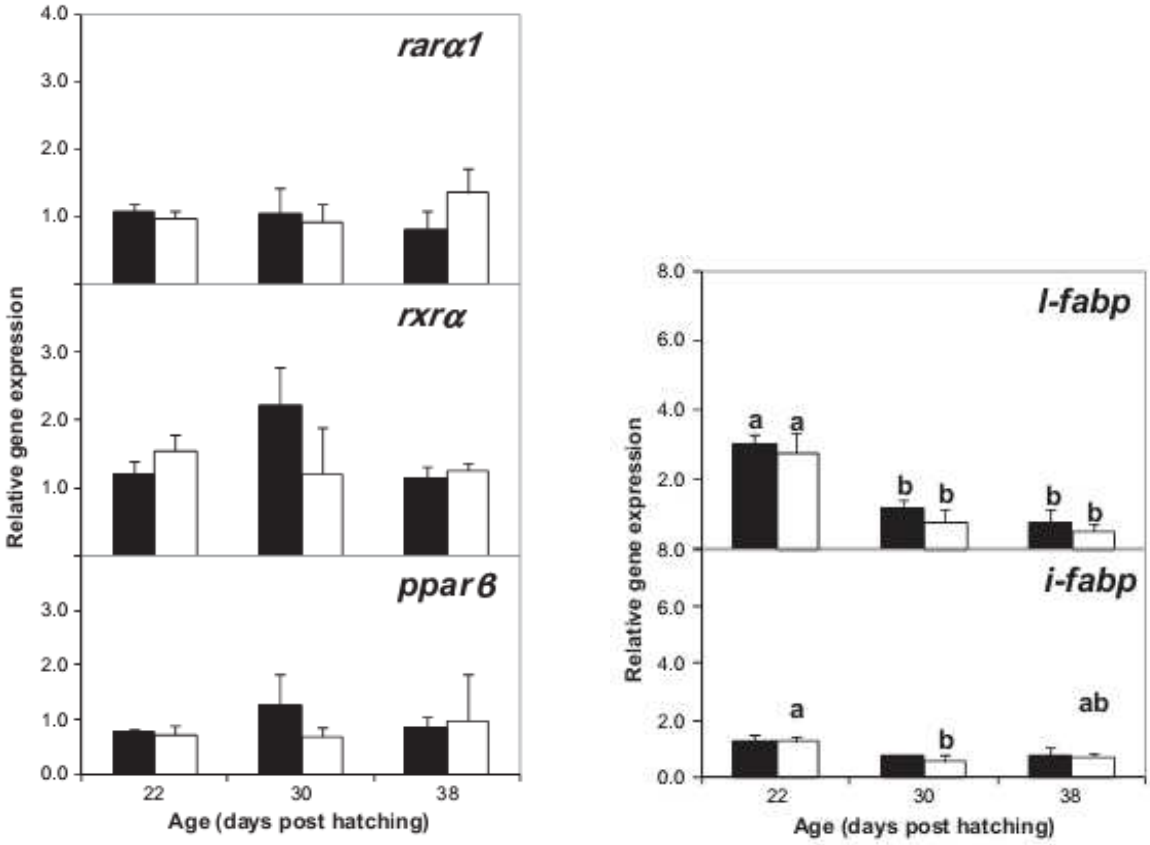


**Figure 2.** Relative gene expression of *rarα1, rxrα, pparβ, l-fabp* and *i-fabp* with respect to the reference gene $ef_{1\text{-}3}$ in Senegalese sole larvae fed with *Artemia* enriched with ES (black bars) or AGG (white bars) at 22, 30 and 38 dph. Different superscripts denote significant differences between days of the same dietary treatments (Two way ANOVA, $P < 0.05$, n = 3).

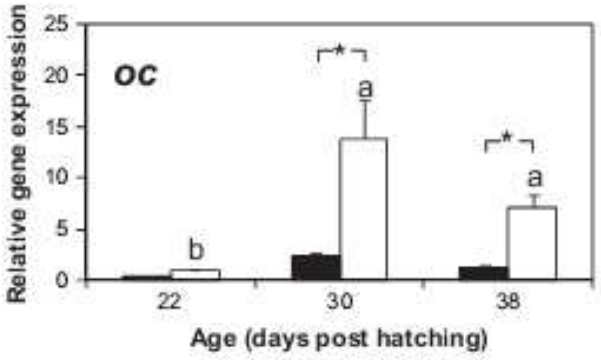


**Figure 3.** Relative gene expression of *oc* with respect to the reference gene $ef_{1\text{-}3}$ in Senegalese sole larvae fed with *Artemia* enriched with ES (black bars) or AGG (white bars) at 22, 30 and 38 dph. Different superscripts denote significant differences between days of the same dietary treatments and asterisks show differences between dietary treatments of the same age (Two way ANOVA, $P < 0.05$, n = 3).

**Discussion**

*Insulin-like growth factors*

Currently, four types of IGFs are known in fish, IGF-Ia, IGF-Ib, IGF-IIa and IGF-IIb (Zou *et al.*, 2009). Both IGF-I and IGF-II regulate differentiation and growth offish (for details of their functions see Peterson *et al.*, 2005; Chao and D'Amore, 2008; Reinecke, 2010). Higher levels of *igf2* than *igf1* transcripts have been detected after hatching and during larval development of Senegalese sole, suggesting a possible role of this gene in flatfish metamorphosis (Funes *et al.*, 2006). In gilthead sea bream (*Sparus aurata*), *igf2* expression level was highest at 1 dph and decreased thereafter, whereasigf1expression was also detected at 1 dph but increased later in development (Duguay *et al.*, 1996) suggesting that both IGFs play different roles during growth. This contrasts with the recent study performed in the same species, where both *igf1* and *2* were expressed at the same relative levels from 2 to 29 dph and, from that time onwards, *igf1* was expressed in higher amounts than *igf2* (Fernández *et al.*, 2011). These variable results for *igf* expression from the same species could reflect different physiological responses to environmental and/or rearing conditions. The insulin-like growth factor receptor (*igf1r*) binds *igf1* with higher affinity than *igf2*, and has a tyrosine-protein kinase activity, needed for the activation of the downstream signaling cascade stimulated by *igf1* (Pandini *et al.*, 2002).

In the present study, no differences in *igf1* (nor in *igf1r*) and *-2* expression levels were observed in Senegalese sole larvae from 22 to 38 dph, being in line with results reported by Funes *et al.* (2006) and complementary to that work, as these authors had analyzed gene expression until 22 dph. Such findings contrasts with those obtained for European sea bass (*Dicentrarchus labrax*) and gilthead sea bream, in which an increase of *igf1* expression during ontogenesis was found, indicating different molecular ontogenetic strategies for each species (Darias *et al.*, 2010b; Fernández *et al.*, 2011). Funes *et al.* (2006) suggested a possible role of *igf2* in mediating the mechanisms that underlie metamorphosis in Senegalese sole larvae. Metamorphosis in this species usually ends at around 20–25 dph, therefore constant levels of *igf2* expression from 22 dph onwards (present study) would be expected.

That nutritional status offish has a profound effect on *igf1* expression is well known (Duan, 1998). Dietary vitamins have been shown to influence skeletogenesis and digestive system development through the modulation of *igf1* expression, in conjunction with other transcription factors (Villeneuve *et al.*, 2006; Mazurais *et al.*, 2008; Darias *et al.*, 2011a, b). A recent study performed with juveniles of Senegalese sole showed that dietary lipid levels have a remarkable impact on the expression of growth-related genes (Campos *et al.*, 2010). However, in our study the gene expression patterns of *igf1 and -2,* and *igf1r*, were not modulated by dietary treatments in Senegalese sole larvae. Considering the isolipidic composition of dietary treatments tested (Boglino *et al.*, 2012a), this finding could indicate that lipid content rather than fatty acid and lipid class composition is the primary

modulator of expression of *igfs*. In addition to this, it seems that similar modulation of *igf* expression occurs during both the larval (present study) and juvenile (Campos *et al.*, 2010) stages.

*Cellular metabolism of vitamin A*

It is well known that VA plays a crucial role in the mechanisms governing cellular proliferation and differentiation, and hence morphogenesis, during development of vertebrates, including fish (Fernández and Gisbert, 2010, 2011). Considering the high VA con-tent contained in both ES and AGG enriching products (1,500,000 IU.kg$^{-1}$ and 6380 IU.kg$^{-1}$, respectively), and the different results in larval performance found in terms of growth and organogenesis (especially in digestive and skeletal systems; Boglino *et al.*, 2012a) we considered it pertinent to investigate the underlying molecular mechanisms of VA metabolism in each larval group in order to better understand the physiological and morphological changes observed.

Vitamin A is present in the lipid fraction of the diet. ES is an oily emulsion, whereas AGG is a drum-dried algal meal. These differences in the form of presentation of the enriching products together with the metabolism of *Artemia* explained the different VA profile observed in enriched *Artemia*. Thus, although retinol was present in both cases, VA in *Artemia* fed ES was mainly represented by retinyl palmitate, whereas no traces of this component were detected in *Artemia* enriched with AGG (data not shown). Therefore, differences in both the amount and forms of VA among dietary treatments would have different consequences in larval VA absorption, digestion, transport to the liver and delivery to target tissues.

Few studies exist on VA metabolism in fish larvae (see review in Fernández and Gisbert, 2011); however, they have equivalent molecular components as other vertebrates (Napoli, 2000) suggesting that they probably have comparable mechanisms of action. Vitamin A is ingested by the larvae through the enriched *Artemia* in the forms of retinyl palmitate, retinol and, in lower proportion, retinoic acid (RA). In the enterocytes, retinol would be transformed into retinyl esters and transported to the liver by chylomicrons (Figure 4). Cellular binding protein 2 (CRBP2) facilitates intestinal VA transport and metabolism (Levin and Davis, 1997). Its binding to retinol has been shown to be necessary for the intestinal esterification of retinol (Figure 4), which suggests that CRBP2 has a role in the initial processing of retinol from food (Napoli, 1996). The down regulation of *crbp2α* gene expression in larvae from the ES group suggested that the intestine could play a key role in controlling dietary VA uptake in Senegalese sole larvae. Proof of this could be the similar VA content observed in larvae from both dietary groups, indicating that tissue accumulation of VA was not excessive in the ES group. Considering that the ES enriching product provided the highest VA content (Boglino *et al.*, 2012a), the low VA accumulation observed in larvae from the ES group could reflect a negative feedback mechanism of larvae to control the required cellular amount of VA. This auto-regulation of VA absorption by dietary and plasma retinoids through the modulation of intestinal *crbp2* mRNA

expression has already been reported in previous studies in higher vertebrates (Rajan *et al.*, 1990; Levin and Davis, 1997). Considering thatcrbp2has been shown to be restricted to, mature enterocytes of the mucosal epithelium in the small intestine of rats (Zetterstrom *et al.*, 1994), the low amount of *crbp2α* transcripts found in larvae from the ES group might also be attributed to their low intestinal maturation degree (Boglino *et al.*, 2012a). In turn, such a situation probably helped to restrict the absorption of excess VA contained in *Artemia* enriched with ES (Boglino *et al.*, 2012a).

Under normal conditions, VA in the liver would be stored mainly in lipid droplets in hepatocytes and released to the blood as needed, with retinol binding protein (*rbp*) expressed in the liver playing a key role in mobilization of VA from this organ to target tissues else-where (Figure 4) (Sauvant *et al.*, 2002; Bellovino *et al.*, 2003). In the target cells, RBP transfers retinol from plasma to the cytoplasmic cellular retinol binding proteins (CRBPs), although the exact mechanism is still unknown (Sundaram *et al.*, 1998). The V-shape profile found in *rbp* expression of the ES group compared to the AGG group suggests different modes of processing of RA, probably related to a difference in availability of RA and/or digestive capacity of larvae of both dietary groups. The increase in *rbp* expression found between the completion of metamorphosis and at post-metamorphic stages (30 and 38 dph, respectively) suggests that during this period there was an increase in physiological demand for VA such as might be needed for the complete ossification of the skeleton (Gavaia *et al.*, 2002), or the achievement of the juvenile-adult physiology and phenotype (Geffen *et al.*, 2007). In line with this is the expression profile of *oc* observed throughout larval development of the AGG group, in which a peak of gene expression was observed at 30 dph. These results were different to those recently reported in gilthead sea bream larvae where *rbp* expression was low and constant during larval development (Fernández *et al.*, 2011), suggesting species-specific ontogenetic differences in retinol mobilization.

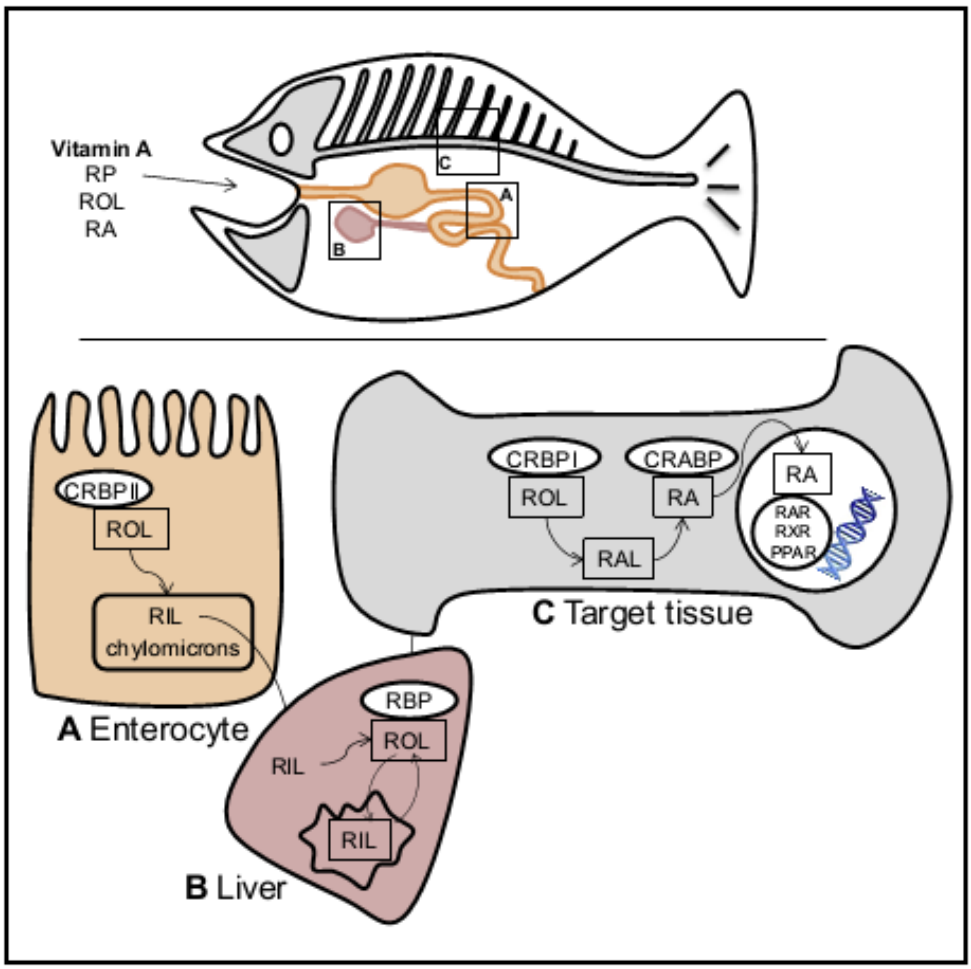


**Figure 4.** Schematic design showing the theoretical metabolism of vitamin A in fish based on the existent knowledge for other vertebrates. Vitamin A digestion, absorption by the intestinal enterocytes (A), transport to the liver (B) and delivering to the target tissues (*i.e.*, bone, C) is represented. The molecular mechanisms involved in each tissue are described in the main text. CRABP, cellular retinoic acid binding protein; CRBP1, cellular retinol binding protein I; CRBP2, cellular retinol binding protein II; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RBP, retinol binding protein; RIL, retinyl; ROL, retinol; RP, retinyl palmitate; retinoic X receptor RXR.

Inside the cells, retinol is transformed into the active metabolite, RA, by a precise and complex regulation. CRABP1 binds RA and is responsible for maintaining adequate concentrations of free RA by inducing its interconversion into other isoforms and its catabolism (Weston *et al.*, 2003) (Figure 4). In the present study, a high variability in *crabp1* gene expression was observed at 30 dph in the ES group. As metamorphosis is not synchronous this could be due to different individuals at somewhat different developmental states, stages of which are strongly influenced by the expression of this gene. Although not significantly, *crabp1* expression tended to be higher at 30 dph in ES group, possibly indicating a regulation of cellular RA levels. This was not the case of larvae from the AGG group,

where *crabp1* expression remained stable throughout the studied period, suggesting that dietary VA levels of AGG are adequate during ontogeny of Senegalese sole.

RA is able to interact in the nucleus with specific transcription factors, such as *rars, rxrs and ppars*, after which these nuclear receptors bind to the retinoic acid responsive elements (RAREs) of specific genes to modulate their expression (Figure 4). These genes could be involved in diverse processes including VA metabolism, but also in lipid metabolism (by activation of *ppars, fabps*) or organogenesis (i.e., skeletogenesis, through the activation of osteocalcin by vitamin D receptors).

*Nuclear receptors*

RA mediates its biological effects through binding to nuclear receptors which transduce the RA signal into appropriate transcription-al changes. Retinoic acid receptors (RARs and RXRs) bind as heterodimers to their target response elements in response to all-trans or 9-cis RA and regulate gene expression in various biological processes. However, the RXRs serve as a common heterodimeric partner for a number of nuclear receptors such as the vitamin D receptor, thyroid hormone receptor or peroxisome proliferator-activated receptors, therefore promoting an environment of competition where heterodimerization is tightly controlled.

In fish, as well as in mammals, RARs and RXRs are distributed in diverse tissues (Joore *et al.*, 1994; Alsop *et al.*, 2001; Villeneuve *et al.*, 2004), justified by the powerful role that RA has on cellular proliferation and differentiation processes. Contradictory results found in fish in regards to *rarα* expression during larval development may be attributed to genetic and environmental factors (i.e., rearing conditions regulating RXR heterodimerization). For instance, an increase in *rarα* expression levels in developing European sea bass versus a de-crease in gilthead sea bream (Villeneuve *et al.*, 2004; Fernández *et al.*, 2011, respectively). In the present study, *rarα1* and *rxrα* expression levels were stable from 22 to 38 dph, which is in line with the results reported for gilthead sea bream within the same developmental period. The abundance of *rxrα* transcripts decreased from 10 to 42 dph in European sea bass larvae (Villeneuve *et al.*, 2004) suggesting that this nuclear receptor is more essential during the early stages of the larval development than later on. In the present study, having the first sampling point at early metamorphosis (22 dph) prevented confirmation of this hypothesis in Senegalese sole larvae.

Peroxisome proliferator-activated receptor beta (PPARβ) is a transcription factor involved in the regulation of fatty acid oxidation and mitochondrial biogenesis and expressed in many tissues due to its involvement in lipid and energy metabolism (Michalik *et al.*, 2002), al-though its precise role and how it functions in cellular differentiation and proliferation, is not yet understood in fish due to species-specific differences reported in the literature (Boukouvala *et al.*, 2004; Batista-Pinto *et al.*, 2005; Tsai *et al.*, 2008).The β-isoform of *ppar* appears to be the first isotype expressed during development in

fish and it is also activated by naturally occurring fatty acids (Leaver *et al.*, 2005). In the present study *pparβ* expression remained constant during the studied period (from 22 to 38 dph), as has also been observed for gilthead sea bream throughout its whole larval development (Fernández *et al.*, 2011). The expression of *pparβ* is highly influenced by nutrition in fish (Vagner *et al.*, 2009; Fernández *et al.*, 2011). In liver of gilthead sea bream, *pparβ* expression is induced by fasting and decreases following feeding, whereas in intestine or adipose tissue its expression was not affected by nutritional status (Leaver *et al.*, 2005). The commercial products used to enrich *Artemia* nauplii include many ligands for RARα, RXRα and PPARβ, such as RA and PUFAs. This might explain, together with the widespread tissue expression of these nuclear factors, the impossibility to detect differences in gene expression throughout larval development and between dietary treatments in Senegalese sole larvae. In addition to this, among PUFAs, PPARβ has a preference for gamma-linoleic acid (GLA) and eicosapentaenoic acid (EPA) (Schmid *et al.*, 1992). *Artemia* enriched with ES and AGG presented similar amounts of GLA but different EPA content, which might have also contributed to the difficulty in detection of differential modulation of this gene.

*Cellular metabolism of fatty acids*

The fatty acid-binding proteins (FABPs) are a family of proteins that are principally located in the cytosol and are characterized by their ability to bind to hydrophobic ligands, such as fatty acids (Glatz and van der Vusse, 1996). FABPs transfer and channel lipidic ligands into the nucleus for initiating nuclear receptor transcriptional activity to provide new lipid nutrient signaling pathways that affect lipid and glucose catabolism and storage (Schroeder *et al.*, 2008). There are several FABPs that have been named according to the tissue from which they were isolated.

The liver-type fatty acid binding protein (*l-fabp*) is expressed exclusively in the liver of the adult zebrafish (Denovan *et al.*, 2000); whereas intestine-type fatty acid-binding protein (*i-fabp*) mRNA was most abundant in intestine (Sharma *et al.*, 2004). As the tissue localization of the expression of these genes was not attempted in this study, it has been assumed that Senegalese sole larvae present a similar distribution pattern of these genes.

The liver plays a major role in lipid metabolism, and large amounts of fatty acids are moved into and out of the liver. The *l-fabp* expression is also influenced by several factors that impact hepatic fatty acid metabolism, including food deprivation, fatty diets and peroxisome proliferators (for review see Bass, 1988; Veerkamp, 1995). It has been proposed that I-FABP is involved in the uptake of dietary fatty acids (Levy *et al.*, 2001) and intracellular fatty acid transport (Baier *et al.*, 1996). To our knowledge, there is no information about *l-fabp* or *i-fabp* expression during the larval development of fish. In the present experiment, *l-fabp* displayed similar mRNA levels in both the ES and AGG groups that decreased from 22 to 30 dph, this being consistent with the decrease of lipid accumulation found in livers of specimens from both dietary groups observed between metamorphic and post-metamorphic stages (Boglino *et al.*, 2012a). The fact that *l-fabp* was not differentially modulated by ES

or AGG dietary treatments suggests that *l-fabp* is sensitive to the dietary long-chain FAs content, which was similar in both treatments, rather than its composition (Boglino *et al.*, 2012a). In contrast, *i-fabp* seemed to be affected by these commercial live prey enrichments. The ES group showed a stable level of *i-fabp* expression, whereas a decrease in expression of this gene from 22 to 30 dph was observed in the AGG group. This could be indicating larval absorptive adaptation responses to the dietary lipids. The dietary EPA could be a possible causative factor, as there is a relatively high difference in EPA content between the two enriching products (7.6 and 3.1% TFA in ES and AGG, respectively). Such differences in EPA content could be responsible for the differences in lipid accumulation in the liver (Boglino *et al.*, 2012a). The expression of *i-fabp* has been considered a useful marker for intestinal development in several vertebrates including fish (Simon *et al.*, 1995; Sonnino *et al.*, 2000; Her *et al.*, 2003). It has been shown that zebrafish presents a cephalo-caudal expression pattern for *i-fabp* during morphogenesis demarcating the anterior fat absorbing processes to the posterior cells of the intestine (Andre *et al.*, 2000). This spatio-temporal gene expression pattern associated with the highest degree of intestinal maturation observed in the AGG group (Boglino *et al.*, 2012a) could explain the decrease in *i-fabp* expression during development observed in larvae from that group as transcripts would be located to cells of the posterior intestine. The stable mRNA expression of *i-fabp* in the ES group from 22 to 38 dph could likely cause excessive fat accumulation in the intestine inducing in turn steatosis in the liver and the large accumulation of lipid droplets in the vascular system in these specimens (Boglino *et al.*, 2012a).

*Effects of ES and AGG dietary treatments on bone ossification*

The major nutritional differences between ES and AGG commercial enrichments are the VA content and fatty acid composition (Boglino *et al.*, 2012a). The harmful effects that RA has on fish larval morphogenesis are well known and especially in regard to skeletogenesis through the RAR, RXR and PPARβpathways (Haga *et al.*, 2002; Villeneuve *et al.*, 2006; Fernández *et al.*, 2011). The RAR and RXR receptor subtypes exhibit dynamic expression patterns through-out skeletal development (Mollard *et al.*, 2000). The widespread and dynamic expression of the *rars* and *rxrs* throughout skeletal development indicates that these receptors are important regulators of this process. No differences in *rxrα, rarα1* and *pparβ* expression were observed among dietary treatments in Senegalese sole from 22 to 38 dph. Many reasons could have contributed to obtain this result such as VA levels contained in ES and AGG enrichments were not harmful enough to induce a notable modulation of these genes. As previously suggested, Senegalese sole larvae could also have regulated the level of VA uptake at the intestine, preventing the entrance of excessive amounts of RA to osteogenic cells. Alternatively, it has been demonstrated that the most powerful effect of vitamins and PUFAs on fish morphogenesis through the modulation of such genes takes place during the early stages of development (Villeneuve *et al.*, 2006; Darias *et al.*, 2010a, 2011a,b) and this could also have been the case for Senegalese sole larvae. Whether CRABPs and CRBPs are critical for normal skeletal development is not yet known, and will require further study.

*Oc* constitutes a gene marker for bone mineralization in developing fish and is responsive to different nutritional conditions, and through influence from VA its over-expression has been implicated in skeletal disorders in gilthead sea bream (Mazurais *et al.*, 2008; Darias *et al.*, 2010a, 2011b; Fernández *et al.*, 2011). Though a less mineralized skeleton contributing to the development of skeletal deformities (Darias *et al.*, 2010a, b) was expected in the present study, there were no differences in the incidence of the total skeletal deformities between the ES and AGG dietary groups (Boglino *et al.*, 2012a). The elevated percentage of malformations observed in the former study, which represented up to 78% of the population, has already been reported in previous studies for the same species suggesting that there must be other causative factors apart from nutrition.

Larvae from the AGG group presented, in concomitance with higher levels of *oc* expression, a more mineralized skeleton at the end of the larval period than larvae fed *Artemia* enriched with ES (Boglino *et al.*, 2012a). Considering the above, this finding indicated that amounts of VA contained in ES and AGG enriching products are not excessive enough to provoke physiological alterations induced by hypervitaminosis A. This supports our previously suggested hypothesis that amounts of VA absorbed by larvae from the ES and AGG groups are controlled at the intestinal level and, hence, the up-regulation of osteocalcin expression in larvae from the AGG group is induced by factors other than RA. ES contains lower levels of ARA and higher levels of EPA than AGG, resulting in lower DHA/EPA, ARA/EPA and ARA/DHA ratios (Boglino *et al.*, 2012a), which are known to be essential for the correct ossification of the skeleton (Lall and Lewis-McCrea, 2007). Raisz *et al.* (1989) demonstrated that diets rich in EPA had less effect on bone remodeling than diets rich in ARA and its precursors. Moreover, ARA supplementation has been associated with an increase in body weight and bone mineral density in pigs (Weiler, 2000). Taking all this into account and considering the low larval growth and mineralization degree obtained for larvae fed ES compared to the AGG group, it seems more likely that the low ARA content of ES could be responsible for the lower *oc* expression, and hence ossification degree, observed in larvae from this dietary group. In this respect, ongoing studies are devoted to analyzing the molecular pathways involving ARA in skeletogenesis of Senegalese sole.

Concerning IGFs, it has been shown that IGF1 stimulates glucose transport in rat bone-derived osteoblastic cells (Zoidis *et al.*, 2011) and IGF2 exhibits osteogenic properties by increasing osteoblast mitogenic activity through phosphor-activation of MAPK1 and MAPK3 (Cornish *et al.*, 2007). In fish, a coordinated decrease inbmp4expression together with an increase inigf1 expression was observed in developing Europe-an sea bass larvae and was suggested to control, concomitant with other growth factors, the differentiation of osteoblastic cells (Mazurais *et al.*, 2008; Darias *et al.*, 2010a, 2011b). Moreover in fish, it has recently been shown that IGF1 has a stimulatory role in pre-osteoblast cell proliferation during the mitogen-activated protein kinase pathway (Tiago *et al.*, 2010). Fernández *et al.* (2011) suggested that the expression of *igf1* and *pparβ* can be modulated by high levels of dietary VA, contributing to an abnormal development of the musculoskeletal system. Senegalese sole larvae fed *Artemia* enriched with ES displayed lower mineralization of the skeleton

than larvae from the AGG group (Boglino *et al.*, 2012a). However, and contrary to what has been found for European sea bass and gilthead sea bream (Mazurais *et al.*, 2008; Darias *et al.*, 2011b), the IGF axis seemed not to play a central role in the mineralization process for Senegalese sole.

**Conclusions**

The present study revealed different strategies of transcriptional regulation in regard to VA and lipid absorption and metabolism depending on the dietary composition. Results suggested that VA absorption may be controlled at intestinal level bycrbp2αin both dietary groups. The decrease *of i-fabp* expression during development observed in larvae from the AGG group was associated with their degree of intestinal maturation (Boglino *et al.*, 2012b). Therefore, the expression of *i-fabp* could be a useful marker for intestinal development in fish larvae. In the liver, the regulation of *rbp* and *fabp* expression reflected the status of the physiological functions demanding VA and lipids (i.e., skeletal ossification). The invariant expression of *l-fabp* suggests that this gene is not sensitive to the dietary long-chain fatty acid composition, but rather to its content which was similar in both diets. Due to the DHA/EPA, ARA/EPA and ARA/DHA ratios importance for correct ossification of the skeleton, and considering the low larval growth and degree of mineralization observed in the ES group as compared to the AGG group, it seems likely that the low ARA content of ES could be responsible for the lower *oc* expression, and hence degree of ossification observed in this dietary group. Ongoing studies are devoted to corroborating this hypothesis. Although few differences in gene expression were found in this study, which has biological sense since ES and AGG have similar com-position, the significant transcriptional modulation that was observed was associated with the differences in VA content existing between both commercial enrichments.

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# 4

# The effect of dietary oxidized lipid levels on growth performance, antioxidant enzyme activities, intestinal lipid deposition and skeletogenesis in Senegalese sole (*Solea senegalensis*) larvae

Anaïs Boglino[1]
Maria J. Darias J.[2]
Alicia Estévez[1]
Karl B. Andree[1]
Carmen Sarasquete[3]
Montserrat Solé[4]
Enric Gisbert[1]

Submitted to Aquaculture Nutrition

[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

[3] Instituto de Ciencias Marinas de Andalucia (CSIC), Campus Universitario Rio San Pedro, Apdo Oficial, 11510 Puerto Real, Cádiz, Spain

[4] Instituto de Ciencias del Mar (ICM-CSIC), Paseo Marítimo de la Barceloneta 37-49, 08003 Barcelona, Spain

**El efecto de lípidos oxidados en la dieta sobre el crecimiento, la actividad de enzimas antioxidantes, la deposición intestinal de lípidos y la esqueletogénesis en larvas de lenguado Senegalés (*Solea senegalensis*).**

**Resumen**

Los tejidos de los peces, particularmente ricos en n-3 ácidos grasos poliinsaturados (AGPI), están propensos a la peroxidación lipídica que puede dañar a las membranas celulares, provocar lesiones severas y ulteriormente incidencias de enfermedades y mortalidades. No obstante, estos tejidos poseen defensas antioxidantes, tal como la vitamina E (VE) y las enzimas antioxidantes que lo protegen contra los daños oxidativos. Este estudio investigó los efectos de un gradiente creciente de lípidos oxidados en la dieta sobre la supervivencia, el crecimiento, la esqueletogénesis y los procesos de defensa antioxidante, las cuales ocurren en larvas de lenguado Senegalés. Cuatro grupos de peces fueron alimentados con rotiferos y metanauplii de *Artemia* enriquecidos con emulsiones experimentales conteniendo un gradiente creciente de aceite oxidado: no oxidado, NO+VE, 34.5 nmol MDA.g-1 w.w; medio oxidado, MO+VE, 43.1 nmol MDA.g-1 w.w; altamente oxidado, HO+VE, 63.3 nmol MDA.g-1 w.w; y altamente oxidado sin VE, HO-VE, 78.8 nmol MDA.g-1 w.w. El nivel de oxidación lipídico en los rotíferos aumento significativamente siguiendo el gradiente creciente de oxidación de las emulsiones, excepto para el tratamiento HO-VE, en cambio, los niveles de oxidación en la *Artemia* no se vieron afectados por los diferentes enriquecimientos. El estado de oxidación de las larvas de lenguado Senegalés aumento durante el desarrollo, pero no fue afectado por los tratamientos. Los niveles crecientes de oxidación en la dieta no afectaron el perfil de ácidos grasos, la supervivencia, el crecimiento y el proceso de metamorfosis en las larvas del lenguado. El lenguado Senegalés, al parecer, activa ciertos mecanismos de defensa antioxidante en respuesta a las cantidades crecientes de lípidos peroxidados suministrados en la dieta y esto resulta suficientemente eficaz para impedir la detección de cualquier alteración de estos procesos fisiológicos. Al parecer las defensas antioxidantes y los mecanismos de detoxificación ocurren a través del consumo de α-tocopherol de la dieta, la activación de las enzimas antioxidantes (catalasa, superóxido dismutasa, glutatión S-transferasa, glutatión reductasa) y de la retención de grasa oxidada en los enterocitos intestinales para una detoxificación antes de su utilización. No obstante, los peces alimentados con la dieta la más oxidada sin VE presentaron una reducción en la mineralización ósea y una mayor incidencia de deformidades vertebrales. Este estudio indicó la importancia utilizar dietas no oxidadas para realizar la crianza temprana de larvas de lenguado Senegalés y con ello disminuir diferentes problemas en el proceso de esqueletogénesis en etapas posteriores. El nivel de oxidación lipídica en la dieta suministrada a las larvas al parecer no estuvo suficientemente elevada para ver un impacto importante sobre su desarrollo. El uso de presas vivas enriquecidas como vector para alimentarlas restringió esta posibilidad, considerando su metabolismo especifico propio. Un posible proceso adaptivo al estrés oxidativo, incluyendo una reducción de la actividad de enzimas antioxidantes ha sido sugerido de suceder cuando los peces estuvieron siendo alimentados con una dieta altamente oxidada durante un periodo prolongado de cultivo.

**Palabras claves:** *Solea senegalensis*, larvas, peroxidación lipídica, enzimas antioxidantes, vitamina E, esqueletogénesis, enriquecimiento de presas vivas.

**Abstract**

Fish tissues, particularly rich in n-3 PUFA, are prone to lipid peroxidation that can damage cellular membranes, cause severe lesions and subsequently incidences of disease and mortality. However, fish possess antioxidant defenses, such as vitamin E (VE) and antioxidant enzymes to protect them against oxidative damage. This study investigated the effects of an increasing gradient of oxidized dietary lipid on the survival, growth performance, skeletogenesis and antioxidant defensive processes occurring in Senegalese sole (*Solea senegalensis*) larvae. Four groups of fish were fed live prey enriched with experimental emulsions containing an increasing gradient of oxidized oil: non-oxidized, NO+VE, 34.5 nmol MDA $g^{-1}$ w.w.; mildly oxidized, MO+VE, 43.1 nmol MDA $g^{-1}$ w.w.; highly oxidized, HO+VE, 63.3 nmol MDA $g^{-1}$ w.w. and highly oxidized without VE, HO-VE, 78.8 nmol MDA $g^{-1}$ w.w. The oxidized fat in rotifers were significantly raised following the increasing oxidation gradient of the emulsions, except for the HO-VE treatment, but oxidation levels in *Artemia* were not affected by the different enrichment. The oxidation status of Senegalese sole larvae increased during development but this was not related to the dietary treatments. The increasing dietary oxidation levels did not affect the fatty acid (FA) profile, survival, growth performance and metamorphosis processes of sole larvae. Senegalese sole seem to activate antioxidant defense mechanisms in response to the increasing amounts of dietary peroxidized lipids, in a manner efficiently enough to prevent detection of any alterations of these physiological processes. Antioxidant systems and detoxification mechanisms appeared to occur through the consumption of dietary α-tocopherol, the activation of the antioxidant enzymes (catalase, superoxide dismutase, glutathione S-transferase, glutathione reductase) and the retention of oxidized fat in the intestinal enterocytes for detoxification prior to their utilization. However, fish fed the highest oxidized diet presented a reduction in bone mineralization and an increased incidence of vertebral deformities. This study exemplifies the importance of rearing Senegalese sole larvae on non-oxidized diets during the early larval development to avoid detrimental consequences in older fish, most notably in the process of skeletogenesis. The dietary levels of oxidized lipids supplied to the larvae were supposedly not high enough to see a strong impact on their development, as the use of enriched live prey as a vector to feed them restricted this possibility, taking into consideration their own specific metabolism. A possible adaptive process to oxidative stress, including a reduction of the activity of antioxidant enzymes, has been suggested to occur when fish are fed a highly oxidized diet during a long period of rearing.

**Key words***: Solea senegalensis* larvae, lipid peroxidation, antioxidant enzymes, vitamin E, skeletogenesis, live prey enrichment.

**Introduction**

Fish oils are the major components of enriching products currently used in commercial marine fish hatcheries to increase the nutritional value of live prey supplied to the larvae. Their richness in polyunsaturated fatty acids (PUFA) makes them highly digestible for fish larvae and promotes their growth and development. In addition, PUFA are essential and vital constituents for cell membrane structure and integrity and precursors of prostaglandins and other essential physiological compounds (Sargent *et al.* 2002). Nevertheless, PUFA are highly susceptible to be damaged by oxygen derivates and free radicals, such as reactive oxygen species (ROS), produced during normal cellular aerobic functioning (Matés, 2000). Lipid peroxidation of these PUFA is a chain reaction that results in deleterious deterioration of cellular membranes with pathological effects on cells, tissues and morphogenesis (Fontagné *et al.* 2006; 2008). Besides, marine fish tissues contain particularly high amounts of n-3 PUFA (Sargent *et al.* 1999), that make them more at risk from peroxidative damage than mammalian tissues. Thus, although PUFA are essential for optimal growth and development in marine fish larvae, they also impose a significant peroxidation burden (Mourente *et al.* 2000).

Under a normal physiological status, harmful effects of oxidative stress on the lipids of cellular membranes are neutralized by effective antioxidant defense systems operating in fish (Jacob, 1995; Martínez-Álvarez *et al.* 2005). According to Halliwell and Gutteridge (2006), an antioxidant molecule is a substance that, present at low concentrations, significantly delays, prevents or removes oxidative damage of a target substrate. Antioxidant systems involve many molecules that differ in the way that they interact with oxidant components to reduce oxidative stress in the organism. Among them, “sacrificial agents” are molecules preferentially oxidized by ROS to preserve more important biomolecules, such as glutathione (GSH), NADH/NADPH, protein with sulfhydryl (-SH) groups, uric acid among others but also dietary antioxidant micronutrients such as α-tocopherol (vitamin E, VE), ascorbic acid (vitamin C) and carotenoids. VE is a lipid-soluble antioxidant compound that is not synthesized by animals and has to be obtained from the diet (Hess *et al.* 1993). The ingestion of very low quantities of dietary VE are sufficient to effectively prevent lipid peroxidation by scavenging lipid peroxyl radicals much faster than these radicals are able to react with adjacent fatty acid (FA) side chains or with membrane proteins (Halliwell and Gutteridge, 2006; Halver, 2002). Increasing the quantities of unsaturated oxidized fat in fish diets has been shown to increase the dietary requirements for VE in many freshwater and marine fish species (Mourente *et al.* 1999; 2000; 2002; Tocher *et al.* 2003; Puangkaew *et al.* 2005; Peng and Gatlin, 2009; Betancor *et al.* 2011; Lebold *et al.* 2011, Abdel-Hameid *et al.* 2012, among others). Antioxidant defenses also include agents that catalytically remove ROS and thus disrupt lipid peroxidation chain reactions, such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione S-transferase (GST) among others (Halliwell and Gutteridge, 2006). CAT and SOD are scavengers of ROS, eliminating hydrogen peroxide ($H_2O_2$) and superoxide anion radical ($O_2^{\cdot-}$), respectively (Miller *et al.* 1993). GR maintains the level of reduced glutathione (GSH) by means of the reduction of glutathione disulfide (GSSG) and the oxidation of NADH and some glutathione S-transferase (GST) isoforms have the capacity to detoxify peroxidized compounds (Halliwell and Gutteridge, 2006). Numerous studies have shown an increase

in the activity of these antioxidant enzymes when fish were fed oxidized diets (Mourente *et al.* 2002; Tocher *et al.* 2003; Trenzado *et al.* 2006; Fontagné *et al.* 2006; 2008; Zambrano and Landines, 2011).

Senegalese sole (*Solea senegalensis*) is a high-value marine flatfish, reared in intensive production systems of the Iberian Peninsula. Although important advances have been recently made with regard to species-specific larval rearing procedures (Imsland *et al.* 2003; Engrola *et al.* 2005), nutrient requirements (Aragão *et al.* 2004; Morais *et al.* 2004; Villalta *et al.* 2005a, b; Fernández and Gisbert, 2011; Ribeiro *et al.* 2012 among others) and digestive physiology (Conceição *et al.* 2007; Rønnestad and Conceição, 2012), there are still important gaps regarding the effect of some nutrients on larval development and performance. Lipids, particularly PUFA and essential fatty acids (EFA), have been shown to be indispensable nutrients for appropriate larval growth, morphogenesis and skeletal formation in sole (Izquierdo *et al.* 2000; Bell and Sargent 2003; Villalta *et al.* 2005a, b; Morais *et al.* 2006; Dâmaso-Rodrigues *et al.* 2010; Boglino *et al.* 2012a, b; Darias *et al.* 2012). However, as these cell membrane components are highly susceptible to lipid peroxidation and oxidative attacks that occur naturally in marine fish, detrimental impacts on developing tissues might occur when sole larvae are fed an oxidized diet.

One of the main objectives of our study was to characterize the antioxidant systems in hatchery reared Senegalese sole larvae, in order to understand how this species is protected against oxidative stress during early stages of larval development. For that, the mechanisms of defense against oxidative stress of Senegalese sole larvae have been explored, using an increasing gradient of oxidized lipids with the presence or not of dietary VE, and studying how larval performance (survival, growth, metamorphosis, morphogenesis and skeletogenesis) was affected.

## Material and methods

*Larval rearing and feeding protocol*

One-day-old Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, A Coruña, Spain), transported and acclimated at IRTA-SCR facilities. Larvae were reared in 16, 100L cylindro-conical tanks (initial density: 100 larvae·$L^{-1}$) connected to a IRTAmar$^{TM}$ water recirculation unit. Water conditions were as follows: temperature 17.4 ± 0.4 ºC, salinity 35 ‰, pH 8.0 ± 0.2, dissolved oxygen 7.5 ± 1.3 ppm and 50% daily water renewal in the recirculation system with individual gentle aeration. Photoperiod was 16L: 8D, and light intensity was 500 lx at the water surface. The effect of increasing oxidized lipid levels on Senegalese sole performance and development was evaluated using the following feeding protocol: larvae were fed twice a day, from 2 days post hatching (dph) to 9 dph, with rotifers (*Brachionus plicatilis*) enriched with four different experimental emulsions, at a density of 10 rotifers $mL^{-1}$ from 2 to 7 dph and of 5 rotifers $mL^{-1}$ from 8 to 9 dph. *Artemia* metanauplii enriched with the same four emulsions were supplied twice a day to larvae from 8 to 19 dph. After this age, larvae were fed *Artemia* metanauplii enriched with a non-oxidized emulsion until the end of the trial (35 dph), at increasing density from 0.5 to 12 metanauplii $mL^{-1}$, adjusted based upon the increase in weight of the larvae and on the daily food ration described by Cañavate *et al.* (2006). From 19 dph onwards,

when larvae began to settle to the bottom of the tank, enriched *Artemia* metanauplii were supplied frozen, following the method of Morais *et al.* (2005) and Villalta *et al.* (2008).

*Experimental diets and live prey enrichment*

The effect of the graded oxidative levels on Senegalese sole performance and development was evaluated during the pre-metamorphic and rotifer feeding period (2-9 dph) and the pro-metamorphic and *Artemia* feeding period (9-19 dph). During the post-metamorphic period, from 19 dph onwards, larvae from all treatments were fed *Artemia* enriched with the non-oxidized emulsion with VE (NO+VE), in order to test their ability to recover a normal development after suppression of the dietary oxidative stress.

A commercially available fish liver oil from *Gadus morhua* (Fluka©, Sigma-Aldrich, Chemie GmBH, Steinheim, Norway) was used to prepare the experimental emulsions to enrich the live prey. This oil was oxidized for 24h under different conditions of temperature (50 and 65ºC) and aeration in order to get oils with medium and high levels of peroxidation. Three emulsions containing a significant increasing gradient of lipid peroxidation values ($P$ = 0.006, Table 1), also adding 2.32% w.w. of VE, were prepared with these oils as follows: NO+VE, non-oxidized emulsion prepared with the original cod liver oil and used as a control emulsion (34.5 nmol MDA $g^{-1}$ w.w.); MO+VE, medium oxidized emulsion prepared with the medium oxidized oil (43.1 nmol MDA $g^{-1}$ w.w.); HO+VE, high oxidized emulsion prepared with the highly oxidized oil (63.3 nmol MDA $g^{-1}$ w.w.). A fourth emulsion was prepared with the high oxidized oil but without VE addition (HO-VE, 78.8 nmol MDA $g^{-1}$ w.w.). Lipid and fatty acid contents of each of the above-mentioned emulsions are shown in Table 1.

**Table 1.** Formulation (mg $g^{-1}$), total lipid and fatty acid contents (mg $g^{-1}$ DW) and fatty acid composition (% of TFA), VE contents (mg·$kg^{-1}$ WW) and oxidation levels (TBARs in nmol MDA.$g^{-1}$ W.W.) of the four experimental emulsions (mean, n = 1). Totals include some minor components not shown.

| | NO+VE | MO+VE | HO+VE | HO-VE |
|---|---|---|---|---|
| **Not oxidized cod liver oil**[1] | 516.2 | - | - | - |
| **Medium oxidized cod liver oil**[1] | - | 516.2 | - | - |
| **High oxidized cod liver oil**[1] | - | - | 516.2 | 539.4 |
| **Total oil** | 516.2 | 516.2 | 516.2 | 539.4 |
| **VE**[2] | 23.2 | 23.2 | 23.2 | - |
| **Supplements**[3] | 460.6 | 460.6 | 460.6 | 460.6 |
| Total lipids | 732.1 | 643.1 | 574.1 | 622.6 |
| Total FA | 375.3 | 351.5 | 309.9 | 440.2 |
| 14:0 | 4.7 | 4.6 | 4.7 | 5.1 |
| 15:0 | 1.4 | 1.2 | 1.1 | 1.6 |
| 16:0 | 14.6 | 14.2 | 14.8 | 14.4 |
| 18:0 | 2.6 | 2.6 | 2.6 | 2.3 |
| Total SFA | 23.5 | 22.8 | 23.2 | 23.4 |
| 16:1n-9 | 6.0 | 6.0 | 5.6 | 7.0 |
| 18:1n-9 | 15.1 | 14.6 | 15.1 | 14.8 |
| 18:1n-7 | 3.6 | 3.5 | 3.7 | 3.7 |
| 18:1n-11 | 0.2 | 0.2 | 0.2 | 0.2 |
| 20:1n-11 | 0.4 | 0.4 | 0.1 | 0.1 |
| 20:1n-9 | 8.8 | 8.9 | 8.1 | 7.7 |
| 20:1n-7 | 1.2 | 1.2 | 1.0 | 1.1 |
| Total MUFA | 37.2 | 35.9 | 35.6 | 36.1 |
| 18:2n-6 | 4.3 | 4.7 | 4.6 | 5.0 |
| 18:3n-6 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20:3n-6 | 0.0 | 0.0 | 0.0 | 0.1 |
| 20:4n-6 | 0.5 | 0.4 | 0.5 | 0.5 |
| 22:5n-6 | 0.1 | 0.2 | 0.1 | 0.2 |
| Total n-6 PUFA | 5.0 | 5.3 | 5.2 | 5.8 |
| 18:3n-3 | 1.3 | 1.4 | 1.4 | 1.4 |
| 18:4n-3 | 2.0 | 2.1 | 2.0 | 2.0 |
| 20:4n-3 | 10.7 | 10.4 | 10.8 | 9.7 |
| 20:5n-3 | 8.0 | 8.6 | 8.6 | 8.3 |
| 21:5n-3 | 0.4 | 0.4 | 0.4 | 0.3 |
| 22:5n-3 | 1.4 | 1.5 | 1.5 | 1.5 |
| 22:6n-3 | 9.8 | 10.5 | 10.4 | 10.5 |
| Total n-3 PUFA | 33.5 | 35.0 | 35.1 | 33.7 |
| Total PUFA | 38.4 | 40.3 | 40.3 | 39.5 |
| (n-6)/(n-3) | 0.1 | 0.1 | 0.1 | 0.2 |
| DHA/EPA | 1.2 | 1.2 | 1.2 | 1.3 |
| ARA/DHA | 0.1 | 0.0 | 0.0 | 0.0 |
| ARA/EPA | 0.1 | 0.0 | 0.1 | 0.1 |
| MUFA/PUFA | 1.0 | 0.9 | 0.9 | 0.9 |
| VE | 18137 | 13005 | 17416 | 15.0 |
| TBARs | $34.47\pm2.42^{c}$ | $43.10\pm1.98^{bc}$ | $63.33\pm5.53^{ab}$ | $78.75\pm5.79^{a}$ |

[1]Fluka® fish liver oil from *Gadus morrhua*, Sigma-Aldrich Chemie GmBH, Steinheim, Norway

[2]VE ≥ 96% (HPLC), Sigma-Aldrich, Germany

[3]Supplements: Soy lecithin, 40.6mg.$g^{-1}$ DW; distilled water, 420mg.$g^{-1}$ DW.

Rotifers and *Artemia* nauplii were enriched with these same four different emulsions in order to obtain four dietary treatments with graded levels of lipid peroxidation (Table 2).

**Table 2.** Total lipid and total fatty acid contents (mg $g^{-1}$ DW), fatty acid composition (in % TFA), VE contents (mg $kg^{-1}$ WW) and oxidation levels (TBARs in nmol MDA.$g^{-1}$ W.W.) in enriched rotifer and *Artemia* metanauplii with the four emulsions (mean ± SD; n = 2). Totals include some minor components not shown. Superscript letters denote significant differences among diets (ANOVA, $P < 0.05$). A 0.0%TFA means content under 0.45%TFA.

| | Enriched rotifer | | | | Enriched *Artemia* nauplii | | | |
|---|---|---|---|---|---|---|---|---|
| | **NO+VE** | **MO+VE** | **HO+VE** | **HO-VE** | **NO+VE** | **MO+VE** | **HO+VE** | **HO-VE** |
| Total lipid | 138.5±49.3 | 189.7±43.0 | 165.4±37.6 | 154.2±58.8 | 263.3±91.7 | 184.0±31.3 | 173.8±0.3 | 188.8±7.6 |
| Total FA | 74.2±18.9 | 105.5±29.9 | 72.9±24.6 | 74.1±42.3 | 143.3±27.1 | 107.6±6.0 | 99.4±7.2 | 109.0±29.5 |
| 14:0 | 3.8±0.3 | 4.1±0.7 | 4.2±0.2 | 4.4±0.1 | 2.1±0.9 | 1.5±0.9 | 1.6±0.7 | 1.8±1.0 |
| 15:0 | 1.3±0.1$^{b}$ | 1.2±0.2$^{b}$ | 1.9±0.2$^{ab}$ | 2.1±0.2$^{a}$ | 1.2±0.9 | 1.1±0.9 | 1.0±0.5 | 1.6±1.3 |
| 16:0 | 15.8±0.2 | 15.1±0.6 | 15.5±1.0 | 15.3±0.6 | 13.2±1.2 | 12.6±0.7 | 12.8±1.1 | 13.9±0.7 |
| 18:0 | 3.3±0.2 | 2.9±0.3 | 3.5±0.1 | 3.4±0.0 | 5.4±1.0 | 6.0±0.9 | 6.0±0.9 | 6.4±1.4 |
| Total SFA | 24.1±0.8 | 23.3±0.1 | 25.1±1.5 | 25.2±0.5 | 22.1±1.9 | 21.4±1.4 | 21.4±1.4 | 23.8±1.5 |
| 16:1n-9 | 7.5±0.7 | 6.2±0.2 | 8.1±0.3 | 9.1±1.6 | 4.1±1.2 | 3.3±0.9 | 3.4±0.8 | 3.4±1.0 |
| 18:1n-9 | 16.0±0.1 | 15.2±0.6 | 16.7±0.8 | 17.0±0.5 | 19.4±4.2 | 18.0±0.6 | 21.1±3.0 | 19.9±3.2 |
| 18:1n-7 | 3.9±0.3 | 3.7±0.0 | 4.3±0.2 | 4.4±0.1 | 2.3±3.3 | 5.7±0.1 | 2.8±4.0 | 2.5±3.6 |
| 18:1n-11 | 0.2±0.0 | 0.2±0.0 | 0.2±0.1 | 0.2±0.0 | 0.0±0.0 | 0.03±0.0 | 0.04±0.1 | 0.04±0.1 |
| 20:1n-11 | 0.2±0.0 | 0.3±0.1 | 0.5±0.3 | 0.2±0.0 | 0.1±0.1 | 0.04±0.1 | 0.1±0.1 | 0.04±0.1 |
| 20:1n-9 | 7.2±0.6 | 8.1±0.2 | 7.2±0.6 | 7.0±0.6 | 2.8±1.8 | 2.1±1.7 | 1.9±1.6 | 2.1±1.4 |
| 20:1n-7 | 0.7±0.1 | 1.1±0.2 | 0.4±0.6 | 0.8±0.2 | 0.3±0.5 | 0.1±0.1 | 0.1±0.1 | 0.0±0.0 |
| Total MUFA | 37.2±0.7 | 36.4±0.6 | 38.1±1.8 | 40.0±1.3 | 30.3±1.9 | 29.5±3.9 | 29.7±4.0 | 28.0±2.8 |
| 18:2n-6 | 7.6±2.7 | 5.5±0.2 | 6.4±0.6 | 6.8±0.8 | 5.5±0.7 | 5.7±1.2 | 6.0±0.6 | 5.9±1.0 |
| 18:3n-6 | 0.0±0.0 | 0.1±0.2 | 0.0±0.0 | 0.0±0.0 | 0.9±0.4 | 0.8±0.6 | 0.9±0.6 | 1.0±0.7 |
| 20:3n-6 | 0.0±0.0 | 0.1±0.1 | 0.0±0.0 | 0.1±0.2 | 0.1±0.0 | 0.2±0.0 | 0.1±0.1 | 0.4±0.5 |
| 20:4n-6 | 0.5±0.0 | 0.5±0.0 | 0.6±0.1 | 0.6±0.1 | 0.3±0.0 | 0.2±0.3 | 0.1±0.2 | 3.8±4.8 |
| 22:5n-6 | 0.1±0.1 | 0.1±0.0 | 0.0±0.0 | 0.0±0.0 | 0.1±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| Total n-6 PUFA | 8.2±2.8 | 6.4±0.4 | 7.0±0.7 | 7.5±0.5 | 7.0±1.2 | 6.9±1.5 | 7.1±0.8 | 11.2±7.0 |
| 18:3n-3 | 3.8±2.7 | 1.9±0.2 | 1.8±0.1 | 1.8±0.3 | 23.2±6.7 | 26.4±7.6 | 26.7±7.3 | 22.5±2.3 |
| 18:4n-3 | 1.4±0.2 | 1.8±0.3 | 1.7±0.4 | 0.8±1.1 | 4.7±1.4 | 4.8±2.0 | 4.9±1.9 | 4.1±0.8 |
| 20:4n-3 | 8.9±0.7$^{ab}$ | 10.7±0.2$^{a}$ | 7.6±0.5$^{b}$ | 8.5±0.6$^{b}$ | 3.4±2.6 | 3.1±2.5 | 3.0±2.1 | 2.7±2.2 |
| 20:5n-3 | 6.7±0.8 | 7.5±0.5 | 7.0±0.4 | 6.5±0.6 | 3.7±1.1 | 3.4±1.7 | 3.0±1.2 | 3.0±1.4 |
| 21:5n-3 | 0.2±0.0 | 0.3±0.0 | 0.3±0.0 | 0.3±0.0 | 0.1±0.1 | 0.1±0.1 | 0.1±0.1 | 0.0±0.0 |
| 22:5n-3 | 1.3±0.1 | 1.3±0.1 | 1.4±0.1 | 1.3±0.2 | 0.5±0.2 | 0.4±0.3 | 0.3±0.3 | 0.4±0.3 |
| 22:6n-3 | 7.0±0.8 | 8.8±0.5 | 7.7±0.5 | 6.7±0.3 | 3.3±1.5 | 2.4±2.0 | 2.2±1.7 | 2.7±2.2 |
| Total n-3 PUFA | 29.3±1.3$^{ab}$ | 32.3±1.8$^{a}$ | 27.6±1.0$^{ab}$ | 25.8±0.3$^{b}$ | 38.8±2.5 | 40.4±3.1 | 40.2±3.8 | 35.4±3.1 |
| Total PUFA | 37.4±1.5 | 38.6±1.4 | 34.6±1.6 | 33.3±0.8 | 45.8±3.7 | 47.3±4.5 | 47.3±4.6 | 46.6±3.9 |
| (n-6)/(n-3) | 0.3±0.1 | 0.2±0.0 | 0.3±0.0 | 0.3±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.3±0.2 |
| DHA/EPA | 1.0±0.0$^{b}$ | 1.2±0.0$^{a}$ | 1.1±0.0$^{ab}$ | 1.0±0.1$^{b}$ | 0.9±0.2 | 0.7±0.3 | 0.7±0.3 | 0.8±0.3 |
| ARA/DHA | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 | 0.1±0.1 | 0.04±0.1 | 3.0±4.2 |
| ARA/EPA | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.04±0.1 | 0.03±0.1 | 1.9±2.5 |
| MUFA/PUFA | 1.0±0.1 | 0.9±0.0 | 1.1±0.1 | 1.2±0.1 | 0.7±0.1 | 0.6±0.1 | 0.6±0.2 | 0.6±0.1 |
| VE | 161.0 | 115.4 | 154.6 | 46.2 | 136.0 | 97.5 | 109.0 | 17.3 |
| TBARs | 34.47±1.28$^{b}$ | 46.42±2.67$^{b}$ | 87.18±13.38$^{a}$ | 46.79±4.12$^{b}$ | 43.29±3.44 | 35.59±1.99 | 33.36±3.21 | 41.85±0.77 |

Rotifers were enriched in 10 L containers at 500 rotifer $mL^{-1}$ at 26ºC with 0.6 g $l^{-1}$ of emulsion. Half of the rotifers were supplied to the larvae after 2h of enrichment and the other half after 6h of enrichment. Preliminary enrichment assays indicated that there was no change in the level of lipid peroxidation and fatty acid composition between rotifers enriched for 2 and 6 hours (data not shown). One-day-old *Artemia* nauplii (EG strain, INVE) were enriched in 20 L containers at 300 nauplii $mL^{-1}$ for

2h at 28 ºC with 0.6 g $l^{-1}$ of emulsion. Enriched *Artemia* were kept at 4 ºC in UV-treated, filtered seawater with aeration until administered to larvae. In order to reduce the bacterial load after enrichment and remove emulsion residues, rotifers and *Artemia* were washed with UV-treated filtered seawater and treated with $H_2O_2$ (40 ppm for 15 min for rotifers and 8000 ppm for 5 min for *Artemia,* according to Giménez *et al.* (2006)) and then rinsed with filtered seawater. The biochemical analysis of lipids and fatty acid (FA) composition of enriched rotifer and *Artemia* are shown in Table 2.

*Determination of the lipid peroxidation levels in live prey and larvae*

Samples of each emulsion, enriched live prey, and larvae at 9, 19 and 35 dph were washed with distilled water and stored at -80ºC until analysis. Approximately 100 mg of tissue per sample was homogenized (Ultra-Turrax D25 basic, IKA©-Werke) for 5 minutes in eight volumes (v/w) for larval samples or ten volumes (v/w) for emulsions and enriched live prey samples with 0.15 M KCl-KOH, 1 mM EDTA (pH 7.5) buffer and then subjected to sonication (Vibra-Cell©, Sonics) for 1.5 minutes, while maintaining them in ice to restrict enzymatic activity. Homogenized samples were centrifuged at 11,000 rpm for 5 minutes at 4ºC and the supernatant was collected for analytical determinations.

Quantification of lipid peroxidation in the emulsions, live prey, and larvae was conducted using the thiobarbituric acid reactive substances (TBARs) method described in Solé *et al.* (2004). In brief, lipid peroxidation was measured using 200 µL of the homogenate mixed with 650 µL of methanol, 1-methyl-2-phenylindole (solution stock of 10.3 mM) in acetonitrile: methanol (1:3; v/v) and 150 µL of 37% HCl. This mixture was incubated for 40 minutes at 45ºC, cooled on ice for 10 minutes and centrifuged at 13000 rpm for 10 minutes to remove protein precipitates. Absorbance was read at 586 nm and the amount of peroxidized lipids (in nmol malondialdehyde (MDA) $g^{-1}$; w/w) was evaluated by means of a calibration curve made of a standard solution of 1,1,3,3-tetramethoxypropane (10 mM).

*Lipid, fatty acid and VE analyses*

Enriched live prey and larvae were sampled at 1 dph (endogenous feeding period before mouth opening), 9 dph (end of the pre-metamorphic and rotifer feeding period), 19 dph (end of the pro-metamorphic period and feeding phase with *Artemia* enriched the four experimental emulsions) and 35 dph (end of the experiment), then washed with distilled water to remove salt and bacteria, and frozen at -80 ºC until analysis. Total lipids were extracted in chloroform: methanol (2:1, v:v) using the method of Folch *et al.* (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform: methanol (2:1, 20mg $mL^{-1}$) containing 0.01% butylated hydroxytoluene at -20 ºC prior to analysis. Fatty acid analysis was conducted by gas-liquid chromatography, acid catalysed transmethylation was carried out according to Christie's method (1982) and fatty acids quantified as previously described in Boglino *et al.* (2012a). VE contents in enriching emulsions, live prey and larvae at 9, 19 and 35 dph were quantified according to Tan *et al.* (2007) following the directive 2000/45/CE fixing methods of VE analysis in food adopted by the EC on July, 6$^{th}$, 2000 (Part B, Official Journal of L174/39-44). In brief, samples were saponified and VE was extracted with mineral ether. The solvent was removed by evaporation and the residue was dissolved in methanol. VE contents were determined by inverse phase-HPLC with UV detection.

Samples were kept in dark during the whole procedure to avoid photo-oxidation of VE. Results were expressed in mg of α-tocopherol per kg of fresh sample.

*Larval growth, survival and metamorphosis success*

Standard length (SL) and dry weight (DW) of larvae were measured at 9, 19 and 35 dph. Thirty larvae from each tank were randomly sampled and euthanized with an overdose of tricaine methane sulphonate (MS-222, Sigma). SL was measured with a digital camera connected to a stereomicroscope (Nikon SMZ 800) and an image analysis system (AnalySIS®, Soft Imaging Systems, GmbH, Olympus, Germany). DW determination was performed by rinsing larvae with distilled water to remove salt and then drying them at 60 ºC for 24 h. Samples were weighed with an analytic microbalance (Sartorius BP211D). Specific growth rate (SGR, in % $d^{-1}$) was calculated as (ln $DW_f$ –ln $DW_i$) / ($t_f$ – $t_i$) * 100; where $DW_f$, $DW_i$, $t_f$ and $t_i$ represented final and initial DW and time of the experiment, respectively. Final survival was evaluated by counting the animals at the end of the experiment and calculated according to Buckley *et al.* (1984), which considers the number of sampled individuals during the experiment. Eye migration in Senegalese sole larvae, used to evaluate the advance of the process of metamorphosis, was assessed in 30 individuals per tank at 9, 15, 19 and 35 dph, according to Villalta *et al.* (2005a). Data are presented as the relative amount of larvae in each stage of development at the same age. Eye migration index ($I_{EM}$ = Σ (%fish in each stage * stage) / 100) was calculated according to Solbakken *et al.* (1999).

*Determination of antioxidant enzyme activities*

Homogenized samples, prepared for the determination of the levels of lipid peroxidation, were used to measure antioxidant enzyme activities. Catalase (CAT, E.C. 1.11.1.6) activity was measured in larvae by the decrease in absorbance at 240 nm (ε = 40 $M^{-1}$ $cm^{-1}$) using 50 mM $H_2O_2$ as substrate (Aebi, 1974). Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was assayed by following the formation of glutathione chlorodinitrobenzene adduct at 340 nm (ε = 9.6 $mM^{-1}$ $cm^{-1}$), using 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione (GSH) as substrates (Habig *et al.* 1974). Glutathione reductase (GR, E.C. 1.8.1.7.) activity was determined by measuring the oxidation of NADPH at 340 nm (ε = 6.22 $mM^{-1}$ $cm^{-1}$), using 20 mM glutathione disulfide and 2 mM NADPH as substrates (Carlberg and Mannervick, 1975). Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured at 550 nm as the degree of inhibition of cytochrome c reduction by $O_2^-$ generated by the xanthine oxidase/hypoxanthine system, according to McCord and Fridovich, (1969). The reaction mixture consisted in 50 mM sodium phosphate buffer pH 7.8, 0.1 mM $Na_2EDTA$, 50 µM hypoxanthine, 10 µM cytochrome c and 0.6 $U.mL^{-1}$ xanthine oxidase. One unit of SOD activity was defined as the amount of sample causing a 50% of inhibition of cytochrome c reduction compared to the baseline record obtained with buffer instead of sample. Soluble protein of crude enzyme extracts was quantified by Bradford's method (Bradford, 1976) using bovine serum albumin as the standard. Enzymatic activities were expressed as specific enzyme activity, in nmol of catalyzed substrate per milligram of protein (nmol $mg^{-1}$ protein) for CAT, GST and GR activities and in $U.mg^{-1}$ protein for SOD activity. All assays

were carried out in triplicate at 25ºC and absorbance was read using a spectrophotometer (Tecan™ infinite M200).

*Accumulation of neutral lipids in the intestine*

For histological purposes, five larvae from each experimental tank (*n* = 20 per dietary treatment) were randomly sampled at 9 and 19 dph, euthanized with an overdose of MS-222, fixed in 4% formalin buffered to pH 7.0 with 0.1 M phosphate buffer during 24 hours and then preserved in 70% ethanol. Afterwards, samples were post-fixed in 1% osmium tetraoxide and 2.5 % potassium dichromate for 8 h, washed in running tap water and dehydrated before the wax embedding procedure. The degree of neutral lipid deposition in the intestinal mucosa was assessed according to previously described in Ortiz-Delgado *et al.* (2008) and Boglino *et al.* (2012c). Histological images were obtained by light microscopy (Leica DM 2000™) connected to a digital camera (Olympus DP70©) and analyzed using the digital image analysis software AnalySIS® (Soft Imaging Systems, GmbH, Olympus, Germany). The quantification of the areas occupied by neutral lipids (stained in black due to their insolubilization by osmium tetraoxide) was conducted as described by Ortiz-Delgado *et al.* (2008). Briefly, fat deposits were assumed to be homogeneous in the intestine and the area of fat deposits was based on the analysis of five randomly chosen fields. Fat accumulation ($F_d$) in each target tissue was determined using the following formula: $F_d$ (%) = ($\Sigma$ $ARF_d$ / $\Sigma$ $ART_t$) * 100, where $ARF_d$ is the area of black stained fat deposits and $ART_t$ the total area of the target tissue in the optical field.

*Calcification degree and skeletal deformities*

Forty larvae per tank were randomly sampled at 9 and 19 dph to evaluate the impact of different dietary treatments on the degree of ossification of the larval skeleton and at 35 dph to identify different typologies of skeletal deformities and quantify their incidence. Sampled larvae were fixed in 4% formalin buffered to pH = 7.0 with 0.1 M phosphate buffer and stored at 4ºC until bone staining. Animals were stained with alizarin red according to Darias *et al.* (2010) and skeletal structures were identified and named according to Wagemans and Vandewalle (2001) and Gavaia *et al.* (2002).

The degree of calcification of skeletal structures was evaluated qualitatively by means of the calcification index (CI) in larvae aged 9 and 19 dph considering the presence/absence of calcified structures for different body regions. At 9 dph, the only detectable calcified structures were in the cranial region; thus the cranial calcification index ($CI_C$; $0 < CI_C < 5$) was calculated as follows: a $CI_C$ of 0 was given to larvae without any calcified structure; a $CI_C$ of 1 for larvae displaying the cleithrum (cl), the operculum (op) and the maxillary (max) calcified; a $CI_C$ of 2 was given for larvae showing the cl, op, max, dentary (dt) and the beginning of the parasphenoid (pasph) calcified; a $CI_C$ of 3 for larvae presenting cl, op, max, dt, pasph, the branchiostegal rays (bs.r) and the preopercular (po) calcified; a $CI_C$ of 4 was given for larvae displaying the cl, op, max, dt, pasph, bs.r, po, the ectopterygoid (ectp), the anguloarticular (an) and the some of the skull bones calcified (frontal (f), supraoccipital (soc), sphenotic (spot), pterotic (ptot), exoccipital (exoc) or basioccipital (boc)), and a $CI_C$ of 5 was given for

larvae showing the cl, op, max, dt, pasph, bs.r, po, ectp, an and the whole skull (f, soc, spot, ptot, exoc and boc) calcified.

At 19 dph, the CI was computed considering the level of calcification in the cranial ($CI_C$), vertebral ($CI_V$) and caudal ($CI_T$) regions. In the cranial region, the $CI_C$ was calculated as explained for larvae aged 9 dph. In the vertebral region, the $CI_V$ was calculated as follows: $CI_V$ = (3*A+2*B+1*C)/100; where A represents the percentage of vertebrae totally calcified (arches, spines and vertebral body); B is the percentage of vertebrae presenting calcified spines but the vertebral body not yet calcified, and C represents the percentage of calcified pairs of neural and haemal spines. Values of A, B and C were expressed as percentages of the total number of vertebrae (generally 45). With regards to the caudal region ($0 < CI_T < 5$), a $CI_T$ value of 1 was attributed to larvae showing only the fin rays (fr) calcified; a $CI_T$ value of 2 was given to larvae with the calcified fr and the hypurals (hyp) starting to calcify; a $CI_T$ value of 3 was given to larvae presenting the fr, hyp and the urostyle partially calcified; whereas a $CI_T$ value of 4 was attributed to larvae displaying the complete tail ossified. While the $CI_C$ ranged between 1 and 5, the $CI_V$ and $CI_T$ only ranged from 1 to 4; consequently, values of $CI_V$ and $CI_T$ were converted into an index from 0 to 5 in order to get the same load in the final calculation of the level of calcified structures from the three considered body regions.

The incidence of skeletal abnormalities was analyzed in the cranium, vertebral column and caudal fin complex of Senegalese sole larvae at 35 dph according to Boglino *et al.* (2012b). However, only a few cranial deformities (mainly jaw deformities) were found in the examined specimens. Vertebral abnormalities were divided into two major classes: vertebral column deformities, affecting the general shape of the vertebral column, such as scoliosis, lordosis or torsion; and vertebral body deformities, affecting a few vertebrae in a region of the vertebral column, such as deformation of vertebral body, compression, fusion or fusion and deformation of the neural and haemal spines.

*Statistics*

Results were expressed as means ± S.E.M. (n = 4). All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test) previously to analysis. A one-way analysis of variance (ANOVA) was performed among treatments and the post-hoc Tukey's test was performed when significant differences were found ($P < 0.05$). All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA).

**Results**

*Lipid peroxidation levels in enriched live prey and larvae*

The level of lipid peroxidation differed between enriched rotifers and *Artemia* metanauplii. TBAR levels in enriched rotifers increased in accordance to lipid peroxidation levels of the experimental enriching products, with the exception of rotifers enriched with the HO-VE emulsion that showed intermediate levels of TBAR ($P < 0.001$, Table 2), while TBAR levels in enriched *Artemia* metanauplii were similar in

all the dietary treatments, regardless the levels of lipid peroxidation in the enriching products ($P$ > 0.05, Table 4). The quantification of TBARs did not show any significant difference in larvae fed the four experimental diets at 9, 19 and 35 dph ($P$ > 0.05, Table 3). However, the TBAR levels in larvae significantly increased during development ($P$ < 0.001).

*Lipid, fatty acid composition and* VE *content in live prey and larvae*

No significant differences were detected in the total lipid and total FA contents among rotifer enriched with the four experimental enriching emulsions ($P$ > 0.05, Table 2). The gradient of oxidation in the emulsion did not affect the contents in total saturated fatty acids (SFA), oleic acid (18:1n-9, OA), total monounsaturated fatty acids (MUFA), nor in arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and total n-6 polyunsaturated fatty acids (PUFA) in enriched rotifers ($P$ > 0.05). Although MO+VE-enriched rotifers contained significantly higher amounts of eicosatetraenoic acid (20:4n-3, ETA) and total n-3 PUFA than rotifers enriched with the HO-VE emulsion ($P$ = 0.017 and $P$ = 0.026, respectively), rotifers contained the same amount of total PUFA and similar ratios of n-6/n-3 PUFA, ARA/EPA, ARA/DHA and MUFA/PUFA ($P$ > 0.05). However, the DHA/EPA ratio was significantly higher in rotifers enriched with the MO+VE emulsion than those enriched with the NO+VE and HO-VE emulsions ($P$ = 0.033). HO-VE-enriched rotifers tended to show the lowest VE amounts, compared to those enriched with the NO+VE, MO+VE and HO+VE emulsions.

Regarding enriched *Artemia* metanauplii, no significant differences were observed in the total lipid and total FA contents among the *Artemia* metanauplii enriched with the four different emulsions ($P$ > 0.05, Table 2). The gradient of lipid peroxidation in the emulsions did not affect the absolute or the relative amounts of total SFA, OA, total MUFA, ARA, DHA, EPA, nor total PUFA in enriched *Artemia* metanauplii ($P$ > 0.05).

The FA composition of tissues in pre-metamorphic larvae aged 9 dph reflected the composition of the diet, with some minor variations (Table 3). Total lipid, total FA and SFA contents in 9 dph-larvae were not significantly affected by dietary treatments ($P$ > 0.05). OA content in the larvae fed the NO+VE diet was significantly higher than in larvae fed the MO+VE diet ($P$ = 0.030), but the amount of total MUFA in larvae was similar among all dietary treatments ($P$ > 0.05). Larvae fed rotifer enriched with the MO+VE emulsion contained significantly higher amounts of linoleic acid (18:2n-6, LA) and n-6 PUFA than larvae fed HO+VE and HO-VE diets ($P$ = 0.003 and $P$ = 0.027, respectively). In addition, they also showed higher contents of docosapentaenoic acid (22:5n-3, DPA) and DHA than larvae from the other groups ($P$ = 0.022 and $P$ = 0.002, respectively), resulting in a higher level of PUFA ($P$ = 0.024) in these larvae, but similar ratios of (n-6)/(n-3) PUFA, DHA/EPA, ARA/DHA and ARA/EPA to those of larvae from the other groups ($P$ > 0.05). Similarly to what was observed in enriched rotifers, the amount of n-3 PUFA tended to be higher in the larvae fed the MO+VE diet. The ratio of MUFA/PUFA was significantly lower in the larvae fed the MO+VE diet than in those fed the NO+VE diet ($P$ = 0.034). Larvae from the NO+VE group contained significantly more VE than larvae from the HO+VE and HO-VE diets ($P$ = 0.008).

**Table 3.** Total lipid and total fatty acid contents (mg $g^{-1}$ DW), VE contents (mg $kg^{-1}$ WW) and fatty acid composition (in % TFA) in Senegalese sole larvae at 1, 9, 19 and 35 dph fed the four dietary treatments (mean ± SD; n = 3). Totals include some minor components not shown. Superscript letters denote significant differences among diets (ANOVA, $P < 0.05$). A 0.0%TFA means content under 0.45%TFA.

| | 1dph | 9 dph | | | | 19 dph | | | | 35 dph | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NO+VE | MO+VE | HO+VE | HO-VE | NO+VE | MO+VE | HO+VE | HO-VE | NO+VE | MO+VE | HO+VE | HO-VE |
| Total lipid (en mg/g DW) | 105.6±16.7 | 104.8±21.2 | 116.5±18.2 | 95.00±36.8 | 115.1±31.6 | 122.6±11.9 | 143.7±7.2 | 139.2±12.6 | 154.7±48.0 | 133.9 | 122.4 | 133.4 | 128.6 |
| Total FA (en mg/g DW) | 36.6±10.0 | 41.6±5.5 | 53.0±9.7 | 39.9±2.2 | 48.3±19.9 | 51.5±2.4 | 66.8±4.1 | 52.2±6.5 | 67.0±14.9 | 43.8 | 41.3 | 70.5 | 46.6 |
| 14:0 | 0.9±0.2 | 1.3±0.1 | 0.9±0.1 | 1.5±0.3 | 1.7±1.0 | 0.5±0.2 | 0.4±0.3 | 0.26±0.04 | 0.4±0.1 | 0.4 | 0.2 | 0.2 | 0.3 |
| 15:0 | 0.56±0.02 | 0.6±0.2 | 0.4±0.01 | 0.8±0.4 | 0.6±0.3 | 0.4±0.2 | 0.3±0.1 | 0.1±0.1 | 0.2±0.1 | 0.4 | 0.3 | 0.2 | 0.2 |
| 16:0 | 18.1±1.0 | 14.4±1.5 | 11.0±0.3 | 14.6±1.2 | 13.8±2.5 | 11.1±0.4 | 11.1±0.2 | 10.2±0.5 | 9.7±0.8 | 12.3 | 10.0 | 7.6 | 10.1 |
| 18:0 | 6.1±0.8 | 6.8±0.3 | 5.6±0.4 | 6.9±0.5 | 6.2±1.4 | 7.8±0.3 | 7.5±0.7 | 8.4±0.3 | 7.6±0.1 | 8.6 | 7.4 | 5.5 | 7.5 |
| Total saturated | 26.2±1.6 | 23.4±2.0 | 18.1±0.5 | 24.1±2.2 | 22.7±4.3 | 20.0±0.5 | 19.6±0.2 | 19.3±0.6 | 18.2±0.9 | 22.1 | 18.4 | 13.6 | 18.5 |
| 16:1n-9 | 3.2±0.5 | 4.4±0.4 | 4.0±0.2 | 4.4±0.2 | 4.8±0.9 | 1.5±0.1 | 1.6±0.1 | 1.2±0.02 | 1.2±0.1 | 1.5 | 1.4 | 1.1 | 1.4 |
| 18:1n-9 | 8.6±0.2 | 16.6±0.1$^{a}$ | 14.6±0.4$^{b}$ | 16.1±0.3$^{ab}$ | 14.9±1.4$^{ab}$ | 19.4±1.1 | 19.7±2.4 | 21.6±0.5 | 18.2±1.0 | 23.4 | 22.8 | 17.9 | 23.5 |
| 18:1n-7 | 3.4±0.2 | 4.6±0.4 | 4.3±0.4 | 4.3±0.1 | 4.1±0.6 | 6.1±0.5 | 6.5±0.8 | 7.1±0.2 | 5.9±0.3 | 6.9 | 6.8 | 5.2 | 6.8 |
| 18:1n-11 | 0.1±0.1 | 0.14±0.02 | 0.1±0.02 | 0.2±0.02 | 0.15±0.03 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20:1n-11 | 0.21±0.02 | 0.5±0.2 | 0.5±0.1 | 0.5±0.1 | 0.4±0.4 | 0.2±0.0 | 0.2±0.1 | 0.3±0.1 | 0.2±0.1 | 0.4 | 0.3 | 0.2 | 0.3 |
| 20:1n-9 | 0.9±0.2 | 5.9±0.9 | 5.5±0.1 | 6.1±0.5 | 6.2±0.9 | 1.17±0.04$^{ab}$ | 1.1±0.1$^{b}$ | 1.4±0.01$^{a}$ | 1.1±0.1$^{ab}$ | 1.4 | 1.3 | 0.9 | 0.0 |
| 20:1n-7 | 0.0±0.0 | 1.1±0.2 | 0.7±0.6 | 1.1±0.1 | 1.1±0.2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0 | 0.0 | 0.0 | 1.3 |
| Total MUFA | 16.3±0.5 | 34.3±2.1 | 30.3±1.5 | 33.6±0.4 | 32.6±2.4 | 28.4±1.5 | 29.0±3.3 | 31.6±0.7 | 26.6±1.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| 18:2n-6 | 2.5±0.2 | 9.5±0.1$^{ab}$ | 10.1±0.7$^{a}$ | 8.6±0.2$^{b}$ | 8.4±0.4$^{b}$ | 6.4±0.7 | 6.6±0.1 | 6.6±0.2 | 6.1±0.3 | 33.6 | 32.6 | 25.4 | 33.3 |
| 18:3n-6 | 0.0±0.0 | 0.0±0.0 | 0.2±0.2 | 0.0±0.0 | 0.1±0.1 | 0.66±0.02 | 0.7±0.01 | 0.7±0.1 | 0.69±0.04 | 6.7 | 7.5 | 7.3 | 7.3 |
| 20:3n-6 | 0.0±0.0 | 0.27±0.03 | 0.3±0.04 | 0.28±0.03 | 0.2±0.2 | 0.2±0.0 | 0.1±0.1 | 0.2±0.01 | 0.17±0.02 | 0.7 | 0.7 | 0.6 | 0.7 |
| 20:4n-6 | 2.8±0.1 | 1.6±0.3 | 1.9±0.2 | 1.6±0.1 | 1.2±1.0 | 0.77±0.04 | 0.8±0.1 | 0.9±0.1 | 0.83±0.03 | 0.2 | 0.1 | 0.3 | 0.2 |
| 22:5n-6 | 0.65±0.03 | 0.2±0.2 | 0.3±0.2 | 0.2±0.2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.6 | 0.6 | 1.0 | 0.7 |
| Total n-6 PUFA | 5.85±0.03 | 11.6±0.5$^{ab}$ | 12.9±1.3$^{a}$ | 10.8±0.2$^{ab}$ | 9.9±1.3$^{b}$ | 8.1±0.7 | 8.1±0.2 | 8.4±0.2 | 7.8±0.3 | 8.2 | 9.0 | 9.2 | 8.9 |
| 18:3n-3 | 0.2±0.3 | 1.6±0.3 | 1.9±0.3 | 1.3±0.1 | 1.6±0.6 | 26.2±0.2 | 27.5±2.4 | 22.9±2.3 | 26.9±0.5 | 20.7 | 23.9 | 33.2 | 24.1 |
| 18:4n-3 | 0.0±0.0 | 0.6±0.6 | 0.5±0.9 | 0.7±0.6 | 0.4±0.7 | 3.7±0.1$^{ab}$ | 3.7±0.3$^{ab}$ | 3.0±0.03$^{b}$ | 4.2±0.3$^{a}$ | 2.7 | 2.7 | 3.1 | 2.7 |
| 20:4n-3 | 0.3±0.5 | 5.7±0.5 | 5.9±0.5 | 6.2±0.9 | 7.2±1.6 | 2.0±0.2 | 1.8±0.2 | 1.9±0.1 | 2.0±0.4 | 1.6 | 1.8 | 2.2 | 1.6 |
| 20:5n-3 | 4.4±0.3 | 5.1±0.9 | 6.7±0.8 | 5.2±0.4 | 5.5±0.7 | 3.0±0.5 | 2.6±0.4 | 2.9±0.3 | 3.3±0.4 | 2.3 | 2.2 | 2.6 | 2.1 |
| 21:5n-3 | 0.0±0.0 | 0.14±0.02 | 0.2±0.1 | 0.16±0.01 | 0.1±0.1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 22:5n-3 | 3.7±0.1 | 2.4±0.3$^{b}$ | 3.2±0.3$^{a}$ | 2.3±0.2$^{b}$ | 2.6±0.3$^{ab}$ | 1.5±0.1 | 1.4±0.1 | 1.42±0.02 | 1.7±0.3 | 1.4 | 1.4 | 1.5 | 1.4 |
| 22:6n-3 | 42.27±0.01 | 12.7±1.2$^{b}$ | 18.1±1.8$^{a}$ | 13.5±0.7$^{b}$ | 14.7±0.4$^{b}$ | 4.3±1.0 | 3.6±0.5 | 4.3±0.7 | 5.1±0.8 | 3.0 | 3.4 | 3.7 | 3.2 |
| Total n-3 PUFA | 50.9±1.2 | 28.4±3.0 | 36.6±4.2 | 29.4±2.5 | 32.0±2.9 | 40.8±1.7 | 40.6±3.8 | 36.4±1.5 | 43.2±2.7 | 31.7 | 35.3 | 46.4 | 35.0 |
| Total PUFA | 56.8±1.2 | 40.0±3.5$^{b}$ | 49.4±3.0$^{a}$ | 40.1±2.7$^{b}$ | 41.9±3.8$^{ab}$ | 48.8±0.9 | 48.7±3.7 | 44.8±1.7 | 50.9±3.0 | 39.9 | 44.3 | 55.6 | 43.9 |
| (n-6)/(n-3) | 0.1±0.0 | 0.4±0.0 | 0.4±0.1 | 0.37±0.03 | 0.31±0.04 | 0.20±0.03 | 0.2±0.02 | 0.2±0.0 | 0.2±0.0 | 0.3 | 0.3 | 0.2 | 0.3 |
| DHA/EPA | 9.7±0.7 | 2.5±0.2 | 2.7±0.1 | 2.6±0.1 | 2.7±0.3 | 1.4±0.1 | 1.4±0.0 | 1.5±0.1 | 1.5±0.1 | 1.3 | 1.5 | 1.4 | 1.5 |
| ARA/DHA | 0.1±0.0 | 0.13±0.01 | 0.1±0.02 | 0.12±0.01 | 0.1±0.1 | 0.2±0.1 | 0.2±0.01 | 0.21±0.02 | 0.17±0.03 | 0.2 | 0.2 | 0.3 | 0.2 |
| ARA/EPA | 0.6±0.1 | 0.31±0.01 | 0.3±0.1 | 0.3±0.1 | 0.2±0.2 | 0.3±0.1 | 0.3±0.01 | 0.30±0.01 | 0.26±0.04 | 0.3 | 0.3 | 0.4 | 0.3 |
| MUFA/PUFA | 0.3±0.0 | 0.9±0.1$^{a}$ | 0.6±0.1$^{b}$ | 0.8±0.1$^{ab}$ | 0.8±0.1$^{ab}$ | 0.58±0.04 | 0.6±0.1 | 0.71±0.04 | 0.5±0.1 | 0.8 | 0.7 | 0.5 | 0.8 |
| VE (mg.kg$^{-1}$ DW) | | 8.7±4.0$^{a}$ | 3.8±2.6$^{ab}$ | 1.5±0.5$^{b}$ | 0.7±0.4$^{b}$ | 0.2±0.1 | 0.2±0.1 | 0.4±0.0 | 0.4±0.4 | 0.3 | 0.9 | 0.6 | 0.7 |

At 19 dph, pro-metamorphic larvae did not show any significant difference in total lipid, total FA and SFA contents among dietary treatments, nor in the OA and MUFA amounts (Table 3, $P > 0.05$), in spite of a slight but significantly higher level of eicosenoic acid (20:1n-9, EA) in larvae fed HO+VE diet than in larvae from the MO+VE group ($P = 0.047$). Although there was a significantly higher amount of stearidonic acid (18:4n-3, SDA) in larvae from the HO-VE group than in those from the HO+VE diet ($P = 0.026$), no significant differences were detected in the absolute amounts and ratios of ARA, n-6 PUFA, EPA, DHA, n-3 PUFA and total PUFA among larvae from the four dietary treatments ($P > 0.05$). Levels of VE in larvae were similar among dietary groups ($P > 0.05$), but tended to be lower in pro-metamorphic larvae than in pre-metamorphic larvae ($P > 0.05$).

At the end of the trial, post-metamorphic larvae fed for 2 weeks with NO-enriched *Artemia* metanauplii tended to show similar FA profiles, with the exception of larvae fed the HO+VE diet that tended to present higher total FA, LNA, SDA, n-3 PUFA, total PUFA contents and lower SFA, OA, MUFA contents, and a lower MUFA/PUFA ratio than larvae from the other groups (Table 3).

*Larval growth, survival and metamorphosis success*

Senegalese sole larval growth in terms of SL and DW was not affected by dietary lipid peroxidation levels at any of the sampling points ($P > 0.05$, Table 4). Final larval survival was similar among dietary treatments ($P > 0.05$), reaching a mean value of 89.6 ± 0.02%. The process of eye migration in Senegalese sole larvae was not significantly affected by any dietary treatment (Table 5). No differences in $I_{EM}$ were detected during the metamorphosis process, among the four experimental groups ($P > 0.05$). No malpigmented fish (pseudo-albinism) were recorded at the end of the experiment in any of the four tested diets.

**Table 4.** Larval size in dry weight (DW, in mg) and standard length (SL, in mm) at 9, 19 and 35 dph, specific growth rate (SGR, in % $d^{-1}$) and survival rate (in %) of Senegalese sole larvae fed the four different dietary treatments. Initial standard length and dry weight of larvae were 3.1 ± 0.1 mm and 53.7 ± 5.5 µg at 1dph. Values are expressed as mean ± S.E.M. (n = 4). No significant differences (ANOVA, $P > 0.05$) were detected among dietary treatments for any sampled day.

| | 9 dph | | 19 dph | | 35 dph | | 1-35 dph | 35 dph |
|---|---|---|---|---|---|---|---|---|
| | DW | SL | DW | SL | DW | SL | SGR | Survival rate |
| **NO+VE** | 0.07±0.01 | 4.00±0.21 | 0.37±0.02 | 5.33±0.32 | 2,5±0.7 | 9,2±1.0 | 0,115±0.006 | 89,5±0.6 |
| **MO+VE** | 0.09±0.01 | 4.12±0.21 | 0.41±0.04 | 5.41±0.23 | 2,1±0.6 | 8,6±0.8 | 0,111±0.004 | 89,6±0.3 |
| **HO+VE** | 0.06±0.01 | 3.83±0.20 | 0.41±0.04 | 5.44±0.32 | 1,9±0.3 | 8,6±0.6 | 0,107±0.053 | 89,5±0.3 |
| **HO-VE** | 0.06±0.01 | 3.87±0.22 | 0.38±0.05 | 5.37±0.27 | 2,0±0.5 | 8,6±0.8 | 0,108±0.062 | 89,6±0.3 |

**Table 5.** Eye migration index ($I_{EM}$) of Senegalese sole larvae fed *Artemia* nauplii enriched the different commercial products at 9, 15, 19 and 35 dph. Values are expressed as mean ± S.E.M. (n = 4). No significant differences (ANOVA, $P > 0.05$) were detected among dietary treatments for any sampled day.

| | Age (days post hatching) | | | |
|---|---|---|---|---|
| | 9 dph | 15 dph | 19 dph | 35 dph |
| **NO+VE** | 0.94±0.04 | 1.50±0.12 | 2.13±0.03 | 4.79±0.11 |
| **MO+VE** | 0.98±0.01 | 1.55±0.11 | 2.16±0.14 | 4.87±0.05 |
| **HO+VE** | 0.96±0.02 | 1.33±0.07 | 2.26±0.13 | 4.91±0.07 |
| **HO-VE** | 0.88±0.03 | 1.50±0.12 | 2.09±0.18 | 4.91±0.04 |

*Antioxidant enzyme activities*

In Senegalese sole, SOD, GST and GR activities significantly decreased during larval development ($P < 0.001$, Table 6), whereas CAT activity remained constant. Regardless of the stage of development considered (9, 19 and 35 dph), CAT and SOD specific activities did not present any significant variations among larvae fed diets with different levels of lipid peroxidation ($P > 0.05$). In contrast, whereas GST specific activity in Senegalese sole larvae was similar among groups at pre- and pro-

metamorphic (9 and 19 dph, respectively) stages ($P > 0.05$), it was significantly higher in larvae fed the NO+VE diet than in larvae fed the MO+VE diet ($P = 0.047$) at the post-metamorphic stage (35 dph). A significant increasing gradient was observed in GR specific activity in 9 dph-larvae fed the four diets ($P = 0.041$), with larvae from the HO-VE group being higher than larvae from the NO+VE group and intermediary in larvae from the MO+VE and HO+VE groups. However, GR specific activity was not significantly different among larvae from the four dietary groups at 19 nor at 35 dph ($P > 0.05$).

**Table 6.** Quantification of the oxidation levels (TBARs in nmol MDA.g$^{-1}$ W.W.) and specific activities of catalase (in µmol/min/mg protein), superoxide dismutase (in U/mg protein), glutathione S-transferase (in nmol/min/mg protein) and glutathione reductase (in nmol/min/mg protein) in Senegalese sole fed the four dietary treatments at 9, 19 and 35 dph (mean ± S.E.M., n = 7). Different superscript letters denote significant differences among diets (ANOVA, $P < 0.05$).

| | NO+VE | MO+VE | HO+VE | HO-VE |
|---|---|---|---|---|
| ***9 dph-larvae*** | | | | |
| **TBARs** | 77.48±11.80 | 80.10±4.37 | 77.04±2.45 | 89.68±5.23 |
| **CAT specific activity** | 30.66±3.05 | 32.00±2.23 | 30.91±2.25 | 27.77±3.30 |
| **SOD specific activity** | 21.03±3.41 | 14.14±1.22 | 24.42±4.59 | 22.15±2.02 |
| **GST specific activity** | 41.13±3.66 | 40.60±2.04 | 43.89±1.56 | 47.88±2.66 |
| **GR specific activity** | 32.44±1.93$^{b}$ | 34.85±1.90$^{ab}$ | 39.23±5.35$^{ab}$ | 42.91±3.83$^{a}$ |
| ***19 dph-larvae*** | | | | |
| **TBARs** | 114.87±3.02 | 114.24±4.28 | 127.76±9.05 | 133.98±7.32 |
| **CAT specific activity** | 34.58±3.55 | 27.89±1.49 | 33.90±2.73 | 27.67±2.61 |
| **SOD specific activity** | 21.79±5.79 | 22.24±4.26 | 21.04±8.26 | 15.60±3.28 |
| **GST specific activity** | 41.78±1.55 | 38.28±2.44 | 39.21±0.90 | 38.25±1.61 |
| **GR specific activity** | 33.04±2.23 | 28.38±1.11 | 27.28±1.07 | 30.73±0.99 |
| ***35 dph-larvae*** | | | | |
| **TBARs** | 257.88±41.72 | 297.95±3.91 | 283.06±15.74 | 331.91±16.84 |
| **CAT specific activity** | 32.57±1.77 | 31.53±1.78 | 30.65±1.53 | 31.84±2.78 |
| **SOD specific activity** | 9.02±3.05 | 6.49±0.58 | 4.50±0.54 | 5.55±1.29 |
| **GST specific activity** | 26.01±1.00$^{a}$ | 21.62±0.47$^{b}$ | 22.92±0.67$^{ab}$ | 22.91±1.71$^{ab}$ |
| **GR specific activity** | 25.33±2.45 | 21.19±1.36 | 20.24±2.17 | 23.15±1.37 |

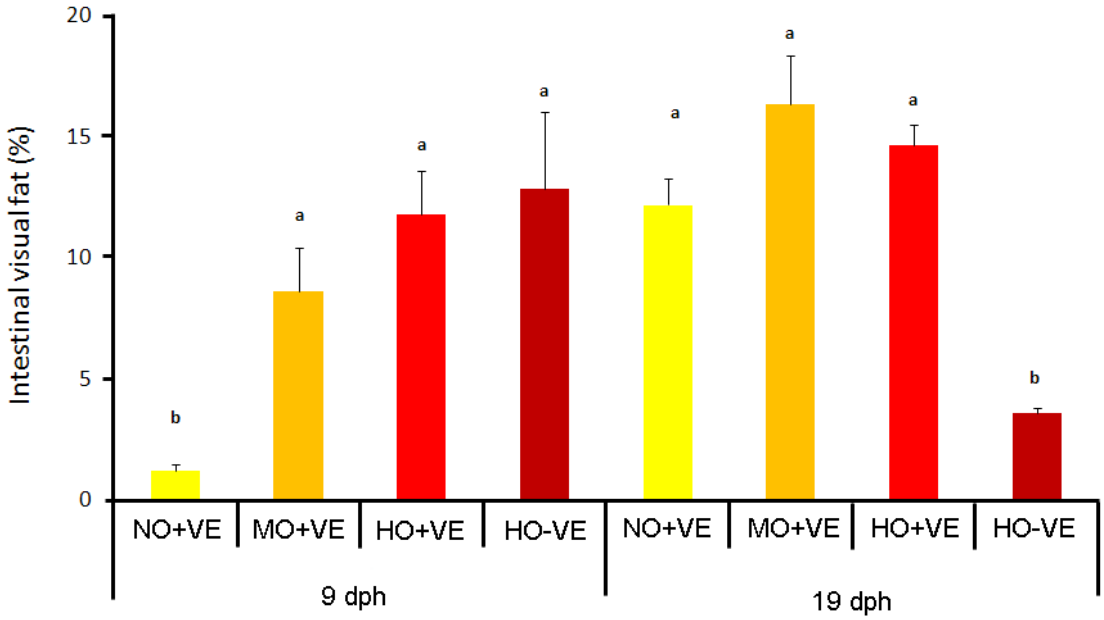


**Figure 1.** Effect of the four dietary treatments on the morphometrical quantitative estimates of accumulated neutral lipids in the intestine (mean ± S.E.M., n = 5) of Senegalese sole larvae at 9 and 19 dph. Different letters indicate significant differences (ANOVA, $P < 0.05$) among dietary treatments within the same age.

*Accumulation of neutral lipids in the intestine*

Profiles of intestinal fat (neutral lipid) accumulation were significantly affected by the diets at 9 and 19 dph ($P = 0.004$ and 0.001, Figures 1 and 2), being lower in 9 dph-larvae fed the NO+VE diet than in those fed the three other diets. In addition, these values were also lower in fish from the HO-VE group than in fish from the three other groups at 19 dph. In relation to hepatic lipid deposits, a slight accumulation of neutral lipids (4%) was only observed in liver of 9 dph-larvae fed the NO+VE diet, whereas in the rest of the dietary groups hepatocytes did not accumulate neutral lipids (data not shown).

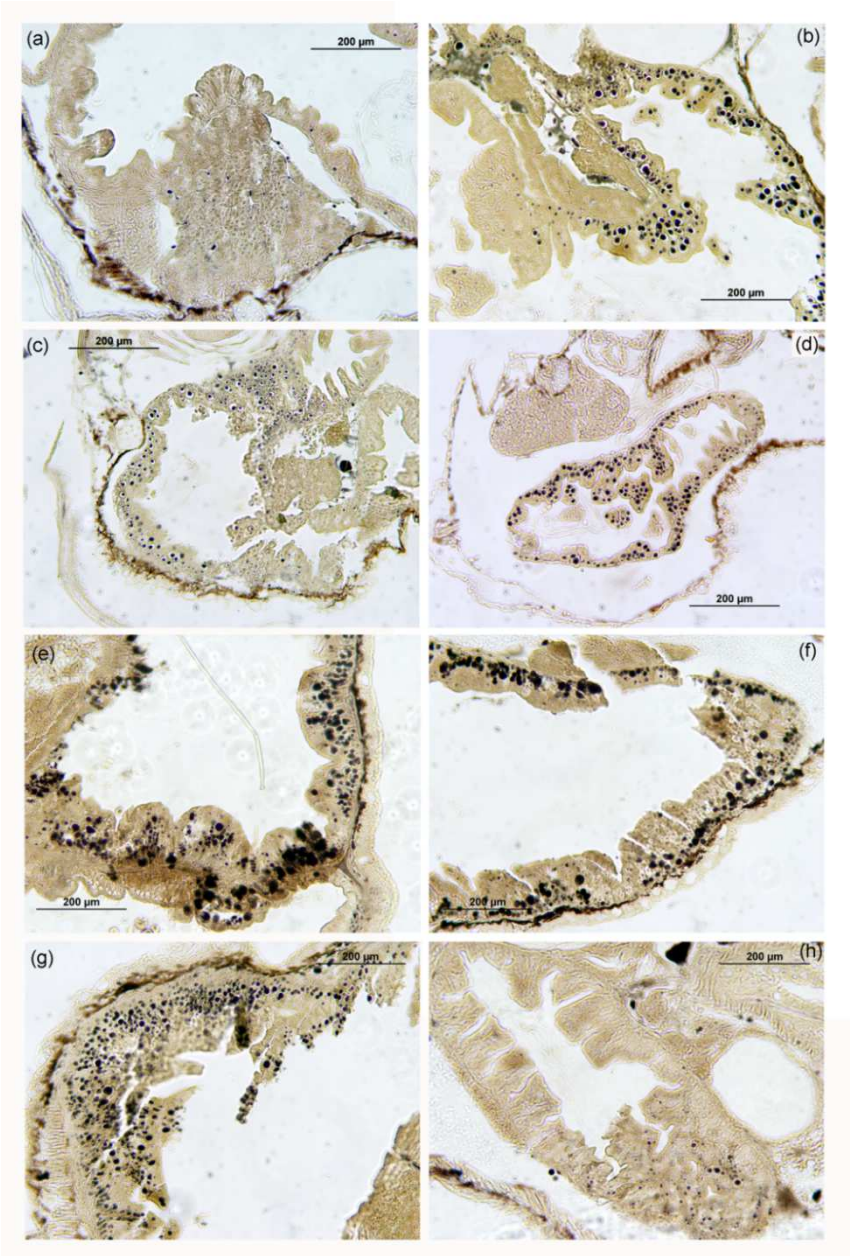


**Figure 2.** Longitudinal paraffin sections of the mid-intestine of pre- (9 dph, A, B, C and D) and pro-metamorphic (19dph, E, F, G and H) Senegalese sole larvae fed the four dietary treatments (NO+VE: A and E; MO+VE: B and E; HO+VE: C and G; HO-VE: D and H), showing different levels of neutral lipid accumulation (in black). Scale bar represents 200 µm.

*Calcification degree and incidence of skeletal deformities*

At 9 dph, the $CI_c$ in Senegalese sole larvae was not significantly affected by the diet ($P > 0.05$, Figure 3). At 19 dph, larvae fed the HO-VE diet showed a significant delay in the calcification process of their cranial structures in comparison to larvae fed with the other diets ($P < 0.001$). The percentage of larvae presenting a CI above 9 was 66.2, 69.6 and 71.0% for the NO+VE, MO+VE and HO+VE diets, but only 35.8% in larvae fed the HO-VE diet.

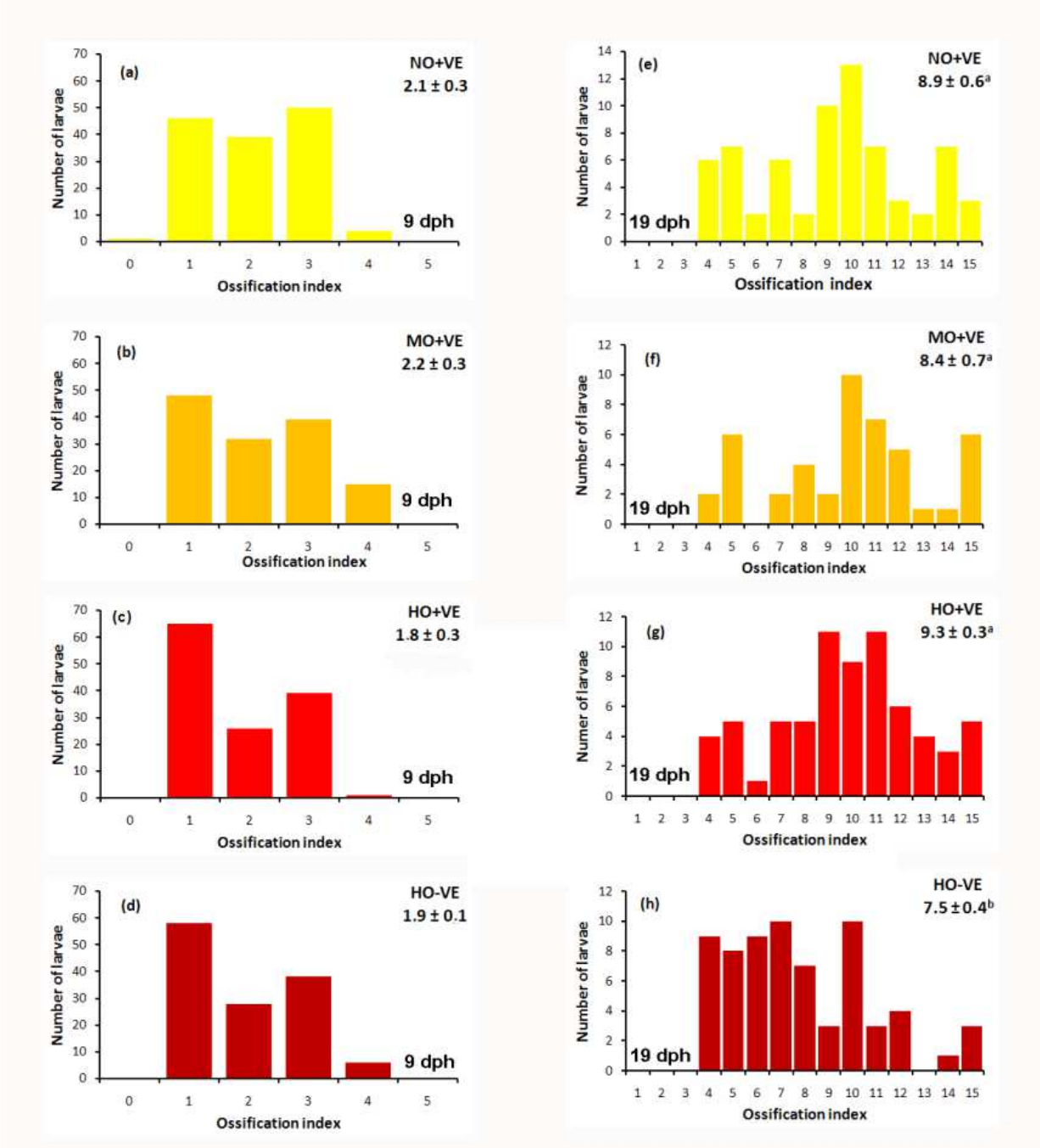


**Figure 3.** Classification of Senegalese sole larvae according to their Calcification Index (CI), calculated considering the order of appearance of the calcified structures in the cranial region (Cranial Calcification Index, $CI_c$) for fish at 9 dph (a, b, c and d) and in the cranial, vertebral and caudal fin complex regions for fish at 19 dph (e, f, g and h) fed NO diet (a, e), MO diet (b, f), HO diet (c, g), HO–VE (d, h). Values indicated above each treatment's initial are the calcification indexes (mean ± S.E.M., n = 4). Superscript letters denote significant differences among diets for the same age (ANOVA, $P < 0.05$).

The levels of lipid peroxidation significantly affected the incidence of skeletal deformities in post-metamorphic Senegalese sole aged 35 dph (Table 7). The frequency of total deformed fish was significantly higher ($P$ = 0.017) in larvae from the MO+VE group (77.1 ± 2.9%) than in those fed NO+VE and HO+VE diets (65.5 ± 0.6% and 65.2 ± 6.5%, respectively), and intermediate with respect to fish fed the HO-VE diet (68.6 ± 2.0%). A very low incidence of malformations (0.6 - 2.1%) was observed in the cranial region for all experimental groups, particularly with respect to both jaws. In all dietary treatments, skeletal deformities mainly affected the haemal vertebral region and the caudal region, particularly the last vertebrae before the urostyle. No significant differences were detected in the percentage of fish with 43 (14.8%), 44 (44.8%), 45 (34.8%) or 46 (5.6%) vertebrae among the four experimental treatments ($P$ > 0.05). The incidence of more than 6 malformations per individual was significantly higher for larvae fed the HO+VE diet (3.2 ± 1.2%) than those fed the HO-VE diet (0.0 ± 0.0%), with values being intermediary for larvae fed the NO+VE and MO+VE diets (0.7 ± 1.4% and 1.9 ± 2.3%, respectively, P = 0.035). The frequency of skeletal abnormalities in the vertebral region (mostly prehaemal and haemal regions) was significantly higher ($P$ = 0.029) in larvae fed HO+VE and MO+VE diets (70.7 ± 8.3 and 66.9 ± 1.4%, respectively) than in larvae fed the HO-VE diet (55.4 ± 3.2%), and intermediate in fish fed the NO+VE diet (57.7 ± 4.0%). Vertebral column deformities concerned 4.4% of fish larvae, while 57.7% of fish were affected by vertebral body deformities. Lipid peroxidation levels significantly affected the frequency of fish presenting skeletal abnormalities in vertebral bodies ($P$ = 0.021), being higher in larvae fed MO+VE and HO+VE diets (64.4 ± 1.4% and 63.0 ± 5.7%, respectively) than in fish fed NO+VE and HO-VE diets (54.9 ± 2.6% and 48.5 ± 4.1%, respectively). Skeletal structures composing the caudal fin complex were significantly affected by dietary lipid peroxidation levels ($P$ = 0.033). The incidence of deformities in these structures were higher in fish fed the MO+VE, NO+VE and HO+VE diets (45.0 ± 4.3%, 38.7 ± 4.7% and 38.1 ± 1.4%, respectively) than those fed the HO-VE diet (30.6 ±1.0%).

**Discussion**

*Effects of the dietary lipid peroxidation levels on the enriched live prey*

An increasing gradient of oxidation was observed among NO+VE, MO+VE, HO+VE and HO-VE emulsions that were used for enriching live prey. Although the HO+VE and HO-VE emulsions were prepared with highly oxidized oil, the addition of VE as an antioxidant in the HO+VE emulsion reduced the peroxidation levels compared to the HO-VE emulsion. However, it is important to mention that maintaining stable peroxidation levels in enriched live prey was not feasible due to their own metabolism. As a result of this in many instances the fatty acid composition of enriched rotifers and *Artemia* metanauplii did not reflect the enriching product given to them as previously reported by Barclay and Zeller (1996) and Navarro *et al.* (1999), nor the level of peroxidation present in the enriching oil. In this study, the FA profiles in neither the enriched rotifers nor *Artemia* were affected by the dietary oxidation levels, with the exception of eicosatetraenoic acid (20:4n-3, ETA), for which the content was higher in rotifers enriched with the MO+VE oil than those enriched with the other emulsions. The capacity of *Artemia* metanauplii to shorten long chain fatty acids from DHA to EPA and

probably to ETA after enrichment was previously reported by Navarro *et al.* (1999). Under the present experimental conditions, the high peroxidation levels may have accelerated this process.

Concerning the unexpected lower TBAR values in rotifers enriched with the highest oxidized emulsion (HO-VE) as compared with those enriched with HO+VE, we hypothesize that when anti-oxidants such as VE are not in limiting quantities in the enriching emulsion, rotifers may normally use them first and foremost to protect themselves against lipid oxidation without the need to activate the enzymatic machinery for detoxification. In contrast, when VE levels were limiting in the diet, other antioxidant defense mechanisms may have been enhanced to replace the role of VE, increasing the strength of the response and reducing by half the oxidation level in the organism as compared with VE alone. The presence and complementarity of both antioxidant systems in reducing the oxidation level in the organism has previously been evidenced in vertebrate and invertebrates (see review in Farooqui and Farooqui, 2012), but this hypothesis needs to be validated for rotifers. On the other hand, enriched *Artemia* metanauplii reflected different peroxidation degrees than enriched rotifers, confirming the species-specificities in fatty acid accumulation in both types of live prey, as previously was shown in the differential capacity of rotifers and *Artemia* to accumulate liposoluble vitamins such as vitamin A (Giménez *et al.* 2006). Moreover, the oxidation levels in enriched *Artemia* metanauplii were not affected by the oxidation levels of the enriching product and remained constant among dietary treatments. Within the available published literature, we have not found any study about the regulation of oxidation levels in *Artemia*; thus, we venture the hypothesis that *Artemia* possess highly efficient anti-oxidant systems to regulate oxidative levels in their tissues. In this sense, several authors have suggested that the carotenoids contained in the pigmented cells might also be involved in the above-mentioned process (Gilchrist and Green, 1960; Hsu and Chichester, 1970; Kumar and Marian, 2006). However, this assumption needs further work to be validated, as well as for determining the functioning of the underlying mechanisms and the threshold of oxidation level allowed in the emulsion before the antioxidant systems are switched on.

In addition, both types of live prey accumulated lower amounts of VE compared to the quantities contained in the emulsions, probably reflecting the utilization of VE as a defense against the oxidized lipids they had incorporated, but could also be due to a limited physiological capacity for VE to be accumulated in these organisms. In this context, rotifers have been proven to accumulate in an almost linear manner, increasing VE concentrations contained in the enriching emulsion (Srivastava *et al.* 2011), while very little information about VE accumulation in *Artemia* metanauplii is available. This lack of information is due to the fact that most of the studies dealing with dietary oxidized lipids have been conducted using inert diets (Mourente *et al.* 2002; Fontagné *et al.* 2006; Zambrano and Landines, 2011; Dong *et al.* 2012). However, sufficient levels of VE can be accumulated and maintained in *Artemia* nauplii, making this live food a delivery system useful for studying dietary requirements as well as the antioxidant effects of VE in marine fish (Huo *et al.* 1996).

*Effects of the dietary lipid peroxidation levels on larval performance and anti-oxidant enzyme activities.* The FA composition in sole larvae reflected the composition of the ingested diet, at all sampled developmental stages, as observed in previous studies (Copeman *et al.* 2002; Villalta et al, 2005a, b; Lund *et al.* 2008; Boglino *et al.* 2012a, b). The levels of dietary peroxidation affected the FA profile of pre-metamorphic larvae fed rotifers enriched with the four different emulsions. Larvae fed the MO+VE diet presented higher contents of LA and n-6 PUFA, DPA and DHA, which was in accordance with the high ETA contents observed in MO+VE-enriched rotifers. However, larvae from all dietary groups presented similar (n-6)/(n-3) PUFA ratios, that restored the balance between contents of both series of PUFA. Larvae fed the MO+VE-enriched rotifers contained lower and higher contents of OA and total PUFA, respectively, than those fed the NO+VE-enriched rotifers, resulting in a higher MUFA/PUFA ratio. However, as the general larval FA profile was relatively stable among diets, this study only focused on the impact of the dietary oxidation levels, rather than on the specific dietary FA composition, on larval growth performance and development. In pro- and post-metamorphic larvae fed enriched *Artemia* metanauplii*,* the general FA profile was similar among dietary groups, reflecting the composition of the diet.

Pre-metamorphic larvae fed rotifers enriched with NO+VE, MO+VE and HO+VE presented decreasing contents of VE along the increasing gradient of oxidation in the live prey, likely reflecting the utilization of this vitamin to protect the organism against the dietary oxidative stress. The same phenomenon has been described in gilthead sea bream (Mourente *et al.* 2002), largemouth bass *Micropterus salmoides* (Chen *et al.* 2011) and spoted murrel *Channa punctatus* (Abdel-Hameid *et al.* 2012). In pro- and post-metamorphic larvae fed *Artemia* metanauplii enriched with the four emulsions, VE levels were lower than in pre-metamorphic larvae, probably due to an increased capacity of tissue VE accumulation, regardless of the dietary treatment, as were the peroxidation levels in the enriched *Artemia*.

The dietary peroxidation levels did not affect larval survival, growth performance nor metamorphosis. These results were similar to those reported in other species. No serious deleterious effects on survival and growth were noticed in in gilthead sea bream fingerlings fed varying dietary PUFA/VE ratios or oxidized oil, with or without VE (Mourente *et al.* 2000; 2002). In largemouth bass, fish fed oxidized diets presented higher feed intake and consequently superior growth than fish fed fresh oil, although no difference in growth was detected among diets containing an increasing dietary oxidation gradient (Chen *et al.* 2011). The growth of rainbow trout *Oncorhynchus mykiss* fry fed different dietary levels of n-3 PUFA and VE was also not affected, although fish fed VE deficient diets tended to present delayed growth (Puangkaew *et al.* 2005). Increasing amounts of oxidation in diets supplemented or not with α-tocopherol acetate in Atlantic halibut *Hippoglossus hippoglossus* juveniles neither affected the growth nor the survival of the fish (Lewis-McCrea and Lall, 2007).

Independently of the dietary treatments, an increase in the lipid peroxidation level of sole larvae during development was observed, in agreement with the results observed by Solé *et al.* (2004)

in wild Senegalese sole larvae. The distinct oxidation levels in enriched rotifers did not affect the oxidative status in 9 dph larvae. As for the oxidation levels in pro- and post-metamorphic larvae, they were similar among dietary treatments, mirroring the equivalent oxidation levels in *Artemia* metanauplii enriched with the four emulsions. In juvenile gilthead sea bream, dietary oxidized oil has also been shown to have no major effect on hepatic lipid peroxidation products (Mourente *et al.* 2002). Under present experimental conditions, it seemed plausible that the levels of dietary lipid peroxidation were not high enough to induce an increase in the oxidation status of Senegalese sole larvae. However, there is the possibility that Senegalese sole larvae engaged anti-oxidant systems and detoxification mechanisms in response to oxidative stress caused by the absorption of oxidized food, thus regulating their own lipid peroxidation levels.

The function of antioxidant enzymes is to catalytically remove ROS and thus disrupt lipid peroxidation chain reaction. The role of SOD is to eliminate $O2^{\cdot-}$ by decomposing it into $O_2$ and $H_2O_2$, while CAT is involved in the detoxification of $H_2O_2$ to $H_2O$ and $O_2$ (Halliwell and Gutteridge, 2006). Some GST isoforms can also detoxify $H_2O_2$ and organic peroxides by oxidizing glutathione (GSH) and GR participates in the recycling of oxidized glutathione molecules. During larval development, the specific activity of most oxidative stress enzymes was observed to decrease, as previously seen in common dentex *Dentex dentex* (Mourente *et al.* 1999) and in turbot *Scophthalmus maximus* (Peters and Livingstone, 1996). In the present study with *S. senegalensis*, we suggested that the decreasing activity of these enzymes might be related to an adaptation to dietary oxidative stress over time. The significant increase in GR specific activity in pre-metamorphic fish was correlated with increasing dietary levels of lipid peroxidation, highlighting the activation of the anti-oxidant defensive response against dietary oxidative stress. In many fish species, such as gilthead sea bream juveniles, common dentex, rainbow trout and largemouth bass, an increase in the hepatic and intestinal activities of most oxidative stress enzymes has been detected with increasing dietary oxidative stress, starvation or increasing dietary levels of n-3 HUFA (Mourente *et al.* 2002; Morales *et al.* 2004; Puangkaew *et al.* 2005; Fontagné *et al.* 2006; Chen *et al.* 2011; Zambrano and Landines, 2011). However, dietary VE has been shown to partially abrogate these nutritionally-induced effects (Mourente *et al.* 2002; Puangkaew *et al.* 2005). On the other hand, CAT, SOD and GST activities were not affected by the dietary oxidation gradient at any developmental stage in Senegalese sole, with the exception of a slight difference among treatments for GST at 35 dph, which may reflect a supportable range of dietary oxidation, neither toxic for sole larvae. As found in juveniles of gilthead sea bream, the weak response of antioxidant enzyme activities to increasing dietary oxidation suggested that more severe conditions may be required to significantly affect these activities (Mourente *et al.* 2000). It seems unlikely that important changes in the activity profiles of the oxidative stress enzymes could be observed in fish being fed during only one week with a diet, even though highly oxidized. Additionally, the fact that we measured enzyme activities in pools of whole larvae could mask the important role these enzymes play in the central tissues and organs (intestine, liver, kidney) where the main detoxification of the ingested peroxidized lipids is realized (Farooqui and Farooqui, 2012).

The activation of detoxifying mechanisms for ingested peroxidized lipids during the digestion was supposed to occur in organs and tissues, especially sensitive to non-optimal feeding conditions or nutritional stress during larval development, and which rapidly respond to nutritional disorders. In this sense, changes in the histological organization of the intestine and the liver was used as histological targets to analyze the nutritional condition of fish larvae and elucidate the effects of different dietary regimes or nutrients on larval physiology at early development (see review in Gisbert *et al.* 2008). The size of lipid vacuoles in enterocytes and hepatocytes has been reported to depend on the dietary fat content and the degree of unsaturation of the lipids ingested, that informs on the capacity to balance the excess of lipid intake (Sheridan, 1988). In the present study, the different patterns of intestinal fat accumulation among larvae from the four dietary treatments did not reflect the response to distinct lipid contents (all diets were isolipidic), but they more likely reflected variations in levels of oxidation. The lowest intestinal fat accumulation in 9 dph sole larvae fed the less oxidized diet (NO+VE), and the increasing intestinal fat accumulation following the dietary oxidation gradient, suggested that the accumulation in enterocytes was proportional to the diet content. The intestinal epithelium has been indicated as a reliable selective physical barrier which detoxifies the peroxidized lipids being fed to the larvae, by retaining them in enterocytes until the mechanisms of defense against lipid peroxidation have been activated, and before being transferred to the blood in the chylomicrons. In Adriatic sturgeon *Acipenser naccarii* and rainbow trout, lipid peroxidation in the intestine has been determined to be higher than in liver or muscle, reinforcing the idea of a primary decontamination step occurring in the intestinal tract (Trendazo *et al.* 2006). At 19 dph, the lower lipid accumulation in the enterocytes of larvae fed the HO-VE diet as compared to the other experimental diets was not related to the dietary oxidation levels (constant in enriched *Artemia*), but more probably to the lower neutral lipid content (cholesterol and triacylglycerol) (data not shown). Nevertheless, in liver, fat accumulation was seldom observed in larvae from any dietary treatments. Liver is one of the main detoxifying organs and accumulation of fat in hepatocytes has previously been associated with an increasing oxidation gradient in rainbow trout, largemouth bass and channel catfish *Ictalurus punctatus* (Puangkaew *et al.* 2005; Chen *et al.* 2011; Dong *et al.* 2012). It seemed that the degree of oxidation of the ingested fat did not affect the hepatic fat accumulation patterns in the present study. This can be related to the pre-metamorphic pelagic larvae (9 dph) being quite active, constantly swimming and hunting live prey, and at 19 dph sole larvae were undergoing metamorphosis that leads to high consumption of dietary lipid for energy, and not so much for storage in hepatic reserves. Once metamorphosis was complete, larvae became benthic, passive feeders with a concomitant reduction in energy demands, and dietary lipids are mainly used for tissue formation (phospholipids) and therefore more fat accumulation should be seen in the liver and intestine (Gisbert *et al.* 2004; Hoehne-Reitan and Kjørsvik, 2004; Boglino *et al.* 2012c).

The impact of a dietary peroxidation gradient on the skeletogenesis process in Senegalese sole was evaluated by comparing the degree of calcification and the incidence of skeletal deformities. A delay of the calcification process was observed in pro-metamorphic larvae fed the highest dietary oxidation level without VE; an effect noticeable from the pre-metamorphic stage, supporting the results

of Lewis-McCrea and Lall (2007) on Atlantic halibut. These authors proved that in the absence of VE in the diet, a lower bone formation activity was found in osteoclasts, associated with reduced bone mineralization and decreased bone strength (Turek *et al.* 2003). All these results demonstrated that osteoblasts are sensitive to oxidative stress and that supplying highly oxidized diets to fish without any antioxidant molecule, such as VE, can cause a reduction in their bone formation.

All experimental groups showed a large incidence of skeletal deformities (from 66 to 77%), particularly vertebral body deformities (57.7% of total larvae), as previously observed in Senegalese sole larvae (Gavaia *et al.* 2002; Engrola *et al.* 2009; Fernández *et al.* 2009; Boglino *et al.* 2012a, b). However, the incidence of total skeletal deformities did not seem to be correlated with the dietary lipid peroxidation levels, as they were higher in fish fed the medium oxidized diet and lower in fish fed the non-oxidized and highly oxidized diets. In relation to vertebral deformities, fish fed the highest dietary oxidation levels during the rotifer feeding period (HO+VE) were more affected, in accordance with the results of Lewis-McCrea and Lall (2007) in Atlantic halibut. The vertebral deformities mainly affecting the fish fed the HO+VE diet were vertebral compression, the fusion of the two last vertebrae before the urostyle (V43-44), the fusion of the neural arches, the deformation of vertebrae and abnormal vertebral ossification. Lewis-McCrea and Lall (2007) determined scoliosis in the prehemal and anterior haemal regions of the vertebral column as the most frequent skeletal deformity in Atlantic halibut fed high levels of oxidized dietary lipids. Fish fed the HO+VE diet also tended to display higher frequency of deformed modified neural spines (Mns) in the caudal fin complex. Curiously, fish fed the MO+VE diet tended to present higher frequencies of deformations of haemal and neural spines and displayed significantly more deformities of the caudal fin complex, particularly higher incidence of deformed modified haemal spines (Mhs), parahypural (Phy) and fin rays.

**Conclusion**

Senegalese sole larvae appeared to be sensitive to the increasing amounts of dietary peroxidized lipids. Although no physiological alterations were detected in their FA profile, oxidation status, survival, growth performance or metamorphosis processes, sole larvae seemed to activate antioxidant defense mechanisms in response to the dietary oxidative stress, through the consumption of the dietary VE provided, the activation of antioxidant enzymes and the accumulation of oxidized fat in the intestinal enterocytes. Feeding fish with highly oxidized diets caused a reduction in bone mineralization and increased the incidence of vertebral deformities. It seemed probable that the dietary levels of oxidation supplied to the larvae were not high enough to see a stronger impact on their development, but the use of enriched live prey to feed them restricted the possibilities. The duration of feeding of diets that promote high oxidative stress seems to have reduced the activity of antioxidant enzymes, possibly the result of an adaptive process, but this requires further investigation.

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# 5

# The effect of dietary arachidonic acid during the *Artemia* feeding period on larval growth and skeletogenesis in Senegalese sole

Anaïs Boglino[1]
Maria J. Darias[1,2]
Alicia Estévez[1]
Karl B. Andree[1]
Enric Gisbert[1]

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[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

**El efecto del ácido araquidónico en la dieta durante el periodo de alimentación con *Artemia* sobre el crecimiento y la esqueletogénesis del lenguado Senegalés.**

**Resumen**

Para mejorar el entendimiento sobre la incidencia de deformidades esqueléticas en larvas de lenguado Senegalés, se realizó una prueba alimenticia con diferentes niveles de ácido araquidónico (20:4n-6, ARA) y se evaluó su efecto sobre el crecimiento y la esqueletogénesis en larvas de 8 a 50 días post-eclosión (dpe). Las larvas fueron alimentadas con nauplios de *Artemia* enriquecidos con tres niveles de ARA: ARA-Bajo, ARA-Medio y ARA-Alto (1.0, 4.5 y 7.0% de ARA del total de ácidos grasos). El incremento de ARA en la dieta no tuvo un efecto significativo sobre la sobrevivencia, no obstante, si lo tuvo sobre el crecimiento. El grupo alimentado con ARA-Medio presentó una mayor longitud estándar y peso seco (11.36 ± 0.47 mm y 5.86 ±1.06 mg a 50 dpe, respectivamente), en comparación con las larvas alimentadas con ARA-Alto que tuvieron los valores más bajos (9.53 ± 0.27 mm y 2.47 ± 0.26 mg a 50 dpe). De igual forma, se observó que las larvas del grupo ARA-Alto presentaron más calcificación esqueletica a los 15 dpe que las larvas de los grupos ARA-Bajo y ARA-Medio. Las larvas del grupo ARA-Medio mostraron una mayor tendencia a fusionar los hipurales 3 y 4 (34.4 ± 3.1%) a los 50 dpe que las larvas de los otros dos grupos (18.7 ± 1.6%, en promedio). Los resultados reflejaron una tendencia a un mayor grado de desarrollo del esqueleto más que una deformidad, en confirmación con el mayor crecimiento de las larvas de este grupo. Asimismo, los tres niveles de ARA que fueron comparados no afectaron significativamente la incidencia de deformidades esqueléticas totales (41.4 ± 1.5% en promedio). Las larvas de todos los tratamientos tuvieron en general una fusión en su vertebras 43 y 44 (32.2 ± 1.3% en promedio). La inclusión de 4.5% de ARA en metanauplii de *Artemia* enriquecidos promovió la mejor tasa de crecimiento y una esqueletogénesis adecuada en las larvas de lenguado Senegalés.

**Palabras claves:** Lenguado senegalés, larvas, ácido arachidonico, crecimiento, esqueletogénesis, osificación

**Abstract**

To improve the understanding of the incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*), a feeding trial was carried out to evaluate the effect of increasing dietary arachidonic acid (ARA) levels during the *Artemia* feeding period on larval growth and skeletogenesis. Larvae were fed from 8 to 50 days post hatching (dph) with *Artemia* nauplii enriched with three different levels of ARA: ARA-Low, ARA-Medium and ARA-High (1.0, 4.5 and 7.0% ARA of total fatty acids, respectively). Increasing levels of dietary ARA did not affect significantly the survival. However, it influenced growth; larvae from the ARA-M group presented the highest values of final standard length and dry weight (11.36 ± 0.47 mm and 5.86 ± 1.06 mg at 50 dph, respectively) and larvae from the ARA-H group the lowest ones (9.53 ± 0.27 mm and 2.47 ± 0.26 mg at 50 dph). The skeleton of larvae fed the ARA-M diet tended to be more calcified at 15 dph than that of larvae fed ARA-L and ARA-H diets. Larvae from the ARA-M group tended to show a higher incidence of fusion of hypurals 3 and 4 (34.4 ± 3.1%) at 50 dph than the larvae from the other groups (18.7 ± 1.6%, in average). The latter results reflected a trend to a higher degree of skeletal development rather than a deformity, this being in agreement with the higher larval growth of this group. Besides, ARA levels did not affect significantly the incidence of total skeletal deformities (41.4 ± 1.5% in average). Larvae from all dietary treatments mostly displayed fusions of the vertebra 43 and 44 (32.2 ± 1.3% in average). The amount of 4.5% of total fatty acid of dietary ARA during *Artemia* feeding period promoted the best growth and proper skeletogenesis in Senegalese sole larvae.

**Keywords:** Senegalese sole, larvae, arachidonic acid, growth performance, skeletogenesis, ossification

**Introduction**

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a high-value flatfish, commonly reared in intensive aquaculture production in Spain and Portugal. Despite many advantages, such as good market price, natural spawning of broodstock in captivity and fast development of larvae and juveniles (Dinis *et al.*, 1999), the rearing of Senegalese sole still presents some constraints, which limit the development of the productive sector. Among them, the larval quality and the high incidence of malpigmentations and skeletal deformities and reduced bone mineralization during larval stages (Gavaia *et al.*, 2002, 2009; Soares *et al.*, 2002) are frequent problems encountered during the larviculture of flatfishes that affect survival, growth and development, and reduce the market value of the final product (see review by Fernández and Gisbert, 2011; and references therein). In Senegalese sole, skeletal deformities may affect up to around 80% of the hatchery reared fish (Gavaia *et al.*, 2009). Larval nutrition at first feeding is known to be one of the key factors affecting skeletogenesis at early developmental stages (Cahu *et al.*, 2003; Lall and Lewis-McCrea, 2007). Among nutrients known to interfere with bone development, dietary lipids and, particularly, highly unsaturated fatty acids (HUFA) and essential fatty acids (EFA), play a crucial role at first feeding in fish larvae (Izquierdo *et al.*, 2000; Tocher, 2003, 2010) . In previous studies on different fish species, attention has mainly been paid to investigate the effects of dietary docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) on bone formation (Kjørsvik *et al.*, 2009; Roo *et al.*, 2009; Dâmaso-Rodrigues *et al.*, 2010; Boglino *et al.*, 2012). Although arachidonic acid (20:4n-6, ARA) is present in fish tissues in lower amounts than DHA and EPA, absolute amounts of dietary ARA may not be neglected, as well as its content relative to EPA and DHA (Moren *et al.*, 2011). ARA is the major precursor for eicosanoids synthesis, enhancing the immune system and resistance to stress (Bell and Sargent, 2003) and alterations in the dietary ARA⁄EPA ratio can affect the production of prostaglandin E2 (PGE2) known to regulate osteoblasts and bone metabolism (Berge *et al.*, 2009). Although the action of ARA on bone turnover is well known in mammals (Watkins *et al.*, 2001), little information on the effects of dietary ARA content on bone development in fish is available (Cahu *et al.*, 2003; Villeneuve *et al.*, 2005, 2006; Berge *et al.*, 2009) and, to our knowledge, no study has been conducted in flatfish. Moreover, live feed organisms used in hatcheries, such as rotifers and *Artemia* nauplii, need to be enriched to meet the nutritional requirements of larval fish for EFA (Harel and Place, 1998). Although ARA is ensured in commercial enriching products at a level of approximately 1% of total fatty acids (TFA; Sargent *et al.*, 2002), there is no evidence to what extent this amount is sufficient, nor is the optimal relative proportion of ARA to DHA and EPA known for marine fish larval feeds. The aim of this dose-response nutritional study was first, to investigate how the dietary ARA level during the *Artemia* feeding period (8–50 days post hatching at 17C) affected larval growth and bone development in Senegalese sole, with a special emphasis on the incidence of skeletal deformities, and secondly to determine the optimal proportion of dietary ARA required by the larvae for their proper development.

**Materials and methods**

*Larval rearing*

Two-day-old Senegalese sole larvae from Stolt Sea Farm SA (Carnota, La Coruña, Spain) were reared in 12white cylindrical tanks of 60 L (initial density: 110 larvae.l$^{-1}$) connected to a water recirculation unit IRTAmar© at the IRTA-SCR facilities. Water conditions were as follows: temperature 17.3 ± 0.01 °C, salinity 35‰, pH 8.0 ± 0.2, dissolved oxygen 7.5 ± 1.3 ppm and 50% daily water renewal in the recirculation system with gentle aeration in each tank. Photoperiod was 16 h L: 8 h D, and light intensity was 500 lux at the water surface. Larvae were fed twice a day, from 2 to 10 days post-hatching (dph), with rotifers (*Brachionus plicatilis*) enriched with microalgae (*Tetraselmis suecica*), at a density of 10 rotifers.ml$^{-1}$ from 2 to 7 dph and of 5 rotifers ml$^{-1}$ from 8 to 10 dph. Enriched *Artemia* metanauplii were supplied to larvae from 8 to 50 dph twice a day, at increasing densities from 0.5 to 12 metanauplii.ml$^{-1}$ as described in Cañavate *et al.* (2006).

*Experimental diets and Artemia enrichment*

The effect of graded levels of dietary ARA on Senegalese sole was only evaluated during the *Artemia* feeding period to avoid introducing variability due to the different patterns of fatty acid accumulation between rotifers and *Artemia*(Giménez *et al.*, 2006). *Artemia* was enriched with three different experimental emulsions (see composition in Table 1) made with commercially available ARA oil obtained from the fungus *Mortierella alpina* (Vevodar©; DSM Food Specialties, The Netherlands) and gradually replaced by corn oil and olive oil. Olive oil contains around 80% of oleic acid (18:1n-9, OA), so it is an appropriate solvent to dilute polyunsaturated fatty acids (PUFA), here particularly ARA, in monounsaturated fatty acids (MUFA), thus keeping the emulsions isolipidic and without altering their absolute and relative levels of EPA and DHA. Three experimental emulsions with graded contents of ARA were obtained: ARA-Low (ARA-L, 0.0% of TFA), ARA-Medium (ARA-M, 5.0% of TFA) and ARA-High (ARA-H, 10.1% of TFA). One-day-old *Artemia* nauplii (EG strain, INVE) were enriched at a density of 300 nauplii.ml$^{-1}$ for 16 h at 28 °C with 0.76 g.l$^{-1}$ of emulsion. After enrichment, *Artemia* metanauplii were washed with UV filtered seawater and freshwater and kept at 4 °C with aeration until administered to larvae. Enriched *Artemia* were supplied frozen to the larvae from 20 dph onwards.

**Table 1.** Formulation of the three experimental emulsions (mg.g$^{-1}$) and total lipid and fatty acids contents and FA composition (% TFA) of the emulsions (n = 1) and the enriched *Artemia* nauplii (mean ± SD; n = 6). Totals include some minor components not shown. Superscripts letters denote significant differences among diets ($P < 0.05$).

| ***Formulation of the emulsions (mg·g$^{-1}$)*** | **ARA-L** | **ARA-M** | **ARA-H** |
|---|---|---|---|
| Vevodar® oil[1] | 0.0 | 36.6 | 73.1 |
| Corn oil | 8.5 | 4.4 | 0.0 |
| Olive oil | 86.5 | 54.1 | 21.9 |
| Aquagrow Gold[2] | 348.8 | 348.8 | 348.8 |
| Supplements[3] | 556.2 | 556.2 | 556.2 |
| Total FA (mg·g$^{-1}$ DW) | 277.7 | 355.8 | 292.4 |

| ***Fatty acid composition*** | **Emulsions** | | | **Enriched *Artemia* nauplii** | | |
|---|---|---|---|---|---|---|
| | **ARA-L** | **ARA-M** | **ARA-H** | **ARA-L** | **ARA-M** | **ARA-H** |
| Total lipids (mg·g$^{-1}$ DW) | 488,19 | 505,30 | 484,64 | 189.3±23.2 | 186.6±26.7 | 190.3±55.7 |
| Total FA (mg·g$^{-1}$ DW) | 277.7 | 355.8 | 292.4 | 137.8±38.5 | 130.9±18.9 | 124.9±39.0 |
| Total saturated | 27.1 | 25.8 | 29.1 | 18.8±2.6 | 16.7±2.8 | 18.1±2.2 |
| 18:1n-9 | 29.9 | 20.6 | 12.0 | 28.6±3.9$^{a}$ | 23.3±3.2$^{b}$ | 18.1±3.1$^{c}$ |
| Total monounsaturated | 30.1 | 21.1 | 12.3 | 32.7±4.7$^{a}$ | 26.7±1.6$^{b}$ | 22.6±2.9$^{b}$ |
| 18:2n-6 | 4.3 | 4.8 | 4.5 | 6.8±0.2 | 6.7±0.7 | 6.2±0.7 |
| 20:4n-6 | 0.0 | 5.0 | 10.1 | 1.0±0.3$^{c}$ | 4.5±0.6$^{b}$ | 7.0±0.8$^{a}$ |
| Total n-6 PUFA | 12.9 | 21.8 | 28.1 | 11.5±3.2$^{b}$ | 15.2±2.3$^{ab}$ | 17.5±3.4$^{a}$ |
| 18:3n-3 | 0.0 | 0.0 | 0.0 | 14.9±2.8 | 15.7±2.5 | 15.7±1.6 |
| 20:5n-3 | 0.6 | 0.9 | 1.0 | 2.8±0.6 | 3.6±0.6 | 3.6±0.6 |
| 22:6n-3 | 27.9 | 28.6 | 27.4 | 12.9±3.7 | 15.3±2.8 | 14.7±2.7 |
| Total n-3 PUFA | 29.9 | 31.4 | 30.6 | 35.7±5.4 | 40.3±2.9 | 39.9±4.0 |
| Total PUFA | 42.8 | 53.1 | 58.6 | 47.2±7.3$^{b}$ | 55.5±3.7$^{a}$ | 57.4±3.7$^{a}$ |
| (n-6)/(n-3) | 0.4 | 0.7 | 0.9 | 0.3±0.1 | 0.4±0.1 | 0.5±0.1 |
| DHA/EPA | 48.2 | 31.2 | 27.9 | 4.6±1.0 | 4.3±0.4 | 4.1±0.8 |
| ARA/DHA | 0.0 | 0.2 | 0.4 | 0.08±0.01$^{b}$ | 0.3±0.1$^{a}$ | 0.4±0.2$^{a}$ |
| ARA/EPA | 0.0 | 5.5 | 10.3 | 0.4±0.1$^{b}$ | 1.3±0.3$^{a}$ | 1.7±0.8$^{a}$ |
| MUFA/PUFA | 0.7 | 0.4 | 0.2 | 0.7±0.8 | 0.5±0.5 | 0.4±0.4 |

[1] Vevodar© oil, DSM Foods Specialties, Delft. Netherlands.

[2] Aquagrow Gold© (*Schizochytrium sp.* based product), Advanced BioNutrition, ABN, USA.

[3] Supplements: Soy lecithin, 17.1 mg; vitamin E, 9.7 mg; distilled water, 52.9 mg.

*Lipids and fatty acids analysis*

Total lipids of enriched live prey and larvae at 2 and 50 dph were extracted using the method of Folch *et al.* (1957) and quantified gravimetrically, as detailed in Boglino *et al.* (2012).Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were analyzed by gas-liquid chromatography as described in Villalta *et al.* (2005a). Results of fatty acid contents were expressed as a percentage of total fatty acids.

*Larval growth and survival rate*

Standard length (SL) and dry weight (DW) of Senegalese sole larvae were measured at 4, 7, 11, 15, 22, 31, 42 and 50 dph on thirty larvae randomly sampled in each tank and euthanized with an overdose of tricaine methane sulphonate (MS-222; Sigma). Final survival was evaluated according to the formula of Buckley *et al.* (1984), considering the number of sampled individuals during the experiment.

*Ossification degree and skeletal deformities analysis*

Forty larvae per tank (160perdiet)were stained with alizarin red to detect bony tissues and the degree of ossification of their skeleton was quantified at 15 dph according to Darias *et al.* (2010a). For this purpose, larvae from all treatments were stained and processed together. For each stained specimen, the red surface corresponding to bone was normalized to the standard length to correct the variability introduced by the differential growth among larvae from different treatments. The incidence of skeletal deformities was determined in the cranium, vertebral column and caudal fin complex of 40 juveniles per replicate (n = 160 per diet) at 50 dph. Skeletal structures were identified and named according to Wagemans and Vandewalle (2001) and Gavaia *et al.* (2002). Caudal deformities included the deformations of epural, hypurals, parahypural, urostyle and modified neural and haemal spines. Fusion of the hypurals 3 and 4 was not considered as a skeletal deformity, but rather as the normal development of the caudal fin complex occurring in juveniles of flatfishes (Barrington, 1937).

*Statistical analysis*

Results were expressed as mean ± SEM (n = 4), except for the fatty acid composition, which was expressed as mean ± SD. All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). One-way ANOVA was performed to analyze differences between dietary treatments followed by Tukey's test when significant differences were found at $P < 0.05$. Sampling size for the evaluation of the incidence of skeletal deformities was confirmed *a posteriori* using the sample size for ANOVA test tool from SigmaStat 3.0 (Systat Software Inc.,) taking into account the difference between the mean values of the incidence of total skeletal deformities, the average SD among groups, a test power of 0.999 and $\alpha = 0.05$. All the statistical analyses were conducted using SigmaStat 3.0.

**Results**

No significant differences were detected in the total lipid and total fatty acid contents, or in their content of saturated fatty acids (SFA), among the *Artemia* nauplii enriched with the three different emulsions (Table 1, $P > 0.05$). The significant increasing levels of ARA among enriched *Artemia* reflected the graduated proportions of ARA in the emulsions. The increasing amounts of OA and MUFA in *Artemia* enriched with the different emulsions was caused by the progressive dilution of the Vevodar© oil with olive and corn oils used to formulate the experimental enriching emulsions. These gradients were also associated with decreasing amounts of ARA, n-6 and total PUFA. *Artemia* nauplii

enriched with the ARA-L emulsion contained significantly higher levels of OA and MUFA and lower levels of n-6 PUFA and total PUFA than *Artemia* enriched with the ARA-H emulsion ($P < 0.05$). *Artemia* nauplii enriched with ARA-M presented a OA content significantly different from ARA-L and ARA-H, values of MUFA and total PUFA not statistically different from the ARA-H group, and an intermediate value of n-6 PUFA, not significantly different from ARA-L and ARA-H groups. Amounts of DHA and EPA were similar among the *Artemia* nauplii enriched with the three different emulsions. Although not significantly ($P = 0.091$), the n-6/n-3 PUFA ratio tended to increase in *Artemia* enriched with graded amounts of ARA, as well as in the experimental emulsions. The ratios of ARA/DHA and ARA/EPA in nauplii were significantly higher in ARA-H and ARA-M groups than in ARA-L group, reflecting the gradients of the experimental emulsions ($P < 0.05$). The ratios of DHA/EPA and MUFA/PUFA did not show any significant difference among the groups ($P > 0.05$).

**Table 2.** Total lipid and total fatty acids contents and fatty acid composition (in % TFA) in Senegalese sole larvae at 2 dph (initial) and in juveniles of 50 dph fed *Artemia* enriched with the three emulsions (mean ± SD; n=4). Totals include some minor components not shown. Different superscripts letters denote significant differences among 50-dph larvae fed the three different diets (ANOVA, $P < 0.05$).

| | | Dietary treatments | | |
|---|---|---|---|---|
| | **Initial** | **ARA-L** | **ARA-M** | **ARA-H** |
| Total lipids (mg·g$^{-1}$ DW) | 80.1±3.4 | 118.1±17.7 | 125.4±3.6 | 121.2±9.0 |
| Total FA (mg·g$^{-1}$ DW) | 28.6±2.6 | 55.6±22.5 | 59.1±0.2 | 60.2±13.4 |
| Total saturated | 26.1±3.2 | 19.6±3.3 | 19.0±0.7 | 20.4±1.0 |
| 18:1n-9 | 12.9±1.7 | 26.8±2.4$^{a}$ | 26.5±0.7$^{a}$ | 20.9±2.3$^{b}$ |
| Total monounsaturated | 23.7±3.5 | 31.9±5.2 | 30.0±1.7 | 25.9±1.6 |
| 18:2n-6 | 1.1±1.6 | 6.0±0.6 | 5.9±1.2 | 6.1±0.3 |
| 20:4n-6 | 2.4±0.2 | 2.0±1.5$^{b}$ | 5.7±0.2$^{a}$ | 7.3±0.2$^{a}$ |
| Total n-6 PUFA | 6.9±0.5 | 14.0±1.7$^{b}$ | 17.8±1.3$^{a}$ | 19.8±0.4$^{a}$ |
| 18:3n-3 | 0.49±0.04 | 9.9±0.9 | 11.2±1.4 | 10.8±1.1 |
| 20:5n-3 | 4.3±0.4 | 1.9±0.4 | 1.6±0.4 | 1.7±0.2 |
| 22:6n-3 | 30.9±5.7 | 15.6±4.8 | 12.7±0.7 | 13.2±0.8 |
| Total n-3 PUFA | 41.1±6.3 | 31.8±6.9 | 30.2±2.9 | 30.7±2.1 |
| Total PUFA | 48.0±6.8 | 45.8±8.6 | 48.0±0.7 | 50.5±2.1 |
| (n-6)/(n-3) | 0.17±0.01 | 0.44±0.04$^{b}$ | 0.6±0.1$^{ab}$ | 0.7±0.1$^{a}$ |
| DHA/EPA | 7.2±0.7 | 8.2±1.1 | 8.3±0.1 | 7.7±0.1 |
| ARA/DHA | 0.08±0.01 | 0.1±0.1$^{b}$ | 0.46±0.03$^{a}$ | 0.57±0.02$^{a}$ |
| ARA/EPA | 0.6±0.0 | 1.1±0.2$^{c}$ | 3.78±0.01$^{b}$ | 4.4±0.1$^{a}$ |
| MUFA/PUFA | 0.5±0.2 | 0.7±0.2 | 0.7±0.3 | 0.5±0.2 |

Larvae at 2 dph contained particularly high proportions of SFA, DHA and EPA and a low content in n-6 PUFA (Table 2) resulting in high amounts of n-3 PUFA and in low n-6/n-3 PUFA, ARA/DHA and ARA/EPA ratios. After 50 days of rearing, the fatty acid composition of larval tissues reflected the fatty acid composition of the diet, showing a gradient of ARA and n-6 PUFA contents, with significantly higher levels in larvae from the ARA-M and ARA-H groups than from the ARA-L group. The amount of ARA contained in ARA-M and ARA-H treatments allowed juveniles aged 50 dph

to increase their initial ARA content. However, larvae fed ARA-L diet slightly depleted their ARA stock during development. Total lipid and total fatty acid contents in larvae were not significantly affected by the dietary treatments, neither SFA nor MUFA amounts ($P > 0.05$), although the content in OA in larvae from ARA-L and ARA-M groups was significantly higher than in larvae from the ARA-H group ($P < 0.05$). Levels of EPA, DHA, n-3 PUFA and total PUFA were not significantly different among larvae from the three experimental groups ($P > 0.05$). Total PUFA content in larvae was not significantly different but reflected the gradient observed in enriched *Artemia* ($P > 0.05$). As in enriched *Artemia*, the ratios of n-6/n-3 PUFA, ARA/DHA and ARA/EPA were significantly higher in larvae fed *Artemia* enriched with ARA-H than in those fed with *Artemia* enriched with ARA-L ($P < 0.05$). In larvae fed the ARA-M diet, the n-6/n-3 ratio was not statistically different from larvae from ARA-H and ARA-L groups, the ARA/DHA ratio was not significantly different from larvae from the ARA-H diet ($P > 0.05$), and the ARA/EPA ratio was significantly different from larvae from ARA-L and ARA-H groups ($P < 0.05$). The ratios of DHA/EPA and MUFA/PUFA in larvae were not significantly affected by the dietary treatments ($P > 0.05$).

In terms of growth performance, Senegalese sole larvae fed the ARA-M diet were significantly longer and heavier than larvae fed the ARA-H diet from 15 dph to the end of the experiment (Figure 1, $P < 0.05$). Larvae fed the ARA-L diet presented intermediate growth values, not significantly different from larvae fed the ARA-M and ARA-H diets, both in terms of SL and DW, during the experimental period. Final survival of Senegalese sole larvae was not affected by any diet, reaching a mean of 96.0 ± 0.8% at 50 dph.

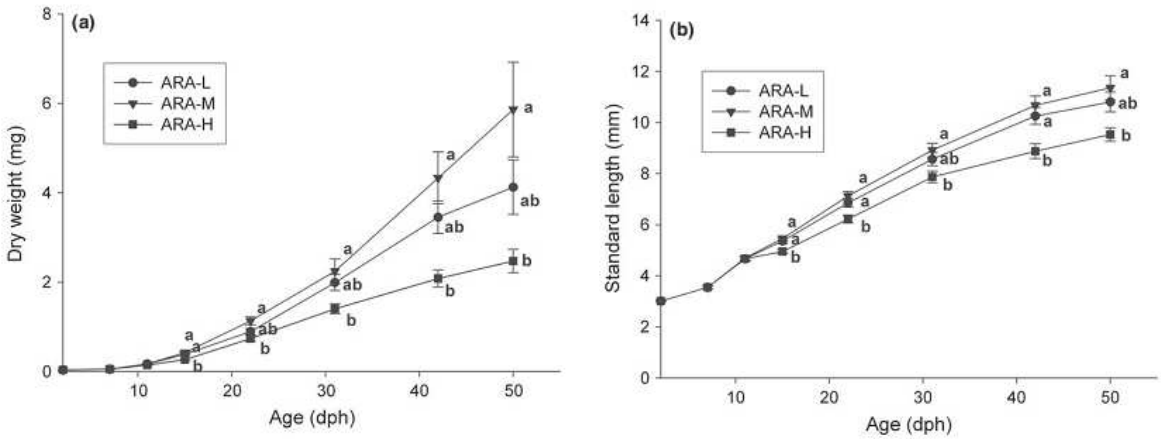


**Figure 1.** Effect of the graded content of dietary ARA on Senegalese sole larvae dry weight (DW; a) and standard length (SL; b) during development. Initial larval SL and DW were 3.01 ± 0.03 mm and 38.3 ± 2.2lg, respectively. Values are expressed as mean ± SEM (n = 4). Different letters within the same column show significant differences ($P < 0.05$).

The ossification process was completed in 50 dph-larvae for all dietary treatments. Therefore, the degree of ossification was measured at 15 dph, when most regions of the skeleton are undergoing mineralization (Gavaia *et al.*, 2002). There was a strong tendency for larvae fed ARA-M diet to be more calcified at 15 dph than larvae fed ARA-L and ARA-H diets (Figures 2 and 3a), although not significant ($P > 0.05$). The incidence of fusions of hypurals 3 and 4 (Figure 3b) at 50 dph, which was considered as a normal event in caudal fin complex development and ossification rather than a skeletal deformity, tended to be higher in larvae from the ARA-M group (34.4 ± 3.1%), lower in larvae from the ARA-H group (15.5 ± 3.6%) and intermediate in larvae from the ARA-L group (21.8 ± 6.9%), although differences were not statistically significant ($P > 0.05$).

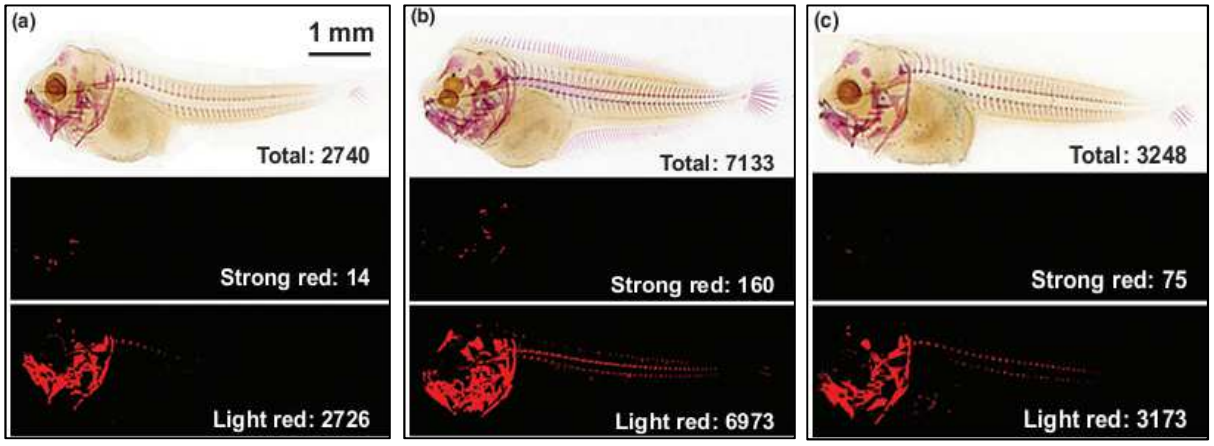


**Figure 2.** Ossification degree in 15 day-old Senegalese sole larvae fed different dietary ARA levels: ARA-L (a), ARA-M (b) and ARA-H (c). The first picture of each panel corresponds to alizarin-red stained larvae. The following pictures show strong red and light red pixels, respectively, that indicate the skeletal ossification degree. The numbers on each photo indicates the average number of pixels per larvae.

The gradient of dietary ARA did not affect the incidence of total skeletal deformities in Senegalese sole ($P = 0.570$, Table 3) and the frequency of deformed fish ranged from 35.7 to 46.2%. Neither cranial deformities, nor severe deformities of the vertebral column (vertebral compression and torsion, scoliosis, lordosis, kyphosis) were observed in any larvae from the different dietary treatments. All dietary treatments affected similarly the incidence of skeletal abnormalities of the vertebral region of larvae ($P = 0.719$). The main vertebral abnormality was the fusion of the last two vertebrae before the urostyle. No significant differences among the dietary groups were detected in the incidence of minor deformities, such as deformities of the haemal spines ($P = 0.382$) or neural spines ($P = 0.477$). Larvae from all dietary groups presented few skeletal deformities in their caudal fin complex, but were not significantly affected by the gradient of dietary ARA ($P = 0.345$).

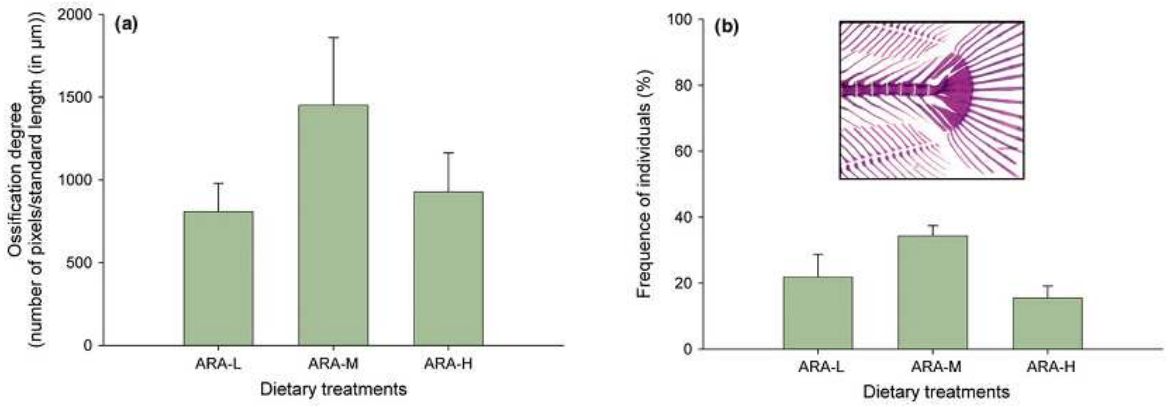


**Figure 3.** Effect of the graded content of dietary ARA on Senegalese sole larvae ossification degree at 15 dph (a) and fusion of hypurals 3-4 at 50 dph (b). Data are expressed as the mean ± SEM (n = 4).

**Table 3.** Effect of the graded content of dietary ARA on the incidence of the main skeletal deformities (in %) in Senegalese sole juveniles of 50 dph fed with the three different dietary treatments, considering the number of abnormal skeletal elements per fish (mean ± SEM, n = 4).

| | Dietary treatments | | |
|---|---|---|---|
| | **ARA-L** | **ARA-M** | **ARA-H** |
| **Total deformities** | 35.7±3.3 | 42.3±6.1 | 46.2±9.7 |
| Vertebral deformities | 35.7±3.3 | 39.6±5.7 | 43.4±9.4 |
| Fusion of vertebral bodies | 33.6±4.1 | 35.7±4.8 | 33.9±9.2 |
| Fusion of vertebra 43-44 | 33.6±4.1 | 31.0±4.4 | 32.1±9.6 |
| Fusion of haemal arches | 12.3±3.2 | 13.9±6.1 | 23.3±7.1 |
| Fusion of neural arches | 12.0±3.2 | 9.4±4.1 | 16.4±4.5 |
| **Caudal fin complex deformities** | 2.1±1.2 | 6.6±3.1 | 3.8±1.5 |

## Discussion

Enriched *Artemia* nauplii from the three dietary treatments were isolipidic. Their fatty acid composition reflected the gradient of ARA in the emulsion, in spite of the characteristic metabolism of this type of live prey that modifies the initial composition of the enriching products (Navarro *et al.*, 1999). The fatty acid profile in whole Senegalese sole larvae at 50 dph closely reflected the composition of their diet. This finding is consistent with previous studies from several flatfish species (Estévez *et al.*, 1999; Copeman *et al.*, 2002; Villalta *et al.*, 2005b; Boglino *et al.*, 2012). This recurrent observation for many species is probably linked to the fact that although some tissues keep a stable and optimal profile as long as the diet meets the minimum requirements, other tissues, like the muscle, mirror the diet unless the profiles in the diets are extreme (Benedito-Palos *et al.*, 2011). The amount of ARA contained in ARA-M and ARA-H diets allowed juveniles of 50 dph to increase their initial ARA content. However, the proportion of 1% TFA of ARA contained in the ARA-L diet, equivalent to the ARA content included in commercial enriching products (Sargent *et al.*, 2002), did not seem sufficient for Senegalese sole

larvae to increase their initial ARA content. In this case, larvae fed the ARA-L diet even slightly depleted their ARA stock during development. According to Sargent *et al.* (1999), who defined larval requirements in ARA as the amount of ARA found in the egg, and as found by Villalta *et al.* (2005b) by comparing ARA contents in yolk-sac larvae and in the diets, this low retention of ARA in larval tissue seems to indicate that providing dietary ARA to Senegalese sole larvae up to 1% TFA may be not enough for this species to increase the original ARA amount. This is not surprising considering that larvae of 2 dph initially contained 2.4% ARA of TFA. This observation points at the species-specificity and the developmental stage-specificity for the requirements in this EFA. Senegalese sole larvae may have specific ARA requirements different from global recommendations for larval requirement that can be found in commercial enriching products.

Larval survival was not affected by the graded dietary ARA amounts tested in this study. High dietary ARA content did not affect larval survival in Senegalese sole, in agreement with Villalta *et al.* (2005b) and with studies on other flatfish species, such as halibut *Hippoglossus hippoglossus* (McEvoy *et al.*, 1998), turbot *Scophthalmus maximus* (Estévez *et al.*, 1999), yellowtail flounder *Limanda ferruginea* (Copeman *et al.*, 2002), and summer flounder *Paralichthys dentatus* (Willey *et al.*, 2003). However, dietary ARA levels had a significant impact on larval growth in Senegalese sole. For the same species fed with enriched *Artemia* containing 0.1, 4.5 or 8.3% ARA of TFA, Villalta *et al.* (2005b) did not find any difference in dry weight at the end of metamorphosis (37 dph). These authors used emulsions made of a DHA-rich oil (Neuromins© Martek Bioscience) and ARA-rich oil (Vevodar©, DSM) in their study, whereas in this work the emulsion was made with lyophilized heterotrophic algae *Schizochytrium sp.* and Vevodar© oil. Thus, although dietary ARA contents in this study were close to those in the work of Villalta *et al.* (2005b), the EPA and DHA contents in enriched *Artemia* were two-fold higher. Under present experimental conditions, this fact has led to lower ratios of ARA/DHA and ARA/EPA, which could have made larvae more sensitive to the gradient of dietary ARA, affecting then their growth. Larvae fed 4.5% ARA of TFA (ARA-M) grew faster than larvae fed 1.0 or 7.0% ARA of TFA (ARA-L or ARA-H). A dose-effect of dietary ARA on larval growth has previously been observed for several species. In European sea bass (*Dicentrarchus labrax*) larvae, a dietary ARA content of 1.2% TFA markedly improved the growth compared to lower contents (Atalah *et al.*, 2011a). In gilthead sea bream (*Sparus aurata*), the increase in the ARA dietary level from 0.1 or 0.3% to 1.0% DW significantly improved larval growth (Bessonart *et al.*, 1999; Atalah *et al.*, 2011b). However, in the range of the dietary doses of ARA used in this experiment, a 1% TFA of dietary ARA did not resulted in the best growth of Senegalese sole larvae and early juveniles. Neither was correlated an increase of the ARA dietary gradient over 4.5% TFA with a better growth; on the contrary, feeding Senegalese sole larvae with 7.0% dietary ARA of TFA resulted in a detrimental effect on growth. However, other authors had previously shown that dietary ARA can be provided in very high amounts in other flatfish species during larval development, including halibut (McEvoy *et al.*, 1998), turbot (Estévez *et al.*, 1999), yellowtail flounder (Copeman *et al.*, 2002), and summer flounder (Willey *et al.*, 2003), with no effect on growth.

Although diets affected growth performance, none of the diets seemed to alter significantly the ossification process. Skeletons of larvae fed the ARA-M diet just presented a strong trend to be more ossified at 15 dph than those of larvae from the other groups. Other studies have highlighted a correlation between growth and ossification in Atlantic cod *Gadus morhua* (Kjørsvik *et al.*, 2009), gilthead sea bream (Fernández *et al.*, 2008), European sea bass (Darias *et al.*, 2010b) and Senegalese sole (Boglino *et al.*, 2012; Darias *et al.*, 2012) larvae.

The absence of evidences in the literature for ARA to be a factor that could hamper growth and bone formation lets the possibility to incriminate other dietary compounds, and their absolute and relative proportions in the diet. An increased n-6/n-3 ratio has been shown to have a negative effect on bone formation in rats (Li and Watkins, 1998; Watkins *et al.*, 1999, 2000). Berge *et al.* (2009) have shown an increased production of PGE2 in blood and a reduced vertebrae mineralization in Atlantic salmon *Salmo salar* juveniles fed high n-6/n-3 ratios (3.8–6.0). Villalta *et al.* (2005b) have also correlated increasing levels of dietary ARA and ARA/EPA ratio with an increased production of prostaglandins (PGE and PGF) in Senegalese sole larvae, which has been related to a higher incidence of malpigmented fish. In this sense, EPA and ARA are both competing precursors for eicosanoids biosynthesis and the resulting n-3 and n-6 series derived eicosanoids (prostaglandins E2 and E3) are potent regulators of bone metabolism, but with opposite effects on bone (Norrdin *et al.*, 1990; Marks and Miller, 1993). The balance in the synthesis of the two series of prostaglandins depends on the dietary intake of ARA relative to EPA (Hamre *et al.*, 2005). In our study, the increase in dietary ARA from 4.5 to 7.0% of TFA increased, although not significantly, the dietary ratios of ARA/EPA and n-6/n-3 PUFA (from 1.3 to 1.7 and from 0.4 to 0.5, respectively). Although the prostaglandin levels were not measured in this study, previous data from Villalta *et al.* (2005b) on this species suggested the high levels of dietary ARA tested might possibly have led to an increased production of PGE2 and then, to a reduction in bone ossification as reported in other species (Watkins *et al.*, 1999; Berge *et al.*, 2009).

The average incidence of skeletal deformities was low compared to other studies with Senegalese sole that obtained between 40 and 80% of deformed fish (Gavaia *et al.*, 2002; Fernández *et al.*, 2009; Boglino *et al.*, 2012). Low frequencies of skeletal malformations were observed in all groups, but none of the observed types of malformation seemed to be linked to ARA levels. Similarly, Berge *et al.* (2009) did not find any differences in the incidence of skeletal abnormalities in Atlantic salmon juveniles fed diets with different n-6/n-3 ratios. The gradient of dietary ARA did not affect the incidence of skeletal deformities in Senegalese sole at this developmental stage. Further, no severe malformations (scoliosis, kyphosis, lordosis) were observed in any larvae, even fed with the ARA-H diet, indicating that 7.0% ARA of TFA did not affect larval skeletogenesis in Senegalese sole, although it delayed their somatic growth.

**Conclusion**

In conclusion, the results of this study suggested 4.5% ARA as the optimal amount of dietary TFA for proper growth and ossification in Senegalese sole larvae during the *Artemia* feeding period. The lowest level of dietary ARA (1% TFA) was insufficient to meet the initial larval requirements (ca. 2.4% TFA) and the highest amount of ARA (7% TFA) led to imbalanced ARA/EPA and n-6/n-3 ratios that, in both cases, delayed somatic growth and skeletal ossification. Nevertheless, none of the dietary ARA contents tested in this study influenced differentially the incidence of skeletal deformities.

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# 6

# High dietary arachidonic acid levels affect the process of eye migration and head shape in pseudo-albino Senegalese sole (*Solea senegalensis*) early juveniles

Anaïs Boglino[1]
Asher Wishkerman[1]
Maria J. Darias[2]
Karl B. Andree[1]
Pablo De la Iglesia[1]
Alicia Estévez[1]
Enric Gisbert[1]

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[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

**Los niveles altos de ácido araquidónico en la dieta afectan al proceso de migración del ojo y la forma de la cabeza en larvas pseudo-albinas y post-metamórficas del lenguado Senegalés (*Solea senegalensis*).**

**Resumen**

En el presente estudio se evaluaron los efectos de adicionar altos niveles de acido araquidónico (ARA) en la dieta sobre la migración de ojo y la remodelación de los huesos del cráneo en el lenguado Senegalés mediante de análisis morfométricos geométricos y de tinción, con rojo alizarin, de los elementos esqueletos del cráneo. La incidencia de larvas post-metamórficas normalmente pigmentadas alimentadas con la dieta control estuvo de 99.1 ± 0.3%, mientras la de los peces alimentados con altos niveles de ARA (ARA-H) estuvo solo de 18.7 ± 7.5%. La frecuencia de peces con deformidades fue significativamente más alta en larvas alimentadas con ARA-H (95.1 ± 1.5%) que en las que fueron alimentadas con la dieta control (1.9 ± 1.9%). Las deformidades del cráneo estuvieron significativamente y negativamente correlacionadas con la incidencia de animales normalmente pigmentados ($R^2$ = -0.88, $P < 0.001$). Además, los peces con desordenes pigmentarios difirieron de los normalmente pigmentados por la posición de los ojos considerando los ejes de la columna vertebral y de la boca, y por una distancia intraocular y altura de la cabeza, cuales fueron más cortas en los peces con desordenes pigmentarios. Además de los cambios en el posicionamiento de los dos ojos, los peces pseudo*albinos mostraron unas diferencias osteológicas inducidas por el ARA, considerando algunos elementos esqueléticos del splanchnocranium y neurocranium en comparación a los especímenes normalmente pigmentados. Otra diferencia pertinente entre los peces pseudo-albinos y normalmente pigmentados fue la presencia de dientes en ambas mandíbulas inferior y superior de los peces pseudo-albinos. Este estudio es el primero entre peces planos que describió la migración defectuosa del ojo del lado ocular a la superficie ventral, el ojo derecho en el caso del lenguado Senegalés, mientras el ojo izquierdo migro dentro del lado ocular casi normalmente.

**Palabras claves**: peces planos, metamorfosis, ácido arachidonico, migración del ojo, cráneo.

**Abstract**

In this study, the effects of high dietary levels of arachidonic acid (ARA) in the eye migration and cranial bone remodeling processes in Senegalese sole early juveniles (age: 50 days post hatch) was evaluated by means of geometric morphometric analysis and alizarin red staining of cranial skeletal elements. The incidence of normally pigmented fish fed the control diet was 99·1 ± 0·3% (mean ± S.E.M.), whereas that of fish fed high levels of ARA (ARA-H) was only 18·7 ± 7·5%. The frequency of fish presenting cranial deformities was significantly higher in fish fed ARA-H (95·1 ± 1·5%) than those fed the control diet (1·9 ± 1·9%). Cranial deformities were significantly and negatively correlated with the incidence of normally-pigmented animals ($R^2$ = -0·88; $P$ < 0·001; $n$ = 16). Thus, fish displaying pigmentary disorders differed from the normally pigmented ones as evidenced by the position of the eyes with regards to the vertebral column and mouth axes, and by the interocular distance and head height, which were shorter in fish displaying pigmentary disorders. In addition to changes in the positioning of both eyes, pseudoalbino fish showed some ARA-induced osteological differences with regards to some skeletal elements from the splanchnocranium (*e.g.* right premaxillary, dentary, angular, lacrimal, ceratohyal and branchiostegal rays) and neurocranium (e.g. sphenotic, left lateral ethmoid and left frontal) in comparison to normally pigmented specimens. Another relevant difference between pseudoalbino and normally pigmented fish was the presence of teeth in both lower and upper jaws in pseudoalbino fish. This is the first study among flatfishes that describes the impaired metamorphic relocation of the ocular side eye, the right eye in the case of Senegalese sole, whereas the left eye migrated into the ocular side almost normally.

**Key words**: flatfish, metamorphosis, arachidonic acid, eye migration, skull

**List of abbreviations**

AGM: Algamac 3050$^{TM}$; AN: angular; ARA: arachidonic acid; BOC: basioccypital; BR.R.: branchiostegal rays; DHA: docosahexaenoic acid; CH: ceratohyal; D: dentary; DW: dry weight; ECTP: ectopterygoid; ENTP: entopterigoid. EP: epural; EPA: eicosapentaenoic acid; EPOT: epioccipital; EXOC: exoccipital; F: frontal; FA: fatty acid; HUFA: highly unsaturated fatty acids; HYP: hypural; HM: hyomandibular; LA: linoleic acid; LAC: lacrimal; L.L.ETHM: lateral left ethmoid; L.R.ETHM: lateral right ethmoid; LM: landmark; MAX: maxillary; METHM: mesethmoid; Mhs: modified haemal spine; Mns: modified neural spine; MPT: metapterygoid; MUFA: mono unsaturated fatty acids; O: opercular.; OA: oleic acid; PA: parietal; PAL: palatine; PASPH: parasphenoid; PGE2: prostaglandin 2; PMAX: premaxillary; PHY: parahypural; PO: preopercular; PTOP: pterotic; Q: quadrate; SL: standard length; SOC: supraoccipital; SPOT: sphenotic; UH: urohyal; V: vertebral body; ∠VO: vertebral-ocular angle; ∠BO: bucco-ocular angle.

**Introduction**

The most spectacular post-embryonic tissue remodeling process in vertebrates is the eye migration in Pleuronectiformes occurring during larval metamorphosis (Sæle *et al.*, 2006). At hatching, flatfish larvae are pelagic and display the principal external morphological features as other teleost larvae: one eye is present on either side of the head, the mouth is horizontal or subhorizontal and the body is bilaterally symmetric (Schreider, 2006). During larval ontogeny, one of the eyes migrates across the midline to the opposite side of the head and its displacement induces an important remodeling of the whole head, whereas the whole body structure is modified accordingly (see reviews in Inui and Miwa, 2012; Schreider, 2013). Thus, the main result of metamorphosis is observed in the bony structures of the skull where there is a pronounced asymmetry of the jaws, suspensoria, ethmoid and orbital regions and to a lesser extent the otico-occipital region (Wagemans and Vandewale, 2001). Eye migration is often used as an indicator for the degree of advancement of the metamorphic process (Solbakken *et al.*, 1999). This whole process, as well as the endocrine mechanisms underlying the morphological and anatomical changes in the external and internal anatomy of fish taking place during metamorphosis, has received extensive attention by fish biologists (Okada *et al.*, 2001; Okada *et al.*, 2003; Sasaki and Yamashita, 2003; Sæle *et al.*, 2003; Sæle *et al.*, 2006; Schreiber, 2006; Power *et al.*, 2008). In addition, this process has also been studied in detail for aquaculture purposes, since impaired eye migration in addition to pigmentary disorders are two of the most important problems affecting the intensive production of flatfish juveniles (Gavaia *et al.*, 2009; Fernández and Gisbert, 2011). The high incidence of abnormal juveniles limits the cost-effectiveness of their production and thus represents a serious industrial bottleneck (Power *et al.*, 2008).

Metamorphosis in flatfish is highly sensitive to intrinsic and extrinsic signals, leading to a tightly regulated climax event required for successful migration of one eye so that both eyes are on the same side of the head (Geffen *et al.*, 2007; Power *et al.*, 2008; Schreider, 2013). This process is primarily controlled by the pituitary-thyroid axis and many biotic and abiotic factors have been described which affect metamorphosis (Inui and Miwa, 2012). Among them, nutrition seems to play a key role in the normal development of the metamorphic process. Thus, an unbalanced macronutrient composition of the diet, a dietary deficiency in iodine, an excess of vitamin A and/or an unbalanced essential fatty acid composition in the diet, are nutritional factors that have been described as affecting the overall process of larval flatfish metamorphosis, and eye migration in particular (Estévez and Kanazawa 1995; Copeman *et al.* 2002; Villalta *et al.*, 2005a; Hamre *et al.*, 2007; Fernández *et al.*, 2009). Several studies have evaluated the effects of dietary docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) on bone formation and metamorphosis in several flatfish species (Estévez and Kanazawa 1995; Villalta *et al.*, 2005a; Hamre *et al.*, 2007; Boglino *et al.*, 2012a). Although arachidonic acid (20:4n-6, ARA) is present in fish tissues in lower amounts than DHA and EPA, absolute amounts of dietary ARA may not be neglected, as well as its content relative to EPA and DHA (Boglione *et al.*, 2013). This essential fatty acid is the major precursor for eicosanoid synthesis, enhancing the immune system and resistance to stress (Bell and Sargent, 2003), whereas changes in the dietary ARA／EPA ratio can affect the production of prostaglandin E2 (PGE2) known to

regulate osteoblasts and bone metabolism (Berge *et al.*, 2009). Although the action of ARA and PGE2 on bone homeostasis and remodeling is known in mammals (Watkins *et al.*, 2001; Zhang and Wang, 2012), little information on the effects of dietary ARA content on bone development in fish is available (Boglino *et al.*, 2012b), especially during the process of eye migration occurring at metamorphosis.

The objective of this study was to evaluate the effects of high doses of dietary ARA on the processes of eye migration and cranial bone remodeling taking place during metamorphosis in Senegalese sole (*Solea senegalensis* Kaup, 1958).

**Materials and methods**

*Larval rearing, feeding protocol and experimental design*

One-day-old Senegalese sole larvae (Stolt Sea Farm SA, Carnota, A Coruña, Spain) were reared in 8, 100 l cylindro-conical tanks (initial density: 110 larvae $l^{-1}$) connected to a IRTAmar™ water recirculation unit at IRTA-SCR facilities. Larval rearing conditions were as follows: temperature 16·7 ± 0·4ºC, salinity 35, pH 8·0 ± 0·2, dissolved oxygen 7·5 ± 1·3 ppm, photoperiod was 16L: 8D (500 lx at the water surface), gentle aeration in each tank and 50% daily water renewal in the recirculation system. Larvae were fed twice a day, from 2 days post hatching (dph) to 10 dph, with enriched rotifers (*Brachionus plicatilis*), at a density of 10 rotifers $ml^{-1}$ from 2 to 8 dph and 5 rotifers $ml^{-1}$ from 9 to 10 dph. Enriched *Artemia* metanauplii were supplied twice a day to larvae from 8 to 50 dph, at increasing densities ranging from 0·5 to 12 metanauplii $ml^{-1}$. The daily food ration was calculated as described by Cañavate *et al.* (2006). From 30 dph onwards, when larvae settled to the bottom of the tank, enriched *Artemia* metanauplii were supplied frozen as described by Villalta *et al.* (2007). Both types of live prey were enriched according to Boglino *et al.* (2012a).

Two experimental groups of larvae (four replicates per group) were fed from 2 to 50 dph on rotifer and *Artemia* nauplii enriched with two different products that resulted in two different levels of ARA. A control group (AGM diet) was fed live prey enriched with a commercial enrichment (AGM, Algamac 3050™, Aquafauna, Biomarine Inc., Hawthorne, CA, USA). The second group was fed live prey enriched with an experimental emulsion containing high levels of ARA (ARA-H diet) that was prepared from a mixture of two oils rich in DHA (cod liver oil, Fluka©, Sigma-Aldrich, Chemie GmBH, Steinheim, Norway) and ARA (Vevodar®, DSM Food Specialties, Delft, The Netherlands). Olive oil was added to the cod liver oil and Vevodar® mixture to dilute and adjust n−3 PUFA concentration in enriched live prey and α-tocopherol was included for preserving the emulsion from oxidation according to Boglino *et al.* (2012). Lipid and fatty acid (FA) composition of enriching products, enriched live prey and early juveniles aged 50 dph was conducted as previously described in Boglino *et al.* (2012a) and their values are shown in Table 1.

**Table 1.** Total lipid and fatty acids contents (mg g $W_D^{-1}$) and fatty acid composition (% of TFA) of the two emulsions (mean ± S.E.M.; *n* = 2), the enriched rotifer (mean ± S.D.; *n* = 5) and *Artemia* nauplii (mean ± S.E.M.; *n* = 3) with the two emulsions and early juveniles aged 50 days post hatching (mean ± S.E.M.; *n* = 4). Totals include some minor components not shown. Superscript letters denote significant differences among diets (ANOVA, $P < 0.05$). A TFA of 0.0% means content under 0.45%.

| | Enriching products | | Enriched rotifer | | Enriched *Artemia* | | Fish | |
|---|---|---|---|---|---|---|---|---|
| | AGM | ARA-H* | AGM | ARA-H | AGM | ARA-H | AGM | ARA-H |
| Total lipid | 368·5±6·1$^b$ | 622·2±2·0$^a$ | 140·6±8·4 | 143·2±2·1 | 164·9±8·5 | 164·9±8·9 | 86·7±7·0 | 100·6±11·0 |
| Total FA | 305·4±5·1 | 328·1±13·7 | 74·2±5·9 | 61·4±6·2 | 87·6±8·5 | 85·0±8·5 | 34·6±4·7 | 46·3±6·0 |
| Total SFA | 23·8±1·8 | 18·1±1·1 | 21·4±1·6$^a$ | 17·2±0·7$^b$ | 17·5±0·5$^a$ | 15·3±0·7$^b$ | 24·6±1·0 | 19·0±0·1 |
| 18:1n-9, OA | 0·07±0·1$^b$ | 25·7±0·1$^a$ | 6·6±1·2$^b$ | 25·6±1·8$^a$ | 14·4±2·2$^b$ | 23·1±1·2$^a$ | 14·3±0·2$^b$ | 22·3±0·5$^a$ |
| Total MUFA | 0·07±0·1$^b$ | 35·3±0·2$^a$ | 14·4±2·1$^b$ | 41·8±1·3$^a$ | 21·7±3·2$^b$ | 33·6±1·7$^a$ | 19·8±1·0$^b$ | 31·0±0·3$^a$ |
| 18:2n-6, LA | 0·0±0·0$^b$ | 7·4±1·0$^a$ | 2·4±0·3$^b$ | 9·3±2·2$^a$ | 4·6±0·7$^b$ | 6·7±0·2$^a$ | 3·6±0·2$^b$ | 6·9±0·2$^a$ |
| 20:4n-6, ARA | 0·3±0·01$^b$ | 15·1±0·6$^a$ | 1·0±0·2$^b$ | 10·2±0·5$^a$ | 1·4±0·8 | 7·1±2·4 | 4·1±0·1$^b$ | 10·2±0·2$^a$ |
| 22:5n-6, DPA | 19·6±0·2$^a$ | 0·9±0·9$^b$ | 13·3±1·0$^a$ | 1·8±0·6$^b$ | 4·3±1·6$^a$ | 0·6±0·2$^b$ | 6·7±0·4$^b$ | 0·9±0·0$^a$ |
| Total n-6 PUFA | 21·1±0·2$^b$ | 26·2±0·7$^a$ | 18·2±0·8$^b$ | 24·2±1·6$^a$ | 11·8±1·7 | 16·4±2·8 | 16·1±0·3$^b$ | 20·6±0·1$^a$ |
| 18:3n-3, ALA | 0·0±0·0$^b$ | 0·8±0·1$^a$ | 0·4±0·1$^b$ | 1·3±0·2$^a$ | 26·8±3·6 | 24·6±3·5 | 10·9±0·5 | 14·4±0·4 |
| 20:5n-3, EPA | 1·6±0·0$^b$ | 11·3±0·4$^a$ | 2·8±0·3$^b$ | 7·4±1·1$^a$ | 4·7±1·8 | 3·7±0·9 | 2·1±0·2$^a$ | 1·6±0·0$^b$ |
| 22:6n-3, DHA | 52·9±2·2$^a$ | 6·5±0·6$^b$ | 40·0±2·1$^a$ | 5·3±0·4$^b$ | 10·6±4·7 | 1·4±0·3 | 20·0±1·3$^a$ | 6·1±0·4$^b$ |
| Total n-3 PUFA | 55·0±2·9$^a$ | 20·4±1·1$^b$ | 46·0±1·8$^a$ | 16·8±0·6$^b$ | 46·4±2·1$^a$ | 33·2±3·1$^b$ | 36·7±2·0$^a$ | 26·2±0·5$^b$ |
| Total PUFA | 76·1±2·1 | 46·6±1·4 | 64·2±2·6$^a$ | 41·0±2·0$^b$ | 58·2±3·8$^a$ | 49·6±0·6$^b$ | 52·8±2·2$^a$ | 46·8±0·5$^b$ |
| (n-3) / (n-6) | 2·6±0·1$^a$ | 0·8±0·01$^b$ | 2·5±0·1$^a$ | 0·7±0·1$^b$ | 4·1±0·3 | 2·3±0·5 | 2·3±0·1$^a$ | 1·3±0·0$^b$ |
| DHA / EPA | 32·8±7·4$^a$ | 0·6±0·1$^b$ | 15·1±0·7$^a$ | 0·9±0·3$^b$ | 2·1±0·5$^a$ | 0·4±0·21$^b$ | 9·5±0·4$^a$ | 3·8±0·3$^b$ |
| ARA / DHA | 0·0±0·0$^b$ | 2·3±0·2$^a$ | 0·03±0·01$^b$ | 2·0±0·2$^a$ | 0·1±0·1$^b$ | 4·7±1·2$^a$ | 0·2±0·0$^b$ | 1·7±0·1$^a$ |
| ARA / EPA | 0·2±0·01$^b$ | 1·3±0·02$^a$ | 0·4±0·1$^b$ | 1·6±0·3$^a$ | 0·3±0·1$^b$ | 1·8±0·2$^a$ | 1·9±0·1$^b$ | 6·3±0·1$^a$ |
| MUFA / PUFA | 0·0±0·0$^b$ | 0·8±0·04$^a$ | 0·2±0·1$^b$ | 1·0±0·1$^a$ | 0·4±0·1$^b$ | 0·7±0·1$^a$ | 0·4±0·1$^b$ | 0·7±0·0$^a$ |

* 100 g of ARA-H contained 29·0 g of Fluka© oil (Sigma-Aldrich, Chemie GmbH, Steinheim, Norway), 17·4 g of Vevodar® (DSM Food Specialties, Netherlands), 5·2 g of olive oil, 2·3 g of vitamin E, 4·1 g of soy lecithin and 42·0 g of distilled water. *Abbreviations*: ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexanoic acid; DPA, docosapentaenoic acid; EPA, eicosapentanoic; FA, fatty acids; LA, ; OA, oleic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

*Larval growth and geometric morphometric analysis*

At the end of the larval rearing (50 dph), 50 fish were sampled from each experimental group, euthanized with an overdose of tricaine methane sulphonate (MS-222, Sigma). Fish were sacrificed

following the protocols of the veterinary committee of IRTA in accordance with EU regulations (EC Directive 86/609/EEC). The ocular side (or upper surface) of each specimen was photographed (300 dpi image) with a digital camera (Olympus DP25©, Olympus Corporation, Germany) connected to a stereomicroscope (Nikon SMZ 800). Fish standard length ($L_S$) was measured to the nearest 0.1 mm using an image analysis system (AnalySIS®, Soft Imaging Systems, GmbH, Olympus, Germany). In addition, dry weight (DW) was determined by rinsing larvae with distilled water to remove salt and then drying them at 60ºC for 24 h.

For each photographed specimen, coordinates of 10 anatomical landmarks were recorded for each larva using tpsDig 2.16 (Rohlf, 2008). Although the separation of camera and fish was equidistant for all images taken, a scale factor was included in every picture to avoid potential scaling differences in further analyses. The landmarks were selected to provide a definition of the fish morphology in which the morphological landmarks are given as *x* and *y* co-ordinates. The distances and angles between specific landmarks were determined from their co-ordinates. The landmarks (LM) were digitized on the ocular side of each individual by the same observer (Fig. 1a): LM1, tip of the snout; LM2, distal point of the dentary bone; LM3, lower edge of the operculum; LM4, center of the right eye; LM5, center of the left (migrated) eye; LM6, maximum head height; LM7, upper edge of the caudal peduncle (posterior end of the modified neural spine); LM8, end of the caudal peduncle (mid posterior end of the hypurals); LM9, lower edge of the caudal peduncle (posterior end of the first modified haemal spine); LM10, most anterior visible point by transparency of the vertebral column; as this point is a semi-landmark, it was not used for the geometric morphometric analyses. Additionally, the following two angular measurements were evaluated in all fish in order to assess differences in eye disposition on the ocular side of the fish between treatments (Fig. 1b): the vertebral-ocular angle ($\angle_{VO}$), which is defined as the angle formed by the vertebral column line (LM8-10) and the axis that bisects both eyes (LM4-5), and the bucco-ocular angle ($\angle_{BO}$), which is defined as the angle formed by the line going through the distal point of the dentary bone (LM2) and between the two eyes and the axis that bisects them (LM4-5).

Body shape was analyzed using the landmark-based geometric morphometric methods as described in Rohlf (1990) and Bookstein (1991). The landmarks (LM1-9) were superimposed to have a common centroid and were rotated to minimize the distances between the corresponding landmarks. Once all the specimens were aligned, the mean configuration of landmarks was computed (consensus or reference shape). The specimens were projected to a tangent space by orthogonal projection where the distances between shapes were linear functions. This process allowed applying multivariate statistical methods on the shape variation. Principal Component Analysis (PCA) was performed after computing the variance covariance matrix of the procruster shape co-ordinates and projecting the data onto the corresponding eigenvectors. All geometric morphometric related analyses were carried out with MorphoJ integrated program package (Klingenberg, 2011).

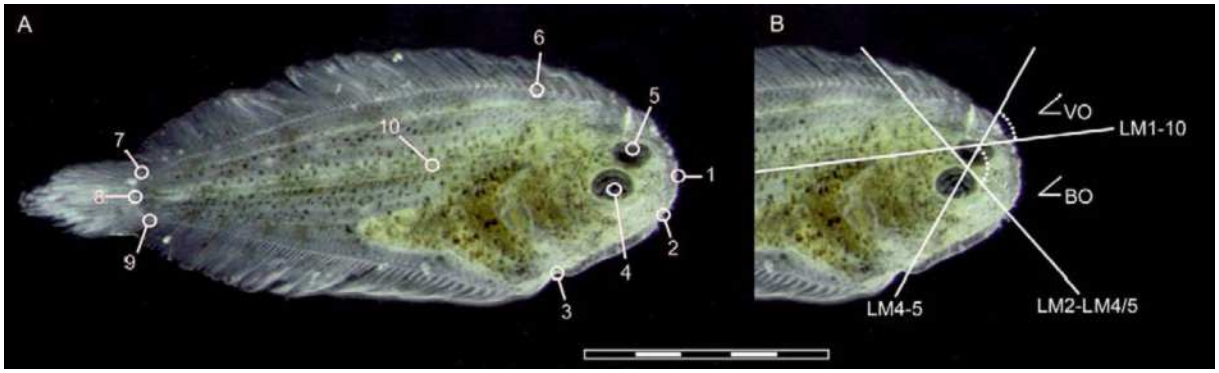


**Figure 1.** General view of a Senegalese sole (*S. senegalensis*) early juvenile aged 50 days post hatching. (a) Ocular side of a specimen showing the 10 landmarks (LM) used for geometric morphometric analysis. (b) Detail of the ocular side of the head indicating how the vertebral-ocular ($\angle_{VO}$) and the bucco-ocular ($\angle_{BO}$) angles were calculated. Scale bar, 4 mm.

*Staining of bones of whole-body samples*

Forty fish were randomly sampled per tank at 50 dph (160 per dietary treatment) to evaluate the impact of the different diets on the incidence of skeletal deformities and the process of eye migration. Specimens were fixed in 4% formalin buffered to pH = 7·0 with 0·1 M phosphate buffer and stored at 4ºC until bone staining. Animals were only stained with alizarin red according to Darias *et al.* (2010) and cranial and vertebral column skeletal structures were identified and named according to Wagemans and Vandewalle (1999, 2001) and Gavaia *et al.* (2002), respectively. In the present study, anomalies in the shape of skeletal elements were defined according to Boglione *et al.* (2013) recommendations; thus, the term abnormality and anomaly were used as synonyms, indicating a difference or deviation from the average or norm; whereas a deformation was considered as an alteration in shape and/or in structure of previously normally formed part (The American HeritageMedical Dictionary®). The incidence of skeletal anomalies and deformities in the cranium (sphachno- and neurocranium; see Figure 2 for details), vertebral column and caudal fin complex, as well as the number of vertebral bodies was determined in Senegalese sole larvae at 50 dph according to Boglino *et al.* (2012a). Vertebral deformities were divided into two major classes: vertebral column deformities, affecting the general shape of the vertebral column, such as scoliosis, lordosis or vertebral torsion; and vertebral body deformities, affecting a few vertebrae in a region of the vertebral column, such as deformation of vertebral bodies, compression, fusion or fusion and deformation of the neural and haemal spines. The size of several cranial bones like the dentary, angular and sphenotic was measured to the nearest 0.1 mm using an image analysis system (AnalySIS®) in Alizarin red-stained photographed (300 dpi) specimens ($n$ = 50 per group). Differences in size of the former cranial bones, between normally pigmented and ARA-induced pseudoalbino fish were expressed as percentages calculated from mean values relative to normal bones. The comparison of different skeletal structures from the skull and other body regions was conducted considering fish at the same developmental stage and size from both experimental groups.

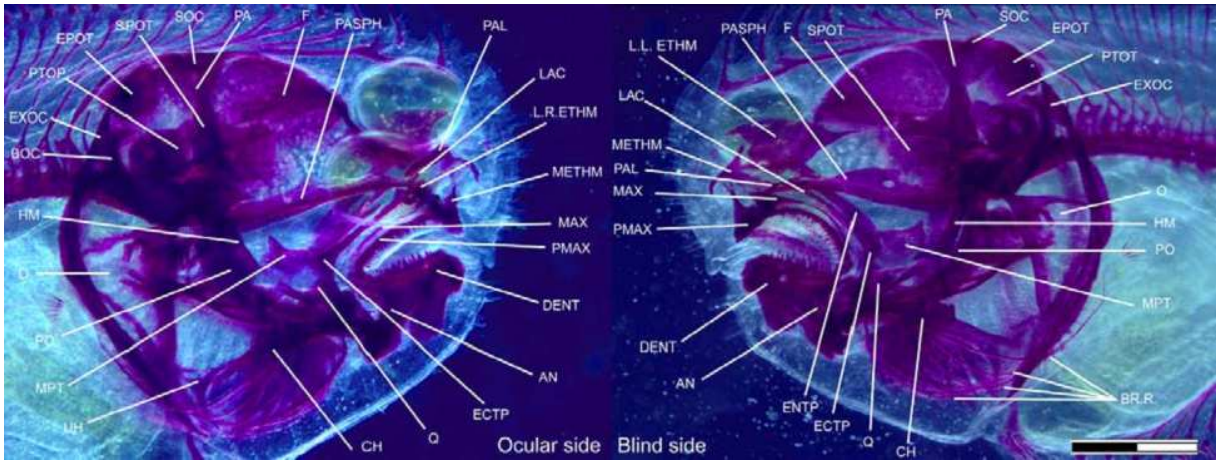


**Figure 2.** Ocular and ventral sides of the head (neurocranium and splanchnocranium) in a Senegalese sole early juvenile (age: 50 days post hatching) stained with Alizarin Red (bone tissue). AN, angular; BOC, basioccypital; BR.R., branchiostegal rays; CH, ceratohyal; ECTP, ectopterygoid; ENTP, entopterigoid; D, dentary; EPOT, epioccipital; EXOC, exoccipital; F, frontal; HM, hyomandibular; LAC, lacrimal; L.L.ETHM, lateral left ethmoid; L.R.ETHM, lateral right ethmoid; MAX, maxillary; PAL, palatine; METHM, mesethmoid; MPT, metapterygoid; O, opercular; PA, parietal; PASPH, parasphenoid; PMAX, premaxillary; PO, preopercular; PTOP, pterotic; Q, quadrate; SOC, supraoccipital; SPOT, sphenotic; UH, urohyal. Scale bar, 1.5 mm.

*Prostaglandin analyses*

Four pools of normally pigmented and pseudoalbino early juveniles aged 50 dph (*n* = 180 fish per pool) were extracted with a high-speed homogenizer (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany) in 4 volumes of Hank's balanced salt solution containing 0·6 ml of absolute ethanol and 0·2ml of 2mM formic acid and then, the homogenates were frozen at -20°C until further analysis. Frozen homogenates were thawed and centrifuged at 3,000g for five minutes to precipitate cell debris. The supernatant (9 ml) was submitted to a clean-up step by solid-phase extraction using octadecyl (C18) "Sep-Pak®" minicolumns (Waters, USA), previously conditioned with 5 ml of methanol and 10 ml of distilled water according to Powell (1982). After loading, samples were washed with 10 ml of distilled water, followed by 5 ml of 15% ethanol and by 5 ml of hexane:chloroform 65:35 (v:v) and eluted with 10 ml of ethyl acetate. After drying samples under nitrogen stream, they were redissolved in 200 µl of methanol, then stored in a glass vial at -20°C before separation of PGE isomers (2 and 3) by reversed-phase high-performance liquid chromatography (RP-HPLC; Bell *et al.*, 1995). The HPLC instrument consisted of a 510 pump coupled with a 996 photodiode array detector (both from Waters Corp., Massachusetts, USA) and a fraction collector FRAC-100 (Pharmacia Biotech., Uppsala, Sweden). An isocratic elution with a mobile phase containing 17 mM phosphoric acid/acetonitrile (70/30, v/v) was performed at a flow rate of 1 ml $min^{-1}$ and room temperature on a Zorbax SB-C18 (4·6 x 250 mm, 5 µm; Agilent Tech.) protected with a SecurityGuard C18 pre-column (4mm×2mm,

Phenomenex). Elution of PGEs was determined between 18-26 minutes (for PGE3) and between 34-41 minutes (for PGE2), by fractionation of the corresponding PGE standards (2- and 3- series) in independent runs under the same conditions. For samples, fractions with PGE2 and PGE3 were colletected and combined, loaded onto a C18 "Sep-Pak" previously conditioned with 5 ml of methanol and 10 ml of distilled water, then washed with 10 ml of distilled water and finally eluted in 5 ml of ethyl acetate. Eluates were dried under nitrogen stream and redissolved in 500µl of immunoassay buffer. Measurement of prostaglandins was performed using a PGE2 enzyme immunoassay kit in accordance to the protocol of the manufacturer (Cayman®, USA).

*Statistics*

Results were expressed as means ± S.E.M ($n$ = 4). All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA). All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). The arcsin square root transformation was conducted on data expressed as a percentage. Variables were compared between treatments by a Student $t$-test. PGE2 levels between normally pigmented and pseudoalbino fish from both dietary groups were compared by means of a one-way ANOVA, followed by the Holm-Sidak post-hoc test. A Pearson product moment correlation test was used to evaluate the correlation between the incidence of specimens with cranial deformities and the incidence of normal pigmented specimens.

**Results**

*Lipid and fatty acid composition in live prey and fish and PGE2 levels in fish*

Rotifer and *Artemia* metanauplii enriched with the AGM enrichment and the ARA-H emulsion presented similar total lipid and total fatty acid contents, but they significantly differed in their fatty acid profiles (Table 1). Both live preys enriched with AGM contained significantly higher amounts of total saturated fatty acids (SFA), and higher amounts of 22:5n-6 and DHA than rotifer and *Artemia* enriched with the ARA-H emulsion. Consequently, live preys enriched with AGM presented higher amounts of total n-3 PUFA and PUFA than those enriched with the ARA-H emulsion. Both live preys enriched with the ARA-H emulsion contained significantly more total monounsaturated fatty acids (MUFA) than those enriched with AGM due to higher amounts of oleic acid (OA) and higher levels of total n-6 PUFA, as they contained higher levels of linoleic acid (LA, 18:2n-6), ARA and EPA. The ratios of (n-3)/(n-6) PUFA and DHA/EPA were higher in rotifer and *Artemia* enriched with AGM, whereas live preys enriched with ARA-H showed higher ratios of ARA / DHA, ARA / EPA and MUFA/PUFA.

The fatty acid composition of early juveniles (50 dph) reflected the fatty acid profile of the enriched rotifer and *Artemia* fed to the larvae (Table 1). Fish fed live feed enriched with ARA contained higher levels of OA, total MUFA, LA, ARA, total n-6 PUFA, and lower contents of EPA, DHA, total n-3 PUFA and total PUFA, and therefore had significantly lower (n-3) / (n-6) PUFA and DHA/EPA ratios, and higher ARA/DHA, ARA/EPA and MUFA / PUFA ratios.

Normally pigmented specimens from both experimental groups (AGM and ARA-H diets) had similar PGE2 levels (33·6 ± 10·9 pg $g^{-1}$ ww and 57·1 ± 2·0 pg $g^{-1}$ ww, respectively; $P > 0·05$). In contrast, ARA-induced pseudoalbino fish contained between 5.0 and 8.4 times higher PGE2 levels (283·2 ± 82·2 pg $g^{-1}$ ww) than normally pigmented fish from ARA-H and the AGM dietary groups (One-way ANOVA, $F = 8·28$, *d.f.* = 8; $P = 0·019$; $n = 9$).

*Dietary ARA effects on larval morphology*

Dietary levels of ARA significantly affected the incidence of specimens with pigmentary disorders (*t*-test, $t = 10.71$, *d.f.* = 6; $P < 0.001$; $n = 8$). The incidence of normally pigmented early juveniles fed the AGM diet was 99.1 ± 0.3%, whereas that of fish fed high levels of ARA was only 18.7 ± 7.5%. No statistically significant differences were observed between both experimental groups fed AGM and ARA-H diets with regards to SL and DW ($P > 0.05$; Table 2) of early juveniles. No significant differences were detected between normally pigmented species fed AGM and ARA-H diets with regards to the VO and BO angles, head height and interocular distance ($P > 0.05$; Table 2). However, normally pigmented and pseudoalbino specimens differed in respect to the postion of the eyes with regards to the vertebral column and mouth axes, as indicated by their different respective $\angle_{VO}$ (*t*-test, $t = 12.70$, *d.f.* = 6, $P < 0.001$; $n = 8$) and $\angle_{BO}$ values (*t*-test, $t = 10.96$, *d.f.* = 6, $P < 0.001$; $n = 8$). In addition, the head height of normally pigmented fish was slightly larger (*t*-test, $t = 21.21$, *d.f.* = 6, $P < 0.001$; $n = 8$) than that of fish displaying pigmentary disorders. The interocular distance in pseudoalbino fish was 25% shorter than that in normally pigmented specimens (*t*-test, $t = 14.14$, *d.f.* = 6, $P < 0.001$; $n = 8$; Table 2).

**Table 2.** Standard length ($L_S$), dry body weight ($W_D$), vertebral-ocular ($\angle_{VO}$) and bucco-ocular ($\angle_{BO}$) angles, head height (L5-L6 distance) and interocular distance (distance L7-L8) in Senegalese sole early juveniles aged 50 days post hatching fed diets containing low (AGM diet) and high levels of arachidonic acid (ARA-H diet). Data are expressed as mean ± S.E.M. ($n = 50$). Superscript letters indicate significant differences among dietary treatments ($P < 0·05$).

| | **AGM diet** | **ARA-H diet** | |
|---|---|---|---|
| | **NP fish** | **NP fish** | **PA fish** |
| $L_S$ (mm) | 9·8 ± 0·1 | 9·6 ± 0·2 | 9·4 ± 0·2 |
| $W_D$ (mg) | 1·92 ± 0·1 | 1·81 ± 0·3 | 1·71 ± 0·2 |
| $\angle_{VO}$ (º) | 63·9 ± 1·0$^a$ | 63·8 ± 1·1$^a$ | 48·4 ± 0·7$^b$ |
| $\angle_{BO}$ (º) | 50·4 ± 0·4$^a$ | 49·8 ± 0·1$^a$ | 45·5 ± 0·2$^b$ |
| Head height (mm) | 3·3 ± 0·0$^a$ | 3·3 ± 0·0$^a$ | 3·0 ± 0·1$^b$ |
| Interocular distance (mm) | 0·8 ± 0·0$^a$ | 0·8 ± 0·0$^a$ | 0·6 ± 0·0$^b$ |

*Abbreviations*: NP, normally pigmented specimen; PA, pseudoalbino specimen.

The principal component analysis (PCA) of the aligned coordinates for the selected 10 landmarks yielded 14 different PCs as shown in Fig 3a. The two first axes (PC1 and PC2) explained

the 50.3% and 20.1% of the total body phenotypic variability found in examined specimens, which accounted for 70.3% of the total variance. In contrast, the third axis (PC3) could only account for 9.2% of the total variance and consequently, this axis and the subsequent ones were not included in further analyses. A transformation grid for visualizing changes in body shape for PC1 and PC2 reflected several changes in both relative shifts and shape of the body in Senegalese sole (Fig. 3c, d). The PC1, which explains 50.3% of the total shape variation, mainly reflected changes affecting the height and position of the head defined by LM3 and 6 with regards to the axis of the vertebral column, as well as slight changes in the position of both eyes (LM4 and 5) and mouth opening (LM2).

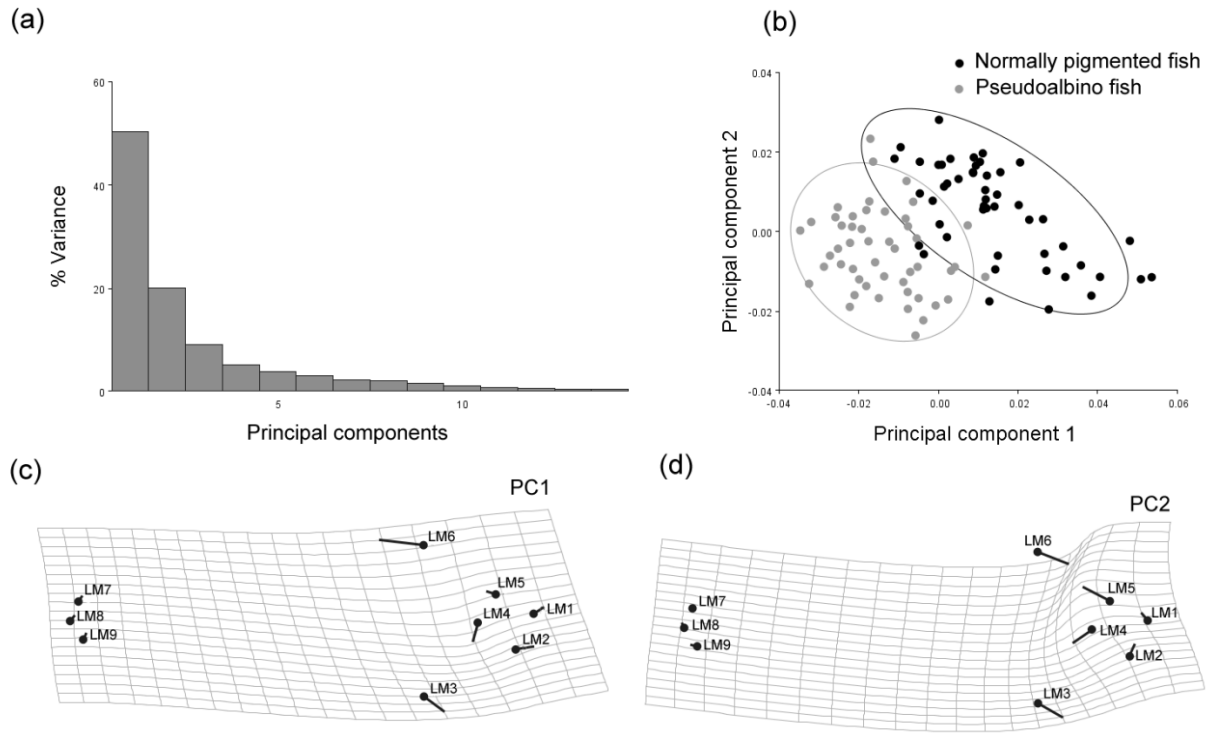


**Figure 3.** Results of the geometric morphological analysis of normally pigmented and pseudoalbino Senegalese sole early juveniles (age: 50 days post hatching) considering the entire set of 10 landmarks. (a) Percentage of variance explained by different principal components obtained by the PCA. PC1 and PC2 explained the 50.3% and 20.1% of the total body phenotypic variability, respectively. (b) Bivariate plot of the PCA scores obtained from normally pigmented and pseudoalbino specimens on the morphospace depicted by PC1 and PC2; 90% ellipses are shown for both fish groups. (c) Thin-plate spline deformation grid for PC1 of fish body shape. (d). Thin-plate spline deformation grid for PC2 of fish body shape.

Additionally, PC2 the thin-plate spline showed important changes in the relative position of both eyes and head height. Minor and not relevant changes were found in the caudal region of examined fish. Considering the distribution of both groups of specimens (normally pigmented and

pseudoalbino fish) shown in the bivariate plot (PC1 versus PC2) of Figure 3b, the morphospace occupied by pseudoalbino fish was smaller compared to the morphospace of normally pigmented fish, which indicated that the diversity of different head shapes and eye positions was larger in fish showing no pigmentary or skeletal developmental defects. In addition, the ellipses shown in Figs. 3b, 4b (confidence interval of 95%) revealed a quite clear separation of pseudoalbino fish from normally pigmented ones, with just minor cases of overlapping between both groups.

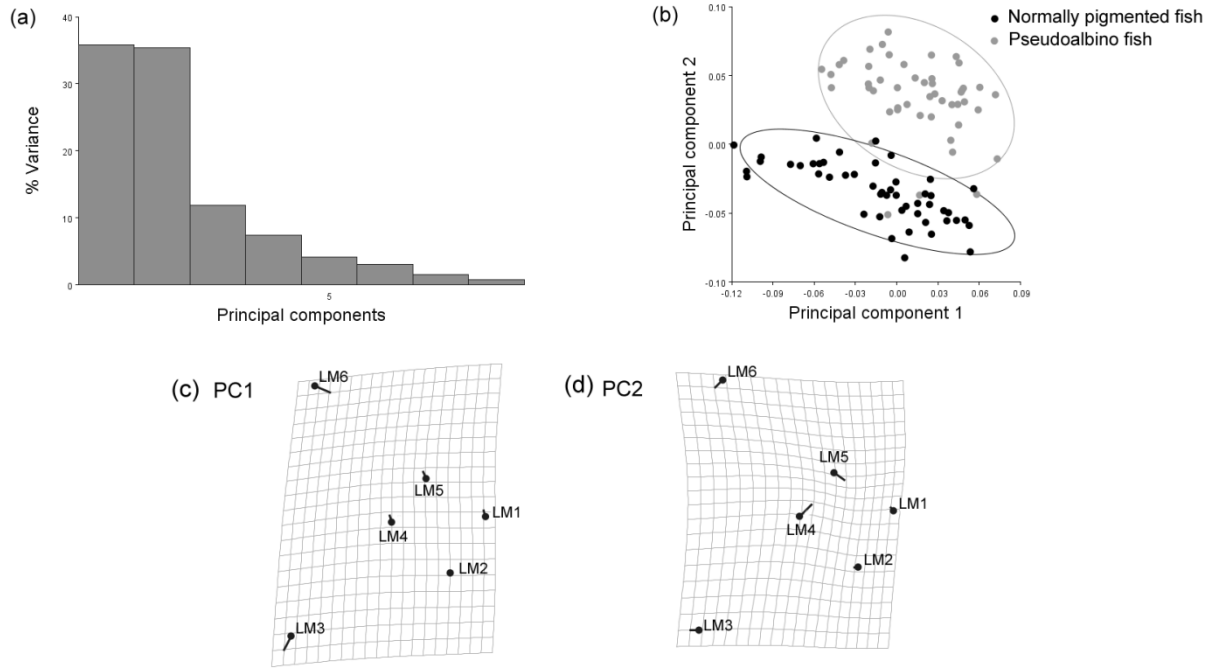


**Figure 4**. Results of the geometric morphological analysis of normally pigmented and pseudoalbino Senegalese sole early juveniles (age: 50 days post hatching) just considering those landmarks affecting the cephalic region (LM1-6). (a) Percentage of variance explained by different principal components obtained by the PCA. PC1 and PC2 explained the 35.8 and 35.4%, of the total head shape phenotypic variability, respectively. (b) Bivariate plot of the PCA scores obtained from normally pigmented and pseudoalbino specimens on the morphospace depicted by PC1 and PC2; 90% ellipses are shown for both fish groups. (c) Thin-plate spline deformation grid for PC1 of fish head shape. (d). Thin-plate spline deformation grid for PC2 of fish head shape.

Considering that most of the changes in shape were found in the cranial region, a further geometric morphological analysis was conducted considering the landmarks from this region (LM1-6). The analysis of the above-mentioned aligned coordinate landmarks yielded only 8 PCs (Fig. 4a). In this case, the first two axes (PC1 and PC2) obtained from the PCA accounted for 70.1% of the total phenotypic head shape variability (35.8 and 35.4%, respectively). The third axis only explained 11.8% of the total variance and it was also excluded from the analyses, as well as the rest of PC axes. The transformation grid for visualizing changes in head shape for PC1 and PC2 showed important

changes in head shape and eye position in fish (Fig. 4c, d). Changes in head shape represented by PC1 were mainly attributed to changes in head height (LM3 and 6), whereas shape variations indicated by PC2 were due to changes in the position of both eyes (LM4 and 5). Regarding both eyes, the right eye (LM4) showed the highest variability in its position as indicated by the larger vector size of this LM. The bivariate plot representing the PCA variance values for PC1 and PC2 showed a better discrimination between normally pigmented and pseudoalbino fish (Fig. 4b), although some minor overlaps between both groups were observed.

*Dietary ARA effcts on skeletal deformities and cranial bones*

Dietary treatments did not significantly affect the incidence of total skeletal deformities in early juveniles at 50 dph (Table 3, $P > 0.05$) with an average value of 84.2 ± 2.4% when the two dietary treatments were considered. The frequency of fish presenting cranial deformities was significantly higher (*t*-test, $t = 38.67$, *d.f.* = 6, $P < 0.001$; $n = 8$) in larvae fed ARA-H (95.1 ± 1.5%) than those fed the control (AGM) diet (1.9 ± 1.9%).

**Table 3.** Incidence of different types of skeletal deformities and anomalies affecting Senegalese sole early juveniles aged 50 days post hatching (dph) fed diets containing low (AGM diet) and high levels of arachidonic acid (ARA-H diet). Data are expressed as mean ± S.E.M. ($n = 4$). Superscript letters indicate significant differences among dietary treatments ($P < 0.05$).

| | Early juveniles (50 dph) | |
|---|---|---|
| | **AGM diet** | **ARA-H diet** |
| **Cranial deformities** | **1·9 ± 1·9$^{b}$** | **95·1 ± 1·5$^{a}$** |
| **Vertebral deformities** | **68·0 ± 4·6** | **68·4 ± 3·5** |
| *Vertebral column deformities* | 2·8 ± 1·6 | 0·6 ± 0·6 |
| *Vertebral body deformities* | 67·4 ± 4·3 | 68·4 ± 3·5 |
| Compression | 9·2 ± 1·6 | 7·3 ± 1·0 |
| Fusion V43-44 | 13·6 ± 2·7 | 15·7 ± 3·4 |
| Fusion other vertebra | 4·0 ± 1·9 | 1·3 ± 0·7 |
| Fusion haemal arches | 3·9 ± 0·9 | 6·0 ± 2·9 |
| Fusion neural arches | 36·3 ± 4·9$^{a}$ | 22·4 ± 0·9$^{b}$ |
| Deformed haemal spines | 8·5 ± 2·1 | 5·0 ± 1·9 |
| Deformed neural spines | 24·2 ± 4·4$^{a}$ | 32·3 ± 1·4$^{b}$ |
| **Caudal fin complex deformities** | **53·1 ± 4 ·5** | **53·7 ± 6·3** |
| Deformed Mns | 20·0 ± 2·9 | 17·6 ± 5·0 |
| Deformed Mhs | 41·6 ± 7·4 | 41·2 ± 5·4 |
| Deformed HYP | 6·0 ± 1·4 | 4·6 ± 2·4 |
| Deformed PHY | 3·5 ± 1·2 | 1·9 ± 1·2 |
| Deformed urostyle | 3·7 ± 1·0 | 6·0 ± 1·5 |
| Deformed EP | 3·2 ± 1·2 | 9·0 ± 3·2 |
| Fusion Mhs-PHY | 2·5 ± 1·0 | 1·1 ± 1·1 |
| Fusion EP-Mns | 1·2 ± 0·7 | 1·7 ± 1·1 |
| **Total deformities** | **83·6 ± 0·7** | **84·7 ± 4·2** |

*Abbreviations*: EP, epural; HYP, hypural; Mhs, modified haemal spine; Mns, modified neural spine; PHY, parahypural; V, vertebral body.

Cranial deformities were significantly and negatively correlated with the incidence of normally pigmented animals ($R^2$ = -0.88; $P$ < 0.001; $n$ = 8). No significant differences were detected in the percentage of fish with 43 (7·1%), 44 (40·5%), 45 (50·2%) or 46 (2·2%) vertebral bodies between both experimental groups ($P$ > 0·05). The frequency of skeletal abnormalities in the vertebral region (prehaemal and haemal regions) was not significantly different between fish fed AGM and ARA-H diets ($P$ > 0·05) with an average value of 67·9 ± 3·9% when both diets were considered (Table 3). Among the different categories of skeletal deformities affecting the vertebral column, both groups differed with regards to the incidence of deformed neural spines in haemal vertebrae (*t*-test, $t$ = 5,580 *d.f.* = 6, $P$ = 0·005; $n$ = 8) and fused neural arches (*t*-test, $t$ = 3,51, *d.f.* = 6, $P$ = 0·032; $n$ = 8), which were higher in fish fed the ARA-H diet.

When considering the different typologies of skeletal deformities affecting the caudal fin complex, no statistically significant differences were observed between both experimental groups ($P$ > 0·05; Table 3). The mean value of total deformities in the caudal fin when considering fish from both experimental groups was 53·4 ± 5·4%. The modified haemal and neural spines were the structures of the caudal fin complex most affected by skeletal malformations. The mean frequencies of deformed modified haemal and neural spines computed from both experimental groups were between 41·4 ± 6·4% and 18·8± 4·0%, respectively, but this result was not due to the diet ($P$ > 0·05).

The organization of the neurocranium and splanchnocranium in Senegalese sole normal early juveniles was similar to that of common sole *S. solea* (Linnaeus, 1758) already described in detail by Wagemans and Vandewalle (1999, 2001). The description of the organization of different skeletal elements forming the skull in Senegalese sole is not performed in this work, since this was not the objective of this study. Skull bones in the ocular and blind sides of a pseudoalbino fish with impaired eye migration are shown in Figures 2 and 5. The main osteological differences between normally pigmented (control diet) and pseudoalbino (ARA-H diet) fish were found in the splanchnocranium and affected the entire population of ARA-induced pseudoalbino fish. One of the most relevant differences between both groups of fish was the presence of dentition in both buccal jaws of each side in the pseudoalbino fish, whereas teeth were absent in the premaxillary and dentary bones of the ocular side in normally pigmented specimens. In addition, the right premaxillary (PMAX) in pseudoalbino fish was more curved and thicker than that of the normally pigmented fish, and the dentary (DENT) and angular (AN) bones in the right side were *ca.* 30-40 40% shorter in comparison to normally pigmented specimens. The shortening of the DENT and AN bones forming the lower right jaw affected the ceratohyal and disposition of the branchiostegal rays (BR. R). In pseudoalbino fish, the right lacrimal (LA) bone was deformed and underdeveloped in comparison to normally pigmented fish.

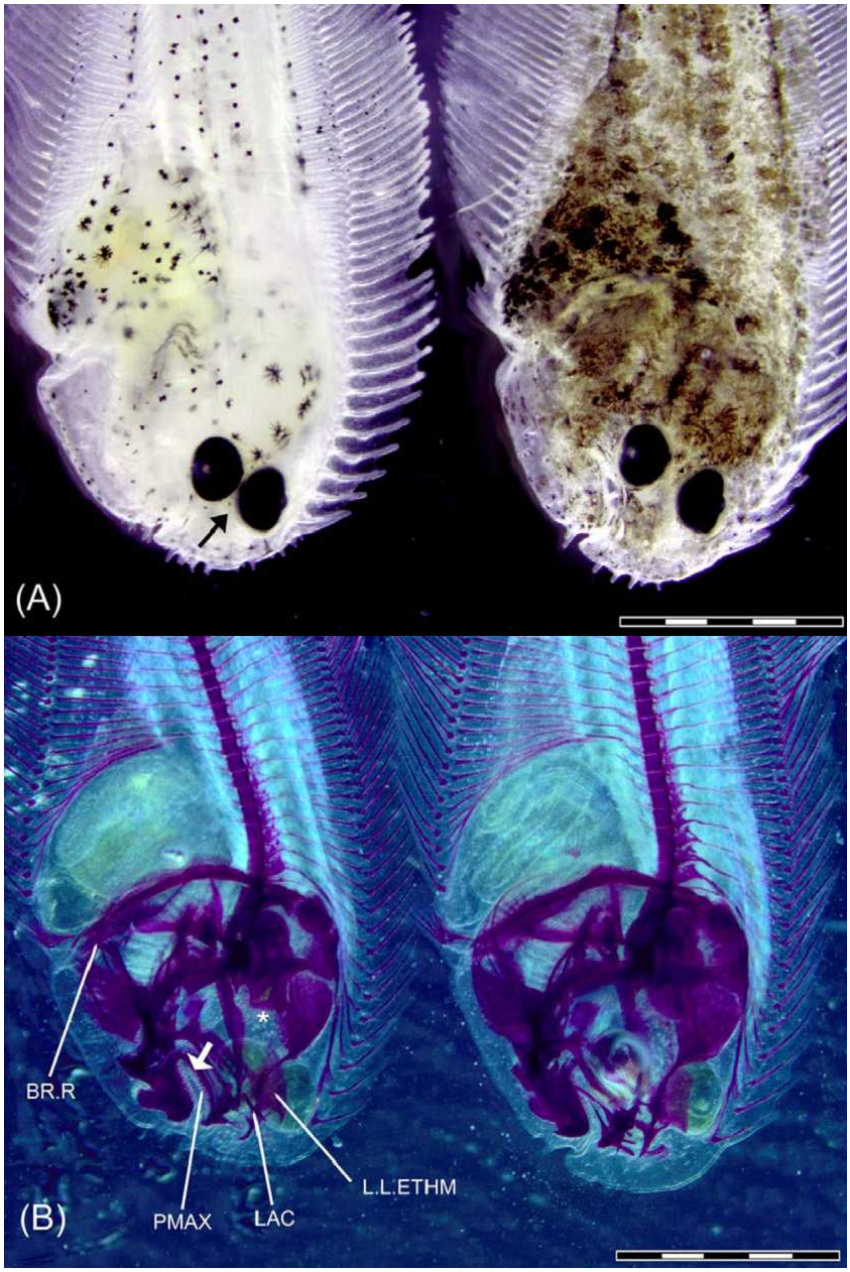


**Figure 5.** Anterior ocular side of Senegalese sole early juveniles aged 50 days post hatching. (a) General view of a normally pigmented (right) and pseudoalbino (left) fish showing different pigmentary patterns and changes in head shape and in the interorbital distance (black arrow). (b) Cranial skeletal structures (Alizarin Red staining) in normally pigmented (right) and pseudoalbino (left) sole showing the most evident osteological differences between these fish. The asterisk denotes the underdevelopment of the sphenotic (SPOT) in pseudoalbino fish with regards a normally pigmented specimen. Note the presence of teeth in right upper and lower jaws (white arrow) in the pseudoalbino specimen, as well as some skeletal disorders in order elements of the splanchnocranium and neurocranium (see text for details). BR. R, branchiostegal rays; LAC, lacrimal; L.L. ETHM, left lateral ethmoid; PMAX, premaxillary. The asterisks denotes the underdevelopment of the sphenotic (SPOT). Scale bar represents 2 mm.

The neurocranium in early juveniles was also affected in pseudoalbino fish. In this sense, the anterior margin of the sphenotic (SPOT) bone in pseudoalbino fish was less mineralized and foreshortened (*ca.* 15-20% shorter) in contrast to that in normally pigmented fish, whereas the left lateral ethmoid (L.L. ETHM) was not completely fused with the left frontal (F) to form the wall of the migrating eye's orbit and it did not have a quadrangular shape as in normal specimens.

**Discussion**

Until now, all the studies describing anomalies in the eye migration process in flatfish species were focused on the description of the impaired migration, of the left or right eye of the fish depending on the symmetry of the species, from the blind to the ocular side of the metamorphic larva, as well as the bone remodeling processes and skeletal disorders in the skull resulting from eye migration (Okada *et al.*, 2001; Okada *et al.*, 2003; Sasaki and Yamashita, 2003; Sæle *et al.*, 2003, 2006; Schreiber, 2006; Cloutier *et al.*, 2011). However, this is the first study among flatfishes that describes the impaired metamorphic relocation of the ocular side eye, the right eye in the case of Senegalese sole, whereas the left eye migrated into the ocular side almost normally.

The postembryonic development of the neurocranium and splanchnocranium observed from hatching to the juvenile stage appears to follow a similar chronological order within different flatfish species (Pleuronectiformes) and this sequence in bone formation is considered to be a response to functional demands (Sæle *et al.*, 2006; Scheriber, 2006). In brief, the only cranial element ossified prior to eye migration is the parasphenoid. After the beginning of eye migration, the ossification of the frontal bones takes place from anterior to posterior, forming a ventral wall to the migrating eye. When the eye reaches its new position, ossification of other cranial elements starts. Cranial asymmetry is mainly established by the relocation of the anterior part of the frontal bone from the blind to the ocular side, and by the enlargement of the lateral ethmoid on the blind side (see reviews in Brewster, 1987; Okada *et al.*, 2001; Wagemans and Vandewalle, 2001; Sæle *et al.*, 2006; Cloutier *et al.*, 2011). Although the eye migration process was initially associated to cranial remodelling (*e.g.* Brewster, 1987; Wagemans *et al.*, 1998; Okada *et al.*, 2001), some studies suggested that the asymmetric skull development alone was insufficient for explaining eye migration (Sæle *et al.*, 2006; Schreiber, 2006). In this context, a recent study from Bao *et al.* (2011) has shown that this process is caused by cell proliferation in the suborbital tissue of the blind side, whereas the twist of frontal bone occurring during metamorphosis is dependent on eye migration. Under the present experimental conditions, ARA-induced pseudoalbino fish differed from the normally pigmented ones by the disposition of the eyes with regards to the vertebral column ($\angle_{VO}$) and mouth ($\angle_{BO}$) axes, and by the interocular distance and head height, which were shorter in fish displaying pigmentary disorders. These results were confirmed by the geometric morphometric analysis of fish body shape in both groups of animals (Figs. 2 and 3). An unbalanced fatty acid composition in the diet, such as high levels of ARA associated with low levels of EPA, have been reported to result in an unbalanced ARA/EPA ratio and changes in the

relative proportions of PGE2 and 3 series (Bell *et al.*, 2003). In the present study, an increase in the dietary ARA levels resulted in an increase in PGE2 levels, confirming previous results from Villalta *et al.* (2007), which in turn resulted in pigmentary and eye migration disorders in Senegalese sole early juveniles. In mammals, high levels of PGE2 disrupted many fibroblast functions, affecting their differentiation and motility (White *et al.*, 2005; Sandulache *et al.*, 2006). Thus, considering the hypothesis of Bao *et al.* (2011) in which the migration of fibroblast cells would be responsible for the proper eye migration in flatfish larvae, the impaired migration of both eyes in Senegalese sole under the present experimental conditions might be due to the increased production of PGE2 or any other ARA-derived metabolite that was also responsible for pigmentary disorders found in juveniles.

In addition to changes in the positioning of both eyes, pseudoalbino specimens showed some ARA-induced osteological differences with regards to some skeletal elements from the splanchnocranium (dentary, ceratohyal, angular, right lacrimal and branchiostegal rays) and neurocranium (sphenotic and left lateral ethmoid) in comparison to normally pigmented specimens. Fernández and Gisbert (2010) recently reported that the ossification process from which different skeletal structures are derived, were differentially affected by the diet (*i.e.* vitamin A). In this sense, those bones directly originated from connective tissue with a preliminary cartilage stage were more sensitive to a nutritional imbalance than those formed by intramembranous ossification. However, present results indicated that those cranial bones displaying anomalies that were derived from endochondral (ceratohyal and sphenotic) or intramembraneous (premaxillary, dentary, angular, lateral ethmoid and lacrimal) ossification, were similarly affected by the high dietary ARA, and PGE2 body levels. Another relevant difference between fish displaying pigmentary disorders and normal ones was the presence of teeth in both lower and upper jaws in pseudoalbino larvae. In common sole, there is a progressive disappearance of teeth on the lower jaw of the ocular side just before the onset of the process of eye migration. The disappearance of teeth is linked to the morphological and functional asymmetries of Soleids, since the right jaws do not participate directly in prey capture, thus rendering teeth unnecessary (Wagemans and Vandewalle, 2001). Present results indicated that high dietary levels of ARA during the larval development might have affected the remodeling of the splanchnocranium, the premaxillary and dentary bones in particular, during the process of larval metamorphosis, resulting in juveniles with teeth in both jaws. The former results are different to those recently reported by Boglino *et al.* (2012b), since the former authors did not find any skeletal deformity affecting the cranial region in Senegalese sole fed high dietary ARA levels. In addition, the incidence of skeletal deformities affecting the vertebral column was different between both studies, since the frequency of vertebral deformities in the present study was between 25 and 32% higher than those reported by Boglino *et al.* (2012b). These different results between both studies might be due to differences in experimental feeding protocols and dietary ARA levels. In the study from Boglino *et al.* (2012b), Senegalese sole were only fed high dietary levels of ARA during the *Artemia* feeding period (8-50 dph), whereas larvae were fed enriched rotifers (control diet) from the onset of exogenous feeding to 10 dph. In contrast, high levels of ARA were maintained during the whole larval rearing

period in the present study (2-50 dph), as both rotifers and *Artemia* metanauplii were enriched with an experimental emulsion containing high doses of ARA. This suggests a probable impact of high dietary ARA supply during the pre-metamorphic period on the correct vertebral column development and skull bone remodeling occurring during metamorphosis. In addition, ARA levels from both studies also slightly differed. For instance, ARA levels in enriched *Artemia* metanauplii from Boglino's *et al.* (2012b) study ranged between 1·0 and 7·0%, whereas in the present study dietary ARA levels were 10·2% in enriched rotifers and 7·1% in enriched *Artemia* metanauplii. However, ARA/EPA ratios between both studies fell within the same range of values. Thus, it seems plausible that the longer and higher exposure to dietary ARA levels of Senegalese sole larvae in this study may be responsible for the development of the above-mentioned skeletal disorders in early juveniles. Diets containing elevated ARA levels can cause an overproduction of PGE2 in bone which finally may lead to a disruption of the rate of bone formation and its mineralization level (Berge *et al.*, 2009), since PGE2 is regarded as a potent regulating agent for bone modeling and remodeling as having both anabolic and catabolic effects on bone (Kagawuchi *et al.*, 1995; Marks and Miller, 1993). Recent *in vitro* models revealed that PGE2 decreased the proliferation of tendon stem cells and their osteogenic differentiation (Zhang and Wang, 2012). Both bone formation and bone resorption are influenced by PGE2, and its effect may be concentration dependant (Berge *et al.*, 2009). Further, high levels of PGE2 measured in ARA-induced pseudoalbino specimens might have increased the activity of cyclooxygenase-2 (COX2) in osteoblasts, resulting in a decrease of their proliferation and increase of their differentiation rates, which might have affected their mineralization level (*i.e.* sphenotic, lateral left ethmoid and left frontal) and also resulted in the alteration of some skeletal elements during the sensitive period of cranial remodeling occuring during metamorphosis, as previously reported in developing mice (Shim *et al.*, 2010). In addition, several studies have shown that COX-2 expression renders cells resistant to apoptosis. The down-regulation of apoptosis by COX-2 is attributed to two possible mechanisms: it could be mediated by generation of prostaglandin products or mediated by the removal of the substrate, ARA, via COX-2 catalytic activity (see review in Cao and Prescott, 2002). Thus, high levels of ARA might have increased the levels of COX-2 in Senegalese sole, affecting the normal process of jaw remodeling and the disruption of the apoptotic process responsible for teeth disappearance in right upper and lower jaws. In this context, further gene expression studies may be of need for confirming the former hypothesis and characterizing the molecular pathway responsible for such morphogenetic process in this species.

**Conclusion**

In conclusion, high dietary ARA levels during larval development of Senegalese sole not only affected the pigmentation success as previously described (Villalta *et al.*, 2005b; Villalta *et al.*, 2007), but also the process of head remodeling during larval metamorphosis, which seemed to be mediated by an increase in PGE2 levels. In particular, dietary ARA induced changes in the eye migration process as well as skeletal defects of some skeletal structures from the neurocranium and splanchnocranium.

These results provide new information about the developmental effects of dietary ARA on fish larvae, information that would be of value for providing insight into fish skeletogenesis and metamorphosis (particularly eye migration), the impact of nutrients on the above-mentioned developmental processes; and aid in the prevention of abnormal phenotypes for the animals of produced commercially through an improved understanding of the nutritional basis of pseudo-albinism and specific types of deformities. Further, the specific induced alteration of normal bone remodeling could prove to be a useful tool for gaining insight into the molecular pathways and specific genetic mechanisms involved and therefore warrants further investigation.

**Acknowledgements**

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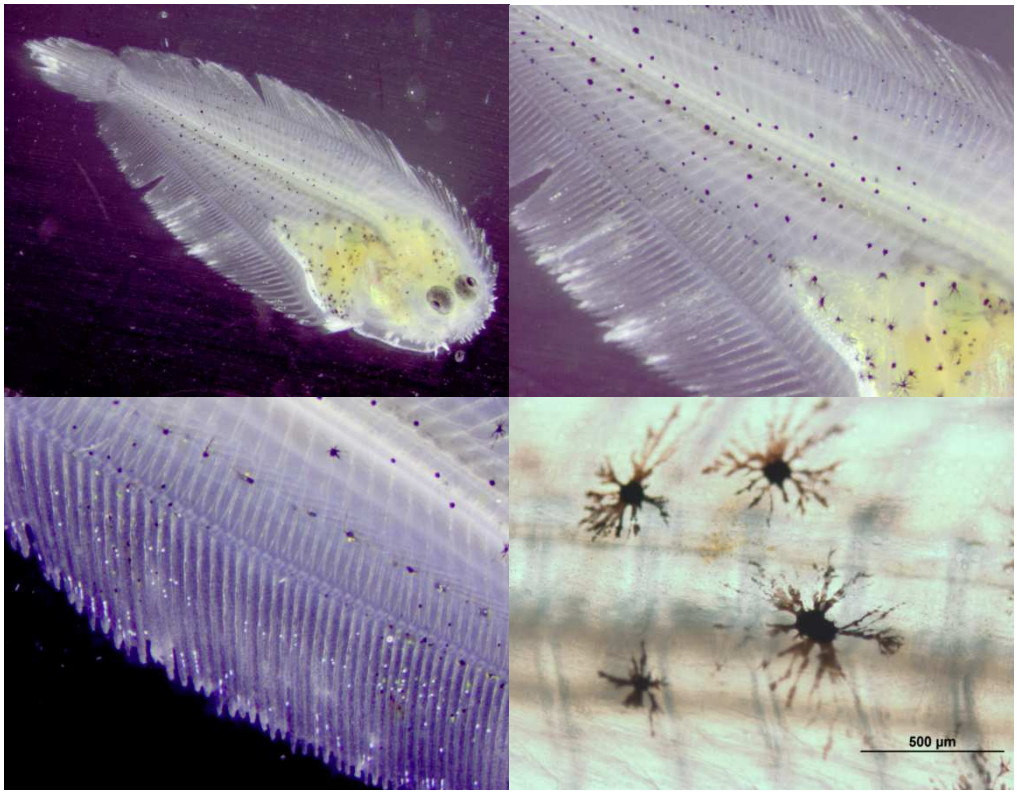
# SECTION 2




# Effects of arachidonic acid (20:4n-6, ARA) on the pigmentation in Senegalese sole

# 1

# Coordinated regulation of chromatophore differentiation and melanogenesis during the ontogeny of skin pigmentation of *Solea senegalensis* (Kaup, 1858)

Maria J. Darias[1,2]
Karl B. Andree[1],
Anaïs Boglino[1]
Ignacio Fernández[3]
Alicia Estévez[1]
Enric Gisbert[1]

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[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

[3] CCMAR, Universidade do Algarve, Campus Gambelas 8000-810, Faro, Portugal

**Regulación coordinada de la diferenciación de los cromatóforos y de la melanogénesis durante la ontogenia de la pigmentación de la piel del *Solea senegalensis* (Kaup, 1858)**

**Resumen**

La pigmentación anormal del lenguado Senegalés ha sido descrita como un problema que es resultado de la explotación intensiva durante su producción comercial. Para mejorar el entendimiento de la pigmentación de esta especie comercialmente importante, evaluamos once genes involucrados en dos procesos diferentes de pigmentación: la diferenciación de los melanóforos y la producción de melanina. La distribución temporal de los picos de expresión génica corresponde a los cambios en los patrones de pigmentación y a la intensidad de la melanization de la piel. Varios ratios de genes fueron también examinados para poner en perspectiva los posibles marcadores genéticos que intervienen en los diferentes estadios de desarrollo de una pigmentación normal. Además, los cambios fenotípicos que ocurren durante la morfogénesis corresponden con las principales transiciones en la expresión génica. Las diversas alteraciones fenotípicas a las cuales los peces están sometidos, incluyendo la coloración asimétrica entre los lados ocular y ciego, y la sincronía de los dos procesos de morfogénesis y de ontogenia de la pigmentación, posicionan a esta especie como un modelo interesante para el estudio de la pigmentación. En este estudio presentamos una primera aproximación para explicar los mecanismos genéticos que regulan la ontogenia de la pigmentación en el lenguado Senegalés, Solea senegalensis.

**Palabras claves:** expresión génica, pigmentación, células pigmentarias, cromatóforo, melanóforo, xantoforo, iridoforo, desarrollo larvario, ontogenia, piel, peces planos, metamorfosis.

**Abstract**

Abnormal pigmentation of Senegalese sole has been described as one problem facing the full exploitation of its commercial production. To improve our understanding of flatfish pigmentation of this commercially important species we have evaluated eleven genes related to two different processes of pigmentation: melanophore differentiation, and melanin production. The temporal distribution of gene expression peaks corresponds well with changes in pigmentation patterns and the intensity of skin melanization. Several gene ratios were also examined to put in perspective possible genetic markers for the different stages of normal pigmentation development. Further, the phenotypic changes that occur during morphogenesis correspond well with the main transitions in gene expression that occur. Given the dramatic phenotypic alterations which flatfish undergo, including the asymmetric coloration that occurs between the ocular and the blind side, and the synchrony of the two processes of morphogenesis and pigmentation ontogenesis, these species constitute an interesting model for the study of pigmentation. In this study we present a first approximation towards explaining the genetic mechanisms for regulating pigmentation ontogeny in Senegalese sole, *Solea senegalensis*.

**Keywords**: gene expression, pigmentation, chromatophore, larval development, ontogeny, skin, flatfish, metamorphosis

**Introduction**

Skin pigmentation of fishes is the result of the spatial combination and changes in number of several types of chromatophores that produce a huge variety of pigment patterns contributing to sex recognition, camouflage and predator avoidance, and speciation (Randall and Randall, 1960; Couldridge and Alexander, 2002; Puebla *et al.*, 2007). These neural crest-derived pigment cells are dermal and epidermal dark (brown-black) colored melanophores (equivalent to mammal melanocytes), yellow-orange xanthophores, red erythrophores, iridescent iridophores, white leucophores and blue cyanophores (Fujii, 2000). However, little is known about how these patterns are generated (Kelsh *et al.*, 1996; Parichy and Turner, 2003). Knowledge of the molecular ontogeny and pigment cell behavior underlying skin coloring is an essential step in understanding not only the origins of naturally occurring trait variation and evolution (Gross *et al.*, 2009), but also the pigmentation disorders appearing in later stages of development (Bolker and Hill, 2000; Nakamura *et al.*, 2010). Insights into the mechanisms underlying these patterns can be gained by analyzing the expression profiles of pigmentation-related genes during the larval development of the fish.

Pigmentation of flatfish has been a subject of special interest since the 19th century because of the remarkable capacity to change skin color to mimic texture and color of the background (Sumner, 1911; Mast, 1914; Kuntz, 1915; Healey, 1999). The first works were devoted to the study of skin morphology (Cunningham and McMunn, 1893), the type of pigments of the skin and their location in tissues. The studies of Burton since 1975 until present, which were more focused on the pleuronectids, have contributed greatly to the knowledge of the chromatic biology and physiology of chromatophores associated to changes in color pattern (Burton, 2002, 2008, 2010). Another notable characteristic of flatfish is that they undergo a complex process of metamorphosis during development that comprises profound morphological and physiological changes associated with eye migration, a 90° rotation in body and asymmetrical pigmentation (Power *et al.*, 2008). After metamorphosis, the most common chromatophores on the blind side of flatfish are the iridophores, whereas on the ocular side there are melanophores and iridophores. The final color of the skin is determined by the amount and distribution of both types of chromatophores (Burton, 2010).

The process of metamorphosis in fish is mirrored in the molecular features (Power *et al.*, 2008; Darias *et al.*, 2008; Fu *et al.*, 2011; Campinho *et al.*, 2012) and changes in gene expression patterns during metamorphosis are necessary to progress from the larval to the adult phenotype. Indeed, malpigmentation in flatfish seems to be the result of a disruption of the development of pigment cells at metamorphosis (Bolker and Hill, 2000; Nakamura *et al.*, 2010). Flatfish can develop pigmentation abnormalities under intensive rearing conditions, which makes them suitable models for the study of the origin of pigmentation disorders. Several environmental factors have shown to induce pigmentation problems, especially related to imbalanced nutrition (Matsumoto and Seikai, 1992; Kanazawa, 1993; Estévez and Kanazawa, 1995; Villalta *et al.*, 2005; Hamre and Harboe, 2008). For instance, it has been shown that excessive amounts of dietary arachidonic acid during the larval development of Senegalese sole, *Solea senegalensis* (Kaup, 1858) could induce up to 90% of pseudo-albinism (Villalta *et al.*, 2005; ). To our knowledge, there is no information about the ontogeny

of pigmentation in this species, although the analysis of the temporal and spatial distribution of chromatophores is essential to elucidate the mechanism of formation of the adult pigmentation pattern (Yamada *et al.*, 2010). Moreover, little is known about the molecular mechanisms underlying pigmentation processes in flatfish, including pigmentation disorders; most of the information found being very recent (Yamada *et al.*, 2010) or coming from medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), and referring to a single or few genes (Inagaki *et al.*, 1994; Dorsky *et al.*, 2000; Curran *et al.*, 2010).

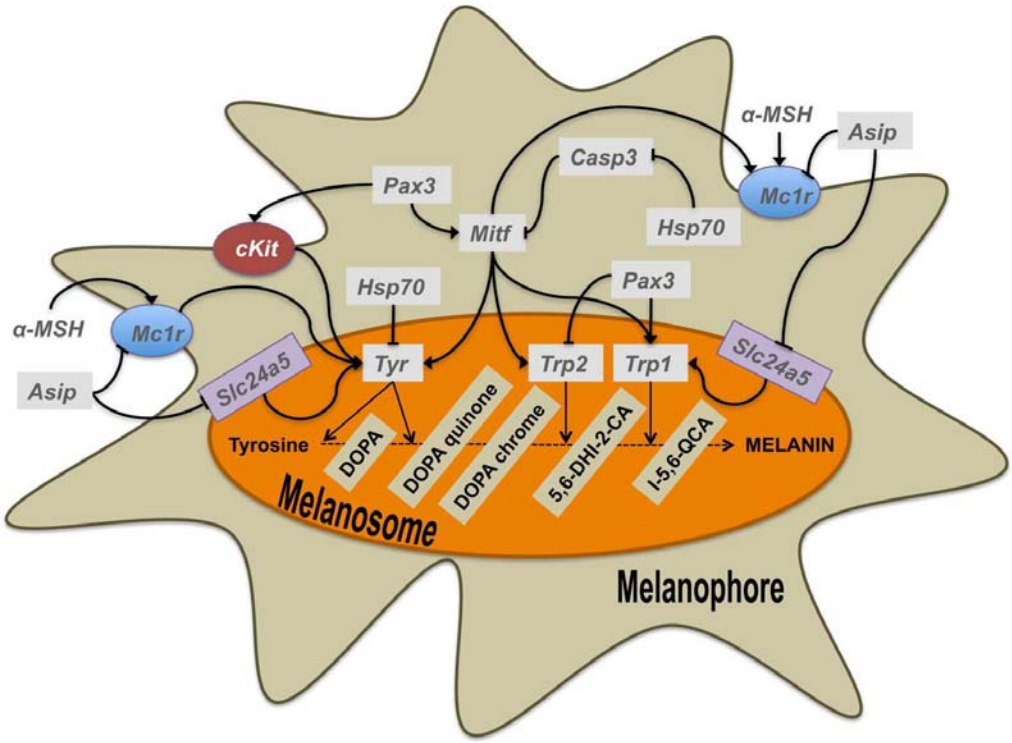


**Figure 1.** Model for the molecular action of the melanophore-differentiating and melanogenic genes within melanophores. Inside melanosomes, the master regulator of melanogenesis, *mitf*, can regulate the action of *tyr*, *trp2* and *trp1,* coding for the main enzymes responsible for the synthesis of melanin. Besides, the action of *Slc24a5,* which is the calcium melanosomal transporter, is crucial for proper melanin synthesis. *Pax3* is a key upstream transcription factor in the cascade that can promote or inhibit melanogenesis through transcriptional regulation *mitf* and *cKit*, the latter being necessary for melanophore differentiation and responsible for the activation of *tyr*. *Pax3* can also modulate the expression of the two other melanogenic enzymes *trp1* and *trp2*. *Mc1r*, located to the melanophore membrane, is activated by *α-MSH* and promotes the activation of *tyr*. *Asip* can inhibit the action of *mc1r* and *slc24a5*. *Hsp70* has been shown to be a negative regulator of *casp3* and the latter a negative regulator of *mitf*. *α-MSH, α*-melanocyte-stimulating hormone; *asip,* agouti signaling protein; *casp3*, caspase 3; *cKit*, mast/stem cell growth factor receptor Kit; *dtc/trp2*, L-dopachrome tautomerase; *mc1r*, melanocyte-stimulating hormone 1 receptor; *mitf*, microphtalmia-associated transcription factor; *pax3*, paired box protein Pax-3; *sl*, somatolactin; slc24a5, sodium/potassium/calcium exchanger; *tyr*, tyrosinase; *trp1*, tyrosinase-related protein 1.

The aim of this work was to 1) describe the morphological development of skin pigmentation and 2) analyze the expression profile of eleven genes involved in melanophore differentiation and melanin synthesis during larval development of Senegalese sole (*Solea senegalensis*).

These genes and their protein products are: I) The melanocyte-stimulating hormone 1 receptor (*mc1r*), which is the "classical" receptor of the *α*-melanocyte-stimulating hormone (*α-MSH*) (Schiöth *et al.*, 1995) and has a key role in determining the pigmentation of skin and hair in mammals (Burchill *et al.*, 1993; Gantz and Fong, 2003). In fish, *mc1r* is involved in skin color changes (Fujii, 2000) and its role in the pigmentation pattern during development has been recently reported (Gross *et al.*, 2009). II) The agouti signaling protein (*asip*), which in mammals regulates the relative proportions of eumelanin (black-brown pigment) and phaeomelanin (yellow-red pigment) by antagonizing the action of *α*-MSH on its receptor MC1R (Hunt *et al.*, 1995) In fish, it has been shown that *asip* is abundantly expressed in the ventral skin, but scarcely in dorsal skin (Cerdá-Reverter *et al.*, 2005), demonstrating the involvement of *α-MSH* and *asip* in the assignment of a dorsal–ventral pigment pattern in fish. III) The paired box protein 3 (*pax3*), a key transcription factor for influencing the development of the neural crest and neural crest-derivatives during mammalian embryogenesis, and that influences melanocytic proliferation, resistance to apoptosis, migration, lineage specificity and differentiation (Kubic *et al.*, 2008). *Pax3* both promotes and inhibits melanogenesis within these cells through transcriptional regulation of microphtalmia-associated transcription factor (*mitf*), L-dopachrome tautomerase (*dct/trp2*), tyrosinase-related protein 1 (*trp1*) (Bertolotto *et al.*, 1998) and the mast/stem cell growth factor receptor Kit*, cKit* (Guo *et al.*, 2010). In zebrafish, *pax3* is required for fate specification of xanthophores and for melanophore development (Minchin and Hughes, 2008). IV) The protein product of *mitf,* one the earliest genes expressed in melanoblast precursors (Lister *et al.*, 1999), which has an essential role in the differentiation and proliferation of melanocytes/melanophores. It is considered the master regulator of melanogenesis due to its ability to activate many melanocyte-specific genes, such as tyrosinase (*tyr*), *trp1* and *dct* (Curran *et al.*, 2010; Levy and Fisher, 2011). V) The protein product of *cKit,* which plays a critical role in melanocyte physiology by influencing melanogenesis, proliferation, migration, and survival of these cells (Alexeev and Yoon, 2006). In zebrafish, *cKit* promotes the processes of larval melanophore migration and survival (Rawls and Johnson, 2000). Activation of *cKit* induces *tyr* gene transcription and melanin synthesis in differentiated cultured melanocytes (Luo *et al.*, 1995). VI) The *tyr* gene, which codes for tyrosinase, the first enzyme of the biosynthetic pathway of melanin that oxidizes the amino acid L-tyrosine to dopaquinone (Ito and Wakamatsu, 2008). VII) The *trp1* gene*,* coding for the last enzyme of melanogenesis in mice, which catalyzes the oxidation of indolic intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to produce eumelanin (Kobayashi *et al.*, 1994). In humans, together with TRP1, TYR can also act at the last step of the melanin synthesis (Olivares *et al.*, 2001). The function of *trp1* in fish is scarcely known, although it seems that its presence is necessary for the formation of melanin (Braasch *et al.*, 2009; Darias *et al.*, unpublished data). *Trp1* has been cloned in several fish species (Camacho-Hubner *et al.*, 2002) but its expression has not been studied during larval development. VIII) The sodium/potassium/calcium exchanger 5

(*slc24a5*), a putative cation exchanger localized to intracellular membranes of melanosomes and their precursors. Its mRNA was first isolated in zebrafish and it is believed to increase uptake and accumulation of calcium in melanophores, necessary for melanogenesis (Lamason *et al.*, 2005). It has been recently demonstrated that *slc24a5* is important for normal melanization processes in all pigmented cells of mice (Vogel *et al.*, 2008). Moreover, it has been suggested to be involved in the development of ocular albinism and macular degeneration in humans and pseudo-albinism in flatfish (Vogel *et al.*, 2008; Darias *et al.*, unpublished data). IX) The enzyme caspase 3 (*casp3*), involved in the activation cascade of caspases responsible for execution of apoptosis (Yamashita *et al.*, 2008). In humans, *casp3* is able to cleave *mitf*, thus conferring proapoptotic functions to this gene to modulate death in melanocytes and melanoma cells (Larribere *et al.*, 2005). No reports have been found regarding the natural influence of this gene in the physiology of melanophores in fish. X) The heat shock 70 kDa protein (*hsp70*), a stress protein that confers cell protection against stressors that is able to suppress melanin production in a mouse melanoma cell line (B16) and in the epidermis of mice through the down-regulation of *tyr* (Yamashita *et al.*, 2010; Hoshino *et al.*, 2010). XII) The somatolactin gene (*sl*), a fish-specific peptide hormone secreted from the pituitary gland (Rand-Weaver *et al.*, 1992) and, regarding pigmentation, is involved in adaptation to background in red drum (*Sciaenops ocellatus*) (Zhu and Thomas, 1996, 1997) and in body color regulation in medaka (Fukamachi *et al.*, 2009).

The present study analyses, for the first time in fish, the expression profile of the above mentioned key genes involved in melanophore differentiation and melanogenesis during the larval development in an integrative physiology approach. Figure 1 shows a schematic design for a better visualization of the action of those genes within melanophores. Increasing the knowledge of the morphological and molecular ontogeny of skin pigmentation would help for a better understanding of this process in vertebrates and of the origin of pigmentation disorders. Indeed, fish have been used as models for melanoma research because it has been shown they share molecular signatures and histopathological features with human cancers (Patton *et al.*, 2010).

## Materials and methods

*Ethics statement*

This study was carried out in accordance with the recommendations in (Kilkenny *et al.*, 2010). Animal experimental procedures were conducted in compliance with the experimental research protocol (reference number 4978-T9900002) approved by the Committee of Ethic and Animal Experimentation of the IRTA and the Departament de Medi Ambient i Habitatge (DMAH, Generalitat de Catalunya, Spain) in accordance with EU regulation (EC Directive 86/609/EEC).

*Animal rearing and sampling procedures*

Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, La Coruña, Spain), acclimated at IRTA-SCR facilities and reared at 17.0 ± 2.8 °C and 35 of salinity in four 500 l cylindrical tanks (initial density: 50 larvae.l$^{-1}$) connected to an IRTAmar$^{TM}$ recirculation unit. Water was renewed daily (20%) with gentle aeration in each tank, pH and dissolved oxygen being 8.0 ± 0.2 and 7.5 ± 1.3 ppm, respectively. Photoperiod was 16L: 8D, and light intensity was 500 lx at the water surface. Larvae were fed twice a day, from 3 to 10 dph, with rotifers (*Brachionus plicatilis*) enriched with Easy Selco$^{TM}$ (ES, INVE, Belgium) following manufacture's recommendations, at a density of 10 rotifers ml$^{-1}$ from 3 to 6 dph and of 5 rotifers ml$^{-1}$ from 7 to 10 dph. *Artemia* metanauplii enriched with ES were supplied to larvae from 6 to 37 dph at increasing density from 0.5 to 12 metanauplii ml$^{-1}$. *Artemia* metanauplii density was adjusted four times per day (at 9, 12, 15 and 18 h) according (Cañavate *et al.*, 2006) to assure the optimal prey density. From 33 dph to the end of the experiment (47 dph), larvae were progressively weaned onto dry feed (Gemma Micro 150–300© Skretting, Spain). Eight larvae were sampled from each tank at 2, 5, 11, 16, 19, 22, 27, 33, 35, 41 and 47 dph to study the morphological development of skin pigmentation. For gene expression analyses, 200 mg wet weight larvae (80 to 3 individuals per sample time depending on fish size) were sampled at 2, 5, 11, 13, 14, 16, 19, 22, 27, 33, and 47 dph, sacrificed with an overdose of anesthetic (Tricaine methanesulfonate, MS-222, Sigma), rinsed in distilled water and preserved in RNAlater© (Ambion) at -80 °C for further analyses. Fish were sacrificed following the protocols of the veterinary committee of IRTA in accordance with EU regulations (EC Directive 86/609/EEC).

*Photography and image analysis*

Alive not anesthetized larvae were examined under stereomicroscope (Nikon SMZ800, Soft Imaging Systems, GmbH) and photographed using a Color View-XS camera at 300 dpi. Skins of larvae were photographed using a DP70 (Olympus) camera attached to DMLB (Leica) microscope. Images were taken under transmitted or incident light and compiled and processed using Analysis®3.1 (Soft Imaging Systems, GmbH). Types of pigment cells were classified by coloration and shape. ImageJ64 software was used to quantify the number of melanophores, xanthophores and iridophores. During pre- and pro-metamorphosis periods, the number of melanophores was quantified in the skin of the left side body trunk of the larvae, excluding the abdominal region. The relative amounts of xanthophores and iridophores were only quantified during the post-metamorphosis period because at earlier stages they were distributed in a dense and thick net that made difficult the proper recognition of single pigment cells. The same happened for leucophores and therefore they were not quantified. During the post-metamorphosis period, when individuals showed flat symmetry, the amount of melanophores, xanthophores and iridophores was quantified in the ocular side of the trunk skin, excluding the abdominal area. Results were represented as the relative proportion, expressed in percentage, of each chromatophore in the analyzed skin area.

**Table 1.** Target genes used in this study with their accession numbers, main biological processes and amplicon size, primers and hydrolysis probes used in qPCR analyses.

| Gene name | GenBank accession no. | Biological process/Activity | Amplicon size | Hydrolysis probes | 5′ to 3′ sequence |
|---|---|---|---|---|---|
| *Ubq* | **AB291588** | Ubl conjugation pathway | 86 | Forward | GCCCAGAAATATAACTGCGACAAG |
| | | | | Reverse | TGACAGCACGTGGATGCA |
| | | | | FAM probe | ACTTGCGGCATATCAT |
| *Tyr* | **JF693907** | Melanin biosynthesis | 73 | Forward | CGTACGCACAGATGGAAAACG |
| | | | | Reverse | CACGTAGTAATGCATCCACACAAAA |
| | | | | FAM probe | ACATCGGCGAATATC |
| *Trp1* | **GU329041** | Melanin biosynthesis; Melanocyte differentiation | 63 | Forward | CGTGTGCAACAATACAGAAACAAGT |
| | | | | Reverse | ATGGGTCGTGCCACGTT |
| | | | | FAM probe | CCTGCCGGGTTCCTT |
| *Mitf* | **GU329042** | Transcription factor for tyr, trp2 and trp1 | 75 | Forward | CGATGACATCATAAGTCTTGAATCCAGTTT |
| | | | | Reverse | CGTGCTGGGCAACTGAAGA |
| | | | | FAM probe | CCGGAGTCAATCAACG |
| *cKit* | **HM100237** | Tyrosine-protein kinase signaling pathway | 69 | Forward | GTGAAGAGAGTGAGATGTTTGACGA |
| | | | | Reverse | CACTTTGGTAGGAGAAGCTCAGAA |
| | | | | FAM probe | CTCGTCACCGAAGATC |
| *Mc1r* | **GU329043** | Melanocyte-stimulating hormone receptor activity | 76 | Forward | CGCCGTCGCCATCATC |
| | | | | Reverse | GCGTTGTCCGTGTGGTAGA |
| | | | | FAM probe | ACCTCCAGCATCCTCT |
| *Scl24a5* | **GU329046** | Ion transport in melanosomes | 66 | Forward | GACGCAGCCTCTGATCGA |
| | | | | Reverse | CCGTCCTGGAGCGAACC |
| | | | | FAM probe | CCAGTCTGCGAAACAT |
| *Casp3* | **GU329040** | Apoptosis | 77 | Forward | CGACAGTGTAGATGACCAAACGT |
| | | | | Reverse | GGAGCAGTGGAATAAGCATAAAGGA |
| | | | | FAM probe | CCTCCACAGGAATCC |
| *Pax3* | **HM100238** | Transcriptional regulation of pigmentation | 68 | Forward | GCATCATGCGCTCCAAGTTC |
| | | | | Reverse | CCCTCTTCACCATTTCATCATCCT |
| | | | | FAM probe | CATCGTCACCAACTCC |
| *Sl* | **U06753** | Hormone activity | 75 | Forward | TTCCCACTGCGGCTTCA |
| | | | | Reverse | GGTAAGGCCTTGGTGATGCA |
| | | | | FAM probe | CCGACCGTGTTTCTC |
| *Asip* | **HE598753** | Regulation of melanogenesis | 81 | Forward | GCTGTGACATCTGTGCCTTCT |
| | | | | Reverse | CCATTCGACAGAAACACACAGTTC |
| | | | | FAM probe | CCAGTGTCGCCTCTTC |
| *Hsp70* | **GU329044** | DNA repair | 73 | Forward | TGGAGTCGTATGCTTTCAACATGA |
| | | | | Reverse | TGCTTGTCGTCGTCACTGAT |
| | | | | FAM probe | CTTGCCAGCCAGTTTC |

*Real Time PCR assays*

Eleven genes involved in the process of pigmentation were selected as markers for melanophore differentiation and melanin synthesis in Senegalese sole larvae (Table 1). The ontogeny of gene expression was analyzed at 2, 5, 11, 13, 14, 16, 19, 22, 27, 33 and 47 dph. Total RNA of a pool of larvae was extracted using TRIzol™ (Invitrogen) following the manufacturers recommended protocol. The quantity of isolated RNA was determined by measuring optical density at 260 nm, using a Gene-

Quant spectrophotometer (Amersham Biosciences), and its purity was evaluated by the absorbance ratio 260/280 nm. RNA samples with a 1.9-2.0 ratio were used for reverse transcription. The quality of the RNA extraction was further examined visually using sample aliquots separated electrophoretically in 1.2% agarose gels. Total RNA (1 µg) from each sample was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen®, GmbH, Germany). Real-time PCR analysis was performed using an ABI PRISM 7300 (Applied Biosystems). Amplification reactions were performed in triplicate in a total volume of 20 µl containing 1 µl of cDNA, 1 µl of Taqman probe, 10 µl of Taqman mix and 8 µl of RNAse and DNAse free water. The gene ubiquitin (*ubq*) was chosen as reference gene since it did not exhibit any significant variation in expression levels among the samples. The amplification conditions were 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Custom Taqman® assays were designed and provided by Applied Biosystems, Life Technologies (Table 1). The average efficiency of amplification for all assays was 99%. Real-time PCR efficiencies were determined for each gene from the slopes obtained with Applied Biosystems software, applying the equation $E = 10^{(-1/slope)}$, where E is PCR efficiency. To determine the relative quantity of target gene-specific transcripts present in the different samples, expression ratios were calculated according to Pfaffl's mathematical model (Pfaffl *et al.*, 2004). Individuals of 2 dph were chosen as reference sample. Gene expression patterns were visualized using supervised hierarchical clustering (Gene Cluster and Tree View, 2002) applied on samples classified according to developmental stage.

*Calculations and Statistics*

All changes to chromatophore percentages were normalized by comparison to a baseline value for iridophores, which were chosen as they were the most abundant. Changes in the percent of chromatophores and melanophores were calculated using the quantity of iridophores counted on day 35 as a baseline, giving consideration to this date of development as the time when adult pigment patterning is normally developed. Results were expressed as mean ± SD (n = 4). All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test) and transformed when necessary. One-Way ANOVA was performed to analyze differences in the relative number of chromatophores and gene expression. When significant differences were found ($P < 0.05$), the post-hoc Holm-Sidak method was used to perform all pairwise multiple comparisons. Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA). ANCOVA was used to compare the slope of *asip*/*mc1r* ratio before and after metamorphosis of Senegalese sole ($P < 0.05$).

**Results**

*Growth, survival, pigmentation and metamorphosis*

Senegalese sole grew adequately throughout the larval stage, weighing 1.92 ± 0.14 mg of dry weight and having a standard length (SL) of 8.75 ± 0.13 mm at the end of the experimental period (47 dph).

Survival rate and pigmentation success was 97.3±0.15 and 99.1±0.3%, respectively. The progress of metamorphosis based on eye migration [61] occurred within the following periods: pre-metamorphosis (until 11 dph), pro-metamorphosis (from 11 to 19 dph) and post-metamorphosis (from 19 to 47 dph).

*Morphological ontogenesis of skin pigmentation*

Morphological development of skin pigmentation in Senegalese sole is shown in Figures 2 and 3. At 2 dph (3.07 ± 0.02 mm), two lines of dendritic black melanophores (M), white leucophores and orange-yellowish xanthophores (X) overlaid the dorsal and ventral flanks of the body skin in the bilateral symmetric larva, with the exception of the future region of the caudal fin (Figure 2A). These chromatophores also covered the skin of the head and abdominal area. Four patches of dendritic leucophores and xanthophores were located in the dorsal fin and one in the anal fin (Figure 2A). The eyes were already pigmented at that time. One or several xanthophores associated to one melanophore could be seen three days later (Figures 2B, 3A). Melanophores were located in the very surface of the skin and overlie the epidermis (Figure 3A). This distribution of chromatophores remained similar until 11 dph (Figures 2C); but round-shaped xanthophores were also observable and the patches of larval chromatophores located in the fins began to disappear at 16 dph (Figure 2D). Metamorphosis was taking place and some larvae already showed a flattened body plan. At 19 dph, the linear pattern of allocation of the body skin chromatophores began to disorganize (Figure 2E). At 22 dph, larvae were flat, although the eye from the blind side had not completely migrated to the ocular side (Figure 2F). Some iridophores could already be seen in the head of some individuals (Figure 3B). The relative amount of skin melanophores remained statistically invariable during pre- and pro-metamorphosis and represented an average of 6.20 % (± 1.23) of the maximum amount of melanophores quantified during the entire studied period (Figure 4A).

**Table 2.** Number of xanthophores (X) associated to one melanophore (M) during the post-metamorphosis period. Values are expressed in means ± SD. Superscript letters denote significant differences in the number of X related to one M between developmental ages (One-way ANOVA, $P < 0.05$).

| Age (dph) | X/M | N | SD |
|---|---|---|---|
| 27 | $1.33^{b}$ | 4 | 0.65 |
| 33 | $4.75^{ab}$ | 4 | 2.43 |
| 35 | $1.81^{b}$ | 4 | 0.99 |
| 47 | $5.35^{a}$ | 4 | 2.20 |

Information about the developmental pattern of other chromatophores such as xanthophores and iridophores were only obtained during the post-metamorphosis period, since they were distributed in a dense and thick net that made difficult the proper recognition of single pigment cells at earlier stages of development. The same happened for leucophores and therefore they were not quantified.

At 27 dph, the amount of iridophores increased and they began to organize to conform to the adult distribution pattern (Figure 3C). In addition, the amount of melanophores significantly increased from 22 to 27 dph (Figure 4A). The skin of the pro-metamorphic larvae contained similar amounts of xanthophores and melanophores, which represented, at this age, around 20% of the amount of pigment cells quantified during the studied period (Figure 4B). Each melanophore was closely associated to one or two xanthophores (Table 2). Some xanthophores seemed to be disintegrating (Figure 4D). The distribution of chromatophores was restricted to two bands on either side of the vertebral column and in the distal parts of the trunk, close to the beginning of the dorsal and anal fins. Some melanophores grouped to form a patch in the middle of the trunk. Two patches of chromatophores could be distinguished in the dorsal fin and another one in the ventral fin (Figure 2G).

The migration of the left eye was completed in most larvae at 33 dph (Figure 2H). On the ocular side, three lines of melanophores and xanthophores could be found in the dorsal and ventral trunk, from both sides of the vertebral column to the end of the trunk. These lines became discontinuous when a patch of iridophores was present. Then, patches of chromatophores, composed of a mixture of melanophores, xanthophores and leucophores, alternated with patches of iridophores, could be observed in the skin of the post-metamorphic larvae (Figure 2H). There were five patches on the trunk, at the level of the vertebral column, five patches in the margin of the dorsal and ventral trunk, at the level of the proximal radials, and a higher number of patches, surrounded by iridophores, in the dorsal and ventral fins (Figures 2H, 3E). This pattern of chromatophore distribution was preserved until the end of the studied developmental period (Figure 2L). Leucophores covered most of the trunk, where there were no iridophores present. In the dorsal and anal fins, leucophores were observed in the patches and also in the border of the dorsal fin (Figures 2H, 3E). At this time of development, two to seven xanthophores were associated with one melanophore (Figure 3F-G, Table 2). Among the pigment cells quantified, xanthophores were the most abundant, followed by melanophores and finally by iridophores (Figures 3F-G, 4B). From this time onwards, the shape of xanthophores was no longer dendritic, but round (Figure 3F-H).

At 35 dph, the number of iridophores increased drastically, reaching the maximum relative percentage of ocular skin chromatophores quantified in this study (Figures 2I, 4B), while the amount of melanophores and xanthophores became statistically equal again and still represented the same 20% of the total pigment cells counted at post-metamorphosis already observed 8 days earlier (Figures 3H, 4B). The X/M ratio was around 2 (Table 2).

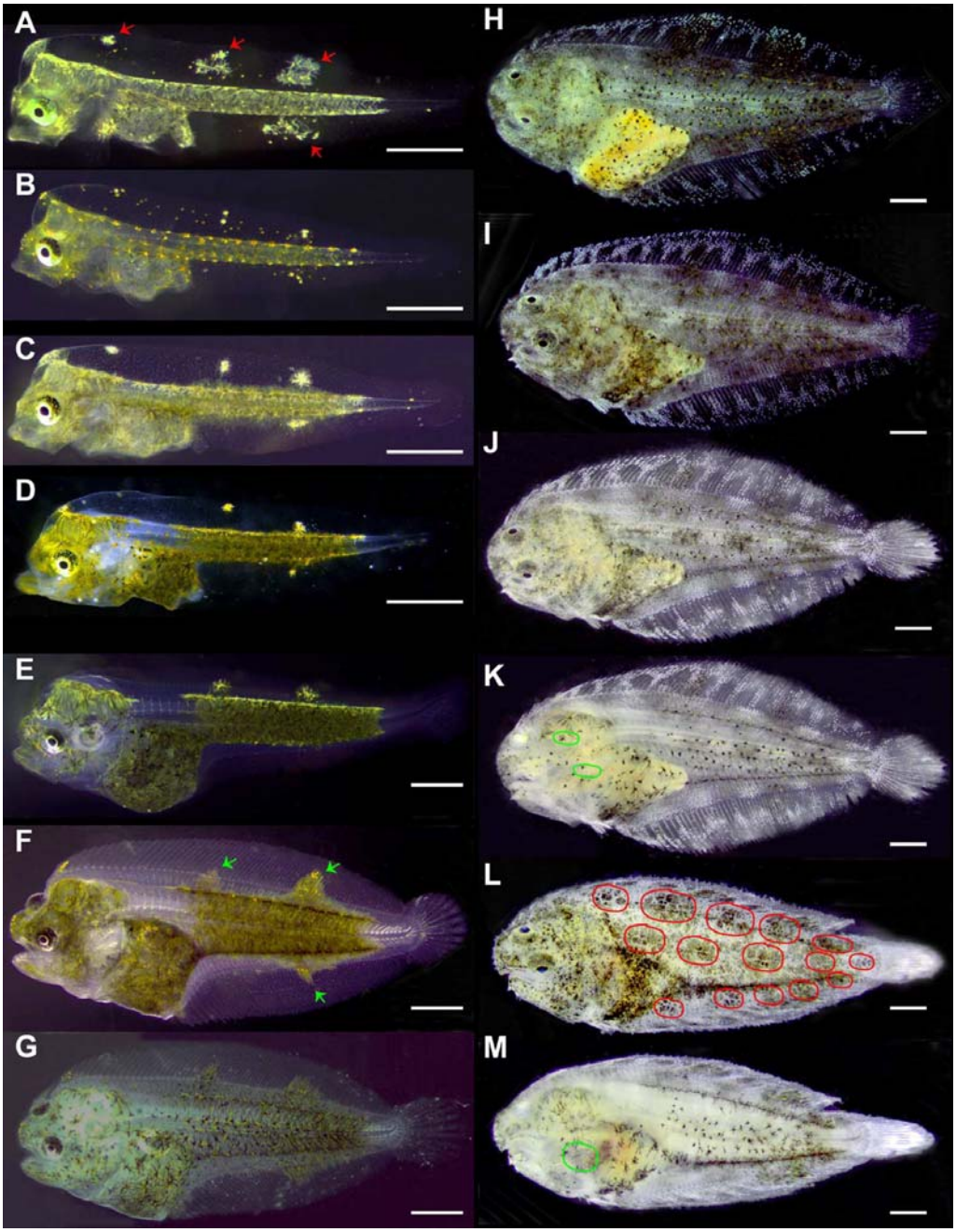


**Figure 2.** Morphological ontogeny of skin pigmentation in Senegalese sole larvae. A) 2 dph, B) 5 dph, C) 11 dph, D) 16 dph, E) 19 dph, F) 22 dph, G) 27 dph, H) 33 dph, I) 35 dph, J-K) 41 dph, L-M) 47 dph. Red arrows indicate patches of leucophores and xanthophores. Green arrows show patches of leucophores, xanthophores and melanophores. Note how the allocation of leucophores and xanthophores in dorsal and anal fins at very early stages of larval development serves as referring point for melanophore migration from the dorsal and ventral flanks of the fish to the fins. Red circles show 3 stripes of 5 patches of chromatophores conforming the juvenile pattern of skin color in the ocular side of the fish. Clusters of iridophores delimit these patches of melanophores, xanthophores and leucophores. Fins also follow the same distribution of chromatophores. Green circles delimit the area where iridophores were found in the skin of the blind side of the fish. Scale bar represent 600 µm.

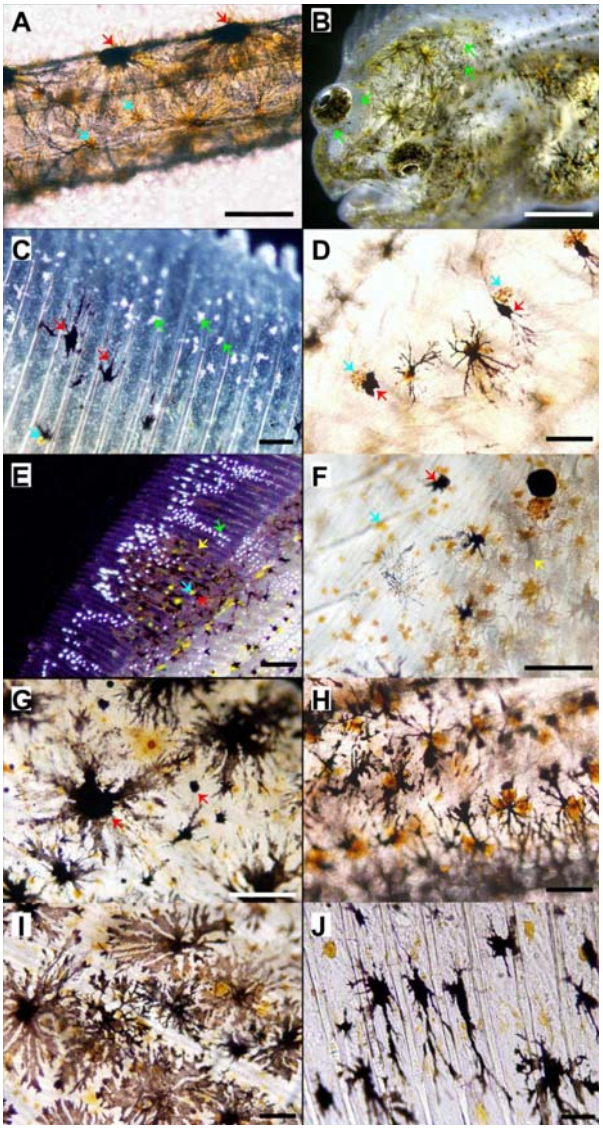


**Figure 3.** Images of the skin of Senegalese sole revealing the presence, shape, patterning and spatial relationships among melanophores, xanthophores, leucophores and iridophores. A) 5 dph, B) 22 dph, C-G) 33 dph, H) 35 dph, I-), 40 dph. A) Epidermal melanophores and xanthophores covered the dorsal and ventral flanks of the fish. B) Iridophores were already present in the skin of the ocular side of the fish at the level of the head. C) Detail of the dorsal fin showing melanophores, xanthophores and iridophores. D) Detail of the skin showing the interaction between xanthophores and melanophores. Note how communication between these cells leads to the disintegration of xanthophores. E) Detail of the distribution of chromatophores in the fins. A patch of melanophores, xanthophores and leucophores is surrounded by iridophores. F-J) Detail of the trunk skin of the ocular side showing the distribution pattern of xanthophores and melanophores. Note that the amount of xanthophores relative to melanophores decreased from 33 to 41 dph (F, J). Red arrows, melanophores; blue arrows, xanthophores; yellow arrows, leucophores; green arrows, iridophores; B, E, stereoscopic images; A, C-D, F-J, microscopic images. Scale bars represent: A, C, G, F, 200 μm; D, H, 250 μm; I, J, 100 μm; E, 500 μm; B, 1 mm.

Between day 27 and 41 (Figure 2J), the amount of melanophores in the skin of the ocular side remained invariable (Figure 4A). However, the number of xanthophores decreased significantly at 41 dph with respect to that of melanophores and it was half the amount than at earlier stages (Figures 3I-J, 4B). Between day 35 and the end of the study the amount of iridophores was higher than that of the other pigment cells (Figures 2J, 4B). The blind side of the larvae was composed of melanophores and few xanthophores, and some iridophores could be observed at the level of the head (Figure 2K).

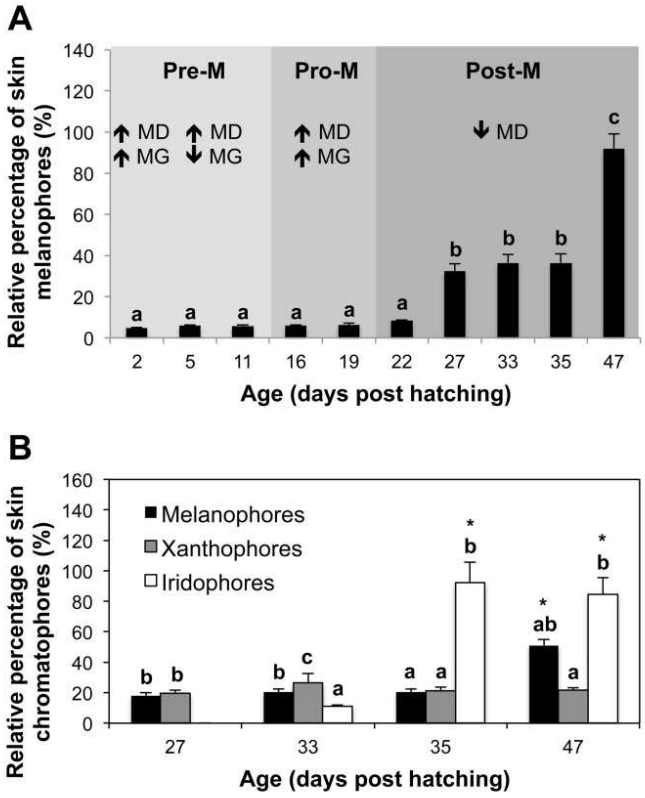


**Figure 4.** Relative amounts of chromatophores (in %) during the ontogeny of the ocular skin pigmentation in Senegalese sole. A) Relative amount of skin melanophores associated to the processes of melanophore differentiation and melanin synthesis governed by tightly controlled molecular signatures (see also Figs. 6 and 7). Values are expressed as mean ± SD (n = 4). Superscript letters denote significant differences among larvae of different age (One-way ANOVA, $P < 0.05$). Pre-M, premetamorphosis; Pro-M, pro-metamorphosis; Post-M, post-metamorphosis; MD, melanophore differentiation (overall responses of *pax3*, *mitf*, *mc1r*, *cKit)*, MG, melanogenesis (overall responses of *tyr*, *trp1*, *slc24a5*, *asip)*. B) Relative amount of skin melanophores, xanthophores and iridophores during post-metamorphosis. Values are expressed as mean ± SD (n = 4). Superscript letters denote significant differences between the relative amounts of chromatophores for a given larval age and asterisks indicate significant differences in the amount of a given chromatophore throughout the post-metamorphosis period (One-way ANOVA, $P < 0.05$).

At 47 dph, the pattern of skin pigmentation began to resemble that of adults (Figure 2L). Chromatophores were organized in well-distinguished patches that covered the whole ocular side, including the head, trunk and fins. The amount of melanophores and xanthophores was higher than at 41 dph (Figures 4A-B). There were no significant differences in the amount of melanophores and xanthophores, and the amount of iridophores was only higher than that of xanthophores (Figure 4B). On the blind side, the skin was composed of a reduced number of melanophores, although there remained a few iridophores at the level of the head (Figure 2M).

*Molecular ontogenesis of skin pigmentation*

The expression patterns of the analyzed genes are represented in Figure 5. The allocation of these genes to the melanogenesis pathway can be found in Figure 1. *Mc1r* expression decreased significantly during development from 2 to 5 dph, from 5 to 16 dph and from 16 to 22 dph. The expression levels of *mc1r* remained constant from 22 dph onwards. *Asip* expression increased from 2 to 14 dph to slightly decrease at 16 dph and remained relatively constant until 33 dph. At 47 dph, the expression of *asip* increased reaching the levels observed at 14 dph. *Pax3* displayed the highest level of gene expression at 2 dph and then decreased until 11 dph (5 fold decrease). From 13 to 16 dph, *pax3* expression was 1.5 times higher than at 11 dph and subsequently decreased until 19 dph to remain stable onwards at the same levels of expression observed at 11 dph. *cKit* and *mitf* showed similar gene expression profiles during ontogeny.

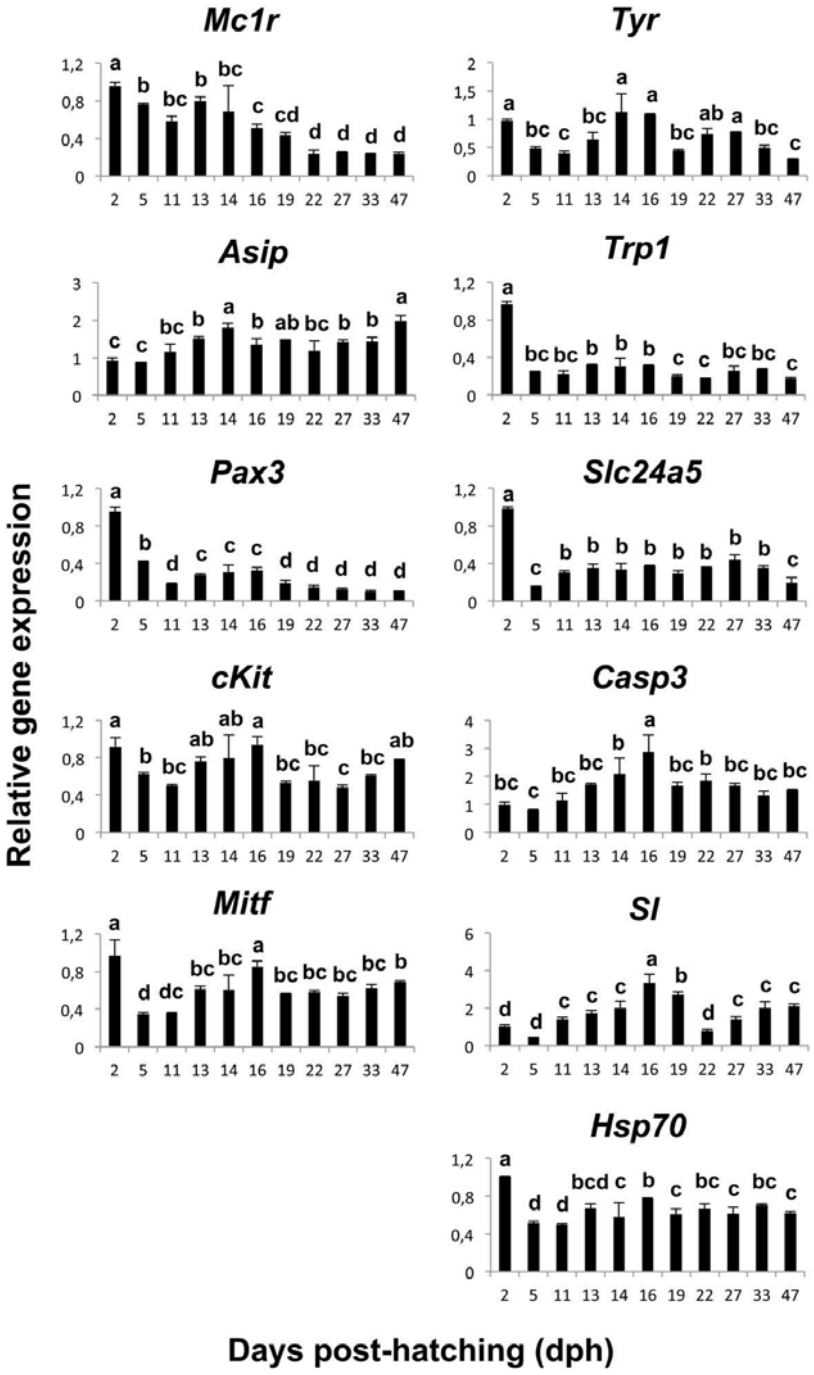


**Figure 5.** Gene expression patterns of pigmentation related genes during the larval development of Senegalese sole. Melanocyte-stimulating hormone 1 receptor (*mc1r*), agouti signaling protein (*asip*), paired box protein Pax-3 (*pax3*), mast/stem cell growth factor receptor Kit, (*cKit*), microphtalmia-associated transcription factor (*mitf*), tyrosinase (*tyr*), tyrosinase-related protein 1 (*trp1*), sodium/potassium/calcium exchanger 5 (*slc24a5*), caspase 3 (*casp3*), somatolactin (*sl*) and heat shock 70 kDa protein (*hsp70*) Data are represented as means of relative gene expression ± SD (n = 3). Values with a different superscript letter denote significant differences between sampling points (One-way ANOVA, $P < 0.05$).

Again, the highest level of expression was detected at 2 dph followed by a decrease until 11 dph. Then, an increase in gene expression was observed between 11 and 16 dph. From 19 dph onwards, the expression level was constant and similar to that observed at 11-13 dph. *Tyr* displayed two peaks of expression, the first one between 14 and 16 dph and the second one between 22 and 27 dph, the level of expression being similar to that observed at 2 dph. *Trp1* displayed the highest level of gene expression at 2 dph. At 5 dph, *trp1* expression decreased 4 times and remained at the same level until 16 dph. Gene expression decreased again from 16 to 19 dph to remain invariable from that day until the end of the studied period. *Slc24a5* showed the highest level of expression at 2 dph. At 5 dph the amount of transcripts was 6 times lower than at 2 dph while at 11 dph it increased 2 fold with respect to 5 dph to remain at a constant level until 33 dph. At 47 dph, the expression level of *slc24a5* decreased to similar values observed at 5 dph. The expression level of *casp3* increased from 2 to 16 dph. The expression level decreased at 19 dph and remained invariable onwards. The amount of *sl* expression increased gradually from 2 dph reaching the highest level at 16 dph (8 fold increase). Subsequently, a 4-fold decrease of *sl* expression was observed from 16 dph to 22 dph. At 27 dph, *sl* increased 1.8 times with respect to day 22 and remain statistically constant afterwards. *Hsp70* showed the highest level of gene expression at 2 dph. From that time onwards, *hsp70* showed lower but fluctuating levels of expression.

The figure 6 shows the global hierarchical clustering of genes based on their expression profile during the larval development. Gene clustering revealed two main groups. The first one included those genes displaying low levels of transcription at early stages of development (from 2 to 11 dph): *casp3*, *sl* and *asip*. The second cluster grouped the rest of the genes, which showed high levels of expression at 2dph. This cluster was divided into two main groups. The first one included *pax3* and *mc1r,* which were highly expressed during pre- and pro-metamorphosis (until 16-19 dph). The second group contained those genes displaying lower expression levels during pre-metamorphosis (5-11 dph). Within this group, *cKit* was placed alone whilst the other genes were grouped together. The latter grouping was divided in two clades. One composed of *tyr* and a second one that was divided again into two closely related sub-groups: *slc24a5* and *trp1,* and *mitf* and *hsp70*.

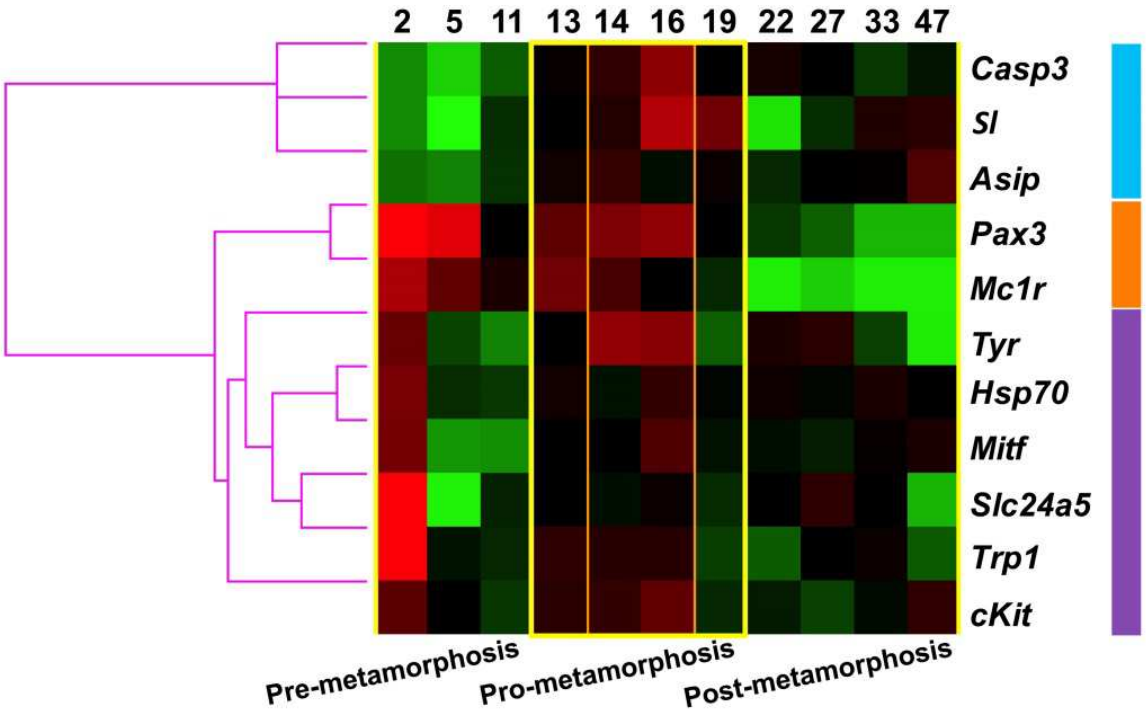


**Figure 6.** Global hierarchical clustering based on similarity of the expression profile for different pigmentation related genes during the larval development of Senegalese sole. Columns represent the mean data values for each sampling point (days post hatching) and rows represent single genes. Expression level of each gene is represented relative to its median abundance across the different stages and is depicted by a color scale: green, black, and red indicating low, medium, and high relative expression levels, respectively. Colored bars to the right margin indicate the three main gene clusters: blue shows genes highly expressed during pro-metamorphosis stage, orange corresponds to genes highly expressed during pre- and pro-metamorphosis and violet to genes highly expressed at 2 dph and at pro-metamorphosis. The three main stages of the larval development are indicated at the bottom of the figure. Note that the expression of all genes was high during the pro-metamorphosis phase (yellow square), most of genes displaying a shift in their level of expression before and/or after that period. The climax of pigmentation development at the molecular level was observed between 14 and 16 dph (orange square). Changes in gene expression profiles coincided with morphological changes in pigmentation, showing that the climax of metamorphosis was achieved at 16 dph and the end of pro-metamorphosis at 19 dph. The transition from the larval to the adult pattern of skin pigmentation could be clearly observed from 22 dph onwards.

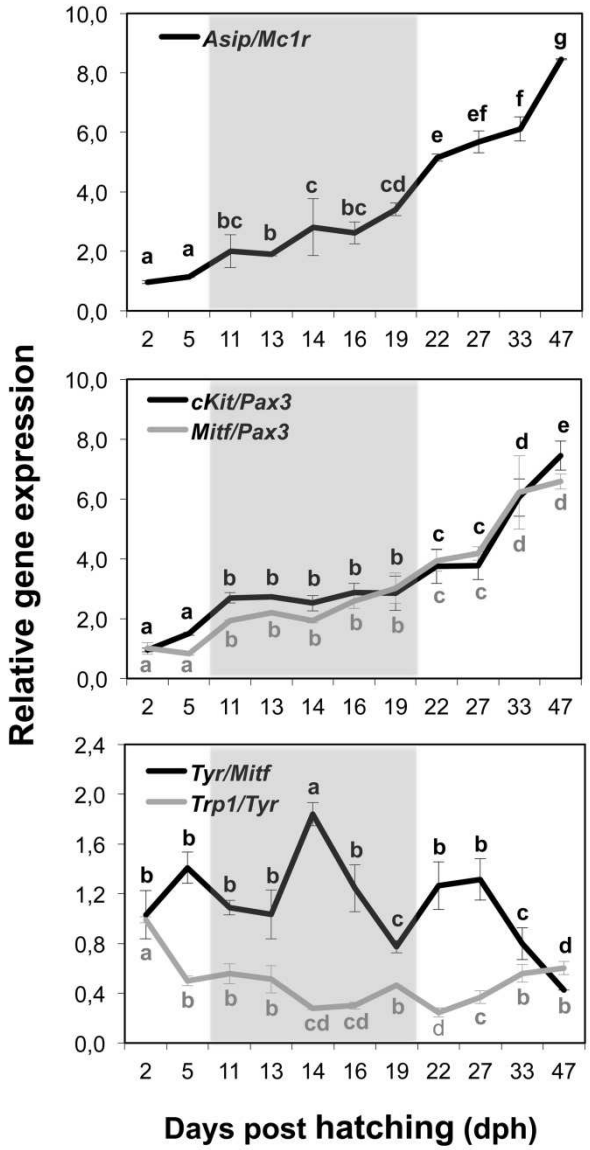


**Figure 7.** Gene expression ratios during the larval development of Senegalese sole illustrating the ontogeny of chromatophores. The *asip/mc1r* ratio shows the relationship between these genes and reflects the pigmentation patterning of Senegalese sole during development. Its increase during post-metamorphosis coincided with the switch from the bilateral to the flat symmetry and with the appearance of iridophores in the skin of the ocular side. The *cKit*/*pax3* and *mitf*/*pax3* ratios indicate the regulation of *cKit* and *mitf* transcription by *pax3.* Note the initial positive regulation of both genes by *pax3* and the subsequent stabilization during the pro-metamorphosis period. The increase of both ratios at post-metamorphosis shows the activation of melanophore migration and melanin synthesis processes during this period. The *tyr/mitf* and *trp1/tyr* ratios display opposite profiles during development and correspond well to the melanin production periods. The peaks of *tyr/mitf* ratio reveal the activation of the melanin synthesis pathway, whereas those of *trp1/tyr* ratio show the production of melanin. Grey regions indicate the pro-metamorphosis stage.

Figure 7 shows *asip/mc1r, cKit/pax3, mitf/pax3, tyr/mitf* and *trp1/tyr* gene expression ratios, which allowed determining the different stages of metamorphosis in terms of pigmentation ontogeny. *Asip/mc1r* ratio increased slightly from 2 to 19 dph ($y = 0.40x + 0.54$; $r = 0.96$) and then it sharply increased from that day onwards ($y = 1.11x + 2.45$; $r = 0.96$) (ANCOVA, $F = 11.64$; $P = 0.002$). The *cKit/pax3* ratio increased from 2 to 11 dph, remained stable from 11 to 19 dph (pro-metamorphosis period), and increased again from 19 to 47 dph (One-way ANOVA, $P < 0.001$). Similarly, the *mitf/pax3* ratio showed a staggered increase from 5 to 11 dph, from 19 to 22 dph and from 27 to 33 dph (One-way ANOVA, $P < 0.001$). *Tyr/mitf* ratio presented two peaks at 14 and 22-27 dph (One-way ANOVA, $P < 0.001$), whereas *trp1/tyr* decreased from 2 to 14 dph, then a peak was observed at 19 dph to subsequently decrease at 22 dph, and it increased gradually again from 22 to 33 dph (One-way ANOVA, $P < 0.001$).

**Discussion**

The regulation of pigmentation in vertebrates is a quite complex process that encompasses the migration of crest-derived stem cells during the embryonic development, their proliferation in target tissues (i.e., skin) and differentiation into mature chromatophores, and a tightly controlled regulation of melanogenesis. Pigmentation biology has been extensively studied and many aspects are today well known, especially in melanocytes (Hoekstra *et al.*, 2006; Lin and Fisher, 2007). However, less is known about the molecular basis of melanogenesis due to the intricate network of pathways regulating this process that includes many multifunctional mechanisms of action (Carlson *et al.*, 2007). Due to the similarities shared with humans, fish have become extremely valuable experimental animal models for vertebrate developmental studies, especially those with underlying genetic components (Patton *et al.*, 2010). The genetics of pigmentation have been explored in several model teleost fish including zebrafish (Kelsh *et al.*, 1996; Parichy and Johnson, 2001), medaka (Fukamachi *et al.*, 2004), fugu (Klovins *et al.*, 2004), goldfish (Cerdá-Reverter *et al.*, 2003, 2005) and, recently, in flatfish (Yamada *et al.*, 2010; Guillot *et al.*, 2012). To our knowledge, this is the first study that combines the characterization of the morphological ontogeny of skin pigmentation with the analysis of the expression profile of a set of key pigmentation-related genes during the larval development of a fish.

It has been proposed that the various fish chromatophores differentiate from a common chromatoblast precursor (Kelsh *et al.*, 1996). This has been supported by recent research on the molecular mechanisms underlying cell fate decisions and differentiation in goldfish (Cerdá-Reverter *et al.*, 2005) and zebrafish (Curran *et al.*, 2010; Minchin and Hughes, 2008). Distinct populations of melanophores and iridophores contribute to pigment patterns before and after metamorphosis (Kelsh *et al.*, 1996; Johnson *et al.*, 1995; Haffter *et al.*, 1996; Parichy *et al.*, 1999). This issue has remained unresolved for xanthophores because of the lack of mutants able to ablate these cells (Puebla *et al.*, 2007), though the existence of two different populations, the “larval xanthophores” and the “adult xanthophores” has been recently suggested (Yamada *et al.*, 2010). In the present study, changes in

the expression of pigmentation related genes were found to be coincident with changes in pigment pattern of Senegalese sole. These changes occurred during pro-metamorphosis (Figure 6) and morphological changes in the population of melanophores, xanthophores and iridophores were evidenced at post-metamorphosis leading to the adult pattern of pigmentation (Figure 4B).

*Morphological ontogeny of skin pigmentation*

Pigment cells were already present in the skin of 2 day-old Senegalese sole larvae. Abundant larval-type xanthophores and melanophores (large dendritic cells) were distributed along the head, abdominal and trunk regions, with the exception of the caudal fin. Xanthophores were often associated to melanophores (Figure 3A). The eye was also pigmented at that time (Figure 2A). This developmental stage was in synchrony with the maturation of the main digestive organs and the mouth and anus opening, which took place at around 3 dph (Sarasquete *et al.*, 1996). At this time, pigmented eyes are crucial for the larvae to be able to succeed in the transition from the endogenous feeding (yolk-sac reserves) to the exogenous feeding period, as prey search and capture mainly involves the vision during the larval stage (Hunter, 1984).

The amount of melanophores and xanthophores remained invariable during pre- and post-metamorphosis in Sengalese sole larvae and were equally represented until 33 dph and, from that day to 41 dph, the amount of xanthophores decreased by a half. In contrast, the density of xanthophores decreased in Japanese flounder (*Paralichthys olivaceus*) before metamorphosis and increased rapidly after metamorphosis (Nakamura *et al.*, 2010). The population of round-shaped xanthophores became more abundant than the dendritic ones during post-metamorphosis and became the main cell type from 33 dph onward (Figures 3F-H). An increase in the number of xanthophores, similar to that found in Japanese flounder (Nakamura *et al.*, 2010), was detected at 47 dph in Senegalese sole. In Japanese flounder and stone flounder (*Kareius bicoloratus*) the same morphological type of xanthophore was observed before and after metamorphosis (Matsumoto and Seikai, 1992). However, recent studies suggested also the existence of two different populations of xanthophores for Japanese flounder (Yamada *et al.*, 2010). Similarly in this study, another population of melanophores appeared at post-metamorphosis (ca. 33 dph) (Figure 4A) in Senegalese sole, coinciding with the increased amount of melanophores quantified in the ocular side during this period. An increase in the number of melanophores after metamorphosis was also observed in flounders (Matsumoto and Seikai, 1992; Nakamura *et al.*, 2010). Previous studies on flounders have identified two distinct populations of melanophores that appeared sequentially during development (Seikai, 1987) and differed in size (Matsumoto and Seikai, 1992). In Senegalese sole larvae, two groups of melanophores were also observed according to their size (Figure 3G). However, because those smaller melanophores were not found again until 47 dph (Figures 2L, 4A), it more likely seems that they corresponded to the morphological stage of newly differentiated and melanized cells rather than the adult-type melanophore described for Japanese flounder. The newly formed small melanophores observed at 47 dph accounted for the increase in the amount of melanophores quantified at that date (Figure 4A).

Melanophores organized in patches in the ocular side of the fish at the end of pro-metamorphosis, whereas the blind side presented few melanophores mainly located in stripes along the margins of the dorsal and ventral flank and in the center of the trunk, and in the abdominal area. Iridophores on the ocular side of Senegalese sole first appeared at the end of pro-metamorphosis and increased in number and distributed in patches thereafter. In Pleuronectiformes (Flounders), iridophores also increased in number from metamorphosis and were restricted to patches in the ocular side while remaining distributed dispersedly in the blind side (Matsumoto and Seikai, 1992; Nakamura *et al.*, 2010). The appearance of iridophores on the blind side was delayed to the late juvenile stage in Japanese flounder (Nakamura *et al.*, 2010). Unfortunately, the extension of the experimental period was not long enough to monitor the ontogeny of iridophores in the blind side of Senegalese sole, since only a few cells could be observed at the level of the head beginning at 41 dph. Another pattern of iridophore allocation was observed in plaice (*Pleuronectes platessa*) were these cells were the most prominent chromatophore on both sides of the body after metamorphosis (Roberts *et al.*, 1971). Leucophores were present in the skin of Senegalese sole at early stages of development. During metamorphosis, leucophores contributed to the conformation of the adult pigmentation pattern by distributing in patches along the trunk and fins. Conversely to what occurred in Japanese flounder (Yamada *et al.*, 2010), leucophores did not disappear after metamorphosis in Senegalese sole. Morphologically, changes in pigment cells types and distribution occurred after metamorphosis in Senegalese sole.

These changes in number and distribution of chromatophores in the skin seem to not occur in an independent manner, but a kind of cellular communication exists between them enabling the pigmentation pattern to mature in a manner characteristic for each species. In this respect, it has been reported that xanthophores regulate melanophore pattern formation (Parichy and Turner, 2003) and that melanophores are required for proper iridophore disposition in the skin of zebrafish (Johnson *et al.*, 1995). These findings suggested the existence of a cascade of interactions among chromatophores in zebrafish: xanthophores -> melanophores -> iridophores (Parichy and Turner, 2003). A similar mechanism of pigment cell interaction seemed to occur in Senegalese sole larvae. After metamorphosis, xanthophores were more abundant than melanophores and closely associated to them, this being in line with the proposed role of the xanthophores in the guidance for melanophore patterning. Yamada *et al.* (2010) demonstrated that pigment cell precursors migrate from the dorsal and ventral margins of the flank to the lateral sides of the body and to the dorsal and ventral fins in Japanese flounder to differentiate into adult-type pigment cells. Similarly, the patches of xanthophores and leucophores observed at 2 dph in Senegalese sole where located at specific points of the dorsal and anal fins and they seemed to function as reference points for the migration of melanophores to the fins at pro-metamorphosis (Figure 2A-F) (Pederzoli *et al.*, 2003). From 33 to 35 dph, a decrease in the proportion of xanthophores versus melanophores was found, with morphological evidences of disintegrating xanthophores (Figure 2D). Then, from 35 dph, the number of xanthophores associated to one melanophore (X/M ratio) varied during post-metamorphosis, switching from 1 to 5 (Table 2).

Concomitantly, the proportion of melanophores versus iridophores decreased and the distribution of iridophores was restricted to those areas free of melanophores (Figures 2F-L, 3E, 4B).

Nevertheless, interaction between xanthophores and melanophores could be bidirectional. Xanthophores are able to eliminate the surrounding melanophores over a short-range in zebrafish (Nakamasu *et al.*, 2009). Similarly, xanthophores in the skin of Senegalese sole pseudo-albinos seemed to be responsible for the degeneration of melanophores (Darias *et al.*, unpublished data). Considering this, analysis of the proportion of melanophores versus xanthophores during the development of Senegalese sole could be a suitable biomarker to evaluate the correct ontogeny of skin pigmentation in this species. Changes in the proportion of these pigment cells are undoubtedly preceded by changes in the molecular signaling. Identification of genes responsible for these specific types of patterning is necessary as a possible means for early detection of pigmentation disorders.

The differences found in the ontogeny of skin pigmentation between Senegalese sole and flounders reveal the complexity of the processes regulating pigmentation within the group of flatfish. Although metamorphosis of Senegalese sole was accomplished largely before the final sampling point, an extended experimental time would be necessary to completely describe the ontogeny of pigmentation in this species. This points to species-specific studies being necessary to find out the mechanisms underlying pigmentation that, in turn, will allow understanding the origin of pigmentation disorders in each species.

*Molecular ontogeny of skin pigmentation*

The morphological features of skin pigmentation ontogeny were mirrored in their molecular features (Figures 2, 5, 6, and 7). As evidenced by the high level of expression of the pigmentation-related genes and the presence of abundant melanophores and xanthophores at 2 dph in Senegalese sole, molecular signaling toward differentiation of these cells occurred even earlier, including the process of melanin synthesis within the larval retina (Hallsson *et al.*, 2004) (Figures 5, 6). The expression patterns of *pax3*, *mitf* and *cKit* followed similar trends. This observation is in line with the known influence of *pax3* in the transcriptional modulation of the other two genes (Prota, 1995; Guo *et al.*, 2010). *Pax3* and *mitf* play a key role in the differentiation of neural crest-derived melanocytes (Kubic, 2008). *Pax3* is also crucial for xanthophore differentiation in zebrafish. Knockdown of *pax3* resulted in a loss of xanthophores and an increase of melanophores, providing more evidence to the existence of a common chromatoblast precursor (Minchin and Hughes, 2008). Indeed, *Pax3* is considered a xanthophore specification gene, whereas *mitf* was described earlier as a melanophore specification factor (Minchin and Hughes, 2008). *cKIT* receptor influences melanogenesis, proliferation, migration, and survival of the pigment-producing cells (Alexeev and Yoon, 2006) (Figure 1). The expression of *mitf* and *cKit* increased progressively during pre- and pro-metamorphosis in Senegalese sole reflecting the differentiation and proliferation of melanophores (Figures 1, 5, 6, and 7). Within the biosynthetic

pathway of melanin, TYR catalyzes the rate-limiting conversions of tyrosine to DOPA, DOPA to DOPA-quinone and possibly 5,6-dihydroxyindole to indole-5,6 quinone. *TRP1* is involved in the oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into indole-5,6-quinone-2-carboxylic acid [80] (Figure 1). The decrease in the amount of chromatophore differentiating *pax3*, *mitf* and *cKit*, and of melanogenic, *tyr* and *trp1,* transcripts between 16 and 19 dph indicated the climax of pigmentation development, and the beginning of the formation of the adult pattern of pigmentation (Figures 2, 5, 6, and 7). These changes in gene expression coincided with the end of the pro-metamorphosis period. This is in agreement with the fact that, in mammals, *pax3* is expressed in early development, but inhibited in adult melanocytes (Hathaway and Haque, 2011), and *cKit* induces *tyr* expression during melanogenesis, but not in mature melanocytes (Alexeev and Yoon, 2006). Considering the gene expression pattern of *pax3* during ontogenesis and its role in the regulation of xanthophore and melanophore differentiation, this gene could be a potential candidate for monitoring the correct development of skin pigmentation in Senegalese sole. The *cKit/pax3* ratio in Senegalese sole reflected the ontogeny of melanophores as demonstrated by an increase in melanogenesis during pre-metamorphosis and a stabilization of melanophore differentiation and melanogenesis processes during pro-metamorphosis. Once metamorphosis was finished, the *cKit/pax3* ratio increased again until the end of the larval period indicating the prevalence of melanogenesis over melanophore differentiation. Similarly, the *mitf/pax3* ratio increased in a staggered way and could be indicating changes in the proportions of melanophores versus xanthophores.

In addition, the expression profile of *mc1r,* involved in the formation of pigmentation pattern during development, decreased during development until 19 dph to remain constant afterwards, indicating the end of melanophore ontogeny and pigment patterning at the transcriptional level. In fact, the stabilization of the gene expression levels of *pax3*, *mitf*, *cKit* and *mc1r* observed at post-metamorphosis coincided with the onset of the adult pigmentation phenotype (Figures 2, 5, and 6).

The expression of the melanogenic genes *tyr* and *trp1* peaked asynchronously during the larval development, where peaks of *tyr* expression preceded peaks of *trp1* expression (Figure 7). The changes in the *tyr/mitf* and *trp1/tyr* ratios clearly showed a cyclic production of melanin in this species during development. Moreover, the opposite profile of both ratios suggests the existence of a regulatory mechanism between *tyr* and *trp1* transcription in Senegalese sole (Figure 7). Indeed, *TRP1* has been demonstrated to be a critical enzyme for the correct trafficking of *TYR* to melanosomes (Brenner and Hearing, 2008). The first peak of *trp1* expression likely reflected the melanin production in larval melanophores, including the retina (2 dph) and the second one could be responsible for the melanin synthesis in the newly differentiated melanophores (19 dph). Therefore, the transition from pro- to post-metamorphosis was also evidenced by the expression profile of melanogenic genes. These results are in line with the thought that new melanophores differentiate from their precursors during metamorphosis (Matsumoto and Seikai, 1992). A new peak of *tyr* expression was observed at 22 dph, followed by a peak of *trp1* expression 10 days later, suggesting a new population of

melanophores was being stimulated into differentiation. This was corroborated by the increase in the amount of melanophores observed at 47 dph (Figure 4A).

*Slc24a5* is a putative cation exchanger localized to intracellular membranes of melanosomes and their precursors and is believed to increase uptake and accumulation of calcium in melanocytes, necessary for melanogenesis (Lamason *et al.*, 2005; Vogel *et al.*, 2008). *Slc24a5* expression is necessary for melanin production in human epidermal melanoblasts stimulated to differentiate (Ginger *et al.*, 2008). This explains the importance of a constant expression of this gene during the larval development of Senegalese sole. In particular, *Slc24a5* is required for *TRP1* protein expression in humans (Ginger *et al.*, 2008) and seems to be the same for Senegalese sole, as both genes were clustered together (Figure 6).

In mammals, *ASIP* inhibits the *α-MSH/MC1R* signaling resulting in the production of phaeomelanin (yellow pigment) instead of eumelanin (brown/black pigment). Moreover, *ASIP* is able to inhibit the differentiation and proliferation of melanoblasts (Sviderskaya *et al.*, 2001). In rodents, *Asip* is expressed only in skin whereas in humans it has a wider pattern of expression, including adipose tissue, testis, ovary, and heart and lower levels of expression in foreskin, kidney, and liver (see review from Ginger *et al.*, 2008) and the physiological role, including hair and skin pigmentation, is not fully understood. In goldfish, *asip* is mainly expressed in the ventral skin and it is thought to be involved in the establishment of the dorsal-ventral pigment pattern by directing chromatophore differentiation, causing production of iridophores (structural pigment cells) and inhibiting production of melanophores (Cerdá-Reverter *et al.*, 2005). *ASIP* has similar role in quails and chickens (Nadeau *et al.*, 2008). In Senegalese sole larvae, the *asip/mc1r* ratio remained constant during pro-metamorphosis and then increased considerably at post-metamorphosis, coinciding not only with the switch from the bilateral to the flat symmetry, but also with the increase in the amount of iridophores in skin of the ocular side of the fish (Figures 2, 4, and 7). The results obtained in the present study show that the establishment of both the new dorsal-ventral body plan and skin pigment pattern is synchronized. Considering the roles of *mc1r* and *asip* in pigmentation, the *asip/mc1r* ratio could be considered as an indicator of the ontogenesis of iridophores and also as a marker for changes in the pigmentation pattern during development in Senegalese sole larvae (Figures 2, 4 and 7). The use of whole larvae for gene expression analyses omitted the possibility to determine whether *asip* was expressed in the skin of the ocular and/or blind sides of Senegalese sole larvae. However, considering that very few iridophores were observed in the blind side during the analyzed period, it is tempting to speculate that *asip* could have different roles at the same time on both sides of the fish. For instance, to first promote iridophore differentiation only on the ocular side while blocking melanin synthesis within melanophores of the blind side, as a step preceding development of iridophores in the blind side (Figure 1). In fact, *asip* expression increased again at 47 dph to the levels observed at pro-metamorphosis, possibly indicating the beginning of iridophore development on the blind side (Figure 5). Matsumoto and Seikai (1992) already suggested that differentiation of adult melanophores at

metamorphosis is blocked in the skin of the blind side of Japanese flounder. Moreover, it has been shown that an excess of *asip* gene expression could be responsible, at least in part, for the pseudo-albinism in Senegalese sole juveniles (Darias *et al.* unpublished data) by causing the down-regulation of *slc24a5* expression and, consequently, of *trp1* expression, thus preventing melanogenesis. This "altered" molecular mechanism occurring in the ocular side of pseudo-albinos could be "normally" happening in the blind side of the well pigmented larvae. Gene expression analyses using isolated skin samples from the ocular and blind sides could be used to test such a hypothesis.

It has been recently demonstrated that melanophores and iridophores are derived from a common precursor in zebrafish and the differentiation is driven by a *foxd3*/*mitfa* transcriptional switch; the role of *foxd3* being both to promote iridophore development and block melanophore differentiation by repressing *mitfa* (Curran *et al.*, 2010). Results of these authors suggested that cell precursors expressing *mitf* are bi-potent, therefore plastic, so those continuing to express this gene become melanophores, while others will repress *mitf* to form iridophores. Matsumoto and Seikai (1992) observed that the most common pigment cells derived from cultured chromatoblasts *in vitro* are iridophores and that the precise moment for differentiation of melanophores responsible for the adult coloration is genetically programmed. Whether these cells have a common precursor in Senegalese sole is unknown, but the molecular (*asip*/*mc1r* ratio) and morphological data indicates that chromatoblasts are first differentiated into melanophores. According to (Curran *et al.*, 2010), the decrease of *mitf* gene expression from 16 to 19 dph could indicate a higher number of bi-potent cells differentiate into iridophores on the ocular side and there is a blockage in melanophore development on the blind side (Figures 2M, 2K, 5).

During metamorphosis of Senegalese sole, many physiological functions, especially growth (Parra and Yúfera, 2001; Geffen *et al.*, 2007; Boglino *et al.*, 2012), are slowed down and stored energy reserves are consumed in switching from bilateral symmetry to the typical body asymmetry of flatfish, including the eye migration and skull remodeling. This study revealed a transcriptional up-regulation of all genes analyzed during the phase of pro-metamorphosis showing that the transition from larval to juvenile pigmentation is also in synchrony with that process (Figure 6). The expression profile of *casp3* indicated that cell apoptosis during larval development of Senegalese sole was especially active during the pro-metamorphosis period. Similar profiles were observed for *asip* and *sl.* The gene expression profile of *asip* is in line with its recently described involvement in the up-regulation of genes that are normally expressed during morphogenesis (Le Pape *et al.*, 2009). *Sl* is a member of the growth hormone/prolactin family, and has been shown to enhance differentiation of light-absorbing pigment cells (melanophores and xanthophores) and suppress differentiation of light-reflecting cells (leucophores and iridophores) in medaka (Fukamachi *et al.*, 2009). In Senegalese sole larvae, the expression profile of *sl* was in line with the sequential development of melanophores and iridohores. Gene expression of *pax3* and *mc1r* was also high during pre-metamorphosis, indicating their early implication in chromatophore differentiation, particularly melanophores and xanthophores,

and patterning. A third gene cluster observed included those highly expressed at 2 dph and which later slowed down during pre-metamorphosis. These genes were involved in chromatophore differentiation and melanogenesis. The high level of *hsp70* expression observed at 2 dph seems to be coherent with the expression profile of *casp3* since it has been shown that HSP70 protects WEHI-S cells from CASPASE-3-induced cell death (Jäättelä *et al.*, 1998). However, most of the stem cells on the blind side of Japanese flounder appeared to undergo cytolysis without evidences for apoptosis (Seikai, 1987; Seikai and Matsumoto, 1994).

Altogether these observations revealed different stages of skin pigmentation and development in Senegalese sole that coincided with the progress of metamorphosis. These stages could be summarized as follows: 1) pre-metamorphosis period (2-11 dph): low expression of apoptotic factor and genes related to melanogenesis (with the exception of day 2), and high expression of melanophore differentiating genes; 2) pro-metamorphosis period (11-19 dph): high expression of apoptotic factors (tissue remodeling) and melanophore differentiating and melanogenic genes; 3) post-metamorphosis (19-47 dph): low expression of all analyzed genes, especially those associated to melanophore differentiation (Figures 4A, 6).

Senegalese sole larvae already presented at 2 dph around 6% of the maximum amount of melanophores counted within the studied period, which remained invariable until 22 dph (Figure 4A). This result illustrates the precocious action of the molecular mechanisms governing pigmentation and explains the high levels of expression of melanophore differentiation- and melanogenic-related genes observed at 2 dph. Interestingly, the expression of melanogenic genes was lower during the rest of the pre-metamorphosis period. Considering that the amount of melanophores remained invariable during pre- and pro-metamorphosis and that larvae already possessed pigmented melanophores (Figure 2), melanogenesis begins quite early. Because melanophore differentiation occurred during both pre- and pro-metamorphosis periods, the increase in the expression of melanogenic-related genes observed at pro-metamorphosis was likely an indicator of melanin synthesis within the new developed melanophores. Likewise, the increased expression of melanophore differentiation-related genes during pro-metamorphosis resulted in an increase in the amount of melanophores during post-metamorphosis. The increased population of melanophores could induce the down-regulation of melanophore differentiating-related genes. Indeed, the relative amount of melanophores significantly increased by 23% at 27 dph and remained constant at post-metamorphosis, which was supported by the low expression of melanophore differentiation-related genes observed after day 27 (Figure 4A). Altogether these results have shown that alternating actions of both melanophore differentiation- and melanogenesis-related genes coordinates melanophore ontogenesis in Senegalese sole. The *cKit/pax3*, *tyr/mitf*, *asip/mc1r* and *trp1/tyr* ratios reflected well these molecular events (Figure 7), suggesting that quantification of the expression of these genes could be a useful tool to evaluate the state of the pigmentation process during early development of Senegalese sole.

While ontogenesis of skin pigmentation in Senegalese sole is genetically programmed, environmental factors can modulate the normal changes in the molecular processes occurring during metamorphosis. However, these alterations are morphologically detectable only after metamorphosis (Darias *et al.*, unpublished data). Knowledge of the molecular mechanisms underlying flatfish pigmentation may help to explain the synergy between genetic, behavioral and environmental influences on this process, aiding in understanding the appearance of pigmentation problems in other vertebrates, including humans. Nutrition is one of the factors affecting pigmentation in flatfish (reviewed by Hamre *et al.*, 2007). Previous studies have demonstrated that high levels of dietary arachidonic acid induced pseudo-albinism in developing Senegalese sole (Villalta *et al.*, 2005). Therefore, nutritional approaches can be suitable for analyzing the origin and mechanisms of some types of pigmentation disorders during the ontogeny of fish. Flatfish are particularly well-suited to this since, as we have seen in this study, morphologic markers have been identified which correspond to underlying molecular mechanisms that are at work during the ontogeny of pigmentation. Future efforts need to focus on contrasting the differences in gene expression that occur in the ocular and blind sides of developing larvae.

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# 2

# Senegalese sole (*Solea senegalensis*) metamorphic larvae are more sensitive to pseudo-albinism induced by high dietary arachidonic acid levels than post-metamorphic larvae

Anaïs Boglino[1]
Asher Wishkerman[1]
Maria J. Darias[2]
Pablo De la Iglesia[1]
Alicia Estévez[1]
Karl B. Andree[1]
Enric Gisbert[1]

Submitted to the British Journal of Nutrition

[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

**Las larvas pro-metamórficas del lenguado Senegalés (*Solea senegalensis*) son más propensas al pseudo-albinismo inducido por altos niveles de ácido araquidónico en la dieta que las larvas post-metamórficas.**

**Resumen**

La inclusión de altos niveles de ácido araquidónico (ARA) en las dietas y la proporción relativa de este con el ácido eicosapentanoico (EPA), suministrados durante los estadios iniciales de desarrollo larvario, han sido asociados con malpigmentaciones de varias especies de peces planos. Este estudio investigó si la inducción nutricional de desórdenes pigmentarios en los estadios larvarios está relacionada con un periodo larvario específico de sensibilidad incrementada al ARA en el lenguado Senegalés (*Solea senegalensis*, Kaup 1858). Las larvas de lenguado fueron alimentadas con dietas con altas concentraciones de ARA durante sus estadios pre- y pro-metamórfico (2 a 15 días post-eclosión, dpe) y/o post-metamórficos (15 a 50 dpe). Los tejidos larvarios reflejaron la composición en ácidos grasos de la dieta. Se encontró que la incidencia de peces con malpigmentaciones en sus tejidos esta significativamente relacionada al suministro de dietas con cantidades altas de ARA y ARA/EPA. Este estudio reportó evidencia de una "ventana de pigmentación", con una mayor sensibilidad de las larvas a la cantidad de ARA adicionado en la dieta durante la pre- y pro-metamorfosis que en la post-metamorfosis. Asimismo, al proveer dietas con alto porcentaje de ARA en larvas en etapa de pre-metamorfosis se observó que su tasa de sobrevivencia se incrementó, no obstante, factores como la tasa de crecimiento y el proceso de migración ocular no tuvieron relación considerable con la inclusión de más ARA durante esta fase de su desarrollo. El aspecto y la densidad de los melanóforos en la piel de lado ocular de los peces pseudo-albinos, inducidos por el ARA, fueron significativamente reducidos en comparación con los individuos normalmente pigmentados y aun más en los peces pseudo-albinos que fueron alimentados con altos niveles de ARA durante el estadio pre-metamórfico. Los peces pseudo-albinos alimentados con altos niveles de ARA durante la post-metamorfosis presentaron concentraciones de prostaglandinas de las serias 2 y 3 (PGE2 y PGE3) más elevadas que los peces normalmente pigmentados alimentados con la misma dieta. El suministro de altas cantidades de ARA desequilibró el ratio ARA/EPA de la dieta, lo que posteriormente afectó las concentraciones relativas de prostaglandinas de las series 2 y 3 en 20-81.7% individuales post-metamórficos de lenguado senegalés, según el tratamiento alimentario, lo que indujo malpigmentaciones.

**Palabras llaves**: *Solea senegalenses*, larvas, pigmentación, ácido araquidónico, prostaglandinas

**Abstract**

High dietary levels of arachidonic acid (ARA) and its relative proportions with eicosapentaenoic acid (EPA), fed during early larval stages, have been associated with malpigmentation in various flatfish species. This study investigated whether the nutritional induction of pigmentary disorders at larval stages was related to a specific larval period of increased sensitivity to ARA in Senegalese sole (*Solea senegalensis* Kaup, 1858). Sole larvae were fed high dietary ARA levels during pre- and pro-metamorphosis (2-15 dph) and/or post-metamorphosis (15-50 dph). Larval tissues reflected the dietary fatty acid composition. Malpigmentations were significantly related to elevated dietary and larval ARA contents and ARA/EPA ratio. This study reports evidence for a "pigmentation window", with a higher larval sensitivity to dietary ARA during pre- and pro-metamorphosis than post-metamorphosis. High dietary ARA fed to larvae during pre-metamorphosis enhanced survival, but did not affect growth nor eye migration. The aspect and density of melanophores in the skin of the ocular side of ARA-induced pseudo-albinos were significantly reduced in comparison to normally pigmented individuals, even more in the pseudo-albino fish fed high dietary ARA levels during the pre-metamorphic stage. Pseudo-albino fish fed high dietary ARA levels during post-metamorphosis showed higher concentrations of 2 and 3-series prostaglandins ($PGE_2$ and $PGE_3$) than normally pigmented specimens fed the same diets. Supplying high ARA amounts imbalanced the dietary ARA/EPA ratio and disrupted the relative concentrations of derived $PGE_2$ and $PGE_3$ in 20 to 81.7% post-metamorphic Senegalese sole individuals, depending on the dietary treatment, resulting in malpigmentation.

**Keywords:** *Solea senegalensis*: larvae: pigmentation: arachidonic acid: prostaglandins

**Introduction**

Flatfish development and ontogenesis are characterized by a crucial event occurring at an early stage, the metamorphosis, a process associated with important morphological, physiological, hormonal, behavioral and ecological changes (Geffen *et al.*, 2007). The functional modifications occurring during the transition of a larva to a juvenile in Pleuronectiformes include many critical processes, from the acquisition of asymmetry with the migration of one eye from one side of the body (blind side) to the other (ocular side), to the maturation of different organs and systems, bone and myomeres remodeling and the differentiation of pigment cells (Geffen *et al.*, 2007; Power *et al.*, 2008). The pigmentation of the ocular side of the fish, occurring during metamorphosis, makes this a critical period for the acquisition of the adult phenotype (Bolker and Hill, 2000; Power *et al.*, 2008; Darias *et al.*, 2013a). Pigmentation development is highly sensitive to epigenetic factors, and their impact on the pigmentary process may vary depending on the stage of larval development (pre-, pro- and post-metamorphosis) at which they were exerted (Power *et al.*, 2008; Darias *et al.*, 2013a).

Lipid and fatty acid nutrition are known to be one of the key factors involved in proper larval development and pigmentation in marine fish (Bolker and Hill, 2000; Izquierdo *et al.*, 2000; Cahu *et al.*, 2003). High dietary contents of arachidonic acid (20:4n-6, ARA) have previously been associated to the incidence of pigmentary anomalies in various flatfish species (McEvoy *et al.*, 1998; Estévez *et al.*, 1999; Copeman *et al.*, 2002; Bell and Sargent, 2003; Villalta *et al.*, 2005b; 2008; Lund *et al.*, 2007; Hamre and Harboe, 2008; Darias *et al.*, 2013b). ARA is the major precursor for eicosanoids synthesis, enhancing the immune system and resistance to stress, among other important physiological processes (Bell and Sargent, 2003) and it directly competes with eicosapentaenoic acid (20:5n-3, EPA) for the enzymes involved in prostaglandin biosynthesis; ARA gives rise to prostaglandins of the 2-series ($PGE_2$), while EPA is the substrate for the synthesis of prostaglandins of the 3-series ($PGE_3$) (Bell *et al.*, 1995). High amounts of dietary ARA have been reported to result in imbalances in the relative content of EPA and DHA (Moren *et al.*, 2011) and therefore, in the relative proportions of $PGE_2$ and $PGE_3$ (Bell and Sargent, 2003). This has been suggested to cause biochemical stress and developmental disorders related to pigmentation patterns (*e.g.* pseudoalbinism) and delayed eye migration in several flatfish species (McEvoy *et al.*, 1998; Estévez *et al.*, 1999, 2001; Copeman *et al.*, 2002; Villalta *et al.*, 2005a, 2008; Lund *et al.*, 2008; Hamre and Harboe, 2008).

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a high-value flatfish commonly reared under intensive aquaculture in Spain and Portugal, which larval quality is still hindered by the incidence of malpigmentation (Dinis *et al.*, 1999). These pigmentary disorders affect fish survival, growth and development, downgrading the market value of the final product. The high incidence of malpigmented fish represents a current major bottleneck in flatfish farming and limits the development of the production sector for this species (Power *et al.*, 2008; Boglione *et al.*, 2013a, b). Pigmentary anomalies in flatfish have previously been described and qualitatively classified (Seikai, 1985). However, the study of pigmentation is complex and require objective and quantitative tools to characterize the different types of pigmentation pattern, such as the skin texture image analysis (Bharati *et al.*, 2004) that we used in this study.

In a recent study, the morphological and molecular ontogeny of skin pigmentation was characterized in the ocular side of Senegalese sole; this work provided the knowledge to elucidate the formation mechanisms of the adult pigmentation pattern and to understand when and how the pseudo-albino phenotype appears (Darias *et al.*, 2013b). This study revealed different stages of skin pigmentation and development that coincided with the progress of metamorphosis and patterns of gene expression. Subsequently, we investigated the morphological development of pseudo-albinism in Senegalese sole larvae induced by high dietary ARA levels supplied at pre-, pro- and post-metamophosis and the molecular signaling accounting for such a pigmentation impairment (Darias *et al.*, 2013b). This study demonstrated that although larval pigmentation was genetically determined, the establishment of the adult pigmentation phenotype could be modified by nutrition (Darias *et al.*, 2013b), which differently affect the pigmentation whether they intervene before, during or after the metamorphosis process (Power *et al.*, 2008). In a study on common sole (*Solea solea*), Lund *et al.* established the concept of the "pigmentation window", revealing that ARA-induced malpigmentation and sensitivity to dietary ARA and EPA relative proportions were higher during pre-metamorphosis than at later stages of development (Lund *et al.*, 2008). In order to deepen the understanding of the sensitivity of larvae to high dietary ARA levels, this study aims to investigate whether the induction of pigmentary disorders during ontogeny is related to a specific larval period of increased sensitivity to ARA in Senegalese sole.

**Materials and Methods**

*Larval rearing and feeding protocol*

One-day-old Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, La Coruña, Spain). Larvae were reared in 16, 100 litres cylindro-conical tanks (initial density: 110 larvae/l) connected to a water recirculation unit IRTA mar$^{TM}$ at IRTA-SCR facilities. Water conditions were as follows: temperature 16.7± 0.4 ºC, salinity 35‰, pH 8.0 ± 0.2, dissolved oxygen 7.5 ± 1.3 ppm, gentle aeration in each tank and 50% daily water renewal in the recirculation system. Photoperiod was 16L: 8D, and light intensity was 500 lx at the water surface. The following feeding protocol was used: larvae were fed twice a day, from 2 days post hatching (dph) to 10dph, with enriched rotifers (*Brachionus plicatilis*), at a density of 10 rotifers/ml from 2 to 8 dph and 5 rotifers/ml from 9 to 10 dph. Enriched *Artemia* metanauplii were supplied twice a day to larvae from 8 to 50 dph, at increasing densities ranging from 0.5 to 12 metanauplii/ml, adjusted based upon the increase of larval weight. The daily food ration was calculated as described by Cañavate *et al.* (2006). From 30 dph onwards, when larvae settled to the bottom of the tank, enriched *Artemia* metanauplii were supplied frozen as previously described (Villalta *et al.*, 2008).

*Experimental design, diets and live prey enrichment*

The effect of high dietary ARA levels on Senegalese sole larval development was evaluated during two different developmental stages, pre-metamorphosis and pro-metamorphosis (2-15 dph at 17 ºC) and

post-metamorphosis (15-50 dph at 17 ºC), in order to determine the differential sensitivity of the larvae to these high levels. Rotifer and *Artemia* metanauplii were enriched with a commercial enrichment (AGM, Algamac 3050™, Aquafauna Biomarine Inc., USA), that was considered as a control diet. In addition, both live prey were enriched with an experimental emulsion containing high levels of ARA (ARA-H) that was prepared using a mixture of commercially available oils rich in DHA (cod liver oil, Fluka©, Sigma-Aldrich, Chemie GmbH, Steinheim, Norway) and ARA (Vevodar®, DSM Food Specialties, Netherlands). Olive oil was added to the cod liver oil and the Vevodar® mixture to dilute and adjust n−3 PUFA concentration in enriched live prey whereas α-tocopherol was included in the emulsion as an antioxidant. Oil mixture was emulsified with soy lecithin and distilled water with an Ultra-turrax T25 at high speed for 60 s. The ingredients used in the formulation of the ARA-H emulsion and the fatty acid composition of both emulsions are shown in Table 1.

Several experimental groups (four replicates each) were constituted in order to evaluate the sensitivity of Senegalese sole to high dietary ARA levels: a control group fed live prey enriched with AGM during the whole experiment (C; 2-50 dph); a second group of fish fed live prey enriched with ARA-H during the whole trial (ARA-H +/+; 2-50 dph); a third group fed live prey enriched with AGM (control diet) during pre- and pro-metamorphic stages (2-15 dph) and then with *Artemia* enriched with the ARA-H emulsion during post-metamorphic stage (15-50 dph) (ARA-H -/+); and a forth group fed live prey enriched with the ARA-H emulsion during pre- and pro-metamorphic stages (2-15 dph) and then with *Artemia* enriched with AGM (control diet) during post-metamorphic stage (15-50 dph) (ARA-H +/-) (Fig. 1).

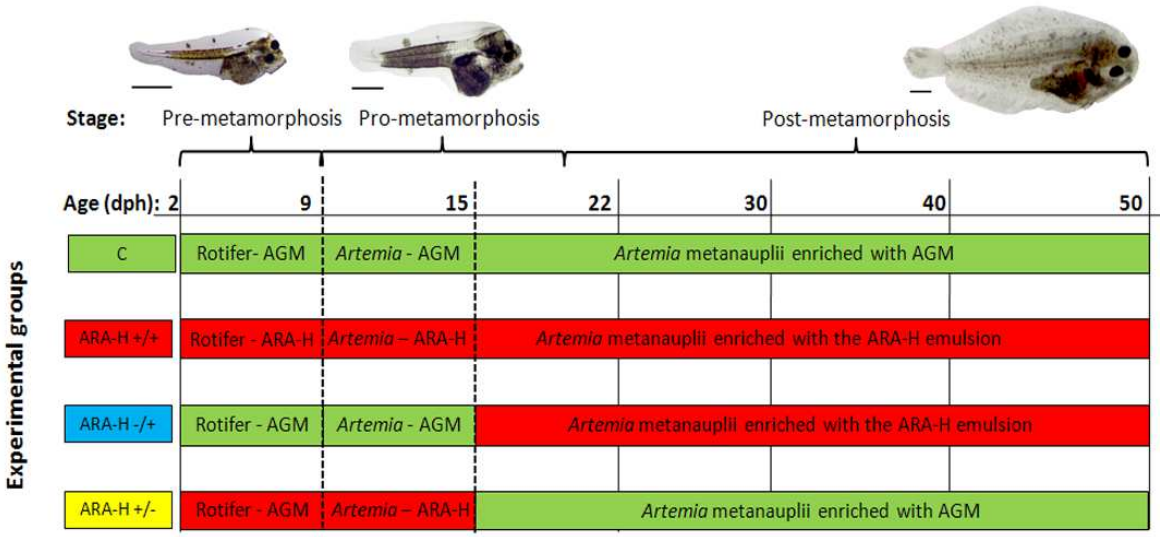


**Figure 1.** Experimental design of the nutritional assay. Rotifer and *Artemia* metanauplii were enriched with a reference commercial product, Algamac (AGM, in green) or with the experimental emulsion containing high dietary levels of ARA (ARA-H, in red) and fed to Senegalese sole larvae at different developmental periods: pre- and pro-metamorphosis (2-15 dph) and post-metamorphosis (16-50 dph), creating four dietary treatments (C, ARA-H +/+, ARA-H -/+, ARA-H +/-) Scale bars represent 2 mm.

Rotifers were enriched in 20 litres containers at 500 rotifer/ml at 26ºC with 0.6 $g.l^{-1}$ of each emulsion. Half of the rotifers were supplied to the larvae after 2h of enrichment and the other half after 6h post enrichment. No significant differences with regards to the biochemical composition and fatty acid profile of rotifers were observed at 2 and 6h postenrichment (data not shown). One-day-old *Artemia* metanauplii (EG strain, INVE) were enriched in 20 litres containers at 300 nauplii/ml for 16h at 28 ºC with 0.6 $g.l^{-1}$ of each emulsion. Enriched *Artemia* were kept at 4 ºC in UV-treated, filtered seawater with aeration until administered to larvae twice a day. In order to reduce the bacterial load after enrichment and remove emulsion residues, rotifers and *Artemia* were washed with UV-treated filtered seawater and treated with $H_2O_2$ (40ppm for 15 min for rotifers and 8000 ppm for 5 min for *Artemia* (Giménez *et al.*, 2006) and then rinsed with filtered seawater. Total lipid content and fatty acid (FA) composition of enriched rotifer and *Artemia* are shown in Table 1.

*Lipid and fatty acid analyses*

Total lipid and fatty acid contents were analyzed in the emulsions, enriched live prey and fish larvae (2 dph, end of endogenous feeding period before the onset of exogenous feeding; 15 dph, end of the pro-metamorphic stage; 50 dph, post-metamorphosis stage and end of the experiment). Samples were washed with distilled water to remove salt and bacteria, and frozen at -80 ºC until analysis. Total lipids were extracted in chloroform: methanol (2:1, v:v) using the method of Folch *et al.* (Folch *et al.*, 1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform: methanol (2:1, 20 mg/ml) containing 0.01% butylated hydroxytoluene at -20 ºC prior to analysis. Fatty acid analysis was conducted by gas-liquid chromatography. Acid catalyzed transmethylation was carried out according to Christie's method (1982) and fatty acids quantified as previously described in Boglino *et al.* (2012a).

*Larval survival, growth and metamorphosis*

At the end of the trial, final survival was evaluated by counting the animals, and calculated according to Buckley *et al.* (1984), which considers the number of sampled individuals during the experiment. Standard length (SL) and dry weight (DW) were measured at 2, 15 and 50 dph. For these purposes, thirty larvae from each tank were randomly sampled and euthanized with an overdose of tricaine methane sulphonate (MS-222, Sigma). Each larvae was photographed (300 dpi image) with a digital camera (Olympus DP25©, Olympus Corporation, Germany) connected to a stereomicroscope (Nikon SMZ 800). SL was measured to the nearest 0.1 mm using an image analysis system (AnalySIS®, Soft Imaging Systems, GmbH, Olympus, Germany). Then, DW was determined by rinsing larvae with distilled water to remove salt and then drying them at 60 ºC for 24 h. Samples were weighed with an analytic microbalance (Sartorius BP211D). Specific growth rate (SGR, in $\%.d^{-1}$) was calculated as $SGR = (\ln DW_f - \ln DW_i) / (t_f - t_i) * 100$; where $DW_f$, $DW_i$, $t_f$ and $t_i$ represented final and initial DW and time of the experiment, respectively.

**Table 1.** Total lipid and fatty acids contents (mg·g $DW^{-1}$) and fatty acid composition (% of total fatty acids, TFA) of the experimental emulsions (mean ± SD; n = 2) and of the rotifers (mean ± SD; n = 5) and *Artemia* nauplii (mean ± SD; n = 3) enriched with the two emulsions. Totals include some minor components not shown. Superscript letters denote significant differences among diets for a given live prey or emulsion (ANOVA, $P < 0.05$). <LOQ means that values were under the lowest limit of quantification (0.45% TFA).

| | **Enrichers** | | **Rotifer** | | ***Artemia*** | |
|---|---|---|---|---|---|---|
| | **AGM** | **ARA-H*** | **AGM** | **ARA-H** | **AGM** | **ARA-H** |
| Total lipid (en mg.g $DW^{-1}$) | 368.5±12.2$^{b}$ | 622.2±0.0$^{a}$ | 140.6±18.5 | 143.2±25.2 | 164.9±14.37 | 164.9±15.1 |
| Total FA (en mg.g $DW^{-1}$) | 305.4±10.3 | 328.1±19.2 | 74.2±13.0 | 61.4±13.6 | 87.6±14.4 | 85.0±14.3 |
| 14:0 | 3.2±0.9 | 0.9±0.2 | 3.3±1.2$^{a}$ | 0.8±0.2$^{b}$ | 0.6±0.2$^{a}$ | 0.3±0.1$^{b}$ |
| 16:0 | 20.2±1.7$^{a}$ | 12.0±1.1$^{b}$ | 16.3±2.5$^{a}$ | 11.8±0.9$^{b}$ | 10.7±0.3$^{a}$ | 9.1±0.6$^{b}$ |
| 18:0 | 0.4±0.1$^{b}$ | 5.1±0.1$^{a}$ | 1.7±0.3$^{b}$ | 4.4±0.5$^{a}$ | 6.2±0.9 | 5.9±0.7 |
| Total saturated | 23.8±2.5 | 18.1±1.6 | 21.4±3.5$^{a}$ | 17.2±1.6$^{b}$ | 17.5±0.9$^{a}$ | 15.3±1.2$^{b}$ |
| 16:1n-9 | <LOQ$^{b}$ | 2.8±0.3$^{a}$ | 5.2±2.0 | 7.1±1.4 | 1.2±0.2$^{b}$ | 1.8±0.2$^{a}$ |
| 18:1n-9 | 0.07±0.1$^{b}$ | 25.7±0.2$^{a}$ | 6.6±2.6$^{b}$ | 25.6±4.0$^{a}$ | 14.4±3.7$^{b}$ | 23.1±2.0$^{a}$ |
| 18:1n-7 | <LOQ | 0.2±0.3 | 1.8±1.0 | 3.7±2.1 | 6.0±1.5 | 6.8±1.4 |
| 20:1n-9 | <LOQ$^{b}$ | 6.6±0.02$^{a}$ | 0.8±0.5$^{b}$ | 5.3±0.5$^{a}$ | <LOQ$^{b}$ | 1.9±0.8$^{a}$ |
| Total monounsaturated | 0.07±0.1$^{b}$ | 35.3±0.4$^{a}$ | 14.4±4.6$^{b}$ | 41.8±2.8$^{a}$ | 21.7±5.5$^{b}$ | 33.6±2.9$^{a}$ |
| 18:2n-6 | <LOQ$^{b}$ | 7.4±1.0$^{a}$ | 2.4±0.6$^{b}$ | 9.3±4.8$^{a}$ | 4.6±1.2$^{b}$ | 6.7±0.4$^{a}$ |
| 18:3n-6 | 0.4±0.1$^{b}$ | 1.6±0.04$^{a}$ | 0.6±0.1$^{b}$ | 1.5±0.3$^{a}$ | 1.3±0.3 | 1.3±0.1 |
| 20:3n-6 | 0.8±0.1$^{b}$ | 1.3±0.04$^{a}$ | 0.9±0.3 | 1.4±0.7 | 0.2±0.2 | 0.9±1.0 |
| 20:4n-6 | 0.3±0.02$^{b}$ | 15.1±0.8$^{a}$ | 1.0±0.4$^{b}$ | 10.2±1.2$^{a}$ | 1.4±1.3$^{b}$ | 7.1±4.1$^{a}$ |
| 22:5n-6 | 19.6±0.3$^{a}$ | 0.9±1.3$^{b}$ | 13.3±2.1$^{a}$ | 1.8±1.4$^{b}$ | 4.3±2.8$^{a}$ | 0.6±0.4$^{b}$ |
| Total n-6 PUFA | 21.1±0.3$^{b}$ | 26.2±1.0$^{a}$ | 18.2±1.7$^{b}$ | 24.2±3.6$^{a}$ | 11.8±2.9 | 16.4±4.7 |
| 18:3n-3 | <LOQ$^{b}$ | 0.8±0.1$^{a}$ | 0.4±0.3$^{b}$ | 1.3±0.4$^{a}$ | 26.8±6.1 | 24.6±5.9 |
| 18:4n-3 | 0.3±0.3$^{b}$ | 1.0±0.04$^{a}$ | 0.4±0.1$^{b}$ | 0.8±0.1$^{a}$ | 4.1±1.5 | 3.3±1.4 |
| 20:5n-3 | 1.6±0.0$^{b}$ | 11.3±0.5$^{a}$ | 2.8±0.6$^{b}$ | 7.4±2.5$^{a}$ | 4.7±3.0 | 3.7±1.5 |
| 22:5n-3 | 0.2±0.3 | 0.8±0.02 | 0.6±0.1 | 0.7±0.1 | 0.3±0.3 | 0.2±0.1 |
| 22:6n-3 | 52.9±3.1$^{a}$ | 6.5±0.9$^{b}$ | 40.0±4.6$^{a}$ | 5.3±0.9$^{b}$ | 10.6±8.0 | 1.4±0.5 |
| Total n-3 PUFA | 55.04±2.9$^{a}$ | 20.4±1.1$^{b}$ | 46.0±4.0$^{a}$ | 16.8±1.2$^{b}$ | 46.4±3.6$^{a}$ | 33.2±5.3$^{b}$ |
| Total PUFA | 76.1±2.6$^{a}$ | 46.6±2.0$^{b}$ | 64.2±5.6$^{a}$ | 41.0±4.3$^{b}$ | 58.2±6.4$^{a}$ | 49.6±1.0$^{b}$ |
| n-3/n-6 | 2.6±0.2$^{a}$ | 0.8±0.01$^{b}$ | 2.5±0.1$^{a}$ | 0.7±0.1$^{b}$ | 4.1±0.6$^{a}$ | 2.3±1.1$^{b}$ |
| DHA/EPA | 32.8±1.3$^{a}$ | 0.6±0.1$^{b}$ | 15.1±3.4$^{a}$ | 0.9±0.6$^{b}$ | 2.1±1.0$^{a}$ | 0.4±0.2$^{b}$ |
| ARA/DHA | <LOQ$^{b}$ | 2.3±0.2$^{a}$ | 0.03±0.01$^{b}$ | 2.0±0.4$^{a}$ | 0.1±0.1$^{b}$ | 4.7±2.0$^{a}$ |
| ARA/EPA | 0.2±0.01$^{b}$ | 1.3±0.02$^{a}$ | 0.4±0.1$^{b}$ | 1.6±0.7$^{a}$ | 0.3±0.1$^{b}$ | 1.8±0.5$^{a}$ |
| MUFA/PUFA | <LOQ$^{b}$ | 0.8±0.04$^{a}$ | 0.2±0.1$^{b}$ | 1.0±0.2$^{a}$ | 0.4±0.1$^{b}$ | 0.7±0.1$^{a}$ |

* 100 g of ARA-H contained 29.0 g of Fluka© oil (Sigma-Aldrich, Chemie GmBH, Steinheim, Norway), 17.4 g of Vevodar® (DSM Food Specialties, Netherlands), 5.2 g of olive oil, 2.3 g of vitamin E, 4.1 g of soy lecithin and 42.0 g of distilled water.

The eye migration in Senegalese sole larvae, used to evaluate the metamorphosis process, was assessed in 30 individuals per tank at 9, 15, 19 and 35 dph, according to Villalta *et al.* (2005a). Data were presented as the relative amount of larvae in each stage of development at the same age. Eye migration index ($I_{EM}$) was calculated as $I_{EM} = \Sigma$ (%fish in each stage * stage) / 100) (Solbakken *et al.*, 1999).

*Pigmentation success, melanophore density and pigmentation texture analysis*

Pigmentation rates were determined in larvae at 50 dph with the exception of those that were sampled for analytical purposes. Abnormally pigmented individuals (totally or pseudo-albino) were considered as a whole group without giving any category or pigmentation index due to the inconveniences of such classifications (Bolker and Hill, 2000).

Image segmentation and texture analysis were performed to determine melanophore density and pigmented skin aspect among different experimental groups. Both image processing approaches were performed using Matlab (The Mathworks Inc., Natick, Massachussetts, USA). Prior to analysis, images of the whole fish (300 dpi) were converted into a gray scale (Fig. 2a, b) and cropped in accordance to fish dimension (height and weight) in order to get a rectangular area of interest centered on the vertebral column, from the basis of head to the tail, and from the dorsal fin to the pelvic fin (Fig. 2c). The cropped image was then sliced into small images based on a fixed number of column and rows (3 x 3), relative to the image dimension (fig. 2d). The middle slice including the center of gravity passing through the line of the vertebral column was chosen for further analysis (Fig. 2e).

Skin melanophores, once sufficiently contrasted with the background, were quantified using the Sobel edge detection method (Gonzales *et al.*, 2009). Then, the melanophore final number was divided by the image area (in pixels) to get a corrected value of density of melanophores per area. Pigmentation texture analysis was performed by statistical methods, using a gray level co-occurrence matrix (GLCM) a robust tool for extracting second-order textural features from a gray level histogram (Haralick *et al.*, 1973; Bharati *et al.*, 2004). Image texture has been described as an attribute representing the spatial arrangement of the gray levels of the pixels in a region of a digital image (IEEE Std 610.4-1990) or as a descriptor of local brightness variation from pixel to pixel in a small neighborhood through an image (Haralick *et al.*, 1973). The purpose of GLCM is to estimate the probability, $p_{(i,j)}$, for the gray-level intensity values of a pair of horizontally adjacent pixels (*i* and *j*) to be statistically similar. This joint probability takes the form of a square matrix, with row and column dimensions equal to the number of discrete gray-level intensities (8 x 8), in which each element (*i,j*) represent the occurrence of spatial relationship between a pair of horizontally adjacent pixels displaying *i* and *j* values of gray-level intensities. The GLCM was then normalized to perform a second-order statistical texture description (Bharati *et al.*, 2004). The GLCM resulting from an entirely flat intensity image (without texture) would result completely diagonal, while its off-diagonal values would become larger as the image texture increases (increases in the variation of local pixel intensity) (Bharati *et al.*, 2004).

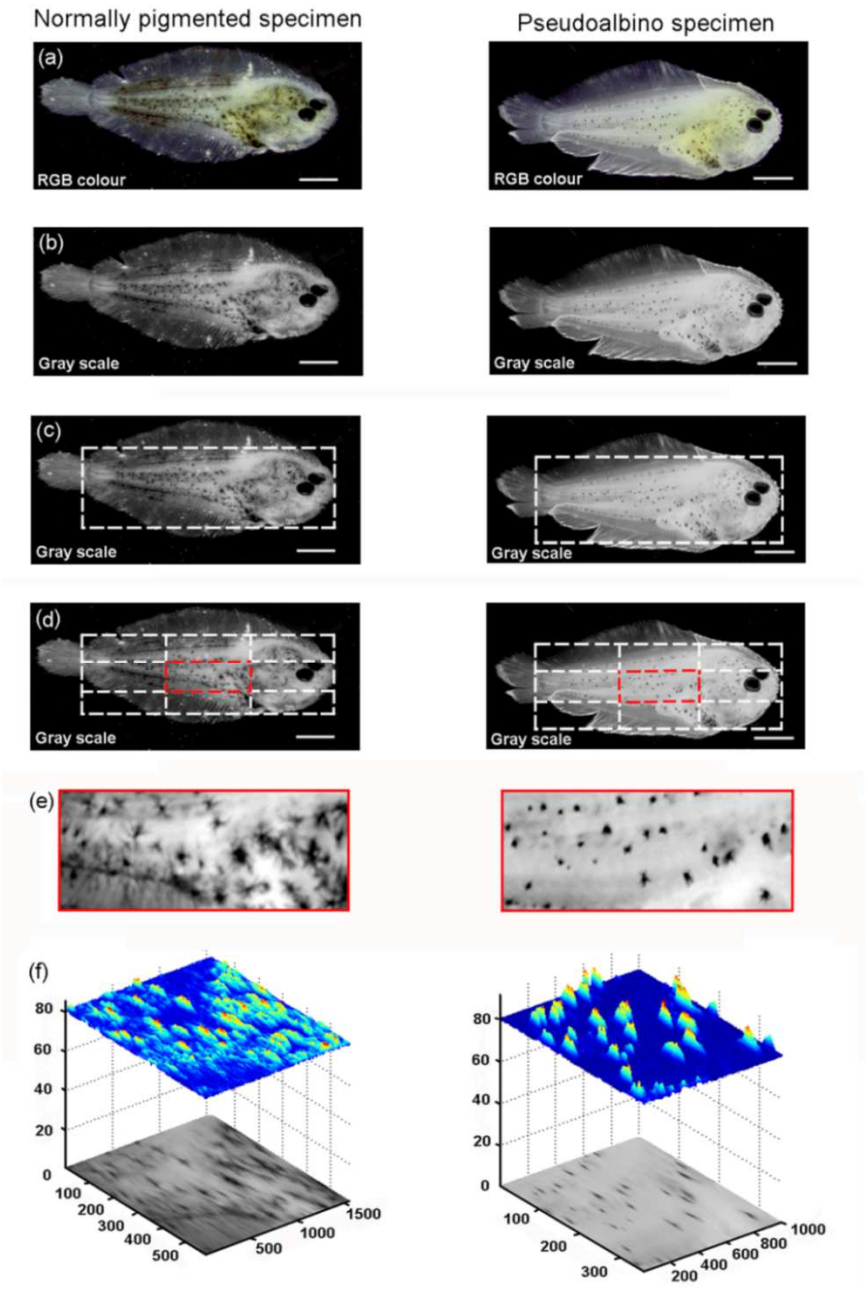


**Figure 2.** Image processing for a normally pigmented (right column) and pseudo-albinos (left column) individuals. (**a**) Picture of a whole larvae in 300 dpi RGB colour image; (**b**) Conversion of the picture to a 300 dpi Gray scale image; (**c**) Image cropping, in accordance to fish dimension (height and weight). A a rectangular area of interest was obtained, centered on the vertebral column, from the basis of head to the tail, and from the dorsal fin to the pelvic fin; (**d**) Image slicing into 9 small areas, based on a fixed number of column and rows (3 x 3), relative to tthe image dimension; (**e**) The chosen area of interest correspond to the middle area including the center of gravity passing through the line of the vertebral column; (**f**) Mesh plots presenting different pigmentation texture and skin intensities (using Matlab meshCanopy).

Four commonly used textural descriptors were chosen to extract textural features from the GLCM (Bharati *et al.*, 2004): 1) the contrast (C, equation 1) indicates the amount of local variations in gray-level intensity between pairs of adjacent pixels, taking into account the heterogeneity and diversity of the repartition of the pixels. A uniform colored skin provides a contrast value of 0, while a value of 1 indicates a skin with a maximum variations of intensity between adjacent pixels mirrored by the highest number of patches of chromatophores as possible; 2) the correlation (R, equation 2) measures how correlated is a pixel to its neighbour over the whole image. In other words, it is a way to measure gray-level linear dependence between the pixels at their specific relative positions. A perfectly positively or negatively correlated image presents a correlation of 1 or -1, while the more irregular is the pattern of chromatophores, the closer to 0 is the correlation value; 3) the energy, also called uniformity or angular second moment (E, equation 3), returns the sum of squared elements in the GLCM. A constant image has an energy value of 1 and this value decreases as the variations of intensity between adjacent pixels increases; 4) the homogeneity (H, equation 4) measures the closeness of the distribution of elements (*i,j*) in the GLCM to the GLCM diagonal. A lower value corresponds to an inhomogeneous image (Fig. 2f).

(1) $\text{Contrast} = \Sigma_{i,j} |i - j|^2 p_{(i,j)}$

(2) $\text{Correlation} = \Sigma_{i,j} \{[(i - \mu_i)(j - \mu_j) p_{(i,j)}] / (\sigma_i \sigma_j)\} |i - j|^2 p_{(i,j)}$

(3) $\text{Energy} = \Sigma_{i,j} p_{(i,j)}^2$

(4) $\text{Homogeneity} = \Sigma_{i,j} (p_{(i,j)}) / (1 + |i - j|)$

where μ and σ represents the means and variances, respectively.

*Prostaglandin analysis*

Pools of larvae at 15 dph (n = 630 larvae) and pools of normally pigmented and pseudo-albino juveniles at 50 dph (n = 180 larvae) were sampled in each replicate tank for each treatment. Samples were extracted with a high-speed homogenizer (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany) in at least 4 volumes of Hank's balanced salt solution containing 0.6 ml of absolute ethanol and 0.2 ml of 2 mM formic acid and the homogenates were frozen at 20 °C. The frozen homogenates were thawed and centrifuged at 3000 g for five minutes to precipitate cell debris. The supernatant (3 and 9 ml for 15 and 50 dph-aged fish, respectively) was submitted to a clean-up step by solid-phase extraction using octadecylsilyl (C18) "Sep-Pak®" minicolumns (Waters, USA), previously conditioned with 5 ml of methanol and 10 ml of distilled water, according to Bell *et al.* (1995) and Powell (1982). After loading, samples were washed with 10 ml of distilled water, followed by 5 ml of 15% ethanol and by 5 ml of hexane:chloroform 65:35 (v:v) and eluted with 10 ml of ethyl acetate. After drying samples under nitrogen, they were redissolved them in 200 µl of methanol and stored in a glass vial at -20 °C before separation of PGE isomers (2 and 3) by reversed-phase high-performance liquid chromatography (RP-HPLC) (Bell *et al.,* 1995). The HPLC instrument consisted of a 510 pump coupled with a 996 photodiode array detector (both from Waters Corp., Massachusetts, USA) and a fraction collector FRAC-100 (Pharmacia Biotech., Uppsala, Sweden). An isocratic elution with a mobile phase containing 17 mM phosphoric acid/acetonitrile (70/30, v/v) was performed at a flow rate of 1

$ml.min^{-1}$ and room temperature on a Zorbax SB-C18 (4·6 x 250 mm, 5 µm; Agilent Tech.) protected with a SecurityGuard C18 pre-column (4mm×2mm, Phenomenex). Elution of PGEs was determined between 18-26 minutes (for $PGE_3$) and between 34-41 minutes (for $PGE_2$), by fractionation of the corresponding PGE standards (2- and 3- series) in independent runs under the same conditions. For samples, fractions with $PGE_2$ and $PGE_3$ were colletected, loaded onto a C18 "Sep-Pak" previously conditioned with 5 ml of methanol and 10 ml of distilled water, then washed with 10 ml of distilled water and finally eluted in 5 ml of ethyl acetate. Eluates were dried under nitrogen stream and redissolved in 500 µl of immunoassay buffer. Measurement of prostaglandins was performed using a $PGE_2$ enzyme immunoassay kit in accordance to the protocol of the manufacturer (Cayman®, USA).

*Statistics*

Results were expressed as mean ± S.E.M. (n = 4). All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA). All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). The arc sine square root transformation was conducted on data expressed as a percentage. A one-way ANOVA was performed among treatments and the post-hoc Tukey's test was performed when significant differences were found ($P < 0.05$). A Chi-squared analysis of frequency was realized on size repartition data. Pearson product moment correlation tests were used to measure the correlation between pigmentation success and dietary and larval contents of selected fatty acids.

**Results**

*Lipid composition of live prey*

Rotifer and *Artemia* metanauplii enriched with AGM and the emulsion containing high levels of ARA (ARA-H) presented similar total lipid and total fatty acid contents, but they significantly differed in their fatty acid profiles (Table 1). Both live prey enriched with AGM contained significantly higher amounts of total SFA, mainly due to major 16:0 content, and higher amounts of 22:5n-6 and DHA than rotifer and *Artemia* enriched with the ARA-H emulsion. Consequently, live prey enriched with AGM presented higher amounts of total n-3 PUFA and PUFA than those enriched with the ARA-H emulsion. Both live prey enriched with the ARA-H emulsion contained significantly higher total MUFA levels than those enriched with AGM due to the higher oleic acid (OA) content, more EPA and higher levels of total n-6 PUFA as they contained higher levels of linoleic acid (LA, 18:2n-6) and ARA. Consequently, the ratios of n-3/n-6 and DHA/EPA were higher in rotifer and *Artemia* enriched with AGM, whereas live prey enriched with the ARA-H emulsion showed higher ratios of ARA/DHA, ARA/EPA and MUFA/PUFA.

**Table 2.** Total lipid and fatty acids contents (mg·g $DW^{-1}$) and fatty acid composition (% of TFA) in Senegalese sole larvae at 2, 15 and 50 dph fed the four dietary treatments (mean ± SD; n = 3). Totals include some minor components not shown. Superscripts letters denote significant differences among diets (ANOVA, $P < 0.05$). <LOQ means that values were under the lowest limit of quantification (0.45% TFA).

| | 2 dph | 15 dph | | | | 50 dph | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | C | ARA-H | ARA-H -/+ | ARA-H | C | ARA-H +/+ | ARA-H -/+ | ARA-H +/- |
| Total lipid | 84.4 | 109.9±19.4 | 82.8±11.8 | 113.4±6.5 | 86.1±3.8 | 86.7±13.9 | 100.6±21.9 | 112.0±6.8 | 104.4±33.0 |
| Total FA | 22.5 | 50.7±12.4 | 31.3±5.6 | 58.2±8.3 | 33.2±5.1 | 34.6±9.4 | 46.3±11.8 | 53.0±4.3 | 46.9±13.2 |
| 14:0 | 0.5 | 0.7±0.2 | 0.4±0.1 | 0.7±0.2 | 0.4±0.1 | 0.7±0.1$^{a}$ | 0.4±0.0$^{b}$ | 0.4±0.0$^{b}$ | 0.6±0.0$^{a}$ |
| 15:0 | 0.0 | 0.1±0.2 | 0.1±0.2 | 0.1±0.1 | 0.2±0.2 | 0.2±0.2 | 0.1±0.1 | 0.2±0.1 | 0.2±0.1 |
| 16:0 | 18.6 | 11.8±0.8 | 11.3±1.1 | 11.0±0.9 | 11.0±1.1 | 14.1±1.0 | 10.8±0.2 | 10.4±1.2 | 12.2±0.9 |
| 18:0 | 10.3 | 7.5±0.4$^{ab}$ | 9.6±0.4$^{a}$ | 7.0±0.5$^{b}$ | 9.1±1.0$^{ab}$ | 9.6±0.8 | 7.7±0.1 | 7.4±0.9 | 8.1±0.5 |
| 20:0 | 0.0 | <LOQ | <LOQ | 4.3±8.7 | 5.1±10.1 | <LOQ | <LOQ | <LOQ | <LOQ |
| Total saturated | 29.4 | 20.2±1.4 | 21.3±1.2 | 23.1±7.7 | 25.8±8.7 | 24.6±2.0 | 19.0±0.2 | 18.4±2.1 | 21.1±1.5 |
| 16:1 | 3.8 | 1.4±0.5 | 2.4±0.6 | 1.5±0.1 | 2.2±0.6 | 1.4±0.5 | 2.1±0.5 | 2.2±0.4 | 1.2±0.3 |
| 18:1n-9 | 10.0 | 13.3±1.9 | 22.2±4.6 | 10.9±1.0 | 18.8±2.4 | 14.3±0.4$^{b}$ | 22.3±0.9$^{a}$ | 22.8±0.5$^{a}$ | 13.1±0.3$^{b}$ |
| 18:1n-7 | 5.6 | 1.3±1.5 | 3.5±2.4 | 3.2±1.1 | 4.9±1.4 | 3.7±1.1 | 5.2±1.0 | 4.9±0.2 | 2.8±0.5 |
| 20:1n-9 | 0.0 | 0.2±0.2 | 1.2±0.7 | 0.2±0.2 | 1.2±0.7 | 0.4±0.1$^{b}$ | 1.4±0.3$^{a}$ | 1.4±0.3$^{a}$ | 0.3±0.1$^{b}$ |
| Total | 19.4 | 16.2±0.6$^{b}$ | 29.4±1.9$^{a}$ | 15.8±1.3$^{b}$ | 27.1±3.8$^{a}$ | 19.8±2.0$^{b}$ | 31.0±0.5$^{a}$ | 31.3±0.9$^{a}$ | 17.4±0.5$^{b}$ |
| 18:2n-6 | 3.2 | 3.1±0.4$^{b}$ | 8.0±0.3$^{a}$ | 3.2±0.3$^{b}$ | 7.8±0.8$^{a}$ | 3.6±0.3$^{b}$ | 6.9±0.4$^{a}$ | 6.6±0.4$^{a}$ | 3.3±0.2$^{b}$ |
| 18:3n-6 | 0.7 | 0.9±0.0 | 1.1±0.1 | 0.9±0.1 | 1.0±0.2 | 1.0±0.1 | 1.2±0.1 | 1.3±0.1 | 1.1±0.1 |
| 20:2n-6 | 0.0 | <LOQ | <LOQ | 0.8±1.6 | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| 20:3n-6 | 0.0 | 0.2±0.2 | 0.6±0.4 | 0.2±0.1 | 0.5±0.4 | 0.4±0.1$^{b}$ | 0.7±0.1$^{a}$ | 0.7±0.1$^{a}$ | 0.4±0.1$^{b}$ |
| 20:4n-6 | 3.2 | 4.4±0.6$^{b}$ | 10.9±0.8$^{a}$ | 4.0±0.8$^{b}$ | 10.3±1.6$^{a}$ | 4.1±0.2$^{b}$ | 10.2±0.3$^{a}$ | 9.6±0.9$^{a}$ | 4.2±0.1$^{b}$ |
| 22:4n-6 | 0.6 | 0.2±0.0 | 0.3±0.1 | 0.2±0.0 | 0.3±0.0 | 0.3±0.0$^{b}$ | 0.6±0.0$^{a}$ | 0.6±0.0$^{a}$ | 0.4±0.0$^{b}$ |
| 22:5n-6 | 0.4 | 8.2±1.1$^{a}$ | 0.8±0.4$^{b}$ | 7.5±0.8$^{a}$ | 0.8±0.2$^{b}$ | 6.7±0.7$^{b}$ | 0.9±0.0$^{a}$ | 1.1±0.3$^{a}$ | 7.5±0.3$^{b}$ |
| Total n-6 PUFA | 8.1 | 17.1±1.3 | 21.7±0.5 | 16.8±2.2 | 20.9±2.8 | 16.1±0.5$^{b}$ | 20.6±0.2$^{a}$ | 19.9±0.8$^{a}$ | 16.8±0.3$^{b}$ |
| 18:3n-3 | 0.6 | 11.9±0.4 | 10.9±1.6 | 11.7±1.6 | 9.9±1.7 | 10.9±0.9 | 14.4±0.8 | 16.0±2.6 | 12.1±0.7 |
| 18:4n-3 | 0.0 | 1.5±0.1$^{a}$ | 0.8±0.1$^{b}$ | 1.5±0.2$^{a}$ | 0.8±0.2$^{b}$ | 1.2±0.2 | 1.6±0.1 | 1.7±0.3 | 1.6±0.1 |
| 20:4n-3 | 0.0 | 0.6±0.4 | 0.3±0.2 | 0.8±0.2 | 0.5±0.1 | 0.7±0.1 | 0.7±0.1 | 0.8±0.1 | 0.8±0.0 |
| 20:5n-3 | 4.7 | 4.2±0.3$^{a}$ | 2.1±0.1$^{b}$ | 3.9±0.4$^{a}$ | 2.0±0.3$^{b}$ | 2.1±0.3$^{a}$ | 1.6±0.0$^{b}$ | 1.6±0.1$^{b}$ | 2.7±0.2$^{a}$ |
| 22:5n-3 | 4.8 | 1.9±0.1 | 1.8±0.1 | 2.0±0.2 | 1.7±0.2 | 1.9±0.3 | 1.8±0.1 | 1.7±0.2 | 2.3±0.1 |
| 22:6n-3 | 33.0 | 25.3±1.0$^{a}$ | 8.7±0.8$^{b}$ | 22.3±2.4$^{a}$ | 8.6±0.7$^{b}$ | 20.0±2.6$^{a}$ | 6.1±0.7$^{b}$ | 5.6±0.9$^{b}$ | 22.8±1.0$^{a}$ |
| Total n-3 PUFA | 43.1 | 45.4±1.5$^{a}$ | 24.7±0.8$^{b}$ | 42.1±4.7$^{a}$ | 23.5±3.1$^{b}$ | 36.7±3.9$^{a}$ | 26.2±0.9$^{b}$ | 27.3±2.1$^{b}$ | 42.2±2.0$^{a}$ |
| Total PUFA | 51.2 | 62.5±2.6 | 46.3±1.2 | 58.9±6.7 | 44.4±5.8 | 52.8±4.4$^{a}$ | 46.8±0.9$^{b}$ | 47.3±2.1$^{b}$ | 59.1±2.1$^{a}$ |
| (n-3)/(n-6) | 5.3 | 2.7±0.1$^{a}$ | 1.1±0.0$^{b}$ | 2.5±0.2$^{a}$ | 1.1±0.0$^{b}$ | 2.3±0.2$^{a}$ | 1.3±0.0$^{b}$ | 1.4±0.1$^{b}$ | 2.5±0.1$^{a}$ |
| DHA/EPA | 7.1 | 6.0±0.3$^{a}$ | 4.2±0.6$^{b}$ | 5.7±0.1$^{a}$ | 4.3±0.4$^{b}$ | 9.5±0.7$^{a}$ | 3.8±0.5$^{b}$ | 3.5±0.7$^{b}$ | 8.4±0.2$^{a}$ |
| ARA/DHA | 0.1 | 0.2±0.0$^{b}$ | 1.3±0.2$^{a}$ | 0.2±0.0$^{b}$ | 1.2±0.1$^{a}$ | 0.2±0.0$^{b}$ | 1.7±0.2$^{a}$ | 1.8±0.2$^{a}$ | 0.2±0.0$^{b}$ |
| ARA/EPA | 0.7 | 1.1±0.1$^{b}$ | 5.2±0.3$^{a}$ | 1.0±0.1$^{b}$ | 5.2±0.1$^{a}$ | 1.9±0.2$^{b}$ | 6.3±0.2$^{a}$ | 6.0±0.7$^{a}$ | 1.6±0.1$^{b}$ |
| MUFA/PUFA | 0.4 | 0.3±0.0$^{b}$ | 0.6±0.0$^{a}$ | 0.3±0.0$^{b}$ | 0.6±0.0$^{a}$ | 0.4±0.1$^{b}$ | 0.7±0.0$^{a}$ | 0.7±0.0$^{a}$ | 0.3±0.0$^{b}$ |

*Dietary effects on lipid and fatty acid composition of larvae*

Larvae at 2 dph contained particularly high proportions of DHA, 16:0, OA, 18:0, EPA and 22:5n−3 (docosapentaenoic acid, DPA) (Table 2), resulting in high n−3/n−6 and DHA/EPA ratios. The fatty acid composition of larvae at the end of both experimental periods (15 and 50 dph) reflected the fatty acid profile of the enriched rotifer and *Artemia* offered to them. At the end of the pro-metamorphic stage (15 dph), larvae fed live prey enriched with AGM (C and ARA-H -/+ treatments) presented a significantly different pattern of fatty acid accumulation than larvae fed live prey enriched with the ARA-H emulsion (ARA-H +/+ and ARA-H +/- treatments). Larvae fed ARA-H-enriched live prey presented significantly higher contents of total MUFA, LA, ARA and lower amounts of EPA and DHA than larvae fed AGM-enriched live prey. This resulted in significantly lower n-3 PUFA amounts and a lower n-3/n-6 ratio, but

higher ARA/DHA, ARA/EPA and MUFA/PUFA ratios. At the end of the experiment (50 dph), this particular fatty acid profile was observed in post-metamorphic larvae fed ARA-H-enriched *Artemia* during the second experimental period. Larvae from the ARA-H +/+ and ARA-H -/+ groups presented significantly higher amounts of OA, total MUFA, LA, ARA, total n-6 PUFA, and lower content of EPA, DHA, total n-3 PUFA and total PUFA than larvae from the C and ARA-H +/- diets, ensuing significantly lower n-3/n-6 and DHA/EPA ratios and higher ARA/DHA, ARA/EPA and MUFA/PUFA ratios.

*Dietary effects on larval survival, growth and metamorphosis*

Survival rates in this study were very high with mean values comprised between 96.4 and 98.4%, depending on the dietary treatment. Survival was significantly higher in larvae from the ARA-H +/+ group than in larvae from the C and ARA-H -/+ treatments ($P < 0.001$; Table 3). Larvae the ARA-H -/+ group were significantly longer than larvae from the C group at 15 dph ($P = 0.012$) and significantly longer than larvae from the other treatments at 50 dph ($P < 0.002$). At 50 dph, the smallest larvae in SL were those from ARA-H +/- treatment. At 15 dph, dry weight was not significantly affected by any dietary treatment at 15dph ($P > 0.05$), whereas larvae from ARA-H -/+ treatment were significantly heavier than the larvae from the ARA-H +/- group at 50 dph ($P = 0.024$). SGR values from the ARA-H -/+ group were significantly higher than those of the other treatments ($P = 0.002$). Size distribution in SL of fish aged 50 dph was significantly affected by the dietary treatments ($\chi^2 = 129.685$; df = 27; $P < 0.001$, Fig. 3). Most of the post-metamorphic fish fed the ARA-H -/+ treatment measured 11 mm, the longest until 15 mm, whereas most of the individuals fed the other treatments measured from 9 to 10 mm and the longest until 12 mm.

**Table 3.** Survival rate (in %), larval size in dry weight (DW, in mg) and standard length (SL, in mm) at 15 and 50 dph and specific growth rate (SGR. in % $d^{-1}$) at pre-metamorphic (2-15 dph) stage, metamorphic (15-50 dph) stage and during the whole period of the experiment (2-50 dph) of Senegalese sole larvae fed the four different dietary treatments (mean ± S.E.M., n = 4). Initial standard length and dry weight of larvae were 3.07 ± 0.02 mm and 36.7 ± 1.6 µg at 2dph. Superscript letters indicate significant differences among dietary treatments (ANOVA, $P < 0.05$).

| | | C | ARA-H +/+ | ARA-H -/+ | ARA-H +/- |
|---|---|---|---|---|---|
| | **Survival rate** | $97.3\pm0.15^{bc}$ | $98.4\pm0.14^{a}$ | $96.4\pm0.45^{c}$ | $98.2\pm0.18^{ab}$ |
| **15 dph** | **DW** | 0.09±0.02 | 0.10±0.01 | 0.11±0.02 | 0.10±0.02 |
| | **SL** | $4.18\pm0.05^{b}$ | $4.36\pm0.05^{ab}$ | $4.41\pm0.06^{a}$ | $4.31\pm0.05^{ab}$ |
| **50 dph** | **DW** | $1.92\pm0.14^{ab}$ | $1.71\pm0.19^{ab}$ | $3.14\pm0.64^{a}$ | $1.53\pm0.07^{b}$ |
| | **SL** | $8.75\pm0.13^{b}$ | $8.71\pm0.11^{bc}$ | $10.40\pm0.14^{a}$ | $8.30\pm0.11^{c}$ |
| **2-15 dph** | | 0.067±0.009 | 0.073±0.010 | 0.082±0.016 | 0.077±0.012 |
| **15-30 dph** | **SGR** | 0.088±0.003 | 0.083±0.002 | 0.097±0.007 | 0.078±0.004 |
| **2-50 dph** | | $0.082\pm0.001^{b}$ | $0.080\pm0.002^{b}$ | $0.093\pm0.004^{a}$ | $0.078\pm0.000^{b}$ |

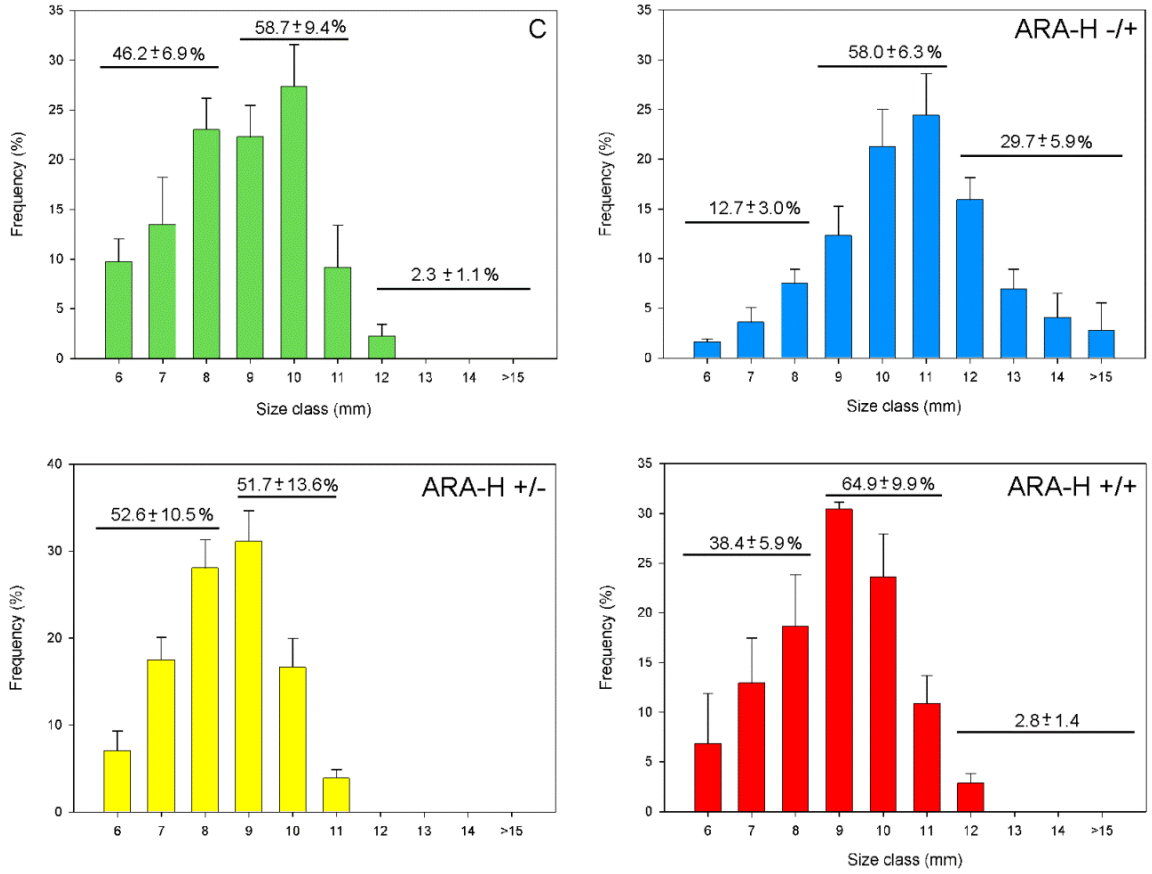


**Figure 3.** Final size (standard length, in mm) distribution of Senegalese sole larvae fed the four dietary treatments. The numbers on each histograms indicate, from left to right, the mean ± S.D. percentage of individuals measuring between 6 and 8 mm; between 9 and 11 mm and above 12 mm.

The process of eye migration in Senegalese sole larvae was not significantly affected by any dietary treatment (Table 4). No differences in $I_{EM}$ were detected during the whole metamorphosis process, among the four experimental groups ($P > 0.05$).

**Table 4**. Eye migration index ($I_{EM}$) of Senegalese sole larvae fed *Artemia* nauplii enriched the different commercial products at 9, 15, 22, 30, 42 and 50 dph ( mean ± S.E.M., n = 4). No significant differences (ANOVA, $P > 0.05$) were detected among dietary treatments for any sampled day.

| Age (days post hatching) | C | ARA-H +/+ | ARA-H -/+ | ARA-H +/- |
|---|---|---|---|---|
| **9** | 0.69±0.18 | 0.86±0.03 | 0.69±0.05 | 0.89±0.04 |
| **15** | 1.04±0.04 | 1.12±0.03 | 1.14±0.05 | 1.15±0.04 |
| **22** | 2.97±0.04 | 3.04±0.06 | 3.25±0.16 | 2.71±0.08 |
| **30** | 4.73±0.16 | 5.10±0.15 | 5.43±0.08 | 4.87±0.17 |
| **42** | 5.94±0.23 | 5.86±0.14 | 5.93±0.06 | 5.80±0.18 |
| **50** | 5.94±0.02 | 5.96±0.02 | 5.98±0.02 | 5.93±0.07 |

*Dietary effects on pigmentation, melanophore aspect and density and pigmentation textural features*

At 50 dph, the frequency of normally pigmented specimens was significantly affected by the dietary regimes ($P < 0.001$, Table 5). Fish from the C and ARA-H -/+ treatments showed the highest rates of normally pigmented specimens (99 and 80%, respectively). In contrast, fish from the ARA-H +/+ and ARA-H +/- groups showed the highest rates of pseudo-albinism (81 and 52% respectively). The incidence of normally pigmented specimens was significantly and positively correlated with the levels of total SFA, DHA, total n-3 PUFA, total PUFA and with the ratios of n-3/n-6 and DHA/EPA in enriched rotifer and *Artemia* metanauplii ($R = 0.87$; $P < 0.001$; $n = 16$), whereas it was significantly and negatively correlated with the content of OA, total MUFA, LA, ARA, total n-6 PUFA, EPA and with the ratios ARA/EPA, ARA/DHA and MUFA/PUFA in enriched rotifer and *Artemia* ($R = -0.87$; $P < 0.001$; $n = 16$).

The correlation analysis between the fatty acid composition of pro-metamorphic larvae aged 15 dph and pigmentation results in post-metamorphic fish at 50 dph revealed that EPA ($R = 0.82$; $P < 0.001$; $n = 16$), DHA ($R = 0.88$; $P < 0.001$; $n = 16$), total n-3 PUFA ($R = 0.83$; $P < 0.001$; $n = 16$), total PUFA ($R = 0.74$; $P < 0.01$; $n = 16$) and n-3/n-6 ratio ($R = 0.81$; $P < 0.001$; $n = 16$) were significantly correlated with a successful pigmentation. In addition, the frequency of normally pigmented post-metamorphic specimens was significantly and negatively correlated with the content in OA ($R = -0.81$; $P < 0.001$; $n = 16$), total MUFA ($R = -0.88$; $P < 0.001$; $n = 16$), LA ($R = -0.89$; $P < 0.001$; $n = 16$), ARA ($R = -0.85$; $P < 0.001$; $n = 16$), total n-6 PUFA ($R = -0.72$; $P < 0.001$; $n = 16$) and with their ratios of ARA/DHA ($R = -0.87$; $P < 0.001$; $n = 16$), ARA/EPA ($R = -0.86$; $P < 0.001$; $n = 16$), MUFA/PUFA ($R = -0.88$; $P < 0.001$; $n = 16$) in larvae aged 15 dph. No significant correlation was found between the incidence of normally-pigmented fish and their body fatty acid composition at 50 dph.

Pseudo-albino post-metamorphic specimens had a lower density of melanophores than normally pigmented ones regardless the dietary treatment considered ($P < 0.001$, Table 5). Normally pigmented larvae presented similar values of melanophore density among dietary treatments, while pseudo-albino fish from the ARA-H +/+ treatment showed a lower density of melanophores in comparison to the pseudo-albino specimens from the ARA-H -/+ and ARA-H +/- treatments ($P < 0.001$; Table 5), which presented similar number of melanophores.

The shape of melanophores differed between normally pigmented and pseudo-albino specimens. Pigmented individuals mainly displayed dendritic melanophores, whereas pseudo-albinos presented abundant round-shaped melanophores. We have suggested that such a diference between both skin pigmentation phenotypes is because melanophores in pseudo-albinos have a reduced ability to disperse melanin within melanosomes (Darias *et al.*, 2013b) This physiological disfunctioning leads to the impression that pesudoalbinos present smaller melanophores. Moreover, normally pigmented post-metamorphic fish from the C group seemed to have more star-shaped melanophores than those from the three other dietary treatments.

**Table 5.** Frequency of normally pigmented larvae (in %), density of melanophores and parameters of texture feature analysis in normal and albino larvae in 50 dph-aged Senegalese sole fed with the four different dietary treatments (mean ± S.E.M., n = 30). Capital superscript letters indicate significant differences in pools of normal larvae among dietary treatments, while lowercase superscript letters indicate significant differences in pools of albinos larvae among dietary treatments (ANOVA, P < 0.05). For all parameters, significant differences were found between normal and albinos larvae within a same dietary treatment. For all significant one-way ANOVA test, P < 0.001. ROI: region of interest.

| | C | ARA-H +/+ | | ARA-H -/+ | | ARA-H +/- | |
|---|---|---|---|---|---|---|---|
| **Pigmentation (%)** | 99.1±0.3$^{a}$ | 18.6±7.5$^{c}$ | | 80.0±8.6$^{a}$ | | 47.8±2.7$^{b}$ | |
| | **Normally Pigmented** | **Normally Pigmented** | **Pseudo-albinos** | **Normally Pigmented** | **Pseudo-albinos** | **Normally Pigmented** | **Pseudo-albinos** |
| **Number of melanophores/ROI** | 2.71±0.014$^{A}$ | 2.74±0.056$^{A}$ | 1.52±0.098$^{b}$ | 2.79±0.076$^{A}$ | 1.98±0.056$^{a}$ | 2.94±0.012$^{A}$ | 1.95±0.097$^{a}$ |
| **"Texture feature analysis"** | | | | | | | |
| **Contrast** | 0.15±0.004$^{B}$ | 0.14±0.004$^{B}$ | 0.09±0.003 | 0.15±0.005$^{B}$ | 0.09±0.004 | 0.18±0.005$^{A}$ | 0.09±0.003 |
| **Correlation** | 0.91±0.003$^{A}$ | 0.92±0.003$^{A}$ | 0.91±0.004$^{b}$ | 0.90±0.003$^{B}$ | 0.92±0.003$^{a}$ | 0.91±0.002$^{A}$ | 0.94±0.002$^{a}$ |
| **Energy** | 0.24±0.006$^{B}$ | 0.24±0.009$^{B}$ | 0.42±0.019$^{a}$ | 0.30±0.102$^{A}$ | 0.36±0.012$^{b}$ | 0.18±0.005$^{C}$ | 0.32±0.010$^{b}$ |
| **Homogeneity** | 0.92±0.002$^{A}$ | 0.93±0.002$^{A}$ | 0.96±0.003 | 0.93±0.002$^{A}$ | 0.96±0.002 | 0.91±0.002$^{B}$ | 0.96±0.001 |

Values for the four parameters obtained in the pigmentation texture analysis – contrast, energy, homogeneity and correlation – were significantly different between pseudo-albino and normally pigmented fish from all dietary treatments ($P$ < 0.001, Table 5). In this sense, contrast values were higher in normally pigmented specimens than in pseudo-albino ones ($P$ < 0.001), while energy and homogeneity values were higher in pseudo-albino specimens ($P$ < 0.001), indicating a more uniform aspect of the skin in the pseudo-albino fish and more heterogeneous and patchiness pigmentation pattern in normally pigmented individuals. Correlation values were close to 1 for normally pigmented and pseudo-albino individuals from all dietary treatments. However, the correlation was higher in pseudo-albino than in normally pigmented specimens from the ARA-H -/+ and ARA-H +/- treatments, and the contrary in individuals from the ARA-H +/+ group ($P$ < 0.001), suggesting a more homogeneous skin in pseudo-albino fish than in normally pigmented post-metamorphic specimens fed the two first mentioned groups. Surprisingly, according to correlation values, pseudo-albino individuals from ARA-H +/+ treatment presented a less homogeneous skin aspect than normally pigmented specimens from this same group. Comparing normally pigmented individuals among different dietary treatments, fish from the ARA-H +/- group presented significantly higher values of contrast and lower values of homogeneity and energy than individuals from the other groups ($P$ < 0.001), reflecting a more heterogeneous texture of their skin. Meanwhile, fish from the ARA-/+ group had significantly lower values of correlation and higher values of energy than individuals from the other groups ($P$ < 0.001), indicating a more uniform skin in these fish. Concerning pseudo-albinos, contrast and homogeneity values were not significantly different among dietary treatments ($P$ > 0.05), while energy

and correlation values were higher and lower ($P$ < 0.001), in specimens from the ARA-H +/+ treatment, respectively, which may indicate a higher uniformity of their skin than in pseudo-albino specimens from the other groups.

*Prostaglandin analysis*

The production of prostaglandins of the 2- and 3-series in 15 dph-aged Senegalese sole larvae was not significantly affected by the four dietary treatments (Table 6, $P$ > 0.05). However, at 50 dph, pseudo-albino specimens fed the ARA-H +/+ and ARA-H -/+ diets presented the highest $PGE_2$ concentrations (Table 6, $P$ < 0.001). Fish fed low dietary ARA levels from 15 to 50 dph presented the lowest $PGE_2$ concentrations, with normally pigmented individuals fed the control diet, normally pigmented and pseudo-albino specimens fed the ARA-H +/- diet showing respectively 7.9, 13.1 and 7.8-fold lower $PGE_2$ concentrations than pseudo-albino specimens fed the ARA-H +/+ and ARA-H -/+ diets. Normally pigmented fish fed the ARA-H +/+ and ARA-H -/+ diets presented intermediate $PGE_2$ concentrations, between 2.3-fold lower than the highest $PGE_2$ concentrations and 6.0-fold higher than the lowest $PGE_2$ production. Pseudo-albino individuals fed the ARA-H -/+ diet showed a significantly 2.3-fold higher $PGE_2$ concentration than normally pigmented individuals fed the same diet.

**Table 6.** $PGE_2$ and $PGE_3$ prostaglandins production (in $pg.g^{-1}$ w.w.) by Senegalese sole larvae at 15 dph and by normally pigmented and pseudo-albinos juveniles at 50 dph, fed the 4 dietary treatments (mean ± S.E.M., n = 4). Superscript letters indicate significant differences in pools of 50 dph-aged normally pigmented and pseudo-albinos specimens fed the 4 different dietary treatments (ANOVA, P < 0.05). Asterisks indicate significant differences (Student t-test, P < 0.05) between normally pigmented and pseudo-albinos fish within a same dietary treatment.

| Age | | Treatment | $PGE_2$ ($pg.g^{-1}$ w.w.) | $PGE_3$ ($pg.g^{-1}$ w.w.) |
|---|---|---|---|---|
| 15 dph | | C | 32.31±8.59 | 138.58±53.18 |
| | | ARA-H +/+ | 12.32±3.40 | 218.32±52.12 |
| | | ARA-H -/+ | 19.09±0.69 | 130.21±9.92 |
| | | ARA-H +/- | 12.58±2.52 | 206.76±41.35 |
| 50 dph | Normally pigmented | C | 30.92±8.53$^{c}$ | 18.26±6.33$^{b}$ |
| | | ARA-H +/+ | 118.28±18.47$^{abc}$ | 7.01±1.40$^{b*}$ |
| | | ARA-H -/+ | 102.28±29.60$^{bc*}$ | 42.15±9.15$^{a}$ |
| | | ARA-H +/- | 18.62±6.37$^{c}$ | 47.49±1.93$^{a}$ |
| | Pseudo-albinos | ARA-H +/+ | 247.35±48.36$^{a}$ | 19.98±3.15$^{b*}$ |
| | | ARA-H -/+ | 238.39±42.65$^{a*}$ | 14.67±5.83$^{b}$ |
| | | ARA-H +/- | 31.48±10.78$^{c}$ | 31.84±5.44$^{ab}$ |

At 50 dph, $PGE_3$ concentration was the highest in normally pigmented fish fed the ARA-H -/+ and ARA-H +/- diets (P = 0.002). Pseudo-albino individuals fed the ARA-H +/- diet presented intermediate $PGE_3$ concentrations. Normally pigmented fish fed the control and the ARA-H +/+ diets

and pseudo-albino specimens fed the ARA-H +/+ and ARA-H -/+ diets showed the lowest $PGE_3$ concentrations, respectively 2.4, 6.4, 2.3 and 3.0-fold lower than normally pigmented fish fed the ARA-H -/+ and ARA-H +/- diets. Pseudo-albino specimens fed the ARA-H +/+ diet had a significantly 2.9-fold higher $PGE_3$ concentration than normally pigmented fish fed the same dietary treatment.

**Discussion**

The fatty acid profile of Senegalese sole larvae at the end of pro-metamorphic (15 dph) and post-metamorphic (50 dph) periods reflected the differences observed in the composition of the diet provided, which was consistent with previous studies in various flatfish species (Copeman *et al.*, 2002; Villalta *et al.*, 2005a, b; Lund *et al.*, 2008; Boglino *et al.*, 2012a, b). In pro- (15 dph) and post-metamorphic (50 dph) specimens, larvae fed live prey enriched with ARA-H emulsion showed higher content of ARA, LA and total n-6 PUFA, but also in OA and total MUFA, due to the inclusion of olive oil in the emulsion used for enriching live prey with high levels of ARA, and lower amounts of EPA, DHA and total n-3 PUFA than in the larvae fed AGM-enriched live prey. As a result, lower n-3/n-6 and DHA/EPA ratios, but higher ARA/DHA, ARA/EPA and MUFA/PUFA ratios were observed in larvae fed the ARA-H diet than in those fed the AGM diet. Due to the competitive interactions between these EFA (DHA, EPA and ARA) and the importance of the balance between EFA requirements and substrates for energy-generating fatty acids (especially SFA and MUFA) in growing larvae, disrupting the relative proportions of these EFA may affect basic functions that lead to the final phenotype (Izquierdo *et al.*, 2000; Sargent *et al.*, 1999). Moreover, shifts in the enriched live prey given to the larvae induced alterations of their fatty acid profile concomitant with the new prey administered, (ARA-H -/+ and ARA-H +/- diets)demonstrating the rapid response of the larvae to the alterations in the dietary fatty acid profile (Lund *et al.*, 2008). This rapid change in fatty acids was most likely related to the relatively fast growth during early stages of development (Conceição *et al.*, 2007).

Larval survival rates were high similarly to what has been obtained in previous studies on this species (Boglino *et al.*, 2012a, b; Morais *et al.*, 2005a, b). Furthermore, a slightly higher survival rate was found in larvae fed high levels of ARA (10.2% of TFA) during at least their pre- and pro-metamorphic stages (ARA-H +/+ and ARA-H +/-) than in larvae fed low levels of ARA (1.0% of TFA) (C and ARA-H -/+) as reported in summer flounder *Paralichthys dentatus* larvae fed 6% ARA during pre-metamorphosis (Willey *et al.*, 2003). In contrast, other studies have shown that high dietary ARA provided during pre-, pro- and post-metamorphic larval stages did not affect survival in Senegalese sole (Villalta *et al.*, 2005b; Boglino *et al.*, 2012b), as in other flatfish species, such as common sole *S. solea* (Lund *et al.*, 2008), halibut *Hippoglossus hippoglossus* (McEvoy *et al.*, 1998), turbot *Scophthalmus maximus* (Estévez *et al.*, 1999), yellowtail flounder *Limanda ferruginea* (Copeman *et al.*, 2002) and Japanese flounder *Paralichthys olivaceus* (Estévez *et al.*, 2001).

Dietary ARA levels provided at pre-, pro- and/or post-metamorphic periods significantly affected Senegalese sole larval growth and size distribution. Thus, larvae fed high levels of ARA during the post-metamorphic period grew faster than those from the other treatments. Dietary ARA provided at 1% during larval development, as commonly used in commercial hatcheries (AGM diet), did not result in the best growth, indicating that ARA requirements for Senegalese sole change gradually during development. In previous studies with Senegalese sole, dietary levels from 0.1 to 8.3% ARA fed to larvae from 3 to 37 dph (Villalta *et al.*, 2005b) or increasing dietary ARA contents from 1.0 to 7% in live prey fed to larvae from 8 to 50 dph (Boglino *et al.*, 2012b) did not affect larval growth. In common sole, increasing ARA dietary levels from 0 to 24%, combined with 0 or 20% EPA in pre- and pro-metamorphic stages also had no affect on larval growth (Lund *et al.*, 2008). High dietary ARA amounts supplied to larvae during pre- and pro-metamorphic periods did not affect the growth in other flatfish species, such as halibut (McEvoy *et al.*, 1998), turbot (Estévez *et al.*, 1999), yellowtail flounder (Copeman *et al.*, 2002) and Japanese flounder (Estévez *et al.*, 2001). Moreover, as MUFA, especially OA, represent a source of energy for developing larvae that can be catabolized via β-oxidation (Sargent *et al.*, 2002), Higher MUFA requirement or availability during metamorphosis might also explain the higher growth of larvae from the ARA-H -/+ treatment, the only group among the four fed such a high MUFA/PUFA ratio from pre-metamorphosis (0.2) to metamorphic and post-metamorphic stages (0.7).

In this study, eye migration rate was not affected by the dietary treatments supplied to the larvae during pre- and pro-metamorphosis and larvae completed metamorphosis around 30 dph, regardless of the dietary treatment used and the developmental stage at which administration of experimental diets began. In other flatfish species growth and eye migration process have frequently been significantly correlated (Lund *et al.*, 2008; Næss and Lie, 1998), but this process was delayed in Senegalese sole when larvae were fed ARA levels from 0.1 to 8.3%, DHA from 0.0 to 14.7%, or SFA from 38.0 to 22.1%, and it was more advanced when the larvae were fed EPA from 0.3 to 29.5% and MUFA from 50.7 to 21.0% (Villalta *et al.*, 2005a, b, 2008).

Larvae fed high dietary ARA levels during the whole experiment showed the highest incidence of pigmentary disorders (81.4% in ARA-H +/+ treatment). The incidence of pigmentary anomalies were positively correlated with ARA dietary levels as well as with ARA/EPA, ARA/DHA, EPA/DHA and MUFA/PUFA ratios. Previous studies considered DHA and the EPA/DHA ratio as the key factors involved in the proper development of juvenile pigmentation in flatfishes (Kanazawa, 1993; Rainuzzo *et al.*, 1994; Reitan *et al.*, 1994; Devresse *et al.*, 1994; Dhert *et al.*, 1994; Venizelos and Benetti, 1999). However, during the last decade, evidence has been found that refutes the implication of DHA in flatfish pigmentation (Copeman *et al.*, 2002; Lund *et al.*, 2007; Villalta *et al.*, 2005a). On the other hand, high dietary ARA levels have been associated to pigmentary disorders in several flatfish species and it has been suggested to be involved in the underlying process of pigment cell differentiation (Darias *et al.*, 2013b). Villalta *et al.* observed 84.2% of abnormally pigmented fish when fed 8.3% ARA

from 3 to 37 dph, whereas only 0.3% of malpigmented larvae were found when they were fed 0.1% ARA (Villalta *et al.*, 2005a). In yellowtail flounder larvae fed 8.9% ARA and 36% DHA for four weeks showed 92% incidence of malpigmentation pointing to the negative effects of high dietary ARA levels on pigmentation (Copeman *et al.*, 2002).

Besides absolute proportions of ARA in the diet, imbalances in the dietary ARA/EPA ratio have also been proved to lead to pigmentation problems (Reitan *et al.*, 1994; Curé *et al.*, 1995; Bell and Sargent, 2003; Villalta *et al.*, 2005a; Hamre *et al.*, 2005). Positive correlations have been found between dietary levels of ARA and the ARA/EPA ratio and the incidence of pseudo-albinism in turbot, halibut and Japanese flounder, with ARA/EPA ratios higher than 1:4, fed to larvae until metamorphosis, being detrimental for proper pigmentation (McEvoy *et al.*, 1998; Estévez *et al.*, 1999; 2001). In the present study, the ARA/EPA ratio in live prey enriched with both enrichments were all above 1:4. However, rotifer and *Artemia* metanauplii displayed a ARA/EPA ratio above 1 when enriched with the experimental emulsion (1.6 and 1.8, respectively) and under 1 when enriched with AGM (0.4 and 0.3, respectively). There was a clear and significant implication of ARA and EPA in the incidence of malpigmented fish, with higher amounts of ARA than EPA leading to the incidence of pigmentary disorders. Indeed, ARA and EPA display competitive interactions as substrates for cyclo-oxygenase and lipo-oxygenase enzymes, which are involved in prostaglandin biosynthesis pathways, especially considering that prostaglandin (PG) series 2 (ARA-derived) and 3 (EPA-derived) are potent regulators of metabolism with opposite effects (Bell and Sargent, 2003; Hamre *et al.*, 2005). The balance in the synthesis, relative concentrations and effects of PGs from the two series is dependent on a balanced dietary intake of both fatty acids (Logue *et al.*, 2000; Hamre *et al.*, 2005). Furthermore, although the mechanisms are yet unknown, excess of $PGE_2$ production has been suggested to cause biochemical stress and to be involved in malpigmentation (Sargent *et al.*, 1999; Brandsen *et al.*, 2005; Villalta *et al.*, 2005a, 2007; Lund *et al.*, 2008). This concurs with our study, where fish fed high dietary ARA levels during the post-metamorphic stage presented higher levels of $PGE_2$ and were associated with malpigmentations, which is also in agreement with a previous study on this species (Villalta *et al.*, 2007). Moreover, pseudo-albino specimens fed high dietary ARA levels during post-metamorphosis displayed higher $PGE_2$ concentrations than normally pigmented fish fed the same diet, suggesting the presence of regulatory mechanisms for controlling excessive levels of ARA in the diet; which when activated influences the production of ARA-derived metabolites leading to normal pigmentation patterns. However, the exact mechanism linking ARA dietary levels, eicosanoid production and malpigmentation in flatfish remain unknown (Planas and Cunha, 1999; Bolker and Hill, 2000; Villalta *et al.*, 2005a; Brandsen *et al.*, 2005). Insights into the gene regulatory network underlying the ontogeny of pigmentation of Senegalese sole have recently been reported (Darias *et al.*, 2013b), but the involvement of ARA and its hormonal derivatives need further investigations (Villalta *et al.*, 2005a; Brandsen *et al.*, 2005; Darias *et al.*, 2013b).

The present study, in agreement with previous work on common sole (Lund *et al.*, 2007, 2008), demonstrated that not only dietary levels of ARA, but the timing of its administration in relation to the larval developmental stage, were responsible for the incidence of pigmentary anomalies, questioning the real involvement of EPA/ARA ratio. Although feeding larvae with high dietary ARA levels during the whole experimental period provided the highest occurrence of pseudo-albino fish (81.4% in the ARA-H +/+ group), a high incidence of pseudo-albinism was also found when larvae were fed high dietary ARA levels during pre- and pro-metamorphic stages (52.2 % in the ARA-H +/- group) rather than in post-metamorphic stage (20.0% in the ARA-H +/- group). In a recent study investigating the morphological and molecular ontogeny of skin pigmentation in the ocular side of Senegalese sole, morphological changes in the population of chromatophores to conform the adult pattern of pigmentation were evidenced at post-metamorphosis, although the molecular signaling occurred during pro-metamorphosis (Darias *et al.*, 2013a). In order to understand when and how the albino phenotype appears, a subsequent study examined the morphological and molecular characteristics of developing ARA-induced pseudo-albino specimens in Senegalese sole larvae (Darias *et al.*, 2013b). Results showed that larvae later becoming pseudo-albino and pigmented individuals developed pigmentation in the same way, but once metamorphosed, the future pseudo-albinos began to show different relative proportions, allocation patterns, shapes and sizes of skin chromatophores. Although the pseudo-albino phenotype could not yet be observable in pre-metamorphic larvae, high dietary ARA levels supplied during pre- and pro-metamorphic stages may have affected molecular mechanisms involved in the pigmentation process, leading to the appearance of pigmentary disorders in post-metamorphic individuals. Lund *et al.* (2008) also found a high incidence of malpigmentation (90.4%) in common sole larvae fed 10.5% ARA during the pre- and pro-metamorphosis stages (3-21 dph), and only 16.7 and 37.3% of pseudo-albinism when 10.5% ARA was provided to larvae from 15 to 21 dph and from 10 to 21 dph, respectively. Therefore, larval sensitivity to ARA and its derivatives was higher during pre- and pro-metamorphosis than during post-metamorphosis. A sensitive “pigmentation window” period (Næss and Lie, 1998) was hypothesized to occur a few days before metamorphosis for larval Japanese flounder and halibut. In these studies, imbalances in dietary ARA levels and the ARA/EPA ratio supplied during their pre-metamorphic stage negatively affecting their correct pigmentation, while no detrimental effects on pigmentation were noticed in the post-pigmentation window (McEvoy *et al.*, 1998; Næss and Lie, 1998; Estévez *et al.*, 1999, 2001), demonstrating that the optimum DHA/EPA/ARA ratio in the early first-feeding period of flatfish larvae is vital for achieving normal metamorphosis and adult dorsal skin pigmentation (Sargent *et al.*, 1999).

Texture analysis of pigmentary patterns in fish was performed in order to characterize the level of heterogeneity of skin intensity between normally pigmented and pseudo-albino post-metamorphic individuals from each treatment. The analysis showed that the skin of pseudo-albino specimens from all dietary treatments displayed a more uniform (lower contrast) and more homogeneous (higher energy, correlation and homogeneity) melanophore pattern than normally pigmented larvae. These

results might be explained by the reduced number and different shape and aspect of melanophores and their allocation in the skin (Darias *et al.*, 2013b). Indeed, it has been shown that the skin of ARA-induced pseudo-albino individuals contained all types of chromatophores at pre and pro-metamorphosis but these progressively disappeared during post-metamorphosis (Darias *et al.*, 2013b). Melanophores were less abundant than in normally pigmented larvae and less aggregated in patches, with a few round-shaped melanophores suggesting their unability to disperse melanin. The pseudo-albino phenotype was then defined as the result of the inhibited differentiation of post-metamorphic populations of chromatophores and the progressive degradation of the already existent larval xanthophores and melanophores (Darias *et al.*, 2013b). These morphological observations supported the results of pigmentation texture analysis of the present study.

We observed a significantly lower number of melanophores, as well as a more uniform skin aspect (higher energy) in pseudo-albino fish from the ARA-H +/+ group, that can be considered totally pseudo-albinos, than in pseudo-albinos from the ARA-H -/+ and +/- groups. These results suggested that dietary ARA differently affect the pigmentation in Senegalese sole depending on the developmental stage when it was supplied to the larvae. Considering the results of the present experiment, we suggest that ARA-induced malpigmentation is a developmental-dependent phenomenon; feeding Senegalese sole larvae with high dietary amounts of ARA before, during and after metamorphosis leads to a higher inhibition of cell differentiation than when ARA was offered during the pre-metamorphic stage or from the onset of metamorphosis. Moreover, dietary levels of ARA were suspected to affect pigmentation pattern and chromatophore aspect even in normally pigmented larvae, as melanophores in normally pigmented fish fed the control diet (1% ARA) during the whole experiment had a more dendritic aspect than those in normally pigmented specimens fed high dietary ARA amounts during pre-, pro- and/or post-metamorphic period. It seems that those individuals, even if they are considered as normally pigmented, showed populations of melanophores affected by the diet, with a loss of ability to change the shape from round to dendritic and to disperse melanin, as observed in some pseudo-albino individuals (Darias *et al.*, 2013b). A comparison of textural features among normally pigmented larvae from the four dietary treatments revealed that even larvae fed with high dietary ARA levels during pre-metamorphosis presented a more patchy and heterogeneous pattern of skin melanophore distribution (higher contrast and lower energy and homogeneity values) than normally pigmented individuals fed high ARA amounts during other developmental periods. This observation once again implicates high levels of ARA supplied at pre- and pro-metamorphosis in the determination of the adult pigmentation pattern, since this fatty acid and its derived metabolites may affect the neural crest cell lines that differentiate into pigmentary cells (Yamguchi *et al.*, 2007).

The morphological alterations in chromatophore populations in pseudo-albino specimens have been associated to the modification of the expression of several pigmentation-related genes involved in the developmental disruption of the new post-metamorphic populations of melanophores,

xanthophores and iridophores and in defects in melanin production (Darias *et al.*, 2013b)). However, further nutrigenomic studies using a holistic approach are required to understand the precise molecular mechanisms underlying the effect of nutritional factors, such as absolute and relative proportions of ARA to other EFA, on the physiological processes leading to the adult phenotype and pigmentation success.

**Conclusion**

The present study revealed that the "sensitivity window" of Senegalese sole larvae to ARA-induced malpigmentation took place during the pre- and pro-metamorphosis periods. High levels of dietary ARA fed during early larval stages enhanced survival, without affecting growth and eye migration. Supplying high ARA amounts in the live prey changed the dietary ARA/EPA ratio, which in turn altered the relative concentrations of prostaglandins of the 2 and 3-series, causing pigmentary disorders. The aspect and density of melanophores in the dorsal skin of ARA-induced pseudo-albinos was reduced compared to normally pigmented individuals, and reduced further when the high dietary ARA levels were supplied during the pre- and pro-metamorphic stages. The use of pigmentation texture analysis has proven to be a valuable tool for studying changes in skin pigmentation patterns among different groups of fish.

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# 3

# Morphological and molecular characterization of dietary-induced pseudo-albinism during post-embryonic development of *Solea senegalensis*

Maria J. Darias[1,2]
Karl B. Andree[1]
Anaïs Boglino[1]
Josep Rotllant[3]
José M. Cerda-Reverter[4]
Alicia Estévez[1]
Enric Gisbert[1]

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[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüicoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] IRD, UMR 226 ISE-M, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

[3] IIM-CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

[4] IATS-CSIC, Torre de la Sal (s/n), Ribera de Cabanes, 12595 Castellón, Spain

**Caracterización morfológica y molecular del pseudo-albinismo inducido por la dieta durante el desarrollo post-embrionario del lenguado Senegalés (Kaup, 1858)**

**Resumen**

La aparición del fenotipo pseudo-albino fue investigada en larvas en desarrollo de lenguado Senegalés (*Solea senegalensis*, Kaup 1858) a niveles morfológico y molecular. Para inducir el desarrollo de pseudo-albinos, larvas de lenguado Senegalés fueron alimentadas con *Artemia* enriquecida con altos niveles de ácido araquidónico (ARA). El proceso de desarrollo de la pigmentación de su piel fue comparado a la de un grupo control alimentado con *Artemia* enriquecida con un producto comercial de referencia. La cantidad relativa de melanóforos (M), xantóforos (X) y iridóforos (I) en la piel reveló que la pigmentación larvaria se desarrolló de manera similar en ambos grupos. No obstante, los resultados de las diferentes proporciones, patrones de distribución, formas y tallas de los cromatóforos de la piel revelaron cambios en los patrones de pigmentación entre los grupos ARA y el control a partir del día 33 post-eclosión (dpe). Las nuevas poblaciones de cromatóforos que deberían aparecer durante la post-metamorfosis no fueron desarrollados en el grupo ARA. Además, los patrones de distribución espacial entre los xantóforos y melanóforos presentes en las larvas fueron sugerentes de una interacción a corto alcance que parecerían implicados en la degradación de estos cromatóforos, conduciendo a la apariencia del fenotipo pseudo-albino. El perfil de expresión de varios genes claves relacionados con la pigmentación reveló que el desarrollo de los melanóforos fue promovido en los pseudo-albinos sin un grado de diferenciación terminal suficiente, impidiendo la melanogenesis. Los resultados presentes sugirieron el papel potencial de los genes asip1 y slc24a5 sobre la regulación negativa de la expresión de trp1, conduciendo a defectos de la producción de melanina. Además, los datos de expresión génica apoyaron la implicación de los genes pax3, mitf y asip1 sobre la perturbación del desarrollo de las nuevas poblaciones post-metamórficas de melanóforos, xantóforos e iridóforos.

**Palabras llaves**: Pseudo-albinismo, larvas, peces planos, Senegaleses ole, ácido araquidónico, pigmentación, melanóforo, xantóforo, iridóforo, ontogenia, piel, regulación génica.

**Abstract**

The appearance of the pseudo-albino phenotype was investigated in developing Senegalese sole (*Solea senegalensis,* Kaup 1858) larvae at morphological and molecular levels. In order to induce the development of pseudo-albinos, Senegalese sole larvae were fed *Artemia* enriched with high levels of arachidonic acid (ARA). The development of their skin pigmentation was compared to that of a control group fed *Artemia* enriched with a reference commercial product. The relative amount of skin melanophores (M), xanthophores (X) and iridophores revealed that larval pigmentation developed similarly in both groups. However, results from different relative proportions, allocation patterns, shapes and sizes of skin chromatophores revealed changes in the pigmentation pattern between ARA and control groups from 33 days post hatching onwards. The new populations of chromatophores that should appear at post-metamorphosis were not formed in the ARA group. Further, spatial patterns of distribution between the already present larval xanthophores and melanophores were suggestive of short-range interaction that seemed to be implicated in the degradation of these chromatophores, leading to the appearance of the pseudo-albino phenotype. The expression profile of several key pigmentation-related genes revealed that melanophore development was promoted in pseudo-albinos without a sufficient degree of terminal differentiation, thus preventing melanogenesis. Present results suggest the potential roles of *asip1* and *slc24a5* genes on the down-regulation*trp1* expression, leading to defects in melanin production. Moreover, gene expression data supports the involvement of *pax3*, *mitf* and *asip1* genes in the developmental disruption of the new post-metamorphic populations of melanophores, xanthophores and iridophores.

**Keywords:** Pseudo-albinism, larvae, flatfish, Senegalese sole, arachidonic acid, pigmentation, melanophore, xanthophore, iridophores, ontogenesis, skin, gene regulation.

**Introduction**

Although more than 378 loci (171 cloned and 207 uncloned genes) have been identified as being involved in vertebrate pigmentation (Montoliu *et al.*, 2011), the underlying bases of pigment pattern development are far from being completely understood. Nevertheless, some pigmentation-related mechanisms have been described in mammals, which represent a relatively straightforward example of genetic color determination among vertebrates (Hofreiter and Schöneberg, 2010). While mammals have only one class of pigment cell, the melanocytes (which produce variations of black, brown, red or yellow pigment), fishes constitute one of the most colorful vertebrates, where color can be determined by up to six different types of chromatophores: melanophores (black), xanthophores (yellow), erythrophores (red), iridophores (iridescent, blue, silver or gold), leucophores (dull, whitish) and cyanophores (blue) (Fujii, 2000). Together, these cells can produce almost any spectacular color combination that can be seen, for instance, in the community of fish from a coral reef. In spite of these added complexities, many of the same genes and control networks found in mammals are conserved in fish (Lister, 2002; Mellgrem and Johnson, 2002); and considering their small size and easy manipulation, fish are suitable models for a better understanding of vertebrate pigmentation. Indeed, fish have been used as models for melanoma research because it has been shown tissues within fish share molecular signatures and histopathological features with human cancers (Patton, 2010). The genetics of pigmentation have been explored in several model teleost fish including zebrafish (Kelsh, 1996; Parichy and Johnson, 2001), medaka (Fukamachi, 2004), fugu (Klovins, 2004), goldfish (Cerdá-Reverter *et al.*, 2003, 2005) and, recently, in flatfish (Yamada *et al.*, 2010; Guillot *et al.*, 2012; Darias *et al.*, 2013). Flatfish are particularly useful to analyze the origin of pigmentation disorders during the ontogeny because altered pigmentation can be induced under intensive rearing conditions (Seikai and Matsumoto, 1994; Villalta *et al.*, 2005, 2008; Lund *et al.*, 2010). Senegalese sole is a flatfish species known to develop pseudo-albinism when fed high levels of dietary arachidonic acid (ARA) during their development (Villalta *et al.*, 2005). However, to our knowledge, there is no information about the possible mechanism that underlies this process. The process of pigmentation development can be seen as a cooperative relationship among three different processes: tissue remodeling (involving apoptosis), cellular differentiation of chromatophores, and pigment production. As a previous step, we have recently studied in the ocular side of this species the morphological and molecular ontogeny of skin pigmentation (Darias *et al.*, 2013), which are essential to elucidate the mechanisms of formation of the adult pigmentation pattern and to understand when and how the albino phenotype appears. Gene markers for the above mentioned processes were seen to alter in a progression that was in synchrony with metamorphosis. The above cited study revealed different stages of skin pigmentation and development in Senegalese sole that coincided with the progress of metamorphosis and patterns of gene expression: i) pre-metamorphosis period (2-11 dph), low expression of a marker of apoptosis (*casp3*) and genes related to melanogenesis and high expression of melanophore differentiating genes; ii) pro-metamorphosis period (11-19 dph), high expression of *casp3* (apoptosis and tissue remodeling) and melanophore differentiating and melanogenic genes; iii) post-metamorphosis (19-47 dph), low expression of all analyzed genes, especially those associated to melanophore

differentiation. Major molecular changes in the pigment pattern occurred during pro-metamorphosis and morphological changes in the population of melanophores, xanthophores and iridophores were evidenced at post-metamorphosis to enable the juveniles to conform to the adult pattern of pigmentation (Darias *et al.*, 2013).

In this report we investigated the morphological development of pseudo-albinism in Senegalese sole and the quantitative expression of eleven pigmentation-related genes to find out if any transcriptional modulation could explain the deviation from normal patterns of pigmentation. In order to obtain the pseudo-albino phenotype, fish larvae were fed during their development with live prey enriched with high levels of ARA, a powerful inhibitor of pigmentation (Villalta *et al.*, 2005). The pseudo-albino phenotype in Senegalese sole was the result of a disruption of the signaling for the dorsal-ventral patterning during metamorphosis and was characterized by the presence of the pigment cells that differentiated during embryogenesis (Kelsh, 2004) and the very early stages of larval development (genetically programmed chromatoblast differentiation) and by the absence of pigment cells that should be formed after metamorphosis for development of the adult pigmentation pattern (environmentally modifiable chromatoblast differentiation). Gene expression results provided evidence that *pax3*, *mitf* and *asip* were involved in the developmental disruption of the new post-metamorphic populations of melanophores, xanthophores and iridophores and that melanogenesis was disrupted through the negative regulatory action of *asip1* and *slc24a5* on *trp1* gene expression.

## Materials and methods

### *Ethics statement*

This study was carried out in accordance with the recommendations in (Kilkenny *et al.*, 2010). Animal experimental procedures were conducted in compliance with the experimental research protocol (reference number 4978-T9900002) approved by the Committee of Ethic and Animal Experimentation of the IRTA and the Departament de Medi Ambient i Habitatge (DMAH, Generalitat de Catalunya, Spain) in accordance with EU regulation (EC Directive 86/609/EEC).

### *Animal rearing and sampling procedures*

Two-day-old Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, A Coruña, Spain), acclimated at IRTA-SCR facilities and reared at 16.7 ± 0.4 ºC and 35 of salinity in 8, 60 l cylindrical white bottomed tanks (initial density: 110 larvae.l$^{-1}$) connected to a IRTAmar$^{TM}$ recirculation unit. Water was daily renewed (50%) with gentle aeration in each tank, pH and dissolved oxygen being 8.0 ± 0.2 and 7.5 ± 1.3 ppm, respectively. Photoperiod was 16L: 8D, and light intensity was 500 lx at the water surface. Larvae were fed twice a day, from 2 days post hatching (dph) to 10 dph, with enriched rotifers (*Brachionus plicatilis*), at a density of 10 rotifers.ml$^{-1}$ from 2 to 8 dph and of 5 rotifers.ml$^{-1}$ from 9 to 10 dph. Enriched *Artemia* metanauplii were supplied to larvae from 8 to 60 dph twice a day, at increasing density from 0.5 to 12 metanauplii.ml$^{-1}$ according to the increase of weight of

the larvae and to the daily food ration (Cañavate *et al.*, 2006). From 20 dph onwards, enriched *Artemia* metanauplii were supplied frozen to larvae settled at the bottom of the tank (Villalta *et al.*, 2008).

**Table 1.** Total lipid and fatty acids contents (mg·g $DW^{-1}$) and fatty acid composition (% of TFA) of the enriched rotifer (mean± SD; n = 5) and *Artemia* nauplii (mean ± SD; n = 3) with the two enriching compounds and in Senegalese sole larvae at 2, 15 and 50 dph fed the two dietary treatments (mean ± SD; n = 3). Totals include some minor components not shown. Superscript letters denote significant differences among diets for a given live prey or larval age (One-way ANOVA, $P < 0.05$). A 0.0%TFA means content under 0.45%TFA.

| | Enriching products | | Enriched rotifer | | Enriched *Artemia* | | Pro-metamophic | Pro-metamorphic larvae | | Post-metamorphic larvae | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AGM | ARA-H* | AGM | ARA-H | AGM | ARA-H | larvae | AGM | ARA-H | AGM | ARA-H |
| Total lipid | 368.5±12.2$^{b}$ | 622.2±0.0$^{a}$ | 140.6±18.5 | 143.2±25.2 | 164.9±14.4 | 164.9±15.1 | 84.4 | 109.9±19.4 | 82.8±11.8 | 86.7±13.9 | 100.6±21.9 |
| Total FA | 305.4±10.3 | 328.1±19.2 | 74.2±13.0 | 61.4±13.6 | 87.6±14.4 | 85.0±14.3 | 22.5 | 50.7±12.4$^{a}$ | 31.3±5.6$^{b}$ | 34.6±9.4 | 46.3±11.8 |
| Total SFA | 23.8±2.5 | 18.1±1.6 | 21.4±3.5$^{a}$ | 17.2±1.6$^{b}$ | 17.5±0.9$^{a}$ | 15.3±1.2$^{b}$ | 29.4 | 20.2±1.4 | 21.3±1.2 | 24.6±2.0 | 19.0±0.2 |
| 18:1n-9, OA | 0.07±0.1$^{b}$ | 25.7±0.2$^{a}$ | 6.6±2.6$^{b}$ | 25.6±4.0$^{a}$ | 14.4±3.7$^{b}$ | 23.1±2.0$^{a}$ | 10.0 | 13.3±1.9$^{b}$ | 22.2±4.6$^{a}$ | 14.3±0.4$^{b}$ | 22.3±0.9$^{a}$ |
| Total MUFA | 0.07±0.1$^{b}$ | 35.3±0.4$^{a}$ | 14.4±4.6$^{b}$ | 41.8±2.8$^{a}$ | 21.7±5.5$^{b}$ | 33.6±2.9$^{a}$ | 19.4 | 16.2±0.6$^{b}$ | 29.4±1.9$^{a}$ | 19.8±2.0$^{b}$ | 31.0±0.5$^{a}$ |
| 18:2n-6, LN | 0.0±0.0$^{b}$ | 7.4±1.0$^{a}$ | 2.4±0.6$^{b}$ | 9.3±4.8$^{a}$ | 4.6±1.2$^{b}$ | 6.7±0.4$^{a}$ | 3.2 | 3.1±0.4$^{b}$ | 8.0±0.3$^{a}$ | 3.6±0.3$^{b}$ | 6.9±0.4$^{a}$ |
| 20:4n-6, ARA | 0.3±0.02$^{b}$ | 15.1±0.8$^{a}$ | 1.0±0.4$^{b}$ | 10.2±1.2$^{a}$ | 1.4±1.3 | 7.1±4.1 | 3.2 | 4.4±0.6$^{b}$ | 10.9±0.8$^{a}$ | 4.1±0.2$^{b}$ | 10.2±0.3$^{a}$ |
| 22:5n-6, DPA | 19.6±0.3$^{a}$ | 0.9±1.3$^{b}$ | 13.3±2.1$^{a}$ | 1.8±1.4$^{b}$ | 4.3±2.8$^{a}$ | 0.6±0.4$^{b}$ | 0.4 | 8.2±1.1$^{a}$ | 0.8±0.4$^{b}$ | 6.7±0.7$^{b}$ | 0.9±0.0$^{a}$ |
| Total n-6 PUFA | 21.1±0.3$^{b}$ | 26.2±1.0$^{a}$ | 18.2±1.7$^{b}$ | 24.2±3.6$^{a}$ | 11.8±2.9 | 16.4±4.7 | 8.1 | 17.1±1.3$^{b}$ | 21.7±0.5$^{a}$ | 16.1±0.5$^{b}$ | 20.6±0.2$^{a}$ |
| 18:3n-3, LNA | 0.0±0.0$^{b}$ | 0.8±0.1$^{a}$ | 0.4±0.3$^{b}$ | 1.3±0.4$^{a}$ | 26.8±6.1 | 24.6±5.9 | 0.6 | 11.9±0.4 | 10.9±1.6 | 10.9±0.9 | 14.4±0.8 |
| 20:5n-3, EPA | 1.6±0.0$^{b}$ | 11.3±0.5$^{a}$ | 2.8±0.6$^{b}$ | 7.4±2.5$^{a}$ | 4.7±3.0 | 3.7±1.5 | 4.7 | 4.2±0.3$^{a}$ | 2.1±0.1$^{b}$ | 2.1±0.3$^{a}$ | 1.6±0.0$^{b}$ |
| 22:6n-3, DHA | 52.9±3.1$^{a}$ | 6.5±0.9$^{b}$ | 40.0±4.6$^{a}$ | 5.3±0.9$^{b}$ | 10.6±8.0 | 1.4±0.5 | 33.0 | 25.3±1.0$^{a}$ | 8.7±0.8$^{b}$ | 20.0±2.6$^{a}$ | 6.1±0.7$^{b}$ |
| Total n-3 PUFA | 55.04±2.9$^{a}$ | 20.4±1.1$^{b}$ | 46.0±4.0$^{a}$ | 16.8±1.2$^{b}$ | 46.4±3.6$^{a}$ | 33.2±5.3$^{b}$ | 43.1 | 45.4±1.5$^{a}$ | 24.7±0.8$^{b}$ | 36.7±3.9$^{a}$ | 26.2±0.9$^{b}$ |
| Total PUFA | 76.1±2.6 | 46.6±2.0 | 64.2±5.6$^{a}$ | 41.0±4.3$^{b}$ | 58.2±6.4$^{a}$ | 49.6±1.0$^{b}$ | 51.2 | 62.5±2.6$^{a}$ | 46.3±1.2$^{b}$ | 52.8±4.4$^{a}$ | 46.8±0.9$^{b}$ |
| n-3 / n-6 | 2.6±0.2$^{a}$ | 0.8±0.01$^{b}$ | 2.5±0.1$^{a}$ | 0.7±0.1$^{b}$ | 4.1±0.6 | 2.3±1.1 | 5.3 | 2.7±0.1$^{a}$ | 1.1±0.0$^{b}$ | 2.3±0.2$^{a}$ | 1.3±0.0$^{b}$ |
| DHA / EPA | 32.8±1.3$^{a}$ | 0.6±0.1$^{b}$ | 15.1±3.4$^{a}$ | 0.9±0.6$^{b}$ | 2.1±1.0$^{a}$ | 0.4±0.2$^{b}$ | 7.1 | 6.0±0.3$^{a}$ | 4.2±0.6$^{b}$ | 9.5±0.7$^{a}$ | 3.8±0.5$^{b}$ |
| ARA / DHA | 0.0±0.0$^{b}$ | 2.3±0.2$^{a}$ | 0.03±0.01$^{b}$ | 2.0±0.4$^{a}$ | 0.1±0.1$^{b}$ | 4.7±2.0$^{a}$ | 0.1 | 0.2±0.0$^{b}$ | 1.3±0.2$^{a}$ | 0.2±0.0$^{b}$ | 1.7±0.2$^{a}$ |
| ARA / EPA | 0.2±0.01$^{b}$ | 1.3±0.02$^{a}$ | 0.4±0.1$^{b}$ | 1.6±0.7$^{a}$ | 0.3±0.1$^{b}$ | 1.8±0.5$^{a}$ | 0.7 | 1.1±0.1$^{b}$ | 5.2±0.3$^{a}$ | 1.9±0.2$^{b}$ | 6.3±0.2$^{a}$ |
| MUFA / PUFA | 0.0±0.0$^{b}$ | 0.8±0.04$^{a}$ | 0.2±0.1$^{b}$ | 1.0±0.2$^{a}$ | 0.4±0.1$^{b}$ | 0.7±0.1$^{a}$ | 0.4 | 0.3±0.0$^{b}$ | 0.6±0.0$^{a}$ | 0.4±0.1$^{b}$ | 0.7±0.0$^{a}$ |

* 100 g of ARA-H contained 29.0 g of Fluka© oil (Sigma-Aldrich, Chemie GmBH, Steinheim, Norway), 17.4 g of Vevodar® (DSM Food Specialties, Netherlands), 5.2 g of olive oil, 2.3 g of vitamin E, 4.1 g of soy lecithin and 42.0 g of distilled water.

In order to induce pseudo-albinism (Villalta *et al.*, 2005), larvae from four tanks were fed with rotifers and *Artemia* nauplii enriched with an experimental emulsion containing high levels of arachidonic acid (ARA). Briefly, the experimental emulsion was prepared from commercially available oils rich in DHA and ARA obtained respectively, from liver of *Gadus morhua* (Fluka©, Sigma-Aldrich, Chemie GmBH, Steinheim, Norway) and from fungus *Mortierella alpina* (Vevodar®, DSM Food Specialties, Netherlands). Olive oil was added to the oil mixture to dilute and adjust n−3 PUFA concentration in enriched live prey and α-tocopherol for preservation of the emulsion. The oil mixture was emulsified with soy lecithine and distilled water by homogenizing with an Ultra-turrax T25 at high speed for 60 s. Larvae from the other four tanks were fed rotifers and *Artemia* nauplii enriched with a commercial enrichment (AGM, Algamac 3050™, Aquafauna, Biomarine Inc., USA) and used as a

control group to monitor the normal pigmentation development. The ingredients used in the formulation ARA emulsion and the fatty acid composition of AGM (control) and ARA enrichments are shown in Table 1.

Rotifers were enriched in 20 l containers at 500 rotifers.ml$^{-1}$ at 26 ºC with 0.6 g.l$^{-1}$ of each emulsion. Half of the rotifers were supplied to the larvae after 2h of enrichment and the other half after 6h of enrichment. One-day-old *Artemia* nauplii (EG strain, INVE) were enriched in 20 l containers at 300 nauplii.ml$^{-1}$ for 16h at 28 ºC with 0.6 g.l$^{-1}$ of each emulsion. Enriched *Artemia* were kept at 4 ºC in UV-treated, filtered seawater with aeration until administered to larvae twice a day. In order to reduce the bacterial load after enrichment and remove emulsion residues, rotifers and *Artemia* were washed with UV-treated filtered seawater and treated with $H_2O_2$ (40ppm for 15 min for rotifers and 8000 ppm for 5 min for *Artemia,* according to (Giménez *et al.*, 2006) and then rinsed with filtered seawater. The biochemical analysis of lipids and fatty acid (FA) composition (Boglino *et al.*, 2012) of enriched rotifer and *Artemia,* as well as in Senegalese sole larvae at 2, 15 and 50 dph are shown in Table 1.

For gene expression analyses, 200 mg wet weight pigmented juveniles and pseudo-albinos were sampled at 60 dph, sacrificed with an overdose of anesthetic (Tricaine methanesulfonate, MS-222, Sigma), rinsed in distilled water and preserved in RNAlater© (Ambion) at -80 ºC for further analyses.

*Metamorphosis*

Eye migration was used as a measure of the progress of the metamorphosis process and consisted of evaluating the eye migration index ($I_{EM}$) in 30 individuals per tank at 15, 30 and 50 dph according to (Villalta *et al.*, 2005). Data were presented as the relative amount of larvae in each stage of development at the same age. The eye migration index ($I_{EM}$=Σ (%fish in each stage*stage)/100) was calculated according to (Solbakken *et al.*, 1999).

*Photography*

Larvae (22, 27, 33, 35 and 41 dph) and juveniles (60 dph) were photographed using a stereomicroscope (Nikon SMZ800, Soft Imaging Systems, GmbH) equipped with a Color View-XS camera. Skins of juveniles were photographed using a DP70 (Olympus) camera attached to DMLB (Leica) microscope. Images were taken under transmitted or incident light and compiled and processed using Analysis®3.1 (Soft Imaging Systems, GmbH, Germany). The amount of melanophores, xanthophores and iridophores in post-metamorphosed larvae was quantified in the ocular side of the trunk skin, excluding the abdominal area, using ImageJ64 software. Results were represented as the relative proportion, expressed in percentage, of each chromatophore in the analyzed skin area. The same software was used to measure, in μm, the size of chromatophores and the extension of dispersed melanin within melanophores in early juveniles.

*Partial cDNA sequences isolation*

Several genes involved in the process of pigmentation were selected as markers for melanophore differentiation and melanin synthesis in Senegalese sole (Table 2). Very few Senegalese sole-specific gene sequences were available from public data bases, therefore multiple sequence homologs for the chosen target genes from extant species were downloaded from GenBank to construct alignments for use in designing primers homologous to conserved regions. Sequences were aligned using the CLUSTAL algorithm embedded in BioEdit ver 7.0.5.2 (T. Hall 1997-2005). Amplification was performed using a gradient thermal cycler (Eppendorf Mastercycler) to optimize specific amplification of target regions. The amplification products were separated and isolated by agarose gel electrophoresis for sequencing in direct sequencing reactions using both forward and reverse primers from the original amplification. The sequences obtained from amplification products were analyzed using BLAST to verify similitude to the intended target sequence.

*Real Time PCR assays*

Gene expression in pigmented and pseudo-albino specimens was analyzed using the skin of 60 day-old juveniles (54 ± 12.6 mg wet weight). Total RNA of a pool (5 individuals) of pigmented and a pool (5 individuals) of pseudo-albino specimens per each tank was extracted in separate using TRIzol™ (Invitrogen®, San Diego, CA, USA). The quantity of RNA isolated was determined using a Gene-Quant spectrophotometer (Amersham Biosciences). The quality of the RNA was examined using 1.2% agarose gel electrophoresis. Total RNA (1 µg) from each sample were reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen®, GmbH, Germany). Genomic DNA was removed using genomic DNA wipe-out buffer included in the Quantitect reverse transcription kit (Qiagen). Selected samples were also run as non-RT negative controls to confirm the efficacy of the DNAse treatment. Real-time PCR analysis was performed using an ABI PRISM 7300 (Applied Biosystems). For each gene, a species-specific Taqman assay was designed (Applied Biosystems) (Table 2). Amplification reactions were performed in triplicate in a total volume of 20 µl containing 1 µl of cDNA, 1 µl of Taqman probe, 10 µl of Taqman mix and 8 µl of sterile water. The gene *Ubq (Ubiquitin)* was chosen as a reference gene since it did not exhibit any significant variation in expression among the samples (Manchado *et al.*, 2007). The amplification conditions were 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Real-time PCR efficiencies were determined for each gene from the slopes obtained with Applied Biosystems software, applying the equation E = 10(-1/slope), where E is PCR efficiency. To determine the relative quantity of target gene-specific transcripts present in the different samples, expression ratios (R) were calculated according to the following formula: R = ($E_{target\ gene}$)ΔCT target gene (mean sample – mean ref sample) / ($E_{Ubq}$)ΔCT *Ubq* (mean sample - mean ref sample), where *Ubq* is the reference gene and mean sample corresponds to quadruplicate average. Pigmented individuals were chosen as ref samples.

**Table 2.** Accession numbers, amplicon size, primers and hydrolysis probes used in qPCR analyses.

| Gene name | GenBank accession no. | Biological process/Activity | Amplicon size | Hydrolysis probes | 5′ to 3′ sequence |
|---|---|---|---|---|---|
| *Ubq* | **AB291588** | Ubl conjugation pathway | 86 | Forward | GCCCAGAAATATAACTGCGACAAG |
| | | | | Reverse | TGACAGCACGTGGATGCA |
| | | | | FAM probe | ACTTGCGGCATATCAT |
| *Tyr* | **JF693907** | Melanin biosynthesis | 73 | Forward | CGTACGCACAGATGGAAAACG |
| | | | | Reverse | CACGTAGTAATGCATCCACACAAAA |
| | | | | FAM probe | ACATCGGCGAATATC |
| *Trp1* | **GU329041** | Melanin biosynthesis; Melanocyte differentiation | 63 | Forward | CGTGTGCAACAATACAGAAACAAGT |
| | | | | Reverse | ATGGGTCGTGCCACGTT |
| | | | | FAM probe | CCTGCCGGGTTCCTT |
| *Mitf* | **GU329042** | Transcription factor for tyr, trp2 and trp1 | 75 | Forward | CGATGACATCATAAGTCTTGAATCCAGTTT |
| | | | | Reverse | CGTGCTGGGCAACTGAAGA |
| | | | | FAM probe | CCGGAGTCAATCAACG |
| *cKit* | **HM100237** | Tyrosine-protein kinase signaling pathway | 69 | Forward | GTGAAGAGAGTGAGATGTTTGACGA |
| | | | | Reverse | CACTTTGGTAGGAGAAGCTCAGAA |
| | | | | FAM probe | CTCGTCACCGAAGATC |
| *Mc1r* | **GU329043** | Melanocyte-stimulating hormone receptor activity | 76 | Forward | CGCCGTCGCCATCATC |
| | | | | Reverse | GCGTTGTCCGTGTGGTAGA |
| | | | | FAM probe | ACCTCCAGCATCCTCT |
| *Scl24a5* | **GU329046** | Ion transport in melanosomes | 66 | Forward | GACGCAGCCTCTGATCGA |
| | | | | Reverse | CCGTCCTGGAGCGAACC |
| | | | | FAM probe | CCAGTCTGCGAAACAT |
| *Casp3* | **GU329040** | Apoptosis | 77 | Forward | CGACAGTGTAGATGACCAAACGT |
| | | | | Reverse | GGAGCAGTGGAATAAGCATAAAGGA |
| | | | | FAM probe | CCTCCACAGGAATCC |
| *Pax3* | **HM100238** | Transcriptional regulation of pigmentation | 68 | Forward | GCATCATGCGCTCCAAGTTC |
| | | | | Reverse | CCCTCTTCACCATTTCATCATCCT |
| | | | | FAM probe | CATCGTCACCAACTCC |
| *Sl* | **U06753** | Hormone activity | 75 | Forward | TTCCCACTGCGGCTTCA |
| | | | | Reverse | GGTAAGGCCTTGGTGATGCA |
| | | | | FAM probe | CCGACCGTGTTTCTC |
| *Asip* | **HE598753** | Regulation of melanogenesis | 81 | Forward | GCTGTGACATCTGTGCCTTCT |
| | | | | Reverse | CCATTCGACAGAAACACACAGTTC |
| | | | | FAM probe | CCAGTGTCGCCTCTTC |
| *Hsp70* | **GU329044** | DNA repair | 73 | Forward | TGGAGTCGTATGCTTTCAACATGA |
| | | | | Reverse | TGCTTGTCGTCGTCACTGAT |
| | | | | FAM probe | CTTGCCAGCCAGTTTC |

*Statistics*

Data are presented as mean ± SD (N=4). Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA). All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). A two-way ANOVA was performed to analyze the amount of chromatophores during development in both groups and a one-way ANOVA to to analyze

the amount of chromatophores of each group at each developmental date. When significant differences were found ($P < 0.05$), the post-hoc Holm-Sidak method was used to perform all pairwise multiple comparisons. For the rest of the analyses, statistical significance was calculated using Student's *t*-test ($P < 0.05$).

**Table 3.** Larval size in dry weight (DW, mg), standard length (SL, mm), specific growth rate (SGR, %.day$^{-1}$), survival rate (%), pigmentation (%) and eye migration index ($I_{EM}$) of Senegalese sole fed the two different dietary treatments. Initial (2 dph) DW and SL of larvae were 3.07 ± 0.02 mm and 36.7 ± 1.6 µg, respectively. Values are expressed as mean ± SEM (n = 4). Superscript letters indicate significant differences among dietary treatments (One-way ANOVA, $P < 0.05$).

| | AGE | CONTROL | ARA |
|---|---|---|---|
| **DW** | 15 dph | 0.09±0.01 | 0.09±0.01 |
| | 30 dph | 0.77±0.07 | 0.89±0.06 |
| | 50 dph | 1.92±0.24 | 1.71±0.32 |
| **SL** | 15 dph | 4.18±0.05 | 4.35±0.05 |
| | 30 dph | 6.401±0.07 | 6.75±0.07 |
| | 50 dph | 8.75±0.22 | 8.71±0.17 |
| **SGR** | 2-50 dph | 0.08±0.00 | 0.08±0.00 |
| **Survival** | 50 dph | 97.3±0.25 | 98.4±0.24 |
| **Pigmentation** | 50 dph | 99.1±0.50$^{a}$ | 18.6±12.9$^{b}$ |
| **Metamorphosis** | 15 dph | 2.97±0.06 | 3.04±0.10 |
| | 30 dph | 5.94±0.39 | 5.86±0.24 |
| | 50 dph | 5.94±0.03 | 5.96±0.03 |

## Results

*Growth, survival, metamorphosis and pigmentation success*

No differences in growth and survival rates were found at the stage of post-embryonic development for both groups in Senegalese sole larvae (control and ARA) (Table 3). The eye migration index, which is used to follow the progress of metamorphosis in flatfish (Fernández-Díaz *et al.*, 2001), was similar in both dietary treatments. However, at the end of the experiment, the control group was composed of 99% pigmented larvae, whereas 80% of the larvae from the ARA group became pseudo-albinos (Table 3).

*Morphological development of pseudo-albino phenotype*

In brief and in terms of skin pigmentation, the progress of metamorphosis of Senegalese sole larvae occurred within the following periods: pre-metamorphosis (until 11 dph), pro-metamorphosis (from 11 to 19 dph) and post-metamorphosis (from 19 to 47 dph). During pre- and pro- metamorphosis, larvae from both dietary groups underwent a normal development of skin pigmentation. At 22 dph, when larvae already had flat symmetry, the pigmentation pattern of individuals from both dietary groups was

similar, showing a very dense net of melanophores, xanthophores and leucophores that covered the head, the digestive cavity and the intermediate region of the trunk. They also presented two patches of chromatophores on the dorsal fin and another one on the anal fin (Figures 1A-A'). At 27 dph, although the relative amounts of melanophores, xanthophores and iridophores were similar in both groups (Figures 1B-B', 2A-C), the skin of larvae from the ARA group appeared less pigmented than that of the larvae from the control group due to the progressive disappearance of leucophores that conferred the greenish aspect to the skin (Figure 1B'). Differences in the amount and shape of melanophores and xanthophores between larvae from both experimental groups were in evidence from 33 dph (Figures 1C-C', 2A, 3A, 4E). The skin of the trunk presented abundant melanophores that were closely associated to 2 to 5 xanthophores in pigmented larvae (Figure 3A-C, Table 4), whereas fewer xanthophores were related to one melanophores in pseudo-albinos (Table 4), most of them showing signs of disintegration (Figure 3A'-C'; Table 5). Patches composed of melanophores, xanthophores and leucophores observed in pigmented individuals were not found in future pseudo-albinos (Figure 1C'). However, iridophores were present in the pelvic fins of specimens from both groups (Figures 1C-C'). At the level of the fins, future pseudo-albinos showed leucophores that appeared pale pink by reflected light, few iridescent iridophores, and round-shaped melanophores and xanthophores that were often associated in pairs and showed signs of degradation later in development (Figures 3A'-C', 4D, E). These chromatophores were progressively disappearing with age and, at 60 dph, the skin of the fin contained only a few leucophores located in the most apical region of the fins (Figure 4F). At 35 dph the pseudo-albino phenotype was already established (Figure 1D'). The differences in the amounts of xanthophores and iridophores between pigmented and pseudo-albino specimens became more evident (Figures 1D-D', 2B-C) and the grouping of iridophores in the fins of pigmented individuals was not found in pseudo-albinos (Figure 1D'). From 35 to 47 dph, the amount of chromatophores in pseudo-albinos remained invariable (Figures 1E'-F', 2A-C).

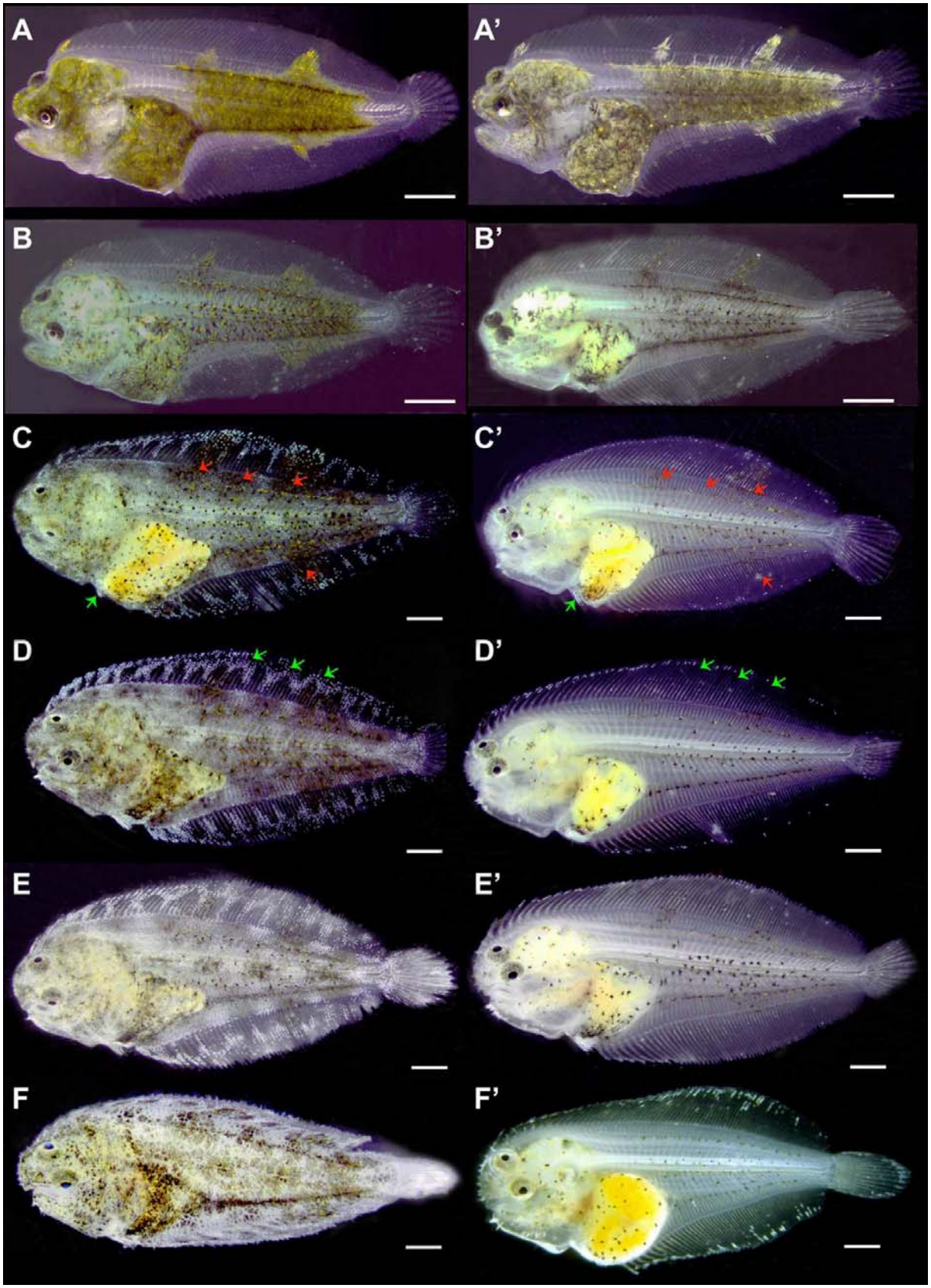
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'

**Figure 1.** Morphological comparison of the ontogeny of skin pigmentation in Senegalese sole larvae from the control (A-F) and ARA (A'-F') groups. A-A') 22 dph, B-B') 27 dph, C-C') 33 dph, D-D') 35 dph, E-E') 41 dph, F-F') 47 dph. At 22 dph, the pigmentation pattern of larvae from both dietary groups is similar, showing a very dense net of melanophores, xanthophores and leucophores that covers the head, the digestive cavity and the intermediate region of the trunk. They have also two patches of chromatophores in the dorsal fin and another one in the anal fin. At 27 dph, although the relative amounts of melanophores, xanthophores and iridophores was similar in both groups (Figure 2), the skin of larvae from the ARA group appeared les pigmented than that of the larvae from the control group due to the progressive disappearance of leucophores that confers the greenish aspect to the skin. At 33 dph, the amount of melanophores in control larvae was higher than in those fed with ARA. Red arrows indicates the allocation of patches of melanophores, xanthophores and leucophores in pigmented individuals (C) and their absence in future pseudo-albino individulas (C'). Green arrows show the presence of iridophores in the pelvic fins of specimens from both groups. At 35 dph the pseudo-albino phenotype is already established. The differences in the amounts of xanthophores and iridophores between pigmented (D) and pseudo-albino (D') specimens became more evident (Figure X). Green arrows show aggrupation of iridophores in the dorsal fin in pigmented individuals (D) and their absence in future pseudo-albino individulas (D'). From 35 to 47 dph, the amount of chromatophores in pseudo-albinos remained invariable. However, because there was an increase in the amount of melanophores and xanthophores in pigmented individuales from 41 to 47 dph there was a significant difference in the amount of all chromatophores between pigmented and pseudo-albino individuals at 47 dph. Scale bar represents 600 µm.

However, because there was an increase in the amount of melanophores and xanthophores in pigmented individuals from 41 to 47 dph, there was a significant difference in the amount of all chromatophores between pigmented larvae and pseudo-albinos at 47 dph (Figure 2A-C). At the level of the trunk, melanophores were dendritic in pigmented individuals while the ability to disperse melanosomes seemed to be reduced in most pseudo-albinos (Figures 4A, B, 6). In the skin of the fins, xanthophores and melanophores differed in shape between both groups: xanthophores were round in pigmented individuals whereas quite deformed in pseudo-albinos; melanophores were dendritic in pigmented individuals while round-shaped in pseudo-albinos (Figure 4C, D). Moreover, there were differences in the distance among xanthophores and melanophores between both groups. Generally, xanthophores were in direct contact with melanophores in pseudo-albinos, whereas they were closely associated, but not in contact, in pigmented specimens (Figure 4A, B).

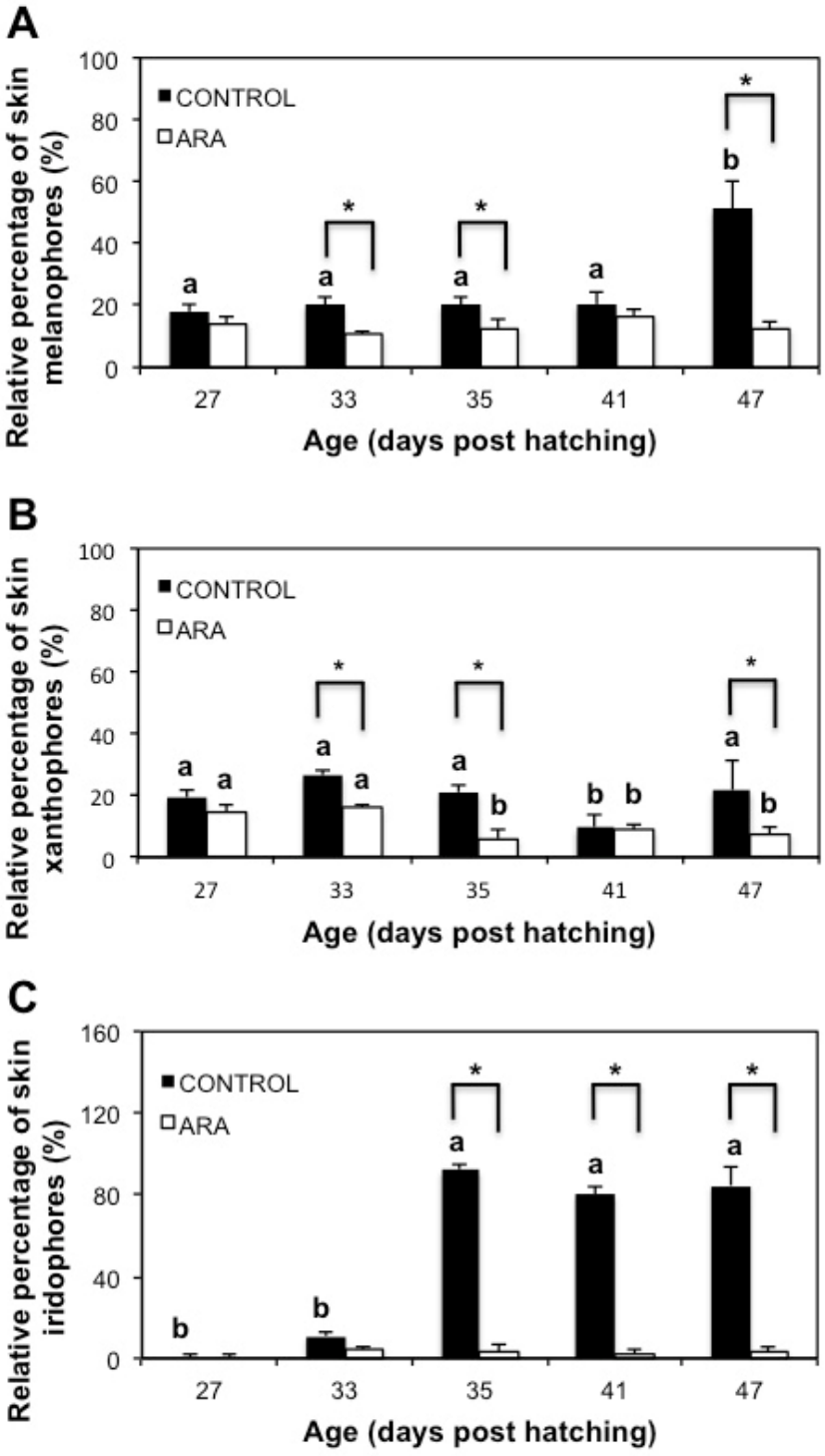


**Figure 2.** Relative percentage of skin melanophores (A), xanthophores (B) and iridophores (C) during post-metamorphosis of pigmented (control) and pseudo-albino (ARA) individuals of Senegalese sole. Values are expressed as mean ± SD (n = 4). Superscript letters denote significant differences in the relative amount of chromatophores with age for a given larval group and asterisks indicate significant differences in the amount chromatophores between pigmented and pseudo-albino specimens for a given larval age (Two-way ANOVA, $P < 0.05$; diet x age interaction $P < 0.006$).

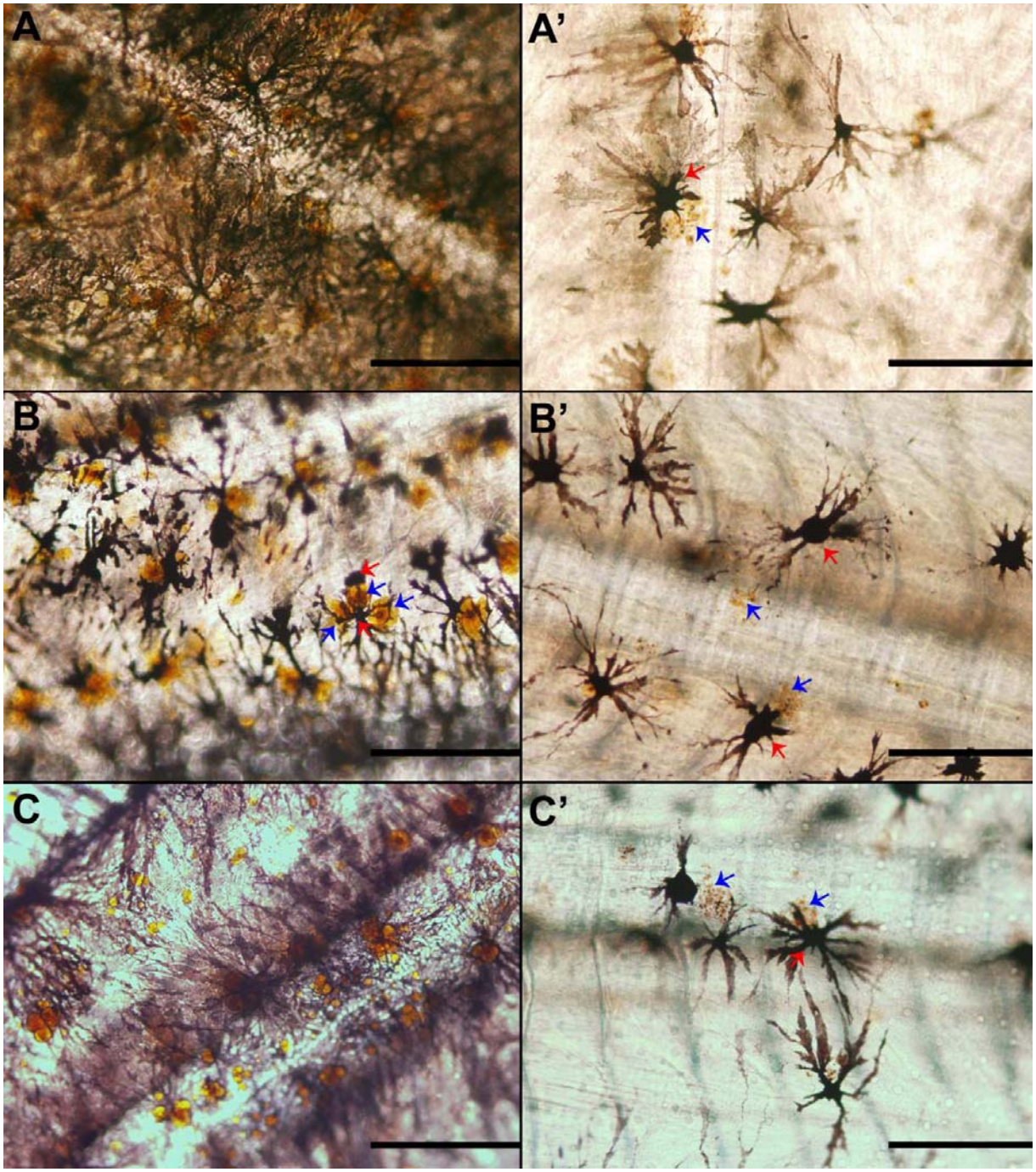


**Figure 3.** Microscopic images of the trunk skin of pigmented (A-C) and pseudo-albino (A'-C') Senegalese sole specimens. A-C) skin showing abundant dendritic melanophores surrounded by several xanthophores. A'-C') skin showing few melanophores and disintegrating xanthophores. A) 33 dph, B) 35 dph, C) 41 dph. Red arrows, melanophores; blue arrows, xanthophores. Scale bar represents 500 µm.

*Skin chromatophores*

Figure 2 shows the relative amount of chromatophores in larvae from control and ARA treatments during post-metamorphosis (from 27 to 47 dph). Specimens from the control group showed 19.75 ± 1.13 % of melanophores from 27 to 41 dph. Then, the relative amount of melanophores increased up to 51% from 41 to 47 dph (Figure 2A). Larvae from the ARA group presented 13.26 ± 2.15 % of melanophores during the whole post-metamorphosis period (Figure 2A). The relative amount of melanophores was significantly lower in larvae from the ARA group compared to those from the control at 33, 35 and 47 dph (Figure 2A). The relative amount of xanthophores in control specimens remained constant from 27 to 35 dph (22.28 ± 3.62 %). From 35 to 41 dph, the relative amount of xanthophores decreased to 9 % and increased again up to 22 % at 47 dph (Figure 2B). Among larval chromatophores from the ARA group from 27 to 33 dph, 15.22 ± 0.82 % were xanthophores. From 33 to 35 dph, the amount of these pigment cells decreased by a half and remained invariable until 47 dph (Figure 2B). The amount of xanthophores in pseudo-albinos was significantly lower than in pigmented individuals at 33, 35 and 47 dph (Figure 2B). The amount of iridophores in pigmented individuals was low from 27 to 33 dph and then it increased 8 times from 33 to 35 dph and remained invariable from that day onwards (85.54 ± 6.14 %) (Figure 2C). In pseudo-albinos, the amount of iridophores was low and stable during the entire period analyzed (2.63 ± 1.64 %) (Figure 2C). The amount of iridophores was significantly lower in pseudo-albinos than in pigmented individuals from 35 dph onwards (Figure 2C).

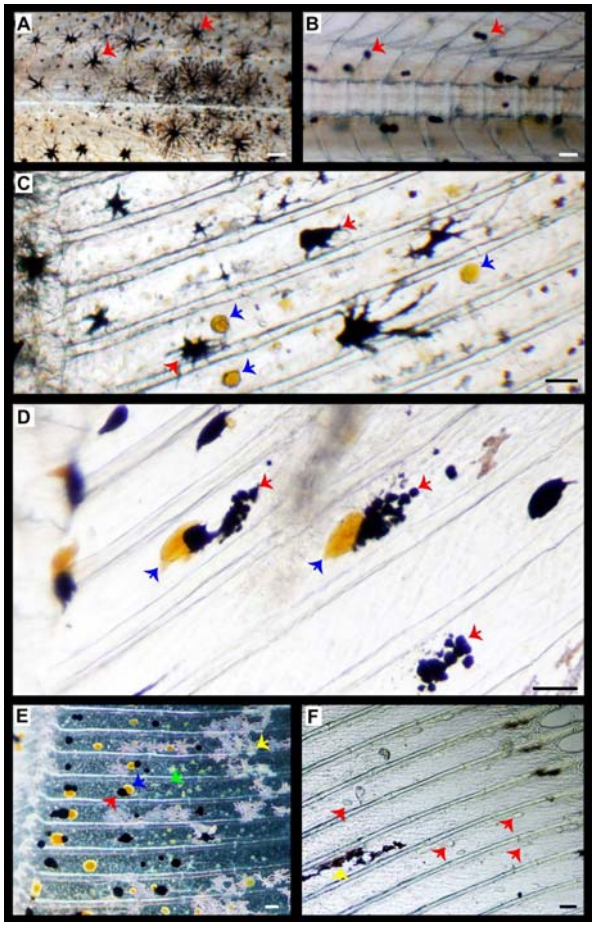


**Figure 4.** Microscopic images showing details of chromatophores distribution in the skin of pigmented larvae and pseudo-albinos in the ocular side of the fish. A-B) trunk skin at the level of the vertebral column in 47 day-old pigmented (A) and pseudo-albino (B) specimens. Melanophores are dendritic in pigmented individuals while the ability to disperse melanosomes is lost in pseudo-albinos. C-D) dorsal fin skin in 60 day-old pigmented (C) and pseudo-albino (D) specimens. Note the different shapes and sizes of xanthophores and melanophores between both groups: xanthophores are round in pigmented individuals whereas quite deformed and bigger in pseudo-albinos; melanophores are dendritic in pigmented individual while the round-shaped in pseudo-albinos. There are differences in the distance among xanthophores and melanophores between both groups. The interaction between these chromatophores was closer in pseudo-albinos and melanophores showed signs of disintegration. E) detail of the dorsal fin skin of a 33 day-old future pseudo-albino showing melanophores (black dendritic cells), xanthophores (yellow round cells), leucophores dendritic cells that appear pale pink under reflecting light) and iridophores (iridescent round cells). Round-shaped melanophores and xanthophores were often associated in pairs, this being associated to the observed phenomenon of disintegration of one of the two chromatophores. F) detail of the dorsal fin skin of a 60 day-old pseudo-albino showing only few leucophores. Some non-identified transparent round structures were appreciated (red arrows). Red arrows, melanophores; blue arrows, xanthophores, green arrows, iridophores, yellow arrows, leucophores. Scale bars represent A-B, 200 µm; C-F, 100 µm.

Figure 5 shows the relative percentage of skin chromatophores in specimens from control (Figure 5A) and ARA (Figure 5B) groups at each sampling day. At 27 dph, larvae from both populations presented similar relative amounts of skin melanophores and xanthophores, and a lower amount of iridophores. At 33 dph, xanthophores were the most abundant pigment cell in ARA group, followed by melanophores and, finally, iridophores. At 35 dph, the amount of iridophores was higher than that of melanophores and xanthophores in pigmented specimes, whereas melanophores were more abundant than xanthophores and iridophores in pseudo-albinos. At 41 dph, iridophores were more abundant than melanophores, followed by xanthophores in pigmented individuals, while melanophores were more abundant than xanthophores, followed by iridophores in pseudo-albinos. These profiles of chromatophore proportions were maintained at 47 dph.

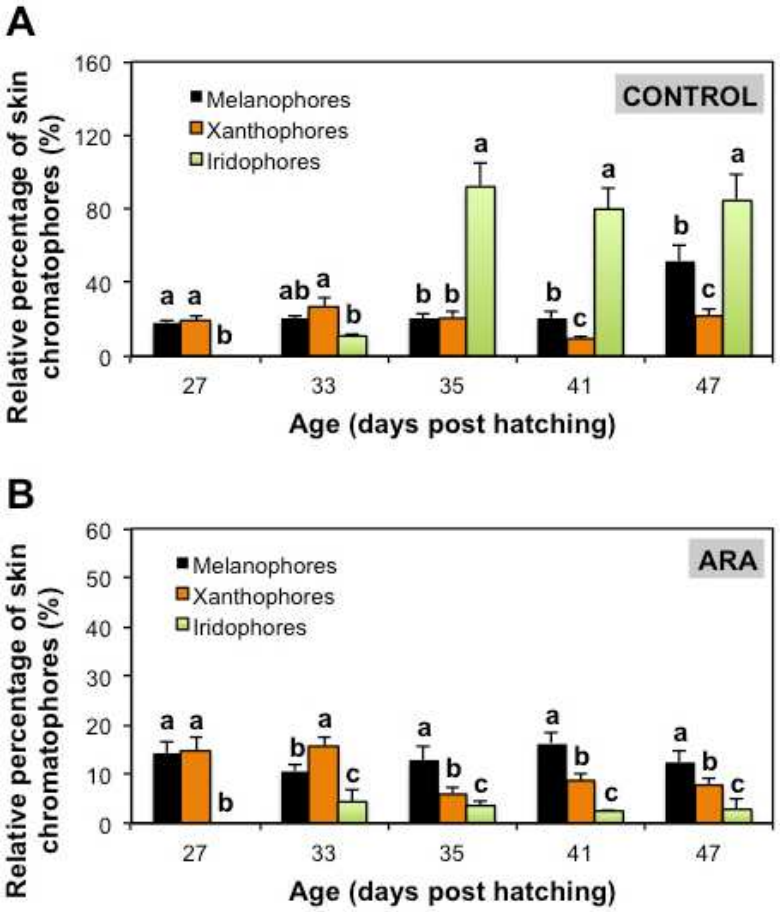


**Figure 5.** Relative percentage of skin chromatophores in pigmented (A) and pseudoalbino (B) specimens of Senegalese sole during post-metamorphosis. Values are expressed as mean ± SD (n = 4). Superscript letters denote significant differences in the relative amount of chromatophores for a given larval age (One-way ANOVA, $P < 0.05$). Note that differences in the relative proportion of the three chromatophores between control and ARA groups were evident from 35 dph onwards and that they became invariable from 41 dph, indicating the establishment of the final skin color phenotype on the ocular side.

The number of xanthophores (X) associated to one melanophore (M) in each group of larvae during post-metamorphosis of Senegalese sole is shown in Table 4. Larvae from the control group showed a X/M ratio close to 1 at 27 dph. The number of xanthophores related to one melanophore increased at 33 dph, and decreased until 2 from 33 to 35 dph. At 41 dph, the X/M ratio increased again displaying an average of 4. The X/M ratio in the skin of larvae from the ARA group was close to 1 at 27 dph and it subsequently decreased to almost 0 until the end of the analyzed period.

**Table 4.** Percentage of disintegrating xanthophores in Senegalese sole specimens from the control and ARA groups during the post-metamorphosis period (n = 4).

| | % Disintegrating xanthophores | |
|---|---|---|
| **Age (dph)** | **CONTROL** | **ARA** |
| 27 | 25 | 25 |
| 33 | 25 | 100 |
| 35 | 25 | 50 |
| 41 | 0 | 57 |

The size of skin chromatophores in pseudo-albinos and pigmented specimens at post-metamorphosis is shown in Figure 6. The size of melanophores was around 50 μm in pseudo-albinos and pigmented specimens. However, their capacity to disperse melanine was significantly reduced in pseudo-albinos (Figures 4A, 4B, 6). The size of xanthophores was higher in pseudo-albinos than in pigmented specimens (90 versus 22 μm in average). Iridophores were the smallest pigment cells and their size was similar in both larval groups (17 μm in average, Figure 6).

From individuals in the control group 25% showed disintegrating xanthophores from 27 to 35 dph, whereas no evidences for xanthophores disintegration were observed at 41 dph (Table 5). From the ARA group 25% of larvae presented disintegrating xanthophores at 27 dph, a 100% at 33 dph, and around a 50% at 35 and 41 dph (Table 5).

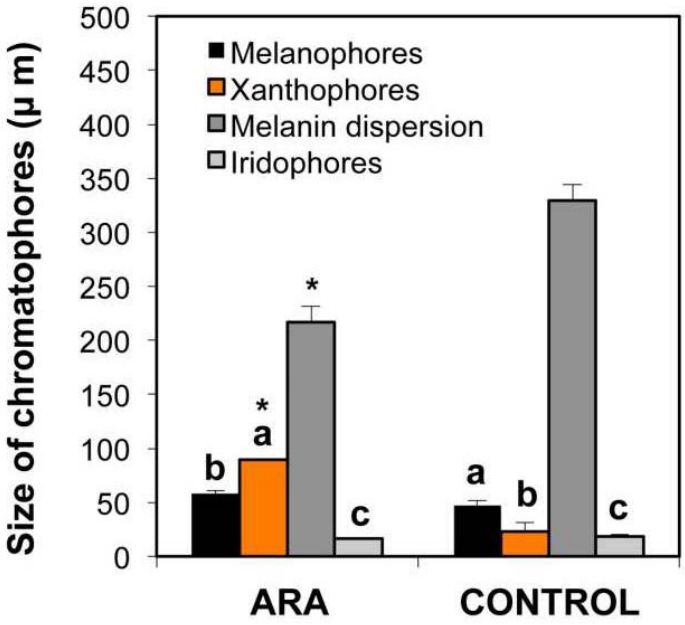


**Figure 6.** Size of skin melanophores, xanthophores and iridophores, and melanin dispersion distance in 60 day-old pigmented (control) and pseudo-albino (ARA) specimens of Senegalese sole. Values are expressed as mean ± SD (n = 4). Superscript letters denote significant differences in size between chromatophores of a given larval group (One-way ANOVA, $P < 0.05$) and asterisks indicate significant differences in size of chromatophores and melanin dispersion distance between pigmented and pseudo-albino specimens (Student's *t*-test, n = 4, $P < 0.001$).

**Table 5.** Number of xanthophores associated to one melanophore in specimens of Senegalese sole from the control and ARA groups during the post-metamorphosis period. Values are expressed in means ± SEM (n = 4). Superscript letters denote significant differences in the number of xanthophores related to one melanophore between developmental ages in each group (One-way ANOVA, $P < 0.05$) and asterisks denote significant differences between groups of the same age (*t*-test, $P < 0.05$).

| | Xanthophores /Melanophores | |
|---|---|---|
| **Age (dph)** | **CONTROL** | **ARA** |
| 27 | 1,36 ± 0,16$^{c}$ | 1,69 ± 0,13$^{a}$ |
| 33$^{*}$ | 3,54 ± 0,82$^{ab}$ | 0,40 ± 0,18$^{b}$ |
| 35$^{*}$ | 1,81 ± 0,16$^{bc}$ | 0,27 ± 0,09$^{b}$ |
| 41$^{*}$ | 4,15 ± 0,64$^{a}$ | 0,45 ± 0,14$^{b}$ |

*Modulation of gene expression in pseudo-albino specimens*

Partial coding sequences from 9 pigmentation-associated genes from Senegalese sole (Table 2) were obtained using consensus primers: melanocyte-stimulating hormone 1 receptor (*mc1r*), agouti signaling protein (*asip*), paired box protein 3 (*pax3*), microphtalmia-associated transcription factor (*mitf*), tyrosinase (*tyr*), tyrosinase-related protein 1 (*trp1*), mast/stem cell growth factor receptor Kit (*cKit*), sodium/potassium/calcium exchanger 5 (*slc24a5*), enzyme caspase 3 (*casp3*) and heat shock 70 kDa protein (*hsp70*). Fragments ranged in size from 272 to 1138 base pairs. Additionally, somatolactin (*sl*) was downloaded from GenBank.

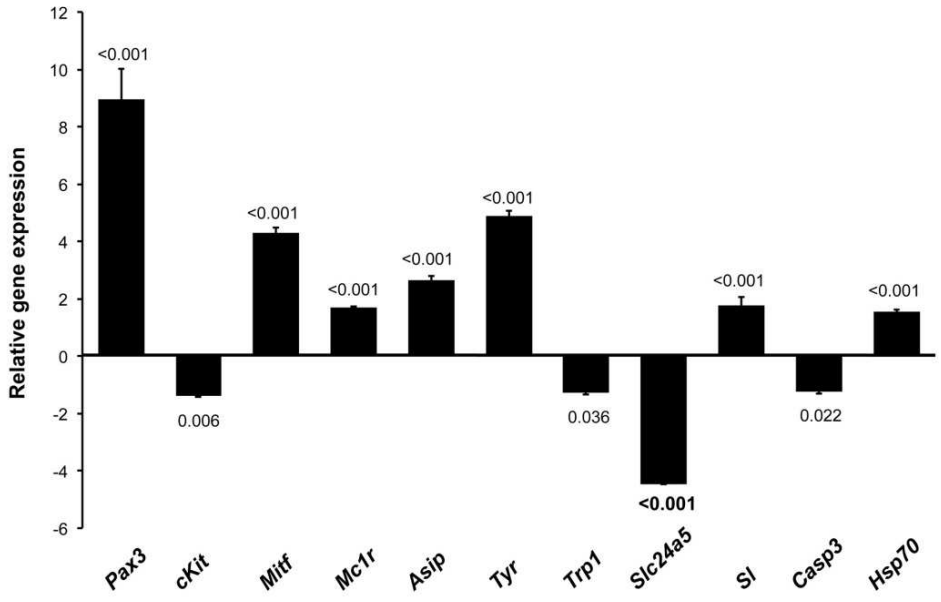


**Figure 7.** Fold change relative gene expression of pigmentation-related genes in 60 day-old pseudo-albino (ARA) specimens of Senegalese sole compared to pigmented specimens. Melanocyte-stimulating hormone 1 receptor (mc1r), agouti signaling protein (asip1), paired box protein 3 (pax3), microphtalmia-associated transcription factor (mitf), tyrosinase (tyr), tyrosinase-related protein 1 (trp1), mast/stem cell growth factor receptor Kit (cKit), sodium/potassium/calcium exchanger 5 (slc24a5), enzyme caspase 3 (casp3), heat shock 70 kDa protein (hsp70) and somatolactin (sl). Student's t-test, n = 4, P-values shown in figure.

The average efficiency of amplification for all assays was 99 %, the slopes of the standard curves being 3.3, 3.1, 3.5, 3.2, 3.2, 3.5, 3.3, 3.5, 3.5, 3.4 and 3.3 for *pax3*, *tyr*, *mitf*, *mcr1*, *asip, hsp70*, *sl*, *casp3*, *trp1*, *slc25a5* and *ckit,* respectively. Results of gene expression in 60 day-old pseudo-albino specimens showed a positive and statistically significant up-regulation of *pax3*, *tyr*, *mitf*, *mcr1*, *asip, hsp70* and *sl* (9, 5, 4, 1.7, 2.6, 1.5 and 1.8 fold changes, respectively) and a down-regulation of *casp3*, *trp1*, *slc24a5* and *ckit* (1.2, 1.3, 4.4 and 1.4 fold changes, respectively) compared to the pigmented individuals (Figure 7).

**Discussion**

*Morphological development of pseudo-albinos*

Morphological data revealed that ARA did not affect larval pigmentation at the pre-metamorphic stage, but prevented chromatophore terminal differentiation at metamorphosis, leading to the appearance of pseudo-albinism. The amount of melanophores and iridophores in pseudo-albinos remained invariable during the entire studied period. However, normally pigmented specimens showed an increase in the population of melanophores and iridophores at post-metamorphosis (47 and 35 dph, respectively). This indicates that the new population of chromatophores that should appear after metamorphosis was not formed (or cells were not pigmented) in pseudo-albinos. While molecular signaling towards the differentiation of new populations of melanophores, xanthophores and iridophores occurs during pro-metamorphosis (11-19 dph), morphological changes occur later at post-metamorphosis (Darias *et al.*, 2013). Considering that the amount and proportions of these chromatophores were similar in pigmented and pseudo-albino specimens until 27 dph, and that their number remained invariable from that day onwards, it seemed that pigment cell precursors were likely influenced by the asymmetric signaling during pro-metamorphosis rather than in mature larval chromatophores (Yamada *et al.*, 2010). The decrease in xanthophores from 33 to 35 dph observed in pseudo-albinos was likely the result of the degradation of already existent xanthophores. Interestingly, and contrary to pigmented specimens, round-shaped melanophores and xanthophores were often coupled in pseudo-albinos and their closer association, together with the increased size of xanthophores, suggested some type of mechanism may be in operation that leads to the collapse of these chromatophores. The different allocation, shape, size and distance between melanophores and xanthophores observed in pseudo-albinos suggested the possible existence of an altered communication between these pigment cells. Interactions between these pigment cells have already been reported and it is believed that xanthophores regulate melanophore pattern (Parichy and Turner, 2003). In fact, this relationship is required to form the Turing pattern of zebrafish (Nakamasu *et al.*, 2009) and has also been suggested for the pigmentation patterning of Senegalese sole (Darias *et al.*, 2013). Moreover, it has been shown that when melanophores and xanthophores are adjacent, these cells exclude each other (Nakamasu *et al.*, 2009). These interactions seem to be necessary for the normal development of pigmentation (Darias *et al.*, 2013), as 25% of the population of pigmented individuals showed disintegrating xanthophores in their skin from 27 to 35 dph. However, at 41 dph, disintegrating xanthophores were not observed in pigmented specimens, probably indicating the end of the action of melanophores over xanthophores for the establishment of the adult pigment pattern. In a previous study we observed that the normal pattern of interaction between xanthophores and melanophores towards the achievement of the adult pigmentation showed a fluctuating X/M (xanthophore /melanophore) ratio from 27 to 47 dph (Darias *et al.*, 2013). Present results suggest that the X/M ratio and cell proximity play a key role in the correct dorsal-ventral pigment patterning. Although the amount was not significant compared to that of xanthophores, disintegrating melanophores were observed from 41 to 60 dph (Figure 4D). In line with this, xanthophores of zebrafish were able to reduce the surrounding melanophores within a short-range, whereas xanthophores that were distally located from melanophores were able to

enhance the development and survival of the latter cells (Nakamasu *et al.*, 2009). This seemed to also be the case for pigmented Senegalese sole specimens in which small xanthophores could be seen surrounding the well-developed melanophores (Figure 4C). Altogether these results show that chromatophores develop and interact in a very delicate equilibrium where xanthophores play a central role in pigment patterning, the relative proportion and behavior (allocation, size, shape) of this pigment cell being critical for the correct ontogeny of pigmentation.

Pseudo-albinos had lost most of their leucophores, with those few remaining being located almost exclusively in the distal part of the fins. Moreover, when analyzing different specimens displaying different degrees of pseudo-albinism, it was noticed that, as the fins grew, tissue lacking pigmented chromatophores appeared basally. This could be an indication of new tissue devoid of chromatophores (or cells devoid of pigment) being produced basally and that the early leucophores located basally disappeared (Figure 4F) (Yamada *et al.*, 2010).

*Modulation of gene expression in pseudo-albino specimens*

The morphological changes in skin pigmentation observed in pseudo-albinos were reflected in their molecular signaling response as seen via analysis of the eleven pigmentation-related genes in which different expression patterns were evident in pseudo-albinos as compared to pigmented specimens. These differences likely prevented the development of post-metamorphic melanophores, xanthophores and iridophores.

Regarding melanophores, two mechanisms (probably related) accounted for the absence of melanin. The first one is that initial but not terminal differentiation of melanophores was promoted and the second one is that melanin synthesis was disrupted at the last step of this process (Figures 7, 8). It has been recently shown that the gene *sl* enhances the differentiation of melanophores in medaka, although the specific molecular action is not yet known (Fukamachi *et al.*, 2009). The up-regulation of *sl* expression in Senegalese sole pseudo-albinos pointed to melanophore differentiation being stimulated. This is further supported by the significant up-regulation of *pax3,* which is one of the first genes expressed in melanoblast/melanophore precursors (Lister, 1999; Minchin and Hughes, 2008) and plays a key role in controlling the development of melanocytes (Kubic *et al.*, 2008). In this capacity, *pax3* maintains the equilibrium between melanocyte differentiation and melanin synthesis. During stem cell differentiation into melanocytes, *pax3* simultaneously induces a melanogenic cascade, through the activation of *trp1* expression, while acting downstream to prevent terminal differentiation (Galibert *et al.*, 1999). Such a mechanism guarantees cellular proliferation, thus sustaining this cell population (Kubic *et al.*, 2008). *Pax3* is able to inhibit the expression of *trp2*, responsible for the conversion of L-dopachrome into 5,6-dihydroxyindole-2-carboxylate. This ensures high levels of *mitf* expression to induce melanocyte differentiation (Bondurand *et al.*, 2000; Guo *et al.*, 2010) but at the same time prevents terminal differentiation. In the presence of β-catenin, the action *of pax3* to block *trp2* transcription is missing (Hathaway and Haque, 2011) and then *trp2* contributes to

melanocyte differentiation (Figure 8). In Senegalese sole pseudo-albinos, *pax3* seemed to promote melanophore differentiation through the up-regulation of *mitf* while preventing its terminal differentiation through the down-regulation of *trp1* expression (Figure 8). As occurs with *mitf, pax3* also promotes and inhibits melanogenesis through transcriptional regulation of *cKit (*Guo *et al.*, 2010). *CKit* plays a critical role in melanocyte physiology by influencing melanogenesis, migration and survival of these cells (Alexeev and Yoon, 2006) and it is primarily expressed in the melanized melanophores (Yamada *et al.*, 2010). In Senegalese sole developing larvae, the profile of *cKit/pax3* and *mitf*/*pax3* ratios reflected the pattern of melanophore ontogeny (Darias *et al.*, 2013). Larvae underwent a process of melanogenesis during pre-metamorphosis and a stabilization of melanophore differentiation and melanogenesis processes during pro-metamorphosis. Once metamorphosis was finished, these ratios increased again indicating the prevalence of melanogenesis over melanophore differentiation. The gene expression profile of *pax3*, *mitf* and *ckit* in metamorphosed pseudo-albinos compared to pigmented individuals indicated that melanophore differentiation prevailed over melanin synthesis, which is surprisingly opposite to what was expected to occur at that period. This suggests that the switch of the molecular events that should take place from pro- to post-metamorphosis to develop the adult pigmentation pattern was disrupted in pseudo-albinos.

*Mitf* is also a master regulator of melanogenesis due to its ability to activate many melanocyte-specific genes, such as *tyr*, *trp1* and *trp2* (Curran *et al.*, 2010; Levy and Fisher, 2011), and *mc1r* (Aoki and Moro, 2002). The melanocyte-stimulating hormone alpha (*α-MSH*) induces the proliferation of melanophores through binding to its receptor, *MC1R* (Fujii, 2000), and activates melanogenesis through the positive regulation of tyr activity (Figure 8). The end of pigmentation metamorphosis in pigmented Senegalese sole specimens was marked by a decrease in *mc1r* expression (Darias *et al.*, 2013). However, pseudo-albinos at post-metamorphosis showed an up-regulation of *mc1r* providing more evidence for altered molecular signatures preventing terminal development of skin pigmentation. The elevated amount of *mitf* transcripts in pseudo-albinos could be responsible for the up-regulation of *mc1r*. As a consequence of this, and as happens in pigmented individuals, an up-regulation of *tyr* induced by *mc1r* could be expected (Chen *et al.*, 2000). It has been shown that *tyr* gene transcription is also induced by the transient activation of the cKIT receptor by its ligand, stem cell factor, in differentiated melanocytes (Luo *et al.*, 1995). Down-regulation of *ckit* observed in pseudo-albinos compared to pigmented specimens likely indicated that *ckit* was not the primary cause for the induction of *tyr* expression. The lower levels of expression of *ckit* observed in pseudo-albinos could reflect the lower amount of differentiated melanophores compared to the pigmented individuals. But it also corroborates that *ckit* was not expressed in what seem to be unmelanized melanophores. Further, *tyr* expression could also be directly induced by *pax3*. The significant up-regulation of *pax3* expression in pseudo-albinos, together with *mitf* and *tyr,* illustrates the above-mentioned role of *pax3* in controlling melanophore differentiation. However, the pseudo-albino phenotype was characterized by the presence of few melanized melanophores. This is in line with the thought that *pax3* in mammals is expressed in early development, but inhibited in adult melanocytes (Hathaway and Haque, 2011).

Therefore, although molecular signaling suggests that melanophore differentiation was favored, melanogenesis was not.

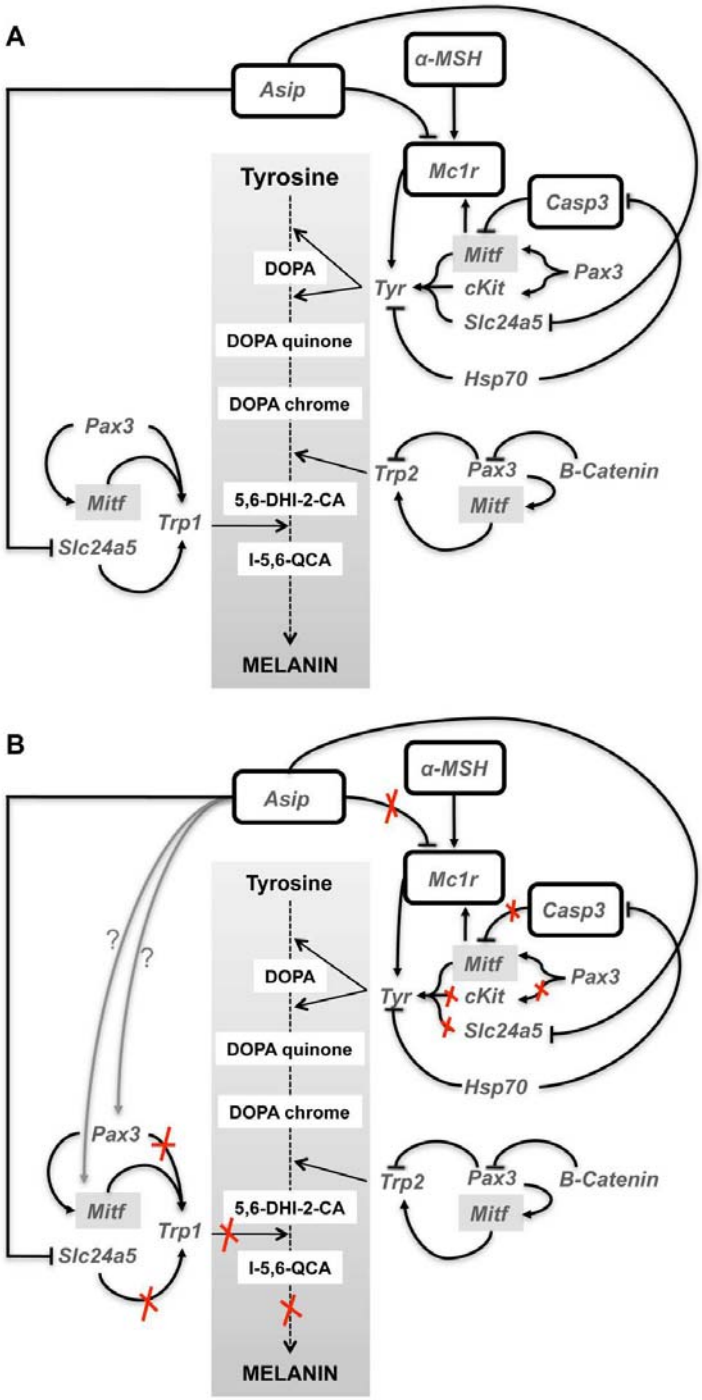


**Figure 8.** Schematic picture showing the known action of the analyzed genes in melanocyte differentiation and melanogenesis in vertebrate melanocytes (A) and the suggested pathways of melanophore differentiation and melanogenesis that were altered in pseudo-albinos of Senegalese sole (B).

*Asip1* is thought to be involved in the establishment of the dorsal-ventral pigment pattern by directing chromatophore differentiation, causing production of iridophores and inhibiting the production of melanophores (Cerdá-Reverter *et al.*, 2005). However, the development of iridophores, which typically occurs at post-metamorphosis (Darias *et al.*, 2013), was distorted in Senegalese sole pseudo-albinos. Our results suggest that ARA affected normal gene expression in a way which blocked the pigment patterning that should have been established on the ocular side of the fish at metamorphosis via *asip1* gene expression; then, the excessive *asip1* expression levels could alter normal chromatoblast differentiation leading to an inhibition of differentiation of new post-metamorphic iridophores. This hypothesis is supported by recent findings on *asip1* regulation in adult Senegalese sole pseudo-albinos that showed a higher expression of this gene within unpigmented patches of the dorsal skin than in pigmented regions, but similar to the levels found in the blind side (Guillot *et al.*, 1992). In developing Senegalese sole larvae, *asip1* seemed to be responsible for the inhibition of melanophore differentiation on the blind side concomitant with the induction of iridophore differentiation on the ocular side before the differentiation of iridophores on the blind side (Darias *et al.*, 2013). We propose that the disruption of the dorsal-ventral pigment patterning during metamorphosis entailed the establishment of the mechanisms for blind side pigment pattern on the ocular side but blocked at the point of inhibition of melanophore differentiation and before the differentiation of iridophores. It is interesting to note that iridophores were present in pelvic fins of pseudo-albinos, suggesting that the molecular signaling towards their differentiation took place before mouth opening, as is the case of the eyes (Darias *et al.*, 2013), or, alternatively, it is independent from the rest of the body. Indeed, little information exists on the molecular mechanisms driving iridophore development. It has been recently demonstrated that *mitf,* the key regulator of melanophore development and melanogenesis (Lister *et al.*, 1999; Steingrimsson *et al.*, 2004), also regulates cell fate plasticity in zebrafish, including the formation of iridophores (Curran *et al.*, 2010). Minchin and Hughes (2008) demonstrated that *mitf* inhibits the iridophore fate in zebrafish. In Senegalese sole pigmented individuals, the expression of *mitf* decreased during development to promote the bi-potent pigment cells precursors to differentiate into iridophores instead of melanophores (Darias *et al.*, 2013). However, this gene was up-regulated in pseudo-albinos, giving more evidence for the disruption of the normal development of iridophores in these specimens. Considering this, it is tempting to speculate that the overexpression of *mitf* in Senegalese sole pseudo-albinos could be blocking the differentiation of precursor cells into iridophores, as melanophore and iridophores have a common precursor and both cell types were not developed in pseudo-albinos. This is suggestive that an excess of *asip1 and mitf* expression are preventing the development of melanophores and iridophores by exerting their inhibitory function at the level of their cellular precursors. Whether *asip1* is able to modulate the expression of *mitf* and/or vice versa needs to be explored. These findings are in line with the evolvement of chromatophore development in Senegalese sole peudo-albinos at post-metamorphosis, where the amount of melanophores was invariable, the amount of xanthophores was reduced and iridophores were not differentiated. The putative implication of *mitf* in xanthophore disintegration deserves to be investigated. Taking into account these findings, Senegalese sole pseudo-albinos

seemed to face a problem of post-metamorphic iridophore and xanthophore development and terminal differentiation of melanophores. Whether the above described molecular signaling contributing to melanophore differentiation is due to signals from the surrounding immature melanophores needs to be further investigated.

Although *tyr* expression was highly induced in pseudo-albinos, the completion of this process was inhibited at the last step of melanin production through the down-regulation of *trp1*. Moreover, the low expression level of *trp1* would be induced by the down-regulation of *slc24a5* (Figure 8). In fact, it has been demonstrated that the knockdown of *slc24a5* provoked dramatic effects on pigmentation in re-differentiating human epidermal melanocytes causing hypomelanosis (Ginger *et al.*, 2008). *Slc24a5* is a putative cation exchanger known to increase uptake and accumulation of calcium in melanocytes, necessary for melanin synthesis (Lamason *et al.*, 2005; Vogel *et al.*, 2008) where calcium plays an important role as a second messenger in the initiation of melanogenesis by stimulating the synthesis of L-tyrosine (Schallreuter *et al.*, 2008). The significant down-regulation of *slc24a5* expression in pseudo-albinos suggests that tyrosine production may be altered in these specimens. Interestingly, expression of *slc24a5* has been found to be negatively regulated by *asip1* (Nadeau *et al.*, 2008) (Figure 8). The reason why and how this gene is down-regulated by ARA to generate pseudo-albinos needs further investigation. Therefore, in addition to the known role of the gene *asip1* in iridophore differentiation in fish (Cerdá-Reverter *et al.*, 2005), our results show that *asip1* also regulates melanophore differentiation and melanogenesis (Sviderskaya *et al.*, 2001). This has been already demonstrated in mammals, where *asip1* regulates the relative proportions of eumelanin (black-brown pigment) and phaeomelanin (yellow-red pigment) by antagonizing the action of α-MSH on its receptor MC1R (Hunt *et al.*, 1995); additionally, *asip1* negatively regulates the expression of *mc1r* (Uong and Zon, 2010) and *slc24a5* ( Nadeau *et al.*, 2008). Fish only produce eumelanin, and *mc1r* expression was up-regulated in pseudo-albinos, therefore the regulation of melanogenesis is likely blocked at some step downstream from *mc1r*. We propose that melanin synthesis is inhibited in newly differentiated melanophores by negatively regulating the expression of *slc24a5,* which is necessary for melanogenesis. Although low, *trp1* expression still existed in these specimens, likely reflecting melanogenesis within the few melanophores remaining in the skin. The primary function of *mc1r* in fish is to mediate distribution of melanin granules in melanophores, allowing the organism to adapt to the background (Logan *et al.*, 2003). The expression of this gene was up-regulated in pseudo-albinos, though the ability to disperse melanin within melanophores was reduced.

Gene expression data suggest that ARA changed the pigment pattern that should have been established in the ocular side of the fish at metamorphosis through its effects on *asip1, pax3* and/or *mitf*; thereafter, the excessive expression levels of these genes could alter their normal action on chromatoblast differentiation leading to an inhibition of terminal differentiation of new post-metamorphic melanophores, xanthophores and iridophores. Abnormal spatial distribution between xanthophores and melanophores were observed in pseudo-albinos. An excessive disintegration of

xanthophores seemed to prevent the normal patterning of melanophores, suggesting that normal pigmentation at metamorphosis depends on some type of communication established between these pigment cells and their proportions. We propose that the unexpected behavior of these pigment cells in the skin of the ocular side of the fish are typically taking place in the blind side during metamorphosis for the development of the asymmetrical dorsal-ventral pigment patterning characteristic of flatfish. The molecular mechanisms underlying these processes need to be further explored. Taking into account the well-known role of *asip1* in the establishment of the dorsal-ventral pigment patterning in this and other species (Cerdá-Reverter *et al.*, 2005; Guillot *et al.*, 2012), and the results obtained in this study, the role of *asip1* in xanthophore physiology deserves investigation. These findings show that the genetically determined pigmentation in larvae can be modulated by environmental factors (i.e., nutrition) and thereby affect changes in the normal molecular signatures occurring during the process of metamorphosis.

**Conclusion**

We conclude that differentiation of melanophores was promoted in pseudo-albinos through *pax3*, *mitf*, *mc1r* and *tyr* signaling with a simultaneous deficiency of terminal differentiation. *Asip1* and *mitf* seemed to play a key role in the prevention of differentiation of pigment cell precursors into melanophores and iridophores. *Slc24a5* seems to play a key role in the disruption of melanogenesis at the end of this process by decreasing *trp1* expression. We propose that the down-regulation of *slc24a5* could be induced by up-regulation of *asip1*, reaffirming the involvement of this gene in melanogenesis. The expression level of *pax3* appeared to be critical for the development of the new populations of melanophores, xanthophores and iridophores at post-metamorphosis. Among these chromatophores, xanthophores seemed to play a central role in pigment patterning, where interaction between pigment cells occurred in a delicate equilibrium. Our results demonstrated that the relative proportions between xanthophores and melanophores, cell proximity, size and shape are critical for the correct ontogeny of pigmentation. Further molecular research on chromatophore interactions would definitely shed light to the mechanisms governing cellular communication leading to the establishment of the pseudo-albino phenotype. Additional insights may also be gained by separation of blind side from ocular side skin for a more detailed look at chromatophore development.

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# GENERAL DISCUSSION

The rapid development of intensive aquaculture production of marine fish in response to an increasing market demand has evidenced the apparition of some limitations linked to the massive production of animals. The optimization of rearing conditions, such as the augmentation of fish densities and daily feeding rates, in order to reduce the duration of production cycles and respond to market needs, directly impacts on the growth and development of the animals. Marine fish larvae are particularly sensitive to rearing conditions, especially during the early larval stages, when they undergo deep morphological, anatomical, and physiological modifications in their different tissues, organs and vital systems. Early larval stages represent a transitional ontogenetic period of growth and differentiation, when the animal switches from endogenous to exogenous feeding, several organs and systems, such as sensory, neuroendocrine, excretory and digestive organs, as well as the skeletal tissue and musculature, further develop and the organism achieves its definitive phenotype (Power *et al.*, 2008). The proper development of these processes during the larval stage is a key step to obtain juveniles of quality and optimize the market value of the final product. The development of reliable and sustainable rearing protocols in industrial hatcheries requires a deep understanding of the critical points of larval development, particularly the nutrition at a stage when the diet is crucial to allow the metabolism to support the intense larval growth. During the early stages of development, larvae are particularly sensitive to non-optimal conditions of feeding and an unbalanced diet can seriously affect the formation of tissues and organs of the future adult.

During the last decades, the Mediterranean finfish larviculture industry has considerably improved its rearing methods under intensive conditions, but larval quality remains one of the main concerns for the proper development of the productive sector (Koumoundouros, 2010; Boglione *et al.*, 2013b). The high incidence of skeletal deformities and pigmentary disorders found in farmed fish (affecting up to 30% of the production) originates from larval rearing stages and indicates that industrial hatcheries still lack the proper knowledge to produce high-quality and healthy larvae under intensive rearing conditions. The incidence of skeletal abnormalities such as scoliosis, lordosis, twisted vertebral column, missing or additional fin rays, bent operculum or jaw malformations, have been reported to affect most part of the production in temperate marine species, such as gilthead sea bream (Matsuoka, 1987; Boglione *et al.*, 2003; Prestinicola *et al.*, 2012), European sea bass (Barahona-Fernandes, 1982) and Senegalese sole (Gavaia *et al.*, 2009). However, this problem does not only affect species culture in temperate waters, but it also affects cold water species, such as rainbow trout *Oncorhynchus mykiss,* cod *Gadus morhua* or Atlantic halibut *Hippoglossus hippoglossus* (Deschamps *et al.*, 2008; Baeverfjord *et al.*, 2009; Lewis-Mc Crea and Lall, 2010). The problem of skeletal deformities does not only affects the European aquaculture industry, it is a recurrent problem world-wide (Hosoya and Kawamura, 1998; Cobcroft and Battaglene, 2013) Moreover, pigmentary disorders (ambicolouration, albinism, pseudo-albinism, hypomelanosis or hypermelanosis) may affect from 5 to 100% of the flatfish species currently reared, such as European plaice *Pleuronectes platesa* (Dickey-Collas, 1993), turbot *Scophthalmus maximus* (Estévez and Kanazawa, 1995; Estévez, 1996), Atlantic halibut (McEvoy *et al.*, 1998a; Pittman *et al.*, 1998), Japanese flounder *Paralichthys olivaceus*

(Takeuchi, 2001), common sole *Solea solea* (Lund, 2007) and Senegalese sole (Soares *et al.*, 2002). Flatfish typically exhibit asymmetrical external pigmentation (Norman, 1934), with the ocular side brown and the blind side entirely white. However, pigmentation is one of the more easily disrupted organogenetic processes (Heuts, 1951). The inherent stress of flatfish larval metamorphosis is further exacerbated in intensive aquaculture conditions, where water quality, diet, substrate, light and noise levels are likely to influence the normal development of the pigmentation (De Veen, 1969). Pigment abnormalities have been reported to occur on both sides of flatfish (Venizelos and Benetti, 1999; Bolker and Hill, 2010). Hypomelanosis consists of white patches or areas devoid of normal pigmentation on the ocular side of the skin, while hypermelanosis has been described as the presence of dark spots on the blind side, normally lacking coloration. Ambicoloration is the occurrence of pigmentation on both sides of flatfish. Although these conditions have no pathogenic effect on the fish (Soutar, 1995), chances of survival in the wild are greatly reduced (Koshiishi *et al.*, 1991). Commercially, abnormally pigmented flatfish are regarded as of inferior quality and not appreciated by consumers (Venizelos and Benetti, 1999). Both morpho-anatomical skeletal and pigmentary abnormalities affect the external aspect and the biological performance (e.g. growth rate, susceptibility to diseases and mortality) of fish, downgrading the market value of the final product and increasing the production costs (Gavaia *et al.*, 2002, 2009; Fernández and Gisbert, 2011). In addition, the presence of deformed fish concerns also ethical issues: fish with deformed mouth, fins or vertebral axis show impaired feeding and swimming performances, with consequent lower feeding rates, slower growth rates, and higher susceptibility to stress and pathogens than healthy well formed individuals. These deformed fish cannot be considered to be in proper welfare conditions (Noble *et al.*, 2012). Thus, reducing the incidence of skeletal and pigmentary abnormalities from the larval stages is a critical challenge for hatcheries, to improve the quality of the product and decrease production costs. In this sense, increasing scientific and zootechnical knowledge about the causes of apparition of these morpho-anatomical anomalies will contribute to resolve current important bottleneck in marine fish larviculture.

The development of skeletal and pigmentary disorders in larval and juvenile fish is linked to a poorly understood relationship between physiological, environmental, genetic, xenobiotic and nutritional factors (Lall and Lewis-McCrea, 2007). Among them, larval nutrition at first feeding has been shown to be one of the key parameters affecting fish development and particularly skeletogenesis and pigmentation processes (Cahu *et al.*, 2003; Lall and Lewis-McCrea, 2007; Boglione *et al.*, 2013b). Several studies have demonstrated that some nutrients are responsible for the appearance of skeletal deformities and pigmentation disorders when they are supplied in inappropriate form or unbalanced levels in the diet (Bolker and Hill, 2000; Cahu *et al.*, 2003; Villalta *et al.*, 2005a, b, 2008; Villeneuve *et al.*, 2005a). Among nutrients interfering with skeletal and skin development, dietary lipids, and particularly highly unsaturated fatty acids (HUFA), have been shown to play a crucial role at first feeding in fish larvae, since they represent the main source of energy in developing fish larvae and a source of essential fatty acids (EFA) needed for building new cellular structures,

required for normal larval growth, bone formation and proper pigmentation development (Izquierdo *et al.*, 2000; Sargent *et al.*, 2002; Cahu *et al.*, 2003; Lall and Lewis-McCrea, 2007; Boglione *et al.*, 2013b). Previous studies have highlighted the negative effects of unbalanced diets with regards to EFA content on flatfish larval growth performance, metamorphosis and survival (Tzoumas, 1988; Dickey-Colas and Geffen, 1992; Izquierdo *et al.*, 1992; Morais *et al.*, 2004; Villalta *et al.*, 2005a), skeletal deformities (Berge *et al.*, 2009; Kjørsvik *et al.*, 2009; Dâmaso-Rodrigues *et al.*, 2010) and pigmentary disorders (Reitan *et al.*, 1994; Estévez and Kanazawa, 1995; Villalta *et al.*, 2005b; Vizcaino-Ochoa *et al.*, 2010). In addition to dietary lipids, retinoids (vitamin A, VA) have important roles in the developing nervous system and notochord, and in many other embryonic structures, as well as in maintenance of epithelial surfaces, immune competence, and reproduction (Ross *et al.*, 2000). However, altered levels of dietary VA reduce collagen synthesis and bone formation, and can lead to the incidence of skeletal deformities (Fernández and Gisbert, 2011) and pigmentary disorders (Estévez, 1996). The quantitative requirements and deficiency signs in EFA and VA have been described in various fish species, but their functional role in bone lipid metabolism and pigmentation establishment remains to be investigated. In this sense, the present research work aimed to **evaluate the combined effect of lipids**, particularly **EFA absolute and relative levels** and **oxidized lipids**, and other important nutrients such as **vitamins A and E** (VA, VE) on fish larvae **skeletogenesis** and **pigmentation** and their implication on the **incidence of skeletal and pigmentary disorders** during early larval development of Senegalese sole, one of the most produced flatfish in the Mediterranean basin.

Most of the nutritional studies investigating how HUFA and EFA affect fish larval growth performance and development presented a **dose-response experimental design**, where the effect of an increasing gradient of one nutrient, or the combination of different proportions of a few of them, is evaluated on larval development. This type of study is particularly indicated to determinate the requirements for specific nutrients in fish larvae fed microdiets. In addition, a few studies have also examined the effect of different types of live prey, displaying different fatty acid profiles (Shields *et al.*, 1999; Hamre *et al.*, 2002) or the effect of live prey enriched with different enriching products on larval development (Morais *et al.*, 2004). However, these studies did not **integrate the whole fatty acid profile of the experimental diet** in the understanding of their impact on larval performance and development, since the strong interactions between HUFA and EFA seriously complicate the interpretation of the results. **The present research work includes both nutritional approaches for a better understanding of the role of each dietary FA and of the interactions among them**.

Since larvae of most marine finfish species do not accept inert diets from the onset of exogenous feeding, they need to be fed with active swimming **live preys** (see review in Øie *et al.*, 2011). Copepods are the natural prey for most marine fish larvae, but their intensive cultivation at high densities is not a commercial industry yet. In this sense, rotifers *Brachionus sp.* and *Artemia sp.* have been produced in marine hatcheries around the world since the early 1960s, because they are easy to

produce in high density all year round and their nutritional composition can be manipulated. The advantages of producing rotifers are (i) their size that fits the mouth of many fish larvae species at first feeding, (ii) their low swimming speed allowing fish larvae to capture them easily, (iii) their tolerance to pronounced variations of salinity, pH and temperature and (iv) their nutritional value that can be manipulated to be adapted to the requirements of fish larvae. The production of *Artemia* presents the following advantages: (i) a good convenience of use, (ii) dormant cysts can be stored for long periods in cans and hatch easily and (iii) their non-selective feeding behavior allows to improve their nutritional value with enriching products.

The nutritional value of both live preys has been found suboptimal for most marine fish larvae. Rotifer and newly hatched *Artemia* are naturally deficient in polar lipids and EFA in comparison to copepods, which constitute a major part of the natural diet of marine larvae and meet the nutritional requirements of fish larvae, particularly in n-3 PUFA. Consequently, live preys need to be enriched in EFA previously to be supplied to fish larvae. By introducing emulsified lipid diets rich in n-3 PUFA in rotifer and *Artemia* nauplii, their nutritional quality can be improved and high-quality live food can be provided to fish larvae. This method of bioencapsulation by different enrichment procedures has led to considerable improvements in the HUFA content of *Artemia*, in particular the content of DHA and EPA (see review in Øie *et al.*, 2011). Through normal enrichment procedures, the content of DHA in *Artemia* might reach 18-22% TFA, a DHA/EPA ratio close to 2 and the total n-3 PUFA content might increase from 10 to 35% TFA, being close to the corresponding values for copepods. In addition to lipids and EFA, the content in other nutrients such as amino acids, minerals or vitamins in live prey is also modified during the enrichment (Aragão *et al.* 2004; Giménez *et al.*, 2007). The content in protein in both live preys is generally reduced after the enrichment, since most of the enriching products contain only lipids; and amino acids profiles in both live preys remain unaffected by the enrichment, with sufficient levels of the 10 essential amino acids. Mineral content of rotifers are generally lower than in copepods, with magnesium and selenium levels particularly under marine fish larvae requirements, while the mineral content of *Artemia* nauplii seems to be sufficient for most marine fish larvae, except for selenium. Regarding vitamins, rotifers fed on microalgae are known to contain sufficient amounts of water-soluble vitamins to meet the nutritional requirements of fish larvae and *Artemia* nauplii has been shown to contain higher vitamin levels than natural zooplankton. Through enrichment procedures, vitamins are efficiently transferred into *Artemia*, with ascorbic acid (vitamin C) being catabolized at a high rate and thiamine content (vitamin B1) remaining constant under starving conditions (see review in Øie *et al.*, 2011).

Improvements in the nutritional manipulation and techniques for enrichment and the offer of new enriching products have allowed to optimize the nutritional value of live preys, resulting in higher survival, growth, successful metamorphosis, reduced incidence of malformations, improved pigmentation and stress resistance in most marine species and thus considerably improving larviculture production (see review in Øie *et al.*, 2011). However, the nutritional composition of

enriched live prey varies largely depending on the enrichment conditions and the emulsion or enrichment used (Monroig *et al.*, 2006). Although the non-selective feeding behavior of rotifers and *Artemia* metanauplii allows the enriching material provided by the enriching products to accumulate in the digestive tract of live prey, many factors affect the enrichment efficiency and repeatability (*i.e.* live prey density, dissolved oxygen, aeration intensity, volume and hydrodynamics of the enrichment containers) (McEvoy *et al.*, 1998a; Conceição *et al.*, 2010). Besides, the own specific metabolism of live prey alter the original formulations that are intended to be supplied to the larvae (Barclay and Zeller, 1996) by retroconverting DHA into EPA and redistributing the incorporated FA among lipid classes with high unpredictability (Navarro *et al.*, 1999). Consequently, *Artemia* metanauplii accumulate higher levels of EPA than DHA, modifying the DHA/EPA ratio from the original enriching product or emulsion and they reincorporate most of the fatty acids provided in the dietary phospholipids mainly in its triacylglycerol (Izquierdo, 1988; McEvoy *et al.*, 1995; Navarro *et al.,* 1999). Similar findings have been found in rotifers (Fernández-Reiriz *et al.,* 1993; Rainuzzo *et al.,* 1994). The retroconversion of DHA into EPA has been demonstrated in rotifer and *Artemia* (Barclay and Zeller, 1996; Navarro *et al.*, 1999), suggesting a preferential oxidation of DHA, although the synthesis of n-3 PUFA including EPA from linoleic acid (18:2n-6, LA) had been demonstrated in *Artemia* (Ito and Simpson, 1996), contributing to the inefficiency of long chain PUFA enrichment. Besides, in enriched *Artemia* metanauplii, the high variability of the fatty acid composition and lipid classes distribution might be due to metabolic variability among individuals reflecting their stage of development, since metanauplii undergo huge developmental and physiological changes during the first hours of life, corresponding to the times in which the enrichment takes place, including a large increase in size, absorption of their yolk reserves and opening of the digestive tract and mouth (Navarro *et al.,* 1999). Moreover, differences in the enrichment conditions, difficult to control (*e.g.* amount of dissolved oxygen, size of the air bubbles, hydrodynamics in the enrichment tubes, size of micelles in the emulsions) may influence the efficiency and repeatability of the enrichment or, secondarily, the developmental rates of the metanauplii and thus their metabolic rates (Navarro *et al.*, 1999). In addition, the diverse physical forms of presentation of the commercially available enriching products (*i.e.* encapsulated fish oil-based emulsions, spray dried products) has been suggested to affect the absorption efficiency of the enriching product and thus, the nutritional value of the final enriched live prey (Chapter 1.1 of the present thesis). Besides, enriched rotifers and *Artemia* metanauplii did not generally reflect the level of lipid peroxidation of the enriching products, due to their own metabolism suggested to allow them to decrease the amount of toxic free radicals included in the diet (Chapter 1.4). In particular, the levels of peroxidation in *Artemia* metanauplii enriched with products containing increasing levels of oxidation were not altered and remained constant, even when enriched with the most oxidized enrichment (Chapter 1.4). This physiological process has not been deeply studied in live prey, but we hypothesize that *Artemia* possess highly efficient anti-oxidant systems that are able to regulate oxidative levels in their tissues, whereas the carotenoids contained in the pigmented cells were suspected to be involved in this process (Gilchrist and Green, 1960; Hsu and Chichester, 1970; Kumar and Marian, 2006). However, further work is needed to test the veracity of these assumptions

and to determine a threshold of oxidation level allowed in the emulsion before the antioxidant systems in live prey are activated. Consequently, this specific property inherent to *Artemia* metanauplii should be taken into account when using live prey as vectors for nutritional studies that aim to examine the effects of increasing dietary levels of lipid peroxidation on fish larval development. Another limiting factor to take into consideration when working with fish larvae is the transition from the rotifer to the *Artemia* feeding phases. Although both live preys are generally enriched with the same product in commercial hatcheries and small-scale trials, they present different patterns of FA, amino acid, VA and peroxides accumulation due to species-specific physiological differences in their developmental stages, filtration rates between adult rotifer and *Artemia* metanauplii and in their respective enriching protocol, particularly the duration of enrichment phase (Giménez *et al.*, 2007). The variability of accumulation patterns in live prey during the transition from rotifer to *Artemia* feeding phase has to be carefully considered when designing nutritional experiments at first feeding, and when transfering them to commercial hatcheries. For all these reasons, a preliminary enrichment of each live prey is recommended to be performed before starting the larval rearing in order to check the final nutritional quality in terms of nutrient composition and to establish standard conditions for enrichment (*i.e.* concentration of the emulsion, duration of the enrichment process, temperature of enrichment and volume of the container where enrichment takes place). These preliminary trials are needed to reach the desired nutrient content in enriched live prey. In the context of the present doctoral thesis, we aimed to determine the effect of lipids and especially EFA on Senegalese sole larval development and morphogenesis, particularly on the incidence of skeletal and pigmentary disorders, by using enriched live preys to feed the larvae, and consequently, the former issues regarding the nutritional value of live prey had to be approached and solved.

*FA composition, retinoid and tocopherol contents*

Studying the effects of nutrition on larval developmental processes leads to consider many dietary factors and nutrients. However, the complex interactions among them make the multinutrient integrated approach difficult for the interpretation of the results; that is why the role of each group of dietary compounds is generally evaluated in independent dose-response studies. The main group of dietary compounds object of study of the present thesis was the **fatty acids**. In addition, the role of the vitamin A was considered in Chapters 1.1, 1.2 and 1.3 as the levels of this morphogenetic nutrient vary among distinct commercially available enriching products. Finally, the evaluation of the effects of dietary oxidized lipids in Chapter 1.4 was conducted to examine how this species is protected against oxidative stress during early stages of larval development.

In the present thesis, the fatty acid profile of Senegalese sole larvae after several weeks of feeding with different experimental diets generally reflected the fatty acid composition of the enriched live prey supplied to them, as previously other researchers working with this species have reported (Morais *et al.*, 2004; Villalta *et al.*, 2005a, b, 2008), as well as in other flatfish species such as turbot

(Estévez *et al.*, 1999), yellowtail flounder *Limanda ferruginea* (Copeman *et al.*, 2002) and common sole (Lund *et al.*, 2008). This observation is recurrent for many other marine fish species and has been linked to the fact that although some tissues keep a stable profile as long as the diet meets the minimum requirements, other tissues such as muscular tissues, mirror the diet (Benedito-Palos *et al.*, 2011). Moreover, the FA composition of Senegalese sole larvae rapidly responded to changes in the dietary FA profile by mirroring the changes in the composition of live preys, as reported in common sole (Lund *et al.*, 2008). Thus, when larvae were fed successively low and high levels of ARA or reciprocally, shifts in the FA composition of enriched live prey provided to the larvae induced alterations of their FA profile in favor of the new prey administered. The fast fatty acid turnover in Senegalese sole tissues was most likely related to the relative fast growth rate during early stages of development (Conceição *et al.*, 2007). Newly-hatched Senegalese sole larvae present particularly higher contents of DHA, 16:0, 18:0, EPA and DPA than at later developmental stages, resulting in high ratios of n-3/n-6 PUFA and DHA/EPA and low ratios of ARA/EPA and ARA/DHA (Morais *et al.*, 2004; Villalta *et al.*, 2005a, b; Chapters 1.1 and 1.5). During early development, larvae rapidly consume these maternal FA for energy and building new cellular structures and once mouth opens, the diet has to provide sufficient and balanced amounts of FA to compensate the progressive depletion of maternal FA resources. In this context, Sargent *et al.* (1999b) defined larval requirements in EFA as the amount of EFA accumulated in the egg. Present results showed in Chapter 1.1 revealed that when EFA contents provided in the diet were not enough to meet Senegalese sole requirements, it affected larval growth performance and development, as observed in larvae fed *Artemia* enriched with Easy Selco© or high dietary levels of ARA.

In contrast to FA, the accumulation of retinoids in Senegalese sole larval tissues did not reflect the retinoid content of the enriched *Artemia*. Feeding larvae with enriched *Artemia* containing the highest amounts of retinoids (24.8 and 13.2 $\mu g.g^{-1}$ D.W.) resulted in the lowest contents in larval tissues (3.2 and 4.6 $\mu g.g^{-1}$ D.W.), while larvae fed the lowest retinoid contents (0.5 and 0.1 $\mu g.g^{-1}$ D.W.) presented the highest amounts in larval tissues (6.3 and 6.5 $\mu g.g^{-1}$ D.W.). Fernández *et al.* (2009) have demonstrated the proportional accumulation of VA in larval tissues regarding the VA amounts in enriched *Artemia*; however, in the range of VA values in our experiment (Chapter 1.1), no difference was found in the VA accumulation pattern of larvae fed enriched *Artemia* containing 11.1 to 24.8 $\mu g.g^{-1}$ D.W. In the present study, low VA contents in enriched *Artemia* compared to the study of Fernández *et al.* (2009) (1,320 – 12,910 $\mu g.g^{-1}$ D.W.) combined with different formulations of the enriching products tested, might explain the distinct VA accumulation pattern in larval tissues between the two studies.

In Chapter 1.4, this thesis showed that larvae fed live prey containing an increasing gradient of lipid peroxidation presented decreasing contents of α-tocopherol, reflecting the utilization of this vitamin to protect the organism against the dietary oxidative stress, as described in gilthead sea bream (Mourente *et al.*, 2002), largemouth bass *Micropterus salmoides* (Chen *et al.*, 2011) and spoted

murrel *Channa punctatus* (Abdel-Hameid *et al.*, 2012). However, no further research has been devoted to this vitamin in this research, since it was not the main nutrient object of study in the current doctoral thesis.

*Survival rates, growth performance and metamorphosis*

Senegalese sole showed high survival rates in all the experimental studies conducted along this thesis (94.7 ± 3.5% in average) and, in most of cases, survival was not affected by any of the dietary treatments tested. Survival rates were higher than reported in other studies in this species (Dinis, 1992; Cañavate and Fernández-Díaz, 1999; Dinis *et al.*, 1999; Morais *et al.*, 2004; Fernández *et al.*, 2008; Villalta *et al.*, 2005a, b; Dâmaso-Rodrigues *et al.*, 2010), but they were equivalent to survival rates found in Senegalese sole larvae fed HUFA-deficient *Artemia* during post-metamorphosis (> 90%, Morais *et al.*, 2005).

Larvae aged 38 dph fed live prey enriched with commercial products and displaying distinct FA profiles presented similar survival rates, as expected according to the nutritional guarantees of commercial products. In larvae fed rotifers and *Artemia* metanauplii enriched with experimental emulsions containing an increasing gradient of lipid peroxidation, survival rate at 35 dph was not affected either, which was in accordance with results reported in fingerlings from other marine species such as gilthead sea bream and Atlantic halibut, where no detrimental effects on survival and growth were observed when fish were fed increasing amounts of dietary lipid peroxides (Mourente *et al.*, 2000; 2002; Lewis McCrea and Lall, 2007). Several studies have highlighted the importance of ARA in survival and stress tolerance (Koven *et al.,* 2001b; Atalah *et al.*, 2011a, b, among others). Under present experimental conditions, survival rates were not affected in Senegalese sole larvae fed high dietary ARA levels (7.0% of TFA) in enriched *Artemia* metanauplii from 8 to 50 dph, in agreement with Villalta *et al.* (2005a) and with studies made on other flatfish species, such as halibut (McEvoy *et al.*, 1998b), turbot (Estévez *et al.*, 1999), yellowtail flounder (Copeman *et al.*, 2002) and summer flounder *Paralichthys dentatus* (Willey *et al.*, 2003). However, higher survival rates were observed in larvae fed high dietary ARA levels (10.2 and 7.1% of TFA in enriched rotifer and *Artemia* metanauplii, respectively) during at least their pre- and pro-metamorphic stages (2-15 dph) than in larvae fed lower levels of ARA (1.0 and 1.4% of TFA in enriched rotifer and *Artemia* metanauplii, respectively), being 98.3 and 96.9% respectively, regardless the dietary ARA levels in enriched *Artemia* metanauplii supplied to the larvae during their post-metamorphic stage (15-50 dph). These results are in agreement with those obtained in summer flounder larvae fed enriched rotifers with 6% ARA during pre-metamorphosis (3-23 dph), showing higher survival and growth rates than those fed 0% ARA, whatever was the level of ARA provided to larvae in enriched *Artemia* metanauplii during post-metamorphosis (33-45 dph) (Willey *et al.*, 2003). Various studies investigating the role of dietary ARA on larval physiological development have highlighted a relationship between this EFA and a higher stress resistance to handling, salinity or air exposure (Koven *et al.,* 2001b; Harel and Koven, 2002;

Atalah *et al.,* 2011b). Although the effects of ARA on stress resistance was out of the scope of the present thesis, Senegalese sole larvae appeared to be particularly sensitive to dietary ARA levels during their pre- and pro-metamorphic periods, highlighting specific high requirements in ARA during these developmental stages.

Present data showed that different effects on growth performances were found depending on the experimental studies. In Chapter 1.1, the study that evaluated the effect of different commercial products containing distinct FA profiles demonstrated the importance of the balance among all FA for a good growth performance and development, specifically with regards to the absolute and relative proportions of dietary DHA and EPA. In this sense, larval growth was higher in larvae fed live prey enriched with Aquagrow Gold©, the commercial product presenting the more balanced FA profile for Senegalese sole among all enriching products tested. *Artemia* metanauplii enriched with this product had intermediate values of total MUFA, ARA, EPA, DHA, total PUFA (32.7, 0.7, 3.1, 9.5 and 51.1% TFA, respectively), and ratios of DHA/EPA and n-3/n-6 of 3.0:1 and 5.2:1, respectively. This subtle balance among all components of this diet may explain the better growth performance of Senegalese sole larvae, as the other diets containing a different proportion of one or several compounds than in Aquagrow Gold© formulation induced less efficient larval growth and development. The surplus of EPA in *Artemia* enriched with Easy Selco© compared to those enriched with Aquagrow Gold© (2.5 times more) resulted in a lower growth performance of larvae fed this diet. Previous studies estimated between 3 and 4% TFA the range of EPA levels suitable for larval growth of various marine organisms (Léger *et al.*, 1986; Izquierdo *et al.*, 2000). In addition, Senegalese sole has been shown to have low requirements in EPA when DHA is present in the diet (Villalta *et al.*, 2008), so that higher dietary EPA levels (7.6% TFA in Easy Selco© diet) may have had a significant detrimental effect on larval growth and morphogenesis in this species.

The importance of balanced levels of DHA in the diet was also highlighted in this study. The lack of DHA in *Artemia* enriched with Easy Selco© diluted by a half with olive oil led to a decreased growth and development compared to larvae fed *Artemia* enriched with Aquagrow Gold©. These results demonstrated the importance of dietary DHA in larval nutrition, despite the low requirements for DHA reported in this species (Morais *et al.*, 2004; Villalta *et al.*, 2005b; Dâmaso-Rodrigues *et al.*, 2010). On the contrary, an excess of DHA, observed in *Artemia* enriched with Multigain©, was compensated by a proportional loss in MUFA, which are more easily catabolized for energy, altering the PUFA/MUFA ratio. Thus, as previously demonstrated in Villalta *et al.* (2008), the disruption of the balance between energy (saturated FA and MUFA) and essentiality (PUFA) may have detrimentally affected Senegalese sole larval growth and development. Finally, the balance of relative dietary proportions of DHA and EPA was showed to play a significant role for adequate growth and development. Higher requirements for dietary DHA than EPA have been demonstrated in red sea bream, gilthead sea bream, striped bass, European sea bass, Atlantic halibut and Senegalese sole (Watanabe *et al.*, 1989; Rodriguez *et al.*, 1998; Harel *et al.*, 2002; Morais *et al.*, 2004). A DHA/EPA

ratio lower than 1 in *Artemia* enriched with Easy Selco© and Easy Selco© diluted by a half was suggested to be sub-optimal for efficient growth performance.

In Chapter 1.3., in order to provide insight into the molecular mechanisms underlying the effect of the diet on larval growth performance, the transcriptional expression of insulin growth factor 1 and 2 (*igf1 and igf2)* and insulin growth factor receptor (*igfr)*, genes that regulate the differentiation and growth (Peterson *et al.*, 2005; Chao and D'Amore, 2008; Reinecke, 2010) was evaluated in larvae presenting the higher (Aquagrow Gold© group) and the lower growth performances (Easy Selco© group). Results indicated that the expression patterns of the above-mentioned genes were stable from pro-metamorphosis (22 dph) to post-metamorphosis (30 and 38 dph) and were not modulated by dietary treatments in Senegalese sole. As all dietary treatments were isolipidic, we suggested that lipid content rather than fatty acid and lipid class composition modulated the expression of *igfs*.

When evaluating the effect of dietary ARA levels on Senegalese sole growth performance (Chapter 1.5), a better growth was found in fish fed 4.5% of dietary ARA than 1.0 or 7.0%, from 8 to 50 dph. Contrary to European sea bass and gilthead sea bream (Bessonart *et al.*, 1999; Atalah *et al.*, 2011a, b), no dose-response effects of dietary ARA on growth were observed in this flatfish species. A proportion of 1% ARA of TFA, equivalent to the ARA content commonly included in commercial enriching products for marine fish larvae (Sargent *et al.*, 1999b), did not result in the best growth performance, highlighting the species-specific requirements of Senegalese sole. In addition, an excess of dietary ARA (7.0%) resulted in lower growth than fish fed 4.5% ARA. In contrast, other studies have shown that growth performance was not affected by high dietary ARA levels in Senegalese sole (Villalta *et al.*, 2005a) and in other flatfish species, such as halibut (McEvoy *et al.*, 1998b), turbot (Estévez *et al.*, 1999) and various species of flounders (Estévez *et al.*, 2001; Copeman *et al.*, 2002; Willey *et al.*, 2003). When examining the requirements in ARA of Senegalese sole larvae along development, larvae fed low levels of ARA (1.2% in average) during pre- and pro-metamorphosis (2-15 dph) and high levels of ARA (7.1%) during the post-metamorphic period (15-50 dph) showed a higher growth performance than larvae fed 1% ARA along all larval development (Chapter 1.5). These results revealed that nutritional requirements in ARA, and by extension to other EFA, may change along larval development according to specific needs. In particular, Senegalese sole larvae may have increasing requirements in ARA from pre- and pro-metamorphosis to post-metamorphosis, and this may be considered in commercial hatcheries for improving and optimizing current rearing protocols.

Concerning the effect of dietary lipid peroxidation levels on larval growth (Chapter 1.4), feeding Senegalese sole larvae from 2 to 9 dph with rotifers enriched with increasing levels of lipid peroxides ($34.4 < TBARs < 87.2$ nmol of malondialdehyde (MDA) $g^{-1}$ W.W.) did not affect larval survival nor their growth performance. These results were in accordance to those reported in other species, such as gilthead sea bream fingerlings (Mourente *et al.*, 2000, 2002), largemouth bass (Chen

*et al.*, 2011), rainbow trout (Puangkaew *et al.*, 2005) and Atlantic halibut (Lewis-McCrea and Lall, 2007), whose survival and growth were not affected by increasing amounts of oxidation in their diets. Senegalese sole larvae seem to possess defense mechanisms, activated by the high amounts of dietary peroxidized lipids, thus preventing the alteration of survival and growth performance. However, the eventuality that the dietary levels of oxidized lipids supplied to the larvae were not high enough to see a strong effect on their development could not be excluded, considering the specific metabolism and of the possible detoxification mechanisms of the enriched live prey used as a vector to feed the larvae.

Flatfish metamorphosis is primarily controlled by hormonal systems, being a high increase of thyroid hormone (TH) levels, corresponding to the climax event, required for successful migration of one eye from the blind to the ocular side (Inui and Miwa, 1985). Unbalanced levels of fatty acids and TH have been shown to affect metamorphosis in flatfish by disrupting the normal pigmentation and eye migration patterns (see reviews by Hamre *et al.*, 2005, 2007). Estévez and Kanazawa (1995) found that a diet deficient in n-3 PUFA stopped metamorphosis in turbot. As long-chain PUFAs are suggested to play an important role in growth related events in the central nervous system (CNS) by modulating the signal transduction pathways (Vaidyanathan *et al.*, 1994), a lack of PUFAs may result in an inadequate development of the CNS and in not fully operational hypothalamus–pituitary–thyroid axis. Consequently, the developing larvae may not receive the correct signals through the increase of TH production (Hamre *et al.*, 2005). In all the experimental studies of the present doctoral thesis, larvae were able to complete metamorphosis, and the progress of metamorphosis, measured in terms of eye migration stages (Fernández-Díaz *et al.*, 2001), was in general not significantly affected by the distinct fatty acid profiles of the different dietary treatments. Nevertheless, the above exposed hypothesis implicating TH could not be validated in the present research work, as TH levels were not quantified. Moreover, when larvae were fed *Artemia* enriched with different commercial products, larvae presenting the faster larval growth (Aquagrow Gold©, Aquagrow DHA© and Multigain© groups) showed the trend to metamorphose earlier too. These reasults were in accordance with other flatfish species in which growth and eye migration process have frequently been significantly correlated (Naess and Lie, 1998; Lund *et al.*, 2008; Geffen *et al.*, 2007). Nevertheless, metamorphosis process was not affected by high dietary peroxidation levels. Likewise, high dietary DHA levels supplied to Senegalese sole larvae during pre- and pro-metamorphosis, when larval requirements are known to be higher for tissue formation and asymmetry acquisition (Power *et al.*, 2008), did not result in a faster growth and eye migration. This reinforces the fact that this species has particularly low DHA requirements at pre-metamorphic stage, when EPA is provided in the diet (Villalta *et al.*, 2005b; Morais *et al.*, 2004). In addition, metamorphosis was not significantly affected when larvae were fed increasing dietary ARA levels (1.0 to 7.0% TFA) in enriched *Artemia* from 8 dph (data not shown), neither when they were fed high dietary ARA levels (10.2% TFA) from their early pre-metamorphosis (2 dph) during the rotifer feeding period. However, Villalta *et al.* (2005a, b, 2008) reported a delay in eye migration in Senegalese sole larvae fed high dietary levels of ARA from 0.1 to 8.3%, DHA from

0.0 to 14.7%, SFA from 38.0 to 22.1%, and an advanced eye migration when larvae were fed EPA from 0.3 to 29.5% and MUFA from 50.7 to 21.0%. The mechanisms by which EPA, DHA and ARA may influence both growth and metamorphosis seem to be interconnected and dependent on the whole FA profile and relative proportions of EFA, although the relationship among them is not well understood. Finally, morphological events altering the eye migration process and the remodeling of cranial bones during metamorphosis – detailed in a section below - were noticed in pseudo-albino larvae fed high dietary ARA levels during pre-, pro- and post-metamorphosis, although they were not considered as an impairment of the metamorphosis.

*Organization and functionality of the digestive tract*

In vertebrates, different segments of the gastrointestinal tract have been shown to employ different cellular mechanisms in response to diet quantity and quality. Thus, the intestine and digestive accessory glands are considered as target organs of the nutritional and physiological status in fish. The intestine is involved in important physiological digestive functions, being the primary site for food digestion and nutrient uptake, while the liver is the central metabolic organ of the body with a predominant role in the intermediary metabolism, with important functions in lipid storage and digestive and detoxification processes. The optimum utilization of dietary nutrients ultimately depends on the effectiveness of functions in the intestine and liver and, consequently, the structural alteration of the histomorphological organization of these organs. The activity of selected digestive enzymes also provides useful information about the quality of the diet, metabolism, and the nutritional status of the fish (see review in Gisbert *et al.*, 2008).

The development and functionality of the digestive tract was evaluated in larvae fed *Artemia* enriched with different commercial products with distinct FA profiles. Generally, the correct maturation of the digestive system allows larvae to digest and assimilate the ingested diet, incorporating required amounts of nutrients for their normal growth and development. Zambonino-Infante *et al.* (2008) have demonstrated that the activity of the pancreatic and intestinal enzymes is a reliable marker for assessing the development of the digestive function in fish larvae. During Senegalese sole larvae development, the specific activity of the brush border enzymes (alkaline phosphatase and N-aminopeptidase) increases with a parallel decrease in the activity of leucine-alanine peptidase (Ribeiro *et al.*, 1999). The ratios between brush border and cytosolic enzyme activities provide an indication of the degree of maturation of the digestive system. In this sense, higher ratios of alkaline phosphatase / leucine-alanine-peptidase and N-aminopeptidase / leucine-alanine peptidase activities were observed in Senegalese sole larvae fed with *Artemia* metanauplii enriched with Aquagrow Gold©, the diet with most balanced FA profile, than in larvae fed the other diets, indicating a more functional and mature intestinal tract. The above-mentioned results were also in accordance with the faster growth and the more advanced metamorphosis trend observed for these larvae. In contrast, Senegalese sole larvae fed the other diets presented lower ratios, showing a perturbed maturational process of the overall

digestive system. Not only intestinal digestive enzymes may be used as markers for assessing the level of maturation of the digestive system, pancreatic enzyme synthesis and secretion appear to be particularly sensitive to food deprivation and dietary composition in teleost larvae and, consequently, pancreatic enzyme activity provides a reliable biochemical marker of larval fish development and condition (Cahu *et al.*, 2004). The pancreatic secretory process matures during the first three or four weeks after hatching in temperate marine fish larvae. This maturational process can be disrupted when larvae are fed diets that do not meet their specific needs: the earlier the feeding with such inadequate diets, the lower the pancreatic secretion level. In this sense, changes in the level of some pancreatic enzymes, such as amylase and trypsin, which normally decrease along the morphogenesis of the digestive system (development of the stomach and intestine, onset of adult-like acidic digestion), is also considered as marker for the development of the digestive tract (Ribeiro *et al.*, 1999; Zambonino-Infante and Cahu, 2007). In this thesis, larvae fed the Aquagrow Gold© diet presented lower amylase activity values than larvae fed the other diets, confirming a more advanced level of maturation of the digestive system, as intestinal enzymes had also evidenced.

In parallel to the above-mentioned physiological changes in the specific activity of selected digestive enzymes, the intestinal mucosa, the main site for nutrient absorption and transport to other tissues, also presented some relevant structural changes. In particular, the activity of the brush border alkaline phosphatase was positively correlated with enterocyte proliferation rates, pointing out that both biochemical and histochemical parameters may be useful biomarkers for evaluation the maturation of the intestine. The enterocytes in larvae fed the Aquagrow Gold© diet showed lower height and higher proliferation rate than in larvae fed the other diets, supporting the digestive enzyme activity results and indicating a more advanced degree of maturation of the digestive system. These results were also in agreement with the faster growth and the trend to an advanced metamorphosis observed in this group of larvae. The imbalances in the dietary FA profile of the other dietary treatments, such as excessive levels of EPA (EasySelco© group), levels of DHA insufficient (Easy Selco© and Easy Selco© diluted by a half) or in excess (Multigain©), levels of ARA in excess (Red Pepper©) or in shortage (Aquagrow DHA©), which ultimately resulted in unbalanced ratios of DHA/EPA and ARA/EPA, may explain the delay in the maturation of the digestive system, along with the lower growth and delayed trend in metamorphosis.

The utilization of dietary lipids by the various tissues and organs of the fish is reflected in their histological organization, such as the intestine, through which dietary lipids are digested and absorbed, the vascular system, which is involved in their transport, and the liver, where lipids are stored and metabolized (Tso, 1994). In this sense, these tissues are considered reliable nutritional and physiological biomarkers because their histological structure is especially sensitive to non-optimal nutrition during larval development and rapidly respond to nutritional disorders (Catalán and Olivar, 2002; Caballero *et al.*, 2003, 2004). Particularly, the accumulation of neutral lipids in these target tissues accurately reflects any physiological disorder originated from nutritional unbalances in the

dietary lipid and fatty acid composition (Segner *et al.*, 1994; Mobin *et al.*, 2000, 2001; Gisbert *et al.*, 2008; Cahu *et al.*, 2009).

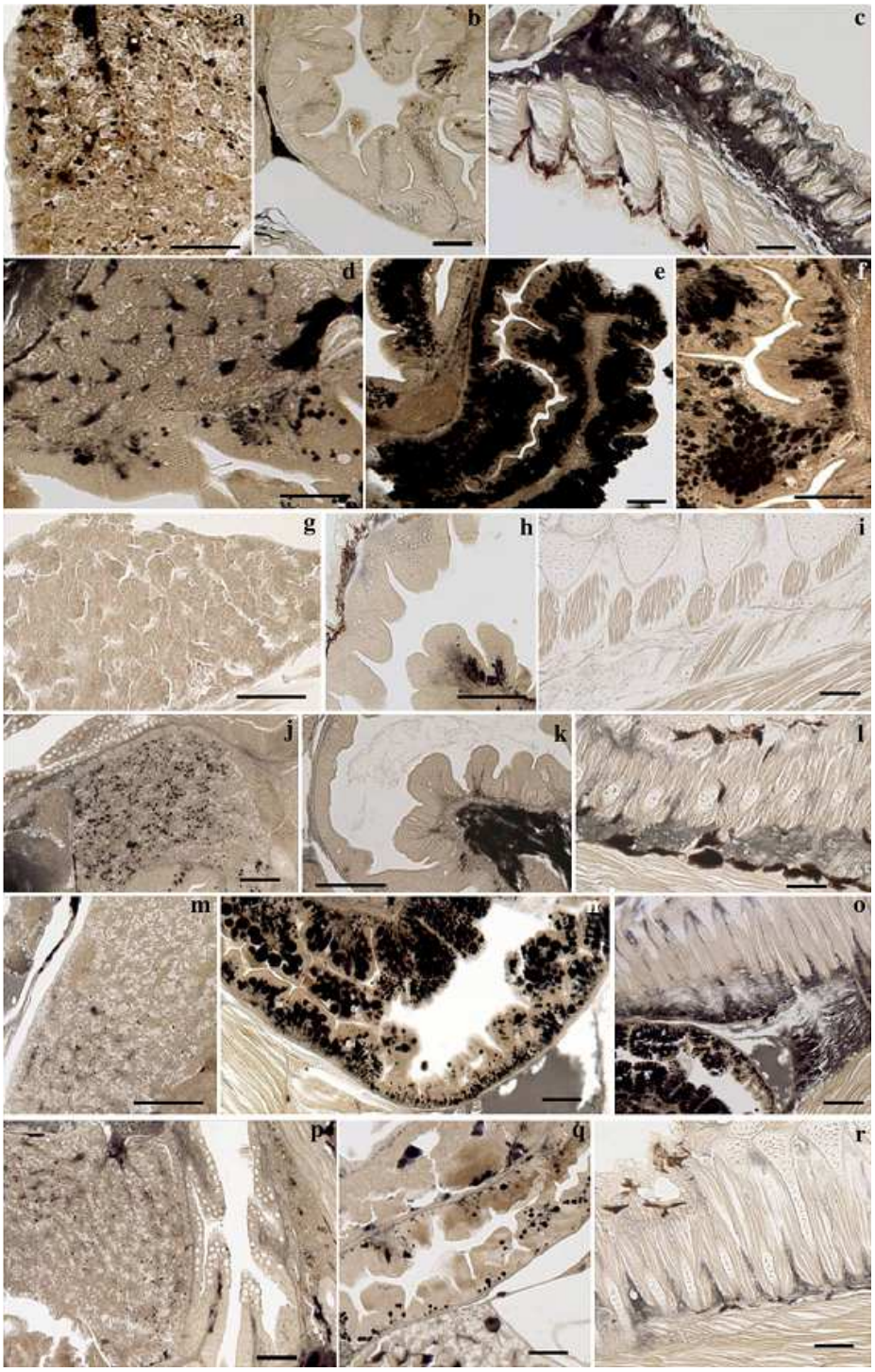


**Figure 1.** Longitudinal paraffin sections of the liver (left side), mid-intestine (middle) and vascular system (right side) of Senegalese sole larvae at the end of metamorphosis (31dph) fed the six dietary treatments (Easy Selco©: a to c; Easy Selco© diluted by a half: d to f; Red Pepper©: g to i; Aquagrow Gold©: j to l; AquaGrow DHA©: m to o and Multigain©: p to r) showing different levels of neutral lipid accumulation (in black). Scale bar represents 50µm (from Chapter 1.2).

Senegalese sole larvae fed *Artemia* enriched with six different enrichers presented different patterns of fat accumulation in the mid-intestine, liver and vascular system during their development. As diets were isolipidic and presented the same amounts of polar and neutral lipids, these differences in the fat deposition patterns in the target tissues were investigated considering the distinct fatty acid profile of the different diets. Thus, fish fed *Artemia* enriched with Aquagrow Gold© showed the best growth, maturation of the digestive tract and skeletal ossification (Chapter 1.1), and for this reason we considered the patterns of fat accumulation in the target tissues in that experimental group as the reference compared to the other treatments. An imbalance in the fatty acid composition of the other diets compared to the Aquagrow Gold© treatment was suggested to be responsible for the modifications in the fat deposition of larvae fed the other treatments, by modifying the larval capacity to absorb lipids in the intestine and to export them through the vascular system towards the liver to be stored and mobilized for larval development when needed (Tso, 1994). Fish fed the Easy Selco© and Red Pepper© diets presented lower fat accumulation in intestine and liver at 31 dph than fish fed the Aquagrow Gold© diet, probably reflecting the delayed maturation of the digestive tract in these larvae (Figure 1; Chapter 1.1). Moreover, the excess in EPA and the deficiency in DHA in *Artemia* enriched with Easy Selco© compared to those enriched with Aquagrow Gold© led to imbalanced dietary ratios of DHA/EPA and ARA/EPA (77 and 70% lower) and also altered the OA/PUFA ratio (38% higher), which might be responsible for the severe hepatic steatosis observed in larvae fed the Easy Selco© diet. Hepatic steatosis did not affect larval survival indicating that the hepatic function was not completely affected, whereas the severe accumulation of neutral lipids in the vascular system at post-metamorphosis was probably due to a problem in the regulation of fat storage (Spisni *et al.*, 1998). Taking into account that OA is generally used for energetic purposes, whereas PUFA are mainly used for structural and physiological functions (Sargent *et al.*, 1999b; Tocher, 2003), an unbalance in the OA/PUFA ratio would have disrupted the balance between energy and essentiality, leading to an excessive fat accumulation in the liver and vascular system in fish fed the Easy Selco© diet. Fish fed *Artemia* enriched with Easy Selco© diluted by a half with olive oil (ES/2) and Aquagrow DHA© both presented severe intestinal steatosis and moderate hepatic steatosis at post-metamorphosis, although they presented different fatty acid profiles. The first diet presented OA/PUFA and ARA/DHA ratios 196% and 175% higher than Aquagrow Gold©, respectively, and n-3/n-6 PUFA, DHA/EPA and ARA/EPA ratios 29, 87 and 61% lower than the Aquagrow Gold© diet, respectively. The partial replacement of fish oil by olive oil rich in OA in this diet may have impaired lipid transport from the intestinal mucosa to the liver, resulting in intestinal steatosis and may be responsible for the moderate hepatic steatosis (Spisni *et al.*, 1998; Caballero *et al.*, 2003, 2004). Moreover, the imbalance in the OA/PUFA ratio coupled with the decrease in the n-3/n-6 PUFA ratio, might also have accounted for the reduced mobilization of lipid reserves in the liver of post-metamorphic fish, leading to lower growth and development (Chapter 1.1). The comparable severe intestinal steatosis and moderate hepatic steatosis in fish fed the Aquagrow DHA© and the Easy Selco© diluted by a half with olive oil diets, and their similar severe fat accumulation in the vascular system as in fish fed the Easy Selco© diet may probably result from the large deficiency in ARA (71% lower than the Aquagrow Gold© diet) and the

subsequently 88% and 83% lower ARA/DHA and ARA/EPA ratios in the Aquagrow DHA© diet, in agreement with Fountoulaki *et al.* (2003). Light alterations in the content of just one essential fatty acid seemed enough to disrupt the profile of the diet, modify the pattern of accumulation of lipids in larval tissues and change their mobilization for proper growth and development. Finally, fish fed *Artemia* enriched with the Multigain© diet presented a relatively similar pattern of fat accumulation in the intestine and liver than fish fed with the Aquagrow Gold© diet, possibly due to the similar nature of both enriching products (spray-dried single cell organisms), except for the lower mobilization of hepatic lipids at post-metamorphic stages, attributed to the 51% lower OA/PUFA ratio than in the Aquagrow Gold© diet.

Changes in fat deposition in target tissues were also correlated with changes in the expression of selected molecular markers chosen to provide insight about the effect of diet on the digestive physiology and metabolism of the organism. However, we decided to limit this analysis to only two dietary groups, those that resulted in the higher (Aquagrow Gold© diet) and lower (Easy Selco© diet) larval performance (Chapter 1.3). Although the expression of the fatty acid binding protein in the liver (*l-fabp*) has been shown to be influenced by nutritional factors that impact hepatic fatty acid metabolism, such as food deprivation, fatty diets and peroxisome proliferators (Veerkamp, 1995), the *l-fabp* expression was not differentially modulated in both dietary treatments during larval development, suggesting that *l-fabp* expression might be sensitive to the dietary long-chain fatty acids content rather than to their composition (Chapter 1.2). In contrast, the expression of the fatty acid binding protein in the intestine (*i-fabp*) was differently affected by both commercial live prey enrichments. Fish fed the Easy Selco© diet showed a stable level of *i-fabp* expression, while those fed the Aquagrow Gold© diet presented a decrease in *i-fabp* expression from their pro- to the post-metamorphic stages (22 to 30 dph). This gene is involved in the uptake of dietary fatty acids (Levy *et al.*, 2001) and intracellular fatty acid transport (Baier *et al.*, 1996); thus, changes in the spatio-temporal gene expression pattern associated with the highest degree of intestinal maturation observed in fish from the Aquagrow Gold© group (Chapter 1.1) could indicate larval absorptive adaptation responses to the dietary lipids. The 2.5-fold higher EPA levels in the Easy Selco© diet than in the Aquagrow Gold© diet and the stable *i-fabp* expression from 22 to 38 dph could probably have caused the excessive fat accumulation in the intestine, which might have induced in turn, hepatic steatosis and severe accumulation of lipid droplets in the vascular system in fish fed the Easy Selco© diet, as shown in Chapter 1.2.

In this doctoral thesis, the effect of the levels of different fatty acids on the overall development and quality of larvae was not the only parameter considered with regards to dietary fatty acid content and larval morphogenesis. Thus, we decided to test the potential effects on oxidized dietary lipids on the above-mentioned parameters. Such approach was conducted in relation to the high content of HUFA in enriching products and live prey and their potential oxidation during the enriching process or enriched live prey maintenance. Generally, when high levels of peroxidized lipids are ingested, the

intestine behaves as a reliable selective physical barrier, especially sensitive to non-optimal feeding conditions or nutritional stress during larval development, which first detoxifies the ingested peroxidized lipids by retaining them in enterocytes until the mechanisms of antioxidant defense have been activated, and before being transferred to the vascular system (Trendazo *et al.*, 2006). In addition, changes in the histological organization of the intestine and the liver can be used as histological targets to analyze the nutritional condition of fish larvae and elucidate the effects of peroxidized lipids on larval physiology at early development (Gisbert *et al.*, 2008). The number and the size of lipid vacuoles in enterocytes and hepatocytes has been reported to depend on the dietary fat content and the degree of unsaturation of the lipids ingested, informing on the capacity of intestine and liver to balance an excess of lipid intake (Sheridan, 1988). Thus, the accumulation of lipids in the intestine of Senegalese sole pre-metamorphic larvae (age: 9 dph) fed diets with increasing lipid peroxidation levels proportionally increased as dietary lipid peroxidation levels increased, while the lipid accumulation in post-metamorphic larvae (age: 19 dph) rather seemed to be related to the neutral lipid content (cholesterol and triacylglycerol) than to the oxidation levels of the diet.

The liver is considered as the central organ of detoxification, and the accumulation of fat deposits has previously been associated with increasing gradient of dietary oxidized lipids in rainbow trout, largemouth bass and channel catfish *Ictalurus puntatus* (Puangkaew *et al.*, 2005; Chen *et al.*, 2011; Dong *et al.*, 2012). As described in Chapter 1.4, the degree of oxidation of the diet did not affect the hepatic fat accumulation patterns in Senegalese sole. Flatfish larvae tend to accumulate a large food reserve in the liver, which they utilize during metamorphosis when they have reduced feeding abilities (Brewster, 1987). In this context, significant energetic costs have been associated with metamorphosis, and this developmental stage could be delayed until the larvae had accumulated sufficient reserves. This accounts for the wide variation in size and age at metamorphosis, and explains why metamorphosis is not size (length)-dependent (Geffen, 2007). In Senegalese sole, pre-metamorphic larvae (9 dph), which are pelagic and active, constantly swimming and foraging live prey, and larvae undergoing metamorphosis (19 dph) are both high consumers of dietary lipids for energetic demands. At both developmental stages, the use of absorbed lipids was in priority involved in cell and tissue growth and differentiation, while hepatic reserves were totally spent for these physiological processes, hence no fat accumulation could be observed in the liver. Once metamorphosis completed, benthic post-metamorphic larvae had reduced energetic demands, dietary lipids were not immediately consumed for energetic purposes and could be stored in the liver, so that hepatic fat deposition was observed to increase (Gisbert *et al.*, 2008; Hoehne-Reitan and Kjørsvik, 2004; Chapter 1.2).

In Chapter 1.4, an increase in the peroxidation levels of Senegalese sole larvae was observed along development (from 9 to 35 dph), in agreement with Solé *et al.* (2004), regardless the dietary level of lipid peroxidation administered to larvae. This could be due to PUFA auto-oxidation as a consequence of increased oxygen presence due to higher metabolism during the metamorphic stage. A shift in the metabolic pathways during this highly demanding period, with higher external oxygen

uptake and overproduction of reactive oxygen species (ROS) could also be responsible for this increase (Solé *et al.,* 2004). Senegalese sole larvae aged 9 dph maintained similar oxidation levels, despite of the increasing oxidation levels in enriched rotifers. In juveniles of gilthead sea bream, dietary oxidized oil has also been shown to have no major effect on hepatic lipid peroxidation products (Mourente *et al.*, 2002). The engagement of antioxidant systems and detoxification mechanisms in response to increasing dietary oxidative stress may explain the constant lipid peroxidation levels in Senegalese sole. Antioxidant defenses include some enzymes, which catabolically remove oxygen derivates and free radicals susceptible to damage lipids and PUFA contained in cell biomembranes and disrupt the peroxidation chain reaction. The specific activity of most antioxidant enzymes in Senegalese sole decreased along larval development, as observed in other species like common dentex *Dentex dentex* (Mourente *et al.*, 1999) and turbot (Peters and Livingstone, 1996), and these changes were attributed to an adaptation to dietary oxidative stress over time/age. In Senegalese sole, the activation of the antioxidant defenses against dietary oxidative stress was illustrated by the increasing activity of glutathione reductase (GR), an enzyme participating in the recycling of oxidized glutathione molecules used for detoxifying $H_2O_2$ and organic peroxides (Halliwell and Gutteridge, 2006), in correlation with the increasing dietary levels of lipid peroxidation during the pre-metamorphic stage. On the contrary, enzymatic activities of superoxide dismutase (SOD), involved in the elimination of $O_2^-$, and catalase (CAT) and glutathione transferase (GST), implied in $H_2O_2$ detoxification, were not affected by the dietary oxidation gradient at any developmental stage, which may indicate that Senegalese sole larvae were able to tolerate such levels of dietary peroxidation. However, we should not exclude the potential hypothesis that tested dietary peroxidation levels were not high enough to observe important changes in all measured oxidative stress enzymes. An increase in hepatic and intestinal activities of most oxidative enzymes has been detected with increasing dietary oxidative stress, starvation or increasing levels of n-3 PUFA, in gilthead sea bream, common dentex, rainbow trout and largemouth bass (Mourente *et al.*, 2002; Morales *et al.*, 2004; Puangkaew *et al.*, 2005; Fontagné *et al.*, 2006; Chen *et al.*, 2011; Zambrano and Landines, 2011). However, a weak response of antioxidant activities to increasing dietary oxidation observed in juveniles of gilthead sea bream suggested that more severe oxidative conditions might be required to significantly affect these activities (Mourente *et al.*, 2000).

*Skeletogenesis*

Larval nutrition has been recognized by many studies as one of the key parameters that affect skeletogenesis during early fish development (Hamre *et al.*, 2012; Rønnestad *et al.*, 2013). Among other nutrients, lipids and fatty acids have been demonstrated to be responsible for the appearance of skeletal anomalies when their level and/or form of supply in the diet are inappropriate or unbalanced (Cahu *et al.*, 2003; Lall and Lewis-McCrea 2007). PUFA and phospholipids play an important role in bone metabolism, as demonstrated in mammals (see review from Poulsen *et al.*, 2007). Some studies have highlighted a relationship between EFA deficiencies and/or imbalances and the incidence of

skeletal deformities (reviewed in Boglione *et al.*, 2013b). However, the precise role of dietary lipids and PUFA on the development of skeletal anomalies remains to be investigated in fish, particularly in flatfish (Lall and Lewis-McCrea 2007). Dietary lipids are suggested to influence the FA composition of fish bones and thus the production of highly biologically active compounds like eicosanoids (prostaglandins and leukotrienes), which may have pathophysiological actions in bone cell metabolism (Bell *et al.*, 2003). The dietary n-3/n-6 PUFA ratio, particularly the ARA/EPA ratio, plays an important role in the production of 2- and 3-series prostaglandins ($PGE_2$ and $PGE_3$), whose relative proportions regulate osteoblasts and bone metabolism, and thus the proper bone mineralization and skeletogenesis (Berge *et al.*, 2009). Moreover, several studies have established a relationship between dietary lipid class and the incidence skeletal anomalies (reviewed in Boglione *et al.*, 2013b). Dietary phospholipids (PL) are known to influence early ontogenesis of fish, specifically skeletal development (reveiwed in Cahu *et al.*, 2009). An increase in dietary PL improved larval performance and prevented skeletal deformities in ayu *Plecoglossus altivelis altivelis* (Kanazawa *et al.*, 1981), common carp (Geurden *et al.*, 1998), Atlantic cod (Finn *et al.*, 2002), European sea bass (Villeneuve *et al.*, 2005b) and Atlantic halibut (Næss *et al.*, 1995; Hamre *et al.*, 2002, 2005, 2006). The stimulating effects of PL in larval fish development have been suggested to be due to the limited ability of fish larvae to biosynthesize phospholipids *de novo* (Coutteau *et al.*, 1997), but it might also be due to a better digestibility and a more effective absorption and utilization of dietary PL than neutral lipids (NL) (Fontagné *et al.*, 1998; Cahu *et al.*, 2009). Besides, fish bones are highly vulnerable to lipid peroxidation as they contain between 24 and 90% of lipids.W.W.$^{-1}$. Lipid peroxidation is an autocatalytic process initiated by free radicals, produced in the body primarily as a result of aerobic metabolism, in which HUFAs in cell membranes undergo degradation via a chain reaction (Lall and Lewis-McCrea 2007). Thus, high levels of PUFA within fish tissues without any suitable antioxidant protection are highly prone to auto-oxidation, leading to diseases and mortalities. Oxidized lipids are suggested to inhibit osteoblast differentiation and induce osteoclast differentiation that contributes to the impairment of the bone remodeling equilibrium, causing bone loss (Watkins *et al.*, 2001) and skeletal deformities in fish (reviewed in Boglione *et al.*, 2013b).

A correlation between a delay in growth and ossification has been previously highlighted in gilthead sea bream (Fernández *et al.*, 2008), Atlantic cod (Kjørsvik *et al.*, 2009) and European sea bass (Darias *et al.*, 2010) larvae fed different diets, often resulting in higher incidence of skeletal disorders. In Senegalese sole, larvae fed *Artemia* enriched with Easy Selco© showed the lowest growth, intestinal maturation and ossification values, while larvae fed *Artemia* enriched with Aquagrow Gold© presented the highest values for the above-mentioned parameters (Chapter 1.1). The decrease in the content of n-3 PUFA in the Easy Selco© diet compared to the Aquagrow Gold© diet disrupted the n-3/n-6 PUFA ratio, since a value of 5.2:1 resulted in better growth and development than a ratio of 3.9:1. Moreover, the deficiency in ARA and DHA and the excess in EPA in *Artemia* enriched with Easy Selco© compared to those enriched with Aquagrow Gold©, also disrupted the balance between different EFA, which has influenced the overall development of Senegalese sole.

Osteocalcin (*oc,* also named *bgp* or *bglap*) constitutes a gene marker for osteoblast mineralization (Pinto *et al.*, 2001) required for the correct maturation of hydroxyapatite crystals during the process of calcification (Boskey *et al.*, 1998) in developing fish. This protein has important signaling functions (bone morphogenetic proteins, growth factors, cytokines and adhesive proteins) and plays a key role during the mineralization process (Sommerfeldt and Rubin, 2001). In mammals, *oc* expression is specific to bone tissue and dentine (Pinto *et al.*, 2001). As this gene is highly responsive to different nutritional conditions, it has been proposed as a reliable marker for detecting disorders in bone formation and mineralization processes (Mazurais *et al.*, 2008; Darias *et al.*, 2010, 2011b; Fernández *et al.*, 2011). Osteocalcin gene expression was examined in Senegalese sole larvae fed *Artemia* enriched with Easy Selco© or Aquagrow Gold© (Chapter 1.3). The expression of this gene has been proved to respond to nutritional conditions and over- or under-expressions linked to vitamin contents have been associated to skeletal disorders in European sea bass and gilthead sea bream (Mazurais *et al.*, 2008; Darias *et al.*, 2010, 2011b; Fernández *et al.*, 2011). Larvae fed the Aquagrow Gold© diet showed higher levels of *oc* expression and a more mineralized skeleton at the post-metamorphic stage than larvae fed the Easy Selco© diet, although no differences in the incidence of total skeletal deformities between fish from both diets were found (Chapter 1.1). The implication of the different dietary VA levels ingested by larvae from both treatments in these distinct molecular and ossification patterns was discarded since expressions of retinoic acid receptor α (*rarα1),* retnoid x receptor α (*rxrα)* and peroxisome proliferator-activated receptor (*pparβ)* were not affected between fish from each dietary treatment, suggesting that the level of VA uptake was probably regulated in the intestine, preventing the accumulation of high amounts of this morphogenetic nutrient that could led to skeletal abnormalities and disrupt other developmental processes (Hathcock *et al.*, 1990; Melhus *et al.*, 1998; Ross *et al.*, 2000). Thus, the stable *oc* expression in fish fed *Artemia* enriched with Easy Selco©, in contrast with the up-regulation in fish fed the Aquagrow Gold© diet, was hypothesized to be due to excessive dietary EPA and deficient dietary ARA levels in the first diet compared to the second one, resulting in lower DHA/EPA, ARA/EPA and ARA/DHA ratios (Chapter 1.1) that were essential for the correct ossification of the skeleton (Lall and Lewis-McCrea, 2007).

Diets containing elevated ARA levels can cause an overproduction of $PGE_2$ in bone, which finally may lead to a disruption of the rate of bone formation and its mineralization level (Berge *et al.*, 2009). In fact, $PGE_2$ is regarded as a potent regulating agent for bone modelling and remodelling as having both anabolic and catabolic effects on bone (Kawaguchi *et al.*, 1995; Marks and Miller, 1993). Recent in vitro models revealed that $PGE_2$ decreased the proliferation of tendon stem cells and their osteogenic differentiation (Zhang and Wang, 2012). Both bone formation and bone resorption are influenced by $PGE_2$, and its effect may be concentration dependant (Berge *et al.*, 2009). In Senegalese sole fed high dietary ARA levels during the *Artemia* feeding period (7% TFA), ossification tended to be reduced compared to fish fed 4.5% TFA of ARA, in relation with their relative growth patterns (Chapter 1.5). Fish fed high dietary ARA levels from mouth opening to 50 dph (8.7% TFA in average) presented reduced ossification of skeletal elements in the cranial region and alterations of

the remodelling process occurring during the metamorphosis (Chapter 1.6). Increasing dietary ARA levels may have disrupted the n-6/n-3 PUFA ratio, in particular the ARA/EPA ratio, leading to unbalanced $PGE_2$ concentrations and to reduced bone mineralization. An optimal balance of the two series of PUFA, n-3 and n-6, is crucial for proper skeletogenesis (Izquierdo, 1996; Sargent *et al.*, 1999b; Izquierdo and Koven, 2011). High n-3/n-6 PUFA ratios are associated with beneficial effects on bone health (Maggio *et al.*, 2009) and a low n-3/n-6 PUFA ratio has been shown to negatively affect bone formation in rats (Li and Watkins, 1998; Watkins *et al.*, 1999, 2000). Marine fish larvae have been proved to have higher requirements in n-3 PUFA than in n-6 PUFA (Sargent *et al.*, 1999b). The dietary ARA/EPA ratio plays an important role in bone formation, since ARA is the major precursor for eicosanoid biosynthesis in fish (Bell and Sargent, 2003), directly competing with EPA for the enzymes involved in prostaglandin synthesis; ARA gives rise to the 2-series prostaglandin ($PGE_2$) and EPA to the 3-series prostaglandin ($PGE_3$) (Bell *et al.*, 1995). Both eicosanoid isoforms are potent regulators of bone metabolism, but with opposite effects on bone (Norrdin *et al.*, 1990; Marks and Miller, 1993), and the relative amounts of each series influence the regulation of osteoblasts and bone metabolism (Berge *et al.*, 2009). High amounts of dietary ARA or excess of EPA in live feeds result in imbalances in the dietary intake of ARA relative to EPA and, consequently, in the relative proportions of $PGE_2$ and $PGE_3$ (Sargent *et al.*, 1997; Bell and Sargent, 2003; Hamre *et al.*, 2005), leading to developmental disorders of fish skeleton. Atlantic salmon *Salmo salar* fed high n-6/n-3 PUFA ratios (3.8 to 6.0) presented reduced vertebrae mineralization combined with increased production of $PGE_2$ in blood (Berge *et al.*, 2009). Villalta *et al.* (2005b) observed a correlation between increasing dietary ARA levels and ARA/EPA ratio with an increasing production of prostaglandins (PGE and PGF) in Senegalese sole larvae, which have been related to a higher incidence of malpigmented fish. Although prostaglandin levels were not measured in the study reported in Chapter 1.5, high dietary ARA levels might have led to an increased production of $PGE_2$ and then possibly to a reduction in bone ossification, as reported in other species (Watkins *et al.*, 1999; Berge *et al.*, 2009) and in Senegalese sole, as described in the Chapter 1.6 of the current thesis. With regards to the effects of high dietary ARA levels on the process of eye migration in pseudo-albino fish (Chapter 1.6), high levels of $PGE_2$ measured in ARA-induced pseudo-albino specimens might have increased the activity of cyclooxygenase-2 (COX2) in osteoblasts, resulting in a decrease of their proliferation and increase of their differentiation rates, which might have affected their mineralization level (i.e. sphenotic, lateral left ethmoid and left frontal) and also resulted in the alteration of some skeletal elements during the sensitive period of cranial remodelling occurring during metamorphosis, as previously reported in developing mice (Greenblatt *et al.*, 2010).

The calcification process tended to be delayed in Senegalese sole pre-metamorphic larvae (9 dph) fed *Artemia* metanauplii enriched with high dietary lipid peroxidation levels and without VE, and was significantly delayed in pro-metamorphic larvae (19 dph). These results were in agreement with those reported by Lewis-McCrea and Lall (2007) for Atlantic halibut fed a diet devoid of VE, where a low bone formation activity by osteoblasts was associated with reduced bone mineralization and bone

strength. Increased lipid peroxidation has been also related to reduced bone mineral density in humans (Basu *et al.*, 2001). In *in vitro* studies and in rodents, free radicals have been proven to be involved in osteoclastogenesis and to increase bone resorption through activation of NF-kB (Garett *et al.*, 1990; Suda *et al.*, 1993; Iotsova *et al.*, 1997). Osteoblasts and osteoclasts appear to be sensitive to oxidative stress and feeding fish with highly oxidized diets without or with low levels of dietary antioxidant compounds can affect their development and reduce the rate of bone formation.

Generally, less mineralized skeleton has been shown to contribute to the development of skeletal disorders in fish, as less mineralized bone tend to be more fragile and prone to abnormally develop or get deformed once formed (Darias *et al.*, 2011a; Boglione *et al.*, 2013a, b). However, no correlation between the degree of ossification and the incidence of skeletal deformities was established in Senegalese sole fed *Artemia* enriched with six different enriching products (Chapter 1.1), neither in those fed increasing gradient of dietary ARA (Chapter 1.5). Senegalese sole larvae are very prone to develop skeletal deformities that ultimately may affect their fitness and quality, since a high incidence of skeletal anomalies has been reported in Senegalese sole juveniles reared in different experimental trials (Fernández *et al.*, 2009; Engrola *et al.*, 2009a; Fernández and Gisbert, 2011; current thesis), in commercial hatcheries where they generally vary between 44 to 80% of produced fish (Gavaia *et al.*, 2002, 2009; Engrola *et al.*, 2009a) or even in wild specimens (Gavaia *et al.*, 2009).

The frequency of deformed individuals in wild populations is lower than in captivity, since skeletal deformities affecting the locomotive capacity of fish, for catching and ingesting live prey and escaping from predators, reduce their probability of survival (Boglione *et al.*, 2003; Gavaia *et al.*, 2009). The lower frequency of deformed animals in wild populations clearly suggests the existence of a strong natural selection towards a normal skeleton and that deformed larvae are gradually eliminated from natural populations (Marino *et al.*1993; Kawamura and Hosoya, 1997; Boglione *et al.* 2003; Gavaia *et al.*, 2009). In captivity, fish are reared under optimal environmental conditions and fresh live food is supplied daily so they do not face with foraging issues, since food availability is never restricted. In addition, the pressure from predators in natural environments is lacking under aquaculture conditions, and this is one of the main reasons why deformed and not fit specimens tend to survive at higher rates that in the wild environment. This high incidence of skeletal deformities has also been highlighted in other fish species reared in commercial flatfish hatcheries. Kawamura and Hosoya (1997) have observed in Japanese flounder only 4% deformed fish from wild population versus 74% in those reared in captivity under standard conditions. A high number of deformed individuals have been registered in European sea bass industrially produced, around 75% presenting vertebral deformities and 45% in the fins. In gilthead sea bream reared under intensive conditions, almost all individuals presented skeletal anomalies, although these proportions are reduced to 55% when reared under semi-intensive conditions (Boglione *et al.*, 2001). However, the incidence of skeletal deformities in Senegalese sole reared under standard feeding protocols is higher than what is

observed in the above-cited species from the Mediterranean region (Boglione *et al.*, 2001; Villeneuve *et al.*, 2005a; Fernández *et al.*, 2008; Mazurais *et al.*, 2009). Two different hypothesis may explain such an elevated incidence of skeletal deformities in Senegalese sole; the first one considering that this species is more prone to develop skeletal disorders under rearing conditions than other species, and the second one postulating that skeletal anomalies in Senegalese sole are not lethal and, consequently, the incidence of deformities appears to be higher in Senegalese sole juveniles than in species for which deformities are lethal at early stages (Divanach *et al.*, 1997; Koumoundouros *et al.*, 1997; Boglione *et al.*, 2001). None of these two hypotheses are totally exclusive, thus, in order to determine which of these two models better explain the observations, further comparative studies are required to identify the most sensitive periods of morphogenesis, and particulary skeletogenesis, to the development of deformities and their impact on larval survival among the different fish species. In Chapters 1.1 and 1.4, all groups of fish fed *Artemia* enriched with different commercial products or with increasing levels of dietary lipid peroxidation presented a high frequency of individuals with skeletal deformities (76.2 and 69.1% in average, respectively), in spite of a generally normal external aspect, coinciding with the results of Fernández *et al.* (2009), who detected 86% of larvae presenting a least one skeletal deformity in the control group fed Easy Selco©.

The trials composing this doctoral thesis showed that the frequency of total skeletal deformities and the incidence of the different typologies of abnormalities were differently affected by the diets, depending on whether Senegalese sole larvae were fed: (i) with *Artemia* enriched with six different enrichers from 8 to 37 dph; (ii) with rotifer enriched with increasing levels of dietary peroxided lipids from 2 to 9 dph (34.5, 46.4, 87.2 and 46.8 nmol MDA.$g^{-1}$ W.W., the last one without VE addition); (iii) with *Artemia* metanauplii enriched with increasing levels of dietary ARA (1.0, 4.5 and 7.0% TFA) from 8 to 50 dph; or (iv) with rotifer and *Artemia* enriched with high dietary ARA amounts (10.2 and 7.1% TFA, respectively) from 2 to 50 dph.

In brief, no significant effects of the dietary treatments on the incidence of total abnormalities and on the different typologies of vertebral deformities were observed in Senegalese sole fed *Artemia* enriched with distinct commercial products. The only minor differences were observed in the incidence of fusions of vertebral centrums, which were higher in larvae fed the Aquagrow DHA© diet than in those fed the Multigain© diet, and in the caudal fin complex, where larvae fed *Artemia* enriched with Aquagrow Gold© and Easy Selco© diluted by a half with olive oil displayed higher incidence of deformed modified neural and haemal spines than larvae fed *Artemia* enriched with easy Selco©. However, considering that emulsions did not only differ in their fatty acid content, but also in their protein (amino acid), vitamin and mineral profiles, and larvae showed similar performances in terms of their quality, it seems plausible that the effects of tested diets were mostly due to the complex interaction among different nutrients than a particular one or a single group of them (Boglione *et al.*, 2013b).

The incidence of total skeletal deformities were different among Senegalese sole fed increasing dietary lipid peroxidation levels during the rotifer feeding period, but not proportionally correlated with these levels, suggesting that the levels of lipid peroxidation tested in this study were not high enough to cause more damages in the skeleton compared to larvae fed with the control non-oxidized diet (Chapter 1.4). In this experiment, fish fed the intermediate and highest dietary lipid peroxidation levels during the rotifer feeding period (46.4 and 87.2 nmol MDA.$g^{-1}$ W.W.) were the most affected by vertebral body deformities, as was observed in Atlantic halibut (Lewis-McCrea and Lall, 2007). The typologies of vertebral deformities most affecting those fish were the compression, the fusion of the two last vertebrae before the urostyle, the fusion of neural arches, the deformation of vertebrae, the deformation of neural and haemal spines and abnormal vertebral ossification. Lewis-McCrea and Lall (2007) found that the scoliosis in the prehaemal and anterior haemal regions of the vertebral column were the most frequent skeletal deformities in Atlantic halibut fed oxidized lipids. Moreover, fish fed live preys enriched with the highly oxidized diet without VE showed the lowest percentage of deformities in the vertebral region (with the non-oxidized control diet) and in the caudal fin complex. In particular, fish fed the highest levels of lipid peroxidation with and without VE tended to present lower frequency of deformations in the skeletal elements that ossified from 18 dph (hypurals, epural, parahypural, and fin rays) and higher percentage of malformations in the skeletal structures that are formed earlier (fusion of the haemal arches, formed at 15 dph) (Gavaia *et al.*, 2002), compared to fish fed lower amounts of peroxidized lipids. It seems that the VE interacted with ossifying cartilage, with an excess in VE in the diet from 2 to 9 dph affecting the those structures formed by endochondral ossification before 18 dph. It was observed that in larvae aged 9 dph fed high levels of lipid peroxidation with VE, part of the dietary VE was used for anti-oxidant detoxification and thus, they presented similar amounts of VE than larvae fed high levels of lipid peroxidation without VE (1.5 and 0.7 mg.$kg^{-1}$ W.W., respectively). The eventuality of an interaction among several nutrients might not be dismissed. The important role of the vitamin C (VC) in the formation of the cartilage and its function in maintaining VE corporal levels (Hamre *et al.*, 1997) suggest a possible interaction between both vitamins. A balance in the dietary amounts of both vitamins allows preventing VE deficiencies. It seems that supplying dietary VE to larvae fed high levels of lipid peroxidation could induce modifications of the normal VC levels required for the correct development of the cartilaginous structures in the caudal fin complex, formed after the period of feeding with oxidized diets.

In Senegalese sole larvae fed increasing dietary gradient of ARA during the *Artemia* feeding period, the average of skeletal deformities was low (41.4%) compared to other studies (Gavaia *et al.*, 2002, 2009; Fernández *et al.*, 2009; Chapter 1.5). Neither the incidence of total skeletal abnormalities nor the typologies of malformations were affected in Senegalese sole by the increasing dietary ARA levels. These results were in agreement with Berge *et al.* (2009) who did not find any differences in the incidence of skeletal deformities in Atlantic salmon juveniles fed increasing n-6/n-3 ratios. For the determination of the incidence of skeletal deformities in the caudal fin complex, the fusion of the hypurals 3 and 4 was not considered as a skeletal deformity, contrary to most of studies on flatfish

larvae, but rather as the normal development of the caudal fin complex occurring in juveniles of flatfishes (Figure 2; Barrington, 1937). This could account for the minor incidence of skeletal deformities found in the caudal fin complex of Senegalese sole in this study (4.2% in average; Chapter 1.5). Besides, no severe deformations (scoliosis, kyphosis, lordosis) were observed in any larvae, even fed with the highest dietary ARA level (7.0% TFA), indicating that this dietary amount of ARA supplied during the *Artemia* feeding period did not affect the skeletogenesis of Senegalese sole, although it delayed their growth performance.

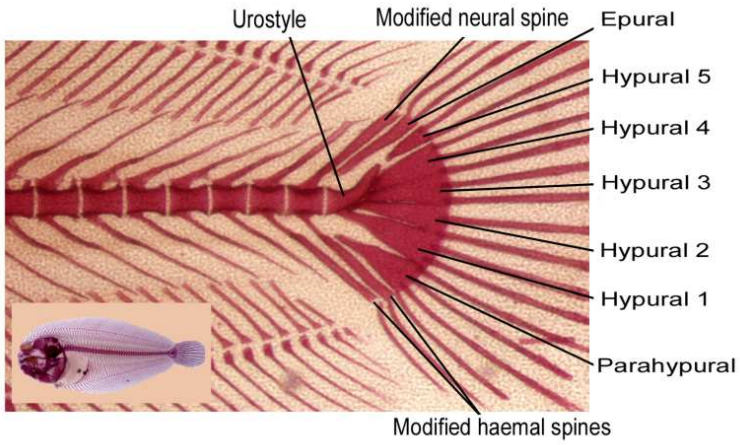


**Figure 2** Structure of skeletal elements in the caudal fin complex of Senegalese sole juveniles (50 dph) stained with Alizarin red.

In post-metamorphic Senegalese sole larvae fed high dietary ARA levels (8.7 % TFA in average) from mouth opening, the high incidence of pseudo-albino individuals (81.3 ± 7.5 %) was significantly correlated with the frequency of cranial deformities (95.1 ± 1.5%), as reported in Chapter 1.6. The incidence of total skeletal deformities was high (84.2 ± 2.4%, in average) and not significantly different between fish fed both diets (live prey enriched with a commercial enrichment, Algamac 3050$^{TM}$, and with an emulsion with high levels of ARA). In addition, the frequency of the different typologies of skeletal abnormalities affecting the vertebral and the caudal regions was similar between fish fed the two diets, except for the incidence of deformed neural and haemal spines that were higher in fish fed high dietary ARA levels than in fish fed the control diet. High levels of dietary ARA impaired the process of eye migration occurring in metamorphosing Senegalese sole larvae. In this sense, pseudo-albino specimens showed differences in head shape and positioning of both eyes with regards to normally pigmented fish. In particular, the head height and the vertebral-ocular and the bucco-ocular angles in pseudo-albino fish fed high dietary ARA levels was significantly lower and their interoccular distance 25% shorter than in normally pigmented specimens (see Chapter 1.6). Moreover, geometric morphometric analyses revealed significant differences in the shape of the cranial region between pseudo-albino and normally pigmented specimens, mainly affecting the height and the position of the head with regards to the axis of the vertebral column, as well as the relative position of both eyes.

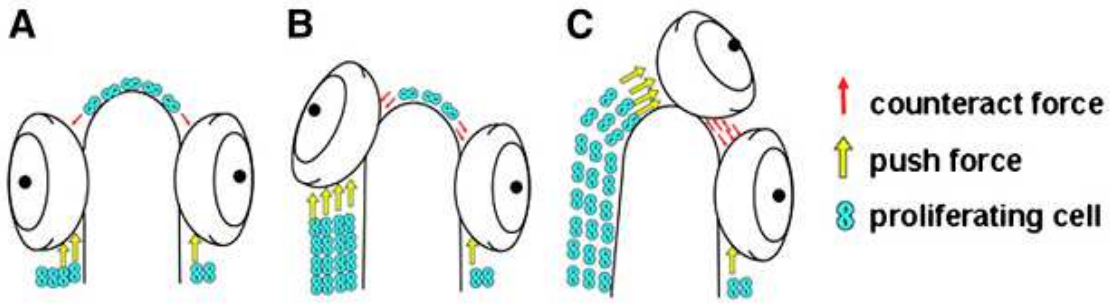


**Figure 3.** Schematic drawing explaining eye migration in flatfish (from Bao *et al.*, 2011). **(A)** Before eye migration initiated, cells located in the suborbital area and along the route of ongoing eye migration begin proliferating. Uneven amounts of proliferating cells exist in the left and right suborbital tissue. Once the eye receives sufficient pushing force from proliferating cells in its suborbital tissue to overcome the main counteracting force from the other eye, along the route of ongoing eye migration, it begins migrating upwards. Once the eye begins migrating upwards, the cartilage of skull closest to the route of migrating eye begins facing the pressing force from the migrating eye and starts twisting towards the other side; **(B)** As it migrates along the route between two eyes, the migrating eye gets more pushing force from additional proliferating cells in its enlarging suborbital area. Meanwhile, the counteracting force grows larger as the two eyes become closer; **(C)** When the migrating eye reaches the place wherein pushing and counteracting force are balanced, it finally stops migration.

Impaired eye migration and pigmentary disorders are two of the most important problems affecting the intensive production of flatfish juveniles (Gavaia *et al.*, 2009; Fernández and Gisbert, 2011). The high incidence of abnormal juveniles limits the cost-effectiveness of their production and thus represents a serious industrial bottleneck (Power *et al.*, 2008). Studies describing anomalies in the eye migration process in flatfish species are generally focused on the description of the impaired migration, of the left or right eye of the fish, depending on the symmetry of the species, from the blind to the ocular side of the metamorphic larva, as well as the bone remodeling processes and skeletal disorders in the skull resulting from eye migration (Okada *et al.*, 2001; Okada *et al.*, 2003; Sæle *et al.*, 2003, 2006; Sasaki and Yamashita, 2003; Schreiber, 2006; Cloutier *et al.*, 2011). However, this is the first study among flatfishes that describes the impaired metamorphic relocation of the ocular side eye, the right eye in the case of Senegalese sole, whereas the left eye normally migrated into the ocular side. Eye migration process is associated to cranial remodeling (Brewster, 1987; Wagemans *et al.*, 1998; Okada *et al.*, 2001), since most of cranial bones start ossifying once eye migration is completed. A recent study of Bao *et al.* (2011) stated that the process of eye migration is caused by the proliferation and migration of fibroblasts in the suborbital tissue of the blind side of the body (Figure 3) and the establishment of the typical cranial asymmetry of this group of fishes during the metamorphosis that is characterized by the relocation of the anterior part of the frontal bone from the blind to the ocular side and the enlargement of the lateral ethmoid on the blind side.

The cause of the impaired eye migration in Senegalese sole was suggested to be due to high levels of dietary ARA, associated with low levels of dietary EPA, which have disrupted the ARA/EPA ratio, resulting in alterations of the relative proportions of PGE of 2 and 3 series (Bell and Sargent, 2003). The former hypothesis was confirmed by the higher levels of $PGE_2$ measured in pseudo-albino fish in comparison to normally pigmented ones. An increase in dietary ARA/EPA ratio has been already correlated with an increased production of $PGE_2$, resulting in pigmentary disorders and impaired eye migration in Senegalese sole. High levels of $PGE_2$ in mammals have been shown to disrupt many fibroblast functions, affecting their differentiation and motility (White, 2006; Sandulache *et al.*, 2007). Thus, considering the hypothesis of Bao *et al.* (2011), in which the migration of fibroblast cells would be responsible for the proper eye migration in flatfish larvae, the impaired migration of both eyes in Senegalese sole under the present experimental conditions might be due to the increased production of $PGE_2$ or any other ARA-derived metabolite that was also responsible for pigmentary disorders found in juveniles.

Another relevant difference between both groups of fish (normally pigmented and pseudo-albinos) was the presence of dentition in both buccal jaws of the pseudo-albino fish; while teeth were totally absent from both premaxillary and dentary bones of the ocular side in normally pigmented individuals. The disappearance of teeth in the lower jaw in normal specimens of common sole occurs just before the eye starts its migration, since the lower jaw do not participate directly in prey capture making teeth unnecessary (Wagemans and Vandewalle, 2001). In addition, this impaired eye migration in pseudo-albino fish was concomitant with deformations of some elements of the splanchnocranium and neurocranium (Figure 4). In the splanchnocranium, the right premaxillary was more curved and thicker in pseudo-albino specimens than in normally pigmented ones, dentary and angular bones were both shortened, affecting the ceratohyal and the disposition of the branchiostegal rays, and the right lacrimal bone was also deformed and underdeveloped. In the neurocranium, the anterior margin of the sphenotic bone was reduced in pseudo-albino fish and the left lateral ethmoid was not completely fused with the left frontal to form the wall of the migrating eye's orbit. A high level of dietary ARA supplied from pre- to post-metamorphosis affected the processes of pigmentation, eye migration as well as remodeling of the splanchnocranium and the neurocranium in Senegalese sole (Chapter 1.6), while no effect of the diet was observed on skin pigmentation and eye migration when high dietary ARA levels were supplied from pro-metamorphosis (Chapter 1.5).

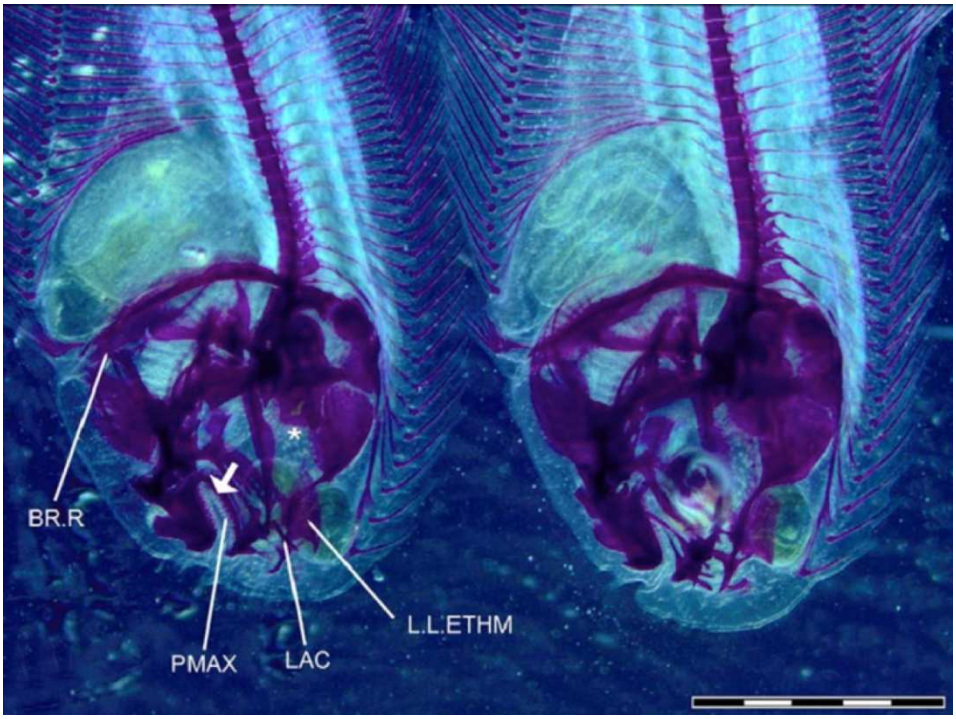


**Figure 4.** Cranial skeletal structures (Alizarin Red staining) in normally pigmented (right) and pseudo-albino (left) post-metamorphic Senegalese sole showing the most evident osteological differences between these fish (from Chapter 1.6). The asterisk denotes the underdevelopment of the sphenotic in pseudo-albino fish with regards a normally pigmented specimen. Note the presence of teeth in right upper and lower jaws (white arrow) in the pseudo-albino specimen, as well as some skeletal disorders in other elements of the splanchnocranium and neurocranium (see text for details). BR. R, branchiostegal rays; LAC, lacrimal; L.L. ETHM, left lateral ethmoid; PMAX, premaxillary. The asterisks denotes the underdevelopment of the sphenotic. Scale bar represents 2 mm (from Chapter 1.6).

Results from both studies were different, since in the study reported in Chapter 1.5, no skeletal deformities affecting the cranial region were found in Senegalese sole fed high dietary ARA levels. In addition, the incidence of skeletal deformities affecting the vertebral column presented in Chapter 1.6 was between 25 and 32% higher than those reported in Chapter 1.5. These different results between both studies might be due to differences in dietary ARA levels and experimental feeding protocols. Dietary ARA levels from both studies slightly differed; they ranged between 1.0 and 7.0% in enriched *Artemia* metanauplii from Chapter 1.5, whereas ARA levels in Chapter 1.6 were 10.2 and 7.1% in enriched rotifers and *Artemia* metanauplii, respectively. However, ARA/EPA ratios fell within the same range of values in both studies (0.4 to 1.7 in Chapter 1.5 and 0.3 to 1.8 in Chapter 1.6). In addition, in Chapter 1.5, Senegalese sole were fed high dietary levels of ARA only during the *Artemia* feeding period (8-50 dph), whereas in chapter 1.6, high levels of ARA were maintained during the whole larval rearing period (2-50 dph), as both rotifers and *Artemia* metanauplii were enriched with high amount of ARA. This suggests a probable impact of high dietary ARA supply during the pre-metamorphic period on the correct vertebral column development and skull bone remodeling occurring during

metamorphosis. Thus, it seems plausible that the longer and higher exposure to dietary ARA levels in Chapter 1.6 may be responsible for the development of the above-mentioned skeletal disorders in early juveniles of Senegalese sole. The pre-metamorphic stage appeared as a particularly sensitive period to dietary unbalances, since the regulation of most of the morphogenic processes might occur during this phase. As previously mentioned, $PGE_2$ is a potent regulator of bone remodeling and metabolism (Kawaguchi *et al.*, 1995). High ARA levels in the diet supplied during the pre-metamorphic stage resulted in the overproduction of $PGE_2$ in bone that may have disrupted the bone formation rate (Berge *et al.*, 2009), during the crucial period of bone formation, affecting the normal process of remodeling of the splanchnocranium and neurocranium and the apoptotic process responsible for teeth disappearance in both jaws.

*Pigmentation*

Skin pigmentation anomalies are commonly found in cultured flatfishes. There are three main types of abnormal skin pigmentation: i) hypomelanosis (pseudo-albinism), characterized as a full or partial lack of pigmentation on the ocular side; ii) hypermelanosis, characterized as abnormal pigmentation on the blind side; and iii) ambicoloration, which means having ocular side pigmentation on both sides of the flatfish. Reduced marketability due to skin malpigmentation is a well-known problem in the production of this group of fishes (Bolker and Hill, 2000; Venizelos and Benetti, 1999). The development of pigmentation abnormalities in flatfish reared under intensive conditions occurs during metamorphosis, when larval pigmentation is replaced by the juvenile-adult pigmentation. In order to understand the mechanisms of the adult pigmentation pattern development and the apparition of pigmentary disorders, the analysis of the spatial and temporal distribution of chromatophores, as well as the molecular ontogeny underlying the development of skin pigmentation has been studied during the larval development of Senegalese sole.

Pigment cells in developing vertebrates are derived from a transient and pluripotent cell population called the neural crest. These cells arise from the border between the neural ectoderm and the epidermis during early development, are then transcription factor *sox10* dependent, as they migrate throughout the embryo, and give rise to widely diverse cell types, including chromatophores, the peripheral and enteric nervous system. The exact ways in which cells migrating along the ventral-medial pathway are specified and differentiate still remains open (Kelsh *et al.,* 1996). Pigmentation patterns arise during different stages of the fish's life. Fish chromatophores differentiate leading to distinct populations of pigment cells like melanophores, xanthophores, iridophores and leucophores, which contribute to pigmentation patterning during the larval stage – "larval chromatophores" - and after metamorphosis – "adult chromatophores". In this sense, changes in the expression of key genes that regulate the pigmentation process occurring during pro-metamorphosis are visible at later stages of development, at post-metamorphosis, when fish display the juvenile-adult pattern of pigmentation. Changes in pigment cell types and distribution generally occurred after metamorphosis. Senegalese

sole larval pigmentation is characterized by abundant large and dendritic larval-type xanthophores and melanophores, distributed along the head, abdominal and trunk regions, and by the association of xanthophores to melanophores. Two populations of xanthophores and melanophores differing in size were observed in post-metamorphic specimens (Figure 5). Firstly, dendritic and small melanophores and xanthophores typical of larval stages disappeared, whereas they were progressively replaced by round-shaped adult-type xanthophores and newly differentiated and melanized melanophores that were organized in patches in the ocular side of the fish, as observed in flounders (Seikai *et al.*, 1987; Matsumoto and Seikai, 1992). In Chapter 2.1, the proportion of melanophores versus xanthophores was proposed as a reliable visual indicator (biomarker) to evaluate the correct ontogeny of skin pigmentation in Senegalese sole, the xanthophores being involved in the guidance for melanophore patterning (Parichy and Turner, 2003). Iridophores first appeared at the end of post-metamorphosis, and melanophores were required for the proper distribution of iridophores in patches in the ocular side of Senegalese sole, as well as in zebrafish (Johnson *et al.*, 1995) and most of flounders (Matsumoto and Seikai, 1992; Nakamura *et al.*, 2010). Leucophores, present in the skin of Senegalese sole from pre-metamorfosis, were redistributed in patches along the trunk and the fins during metamorphosis and did not disappear after metamorphosis, contrary to what happened in Japanese flounder (Yamada *et al.*, 2010).

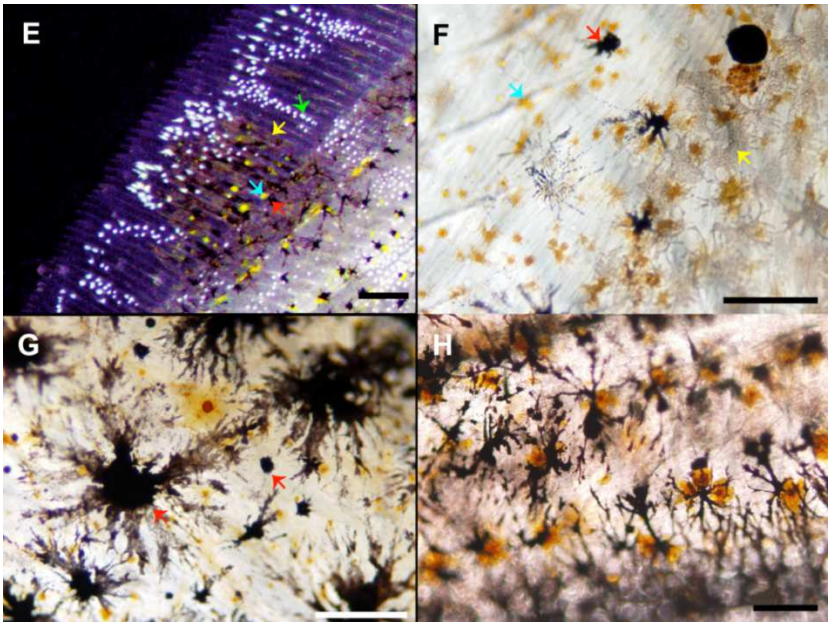


**Figure 5.** Images of the skin of Senegalese sole at 33 dph (E-G) and 35 dph (H) revealing the presence, shape, patterning and spatial relationships among melanophores (red arrows), xanthophores (blue arrows), leucophores (yellow arrows) and iridophores (green arrows). **(E)** Detail of the distribution of chromatophores in the fins. A patch of melanophores, xanthophores and leucophores is surrounded by iridophores; **(F-H)** Detail of the trunk skin of the ocular side showing the distribution pattern of xanthophores and melanophores. Scale bars represents: E, 500 µm; F, 200 µm; G, H, 250 µm (from Chapter 2.1)

The morphological characteristics of skin pigmentation ontogeny were reflected in the expression profiles of eleven genes involved in melanophore differentiation and melanin synthesis of Senegalese sole. Data shown in Chapter 2.1 revealed that the transition from larval to juvenile pigmentation coincided with the progress of metamorphosis: (i) during the pre-metamorphic period (2-11 dph), a low expression of apoptic factors and genes related to melanogenesis and a high expression of melanophore differentiating genes were recorded; (ii) during the pro-metamorphic period (11-19 dph), a high expression of apoptic factors for tissue remodeling and melanophore differentiating and melanogenic genes were measured and; (iii) during the post-metamorphic stage (19-47 dph), a low expression of all analyzed genes was observed, especially those associated to melanophore differentiation.

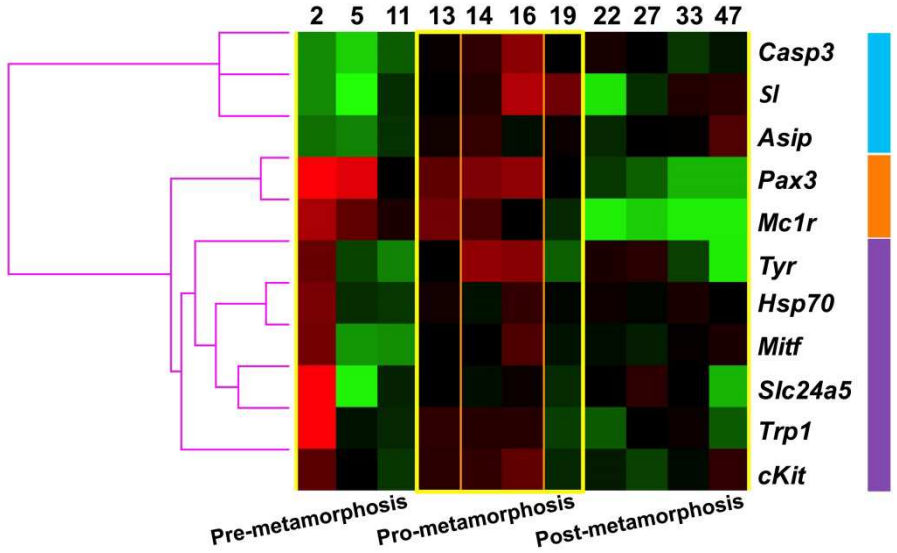


**Figure 6.** Global hierarchical clustering based on similarity of the expression profile for different pigmentation related genes during the larval development of Senegalese sole. Columns represent the mean data values for each sampling point (in dph) and rows represent single genes. Expression level of each gene is represented relative to its median abundance across the different stages and is depicted by a color scale: green, black, and red indicating low, medium, and high relative expression levels, respectively. Colored bars to the right margin indicate the three main gene clusters: blue shows genes highly expressed during pro-metamorphosis, orange corresponds to genes highly expressed during pre- and pro-metamorphosis and violet to genes highly expressed at 2 dph and at pro-metamorphosis. The expression of all genes was high during the pro-metamorphosis phase (yellow square) and the climax of pigmentation development at the molecular level was observed from 14 to 16 dph (orange square). Changes in gene expression profiles coincided with morphological changes in pigmentation: the climax of metamorphosis occurred at 16 dph, the end of pro-metamorphosis at 19 dph and the transition from the larval to the adult pattern of skin pigmentation from 22 dph onwards (from Chapter 2.1).

In Chapter 2.1, it has been established that during pre-metamorphosis, the increasing gene expression of paired box protein 3 (*pax-3),* microphtalmia-associated transcription factor *(mitf)* and *cKit* reflected the differentiation and proliferation of melanophores and the speciation of xanthophores (Figure 6). The expression of melanogenic genes like the tyrosinase (*tyr)* and the last enzyme of melanogenesis (*trp1)* was correlated during the larval development and regulated coordinately, whereas changes in the *tyr/mitf* and *tyr/trp1* ratios along development revealed a cyclic production of melanin in Senegalese sole. The peak of *trp1* expression at 2 dph reflected the melanin production in larval melanophores. During the pro-metamorphosis, the high expression of caspase 3 (*casp3)* and somatolactin (*sl)* reflected the apoptosis of larval chromatophores and the enhancement of melanophore differentiation, respectively. The second peak of *trp1* expression was in line with the melanin synthesis in newly differentiated melanophores. At the climax of pigmentation development coinciding with the end of the pro-metamorphic stage, the expression of *tyr* and *trp1* (melanogenic genes) and *pax-3, mitf* and *cKit* genes decreased, indicating the stabilization of melanogenesis process and melanophore differentiation. The stable expression of the melanocyte-stimulating hormone 1 receptor (*mc1r)* gene at post-metamorphosis indicated the end of the melanophore ontogeny and patterning at a transcriptional level that morphologically coincided with the onset of the adult pigmentation phenotype. Once metamorphosis completed, the prevalence of melanogenesis over melanophore differentiation was highlighted by the increase of *cKit/pax3* ratio. The similar increase of the *mitf/pax3* ratio at post-metamorphosis indicated changes in the relative proportions of melanophores and xanthophores. The expression of the sodium/potassium/calcium exchanger 5 (*Slc24a5)*, necessary for melanin production was constant during the larval development of Senegalese sole and was required for *trp1* expression. In addition, agouti signaling protein (*asip)* expression was suggested to inhibit melanin production and melanoblasts differentiation and proliferation in the blind side of the fish and the *asip/mc1r* ratio is an indicator of the ontogenesis of iridophores and a marker for changes in the pigmentation pattern during the development of Senegalese sole. The considerable increase of the *asip/mc1r* ratio at post-metamorphosis coincided with an increase in the amount of iridophores in the ocular side of the fish and with the switch for bilateral to the flat symmetry, showing that the establishment of the new asymmetry was synchronized with skin pigment pattern. Moreover, melanophores and iridophores derive from a common precursor and the differentiation of bi-potent chromoblasts in melanophores or iridophores seemed to depend on the expression or repression of *mitf*. In Senegalese sole, the decrease of *mitf* expression during the metamorphosis could indicate the differentiation of chromoblasts into iridophores in the ocular side and the blockage of melanophore development in the blind side. Altogether, these results presented in Chapter 2.1 showed that a combined action of both melanophore differentiating- and melanogenic-related genes coordinated melanophore ontogenesis in different molecular events that resulted in the establishment of the juvenile/adult pigmentation phenotype.

Although the ontogenesis of skin pigmentation in Senegalese sole larvae is genetically programmed, epigenetic factors can modulate and affect the expression of genes that regulate

pigmentation, even if the result of these alterations is detectable only after metamorphosis. Several environmental factors have been reported to induce pigmentary problems, especially related to an imbalanced nutrition (Kanazawa, 1993; Estévez *et al.*, 1995; Matsumoto and Seikai, 1992; Villalta *et al.*, 2005a; Hamre *et al.*, 2007, 2008). Excessive contents of dietary ARA supplied to Senegalese sole during larval development have been associated with up to 90% of pseudo-albinism (Villalta *et al.*, 2005a; Chapter 2.2). Whether the induction of pigmentary disorders during Senegalese sole ontogeny was related to a specific larval period of increased sensibility to dietary ARA imbalances was investigated by feeding fish with high ARA levels (10.2 and 7.1% TFA) versus a control diet based on live prey enriched with a commercial product (Algamac 3050$^{TM}$, 1.2% of ARA in average), during the pre- and pro- metamorphic stages and/or the post-metamorphic stage (Chapter 2.2). Thus, larvae fed high dietary ARA levels during pre-, pro- and post-metamorphic stages showed 81.4% of incidence of pigmentary disorders versus 0.9% in fish fed the control diet. In accordance with these results, Villalta *et al.* (2005a) found 84.2% of abnormally pigmented Senegalese sole when fed 8.3% of ARA from 3 to 37 dph and only 0.3% when fish were fed 0.1% of ARA. Similarly, Copeman *et al.* (2002) found 92% of incidence of malpigmentations in yellowtail flounder larvae fed 8.9% ARA and 36% DHA for 4 weeks, pointing to the negative effects of high dietary ARA levels on pigmentation.

In addition to the absolute proportions of ARA in the diet, imbalances in the dietary ARA/EPA ratio have also previously been related to pigmentary disorders in several flatfish species (Reitan *et al.*, 1994; Curé *et al.*, 1995; Bell *et al.*, 2003; Villalta *et al.*, 2005a; Hamre *et al.*, 2005). In Senegalese sole, the incidence of pigmentary disorders was positively correlated with ARA dietary levels and with ARA/EPA, ARA/DHA, EPA/DHA and MUFA/PUFA ratios (Chapter 2.2). High dietary ARA levels have been associated to pigmentary disorders in several flatfish species and these ARA levels have been suggested to be involved in the underlying process of pigment cell differentiation (Chapter 2.3). Positive correlations between dietary levels of ARA and the ARA/EPA ratio and the incidence of pseudo-albinism have been found in turbot, halibut and Japanese flounder, where a dietary ARA/EPA ratio up to 1:4 fed to larvae until metamorphosis affected the proper pigmentation of the fish (McEvoy *et al.*, 1998a; Estévez *et al.*, 1999, 2001). Marine fish larvae have higher requirements for n-3 PUFA than for n-6 PUFA (Sargent *et al.*, 2002); thus, unbalancing the ARA/EPA ratio above 1 in rotifer and *Artemia* metanauplii enriched with the experimental emulsion (ARA/EPA = 1.6 and 1.8, respectively) may have led to pigmentary disorders. There was a clear and significant implication of ARA and EPA in the incidence of malpigmented fish, with higher amounts of ARA than EPA leading to the incidence of pigmentary disorders. Indeed, ARA and EPA display competitive interactions as substrates for cyclo-oxygenase and lipo-oxygenase enzymes, involved in prostaglandin biosynthesis pathways (Bell and Sargent, 2003). Imbalanced dietary ARA/EPA ratio has been reported to disrupt the relative concentrations and effects of $PGE_2$ and $PGE_3$ in target tissues (Reitan *et al.*, 1994; Curé *et al.*, 1995; Logue *et al.*, 2000; Bell and Sargent, 2003; Bell *et al.*, 2003; Hamre *et al.*, 2005; Villalta *et al.*, 2005a; present thesis). Furthermore, although the mechanisms are yet unknown, excess of $PGE_2$ production has been suggested to cause biochemical stress and to be involved in malpigmentation (Sargent *et*

*al.*, 1999b; Tocher, 2003; Brandsen *et al.*, 2005; Villalta *et al.*, 2005a, 2007; Lund *et al.*, 2008), although in the study of Villalta *et al.* (2007) no differences in PGE levels were detected between normally pigmented and pseudo-albino fish. In the study exposed in Chapter 2.2, fish fed high dietary ARA levels during the post-metamorphic stage presented higher levels of $PGE_2$ and were associated with malpigmentations, being in agreement with the results of Villalta *et al.* (2007). Moreover, pseudo-albino specimens fed high dietary ARA levels during post-metamorphosis displayed higher $PGE_2$ concentrations than normally pigmented fish fed the same diet, suggesting the presence of regulatory mechanisms for controlling excessive levels of ARA in the diet; which, when activated, influences the production of ARA-derived metabolites leading to normal pigmentation patterns. However, the exact mechanism linking ARA dietary levels, PGE production and malpigmentation in flatfish remains unknown (Planas and Cunha, 1999; Bolker and Hill, 2000; Villalta *et al.*, 2005a; Brandsen *et al.*, 2005). Insights into the gene regulatory network underlying the pigmentation ontogeny of Senegalese sole have recently been reported (Chapter 2.1), but the involvement of ARA and its hormonal derivatives need further investigations (Villalta *et al.*, 2005a; Brandsen *et al.*, 2005; Chapter 2.2).

Although feeding Senegalese sole larvae with high dietary ARA levels during the whole larval development induced a 81.4% of pseudo-albino fish, whereas a 52.2% of pseudo-albinism was also observed when fish were fed high ARA levels only during pre- and pro-metamorphic stages (2-15 dph), versus only a 20.0% when fish were fed high ARA amounts from post-metamorphosis (15-50 dph). These results, in agreement with those of Lund *et al.* (2007, 2008) on common sole, demonstrated that not only excessive dietary ARA levels were responsible for the incidence of pigmentary disorders, but also the developmental period at which the nutritional imbalance was exerted. These results indicated that nutritional requirements of larvae are not uniform during larval ontogeny, and they change depending on the stage of development of the organism. In Senegalese sole, larval sensitivity to excessive amounts of dietary ARA was higher during pre- and pro-metamorphosis stages, when the adult pigmentation pattern was being determined. In common sole, larvae fed 10.5% ARA during the pre- and pro-metamorphic stages (3-21 dph) were malpigmented (90.4%), whereas only 16.7 and 37.3% of them showed problems of pseudo-albinism when the high dietary ARA levels were provided from 15 to 21 dph or 10 to 21 dph, respectively (Lund *et al.*, 2008). In this context, Næss and Lie (1998) described a sensitive “pigmentation window” occurring just a few days before the metamorphosis in Japanese flounder and halibut. In those species, proper pigmentation was negatively affected by excessive dietary ARA levels and ARA/EPA ratio supplied during their pre-metamorphic stage, but not during the post-metamorphic stage.

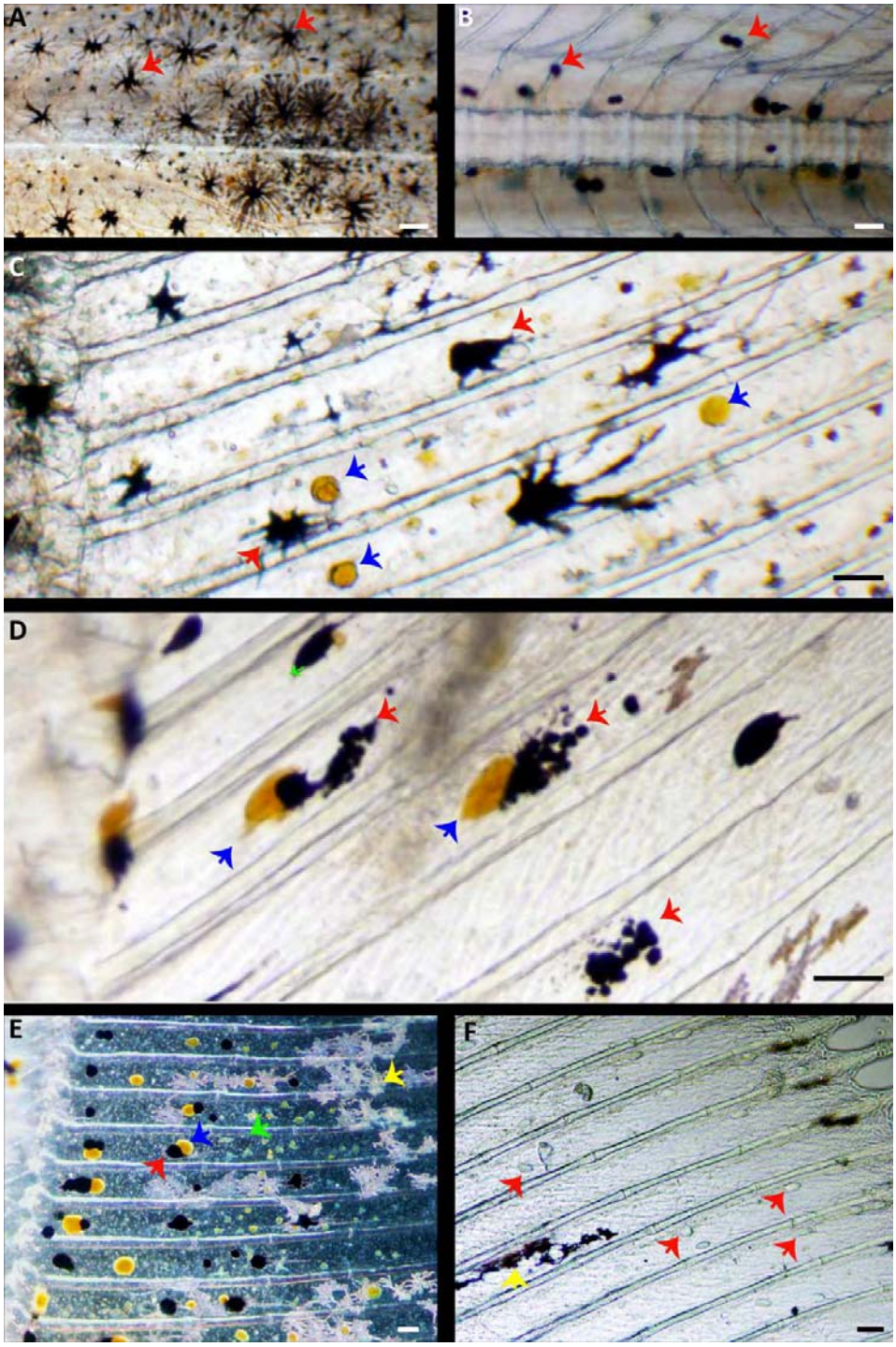


**Figure 7.** Microscopic images showing details of chromatophores distribution in the skin of pigmented larvae and pseudo-albinos in the ocular side of the fish. A-B) trunk skin at the level of the vertebral column in 47 dph pigmented (A) and pseudo-albino (B) fish. C-D) dorsal fin skin in 60 dph pigmented (C) and pseudo-albino (D) fish. E-F) detail of the dorsal fin skin in 33 dph dph € and 60 dph (F) pseudo-albino. Red arrows: melanophores; blue arrows: xanthophores; yellow arrows: leucophores: green arrows: iridophores. Scale bars represent A-B, 200 µm; C-F, 100 µm (from Chapter 2.3).

The development of skin pigmentation during pre-metamorphic stages is morphologically similar between normally pigmented and pseudoalbino post-metamorphic fish, although once metamorphosed, the future pseudo-albino specimens began to present differences with regards to the relative proportions, allocation patterns, shapes and sizes of skin chromatophores (Figure 7). The texture analysis of pigmentary patterns in fish developed under this doctoral thesis showed that the skin of pseudo-albino specimens fed high dietary ARA amounts during the pre- and pro-metamorphosis and/or the post-metamorphosis displayed a more uniform and homogeneous melanophore pattern than normally pigmented fish. In Chapter 2.3, it has been shown that the skin of Senegalese sole pre-metamorphic larvae that turned into pseudo-albinos contained all types of chromatophores, but these cells progressively disappeared after metamorphosis to form the adult pseudo-albino phenotype. Normal individuals displayed many melanophores aggregated in patches throughout the body with the ability to change the shape from round to dendritic to imitate the color background. However, melanophores in pseudo-albinos were less abundant, less aggregated in patches and round-shaped, suggesting their inability to disperse melanin, whereas they progressively disappeared during the post-metamorphosis stage. Besides, the amount of melanophores and iridophores, which increased during the post-metamorphic stage in normally pigmented fish, remained invariable in pseudo-albinos, indicating that the new population of pigmentary cells was not differentiated. Pseudo-albinos also lost most of their leucophores, whereas the few remaining cells were located in the distal part of the fins. The ARA-induced pseudo-albino phenotype was defined as the result of inhibited differentiation of post-metamorphic populations of chromatophores and the progressive degradation of the already existent larval chromatophores (Chapter 2.3). These findings showed that larval pigmentation is determined genetically, but that environmental factors can modulate the normal molecular changes occurring during the process of metamorphosis, modifying the adult pigmentation pattern at post-metamorphosis. Moreover, pseudo-albino fish fed high dietary ARA levels (10.2 and 7.1% TFA in enriched rotifer and *Artemia* metanauplii, respectively) during their whole larval development presented lower density of melanophores and a more uniform aspect of their ocular pigmented side compared to pseudo-albino specimens fed high dietary ARA amounts during only the pre- and pro-metamorphosis or only the post-metamorphosis. This observation suggested that ARA-induced pseudo-albinism is a developmental-dependent phenomenon. Feeding Senegalese sole larvae with high dietary amounts of ARA before, during and after metamorphosis lead to a higher inhibition of chromatophore differentiation than when ARA was supplied only during one of these periods.

As explained above, major molecular changes in the pigment cell pattern occurred during pro-metamorphosis, while morphological changes in the population of the different chromatophores were evidenced at post-metamorphosis. This means that, although the pseudo-albino phenotype could not be detected in pre-metamorphic larvae, high dietary ARA levels supplied during the pre- and pro-metamorphic periods might have affected the molecular mechanisms involved in the pigmentation process, leading to the appearance of pigmentary disorders in post-metamorphic individuals. Feeding Senegalese sole with high dietary ARA levels during pre- and pro-metamorphosis induced an over-

expression of *asip, pax3, cKit* and *mitf* during metamorphosis (Figure 8), which altered the differentiation of chromatoblasts in the ocular side of the fish and concomitantly inhibited the terminal differentiation of new post-metamorphic melanophores, xanthophores and iridophores. In addition, the up-regulation of *asip* and *mitf* in pseudo-albinos seemed to play a key role in the prevention of differentiation of pigment precursor cells into melanophores and xanthophores. The down-regulation of *slc24a5* could be induced by *asip*, disrupting the melanogenesis at the end of the process by also reducing *trp1* expression and, therefore, melanin synthesis. The expression level of *pax3* was demonstrated to be critical for the development of the new populations of melanophores, xanthophores and iridophores. Pigmentation success and pigment patterning relied on the communication between xanthophores and melanophores, their relative proportions, cell proximity, allocation, size and shape (Parichy and Turner, 2003, Nakamasu *et al.*, 2009; Chapter 2.1), and this balance might have been nutritionally disrupted in Senegalese sole pseudo-albinos. An altered communication between melanophores and xanthophores was observed in pseudo-albinos, with an excessive disintegration of xanthophores that possibly prevented the normal patterning of melanophores, leading to the disruption of the dorsal-ventral pigmentation signaling patterning during metamorphosis and resulting in the apparition of the pseudo-albino phenotype. This altered behavior of pigment cells in the ocular side of pseudo-albino specimens was suggested to normally occur in the blind side during metamorphosis, progressively forming the dorsal-ventral pigment pattern characteristic of flatfish. The role of *asip* in the establishment of dorsal-ventral pigment patterning has previously been studied in other species (Cerdá-Reverter *et al.*, 2005), but its implication in xanthophores physiology requires further investigation in Senegalese sole.

These pigmentation related results allowed increasing the current knowledge about morphologic and molecular processes related to the appearance of pigmentary disorders in flatfish and about the effect of ARA and its derived metabolites in the proper pigmentation development in Senegalese sole.

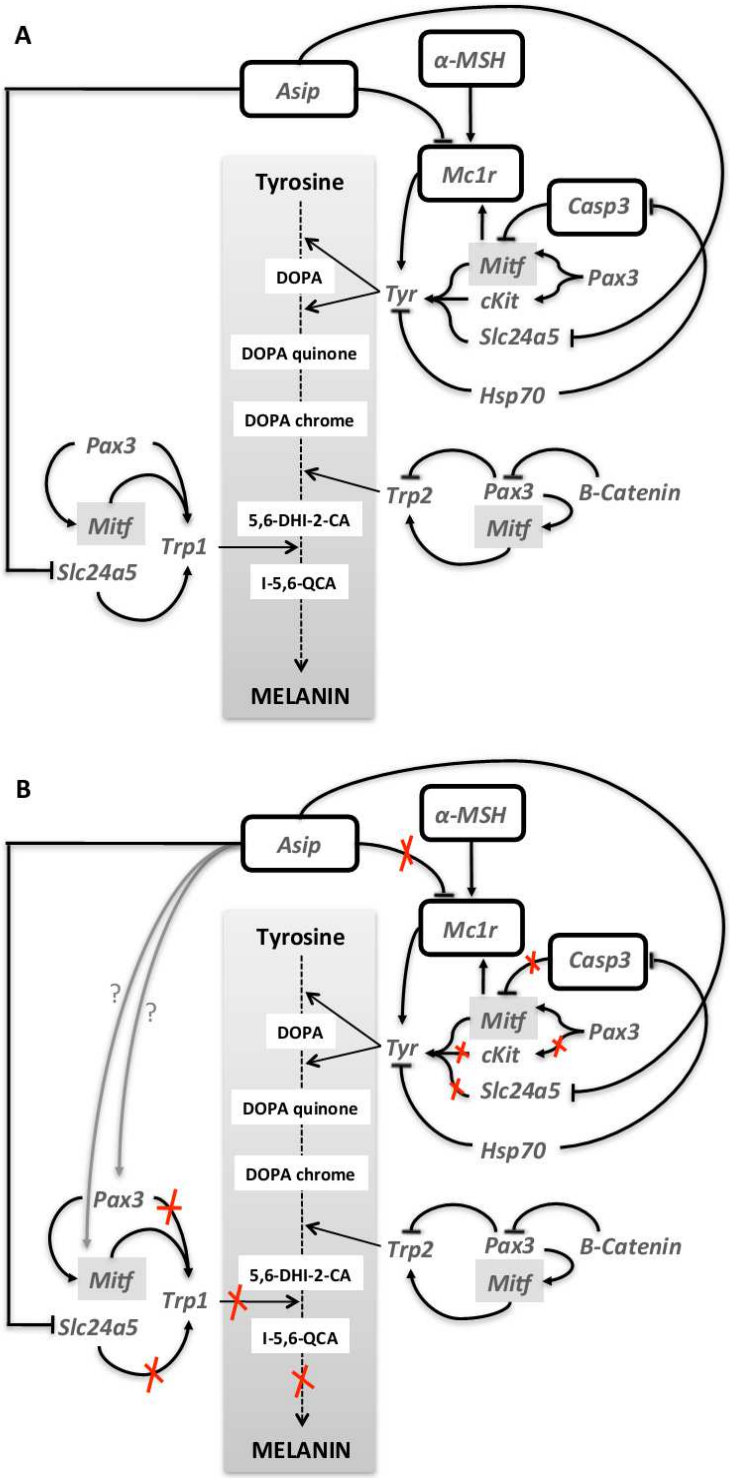


**Figure 8.** Schematic picture showing the known action of the analyzed genes in melanocyte differentiation and melanogenesis in vertebrate melanocytes **(A)** and the suggested pathways of melanophore differentiation and melanogenesis that were altered in pseudo-albinos of Senegalese sole **(B)** (from Chapter 2.3).

# DISCUSIÓN

## (en castellano)

La producción acuícola intensiva de peces marinos se ha desarrollado rápidamente en respuesta a la demanda creciente de los mercados; no obstante, este hecho ha puesto de manifiesto diversas deficiencias relacionadas con la producción masiva de peces. La optimización de las condiciones de cultivo, tales como el aumento de las densidades de cultivo y de las tasas diarias de alimentación, estrategias destinadas a reducir la duración de los ciclos de producción y responder así a las necesidades del mercado, impacta directamente sobre el crecimiento y el desarrollo de los animales. Las larvas de peces marinos son particularmente sensibles a las condiciones de cultivo, ya que sufren importantes modificaciones morfológicas, anatómicas y fisiológicas en sus diferentes tejidos, órganos y sistemas funcionales. Las etapas iniciales de la vida representan un periodo ontogenético de transición a nivel de crecimiento y diferenciación, en las que los animales pasan de la alimentación endógena a la exógena, varios órganos y sistemas, como los órganos sensoriales, neuroendocrinos, excretorios y digestivos, así como también el tejido esquelético y la musculatura, se desarrollan definitivamente y, como consecuencia, el organismo adquiere su fenotipo definitivo (Power *et al.*, 2008). El desarrollo correcto de los citados procesos durante los estadios larvarios es un punto clave para obtener juveniles de calidad y optimizar el valor del producto final en el mercado. El desarrollo de protocolos de cultivo fiables y sostenibles en los criaderos industriales necesita de un buen conocimiento de los puntos críticos del desarrollo larvario, particularmente la nutrición, en un estadio en el que la dieta es crucial para permitir al metabolismo soportar el intenso crecimiento larvario. Durante las fases iniciales del desarrollo, las larvas son particularmente sensibles a las condiciones inadecuadas de alimentación y una dieta desequilibrada puede afectar seriamente a la formación de los tejidos y órganos del futuro adulto.

Durante las últimas décadas, los productores de larvas de peces marinos del Mediterráneo han mejorado considerablemente sus métodos de cultivo mediante la aplicación de condiciones intensivas, pero la calidad larvaria sigue siendo una de las mayores preocupaciones para el correcto desarrollo de este sector (Koumoundouros, 2010; Boglione *et al.*, 2013b). La alta incidencia de malformaciones esqueléticas y desordenes pigmentarios que se dan en peces de cultivo (que pueden llegar a afectar a más del 30% de la producción) se originan principalmente durante los estadios larvarios e indican que los criaderos aún carecen del conocimiento necesario para producir larvas de calidad óptima y saludables en condiciones de cultivo intensivo. La incidencia de deformaciones esqueléticas, como la escoliosis, lordosis, quifosis, ausencia o exceso de aletas, opérculo subdesarrollado o malformaciones de la mandíbula, han sido descritos en la mayor parte de especies marinas cultivadas en climas templados, como la dorada (Matsuoka, 1987; Boglione *et al.*, 2003b; Prestinicola *et al.*, 2012), la lubina (Barahona-Fernandes, 1982) y el lenguado senegalés (Gavaia *et al.*, 2009). No obstante, este problema no sólo afecta al cultivo de las especies de aguas templadas sino también a las de agua fría, como la trucha, el bacalao y el fletán (Deschamps *et al.*, 2008; Baeverfjord *et al.*, 2009; Lewis-Mc Crea y Lall, 2010). Asimismo, el recurrente problema de las deformidades esqueléticas no sólo afecta a la industria acuícola europea, sino que se trata de un problema a nivel mundial (Hosoya y Kawamura, 1998; Cobcroft y Battaglene, 2013). De igual forma, los problemas de pigmentación (bicoloración, pseudo-albinismo, hipomelanosis e hipermelanosis)

pueden afectar a entre el 5 y el 100% de las diferentes especies de peces planos cultivados en la actualidad, como es el caso de la platija europea (Dickey-Collas, 1993), el rodaballo (Estévez y Kanazawa, 1995; Estévez, 1996), el halibut (McEvoy *et al.*, 1998a; Pittman *et al.*, 1998), la platija japonesa (Takeuchi, 2001), el lenguado común (Lund, 2007) y el lenguado senegalés (Soares *et al.*, 2002). Este grupo de peces presenta una pigmentación externa asimétrica (Norman, 1934), con el lado ocular marrón (pigmentado) y el lado ciego completamente blanco. No obstante, la pigmentación es uno de los procesos organogénicos más sensibles a desequilibrios bióticos y abióticos (Heuts, 1951). El estrés inherente en las larvas de peces planos durante la metamorfosis se agrava en condiciones de cultivo intensivo, donde la calidad del agua, la alimentación, el sustrato, la luz y los niveles de ruido, son susceptibles de afectar al proceso de pigmentación (De Veen, 1969). Las anormalidades de pigmentación han sido descritas en ambos lados del cuerpo del animal (Venizelos y Benetti, 1999; Bolker y Hill, 2010). Entre los distintos tipos de anomalías pigmentarias descritas, es importante destacar la hipomelanosis, que consiste en zonas blancas o áreas libres de pigmentación en el lado ocular de la piel, y la hipermelanosis, que ha sido descrita como la aparición de zonas hiperpigmentadas en lado ciego del animal, zona que normalmente carece de pigmentación. La ambicoloración es la presencia de pigmentación en ambos lados de los peces planos, si bien su incidencia es mucho menor que las dos anteriores. Aunque estas alteraciones de la pigmentación de la piel no afectan al desarrollo y bienestar de los peces (Soutar, 1995), los individuos con anomalías pigmentarias presentan menores probabilidades de sobrevivir en la naturaleza debido a la pérdida de la capacidad críptica de la piel en relación al entorno (Koshiishi *et al.*, 1991). Comercialmente, los peces planos mal pigmentados y que se comercializan en pieza (rodaballo, lenguado común y senegalés) se consideran de baja calidad, pues se alejan de los estándares de calidad marcados por los animales procedentes de la pesca, hecho que ocasiona que su precio se reduzca en los mercados (Venizelos y Benetti, 1999). En las especies que se comercializan fileteadas (fletán, platija japonesa), este problema tiene menores consecuencias, pues el pescado es presentado al consumidor desprovisto de piel y, en muchas ocasiones, sin espinas. En todo caso, ambos problemas morfo-anatómicos (esqueléticos y de pigmentación) afectan al aspecto externo y rendimiento biológico del organismo (*p. ej.,* tasa de crecimiento, uso del alimento, susceptibilidad a enfermedades y mortalidad), reduciendo el valor del producto final en el mercado e incrementando los costes de producción asociados (Gavaia *et al.*, 2002, 2009; Fernández y Gisbert, 2011). Además, la presencia de peces con deformaciones conlleva también implicaciones éticas puesto que los peces con deformidades esqueléticas pueden presentar problemas de natación y un menor bienestar (Boglione *et al.*, 2013a), situación que puede traducirse en reducidas tasas de alimentación y crecimiento, una mayor susceptibilidad al estrés y patógenos en comparación con sus congéneres sanos y con un correcto desarrollo. Por ello se considera que estos peces no se encuentran en condiciones adecuadas de bienestar (Noble *et al.*, 2012). Por lo anteriormente explicado, es de vital necesidad, desde un punto de vista industrial, poder reducir la incidencia de malformaciones esqueléticas y problemas pigmentarios en peces de cultivo. En este contexto, el incremento del conocimiento científico y zootécnico sobre las causas de la aparición de estas anomalías morfo-anatómicas

contribuirá a resolver uno de los mayores cuellos de botella en el cultivo de larvas de peces marinos actual.

El desarrollo de las malformaciones esqueléticas y problemas de pigmentación en larvas y juveniles de peces marinos viene determinado por una amplia variedad de factores fisiológicos, ambientales, genéticos, xenobióticos y nutricionales, los cuales pueden alterar el desarrollo normal del organismo (Lall y Lewis-McCrea, 2007). Entre ellos, la nutrición larvaria ha sido identificada como un factor clave en el desarrollo del pez y particularmente influyente sobre los procesos de esqueletogénesis y pigmentación (Cahu *et al.*, 2003; Lall y Lewis-McCrea, 2007; Boglione *et al.*, 2013a). Diversos estudios han demostrado que algunos nutrientes son responsables de la aparición de malformaciones esqueléticas y desordenes pigmentarios cuando se suministran de manera inapropiada o con niveles desequilibrados (Bolker y Hill, 2000; Cahu *et al.*, 2003; Villalta *et al.*, 2005a, b, 2008; Villeneuve *et al.*, 2005a). Entre los nutrientes que interfieren en el desarrollo del esqueleto y de la piel, los lípidos, en particular los ácidos grasos altamente insaturados (AGAI), juegan un papel muy importante en las primeras etapas del desarrollo de larvas de peces marinos, ya que representan la mayor fuente de energía y una fuente de ácidos grasos esenciales (AGE). Los elevados requerimientos nutricionales en AGE están relacionados con el rápido crecimiento de las larvas y en la formación de nuevas estructuras celulares que son necesarias para el correcto crecimiento y desarrollo del organismo (Izquierdo *et al.*, 2000; Sargent *et al.*, 2002; Cahu *et al.*, 2003; Lall y Lewis-McCrea, 2007; Boglione *et al.*, 2013a). Estudios previos han ilustrado que un desequilibrio nutricional en AGE tiene un efecto negativo sobre diversos procesos en larvas de peces planos, afectando de forma importante a su crecimiento, metamorfosis y supervivencia (Dickey-Colas y Geffen, 1992; Izquierdo *et al.*, 1992; Tzoumas, 1988; Morais *et al.*, 2004; Villalta *et al.*, 2005a), al desarrollo del esqueleto (Berge *et al.*, 2009; Kjørsvik *et al.*, 2009; Dâmaso-Rodrigues *et al.*, 2010) y de la piel (Reitan *et al.*, 1994; Estévez y Kanazawa, 1995; Villalta *et al.*, 2005b; Vizcaino-Ochoa *et al.*, 2010). Además de los lípidos de la dieta, los retinoides (vitamina A, VA) desempeñan un papel importante en el desarrollo del organismo, así como en la mayor parte de procesos fisiológicos de éste (Ross *et al.*, 2000). En cuanto al tema objeto de estudio de la presente tesis doctoral, es importante mencionar que si bien la VA es considerada como un nutriente esencial, unos niveles alterados de VA en la dieta (hipo e hipervitaminosis A) son capaces de reducir la síntesis del colágeno y la formación del hueso y, por consiguiente, pueden conducir al desarrollo de deformidades esqueléticas (Fernández y Gisbert, 2011), además de desórdenes pigmentarios (Estévez, 1996). Los requerimientos cuantitativos, así como signos de deficiencia en VA y AGE, han sido descritos en varias especies de peces, pero su papel funcional en el metabolismo lipídico del hueso y en el desarrollo de la pigmentación aún es poco conocido. En este sentido, el presente trabajo de investigación tuvo como objetivo evaluar el efecto combinado de lípidos, particularmente los niveles absolutos y relativos de AGE y de los lípidos oxidados, y otros nutrientes importantes como las vitaminas A y E (VA, VE) sobre la esqueletogénesis y la pigmentación, así como su implicación en la incidencia de desórdenes esqueléticos y pigmentarios durante el desarrollo larvario del lenguado senegalés.

La mayor parte de los estudios nutricionales que han investigado cómo los AGAI y los AGE afectan al crecimiento y desarrollo larvario en peces están basados en diversos diseños experimentales de tipo dosis-respuesta, donde el efecto de un gradiente creciente de un nutriente, o bien de la combinación de diferentes proporciones de algunos de ellos, son evaluados sobre el desarrollo del animal. Este tipo de estudios está particularmente indicado para determinar los requerimientos nutricionales en larvas de peces alimentadas con microdietas. Adicionalmente, algunos estudios han examinado también el efecto de diferentes tipos de presas vivas con distintos perfiles de ácidos grasos (Shields *et al.*, 1999; Hamre *et al.*, 2002), así como también y el efecto de presas vivas enriquecidas con diferentes emulsiones sobre el desarrollo larvario (Morais *et al.*, 2004). No obstante, la mayor parte de estos estudios no integran el perfil completo de ácidos grasos de las dietas experimentales para comprender su impacto sobre el desarrollo de la larva, ya que las interacciones entre los AGAI y AGE complican sumamente la interpretación de los resultados. El presente trabajo de investigación doctoral incluye las anteriormente citadas aproximaciones nutricionales para mejorar la comprensión del efecto de cada ácido graso de la dieta y de las interacciones entre ellos sobre los procesos de morfogénesis en larvas de lenguado senegalés.

La mayoría de larvas de especies marinas no aceptan alimentos inertes al inicio de la fase de alimentación exógena y, por lo tanto, el uso de presas vivas que nadan activamente en la columna de agua es el recurso empleado mayoritariamente en criaderos comerciales (ver revisión en Øie *et al.*, 2011). Si bien los copépodos representan la presa viva ideal para la alimentación de larvas de peces marinos, su uso a escala industrial es casi nulo dado que su cultivo intensivo y en altas cantidades conlleva altos costes y no resulta ser tarea fácil. En este sentido, los rotíferos (*Brachionus sp.*) y la *Artemia* (*Artemia sp.*) son los dos tipos de presa viva más ampliamente utilizados a nivel mundial desde los años 60 debido a su facilidad de producción a densidades y cantidades elevadas a lo largo del año y a la capacidad de poder modular su calidad nutricional mediante el uso de emulsiones y/o enriquecedores. En términos generales, las principales ventajas que conlleva el uso de rotíferos en la alimentación de larvas de peces son (i) su tamaño óptimo en relación al tamaño de la boca de la larva, (ii) su lenta velocidad de natación que permite a las larvas su fácil captura, (iii) su tolerancia a variaciones de salinidad, pH y temperatura, facilitando así su cultivo bajo distintas condiciones ambientales, y (iv) la facilidad para manipular su valor nutricional. Asimismo, la producción de *Artemia* presenta las siguientes ventajas: (i) facilidad de uso, (ii) sus quistes pueden ser almacenados por largos periodos de tiempo y son fáciles de eclosionar y (iii) se puede incrementar su valor nutricional mediante el uso de enriquecedores gracias a su comportamiento alimentario no selectivo.

El valor nutricional de ambas presas vivas ha sido demostrado ser sub-óptimo para la mayoría de larvas de peces marinos, pues tanto el rotífero como la *Artemia* son deficientes en lípidos polares y AGE en comparación con los copépodos, quienes constituyen la dieta natural principal de las larvas de peces marinos, y cumplen los requerimientos nutricionales de las mismas, en particular los de la serie AGAI n-3. En consecuencia, el rotífero y la *Artemia* necesitan ser enriquecidos en AGE y AGAI n-3 antes de ser administrados a las larvas de peces marinos, mejorando así su calidad

nutricional. El enriquecimiento de la presa viva mejora el contenido de AGAI en la *Artemia*, en particular el de DHA y EPA (revisado en Øie *et al.*, 2011). Generalmente, el procedimiento de enriquecimiento de presas vivas permite que el contenido de DHA alcance entre el 18% y el 22% de ácidos grasos totales (AGT), que la relación de DHA/EPA se encuentre cerca de 2 y que el contenido en AGAI n-3 incremente de un 10% a un 35% AGT, valores cercanos a los encontrados en copépodos en condiciones naturales. Además de los lípidos y los AGE, el contenido de otros nutrientes, tales como los aminoácidos, minerales o vitaminas, en las presas vivas también se modifica durante el proceso de enriquecimiento (Aragão *et al.* 2004; Giménez *et al.*, 2007). El contenido proteico de ambas presas vivas (rotífero y *Artemia*) disminuye después del enriquecimiento debido a que estos productos contienen un elevado contenido en lípidos, si bien los perfiles de aminoácidos en ambas presas vivas no se ven perturbados por el enriquecimiento, pues en ambos casos la presa viva enriquecida presenta niveles suficientes para cada uno de los diez aminoácidos esenciales. Los niveles de minerales son generalmente más bajos en rotíferos que en copépodos, especialmente los de magnesio y selenio, los cuales se encuentran por debajo de los requerimientos recomendados para la mayoría de larvas de peces marinos. En cambio, el contenido de minerales en los nauplios de *Artemia*, parece encontrarse en cantidades suficientes para la mayoría de las larvas de peces marinos, con excepción del selenio. Respecto a las vitaminas, se sabe que los rotíferos alimentados con microalgas contienen cantidades suficientes de vitaminas hidrosolubles para cubrir las necesidades de los requerimientos nutricionales de las larvas de peces; asimismo, se ha demostrado que los nauplios de *Artemia* contienen mayores niveles de vitaminas que el zooplancton natural. A través del proceso de enriquecimiento, las vitaminas son incorporadas de manera eficiente en la *Artemia* (revisado en Øie *et al.*, 2011).

La mejora en el diseño de los distintos enriquecedores nutricionales, de las distintas técnicas de enriquecimiento y la oferta de nuevos productos para enriquecer presas vivas ha permitido en estas últimas décadas optimizar el valor nutricional del alimento vivo, lo que resulta en mejores tasas de supervivencia, crecimiento, metamorfosis, una reducción en la incidencia de malformaciones, mejoras en la pigmentación y resistencia al estrés en la mayoría de las especies marinas (ver revisión en Øie *et al.*, 2011). No obstante, la composición nutricional de la presa viva puede variar considerablemente dependiendo de las condiciones en las que se lleva a cabo el proceso de enriquecimiento y de las características propias de la emulsión o enriquecedor utilizado (ver revisión en Monroig *et al.*, 2006). Si bien los rotíferos y los metanauplios de *Artemia* acumulan fácilmente los enriquecedores en su tracto digestivo, diversos factores pueden afectar a la eficiencia y repetitividad del enriquecimiento (densidad, oxígeno disuelto, intensidad de aireación, volumen e hidrodinámica de los tanques donde se realiza el proceso de enriquecimiento) (McEvoy *et al.*, 1998a; Conceição *et al.*, 2010). Además, el metabolismo específico de la presa viva altera las formulaciones originales que se intentan suministrar a las larvas (Barclay y Zeller, 1996). Así, por ejemplo, la presa viva es capaz de retro-convertir el DHA en EPA, así como de variar los niveles de las distintas clases de lípidos al transformar los fosfolípidos de la emulsión en triglicéridos (Navarro *et al.*, 1999). Consecuentemente, los metanauplios de *Artemia* acumulan mayores niveles de EPA que de DHA, lo que modifica la

relación DHA/EPA de la emulsión o enriquecedor original, y transforma la mayoría de los ácidos grasos suministrados como fosfolípidos en lípidos neutros (Izquierdo, 1988; McEvoy *et al.*, 1995; Navarro *et al.,* 1999). Resultados similares han sido descritos en rotíferos (Fernández-Reiriz *et al,* 1993; Rainuzzo *et al.,* 1994). Además, la alta variabilidad en la composición en ácidos grasos y la distribución de las diferentes clases de lípidos en metanauplios de *Artemia* enriquecidos en relación al rotífero se debe en parte también al distinto metabolismo de ambos tipos de presas al encontrarse en estadios de desarrollo muy distintos. En este sentido, los nauplios de *Artemia* sufren importantes cambios fisiológicos y de desarrollo durante las primeras horas de vida, que coinciden con el periodo en el que se lleva a cabo el enriquecimiento, incluyendo un elevado incremento en su tamaño, la absorción de la reservas del vitelo, la abertura del tracto digestivo y de la boca y el inicio de su alimentación exógena (Navarro *et al.,* 1999). Además, dado que las condiciones de enriquecimiento son difícilmente controlables (*p. ej.,* cantidad de oxígeno disuelto, tamaño de las burbujas de aire, hidrodinámica en los tanques de enriquecimiento, tamaño de las micelas en la emulsión) las diferencias entre éstas pueden influir tanto en la eficiencia y repetitividad del enriquecimiento, como en el nivel de desarrollo de los nauplios y su tasa metabólica (Navarro *et al.*, 1999). Las diversas formas físicas de presentación de los productos de enriquecimiento disponibles en el mercado (*p. ej.,* emulsiones basadas en aceite de pescado, encapsulados o productos en polvo) también pueden afectar a la eficiencia de absorción del enriquecedor que, a su vez, modifica el valor nutricional de la presa viva enriquecida (Capítulo 1.1 de la presente tesis). En este contexto, es importante mencionar que tanto el rotífero como los metanauplios de *Artemia* enriquecidos no reflejan el nivel de peroxidación lipídica de los enriquecedores que se les han suministrado, dado que el propio metabolismo de ambos tipos de presa viva es capaz de disminuir la cantidad de radicales libres tóxicos incluidos en la dieta (Capítulo 1.4). En concreto, los metanauplios de *Artemia* que fueron enriquecidos con productos que contenían niveles de oxidación creciente no mostraron variaciones ni alteraciones en los niveles de oxidación de sus tejidos, incluso en aquellas enriquecidas con la emulsión que presentaba el mayor grado de oxidación (Capítulo 1.4). Este proceso fisiológico no ha sido estudiado aun en presas vivas, pero la hipótesis de que la *Artemia* posee sistemas antioxidantes altamente eficientes, capaces de regular los niveles de oxidación en sus tejidos, es altamente plausible, así como también la implicación de los carotenoides en los mecanismos de detoxificación frente a lípidos oxidados (Gilchrist y Green, 1960; Hsu y Chichester, 1970; Kumar y Marian, 2006). No obstante, es conveniente realizar más investigaciones para probar la veracidad de estas presunciones y establecer un nivel límite de oxidación permitido en la emulsión antes de que los sistemas antioxidantes de las presas vivas se activen. Consecuentemente, esta propiedad específica de los metanauplios de *Artemia* debería ser considerada cuando se decida utilizar este tipo de presa viva para estudios nutricionales con distintos niveles de grasa oxidada.

Otro factor importante que debe ser considerado al trabajar con larvas de peces marinos es la transición de la fase de alimentación con rotíferos a la de nauplios y metanauplios de *Artemia*. A pesar de que la presa viva se enriquece con los mismos productos comerciales en distintos criaderos y en ensayos a pequeña escala, estos presentan diferentes perfiles de ácidos grasos, aminoácidos

VA y de acumulación de peróxidos, según el organismo que se enriquezca. Como ya se ha mencionado anteriormente, este hecho se debe a las diferencias fisiológicas entre la presa viva, la distinta tasa de filtración entre rotíferos adultos y metanauplios de *Artemia* y a las diferencias en sus protocolos de enriquecimiento, especialmente la duración de la fase de enriquecimiento (Giménez *et al.*, 2007). Se recomienda que esta variabilidad de los patrones de acumulación de nutrientes en la presa viva durante la transición de la fase de alimentación de rotífero a la de *Artemia* se tenga en cuenta a la hora de diseñar experimentos nutricionales, así como también en condiciones de producción industrial intensiva. Por todas estas razones, antes de llevar a cabo un trabajo de cultivo larvario es aconsejable realizar un estudio preliminar del enriquecimiento de la presa viva en función de su perfil nutricional post enriquecimiento. Dicha información es de gran utilidad para verificar la calidad nutricional final de la presa viva y para establecer condiciones estándares para el enriquecimiento (*p. ej.,* concentración de la emulsión, duración del enriquecimiento, temperatura del enriquecimiento y volumen del tanque en el que se lleva a cabo este proceso). En el contexto general de la presente tesis doctoral, el objetivo fue determinar el efecto de los lípidos y, especialmente, de los AGE en el desarrollo y morfogénesis larvaria del lenguado senegalés, particularmente su implicación en la incidencia de desórdenes esqueléticos y pigmentarios, mediante el uso de presas vivas enriquecidas, teniendo en cuenta los factores anteriormente descritos.

*Composición de ácidos grasos y contenido en retinoides y α-tocoferol.*

El estudio de los efectos de la nutrición sobre los procesos de desarrollo de larvas de peces conlleva tener presente una amplia variedad de factores y nutrientes de la dieta. Sin embargo, las complejas interacciones entre ellos hacen difícil la interpretación de los resultados; por ello, el papel de cada grupo de compuestos de la dieta es generalmente evaluado de manera independiente en estudios de tipo dosis-respuesta. El principal grupo de compuestos de la dieta objeto de estudio en la presente tesis fueron los ácidos grasos esenciales. Adicionalmente, se ha considerado el papel de la VA en los Capítulos 1.1, 1.2 y 1.3, ya que sus niveles varían de un enriquecedor comercial a otro, así como la evaluación del efecto de los lípidos oxidados en la dieta sobre el desarrollo larvario en el Capítulo 1.4, teniendo en cuenta el efecto antioxidante de la vitamina E (α-tocoferol) en la dieta.

En la presente tesis, el perfil de ácidos grasos de las larvas de lenguado senegalés, después de varias semanas de alimentación con diferentes dietas experimentales, reflejó generalmente la composición de ácidos grasos de la dieta, tal y como ya se había comprobado en trabajos anteriores con la misma especie (Morais *et al.*, 2004; Villalta *et al.*, 2005a, b, 2008), y en otros peces planos como el rodaballo (Estévez *et al.*, 1999), la platija (Copeman *et al.*, 2002) y el lenguado común (Lund *et al.*, 2008). Esta observación es recurrente para otras especies de peces marinos y está asociada al hecho de que, aunque algunos tejidos conservan un perfil estable cuando las dietas cumplen los requerimientos mínimos, otros tejidos, como por ejemplo el tejido muscular, reflejan el perfil de la dieta, salvo si sus perfiles son extremos (Benedito-Palos *et al.*, 2011). Además, la composición de ácidos grasos de las larvas de lenguado senegalés responde rápidamente a los cambios en el perfil

de ácidos grasos de la dieta, reflejando así los cambios en la composición de las presas vivas. Así, cuando las larvas fueron alimentadas sucesivamente con niveles bajos y altos de ARA o viceversa, los cambios en la composición de AG de las presas vivas enriquecida indujeron cambios en los perfiles de AG de los tejidos de las larvas en favor de las nuevas presas administradas. El rápido reemplazo de los AG en tejidos del lenguado senegalés se considera que está mayormente relacionado con su tasa de crecimiento, que es relativamente rápida durante las etapas iniciales de desarrollo (Conceição *et al.*, 2007). Las larvas de lenguado senegalés recién eclosionadas presentaron generalmente niveles de DHA, 16:0, 18:0, EPA y DPA más elevados que en estadios posteriores de su desarrollo, lo que resultó en un incremento de los ratios n-3/n-6 AGAI y DHA/EPA y una disminución de los ratios ARA/EPA y ARA/DHA (Morais *et al.*, 2004; Villalta *et al.*, 2005a, b; Capítulos 1.1 y 1.5). Durante las etapas iniciales del desarrollo, las larvas consumieron rápidamente los AG maternales contenidos en el vitelo con el fin de obtener la energía necesaria para crecer y crear nuevas estructuras celulares antes del inicio de la alimentación exógena. En este sentido, Sargent *et al.* (1999) sugirió que los requerimientos nutricionales en AGE de las larvas de peces eran similares a la cantidad de AGE acumulados en el huevo. Los resultados obtenidos en la presente tesis (Capítulo 1.1) mostraron que cuando los contenidos de AGE en la dieta no fueron suficientes para satisfacer los requerimientos de la larva, su crecimiento y desarrollo larvario se vieron afectados, tal y como se observó en larvas alimentadas con *Artemia* enriquecida con Easy Selco© o con altos niveles de ARA en la dieta.

A diferencia de los AG, la acumulación de retinoides en los tejidos de las larvas de lenguado senegalés no reflejó el contenido de retinoides de la *Artemia* enriquecida. Las larvas de lenguado senegalés alimentadas con *Artemia* enriquecida con niveles elevados de retinoides (24.8 y 13.2 $\mu g.g^{-1}$ de peso seco, P.S.) mostraron niveles más bajos de acumulación de estos compuestos (3.2 y 4.6 $\mu g.g^{-1}$ P.S.) que aquellas alimentadas con los contenidos más bajos de retinoides (0.5 y 0.1 $\mu g.g^{-1}$ P.S. en presa viva, 6.3 y 6.5 $\mu g.g^{-1}$ P.S. en larvas). Fernández *et al.* (2005) demostraron que la acumulación de VA en los tejidos de la larva era proporcional a los niveles de VA en la dieta. Sin embargo, en nuestro trabajo (Capítulo 1.1) no se encontró dicho patrón de acumulación de VA en larvas alimentadas con *Artemia* enriquecida con niveles de retinoides que variaron entre 11.1 y 24.8 $\mu g.g^{-1}$ P.S. En el presente estudio, los bajos contenidos en VA en la *Artemia* enriquecida utilizados, en comparación con los del estudio de Fernández *et al.* (2009) (1,320 – 12,910 $\mu g.g^{-1}$ P.S.), junto a la distinta composición de los productos de enriquecimiento evaluados, podrían explicar la discrepancia entre ambos estudios. En el Capítulo 1.4, se ha mostrado cmo las larvas alimentadas con presas vivas enriquecidas con un gradiente creciente de peroxidación lipídica presentaron unos niveles decrecientes de α-tocoferol, lo que refleja la utilización de esta vitamina en la protección del organismo frente a situaciones de estrés oxidativo, tal y como se ha descrito en la dorada (Mourente *et al.*, 2002), en la perca americana *Micropterus salmoides* (Chen *et al.*, 2011) y en el pez cabeza de serpiente *Channa punctatus* (Abdel-Hameid *et al.*, 2012). No obstante, dado que no se consideró la vitamina E como principal nutriente de estudio en la presente tesis doctoral, no se ha profundizado en la evaluación del efecto de la misma sobre el desarrollo de las larvas de lenguado senegalés.

*Tasas de supervivencia, crecimiento y metamorfosis*

En todos los estudios experimentales que se realizaron en el marco de la presente tesis, las tasas de supervivencia del lenguado senegalés fueron muy elevadas (94.7 ± 3.5% en promedio). Además, la supervivencia no se vio afectada por la mayor parte de los tratamientos nutricionales evaluados. A título comparativo, las tasas de supervivencia fueron mayores que las reportadas en otros estudios sobre esta especie (Cañavate y Fernández-Díaz, 1999; Dinis, 1992; Dinis *et al.*, 1999; Morais *et al.*, 2004; Villalta *et al.*, 2005a, b; Fernández *et al.*, 2008; Dâmaso-Rodrigues *et al.*, 2010), aunque similares a los valores encontrados en larvas de lenguado senegalés alimentadas con *Artemia* deficiente en AGAI durante la post-metamorfosis (> 90%, Morais *et al.*, 2005). En la presente tesis, las larvas de 38 días después de la eclosión (dde) alimentadas con presa viva enriquecida con cinco productos comerciales con distintos perfiles de AG presentaron tasas de supervivencia similares, tal y como era de esperar de acuerdo a las garantías nutricionales de dichos productos. En las larvas alimentadas con rotíferos y metanauplios de *Artemia* enriquecidos con emulsiones experimentales que contenían un gradiente creciente de peroxidación lipídica, la tasa de supervivencia a los 35 dde tampoco se vio afectada por los diferentes tratamientos. Se han descrito resultados similares en juveniles de otras especies de peces marinos, como en la dorada y el fletán, cuyas tasas de crecimiento y supervivencia no se vieron afectadas por las cantidades crecientes de lípidos peroxidados incorporados en sus dietas (Mourente *et al.*, 2000; 2002; Lewis McCrea y Lall, 2007).

Varios estudios han evidenciado la importancia del ARA en la supervivencia y la tolerancia al estrés en larvas de peces marinos (Koven *et al.,* 2001; Atalah *et al.*, 2011a, b, entre otros). Bajo las condiciones experimentales utilizadas en la presente tesis, las tasas de supervivencia de las larvas de lenguado senegalés no se vieron afectadas cuando los animales fueron alimentados con altos niveles de ARA (7.0% de AGT) durante las fases pro- y post-metamórfica de alimentación con metanauplios de *Artemia* enriquecidos (de 8 a 50 dde). Estos resultados son similares a los encontrados por Villalta *et al.* (2005a) para esta misma especie y en estudios con otras especies de peces planos, tal como el halibut (McEvoy *et al.*, 1998b), el rodaballo (Estévez *et al.*, 1999), la platija amarilla (Copeman *et al.*, 2002) y el falso halibut de Canadá *Paralichthys dentatus* (Willey *et al.*, 2003). No obstante, cuando las larvas de lenguado fueron alimentadas con niveles elevados de ARA (10.2 y 7.1% AGT en el rotífero y la *Artemia*, respectivamente) durante sus estadios pre- y pro-metamórfico, la supervivencia de los animales a los 50 dde se vio significativamente incrementada en relación a las dietas con niveles de ARA más bajos (1.0 y 1.4% AGT en el rotífero y la *Artemia* enriquecidos, respectivamente). Estos resultados están de acuerdo con los obtenidos por Willey *et al.* (2003) en larvas de falso halibut de Canadá alimentadas con rotíferos con un 6% AGT de ARA durante la etapa de pre-metamorfosis (3-23 dde), los cuales presentaron tasas de supervivencia y de crecimiento más altas que aquellos animales alimentados con un 0% AGT de ARA. Aunque los efectos del ARA sobre la resistencia y tolerancia al estrés no fueron el objeto de estudio de la presente tesis, se observó que las larvas de lenguado senegalés fueron particularmente sensibles a altos niveles de ARA de la dieta durante sus estadios pre- y pro-metamórfico, lo que resalta los importantes requerimientos específicos en ARA durante estos estadios de desarrollo. El conjunto de los resultados anteriormente descritos muestran

el gran potencial de cultivo de las larvas de lenguado, con elevadas tasas de supervivencia y buen crecimiento, garantizándose así una de las primeras premisas del cultivo intensivo de cualquier especie, la producción de animales en cantidad.

Los diferentes estudios experimentales llevados a cabo en el marco de la presente tesis afectaron al crecimiento de las larvas de lenguado senegalés. En el Capítulo 1.1, el trabajo dedicado a investigar el efecto de diferentes productos comerciales con distintos perfiles de AG en el desarrollo de las larvas mostró la importancia del equilibrio entre todos los AG de la dieta para alcanzar un crecimiento y desarrollo óptimo del animal. En este sentido, el crecimiento fue mejor en larvas alimentadas con presa viva enriquecida con Aquagrow Gold© siendo, entre todos los productos de enriquecimiento testados, el producto comercial que presentó el perfil de AG más equilibrado para el lenguado senegalés. Los metanauplios de *Artemia* alimentados con este enriquecedor presentaron unos valores intermedios de AG monoinsaturados (AGMI), ARA, EPA, DHA, AG poliinsaturados (AGPI) (32.7, 0.7, 3.1, 9.5 y 51.1% AGT, respectivamente), y unas relaciones de DHA/EPA y n-3/n-6 AGPI de 3.0:1 y 5.2:1, respectivamente. Este equilibrio sutil entre todos los AG de la dieta Aquagrow Gold© podría explicar el mejor crecimiento de las larvas, ya que las otras dietas que se evaluaron mostraron proporciones diferentes en cuanto a uno o varios de los citados nutrientes y un crecimiento y desarrollo de la larva menor. El exceso de EPA en la *Artemia* enriquecida con Easy Selco© en comparación con la *Artemia* enriquecida con Aquagrow Gold© (2.5 veces mayor) resultó en un crecimiento más bajo de las larvas alimentadas con esta última dieta. Estudios previos han estimado que el rango de EPA apropiado para el crecimiento de peces marinos se encuentra entre el 3 y el 4% AGT (Léger *et al.*, 1986; Izquierdo *et al.*, 2000). Adicionalmente, Villalta *et al.* (2008) demostraron que el lenguado senegalés posee unos requerimientos nutricionales muy bajos en EPA cuando el DHA está presente en la dieta, por lo que los niveles elevados de la dieta Easy Selco© (7.6% AGT) pudieron afectar al crecimiento larvario y a la morfogénesis en nuestros trabajos.

En este estudio se consideró también la importancia de unos niveles equilibrados de DHA en la dieta. La deficiencia de DHA en la *Artemia* enriquecida con Easy Selco© diluido a la mitad con aceite de oliva condujo a un menor crecimiento y desarrollo comparado con las larvas alimentadas con *Artemia* enriquecida con Aquagrow Gold©. Estos resultados confirman la importancia del DHA en la nutrición de la larva, a pesar de que en esta especie se haya descrito que los requerimientos en DHA son muy bajos en comparación con otras especies marinas (Morais *et al.*, 2004; Villalta *et al.*, 2005b; Dâmaso-Rodrigues *et al.*, 2010). Por el contrario, un exceso de DHA, como el observado en la *Artemia* enriquecida con Multigain©, fue compensado por la larva con una pérdida proporcional en AGMI, que son los AG más fáciles de catabolizar con fines energéticos, lo que, consecuentemente, alteró la relación AGPI/AGMI. Tal y como ha sido demostrado anteriormente por Villalta *et al.* (2008), estos resultados mostraron que la alteración del equilibrio entre la energía (AG saturados y AGMI) y la esencialidad de los distintos ácidos grasos (AGAI) pudieron perjudicar el crecimiento y desarrollo de las larvas de lenguado senegalés. Finalmente, es importante mencionar que el equilibrio entre las proporciones relativas de DHA y EPA es esencial para un crecimiento y desarrollo adecuado del

animal. Así, se ha demostrado que esta especie posee unos requerimientos más altos en DHA que en EPA (Morais *et al.*, 2004), así como otras especies de peces marinos como el pargo japonés *Pagrus major*, la dorada, la lubina estriada *Morone saxatilis*, la lubina y el halibut (Watanabe *et al.*, 1989; Rodriguez *et al.*, 1998; Harel *et al.*, 2002). Una relación DHA/EPA inferior a 1 en la *Artemia* enriquecida con Easy Selco© e Easy Selco© diluido a la mitad con aceite de oliva estuvo por debajo de los niveles óptimos para el buen crecimiento del animal.

En el Capítulo 1.3, con el fin de profundizar en los mecanismos moleculares subyacentes al efecto de la dieta sobre el crecimiento del lenguado senegalés, se evaluó la expresión de los factores de crecimiento insulínico de tipo 1 y 2 (*igf1* y *igf2)* y el receptor de los factores de crecimiento insulínico (*igfr)*, genes reguladores de la diferenciación y del crecimiento celular (Peterson *et al.*, 2005; Chao y D'Amore, 2008; Reinecke, 2010). Dicha comparación se llevó a cabo únicamente entre aquellos grupos experimentales que mostraron el mejor y el peor crecimiento (grupos Aquagrow Gold© e Easy Selco©, respectivamente). Los resultados indicaron que los perfiles de expresión de dichos genes fueron estables desde la pro-metamorfosis (22 dde) hasta la post-metamorfosis (30 y 38 dde) y que no se vieron afectados por la dieta. Dado que ambas dietas fueron isolipídicas, se podría deducir que es más bien el contenido lipídico el que regula la expresión de dichos genes, que la composición en AG y clases lipíidicas, si bien estudios más detallados son necesarios para validar dicha hipótesis.

Cuando se evaluó el efecto de los niveles de ARA en la dieta sobre el crecimiento del lenguado senegalés (Capítulo 1.5), los resultados mostraron un mayor crecimiento de los peces alimentados con un 4.5% AGT de ARA en comparación con aquellos alimentados con un 1.0 ó un 7.0% AGT durante el periodo de alimentación con *Artemia* comprendido entre los 8 y 50 dde. No se observó ningún efecto en el crecimiento de tipo dosis-respuesta referente a los niveles del ARA, contrariamente a los resultados obtenidos en lubina y dorada (Bessonart *et al.*, 1999; Atalah *et al.*, 2011a, b).Una proporción del 1% AGT de ARA, que equivale a los contenidos en ARA incluidos comúnmente en los productos de enriquecimiento comerciales para las larvas de peces marinos (Sargent *et al.*, 1999), no resultó en el mejor crecimiento, lo que demuestra que los requerimientos nutricionales de ARA son específicos para el lenguado senegalés. Como se mencionó anteriormente, un exceso de ARA en la dieta (7.0% AGT) resultó en un crecimiento larvario menor que en el grupo alimentado con un 4.5% AGT de ARA. Sin embargo, otros estudios han demostrado que el crecimiento del lenguado senegalés no se ve afectado por niveles altos de ARA en la dieta (Villalta *et al.*, 2005a), así como tampoco en otras especies de peces planos, tales como el halibut (McEvoy *et al.*, 1998b), el rodaballo (Estévez *et al.*, 1999) y varias especies de platijas (Estévez *et al.*, 2001; Copeman *et al.*, 2002; Willey *et al.*, 2003). Cuando se examinaron los requerimientos en ARA del lenguado senegalés a lo largo de su desarrollo, las larvas alimentadas con bajos niveles de ARA (1.2% AGT en promedio) durante las etapas de pre- y pro-metamorfosis (2-15 dde) y elevados niveles de ARA (7.1% AGT) durante el periodo post-metamórfico (15-50 dde) presentaron un crecimiento mayor que aquellas larvas alimentadas con un 1% AGT de ARA durante todo el desarrollo larvario

(Capítulo 1.5). Estos resultados revelaron que los requerimientos nutricionales en ARA y, por extensión, de otros AGE pueden cambiar a lo largo del desarrollo larvario en función de las necesidades específicas del organismo. En particular, los requerimientos en ARA de las larvas de lenguado senegalés pueden aumentar de la pre- y pro-metamorfosis a la post-metamorfosis, y es recomendable que estos resultados sean considerados a la hora de optimizar los protocolos de alimentación de larvas en criaderos comerciales.

En relación al estudio destinado a evaluar el efecto de distintos niveles de peroxidación lipídica de la dieta en el crecimiento y desarrollo de la larva (Capítulo 1.4), los resultados indicaron que alimentar las larvas de lenguado senegalés de 2 a 9 dde con rotíferos enriquecidos con niveles crecientes de lípidos peroxidados (34.4 < TBARs < 87.2 nmol de malondialdehído, MDA.$g^{-1}$ de peso fresco, PF) no afectó a su supervivencia, ni a su crecimiento. Se han descrito resultados similares en juveniles de otras especies como la dorada (Mourente *et al.*, 2000, 2002), la perca americana (Chen *et al.*, 2011), la trucha arco-iris (Puangkaew *et al.*, 2005) y el halibut (Lewis-McCrea y Lall, 2007). En este contexto, las larvas de lenguado senegalés poseen los mecanismos de defensa (enzimas del estrés oxidativo) necesarios para contrarrestar los efectos negativos ocasionados por la presencia de altas cantidades de lípidos peroxidados en la dieta, lo que explicaría que dichos niveles de peroxidación no alteraran la supervivencia y el crecimiento de las larvas. No obstante, si se tiene en cuenta el metabolismo especifico y los posibles mecanismos de detoxificación de la presa viva enriquecida utilizada como vector para alimentar a las larvas, existe también la posibilidad de que los niveles de lípidos oxidados de la dieta no fueran lo suficiente elevados, ni suministrados a las larvas durante un tiempo lo suficientemente prolongado, como para detectar un impacto notable de éstos sobre el organismo.

El proceso de metamorfosis en peces planos está principalmente controlado por las hormonas tiroideas (Inui y Miwa, 1985). Unos niveles desequilibrados de ácidos grasos y hormonas tiroideas han sido descritos como capaces de afectar a la metamorfosis en peces planos, alterando el desarrollo normal de la pigmentación y la migración del ojo (ver revisión en Hamre *et al.*, 2005, 2007). En este sentido, Estévez y Kanazawa (1995) encontraron que una dieta deficiente en AGPI n-3 provocó el cese de la metamorfosis en el rodaballo. Los AGPi n-3 de cadena larga juegan un papel importante en los procesos relacionados con el desarrollo del sistema nervioso central (SNC) (Vaidyanathan *et al.*, 1994), por lo que una carencia en AGPI puede resultar en un desarrollo inadecuado del SNC y, por tanto, del eje hipotálamo – hipófisis – tiroideo, afectando así a su funcionalidad (Hamre *et al.*, 2005). En todos los estudios de la presente tesis doctoral, las larvas fueron capaces de completar satisfactoriamente el proceso de metamorfosis, de lo que se deduce que los distintos perfiles de AG evaluados (Capítulo 1.1) no afectaron al desarrollo del sistema tiroideo de los especímenes. Sin embargo, la anterior hipótesis no pudo ser validada al no haberse cuantificado los niveles de hormonas tiroideas en larvas de los distintos grupos experimentales. Además, cuando se alimentaron las larvas con *Artemia* enriquecida con diferentes productos comerciales, las larvas que presentaron el mayor crecimiento larvario (grupos Aquagrow Gold©,

Aquagrow DHA© y Multigain©), también presentaron una tendencia a metamorfosearse más rápido, resultados que están de acuerdo con los obtenidos en otras especies de peces planos, donde el crecimiento y el proceso de migración del ojo estaban correlacionados (Naess y Lie, 1998; Lund *et al.*, 2008; Geffen *et al.*, 2007). Asimismo, el proceso de metamorfosis no se vio afectado por los niveles altos de lípidos peroxidados de la dieta. Los niveles elevados de DHA en la dieta suministrada a las larvas de lenguado senegalés durante las etapas de pre- y pro-metamorfosis, periodos del desarrollo en los que se ha visto que los requerimientos nutricionales son más altos debido a la intensa formación de tejidos en el organismo (Power *et al.*, 2008), no afectaron al crecimiento, ni al proceso de migración del ojo. Estos resultados refuerzan la idea de que esta especie presenta unos requerimientos en DHA particularmente bajos durante el estadio pre-metamórfico, cuando se proporcionan suficientes cantidades de EPA en la dieta (Morais *et al.*, 2004; Villalta *et al.*, 2005b). El proceso de metamorfosis no se vio afectado significativamente cuando se alimentaron las larvas con niveles crecientes de ARA (1.0% < ARA < 7.0% AGT) en la *Artemia* enriquecida desde los 8 dde (datos no presentados), ni tampoco cuando se alimentaron con altos niveles de ARA (10.2% AGT) desde el inicio de la alimentación exógena (2 dde) y durante todo el periodo de alimentación con rotíferos (Capítulo 1.6). No obstante, Villalta *et al.* (2005a, b, 2008) encontraron un retraso en la migración del ojo de larvas de lenguado senegalés alimentadas con niveles de ARA del 0.1% al 8.3% AGT, de DHA del 0.0% al 14.7% AGT y de AG saturados del 38.0% al 22.1% AGT, mientras que dichos autores encontraron un adelanto en el proceso de migración del ojo cuando las larvas fueron alimentadas con niveles de EPA del 0.3% al 29.5% AGT y de AGMI del 21.0% al 50.7% AGT. Los mecanismos de acción del EPA, DHA y ARA sobre el crecimiento y la metamorfosis parecen estar interconectados y ser dependientes del perfil completo de AG y de las proporciones relativas entre los AG de la dieta, aunque las relaciones entre ellos todavía no se comprendan bien. Finalmente, se observaron alteraciones de los procesos de migración del ojo y remodelación de los huesos craneales durante la metamorfosis – detallados en una sección más abajo – en larvas pseudo-albinas alimentadas con altos niveles de ARA en la dieta durante su pre-, pro- y post-metamorfosis, aunque no fue considerado como una disfunción de su metamorfosis.

*Organización y funcionalidad del tracto digestivo*

En vertebrados, las diferentes regiones del tracto digestivo utilizan diferentes mecanismos celulares en respuesta a la cantidad y calidad de la dieta. Por ello se suelen utilizar el intestino y las glándulas digestivas accesorias como tejidos diana para el estudio y caracterización de la condición nutricional y fisiológica de los peces. El intestino está implicado en funciones fisiológicas importantes, siendo el primer lugar donde se realiza la digestión de los alimentos y la absorción de los nutrientes, mientras que el hígado representa el órgano metabólico central del cuerpo, con un papel predominante en el metabolismo intermediario y con importantes funciones en el almacenamiento de los lípidos y en procesos digestivos y de detoxificación. La utilización óptima de los nutrientes depende de la eficiencia de las funciones del intestino y del hígado y, por tanto, la alteración estructural de la organización histo-morfológica de estos órganos, así como la actividad de enzimas seleccionadas,

proporcionan información útil sobre la calidad de la dieta, el metabolismo y la condición nutricional de los peces (ver revisión en Gisbert *et al.*, 2008).

En la presente tesis doctoral se evaluó el desarrollo y la funcionalidad del tracto digestivo en larvas alimentadas con *Artemia* enriquecidas con diferentes productos comerciales con distintos perfiles de AG con el fin de profundizar en los efectos de la dieta sobre el desarrollo del organismo. Generalmente, la maduración correcta del sistema digestivo permite a las larvas digerir y asimilar la dieta ingerida, incorporando así las cantidades requeridas para su crecimiento óptimo y desarrollo normal. En este contexto, Zambonino-Infante *et al.* (2008) han demostrado que la actividad de las enzimas pancreáticas e intestinales puede servir como marcador fiable para evaluar el grado de desarrollo de la función digestiva en larvas de peces. Durante el desarrollo, las enzimas del borde en cepillo del intestino (*p. ej.*, fosfatasa alcalina y N-aminopeptidasa) presentan un aumento de su actividad específica, incremento que va acompañado con una disminución de la actividad de una peptidasa citosólica, la leucina-alanina-peptidasa (Ribeiro *et al.*, 1999). La relación entre las actividades de las enzimas del borde en cepillo y las citosólicas es informativa sobre el grado de maduración del sistema digestivo. En este sentido, los ratios de las actividades de fosfatasa alcalina y leucina-alanina-peptidasa y de la N-aminopeptidasa y leucina-alanina peptidasa en larvas de lenguado senegalés alimentadas con *Artemia* enriquecida con la dieta con el perfil de AG más equilibrado (Aquagrow Gold©) mostraron valores más elevados que los de las larvas alimentadas con el resto de tratamientos, indicando que las larvas del grupo Aquagrow Gold© contaban con un tracto intestinal más maduro que el del resto de grupos. Estos resultados coinciden con el crecimiento más rápido y la tendencia a un avance en la metamorfosis observados en este grupo de larvas. Las enzimas digestivas intestinales no son las únicas que pueden servir de marcador para evaluar el nivel de maduración del sistema digestivo. La síntesis y la secreción de las enzimas pancreáticas es particularmente sensible a la privación de alimentos y a la composición de la dieta en las larvas de teleósteos y, por consiguiente, la actividad enzimática pancreática constituye un marcador bioquímico fiable del desarrollo y de la condición de las larvas de peces marinos (Cahu *et al.*, 2004). El proceso de secreción pancreático madura durante las tres o cuatro semanas después de la eclosión en larvas de peces marinos templados. Este proceso de maduración puede ser alterado cuando las larvas son alimentadas con dietas que no cumplen los requerimientos específicos del animal. En este sentido, los cambios en el nivel de algunas enzimas pancreáticas, tales como la amilasa y la tripsina, que normalmente disminuyen a lo largo del desarrollo del sistema digestivo, junto con la formación del estómago (digestión ácida) y la maduración del intestino, son considerados también excelentes marcadores del desarrollo del tracto digestivo (Ribeiro *et al.*, 1999; Zambonino-Infante y Cahu, 2007). En esta tesis, las larvas alimentadas con la dieta Aquagrow Gold© presentaron valores de actividad de la amilasa más bajas que larvas alimentadas con otras dietas, lo que confirma un nivel más avanzado de maduración de su sistema digestivo, tal y como evidenciaron los resultados de las enzimas intestinales. En paralelo a estos cambios fisiológicos de la actividad específica de las enzimas digestivas seleccionadas, la mucosa intestinal, que representa el mayor lugar de absorción de nutrientes y de transporte a los demás tejidos, también presentó cambios estructurales importantes.

En particular, la actividad de la fosfatasa alcalina del borde en cepillo fue positivamente correlacionada con las tasas de proliferación de los enterocitos, lo que indica que ambos parámetros bioquímicos e histoquímicos pueden servir como marcadores útiles y fiables para la evaluación de la maduración del intestino. Larvas alimentadas con la dieta Aquagrow Gold© presentaron una altura y una tasa de proliferación de los enterocitos más bajas que en larvas alimentadas con las otras dietas, lo que coincide con los resultados de actividad enzimática intestinal e indica un grado de maduración del sistema digestivo más avanzado. Estos resultados también concordaron con el crecimiento más rápido y la tendencia observada a una metamorfosis más avanzada en este grupo de larvas. Los desequilibrios en los perfiles de AG (DHA/EPA y ARA/EPA) de la dieta, como por ejemplo: (i) los niveles excesivos de EPA (grupo EasySelco©); (ii) los niveles insuficientes de DHA (grupos Easy Selco© y Easy Selco© diluido a la mitad) o bien en exceso (grupo Multigain©); (iii) los niveles de ARA en exceso (Red Pepper©) o insuficientes (grupo Aquagrow DHA©), podrían explicar el retraso observado en la maduración del sistema digestivo de dichos grupos experimentales.

Tejidos como el intestino y el hígado están considerados como marcadores nutricionales y fisiológicos fiables, ya que su organización histológica es especialmente sensible a las condiciones de nutrición durante el desarrollo larvario y porque responden rápidamente a los desórdenes nutricionales y/o situación de ayuno (Catalán y Olivar, 2002; Caballero *et al.*, 2003, 2004). Particularmente, la acumulación de lípidos neutros en los citados tejidos refleja los desórdenes fisiológicos originados por desequilibrios en la composición de lípidos y ácidos grasos de la dieta (Segner *et al.*, 1994; Mobin *et al.*, 2000, 2001; Gisbert *et al.*, 2008; Cahu *et al.*, 2009).

Las larvas de lenguado senegalés alimentadas con *Artemia* enriquecida con los distintos enriquecedores evaluados presentaron diferentes perfiles de acumulación de grasa en el intestino medio, en el hígado y en el sistema vascular a lo largo de su desarrollo. Como las dietas fueron isolipídicas y presentaron las mismas cantidades de lípidos polares y neutros, estas diferencias en los perfiles de deposición de grasa en los tejidos estudiados fueron investigados considerando los distintos perfiles de AG de las diferentes dietas. Así, los peces alimentados con *Artemia* enriquecida con Aquagrow Gold© presentaron el mayor crecimiento, maduración del tracto digestivo y osificación de esqueleto (Capítulo 1.1) y, por esta razón, consideramos los perfiles de acumulación de grasa en los tejidos estudiados de este grupo experimental como referencia, en comparación con el resto de tratamientos. Se ha sugerido que un desequilibrio en la composición en AG del resto de dietas comparado con el tratamiento Aquagrow Gold© es el responsable de las alteraciones en la deposición de grasa en dichos grupos experimentales. Dichas modificaciones vendrían dadas por la modificación de la capacidad de la larva de absorber los lípidos en el intestino y exportarlos a través del sistema vascular hasta el hígado, para que sean almacenados y movilizados cuando sea necesario para el desarrollo del organismo (Tso, 1994). Los peces alimentados con las dietas Easy Selco© y Red Pepper© presentaron una acumulación más baja de grasa en el intestino e hígado a los 31 dde que los peces alimentados con la dieta Aquagrow Gold©, lo que probablemente refleja la maduración retrasada del tracto digestivo descrito en este grupo de larvas (Capítulo 1.1). Además, el

exceso de EPA y la deficiencia de DHA en la *Artemia* enriquecida con Easy Selco© en comparación a la *Artemia* enriquecida con Aquagrow Gold© resultó en unos ratios desequilibrados de DHA/EPA y ARA/EPA (77 y 70% más bajos), alterándose también la relación AO/AGPI (38% más alta). Estas alteraciones podrían ser las responsables de la esteatosis hepática severa observada en larvas alimentadas con la dieta Easy Selco©. La esteatosis hepática no afectó a la supervivencia larvaria, lo que indica que la función hepática no se vio afectada, mientras que la acumulación severa de lípidos neutrales en el sistema vascular de los ejemplares post-metamórficos fue probablemente debido a un problema de regulación del almacenamiento de grasas (Spisni *et al.*, 1998). Tomando en consideración que el contenido en ácido oleico (AO) de la dieta es generalmente utilizado con fines energéticos, mientras que las AGPI son utilizadas particularmente para funciones estructurales y fisiológicas (Sargent *et al.*, 1999; Tocher, 2003), un desequilibrio en la relación AO/AGPI pudo haber alterado el equilibrio entre la energía y esencialidad y conllevar una acumulación excesiva de grasa en el hígado y en el sistema vascular de los peces alimentados con la dieta Easy Selco©. Los peces alimentados con la dieta Easy Selco© diluida a la mitad con aceite de oliva (ES/2) y con Aquagrow DHA© presentaron, una vez completada su metamorfosis, una esteatosis intestinal y hepática severa. La primera dieta presentó unos ratios AO/AGPI y ARA/DHA 196% y 175% más altos, respectivamente, que los de la dieta Aquagrow Gold©, y unos ratios n-3/n-6 AGPI, DHA/EPA y ARA/EPA 29%, 87% y 61% más bajos, respectivamente, que los de la dieta Aquagrow Gold©. El remplazo parcial de aceite de pescado por aceite de oliva, rico en AO, en esta dieta pudo haber afectado el transporte de lípidos de la mucosa intestinal hacia el hígado, resultando en una esteatosis intestinal y una esteatosis hepática moderada (Spisni *et al.*, 1998; Caballero *et al.*, 2003, 2004). Más aun, el desequilibrio en la relación AO/AGPI junto con la diminución de la relación n-3/n-6 AGPI también pudo ser responsable de una menor movilización de las reservas lipídicas en el hígado de los peces post-metamórficos, originando así un menor crecimiento y desarrollo (Capítulo 1.1). Al comparar la esteatosis intestinal severa y moderada de los peces alimentados con las dietas Aquagrow DHA© e Easy Selco© diluido a la mitad con aceite de oliva, respectivamente, así como la acumulación severa de grasa en el sistema vascular de los peces alimentados con la dieta Easy Selco©, se consideró que dicho proceso fue posiblemente el resultado de una alta deficiencia de ARA (un 71% inferior a la dieta referencia) y de una baja relación de ARA/DHA y ARA/EPA (un 88% y un 83% inferior a la dieta Aquagrow Gold©, respectivamente), tal y como ha sido sugerido por Fountoulaki *et al.* (2003). Las ligeras alteraciones en el contenido de un sólo AGE parecen ser suficientes para alterar el perfil de la dieta, modificando la acumulación de lípidos en los tejidos de las larvas y perturbando su movilización para un crecimiento y desarrollo adecuados. Finalmente, los peces alimentados con la dieta Multigain© mostraron un patrón de acumulación de grasa en el intestino y en el hígado similar al de los peces alimentados con la dieta Aquagrow Gold©, posiblemente debido a la naturaleza de ambos enriquecedores (organismos unicelulares en polvo), exceptuando los peces post-metamórficos, que mostraron una movilización de lípidos hepáticos inferior, hecho atribuido a una relación AO/AGPI 51% más baja que la de la dieta Aquagrow Gold©.

Los cambios en la deposición de grasa en el intestino, hígado y sistema vascular también fueron correlacionados con cambios en la expresión de distintos marcadores moleculares seleccionados con el fin de profundizar en el conocimiento del efecto de la dieta sobre la fisiología digestiva y el metabolismo del organismo. No obstante, y tal y como se mencionó anteriormente, decidimos limitar este análisis a sólo dos grupos experimentales (grupos Aquagrow Gold© e Easy Selco©) (Capítulo 1.3). Aunque se ha visto que la expresión de la proteína de unión a los ácidos grasos en el hígado (*l-fabp*) está afectada por factores nutricionales, como el ayuno o un exceso de grasa en la dieta, que inciden sobre el metabolismo de los ácidos grasos en el hígado (Veerkamp, 1995), la expresión de *l-fabp* no se vio diferencialmente modulada en ninguno de los dos grupos experimentales analizados en el presente trabajo. Estos resultados sugieren que la expresión del *l-fabp* es más sensible al contenido en AG de cadenas largas de la dieta que a su composición (Capítulo 1.2). Por el contrario, la expresión de la proteína de unión a los ácidos grasos en el intestino (*i-fabp*) se vio afectada de forma diferencial entre los dos grupos. Los peces alimentados con la dieta Easy Selco© mostraron una expresión del gen *i-fabp* más estable, mientras que los peces alimentados con la dieta Aquagrow Gold© presentaron una menor expresión del gen *i-fabp* durante las etapas de pro y post-metamorfosis (periodo comprendido entre los 22 y 30 dde). La proteína codificada por este gen se encuentra involucrada en la absorción de ácidos grasos de la dieta (Levy *et al.*, 2001) y en el transporte de ácidos grasos a nivel intracelular (Baier *et al.*, 1996); por lo tanto, los cambios del patrón de la expresión genética a nivel ontogenético, asociados con el mayor grado de maduración intestinal observados en los peces del grupo Aquagrow Gold© (Capítulo 1.1), podrían ser una respuesta adaptativa de las larvas a la absorción de lípidos en la dieta. Los niveles de EPA, 2.5 veces mayores en el tratamiento Easy Selco© que en el tratamiento Aquagrow Gold©, y la expresión estable de *i-fapb* de los 28 a los 38 dde habrían inducido la acumulación excesiva de grasa en el intestino, lo que a su vez daría lugar a un episodio de esteatosis hepática y una acumulación severa de gotas lipídicas en el sistema vascular de los peces alimentados con la dieta Easy Selco© (Capítulo 1.2).

En esta tesis doctoral, además del efecto de los niveles de los diferentes AG sobre el desarrollo y la calidad larvaria, también se evaluaron los efectos potenciales de los lípidos oxidados de la dieta sobre los parámetros previamente indicados. Este estudio se realizó en base al alto contenido de AGAI en los enriquecedores y en la presa viva y su posible oxidación durante el proceso de enriquecimiento. Generalmente, cuando hay altos niveles de ingesta de lípidos peroxidados, el intestino se comporta como una barrera física selectiva, especialmente sensible a las condiciones sub-óptimas de alimentación o al estrés nutricional. Así, el intestino es capaz de reducir y/o eliminar la toxicidad de los lípidos peroxidados ingeridos mediante su retención en los enterocitos hasta que los mecanismos de defensa antioxidante sean activados, proceso que se da antes de que la grasa absorbida sea transferida al sistema vascular y a otros órganos (Trendazo *et al.*, 2006). El número y tamaño de las vacuolas lipídicas en los enterocitos y los hepatocitos dependen del contenido de grasa en la dieta y del grado de instauración de los lípidos ingeridos, lo que indica la capacidad del intestino e hígado de equilibrar los excesos de absorción de lípidos (Sheridan, 1988). Asimismo, la acumulación de lípidos en el intestino de las larvas pre-metamórficas de lenguado senegalés (edad: 9

dde) alimentadas con dietas que contenían niveles crecientes de peroxidación lipídica, aumentó proporcionalmente al incrementar la cantidad de TBARS de la dieta, mientras que la acumulación de lípidos en la larvas post-metamórficas (edad: 19 dde) estuvo más asociada al contenido de lípidos neutros (colesterol y triacilglicerol) de la dieta que a sus niveles de oxidación.

El hígado es considerado como el órgano central encargado de la eliminación de los productos tóxicos y la acumulación de depósitos grasos ha sido previamente asociada al incremento del gradiente de lípidos oxidados en la dieta (Puangkaew *et al.*, 2005; Chen *et al.*, 2011; Dong *et al.*, 2012). Tal y como se ha descrito en el Capítulo 1.4, el grado de oxidación de la dieta no afectó a los perfiles de acumulación de grasa en el hígado. Las larvas de peces planos acumulan una gran reserva de nutrientes en el hígado que utilizarán durante la metamorfosis, periodo durante el cual los animales dejan de alimentarse (Brewster, 1987). En este contexto, el proceso de metamorfosis está asociado a un elevado coste energético para el animal, el cual no da comienzo si el organismo no dispone de las reservas energéticas suficientes para completar dicho proceso con éxito (Geffen, 2007). Durante el período pre-metamórfico, los lípidos absorbidos estuvieron prioritariamente destinados al crecimiento y diferenciación celular y a la formación de nuevos tejidos, por lo que no se observaron diferencias en cuanto a las reservas hepáticas de grasa. Una vez completada la metamorfosis, los ejemplares post-metamórficos reducen su demanda energética y, por tanto, no todos los lípidos de la dieta fueron directamente transformados en energía, sino almacenados, en parte, en el hígado. Este cambio se vio reflejado en la fisiología y metabolismo del organismo a través de un incremento en la deposición de grasa hepática (Gisbert *et al.*, 2008; Hoehne-Reitan y Kjørsvik, 2004; Capítulo 1.2).

En el Capítulo 1.4, destinado a evaluar los efectos de distintos niveles de peroxidación lipídica sobre el desarrollo de las larvas, se observó un incremento en los niveles de peroxidación en los tejidos del lenguado senegalés a lo largo de su desarrollo (de los 9 a los 35 dde), independientemente del nivel de peroxidación lipídica administrado por la dieta. Estos resultados son similares a los descritos por Solé *et al.* (2004). Un cambio en las vías metabólicas durante este periodo de alta demanda energética, junto con una mayor demanda de oxígeno y una sobreproducción de especies reactivas de oxígeno (ERO), pudo haber sido responsable de este incremento en los niveles de peroxidación tisular (Solé *et al.*, 2004). Las larvas de lenguado senegalés de 9 dde mantuvieron niveles de oxidación similares a pesar del incremento de los niveles de oxidación de la dieta (rotíferos enriquecidos). En juveniles de dorada, las dietas con aceites oxidados no tuvieron efectos importantes sobre los niveles de peroxidación en el hígado de dichos animales (Mourente *et al.*, 2002). Diversos trabajos han puesto de manifiesto que la actividad hepática e intestinal de la mayoría de las enzimas antioxidantes aumenta en casos tales como el incremento del estrés oxidativo de la dieta, la inanición o el increment los niveles de AGPI n-3 (Mourente *et al.*, 2002; Morales *et al.*, 2004; Puangkaew *et al.*, 2005; Fontagné *et al.*, 2006; Chen *et al.*, 2011; Zambrano y Landines, 2011). No obstante, se ha observado una débil respuesta de las actividades antioxidantes al incrementar la oxidación en la dieta en juveniles de dorada, lo que

sugiere que se necesitan condiciones más severas de oxidación para afectar de manera significativa dichas actividades (Mourente *et al.*, 2000). En el caso del lenguado senegalés, la participación del sistema antioxidante y de los mecanismos de detoxificación de las larvas, en respuesta al incremento del estrés oxidativo de la dieta, explicaría los niveles constantes de peroxidación observados. Las defensas antioxidantes incluyen diversas enzimas que se encargan de catabolizar los productos tóxicos derivados del oxígeno y eliminar los radicales libres susceptibles de dañar los lípidos y los AGPI contenidos en las membranas celulares. La actividad específica de la mayoría de estas enzimas antioxidantes en el lenguado senegalés disminuyó a lo largo del desarrollo larvario, tal y como se ha observado en otras especies de peces, como el dentón (Mourente *et al.*, 1999) y el rodaballo (Peters y Livingstone, 1996). En nuestro caso, dichos cambios fueron atribuidos a una adaptación al estrés oxidativo de la dieta a través del tiempo y la edad. En el lenguado senegalés, la activación de las defensas antioxidantes contra el estrés oxidativo de la dieta fue demostrado al incrementarse la actividad de la glutatión reductasa (GR), enzima que participa en el reciclaje de las moléculas de glutatión oxidadas utilizadas para eliminar la toxicidad del $H_2O_2$ y los peróxidos orgánicos (Halliwell y Gutteridge, 2006). Por el contrario, la actividad de las enzimas superóxido dismutasa (SOD), involucrada en la eliminación de $O_2^-$, de la catalasa (CAT) y de la glutatión transferasa (GST), implicadas en la detoxificación de $H_2O_2$, no se vieron afectadas por el gradiente de oxidación en la dieta en ninguno de los estadios de desarrollo. Estos resultados indican que las larvas de lenguado senegalés fueron capaces de tolerar dichos niveles de peroxidación de las dietas. No obstante, no se debe excluir la posibilidad de que los niveles de peroxidación en las dietas evaluadas en dicho experimento no fueran lo suficientemente elevados como para detectar cambios importantes en cuanto a la actividad de todas las enzimas del estrés oxidativo evaluadas.

*Esqueletogénesis*

La nutrición larvaria ha sido reconocida por diversos estudios como un factor clave en el proceso de esqueletogénesis en larvas de peces (Hamre *et al.*, 2012; Rønnestad *et al.*, 2012; Boglione *et al.*, 2013b). Dentro de los nutrientes, los lípidos y los ácidos grasos han sido identificados como responsables de la aparición de anomalías esqueléticas cuando su nivel y/o forma de suministro en la dieta es inapropiado o desequilibrado en relación a los requerimientos nutricionales de la especie o estadio de desarrollo considerado (Cahu *et al.*, 2003; Lall y Lewis-McCrea 2007). Los AGPI y los fosfolípidos juegan un papel importante en el metabolismo óseo, como se ha demostrado en mamíferos (ver revisión en Poulsen *et al.*, 2007). Asimismo, diversos estudios han determinado una relación entre las deficiencias y/o los desequilibrios en AGE de la dieta y la incidencia de deformidades esqueléticas (Boglione *et al.*, 2013b). No obstante, el papel exacto que juegan los lípidos y los AGPI de la dieta sobre el desarrollo de anomalías esqueléticas está todavía lejos de ser esclarecido en peces (Lall y Lewis-McCrea 2007). Los lípidos de la dieta son capaces de influenciar la composición de AG en los huesos de los peces, lo que a su vez, altera la producción de compuestos alta y biológicamente activos, como los eicosanoides (prostaglandinas y leucotrienos), los cuales tienen diversos efectos fisiológicos sobre el metabolismo de las células óseas (Bell *et al.*, 2003). El

ratio n-6/n-3 AGPI de la dieta, y, en especial, la relación ARA/EPA, juega un papel importante en la producción de prostaglandinas de las series 2 y 3 ($PGE_2$ y $PGE_3$), cuyas proporciones relativas se encargan de regular el metabolismo de los osteoblastos y del hueso en general y, consiguientemente, el proceso de esqueletogénesis y la correcta mineralización del hueso (Berge *et al.*, 2009). Diversos estudios han establecido una relación entre las clases de lípidos y la incidencia de anomalías del esqueleto (ver revisión en Boglione *et al.*, 2013b). Los fosfolípidos (FL) de la dieta son conocidos por tener un papel muy importante durante los procesos de morfogénesis del organismo (Cahu *et al.*, 2009). Un incremento de los niveles de FL en la dieta está asociado con un mejor crecimiento y desarrollo del organismo, así como también con una menor incidencia de deformidades esqueléticas, tal y como ha sido descrito en el ayu (Kanazawa *et al.*, 1981), la carpa común (Geurden *et al.*, 1998), el bacalao (Finn *et al.*, 2002), la lubina (Villeneuve *et al.*, 2005b) y el halibut (Næss *et al.*, 1995; Hamre *et al.*, 2002, 2005, 2006). Los FL tienen efectos positivos sobre el desarrollo larvario de los peces, debido a que las larvas de peces marinos tienen una limitada capacidad para sintetizar los FL *de novo* con lo que éstos han de ser aportados mayoritariamente por la dieta (Coutteau *et al.*, 1997). También puede deberse a su mejor digestibilidad y mayor absorción y utilización en comparación con los lípidos neutros (LN) (Fontagné *et al.,* 1998; Cahu *et al.*, 2009). Además, es importante considerar que los huesos de los peces son muy vulnerables a procesos de peroxidación lipídica ya que contienen entre un 24 y un 90% de lípidos. La peroxidación de lípidos es un proceso de auto-catálisis iniciado por los radicales libres que son producidos en el cuerpo como resultado del metabolismo aeróbico, durante el cual los AGAI de las membranas celulares se degradan vía una reacción en cadena (Lall y Lewis-McCrea 2007). Así, unos niveles altos de AGAI en los tejidos de los peces sin ningún tipo de protección antioxidante están muy expuestos a una auto-oxidación, pudiendo inducir alteraciones en su estructura. A su vez, se ha sugerido que los lípidos oxidados inhiben la diferenciación de los osteoblastos e inducen la diferenciación de los osteoclastos, lo que contribuye a alterar el equilibrio de la remodelación del hueso, causando pérdidas de densidad mineral ósea (Watkins *et al.*, 2001) y deformidades esqueléticas en los peces (ver revisión en Boglione *et al.*, 2013b).

Se ha descrito una correlación entre el retraso del crecimiento y la osificación en larvas de dorada (Fernández *et al.*, 2008), bacalao (Kjørsvik *et al.*, 2009) y lubina (Darias *et al.*, 2010) alimentadas con diferentes dietas, dando con frecuencia como resultado una mayor incidencia de deformaciones esqueléticas. Las larvas de lenguado senegalés alimentadas con *Artemia* enriquecida con Easy Selco© mostraron un crecimiento, maduración intestinal y valores de osificación menores que las larvas alimentadas con *Artemia* enriquecida con Aquagrow Gold©, las cuales presentaron valores superiores de todos estos parámetros (Capítulo 1.1). Al disminuir el contenido de n-3 AGPI de la dieta Easy Selco©, en comparación con la dieta Aquagrow Gold©, se alteró la relación n-3/n-6 AGPI, ya que los valores de 5.2:1 condujeron a un mejor crecimiento y desarrollo que la relación 3.9:1. Más aun, la deficiencia de ARA y de DHA y el exceso de EPA en la *Artemia* enriquecida con Easy Selco©, en comparación con las larvas enriquecidas con Aquagrow Gold©, también alteraron el balance de los AGE, lo que influyó en el desarrollo del lenguado senegalés.

El gen de la osteocalcina (*oc*, también llamada *bgp* o *bglap*) es un marcador de la mineralización de los osteoblastos (Pinto *et al.*, 2001), dado que esta proteína es necesaria para la correcta maduración de los cristales de hidroxiapatita durante el proceso de calcificación y formación de la matriz extracelular del hueso (Boskey *et al.*, 1998). El gen *oc* también tiene funciones de señalización (proteínas morfo-genéticas del hueso, factores de crecimiento, citoquinas y proteínas adhesivas) y juega un papel clave durante el proceso de mineralización (Sommerfeldt y Rubin, 2001; Pinto *et al.*, 2001). Como la expresión de este gen responde a diferentes condiciones nutricionales, ha sido propuesto como un marcador fiable para detectar desórdenes durante la formación del hueso y el proceso de mineralización (Mazurais *et al.*, 2008; Darias *et al.*, 2010, 2011; Fernández *et al.*, 2011). La expresión del gen *oc* fue examinada en las larvas de lenguado senegalés alimentadas con Easy Selco© y Aquagrow Gold©, ambas dietas con distintos perfiles de AG y contenidos de VA (Capítulo 1.3). Estudios previos han mostrado que la expresión de este gen está relacionada con la dieta y su sobre- o sub-expresión, correlacionada con el contenido de vitaminas y de otros nutrientes en la dieta, está asociadao a un aumento de deformaciones esqueléticas (Mazurais *et al.*, 2008; Darias *et al.*, 2010, 2011; Fernández *et al.*, 2011). Las larvas alimentadas con Aquagrow Gold© presentaron mayores niveles de expresión de *oc* y un esqueleto más mineralizado durante la fase post-metamórfica en comparación con las larvas alimentadas con la dieta Easy Selco©. En cambio, no hubo diferencias significativas en cuanto a la incidencia de deformaciones esqueléticas entre los peces de ambas dietas (Capítulo 1.1). La implicación de los diferentes niveles de VA de la dieta en ambos grupos experimentales y su impacto sobre los niveles de osificación del esqueleto de las larvas fue descartado, ya que la expresión de los genes de los receptores al ácido retinoico (*rar* y *rxr)* y del gen del receptor activado por el proliferador peroxisomal *β* (*pparβ)* no se vio afectada por dichas dietas. Estos resultados parecen indicar que la asimilación de la VA por el organismo fue regulada a nivel del intestino, lo que previno la acumulación de altas cantidades de este nutriente en el organismo, pudiendo así evitar el desarrollo de anormalidades esqueléticas derivadas de un exceso de vitamina A en la dieta (Hathcock *et al.*, 1990; Melhus *et al.*, 1998; Ross *et al.*, 2000). Así, la expresión estable del gen *oc* en los peces alimentados con *Artemia* enriquecida con Easy Selco©, en contraste con su activación en larvas alimentadas con Aquagrow Gold©, vendría dada por unos elevados niveles de EPA y una deficiencia de ARA en la primera dieta en comparación con la segunda, resultando en unos ratios bajos de DHA/EPA, ARA/EPA y ARA/DHA (Capítulo 1.1), siendo éstos esenciales para la correcta formación del esqueleto (Lall y Lewis-McCrea, 2007).

Las dietas con elevados niveles de ARA pueden ser responsables de una sobreproducción de $PGE_2$ en el hueso, lo que puede alterar la formación del hueso y sus niveles de mineralización (Berge *et al.*, 2009). De hecho, la $PGE_2$ es un potente agente regulador de la formación y remodelación del tejido óseo (Kawaguchi *et al.*, 1995; Marks y Miller, 1993). Resultados recientes obtenidos a partir de modelos *in vitro* revelaron que la $PGE_2$ disminuyó la proliferación de células madre en el tendón y su diferenciación osteogénica (Zhang y Wang, 2012). Tanto la formación como la reabsorción del hueso están influenciados por los niveles de $PGE_2$ (Berge *et al.*, 2009). Los niveles de osificación en

ejemplares de lenguado senegalés alimentados con niveles elevados de ARA durante el periodo de alimentación con *Artemia* (7% AGT) fueron menores que los observados en animales alimentados con 4.5% AGT de ARA (Capítulo 1.5). Los peces alimentados con altos niveles de ARA en la dieta desde el inicio de la alimentación exógena hasta los 50 dde (8.7% AGT en promedio) presentaron una reducida osificación de los elementos esqueléticos en la región craneal y algunas alteraciones en el proceso de remodelación ósea ocurrido durante la metamorfosis (Capítulo 1.6). Al incrementar los niveles de ARA en la dieta, se alteró la relación n-6/n-3 AGPI, en particular la relación ARA/EPA, llevando a concentraciones desequilibradas de $PGE_2$ y a una mineralización ósea reducida. Un equilibrio óptimo de estas dos series de AGPI, n-3 y n-6, es crucial para la correcta esqueletogénesis del organismo (Izquierdo, 1996; Sargent *et al.*, 1999; Izquierdo y Koven, 2011). Se han asociado ratios n-6/n-3 AGPI bajos con efectos benéficos sobre la condición de los huesos (Maggio *et al.*, 2009), mientras que ratios n-6/n-3 AGPI elevados afectan negativamente la formación del hueso en ratas (Li y Watkins, 1998; Watkins *et al.*, 1999, 2000). Dado que las larvas de peces marinos requieren mayores cantidades de n-3 AGPI que de n-6 AGPI (Sargent *et al.*, 1999), la relación ARA/EPA en las dietas juega un papel importante en la formación del hueso, ya que ARA es el principal precursor de la biosíntesis de los eicosanoides en peces (Bell y Sargent, 2003) y también compite directamente con el EPA por las enzimas involucradas en la síntesis de prostaglandinas. Si bien la $PGE_2$ se forma a partir del ARA y la $PGE_3$ del EPA (Bell *et al.*, 1995), ambas isoformas de eicosanoides son potentes reguladoras del metabolismo óseo, pero con efectos opuestos sobre el hueso (Norrdin *et al.*, 1990; Marks y Miller, 1993), y las cantidades relativas de cada serie influyen en la regulación de los osteoblastos y del metabolismo del hueso (Berge *et al.*, 2009). Altas cantidades de ARA en la dieta o un exceso de EPA en la misma resultan en desequilibrios en la absorción de ARA en relación al EPA y, por consiguiente, en las proporciones relativas de $PGE_2$ y $PGE_3$ (Sargent *et al.*, 1997; Bell y Sargent, 2003; Hamre *et al.*, 2005), que pueden derivar en desórdenes esqueléticos en peces. En salmón del atlántico alimentado con altas relaciones de n-6/n-3 AGPI (3.8 a 6.0) se observó un menor grado de mineralización de los cuerpos vertebrales asociado a un incremento de los niveles de $PGE_2$ en sangre (Berge *et al.*, 2009). Villalta *et al.* (2005b) observaron una correlación entre el incremento de los niveles de ARA en la dieta, y de la relación ARA/EPA, y la producción más elevada de prostaglandinas (PGE y PGF) en las larvas de lenguado senegalés, lo que fue relacionado con una incidencia mayor de peces malpigmentados, si bien dichos autores no encontraron diferencias en los niveles de prostaglandinas entre peces correctamente pigmentados y pseudo-albinos. Aunque los niveles de prostaglandinas no fueron medidos en el Capítulo 1.5, los elevados niveles de ARA de la dieta podrían haber resultado en un aumento de la producción de $PGE_2$ y, posteriormente, en una reducción de la osificación del tejido óseo en formación (Watkins *et al.*, 1999; Berge *et al.*, 2009; Capítulo 1.6). En cuanto al efecto de los elevados niveles de ARA de la dieta sobre el proceso de migración del ojo en peces pseudo-albinos (Capítulo 1.6), los elevados niveles de $PGE_2$ medidos en dichos ejemplares podría haber incrementado la actividad de la ciclooxigenasa-2 (COX2) en los osteoblastos, resultando en una disminución de su proliferación y en un incremento de su tasa de diferenciación, lo que habría afectado al nivel de mineralización de diversas estructuras craneales (*p. ej.*, huesos esfenótico, etmoides lateral izquierdo y frontal

izquierdo). Asimismo, dicho proceso pudo también resultar en la alteración de determinados elementos esqueléticos durante el periodo de remodelación craneal que ocurre durante la metamorfosis de la larva, como ya se ha descrito en ratones (Greenblatt *et al.*, 2010).

Las larvas pre-metamórficas de lenguado senegalés (9 dde) alimentadas con metanauplios de *Artemia* enriquecidos con una dieta con elevados niveles de peróxidos y sin VE mostraron una ligera tendencia a presentar un menor grado de mineralización de su esqueleto, mientras que éste estuvo significativamente retrasado en ejemplares pro-metamórficos (19 dde). Estos resultados están de acuerdo con los descritos por Lewis-McCrea y Lall (2007) en ejemplares de halibut alimentados con una dieta sin VE, en donde una baja actividad de formación del hueso estuvo asociada con una mineralización y densidad ósea reducidas (Basu *et al.*, 2001). Estudios *in vitro* en roedores han demostrado que los radicales libres están involucrados en la osteoclastogénesis y en el aumento de la reabsorción del tejido óseo a través de la activación del gen NF-κB (Garett *et al.*, 1990; Suda *et al.*, 1993; Iotsova *et al.*, 1997). Los osteoblastos y los osteoclastos parecen ser sensibles al estrés oxidativo producido por la dieta y, por tanto, alimentar los peces con dietas altamente oxidadas, con o sin niveles bajos de compuestos antioxidantes, puede afectar su desarrollo y reducir la tasa de formación del hueso.

Generalmente, se ha demostrado en peces que un esqueleto menos mineralizado es más susceptible a desarrollar deformidades esqueléticas, ya que un hueso menos mineralizado es más frágil y susceptible a un desarrollo anormal durante su formación (Darias *et al.*, 2011; Boglione *et al.*, 2013a, b). No obstante, en la presente tesis doctoral no se estableció ninguna correlación entre el grado de osificación y la incidencia de deformidades esqueléticas en aquellas larvas de lenguado senegalés que fueron alimentadas con *Artemia* enriquecida con seis diferentes productos comerciales (Capítulo 1.1) o con un gradiente creciente de ARA (Capítulo 1.5). Tales diferencias entre los resultados presentes y los reportados en la literatura podrían ser debidas a la elevada incidencia de deformaciones encontradas en los distintos estudios, hecho que hubiera podido enmascarar la anteriormente citada relación entre el nivel de mineralización del hueso y la incidencia de anomalías esqueléticas. La elevada frecuencia de animales deformes no es un caso aislado de esta tesis doctoral, diversos trabajos han puesto de manifiesto que las larvas de lenguado senegalés son muy sensibles a desarrollar deformidades esqueléticas, ya sea en estudios experimentales (Fernández *et al.*, 2009; Engrola *et al.*, 2009; Fernández y Gisbert, 2011), en criaderos comerciales, donde generalmente varían de 44 a 80% de los peces producidos (Gavaia *et al.*, 2002, 2009; Engrola *et al.*, 2009), o incluso en el medio natural (Gavaia *et al.*, 2009).

La frecuencia de peces deformados en poblaciones naturales es más baja que en cautividad, debido a que las deformidades esqueléticas que afectan la capacidad locomotora de los peces (*p. ej.,* para capturar e ingerir las presas y escapar de posibles depredadores) reducen, por consecuencia, su probabilidad de supervivencia (Boglione *et al.*, 2003; Gavaia *et al.*, 2009). La frecuencia más baja de animales deformados en las poblaciones salvajes sugiere claramente la existencia de una selección

natural hacia un esqueleto normal y que las larvas con deformidades son eliminadas gradualmente de la población natural (Marino *et al.*1993; Kawamura y Hosoya, 1997; Boglione *et al.* 2003; Gavaia *et al.*, 2009). En cautividad, los peces son cultivados mediante condiciones medio-ambientales óptimas y alimentadas diariamente con dietas frescas, por lo cual, no se enfrentan a estos problemas de buscar su alimento porque la comida siempre está disponible. Adicionalmente, la presión de depredación en el medio ambiente natural no está presente en la acuicultura, y es una de las principales razones por cual los peces deformados tienen mayor probabilidad de sobrevivir en comparación con los peces salvajes. También se ha encontrado una alta incidencia de deformidades esqueléticas en otras especies cultivadas en criaderos de peces planos. Kawamura y Hosoya (1997) observaron que sólo un 4% de los peces presentaron deformaciones en poblaciones naturales de platija japonesa, contra un 74 % en los cultivados. Se ha registrado un elevado número de peces deformes en lubina a nivel industrial, de los cuales un 75% de los ejemplares producidos presentaron deformidades vertebrales y un 45% en las aletas. En el caso de la dorada, casi todos los peces producidos a escala industrial presentan anomalías esqueléticas de diversa magnitud, aunque estas proporciones se reducen hasta un 55% cuando los animales se cultivan en condiciones semi-intensivas (Boglione *et al.*, 2001; Prentiscola *et al*., 2013). No obstante, la incidencia de deformidades esqueléticas en el lenguado senegalés cultivado mediante protocolos de alimentación estándares es más alta que la observada en las especies anteriormente citadas (Boglione *et al.*, 2001; Villeneuve *et al.*, 2005a; Fernández *et al.*, 2008; Mazurais *et al.*, 2009). Dos posibles hipótesis podrían explicar dicha elevada incidencia de deformidades esqueléticas en el lenguado senegalés; la primera consideraría que esta especie es más susceptible de desarrollar desórdenes esqueléticos en comparación con otras especies que se cultivan en la actualidad; y la segunda postularía que las anomalías del esqueleto del lenguado senegalés no son letales y, consecuentemente, la incidencia de deformidades parece ser más alta en juveniles de lenguado senegalés que en otras especies para las que dichas deformidades son letales en los estadios iniciales de desarrollo y que, por tanto, no alcanzan la talla comercial (Divanach *et al.*, 1997; Koumoundouros *et al.*, 1997; Boglione *et al.*, 2001). Ninguna de estas dos hipótesis puede excluir la otra; por consiguiente, y con el fin de determinar cuál de estos dos modelos explicaría mejor la situación actual, es necesario realizar estudios comparativos complementarios para identificar los periodos más sensibles de la morfogénesis, y, particularmente, de la esqueletogénesis, relacionados con el desarrollo de deformidades. En los Capítulos 1.1 y 1.4, todos los grupos de peces alimentados con *Artemia* enriquecida con diferentes productos comerciales o con niveles crecientes de peroxidación lipídica en la dieta presentaron una alta frecuencia de individuos con deformidades esqueléticas (76.2 y 69.1 % en promedio, respectivamente), a pesar de tener un aspecto externo generalmente normal, lo que coincide con los resultados de Fernández *et al.* (2009), que mostraron un 86% de larvas que presentaban, por lo menos, una deformación esquelética en el grupo control alimentadas con EasySelco©.

Los diferentes ensayos realizados en esta tesis doctoral mostraron que las diferentes dietas utilizadas afectaron de manera diferente la frecuencia de deformidades esqueléticas totales y la incidencia de los diferentes tipos de anormalidades dependiendo de cómo las larvas de lenguado

senegalés fueron alimentadas: (i) con *Artemia* enriquecida con seis diferentes productos en larvas de 8 a 37 dde; (ii) con rotíferos enriquecidos con diferentes niveles de lípidos peroxidados en la dieta en larvas de 2 a 9 dde (34.5, 46.4, 87.2 y 46.8 nmol MDA.g$^{-1}$ P.F., la última sin adicionarle VE); (iii) con metanauplios de *Artemia* enriquecidos con niveles crecientes de ARA en la dieta (1.0, 4.5 y 7.0 % AGT) en larvas de 8 a 50 dde; o (iv) con rotífero y *Artemia* enriquecidos con altas cantidades de ARA en la dieta (10.2 y 7.1 AGT, respectivamente) en larvas de 2 a 50 dde. No se observaron efectos significativos de la dieta sobre la incidencia de anormalidades totales y sobre las diferentes tipologías de deformidades vertebrales en el lenguado senegalés alimentados con *Artemia* enriquecida con distintos productos comerciales (Capítulo 1.1). Las únicas diferencias, aunque menores, fueron observadas en la incidencia de fusiones entre cuerpos vertebrales adyacentes, que fue más elevada en larvas alimentadas con la dieta Aquagrow DHA© que en las alimentadas con la dieta Multigain©, así como también en aquellas que hacen referencia al complejo de la aleta caudal, donde las larvas alimentadas con Aquagrow Gold© e Easy Selco© diluido a la mitad con aceite de oliva presentaron mayor incidencia de deformidades en las espinas neural y haemal modificadas que las larvas alimentadas con Easy Selco©. No obstante, si consideramos que las emulsiones no solamente difirieron en su contenido de ácidos grasos sino también en sus perfiles de proteínas (aminoácidos), vitaminas y minerales, parece posible que los efectos observados fueran debidos principalmente a la compleja interacción entre sus diferentes nutrientes más que a un nutriente en particular o a un grupo de ellos (Boglione *et al.*, 2013b).

La incidencia de deformidades esqueléticas totales fue diferente entre los lenguados alimentados con niveles crecientes de peroxidación lipídica en la dieta durante el periodo de alimentación con rotíferos, pero no fue proporcionalmente correlacionada con estos niveles, lo que sugirió que los niveles de peroxidación lipídica evaluados en este estudio no fueron suficientemente elevados para causar un mayor daño al esqueleto en comparación con las larvas alimentadas con la dieta control no oxidada (Capítulo 1.4). En este experimento, los peces alimentados con niveles intermedios y elevados de peroxidación lipídica durante el periodo de alimentación con rotíferos (46.4 y 87.2 nmol MDA.g$^{-1}$ P.F.) fueron los más afectados por deformidades en los cuerpos vertebrales, al igual que ha sido observado en el halibut (Lewis-McCrea y Lall, 2007). Las tipologías de deformidades vertebrales que afectaron más a estos peces fueron la compresión, la fusión de las dos últimas vertebras antes del urostilo, la fusión de los arcos neurales, la deformación de las vértebras, la deformación de las espinas neurales y haemales y la osificación anormal de alguna de las vértebras. Lewis-McCrea y Lall (2007) encontraron que la escoliosis en las regiones pre-haemal y anterior-haemal de la columna vertebral fueron las deformidades esqueléticas más frecuentes en el halibut alimentado con lípidos oxidados. Además, los peces alimentados con presas vivas enriquecidas con la dieta altamente oxidada y sin VE presentaron el porcentaje más bajo de deformidades en la región vertebral (junto con la dieta control no oxidada) y en el complejo de la aleta caudal. En particular, los peces alimentados con altos niveles de peroxidación lipídica, con o sin VE, tuvieron la tendencia a presentar frecuencias más bajas de deformaciones en los elementos esqueléticos que osifican a partir del día 18 de edad (hipurales, epurales, parahipurales y radios

caudales) y un alto porcentaje de malformaciones en las estructuras esqueléticas que se forman antes (arcos haemales) (Gavaia *et al.*, 2002), comparados con los peces alimentados con menores cantidades de peroxidación lipídica. Teniendo en cuenta esto, la VE pudo interactuar con la osificación del cartílago (osificación endocondral) y un exceso de VE en la dieta en larvas de 2 a 9 dde pudo afectar a las estructuras cartilaginosas formadas antes de los 18 dde. Estos resultados se apoyan en que las larvas de 9 dde alimentadas con altos niveles de peroxidación lipídica con VE utilizaron una parte de la VE de la dieta para la eliminar los productos tóxicos y oxidados, y, como consecuencia, las larvas presentaron cantidades similares de VE a las larvas alimentadas con altos niveles de peroxidación lipídica sin VE (1.5 y 0.7 $mg.kg^{-1}$ P.S., respectivamente). Tampoco se puede descartar la posibilidad de que exista una interacción entre varios nutrientes de la dieta. Por ejemplo, la importancia del papel de la vitamina C (VC) en la formación del cartílago y su función en el mantenimiento de los niveles de VE en el cuerpo (Hamre *et al.*, 1997), sugiere una posible interacción entre ambas vitaminas. Un equilibrio en las cantidades en la dieta de ambas vitaminas permite impedir la deficiencia en VE. El hecho de añadir VE a la dieta de las larvas alimentadas con altos niveles de peroxidación lipídica pudo inducir modificaciones de los niveles normales de VC requeridos para el correcto desarrollo de las estructuras del complejo de la aleta caudal que se forman por osificación endocondral después del periodo de alimentación con las dietas oxidadas.

El promedio de deformidades esqueléticas en larvas de lenguado senegalés alimentadas con un gradiente creciente de ARA en la dieta durante el periodo de alimentación con *Artemia*, fue bajo (41.4%) en comparación con otros estudios (Gavaia *et al.*, 2002, 2009; Fernández *et al.*, 2009; Capítulo 1.5). Ni la incidencia de anormalidades esqueléticas totales ni las tipologías de malformaciones se vieron afectadas en el lenguado senegalés por los niveles crecientes de ARA en la dieta, resultados que están en consonancia con los presentados por Berge *et al.* (2009), quienes no encontraron ninguna diferencia en la incidencia de deformidades esqueléticas en juveniles de salmón del atlántico alimentados con una relación creciente de n-6/n-3 AGPI. En nuestro caso, y en relación a los datos presentados en el Capítulo 1.5, para la determinación de la incidencia de deformidades esqueléticas en el complejo de la aleta caudal, la fusión de las hipurales 3 y 4 no se consideró como una malformación esquelética, al contrario de la mayoría de los estudios previos con larvas de peces planos, sino como el desarrollo normal del complejo de la aleta caudal que se observa en ejemplares juveniles y adultos de este grupo de especies (Barrington, 1937). La presente asunción podría explicar la menor incidencia de deformidades esqueléticas encontradas en el complejo de la aleta caudal del lenguado senegalés en este estudio (4.2 % en promedio; Capítulo 1.5). Adicionalmente, no se observó ninguna deformación severa (escoliosis, cifosis, lordosis), ni siquiera en las alimentadas con el nivel más alto de ARA en la dieta (7.0% AGT), lo que indica que dicha cantidad de ARA en la dieta suministrada a la larva durante el periodo de alimentación con *Artemia* no afectó a su esqueletogénesis, aunque sí retrasó su crecimiento.

En larvas post-metamórficas de lenguado senegalés alimentadas con altos niveles de ARA en la dieta (8.7% AGT en promedio) desde el inicio de la alimentación exógena hasta completar su

metamorfosis, la alta incidencia de animales pseudo-albinos (81.3 ± 7.5 %) estuvo significativamente correlacionada con la frecuencia de deformidades craneales (95.1 ± 1.5%), tal y como se describe en el Capítulo 1.6. La incidencia de deformidades esqueléticas totales fue elevada (84.2 ± 2.4% en promedio) y similar a la del grupo control (presa viva enriquecida con un producto comercial, Algamac 3050$^{TM}$). Adicionalmente, la frecuencia de las diferentes tipologías de anomalías esqueléticas que afectan a las regiones vertebrales y caudal fue similar entre los peces de ambos grupos, con la excepción de las espinas neurales y haemales, las cuales presentaron una mayor frecuencia de deformaciones entre los peces alimentados con elevados niveles de ARA. Los elevados niveles de ARA en la dieta alteraron también el proceso de migración del ojo, proceso que ocurre durante la metamorfosis de la larva. En este sentido, los animales pseudo-albinos presentaron diferencias en la forma de la cabeza y en la posición de ambos ojos en comparación a los peces de pigmentación normal. En particular, la altura de la cabeza y los ángulos vertebral-ocular y buco-ocular en los peces pseudo-albinos alimentados con altos niveles de ARA fueron significativamente más bajos y su distancia interocular un 25% más corta que en peces de pigmentación normal (Capítulo 1.6). Los análisis geomorfométricos revelaron diferencias significativas en la forma de la región craneal entre los albinos y los peces de pigmentación normal. La alteración del proceso de migración del ojo y los desórdenes pigmentarios son dos de los problemas más importantes que afectan la producción intensiva de peces planos (Gavaia *et al.*, 2009; Fernández y Gisbert, 2011). La alta incidencia de juveniles anormales limita el beneficio de su producción y representa un serio cuello de botella a nivel industrial (Power *et al.*, 2008). Los estudios que describen las anomalías en el proceso de migración del ojo en peces planos generalmente se basan en la descripción de los problemas de migración del ojo izquierdo o derecho de los peces, dependiendo de la simetría de las especies, desde el lado ciego hasta el lado ocular del animal durante su metamorfosis, así como también en las alteraciones de los huesos del cráneo que se generan a causa de dicha migración imperfecta del ojo (Okada *et al.*, 2001; Okada *et al.*, 2003; Sasaki y Yamashita, 2003; Sæle *et al.*, 2003, 2006; Schreiber, 2006; Cloutier *et al.*, 2011). No obstante, este es el primer estudio en el que se describen los problemas de migración del ojo durante la metamorfosis que afectan al ojo inicialmente posicionado en el lado ocular del animal, el ojo derecho en el caso del lenguado senegalés, mientras que es el ojo izquierdo el que migra normalmente hacia el otro lado del cuerpo del animal. El proceso de migración del ojo se encuentra asociado a la remodelación craneal (Brewster, 1987; Wagemans *et al.*, 1998; Okada *et al.*, 2001), ya que la mayoría de los huesos craneales comienzan su osificación una vez completada la migración del ojo. Un estudio reciente realizado por Bao *et al.* (2011) determinó que el proceso de migración ocular viene dado por la proliferación y migración de fibroblastos en el tejido suborbital del lado ciego del cuerpo, mientras que el establecimiento de la asimetría craneal en este grupo de peces, durante la metamorfosis, se caracteriza por la reubicación de la parte anterior del hueso frontal, del lado ciego al lado ocular, y por el alargamiento del hueso etmoides lateral del lado ciego del animal.

El origen de los problemas de migración del ojo en el lenguado senegalés parece deberse a los elevados niveles de ARA en la dieta, asociados con los niveles bajos de EPA, que alteran la

relación ARA/EPA, generando cambios en la proporciones relativas de la $PGE_2$ y $PGE_3$ (Bell y Sargent, 2003). Esta hipótesis fue confirmada al cuantificar altos niveles de $PGE_2$ en peces pseudo-albinos en comparación con aquellos de pigmentación normal. Los resultados del presente trabajo muestran que el incremento en la relación ARA/EPA en la dieta se correlacionó con un incremento de $PGE_2$, lo que conllevó en desordenes pigmentarios y problemas de migración ocular en larvas de lenguado senegalés. Diversos trabajos han demostrado que elevados niveles de $PGE_2$ en mamíferos afectan a la función de los fibroblastos alterando su diferenciación y motilidad (White, 2006; Sandulache *et al.*, 2007). Así, considerando la hipótesis de Bao *et al.* (2011), que indica que la migración de los fibroblastos es la responsable de la correcta migración del ojo en larvas de peces planos, los problemas de migración del ojo en el lenguado senegalés parecen deberse al incremento de la producción de $PGE_2$ o a cualquier otro metabolito derivado del ARA.

Otra diferencia relevante entre ambos grupos de peces (de pigmentación normal y pseudo-albinos) fue la presencia de dentición en ambas mandíbulas en los ejemplares de pseudo-albinos, mientras que los peces de pigmentación normal tienden a perder la dentición en la mandíbula superior. La desaparición de los dientes de la mandíbula superior en especímenes de lenguado común de pigmentación normal ocurre justo antes de que el ojo comience su migración, ya que dicha estructura no participa directamente en la captura de presas (Wagemans y Vandewalle, 2001). Adicionalmente, los problemas de migración del ojo en peces pseudo-albinos estuvieron acompañados de deformaciones en algunos elementos del esplacnocráneo y del neurocráneo. En el esplacnocráneo, el premaxilar derecho mostró una mayor curvatura y delgadez en los animales pseudo-albinos, mientras que los huesos dental y angular fueron más cortos, lo que afectó al ceratohial y a la disposición de los radios branquiostegales. Además, el lacrimal derecho también se deformó, presentado un menor grado de desarrollo que en los peces de pigmentación normal. En el neurocráneo, el margen anterior del hueso esfenótico se redujo en los peces pseudo-albinos y el etmoides lateral izquierdo no se fusionó completamente con el hueso frontal izquierdo para formar la pared de la órbita ocular del ojo.

Un alto contenido de ARA en la dieta desde la pre- hasta la post-metamorfosis afectó al proceso de pigmentación, de migración del ojo, así como a la remodelación del esplacnocráneo y del neurocráneo en el lenguado senegalés (Capítulo 1.6), mientras que no se observó ningún efecto sobre la pigmentación de la piel y la migración del ojo cuando se administraron dietas con altos contenidos de ARA desde la pro-metamorfosis (Capítulo 1.5). Los resultados de ambos estudios fueron diferentes, ya que en el estudio reportado en el Capítulo 1.5 se encontró que niveles altos de ARA suministrados a las larvas de lenguado senegalés no indujeron deformidades esqueléticas en la región craneal. Adicionalmente, la incidencia de deformidades esqueléticas que afectaron a la columna vertebral, presentadas en el Capítulo 1.6, fueron entre un 25% y 32% mayores que las descritas en el Capítulo 1.5. Estas diferencias entre los resultados de ambos estudios pueden deberse a las diferencias en las concentraciones de ARA y en los protocolos de alimentación. Los niveles de ARA de ambos estudios difirieron ligeramente; sus rangos fueron entre el 1.0% y 7.0%

AGT en los metanauplios de *Artemia* enriquecida en el Capítulo 1.5, mientras que los niveles de ARA del trabajo descrito en el Capítulo 1.6 fueron del 10.2% y 7.1 % AGT en los rotíferos y metanauplios de *Artemia* enriquecidos, respectivamente. Sin embargo, la relación ARA/EPA estuvo dentro el mismo rango de valores en ambos estudios (0.4 - 1.7 en el Capítulo 1.5 y 0.3 - 1.8 en el Capítulo 1.6). Las larvas de lenguado senegalés del Capítulo 1.5 fueron alimentadas con altos niveles de ARA solamente durante el periodo de alimentación con *Artemia* (8-50 dde), mientras que en el Capítulo 1.6 se mantuvieron elevados los niveles de ARA durante todo el periodo de cultivo larvario (2-50 dde). Esto indica el posible efecto del suministro de elevados niveles de ARA en la dieta durante la etapa pre-metamórfica sobre el desarrollo correcto de la columna vertebral y sobre el proceso de remodelación del cráneo. El estadio pre-metamórfico apareció como un periodo particularmente sensible a los desequilibrios de la dieta, ya que la regulación de la mayoría de los procesos morfogénicos deben ocurrir durante esta etapa. Como previamente se comentó, los $PGE_2$ son potentes reguladores de la remodelación y del metabolismo de los huesos (Kawaguchi *et al.*, 1995), por lo que niveles elevados de ARA en la dieta suministrados durante el estadio pre-metamórfico resultaron en la sobreproducción de $PGE_2$, que pudo haber alterado la tasa de formación del hueso (Berge *et al.*, 2009).

*Pigmentación*

Los problemas de pigmentación son comunes en peces planos de cultivo. Existen tres tipos principales de anomalías pigmentarias: (i) hipomelanosis (pseudo-albinismo), caracterizado por una ausencia parcial o total de pigmentación en el lado ocular del animal; (ii) hipermelanosis, que consiste en una pigmentación anormal en el lado ciego y (iii) ambicoloración, caso en el que se observa pigmentación en ambos lados del cuerpo del animal. En cualquiera de estos casos, los peces con desórdenes pigmentarios tienen un menor valor de mercado (Venizelos y Benetti, 1999; Bolker y Hill, 2000). El desarrollo de los problemas de pigmentación de los peces planos en condiciones intensivas de cultivo tiene lugar durante la metamorfosis, cuando la pigmentación larvaria es reemplazada por la pigmentación adulta. Para entender los mecanismos responsables del establecimiento de los patrones de pigmentación en adultos del lenguado senegalés y la aparición de dichos problemas pigmentarios, se llevó a cabo el análisis de la distribución espacial y temporal de los distintos cromatóforos de la piel, así como de los cambios en la expresión de distintos marcadores moleculares relacionados con el proceso de pigmentación.

Las células pigmentarias en vertebrados derivan de una población celular pluripotente, la cresta neural. Estas células nacen del borde entre el ectodermo neural y la epidermis durante el desarrollo inicial del embrión, migrando a lo largo de éste y dando origen a diversos tipos celulares, incluyendo los cromatóforos y el sistema nervioso periférico y entérico. En peces existen distintos tipos de cromatóforos: melanóforos, xantóforos, iridóforos, leucóforos y cianóforos, los cuales contribuyen a la formación de los patrones de pigmentación durante el desarrollo del organismo. En este sentido, los cambios en la expresión de diversos genes que regulan el proceso de pigmentación

durante la pro-metamorfosis son visibles en los estadios posteriores de desarrollo, durante el estadio post-metamórfico, cuando los peces presentan el típico patrón de pigmentación del juvenil-adulto. Los cambios en los tipos de células pigmentarias y en su distribución suceden después de la metamorfosis. La pigmentación de las larvas de lenguado senegalés se caracterizó por la presencia de abundantes xantóforos y melanóforos de tipo larvario, grandes y dendríticos, distribuidos a lo largo de la cabeza y de las regiones abdominales y del tronco, y por la asociación de xantóforos con melanóforos, mientras que se observaron dos poblaciones de xantóforos y melanóforos de diferente tamaño en peces post-metamórficos. Los melanóforos y xantóforos dendríticos típicos de los estadios larvarios, desaparecieron y fueron reemplazados progresivamente por xantóforos de tipo adulto y con forma redonda y por melanóforos recién diferenciados y melanizados, que se organizaron formando parches en el lado ocular de los peces, como se ha observado en platijas (Seikai *et al.*, 1987; Matsumoto y Seikai, 1992). En el Capítulo 2.1, la proporción de melanóforos respecto a la de xantóforos fue propuesta como un indicador visual fiable para evaluar la correcta ontogenia de la pigmentación de la piel en el lenguado senegalés, ya que los xantóforos están también involucrados en guiar los patrones de distribución de los melanóforos a lo largo de la piel (Parichy y Turner, 2003). Los iridóforos, aparecieron por primera vez al final de la post-metamorfosis, siendo requerida la presencia de los melanóforos para su correcta distribución en parches en el lado ocular del lenguado senegalés, así como en el pez cebra (Johnson *et al.*, 1995) y en la mayoría de las platijas (Matsumoto y Seikai, 1992; Nakamura *et al.*, 2010). Los leucóforos, presentes en la piel del lenguado senegalés desde la pre-metamorfosis, se redistribuyeron en parches a lo largo del tronco y de las aletas durante la metamorfosis y no desaparecieron después de ésta, al contrario de lo observado con la platija japonesa (Yamada *et al.*, 2010).

Las características morfológicas de la ontogenia de la pigmentación de la piel ocular del lenguado senegalés se vieron reflejadas en los perfiles de expresión molecular de 11 genes involucrados en la diferenciación de los melanóforos y en la síntesis de la melanina. Los datos presentados en el Capítulo 2.1 revelaron que la transición de la pigmentación de larvas a juveniles coincidió con el proceso de la metamorfosis: (i) el periodo pre-metamórfico (2-11 dde) estuvo caracterizado por una baja expresión de los factores apoptóticos y de los genes relacionados con la melanogénesis y una expresión elevada de los genes de la diferenciación de melanóforos; (ii) durante el periodo pro-metamórfico (11-19 dde) se observó una alta expresión de los factores apoptóticos involucrados en la remodelación de los tejidos, así como también de los genes implicados en la diferenciación de melanóforos y síntesis de melanina y, finalmente, (iii) durante el periodo post-metamórfico (19-47 dde) se observó una reducción en la expresión de todos los genes analizados, y en especial de aquellos asociados con la diferenciación de los melanóforos.

En el Capítulo 2.1 se estableció que, durante la pre-metamorfosis, los incrementos en la expresión de los genes de la proteína “paired box 3” (*pax-3*), del factor de transcripción asociado con la microftalmia (*mitf*) y el gen *cKit* reflejaron los procesos de diferenciación y proliferación de los melanóforos, así como también la especiación de los xantóforos. La expresión de los genes

melanogénicos, como el de la tirosinasa (*tyr*) y el de la última enzima de la melanogénesis (*trp1*), estuvo correlacionada durante todo el desarrollo larvario, mientras que los cambios en las relaciones *tyr/mitf* y *tyr/trp1* a lo largo del desarrollo reflejaron una producción cíclica de melanina. El pico de expresión de *trp1* en larvas de dos días de edad reflejó la producción de melanina en los melanóforos larvarios. Durante la pro-metamorfosis, la elevada expresión de los genes caspasa 3 (*casp3*) y de la somatolactina (*sl*) reflejaron la apoptosis de los cromatóforos larvarios y la estimulación de la diferenciación de los melanóforos, respectivamente. El segundo pico de expresión de *trp1* estuvo asociado con la síntesis de melanina en los melanóforos recién diferenciados. Durante el clímax del desarrollo de la pigmentación y al final del periodo pro-metamórfico, la expresión de los genes melanogénicos, como *tyr* y *trp1,* y de los genes *pax-3, mitf* y *cKit* disminuyó, indicando la estabilización del proceso de melanogénesis y de la diferenciación de los melanóforos. La expresión estable del gen receptor de la hormona estimuladora de melanocitos de tipo 1 (*mc1r*) durante la post-metamorfosis indicó el final de la ontogenia y de la especificación de los patrones de los melanóforos a nivel transcripcional, lo que coincidió morfológicamente con el inicio del fenotipo de pigmentación adulta. Una vez completada la metamorfosis, la prevalencia de la melanogénesis sobre la diferenciación de los melanóforos fue indicada por el aumento de la relación *cKit/pax-3*. El incremento similar de la relación *mitf/pax-3* en la post-metamorfosis indicó cambios en las proporciones relativas de melanóforos y xantóforos. La expresión del gen intercambiador de sodio/potasio/calcio (*slc24a5)*, necesario para la producción de melanina, fue constante durante el desarrollo larvario del lenguado senegalés, siendo un gen necesario para la correcta expresión de *trp1*. La expresión del gen de la proteína de señalización agouti (*asip)* fue responsable de la inhibición de la producción de melanina y de la diferenciación y proliferación de melanoblastos en el lado ciego del animal. Asimismo, la relación *asip*/*mc1r* fue un buen indicador del proceso de ontogénesis de los iridóforos y un marcador para los cambios en los patrones de pigmentación durante el desarrollo del lenguado senegalés. El incremento considerable de la relación *asip*/*mc1r* en ejemplares post-metamórficos coincidió con un aumento en la cantidad de iridóforos del lado ocular de los peces y con el cambio de la simetría bilateral a la asimetría plana, mostrando que el establecimiento de la nueva asimetría estuvo sincronizada con los patrones de pigmentación en la piel. Además, es importante considerar que tanto los melanóforos como los iridóforos proceden de un precursor común y que la diferenciación de los cromatoblastos bipotentes en melanóforos o iridóforos parece depender de la expresión o de la represión de *mitf*. En el lenguado senegalés, la disminución de la expresión de *mitf* durante la metamorfosis podría indicar la diferenciación de los cromatoblastos en iridóforos en el lado ocular y el bloqueo del desarrollo de los melanóforos en el lado ciego. El conjunto de estos resultados expuestos en el Capítulo 2.1 muestra la acción combinada de los genes implicados en la diferenciación de los melanóforos y en la melanogénesis coordinada en diferentes acontecimientos moleculares que resultaron en el establecimiento del fenotipo de pigmentación del adulto.

Aunque la ontogenia de la pigmentación de la piel del lado ocular de las larvas del lenguado senegalés está programada genéticamente, diversos factores epigenéticos pueden modular y afectar la expresión de genes que regulan la pigmentación, a pesar de que el resultado morfológico de estas

alteraciones sólo se detecta después de la metamorfosis. Diversos factores ambientales han sido descritos como capaces de inducir problemas en la pigmentación, destacando entre ellos la nutrición del animal como uno de los factores más importantes para el correcto desarrollo de la pigmentación en peces planos (Kanazawa, 1993; Estévez *et al.*, 1995; Matsumoto y Seikai, 1992; Villalta *et al.*, 2005a; Hamre *et al.*, 2007, 2008). Se han asociado niveles excesivos de ARA suministrados al lenguado senegalés durante su desarrollo larvario con una incidencia de pseudo-albinismo del 90% o más (Villalta *et al.*, 2005a; Capítulo 2.2). Con el fin de esclarecer si la inducción de dichos desórdenes pigmentarios durante la ontogenia del lenguado senegalés estuvo relacionada con algún periodo larvario especifico, se evaluó si las larvas de lenguado alimentadas con dosis elevadas de ARA (10.2 y 7.1 % AGT) durante las etapas pre-, pro- y post-metamórficas eran más sensibles a dichos niveles de ARA que aquellos animales alimentados con una dieta control basada en presas vivas enriquecidas con un producto comercial (Algamac 3050$^{TM}$) y bajos niveles de ARA (1.2% AGT en promedio; Capítulo 2.2). Los resultados de dicho ensayo mostraron que las larvas alimentadas con elevados niveles de ARA durante la pre-, pro- y post-metamorfosis presentaron un 81.4% de animales con desórdenes pigmentarios frente a un 0.9% observado en los peces alimentados con la dieta control. De forma similar, Villalta *et al.* (2005a) encontraron un 84.2% de lenguados senegaleses de pigmentación anormal cuando éstos se alimentaron con un 8.3% AGT de ARA desde los 3 a los 37 dde y sólo un 0.3% cuando los peces fueron alimentados con un 0.1% AGT de ARA. Copeman *et al.* (2002) mostraron un 92% de animales malpigmentados en larvas de platija amarilla alimentadas con un 8.9% AGT de ARA y un 36% AGT de DHA durante 4 semanas, indicando los efectos negativos de altos niveles de ARA en la dieta sobre la pigmentación.

En relación a las proporciones absolutas de ARA en la dieta, se han relacionado previamente desequilibrios en la relación ARA/EPA con problemas de pigmentación en varias especies de peces planos (Reitan *et al.*, 1994; Curé *et al.*, 1995; Bell *et al.*, 2003; Villalta *et al.*, 2005a; Hamre *et al.*, 2005). En el lenguado senegalés, la incidencia de desórdenes pigmentarios está posiblemente correlacionada con los niveles de ARA en la dieta y con la relación de ARA/EPA, ARA/DHA, EPA/DHA y AGMI/AGPI (Capítulo 2.2). Asimismo, niveles elevados de ARA en la dieta han sido asociados a desequilibrios en la pigmentación de diversos peces planos, pudiéndose ver afectados los procesos subyacentes a la diferenciación de células pigmentarias (Capítulo 2.3). Se han encontrado también correlaciones positivas entre distintos niveles de ARA y la relación ARA/EPA y la incidencia de pseudo-albinismo en el rodaballo, el halibut y la platija japonesa, donde una dieta que contiene una relación de ARA/EPA superior a 1:4 administrada hasta la metamorfosis afectó a la correcta pigmentación de los peces (McEvoy *et al.*, 1998a; Estévez *et al.*, 1999, 2001). Las larvas de peces marinos requieren mayores cantidades de AGPI n-3 que de AGPI n-6 (Sargent *et al.*, 2002), por lo que un desequilibrio en la relación ARA/EPA en la presa viva (ARA/EPA = 1.6 en rotífero y 1.8 en *Artemia*) podrían ser responsables de desórdenes en la pigmentación. Además, el ARA y el EPA muestran interacciones competitivas como sustratos para las enzimas ciclo-oxigenasa y lipo-oxigenasa, involucradas en la biosíntesis de las prostaglandinas (Bell y Sargent, 2003). Un desequilibrio en la relación de ARA/EPA en la dieta ha sido descrito como responsable de afectar a

las concentraciones de $PGE_2$ y $PGE_3$ en diversos tejidos (Reitan *et al.*, 1994; Curé *et al.*, 1995; Logue *et al.*, 2000; Bell y Sargent, 2003; Bell *et al.*, 2003; Hamre *et al.*, 2005; Villalta *et al.*, 2005a; presente tesis). Asimismo, a pesar de que estos mecanismos aún no se encuentran bien caracterizados, se ha sugerido que un exceso en la producción de $PGE_2$ podría ser responsable de un estrés bioquímico que podría generar malpigmentaciones (Sargent *et al.*, 1999; Brandsen *et al.*, 2005; Villalta *et al.*, 2005a, 2007; Lund *et al.*, 2008). En el estudio presentado en el Capítulo 2.2, los peces alimentados con niveles elevados de ARA durante la fase post-metamórfica presentaron elevados niveles de $PGE_2$, los cuales estuvieron asociados con desordenes pigmentarios. Simultáneamente, los peces pseudo-albinos alimentados con elevados niveles de ARA durante la post-metamorfosis presentaron mayores concentraciones de $PGE_2$ que los peces alimentados con la misma dieta, sugiriendo la presencia de mecanismos que regulan y controlan los niveles excesivos de ARA en la dieta. Estos mecanismos, cuando fueron activados, pudieron influenciar la producción de metabolitos derivados del ARA y, a su vez, afectar al proceso de diferenciación de los distintos cromatóforos de la piel. No se conocen aún los mecanismos precisos que relacionan los niveles de ARA, la producción de eicosanoides y el desarrollo de malpigmentaciones en peces planos (Planas y Cunha, 1999; Bolker y Hill, 2000; Villalta *et al.*, 2005a; Brandsen *et al.*, 2005). En el caso del lenguado senegalés, se ha avanzado en el conocimiento de los mecanismos moleculares subyacentes a la ontogenia de la pigmentación, pero la implicación del ARA y sus derivados en este proceso está por investigar (Capítulo 2.2).

Se obtuvo un 81.4% de peces pseudo-albinos cuando se alimentaron con niveles elevados de ARA durante toda la etapa larvaria, un 52.2% cuando se alimentaron con dichos niveles de ARA durante los estadios pre- y pro-metamórficos y solamente un 20.0% cuando se alimentaron con altas cantidades de ARA una vez completada su metamorfosis. Estos resultados, similares a los descritos por Lund *et al.* (2007, 2008) en el lenguado común, demostraron que no solamente los niveles excesivos de ARA en la dieta pueden ser responsables de la incidencia de desórdenes pigmentarios, sino también el periodo de desarrollo durante el cual se ejerció el desequilibrio nutricional. Estos resultados indican que los requerimientos nutricionales de las larvas no son uniformes durante su ontogenia y cambian dependiendo del estadio de desarrollo del organismo. En el caso del lenguado senegalés, la sensibilidad de la larva a un exceso de ARA en la dieta fue mayor durante los estadios pre- y pro-metamórficos, periodos durante los que se determinan los patrones de pigmentación adulta que se observan una vez completada la metamorfosis del animal. En el lenguado común, las larvas alimentadas con un 10.5% AGT de ARA durante los estadios pre- y pro-metamórficos (3-21 dde) estuvieron malpigmentadas (90.4%), mientras que sólo el 16.7% y el 37.3% de ellas fueron pseudo-albinas cuando se suministraron los niveles altos de ARA en la dieta de los 15 a 21 dde o de los 10 a 21 dde, respectivamente (Lund *et al.*, 2008). En este contexto, Næss y Lie (1998) definieron lo que se conoce como “ventana de pigmentación”, periodo en el que el animal es altamente sensible a factores ambientales que afectan a su pigmentación normal. La pigmentación normal de la platija japonesa y del halibut se vio afectada negativamente por niveles excesivos de ARA y del ratio ARA/EPA de la

dieta cuando se suministraron durante el estadio pre-metamórfico, pero no durante el estadio post-metamórfico.

El desarrollo de la pigmentación de la piel durante la pre-metamorfosis es morfológicamente similar en peces que pueden presentar más adelante una pigmentación normal o pseudo-albina, pero, una vez metamorfoseados, los futuros pseudo-albinos empiezan a mostrar diferencias en cuanto a las proporciones relativas, los patrones de repartición, las formas y los tamaños de los cromatóforos. El análisis de textura de los patrones de pigmentación de la piel de los peces, método desarrollado en la presente tesis doctoral, mostró que la piel de los animales pseudo-albinos alimentados con altos niveles de ARA durante la pre- y pro-metamorfosis y/o la post-metamorfosis presentó un patrón de melanóforos más uniforme y homogéneo que los peces de pigmentación normal. En el Capítulo 2.3, se ha mostrado que la piel de las larvas pre-metamórficas del lenguado senegalés que se volvieron pseudo-albinas tras su metamorfosis, presentó todos los tipos de cromatóforos, pero estas células progresivamente desaparecieron después de la metamorfosis dando lugar al fenotipo adulto típico de ejemplares pseudo-albinos. Los peces de pigmentación normal mostraron mayor número de melanóforos agregados en parches a lo largo de todo el cuerpo del animal, con la capacidad de cambiar su forma de redonda a dendrítica, hecho que les permite mimetizarse con el color del fondo. No obstante, las cantidades de melanóforos e iridóforos, las cuales fueron incrementadas durante el estadio post-metamórfico en los peces de pigmentación normal, no cambiaron en los ejemplares pseudo-albinos, indicando que no se diferenciaron nuevas poblaciones de células pigmentarias. Los animales pseudo-albinos también perdieron la mayoría de sus leucóforos, mientras que las pocas células restantes fueron localizadas en la parte distal de las aletas. El fenotipo pseudo-albino inducido por el ARA fue definido como el resultado de la inhibición de la diferenciación de las poblaciones post-metamórficas de cromatóforos y de la progresiva degradación de los cromatóforos larvarios aún existentes (Capítulo 2.3). Estos resultados mostraron que la pigmentación larvaria está determinada genéticamente, pero que los factores ambientales pueden modular los cambios moleculares normales que ocurren durante el proceso de metamorfosis y modificar el patrón de pigmentación adulto. Además, los peces pseudo-albinos alimentados con niveles elevados de ARA en la dieta (10.2 y 7.1% AGT en los rotíferos y metanauplios de *Artemia* enriquecidos, respectivamente) durante todo el desarrollo larvario presentaron una densidad de melanóforos de su lado ocular más baja y un aspecto más uniforme en comparación con los peces de pigmentación normal alimentados con altas cantidades de ARA en la dieta solamente durante la pre- y pro-metamorfosis o durante la post-metamorfosis. Esta observación sugiere que el pseudo-albinismo inducido por el ARA es un fenómeno dependiente del estadio de desarrollo. Al alimentar las larvas de lenguado senegalés con altas cantidades de ARA antes, durante y después de la metamorfosis, se observó una mayor inhibición de la diferenciación de los cromatóforos que cuando el ARA fue suministrado durante solamente uno de estos periodos.

Como se ha explicado previamente, los mayores cambios en el patrón molecular de las células pigmentarias ocurren durante la pro-metamorfosis, mientras que los cambios morfológicos en

la población de los diferentes cromatóforos se evidenciaron en la etapa post-metamórfica. Esto significa que, aunque el fenotipo pseudo-albino no se puede detectar en las larvas pre-metamórficas, los altos niveles de ARA en la dieta suministrada durante los periodos pre- y pro-metamórficos afectaron, probablemente, a los mecanismos moleculares involucrados en el proceso de pigmentación, lo que lleva a la aparición de desórdenes pigmentarios en los peces post-metamórficos. Al alimentar al lenguado senegalés con niveles altos de ARA en la dieta, se observó una sobreexpresión de los genes *asip, pax-3, cKit* y *mitf* durante la metamorfosis, la cual alteró la diferenciación de los cromatoblastos en el lado ocular de los peces y, al mismo tiempo, inhibió la diferenciación terminal de los melanóforos, xantóforos e iridóforos adultos. Además, la regulación positiva de los genes *asip* y *mitf* en los peces pseudo-albinos parece jugar un papel clave en el impedimento de la diferenciación de las células pigmentarias precursoras en melanóforos y xantóforos. La regulación negativa del gen *slc24a5* podría ser inducida por el gen *asip*, alterando la melanogénesis al final del proceso a través de la reducción de la expresión del gen *trp1* y, por tanto, de la síntesis de melanina. A su vez, se ha demostrado que el nivel de expresión del gen *pax-3* es crítico para el desarrollo de nuevas poblaciones de melanóforos, xantóforos e iridóforos. Finalmente cabe mencionar que el éxito del establecimiento del patrón de la pigmentación depende de la comunicación entre los xantóforos y los melanóforos, sus proporciones relativas, la proximidad de las células, su localización, su forma y su tamaño (Parichy y Turner, 2003; Nakamasu *et al.*, 2009; Capítulo 2.1). Este equilibrio pareció estar perturbado en los peces pseudo-albinos del presente estudio, en los que se observó una comunicación alterada entre los melanóforos y los xantóforos asociado a una desintegración excesiva de xantóforos. Esto posiblemente impidió la especificación correcta del patrón de melanóforos, lo que originó una alteración en el patrón de señalización dorso-ventral de la pigmentación durante la metamorfosis, dando lugar al fenotipo pseudo-albino. Se ha sugerido que este comportamiento alterado de las células pigmentarias en el lado ocular de los peces pseudo-albinos ocurre normalmente en el lado ciego durante la metamorfosis, formando progresivamente el patrón dorso-ventral de pigmentación característico de los peces planos. El papel del gen *asip* en el establecimiento del patrón dorso-ventral de pigmentación ha sido previamente estudiado en otras especies (Cerdá-Reverter *et al.*, 2005), pero su implicación en la fisiología de los xantóforos requiere más investigación, especialmente en el lenguado senegalés.

Estos resultados relativos a la pigmentación permitieron aumentar eln conocimiento actual sobre los procesos morfológicos y moleculares relacionados con la aparición de desórdenes pigmentarios y sobre el efecto del ARA y de sus metabolitos derivados sobre el desarrollo correcto de la pigmentación en el lenguado senegalés.

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# CONCLUSIONS

1. The absolute dietary levels of DHA, EPA and ARA recommended for proper Senegalese sole growth performance and development were 9.5%, 3.1% and 0.7% TFA, respectively, and the n−3/n−6 PUFA, DHA/EPA, ARA/DHA and OA/PUFA recommended ratios of 5.2, 3.0, 0.1 y 0.5, respectively.

2. Optimizing larval diets requires a careful balance of all nutrients. A variation, even slight, in the EPA, DHA or ARA levels or in the EPA/DHA, ARA/EPA, ARA/DHA, (n-3)/(n-6) HUFA ratios, particularly in the OA/PUFA ratio, might have modified the metabolism of lipids and disrupted the profile of lipid accumulation in the target tissues, leading to intestinal and hepatic steatosis.

3. Senegalese sole larvae were able to activate antioxidant defense mechanisms in response to a dietary oxidative stress, through the provided dietary vitamin E and the activation of antioxidant stress enzymes (catalase, superoxide dismutase, glutathione transferase and glutathione reductase). These mechanisms were efficient enough to prevent the detection of any dietary physiological alterations in larvae. However, the highly oxidized diet without VE supplementation delayed larval bone mineralization, highlighting the impact that oxidative stress has in skeletogenesis.

4. The optimal dietary level of ARA in Senegalese sole larvae was estimated at 4.5% TFA, based on growth performance and ossification results.

5. High dietary levels of ARA (8.7% TFA) fed to Senegalese sole during the pre-, pro- and/or post-metamorphosis stages improved larval survival, without affecting growth performance or the incidence of skeletal and pigmentary disorders.

6. Feeding larvae with high ARA levels (10.2% TFA in rotifer and 7.1% TFAin *Artemia*) in the live prey affected the ARA/EPA ratio in larval tissues, altered the relative concentrations of prostaglandins of the 2 and 3-series, induced pigmentary disorders and impaired the processes of eye migration and cranial bone remodeling during larval metamorphosis. A “sensitive developmental window” with regards to dietary ARA was identified during pre- and pro-metamorphosis stages, an excess of this PUFA resulting in pseudo-albino specimens with reduced density and altered aspect of melanophores in their dorsal skin compared to normally pigmented individuals.

7. Morphological changes in pigment cell types and distribution in the skin of the ocular side that establish the adult pattern of pigmentation occurred at post-metamorphosis and were the consequence of changes in the expression profiles of the genes involved in melanophore differentiation and melanin synthesis from pre- to pro-metamorphic stage.

8. The transition from larval to juvenile pigmentation coincides with the progress of metamorphosis: (i) the pre-metamorphic period (2-11 dph) was characterized by a low expression of apoptotic factors and genes related to melanogenesis (*tyr* and *trp1)* and a high expression of melanophore proliferating and differentiating genes (*pax-3, mitf* and *cKit)*; (ii) the pro-metamorphic period (11-19 dph) was marked by a high expression of apoptotic factors (tissue remodeling) and melanophore differentiating and melanogenic genes (*tyr* and *trp1*) and (iii) the post-metamorphic stage (19-47 dph) showed a low expression of all analyzed genes, especially those associated to melanophore differentiation *(pax-3, mitf, cKit* and *mc1r)*.

9. While the ontogeny of skin pigmentation in Senegalese sole larvae is genetically programmed, environmental factors, such as nutrition, can modulate the normal changes in the molecular processes occurring during metamorphosis, but these alterations were only morphologically detectable after metamorphosis.

10. The ARA-induced pseudo-albino phenotype was the result of inhibited differentiation of post-metamorphic populations of chromatophores and the progressive degradation of the already existent larval chromatophores, mirrored in the over-expression of *asip, pax3, cKit* and *mitf* and the down-regulation of *slc24a5* and *trp1.* These alterations of the normal gene expression patterns likely accounted for the different relative proportions, allocation patterns, shapes and sizes of skin chromatophores, compared to a normally pigmented fish, and for the excessive disintegration of xanthophores that prevented the normal patterning of melanophores and the proper dorsal-ventral patterning signaling during metamorphosis.

# CONCLUSIONES

## (en castellano)

1. Los niveles absolutos de DHA, EPA y ARA en la dieta recomendados para el correcto crecimiento y desarrollo del lenguado senegalés son 9.5%, 3.1% y 0.7% AGT, respectivamente, y los ratios n−3/n−6 AGPI, DHA/EPA, ARA/DHA y OA/AGPI recomendados son 5.2, 3.0, 0.1 y 0.5, respectivamente.

2. La composición óptima de las dietas para larvas requiere de un balance cuidadoso de todos los nutrientes. Una variación, aunque ligera, de los niveles de EPA, DHA o ARA o de los ratios EPA/DHA, ARA/EPA, ARA/DHA, (n-3)/(n-6) AGPI o, especialmente, OA/AGPI pudo haber modificado el metabolismo lipídico y alterado el perfil de acumulación de los lípidos en los tejidos diana, dando lugar a esteatosis intestinal y hepática.

3. Las larvas de lenguado senegalés fueron capaces de activar los mecanismos de defensa antioxidante, en respuesta al estrés oxidativo ocasionado por la dieta, a través del uso de la VE suministrada y de la activación de las enzimas del estrés oxidativo (catalasa, superóxido dismutasa, glutatión transferasa y glutatión reductasa). Estos mecanismos fueron lo suficientemente eficientes como para impedir la detección de posibles alteraciones fisiológicas ocasionadas por las dietas oxidadas en las larvas. Sin embargo, la dieta con el nivel de oxidación más alto y sin suplemento de VE retrasó la mineralización del esqueleto de las larvas, lo que pone de manifiesto que el estrés oxidativo tiene un impacto negativo en la esqueletogénesis.

4. Se estimó, en base a los resultados de crecimiento y osificación del esqueleto, que el nivel óptimo de ARA en la dieta para las larvas de lenguado senegalés es de un 4.5% AGT.

5. La alimentación de las larvas de lenguado senegalés con niveles elevados de ARA (8.7% AGT) durante los periodos de pre-, pro- y/o post-metamorfosis mejoró la supervivencia larvaria sin afectar al crecimiento, ni a la incidencia de deformaciones esqueléticas.

6. La alimentación de larvas de lenguado senegalés con niveles elevados de ARA (10.2% AGT en el rotífero y 7.1% AGT en la *Artemia*) afectó al ratio ARA/EPA de los tejidos, alteró las concentraciones relativas de prostaglandinas de las series 2 y 3, indujo problemas pigmentarios e impidió el proceso correcto de migración del ojo y de la remodelación de los huesos craneales durante la metamorfosis. Se identificó una “ventana de desarrollo sensible” durante la pre- y la pro-metamorfosis, donde un exceso de ARA provocó la aparición de especímenes pseudo-

albinos, caracterizados por poseer una densidad menor y un aspecto alterado de los melanóforos de la piel del lado ocular en comparación con individuos de pigmentación normal.

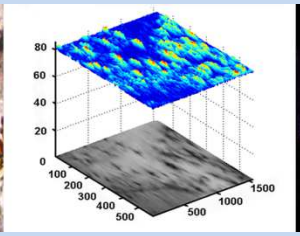
7. Los cambios morfológicos y la distribución de los diferentes tipos de células pigmentarias en la piel del lado ocular del lenguado senegalés, que dan lugar al patrón de pigmentación adulto, ocurrieron durante la post-metamorfosis y fueron consecuencia de cambios en el perfil de expresión de genes implicados en la diferenciación de melanóforos y en la síntesis de melanina detectados durante la transición de la etapa pre- a la pro-metamórfica.

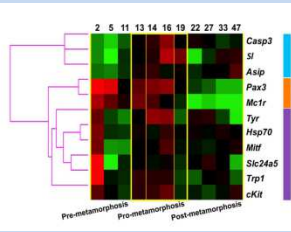
8. La transición de la pigmentación larvaria a la juvenil coincidió con el progreso de la metamorfosis: (i) el periodo pre-metamórfico (2-11 dde) se caracterizó por una expresión baja de factores apoptóticos y genes relacionados con la melanogénesis (*tyr* y *trp1*) y una expresión elevada de los genes implicados en la diferenciación y proliferación de melanóforos (*pax-3, mitf* y *cKit)*; (ii) el periodo pro-metamórfico (11-19 dde) fue marcado por una expresión elevada de factores apoptóticos (remodelación de tejidos) y de genes asociados a la diferenciación de melanóforos y a la melanización (*tyr* y *trp1*) y (iii) el periodo post-metamórfico (19-47 dde) mostró una expresión baja de todos los genes analizados, especialmente aquellos asociados con la diferenciación de melanóforos (*pax-3, mitf, cKit* y *mc1r*).

9. Mientras que la ontogenia de la pigmentación de la piel del lenguado senegalés está genéticamente programada, los factores ambientales, tales como la nutrición, pueden alterar los cambios normales de los procesos moleculares que tienen lugar durante la metamorfosis, aunque dichas alteraciones sólo fueran morfológicamente detectables después de la misma.

10. El fenotipo pseudo-albino, inducido por el ARA presente en la dieta, fue el resultado de la inhibición de la diferenciación de las poblaciones post-metamórficas de cromatóforos y de la degradación progresiva de los cromatóforos larvarios ya existentes como consecuencia de la sobre-expresión de *asip, pax3, cKit* y *mitf, y* de la sub-expresión de *slc24a5* y *trp1.* La alteración de la expresión normal de estos genes fue probablemente la responsable de los cambios en la forma, tamaño, proporciones relativas y patrones de localización de los cromatóforos y de la desintegración excesiva de los xantóforos, lo que impidió la formación del patrón normal de melanóforos y la correcta señalización del patrón de pigmentación dorso-ventral durante la metamorfosis.



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