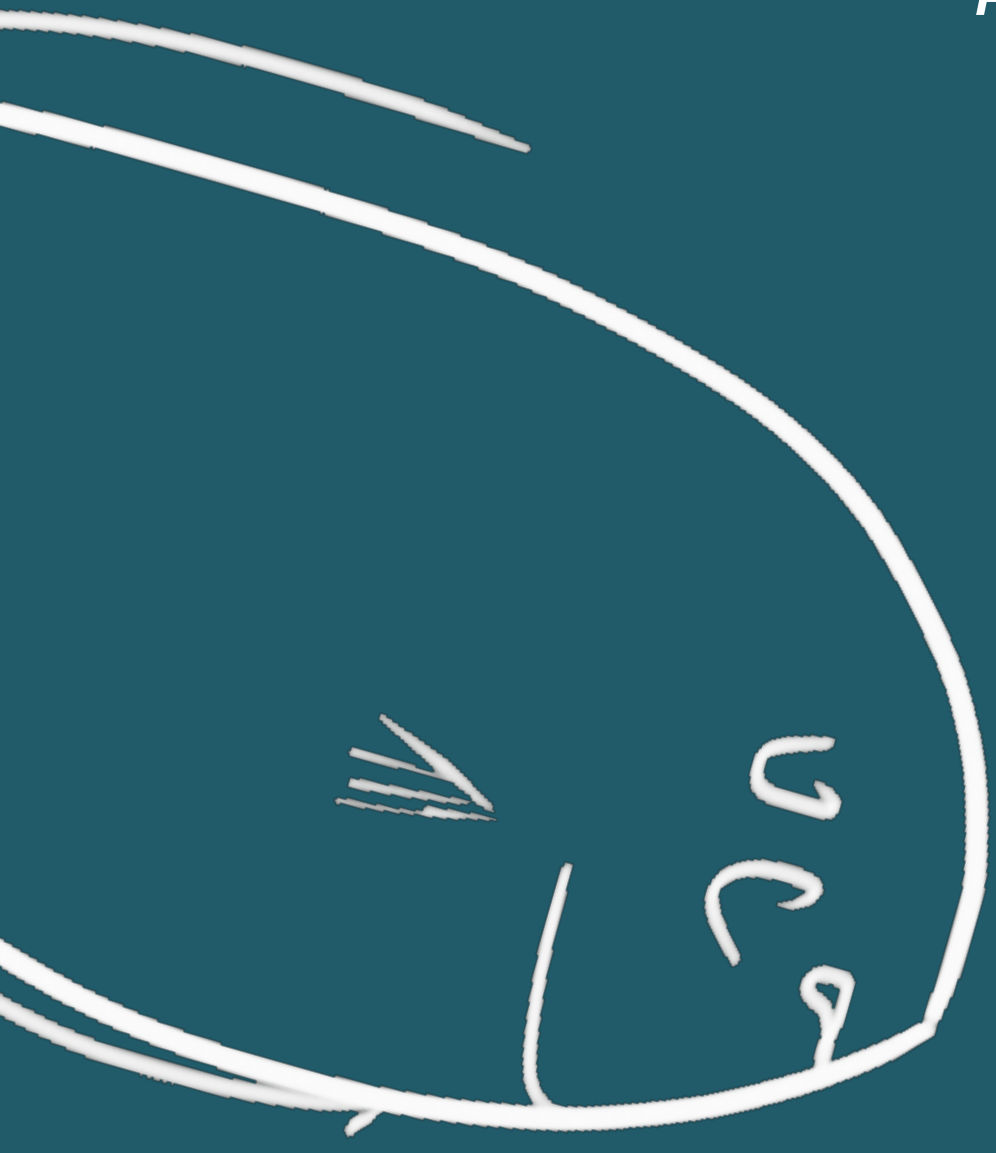
Growth and size variation of Senegalese sole (*Solea senegalensis*)

PhD Dissertation



Pablo Sánchez Fernández

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Xarxa de Referència de R+D+I en
Aqüicultura de la Generalitat de
Catalunya

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Agraïments

Aquesta és potser una de les pàgines més difícils d'escriure de la tesi. Segurament per què un té la certesa que els agraïments és l'únic que tothom que tingui aquest document entre les mans llegirà i clar, un sent les mirades clavades. Malgrat tot, i encara que la literatura suggereixi deixar aquest apartat en blanc (Schulman & Cox, 1997), no puc ni vull deixar passar l'oportunitat d'esmentar i agrair a les persones que han fet possible aquest treball d'una o altra manera.

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Abstract

Senegalese sole (*Solea senegalensis*) is a flatfish of high commercial importance in Southern Europe that has been a promising species for diversifying Mediterranean aquaculture since early 1980's. It has failed to reach successful farming development due to lack of full control over spawning, poor fry quality and high mortality during the weaning stage. Furthermore, optimization of production has not been possible due to high heterogeneity in growth rates within cultured stocks that result in high body size variance at harvest.

Among the several aspects needing wider knowledge in order to reach optimization of sole aquaculture, the ongrowing stage from juveniles to the commercialization size has been overlooked until the last years. It includes the relationships between individual growth and rearing parameters as stocking density, or the size composition of stocks. It is also important to evaluate growth according to the feeding rhythms, offering feed at adequate times for this species. Not less important is to assess the genetic variability of farmed stocks, and compare it to the wild populations, as a stepping stone to link genetic traits to the performance of aquaculture stocks.

The aims of this thesis were to take an individual-based approach to growth, and growth sexual dimorphism, of Senegalese sole reared at high and low densities, with different levels of initial size variation, and under different feeding strategies. Moreover, the level of genetic variability of this species was compared between farmed and wild stocks through molecular genetic markers, and their sibship relationships were also assessed.

Three experiments were conducted, involving a) rearing soles under high density (180% of bottom coverage) and low density (60% of bottom coverage); b) rearing soles under the same high and low densities, but also under homogeneous or heterogeneous initial size composition; c) rearing soles under a medium/high initial density (130% of bottom coverage) fed either during nocturnal hours, similar to their natural feeding rhythms, or during daylight hours. Additionally, the genetic variability of a farmed sample was compared to its wild donor population.

Stocking density affects growth of Senegalese sole in two ways. First, sudden and steep increases in density could lead to poorer or no growth of fish until re-acclimatization to new high density conditions. Second, it seems that there is a size-dependent component on how stocking density affects growth, with smaller fish (sub-adults) growth being suppressed by high stocking density.

Size composition of reared Senegalese sole does not affect individual growth. Sole doesn't show aggressive behavior and apparently, competition between individuals is low and size independent. Grading Senegalese sole does not guarantee the improvement of growth, and if so, it would be in high density conditions.

Senegalese sole females grow faster than males, but after attaining certain body weight (between 40 and 80 g). It could be hypothesized that sexual maturation may be involved in the onset of sexual growth dimorphism in this species.

Besides being an eminently nocturnal species, Senegalese sole fed during the light phase may yield specific growth rates and feed conversion ratios that are comparable to those of fish fed during the dark phase, suggesting the feasibility of feeding during normal business hours in commercial facilities.

There is a loss in genetic variability in a single generation of Senegalese sole rearing, as evidenced by lower values of mitochondrial haplotypic diversity and nuclear diversity in the farmed sample compared with the wild donor population. Comparing wild Atlantic and Mediterranean samples suggests a limited gene flow between the populations inhabiting these basins. An accurate knowledge of the genetic composition of farmed stocks is essential both for maintaining the cultured stocks and for potential future restocking purposes.

Resum

El llenguado senegalès (*Solea senegalensis*) és un peix pla de gran importància comercial al sud d'Europa que ha estat una espècie prometedora per a la diversificació de l'aqüicultura mediterrània des de principis dels 80. L'aqüicultura de llenguado no està encara totalment desenvolupada a causa de la falta de control sobre les postes, la baixa qualitat dels alevins i una alta mortalitat durant la fase de deslletament. D'altra banda, l'optimització de la producció no ha estat possible causa de l'alta heterogeneïtat en les taxes de creixement dins de les poblacions cultivades, que donen lloc a una alta dispersió de talles en arribar a la mida comercial.

Entre els diversos aspectes que requereixen un coneixement més ampli per tal d'arribar a l'optimització del cultiu del llenguado, els darrers anys s'ha passat per alt la fase d'engreix. Això inclou les relacions entre el creixement individual i els paràmetres de cultiu, com la densitat de cultiu o la composició per talles de les poblacions. També és important avaluar el creixement en funció dels ritmes d'alimentació, oferint l'aliment al moment adequat per a aquesta espècie. No menys important és avaluar la variabilitat genètica dels stocks de cultiu, i comparar-la amb la de les poblacions salvatges, com a punt de partida per vincular els trets genètics amb el seu rendiment en cultiu.

Els objectius de la tesi van ésser l'aproximació individual al creixement, i al dimorfisme sexual del creixement, del llenguado cultivat a altes i baixes densitats, amb diferents nivells de dispersió de talles inicial i sota diferents estratègies alimentàries. D'altra banda, el nivell de variabilitat genètica d'aquesta espècie es va comparar entre les poblacions de cultiu i salvatges a través de marcadors genètics moleculars, i es van avaluar les seves relacions de parentiu.

Es van dur a terme tres experiments, implicant a) el cultiu de llenguado a alta densitat (180% de cobertura del fons del tanc) i baixa densitat (60% de cobertura); b) el cultiu de llenguado sota les mateixes densitats, però també sota condicions d'homogeneïtat o heterogeneïtat de talles inicials; c) cultiu de llenguado a densitat mitja/alta (130% de cobertura) alimentats durant les hores nocturnes, de manera semblant als seus ritmes alimentaris naturals, o durant les hores diürnes. Addicionalment, es va comparar la variabilitat genètica d'una mostra de cultiu amb la seva població salvatge donant.

La densitat de cultiu afecta el creixement del llenguado senegalès de dues maneres. En primer lloc, els augments sobtats i pronunciats de la densitat podrien conduir a un creixement pobre o nul fins la re-aclimatació a les noves condicions d'alta densitat. En segon lloc, sembla que hi ha una component mida-dependent de com la densitat de cultiu afecta el creixement, amb els peixos més petits (sub-adults) experimentant una supressió del seu creixement degut a l'alta densitat.

La composició de talles del llenguado senegalès de cultiu no afecta el creixement individual. El llenguado no mostra un comportament agressiu i, pel que sembla, la competència entre els individus és baixa i independent de la mida. La classificació de talles en el llenguado no garanteix la millora del seu creixement, i si és així, seria en condicions d'alta densitat.

Les femelles de llenguado senegalès creixen més ràpid que els mascles, però després d'assolir cert pes corporal (entre 40 i 80 g). Es podria hipotetitzar que la maduració sexual pot estar implicada en l'aparició de dimorfisme sexual en el creixement d'aquesta espècie.

A pesar de ser una espècie eminentment nocturna, el llenguado senegalès alimentat durant les hores de llum pot experimentar taxes de creixement específiques i ràtios de conversió de l'aliment que són comparables a les dels peixos alimentats durant les hores nocturnes, el que suggereix la viabilitat de l'alimentació durant les hores normals de treball a les instal·lacions comercials.

Hi ha una pèrdua de la variabilitat genètica en una sola generació de cultiu de llenguado senegalès, com s'evidencia pels valors més baixos de diversitat haplotípica mitocondrial i diversitat nuclear de la mostra de cultiu en comparació amb la població salvatge donant. La comparació de mostres salvatges de l'Atlàntic i del Mediterrani suggereix un flux limitat de gens entre les poblacions que habiten en aquestes conques. Un coneixement exacte de la composició genètica de les poblacions de cria és essencial per al manteniment de les poblacions cultivades, i per a possibles programes de repoblació en el futur.

Resumen

El lenguado senegalés (*Solea senegalensis*) es un pez plano de gran importancia comercial en el sur de Europa, que ha sido una especie prometedora para la diversificación de la acuicultura mediterránea desde principios de los 80. La acuicultura de lenguado no está todavía totalmente desarrollada debido a la falta de control sobre las puestas, la baja calidad de los alevines y una alta mortalidad durante la fase de destete. Por otro lado, la optimización de la producción no ha sido posible debido a la alta heterogeneidad en las tasas de crecimiento dentro de las poblaciones cultivadas, que dan lugar a una alta dispersión de tallas al llegar al tamaño comercial.

Entre los diversos aspectos que requieren un conocimiento más amplio para llegar a la optimización del cultivo del lenguado, en los últimos años se ha pasado por alto la fase de engorde. Esto incluye las relaciones entre el crecimiento individual y los parámetros de cultivo, como la densidad de cultivo o la composición de tallas de los stocks. También es importante evaluar el crecimiento en función de los ritmos de alimentación, ofreciendo el alimento en el momento adecuado para esta especie. No menos importante es evaluar la variabilidad genética de los stocks de cultivo, y compararla con la de las poblaciones salvajes, como punto de partida para vincular los rasgos genéticos con su rendimiento en cultivo.

Los objetivos de la tesis fueron la aproximación individual al crecimiento, y al dimorfismo sexual del crecimiento, del lenguado cultivado a altas y bajas densidades, con diferentes niveles de dispersión de tallas inicial y bajo diferentes estrategias de alimentación. Por otro lado, el nivel de variabilidad genética de esta especie se comparó entre las poblaciones de cultivo y salvajes a través de marcadores genéticos moleculares, y se evaluaron sus relaciones de parentesco.

Se llevaron a cabo tres experimentos, implicando a) el cultivo de lenguado a alta densidad (180% de cobertura del fondo del tanque) y baja densidad (60% de cobertura), b) el cultivo de lenguado bajo las mismas densidades, pero también bajo condiciones de homogeneidad o heterogeneidad de tallas iniciales; c) cultivo de lenguado a densidad media/alta (130% de cobertura) alimentados durante las horas nocturnas, de manera similar a sus ritmos alimentarios naturales, o durante las horas diurnas. Adicionalmente, se comparó la variabilidad genética de una muestra de cultivo con su población salvaje donante.

La densidad de cultivo afecta el crecimiento del lenguado senegalés de dos maneras. En primer lugar, los aumentos repentinos y pronunciados de la densidad podrían conducir a un crecimiento pobre o nulo hasta la re-aclimatación a las nuevas condiciones de alta densidad. En segundo lugar, parece que hay un componente tamaño-dependiente de cómo la densidad de cultivo afecta al crecimiento, con los peces más pequeños (sub-adultos) experimentando una supresión de su crecimiento debido a la alta densidad.

La composición de tallas del lenguado senegalés de cultivo no afecta al crecimiento individual. El lenguado no muestra un comportamiento agresivo y, al parecer, la competencia entre los individuos es baja e independiente del tamaño. La clasificación de tallas en el lenguado no garantiza la mejora de su crecimiento, y si es así, sería en condiciones de alta densidad.

Las hembras de lenguado senegalés crecen más rápido que los machos, pero después de alcanzar cierto peso corporal (entre 40 y 80 g). Se podría hipotetizar que la maduración sexual puede estar implicada en la aparición de dimorfismo sexual en el crecimiento de esta especie.

A pesar de ser una especie eminentemente nocturna, el lenguado senegalés alimentado durante las horas de luz puede experimentar tasas de crecimiento específicas y ratios de conversión del alimento que son comparables a las de los peces alimentados durante las horas nocturnas, lo que sugiere la viabilidad de la alimentación durante las horas normales de trabajo en las instalaciones comerciales.

Hay una pérdida de la variabilidad genética en una sola generación de cultivo de lenguado senegalés, como se evidencia por los valores más bajos de diversidad haplotípica mitocondrial y diversidad nuclear de la muestra de cultivo en comparación con la población salvaje donante. La comparación de muestras salvajes del Atlántico y del Mediterráneo sugiere un flujo limitado de genes entre las poblaciones que habitan en estas cuencas. Un conocimiento exacto de la composición genética de las poblaciones de cría es esencial para el mantenimiento de las poblaciones cultivadas, y para posibles programas de repoblación en el futuro.

Chapter 1

Introduction

1.1 Sole as an aquaculture species

Senegalese sole (*Solea senegalensis*, Kaup, 1858) is a flatfish of high commercial importance that is indistinguishable by consumers from common sole (*S. solea*, Linnaeus, 1758), so it is rated as the same species in marketing statistics (Reig & Oca, 2000). These two species, however, display marked differences in biological requirements that are important for its rearing in captivity. Traditionally, Southern Europe countries have been more focused in *S. senegalensis* aquaculture due to the lower spawning temperature requirements of *S. solea* (Howell, 1997), and the high abundance of *S. senegalensis* in Mediterranean and Southern Atlantic waters (Dinis et al., 1999), that makes of *S. senegalensis* the only sole species reared in Spain or Portugal nowadays.

Although interest in farming Senegalese sole intensively in southern Europe dates back to the early 1980s, it has failed to reach successful commercial development (Flos et al., 2001; Imsland et al., 2003; FAO Fisheries and Aquaculture Information and Statistics Service, 2011). The reasons for this include lack of full control over spawning, poor fry quality and high mortality rates during the weaning stage (Cañavate & Fernández-Díaz, 1999; Anguís & Cañavate, 2005), all leading to juvenile scarcity for stocking purposes. This problem is compounded by a high incidence of skeletal malformations and pigmentation abnormalities in post-larvae and juveniles (Gavaia et al., 2002; Soares et al., 2002; Villalta et al., 2005a) and of disease outbursts affecting all ontogenetic stages caused by multiple pathogenic agents (Zorrilla et al., 2003). Furthermore, optimization of production has not been possible due to high heterogeneity in growth rates within cultured stocks that result in high body size variance at harvest (Dinis et al., 1999; Flos et al., 2001).

High market demand, high market value and the adaptability of existent facilities to accommodate its rearing (Imsland et al., 2003) presents Senegalese sole as an interesting new species for diversifying Mediterranean marine aquaculture.

1.2 Market

Sole is a species of high acceptance by the consumer that reaches high commercial value not only in Spanish markets, but also in European markets (Reig & Oca, 2000). Actually, what the consumer perceives as *sole*, is a mix of two similar species: Senegalese sole and common sole. These two species are commonly fished along the Atlantic coasts of Portugal and Spain, and also along all the Mediterranean basin, in gillnets or trawling nets, and are often sold under the same label. As a result, both species get mixed in the fisheries statistics, appearing as a single cluster.

The commercialization statistics from the Central Fish Market at Mercabarna (the most important food distribution hub in Catalonia) may give some information about the market status of these species. In 2010, fresh sole was the 11th finfish species in importance in terms of sales volume (1384 Tn) according to MERCABARNA (2011), behind species like European hake (*Merluccius merluccius*), Atlantic salmon (*Salmo salar*), monkfish (*Lophius piscatorius*), sardine (*Sardina pilchardus*), or farmed European sea bass (*Dicentrarchus labrax*) and farmed gilthead sea bream (*Sparus aurata*). Additionally, sole was the 9th species in the price ranking (13.6 €·kg^{-1}), only behind wild high valued species as European sea bass, gilthead seabream, turbot (*Scophthalmus maximus*), red porgy (*Pagrus pagrus*), blackspot seabream (*Pagellus bogaraveo*) or dusky grouper (*Epinephelus marginatus*).

Aquaculture production of Senegalese sole in Spain is well behind that of gilthead seabream, European sea bass or turbot, which are the classic marine reared species in the Mediterranean. In 2009, 63 Tn of Senegalese sole were produced in Spain for 12656 Tn of European sea bass, 23219 Tn of gilthead sea bream and 7188 Tn of turbot, while another emerging species in marine aquaculture, the blackspot sea bream, had a production of 183 Tn (FAO Fisheries and Aquaculture Information and Statistics Service, 2011). As it has been mentioned above, too often common sole and Senegalese sole are treated as a single species in fisheries and aquaculture statistics, and some times it is difficult to obtain reliable data regarding its production. Global aquaculture production of soles is depicted in table 1.1, whereas global revenue *ex farm* is shown in table 1.2.

The commercialization of soles in the central market of Barcelona in 2008 was of 17892 Tn (MERCABARNA, 2011), indicating that the farming production of this species is residual when faced with the actual consumer demand of this species. Sole aquaculture has clearly some room for improvement. In terms of economic revenue, total production of Senegalese sole in Spain in 2008 yielded 533572 €, at an average price of 8.98 €·kg^{-1}. Such a commercial price is the highest of all the marine species produced in Spain (with the exception of the bluefin

Table 1.1: Global production of *Solea* spp. in tons (Tn) by country from 2000 to 2010 according to FAO Fisheries and Aquaculture Information and Statistics Service (2011)

		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
S. senegalensis	Spain	–	–	–	–	–	8	32	36	60	63	–
	Subtotal	**–**	**–**	**–**	**–**	**–**	**8**	**32**	**36**	**60**	**63**	**224***
S. solea	Italy	–	17	–	–	–	–	–	–	19	14	–
	Portugal	10	43	6	4	4	11	9	8	13	14	–
	Spain	13	–	4	20	70	–	–	11		2	–
	Subtotal	**23**	**60**	**10**	**24**	**74**	**11**	**9**	**19**	**32**	**30**	**116***
Soles nei[a]	Spain	–	–	39	44	34	28	11	6	–	–	–
	Subtotal	**–**	**–**	**39**	**44**	**34**	**28**	**11**	**6**	**–**	**–**	**20***
	Total	**23**	**60**	**49**	**68**	**108**	**47**	**52**	**61**	**92**	**93**	**360**

* FAO estimate from available sources of information or calculation based on specific assumptions.
[a] Not elsewhere included.

Table 1.2: Global value of *Solea* spp. production *ex farm* in 1000 USD from 2000 to 2010, according to FAO Fisheries and Aquaculture Information and Statistics Service (2011)

	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
S. senegalensis	–	–	–	–	–	56	224	252	793	881	2954
S. solea	247	608	92	192	550	179	130	203	611	535	1794
Soles nei[a]	–	–	312	352	272	224	88	48	–	–	239
Total	**247**	**608**	**404**	**544**	**822**	**459**	**442**	**503**	**1404**	**1416**	**4987***

* FAO estimate from available sources of information or calculation based on specific assumptions.
[a] Not elsewhere included.

tuna (*Thunnus thynnus*, 13.73 €·kg^{-1}, which is not a farmed species *sensu stricto*). Sole prices are well ahead of those of more developed species like European sea bass (5.43 €·kg^{-1}), gilthead sea bream (3.59 €·kg^{-1}), or turbot (6.96 €·kg^{-1}). Blackspot sea bream, as an example of a developing species, reached 7.49 €·kg^{-1} during the same period (JACUMAR, 2011).

Until recent years, Senegalese sole aquaculture has been intimately linked to the production of old salt marshes in the South of Spain (Cádiz), that typically recruited wild sole seed with few control. Progressively, these facilities started to be stocked with hatchery reared juveniles, mainly produced in scientific institutions.

According to Reig & Oca (2000), due to the high acceptance of Senegalese sole by the consumer, as well as the wide knowledge of this species, Spanish market could assume an important volume of farmed Senegalese sole without presumable differences in price from the wild specimens, while benefitting of not needing any especial promotion campaigns, which would be the case for a new and unknown species.

1.3 Genetics

Good management of broodstock is essential in any form of livestock production, since loss of genetic variability typically leads to detrimental effects in various performance traits as survival or growth (Falconer, 1960). Modern aquaculture breeding strategies should, accordingly, rely on the accurate knowledge of genetic composition of wild and farmed stocks in order to assure that the bred families keep the most of the variability present in the wild, thus improving yields from fry production to growth rates. Ultimately, this information can be used to prevent inbreeding in captivity and to minimize the potential genetic dilution of natural populations in accidental escapes of hatchery fish, or as part of stock enhancement programmes (Sekino et al., 2002; Fjalestad et al., 2003; Porta et al., 2007), a practice that is being considered as aquaculture industries increase their activity (Cognetti et al., 2006). In addition, the characterization of genetic variation is a prime requirement for marker-assisted selection (MAS) programmes aimed at increasing production (Fjalestad et al., 2003).

Genetic studies in Senegalese sole are scarce compared to those carried out in other aquaculture species as gilthead seabream (Alarcón et al., 2004) or turbot (Coughlan et al., 1998) among others, and only recently the composition of farmed stocks, both of breeders and reared batches, has been assessed through molecular markers (Porta et al., 2006b, 2007; Blonk et al., 2009). Among soles, the characterization of genetic variability has focused primarily on elucidating the genetic population structure of wild populations of *S. solea* and *S. senegalensis* using allozyme (Exadactylos et al., 1998) and mitochondrial DNA (mtDNA) control region (CR) data (Tinti et al., 2000; Guarniero et al., 2002). In addition, genetic studies have been conducted to clarify the phylogenetic relationships among members of this genus (Tinti & Piccinetti, 2000; Infante et al., 2004).

The genetic structure of wild soles has been studied mainly in *S. solea* using allozymes as a marker. Assuming that both species share some biological traits, some of the data referred to population genetics of common sole could be cautiously applied to Senegalese sole.

Exadactylos et al. (1998) used 33 allozyme loci to observe that mean heterozygosity in common sole was inversely correlated to latitude, and that mean heterozygosity was higher than that of the average for marine teleosts, and even for vertebrates (Exadactylos, 2001). *Solea solea* larvae originating from different locations in the Irish sea and from the Skagerrak-Kattegat in Norway were indistinguishable by means of genetic traits as single-locus heterozygosity or allelic or genotype frequencies (Exadactylos et al., 1999).

On the other hand, Cabral et al. (2003) observed in several estuarine systems of the Portuguese coast that there was a significant correlation between genetic and geographic distances in common sole, that supported a non-random mating system and an isolation by distance model. Nevertheless, the genetic differentiation between samples was very low, de-emphasizing the importance of estuarine systems as structuring forces of genetic differentiation.

Guinand et al. (2008) investigated temporal patterns of genotypic diversity in common sole at three intronic markers over nursery grounds in the bay of Biscay, finding panmixia in practically all the samples, suggesting that there was a gene flow between the offshore spawning grounds and the nurseries.

Exceptions to the high gene flow showed by different genetic markers can be found in works by Guarniero et al. (2002), who used the mitochondrial DNA (mtDNA) control region (CR), a molecular marker widely used in phylogenetic studies, to investigate the population structure of common sole in the central Mediterranean. This

marker, more sensitive than allozymes, allowed to find two partially subdivided or nearly panmictic population units, that should be managed separately according to the authors.

In a broader geographic scale, allozyme data showed a differentiation between an Aegean Mediterranean population and several locations from the Irish and North seas in the Atlantic Ocean (Exadactylos et al., 1998; Exadactylos, 2001). The authors suggested that Atlantic and Mediterranean *Solea solea* populations were separated during one of the regressions associated to one of the interglacial periods occurring after the Pleistocene.

Genetic structure of Wild Senegalese sole has been even less studied than for common sole. Cabral et al. (2003) observed that *S. senegalensis* also presented an isolation by distance model based in allozyme electrophoresis. According to these authors Senegalese sole showed a lower genetic diversity between populations than common sole, probably due to some biological characteristics of this species, namely the longer planktonic stage of *S. senegalensis* larvae.

Taking into account that the information about genetic variability and population structure of Senegalese sole is scarce, it is foreseeable that there are few studies comparing wild and hatchery reared genetic variability in sole. Besides that, often it is difficult to compare results between studies, as there is a diversity of markers used, from allozymes to mtDNA-CR and microsatellite loci. This type of marker has been successfully used to trace offspring individuals to a single pair of parentals (Castro et al., 2006), and have been successfully developed for both Senegalese and common soles (Iyengar et al., 2000; Porta & Álvarez, 2004). Microsatellite loci developed for Senegalese sole showed low cross-amplification within Pleuronectiformes, even within congeneric species, suggesting a low conservation of the flanking regions of the tandem repeat (Castro et al., 2006), and making them suitable for population genetic studies in this species.

As it happens in many aquaculture species, more so if no breeding programmes are established as is the case of sole, genetic variability levels decrease after some (or even one) generations in captivity. This effect is highly amplified in Senegalese sole, due to the reported reproductive problems experienced in the hatcheries, where some individuals breed more than others and even a single fish monopolizes the breeding season Porta et al. (2006b). Blonk et al. (2009) observed a non-uniform mating pattern in wild caught common sole breeders, where some males mated more frequently than others. In the parental populations most loci were observed to be in Hardy-Weinberg equilibrium. The F_1 showed an excess of heterozygotes, implying that genetic diversity in terms of number of alleles and a minimum of heterozygosity was relatively well preserved, despite that 5 parental pairs or less produced all progeny. The mean co-ancestry coefficient increased in the offspring, suggesting that after some generations there could be a considerable loss of genetic variability due to inbreeding.

Porta et al. (2006b) also observed reductions of heterozygosity and a 50% in allelic richness in the offspring of wild Senegalese sole broodstock, finding that only few individuals had contributed to the F_1, composed mostly of related individuals, mainly full sibs. Accordingly, wild breeders showed higher levels of allelic richness than hatchery reared breeders (Porta et al., 2007), possibly due to bottleneck effects that could be produced in selecting the F_1 individuals to set up the broodstocks. Similarly, Sekino et al. (2002) documented a marked reduction in genetic variability in both nuclear DNA (nDNA) and mtDNA in hatchery strains of the flatfish Japanese flounder (*Paralichthys olivaceus*) compared with wild populations. Because of the high resolution of the hypervariable mtDNA CR nucleotide sequence data, the authors were able to conclude that only a small fraction of the stocked females contributed to the reared population.

To maintain genetic variation within farmed strains at levels comparable with those of wild source populations, good broodstock management practices are required, especially the use of adequate numbers of effective parents and broodstock from different locations, or more locally if there are significant differences in the wild (Exadactylos et al., 2007)

1.4 Reproduction

Reproduction of Senegalese sole is one of the main hurdles that its domestication is still facing nowadays. There are severe problems to obtain fertile spawnings from second generation individuals, and this is a huge drawback, as broodstocks have to be collected from the wild as they loose reproductive performance or die. It is also a problem to establish selection programmes, as aquaculture reared Senegalese sole fail to reproduce in captivity even tough they successfully spawn in the broodstock tanks (Cabrita et al., 2006; Guzmán et al., 2008, 2009a, 2011; Oliveira et al., 2011)

Breeders are captured by beam trawls or beach seines and they are kept at low densities, up to 5 $kg \cdot m^{-3}$ in the stocking tanks. They are fed with fresh food with mussels, squid and polychaetes (Dinis et al., 1999).

Males produce sperm all year round, with peaks in spring (Anguís & Cañavate, 2005). Testicular recrudescence begins in autumn, with peaks of testosterone and 11-ketotestosterone during winter, followed by an increase of the proportion of running males thereafter and throughout spring (García-López et al., 2006b). Sperm quality, in terms of motility and linear speed, decrease from march to july (Beirão et al., 2011). Testis are of the semi-cystic type, and the transformation of spermatids to spermatozoa follows a group-synchronous fashion (batch maturation), consistent with the small quantity of sperm that can be manually stripped in this species (García-López et al., 2006b). This is also reflected in the males showing sperm quality peaks at different times, which may result in unequal contributions to the next generation (Beirão et al., 2011).

There are differences in terms of sperm production between wild (10-80 µl) and hatchery-reared individuals (5 to 20 µl; Cabrita et al., 2006). Recent studies carried out through real-time quantitative reverse transcription polymerase chain reaction (rtPCR) suggest that follicle stimulating hormone (FSH) and luteinizing hormone (LH) may regulate spermatogenesis in Senegalese sole (Cerdà et al., 2008), as levels of its transcripts increased during winter and spring. FSH appears to be also related to the early reproductive axis, as its transcripts can be detected from day 1 after hatching, peaking at mid metamorphosis.

Females show more developed gonads from october to may, with a partial regression during the summer (Anguís & Cañavate, 2005) and a maximum at the end of the winter and early spring (García-López et al., 2007). Profiles of 17,20 β-dihydroxy-4-pregen-3-one correlate with gonadal maturation in Senegalese sole females, likely being the steroid inducing maturation in this species (García-López et al., 2006a). Plasma vitelogenin also correlates with levels of sexual steroids and spawning performance, also peaking at pre-spawning in early spring (Guzmán et al., 2008). Maturation can be assessed through visual inspection of the swelling of the abdominal cavity and condition index (Anguís & Cañavate, 2005).

Spawning takes place naturally in communal tanks, primarily from March to July (Dinis et al., 1999), with a secondary spawning in autumn (5.4% of eggs; Anguís & Cañavate, 2005). Yearly fecundity rate varies between 1.15 to $1.65 \cdot 10^6$ $eggs \cdot kg^{-1}$ of body mass in natural spawnings in captivity. Egg fertilization rate is around $44.9 \pm 18\%$ and $86.5 \pm 14.2\%$ (Dinis et al., 1999; Anguís & Cañavate, 2005). Mating behavior is similar to the one observed in common sole (Baynes et al., 1994), with males swimming underneath a female towards the surface with synchronized movements, although this behavior has not been observed in F_1 Senegalese sole breeders (Howell et al., 2009).

Spawning in sole has an endogenous rhythmicity that synchronizes with dusk periods. It starts after dusk and it peaks about 4 hours later. It also shows synchronization with lunar phases, peaking at the new moon (Oliveira et al., 2009b). Spawning takes place between 13 and 23°C, with higher fecundities between 15 and 21°C (Anguís & Cañavate, 2005). Some authors report the interruption of the emission of eggs below 16°C (Dinis et al., 1999). It is unquestionable that temperature plays a major role in Senegalese sole reproduction (Dinis et al., 1999; Anguís & Cañavate, 2005; García-López et al., 2006c), not only in spawning, but it also seems to be the most important environmental cue for gonadal maturation in this species. Females subjected to natural photoperiod, but to constant temperature showed disrupted gonadal maturation assessed by levels of 17β-estradiol, revealing that temperature, and not photoperiod, is the crucial factor in gonadal development (García-López et al., 2006c). There is a natural yearly rhythm of 17β-estradiol and testosterone, with maximum values in march. An attenuated thermal cycle suppressed the steroid rhythm and these fish did not spawn, despite being subjected to natural photoperiod conditions (Oliveira et al., 2008). Temperature may also be involved in sex differentiation, as daily thermocycles can determine sex ratios during the larval development (Blanco-Vives et al., 2011b).

Despite this main role of temperature in the success of spawning of Senegalese sole in captivity, recent studies show that keeping a constant photoperiod after winter solstice deters the increase in melatonin production, characteristic of shorter days and longer nights, and also increases the production of steroid hormones and vitellogenin in the pre-spawning phase, also advancing spawning (Oliveira et al., 2011). These findings point out to the possible feasibility of advancing/controlling spawning season of Senegalese sole in fish farms.

A crucial step in the rearing of an aquaculture species is the ability to control maturation and, ultimately, spawning. Spawning of Senegalese sole females have been successfully induced through hormonal manipulation, although results have not been conclusive in suggesting a dosage, administration method or administration timing. The steroid of choice is an analogue of the gonadotropin releasing hormone (GnRHa), administered either via repetitive injections or through sustained-release implants (Agulleiro et al., 2006). Some studies show that the

GnRHa implant yields higher fecundity rates than repetitive injections (Guzmán et al., 2009a), while others claim that injections provide better results (Rasines et al., 2012). Treatment with GnRHa in males does not increase spermiation or milt production, although it increased the levels of plasma 11-ketotestosterone (Agulleiro et al., 2006).

As it has already been mentioned, reproduction of second generations of Senegalese sole fails completely. Many approaches have been taken to investigate this problem It has been seen that F_1 females show normal vitelogenin and steroid profiles and spontaneous spawning (Guzmán et al., 2008), with egg quality parameters within normal ranges (Guzmán et al., 2009a) but typically these eggs show no fertilization in the communal reproduction tanks.

Some authors have also detected sexual steroid hormones levels to be higher in wild breeder females compared to cultured breeder females, suggesting that it might cause extensive atresia without final oocyte maturation (García-López et al., 2007) and ultimately, endocrine differences between wild and cultured breeders during the spawning period could be behind the unsuccessful egg fertilization of F_1 broodstocks (Guzmán et al., 2009b). On the other hand, faulty fertilization of F_1 eggs could also be due to delayed release of overripe eggs after induced ovulation (Rasines et al., 2012).

A transcriptomic and proteomic approach has recently highlighted decreased efficiency in sperm production and sperm functionality in F_1 males. Some proteins differ in abundance between wild and F1 fish, pointing toward alterations in cytoskeleton, sperm motility, ubiquitin-proteasome system and redox state during spermiogenesis as possible causes for the decreased fertility of F_1 males (Forné et al., 2011).

The absence of F_1 egg fertilization has been suggested to be due to possible dysfunctions of F_1 males or the reproductive behaviour of the broodstock (Guzmán et al., 2009a), as viable fertilized spawning can be obtained from F_1 females crossed with wild males, but the opposite crossings, F_1 males and wild females, yield unfertilized eggs (Mañanós et al., 2007).

Finally, artificial fertilization looks as a promising strategy to overcome these problems and to provide a tool for actual genetic selection of breeders and improvement of culture yields. Recently Rasines et al. (2012) achieved to collect eggs by stripping hormonally treated F_1 females, and successfully fertilized them with cryopreserved sperm.

1.5 Larval rearing

Incubation of Senegalese sole eggs lasts for approximately 42 hours between 18° C and 20° C, with slight aeration and water renewal until hatching (Imsland et al., 2003; Conceição et al., 2007). During this stage, free amino acids are the most important energy substrate for the embryo (86%) (Parra et al., 1999).

Lighting is crucial during the early life stages of Senegalese sole. Light-darkness photoperiods are essential for the correct hatching and development of eggs and larvae. Light-darkness cycles using blue light maximizes hatching rate 94.5%$\pm$1.9, growth and development speed. On the other hand, complete darkness and light-darkness cycles 12L:12D with red light result in lower hatching rate and poorer larval performance. Hence, it is important to mimic the environment lighting conditions during incubation and early larvae development (Blanco-Vives et al., 2011a).

After hatching, larvae are stocked at 30 to 100 individuals/l in 70 to 700 l cylindro-conical tanks, at a temperature ranging between 18 to 21° C, keeping salinity between 33 and 35‰. Larval rearing using green water is common, using *Isochrysis galbana*, *Tetraselmis chui* or *Nannochloropsis gaditana*. Post-larvae are stocked at 3000 fish/m^2 under 12D:12L photoperiods at intensities lower than 400 lx. Feeding takes place 2-4 times a day during light hours (Cañavate & Fernández-Díaz, 1999; Dinis et al., 1999; Imsland et al., 2003; Conceição et al., 2007).

Light also plays a role in the first days of larval rearing. Light intensity needs to be at least 1200 lx at the surface of the tanks, but can be reduced to 500 lx in white colored tanks. Photoperiod of 16L:8D or continuous light is used (Conceição et al., 2007), as pre-metamorphic larvae depend on light to capture live prey. Under permanent light, only 25% of larvae showed feeding activity during morning hours, and the use of a 10 hour dark phase photoperiod is recommended (Cañavate et al., 2006).

Senegalese sole larvae suffer a dramatic metamorphosis during the first 20-30 days, involving the migration of the left eye, and the transition from a pelagic life to the settlement in the bentos. Larval development experiences 5 stages according to morphological criteria (Padrós et al., 2011). Recently hatched larvae measure 2.2$\pm$0.1 mm to

2.8±0.01 mm of total length (Dinis et al., 1999). Two days after hatching (DAH) the mouth and the anus become functional (Ribeiro et al., 1999a). On 11 DAH the metamorphosis begins and larvae start to settle and have to be transferred to bottom tanks. On 15 DAH larvae measure 8 mm in total length (Dinis et al., 1999). Metamorphosis is complete between days 20 to 37 (Padrós et al., 2011). Gastric glands appear around 27 DAH, and at 31 DAH sole larvae are capable of ingesting, digesting and absorbing nutrients, with a morphologically and enzymatically complete digestive tract (Ribeiro et al., 1999a). On 40 DAH, larvae measure 16±0.84 mm and may be transferred to the ongrowing tanks (Dinis et al., 1999).

Much research has been devoted to the ontogeny of the gastrointestinal tract of sole larvae at the histological and physiological level. Acid and alkaline proteases are identified at early stages of development, and the former increase its activity as development advances. Lipase activity peaks between 6 to 10 DAH, related to the development of the exocrine pancreas and the onset of metamorphosis (Martínez et al., 1999). From 21 DAH to 27 DAH, alkaline phosphatase increases importantly, reflecting the development of the brush border membranes of enterocytes, indicating that from 27 DAH the enterocytes are mature and fish acquire adult mode of digestion (Ribeiro et al., 1999b).

Amino acid profile changes during ontogeny (especially metionine, tyrosine and cysteine), until larvae settle (Aragão et al., 2000, 2004a). Lysine and arginine are indispensable amino acids (IAA) in this species (more than 80% of those amino acids are retained in 32 DAH post-larvae when administered in the diet), and larvae use dispensable amino acids as energy source instead (Rønnestad et al., 2001).

Survival of larvae vary greatly depending on feeding strategies, diet, environmental parameters or weaning strategies. Nevertheless, Senegalese sole shows low mortalities during the larval stage compared to other marine fish species (Conceição et al., 2007). Standard figures of survival are in the range of 74.5%±2.9 to 81.1%±7.8 in aquaculture tanks fed with enriched *Artemia* and rotifers (Cañavate et al., 2006), although larvae fed on natural prey in net cages in the bottom of earthen ponds showed good survival (65%±26%), reaching 90-99% in some occasions (de Castelo Branco et al., 2006).

Growth rates of larvae vary greatly depending on metamorphosis state. Cañavate et al. (2006) observed specific growth rates (SGR) of 1.18% for pelagic larvae and 0.81% for metamorphic stages for larvae fed on enriched live prey. Two month-old post-larval sole stocked in net cages at the bottom of earthen ponds in the Tagus and Sado estuaries reached a SGR of 3.18% feeding on natural prey. After 7 months these soles were 11.78±2.26 cm (an estimated of 13.98 g) (de Castelo Branco et al., 2006).

An important problem that faces larval rearing is the occurrence of malformations and pigmentation abnormalities that are observable in varying degrees in many facilities (Flos et al., 1998). In some cases, up to 44% of larvae present at least one skeletal malformation, mainly in the caudal region and in the vertebral column. These deformities are likely reflecting rearing and/or feeding conditions (Gavaia et al., 2002). For example, eye migration was affected by docohexanoic acid (DHA) concentration (Villalta et al., 2005b) and by lighting, as 1.8% of the larvae reared under permanent lighting showed an incomplete eye migration after metamorphosis (Cañavate et al., 2006).

Although some authors report negligible levels of altered pigmentation under routine feeding procedures (Cañavate et al., 2006), in some cases up to 11% of juveniles show pigmentation problems in form of albinism, partial depigmentation (most of the cases) or abnormal nadiral pigmentation (Soares et al., 2002). It has been suggested that excessive eicosanoid production, or inadequate regulation of tyrosinase, could be related to deficiencies in pigmentation, as growing dosages of arachidonic acid were correlated with depigmentation (Villalta et al., 2005a).

Senegalese sole larvae are usually fed with *Artemia* nauplii, and after 9 DAH nauplii enriched with commercial lipid emulsions or micro-algae. From 12 DAH frozen enriched metanauplii are offered, which are easier to catch by benthic larvae. There is no need to feed rotifers, although sometimes they are offered until 4 DAH to ensure high levels of highly unsaturated fatty acids (HUFA) (Dinis et al., 1999; Aragão et al., 2000; Imsland et al., 2003; Conceição et al., 2007).

Nutritional requirements for sole larvae are still poorly known, and big efforts are being made to maximize survival and growth, but also to minimize common problems as skeletal malformations and pigmentation abnormalities. Several dietary amino acid deficiencies have been detected during the ontogenic development, not only due to different requirements depending on the development stage, but also because of diet composition heterogeneity (Aragão et al., 2004a). Feeding with a balanced dietary amino acid profile increased the amino acid retention and might improve growth and nitrogen utilisation (Aragão et al., 2004b; Conceição et al., 2007).

There is a likely requirement for long-chain poly-unsaturated fatty acids (PUFA) such as eicosapentanoic acid (20:5n-3; EPA) and docosahexanoic acid (22:6n-3), since there are no evidences of bioconversion from their precursors, and also a requirement for arachidonic acid (20:4n-6; ARA) as it is necessary during whole development (Vázquez et al., 1994) and are strongly retained in the tissues relative to the diet (Villalta et al., 2005a). Imbalances of essential fatty acid levels (EPA, DHA, ARA) and their ratios can cause intestinal problems (i.e. steatosis) (Boglino et al., 2012b). Requirements of DHA in Senegalese sole are low compared to other pelagic larvae, maybe related to a predominance of EPA instead of DHA in the benthic fauna that constitutes settled larvae food items in the wild (Villalta et al., 2005b). Lipid levels in the diet do not affect amino acid absorption, suggesting that absorption mechanisms for lipids and amino acids are fundamentally different (Morais et al., 2005).

There are several commercial *Artemia* enrichment formulae. The election of this formula may influence growth, intestinal maturation, metamorphosis and osteogenesis processes, although from 6 formulae tested, none promoted significant differences in survival or incidence of skeletal deformities, and none of them was optimal for proper larval performance and survival (Boglino et al., 2012a).

Co-feeding involves offering growing amounts of inert feed accompanying live prey at some point of the larval rearing. Early introduction of co-feeding microdiets with *Artemia* improve survival rates, but it also promotes lower growth rates and higher size dispersal than larvae fed only live prey (Conceição et al., 2007; Gamboa-Delgado et al., 2011). Opposite results have also been found (Cañavate & Fernández-Díaz, 1999), and in some cases inert diet-fed larvae have shown histological alterations in hepatic and gastrointestinal structures and/or activities, influencing nutrient assimilation (Fernández-Díaz et al., 2006). Generally, sole digestive maturation improves with early co-feeding (from mouth opening) and at the end of weaning (68 DAH). Co-fed larvae were larger and had better tail condition, as an indicator of better nutritional status and physiological condition, with no effects on survival rates or skeletal deformities whatsoever (Engrola et al., 2009).

Some approaches to alternative larval nutrition have been undertaken as substitution of fish meal by vegetal protein (soy protein concentrate; Aragão et al., 2003) or co-feeding with preserved (-80° C) copepods, that have shown improved lipid assimilation, growth, survival and tolerance to captivity (Piccinetti et al., 2012).

Weaning is more difficult and less reproducible in sole than in other marine fish species. Usually 2 approaches can be taken: co-feeding with both enriched *Artemia* and inert diet and progressive substitution of the live prey, or sudden weaning. The latter consists in offering inert diet to one or two-day fasting post-larvae. It has been successfully applied to common sole post-larvae with high survival rates when incorporating hydrolysed fish protein concentrates in the diet (Day et al., 1997). Weaning has to be done with larvae less than 10mg dry weight. The relatively high mortality in this stage may be related to hygiene problems, as the larvae are in close contact with the bottom of the tank (Conceição et al., 2007).

1.6 Ongrowing

Ongrowing of sole is carried out under 2 major strategies. Traditionally, soles were reared in earthen ponds in old salt marshes in Spain and the South of Portugal, or other deltaic or estuarine environments. Fingerlings were either passively captured in these ponds, or stocked after larval rearing in the hatchery. Feeding of soles could rely in the occurrence of natural prey in the bottom of the ponds, with few or nil effort from the aquaculturist, or supplementing or replacing these feed items with inert feed, taking a semi-intensive approach. This strategy has been used simultaneously as a complement to intensive gilthead sea bream rearing. In such cases, variable survival and culture yield may be obtained. Unweaned juveniles stocked in earthen ponds at 2 individuals$\cdot$m^{-2} showed a survival of 20% and a final weight of 40.3$\pm$2.5 g after 1 year, while 8% of stocked fish in gilthead sea bream ponds, with feed supplementation, reached weights of 456.1$\pm$3.6 g (Dinis et al., 1999).

On the other hand, although salt marshes are still used for semi-intensive and intensive sole aquaculture, the intensive approach is nowadays the leading trend. Sole is stocked in fiberglass or concrete tanks, often in shallow raceways (Imsland et al., 2003), for the whole production cycle, and fed inert feeds in highly controlled environments.

1.6.1 Environmental conditions

There are no studies about the optimal rearing temperature of Senegalese sole, but generally it follows natural thermoperiod or is kept around 20° C (Ambrosio et al., 2008; Salas-Leiton et al., 2008; Borges et al., 2009; Costas et al., 2011). As an approximation, in *S. solea* under natural thermoperiod, maximum growth occurs between May and June, at temperatures ranging 20 to 25° C, and growth during the winter months, with temperatures reaching up to 3° C, is negligible (Palazzi et al., 2006). It has been observed that temperatures above 25 ° C entail higher risks of pathologies, as viral encephalopathy and retinopathy in common sole (Palazzi et al., 2006), but it has also been observed in Senegalese sole, although the causative pathogens were not identified.

Sole is an euryhaline species and can tolerate a range of salinities from 30‰ (Rueda-Jasso et al., 2004) to 38‰ (Salas-Leiton et al., 2008; Ambrosio et al., 2008). It is also a species that tolerates low levels of dissolved oxygen and has grown optimally in undersaturated environments (Salas-Leiton et al., 2008).

There are no studies devoted to assess the optimal ongrowing photoperiod for sole, although it has been observed that sole is strongly influenced by it, as seen from a clearly nocturnal activity pattern, with locomotor activity peaking in the first part of the dark period, and progressively decreasing during the night. Light also influences daily plasma melatonin rhythm, that shows similar dynamics to the typical melatonin profile, with low values during the day and high values during the night (Bayarri et al., 2004).

Photoperiods for ongrowing of sole indoors are usually 12L:12D (Rueda-Jasso et al., 2004; Salas-Leiton et al., 2008; Valente et al., 2011), although simulations of natural photoperiod have also been used (Ambrosio et al., 2008) in the laboratory and in outdoor tanks. Independently of the rearing system, usually tanks are provided with some shading or some means to keep light intensity at the surface between 80 and 350 lx (Salas-Leiton et al., 2008; Boluda Navarro et al., 2009).

Diel rhythms have been widely studied in many species of vertebrates including fish (Iigo & Tabata, 1996; Thetmeyer, 1997; Okimoto & Stetson, 1999; Reebs, 2002). The presence of an endogenous circadian oscillator allows the animal to synchronize its activity periods to the time of day when food is more available, or when risk of predation is lower (Álvarez & Nicieza, 2003), and even to set up a sleep period in some fish species to avoid excessive spending of energy when food items are less likely to be present in the environment (Reebs, 2002). This internal oscillator can be synchronized by an external input, the so-called *zeitgeber*, in the form of darkness-light succession (Aranda et al., 2001; Herrero et al., 2005), or the periodic availability of some food resource as it clearly occurs in a fish farming facility (Sánchez-Vázquez et al., 1995; Reebs, 2002 and references therein).

This aspect of fish biology becomes of a crucial importance when a particular species is going to be farmed, as adjusting the environmental conditions (lighting, temperature) or feeding schedules can have a huge impact in the performance of the culture, from spawning (Bromage et al., 1984; Blanco-Vives & Sánchez-Vázquez, 2009; Oliveira et al., 2009a; Vinagre et al., 2009; Oliveira et al., 2011) to larval rearing (Cañavate et al., 2006; Harboe et al., 2009; Villamizar et al., 2009; Blanco-Vives et al., 2011b) or growth efficiency (Jørgensen & Jobling, 1992; Azzaydi et al., 2000). Particularly in this aspect, feeding European sea bass during night hours in winter, when this species shows nocturnal behaviour (Sánchez-Vázquez et al., 1998), showed better SGR and feed conversion ratio (FCR) then sea bass fed during the photophase (Azzaydi et al., 2000), suggesting that adjusting feeding schedules to the biological rhythms requirements of the species can improve growth in culture. Differences of growth revealed by changes of the feeding schedule have also been linked to the foraging/feeding strategy of fish, that can also change in different periods of the year. For example, juvenile Atlantic salmon, a species that feeds mainly in the water column during the daylight hours, can shift from a visual to an olfactory feeding strategy, being able to locate food in the bottom of the tank during the dark periods in the winter (Jørgensen & Jobling, 1992).

Many fish species show a particular feeding behavior, with activity and ingestion patterns that are essentially diurnal, nocturnal or indifferent, and that remain stable during time (Björnsson, 2001; Herrero et al., 2005; Sunuma et al., 2007; Boluda Navarro et al., 2009), or that can vary depending on the season of the year (Sánchez-Vázquez et al., 1998; Metcalfe et al., 1999; Azzaydi et al., 2000; Rubio et al., 2004). Both laboratory trials and fish farming experiences have shown that some fish species can be fed at different times of the day independently of their biological preference, often keeping similar specific growth rates (Champalbert & Le Direach-Boursier, 1998; Harpaz et al., 2005; Herrero et al., 2005).

Senegalese sole has shown clear nocturnal habits for spawning (Oliveira et al., 2009a), locomotor activity (Bayarri et al., 2004), and even a higher metabolic rate during the dark phase (Castanheira et al., 2011). Sole has also a marked nocturnal feeding behavior (Boluda Navarro et al., 2009). Fish farming operations, including

feeding, occur mainly during the light phase, but it has been suggested that this practice could interfere with the natural rhythms and the welfare of this species (Boluda Navarro et al., 2009).

1.6.2 Feeding

As has been mentioned above, Senegalese sole displays clearly nocturnal self-feeding patterns under laboratory conditions, but also under farming conditions, with 77% to 85% of feed demands occurring at night. Therefore, feeding during the photophase could be incompatible with the natural feeding rhythm of sole (Boluda Navarro et al., 2009).

Feeding is usually offered in 4 to 8 meals during daylight hours in the laboratory by automatic feeders (Rema et al., 2008; Valente et al., 2011), or either continuously throughout 24 hours a day (Silva et al., 2009, 2010; Costas et al., 2011), that has been recommended as a strategy to achieve maximal growth and improve FCR (Borges et al., 2009). In some experiments, strictly nocturnal feeding has been offered with automatic feeders in order to adapt it to the natural rhythms of this species (Ambrosio et al., 2008).

Some experiments demonstrated the ability of sole to self-feed successfully. Fish learned to operate self-feeding sensors in the laboratory, being the string sensor model the one that showed more efficiency, leading to lowest food wastes and higher demands. The same sensor proved to be operative under farming conditions, in tanks of 5.6 m of diameter. Self-feeding demand level was attained from the first day, and when reward level was modified, fish modified their feeding activity accordingly, showing an accurate compensatory feeding behaviour (Boluda Navarro et al., 2009).

1.6.3 Nutrition

Senegalese sole in its wild habitat feeds preferentially on polychaetes (i. e. *Hediste diversicolor, Capitella capitata*), crustaceans (orders Tanaidacea, Amphipoda and Decapoda) and bivalve mollusks (i. e. *Scrobularia plana*), all prey sharing the same type of sandy bottom habitat and age has an important influence on type of prey consumption (Garcia-Franquesa et al., 1996). In semi-intensive conditions in earthen ponds in the Sado estuary (Portugal) its diet was composed also by the insect larvae *Chironomus salinarum*. Soles appear to be very selective with their food items, and fish stocked in feed supplemented ponds still fed on benthic organisms (Branco et al., 2010).

Soles display nocturnal feeding habits, and thus rely heavily in olfaction and other chemotactic stimuli for food-search behaviour. When whole homogenates and solid-phase extraction fractions of the polychaetes *Diopatra neapolitana* were added to commercial feeds, the number of movements, time moving, linear velocity, distance travelled and time swimming upstream in a fluviarium increased, while the ablation of the olfactory epithelia results in the disruption of these behavioural responses (Barata et al., 2009).

Thus, efforts have been made in incorporating bivalve flavours, polychaete homogenates and squid meal to the feed, which have had positive effects on feed ingestion, acting as attractants or improving feed palatability in both Senegalese and common soles (Day et al., 1997; Reig et al., 2003a; Barata et al., 2009; Silva et al., 2009, 2010).

Artificial feeds for sole are not well developed, as nutritional requirements and growth performance of soles over 100 g are still poorly known. As the availability of feed formulated for sole is somewhat unreliable, feeding of this species is commonly done with feed pellets developed for other flatfish species like turbot, or formulated *ad hoc* with the composition specified by the aquaculturist.

Regarding the nutritional requirements of Senegalese sole, it has been observed that sole can select from unevenly balanced diets to compose a protein-rich balanced diet, reflecting their carnivorous feeding behavior. When offered 3 experimental diets composed of pure macronutrients, soles selected a diet composed of 68% prot, 15.7% crude fat and 16.3% carbohydrates. When diets were diluted with indigestible carbohydrates (cellulose), fish increased intake to sustain energy levels (5 to 5.2 $kJ \cdot kg^{-1} \cdot day^{-1}$), keeping fat levels unchanged (Rubio et al., 2009).

It has been observed that lipid and carbohydrate energy sources affect its oxidative status. Diets with low levels of lipid (under 11%) and digestible starch reduce the susceptibility of the fish to oxidation and may enhance growth rate. Diets with high lipid levels seem not to be suitable for Senegalese sole, leading to higher susceptibility to oxidation, poor physiological condition, altered liver histology and lipid accumulation in liver, viscera and muscle (Dias et al., 2004; Rueda-Jasso et al., 2004). Borges et al. (2009) observed better growth and FCR of 10 g juveniles with low lipid levels in 5 isonitrogenous diets (56% of protein). Lipid levels under 12% improved nutrient retention and growth, suggesting a maximum level of 8% of lipids in fish, while Valente et al. (2011) described that diets with

15% of lipidic content lead to higher liver vacuolization degree and signs of hepatocyte necrosis, while reduction of lipid levels to 8% had no impact on growth or feed efficiency.

As a carnivorous species, sole needs a high percentage of protein in its diet. Rema et al. (2008) assayed diets with constant lipid levels (100-130 $g \cdot kg^{-1}$) and growing percentage of protein (from 43% to 60%). Fish fed diets under 53% of protein showed lower daily weight gain, SGR, feed efficiency and protein content than fish fed 53% to 60% protein, suggesting that diets for sole should include at least 53% of crude protein.

Substitution of fish meal and protein from marine origin has been already described for sole larvae. Economic cost of fish meal and fish oil, but also the ecologic toll that it may suppose in the long run, has encouraged a series of experiments aimed to assess the feasibility of incorporating larger percentages of plant-origin protein in fish diets. Valente et al. (2011) observed no significant differences in final weight, size dispersion, growth index, FCR nor body composition of adult soles reared during 8 months fed on 3 isonitrogenous diets (59% crude protein) either with a) 15% crude fat and 46% of protein from marine source, mainly fish meal, b) 15% crude fat and 15% of protein from marine origin and the rest from plant origin (soybean meal, soy protein concentrate, corn and wheat gluten), supplemented with calcium phosphate and crystallized arginine, threonine, methionine and lysine, and c) a low energy diet, identical to a) but with lower lipid levels (8%) and less energy. Combining adequate plant protein sources with the inclusion of palatability enhancing feed ingredients (squid meal or fish protein hydrolysate) was proved to be effective in large-sized senegalese sole in terms of growth, survival and feed intake.

Other studies have demonstrated that fish meal can be replaced by plant-source protein without any negative effect on growth, provided that dietary amino acids are balanced, adding crystalline indispensable amino acids when necessary and improving feed palatability with squid meal (Silva et al., 2009, 2010), and that substitution of fish meal by plant protein does not have major effects on lipid content, fatty acid profile and volatile compounds composition of sole's muscle (Fernandes et al., 2012; Silva et al., 2012).

1.6.4 Growth

Wild Senegalese soles from cohorts 0+ and 1+ captured in the Tagus estuary from May 2003 to July 2004 showed growth rates that varied between 0.40 to 1.38 mm/day (Fonseca et al., 2006). Besides that study, the only information of growth of sole is taken from rearing experiments, often designed to test a nutritional or management condition. Thus, there is little *raw* information about the true growing potential of farmed Senegalese sole. The best approaches to assess the potential growth rates of sole could be found in studies like the one of Dinis et al. (1999) mentioned before, with unweaned juveniles reaching more than 450 g in one year reared in a polyculture with gilthead sea bream (although with poor survival). Nevertheless, the lack of control of food sources makes it difficult to analyze the results.

A summary of some data regarding Senegalese sole growth is presented in table 1.3.

Growth is also modulated by sexual dimorphism in many species as European sea bass (Gardeur et al., 2001a; Saillant et al., 2001) and flatfish species as turbot (Imsland et al., 1997) or half-smooth tongue sole (*Cynoglossus semilaevis*; Ji et al., 2011), with females growing faster than males.

In turbot differences of growth between both sexes are attributed to differences in food intake and sexual maturation, with mature females growing faster than males (Imsland et al., 1997). Something similar occurs in half-smooth tongue sole, as immature individuals didn't show differences of growth between sexes (up to 5 months after hatching). After maturation (9 months) sexual growth dimorphism was evident, with females growing faster than males and growth hormone mRNA levels being significantly higher in females than in males. It is postulated that early maturation of males is behind the sexual growth dimorphism in this species (Ji et al., 2011). Nevertheless, up to our knowledge, no data are available about this particular issue for Senegalese sole, although there are studies that revealed different prey items in the stomach contents of same age class females and males *S. senegalensis* living in the same area of the Ebro delta (Tarragona, Spain), perhaps revealing different nutritional requirements between sexes (Molinero & Flos, 1991; Garcia-Franquesa et al., 1996).

1.6.5 Stocking density

Stocking density has been demonstrated as a crucial variable regarding growth performance of cultured fish. The effects of density on growth are diverse, usually showing a negative correlation in several finfish species as rainbow trout (*Oncorhynchus mykiss*; Refstie, 1977), Atlantic cod (*Gadus morhua*; Lambert & Dutil, 2001) or in flatfish

species like turbot (Irwin et al., 1999). Nevertheless, it has been noted that too low densities can also have a negative effect on growth in species that present schooling behavior as Arctic charr (*Salvelinus alpinus*; Jørgensen et al., 1993) or European sea bass (Papoutsoglou et al., 1998). Stocking density also has an important role during the settling of larvae of Japanese flounder (Bolasina et al., 2006), and it is also involved in fish welfare in many species (Ashley, 2007), like rainbow trout (Ellis et al., 2002; North et al., 2006), Atlantic salmon (Turnbull et al., 2005) or Atlantic halibut (*Hippoglossus hippoglossus*; Kristiansen et al., 2004). Senegalese sole shape can also be significantly affected by stocking density (Ambrosio et al., 2008) with fish reared at high density (180% of initial bottom coverage; 26.6 $kg \cdot m^{-2}$) showing a different evolution of shape compared to low density fish (60% of initial bottom coverage; 8.6 $kg \cdot m^{-2}$).

Mechanisms relating stocking density and growth are not fully understood, but it is generally accepted that, when water quality is not affected by the increased number of fish per cubic meter, and food items are provided in sufficient amounts, differences in growth performance could be attributed to the onset of hierarchies and dominance relationships (Papoutsoglou et al., 1998; Bolasina et al., 2006). Moreover, intrinsic internal factors such as genotype or the interaction among genotype and ongrowing environment, could as well be related to growth performance (Bagley et al., 1994).

In soleid fish contradictory results on how density affects growth have been given by different authors. Schram et al. (2006) found significant effects of density on growth of 35-40 g common soles, stocked at 6 densities between 0.56 and 12.6 $kg \cdot m^{-2}$ for 55 days. SGR decreased with increasing stocking density, and the difference between initial and final coefficient of variation increased with density. The authors found that productivity peaked at 7.4 $kg \cdot m^{-2}$.

Contrarily, Salas-Leiton et al. (2008) assayed four stocking densities between 2 and 30 $kg \cdot m^{-2}$ with 70 g Senegalese sole for 60 days, finding no significant differences in biomass production or growth rates, concluding that Senegalese sole is compatible with high densities under intensive culture. They observed that crowding lead to higher feed ingestion and higher metabolic rates (higher O_2 consumption, correlated to the amount of feed ingested), and that density did not affect size hierarchies concerning feeding.

Costas et al. (2008) stocked 78.8$\pm$18.9 g juveniles at final densities of 4, 9 and 16 $kg \cdot m^{-2}$ during 63 days, finding no differences in growth or feed consumption. However, he found higher plasma cortisol levels and less free amino acids (FAA) in fish held at higher densities, indicating higher stress levels, accompanied by higher occurrence of pathologies. They suggested that high density fish used more FAA, maybe due to higher demands of energy, or for energy production to cope with stressful rearing conditions or due to the synthesis of other metabolites related to the stress response. Accordingly, the authors recommended rearing densities below 9 $kg \cdot m^{-2}$ although they found a great variability in the stress response of different individuals, suggesting that some fish may or may not adapt to high density conditions. On the other hand, crowding may affect IAA requirements in this species.

Salas-Leiton et al. (2010a) also found that stocking soles at high density (30 $kg \cdot m^{-2}$) increased stress 45-fold compared with fish stocked at low density, as showed by higher cortisol levels and by lower expression of some immune response-associated genes, but it didn't affect growth. Concurring with these results, Costas et al. (2011) observed that handling or induced stress did not affect growth of juvenile soles, although stressed fish showed higher levels of cortisol, plasma glucose and lactate levels.

1.6.6 Size variability

Large size variation is an important issue in the aquaculture of Senegalese sole (Flos et al., 1995, 1998, 2001). Under controlled laboratory experiments size variation in sole, measured as standard deviation or as coefficient of variation (CV), usually increases with time, leading to higher differences between fast and slow growers. Borges et al. (2009) found standard deviations ranging from 0.56 to 6.54 in soles of 28.54 to 43.66 g of final weight after 84 days of growth fed with 4 isonitrogenous diets and different lipid levels. Similar results were obtained by Rema et al. (2008) assaying different diets with variable compositions of protein and lipids, finding standard deviations up to 2.09 in 31.6 g soles. With larger fish, these results are amplified, as soles with a final weight between 113 and 121 g showed standard deviations from 24.6 to 27.7 (Costas et al., 2008), and soles with a final weight between 323 and 354 g, fed on different isonitrogenous diets for 8 months (including a plant protein substitution diet), showed standard deviations ranging 28.75 to 51.61 (Valente et al., 2011). Salas-Leiton et al. (2010b) observed size dispersions between 20.5 and 51.8 (standard deviation) in 3 graded groups of soles (from 155 to 212 g of initial weight).

Table 1.3: Senegalese sole growth data according to the bibliography

Initial weight (g)	Final weight (g)	SGR[a]	FCR[b]	Time (days)	Temp (° C)	Protein (%)	Lipid (%)	SD[c] (kg·m^{-2})	Author
23.6±1.2	37.72±1.35 to 43.79±1.2	0.69 to 0.88	2.46 to 3.12	67	20±1	51	11 to 21	–	Rueda Jasso *et al.* 2004
39.7±1.4	–	0.3	0.83±0.2	21	22.5±0.5	68*	15.7*	–	Rubio *et al.* 2009
9.9±1.8	43.7±6.54 to 42.5±1.51	1.2	1.04±0.05 to 1.15±0.03	84	20±1	56	4, 8	1	Borges *et al.* 2009
11.9±0.5	31.6±2.09 to 33.0±0.35	1.17±0.08 to 1.22±0.02	1.02±0.11 to 1.00±0.04	84	21±1	53, 57, 59	10-13	–	Rema *et al.* 2008
180	354±51.61 to 323±28.75	0.5 to 0.6	2.0 to 2.4	240	19±1	59**	15-8	3	Valente *et al.* 2011
9.5	39.8±1.5	1.54±0.05	0.85±0.03	84	20±1	57	6	–	Silva *et al.* 2009
78.8±18.9	113±24.6 to 121.3±27.7	0.63±0.15 to 0.66±0.14	–	63	20±1	–	–	4, 9, 14	Costas *et al.* 2008
70	0ver 100	–	0.99±0.08 to 1.45±0.08	60	19.4±0.27	–	–	2, 7, 15, 30	Salas-Leiton *et al.* 2008
148.32±50.3	–	0.59±0.01 to 0.65±0.01	–	60	18.4±0.09	–	–	7, 30	Salas-Leiton *et al.*2010
317.0±17.67	495.9±28.48	0.35±0.020	–	126	20±1	55	15	8.6±0.17	Ambrosio *et al.* 2008

[a] SGR=Specific growth rate.
[b] FCR=Feed conversion ratio (Feed offered/Weight gain).
[c] Stocking density.
[*] Diet selected by fish based on 3 experimental diets composed of macronutrients.
[**] One diet with fish meal replaced by plant protein.

Stocking density among other environmental parameters such as feed availability (Hatlen et al., 2006), and intrinsic genetic differences between individuals, could be behind differences in growth through the development of complex social interactions, from which strong hierarchization can emerge (Irwin et al., 1999). Size composition in the rearing stocks can also be behind aggressive behavior and competition over resources as food or territory, promoting the onset of hierarchies and affecting fish growth in several ways.

For example, in turbot the CV of fish stocked at 0.39 and 0.77 $kg \cdot m^{-2}$ increased throughout 82 days, indicating the formation of hierarchies with high levels of competition, and leading to bigger fish consistently consuming more feed than smaller fish, and thus consolidating growth differences and size variation (Irwin et al., 2002).

In Japanese flounder, stocking small and large fish lead to growth of small individuals being suppressed by the presence of large ones, by inhibiting their locomotor and feeding activity through constant attacks. Large flounder increased their size differential compared to smaller fish throughout the experiment. When feed was offered in excess, growth differences persisted. It is suggested that physiological stress, caused by dominance-related social interactions, could be an alternative cause of growth depensation in fish, with only subordinates maintaining the stress level after aggressive encounters (Dou et al., 2004).

In Atlantic cod, feed restriction lead to the occurrence of more fin wounds, with fast growing fish showing less damages. The variation of growth rates increased when feed was restricted, evidencing that competition is an important limiting factor in growth of farmed cod (Hatlen et al., 2006).

Apparently, Senegalese sole doesn't show aggressive behaviours under culture conditions (Salas-Leiton et al., 2008), and larger size individuals don't monopolize the food source when stocked in ungraded conditions, where smaller fish usually access feed earlier (Duarte et al., 2006). Size distribution and its influence on growth has been, nevertheless, little studied in Senegalese sole. Salas-Leiton et al. (2008) graded soles in 3 groups according their size, plus one ungraded group. After 60 days they found that, although grading affected the distribution of individual SGRs, being narrower in the graded groups, individual SGR was independent of fish size. All populations tended to reach the same size variation disregarding whether or not they were graded. These authors suggested that group heterogeneity improves overall growth in sole, probably associated to more efficient social arrangements implying that there is a hierarchical structure caused by fish size distribution, instead being hierarchy which causes size variability.

Salas-Leiton et al. (2011) also observed that soles reared at 31 $kg \cdot m^{-2}$ showed higher standard deviations of individual SGRs than soles reared at 6 $kg \cdot m^{-2}$, although density didn't affect size variation, and that soles fed either 0.25% or 1% of body weight per day had equivalent individual SGR CVs. These authors suggested that there is a strong social effect behind growth of soles under culture conditions.

1.6.7 Growth experiments

Gardeur et al. (2001b) postulated that often growth experiments fail in finding significant differences between treatments due to the inter-individual variation in growth potentiality, which diminishes the statistical power of many growth studies approached from the classical analysis of variance point of view. This can be especially relevant in species like sole, that as has been previously noted, typically displays high size variation and a variable individual SGR.

A way to overcome these problems is to work with individualized fish and to apply a proper statistical methodology to extract as much information from the data as possible.

There is an increase of the reliability of change (e. g. growth) measurement through the collection of successive waves of data on the same individual. Such multi-wave approach allows to fit a model for each individual set of growth records, summarizing its individual growth (Willett, 1989).

Mixed-effects models are a refinement of generalized linear models that take into account random effects as well as fixed effects to better describe the variance and covariance of the sample, thus providing a better resolution than generalized linear models. Fixed effects are unknown constants to be estimated from the data, while random effects influence the variance-covariance structure of the response variable. This method can be used when data present temporal pseudoreplication as each individual is measured several times as it grows during the course of an experiment (Crawley, 2007).

Fish tagging for individual identification purposes has been widely used in aquaculture experiments with passive transponder tags (Imsland et al., 1997; Anguís & Cañavate, 2005), ink marks (Reig et al., 2003b; Ambrosio et al., 2008), or diverse external tags like elastomers or polymeric clay (Reig et al., 2010; Salas-Leiton et al., 2010b)

among other methods. Other approaches to individual identification, as the less invasive photo-identification method, have been implemented both in the field and in the laboratory in several animal species, taking advantage of conspicuous singularities like the spots of the whale shark skin (*Rhincodon typus*; Arzoumanian et al., 2005), facial markings in loggerhead sea turtles (*Caretta caretta*; Schofield et al., 2008), color patterns in painted crayfish (*Palinurus versicolor*; Frisch & Hobbs, 2007) or fin edges in cetacean species (Parra et al., 2006).

Senegalese sole could be a fish species to be easily identified visually, mainly through the comparison of the spot patterns of their skin, but also comparing skeletal deformities and pigmentation abnormalities, which are rather common in hatchery reared sole (Gavaia et al., 2002; Soares et al., 2002; Villalta et al., 2005a). This species is thus a potential candidate to be individualized in growth experiments through photo-identification.

1.7 Justification of the thesis

As it has been presented earlier, Senegalese sole is a species that has all in its favor to be a successful new product in Mediterranean aquaculture. It is a valued species, known by the consumer and some trials show its interesting growth potential.

Unfortunately, some aspects of Senegalese sole rearing have eluded full control and repeatability, and more research is still necessary to reach a level of technification comparable to that of other Mediterranean species.

Among the several aspects needing wider knowledge in order to reach optimization of sole aquaculture (reproduction, nutrition), there is an important area that has been overlooked until the last years, which is the ongrowing stage from juveniles to the commercialization size. The difficulty in obtaining enough individuals to set up growth experiments of enough duration, has biased research to working with larvae and post-larvae. Eventually reproduction and larval rearing will succeed and get to an *industry* level, but there are still management issues that needs to be elucidated to take that post-larvae to the commercial size, like the relationships between individual growth and rearing parameters as stocking density, or the size composition of stocks. It is also important to evaluate growth according to the feeding rhythms to maximize growth, but also welfare, offering feed at adequate times for this species.

Not less important is to assess the genetic variability of farmed Senegalese sole stocks, and compare it to the wild populations, as a stepping stone to link genetic traits to the performance of aquaculture stocks.

1.8 Objectives

The aims of the present thesis were:

1) To take an individual-based approach to growth, and growth sexual dimorphism, of Senegalese sole reared at high and low stocking densities, by fitting linear mixed-effects models on individually tagged fish, and to link growth parameters to physiological growth indicators such as the RNA/DNA ratio.

2) To assess the influence of initial size variability on growth of individualized Senegalese sole under high and low stocking densities.

3) To assess the feasibility of an individual photo-identification method in Senegalese sole.

4) To assess whether feeding soles during the dark phase could improve growth compared to fish fed during the light phase.

5) To compare the level of genetic variability of a farmed Senegalese sole population with its wild donor population by determining the nucleotide sequence of the hypervariable mtDNA control region I (CR-I), and by analyzing six microsatellite loci, and additionally, to perform a preliminary exploration of the genetic relationships between Atlantic and Mediterranean Senegalese sole populations.

6) To use mtDNA and microsatellite data to infer sibship relationships within wild and farmed sole samples.

1.9 Description of the structure of the thesis

This thesis consists of 8 chapters. A first introductory chapter, plus 4 chapters, each one presenting a research article, published in –or submitted to– international journals of relevance in the aquaculture and fisheries sciences,

a chapter for general discussion of the results, a chapter with the conclusions and a chapter with the bibliographic references.

Objective 1 has been studied in chapter 2 and has been published in: Stocking density and sex influence individual growth of Senegalese sole (*Solea senegalensis*). Sánchez, P., Ambrosio, P. P., & Flos, R. (2010) *Aquaculture*, 300, 93–101.

Objectives 2 and 3 have been studied in chapter 3 and have been published in: Stocking density affects Senegalese sole (*Solea senegalensis*, Kaup) growth independently of size dispersion, evaluated using an individual photo- identification technique. Sánchez, P., Ambrosio, P. P., & Flos, R. (2011) *Aquaculture Research, In Press*.

Objective 4 has been studied in chapter 4 and has been submitted to the journal *Aquaculture* Growth of the nocturnal Senegalese sole (*Solea senegalensis*) is light independent. Sánchez, P., Ambrosio, P. P., & Flos, R. *Aquaculture. Submitted*.

Objectives 5 and 6 have been studied in chapter 5 and have been published in: Loss of genetic variability in a hatchery strain of Senegalese sole (*Solea senegalensis*) revealed by sequence data of the mitochondrial DNA control region and microsatellite markers. Sánchez, P., Viñas, J., Alvarado-Bremer, J. R., Ambrosio, P. P., & Flos, R. (2012) *Scientia Marina*, 76(2), 225–235.

Chapter 2

Stocking density and sex influence individual growth of Senegalese sole (*Solea senegalensis)*

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Stocking density and sex influence individual growth of Senegalese sole (*Solea senegalensis*)

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ABSTRACT

Growth is usually inversely correlated with stocking density of fish in culture. Senegalese sole aquaculture is affected by a high size variability and thus, this work tried to investigate the relationship of growth with density of two populations of 96 individually tagged Senegalese sole (318.7 ± 7.9 g; mean ± standard error of the mean). Fish were reared at low (LD) and high (HD) density (60% and 180% of bottom coverage respectively) for 195 days. After 134 days (period 1), density conditions were exchanged between groups. Mean weight, standard length, maximum width and centroid size were calculated for each of the 11 census days of the experiment. White muscle biopsies were taken in 7 of the census days in order to assess the RNA/DNA ratio, as a biochemical indicator of growth. Stocking density had an important effect on growth, as fish reared under HD showed poor or no growth during a 'lag phase' on the first 61 days of the experiment, leading to a significantly lower specific growth rate (0.23 ± 0.014) for period 1 compared with LD fish (0.34 ± 0.016). Fitting of linear mixed-effects (LME) models for the first 134 days of experiment showed a significant effect of density and sex on all the assessed biometric parameters. These results could be attributed mainly to the first 61 days of the experiment, as no differences were observed between days 61 and 134 in all the measurements, except for standard length, that showed to be lower for HD fish throughout the whole period. Fish reared under high density tended to grow slower than fish held at low density, while females showed faster growth than males, particularly in HD. Nevertheless, due to high size variability, no significant differences could be found in the mean values of weight or standard length after 134 days (467.2 ± 21.6 g and 28.7 ± 4.2 cm; 502.6 ± 22.5 g and 29.7 ± 4.5 cm for HD and LD fish respectively). Size variability could be an indicator of the onset of hierarchies, being stronger and with more females as dominant individuals than males in HD. After exchanging densities, and up to day 195, a similar lagging effect could be observed in LD fish exposed to high density, suggesting that a sudden change in density, more than density itself, could be the responsible for a detrimental effect on growth. RNA/DNA ratios, were significantly lower for HD fish between days 20 and 61.

1. Introduction

Senegalese sole has been the subject of thorough research in the last two decades, because of its high market demand, high market value and the adaptability of existent facilities to accommodate its rearing (Imsland et al., 2003). These facts present sole as an interesting new species for diversifying Mediterranean marine aquaculture.

Consumers purchase as sole indistinctly *Solea solea* or *S. senegalensis* (Reig et al., 2000), and different results have been obtained to date in terms of reproduction, growth or, in general, success in the consistent and reliable supply of farm-reared sole of both species to the market. Traditionally, Southern Europe countries have been more focused in *S. senegalensis* aquaculture among both species of sole, due to the lower spawning temperature requirements of *S. solea* (Howell, 1997) and the high abundance of *S. senegalensis* in Mediterranean and Southern Atlantic waters (Dinis et al., 1999), the former being nowadays the only sole species reared in Spain or Portugal.

Following some promising trials in early 80s, rearing of *S. senegalensis* has succeeded in key points such as reproduction (Anguis and Cañavate, 2005), weaning (Cañavate and Fernández-Díaz, 1999; Engrola et al., 2009), or nutrition (Rønnestad et al., 2001; Aragão et al., 2003; Morais et al., 2006; Conceição et al., 2007). However, diverse growth performance and high size variability is still an important issue when rearing Senegalese sole in captivity (Dinis et al., 1999; Flos et al., 1995, Flos et al., 2001; Rueda-Jasso et al., 2004).

Stocking density has been demonstrated as a crucial variable regarding growth performance of cultured fish. The effects of density on growth are diverse, usually showing a negative correlation in several finfish species as rainbow trout (*Oncorhynchus mykiss*) (Refstie 1977), Atlantic cod (*Gadus morhua*) (Lambert and Dutil, 2001) or in flatfish species like turbot (*Scophthalmus maximus*) (Irwin et al., 1999). Although, it has been noted that too low densities can also have a

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negative effect on growth in species that present schooling behavior as Arctic charr (*Salvelinus alpinus*) (Jørgensen et al., 1993) or sea bass (*Dicentrarchus labrax*) (Papoutsoglou et al., 1998). Stocking density also has an important role during the settling of larvae of Japanese flounder (*Paralichthys olivaceus*) (Bolasina et al., 2006), and it is also involved in fish welfare in many species (Ashley 2007), like rainbow trout (Ellis et al., 2002; North et al., 2006), Atlantic salmon (*Salmo salar*) (Turnbull et al., 2005) or Atlantic halibut (*Hippoglossus hippoglossus*) (Kristiansen et al., 2004). Senegalese sole shape can also be significantly affected by stocking density (Ambrosio et al., 2008).

Mechanisms relating stocking density and growth are not fully understood, but it is generally accepted that, when water quality is not affected by the increased number of fish per cubic meter, and food items are provided in sufficient amounts, differences in growth performance could be attributed to the onset of hierarchies and dominance relationships (Papoutsoglou et al., 1998, Bolasina et al., 2006). Moreover, intrinsic internal factors such as genotype or the interaction among genotype and ongrowing environment, could as well be related to growth performance (Bagley et al., 1994).

Growth is also modulated by sexual dimorphism in many species as sea bass (Gardeur et al., 2001a; Saillant et al., 2001) and turbot (Imsland et al., 1997), with females growing faster than males, although, up to our knowledge, no data are available about this particular issue for Senegalese sole.

In soleid fish contradictory results on how density affects growth have been given by different authors. Schram et al. (2006) found significant effects of density on growth in common sole, but Salas-Leiton et al. (2008) assaying four stocking densities between 2 and 30 kg m^{-2} with Senegalese sole did not find any significant differences in biomass production or growth rates.

Gardeur et al. (2001b) postulated that often growth experiments fail in finding significant differences between treatments due to the inter-individual variation, which diminishes the statistical power of many growth studies approached from the classical analysis of variance point of view. A way to overcome these problems is to work with individualized fish and to apply a proper statistical methodology to extract as much information from the data as possible. Mixed-effects models are a refinement of generalized linear models that take into account random effects as well as fixed effects to better describe the variance and covariance of the sample, thus providing a better resolution than generalized linear models. Fixed effects are unknown constants to be estimated from the data, while random effects influence the variance–covariance structure of the response variable. This method can be used when data present temporal pseudoreplication as each individual is measured several times as it grows during the course of an experiment (Crawley, 2007).

The aim of the present work was to take an individual-based approach to growth, and growth sexual dimorphism, of Senegalese sole reared at high and low stocking densities, by fitting linear mixed-effects models on individually tagged fish.

2. Material and methods

2.1. Density definition and experimental layout

Sole life habits are closely related to the sea bottom, including burrowing in sandy substrates to avoid predators or browsing for food items. Thus, a surface/surface criterion was chosen to define different densities over the mass/volume or mass/surface criterion, widely used in other fish species of symmetrical body. Density was thus calculated as a percentage of tank bottom covered by fish body surface. Following this criterion, 2 densities were defined: a low stocking density (LD) where enough bottom surface should make fish overlapping unnecessary (set at 60% of bottom surface covered by fish) and a high stocking density (HD) where fish overlapping was granted (set at 180% of bottom surface covered by fish). Fish surface estimation was individually calculated assuming that sole shape could be assimilated to an ellipse.

The three experimental tanks (0.88 m^2 of bottom surface and a volume of 700 l) were each equipped with two movable dividers that split each tank in 2 experimental units of independent and adjustable surface, and thus, allowing for the control of stocking density.

Fish were obtained from a fish farm in the Ebro river delta, in the NE coast of Spain, and conditioned to the experimental tanks at a low density (50% of bottom occupation) for 62 days at the Mediterranean Marine and Environmental Research Centre (CMIMA) in Barcelona, Spain. A total of 96 Senegalese sole (318.7 ± 7.9 g; mean ± standard error of the mean) were individually color-tagged (Reig et al., 2003), weighed, measured, and randomly distributed among the 6 experimental units (16 fish each). Subsequently, HD and LD treatments were randomly assigned to the 2 experimental units of each tank, and the available bottom surface for each treatment was set with the movable dividers. However, at day 77, due to fish size and tank size constraints, it was necessary to set a definitive value for available area and, thus, stocking density grew proportionally from then on. Stocking density at days 1, 134 and 195 are shown in Table 1.

The experiment lasted a total of 195 days and biometric data (weight (WG), standard length (SL), total length (TL) and maximum width (WD)) were gathered for each individual on days 1, 20, 40, 61, 77, 103, 126, 134, 147, 161 and 195. From days 134 to 195 treatments were reverted, in a way that fish under low density conditions were then under a high stocking density and vice-versa (albeit keeping their original names LD and HD), thus defining 2 experimental periods: period 1 (P1) from day 1 through day 134, and period 2 (P2) from day 134 through day 195.

On census days, all fish were anesthetized by immersion in sea water with MS-222 (200 mg l^{-1}), individually identified, measured and then digitally photographed, perpendicularly to their zenithal side, against a highly contrasted background provided with a printed scale.

To obtain a small sample of tissue to determine the RNA/DNA ratio throughout the experiment, a small biopsy of white epaxial muscle tissue was carried out with a 18 gauge cutting biopsy needle (Biopince, Amedic) at every census day except on days 1, 161 and 195 (Sánchez et al., 2003).

Besides the standardized biometric measures, centroid size (CS) for each individual was also computed from the digitalized images using the software tpsRegr v. 1.28 (Rohlf, 2003). Centroid size is a potentially interesting way of assessing fish growth, as it is a measure that is mathematically independent of shape in the absence of allometry (Zelditch et al., 2004).

At the end of the experiment, fish were sacrificed by anesthetic overdose and immersion in chilling water. Sex was determined by dissection and visual inspection of the gonad in all fish except 2 LD individuals.

2.2. Environmental conditions

Fish were kept in a flow-through circuit of sea water that flow into the tanks through a vertical pipe perforated every ten cm from the surface to the bottom of the tank in order to homogenize the environmental conditions as much as possible. Water flow (30% of the tank volume per hour), temperature (20 ± 1 °C), salinity (38.2 mg l^{-1}) and O_2 (>5.0 mg l^{-1}) were monitored daily. Photoperiod for 41.23° N latitude from July to January with artificial dusk and dawn was simulated with fluorescent light dimmed by shading covers laid over the tanks.

On day 77, a disease burst affected most of the individuals of one tank. This replicate was eliminated and, as a prophylactic measure, siliceous aquarium gravel (2 to 4 mm diameter) was added to the remaining tanks.

Table 1
Initial and final stocking density in periods 1 (days 1 to 134) and 2 (days 134 to 195) for both high density (HD) and low density (LD) treatments in % of covered bottom and in kg·m^{-2} (mean ± standard error of the mean).

	N	Initial stocking density		Final stocking density (day 134)		Stocking density day 134 (density change)		Final stocking density day 195	
		%	kg m^{-2}	%	kg m^{-2}	%	kg m^{-2}	%	kg m^{-2}
HD	28	180%	26.6 ± 0.2	233%	39.8 ± 0.8	64%	10.9 ± 0.6	65%	11.9 ± 0.6
LD	26	60%	8.6 ± 0.2	65%	11.5 ± 0.2	242%	42.8 ± 1.5	241%	41.4 ± 2.2

2.3. Feeding

Sole is a species that mainly shows an activity pattern during night hours (Bayarri et al., 2004), thus feeding was scheduled in four feed takes, spread from dusk to dawn (at 30 min before dusk, 00:00 h, 03:00 h and 30 min before dawn) with electronic programmable feeders. Daily ratio was set to 0.6% of tank biomass day^{-1}, after being optimized during the conditioning period to minimize uneaten feed. A commercial feed for sole (ProAqua: 55% of gross protein, 15% of gross fat, 12% of ashes, 1% of gross fiber and 12% of carbohydrates; 20.3 MJ kg^{-1}, 3 and 5 mm pellet diameter) was fed throughout the experiment. Uneaten feed remains, when visible, were retired every morning.

2.4. Production parameters

In order to give the most powerful resolution to statistical procedures, fish that died at any point of the experiment were not taken into account for calculations.

Growth was described for each stocking density treatment and period averaging the specific growth rate (SGR) of each individual fish calculated as follows,

$$SGR = \frac{\ln(W_f) - \ln(W_i)}{t} \times 100$$

where W_f and W_i stand for the value of weight at the end and at the beginning of each analyzed period respectively, and t stands for the total of days of such period.

Individual fish growth was described for each fish as their individual specific growth rate (IGR) for the whole period 1 (days 1 to 134), and by the intra-individual coefficient of variation (CV_{IGR}) of IGR calculated for each inter-biometry period as follows:

$$CV_{IGR} = \left(\frac{\sigma_i}{\overline{IGR_i}} \times 100\right)$$

where σ_i = standard deviation of all IGRs of the same individual between 2 consecutive measures, and $\overline{IGR_i}$ = average of all IGRs of that individual.

2.5. RNA/DNA ratio

RNA/DNA ratio, as a biochemical indicator of growth, was determined for each fish on biometry days 20, 40, 61, 77, 103, 126, 134 and 147. This method assumes the quantity of DNA per cell is constant, but the quantity of RNA, and particularly rRNA, increases as the cell exhibits a higher rate of protein synthesis. Comparing samples of the same tissue, RNA/DNA will be higher in the ones with higher synthetic activity.

White muscle has not a significant metabolic activity and it is assumed that RNA/DNA ratios of this tissue indicate the synthesis of structural protein of the own muscle. It makes white epaxial muscle in fish a tissue of choice for assessing RNA/DNA ratios in growth experiments (Bulow, 1987). White muscle has been used as a suitable tissue for correlating RNA/DNA ratios with juvenile and adult fish growth (Lied and Roselund, 1984; Grant, 1996).

Normally, analysis of RNA/DNA ratio implies sacrificing the experimental specimens. The fluorescence method used in the present work, needing much less tissue sample than traditional protocols, allowed to assess RNA/DNA ratio of the same individuals along time.

Accordingly, white muscle biopsies of the experimental fish were assayed for RNA/DNA ratios following the fluorescence method proposed by Caldarone et al. (2001). A 0.5 g homogenate of white epaxial muscle of Senegalese sole was used as a control sample while DNA from calf thymus and 18S and 28S rRNA from calf liver (Sigma) were used as nucleic acids standards. Fluorescence readings were carried out in a Synergy HT microplate fluorimeter.

2.6. Data analysis

The effects of stocking density on average growth (weight, standard length, maximum width and SGR) were assessed by the Student's t-test at a significance level of $\alpha = 0.05$.

A cluster analysis (Euclidean distance linked to Ward's association criteria) was performed for initial and final weight of each individual to look for different growth profiles.

Individual and combined effects of stocking density and sex, as independent categorical variables, on individual growth were analyzed by linear mixed-effects models (LME) using the package *nmle* for R software (Pinheiro et al., 2008) for weight, standard length, maximum width, condition index and centroid size as response variables. When data are collected over time on the same individuals, as was the case in the present study, as well as when data are gathered hierarchically, or on related individuals, mixed-effects models are a useful tool that could provide a better fit than generalized linear models (Willett, 1989; Crawley, 2007). LME models take into account the so called fixed effects and random effects to calculate the different coefficients of the model and their significance.

All statistical procedures were carried out with the statistics software environment R (R Development Core Team, 2008).

3. Results

3.1. Effect of stocking density and sex on growth

3.1.1. Analysis of mean growth descriptors

The evolution of weight during the whole experimental period (including P1 and P2) is shown in Fig. 1a and b, for average weight curves and individual weight curves respectively, for both HD and LD. An apparent delay of growth regarding weight could be observed for HD fish from day 1 to day 40, while the mean slope for some time between day 40 and day 61 until the end of P1 behaved similarly for both densities. Such observation led us to consider two new sub-periods for further statistical analysis (sub-period 1a, SP1a, from day 1 to day 61, and sub-period 1b, SP1b, from day 61 to day 134). Nevertheless, the great variability that can be seen in Fig. 1b, made unfeasible to find significant differences in mean weight between densities at the end of both P1 and P2 (Table 2; Fig. 1).

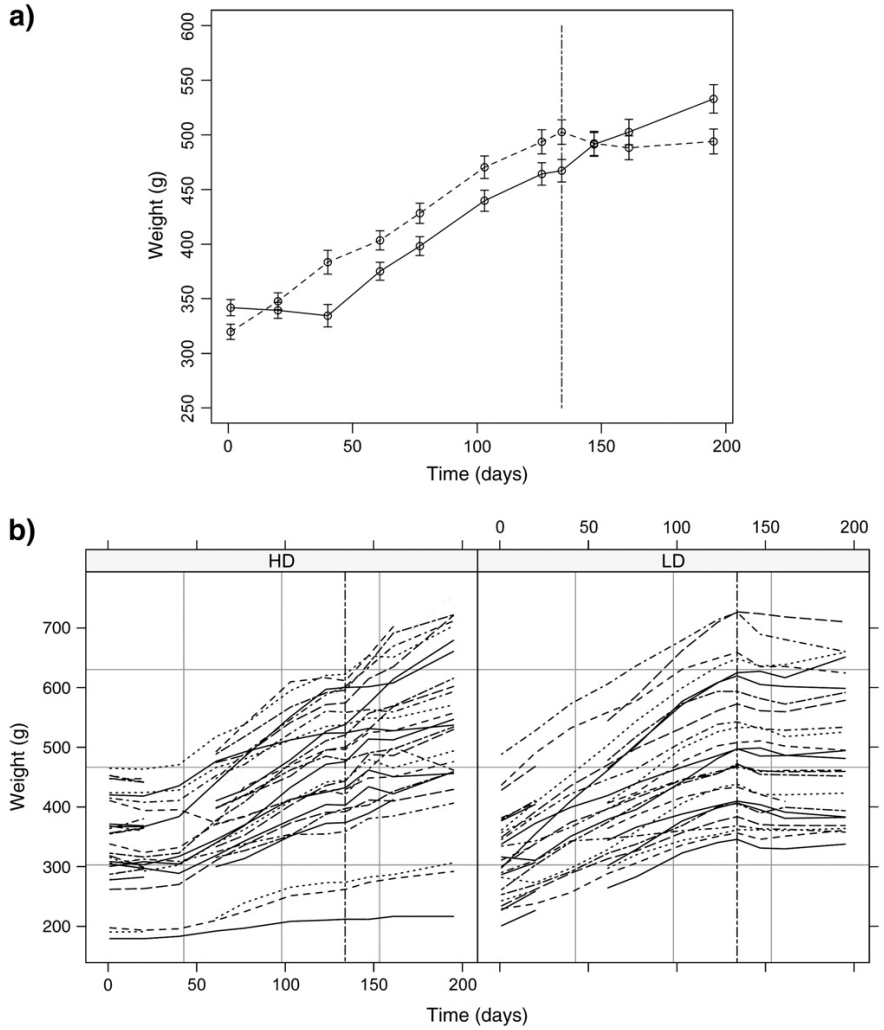


Fig. 1. a) Mean weight ± standard error of the mean over time of both low (LD, dashed line) and high (HD, solid line) stocking densities. b) Individual weight curves for HD (left) and LD (right) during the 195 days of experiment. Vertical dashed lines points to day 134, when density treatments were reverted between groups.

Table 2
Mean values for weight, standard length and maximum width (mean ± standard error of the mean) at the beginning of the experiment (day 1), at the end of sub-period 1a (day 61), at the end of period 1 (day 134) and at the end of period 2 (day 195).

			N	Day 1	Day 61	Day 134	Day 195
Weight	HD	Global	28	341.8 ± 14.7	375.1 ± 17.3	467.2 ± 21.6	533.0 ± 26.0
		Females	12	376.3 ± 16.5*	419.3 ± 19.1*	531.9 ± 24.2**	618.4 ± 31.0**
		Males	16	316.0 ± 21.0*	341.9 ± 22.0*†	419.5 ± 25.7**	468.9 ± 30.9**
	LD	Global	26[a]	319.7 ± 15.4	403.5 ± 17.5	502.6 ± 22.5	494.0 ± 22.8
		Females	8	345.0 ± 25.5	442.4 ± 33.9	563.1 ± 42.9	558.2 ± 41.4
		Males	16	312.3 ± 18.0	393.0 ± 21.2†	485.5 ± 26.6	470.0 ± 25.5
Standard length	HD	Global	28	26.3 ± 3.7	27.1 ± 3.7	28.7 ± 4.2	29.7 ± 4.7
		Females	12	27.2 ± 3.8	28.0 ± 3.9	29.8 ± 4.3*	31.1 ± 4.6
		Males	16	25.7 ± 5.3	26.4 ± 5.3	27.9 ± 5.9*	28.7 ± 6.4
	LD	Global	26[a]	26.1 ± 4.0	27.7 ± 4.3	29.7 ± 4.5	29.8 ± 4.6
		Females	8	26.7 ± 6.3	28.7 ± 7.7	30.8 ± 8.0	31.1 ± 8.1
		Males	16	25.8 ± 5.4	27.4 ± 5.4	29.3 ± 5.5	29.3 ± 5.2
Maximum Width	HD	Global	28	11.2 ± 2.0	11.6 ± 2.0	12.2 ± 2.2	12.5 ± 2.3
		Females	12	11.6 ± 2.2	11.9 ± 2.0	12.7 ± 2.3*	13.2 ± 2.5*
		Males	16	10.8 ± 2.9	11.0 ± 2.9	11.7 ± 2.9*	12.0 ± 3.1*
	LD	Global	26[a]	10.9 ± 1.7	11.4 ± 1.6	12.2 ± 1.8	12.3 ± 1.9
		Females	8	11.3 ± 2.7	11.8 ± 3.3	12.6 ± 3.5	12.8 ± 3.4
		Males	16	10.8 ± 2.4	11.3 ± 1.9	12.1 ± 2.0	12.1 ± 2.2

HD = High density, LD = Low density; significant differences (Student's *t*-test): *between sexes within density (*$P<0.05$; **$P<0.01$) † between males across densities ($P<0.05$).
[a] In LD treatment, sex of 2 fish could not be assessed.

Similar results as those commented above were found when analyzing mean weight according to fish sex. Sex ratio was biased to more males than females in both densities (9:5; male:female, Table 2), being similar to previous experiments. This disequilibrium in numbers of males and females was due to the difficulty of assessing fish sex visually before the onset of sexual maturation.

Mean weight of females was significantly higher than that of males in HD, but not in LD (Table 2), at the end of both P1 and P2. Considering all fish grouped by sex, mean weight was significantly higher for females than for males at days 134 and 195. Although females were in general larger in standard length and maximum width than males, these differences became significant for HD at the end of period 1 ($P<0.05$, Table 2).

Growth studies benefit greatly from individual data, as it allows to compare all individual SGRs, instead of an average calculation describing one SGR per replicate. In the present experiment, when approaching the analysis of growth through average individual SGRs (Table 3, Fig. 2) it could be observed that LD fish presented a significantly higher overall specific growth rate for period 1 than HD fish ($P<0.0001$). Dissecting period 1 in the two sub-periods defined above, resulted in a significantly lower growth of HD fish from day 1 to 61 ($P<0.0001$), but from day 61 mean specific growth rates for both treatments evolved similarly up to day 134 ($P=0.95$). After reverting stocking density treatments between groups, a similar effect could be observed, as fish that originally were stocked at low density showed a significant drop in their mean SGR when exposed to high density stocking conditions. Conversely, fish originally stocked at high density presented a sudden peak in their mean SGR, resulting in a higher overall mean SGR for period 2 ($P<0.0001$).

Specific growth rates analyzed by sex are shown in Table 3. High density males showed significantly lower SGR for sub-period 1a and for the whole period 1 than LD males, and this effect was reverted after switching density conditions. Specific growth rate of females was significantly lower for HD fish only in SP1a and, as above, HD females presented a higher SGR than LD females after the density change.

The analysis of the growth variation showed that weight's standard error of the mean increased linearly throughout the experiment for both densities ($r^2=0.80$, $P<0.001$; $r^2=0.67$, $P<0.01$ for HD and LD fish respectively). Nevertheless, both slopes were not statistically different, indicating no differences in the behavior of size dispersion between densities along time. Moreover, no differences were found between densities in the coefficients of variation of weight and standard length over time (0.23 ± 0.00 and 0.22 ± 0.00 for HD and LD respectively).

3.1.2. Fitting of linear mixed-effects models

Linear mixed-effects models were fitted for each density treatment for period 1 and sub-periods 1a and 1b for weight, standard length, width, condition index and centroid size. The initial model for each variable took into account as fixed effects time, density, sex and their interactions, while time, given the individual, was set as random effect. All models could be simplified either eliminating non-significant interactions or non-significant fixed effects. Significance level was set at 0.05. Plots for the estimated growth models for weight, standard length and width for the whole period 1 are depicted in Fig. 3, while model equations for each variable and sub-period are shown in Table 4.

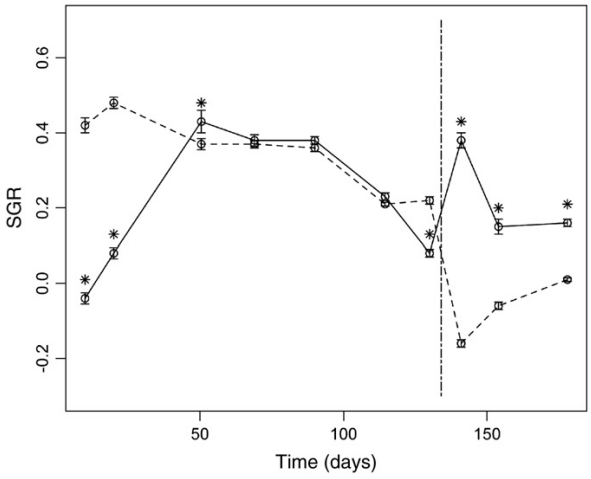


Fig. 2. Mean specific growth rate (SGR) ± standard error of the mean over time of both low (LD, dashed line) and high (HD, solid line) stocking densities. Mean values are calculated between two successive census days. A vertical dashed line points to day 134, when density treatments were reverted between groups. Significant differences (Student's *t*-test $P<0.05$) are marked with an asterisk.

As expected from the previous analysis, in P1 sex had a significant effect on the initial weight ($P<0.05$) and over time ($P<0.001$), with females starting at higher initial weights, and their slope being also higher. Stocking density had also a significant effect on the slope of the model, being higher for LD fish than for HD fish ($P<0.001$). No significant interaction between density and sex was found. For sub-period 1a, the effect of stocking density is significant ($P<0.0001$), and markedly higher than for the whole P1, and sex effects are also significant. Analyzing sub-period 1b, only sex had a significant effect over time ($P<0.01$), and no differences due to stocking density could be found from day 61 to 134.

When analyzing P1 for standard length, a similar result was found. Density and sex significantly affected standard length values over time, being favorable for a faster growth of LD fish and also determining a steeper slope for females than for males. Sex also had a significant effect on initial standard length, females being larger than males.

The separate analysis of periods 1a and 1b for standard length showed somewhat different results than the ones obtained for weight, confirming that density and sex significantly affected standard length during both sub-periods, and not only in sub-period 1a. Nevertheless, density effect was two times higher for sub-period 1a than for sub-period 1b.

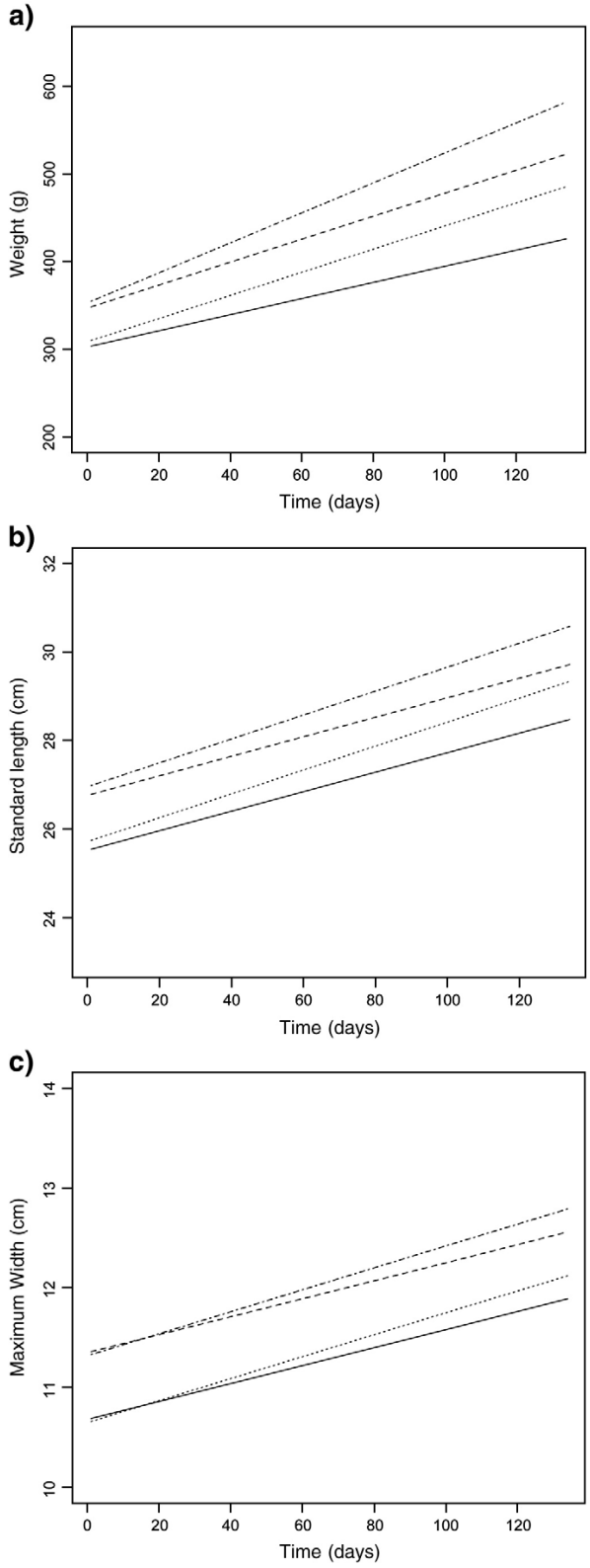
Density had also a significant effect on the evolution of maximum width, that tended to increase faster for LD fish than for HD fish. Although the effect of sex in the intercept of the models was significant, it did not affect growth measured as width. Analyzing sub-periods 1a and 1b, density significantly affected width during SP1a, but, as it was the case with weight, it did not have any significant effect during SP1b.

Highly significant differences in the intercept of centroid size models, as a size measure independent of shape, were found according to sex, being females' centroid sizes higher than males'. Nevertheless, sex did not influence CS evolution over time, but density did. Low density fish presented a significantly higher slope than high density fish.

Table 3
Mean specific growth rate (SGR ± standard error of the mean) calculated from weight data for sub-period 1a (SGR_{1-61}), for sub-period 1b ($SGR6_{1-134}$) for period 1 (SGR_{1-134}), and for period 2 ($SGR_{134-195}$).

		N	SGR_{1-61}	SGR_{61-134}	SGR_{1-134}	$SGR_{134-195}$
HD	Global	28	$0.15\pm0.018^{*}$	0.30 ± 0.017	$0.23\pm0.014^{*}$	$0.21\pm0.017^{*}$
	Females	12	$0.18\pm0.026^{\ddagger}$	0.32 ± 0.022	0.26 ± 0.023	$0.25\pm0.024^{\ddagger\ddagger}$
	Males	16	$0.13\pm0.024^{\dagger\dagger}$	0.28 ± 0.025	$0.21\pm0.016^{\dagger}$	$0.17\pm0.022^{\dagger\dagger\dagger}$
LD	Global	26	0.38 ± 0.025	0.30 ± 0.015	0.34 ± 0.016	-0.04 ± 0.010
	Females	8	0.40 ± 0.054	0.33 ± 0.025	0.36 ± 0.029	-0.01 ± 0.017
	Males	16	0.38 ± 0.030	0.29 ± 0.021	0.33 ± 0.020	-0.05 ± 0.011

HD = High density, LD = Low density, SGR_{j-k} = Specific growth rate for the period comprised between days *j* and *k*. Significant differences (Student's *t*-test): * between global values across densities ($P<0.00001$), ‡ between females across densities ($^{\ddagger}P<0.01$;$^{\ddagger\ddagger}P<0.00001$), † between males across densities ($^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.001$; $^{\dagger\dagger\dagger}P<0.00001$).



Condition index was independent of either stocking density or sex, when taking the whole P1 into account. When analyzing SP1a, there was a significant influence of density, with LD fish presenting a faster increase of CI than HD fish. Conversely, in SP1b, HD fish showed a significantly steeper slope of CI over time.

3.2. Cluster analysis and growth profiles

Cluster analyses computing Euclidean distances among individual initial and final weights for each density were carried out using Ward's association criteria. In both stocking densities the analysis initially distributed all individuals in 4 weight categories, but due to the under-representation of some of the weight categories, and in order to be able to strengthen the statistical analysis, 3 weight groups (large, medium and small) were set up accordingly to the obtained clusters. It was contrasted if the frequencies of females within densities in each weight class at day 134 were the ones expected from the initial frequencies (chi-square test). In both stocking densities the number of females present in the large weight class increased with time, but only in HD it significantly increased from 4 to 8 ($P<0.05$). Conversely, no males initially belonging to small and medium weight classes reached the large weight class at the end of the experiment.

3.3. Inter-individual variability in growth rate

According to Gardeur et al. (2001a) a high individual growth rate (IGR), coupled with a low intra-individual coefficient of variation (CV_{IGR}) could be indicating a dominant fish that is able of a sustained and consistent growth throughout the assessed period. The CV_{IGR} at the end of period 1 was calculated for each fish. As IGR range, but mainly CVI_{GR} values were very different between treatments, a cluster analysis (Euclidean distance, Ward's association method) for the ratio between IGR to CV_{IGR} was performed for each density. In LD up to 11 individuals were considered dominant (3 females and 8 males), with IGR ranging from 0.30 to 0.55 (minimum: 0.18, maximum: 0.55) and CV_{IGR}s from 21.7% to 45.1% (minimum: 21.7%, maximum: 95.9%) while in HD 10 individuals were cataloged as dominant (6 females and 4 males), with IGRs that ranged from 0.22 to 0.38 (minimum: 0.10, maximum: 0.38) and CV_{IGR}s from 36.6% to 84.9% (minimum: 36.6%, maximum: 323.3%). Thus, 37.5% of the females and 50% of the males could be considered dominant fish in LD, while in HD, 50% of the females and 25% of the males could be considered with such a status.

3.4. RNA/DNA ratios

Recovery of RNA and DNA spikes from the control homogenate in Senegalese sole was 91.6% and 93.4% respectively. Mean RNA/DNA ratios for Senegalese sole reared at two stocking densities showed a similar evolution as the observed specific growth rate (Fig. 4). Within sub-period 1a, from day 20 to 61, HD fish showed a significantly lower mean RNA/DNA ratio than LD. These differences disappeared for the rest of period 1, and, similarly to what happened with SGR, an opposite tendency is observed after density exchange between groups.

4. Discussion

Stocking density is an important parameter in fish culture, not only because it has strong implications on growth performance, but also because it can affect fish welfare (Ellis et al., 2002; Turnbull et al.,

Fig. 3. Fitting of linear mixed-effects models for the whole period 1 (days 1 to 134) for a) weight, b) standard length, and c) maximum width, for high density (HD) males (solid line), HD females (small dashed line), low density (LD) males (regular dashed line), and LD females (dashed line with dots).

Table 4
Fitted linear mixed-effects models for weight, standard length, maximum width, centroid size and condition index of Senegalese sole reared under high and low stocking densities (180% and 60% of bottom coverage respectively), during 134 days, and the sub-periods comprised from days 1 to 61 and from 61 to 134.

	Period 1 (days 1 to 134)	Sub-period 1a (days 1 to 61)	Sub-period 1b (days 61 to 134)
Weight	$346.99 + 1.31 \cdot t + 5.97 \cdot sd - 44.35 \cdot s + 0.40 \cdot t \cdot sd - 0.39 \cdot t \cdot s$	$361.48 + 0.72 \cdot t - 7.5 \cdot sd - 47.09 \cdot s + 0.91 \cdot t \cdot sd - 0.28 \cdot t \cdot s$	$340.30 + 1.57 \cdot t - 37.30 \cdot s - 0.42 \cdot t \cdot s$
Standard length	$26.76 + 0.022 \cdot t + 0.19 \cdot sd - 1.24 \cdot s + 0.009 \cdot t \cdot sd - 0.004 \cdot t \cdot s$	$26.76 + 0.022 \cdot t + 0.19 \cdot sd - 1.24 \cdot s + 0.009 \cdot t \cdot sd - 0.004 \cdot t \cdot s$	$26.44 + 0.025 \cdot t + 0.64 \cdot sd - 1.17 \cdot s + 0.005 \cdot t \cdot sd - 0.005 \cdot t \cdot s$
Maximum width	$11.35 + 0.009 \cdot t - 0.03 \cdot sd - 0.67 \cdot s + 0.002 \cdot t \cdot sd$	$11.46 + 0.004 \cdot t - 0.10 \cdot sd - 0.66 \cdot s + 0.005 \cdot t \cdot sd$	$11.32 + 0.01 \cdot t - 0.65 \cdot s$
Centroid size	$43.11 + 0.042 \cdot t - 0.03 \cdot sd - 2.10 \cdot s + 0.012 \cdot t \cdot sd$	$42.95 + 0.053 \cdot t - 2.06 \cdot s$	$41.52 + 0.05 \cdot t$
Condition index	$1.81 + 0.001 \cdot t$	$1.83 + 0.0002 \cdot t - 0.039 \cdot sd + 0.001 \cdot t \cdot sd$	$1.83 + 0.001 \cdot t + 0.035 \cdot sd - 0.0007 \cdot t \cdot sd$

t = time (days); sd = stocking density (categorical variable that takes values 0 or 1 for high density and for low density respectively); s = sex (categorical variable that takes values 0 or 1 for females and males respectively).

2005) and has an economical impact. Social interactions could be behind the differences observed in growth efficiency of several fish species. For example, gilt-head sea bream feeding efficiency has been observed to be affected by ration size, with lower rations leading to increased competition, faster swimming speeds and higher densities under the feeder (Andrew et al., 2004). Similarly, common sole reared at different stocking densities between 0.5 and 12 kg m^{-2} showed a density-dependent growth performance, with productivity maxima at intermediate densities (7.4 kg m^{-2}; Schram et al., 2006). On the other hand, growth efficiency of species presenting schooling behavior can be improved rising stocking densities (Gardeur et al., 2001a). In the present study, different effects of a high stocking density (180% of initial bottom coverage, 26.6 kg m^{-2}) could be identified in growth of cultured Senegalese sole.

Fish reared under HD showed a latency period that resulted in almost no mean biomass gain for the first 40 to 61 days of the experiment. This delay in growth accounted for a subsequent and significant lower specific growth rate for HD fish after 134 days of experiment. As no differences in growth rate could be observed from days 61 to 134, such a slow start following a sudden increase in stocking density (from the low density of acclimatization to the experimental HD) could be greatly responsible for the final differences observed. After day 134, stocking densities were exchanged between tanks and a similar effect could be verified, seeming that, in our conditions, a sudden change in stocking density, instead or added to density itself, could be responsible for the observed period of poor or no growth. However, due to the high variation in growth rate, no differences in mean weight, standard length or maximum width could be verified neither at the end of period 1, nor at the end of period 2. Similarly, Salas-Leiton et al. (2008) did not find any significant differences in final weight in Senegalese sole reared at 4 stocking densities, the highest of which (30 to 45 kg m^{-2} initial and final respectively) was very close to the high density presented in this work (26.6 to 39.8 kg m^{-2} initial and final respectively).

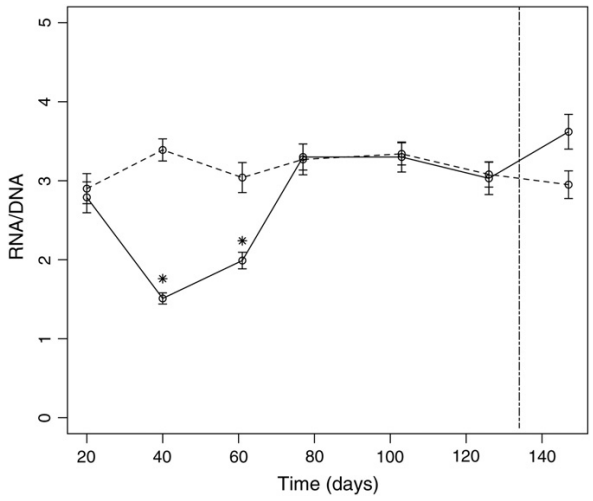


Fig. 4. Mean RNA/DNA ratio ± standard error of the mean over time for both low (LD, dashed line) and high (HD, solid line) stocking densities. A vertical dashed line points to day 134, when density treatments were reverted between groups. Significant differences (Student's t-test, $P<0.05$ are marked with an asterisk).

Nevertheless, the present work was designed as an individual, and more powerful, approach to the analysis of growth of Senegalese sole by gathering biometric data of each tagged fish. With this data, a linear mixed-effects model could be fitted for each biometric parameter, for both period 1, from the beginning of the experiment to day 134, and the sub-periods within, and for period 2, from day 134, when density conditions were exchanged among groups, to day 195.

Weight evolution fitting by LME models showed a significant effect of both density and sex on growth. Again, the first 61 days of growth from the onset of the high density conditions proved crucial for HD fish, as a separate analysis for the sub-periods showed that stocking density had such effect only during SP1a. The significant effect of density for the whole period 1 could be then assigned to the above mentioned lag phase, that handicapped HD fish for the rest of the ongrowing period up to day 195. Such were the results for LME models fitting for maximum width and centroid size, but for standard length, both sub-periods showed a significant effect of density. So, fish reared in low density kept a higher growth rate measured as the increase of standard length, than HD fish during the whole period 1. Although, generally, production parameters are calculated from weight data, it is worth highlighting this result, as standard length could have been reacting more slowly than weight to density changes. This result could imply that fish under the potentially stressful conditions associated to high densities could be trailing such detrimental effects long after those first 61 days of acclimatization to a crowded environment. Also, it could be a hint that high densities, indeed, affect growth of Senegalese sole during the whole time that fish are reared under such conditions, but statistical analysis fail in detecting so due to the high variability in biometric measures as weight (Gardeur et al., 2001b).

The increase of the differences in size within a stock of fish cultured in the same tank is usually associated to the onset of hierarchies, due to competition for food items, space or other resources. However, no differences in the evolution of CV over time, neither between densities, could be found in this study. As food was provided in excess, and no signs of lesions due to aggressive behavior could be detected, differences in growth could be due to factors lying behind the genetic background, or due to differences in metabolic efficiency elicited by genetic variability and/or gender. Saillant et al. (2001) described a substantial drop in the growth coefficient of variation of European sea bass in their second year of life, when smaller individuals (mainly males), started to

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Standard length	$26.76 + 0.022 \cdot t + 0.19 \cdot sd - 1.24 \cdot s + 0.009 \cdot t \cdot sd - 0.004 \cdot t \cdot s$	$26.76 + 0.022 \cdot t + 0.19 \cdot sd - 1.24 \cdot s + 0.009 \cdot t \cdot sd - 0.004 \cdot t \cdot s$	$26.44 + 0.025 \cdot t + 0.64 \cdot sd - 1.17 \cdot s + 0.005 \cdot t \cdot sd - 0.005 \cdot t \cdot s$
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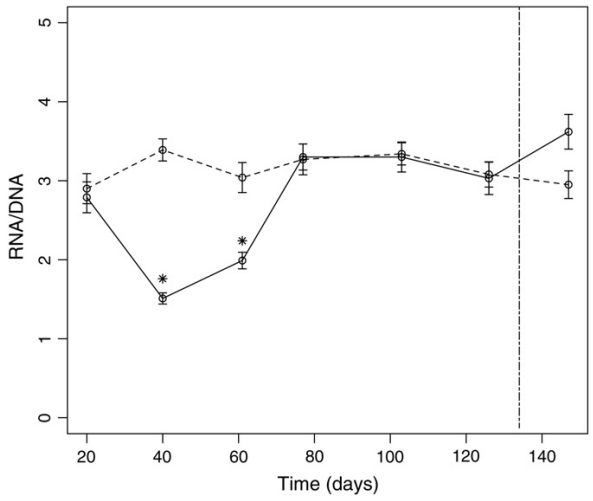

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stocking density, and then to be hierarchically dominant over males. Further studies should focus in size composition of cultured stocks in order to improve production. More research is needed to identify the factors involved with growth sexual dimorphism in Senegalese sole, as a first step of assessing the feasibility of monosex culture. Finally, RNA/DNA ratio has proved to be a sensitive biochemical indicator of growth in Senegalese sole.

Acknowledgements

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Chapter 3

Stocking density affects Senegalese sole (*Solea senegalensis*, Kaup) growth independently of size dispersion, evaluated using an individual photo-identification technique

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Stocking density affects Senegalese sole (*Solea senegalensis*, Kaup) growth independently of size dispersion, evaluated using an individual photo-identification technique

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Abstract

To assess how initial size dispersion affects Senegalese sole (*Solea senegalensis*, Kaup) growth, 128 fish were stocked either under homogeneous (HOM) or heterogeneous (HET) initial size dispersion (standard length, CV 11.8% and 29.7% respectively), and also under low (LD) and high (HD) stocking density (60% and 180% of bottom occupation). After 105 days, growth parameters, evaluated at an individual level using linear mixed-effects models, were not significantly affected by initial size dispersion, LD fish growing significantly faster than HD fish. Variance of individual growth rate distributions of weight and standard length were lowest for LD-HOM fish, indicating the most regular growth, and highest for LD-HET. The diminution of the coefficient of variation along time in HET groups (between 13% and 15.4% for weight, and between 4.3% and 5.8% for standard length), and its slight decrease in the HOM groups (between 1% and 3% for both parameters), is consistent with the absence of strong competition between individuals.

Keywords: Senegalese sole, stocking density, individual growth, social hierarchy

Introduction

Stock size composition can be behind aggressive behaviour and competition over resources as food or territory, promoting the onset of hierarchies and affecting fish growth in several ways (Gunnes 1976; Irwin, OHalloran & Fitzgerald 1999, 2002; Dou, Masuda, Tanaka & Tsukamoto 2004; Hatlen, Grisdale-Helland & Helland 2006). Large size variation is still an issue in the aquaculture of Senegalese sole (*Solea senegalensis*, Kaup; Flos, Reig, Fernández, Ambrosio & Carbó 1995; Dinis, Ribeiro, Soares & Sarasquete 1999; Flos, Reig & Ambrosio 2001; Rueda-Jasso, Conceição, Dias, De Coen, Gomes, Rees, Soares, Dinis & Sorgeloos 2004; Sánchez, Ambrosio & Flos 2010), and the influence of initial size distribution on growth has been little studied (Salas-Leiton, Anguis, Rodríguez-Rúa & Cañavate 2010).

Besides genetic differences between individuals, environmental parameters such as feed availability (Hatlen *et al.* 2006) or stocking density (Irwin *et al.* 1999) among others could be behind differences in growth through the development of complex social interactions, from which strong hierarchization can emerge.

Mechanisms linking stocking density and growth are not fully understood, but it is generally accepted that, when water quality is not affected by density, and food is provided in sufficient amounts, differences in growth performance could be attributed to the onset of hierarchies and dominance relationships (Papoutsoglou, Tziha, Vrettos & Athanasiou 1998; Bolasina, Tagawa, Yamashita & Tanaka 2006).

The effect of stocking density on growth has been investigated before in soles with contradictory outcomes. Some studies have shown that higher densities negatively affects growth in common sole (Schram, Van der Heul, Kamstra &

Verdegem 2006), while such effect could not be found in Senegalese sole reared at four stocking densities (Salas-Leiton, Anguis, Manchado & Cañavate 2008). Mixed results have been recently observed in Senegalese sole (Sánchez *et al.* 2010), with adult fish showing low growth phases after sudden increases in density.

Fish tagging for individual identification purposes has been widely used in aquaculture experiments with passive transponder tags (Anguis & Cañavate 2005; Imsland, Folkvord, Grung & Stefansson 1997) ink marks (Sánchez *et al.* 2010), or diverse external tags (Reig, Duarte, Valero & Oca 2010; Salas-Leiton *et al.* 2010) among other methods. Other approaches, as the less invasive photo-identification method, have been implemented both in the field and in the laboratory in several animal species, taking advantage of conspicuous singularities like the spots of the whale shark skin (*Rhincodon typus*, Smith; Arzoumanian, Holmberg & Norman 2005), facial markings in loggerhead sea turtles (*Caretta caretta,* L.; Schofield, Katselidis, Dimopoulos & Pantis 2008), colour patterns in painted crayfish (*Palinurus versicolor*, Latreille; Frisch & Hobbs 2007) or fin edges in cetacean species (Parra, Corkeron & Marsh 2006).

Senegalese sole could be a fish species to be easily identified visually, mainly through the comparison of the spot patterns of their skin, but also comparing skeletal deformities and malpigmentations, which are rather common in hatchery-reared sole (Gavaia, Dinis & Cancela 2002; Soares, Engrola & Dinis 2002; Villalta, Estévez & Bransden 2005).

The aim of this work was to assess the influence of size dispersion on growth of Senegalese sole under high (HD) and low stocking density (LD) through an individual photo-identification method.

Materials and methods

Experimental layout

Fish were obtained from the Centre d'Aqüicultura (IRTA, Sant Carles de la Ràpita, Spain), and stocked for 6 months under conditions of low density (43% of bottom coverage, 4.8 kg m^{-2}) at the Mediterranean Marine and Environmental Research Centre (CMIMA-CSIC) in Barcelona, Spain.

After the acclimation period, and 7 days before the starting of the experiment, fish were measured and weighed to determine the size range and dispersion of the population, in order to be able to design two size dispersion categories: homogeneous (HOM) and heterogeneous (HET), using standard length as the biometrical parameter of choice.

On the first day of the experiment, fish were again measured, weighed and photographed by their zenithal side. A total of 128 fish were assigned randomly to eight experimental groups of 16 fish each: 4 HOM groups and 4 HET groups, with coefficients of variation (CV) of standard length of 11.8% (HOM) and 29.7% (HET).

A surface/surface criterion was chosen to define the two experimental densities, as Senegalese sole lives in close interaction with the tank or sea bottom, often using its conspecifics body mass to carry on the same burrowing behaviour seen in sandy substrates. Density was calculated as a percentage of tank bottom covered by fish body surface. Following this criterion, a LD, where enough bottom surface made unnecessary fish overlapping (set at 60% of bottom surface covered by fish; 4.97 ± 0.16 kg m^{-2}) and a HD, where fish overlapping was granted (set at 180% of bottom surface covered by fish; 15.72 ± 0.79 kg m^{-2}) were defined. Fish surface was individually estimated from standard length and maximum width data, assuming that sole shape could be assimilated geometrically to an ellipse. Two HOM groups were randomly assigned to LD and two to HD conditions, and equivalently for HET groups. Afterwards they were stocked, also randomly, in eight tanks with the same length to wide ratio, but with different surface to get the designed LD and HD.

Summarizing, four experimental conditions were assayed: LD-HOM, LD-HET, HD-HOM and HD-HET, all of them duplicated totalling 128 Senegalese soles. Stocking density for each treatment at days 1 and 105 are shown in Table 1.

The experiment lasted a total of 105 days and biometric data [weight (WG), standard length (SL), total length (TL) and maximum width (WD)] were gathered for each individual on days 1, 16, 28, 49, 66, 79 and 105. On census days, all fish were anaesthetized by immersion in sea water with 2-phenoxyethanol (0.3 mL L^{-1}; Sigma, Tres Cantos, Spain), individually photo-identified, weighed, measured and then digitally photographed, perpendicularly to their zenithal side, against a millimetre paper background.

At the end of the experiment, most of the fish were sacrificed by anaesthetic overdose and sex was visually determined by dissection and direct observation of the gonad.

Table 1 Initial and final density, measured as percentage of bottom occupation and as kg m^{-2} (mean ± standard error of the mean)

	Initial density		Final density	
	% of bottom occupation	kg m^{-2}	% of bottom occupation	kg m^{-2}
LD-hom	60	4.9 ± 0.04	98 ± 0.8	10.9 ± 0.01
LD-het	60	5.1 ± 0.18	94 ± 0.02	10.8 ± 0.02
HD-hom	180	15.1 ± 0.58	244 ± 0.5	26.1 ± 0.53
HD-het	180	16.3 ± 0.37	230 ± 2.7	25.2 ± 2.7

Photo-identification

Some fish in the present work were considered too small to be subjected to an invasive tagging procedure, so a photo-identification method was implemented to gather individual biometric measures throughout the experiment.

After taking a digital picture of each fish on day 1, a 'family' photo album was printed for each of the experimental tanks (16 fish each). On following census days, the photo album corresponding to each tank was kept at hand when taking the biometric measures. Fish were identified *in situ* looking for the conspicuous particularities that make each fish singular and comparing them against the pictures of the album, so biometric data could be assigned to a particular fish. This technique was assayed previously with the ink-marked fish in Sánchez *et al.* (2010), consisting in matching 'before' and 'after' pictures of the same individuals by both trained and untrained observers. This trial yielded a successful identification rate of 97.4 ± 2.8% (mean ± SEM; P. Sánchez, P.P. Ambrosio & R. Flos, unpublished data).

As means of backup and disambiguation, each fish was photographed on every census day, with a label stating the original tank of the fish, its weight and a numerical code that allowed to double check the ID when processing the data.

Environmental conditions

Fish were kept in a flow-through circuit of sea water that flow into the tanks through an atmospheric pressure inlet pipe. Water flow (30% of the tank volume per hour), temperature (natural temperature for October through January, 41.23°N latitude), salinity (38 mg L^{-1}) and O_2 (>5.0 mg L^{-1}) were monitored daily. Photoperiod, 41.23°N latitude from October through January, was simulated with fluorescent light dimmed by shading covers laid over the tanks, with artificial periods of dusk and dawn. Cleaning protocols were carried out daily, involving siphoning feed leftovers, washing the automatic belt feeders and purging outlet pipes.

Feeding

Sole is a species that mainly shows an activity pattern during night hours (Bayarri, Muñoz-Cueto, López-Olmeda, Vera, Rol de Lama, Madrid & Sánchez-Vázquez 2004; Boluda Navarro, Rubio, Luz, Madrid & Sánchez-Vázquez 2009), thus feed was offered continuously from dusk to dawn with automatic belt feeders 7 days a week. Feed was offered in excess, and ratio was continuously optimized during the conditioning period in order to minimize uneaten feed in the bottom of the tank. A commercial feed for sole (ProAqua: 55% of gross protein, 15% of gross fat, 12% of ashes, 1% of gross fibre and 12% of carbohydrates; 20.3 MJ kg^{-1}) was fed throughout the experiment at a suitable pellet size for fish size.

Production parameters

Individual fish growth was described for each fish as their individual specific growth rate (IGR),

$$\mathrm{IGR} = 100x[\ln(X_f) - \ln(X_i)]xt^{-1}$$

where X_f and X_i stand for the value of either weight, standard length or maximum width at the end and at the beginning of the experiment respectively, and t stands for the total number of days.

Data analysis

When data are collected over time on the same individuals, as well as when data are gathered hierarchically or on related individuals, linear mixed-effects (LME) models are a useful tool that

can provide a better fit than the wide-spread linear models (Willett 1989; Crawley 2007). According to the particular design of the present experiment, classic parametric statistics were not suitable for analysing the data, as the condition of homocedasticity could not be satisfied due to the imposed differences in variance among groups. Consequently, an approach through LME models was selected for analysing growth. LME models take into account the so-called fixed effects and random effects to calculate the different coefficients of the model and their significance. While fixed effects are unknown constants that have to be estimated from the data, random effects are components of the variance and covariance matrix associated to the response variable.

Individual and combined effects of stocking density and size dispersion on individual growth, as independent categorical variables, were analysed by LME using the package *nmle* in R software (Pinheiro *et al.*, 2008). Weight, standard length and maximum width were used as response variables. All models were simplified discarding any non-significant fixed effect or interaction. Candidate models were then compared using ANOVA and when no significant differences were found, the best model was selected according to the Akaike Information (Akaike 1974) and log-likelihood criteria.

The distributions of IGR for weight and standard length were checked for normality (Shapiro-Wilk normality test) and for equality of variances performing an *F*-test. Mean values of each distribution were then compared using a Student's *t*-test. When differences in variances were found, the Welch approximation to the degrees of freedom was used. A lower variance in the IGR distribution of certain experimental condition would indicate a more regular growth of the majority of the individuals of such treatment.

In order to give the most powerful resolution to statistical procedures, fish that died at any point of the experiment were not taken into account for calculations.

All statistical procedures were carried out with the statistics software environment R (R Development Core Team, 2008) and significance level for all tests was set at 0.05.

Results

Growth

Individual growth, understanding as growth the slope of the evolution of each biometric parameter along time (Table 2, Fig. 1), of Senegalese soles reared in HOM and HET populations, and under

Table 2 Growth of Senegalese soles reared for 105 days under low or high stocking density and under homogeneous or heterogeneous size populations (mean; CV)

	Day						
	1	16	28	49	66	79	105
LD-hom							
WG	36.38 (34.6)	45.37 (35.6)	52.71 (35.1)	63.73 (34.3)	69.88 (33.6)	73.81 (34.0)	81.59 (33.7)
SL	12.64 (12.3)	13.42 (12.7)	14.08 (12.7)	14.87 (11.9)	15.31 (11.8)	15.59 (11.8)	15.93 (11.9)
WD	4.45 (13.0)	4.77 (13.0)	5.03 (13.0)	5.25 (13.3)	5.52 (12.6)	5.64 (13.1)	5.79 (12.5)
LD-het							
WG	35.53 (84.9)	43.64 (82.5)	49.51 (80.1)	59.41 (78.5)	65.36 (77.3)	68.66 (75.2)	75.41 (71.9)
SL	11.77 (28.9)	12.47 (28.9)	13.05 (27.7)	13.83 (26.2)	14.26 (25.6)	14.53 (25.2)	14.93 (24.2)
WD	4.17 (32.0)	4.46 (31.9)	4.63 (30.6)	4.95 (29.6)	5.14 (29.6)	5.27 (29.4)	5.38 (27.5)
HD-hom							
WG	39.73 (36.2)	47.41 (34.7)	51.81 (33.3)	59.58 (33.4)	64.67 (33.2)	66.77 (33.1)	71.5 (33.5)
SL	12.83 (11.4)	13.36 (11.4)	13.8 (11.0)	14.43 (11.1)	14.73 (11.1)	14.89 (10.9)	15.13 (11.0)
WD	4.59 (13.7)	4.82 (13.7)	5.00 (12.7)	5.28 (13.4)	5.40 (13.3)	5.49 (12.5)	5.57 (12.4)
HD-het							
WG	39.57 (97.5)	45.06 (94.7)	49.82 (93.2)	57.14 (85.6)	58.12 (86.4)	60.42 (84.7)	64.9 (82.1)
SL	11.97 (30.3)	12.45 (29.6)	12.86 (28.3)	13.62 (26.5)	13.67 (26.7)	13.81 (26.8)	14.06 (26.0)
WD	4.2 (34.6)	4.36 (33.3)	4.63 (30.1)	4.85 (27.3)	4.80 (32.6)	4.97 (29.7)	5.08 (29.5)

Values in parentheses are percentages.
LD-hom = low density + size homogeneity; LD-het = low density + size heterogeneity; HD-hom = high density + size homogeneity; HD-het = high density + size heterogeneity; WG = weight (g); SL = standard length (cm); WD = maximum width (cm).

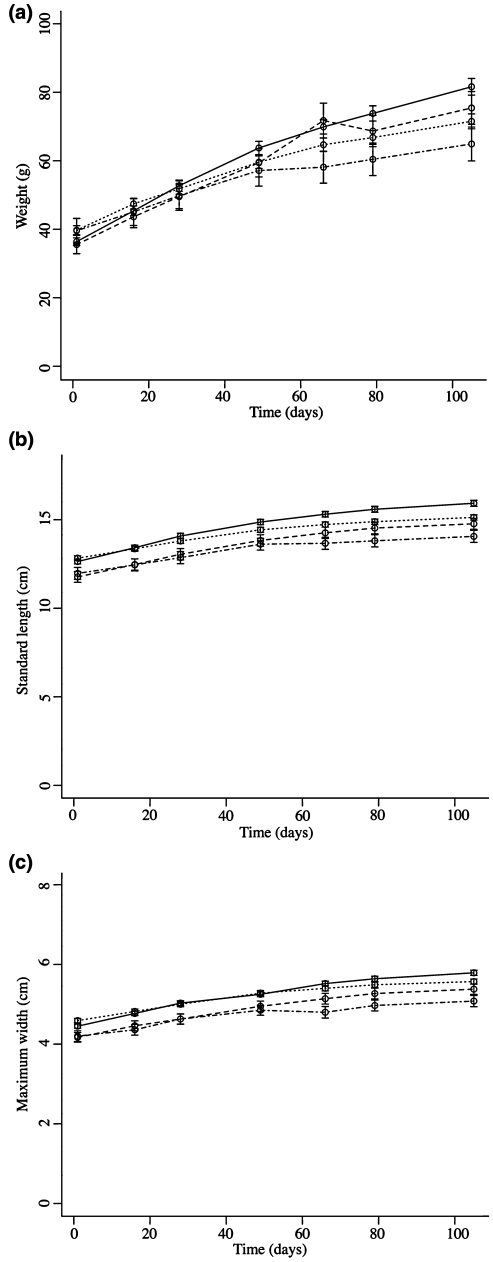

Figure 1 Evolution of the mean values for each growth descriptors: (a) weight, (b) standard length and (c) maximum width, for all four treatments during 105 days of growth trial. Solid lines: low density/size homogeneity; dashed lines: low density/size heterogeneity; dotted lines: high density/size homogeneity; dashed-dotted lines: high density/size heterogeneity. Error bars depict the standard error of the mean.

LD and HD, was significantly affected by density, with LD fish growing faster than HD fish (Fig. 2). Conversely, growth was not significantly affected by the size composition of the population in all studied parameters. The equations obtained for all models are depicted in Fig. 2.

Individual specific growth rates were negatively correlated with both initial weight ($R^2 = 0.26$; $P < 0.001$) and standard length ($R^2 = 0.31$; $P < 0.001$) (Fig. 3).

Mean values of IGR calculated from weight data are shown on Fig. 4, and plots of the distributions of IGR for each treatment are depicted in Fig. 5. Despite there was no significant influence of size dispersion on individual growth, different tendencies could be observed on the IGR for LD and HD groups. The variance of IGR of weight, standard length and maximum width (Table 3) of LD-HOM was significantly lower than the rest of treatments. Conversely, fish also under LD, but in size heterogeneity conditions, showed a significantly higher variance of weight IGR compared to both HOM groups (LD and HD) and a higher value of variance of standard length IGR than both HD groups (HOM and HET).

It was only possible to determine the sex of 85 out of 128 fish. The sex ratio, to be taken carefully, was 4:3 (♂:♀). A set of LME analyses were carried out with a sub-sample of the 85 fish with known sex and similar results were obtained, with no significant effect of either size dispersion or sex on growth whatsoever, but with a significant effect of density, similar to the one observed for the full sets of individuals described above.

Photo-identification

In situ identification of experimental individuals was easily accomplished in census days with the 'family' albums compiled for each tank after fish distribution on day 1. Only 11 out of 738 observations (1.49%) needed disambiguation after the identification of fish in the wet laboratory, although all individual identifications were double checked posteriorly to assess the accuracy of the method.

The more conspicuous particularities used for individual identification were, in order of usefulness to the naked eye, skin malpigmentations (when present), caudal fin morphology, overall body shape, pectoral fin morphology, and spot pattern of the skin.

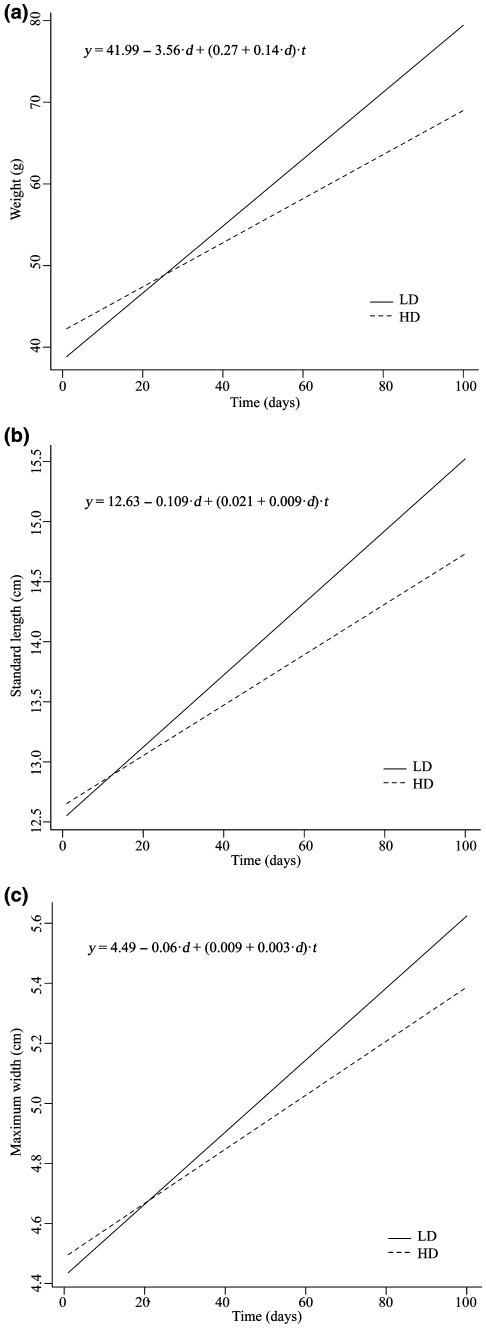

Figure 2 Linear mixed-effects models and their respective equations for each growth descriptor: weight (a), standard length (b) and maximum width (c). LD = low density (solid lines), HD = high density (dashed lines). At the equations: t = time (days); d = stocking density (categorical variable that takes values 0 or 1 for high density and for low density respectively).

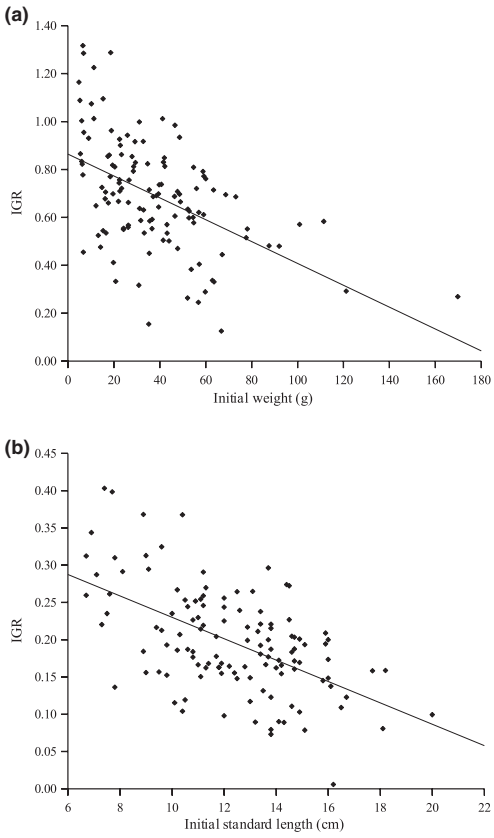

Figure 3 Correlation of individual growth rate (IGR) with initial weight (a; $R^2 = 0.26$; $P < 0.001$) and initial standard length (b; $R^2 = 0.32$; $P < 0.001$).

Discussion

It is widely accepted that stocking density plays a key role in intensive fish farming, as it not only may influence growth, but also welfare (North, Turnbull, Ellis, Porter, Migaud, Bron & Bromage 2006; Ashley 2007). In the present work stocking density was the most determinant rearing parameter on Senegalese sole growth after 105 days of rearing under conditions of either LD or HD, and either HOM or HET size distribution. Independently of initial size dispersion, LME models for fish reared under LD (60% of initial bottom occupation) showed a steeper slope of growth in all studied biometric parameters than fish reared under high density (180% of initial bottom occupation). Fish started on a mean weight of 37.7 g and CV of 68.4%, and ended up averaging 73.6 g (CV 57.1%). Final densities achieved in the HD groups

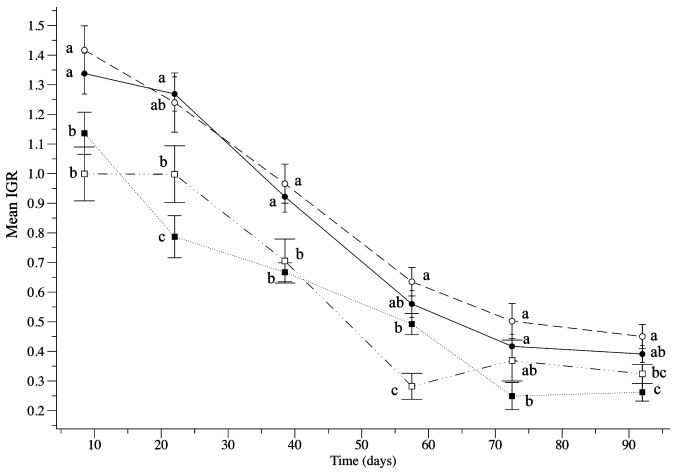

Figure 4 Evolution of the mean individual growth rate (IGR) for each of the four treatments during 105 days of growth trial. Solid lines: low density/size homogeneity; dashed lines: low density/size heterogeneity; dotted lines: high density/size homogeneity; dashed-dotted lines: high density/size heterogeneity. Different letters indicate significant differences of mean IGR within each analysed period.

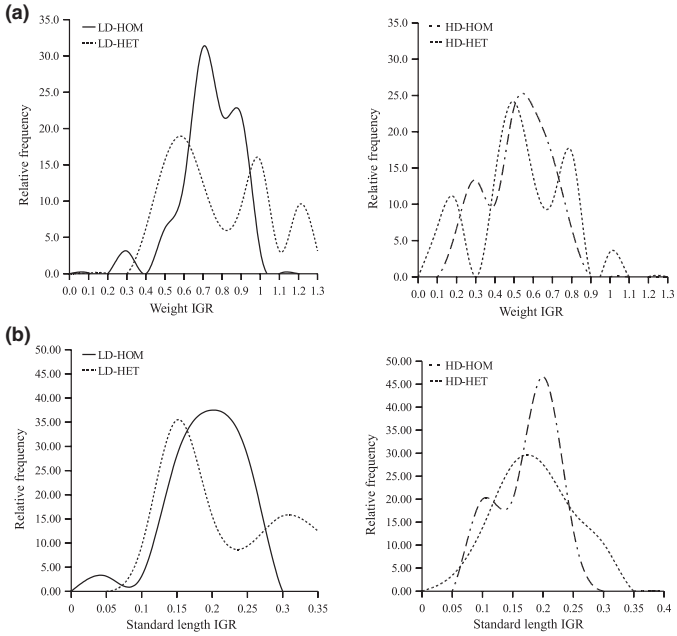

Figure 5 Distribution of individual growth rate (IGR) relative frequencies for weight (a) and standard length (b).

were similar to the ones reported previously for this species in terms of bottom occupation (Salas-Leiton *et al.* 2008; Sánchez *et al.* 2010).

Many studies regarding the interaction between density and production in Senegalese sole (Costas, Aragão, Mancera, Dinis & Conceição 2008; Salas-Leiton *et al.* 2008) and other flatfish species like Winter flounder (*Pseudopleuronectes americanus*, Walbaum; Fairchild & Howell 2001) did not show a relationship between stocking density and

Table 3 Variance of individual growth rate (IGR) of Senegalese soles reared for 105 days under low or high stocking density and under homogeneous or heterogeneous size populations

		Variance		
	N	**Weight**	**Standard length**	**Maximum width**
LD-hom	32	0.029a	0.003a	0.003a
LD-het	32	0.067c	0.007a	0.007b
HD-hom	30	0.031b	0.004b	0.009b
HD-het	29	0.047bc	0.005bc	0.008b

LD-hom = low density + size homogeneity; LD-het = low density + size heterogeneity; HD-hom = high density + size homogeneity; HD-het = high density + size heterogeneity. Different letters indicate significant differences between groups.

growth, although growth depensation following increased stocking density has been also verified in other flatfish species like common sole (Schram *et al.* 2006) and turbot juveniles (*Scophthalmus maximus*, L., Irwin *et al.* 1999, 2002).

In previous studies (Sánchez *et al.* 2010), 319 g adult Senegalese soles reared at high densities (233% of bottom occupation, 39.8 kg m^{-2}) during 134 days showed a lower growth rate than fish reared under low density, presumably due to a lag phase in growth at the beginning of the experiment. After lagging for 60 days, both densities grew up showing similar slopes. In the present work, 38 g soles showed slower growth at high density in the absence of any adaptation phase, suggesting that, at least in this size range, there is an inverse relationship between stocking density and growth. These differences on how stocking density affects growth depending on fish size were already pointed out by Salas-Leiton *et al.* (2008), that did not find any influence of stocking density on growth of 70 g Senegalese sole juveniles, and postulated that the detrimental effects of density on growth of this species could decrease as they approach the adult size.

Contrasting with previous studies on adult soles (fish over 300 g; Sánchez *et al.* 2010), no influence of sex on growth was found in this experiment. Although the missing data on some individuals advises to be cautious in this aspect, soles weighing 38 g could be in an early life stage for sexual growth dimorphism to be observed.

In this experiment, fish were graded in order to build HOM and HET size groups. By mixing small and large fish in the same population it could be hypothesized that hierarchic relationships of dominance would have emerged, and larger fish could have exerted a detrimental effect on growth of smaller fish (Gunnes 1976; Jobling & Reinsnes 1987; Irwin *et al.* 1999; Dou *et al.* 2004). Nevertheless, the LME models showed that size composition had no significant influence on sole growth. Additionally, the CV of weight and standard length in the HET groups diminished during the experiment (between 13% and 15.4%, and between 5.8% and 4.3% respectively). These data are consistent with the absence of strong competition between individuals (Wallace & Kolbeinshavn 1988; Kamstra 1993) as the increase in weight CV has been related to hierarchy formation in turbot (Irwin *et al.* 1999, 2002). Homogeneous groups kept their weight CV under 37% and, although it decreased between 1% and 3%, it remained fairly stable during the whole experiment, also indicating a low level of hierarchic competition. Similarly, Salas-Leiton *et al.* (2010) found that the CV of ungraded 155 g Senegalese sole (CV 37.4%; similar to the one used here for HOM groups), showed a slight non-significant tendency to increase during 60 days of trial.

As specific growth rate assumes that growth is exponential, if fish reared together do not interact competitively, there should be a negative correlation between body size and individual SGR (Jobling 1985). Such a significant negative correlation was observed in this experiment, adding up to the suggestion that smaller fish had not their growth suppressed in these conditions.

Individual growth rate distributions of HOM and HET groups were significantly different in LD, with HET showing a higher variation for both weight and standard length IGRs than HOM. But in HD, both HOM and HET groups showed similar IGR distributions. High density promotes a higher amount of activity in sole (Duarte, Reig & Oca 2009) and increases social interactions, which could generate some sort of physiological stress in sole. This stress could be higher for fish stocked at high density in HOM groups and, as also suggested by Salas-Leiton *et al.* (2010), more fish could be in position to exert some pressure over the resources, leading to increased social interaction and slower growth in populations of similar sized soles.

Social-induced growth depensation could be a major problem in aquacultured fish, as individuals low in the dominance hierarchy display chronic stress that in turn leads to appetite diminution, reduced food intake and low SGR (Jobling 1985).

Feed access can be more difficult for subordinate fish (Irwin *et al.* 2002; Dou *et al.* 2004), and even subordinates can 'voluntarily' reduce their food intake, and hence their growth, when conflict over rank intensifies to avoid social conflict (Wong, Munday, Buston & Jones 2008). But differences in feed consumption are not the only factor linked to the lower growth of low-rank fish, as poorer growth can be observed even when feed is not a limiting factor (Irwin *et al.* 1999; Dou *et al.* 2004). Cubitt, Winberg, Huntingford, Kadri, Crampton and Øverli (2008) suggested that social position is related to the brain neurochemistry, and thus potentially animal welfare, in large and densely populated rearing units of fish. Altered serotoninergic activity was present in subordinate fish and failed to grow adequately even when feed was available in excess. Moreover, findings by Mackenzie, Ribas, Pilarczyk, Capdevila, Kadri and Huntingford (2009) in the common carp (*Cyprinus carpio*) suggest that other factors, such as adaptive inherited variability, including differences in stress coping style, may influence the response to challenging conditions.

Senegalese sole has no records of aggressive behaviour. When reared in experimental tanks at low densities (5–50% of bottom occupation), smaller fish have been observed to gain access to feed particles before the largest fish (De Wolf, Lenzi & Lenzi 2011) that preferred to stay close to the water inlet. Apparently, if feed is provided in sufficient amounts, no competition for food is established, although maybe the real dominance struggle could be for other resources like the best water quality, or to reach a favourable position over or under conspecifics. It is a fact that some soles coming from the same broodstock grow slower than their siblings. Whether it is a genetic predisposition or an environmental fact is still unknown and an interesting question to be investigated.

Should the producer grade the stocks? According to our results, lowering size dispersion would not improve growth, mainly in high stocking density conditions.

Anyhow, keeping the runts would not have a beneficial effect in production either, so maybe it could be interesting to develop a market for such small fish, commercializing these slow-growing individuals as '*fritada*', a very appreciated way of frying small fish, or as pre-cooked dishes. In this experiment, some guidelines were proposed to identify individual soles *in situ* through digital photographs. The particular morphometric and pigmentation traits of each sole proved to be sufficient to successfully identify individual fish along time. Especially useful clues were traits like malformations and malpigmentations, unfortunately very common in hatchery-reared Senegalese sole (Gavaia *et al.* 2002; Soares *et al.* 2002; Villalta *et al.* 2005). The spot pattern of sole skin apparently did not change for the whole length of the experiment and could be used as a fingerprint, but the difficulty to operate with this parameter to the naked eye made it the last resource to disambiguate fish identity when confronted with similar fish. Nevertheless, it looks like relationships of length and angles between spots could remain unchanged for a long period of time, being the most promising candidate for developing an automated individual identification technique in soles.

Summarizing, a high stocking density negatively affected Senegalese sole juveniles growth, while initial size dispersion did not, suggesting that other factors, including social interactions or stress, might be behind the differential growth profiles of soles. Some guidelines were also proposed to successfully identify individual soles through digital photographs.

Acknowledgments

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Chapter 4

Growth of the nocturnal flatfish Senegalese sole (*Solea senegalensis)* is light independent

Growth of the nocturnal flatfish Senegalese sole *Solea senegalensis* is light independent.*

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Abstract

Growth performance of the nocturnal flatfish *Solea senegalensis* was assessed feeding fish stocked at high density (139% of bottom occupation or 13.55 kg·m^{-2}; CV = 9.58%) fed a commercial diet in excess either during the daylight hours (30 minutes after dawn to 30 minutes before dusk) or during the night hours (30 minutes after dusk to 30 minutes before dawn). There were no significant differences between mean individual growth rates for weight (0.63; CV=25.01%), standard length (0.18; CV=25.24%) and maximum width (0.23; CV=28.61%) of daylight-fed or night-fed fish. Growth analyzed through linear mixed effects (LME) models showed significant steeper slopes for standard length and maximum width for night-fed fish, although very small and biologically irrelevant. All mean biometric parameters were higher for females than for males during the whole experiment and LME models showed significantly higher slopes for females than for males, although they were not affected by feeding strategy, indicating that 80 gram Senegalese sole already show sexual growth dimorphism. There were no differences of apparent feed conversion ratio (FCR) between treatments (1.36±0.122 and 1.27±0.097 for day-fed and night-fed fish respectively). Our results show that, besides being a nocturnal species, Senegalese sole fed during the light phase may yield similar growth rates and FCR than fish fed during the dark phase, suggesting that this species can be successfully adapted to feed during normal business hours in commercial facilities.
—*Keywords*: diel rhythms, feeding, growth, Senegalese sole, sexual dimorphism.

1 Introduction

Diel rhythms have been widely studied in many species of vertebrates including fish (Iigo and Tabata, 1996; Thetmeyer, 1997; Okimoto and Stetson, 1999; Reebs, 2002). The presence of an endogenous circadian oscillator allows the animal to synchronize its activity periods to the time of day when food is more available, or when risk of predation is lower (Álvarez and Nicieza, 2003), and even to set up a sleep period in some fish species to avoid excessive spending of energy when food items are less likely to be present in the environment (Reebs, 2002). This internal oscillator can be synchronized by an external input, the so-called 'zeitgeber', in the form of darkness-light succession (Aranda et al., 2001; Herrero et al., 2005), or the periodic availability of some food resource as it clearly occurs in a fish farming facility (Sánchez-Vázquez et al., 1995; Reebs, 2002 and references therein).

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This aspect of fish biology becomes of a crucial importance when a particular species is going to be farmed, as adjusting the environmental conditions (lighting, temperature) or feeding schedules can have a huge impact in the performance of the culture, from spawning (Bromage et al., 1984; Blanco-Vives and Sánchez-Vázquez, 2009; Oliveira et al., 2009; Vinagre et al., 2009; Oliveira et al., 2011) to larval rearing (Cañavate et al., 2006; Harboe et al., 2009; Villamizar et al., 2009; Blanco-Vives et al., 2011) or growth efficiency (Jørgensen and Jobling, 1992; Azzaydi et al., 2000).

Many fish species show a particular feeding behavior, with activity and ingestion patterns that are essentially diurnal, nocturnal or indifferent, and that remain stable during time (Björnsson, 2001; Herrero et al., 2005; Sunuma et al., 2007; Boluda Navarro et al., 2009), or that can vary depending on the season of the year (Sánchez-Vázquez et al., 1998; Metcalfe et al., 1999; Azzaydi et al., 2000; Rubio et al., 2004). Both laboratory trials and fish farming experiences have shown that some fish species can be fed at different times of the day independently of their biological preference, often keeping similar specific growth rates (Champalbert and Le Direach-Boursier, 1998; Harpaz et al., 2005; Herrero et al., 2005).

Senegalese sole (*Solea senegalensis*) is a species that has been the subject of thorough research in the last three decades, because of its high market demand and the general idea that inland facilities, initially devoted to gilt-head sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) aquaculture in the Mediterranean, could be easily converted to accommodate its rearing, thus making sole an interesting new species for marine aquaculture, while keeping the initial development costs to a minimum (Imsland et al., 2003).

Following some promising trials in early 80's, rearing of *S. senegalensis* has succeeded in key points such as reproduction (Dinis et al., 1999), weaning (Cañavate and Fernández-Díaz, 1999; Ribeiro et al., 1999), or nutrition (Vázquez et al., 1994; Rønnestad et al., 2001; Morais et al., 2006). However, still diverse growth performance and high size variability have been reported (Dinis et al., 1999; Flos et al., 2001; Rueda-Jasso et al., 2004; Sánchez et al., 2010; Salas-Leiton et al., 2011).

Senegalese sole has shown clear nocturnal habits for spawning (Oliveira et al., 2009), locomotor activity (Bayarri et al., 2004), and even a higher metabolic rate during the dark phase (Castanheira et al., 2011). Sole has also a marked nocturnal feeding behavior (Boluda Navarro et al., 2009). Fish farming operations, including feeding, occur mainly during the light phase, but it has been suggested that this practice could interfere with the natural rhythms and the welfare of this species (Boluda Navarro et al., 2009). Therefore, considering all the evidences pointing to nocturnal habits in Senegalese sole, the aim of this study was to assess whether feeding soles during the dark phase could improve growth compared to fish fed during the light phase.

2 Material and Methods

2.1 Fish and experimental conditions

Senegalese soles (mean weight 37.9 g, coefficient of variation, CV=36.65%) were obtained from a fish farm located in Almería, Spain, and stocked for 3 months under conditions of low density (43% of bottom coverage, 4.8 $kg \cdot m^{-2}$) at the Mediterranean Marine and Environmental Research Centre (CMIMA-CSIC) in Barcelona, Spain. Stocking density was estimated calculating the ratio between tank surface and total fish area, assuming that sole shape could be geometrically assimilated to an ellipse.

A total of 666 Senegalese soles were randomly distributed among 6 experimental tanks (111 fish each) and acclimatized for 88 days to new density conditions (101.8% bottom coverage, CV=2.81%; 7.39 $kg \cdot m^{-2}$, CV=3.01%) and to 2 different feeding strategies: three randomly chosen tanks were fed during daylight hours, from 30 minutes after dawn to 30 minutes before dusk, and the remaining tanks were fed during night hours, from 30 minutes after dusk to 30 minutes before dawn. Feed pellets (ProAqua, Solea 3 to Solea 7; 55% of gross protein, 15% of gross fat, 12% of ashes, 1% of gross fiber and 12% of carbohydrates; 20.3 $Mj \cdot kg^{-1}$) were administered in excess by automatic belt feeders. Daily ratio was

adjusted to an initial 1% of body weight to avoid the accumulation of feed leftovers, while granting food access to all individuals, and was adjusted throughout the experiment in order to minimize uneaten feed in the bottom of the tank.

The experiment lasted a total of 105 days. Biometric measures (weight, standard length, total length and maximum width) were gathered on days 1, 29, 70 and 105. All biometrical measures were taken in fish after being anesthetized by immersion in seawater with 2-phenoxyethanol (Sigma, 0.3 $ml \cdot l^{-1}$). Fish were weighed to the nearest 0.01 g, measured to the nearest 0.1 cm, and photographed by their zenithal side against a millimeter paper background with a label indicating a unique code, the tank of origin, and its measured weight. These pictures allowed to identify every fish later on in the laboratory (Sánchez et al., 2011), and hence to assign the measured biometric data to each individual along time.

At the end of the experiment, 50 fish of each tank were randomly sacrificed by immersion in ice-cold water and dissected to record sex by direct observation of the gonad.

2.2 Experimental facilities

Six experimental tanks (1.5 x 0.45 x 0.45 m) were set for the purposes of this work at the CMIMA-CSIC facility. Two tangential water inlets, which provided seawater in a flow-through regime, were placed in the center of one of the longer lateral walls of each tank. This setting allows to maximize the uniformity of water velocity across the tank and contributes to the self-cleaning of the tank (Oca et al., 2004). Temperature (17.7° C$\pm$0.89), salinity (38 $mg \cdot l^{-1}$) and O_2 (>5.0 $mg \cdot l^{-1}$) were monitored daily. Photoperiod corresponding to 41.23° N latitude from July through January was simulated with fluorescent light dimmed by shading covers laid over the tanks, with artificial periods of dusk and dawn. Cleaning protocols were carried out daily, involving siphoning feed leftovers, washing the automatic belt feeders, skimming the water surface when necessary and purging outlet pipes.

2.3 Data analysis

Individual fish growth was described for each fish as its individual specific growth rate (IGR),

$$IGR = \left(\frac{(ln(X_f) - ln(X_i)}{t} \right) x100$$

where X_f and X_i stand for the value of either weight, standard length or maximum width at the end and at the beginning of the experiment respectively, and t stands for the total number of days.

When data are collected over time on the same individuals, linear mixed-effects (LME) models are a useful tool that can provide a better fit than the wide-spread linear models (Willett, 1989; Crawley, 2007). An approach through LME models, taking advantage of the individual data gathered throughout the experiment, was selected for analyzing growth.

The effect of feeding strategy on individual growth was analyzed by LME models using the package *nmle* in R software (Pinheiro et al., 2011). Weight, standard length and maximum width were used as response variables. All models were simplified discarding any non-significant fixed effect or interaction. The so obtained candidate models were recalculated through the maximum likelihood method and compared by ANOVA. When no significant differences were found, the best model was selected according to the Akaike Information (Akaike, 1974) and log likelihood criterions. Mean growth descriptors were tested for normality (Shapiro-Wilk normality test) and compared either through Student's t-test or through Mann-Whitney's U when normality was not met.

Apparent feed conversion ratio (FCR) for each tank was calculated as

$$FCR = \left(\frac{F_t}{(B_f - B_i)} \right)$$

Table 1: Acclimation (day -88), initial (day 1) and final (day 105) average stocking density for Senegalese sole fed either during the daylight hours or during the night.

	Acclimation SD_{day-88}	*Initial* SD_{day1}	*Final* SD_{day105}
Day	103.4% (3.20%)	139.0% (1.08%)	201.5% (1.14%)
	7.53 kg·m^{-2} (3.07%)	14.4 kg·m^{-2} (1.55%)	26.2 kg·m^{-2} (2.34%)
Night	100.2% (1.32%)	122.2% (10.39%)	182% (12.35%)
	7.50 kg·m^{-2} (3.07%)	12.7 kg·m^{-2} (11.27%)	23.8 kg·m^{-2} (11.82%)
Total	101.8% (2.81%)	130.6% (9.38%)	191.8% (9.29%)
	7.39 kg·m^{-2} (3.01%)	13.55 kg·m^{-2} (9.58%)	25.01 kg·m^{-2} (8.96%)

Day = fish fed during the photophase; *Night* = fish fed during the scotophase; *Total* = pooled fish; *SD* = stocking density. Values are average (% coefficient of variation).

whereF_t is the total amount of feed offered to each tank for the duration of the experiment, B_f is total biomass of fish at day 105, and B_i is total biomass at day 1.

All statistical procedures were carried out with the statistics software environment R (Team, 2011) and significance level for all tests was set at 0.05.

3 Results

Fish stocked at an initial mean density of 101.8% (CV=2.81%) of bottom coverage ended the experiment at an average 191.8% (CV=0.09%) of bottom occupation (Table 1). Mean growth of Senegalese sole, expressed as the evolution of each biometric parameter along time (Table 2, Fig. 1) was not significantly affected by the feeding strategy, with fish fed throughout the nocturnal hours growing at the same rate as fish fed during daylight hours. This result was also backed up comparing mean individual growth rates (Table 3).

No differences of apparent feed conversion ratio (Student's t-test) were found between day-fed fish (1.36$\pm$0.122) and fish fed during the night hours (1.27$\pm$0.097).

Individual growth analyzed through linear mixed effects models showed no significant differences between treatments for weight, although models for standard length and maximum width presented significant steeper slopes for night-fed fish, although very small (Table 3, Fig. 2).

All mean biometric parameters comparing sexed fish were consistently higher for females than for males during the whole experiment. Nevertheless, comparing growth performance of sexed fish within treatments, only final standard length was significantly higher for females than for males in daylight-fed fish. When fish from both treatments were pooled, mean standard length and mean maximum width were also significantly higher for females than for males (Table 2).

Differences between males and females became more evident through the analysis of individual growth with linear mixed effects models, with all biometric parameters showing significantly higher slopes in females (Table 4, Fig. 3). Additionally, the intercept of the model for weight was also significantly higher for females than for males. Nevertheless, LME models could not find any significant differences between males and females fed either during the photophase or the scotophase.

4 Discussion

Senegalese sole is a flatfish species that is promising for Mediterranean aquaculture. Several works have clearly shown that this species has preeminently nocturnal biological rhythms, as sole displays higher

Table 2: Initial and final mean biometric parameters of individually identified Senegalese soles fed either during daylight hours or during nocturnal hours, both regarding sex and in total.

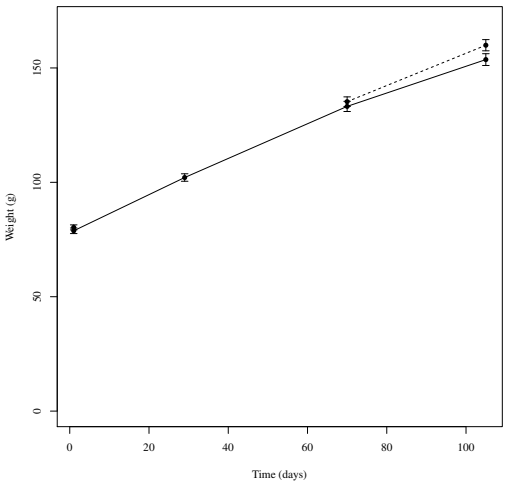
	Sex	*N*	WG_1	WG_{105}	SL_1	SL_{105}	WD_1	WD_{105}
Day	♀	22	88.4 (44.6%)	168.8 (50.6%)	16.3 (12.7%)	19.6 (15.1%)[a]	6.3 (17.2%)	8.1 (19.8%)
	♂	49	72.9 (55.7%)	135.5 (65.0%)	15.4 (14.9%)	18.2 (18.4%)[a]	5.8 (20.8%)	7.4 (24.9%)
	All	144	78.8 (46.5%)	153.6 (50.2%)	15.9 (12.9%)	19.1 (14.8%)	6.1 (16.5%)	7.7 (19.5%)
Night	♀	23	91.1 (41.6%)	181.3 (49.5%)	16.3 (15.8%)	19.6 (19.0%)	6.3 (16.0%)	8.2 (20.7%)
	♂	26	79.0 (44.5%)	150.0 (54.3%)	15.5 (15.6%)	18.4 (17.9%)	6.1 (15.6%)	7.7 (19.8%)
	All	148	80.3 (33.4%)	159.9 (37.3%)	16.0 (11.9%)	19.4 (13.1%)	6.1 (12.1%)	7.9 (14.2%)
Total	♀	45	89.8 (42.6%)	175.2 (49.6%)	16.3 (14.2%)	19.6 (17.0%)[b]	6.3 (16.4%)	8.2 (20.0%)[c]
	♂	75	75.0 (47.9%)	140.6 (55.8%)	15.5 (13.8%)	18.3 (16.4%)[b]	5.9 (16.7%)	7.5 (20.5%)[c]
	All	292	79.6 (35.2%)	157.1 (38.6%)	15.9 (11.4%)	19.3 (12.5%)	6.1 (12.8%)	7.8 (14.9%)

N = number of fish in each analyzed sample; ♀= females; ♂= males; *All* = all identified fish for each treatment (females + males + unsexed fish); *WG* = weight; *SL* = standard length; *WD* = maximum width; The subscripts indicate the day of measurement. Values are average (% coefficient of variation). Same letters indicate significant differences between values $p < 0.05$.

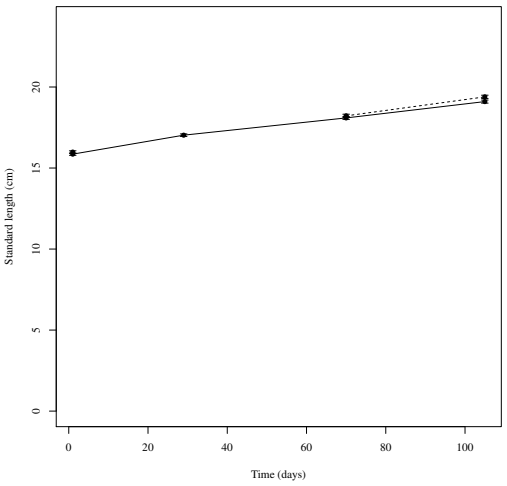
Table 3: Mean individual growth rate of Senegalese sole fed either during daylight hours or during nocturnal hours, both regarding sex and in total.

	Sex	*N*	IGR_{WG}	IGR_{SL}	IGR_{WD}
Day	♀	22	0.58 (25.19%)	0.17 (24.61%)	0.24 (27.00%)
	♂	49	0.55 (26.25%)	0.16 (24.04%)	0.21 (27.45%)
	All	144	0.62 (28.26%)	0.18 (30.65%)	0.23 (34.84%)
Night	♀	23	0.59 (33.71%)	0.17 (31.06%)	0.24 (29.07%)
	♂	26	0.56 (31.23%)	0.16 (33.89%)	0.21 (36.87%)
	All	148	0.64 (24.45%)	0.18 (24.04%)	0.24 (26.13%)
Total	♀	45	0.58 (29.54%)	0.17 (27.72%)	0.24 (27.75%)[a]
	♂	75	0.55 (31.44%)	0.16 (33.71%)	0.21 (36.73%)[a]
	All	292	0.63 (25.01%)	0.18 (25.24%)	0.23 (28.61%)

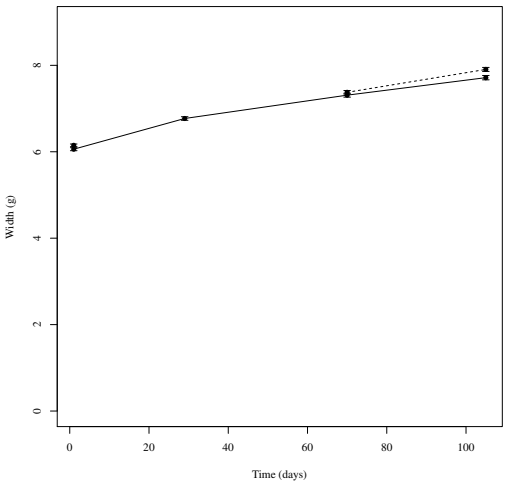
N = number of fish in each analyzed sample; ♀= females; ♂= males; *All* = all identified fish for each treatment (females + males + unsexed fish); *IGR* = individual growth rate. In subscripts after IGR: *WG* = weight; *SL* = standard length; *WD* = maximum width. Values are average (% coefficient of variation). Same letters indicate significant differences between values $p < 0.05$.



(a) Weight

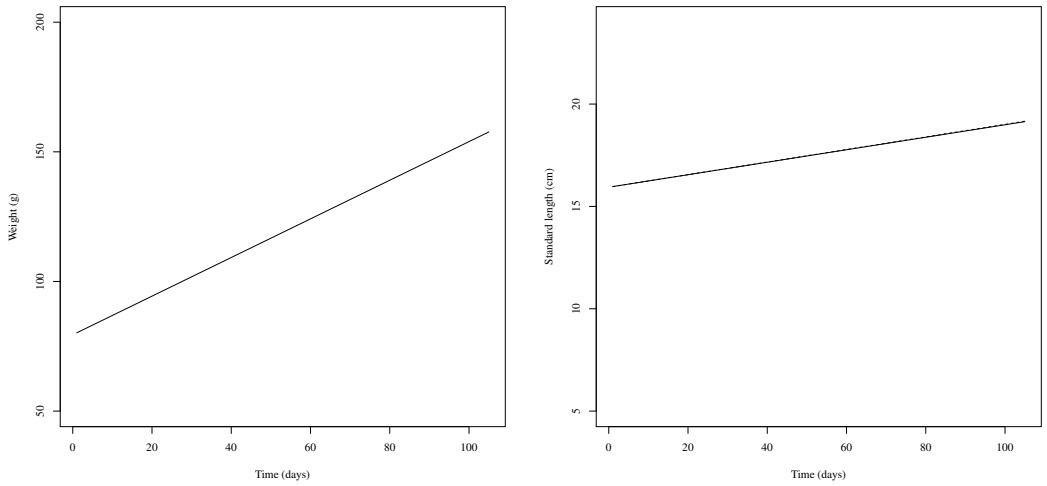


(b) Standard length



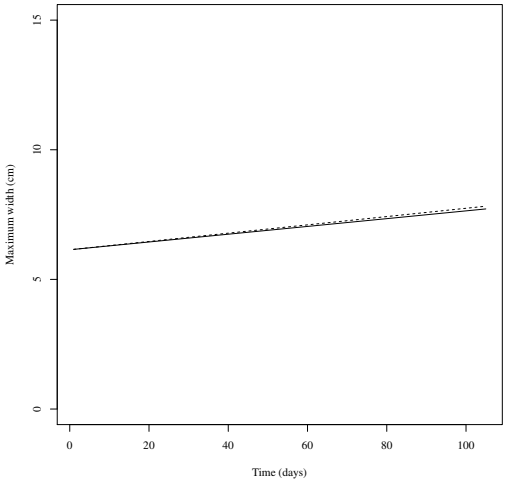
(c) Maximum width

Figure 1: Evolution of biometric parameters (a=weight, b=standard length and c=maximum width) Senegalese soles fed either throughout the photophase or throughout the scotophase for 105 days. Solid lines represent daylight-fed fish and dashed lines show nocturnal-fed fish.



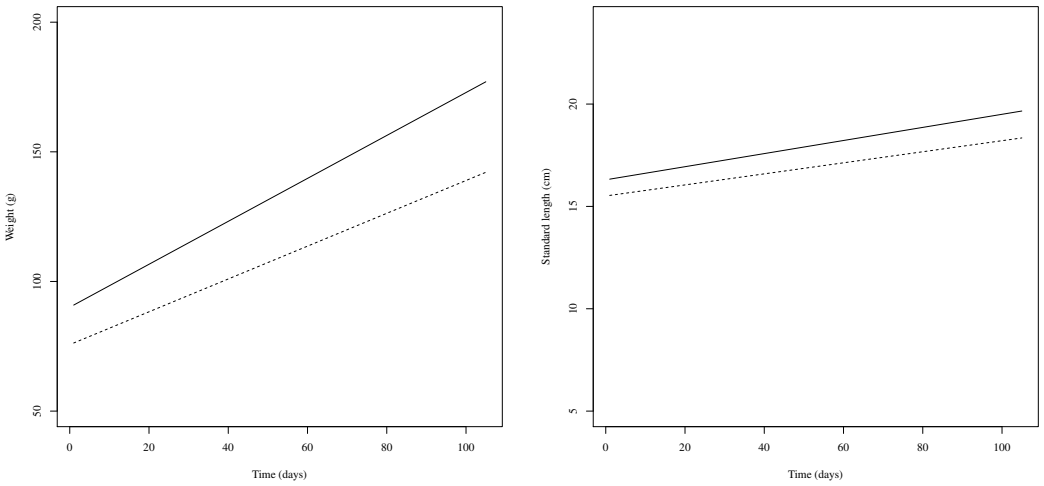
(a) Weight

(b) Standard length



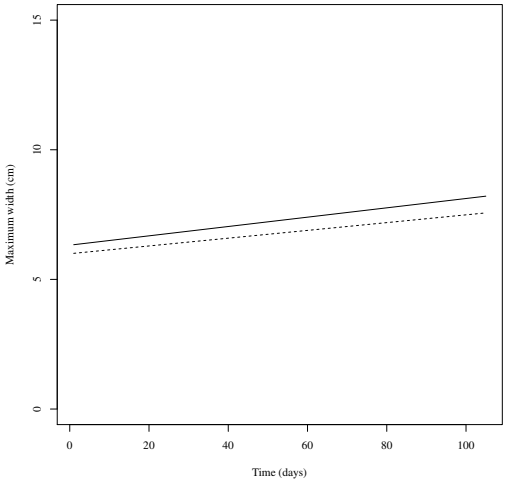
(c) Maximum width

Figure 2: Linear mixed effects models for each growth descriptor: a) weight, b) standard length, and c) maximum width of Senegalese soles fed either throughout the photophase or throughout the scotophase for 105 days. Solid lines represent daylight-fed fish and dashed lines show nocturnal-fed fish.



(a) Weight

(b) Standard length



(c) Maximum width

Figure 3: Linear mixed effects models for each growth descriptor: a) weight, b) standard length, and c) maximum width of 45 female and 75 male Senegalese soles fed either throughout the photophase or throughout the scotophase for 105 days. Solid lines represent females and dashed lines show males.

Table 4: Equations of the linear mixed effects models of growth of Senegalese sole fed either during daylight hours or during nocturnal hours.

All fish $_{(N=292)}$	
Weight	$y = 79.44 + 0.75 \times time$
Standard length	$y = 15.938 + 0.0305 \times time + 0.002 \times time \times feed$
Maximum width	$y = 6.15 + 0.15 \times time - (0.021 - 0.001 \times time) \times feed$
Sexed fish $_{(N=120)}$	
Weight	$y = 90.07 + 0.83 \times time - (14.43 + 0.195 \times time) \times sex$
Standard length	$y = 16.30 + 0.032 \times time - (0.79 + 0.005 \times time) \times sex$
Maximum width	$y = 6.32 + 0.018 \times time - (0.33 + 0.003 \times time) \times sex$

All fish = individually identified fish. *Sexed fish* = fish that were sexed at the end of the experiment, among all individually identified fish. *Feed* and *sex* are categorical variables that take values 1 or 0. In *feed*, it takes 0 for daylight fed fish and 1 for night fed fish. In *sex*, it takes 0 for females and 1 for males.

locomotor activity and oxygen consumption during dark hours (Bayarri et al., 2004; Castanheira et al., 2011), and that it can also be entrained to self feed, concentrating the majority of feed demands during the scotophase (Boluda Navarro et al., 2009). Such a tenacious nocturnal behavior, combined with the usual practices of fish farms, that tend to work during daylight hours for obvious reasons, has raised some concerns about the welfare of this species in culture (Boluda Navarro et al., 2009). Usual daylight feeding in commercial facilities also poses the question of whether soles fed against their biological preferences might be doing the most of the energy and nutrients they are getting.

Two groups of individually photo-identified Senegalese sole were reared for 105 days, fed continuously either throughout the light phase or the dark phase, to assess if commercial feeding practices yielded poorer growth than feeding according to the biology of the species.

In our experiment, Senegalese sole stocked at a moderately high density grows at the same pace independently of being fed during the light phase or, according to their biological rhythms, during the dark phase. It has been seen that despite being diurnal or nocturnal feeders, some fish species can sustain similar growth rates when fed against the schedule found in self-feeding experiments. Such is the case of tench (*Tinca tinca*; Herrero et al., 2005) or the Asian sea bass (*Lates calcarifer*; Harpaz et al., 2005) and now apparently the case of Senegalese sole. Contrarily, Azzaydi et al. (2000) found that European sea bass fed during the light phase in winter, season when this species feeds at night (Sánchez-Vázquez et al., 1998), resulted in poorer growth than fish fed at night. In that case, feed ratio was offered differentially by automatic feeders, but nocturnal ratio was offered in two meals, concentrating 66.7% post-dusk and 33.3% pre-dawn, while in the light phase was offered in 3 equal meals in the morning, afternoon and evening. Our feeding schedule consisted in continuous feeding either during day or night, according to the results by Boluda Navarro et al. (2009), who observed that sole shows a steady feed demand throughout the night. Hence, in order to maximize growth it is important not only to choose the time of the day to offer the feed, but also, when no self-feeding is involved, to adjust the size and number of meals to the biology of the species.

Fish feeding behavior shows plasticity to some extent. European sea bass can shift its feeding activity to tailor external factors like forced exercise or social interactions between individuals within a group (Valverde et al., 2005). In sole, environmental and social factors could also affect feeding rhythms. For example, rearing soles in high size heterogeneity (large and small) and in two stocking densities forced smaller soles to feed first in all cases, but also both fish sizes in higher densities tended to show higher

swimming activity and faster access to feed than low density fish (Duarte et al., 2006). In our study, fish were stocked to a considerably initial high density, finishing the experiment at nearly 200% of bottom coverage, roughly in the order of the high densities defined in Ambrosio et al. (2008), Salas-Leiton et al. (2010) and Sánchez et al. (2010). The elevated social interactions involved in such high stocking densities might have exerted some effects in the swimming activity, and also might have forced a faster response to feed administration, thus diminishing the possible effect that diurnal feeding could have had on growth.

There are abundant studies on feeding rhythms on fish in the laboratory, many of them dealing with self-feeding systems when fish are able to operate them. There are some species, like tench, that present strict preferences to feed during a particular time of the day (Herrero et al., 2005) and others, like European sea bass, that change their feeding rhythms according to the season (Sánchez-Vázquez et al., 1998), switching from diurnal to nocturnal feeders for the winter months or other species like red porgy (*Pagrus pagrus*) or gilthead sea bream that increase feed demands during the dark hours in the colder months (Paspatis et al., 2000). Due to the effort and resources needed to check a full year of feeding rhythms, few studies like the one conducted in sea bass (Sánchez-Vázquez et al., 1998), or in yellowtail (*Seriola quinqueradiata*; Kohbara et al., 2003) are available for most of the commercial species. This applies also to sole, where locomotor, metabolic and feeding rhythms have been conducted for a limited amount of time (35 and 44 days; Boluda Navarro et al., 2009; Castanheira et al., 2011, and 163 days; Bayarri et al., 2004) and under different temperature ranges. Our experiment was not designed to see whether sole has a strict preference for diurnal or nocturnal feeding, as this condition was imposed from the design of the experiment, nor to observe an eventual switch from diurnal to nocturnal feeding or vice-versa. Our experiment was conducted under natural photoperiod from early October, just after the autumn equinox, in similar environmental conditions as the ones described by Castanheira et al. (2011), and at the end of our experimental period, similar to the conditions of the experiments in Boluda Navarro et al. (2009). More studies, targeted specifically to assess if there is any stationality in Senegalese sole feeding rhythms should be carried out under natural photoperiod and temperature for a whole year.

European sea bass fed at night in the laboratory missed 3 times more pellets than fish fed during the day (Rubio et al., 2003) and it has been seen that, despite being visual predators, sea bass must rely in other sensory systems, mainly chemoreception (Faucher et al., 2006), to feed during winter. In the case of soles, the olfactory system has been demonstrated to play a critical role in feeding of these species, as trials involving bivalve commercial flavor (Reig et al., 2003) and homogenates of polychaetes added to feed (Barata et al., 2009) have been correlated with the velocity of the response of sole to food. Additionally, ablation of the olfactory epithelia suppressed the response towards the homogenates (Barata et al., 2009), underlining the importance of chemoreception in Senegalese sole feeding activity. In conditions of high density used in the present experiment, where fish commonly overlap, soles fed during the day could be relaying in the same sensory systems they use to feed at night to seek feed pellets in the bottom of the tank.

Sole, as most vertebrates, reacts to photoperiod and shows a typical plasma melatonin profile, with high levels during the scotophase and lower levels during the light phase (Bayarri et al., 2004). Melatonin has been demonstrated to have effects on food ingestion inhibition in goldfish (*Carassius auratus*; Pinillos et al., 2001; López-Olmeda et al., 2006) and tench (López-Olmeda et al., 2006), either by endocrine function, stimulating the release of growth hormone, and inhibiting the secretion of prolactin by the pituitary gland in rainbow trout (*Oncorhynchus mykiss*; Falcón et al., 2003) or by interacting with melatonin binding sites in peripheral tissues (Pinillos et al., 2001). Also, results linking melatonin to feed ingestion might be only indicating that feeding and growth could follow a seasonal pattern modulated by daylight length (Falcón et al., 2010). Recent studies in Senegalese sole have cloned different melatonin receptor subtypes. Subtype MT1 was present in light sensitive structures, like the retina or the optical tectum, but also in peripheral tissues like gills, kidney, liver or intestine (Confente et al., 2010). Whether melatonin has a direct effect on feed intake in sole is yet to be investigated.

As observed in other studies in sole (Sánchez et al., 2010), females in the present work showed

faster growth than males, showing higher weights already in the beginning of the experiment. Such sexual growth dimorphism has been already observed in other commercial species like turbot (*Scophthalmus maximus*; Imsland et al., 1997), Atlantic halibut (*Hippoglossus hippoglossus*; Hagen et al., 2006) or European sea bass (Saillant et al., 2001), suggesting that ovary physiology, including vitellogenesis, might be involved in the onset of sexual dimorphism. Females of those species present higher growths from the 9th or 10th month after hatching. It has been suggested that sexual steroids, particularly 17α-methyltestosterone, may be involved in reducing feed intake and feed conversion ratio in Eurasian perch juveniles (*Perca fluviatilis*), leading to lower weight males (Mandiki et al., 2004). Also, the expression of growth hormone mRNA was higher in immature half-smooth tongue sole females (*Cynoglossus semilaevis*) than in mature males from the same age, suggesting that the early maturation of males is behind the sexual growth dimorphism in this species (Ji et al., 2011). Growth dimorphism has been showed to be related to phenotypic sex in European sea bass (Saillant et al., 2001), reinforcing the role of the brain-hypothalamus-gonad axis as a key player in differential growth between males and females. Sexual steroid hormones also affect growth of Atlantic halibut before and after maturation. Females recruit more fast muscular fibers than males before puberty, and hyperplastic growth of muscle in males stops at the point of reaching maturation. These two effects, combined with the later maturation of females, results in sexual growth dimorphism in this species (Hagen et al., 2006). Sánchez et al. (2011) didn't find sexual growth dimorphism in soles of 38 g of initial weight, so it can be inferred that somewhere between 40 and the 80 g of initial weight of this experiment, where differences in weight between sexes were observed since the beginning, female soles start growing faster than males. Onset of puberty in sole is still poorly known, although spermatogenesis has been observed in 1-1.5 year old soles belonging to the F_1 of wild males reared in captivity (Agulleiro et al., 2007) and response of the testis to human chorionic gonadotropin was observed in 60 g male soles (Marín-Juez et al., 2011). Growth dynamics of female and male Senegalese sole, compared to data obtained in other flatfish species, suggest that the onset of puberty, probably beginning between 40 and 80 g of body weight, could play a major role in differential growth between males and females. More studies, focused directly to clarify this issue, should be carried out to confirm at which point of the life history of sole does sexual maturation occur and its exact relationship with differential growth between males and females. Additionally, as it has been discussed before, endogenous physiological factors are not the only ones that can shape growth in farmed fish, as factors as stocking density or feeding schedule (ratio and feeding frequency) can also affect growth and sexual dimorphism. In Eurasian perch, feeding in excess triggered a differential growth between females and males that was not seen offering 100% of the recommended ratio for this species (Fontaine et al., 1997). Independently of the influence of physiology on dimorphism of sole, our experiment was designed to offer feed in excess so, could feed availability have set the ground for female sole to grow to the maximum extent of their genetics/physiology?

Despite feeding in excess, apparent feed conversion ratios (1.36$\pm$0.122 for day-fed fish and 1.27$\pm$0.097 for night-fed fish) were similar to the ones presented by Salas-Leiton et al. (2011, in the order of 1.27 for graded fish and 1.12 for ungraded fish fed at 1.0% of body weight during the dark phase), between the several FCRs obtained by Borges et al. (2009) comparing isonitrogenous diets (FCRs of 1.04 and 1.15 for diets with 4-8% of lipid content, and FCRs from 1.66 to 2.16 for diets with 12-20% of lipid content) and almost two-fold better than the ones obtained by Valente et al. (2011) using experimental diets on market-size soles (between 2.03 and 2.35).

In the present work size dispersion was high, like in Sánchez et al. (2010), Salas-Leiton et al. (2011), and Sánchez et al. (2011). From the production point of view, grading of soles of 80 g onwards would tend to bias sex ratios of heads and tails, as more males would be expected in the smaller sizes. Recent findings by Salas-Leiton et al. (2011) show that there is a strong social effect in shaping growth dynamics on sole, with individual growth being strongly affected by social composition when grading not by size, but by specific growth rate (fast, medium and slow growers). It would have been interesting to know the sex composition of these groups to verify this hypothesis and to assess whether the fast growers within

the newly formed growth hierarchies were females or males.

Besides being an heterogeneous size population, the coefficient of variation augmented for all the biometric parameters studied throughout the experiment. Our conditions favored an increase of CV along time. In Salas-Leiton et al. (2011) the ungraded population was similar to our ungraded conditions, and its CV also increased during the 60 days of experiment, while CV of fish graded according to previous growth rate decreased throughout the experiment. An increase of CV usually denotes increased social interactions and hierarchies (Irwin et al., 1999, 2002). In this study the increase of CV was very similar between treatments, indicating that maybe the stocking density level was promoting competition, not necessarily by the food resource, offered in excess, but for space, as in high density fish tend to look for favored spots in the tank where they feel more protected, including beneath conspecifics.

Feeding activity in cultured sole is a complex issue that can involve physiological factors, as response to light, either in form of photoperiod length, time of the photoperiod in which feed is administered, maturity state and sex, or rearing conditions such as stocking density or size heterogeneity that can shape the behavior of the stocked fish. In conclusion, besides being an eminently nocturnal species, Senegalese sole fed during the light phase may yield specific growth rates and feed conversion ratios that are comparable to those of fish fed during the dark phase, suggesting the feasibility of feeding during normal business hours in commercial facilities.

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Chapter 5

Loss of genetic variability in a hatchery strain of Senegalese sole (*Solea senegalensis*) revealed by sequence data of the mitochondrial DNA control region and microsatellite markers

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Loss of genetic variability in a hatchery strain of Senegalese sole (*Solea senegalensis*) revealed by sequence data of the mitochondrial DNA control region and microsatellite markers

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SUMMARY: Comparisons of the levels of genetic variation within and between a hatchery F_1 (FAR, n=116) of Senegalese sole, *Solea senegalensis*, and its wild donor population (ATL, n=26), both native to the SW Atlantic coast of the Iberian peninsula, as well as between the wild donor population and a wild western Mediterranean sample (MED, n=18), were carried out by characterizing 412 base pairs of the nucleotide sequence of the mitochondrial DNA control region I, and six polymorphic microsatellite loci. FAR showed a substantial loss of genetic variability (haplotypic diversity, h=0.49±0.066; nucleotide diversity, π=0.006±0.004; private allelic richness, pAg=0.28) to its donor population ATL (h=0.69±0.114; π=0.009±0.006; pAg=1.21). Pairwise F_{ST} values of microsatellite data were highly significant (P<0.0001) between FAR and ATL (0.053) and FAR and MED (0.055). The comparison of wild samples revealed higher values of genetic variability in MED than in ATL, but only with mtDNA CR-I sequence data (h=0.948±0.033; π=0.030±0.016). However, pairwise Φ_{ST} and F_{ST} values between ATL and MED were highly significant (P<0.0001) with mtDNA CR-I (0.228) and with microsatellite data (0.095), respectively. While loss of genetic variability in FAR could be associated with the sampling error when the broodstock was established, the results of parental and sibship inference suggest that most of these losses can be attributed to a high variance in reproductive success among members of the broodstock, particularly among females.

Keywords: *Solea senegalensis*, genetic variability, mitochondrial DNA Control Region, microsatellite loci, broodstock management, variance in reproductive success, flatfish.

RESUMEN: PÉRDIDA DE VARIABILIDAD GENÉTICA EN UNA POBLACIÓN DE CULTIVO DE LENGUADO SENEGALÉS (*SOLEA SENEGALENSIS*) REVELADA MEDIANTE DATOS DE SECUENCIA DE LA REGIÓN CONTROL DEL ADN MITOCONDRIAL Y DE MARCADORES MICROSATÉLITE. – Se compararon los niveles de variabilidad genética de la F_1 de una población de cultivo (FAR, n=116) de lenguado senegalés, *Solea senegalensis*, y de una muestra de su población de origen (ATL, n=26), ambas provenientes del SO de la península ibérica (Atlántico), así como entre esta última y una muestra de individuos salvajes del Mediterráneo occidental (MED, n=18), mediante la caracterización de una secuencia de 412 pares de bases de la Región Control-I del ADN mitocondrial, y de seis loci microsatélite. FAR experimentó una reducción sustancial de variabilidad genética (diversidad haplotípica, h=0.49±0.066; diversidad nucleotídica, π=0.006±0.004; riqueza de alelos privados, pAg=0.28) respecto a su población original ATL (h=0.69±0.114; π=0.009±0.006; pAg=1.21). Los valores de F_{ST} entre poblaciones, calculados a partir del análisis de los microsatélites, fueron altamente significativos (p<0.0001) para FAR y ATL (0.053), y para FAR y MED (0.055). La comparación de las dos muestras salvajes mostró mayores niveles de variabilidad genética en MED que en ATL, pero únicamente en marcadores mitocondriales (h=0.948±0.033; π=0.030±0.016). Sin embargo, los valores de Φ_{ST}

y F_{ST} presentaron diferencias significativas (p<0.0001) respectivamente tanto a partir de datos mitocondriales (0.228) como de microsatélites (0.095), aunque la pérdida de variabilidad en FAR podría estar asociada al error de muestreo al establecer el lote de reproductores a partir de peces salvajes, los resultados de la inferencia de parentesco sugieren que la mayor parte de esta pérdida podría estar relacionada con la varianza reproductiva entre los miembros de la generación parental, particularmente entre las hembras.

Palabras clave: *Solea senegalensis*, variabilidad genética, región control mitocondrial, loci microsatélite, gestión de reproductores, peces planos, varianza reproductiva.

INTRODUCTION

Senegalese sole (*Solea senegalensis*, Kaup, 1858) is a flatfish of high commercial importance that is indistinguishable by consumers from common sole (*S. solea*, Linnaeus, 1758), so it is rated as the same species in marketing statistics (Reig *et al.* 2000). These two species, however, display marked differences in biological requirements that are important for rearing in captivity, such as spawning timing and temperature tolerance (Imsland *et al.* 2003). Although interest in farming Senegalese sole intensively in southern Europe dates back to the early 1980s, it failed to reach successful commercial development (Imsland *et al.* 2003, Reig *et al.* 2003). The reasons for this include lack of full control over spawning, poor fry quality and high mortality rates during the weaning stage (Cañavate and Fernández-Díaz 1999; Anguís and Cañavate 2005), all leading to juvenile scarcity for stocking purposes. This problem is compounded by a high incidence of skeletal malformations in post-larvae and juveniles (Gavaia *et al.* 2002) and of disease outbursts affecting all ontogenetic stages caused by multiple pathogenic agents (Zorrilla *et al.* 2003). Furthermore, optimization of production has not been possible due to high heterogeneity in growth rates within cultured stocks that result in high body size variance at harvest (Dinis *et al.* 1999, Flos *et al.* 2001).

Because losses in genetic variability are known to negatively affect survival and growth (Falconer 1960), a good benchmark during the establishment of breeding programmes is to characterize the genetic composition of wild stocks in order to maintain similar levels of genetic variability in captive populations. Ultimately, this information can be used to prevent inbreeding in captivity and to minimize the potential genetic dilution of natural populations in accidental escapes of hatchery fish, or as part of stock enhancement programmes (Sekino *et al.* 2002, Fjalestad *et al.* 2003, Porta *et al.* 2007), a practice that is being considered as aquaculture industries increase their activity (Cognetti *et al.* 2006). In addition, the characterization of genetic variation is a prime requirement for marker-assisted selection (MAS) programmes aimed at increasing production (Fjalestad *et al.* 2003).

Genetic variability between wild and hatchery-reared marine fishes has been compared for several species (Coughlan *et al.* 1998, Tinti and Piccinetti 2000, Alarcón *et al.* 2004, Nguyen *et al.* 2006). Among soles, the characterization of genetic variability has focused primarily on elucidating the genetic population structure of wild populations of *S. solea* and *S. senegalensis* using allozyme (Exadactylos *et al.* 1998) and mitochondrial DNA (mtDNA) control region (CR) data (Tinti *et al.* 2000, Guarniero *et al.* 2002). In addition, genetic studies have been conducted to clarify the phylogenetic relationships among members of this genus (Tinti *et al.* 2000, Borsa and Quignard 2001).

Studies of genetic diversity of farmed soles are limited to the characterization of variation in *S. solea* with allozymes (Exadactylos *et al.* 1999), and recent studies in *S. senegalensis* using microsatellite loci by Porta *et al*. (2006, 2007). These studies revealed substantial reductions in genetic variability in just one generation in stocks composed of F_1 individuals compared with wild donor populations. Similarly, Sekino *et al*. (2002) documented a marked reduction in genetic variability in both nuclear DNA (nDNA) and mtDNA in hatchery strains of the flatfish Japanese flounder (*Paralichthys olivaceus*) compared with wild populations. Because of the high resolution of the hypervariable mtDNA CR nucleotide sequence data, Sekino *et al.* (2002) were able to conclude that only a small fraction of the stocked females contributed to the reared population.

The goal of this study was to compare the level of genetic variability of a farmed Senegalese sole population with its wild donor population by determining the nucleotide sequence of the hypervariable mtDNA control region I (CR-I), and by characterizing six microsatellite loci. Both mtDNA and microsatellite data were then used to infer sibship relationships within samples. Additionally, this study aimed to perform a preliminary exploration of the genetic relationships between Atlantic and Mediterranean Senegalese sole populations.

MATERIALS AND METHODS

A total of 160 specimens of *S. senegalensis* were obtained from the following sources:

1) A farmed sample (FAR, n=116) supplied by an aquaculture operation located in the vicinity of the Ebro River delta (NE coast of Spain) in 2003. These farmed specimens were the F_1 progeny of a broodstock kept at the "El Toruño" CICEM research centre on the SW Atlantic coast of Spain. This facility obtains its broodstock from the nearby ponds, which are communicated with the waters of the Cadiz Bay (Anguís and Cañavate 2005).

2) A wild Mediterranean sample (MED, n=18) caught by local fishermen from the Mediterranean fishing harbor of Torredembarra, located north of the Ebro river, along the NE coast of Spain, in January 2004.

3) A wild Atlantic sample (ATL, n=26) captured in December 2005 by local fishermen from the Atlantic fishing harbours of Barbate and Sanlúcar de Barrameda, both in the Gulf of Cadiz, along the SW coast of the Iberian Peninsula. These two Atlantic locations were pooled to increase the sample size, taking into account that their geographical proximity and the lack of geographic differentiation shown for other flatfish species (Borsa *et al.* 1997).

Tissue samples consisted of white skeletal muscle obtained either by needle biopsy (Sánchez *et al.* 2003) from farmed fish or by necropsy from the wild fish samples. Tissue was preserved in 96% ethanol until assayed in the laboratory. Total DNA was extracted overnight using the modified version of the Proteinase K-ethanol precipitation method without organic extractions described by Greig (2000).

Characterization of the mtDNA Control Region-I (CR-I)

The entire mtDNA CR-I of 110 Senegalese soles (74 FAR, 18 MED and 18 ATL) was amplified using the primer L15998-Pro (5' TACCCCAAACTCCCAAAGCTA 3') (NCBI accession number AY270154; Alvarado Bremer *et al.* 1995) and the universal H-strand primer H00598-Phe (5' GCATCTTCAGTGCTATGCTTT 3'; Vu, 1997). All polymerase chain reactions (PCR) were carried out in 12.5 µl volumes containing 1.25 µl of 10X PCR buffer (Invitrogen, 20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each primer, 0.2 mM of each dNTP, 0.31 U of *Taq* polymerase (Invitrogen, Platinum *Taq*) and 1 µl of template DNA, except for ATL samples, which were amplified using Sigma JumpStart *Taq* DNA polymerase and its corresponding buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl).

Amplicons were sequenced with L15998 primer using the dideoxy terminators method (Sanger *et al.* 1977) and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Nucleotide sequences were read using an ABI Prism 310 Genetic Analyzer. Nucleotide sequences were visualized in Chromas 1.45 (Technelysium Pty Ltd) and multiple sequence alignments were manually optimized in BioEdit (Hall, 1999), using the orthologous sequence of common sole (NCBI accession number AF034262) as reference.

The number of haplotypes (M), haplotypic diversity (*h*) (Nei and Tajima, 1981) and nucleotide diversity (π) (Nei 1987) and mean number of pairwise differences (mPD) (Nei and Li, 1979) were estimated with ARLEQUIN 3.5 (Excoffier and Lischer 2010). The proportion of variance distributed between samples (F_{ST}) was estimated by an analysis of the molecular variance (AMOVA) (Excoffier *et al.* 1992) as implemented in ARLEQUIN. Significance levels were determined by conducting a non-parametric permutation procedure 1023 times.

TABLE 1. – PCR conditions for six polymorphic microsatellite loci used to characterize three samples of Senegalese sole.

Locus	A_T (ºC)	PCR	Co-amplified locus
Sol12D	56	*Simplex*	
Sol13B	60	*Multiplex*	*SolCA13*
SolCA13	60	*Multiplex*	*Sol13B*
Sol14	50	*Simplex*	
Sol19A	60	*Multiplex*	*SolMII*
SolMII	60	*Multiplex*	*Sol19A*

A_T = annealing temperature of the PCR. *PCR* = whether polymerase chain reaction amplified a single locus (*simplex*) or two loci (*multiplex*). *Co-amplified locus* = locus amplified in the same multiplex reaction.

A statistical parsimony cladogram incorporating haplotype frequencies was estimated in TCS (Clement *et al.* 2000), where the probability of parsimony is calculated for DNA pairwise differences until the probability exceeds 95%. The number of mutational differences associated with this probability is then the maximum number of mutational connections justified by the parsimony criterion. This approach allows genealogical relationships to be observed as a network instead of a tree, depicting loops wherever such relationships cannot be unambiguously resolved.

Microsatellite loci

Six microsatellite loci, described for *S. senegalensis* by Porta and Álvarez (2004), were amplified for all the 160 fish, using fluorescently labelled primers (IDT DNA and Applied Biosystems). All PCR reactions were performed following the same procedure as the one described for the mtDNA CR-I, and four reactions were necessary for genotyping each individual: 2 simplex PCR and 2 multiplex PCR with 2 markers each. In all cases, PCR volume was adjusted for a final reaction volume of 12.5 µl. The amplified loci, annealing temperature of each reaction, as well as if amplification was on a simplex or multiplex PCR (with the corresponding multiplex pair for each locus), are presented in Table 1. All PCR products were mixed and diluted in 1:30 bi-distilled water, and denatured in highly deionized formamide at 95°C for 2 min. Molecular weight of the different fragments was assessed through capillary electrophoresis in an ABI Prism 310 Genetic Analyzer and analyzed in Genescan 3.7 and GeneMapper 4.0 (Applied Biosystems) using GENESCAN™-500 TAMRA™ as the molecular weight marker.

Genetic variability estimators, including observed heterozygosity (Ho) and expected heterozygosity (He), F_{IS} and F_{ST} for each population, as well as the assessment of the Hardy-Weinberg equilibrium for each locus within each population, were performed in Genepop 3.34 (Raymond and Rousset, 1995). Allelic richness and private allelic richness were calculated through statistic rarefaction to compensate for the bias in sample size in HP-Rare 1.0 (Kalinowski 2005). Genic and genotypic differentiation, as well as linkage disequilibrium between loci, was estimated in Genep-

TABLE 2. – *Solea senegalensis* mtDNA CR-I haplotypes (*Ssen#*) defined by the variable nucleotide positions (numbers above sites), their corresponding NCBI's GenBank accession numbers, and relative frequencies in farmed (FAR), wild Atlantic (ATL) and wild Mediterranean (MED) sole. Sampling details in Materials and Methods. Identity with sequence *Ssen001* is indicated by dots (·), and ambiguous nucleotides positions by the letter N.

	033	054	071	082	089	090	092	105	106	125	132	139	143	144	159	161	164	167	168	176	185	187	222	223	226	317	GenBank accession number	Haplotype relative frequencies FAR *n*=74	ATL *n=18*	MED *n*=18
Ssen001	T	G	C	A	C	C	A	T	G	T	T	A	C	T	A	C	A	A	A	T	A	G	A	A	T	C	DQ523428	0.703	0.556	0.000
Ssen002	·	C	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	DQ523429	0.122	0.000	0.000
Ssen003	C	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	G	·	·	DQ523430	0.040	0.000	0.000
Ssen004	·	·	·	·	T	T		·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	G	·	C	·	DQ523431	0.000	0.000	0.111
Ssen005	·	·	·	·	T	·	·	·	·	·	A	·	·	·	·	·	·	·	·	N	·	·	G	·	·	·	DQ523432	0.000	0.000	0.056
Ssen006	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	T	·	G	·	C	·	·	G	·	·	T	DQ523433	0.068	0.000	0.000
Ssen007	·	·	·	G	T	·	·	·	·	·	A	·	·	·	·	·	·	·	·	C	·	·	G	·	·	·	DQ523434	0.000	0.000	0.056
Ssen008	·	·	·	G	T	·	·	·	·	·	A	·	·	·	·	·	·	G	·	C	·	·	G	·	·	·	DQ523435	0.000	0.000	0.111
Ssen009	·	·	·	G	T	·	·	·	·	·	A	·	·	·	·	·	·	G	·	C	·	·	G	·	·	T	DQ523436	0.000	0.000	0.056
Ssen010	·	·	·	G	T	·	·	·	·	C	A	·	·	·	·	·	·	G	·	C	·	·	G	·	·	·	DQ523437	0.000	0.000	0.056
Ssen011	·	·	A	G	T	·	·	·	·	·	A	·	·	·	·	·	·	G	·	C	·	·	G	·	·	·	DQ523438	0.000	0.000	0.167
Ssen012	·	·	·	G	T	·	·	·	·	·	A	·	·	C	·	·	·	G	·	C	·	·	G	·	·	·	DQ523439	0.014	0.000	0.167
Ssen013	·	·	·	·	T	·	·	·	·	·	·	G	·	·	·	·	G	·	·	N	G	·	·	·	·	T	DQ523440	0.000	0.000	0.056
Ssen014	·	·	·	·	T	·	·	C	A	·	·	·	·	·	·	·	G	·	·	C	G	A	·	·	·	T	DQ523441	0.054	0.000	0.056
Ssen015	·	·	·	·	T	·	·	C	A	·	·	·	T	·	·	·	G	·	·	C	G	A	·	·	·	T	DQ523442	0.000	0.000	0.056
Ssen016	·	·	·	·	T	·	G	C	A	·	·	·	·	·	·	·	G	·	·	C	G	A	·	·	·	T	DQ523443	0.000	0.000	0.056
Ssen017	·	·	·	·	T	T	·	·	·	·	·	·	·	·	·	G	·	·	·	C	·	·	·	·	·	·	FJ839912	0.000	0.056	0.000
Ssen018	·	·	·	·	T	T	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	G	·	·	·	FJ839913	0.000	0.111	0.000
Ssen019	·	·	·	·	N	N	N	C	·	·	·	·	·	·	G	·	G	·	·	C	G	A	·	·	·	T	FJ839914	0.000	0.056	0.000
Ssen020	·	·	·	G	T	·	·	·	·	·	A	·	·	·	·	·		G	·	C	·	·	·	·	·	·	FJ839915	0.000	0.111	0.000
Ssen022	·	·	·	·	T	·	·	C	A	·	·	·	·	·	·	·	G	·	·	C	G	A	·	G	·	T	FJ839916	0.000	0.056	0.000
Ssen023	·	·	·	·	N	N	N	N	N	·	·	·	·	·	·	·	G	·	G	C	G	A	·	·	·	T	FJ839917	0.000	0.056	0.000

op 3.4 (Markov chain: 1000 batches, 10000 iterations). Average gene diversity was estimated in ARLEQUIN 3.5. All loci were checked for null alleles with Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004). Genetic differentiation among populations was also assessed using a locus-by-locus AMOVA with the number of alleles as distance, as implemented in ARLEQUIN 3.5.

Parentage and sibship inference

As the exact composition of the original broodstock was unknown, the number of parental individuals for FAR was inferred using COLONY 2.0 (Wang, 2004). Parental genotypes were inferred through maximum likelihood, assuming polygamous mating for both males and females, using the allele frequencies observed in the donor sample (ATL). The likelihood that each pair of individuals was or was not related was also estimated. In these analyses those individuals that did not share the same mtDNA CR-I haplotype were excluded as potential maternal siblings. Similar analyses were performed for ATL and MED samples, using the respective observed allele frequencies for each sample.

Average individual maximum likelihood estimates of relatedness for each sample were computed on ML-RELATE (Kalinowski *et al.* 2006). Mean variability estimators were compared among samples through a Student *t* test (α=0.05) with the Welch correction for 2 independent samples, except when a Shaphiro-Wilk test failed to detect normality within the sample. In such cases, a non-parametric Wilcoxon rank sum test with continuity correction was used instead. All tests were performed on R (R Development Core Team, 2010).

RESULTS

mtDNA CR-I analysis

In total, 412 base pairs (bp) of nucleotide sequence of the mtDNA CR-I were determined for 74 farmed and 36 wild Senegalese sole (Table 2). A total of 26 segregating sites (K), 12 of which were parsimony-informative, defined 22 distinct Senegalese sole CR-I haplotypes. Haplotype frequencies, the number of segregating sites, and the NCBI's GenBank accession numbers for each sample are also included in Table 2.

Genetic variation was lower in the farmed sample than in the wild Atlantic sample. Accordingly, the probability of selecting two different haplotypes from a random pair was higher in ATL (h=0.69±0.114) than in FAR (h=0.49±0.066). In FAR 83% of the sample was accounted for by the two most common haplotypes (*Ssen001* and *Ssen002*), with *Ssen001* accounting for 70% and 56% of FAR and ATL, respectively, but absent from MED (Table 2). Estimates of nucleotide di-

TABLE 3. – Genetic variability estimators from the analysis of 412 bp of the mtDNA Control Region-I sequences for three samples of Senegalese sole.

	N	M	Mp	h	π	mPD
FAR	74	6	3	0.489±0.067	0.006±0.004	2.467±1.35
ATL	18	7	6	0.693±0.114	0.009±0.006	2.771±1.54
MED	18	12	10	0.948±0.033	0.030±0.016	11.963±5.68

Sample acronyms as in Table 2. N, number of sequenced individuals; M, number of different haplotypes; Mp, number of private haplotypes; h, haplotypic diversity; π, nucleotide diversity; mPD, mean number of pairwise differences.

TABLE 4. – F_{ST} values of the analysis of the molecular variance (AMOVA) for three Senegalese sole populations.

a)	FAR	ATL	MED	b)	FAR	ATL	MED
FAR	0	0.024	0.395**	FAR	0	-	-
ATL	0.127**	0	0.228**	ATL	0.053**	0	-
MED	0.055**	0.095**	0	MED	0.053**	0.054**	0

FAR, farmed fish; ATL, wild Atlantic fish; MED, wild Mediterranean fish. a) Above diagonal: Φ_{ST} values corresponding to the AMOVA of mitochondrial DNA data. Below the diagonal: F_{ST} values corresponding to the AMOVA of microsatellite data including all six studied markers. b) Below the diagonal only: F_{ST} values corresponding to the AMOVA of microsatellite data excluding locus *Sol14*. * P<0.01; ** P<0.001.

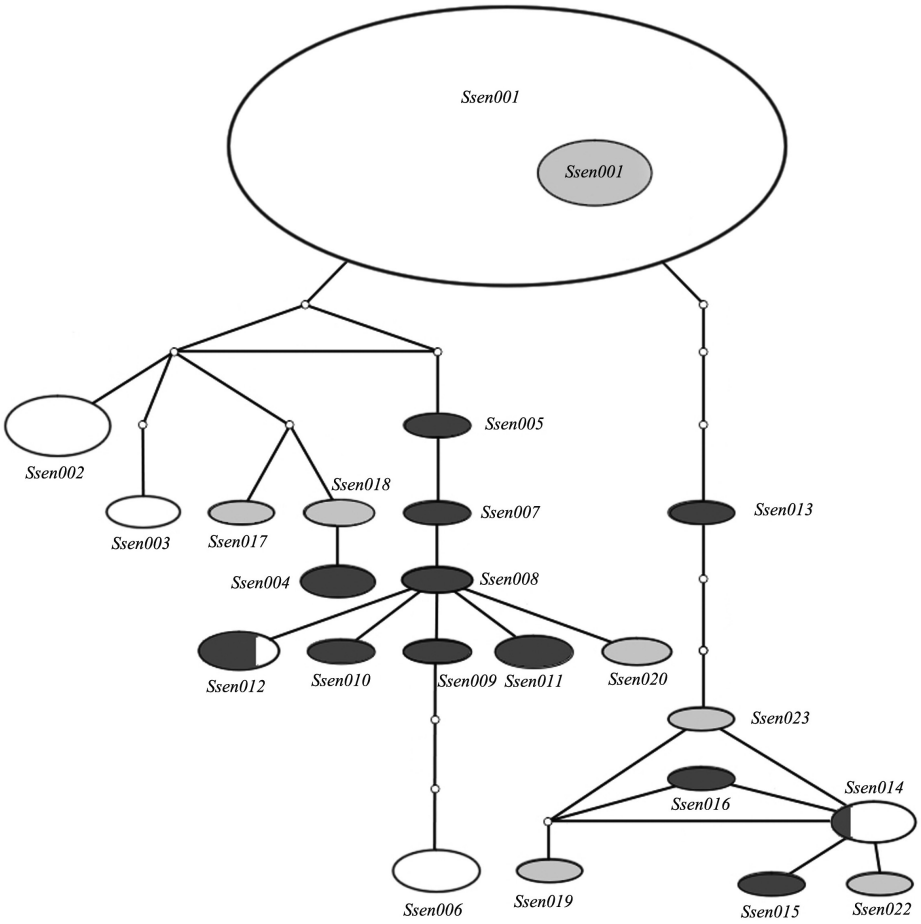

FIG. 1. – Parsimony network estimated in TCS v1.21 for FAR, ATL and MED haplotypes (FAR = farmed Atlantic sample; ATL = wild Atlantic sample; MED = wild Mediterranean sample). The area of the ellipsoids is proportional to the frequency of each haplotype within the pooled populations. Un-sampled or non-existent nodes are represented by small white dots. White, grey and black ellipsoids stand for FAR, ATL and MED, respectively.

versity and mean number of pairwise differences were also higher in ATL than in FAR (Table 3). An AMOVA comparing FAR and ATL identified most of the genetic variation within populations, with only 2.35% of the mtDNA variance corresponding to non-significant differences between these two populations (Table 4).

There were substantial differences in genetic variability between wild Atlantic (ATL) and wild Mediterranean fish (MED) samples (Table 3), with a higher diversity in MED (h=0.95±0.03) than in ATL (h=0.69±0.11). Similarly, estimates of nucleotide diversity and mean number of pairwise differences were three and four times higher in MED than in ATL (Table 3). There were 12 different haplotypes in MED, ten of these private and including *Ssen011*, one of two most common haplotypes in this population at 16.7%, with the other (*Ssen012*) shared with FAR (Table 2). An AMOVA of mtDNA CR-I data of the two wild samples revealed that roughly one fourth (Φ_{ST}=0.228; P<0.0001) of the total genetic variability corresponded to differences between ATL and MED (Table 4), with the rest of the variance within samples.

TABLE 5. – Genetic variability estimators for six polymorphic microsatellite loci used to characterize the three samples of Senegalese sole.

Locus	Sample	N	#alleles	#private alleles	A_g	Private A_g	He	Ho	Fis
Sol12D	FAR	114	5		4.9	0.01	0.73	0.70	0.0413**
	ATL	26	6	1	5.3	0.77	0.72	0.58	0.2013
	MED	18	5	1	5.0	1.00	0.74	0.72	0.0286
Sol13B	FAR	115	14	1	9.2	0.22	0.85	0.90	-0.0684**
	ATL	25	11		10.0	1.27	0.89	0.96	-0.0557
	MED	18	90		8.9	0.91	0.87	0.94	-0.0804
SolCA13	FAR	113	10		7.8	0.61	0.80	0.75	0.0623**
	ATL	26	13	2	11.1	2.32	0.89	0.85	0.0542
	MED	18	9	1	8.9	1.90	0.84	0.83	0.0135
Sol14	FAR	111	5	1	3.2	0.15	0.41	0.42	-0.0535
	ATL	26	6	1	5.7	0.94	0.56	0.35	0.3882*
	MED	18	7	2	6.9	2.11	0.69	0.72	-0.0400
Sol19A	FAR	114	7		5.3	0.12	0.73	0.85	-0.1669
	ATL	26	7	1	6.8	1.96	0.82	0.73	0.1071
	MED	17	6		6.0	1.57	0.76	0.82	-0.0900
SolMII	FAR	113	9	2	6.8	0.54	0.78	0.85	-0.0909
	ATL	26	7		7.0	0.00	0.86	0.88	-0.0305
	MED	17	7		7.0	0.03	0.83	0.71	0.1504

FAR, farmed fish; ATL, wild Atlantic fish; MED, wild Mediterranean fish; N, number of individuals genotyped for each locus; Ag, allelic richness; Ho, observed heterozygosity; He, expected heterozygosity.* $P<0.01$; ** $P<0.001$

The relationship among the 22 haplotypes is depicted with a genetic network obtained by statistical parsimony (Fig. 1). Haplotypes of both farmed and wild samples are interspersed throughout the branches, and include one haplotype (*Ssen001*) shared between ATL and FAR, and two haplotypes (*Ssen012* and *Ssen014*) shared between FAR and MED.

Microsatellite data analysis

Analysis of a sample of 160 Senegalese sole from three populations (116 FAR, 26 ATL and 18 MED) identified a total of 64 alleles for the six studied loci, ranging from 7 to 14 alleles per locus, with a mean value of 10.6 alleles per locus. The mean number of private alleles per locus was 1.3. Mean allelic richness was 7.0 and mean private allelic richness was 0.9. Mean values of expected and observed heterozygosity were 0.77 and 0.75, respectively. The values of genetic variability estimators for all three samples are shown in Table 5.

The mean values for expected and observed heterozygosity in FAR were 0.72 and 0.75, showing significant departures from the Hardy-Weinberg equilibrium at three loci. *Sol12D* and *SolCA13* showed heterozygote deficiency, while *Sol13B* showed heterozygote excess. F_{IS} values for these loci were significant, although low (between –0.07 and 0.06). Significant linkage disequilibrium ($P<0.05$) was detected between all pairs that included *Sol13B* or *Sol19A*, and also between *SolCA13* and *Sol14*, and between *SolCA13 and SolMII*. Accordingly, in FAR 11 out of the 15 possible pairs of loci were linked. No null alleles were detected in FAR.

Observed indices of genetic variability for FAR are similar to those obtained for its donor population (ATL), including similar values of allelic richness and allele size ranges. FAR and ATL had a total of 50 alleles each, accounting respectively for 78.1% of all the polymorphism observed in the pooled sample of the three localities. Although average allelic richness was lower in FAR (6.2±2.16) than in ATL (7.7±2.35), and in MED (7.1±1.56), these estimates were not significantly different from each other (Student *t* test; $P<0.05$). However, mean private allelic richness was significantly higher (Student *t* test; $P<0.05$) in ATL (1.21) than in FAR (0.28). Mean values of expected and observed heterozygosity were 0.79 and 0.73, respectively, with only one microsatellite locus (*Sol14*) significantly departing from the Hardy-Weinberg equilibrium in ATL, where a high (0.388) and significant F_{IS} value ($P<0.01$), indicative of heterozygote deficit, was detected. Null alleles were detected solely at *Sol14* in ATL. No linkage disequilibrium was found in ATL among any of the loci pairs ($P<0.05$).

MED, the smallest sample analyzed, showed similar allelic richness to that in FAR and ATL. A total of 43 alleles were found in MED, accounting for 67.2% of the total allele count for the three localities, with a mean of 7.1 alleles per locus. In addition, mean private allelic richness for MED was significantly higher (1.25; Student *t* test; $P<0.05$) than for FAR, but not significantly different from ATL. Mean expected and observed heterozygosity values for MED equalled 0.79, with no significant departures from the Hardy-Weinberg equilibrium or significant linkage disequilibrium among loci pairs ($P<0.05$). No null alleles were detected in MED.

Average gene diversities over loci using the number of different alleles as distance were similar among ATL (0.78), FAR (0.69) and MED (0.79). Additionally no differences were observed for mean expected heterozygosity nor expected heterozygosity among all three samples (Student *t* test; $P>0.05$). Several alleles in FAR occurred at higher frequencies than in ATL (Fig. 2), including alleles 149 and 161 in *Sol13B*, allele 229 in *Sol14*, and alleles 128 and 132 in *Sol19A*, all rare in ATL (Fig. 2). The reciprocal condition was observed, specifically, allele 226 in *Sol14* accounted for 65.4% in ATL and only 0.5% in FAR (Table 5).

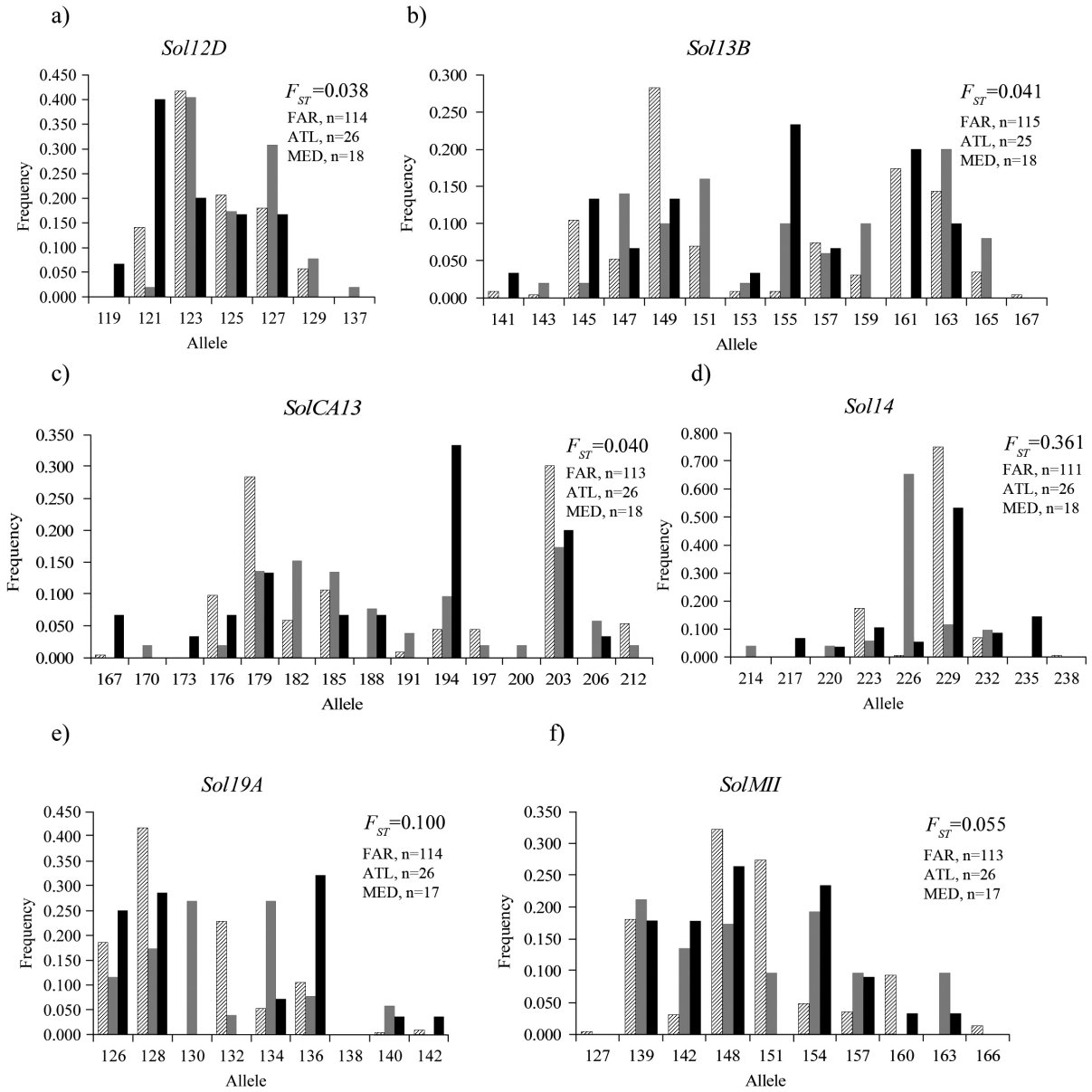

FIG. 2. – Allele frequencies for each of the six microsatellite loci characterized in this study. a) *Sol12D*, b) *Sol13B*, c) *SolCA13*, d) *Sol14*, e) *Sol19A* and f) *SolMII* for the three Senegalese sole samples: FAR (pattern bars); ATL (grey bars); MED (black bars). The number (n) of individuals per sample characterized for each locus is given. On each x-axis, the allele sizes (bp) for each locus, and on each y-axis the corresponding relative frequency. F_{ST} for each locus estimated among the three samples.

Multilocus AMOVA identified FAR and ATL as the sample-pair with the highest proportion of variation between groups, with a F_{ST} value of 0.127 (P<0.0001) compared with the F_{ST} for FAR and MED and for ATL and MED at 0.055 and 0.095, respectively (Table 4). F_{ST} values by locus inferred from the AMOVA among all three samples ranged from 0.038 for *Sol12D* to a substantially higher value of 0.361 for *Sol14* (Fig. 2). A locus-by-locus AMOVA between all sample-pairs yielded F_{ST} values that were four to 50 times larger for *Sol14* than for other loci (data not shown), reaching 0.298 for ATL to MED and 0.492 for ATL to FAR, compared with 0.070 between FAR and MED. Excluding *Sol 14* from the multi-locus AMOVA yielded considerably lower F_{ST} values in all comparisons involving ATL (Table 4).

Parentage, sibship inference and relatedness

A total of 18 male and 16 female parental genotypes were inferred in the FAR sample. The combination of these inferred broodstock genotypes could yield a total

of 58 full-sib groups or families (220 full-sib dyads), ranging from two to 15 full-sib individuals per family, and up to 750 half-sib dyads. All the offspring could be grouped in a single cluster (understanding as a cluster all offspring that, regardless of whether or not they share a parent, are still linked in a pedigree).

Three of the inferred female genotypes were identified as particularly prolific, accounting for 50% of the offspring and producing 22, 19 and 16 offspring. By contrast, the remaining females contribution ranged between one and 12 descendants, with an average of 4.5 offspring per female. Furthermore, FAR specimens sharing haplotype *Ssen001* (n=52) could have been generated by seven females, with the two most prolific accounting for 79% (41/52) of those individuals. These two females could have mated with up to 13 and four different males, respectively. The reproductive variance in paternal contribution was less pronounced, with only one inferred male as prolific as the dominant maternal genotypes, potentially siring 19 individuals. The remaining males sired on average 5.7 individuals, with a range of between 2 and 13 offspring per male, a contribution comparable with that of non-dominant females.

The whole ATL sample could have been bred by the 11 male and 13 female inferred parental genotypes, which could be grouped into four different clusters , although three of them included only one individual, and revealed two full-sib individuals as well as up to 24 half-sib families (47 half-sib dyads). A minimum of four females could have produced those individuals carrying haplotype *Ssen001*, corresponding to about 46% of the sample.

Sibship and parental inference for MED identified up to 15 possible half-sib families (15 half-sib dyads) as the product of the inferred genotypes of 7 males and 14 females, grouping all the offspring into six clusters. No full sibs were found in MED and only two fish, sharing haplotype (*Ssen008*), could be grouped as maternal siblings. Mating of different parents likely produced the rest of the individuals in that sample.

A parental variability index (P_V) that takes into account the differences in size between samples is proposed as the ratio between the number of inferred parental genotypes and the number of candidate offspring as follows:

$$P_V = [(N_M + N_P)/2] * 1/N_O$$

where N_M stands for the number of maternal genotypes, N_P for the number of paternal genotypes and N_O for the number of the offspring. An offspring in which each individual is bred by a single couple of parental genotypes would have an index of P_V=1, indicating the highest level of parental variability. Accordingly, P_V values for FAR, ATL and MED calculated through the inferred number of parental genotypes in COLONY were 0.150, 0.462 and 0.583, respectively.

Mean individual relatedness within samples (r) was 0.06±0.101 for both ATL and MED, and 0.11±0.179 for FAR. No significant differences of these values were found between ATL and MED, but the relatedness value for the farmed sample was significantly higher than for both wild populations (Wilcoxon rank sum test; P<0.001).

DISCUSSION

The analysis of 412 bp of nucleotide sequence of the mtDNA CR-I from two wild samples and a farmed F_1 sample of Senegalese sole revealed a substantial loss of genetic variability in one generation of aquaculture rearing, as shown by haplotypic diversity values and the haplotypic composition of each sample. FAR and ATL shared only haplotype *Ssen001*, the most common haplotype in both populations, respectively at a frequency of 70% and 56% but absent from MED. Similarly, the second most common haplotypes in FAR (*Ssen002*) and ATL (*Ssen018*) were private to each sample and absent in MED. It should be noted that an AMOVA using mtDNA CR-I data failed to find significant differences between ATL and FAR, presumably because of the high frequency of haplotype *Ssen001* in these samples. With the exception of *Ssen001*, the rest of ATL haplotypes were private to that sample.

Lower estimates of mtDNA diversity have been reported in farmed flatfish compared with wild populations. In Japanese flounder, Sekino *et al*. (2002) found an average haplotypic diversity of 0.761 for three farmed samples compared with 0.998 for a wild sample. The value of haplotypic diversity for the wild ATL sample reported here is lower than those of farmed Japanese flounder. Although the lower mtDNA variability in FAR could have been caused by sampling error during the establishment of the broodstock, it could also be the result of the differential reproductive performance among parents, as suggested in this species by Porta *et al*. (2007). By contrast, the value of haplotypic diversity of MED was higher (h=0.948), and while this estimate is lower than the value reported in wild Japanese flounder, it is similar to haplotypic diversity values reported for the wild Mediterranean populations of albacore tuna, *Thunnus alalunga* (h=0.961±0.018), (Viñas *et al.* 2004) and swordfish, *Xiphias gladius*, (h=0.942±0.009) (Alvarado Bremer *et al.* 2005).

Consistent with the reduction in mtDNA variability, private allelic richness was an indicator of allele loss in FAR, being significantly lower than in both wild samples (Table 5). Additionally, *Sol14* showed significant homozygote excess in ATL due to this high proportion of allele 226, which was likely responsible for the high fixation index of this locus among populations. Porta and Álvarez (2004) also reported a significant excess of homozygotes at this locus for a hatchery stock. Since the analysis with Micro-Checker strongly suggested the presence of null alleles at this locus, it was excluded from the analyses, resulting in a reduction in

half of the F_{ST} values for the comparisons that involved ATL, while at the same time lowering the p-value for comparisons of private allelic richness to 0.07, close to the threshold of significance adopted here. These F_{ST} values calculated without *Sol14* explain the apparent contradiction of a larger differentiation between FAR and ATL as opposed to MED and ATL that came from geographically more distant locations. Altogether, the differences in allele frequency resulted in significant allelic and genotypic differentiation between FAR and ATL. Despite these differences, the values of microsatellite heterozygosity were similar in FAR (0.75), ATL (0.73) and MED (0.79), with these values comparable to the average heterozygosity (0.79±0.19) reported by DeWoody and Avise (2000) for a pool of 12 marine fish species. However, all three Senegalese sole samples showed a low average value of allelic richness per locus, ranging from 6.2 in FAR to 7.7 in ATL, compared with the average value of 19.9±6.6 for marine fishes (DeWoody and Avise 2000). Porta *et al*. (2007) reported an average allele number for eight microsatellites loci ranging between 7.9 and 16.7, depending on the size of the sample and whether the fish were hatchery-reared or wild. However, of the eight loci analyzed by Porta *et al*. (2007), only four were characterized in here (*Sol13B*, *Sol19A*, *SolCA13* and *SolMII*). Restricting the comparison to these four loci yields similar ranges in the average number of alleles, between 7.5 and 12.5 for Porta *et al*. (2007) and 7.8 and 10.0 for this study.

Mean relatedness was higher in the cultured sample than in the wild samples. This is consistent with the data presented above for both mtDNA and microsatellite loci, and with relatedness values found in many reared fish species, including Senegalese sole (Porta *et al.* 2007). It is interesting to observe that while ATL showed lower haplotypic diversity than MED, both samples had similar mean relatedness and gene diversity values estimated from nDNA data. Apparent discrepancies between variability levels detected with microsatellite loci and mtDNA CR-I analysis have already been described by Alarcón *et al*. (2004) in gilthead sea bream (*Sparus aurata*) and were attributed to presence of rare haplotypes in high frequencies. The authors suggested that this pattern could be explained by drift in subdivided populations. In the present study, the small sizes of the wild samples, together with their restricted geographic origin, precludes reaching a definitive conclusion regarding this issue, but suggests the need for further studies.

Biases in both mtDNA haplotype and nDNA allele frequencies in farmed fish compared with the wild population of origin are not uncommon (Coughlan *et al.* 1998, Alarcón *et al.* 2004; Aho *et al.* 2006). Such biases result from random sampling error of the wild population when broodstocks are established using a small number of individuals (founder effect), together with the ensuing genetic drift experienced by successive generations of inbreeding in small effective size populations. The founder effect is particularly important in the interpretation of the results of this study, as the performance of hatchery-reared individuals of Senegalese sole as broodstock has been marginal. Accordingly, it is customary to utilize only wild-caught individuals as broodstock in aquaculture operations of this species, including the broodstock at "El Toruño" that produced FAR. A biased haplotypic profile in FAR would be expected exclusively from such a founder event even if all individuals in the broodstock contributed equally to this F_1. However, Porta *et al.* (2006) reported substantial losses in genetic variability at eight microsatellite loci in three Senegalese sole broodstock groups, two of which were F_1 progenies. Specifically, these authors found that alleles that were common in the broodstock were absent in the progeny, suggesting a large variance in reproductive success among potential parents. Such biases in parental contribution have been reported in other flatfishes in aquaculture operations. Blonk *et al.* (2009) showed that out of two *S. solea* broodstocks consisting of 28 and 20 individuals only five or fewer parental pairs, respectively, produced more than half of the total progeny. Similarly, only 25% of the females in two strains of Japanese flounder apparently contributed to the offspring of two strains founded by 50 and 30 candidate female parents (Sekino *et al.* 2002). The high linkage disequilibrium observed between microsatellite loci in FAR could also be related to possible non-random mating or the bottlenecks that occur in aquaculture breeding in communal tanks where fish mating is not fully controlled, and thus concordant with the results from Porta and Álvarez (2004) and Porta *et al*. (2006, 2007) regarding important biases in the expected allele combinations of F_1 generations.

In this study, estimates of reproductive variance among members of the broodstock were assessed by estimating the maternal contribution from the highly informative mtDNA CR-I sequence data, in combination with sibship and parental inference from microsatellite data. Since six mtDNA CR-I haplotypes were identified among 74 FAR individuals, it follows that the maternal stock consisted of a minimum of six females. Although the exact number of parents that produced the F_1 analyzed in this study is unknown, studies carried out at the same experimental hatchery (Cañavate and Fernández-Díaz 1999, Anguís and Cañavate 2005), suggest that between 8 and 15 females and 7 and 10 males were used to establish the broodstock at this operation. Given that with the exception of *Ssen001*, present in ATL at 56% and in FAR at 70%, no other haplotype was shared between FAR and ATL, it is plausible to assume that these non-shared haplotypes do not occur at a high frequency in the wild. In fact, excluding the common haplotype *Ssen001* from ATL increased the probability of sampling a different haplotype from 0.693 to 0.929, a value of h similar to that of MED (h=0.948). Thus, each of the fish in FAR carrying a distinct haplotype, except those carrying *Ssen001*, might have been produced by only five dams.

Incorporating the mtDNA CR-I data in the results from COLONY identifies seven females producing the F_1 individuals carrying haplotype *Ssen001*. Thus, no fewer than 12 females in the parental stock produced FAR. However, inference of parentage and sibship indicates that 16 females account for all the offspring analyzed in FAR. Thus, in addition to the seven females in the broodstock sharing *Ssen001*, three had to share *Ssen002*, two *Ssen003* and three *Ssen006*. These estimates of the number of females in the original broodstock are consistent with the higher estimate of 15 females recalled to have been used to establish the original operation at "El Toruño". These results are particularly revealing of the reproductive variance associated with each sample. While in FAR a total of 58 full-sib groups were identified, with 2-15 full sibs per family, in ATL only two full-sib individuals were identified and none in MED. Such dramatic differences in the level of inferred sibship among samples is most likely the result of the large variance in reproductive success among members of the original broodstock, particularly among females, with the four most prolific dams producing 60% of fish in FAR. The parental variability index also underlines the variability loss of the FAR sample compared with the donor sample (P_V =0.462 for ATL and 0.150 for FAR). It thus appears that the variance in reproductive success among breeders is an important factor responsible for loss of genetic variability among the offspring, and confirming results by Sekino *et al*. (2002) and Porta *et al*. (2006, 2007).

Phylogeographic studies of common sole characterizing the mtDNA Control Region revealed geographic isolation among samples within the central Mediterranean (Guarniero *et al.* 2002). Although the present study is the first to assess the genetic variability of Senegalese sole across basins using mtDNA CR-I data, caution should be used in the interpretation of the results because of the small sample sizes and limited sampling coverage. Nevertheless, the particularly pronounced mtDNA differentiation recorded here between the two wild populations from different basins (ATL and MED) is worth noting. There were nearly twice as many haplotypes in the wild Mediterranean sample as in the wild Atlantic sample, and significant differentiation in both nucleotide diversity values and the mean number of pairwise differences. Values of differentiation between ATL and MED samples with microsatellite data (F_{ST}=0.054) were similar to the average reported by Ward (1994) for sub-populations of marine fish species (F_{ST}=0.062). These results are a preliminary approximation for further studies, which should be carried out by increasing the number of sampling locations and the size of the samples to determine the degree of differentiation between Atlantic and Mediterranean Senegalese sole populations. Accordingly, it would be possible to elucidate the presence of a barrier between Atlantic and Mediterranean populations similar to the one found between the west of the Gibraltar Strait and the Ebro Delta–Gulf of Lion in European flounder (*Platichthys flesus*) using allozymes and mtDNA cytochrome b data (Borsa *et al.* 1997).

In conclusion, this study documents a loss in genetic variability in a single generation of Senegalese sole rearing, as evidenced by lower values of mitochondrial haplotypic diversity and nuclear diversity of microsatellites in the farmed sample compared with the wild donor population.

The comparison of wild Atlantic and Mediterranean samples in both mitochondrial and microsatellite DNA suggests a limited gene flow between the populations inhabiting these basins, although the exact nature of the isolation mechanism requires further investigation.

Finally, it is clear that an accurate knowledge of the genetic composition of farmed stocks is essential both for maintaining the cultured stocks and for potential future restocking purposes because the genetic composition of the wild populations could be severely affected.

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Chapter 6

Discussion

6.1 Growth

6.1.1 Stocking density

Stocking density is an important parameter in fish culture, not only because it has strong implications on growth performance, but also because it can affect fish welfare through the development of social interactions and hierarchies (Ellis et al., 2002; Turnbull et al., 2005) and it has an economic impact, conditioning the performance of the production. Social interactions could be behind the differences observed in growth efficiency of several fish species. For example, gilt-head sea bream feeding efficiency has been observed to be affected by ration size, with lower rations leading to increased competition, faster swimming speeds and higher densities under the feeder (Andrew et al., 2004). On the other hand, growth of species presenting schooling behavior can be improved by rising stocking densities (Gardeur et al., 2001a).

Throughout the experiments of the present thesis, stocking density has been one of the culture management parameters that has been more thoroughly studied. In chapter 2, two different densities were assayed (HD; 180%, of initial bottom coverage, 26.6 kg·m^{-2}. LD; 60% of initial bottom coverage,8.6 kg·m^{-2}), to investigate whether this parameter had any significant influence on growth, but also in size variation. In chapter 3, the same initial densities were also assayed, but this time, initial size composition of the experimental groups were also taken into account, in order to assess whether it could afterwards modulate the evolution of size variation. Finally, an intermediate density was chosen (at 130% of bottom coverage, 13.5 kg·m^{-2}), knowing that it would increase to a high density towards the end of the rearing period, to assess whether fish fed at night, according to the natural rhythms of this species, grew faster than fish fed during daylight hours.

In the experiment described in chapter 2, different effects of a high stocking density could be identified in growth of cultured Senegalese sole. Soles were reared for 134 days in LD or HD conditions. Fish reared under high stocking density showed a growth latency period that resulted in almost no weight gain for the first 40 to 61 days of the experiment. After that initial phase, HD fish started to grow at a similar rate to LD fish up to day 134. The lower specific growth rate of HD fish could be the result of the ”cold-start” associated to this treatment, presumably due to the sudden increase in stocking density experimented after crowding fish that were being held in a low acclimatization density. The same effect could be observed when, after 134 days of trial, stocking density treatments were permuted between LD and HD fish up to day 195. Hence, in our conditions, it seemed that extreme and sudden increases in stocking density, instead of high density itself after proper acclimatization, could be responsible of the effects observed in SGR, particularly, the initial lack of growth or lag phase.

Growth analyzed by LME models was significantly affected by density, but a separate analysis for days 1-61 and 61-134 showed that stocking density had such effect only during the first period, that handicapped HD fish for the rest of the ongrowing period. Contrarily, standard length was significantly affected by density throughout the 134 days, with LD fish showing higher growth in terms of length. Generally, production parameters are calculated from weight data, although it is worth highlighting this result, as standard length could have been reacting more slowly than weight to density changes. This result could imply that fish under the potentially stressful conditions associated to high densities could be trailing such detrimental effects long after the acclimatization to a crowded environment.

In chapter 3, two densities with the same values in % of bottom coverage as those in chapter 2 were assayed, combined with different initial body size distributions of the stocks, in form of homogeneous or heterogeneous size groups. Under these conditions, after 105 days of trial, stocking density was the most determinant rearing parameter on Senegalese sole growth. Independently of initial size dispersion, LME models for fish reared under low stocking density showed a steeper slope of growth in all studied biometric parameters than fish reared under high density. Contrarily to fish in chapter 2, where all individuals in average started the trial close to the market size at 318.7$\pm$7.9 g, in this experiment fish started on a mean weight of 37.7 g and a coefficient of variation of 68.4%, and ended up averaging 73.6 g (CV 57.1%), so it could be considered that they were sub-adult individuals. Despite the differences in size, final densities achieved in the HD groups were similar to the ones obtained in chapter 2 in terms of bottom occupation, and also to the ones reported by Salas-Leiton et al. (2008, 2010a, 2011).

The experiment in chapter 4 was designed with an intermediate starting density, in order to end the ongrowing period in the high stocking densities considered for the previous experiments. Fish started at a density of 130.6% (CV 1.08%) or 13.6 kg·m^{-2} (CV 9.58%), and ended at 191.8% (CV 9.29%) or 25.1 kg·m^{-2} (CV 8.96%). In these conditions, fish were fed either during the photophase, similarly to regular fish farming operations, or during the

scotophase, following more closely the feeding and activity rhythms observed in the laboratory for this species (Bayarri et al., 2004; Boluda Navarro et al., 2009; Castanheira et al., 2011).

Some studies regarding the interaction between density and production in Senegalese sole (Costas et al., 2008; Salas-Leiton et al., 2008) and other flatfish species like Winter flounder (*Pseudopleuronectes americanus*; 84 DAH juveniles, Fairchild & Howell, 2001) did not show a relationship between stocking density and growth.

On the other hand, growth depensation following increased stocking density has been also verified in other flatfish species like common sole; Schram et al. (2006) found an inverse proportionality between stocking density and growth in this species, rearing 35.1-40.2 g *S. solea* at stocking densities ranging from 7.7% to 194.9% of bottom coverage, up to a maximum density of 205.7% of bottom coverage (13.3 $kg \cdot m^{-2}$). Additionally, Irwin et al. (2002) described that higher densities not only affected growth of juvenile turbot, but also increased size dispersion.

Our results suggest that there is a size-dependent component on how stocking density affects growth of Senegalese sole. Adult individuals reared at high densities (up to 233% of bottom occupation, 39.8 $kg \cdot m^{-2}$) during 134 days showed a lower growth rate than fish reared under low density, presumably due to the observed lag phase in growth at the beginning of the experiment. After lagging for 60 days, fish from both density treatments grew up showing similar slopes.

Soles of an intermediate initial size (89.8 g; CV 42.6%), as those in chapter 4, stocked at moderate initial densities, but ending at a high density of 191.9% of bottom coverage (25.01 $kg \cdot m^{-2}$) showed specific growth rates of 0.63, comparable to SGRs obtained for soles of similar size stocked at 30 $kg \cdot m^{-2}$ (approximately 0.6; Salas-Leiton et al., 2008), 31 $kg \cdot m^{-2}$ and 7.45 $kg \cdot m^{-2}$ (0.59 and 0.65 respectively; Salas-Leiton et al., 2010a), 31 $kg \cdot m^{-2}$ and 6.2 $kg \cdot m^{-2}$ (0.62 and 0.64 respectively; Salas-Leiton et al., 2011) or 14 $kg \cdot m^{-2}$ and 4 $kg \cdot m^{-2}$ (0.66 and 0.63 respectively; Costas et al., 2008), and apparent FCR resembling the ones obtained by Borges et al. (2009) and Salas-Leiton et al. (2011). It could be assumed, then, that fish in chapter 4 grew with similar efficiency to other fish stocked at high and low densities that did not show differences in performance between them, henceforth suggesting that stocking density was not a constraint during the experiment.

When sub-adult individuals (38 g) were stocked at a high density, a detrimental effect on growth could be observed, suggesting that, at least in this size range, there is an inverse relationship between stocking density and growth. These differences on how stocking density affects growth depending on fish size were already pointed out by Salas-Leiton et al. (2008), who postulated that the detrimental effects of density on growth of this species could decrease as they approach the adult size.

Obliterating the first 61 days of the experiment in chapter 2, our data seem to support that Senegalese sole over 90 g could sustain high stocking densities, from 25 $kg \cdot m^{-2}$ (230% of bottom coverage) up to 43 $kg \cdot m^{-2}$ (240% of bottom coverage), without detrimental effects on weight gain, providing that no sudden and steep changes in density are applied. This has clear implications on fish husbandry, as care should be taken when performing grading or splitting operations. Sub-adult and, presumably immature fish, should be kept at lower densities to maximize growth in a sort of pre-ongrowing phase. Although it would imply the need for larger facilities, it might not be a heavy obstacle for producers due to fish size and the possibility of stacking shallow tanks (i. e. raceways) in this stage.

Stocking density could have other implications, as it has been shown that it could have an influence on the shape of Senegalese sole adults Ambrosio et al. (2008), which is also needed to be taken into account looking forward to the acceptance of the product by the consumers. Also, moderate or high densities could have an impact on stress and fish welfare, as fish exposed to crowded conditions may present higher levels of cortisol (Tort et al., 1996; Rotllant et al., 1997, 2003; Costas et al., 2008) and affect the immunological status in this species (Salas-Leiton et al., 2010a). In gilthead sea bream it has been observed a phase of immune depression from day 9 out of 23 days of crowding, that eventually stabilizes, showing that this species may adapt to such stressful conditions (Tort et al., 1996). Such adaptation to crowding conditions was absent in similar experiments with red porgy (Rotllant et al., 1997). It suggests that experiments specifically designed to assess whether the lack of growth from the first 60 days in chapter 2 are associated to stress-related immune depression, would be needed.

6.1.2 Size variation and hierarchic relationships

The increase of the size variation within a stock of fish cultured in the same tank is usually associated to the onset of hierarchies, due to competition for food items, space or other resources. However, in chapter 2 no differences

in the evolution of CV could be found neither over time nor between densities. As food was provided in excess, and no signs of lesions due to aggressive behavior could be detected, differences in growth could be due to factors lying behind the genetic background, or due to differences in metabolic efficiency elicited by genetic variability and/or gender.

Fish showing a fast growth, which is also steady throughout time (low CV_{IGR}), are candidate fish to be dominant individuals within a tank (Gardeur et al., 2001a). It has also been shown that the increase of the size variation through time can be an indication of the onset of hierarchies and dominance relationships within a fish population. According to the above mentioned criterion, in HD 50% of the females in front of 25% of the males were estimated to be dominant. Interestingly, not all the bigger females in HD were considered dominant, while some of the medium weight class could claim such status. Conversely, in LD 37% of the females and 50% of the males were estimated as dominant. In LD the bigger fish were found to be dominant, but also fish from lower weight classes appeared in this hierarchy.

In chapter 3, fish were graded in order to build homogeneous (HOM) and heterogeneous (HET) size groups. By mixing small and large fish in the same population it could be hypothesized that hierarchic relationships of dominance would have emerged, and larger fish could have exerted a detrimental effect on growth of smaller fish (Gunnes, 1976; Jobling & Reinsnes, 1987; Irwin et al., 1999; Dou et al., 2004). Nevertheless, the LME models showed that size composition had no significant influence on sole growth. Additionally, the coefficient of variation of weight and standard length in the heterogeneous groups diminished during the experiment (about 14% and 5% respectively). These data are consistent with the absence of strong competition between individuals (Wallace & Kolbeinshavn, 1988; Kamstra, 1993) as the increase of weight CV has been related to hierarchy formation in turbot (Irwin et al., 1999, 2002). Homogeneous groups kept their weight CV under 37% and, although it decreased between 1 and 3%, it remained fairly stable during the whole experiment, also indicating a low level of hierarchic competition. Similarly, Salas-Leiton et al. (2010b) found that the CV of ungraded 155 g Senegalese sole, (CV 37.4%; similar to the one used here for HOM groups), showed a slight non-significant tendency to increase during 60 days of trial.

As specific growth rate assumes that growth is exponential, if fish reared together do not interact competitively, there should be a negative correlation between body size and individual SGR (Jobling, 1985). Such a significant negative correlation was observed in chapter 3, adding up to the suggestion that smaller fish hadn't their growth suppressed in these conditions.

Individual growth rate distributions of homogeneous and heterogeneous groups were significantly different in LD, with HET showing a higher variation for both weight and standard length IGRs than HOM. But in HD, both HOM and HET groups showed similar IGR distributions. High density promotes a higher amount of activity in sole (Duarte et al., 2009) and increases social interactions, which could generate some sort of physiological stress in sole. This stress could be higher for fish stocked at high density in homogeneous groups and, as also suggested by Salas-Leiton et al. (2010b), more fish could be in position to exert some pressure over the resources, leading to increased social interaction and slower growth in populations of similar sized soles.

Soles in chapter 4 started the growth trial being already an heterogeneous population (CV 35.2%), and the coefficient of variation increased for all the biometric parameters studied throughout the experiment. In Salas-Leiton et al. (2011) the ungraded population was similar to our ungraded conditions, and its CV also showed a tendency to increase during the 60 days of experiment, while CV of fish graded according to previous growth rate decreased throughout the experiment. In this study the increase of CV was very similar between treatments, indicating that maybe the stocking density level was promoting competition, not necessarily for the food resource, offered in excess, but for space, as in high density, fish tend to look for favored spots in the tank, including beneath conspecifics, where they may feel more protected.

Social-induced growth depensation could be a major problem in cultured fish, as individuals low in the dominance hierarchy display chronic stress that in turn leads to appetite diminution, reduced food intake and low SGR (Jobling, 1985). Feed access can be more difficult for subordinate fish (Irwin et al., 2002; Dou et al., 2004), and even subordinates can "voluntarily" reduce their food intake, and hence their growth, when conflict over rank intensifies (Wong et al., 2008). But differences in feed consumption are not the only factor linked to the lower growth of low-rank fish, as poorer growth can be observed even when feed is not a limiting factor (Irwin et al., 1999; Dou et al., 2004). Cubitt et al. (2008) suggested that social position is related to the brain neurochemistry, and thus potentially animal welfare, in large and densely populated rearing units of fish. Altered serotoninergic activity was present in subordinate fish, that failed to grow adequately even when feed was available in excess.

Moreover, findings by MacKenzie et al. (2009) in the common carp (*Cyprinus carpio*) suggest that other factors, such as adaptive inherited variability, including differences in stress coping style, may influence the response to challenging conditions.

Taking into account that size composition did not affect growth in chapter 3, and that dominant fish from chapter 2 were both large and smaller fish, size may not be the only factor involved in the development of social interactions when rearing Senegalese sole. Apparently, competition in Senegalese sole seems to be low, either in conditions of high or low density and under different size compositions. Senegalese sole has no records of aggressive behavior, nor any aggressive behavior was observed in the experiments carried out for this thesis. When reared in experimental tanks at low densities (5-50% of bottom occupation), smaller fish have been observed to gain access to feed particles before the largest fish (Duarte et al., 2006) that preferred to stay close to the water inlet. Apparently, if feed is provided in sufficient amounts, no competition for food is established, although maybe the real dominance struggle could be for other resources like the best water quality, or to reach a favorable position over or under conspecifics, as it could be the case in fish from chapter 4.

It is a fact that some soles coming from the same broodstock grow slower than their siblings. Whether it is a genetic predisposition or an environmental condition is still unknown and an interesting question to be investigated. Should the producer grade the stocks to maximize growth or modulate competition? According to our results, lowering size dispersion would not improve growth, mainly in high stocking density conditions. Looking at the individual growth curves (i. e. Chapter 2, Fig. 1b) it can be seen that some individuals, particularly in HD, had their growth dramatically suppressed. If this slow-growers could be identified in earlier stages of the culture and eliminated, yields and production costs could be optimized. In other possible scenario, it might be interesting to develop a market for such small fish, commercializing these slow-growing individuals as "*fritada*", a very appreciated way of frying small fish, or as pre-cooked dishes.

6.1.3 Sexual growth dimorphism

Many fish species show a differential growth between sexes and several cultured species like turbot (Imsland et al., 1997), European sea bass (Saillant et al., 2001) or Atlantic halibut (Hagen et al., 2006) also display this sexual dimorphism. This is of particular interest in aquaculture, as when one of the sexes grows faster or bigger than the other, in some occasions it might be interesting to develop strategies to achieve monosex populations, thus reducing size variation and attaining higher production (Piferrer, 2001).

In the experiments of this thesis, a sub-sample of each group of fish was analyzed to assess the sex of the individuals and whether it had any influence on growth. It has been seen that same age-group wild Senegalese sole show different consumption of prey items according to sex (Garcia-Franquesa et al., 1996) that could influence growth profiles in the wild. According to our experimental data, Senegalese sole shows a clear growth dimorphism, with females growing faster than males, but apparently, only after certain body size. As soles fed artificial diets can't choose its composition, we suggest that there is presumably a mechanism involving sexual maturation that might influence the onset of the differential growth between sexes. Similarly, Imsland et al. (1997) found sexual growth dimorphism in turbot, with females growing larger than males at three out of four different temperatures, from 9 months after hatch onwards.

In chapter 2, pooled females, of both HD and LD, showed a significantly higher mean weight than pooled males throughout the experiment, and LME showed higher growth of females throughout the 134 days of experiment. Moreover, females in lower weight classes tended to move to higher weight classes along the experiment, particularly in HD, where the number of females that climbed to the upper weight category was statistically significant.

Fish in chapter 2 and 4 were coetaneous siblings, but females were in general larger than males from the beginning of the experiments. This could not be detected until the end of the experiment as it is difficult to differentiate males from females when they are immature (Dinis et al., 1999). Although fish in these experiments were not sexually mature, taking into account the higher initial weight of females, the process leading to a differential growth between sexes had already begun.

Contrarily, fish from chapter 3 did not show any influence of sex on growth, suggesting that soles weighing 38 g could be in an early life stage for sexual growth dimorphism to be observed.

Imsland et al. (1997) tracked back the differences of growth between mature and immature turbot up to 16 months before the first spawning, or 9 months post-hatch, suggesting that ovary physiology, including vitellogenesis, might be involved in the onset of sexual growth dimorphism. Although it is still an issue when this apparent

sexual dimorphism begins in the life cycle of Senegalese sole, it would appear that it might be some time between 40 and 80 g of body mass.

It has been suggested that sexual steroids, particularly 17α-methyltestosterone, may be involved in reducing feed intake and feed conversion ratio in Eurasian perch juveniles (*Perca fluviatilis*), leading to lower weight males (Mandiki et al., 2004). Also, the expression of growth hormone mRNA was higher in immature half-smooth tongue sole females than in mature males from the same age, suggesting that the early maturation of males is behind the sexual growth dimorphism in this species (Ji et al., 2011). Growth dimorphism has been showed to be related to phenotypic sex in European sea bass (Saillant et al., 2001), reinforcing the role of the brain-hypothalamus-gonad axis as a key player in differential growth between males and females. Sexual steroid hormones also affect growth of Atlantic halibut before and after maturation. Females recruit more fast muscular fibers than males before puberty, and hyperplastic growth of muscle in males stops at the point of reaching maturation. These two effects, combined with the later maturation of females, results in sexual growth dimorphism in this species (Hagen et al., 2006).

Onset of puberty in sole is still poorly known, although spermatogenesis has been observed in 1-1.5 year old soles belonging to the F_1 of wild males reared in captivity (Agulleiro et al., 2007) and response of the testis to human chorionic gonadotropin was observed in 60 grams male soles (Marín-Juez et al., 2011). Growth dynamics of female and male Senegalese sole, compared to data obtained in other flatfish species, suggest that the onset of puberty, probably beginning between 40 and 80 grams of body weight, could play a major role in differential growth between males and females. More studies, focused directly to clarify this issue, should be carried out to confirm at which point of the life history of sole does sexual maturation occur and its exact relationship with differential growth between males and females.

Additionally, endogenous physiological factors are not the only ones that can shape growth in farmed fish, as factors as stocking density or feeding schedule (ratio and feeding frequency) can also affect growth and sexual dimorphism. In Eurasian perch, feeding in excess triggered a differential growth between females and males that was not seen offering 100% of the recommended ratio for this species (Fontaine et al., 1997).

Sexual growth dimorphism besides fish age, as seen before, could also play a role in the size variation of sole, in close relationship with the physiological factors involved with sexual maturation. Saillant et al. (2001) described a substantial drop in the growth coefficient of variation of European sea bass in their second year of life, when smaller individuals (mainly males), started to grow faster than the bigger fish (mostly females), although more experiments should be designed to elucidate this issue.

In the present thesis size dispersion was high. From the production point of view, grading of soles of 80 grams onwards would tend to bias sex ratios of heads and tails, as more males would be expected in the smaller sizes. Recent findings by Salas-Leiton et al. (2011) show that there is a strong social effect in shaping growth dynamics on sole, with individual growth being strongly affected by social composition when grading not by size, but by specific growth rate (fast, medium and slow growers). It would have been interesting to know the sex composition of these groups to verify this hypothesis and to assess whether the fast growers within the newly formed growth hierarchies were females or males.

6.1.4 RNA/DNA ratio

The RNA/DNA ratio has been correlated with growth in many studies with fish larvae, and less frequently with fish adults (Lied & Roselund, 1984; Grant, 1996). Up to date, there was not any experiment that followed the evolution of RNA/DNA at an individual level in Senegalese sole.

A technique was developed to be able to extract white muscle tissue samples on the same individuals along time. A 18 G biopsy needle (BIOPINCE, Amedic), fitted on a co-axial cannula to avoid accidental sampling of skin, was used to get the tissue samples. To test whether this procedure could affect growth and survival of fish, 30 individually ink-tagged Senegalese soles were stocked in the same tank for 82 days, divided in two groups: fish to be biopsied repeatedly throughout the experiment (6 times during census days), and control fish. Soles were fed *ad libitum* and measured and weighed on all 6 census days. No significant differences were found in average weight, standard length or SGR (ANOVA; $P< 0.05$), neither on survival of both groups (Kaplan-Meier; $P< 0.05$), suggesting that successive muscular biopsies did not affect nor growth nor survival of sole.

Interestingly, RNA/DNA ratio seemed to respond to the change in stocking density in chapter 2, as it appears to descend from 20 days of first applying high density conditions. Similarly to SGR evolution, after 60 days of

experiment RNA/DNA ratios of HD fish followed a similar behavior to that of LD fish.

Starvation and low rations have been described as important factors controlling protein production in white muscle of fish (Grant, 1996). Although no records regarding feed intake were available for this experiment, the lower growth of HD fish during the first 60 days of experiment could be an indicator of lower feed consumption or poor profitability of nutrients during this stage, triggered by the sudden increase of stocking density.

The lower RNA found in HD fish during the first 60 days could be indicating a loss in the capacity for protein production and, thus, the slower or null growth registered during that period. From day 61 to day 134, both SGR and RNA/DNA ratio of HD fish closely resemble those of LD fish, suggesting that the factors affecting protein synthesis of HD fish (crowding stress, social interactions leading to feeding hierarchies...) disappeared or were less active. Within 13 days from the density exchange between groups there seems to be a tendency, although non significant, towards the same phenomenon. Unfortunately, no data from day 147 onwards were available to confirm this.

As RNA/DNA ratio in white muscle is closely related to its growth associated to protein synthesis, this study supported that it could be, following proper calibration, a good instant-growth rate indicator for fisheries and aquaculture research (Carter et al., 1998). To do so, it is necessary to extract a sample of tissue periodically, as it was done in chapter 2, or to sacrifice a sample of the population in order to analyze RNA/DNA ratios. The smaller size of fish in chapters 3 and 4 discouraged us to carry on the same muscular biopsy procedures assayed in chapter 2. Additionally, the large size variation, and the low number of available fish also made impractical to sacrifice a significant sample of individuals to obtain RNA/DNA information, which we are confident it would have been of great interest.

6.2 Feeding rhythms

Several works have clearly shown that Senegalese sole has preeminently nocturnal biological rhythms, as it displays higher locomotor activity and oxygen consumption during dark hours (Bayarri et al., 2004; Castanheira et al., 2011), and that it can also be entrained to self feed, concentrating the majority of feed demands during the scotophase (Boluda Navarro et al., 2009). Such a tenacious nocturnal behavior, combined with the usual practices of fish farms, that tend to work during daylight hours for practical reasons, has raised some concerns about the welfare of this species in culture (Boluda Navarro et al., 2009). Usual daylight feeding in commercial facilities also poses the question of whether soles fed against their biological preferences might be doing the most of the energy and nutrients they are getting. In accordance, fish in chapters 2 and 3 were fed either continuously or in several ratios, but always during the nocturnal hours. In order to assess if commercial feeding practices yielded poorer growth than feeding according to the biology of the species, in chapter 4 two groups of individually photo-identified Senegalese sole were reared for 105 days, fed continuously either throughout the light phase or the dark phase.

Senegalese sole stocked at a moderately high density grows at the same pace independently of being fed during the light phase or, according to their biological rhythms, during the dark phase. It has been seen that despite being diurnal or nocturnal feeders, some fish species can sustain similar growth rates when fed against the schedule found in self-feeding experiments. Such is the case of tench (*Tinca tinca*; Herrero et al., 2005) or the Asian sea bass (*Lates calcarifer*; Harpaz et al., 2005). Contrarily, Azzaydi et al. (2000) found that European sea bass fed during the light phase in winter resulted in poorer growth than fish fed at night. In that case, feed ratio was offered differentially by automatic feeders, but nocturnal ratio was offered in two meals, concentrating 66.7% post-dusk and 33.3% pre-dawn, while in the light phase was offered in 3 equal meals in the morning, afternoon and evening. Our feeding schedule consisted in continuous feeding either during day or night, according to the results by Boluda Navarro et al. (2009), who observed that sole shows a steady feed demand throughout the night. Hence, in order to maximize growth it is important not only to choose the time of the day to offer the feed, but also, when no self-feeding is involved, to adjust the size and number of meals to the biology of the species.

There are abundant studies on feeding rhythms on fish in the laboratory, many of them dealing with self-feeding systems when fish are able to operate them. There are some species, like tench, that present strict preferences to feed during a particular time of the day (Herrero et al., 2005) and others, like European sea bass, that change their feeding rhythms according to the season (Sánchez-Vázquez et al., 1998), switching from diurnal to nocturnal feeders for the winter months or other species like red porgy or gilthead sea bream that increase feed demands

during the dark hours in the colder months (Paspatis et al., 2000). Due to the effort and resources needed to check a full year of feeding rhythms, few studies like the one conducted in European sea bass (Sánchez-Vázquez et al., 1998), or in yellowtail (*Seriola quinqueradiata*; Kohbara et al., 2003) are available for most of the commercial species. This applies also to sole, where locomotor, metabolic and feeding rhythms have been conducted for a limited amount of time (35, 44 and 163 days; Boluda Navarro et al., 2009; Castanheira et al., 2011; Bayarri et al., 2004, respectively) and under different temperature ranges. Our experiment was not designed to see whether sole has a strict preference for diurnal or nocturnal feeding, as this condition was imposed from the design of the experiment, nor to observe an eventual switch from diurnal to nocturnal feeding or vice-versa. Our experiment was conducted under natural photoperiod from early October, just after the autumn equinox, in similar environmental conditions as the ones described by Castanheira et al. (2011), and at the end of our experimental period, similar to the conditions in Boluda Navarro et al. (2009). More studies, targeted specifically to assess if there is any stationality in Senegalese sole feeding rhythms, should be carried out under natural photoperiod and temperature for a whole year.

European sea bass fed at night in the laboratory missed 3 times more pellets than fish fed during the day (Rubio et al., 2003) and it has been seen that, despite being visual predators, sea bass must rely in other sensory systems, mainly chemoreception (Faucher et al., 2006), to feed during winter. In the case of soles, the olfactory system has been demonstrated to play a critical role in feeding of these species, as trials involving bivalve commercial flavor (Reig et al., 2003a) and homogenates of polichaetes added to feed (Barata et al., 2009) have been correlated with the velocity of the response of sole to food. Additionally, ablation of the olfactory epithelia suppressed the response towards the homogenates (Barata et al., 2009), underlining the importance of chemoreception in Senegalese sole feeding activity. In conditions of moderate to high density used in chapter 4, where fish commonly overlap, soles fed during the day could be relaying in the same sensory systems they use to feed at night to seek feed pellets in the bottom of the tank.

Sole, as most vertebrates, reacts to photoperiod and shows a typical plasma melatonin profile, with high levels during the scotophase and lower levels during the light phase (Bayarri et al., 2004). Melatonin has been demonstrated to have effects on food ingestion inhibition in goldfish (*Carassius auratus*; Pinillos et al., 2001; López-Olmeda et al., 2006) and tench (López-Olmeda et al., 2006), either by endocrine function, stimulating the release of growth hormone, and inhibiting the secretion of prolactin by the pituitary gland in rainbow trout (Falcón et al., 2003) or by interacting with melatonin binding sites in peripheral tissues (Pinillos et al., 2001). Also, results linking melatonin to feed ingestion might be only indicating that feeding and growth could follow a seasonal pattern modulated by daylight length (Falcón et al., 2003). Recent studies in Senegalese sole have cloned different melatonin receptor subtypes. Subtype MT1 was present in light sensitive structures, but also in peripheral tissues including the digestive tract (Confente et al., 2010). Whether melatonin has a direct effect on feed intake in sole is yet to be investigated.

Feeding activity in cultured sole is a complex issue that can involve physiological factors, as response to light, either in form of photoperiod length, time of the photoperiod in which feed is administered, maturity state and sex. Additionally, rearing conditions such as stocking density or size heterogeneity can also shape the behavior of the stocked fish. In the high density conditions of chapter 4, the elevated social interactions involved might have exerted some effects in the swimming activity, and also might have forced a faster response to feed administration, thus diminishing the possible effect that diurnal feeding could have had on growth.

6.3 Tools for individual growth assessment in Senegalese sole

All the experiments in this thesis relied on the individualization of fish, in order to be able to apply longitudinal data analysis (linear mixed-effects models), and get a better insight to growth of sole in different management conditions such as stocking density, initial size variation or feeding strategy. In order to individually identify fish, ink marking as described in Reig et al. (2003b), and newly developed photo-identification techniques were assayed.

Ink marks have been routinely used in flatfish aquaculture. And showed to be a good tagging technique for larger fish. Three colors were used to generate enough combinations for the experiment in chapter 2 and unequivocally identify individual soles.

Two different approaches were assayed to identify individual soles through digital photographs: an *in situ* (chapter 3) and an *a posteriori* (chapter 4) photo-identification. Both methods proved to be reliable and easy to

implement. When dealing with lower numbers of fishes, the *in situ* method may be advisable, as it is easier to solve ambiguities and makes the workflow faster, relying in a previously printed *family album*. The *a posteriori* approach is to be taken when the identification would take too much time with the fish outside of the water, and it is based on comparing a picture of each fish in each census day with a reference image in the computer afterwards. This method is more time consuming.

The particular morphometric and pigmentation traits of each sole proved to be sufficient to successfully identify individual fish along time. Especially useful clues were traits like malformations and malpigmentations, unfortunately very common in hatchery-reared Senegalese sole (Gavaia et al., 2002; Soares et al., 2002; Villalta et al., 2005a). The spot pattern of sole skin apparently didn't change for the whole length of the experiments and could be used as a fingerprint, but the difficulty to operate with this parameter to the naked eye made it the last resource to disambiguate fish identity when confronted with similar fish. Nevertheless, it looks like relationships of length and angles between spots could remain unchanged for a long period of time, being the most promising candidate for developing an automated individual identification technique in soles.

6.4 Genetic variability

As it has been seen in the growth experiments of this thesis, Senegalese sole from the same cohort suffers from a high size variation when reared in captivity. This phenomenon is of paramount importance to producers, as it implies that some action must be taken: either grading (which seems not to be very efficient in homogenizing sizes or growth rates; Salas-Leiton et al., 2010b) or discarding the runts in order to eliminate the costs of feeding fish that may not reach commercial size and may act as vectors for pathogens. The underlying cause of such variation is still unknown, but it occurs in varied rearing conditions and with diverse social arrangements.

It could be hypothesized that genetic composition of the cultured stocks, as it has been seen that Senegalese sole reproduction has some problems mainly related to the F_1 ability to produce fertile spawns, might be involved with the final performance of the culture. In order to take a first approach to this issue, the levels of genetic variability of a farmed Senegalese sole population (FAR) was compared with its wild donor population (ATL) using molecular markers (mtDNA control region I and 6 microsatellite loci). Additionally, a preliminary exploration of the genetic relationships between wild Atlantic (ATL) and wild Mediterranean (MED) Senegalese sole populations was carried out using both markers. Microsatellite loci were also used to infer sibship relationships within wild and farmed samples.

The analysis of 412 bp of nucleotide sequence of the mtDNA CR-I from two wild samples and a farmed F_1 sample (fish from the experiment described in chapter 2) of Senegalese sole revealed a substantial loss of genetic variability in one generation of aquaculture rearing, as shown by haplotypic diversity values and the haplotypic composition of each sample. FAR and ATL shared only haplotype *Ssen001*, the most common haplotype in both populations, respectively at a frequency of 70% and 56% but absent from MED. Similarly, the second most common haplotypes in FAR (*Ssen002*) and ATL (*Ssen018*) were private to each sample and absent in MED. It should be noted that an AMOVA using mtDNA CR-I data failed to find significant differences between ATL and FAR, presumably because of the high frequency of haplotype *Ssen001* in these samples. With the exception of *Ssen001*, the rest of ATL haplotypes were private to that sample.

Lower estimates of mtDNA diversity have been reported in farmed flatfish compared with wild populations. In Japanese flounder, Sekino et al. (2002) found an average haplotypic diversity of 0.761 for three farmed samples compared with 0.998 for a wild sample. The value of haplotypic diversity for the wild ATL sample reported here (0.693) is lower than those of farmed Japanese flounder. Although the lower mtDNA variability in FAR could have been caused by sampling error during the establishment of the broodstock, it could also be the result of the differential reproductive performance among parents, as suggested in this species by Porta et al. (2007). By contrast, the value of haplotypic diversity of wild MED was higher (h = 0.948), and while this estimate is lower than the value reported in wild Japanese flounder, it is similar to haplotypic diversity values reported for the wild Mediterranean populations of albacore tuna, *Thunnus alalunga* (h = 0.961±0.018; Viñas et al., 2004) and swordfish, *Xiphias gladius*, (h = 0.942±0.009; Alvarado Bremer et al., 2005).

Consistent with the reduction in mtDNA variability, private allelic richness was an indicator of allele loss in FAR, being significantly lower than in both wild samples. The differences in allele frequency resulted in significant allelic and genotypic differentiation between FAR and ATL. Despite these differences, the values of microsatellite

heterozygosity were similar in FAR (0.75), ATL (0.73) and MED (0.79), with these values comparable to the average heterozygosity (0.79±0.19) reported by Dewoody & Avise (2000) for a pool of 12 marine fish species. However, all three Senegalese sole samples showed a low average value of allelic richness per locus, ranging from 6.2 in FAR to 7.7 in ATL, compared with the average value of 19.9±6.6 for marine fishes (Dewoody & Avise, 2000). Porta et al. (2007) reported an average allele number for eight microsatellite loci ranging between 7.9 and 16.7, depending on the size of the sample and whether the fish were hatchery-reared or wild. However, of the eight loci analyzed by Porta et al. (2007), only four were used in this thesis to characterize our populations (*Sol13B*, *Sol19A*, *SolCA13* and *SolMII*). Restricting the comparison to these four loci yields similar ranges in the average number of alleles, between 7.5 and 12.5 for Porta et al. (2007) and 7.8 and 10.0 for this study.

Mean relatedness was higher in the cultured sample than in the wild samples. This is consistent with the data presented above for both mtDNA and microsatellite loci, and with relatedness values found in many reared fish species, including Senegalese sole (Porta et al., 2007).

Biases in both mtDNA haplotype and nDNA allele frequencies in farmed fish compared with the wild population of origin are not uncommon (Coughlan et al., 1998; Alarcón et al., 2004; Aho et al., 2006). Such biases result from random sampling error of the wild population when broodstocks are established using a small number of individuals (founder effect), together with the ensuing genetic drift experienced by successive generations of inbreeding in small effective size populations. The founder effect is particularly important in the interpretation of the results of this study, as the performance of hatchery-reared individuals of Senegalese sole as broodstock has been marginal. Accordingly, it is customary to utilize only wild-caught individuals as broodstock in aquaculture operations of this species, including the broodstock at "El Toruño" that produced FAR. A biased haplotypic profile in FAR would be expected exclusively from such a founder event even if all individuals in the broodstock contributed equally to this F_1. However, Porta et al. (2006b) reported substantial losses in genetic variability at eight microsatellite loci in three Senegalese sole broodstock groups, two of which were F_1 progenies. Specifically, these authors found that alleles that were common in the broodstock were absent in the progeny, suggesting a large variance in reproductive success among potential parents. Such biases in parental contribution have been reported in other flatfishes in aquaculture operations. Blonk et al. (2009) showed that out of two common sole broodstocks consisting of 20 and 28 individuals, only 5 and 2 parental pairs, respectively, produced more than half of the total progeny. Similarly, only 25% of the females in two strains of Japanese flounder apparently contributed to the offspring of two strains founded by 50 and 30 candidate female parents (Sekino et al., 2002). The high linkage disequilibrium observed between microsatellite loci in FAR could also be related to possible non-random mating or the bottlenecks that occur in aquaculture breeding in communal tanks where fish mating is not fully controlled, and thus concordant with the results from Porta et al. (2006a) and Porta et al. (2006b, 2007) regarding important biases in the expected allele combinations of F_1 generations.

In this study, estimates of reproductive variance among members of the broodstock were assessed by estimating the maternal contribution from the highly informative mtDNA CR-I sequence data, in combination with sibship and parental inference from microsatellite data. Since six mtDNA CR-I haplotypes were identified among 74 FAR individuals, it follows that the maternal stock consisted of a minimum of six females. Although the exact number of parents that produced the F_1 analyzed in this study is unknown, studies carried out at the same experimental hatchery (Cañavate & Fernández-Díaz, 1999; Anguís & Cañavate, 2005), suggest that between 8 and 15 females and 7 and 10 males were used to establish the broodstock at this operation. Given that with the exception of *Ssen001*, present in ATL at 56% and in FAR at 70%, no other haplotype was shared between FAR and ATL, it is plausible to assume that these non-shared haplotypes do not occur at a high frequency in the wild.

Incorporating the mtDNA CR-I data in the results from the sibship inference software (COLONY), it identifies seven females producing the F_1 individuals carrying haplotype *Ssen001*. Thus, no fewer than 12 females in the parental stock produced FAR. However, inference of parentage and sibship indicates that 16 females account for all the offspring analyzed in FAR. Thus, in addition to the seven females in the broodstock sharing *Ssen001*, three had to share *Ssen002*, two *Ssen003* and three *Ssen006*. These results are particularly revealing of the reproductive variance associated with each sample.

Phylogeographic studies of common sole characterizing the mtDNA Control Region revealed geographic isolation among samples within the central Mediterranean (Guarniero et al., 2002). Although the present study is the first to assess the genetic variability of Senegalese sole across basins using mtDNA CR-I data, caution should be used in the interpretation of the results because of the small sample sizes and limited sampling coverage. Nevertheless, the particularly pronounced mtDNA differentiation recorded here between the two wild populations from

different basins (ATL and MED) is worth noting. There were nearly twice as many haplotypes in the wild Mediterranean sample as in the wild Atlantic sample, and significant differentiation in both nucleotide diversity values and the mean number of pairwise differences. Values of differentiation between ATL and MED samples with microsatellite data (F_{ST} = 0.054) were similar to the average reported by Ward et al. (1994) for sub-populations of marine fish species (F_{ST} = 0.062). These results are a preliminary approximation for further studies, which should be carried out by increasing the number of sampling locations and the size of the samples to determine the degree of differentiation between Atlantic and Mediterranean Senegalese sole populations. Accordingly, it would be possible to elucidate the presence of a barrier between Atlantic and Mediterranean populations similar to the one found between the west of the Gibraltar Strait and the Ebro Delta–Gulf of Lion in European flounder (*Platichthys flesus*; Borsa et al., 1997).

Chapter 7

Conclusions

Conclusions

- Stocking density affects growth of Senegalese sole in two ways. First, sudden and steep increases in density could lead to poorer or no growth of fish until re-acclimatization to new high density conditions. Second, it seems that there is a size-dependent component on how stocking density affects growth, with smaller fish (sub-adults) growth being suppressed by high stocking density.
- Size composition of reared Senegalese sole does not affect individual growth. Sole doesn't show aggressive behavior and apparently, competition between individuals is low and size independent. Grading Senegalese sole does not guarantee the improvement of growth, and if so, it would be in high density conditions.
- There are groups of slow growers (or zero-growers) that, if identified in earlier phases of the culture, could be eliminated or commercialized as a derived product.
- Senegalese sole females grow faster than males, but only after attaining certain body weight (between 40 and 80 g). It could be hypothesized that sexual maturation may be involved in the onset of sexual growth dimorphism in this species.
- RNA/DNA ratio has proved to be a sensitive biochemical indicator of growth in Senegalese sole, although to be assessed in a non-destructive manner, fish must be over 150 g.
- Despite being an eminently nocturnal species, Senegalese sole fed during the light phase may yield specific growth rates and feed conversion ratios that are comparable to those of fish fed during the dark phase, suggesting the feasibility of feeding during normal business hours in commercial facilities.
- Senegalese sole can be successfully individually identified through digital photographs using characteristic morphological and pigmentation traits.
- There is a loss in genetic variability in a single generation of Senegalese sole rearing, as evidenced by lower values of mitochondrial haplotypic diversity and nuclear diversity of microsatellites in the farmed sample compared with the wild donor population.
- The comparison of wild Atlantic and Mediterranean samples in both mitochondrial and microsatellite DNA suggests a limited gene flow between the populations inhabiting these basins, although the exact nature of the isolation mechanism requires further investigation.
- An accurate knowledge of the genetic composition of farmed stocks is essential both for maintaining the cultured stocks and for potential future restocking purposes because the genetic composition of the wild populations could be severely affected.

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