The role of stress coping style in reproduction and other biological aspects in the aquaculture species, Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*)

PhD thesis

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Para mi madre

Mi esposa

E hijo

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Abstract

Individuals of a same population consistently differ in their behavioural strategies to cope with stressors, commonly labelled as stress coping styles (SCS). SCS are typically characterized by two extreme behaviours: proactive and reactive. Proactive fish have been recognized to have higher activity in novel situations, to be more impulsive in decision making, to take higher risk when facing a potential danger, to be novelty seekers and to show lower glucocorticoids levels than reactive fish. Numerous studies have reported that SCS influence important biological aspects in fish, such as growth, health, resistance to diseases, welfare and reproduction. Thus, the present study aimed to characterize SCS in Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*) to: i) establish some reliable and operational SCS tests to characterize behaviours in Senegalese sole juveniles and breeders (**Chapter 2**); ii) determine whether SCS were repeatable and consistent over time and across contexts in Senegalese sole juveniles and breeders (**Chapter 3**) and gilthead seabream breeders (**Chapter 6**) and whether SCS differed between Senegalese sole juveniles with and without gametogenesis (**Chapter 3**); iii) evaluate whether reproduction (spawned/did not spawn), sex (males/females) and origin (wild/hatchery-reared) were related to proactive or reactive SCS in Senegalese sole **(Chapter 4)** and gilthead seabream **(Chapter 6)**; iv) assess the influence of four different dietary emulsions for *Artemia* enrichments (based on cod liver, soybean, olive oil and linseed oil) on Senegalese sole larvae behaviour (**Chapter 5**); v) describe the spawning behaviour of gilthead seabream (**Chapter 7**). Three SCS individual tests (restraining, new environment and confinement) were selected that efficiently characterize SCS of Senegalese sole juveniles and breeders into two clusters of individuals differing in activity rates, latency to explore novel environments, risk taking and glucocorticoids levels. The selected tests are operational for aquaculture farms, since they are easy to perform, can be applied in large number of fish in a relative short time period and do not require special skills to be interpreted. Additionally, these tests explained over 70% of total behavioural variance, were cross-context correlated and identified two axes of personality defined as "*fearfulness-reactivity*" and "*activity-exploration*". Senegalese sole and gilthead seabream showed high intra-individual behavioural repeatability over time (from $\alpha = 0.989$, $P < 0.001$ to $\alpha = 0.704$, $P = 0.047$), high correlations over time (from intra-class correlations ICC = 0.978 to ICC = 0.285) and high correlations between tests (from $R = 0.285$, $P = 0.035$ to $R = 0.939$, $P = 0.001$) or across context. Senegalese sole

juveniles starting gametogenesis showed higher activity, risk taking predisposition and produced lower glucocorticoids than fish without gonadal development; being classified as more proactive than fish without gametogenesis. In gilthead seabream, proactive SCS were significantly and positively associated with the reproductive success and behaviours of males were related with proactive SCS, while those of females with reactive SCS. On the contrary, SCS of Senegalese sole were not linked to spawning success, sex or origin, suggesting different life strategies for both fish species that led to different SCS tactics. Senegalese sole post-larvae showed defined proactive and reactive SCS from early ontogenesis and oils enrichments influenced SCS and risk disposition of larvae (*e.g.* cod oil induced proactive SCS, while vegetable oils reactive traits). Finally, the spawning behaviour of gilthead seabream was characterized by two specific patterns: a pre-spawning behaviour (schooling and coordinated swimming patterns) and a spawning behaviour *stricto sensu* (aggregations, courtships and spawning rush) and pair-spawning (71.6%) were more frequent than group-spawning (28.4%). The findings of this study provided valuable information to the industry for the management of these two aquacultured species that ultimately could improve welfare and production.

Resumen

Los individuos de una misma población consistentemente difieren en sus respuestas al estrés y comúnmente se reconocen como estilos afrontamiento al estrés (EAE). Los EAE se caracterizan por dos tipos comportamientos: proactivo y reactivo. Los peces proactivos se reconocen por su mayor actividad, son impulsivos en sus decisiones, se arriesgan más en situaciones de peligro y muestran bajos niveles de glucocorticoides, en comparación con los peces reactivos. Numerosos estudios han demostrado que los EAE influyen en importantes aspectos biológicos de los peces, como crecimiento, salud, resistencia a enfermedades, bienestar y reproducción. Por ello, en esta tesis se caracterizaron los EAE del lenguado Senegalés (*Solea senegalensis*) y la dorada (*Sparus aurata*) y: i) se establecieron pruebas de EAE fiables y operacionales para juveniles y reproductores de lenguado (**capitulo 2**); ii) se determinó si los EAE se repetían de forma consistente en el tiempo y en distintas situaciones tanto en juveniles como reproductores de lenguado (**capitulo 3**) y en reproductores de dorada (**capitulo 6**) y se evaluó si los EAE difieren entre juveniles de con y sin desarrollo gonadal (**capitulo 3**); iii) se evaluó la posible relación entre EAE y la reproducción (desove/no desove), el sexo (macho/hembra) y el origen (cultivo/salvaje) en el lenguado (**capitulo 4**) y la dorada (**capitulo 6**); iv) se analizó el efecto de cuatro emulsiones enriquecedoras de *Artemia* (aceite de bacalao, soja, oliva y linaza) en el comportamiento de larvas de lenguado (**capitulo 5**); v) se describió el comportamiento reproductivo de la dorada (**capitulo 7**). Tres tipos de pruebas (retención en un salabre, reacción a un nuevo ambiente y confinamiento) demostraron ser apropiadas para caracterizar los EAE en juveniles y reproductores de lenguado, ya que identificaron dos grupos de individuos que variaron en niveles de actividad, latencia para explorar nuevos ambientes, disposición de riesgo y producción de glucocorticoides. Adicionalmente, estas pruebas explicaron más del 70% de variación total, se correlacionaron positivamente e identificaron dos ejes de la personalidad definidos como "*miedo-reactividad*" y "*actividad-exploración*". Estas pruebas son operativas para granjas acuícolas, ya que son fáciles de realizar, pueden ser aplicadas a muchos individuos en un tiempo corto, además de que no requieren habilidades específicas a la hora de ser interpretadas. El comportamiento individual del lenguado y la dorada fue repetible (desde $\alpha = 0.989$, $P < 0.001$ a $\alpha = 0.704$, $P = 0.047$) y correlacionado (intra-individuales desde ICC = 0.978 a ICC = 0.285) en el tiempo y correlacionada entre test o en diferentes situaciones (desde $R = 0.285$, $P = 0.035$ a $R = 0.939$, $P = 0.001$). Los juveniles de

lenguado con inicio de desarrollo gonadal presentaron una mayor actividad, mayor disposición al riesgo y segregaron menos glucocorticoides que los juveniles sin desarrollo gonadal, por lo que fueron clasificados como proactivos. En la dorada, los peces con mayor éxito reproductivo así como los machos estuvieron significativa y positivamente asociados con EAE proactivos. Por el contrario, los EAE del lenguado Senegalés no estuvieron vinculados al éxito reproductivo, sexo, ni origen, lo que indicó que estas dos especies diferentes estrategias vitales con diferentes tácticas de EAE. Las larvas de lenguado mostraron comportamientos proactivo y reactivo y los enriquecedores influyeron sus EAE (*ejem.* aceite bacalao indujo más larvas proactivas y emulsiones vegetales más reactivas) y su predisposición al riesgo. Finalmente, el comportamiento reproductivo de la dorada se caracterizó por dos patrones: el pre-desove (natación coordinada) y el desove (agregaciones, cortejos y desoves) y realizaron mayor número de desoves en pareja (71.6%) que en grupo (28.4%). Los resultados descritos son muy valiosos para el cultivo de estas dos especies ya que pueden ayudar a mejorar bienestar y su producción comercial.

Chapter 1

General introduction

1. Aquaculture: trends and status

During the past 60 years, aquaculture sector has expanded, diversified and intensified (Figure 1). According to FAO (2014), fish aquaculture has also considerably increased its production over the last 30 years and factors such as the fast world demographical expansion, the significant augmentation of the demand for seafood, the efficient distribution channels and the overexploitation and exhaustion of some marine fisheries resources, has contributed to this bloom. Additionally, the comprehension of fish biology (*e.g.* physiology, nutrimental requirements, genetics, etc.) and the development of new rearing techniques have also contributed to increase marine fish aquaculture. Moreover, aspects such as the control of reproduction, the development of optimal rearing protocols (to improve larval and broodstocks rearing), the selection of breeding lines and the control of diseases are bottlenecks that still need to be investigated to improve fish aquaculture production (Naylor et al., 2000; Bostock et al., 2010).

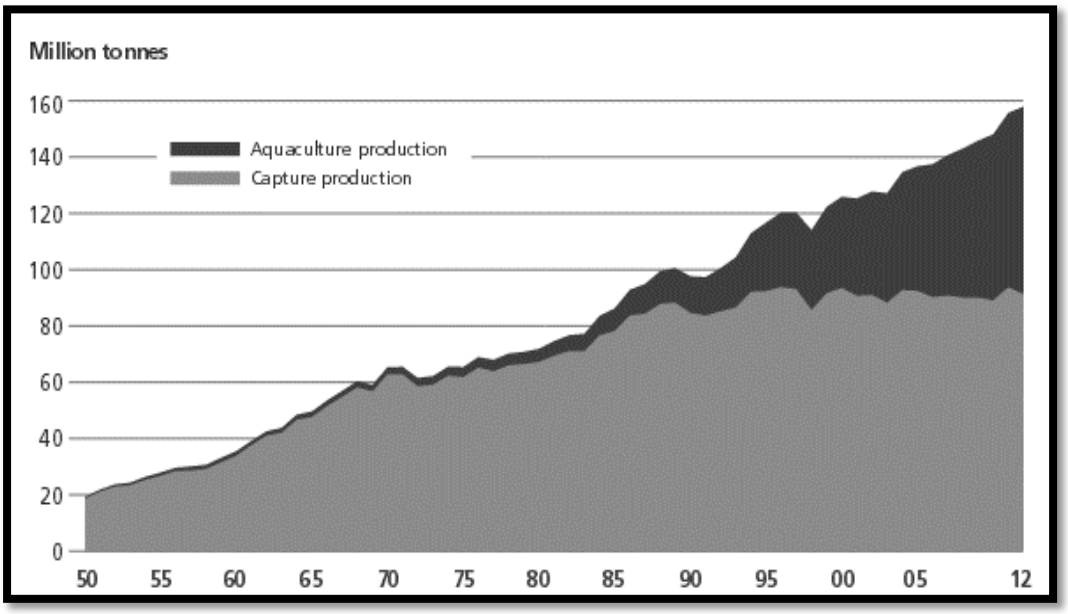

Figure 1. Increase of world aquaculture production (millions of tonnes) over last 60 years. (Graphic credit: FAO, 2014).

The world apparent fish consumption increased from an average of 9.9 kg per capita in the 1960s to 19.2 kg per capita in 2012. In terms of production, FAO (2014) showed that marine fish aquaculture increased 6.2 % per year in average in the period 2000–2012 that is more slowly than in the periods 1980–1990 (10.8 %) and 1990–2000 (9.5 %). Between 1980 and 2012, world aquaculture production volume increased at an average rate of 8.6 % per year. World food fish aquaculture production more than doubled from 32.4 million

tonnes in 2000 to 66.6 million tonnes in 2012. Asia as a whole has been producing more farmed fish than wild catches since 2008, and its aquaculture share in total production reached 54 % in 2012, while Europe represented 18 % and other continents less than 15 %.

To date there are an approximately of 350 fish species under intensive production systems in world. In Europe, the number of species produced is limited to only 35. Two species are of special interest for the European aquaculture and there were studies in the present thesis are: the Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*). In Spain, the Senegalese sole and gilthead seabream production in 2014 was 551 tons and 16, 230 tons, respectively (APROMAR, 2015).

2. Studied species

2.1. Biology, characteristics and rearing of Senegalese sole (*Solea senegalensis*)

Senegalese sole (*Solea senegalensis*, Kaup 1858) (Figure 2) is a benthonic fish species that occasionally inhabit estuaries. This species can be found from Senegal, Africa to La Rochelle, France and in the Western Mediterranean Sea in its natural environment (Ben-Tuvia, 1990). The wild diet of sole mainly consists in polychaetes, small fish, molluscs and small crustaceans. Until recent years, sole aquaculture was linked to extensive earthen ponds along the south coasts of Portugal and Spain as an added value product in polyculture with semi-intensive seabream and sea bass cultivation (Morais et al., 2014). Nowadays, the trend is for more intensive production using recirculation systems with fibreglass or cemented tanks, using commercial feds and maintaining controlled physicochemical conditions.

The reproduction of Senegalese sole in captivity has been the focus of research in Spain and Portugal since the early 1980´s (Dinis et al., 1999). This species are gonochoric, and females mature faster than males. The main spawning season is in spring (March to June), however, there is another less important period of spawning in autumn (oct-nov). Fecundity ranges between 500 – 530 eggs/g (500,000 eggs/kg), but this number can vary with temperature (Anguis and Cañavate, 2005). The mean diameter of eggs is 0.90 to 0.95 mm and fertilization rate is around 87 – 90% (Anguis and Cañavate, 2005). Artificial induction of sole reproduction by hormone-based treatments has been standardized and

ova were stripped and fertilized (Rasines et al., 2012). However, the most common method is natural spawning often in association with controlled photoperiod and temperature (Morais et al., 2014).

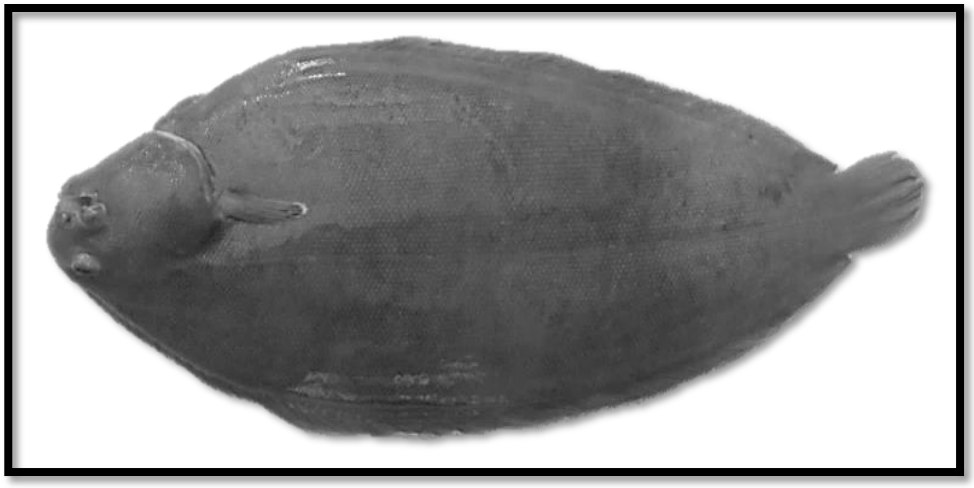

Figure 2. Senegalese sole breeder. (Photo credit: Z. Ibarra-Zatarain, 2013)

Despite the significant advances in protocols and rearing techniques for Senegalese sole production, some bottlenecks are still limiting the expansion of sole aquaculture. Some of these difficulties results from high growth dispersion, incidence of pathologies and absence of knowledge on digestive physiology and nutritional requirements, high mortality during larval weaning and larval quality (Morais et al., 2014). Nonetheless, recent studies have been investigating alternatives to solve these problems. The development of recirculated water systems with optimal water parameters significantly contributed to the control of pathologies of this species (Morais et al., 2014). Similarly, clarifying Senegalese sole digestive physiology and nutritional requirements in essential fatty acids, such as Docosahexaenoic acid (DHA), Eicosapentaenoic acid (EPA) and Arachidonic acid (ARA) (Ribeiro et al., 1999; Boglino et al., 2012; Morais et al., 2014), helped to create new feeds that increased growth rates, improved weaning protocols and reduced size dispersion in larvae. However, the major constraint that is still limiting the cultivation of Senegalese sole and remains unclear is the failure of breeders born in captivity (G1) to reproduce and the dependence on breeders caught from the wild for spawning and for larval/juvenile production.

G1 breeders have been reported to produce few spontaneous spawns, but eggs were unfertilized and of low quality (Duncan et al., 2013; Carazo, 2013; Morais et al., 2014).

These observations led the previous authors and others (Agullero et al., 2007; Howell et al., 2009; Rasines et al., 2012) to hypothesize that some physiological, nutritional or genetic aspects during the early rearing might influence culture broodstock fitness to spawn viable eggs. Nonetheless, G1 reproduction failures have been demonstrated to not be attributed to hormonal dysfunctions, since this species successfully completes gametogenesis and produces viable gametes (Guzman et al., 2009; Rasines et al., 2012; Morais et al., 2014). As well, nutrition has been suggested to be a lesser constraint for G1 breeder's reproduction (Norambuena, 2012; Morais et al., 2014). Other studies reported that the reproductive problem of this fish species relies on the behaviour of males from the first generation, since they do not display the typical reproductive behaviour defined by accompanying and chasing females, placing themselves under females and synchronizing swimming towards water surface to liberate gametes (Carazo, 2013). Besides, females have been shown to produce fertilized eggs only in presence of wild males (Carazo et al., 2011; Mañanos, 2011; Carazo, 2013; see review Morais et al., 2014). The reasons of this inability to achieve a successful reproductive behaviour and, therefore, to fertilize eggs remain unclear. Thus, an interesting and innovating approach that may provide valuable information to solve this impossibility of males to reproduce and, which have to date not been considered, is to characterize **stress coping styles** of Senegalese sole broodstock and to determine their impact on the reproductive success. Studies in a wide variety of fishes have demonstrated that coping styles have significant individual variations and influence the reproductive ability of fishes (Smith and Blumstein, 2008; Castanheira et al., 2015). Therefore, the present study proposed for the first time to investigate whether individual differences in coping styles, characterized by proactive – reactive behaviours, may favour some individuals than others (*e.g.* wild male breeders over G1 male breeders) in reproduction.

2.2. Biology, characteristics and rearing of gilthead seabream (*Sparus aurata*)

Gilthead seabream (*Sparus aurata*, Linnaeus 1758) (Figure 3) is a common species from Eastern and South-Eastern Mediterranean sea, Black sea, Atlantic ocean or British isles (Basurco et al., 2011). It is a sedentary fish, or forming small aggregations. It is mainly carnivorous (mussels, fish, crustaceans, others), but accessorily herbivorous. Before seabream aquaculture started, fish were traditionally reared in Mediterranean coastal lagoons and brackish/salt water ponds. Later, seabream was farmed extensively in

lagoons and is actually intensively cultured in tanks or cages. At first, broodstocks all came from the wild, with a substantial division among two different stocks, the Mediterranean and the Atlantic ones.

Figure 3. Gilthead seabream breeder. (Photo credit: Z. Ibarra-Zatarain, 2012)

Gilthead seabream spawning season typically occurs from December to April (13 to 17ºC). Females present an asynchronous ovary development and spawn about 20,000 to 30,000 egg kg^{-3} for a period of 3-4 months (around 1 million eggs). Eggs present a mean size of 0.9 – 1.1 mm, and the normal fertilization ratio is 90 – 95% (Sola et al., 2006). However, Basurco et al. (2011) suggested that fertility and eggs quality of this species are strictly related to a calm environment and a rich balanced diet. In intensive conditions of production, broodstocks are normally kept in tanks (10 – 20 m^3) at a density of 4 – 8 kg m^{-1} and with sex ratio of 1 males to 3 female (Basurco et al., 2011). The use of hormones (human chorionic gonadotropin HCG and, later, luteinise hormones LH) were used at first to induce spawning (Mylonas et al., 2011), and then replaced by modulation of environmental rearing conditions, in particular temperature and photoperiod, that enabled to extend the spawning season to all year round (Sola et al., 2006; Basurco et al., 2011). Gilthead sea bream are protandrous hermaphrodites, that means that new males have to be added to the broodstock every year, as they turn into females at 2 years old (33 - 40 cm), so that a 5 to 20 % per year renewal occurs, whenever possible with wild fishes. However, males are often taken from the first generation born in captivity (F1) and farmers usually select among their stock the best performing specimens for reproduction (Sola et al., 2006).

However, the reproductive behaviour of gilthead seabream has not been reported yet, despite an increasing need to understand the factors that influence breeder's participation in spawning, in order to control the families produced (Chavanne et al., 2012). Therefore, the study of gilthead seabream spawning behaviour is needed to increase the understanding of spawning in Sparidae and to enable geneticists and broodstock managers to understand the parental contributions obtained for genetic improvement programmes. In addition, characterizing fish coping style behaviours in relation to their reproductive success may be considered as good alternative for aquaculture to establish the production of specific breeding and fingerlings lines with specific behaviours.

3. Welfare, stress responses and coping styles

3.1. The welfare of fish

Welfare refers to quality of life and is related to experience, a phenomenon emerging from brain's motivational and cognitive systems and developed through evolution as an integrated part of the survival mechanisms in animals with their central neural systems (Prunet et al., 2011). Nonetheless, the term welfare in relation to fish has an absence of clear accepted definitions, because of the complexity of the concept and the application to an aquatic species. However, numerous specialists have proposed that an optimal welfare status is reached if fish deal with one of the following three concepts: a) feelings-based definitions (*i.e.* animals experience positive states and are free of hunger, thirst, pain or fear), b) functions-based definitions (*i.e.* centre on the animals ability to adapt to its present environment by showing good health and low stress levels), and c) nature-based definitions (*i.e.* animals are able to lead a natural life and express its natural behaviour) (Huntingford et al., 2006; Galhardo and Oliveira, 2009; Prunet et al., 2011). To date definition related to these approachs are still being defined and a particular problem is what indicators to use to measure fish welfare. Despite of these uncertainties, fish welfare assessments have been developed that focus on holding conditions and husbandry practices that provide conditions of good health and normal behaviour and are being applied to enable consumers to select salmon reared in good welfare (RSPCA; Duncan et al., 2013).

Intensive aquaculture may affect fish welfare in many ways, including physical injuries, increased susceptibility to diseases, skeletal deformities and high mortality rates. In

practice, fish welfare can be compromised by deficient housing conditions, high stocking densities, poor water quality, unbalanced feeds, improper design of tanks, among others factors (Huntingford et al., 2006; Ashley, 2007). Thus, providing fish with appropriate rearing conditions, such as clean water with renovation, temperature optimal to the season, tanks should be clean, high quality diets, reduced handling (or performe as quick as possible), will improve welfare and leads to increased survival rates, improved physiological status, behavour and unltimatel improved aquaculture productivity (Conte, 2004; Huntingford et al., 2006; Ashley, 2007; Duncan et al., 2013).

3.2. Stress and stress responsiveness

The word stress is commonly used in our daily lives, but its meaning can be interpreted in different ways. Stress could be simply defined as any influence or situation that threats homeostasis which is re-established by a complex suite of adaptive responses (Chrousos, 1998). According to McEwen and Wingfield (2003), homeostasis is interpretated as the *stability through change*. Fish stress responses are considered to be part of adaptive behavioural and physiological strategies that have evolved to cope with a perceived threat and that basically consist in three fundamental phases (Barton, 2002; Huntingford et al., 2006; Galhardo and Oliveira, 2009):

i) ***Primary responses***, which are endocrine responses, involve the release of catecholamine and the activation of the hypothalamic–pituitary–interenal (HPI) axis, with the production of corticosteroids (*i.e.* cortisol) (Figure 4).
ii) ***Secondary responses***, which are physiological and behavioural adjustments, including changes in plasma, alterations of tissue ions and metabolite levels, immune function and cellular responses (Figure 4).
iii) ***Tertiary responses***, which refer to changes in the whole animal performance, gather growth and reproduction inhibition, immune suppression and mortality and depend on the magnitude of stress and its duration (Figure 4).

Stress concept is extremely linked to that of welfare: depending on the severity of stressors, animal's neuroendocrine system will respond differently in speed, magnitude and patterning of hormones, as well as in behavioural patterns. Barton (2002) proposed two factors influencing stress response: (i) the individual-related response, which

suggests that the state of the internal and external individual's environment may alter stressors effect and response (*i.e.* rainbow trout females produce more cortisol than males, Øverli et al., 2006) and (ii) the stressor-related response, which suggests that the relevance of a stressor to an individual has not only to do with its nature, but also with the pattern of exposure, intensity and duration (*i.e.* predation induces different social patterns, Brown et al., 2005).

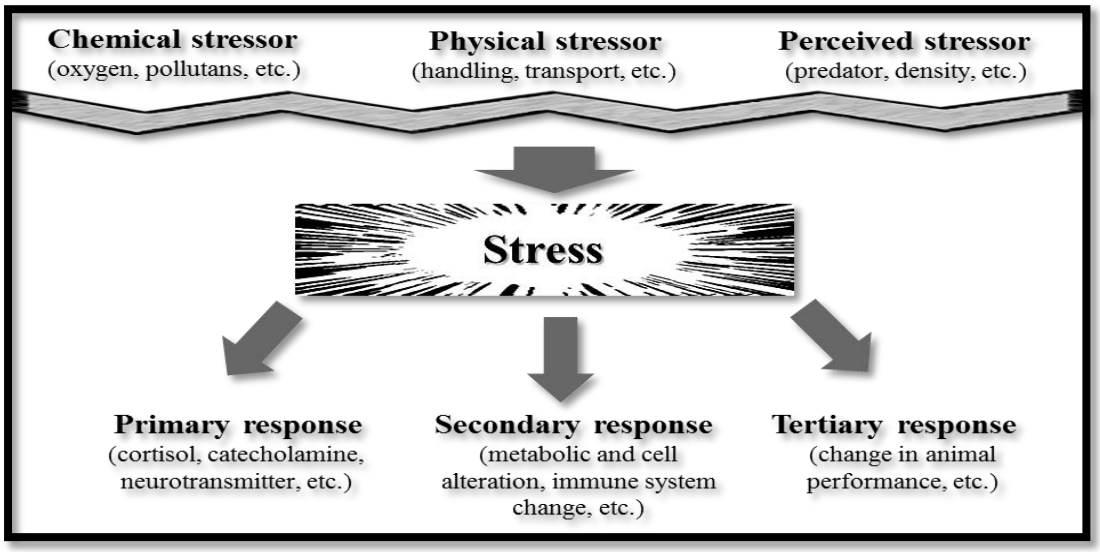

Figure 4. Factors altering fish normal homeostasis and their physiological responses.

3.3. Stress coping styles

Despite the now well-established connection between animal welfare and stress, the implications of these factors for farmed fish health and productivity need further investigations on their behavioural responses to hazard situations (Huntingford and Adams 2005), which are essential for fitness and quality. An adaptive stress response involves the combination of physiological and behavioural processes (Wingfield et al., 1998; Boonstra et al., 2001) with potentials correlations between them (LaRowe et al., 2006). It is also acceptable that stress responses may vary among individuals within a population due to underlying differences in age, sex, genetic factors, life experience and/or environmental conditions (*i.e.* conditions that are well tolerated by some individuals may be detrimental for others). Indeed, intra-individual variation in stress response traits has proven to be a ubiquitous and common phenomenon (Huntingford, 1976; Verbeek et al., 1996), and this has led to the theory of stress coping styles (SCS).

Coping styles have been documented since late twenties to establish the reaction of individuals to threatening situations and was initially represented by the fight/scape model (Canon, 1929). Since that, possible, first definition diverse modifications and improvement have re-orientated the coping style theory by including changes in neuroendocrine and central nervous system of individuals (Henry and Stephens, 1977), creation of routines and fixed comportments (Benus et al., 1987) to the most new and accepted definition given by Koolhaas et al. (1997) and confirmed by Coopens et al. (2010), whom stated that SCS establish *the individual differences in behaviour in reaction to challenges that are stable over time and across various situations*. Throughout the present thesis this will be the definition of SCS. Indeed, this concept of stress coping style has been defined in a wide range of taxa, such as birds, insects and mammals (including humans) (Van Oers et al., 2005; LaRowe et al., 2006; Réale, 2007), and fishes (Toms et al., 2010; Conrad et al., 2011; Castanheira et al., 2015).

To clearly understand the SCS concept, is necessary to comprehend that the individual differences in behaviour are characterized along two behavioural axis of characters defined as proactive and reactive (Koolhaas et al., 1999). Behaviorally, proactive animals are highly aggressive toward conspecifics, take risk in the face of potential danger, are novelty seekers, present higher rates of activity and readily develop rigid learned routines. The reactive SCS is characterized by low levels of conspecific aggression, avoid taking risk in unknown environments, show lower rates of activity, flexible cue-dependent learning routines, and passive behaviors such as immobility in response to stressful stimuli (Koolhaas et al., 1999; Koolhaas et al., 2007; Coopens et al., 2010) (Figure 5). Moreover, fish physiology in relation to stress coping styles show significant differences, since proactive fish present a lower hypothalamic–pituitary–interrenal (HPI) axis reactivity than reactive animals, leading to a lower production of glucocorticoids (*i.e.* catecholamines or cortisol) and a higher sympathetic activity than reactive animals, leading to a higher increase of noradrenaline and adrenaline in blood (Øverli et al., 2007). A number of studies have contributed to confirm these approaches by showing two clusters of individuals with different degree of activity, risk taking, aggression, cortisol as other behavioural patterns (see review of Toms et al., 2010; Conrad et al., 2011; Mittelbach et al., 2014; Castanheira et al., 2015). A reasonable hypotheses for these two distinct behavioural characteristics are, in accordance to Coppens et al. (2010), motivational reflections of how animals respond to challenges (qualitative dimension) and

how strong are their reactions (quantitative dimension) to allow the characterization of individuals and depend on natural selection.

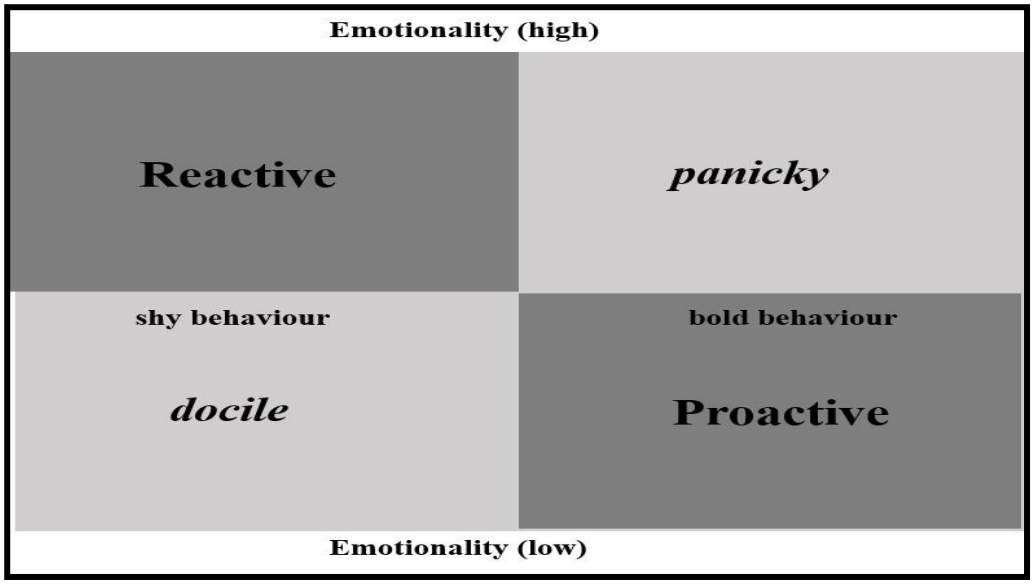

Figure 5. Differentiation between personalities of stress coping style (Figure credit: Koolhaas et al., 2007).

Another consideration required to confirm the existence of SCS is that the stress coping response is consistent across-contexts and repeatable over certain periods of time. When individuals display the same SCS *across different contexts* suggest that individuals behave consistently similar in different situations (*e.g.* some animals showing proactive behaviour in a particular situation are also proactive in other contexts). For instance, fishes showing suites of behavioural traits that co-vary in multiple situations have been recognized in the pumpkinseed sunfish *Lepomis gibbosus* (Coleman and Wilson, 1998), trout *Oncorhynchus mykiss* (Wilson and Stevens, 2005), perch *Perca fluviatilis* (Magnhangen and Staffan, 2005), speckled damsel *Pomacentrus bankanensis* (Biro et al., 2010), as other animal species (see review of Wolf and Weissing, 2010; Conrad et al., 2011). From an evolutionary view, the correlation between SCS corresponds to the existence of "behavioural syndromes", which is analogous to coping styles theory to represent the variations of personality (Sih et al., 2004). Lastly, the third type of behavioural patterns that represent coping styles is *repeatability*. In accordance to Bell et al. (2009) and Sih et al. (2015), the repeatability represents the behavioural stability of individuals over the time in similar contexts, and is also applied when individuals behave consistently differently from each other. Statistically, the repeatability of characters are measured as the intra-individual variance, remarking that low intra-individual variability

represents high repeatability, while high intra-individual variations exhibit low repeatability. The implications for individuals by exhibiting repeatable behaviours may affect or provide benefits in their actions, since it may generate important information of fish physical (*e.g.* energy, reproduction status and metabolism) and will be possible to assess the fish rank status, role in group, skill set, etc. (Sih et al., 2015). For instance, predictability of behaviours have been documented in several fish species (Wilson and Godin, 2009; Castanheira et al., 2013; Ferrari et al., 2015).

The ecological and biological consequences of distinct coping styles include potential effects on individual survival and reproductive success, population dynamics (through influences on species vital rates, *i.e.*, growth, fecundity and survival), community organisation, species diversity (through influences on species interactions), and the conservation and management of natural resources (Wilson et al., 1993; Mittlebach et al., 2014). However, one of the challenges when assessing personality traits in fish and other taxa's is the fact that behaviour can be extremely plastic and is often context dependent: individuals often respond to changes in their environment by adjusting their behaviour to the present situation (Coopens et al., 2010; Wolf and Weissing, 2010). Therefore, assessing the behaviour of individuals in several contexts would allow the development of reaction types that would more accurately to characterize the individual's personality.

In aquaculture, different studies have proposed the advantages of characterizing proactive or reactive coping strategies (see review of Huntingford and Adams, 2005; Conrad et al., 2011; Castanheira et al., 2015). For instance, proactive fish frequently recover the appetite faster after a stressful situation (Ward et al., 2004; Mas-Muñoz et al., 2011), show higher resistance to diseases (MacKenzie et al., 2009), usually present higher growing rates (Basic et al., 2012) and frequently possess higher reproductive success (Wilson et al., 2010; King et al., 2013) than reactive individual. Nonetheless, reactive individuals had a higher life span, possess higher control of hormonal regulation and has a developed neural plasticity in comparison to proactive fish (see review of Castanheira et al., 2015). However, according to Biro and Stamps (2008) and Sih et al. (2004), the maintenance of both coping styles strategies in populations are fundamentals because each trait represent different adaptive solutions to complex situations or environments and promote the cooperation between individuals.

Lastly, the implications of assessing the coping style of fish with commercial importance or those destined to aquaculture (like in the present study) relies on the fact that behaviours, as previously described, may influence different aspects of fish, such as feeding efficiency, stress responsiveness, disease tolerance, adaptation, growth, survival, reproduction success and fingerling quality, as others, and in consequence could improve the productivity (see review of Huntingford and Adams, 2005; Castanheira et al., 2015).

3.4. Fish as stress coping style model

Despite of their phylogenic distance from mammals, fish are valuable to ecological and biological research in many different aspects. Fish have relatively fast developmental stages, reproductive and developmental timeframes, are sensitive to environmental and social variables, are relatively easy to handle and their maintenance do not requires much attention. Indeed, fish have substancial differences between conspecifics in feeding, defensive, sexual, and other behaviours (Budaev and Zworykin, 2002). For instance, altered patterns of swimming speed, direction and activity are a common response to numerous stressors (Schjolden et al., 2005). Moreover, when fish are attacked by conspecifics, their behaviour change to include fleeing or hiding and changing colour (Backström et al., 2014). Therefore they make excellent model organisms for investigating specific cognitive abilities, since they show consistent individual differences. Hence, fish are practical organisms for generation-long research and developmental questions. Even when studies do not test many species simultaneously, such as in this thesis, the results from fish behavioural studies alone can be used as a good tool to demonstrate the importance of showing a specific behaviour.

4. Thesis overview

The main goal of this thesis was to characterize the stress coping style behaviours and establish the consequences in reproductive fitness of two commercial species: Senegalese sole (*Solea senegalensis*) and Gilthead seabream (*Sparus aurata*). In Senegalese sole, the stress coping style behaviours of larvae, juveniles and breeders were characterized, considering different variables (*i.e.* nutrition, sex, origin, etc.) and contexts. In seabream, the spawning behaviour was described and the stress coping style of breeders was characterized with relation to reproductive success and sex.

- **Chapter 2** focused on the establishment of some specific coping styles tests for Senegalese sole juveniles and breeders (in review for publication). Senegalese sole were submitted to different individual and grouping tests. Three tests were selected to easily characterize coping style behaviours in this species. Moreover, sole juveniles and breeders presented both proactive (more active and take more risk) and reactive (low activity and avoid risk) behaviours. The present results met some basic criteria that could result attractive for the aquaculture industry, since tests were easy to apply, require short time periods to be achieved and were not complicated to evalute.

- **Chapter 3** explored the repeatability, consistency and correlations of coping styles in Senegalese sole juveniles and breeders (in review for publication). Both juveniles and breeders were submitted to different coping style tests along three years. The main results of the present study were to observe that both groups of fish presented high stability and consistency in behaviour. This result is the first report for this species at this age and one of the first in fish field that demonstrated that behaviours are stable over consecutive years.

- **Chapter 4**, investigated the relation between coping styles and reproductive success in Senegalese sole for the first time in this species (in review for publication). Unlike our expectations, the coping style behaviour of Senegalese sole breeders was not related with the reproduction success. Moreover, it was also observed that sex and fish origin (wild vs. cultured) were not related to proactive or reactive behaviours. Reproduction success in sole seemed to not be linked to a specific behavioural syndrome. Moreover, both coping styles (proactive-reactive) seemed to be be essential from an evolutionary approach, since they may favour the maintenance of different life-histories strategies to face novel situations or challenging stimuli.

- **Chapter 5**, evaluated the influence of different dietary treatments on the stress coping styles responses of Senegalese sole post-larvae (published in Applied Animal Behaviour, Ibarra-Zatarain et al., 2015). Post-larvae were fed dietary treatments of *Artemia* enriched with either one of three different vegetables oils

or fish oil. The study showed that sole larvae presented defined proactive and reactive behaviours and that sole larvae fed *Artemia* enriched with fish oil grew faster and tended to present a more proactive behaviour (*i.e.* higher activity and take more risk)than larvae fed with vegetable oils.

- **Chapter 6** described the coping style behaviour in sea bream in relation with reproduction (in review for publication). The principal findings of this study were highlighting the presence of both coping style strategies in sea bream breeders, repeatability along time of stress coping style and relation with reproductive success. Males were more active than females and, remarkably, the reproductive success in this species was correlated with a proactive behaviour.

- **Chapter 7**, described the spawning behaviour of seabream (published in Spanish Journal of Agricultural Research, Ibarra-Zatarain and Duncan, 2015). This first description of gilthead seabream spawning behaviour provides an important base for genetic improvement studies as for the first time researchers and broodstock managers have a clear idea of the spawning behaviour when considering physical and social manipulations to increase parental contribution for breeding programs.

- Finally, in **chapter 8**, the main findings of the present work were summarized and discussed. Some concluding remarks, limitations and recommendations for potential future research and for the industry with the present fishes were also exposed.

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Chapter 2

Characterization of stress coping style in Senegalese sole (*Solea senegalensis*) juveniles and breeders for aquaculture

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Neil Duncan

In review

Abstract

The aim of the present work was to characterise stress coping styles of Senegalese sole (*Solea senegalensis*) juveniles and breeders and to select operational screening that can be used by the aquaculture industry to classify and select between behavioural phenotypes to enable the industry to improve production indicators. A total of 61 juveniles and 59 breeders were submitted to five individual behavioural tests and two grouping tests. At the end of the individual tests, all animals were blood sampled in order to measure cortisol, glucose and lactate. Three tests (restraining, new environment and confinement) characterized the stress coping style behaviour of Senegalese sole juveniles and breeders and demonstrated inter-individual consistency. Further the tests (a) identified two principal axis of personality traits were found after PCA analysis of the data: "fearfulness-reactivity" and "activity-exploration", (b) were representative of the physiological axis of stress coping style and (c) were validated by established group tests. The present study proposed for the first time three individual coping styles tests that reliably represented proactive and reactive personalities of Senegalese sole juveniles and breeders. In addition, the proposed tests met some basic criteria that could result attractive for the aquaculture industry.

***Keywords**: Solea senegalensis, animal personality, behavioural syndromes, exploratory behaviour*

1. Introduction

Individuals submitted to hazardous conditions exhibit a wide range of different individual responses or stress coping styles that range from proactive to reactive behaviours (Koolhaas et al., 1999). Compared to reactive individuals, proactive individuals are usually more bold and active, take more risk when faced with potential threats, but show lower flexibility and sensitivity to changes in the environmental (Koolhaas et al., 1999, 2010; Sih et al., 2004; Coopens et al., 2010; Wilson and Godin, 2009). Physiologically, proactive fishes are characterised by a lower hypothalamus-pituitary-adrenal/interrenal (HPI) axis activity compared to reactive fish, leading to lower post-stress levels of glucocorticoids (*i.e.* cortisol) (Koolhaas et al., 2010; Braithwaite et al., 2011). Nevertheless, coping strategies may be influenced by life experience and contexts, such as confinement, predation, novel situations or others environmental parameters (Frost et al., 2007; Coopens et al., 2010; Braithwaite et al., 2011). Therefore, reliable tests are essential in order to characterize the stress coping styles of different fish species (Carter et al., 2013).

The assessment of proactive and reactive individuals is of interest for aquaculture, in order to increase the productivity and to establish genetic breeding lines with improved growth, survival and resistance to diseases. In this sense, several studies have shown the significance of both behaviours in different fish species under different rearing conditions. For instance, some authors found that proactive fish tended to grow faster (Brown et al., 2007; Millot et al., 2009), possessed a higher immune response (MacKenzie et al., 2009) and a higher reproductive success (Godin and Dugatkin, 1996; King et al., 2013), but relied on routines and presented a short life expectancy in the presence of predators (Huntingford et al., 2010). On the other hand, reactive fish seemed to pay more attention to external stimuli, possessed high flexibility to changing environments (Adriaenssens and Johnson, 2011; Ruiz-Gomez et al., 2011) and presented higher anti-predatory responses (Biro et al., 2006). Thus, identifying proactive and reactive behaviours appears to play an important role in fish fitness and performance in captivity and could, therefore, influence productivity in aquaculture.

Different tests have been used to characterize fish stress coping style, often with the objective to asses stress coping style from an ecological perspective. These include

evaluating the fish reaction when confronted to predators (Webster et al., 2007), observing the feeding behaviour (Mas-Muñoz et al., 2011) and assessing the latency to feed after disturbance (Moretz et al., 2007), observing successes and failures in fights (Frost et al., 2007), evaluating the competition for a food resource (MacKenzie et al., 2009) and determining the willingness to take decisions (Mamuneas et al., 2014). Most of the previous examples were designed for an ecological approach and have been generally applied in experimental conditions that required specifically designed tanks with divisions and monitoring equipment and all have consumed long time periods to obtain results and demanded special technical skills to interpret the animal behaviour ("body language"). These reasons make the cited tests difficult to apply to large numbers of fish in aquaculture and, for example, reactions in relation to predators are of questionable relevance to aquaculture. Therefore, tests that can be quickly performed, with relative little modifications of the rearing environment, easily achievable and with results that could be easily transformed into quantitative variables by farmers with minimal expertise, would be suitable for the aquaculture industry.

Senegalese sole (*Solea senegalensis*) is currently reared under intensive production systems in Spain, France and Portugal (Morais et al., 2014). However, there still exist some bottlenecks that affect production such as mortalities during weaning, variable growth and poor juvenile quality and complete reproductive failure of first generation males (G1) reared in captivity to court females and fertilize eggs (Morais et al., 2014). Therefore, the development of specific and reliable tests to characterize stress coping styles in juveniles and breeders of Senegalese sole may be useful for the aquaculture industry. Such tests could be used to select individuals with a particular behavioural characteristic or trait that may give advantages for reproductive fitness or be used to establish a selection-based breeding program in order to improve domestication and produce fingerlings with a specific behavioural trait. In this context, the aim of the present study was i) to confirm the existence of stress coping styles in juveniles and breeders and ii) to select some reliable tests to easily characterize the stress coping style behaviours of Senegalese sole juveniles and breeders reared in captivity.

2. Materials and methods

2.1. Ethic statement

All experimental procedures on fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee of IRTA.

2.2. Fish maintenance and identification

Senegalese sole juveniles (n = 61; weight= 46 ± 2 g; length= 15.2 ± 0.2 cm) and breeders (n = 59; weight = 1189 ± 50 g; length= 45.8 ± 0.7 cm) were used for the present study. Juveniles were housed in eight 500L rectangular tanks, while breeders were stocked in four 13 m^3 rectangular tanks. All tanks were located in a greenhouse structure and included in a recirculation system (IRTAmar®) to maintain a simulated natural water temperature (9 - 19ºC: winter to summer) and oxygen (5 - 6 mg/L) levels. Photoperiod was natural ranging from approximately light dark (L: D) 14: 10 in the summer to 10: 14 in the winter for breeders and juveniles.

All individuals were Pit-tagged (ID-100 Unique, Trovan-Zeus, Madrid, Spain) for identification. Juveniles and breeders were fed *ad libitum* every morning (10:00 h) as follow: i) juveniles: daily with balanced feed (LE-3 mm ELITE, Skretting Co.); and ii) breeders: on Monday and Sunday balanced feed (Repro-Vitalis, LE-7 mm ELITE, Skretting Co.), on Wednesday cooked mussels (Sariego Intermares, Spain), and on Friday, marine polychaetes (Topsy-Baits, Holland). One hour after feeding, uneaten food was removed from all tanks to maintain optimal physicochemical water conditions.

2.3. Stress coping styles tests

Individual and group coping style tests were performed at different periods for juveniles and breeders. For juveniles, individual coping style tests were performed from July, 23rd to 26th, and group tests from August, 13th to 17th. For breeders, individual stress coping style tests were made from October, 06th to 10th, and group tests from November, 06th to 11th. The individual coping style tests, which were applied on juveniles and breeders with the same behavioural criteria, consisted in: i) restraining, ii) evaluating the reaction to a new environment, iii) evaluating the reaction to a confinement situation, iv) flipping over

the fish, v) inducing anesthesia. Subsequently, a sample of blood was obtained from each individual to measure cortisol, glucose and lactate. The group tests consisted in: i) evaluating fish reaction to a novel object introduced in the tank and ii) a risk taking in group test.

2.4. Individual coping style tests

Test 1. Restraining test

Fish behaviour was evaluated by holding fish in a net in water (90s) and then in air out of water (90s). Two behavioural variables were evaluated for both phases: **i)** the total activity time that fish spent moving inside the net, considered as the swimming activity inside the water, **NetActW**, or the contortions or shivers in the air outside the water, **NetActA**, and **ii)** the total number of escape attempts, that is the number of body torsions resulting in an elevation of the body from the net, inside the water **NetEscW** or outside the water **NetEscA**. Selected variables were adapted from previous studies performed with this species (Silva et al., 2010; Martins et al., 2011a), and other fish species, such as zebrafish *Danio rerio* (Tudorache et al., 2013) and gilthead seabream *Sparus aurata* (Castanheira et al., 2013). The abbreviations used for these tests indicate the parameter measured in the test, Net = net, Act = total activity time, W = water, A = Air (out of the water) and Esc = escape attempts.

Test 2. New environment test

Once the first net test was completed, each fish was individually placed in a plastic tank that simulated a new environment. Dimensions of the tank were 56.5 x 36.5 x 30 cm for juveniles and 114 x 95 x 57 cm for breeders. Two behavioural parameters were registered during 5 minutes: **i)** the latency time to move from being introduced to the new environment until the first movement, **NewLat**, considered as the first moment that fish started to explore the new environment, if fish did not move at all during the 5 minutes period, then 300s was recorded for statistical analysis (Farwell and McLaughlin, 2009), and **ii)** the total activity time, **NewAct**, referring to the total time each fish spent swimming forward in the tank. During the test, observers stood completely stationary 1 m away from the tank to cause minimal disturbance to fish (Øverli et al., 2002). Tanks were provided with constant water that maintained > 5-6 mg/L oxygen levels. Methodology was adapted from tests documented in bluegill sunfish *Lepomis macrochirus* (Wilson and

Godin, 2009), common sole *Solea solea* (Mas-Muñoz et al., 2011) and stickleback *Gasterosteus aculeatus* (Bell et al., 2005). The abbreviations used indicate the parameter measured, New = new environment, Lat = latency time and Act = total activity time.

Test 3. Confinement situation test

Fish were individually placed in a plastic tank that simulated a confinement situation. Dimensions of tanks were 25 x 14 x 8 cm for juveniles and 56 x 36 x 30 cm for breeders. Two behavioural parameters were registered for 5 minutes: **i)** the latency time to move from being introduced into confinement until the first movement, **ConLat**, considered as the first moment that fish started to explore, if fish did not move at all during the 5 minutes period, then 300s was recorded for statistical analysis (Farwell and McLaughlin, 2009), and **ii)** total activity time, **ConAct**, that was restricted to active locomotion against the walls of the confinement container. As for the previous test, observers stood stationary 1 m away from the container to not disturb fish. Methodological procedures were adapted from those documented in brown trout *Salmo trutta* (Brelin et al., 2005) and rainbow trout *Oncorhynchus mikyss* (Øverli et al., 2006). The abbreviations used indicate the parameter measured, Con = confinement, Lat = latency time and Act = total activity time.

The number of opercula openings, **NumOper**, as a breathing rate approach, was counted and registered for juveniles during the first minute of this test (Barreto and Volpato, 2011).

Test 4. Flipping fish over

This test consisted in flipping fish over (eyes down position) and in recording the time (in seconds) the fish needed to recover its normal ventral position (**Flip**). Fish were laid on a rubber-foam carpet to cause minimal skin damage. Fish unable to recover normal ventral position after 3 minutes were turned over by hand and a maximum time of 180s was assigned for statistical analysis.

Test 5. Anesthesia induction

The time needed to induce three anaesthesia levels was determined in sole breeders. Sedation levels were selected in accordance to Schoettger and Julin (1967) and were named as i) Light Sedation **LSed**, characterized by the partial loose of reactivity; ii) Total

Loss of Equilibrium **TSed**, described as the impossibility to recover a normal position once turned over by hand; and iii) Deep Anesthesia **DSed**, when fish completely lost reflex to external stimuli. The anesthetic agent consisted in tricaine methanesulfonate (MS-222; Acros-Organic, New Jersey, USA). Anaesthesia was prepared by adding 20mg of MS-222 to 1L of distilled water. A final dose of 60 µg L^{-1} in 45 L bath was used for the test (Norambuena et al., 2011).

2.5. Group coping style tests

Test 1. Reaction to a novel object

Fifteen days after completion of individual tests, juveniles and breeders were submitted to a novel object test. For juveniles, the novel object consisted of a square wooden frame (30 x 30 x 20 cm), through which fish could pass, with an identification antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) mounted in the interior. For breeders, the novel object consisted of a grey plastic cube (56.5 x 36.5x 30 cm), supplied with an identification antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) located in the bottom of the cube.

Test started immediately after introducing the novel object in the middle of tank and lasted 24 hours for both fish populations. Whether the fish passed through the novel object (juveniles) or enter into the cube (breeders) was recorded and posteriorly analysed (Frost et al., 2007; Castanheira et al., 2013). Those fish that successfully passed through or stayed in the antenna were registered and identified.

Test 2. Risk taking test

This test was performed on juveniles and breeders with the same behavioural criteria. The risk taking test was realized one month after individual tests to allow juveniles and breeders to recover. This test aimed to determine fish capacity to cross from a known area (safe zone) to an unknown area (risky zone). The safe zone was isolated from light (2 and 3 lux on the surface for juveniles and breeders, respectively) and covered with sand, to provide a comfortable and secure space for fish. On the contrary, the risky area had more illumination (15 lux on the surface for juveniles and 11 lux on the surface for breeders; OSRAM DULUX 48 and 150W, respectively) and the bottom was devoid of sand. For juveniles, a 500 L tank was divided into two equal zones by a rigid plastic screen. A small window (5 cm high x 20 cm width) was opened at the bottom of the dividing screen, to

allow fish to cross between both areas. For breeders, the test was realized in a 16 m^3 tank (6 m length x 3 m width x 0.9 m depth), divided into two equal areas by a wooden screen. A window (30 cm width x 15 cm tall) was placed at the base of the dividing screen with a door that could be opened to allow fish to pass from an area to another. The window for both juveniles and breeders tanks was at the centre of a PIT (passive integrated transducer) tag reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that was positioned to read the tag number of the fish that passed through the window to the risk zone. Additionally, two submersible black and white digital cameras (F60B/NIR580-50G model, Korea Technology Co. Ltd, supplied by Praentesis S.L., Barcelona) connected to a recorder (DVR- 0404HB model, Dahua Technology Co. Ltd, supplied by Praentesis S.L., Barcelona) were installed 10 cm below the water surface in both the safe and risky zone, to corroborate information obtained from the antenna. Constant water and aeration flows were provided during the period of the test to maintain optimal water conditions.

Before the beginning of the test, juveniles and breeders were submitted to a 24 hour acclimation in the safe zone, by keeping the window closed until the beginning of the test, which started at 10:00 hours and lasted 24 hours. Juveniles were tested by groups of 15 individuals and breeders in groups of 10 individuals to avoid inducing stress due to high stocking densities. Fish that successfully crossed from the safe zone to the risky zone were defined as “proactive”, while fish that did not cross were recognized as “reactive” (Budaev, 1997a, b; Frost et al., 2007; Wilson and Godin, 2009; Huntingford et al., 2010; Tudorache et al., 2013). The latency time of each organism to cross from one area to another was recorded. A maximum time of 1440 min was assigned to fish that did not cross during the 24 h period of the test.

2.6. Cortisol, glucose and lactate analysis

A blood sample (0.5 ml) was extracted from the caudal vein of anesthetized fish (MS-222; Argent, USA, 100 ppm) to measure cortisol, lactate and glucose concentrations. In juveniles, blood extractions were performed twice, one month before the stress coping styles tests (control), and approximately 35 minutes after completing all the individual tests (post-stress). In breeders, blood extraction was performed once, approximately 40 minutes after completing individual tests (post-stress). To avoid blood coagulation, a solution of 10 µl sodium heparin (5%, 25.000 UI; HOSPIRA) and 15 µl aprotinin (from

bovine lung; 0.9% NaCl, 0.9% benzyl alcohol and 1.7 mg of protein; SIGMA) was placed inside eppendorf´s tubes, while the syringes and needles used were coated with heparin. Blood samples were centrifuged (ThermoScientific centrifuge, M23i; Thermo rotor AM 2.18; 24 x 1.5 ml) at 3000 G and 4ºC during 15 min and plasma supernatant was removed and stored in triplicates at −80ºC prior to analysis (Martins et al., 2011a). Cortisol was measured by a competitive conjugated binding ligand by means of a commercial ELISA kit (Range of detection: 0-800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany), whereas glucose and lactate were measured by means of commercial enzymatic colorimetric kits (SPINREACT, Gerona, Spain). Cortisol, glucose and lactate absorptions were read by a spectrophotometer (Infinite M-200; TECAN, Switzerland), at 23ºC and 505 nm.

2.7. Statistical analysis

Statistical analyses were performed using SPSS Statistics 18.0 software (IBM Co., Hong Kong) and Sigmaplot 12.0 software (Systat, Inc.). Data was checked for normality by means of a Kolmogorov-Smirnov normality test. A Principal Components Analysis (PCA), with a Kaiser-Meyer-Olkin adequacy test, Bartlett's test of sphericity and an orthogonal varimax rotation was performed in two steps: i) individually on tests including the measurement of several variables, such as restraining, novel environment and confinement tests, in order to select the most representative variable from each test and ii) on all variables, including cortisol, lactate and glucose concentrations, in order to select the tests that best characterized fish stress coping styles. Next, a principal component regression analysis was performed on the selected variables to generate a "principal component score" (PCS) for each individual. This PCS represented the individual stress coping style behaviour of each fish for each of the most representative selected variables. Subsequently, a general linear multivariate model analysis (GLM) and Kolmogorov Smirnov-test (KS-test), for independent populations, were performed to examine the between individual consistency between the group of juveniles and breeders for the selected tests resulted from the PCA.

In the group tests, the PCS that was assigned to fish that successfully crossed in the risk taking test was compared to the PCS of fish that did not cross, by means of Student t-test. Such comparisons were made in order to support the effectiveness of the risk taking test to discriminate proactive fish from reactive fish within the selected tests. Whilst, the

consistency was checked by comparing proportion of fish (juvenile and breeders) that crossed and those that did not cross in the risk taking test by a Chi-square (X^2) test. To check if the latency time to cross into the risk area in the risk taking test was correlated with the latency time in the new environment and confinement tests, a Pearson correlation analysis was performed. Lastly, the coefficient of variation (CV % = SD/mean*100) was averaged for each test and represented the inter-individual variability of sole. For each parameter of the selected coping style tests, fish were separated into four quartiles that represented low to high activity categories, by an Ntiles rank analysis and the mean glucocorticoids production were compared by ANOVAs between the low and high activity groups, for initial and post-stress levels in juveniles and for post-stress levels in breeders. Across-context correlations between the PCS and fish weight, length, and cortisol, glucose and lactate concentrations were performed using Intraclass correlation coefficient analysis (ICC). A *P*-value < 0.05 was established as statistically significant for all tests realized.

3. Results

3.1. Senegalese sole behavioural responses in individual tests

Tests induced different responses in Senegalese sole juveniles and breeders. In the restraining test, behavioural responses of both juveniles and breeders ranged from fish that that did not move and remained static in the net to fish that immediately attempted to escape with a high level of activity (escape attempts and time of activity) during the 90s. Juveniles showed different behavioural motivation than breeders. Indeed, juveniles showed significant higher activity ($P < 0.016$), escape attempts ($P < 0.001$) and less variability (CV NetActA = 57.4%; CV NetEscA = 65.8%) outside the water, while breeders presented significant higher activity ($P < 0.001$), escape attempts ($P < 0.001$) and less variability (CV NetActW = 94.9%; NetEscW = 133.9%) inside the water (Table 1 and 2). The new environment and confinement tests were the only trials in which behaviour of juveniles and breeders adjusted to bimodal distribution (Figure 1A and B).

Table 1. General results for individual coping style tests of Senegalese sole juveniles (n = 61). NewLat and ConLat CV were calculated with the total averaged mean. Superscript letters reveal significant differences (analyzed by Student t-test).

Coping style tests	Variables	Type of distribution	Mean ± SD (1st mode)	Mean ± SD (2nd mode)	Min	Max	Coef. Variance (%)
Netting the fish	NetActW (sec)	Normal	14.80 ± 14.8^{A}	-	0	76	100
	NetEscW	Skew positive	2.9 ± 4.1^{A}	-	0	16	141.4
	NetActA (sec)	Normal	10.1 ± 5.8^{B}	-	1	23	57.4
	NetEscA	Normal	27.5 ± 18.1^{B}	-	0	67	65.8
New environment	NewLat (sec)	Bimodal	28.32 ± 6.6	295.5 ± 3.1	2	300	88.6
	NewAct (sec)	Skew positive	11.6 ± 19.4	-	0	157	167.2
Confinement	ConLat (sec)	Bimodal	31.2 ± 7.1	294.6 ± 3.9	1	300	105.2
	ConAct (sec)	Skew positive	31.3 ± 42.5	-	0	70	135.8
	NumOper	Skew positive	49.6 ± 47.2	-	1	154	95.2
Flip over the fish	Flip (sec)	Skew positive	115.6 ± 110.2	-	1	180	95.3
Novel object	NO	Skew	71.6 ± 97.1	-	0	428	135.6
Blood hormones (control)	Cortisol (ng/ml)	Normal	32.1 ± 25.1	-	8.3	132.7	78.2
	Lactate (mmol/l)	Normal	19.4 ± 5.1	-	11.5	30.1	26.3
	Glucose (mmol/l)	Normal	4.0 ± 1.7	-	1.6	12.2	42.5
Blood hormones (post-stress)	Cortisol (ng/ml)	Skew positive	79.6 ± 64.8	-	12.9	265.6	81.1
	Lactate (mmol/l)	Normal	26.8 ± 5.6	-	15.3	47.1	20.9
	Glucose (mmol/l)	Normal	6.2 ± 3.1	-	2.8	20.1	50

Table 2. General results for individual coping style tests of Senegalese sole breeders (n = 59). NewLat and ConLat CV were calculated with the total averaged mean. Superscript letters reveal significant differences (analyzed by Student t-test)

Coping style tests	Variables	Type of distribution	Mean ± SD (1st mode)	Mean ± SD (2nd mode)	Min	Max	Coef. Variance (%)
Netting the fish	NetActW (sec)	Skew positive	17.80 ± 16.89^{A}	-	0	73	94.9
	NetEscW	Skew positive	5.30 ± 7.18^{A}	-	0	31	133.9
	NetActA (sec)	Skew positive	3.40 ± 6.13^{B}	-	0	26	180.2
	NetEscA	Skew positive	3.00 ± 6.0^{B}	-	0	30	200
New environment	NewLat (sec)	Bimodal	28.44 ± 6.05	291.72 ± 6.01	0	300	117.8
	NewAct (sec)	Skew positive	26.10 ± 35.8	-	0	166	137.6
Confinement	ConLat (sec)	Bimodal	22.55 ± 3.95	291.74 ± 5.75	0	300	115.7
	ConAct (sec)	Skew positive	24.20 ± 36.3	-	0	184	150
Flip over the fish	Flip (sec)	Skew negative	143.70 ± 65.1	-	1	180	45.3
Novel object	NO	-	0		0	0	
Anaesthesia	LSed (sec)	Normal	66.6 ± 29.9	-	14	160	44.9
	TSed (sec)	Normal	146.70 ± 81.15	-	4	559	55.3
	DSed (sec)	Normal	251. 81 ± 145.10	-	68	980	57.5
Blood hormones (post-stress)	Cortisol (ng/ml)	Skew	20.60 ± 55.17	-	0.1	318	267.4
	Glucose (mmol/l)	Normal	4.70 ± 2.67	-	0.91	10.36	55.4
	Lactate (mmol/l)	Normal	6.63 ± 6.39	-	0.05	25.65	96.4

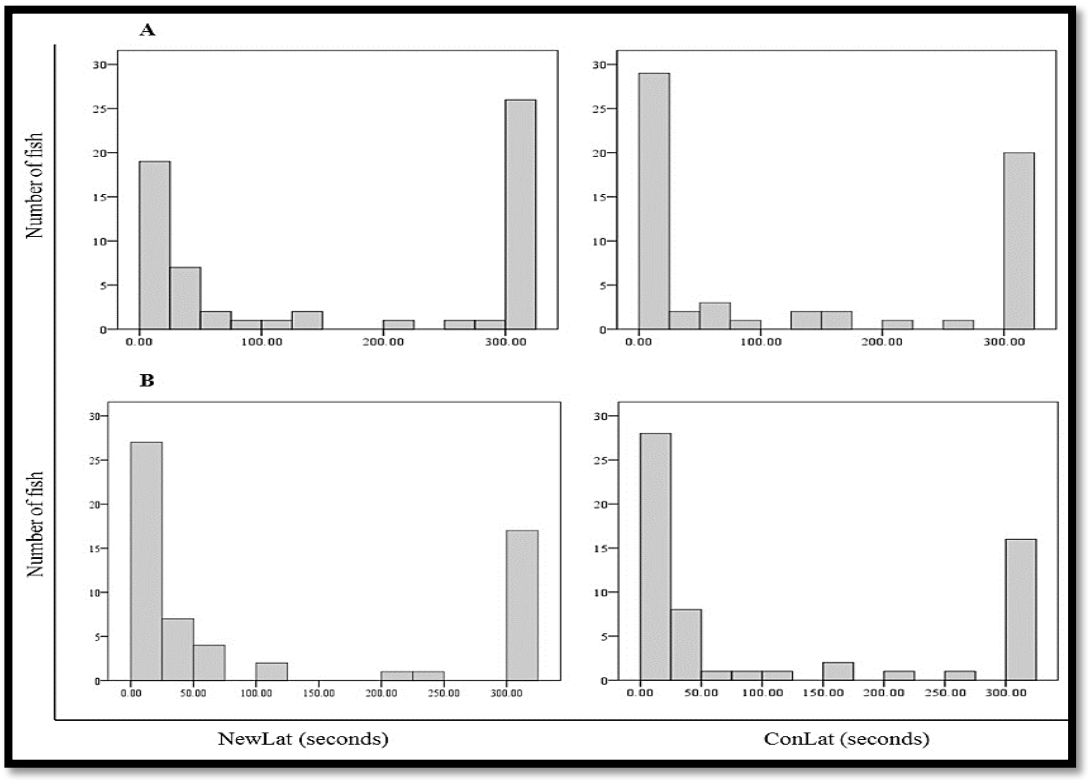

Figure 1. Frequency distribution of Senegalese sole juveniles (A) and breeders (B) for the variables first activity time (latency to move) in new environment (NewLat) and first activity time (latency to move) in confinement (ConLat) tests.

In both tests, behavioural responses ranged from fish that that did not move and appeared to freeze on being introduced into the container to fish that immediately moved (low latency time) with a high level of exploratory activity (time of activity) during the 300s and consequentially the variation was high ranging from CV = 88.6%; (juvenile NewLat) to 167.2% (juvenile NewAct) (Table 1 and 2). Moreover, the GLMM showed high inter-individual consistency, since latency time and total activity time in new environment and confinement tests of juveniles and breeders were not significantly different (F_{116} = 2.00, P = 0.159 and F_{116} = 0.118, P = 0.732; F_{116} = 1.00, P = 0.319 and F_{116} = 0.066, P = 0.798, respectively) and showed similar distributions (KS-test, NewLat P = 0.785, NewAct P = 0.430, ConLat P = 0.110 and ConAct P = 0.158). Juveniles showed significant correlations between NetActA and ConAct (R = 0.491; P < 0.001), between NetEscA and ConLat (R = 0.439; P < 0.001), between NetEscA and ConAct (R = 0.556; P < 0.001). In breeders, the highest correlations were noticed between NetActW and NewLat (R = 0.455; P < 0.001) and between NetActW and NewAct (R = 0.465; P < 0.001). These correlations suggested that more active individuals in the restraining test were also more

active in the new environment test, showed lower latency to start exploration and indicated inter-individual consistency across contexts. No significant correlations ($P > 0.05$) were observed between the variables from the restraining, new environment or confinement tests with cortisol, glucose or lactate levels, weight and length, neither for juveniles nor for breeders.

In the opercula openings variable (Table 1), juveniles averaged 49.6 ± 47.2 movements during one minute and presented considerable variation (CV = 95.2%). However, this test was difficult to assess in this flatfish species because of the position of gill. In the flip over test (Table 1 and 2), only 14 juveniles from a total of 61 recovered their normal position after an average time of 60.4 ± 48.65 s (the average time for all juveniles ($n = 61$) was 115.6 ± 110.2 s and CV = 95.3%), while 17 breeders of 59 recovered their normal position after an average time of 47.1 ± 39.3 s and in comparison to other tests variation in individual responses was low (the average time for all breeders ($n = 59$) was 143.7 ± 65.1 s and CV = 45.3%). Fish weight appeared to be an issue for this test, since the heaviest fish showed more difficulties in flipping over than lighter fish. In the anaesthesia test breeders reached the LSed anaesthesia level after 66.6 ± 29.9 s, the TSed anaesthesia level after 146.7 ± 81.1 s and the DSed level after 251.8 ± 145.1 s. This test did not showed much behavioural variability (CV Lsed = 44.9, CV Tsed = 55.3; CV Dsed = 57.3%) and, therefore, may not express a large variation in relation to behavioural syndromes (Table 2). Lastly, the juveniles post-stress cortisol concentration (79.6 ± 64.8 ng/mL) was significantly higher ($P < 0.001$) than their basal level (32.1 ± 25.1 ng/mL), but the glucose and lactate concentrations were not statistically different ($P > 0.05$) and variation was relative low. In breeders, the post-stress levels were 20.60 ± 55.17 ng/mL for cortisol, 4.70 ± 2.67 mmol/L for glucose and 6.63 ± 6.39 mmol/L for lactate (Table 1 and 2). Breeders individual response variation was higher than in juveniles, especially for cortisol (CV = 267.4%).

Further, the fish from the high activity quartile (ranked by an Ntiles-analysis) had significantly lower cortisol than fish in the low activity quartile in juveniles for NewAct (control cortisol $F_{1, 39} = 4.10$, $P = 0.050$), NetEscA (post-stress cortisol $F_{1, 28} = 6.16$, $P = 0.019$) and ConAct (control cortisol $F_{1, 33} = 4.75$, $P = 0.038$ and post-stress cortisol $F_{1, 33}$

= 6.58, P = 0.015) and in breeders also for NewAct (post-stress cortisol $F_{1, 28}$ = 4.26, P = 0.048) and ConAct (post-stress cortisol $F_{1, 29}$ = 6.89, P = 0.014). This indicated that selected tests were in line with the differences in cortisol production observed between proactive and reactive fish in both juveniles and breeders.

3.2. Behavioural responses of sole in the group tests

Fifty-nine juveniles of the 61 passed through the novel object and 2 fish never passed, while none of the 59 breeders entered or even get close to the novel object (Table 1 and 2). In addition, a high variability was observed in the number of juveniles passing through the novel object (CV = 135.6%).

In the risk taking test, 22 juveniles of 61 crossed from the safe area to the risky area (36%) in an average time of 337.5 ± 177.1 min after starting the test (Table 3), while 17 breeders of 59 (29%) crossed in an average time of 414.1 ± 187 min after starting the test (Table 4). The observed proportion of juveniles that successfully crossed was consistent with the proportion of breeders that crossed (X^2 = 0.719, df = 1, P = 0.396). The PCS of juvenile that successfully crossed showed significantly higher activity levels (NetActA, t = 5.94, df = 59, P = 0.001; NetEscA, t = 7.23, df = 59, P = 0.008; ConAct, t = 6.98, df = 59, P = 0.001 and NewAct, t = 5.50, df = 59, P = 0.024) and significant lower latencies (ConLat; t = 3.12, df = 59, P < 0.001) than juveniles that did not cross (Figure 2).

Table 3. Average values of coping style variables measured for juveniles that crossed (n = 22) versus those that did not cross (n = 39) in the risk taking test. Superscript letters indicates significant differences.

Coping style tests	Variable	Crossed	Did not cross
Netting the fish	NetActW (sec)	20.5 ± 3.8 [a]	11.5 ± 1.8 [b]
	NetEscW	4.1 ± 1.1	2.3 ± 0.5
	NetActA (sec)	11.5 ± 1.2 [a]	9.2 ± 0.9 [b]
	NetEscA	39.7 ± 3.6 [a]	20.6 ± 2.3 [b]
New environment	NewLat (sec)	142.8 ± 27.7	162.5 ± 22.9
	NewAct (sec)	17.4 ± 5.2 [a]	8.2 ± 24 [b]
Confinement	ConLat (sec)	50.3 ± 16.8 [a]	168.9 ± 22.1 [b]
	ConAct (sec)	61.1 ± 10.3 [a]	14.6 ± 4.4 [b]
	OBR	54 ± 10.6	47.1 ± 7.3
Flip over the fish	Flip (sec)	125.6 ± 24.3	109.5 ± 17.4
Novel object	NO	84.9 ± 27.1	64.2 ± 12.2
Blood hormones (control)	Cortisol (ng/mL)	21.1 ± 1.8 [a]	38.3 ± 4.6 [b]
	Lactate (mmol/L)	20.1 ± 1.1	19.1 ± 0.8
	Glucose (mmol/L)	3.7 ± 0.2	4.1 ± 0.2
Blood hormones (post-stress)	Cortisol (ng/mL)	41.8 ± 6.2 [a]	100.9 ± 11.1 [b]
	Lactate (mmol/L)	25.9 ± 1.1	27.3 ± 0.9
	Glucose (mmol/L)	5.8 ± 0.7	6.4 ± 0.4

Besides, PCS of breeders that successfully crossed showed significantly higher activity levels (NetActW, t = 3.63, df = 57, P = 0.012; NetEscW, t = 3.62, df = 57, P = 0.011; NewAct, t = 3.28, df = 57, P = 0.014 and ConAct, t = 1.28, df = 57, P = 0.042) than fish that did not cross (Figure 3). Moreover, both juveniles and breeders that successfully crossed in the risk taking test showed lower post-stress cortisol levels than fish that did not cross (Tables 3 and 4). This result suggest that proactive (also named "bold" fish) - assumed to be those that successfully crossed in the risk taking test - were confirmed to present higher activity in the restraining, new environment and confinement tests than reactive (also named "shy" fish) - assumed to be those that did not cross in the risk taking

test. Lastly, the Pearson analysis showed significant correlation between the latency time to cross with NewLat (R = 0.304, P = 0.001), but it was not correlated with ConLat (R = 0.025, P = 0.788).

Table 4. Average values of coping style variables for breeders that cross (n = 17) versus those that did not cross (n = 42). Superscript letters indicates significant differences.

Coping style tests	Variables evaluated	Crossed	Did not cross
Netting the fish	NetActW (sec)	21.5 ± 3.8[a]	16.2 ± 2.6 [b]
	NetEscW	6.6 ± 1.8	4.8 ± 1.1
	NetActA (sec)	5.5 ± 1.9	2.6 ± 0.8
	NetEscA	4.14 ± 1.7	2.5 ± 0.9
New environment	NewLat (sec)	100.5 ± 30.4	113.2 ± 20.3
	NewAct (sec)	35.2 ± 8[a]	22.4 ± 5.6 [b]
Confinement	ConLat (sec)	113.2 ± 31.7	108 ± 19.5
	ConAct (sec)	19.5 ± 8.2	26.1 ± 5.8
Flip over the fish	FLIP (sec)	116.1 ± 17.7[b]	155 ± 9.1 [a]
Anaesthesia	LS (sec)	64.5 ± 8.2	67.3 ± 4.4
	TSed (sec)	138.6 ± 16	150 ± 13.4
	DSed (sec)	281.1 ± 50.2	239.3 ± 17.2
Blood hormones (post-stress)	Cortisol (ng/mL)	3.3 ± 1.1[b]	27.6 ± 9.9 [a]
	Glucose (mmol/L)	5.0 ± 0.8	4.6 ± 9.9
	Lactate (mmol/L)	6.1 ± 1.1	6.8 ± 1.1

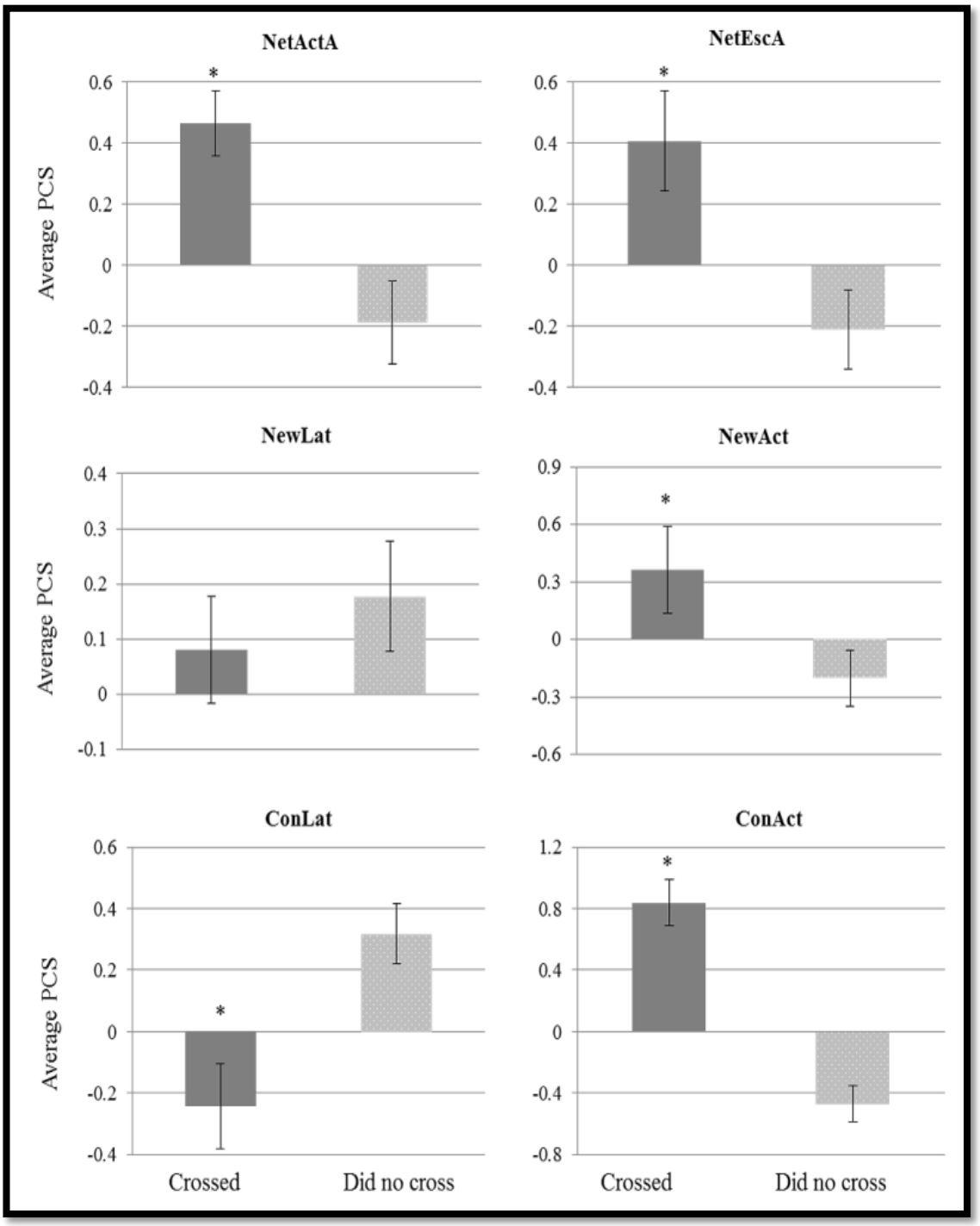

Figure 2. Principal component scores (PCS) of juveniles that successfully crossed versus those that did not cross in the risk taking test, for the 6 selected variables. Asterisks indicate statistical differences.

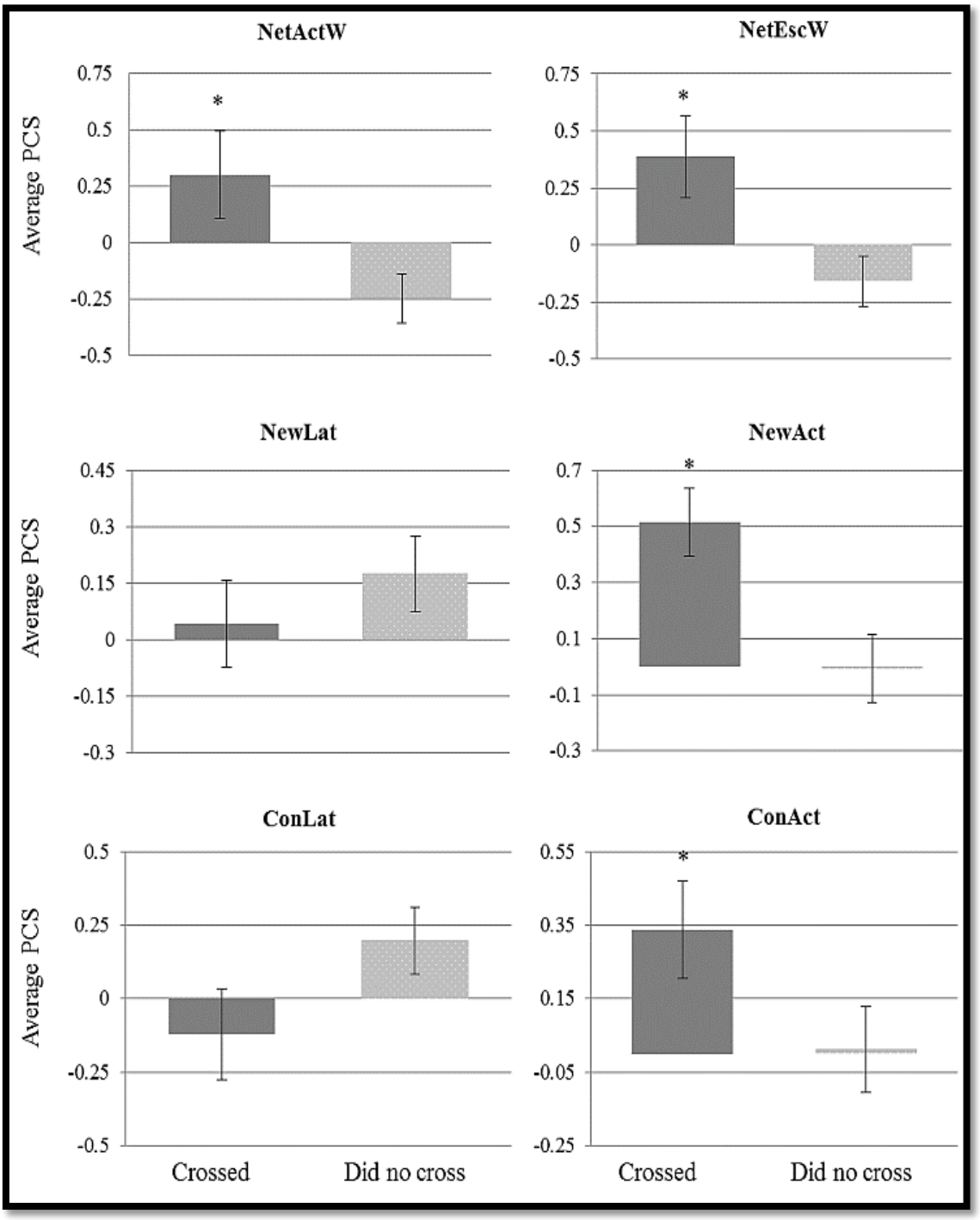

Figure 3. Principal component scores (PCS) of breeders that successfully crossed versus those that did not cross in the risk taking test, for the 6 selected variables. Asterisks indicate statistical differences.

3.3. Coping style tests selection

The efficacy of the PCA was confirmed by the significance of the results of Kaiser-Meyer-Olkin test and Bartlett's test of sphericity results (KMO = 0.574 and 0.588; χ^2 = 229.80, *df* = 105, *P* < 0.001 and χ^2 = 102.43, *df* = 55, *P* < 0.001, for juveniles and breeders respectively). From all behavioural variables evaluated in the PCA, three main tests resulted appropriate to characterize Senegalese sole juveniles and breeders stress coping styles: **i)** restraining test, **ii)** novel environment test and **iii)** confinement test (Figure 4A and B).

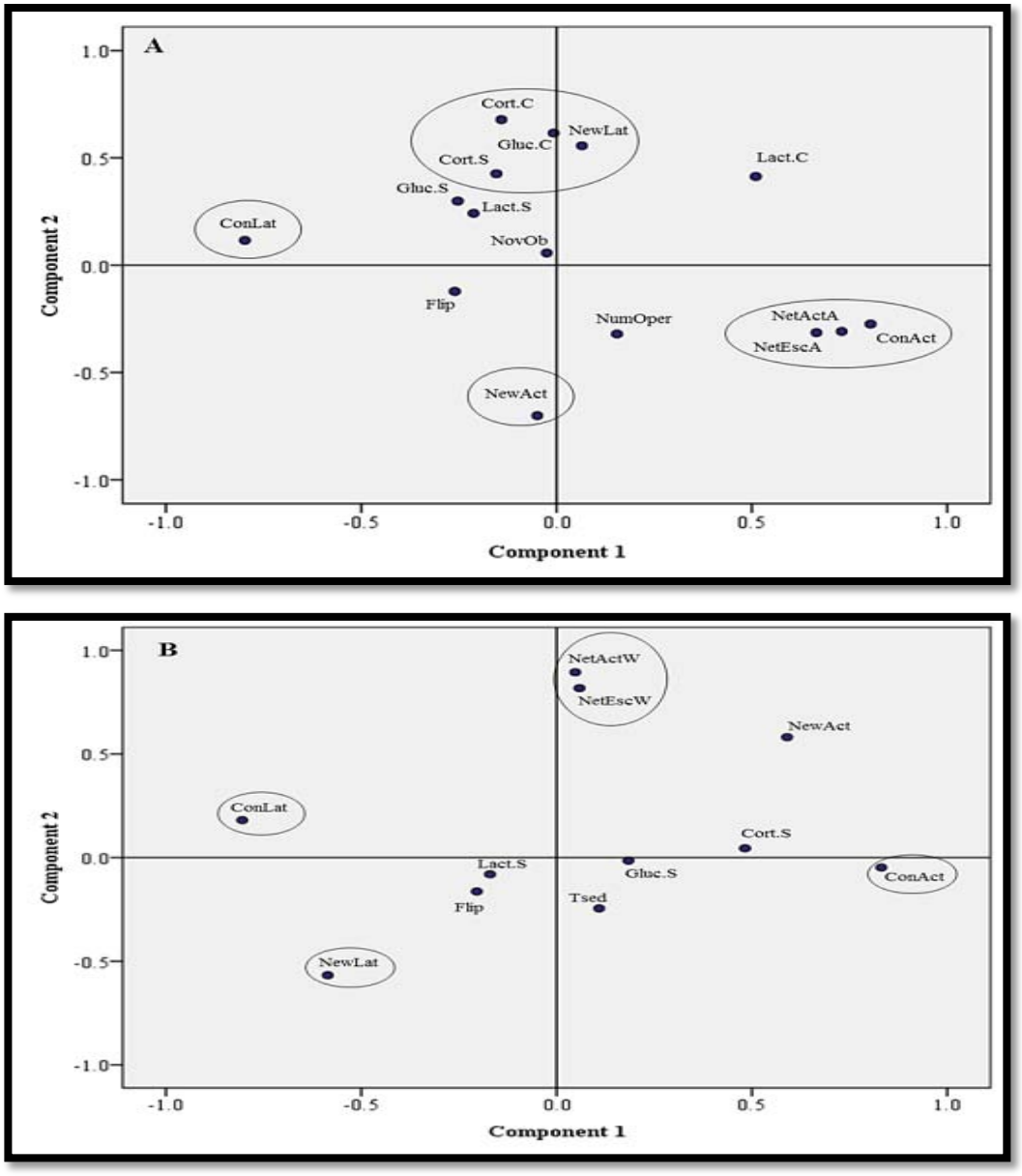

Figure 4. Component matrix diagrams of variables selected (in circled) to characterize stress coping style of Senegalese sole juveniles (A) and breeders (B).

These three selected tests described more than 70% of the total variance for juvenile's behavioural differences and 74% for breeders in the principal component matrix, when represented by two principal components (PC1 and PC2). Those tests also had the highest communalities and eigenvalues (Table 5 and 6). Component one of PCA showed high context correlations between NewLat-NewAct and ConLat-ConAct (new environment and confinement variables) for juveniles and breeders. In component two, the highest context correlations observed for juveniles corresponded to variables NetActA-NetEscA (netting the fish outside the water) and cortisol concentration before and after performing the stress coping style tests, while in breeders the highest correlations corresponded to variables NetActW-NetEscW (netting the fish inside the water) and cortisol levels (Table 5 and 6).

Table 5. PCA scores, eigenvalues and percentage of variance explained of the different coping style tests performed in Senegalese sole juveniles. Variables marked in bold were selected. * Not considered since eigenvalue was less than 1.

Coping style tests	Variable	Component matrix score PC1	Component matrix score PC2	Communality	Eigenvalue	% Variance explained
Netting the fish	NetActW *		0.253			
	NetEscW *		-0.422			
	NetActA		**0.744**	**0.628**	**1.871**	**12.47**
	NetEscA		**0.708**	**0.641**	**1.641**	**10.94**
New environment	**NewLat**	**0.665**		**0.514**	**1.135**	**7.565**
	NewAct	**-0.562**		**0.494**	**1.107**	**7.379**
Confinement	**FAC**	**0.679**		**0.650**	**1.351**	**9.01**
	TAC	**-0.787**		**0.721**	**3.444**	**22.96**
	OBR *	-0.327				
Flip over the fish	FLIP *	0.117				
Novel object	NO *	0.057				
Blood hormones (control)	Cortisol*		0.678	0.556	0.981	
	Lactate *		0.413			
	Glucose *		0.141			
Blood hormones (post-stress)	Cortisol*		0.426	0.480	0.810	
	Lactate *		0.242			
	Glucose *		0.299			

Table 6. PCA scores, eigenvalues and percentage of variance explained of the different coping style tests performed in Senegalese sole breeders. Variables marked in bold were selected. * Not considered since eigenvalue was less than 1.

Coping style tests	Variable	Component matrix score PC1	Component matrix score PC2	Communality	Eigenvalue	% Variance explained
Netting the fish	**NetActW**		**0.735**	**0.802**	**2.946**	**10.96**
	NetEscW *		0.682	0.672	0.876	
	NetActA *		-0.482			
	NetEscA *		0.344			
New environment	**NewLat**	**-0.815**		**0.665**	**1.298**	**15.43**
	NewAct	**0.827**		**0.686**	**1.698**	**26.77**
Confinement	**ConLat**	**-0.692**		**0.693**	**1.006**	**9.14**
	ConAct	**0.785**		**0.680**	**1.206**	**11.81**
Flip over the fish	FLIP *	0.261				
Anaesthesia	LS *	0.206				
	TSed *	0.083				
	DSed *	0.101				
Blood hormones (post-stress)	Cortisol *		**0.485**	0.465	0.764	-
	Glucose *		-0.181			
	Lactate *		0.126			

4. Discussion

Three tests were demonstrated for the first time to enable the characterization of stress coping style of Senegalese sole juveniles and breeders: i) restraining test, ii) new environment test, and iii) confinement test. Each test found inter-individual consistency in the behavioural responses across context within each group, juveniles and breeders. The restraining test, which measured the ability of fish to respond to an invasive and aversive situations, induced two divergent behavioural reactions in juveniles and breeders characterized by high and low: activity and escapes attempts. Thus, proactive juveniles and breeders made many escape attempts and were active in the net while reactive individuals did not make escape attempts or move in the net. Therefore, indicating the test was reliable to sort proactive and reactive behaviours and was in agreement with other studies performed with this and other fish species (Bell, 2005; Silva et al., 2010;

Castanheira et al., 2013; Tudorache et al., 2013). Similarly, the new environment and confinement tests induced high amplitude of individual behavioural variability in the latency time and total activity time responses that were consistent with coping styles. Thus, proactive juveniles and breeders tended to resume activity earlier and showed higher activity than reactive individuals, suggesting a higher explorative behaviour and reactivity to stressful situations. These behavioural observations were similar to those reported in bluegill sunfish *Lepomis macrochirus* (Yoshida et al., 2005; Wilson and Godin, 2009), gilthead seabream (Castanheira et al., 2013) and swordtail *Xiphophorus birchmanni* (Boulton et al., 2014), with proactive fish displaying higher activity and explorative behaviour in comparison to reactive fish. Additionally, the first activity time in new environment (NewLat) and the first activity time in confinement (ConLat) adjusted to a bimodal frequency distribution in both juveniles and breeders Senegalese sole, since some fish resumed activity almost immediately after being introduced into the new environment and others did not move during the entire 5 minutes of the test. Hence, such bimodality appeared to represent the two extremes of personalities and possibly explained how fast proactive fish acclimate to a new environment and/or to a confinement situation. This assumption agrees to the concept proposed by Fox et al. (2009) and Budaev and Brown (2011). These authors indicated that bimodality in behavioral responses to coping style tests is a common factor in several fish species, since individuals usually tend to form clusters of similar traits rather than continuously distributed traits or dimensions. These two tests new environment and confinement also exhibited a degree of correlation with the restraining test for juveniles and breeders (R = 0.439 to R = 0.556) indicating inter-individual consistency across these contexts. Perhaps contradicting this cross context correlations were poor amongst parameters with the physiological parameters indicating that behaviour was not consistent over all contexts. However, it should be noted that even though correlations were poor with physiological parameters, the fish that were identified as proactive (high activity quartile) with the three tests, restraining, new environment and confinement had significantly lower levels of cortisol compared to fish identified as reactive (low activity quartile), which suggests there was a degree of cross context inter-individual consistency and that these tests identify stress coping styles in agreement to the physiological dimension. Flip over the fish, anesthesia and opercula breathing rate tended not to exhibit sufficient variation in responses and / or had technical complications such as observing slight opercula movements or results appeared to be compromised by fish size as in flip over the fish.

The risk taking test has been widely accepted as a test that discriminated proactive from reactive individuals in other fish species, such as bluegill sunfish (Wilson and Godin, 2009), common carp (Huntingford et al., 2010) and gilthead seabream (Castanheira et al., 2013). In the present study, the risk taking test was successfully used to both (a) discriminate proactive from reactive individuals in both groups of fish (juveniles and breeders) and (b) validate the identification of proactive and reactive fish identified by other tests. Indeed, the tests revealed that those juveniles and breeders with higher activity in the three selected tests were those with higher predisposition to take risk (*e.g.* cross from safe to risk zone). However, the inadequacy of the risk test for aquaculture, in comparison of the three individual tests selected, is the long time period required to be performed. In addition, the hypothalamic-pituitary-interrenal (HPI) axis of fish with high overall activity in the three selected was lower than fish with reduced activity, this lead to differences in cortisol concentrations. Moreover, control and post-stress levels of cortisol in juveniles and post-stress levels of cortisol in breeders were consistent to those reported for other fish species, such as gilthead seabream (Castanheira et al., 2013) and sea bass (*Dicentrarchus labrax*) (Yildiz and Ergonul, 2010). These results are in agreement with other studies that established cortisol as stress coping style predictor and confirmed the lower HPA/HPI axis reactivity to stress in proactive fish than in reactive fish (Øverli et al., 2006; Castanheira et al., 2013).

Additionally, the behavioural profiles of juveniles and breeders were similar among two selected tests the new environment and confinement, since the general linear model and the Kolmogorov-Smirnov tests showed no differences in the between individual variation between the two groups. In addition, the Chi-square test showed no statistical differences between the proportion of juveniles and breeders crossing from the safe to risky area in the risk taking. These results demonstrated inter-individual consistency across context (juveniles compared to breeders) and confirmed the existence of behavioural syndromes in different situations (*e.g.* high activity and escapes attempts in juveniles and breeders resulted in low latency and high total activity) in this fish species (Sih et al., 2004; Conrad et al., 2011). Correlations or consistency between behaviours in different contexts or situations may have important implications in animals, since they might generate trade-offs between different behaviours (Sih et al., 2004; Bell, 2005; Conrad et al., 2011). Therefore, is possible that this consistency between juveniles and breeders rely in the fact

that behaviours are linked because they are both governed by a common physiological mechanism.

It is worth noting that the PCA analysis represented a practical statistical method to reduce variables and characterized coping styles of Senegalese sole juveniles and breeders. The three selected tests presented the highest weight in the first two components (PC1 and PC2) of the PCA and explained the highest variance. Studies using PCA to characterize animal behaviour have been performed not only in fish (Budaev, 1997a, b; Wilson et al., 2010; Castanheira et al., 2013) but also in other vertebrates such as birds (Laiolo et al., 2009) and mammals (Constantini et al., 2012). In relation to the two main extracted PCA components, it seemed that PC1 represented the fish "activity-exploration behaviour" due to the strong correlations observed between NewAct and ConAct and between NewLat and ConLat, while PC2 characterised Senegalese sole "fearfulness-reactivity behaviour" because of the solid correlations between NetActA and cortisol concentrations (control and post-stress) for juveniles and between NetActW and cortisol concentrations (post-stress) for breeders. The "activity-exploration behaviour" was defined by the high activity and explorative behaviour showed by juveniles and breeders when submitted to new situations, which closely resembles curiosity, impulsiveness or reactiveness to the presence-absence of conspecifics and sociability. The "fearfulness-reactivity behaviour" was interpreted as the reaction of fish in relation to an aversive situation that resembles fear, stimulation and anxiety. These axis of personality has been previously confirmed in others fish species, such as guppy (*Poecilia reticulata*) (Budaev et al., 1997a), Nile tilapia (Martins et al., 2011b), gilthead seabream (Castanheira et al., 2013) and brown trout (Korte et al., 2014), by means of similar tests to those used in the present study to characterize fish stress coping styles. In addition, the behavioural dimensions interpreted in the present work are two of the main behavioural traits documented in fish and are supposed to cover all fish behaviour and temperament (Budaev and Brown, 2011; Conrad et al., 2011; Castanheira et al., 2013). Furthermore, the principal component score (PCS), which represented the behavioural score of each organism, allowed to confirm the presence of significant differences between proactive and reactive Senegalese sole juveniles and breeders in the three selected individual coping style tests. Similar methodology and results have been reported in other fish species such as bluegill sunfish, gilthead seabream (Castanheira et al., 2013), crucian carp (*Carassius*

langsdorfii), goldfish (*Carassius auratus*) (Yoshida et al., 2005) and poeciliid fish (*Brachyrhaphis episcopi*) (Archard et al., 2012).

In aquaculture, the operational success of culturing fish depends, among other things, on providing optimal rearing conditions to improve their behaviour, which is essential for increase production, survival, growth, disease resistance and, ultimately, their reproduction. Further, the importance to understand why animals behave as they do should not be neglected, since it is through their behaviour that animals interact with and cope with the environment (Huntingford, 2004; Castanheira et al., 2015). The effect of external stimuli (*i.e.* human presence, fish manipulation, etc.) might result in different coping styles behavioural responses, since animals react differently to the same stimulus in function of their different personalities or stress coping styles. For instance, it has been documented that fish with a proactive personality, and held in rearing conditions, frequently tend to recover the appetite faster after a stressful situation (Mas-Muñoz et al., 2011; Basic et al., 2012), show a higher immune response (MacKenzie et al., 2009), present higher growing rates (Ward et al., 2004) and possess higher reproductive success (Budaev and Brown, 2011; King et al., 2013; Castanheira et al., 2015) than reactive individuals. Therefore, the selection of certain personality traits might improve the efficiency of aquaculture productivity. The three coping style tests proposed in the present study might result attractive and feasible for sole farmers, since: they are easy to perform, many individuals can be tested in a short amount of time, are relatively inexpensive and do not need modifications of the rearing systems. These tests are individually based and can be performed quickly on a low or high number of fish in comparison to other tests. Moreover, these tests could be easily achieved and interpreted by farmers without experience in fish behaviour. In addition, the selected tests (a) confirmed two personality axes that are well accepted in literature, being the fearfulness-reactivity behaviour and the activity-exploration behaviour and both axes exhibited significant differences between each other, (b) the selected tests exhibited inter-individual consistency, (c) the tests were representative of the physiological stress coping style response as the proactive fish that were identified had significantly higher cortisol levels than reactive fish, and (d) the selected tests were validated by the risk taking test for identifying proactive and reactive individuals. Incorporating suitable and reliable tests to characterize stress coping styles of Senegalese sole juveniles and breeders under rearing

conditions to rearing protocols may generate valuable information to fish farmers in order to detect, by example, how efficiently fish feed and adapt to their environment.

5. Conclusions

The present study confirmed the existence of proactive and reactive behaviours in Senegalese sole breeders and proposed, for the first time, three individual stress coping styles tests that identify individual behavioural differences in Senegalese sole juveniles and breeders, kept under aquaculture rearing conditions. Moreover, the three selected tests might be used as an operational screening method in the aquaculture industry to select proactive and reactive individuals. Understanding Senegalese sole stress coping styles may benefit fish farmers to improve fish welfare and production systems, which ultimately may result in the establishment of selection-based breeding programs, to improve domestication and produce fingerlings with specific behavioural trait. Finally, more studies should be performed in order to increase the knowledge on Senegalese sole coping styles in relation to growth, food conversion, disease resistance, reproductive success, fitness, repeatability and dominance.

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Chapter 3

Senegalese sole (*Solea senegalensis*) show repeatable and consistent behavioural responses across different contexts and over considerable long time periods

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Abstract

Individuals differ in how they deal with stressful situations along a behavioural continuum defined as proactive and reactive. Proactive individuals are usually bold, highly active and take risks, while reactive organisms are generally shy, exhibit low activity and avoid risk. Moreover, models have suggested that proactive and reactive traits are consistent over time and across contexts. The present study evaluated the stress coping styles and physiological changes in Senegalese sole juveniles and breeders over three and two year period, respectively. Fish were submitted to three individual (restraining, new environment, confinement) and one group test (risk taking) and each individual was sampled at least two times during the whole period of the study. On the third year, juveniles were sexed and their gametogenesis was assessed and compared with their behavioural responses. Results demonstrated consistent individual differences that were consistent with proactive and reactive traits in juveniles and breeders. However, the main result was the significant intra-individual repeatability and consistency of juveniles and breeders behavioural responses over the time and across situations. Nevertheless, when the behavioural responses of both groups of juveniles and breeders were compared, only two variables were repeatable and consistent between groups, suggesting significant behavioural differences between groups. Indeed, glucocorticoids levels were predictable intra-individuals but not between inter-individuals. Another key result was that juveniles that started gametogenesis showed significant higher activity, risk predisposition and lower glucocorticoids production than immature fish.

***Keywords**: Solea senegalensis, coping styles, consistency, contexts*

1. Introduction

Individuals of a same population commonly differ in how they deal to stressful stimulus and novel contexts along two behavioural continuum defined as proactive and reactive (Wilson et al., 1993; Koolhaas et al., 1999). The most striking differences between proactive and reactive individuals are how they use the internal and external information to organize their behaviour associated with the environmental stimulus. Proactive individuals tend to be bold, active, dominant, aggressive and risk prone without considering the danger, while reactive organisms tend to be are shy, exhibit lower levels of activity, are less aggressive and avoid risk (Koolhaas et al., 1999; Sih et al., 2004a; Brown et al., 2007). In addition, models have proposed that animals with proactive behaviours tend to create routines, while reactive individuals seem to adapt more easily to environmental changes (Benus et al., 1991; Koolhaas et al., 1999). Physiologically, the proactive strategy has been associated with low hypothalamus–pituitary–interrenal (HPI) axis responsiveness, and hence produce lower levels of glucocorticoids (*e.g.* cortisol, glucose), while reactive fish present high HPI axis and high levels of glucocorticoids (Øverli et al., 2007; Koolhaas et al., 2010). Further, behavioural differences may not be simple fixed adaptations, but flexible and plastic responses influenced by age, sex, social status, environment, life experience and selection pressure (Réale et al., 2007; Wolf and Weissing, 2010; Sih et al., 2015) and might be heritable (Dingemanse et al., 2002). Such behavioural differences have been commonly referred as behavioural syndromes (Sih et al, 2004a), temperaments (Réale et al., 2007), and coping styles (Koolhaas et al., 1999), this last term will be the reference term for the present study.

From ecological and evolutionary points of view, the coping style concept refers to the inter-individual differences in behaviours and to suites of correlated behaviours including those expressed either within a given behavioural context (*e.g.* exploratory behaviours in different environments) or across different contexts (*e.g.* mating, competition, and dispersal contexts) (Koolhaas et al., 1999; Wolf and Weissing, 2010; Sih et al., 2015). To date, the existence of coping styles have been confirmed in a number of taxa, such as birds (Dingemanse et al., 2002), mammals (Fernández et al., 2009) and fish (see reviews of Conrad et al., 2011; Mittelbach et al., 2014; Castanheira et al., 2015). Likewise, it have been demonstrated that proactive and reactive traits can influence social relationships, reproduction value, social dynamics, and many other physiological and behavioural

aspects of an individual's life fitness that can have profound costs or benefits depending upon environmental contexts (Dingemanse and Réale, 2005; Smith and Blumstein, 2008; Mittelbach et al., 2014; Castanheira et al., 2015). Indeed, it has been hypothesized that coping styles may be repeatable (*e.g.* refers to a stable individual behaviour over time), consistent (*e.g.* refers to the predictability of repeated measures within individuals and between individuals in groups) and correlated (*e.g.* refers to individual consistency across different situations or contexts) over periods of time or contexts (for further detail of definitions see Dall et al, 2004; Sih et al., 2004b; Réale et al., 2007; Bell et al., 2009).

Individual repeatability and consistency of a specific behavioural trait can be assessed with multiple measures on the same individuals across different situations and over time (Lessels and Boag, 1987). Measuring the repeatability and consistency of coping styles is of particular importance when evaluating the behaviour of animals in novel, open field or risky situations, since environmental factors have been observed to potentially mask individual behavioural differences (Martin and Réale, 2008). Hence, one way to reduce this slant is to repeat tests several times for each individual to reliably estimate the intra-individual behavioural variation and once the intra-individual variation has been establish the inter-individual behavioural variation can be reliably assessed (Dingemanse et al., 2002). Being able to forecast whether individuals in a group behave predictably over certain period of time would be valuable for diverse areas, such as behavioural ecology, conservation biology or aquaculture, since it could increases the possibility to reduce competition, would allow to characterize individual status (*e.g.* active, aggressive) and could provide suitable habitats for individuals. To date, several studies have investigated the repeatability and consistency of coping style behaviours over time and across different tests or situations in several fish species (Bell and Stamps, 2004; Cummings and Mollaghan, 2006; Bell et al., 2009; Millot et al., 2009; Chervet et al., 2011; Boulton et al., 2014; Ferrari et al., 2015). Nevertheless, most of previous studies have investigated fish behavioural traits over a relatively short (days - weeks) and intermediate (week - months) time periods, and only few studies were carried out over long time scales (near or more than a year) and have evaluated life-long repeatability and consistency.

Senegalese sole (*Solea senegalensis*), a flatfish species of high commercial importance that has been demonstrated to exhibit proactive and reactive coping styles, with significant differences in activity, risk taking and HPA axis responsiveness (Mota-Silva et

al., 2010; Martins et al., 2011; Ibarra-Zatarain et al., 2015). Nevertheless, no information is available yet on the temporal behavioural repeatability or consistency in this species for neither juveniles nor adults. Therefore, in this work it was proposed to evaluate the repeatability and consistency of Senegalese sole juveniles and breeders across different contexts (three individual tests and one group test) and over long time period (juveniles tested three times in three years and breeders tested three times in two years). The aims of the present study were to a) characterize the coping style behaviours in Senegalese sole juveniles and breeders, b) investigate the intra-individual behavioural repeatability and consistency of juveniles and breeders over the time and across contexts, c) determine the existence of repeatability and inter-individual consistency between juveniles and breeders, and d) compare the possible behavioural differences over time between juveniles of the same year class that started gametogenesis (entered puberty) and those that did not begin gametogenesis (pre-pubescent) over the time.

2. Materials and methods

2.1. Ethic statement

All experimental fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee of IRTA, Spain.

2.2. Experimental animals, housing and feeding.

Sixty one Senegalese sole juveniles and 59 breeders were used as experimental animals. Sole juveniles presented an initial average weight of 45.6 ± 1.8 g and length of 15.2 ± 0.2 cm, while breeders initial average weight was 1238 ± 55.2 g and length 45.8 ± 0.6 cm. Juveniles were housed in three 500 L square tanks, while four 13m^3 tanks were used for breeders and both systems were located in a greenhouse structure. A recirculation system (IRTAMAR®) with a daily total water exchange rate of 50% day^{-1} was used to maintain optimal water parameters for both groups of fish. Juvenile were fed *ab libitum* twice a day (10:00 and 15:00 h) with a commercial balanced food (Elite LE-2mm, Skretting, Co), while breeders feeding regime was as follow: Monday: balanced feed (Vitali Repro-7 mm and LE-7 ELITE, Skretting Co.), Wednesday: cooked mussels *Mytilus edulis* (Sariego Intermares, Spain) and Friday: frozen marine polychaetes *Nereis virens* (Topsy-Baits, Wilhelminadorp, Holland). One hour after feeding, uneaten food was removed from tanks

to maintain optimal physicochemical conditions. Juveniles and adult fish were PIT-tagged (ID-100 Unique, Trovan-Zeus, Madrid, Spain) for identification.

2.3. Experimental procedures

Three runs of coping styles tests were performed. Each run started and finished at the same hour and the same material was used (*i.e.* tanks, nets, etc.). The stress assays consisted in three individual (restraining, new environment and confinement) and one grouping test (risk taking) for both groups (juveniles and breeders). Individual tests were performed in sequence, while the risk taking test was realized one month later to allow fish recovery (see below). Two blood extractions were realized to quantify glucocorticoids levels (see below). At the end of round 3, the sex and the gonadal maturity of juveniles were assessed following the methodology of Anguis and Cañavate (2005) (see below).

a) In juveniles, the restraining and confinement tests were performed in July 2012 (run 1), 2013 (run 2) and 2014 (run 3), the new environment test in July 2012 (run 1) and 2014 (run 3) and the risk taking tests in August 2012 (run 1) and 2014 (run 3).
b) In breeders, the restraining and confinement tests were performed in October 2012 (run 1), June 2013 (run 2) and October 2013 (run 3), the new environment test in October 2012 (run 1) and October 2013 (run 3) and the risk taking tests in November 2012 (run 1) and November 2013 (run 3).
c) The extraction of the blood was performed in July 2012 (run 1) and 2014 (run 3) in juveniles and in October 2012 (run 1) and October 2013 (run 3) for breeders.
d) Females with gametogenesis were detected if presented a degree of gonadal maturation as follow: stage I the ovary was detected by touching the ventral area of the female; stages II and III was reached when different degrees of gonad swelling were visible externally (initial and intermediate, respectively), and fish were in stage IV when maximum ovarian swelling was observed as a result of oocyte hydration. Males with gametogenesis were identified according to the shape of their testis and, with a gentle pressure on the abdomen, the production of small amounts of milt was recorded and the percentage of active sperm cells was evaluated.

Test 1. Restraining test

The behavioural responses of juveniles were evaluated by holding each individual in a net out of the water for 90 s, while the behaviour of breeders was determined in a net inside of the water for the same time period. Tests were adapted from Martins et al., 2011; Hori et al., 2012; Castanheira et al., 2013 and validated in chapter 2. Two variables were measured in both groups: **i)** the total activity time, in the air for juveniles (**NetActA**), and in the water for breeders (**NetActW**), and **ii)** the total number of escape attempts, in the air for juveniles (**NetEscA**) and in the water for breeders (**NetEscW**).

Test 2. New environment test

Each individual was placed for five minutes in a plastic tank (56.5 x 36.5 x 30 cm for juveniles and 114 x 95 x 57 cm for breeders) that simulated a new environment. Tests were adapted from Wilson and Godin, 2009; Martins et al., 2012; Carter et al., 2013 and validated in chapter 2. Two parameters were measured for juveniles and breeders: **i)** the latency time to move, **NewLat**, considered as the first moment that fish started to explore the new environment and **ii)** the total activity time, **NewAct**, being the total time each fish spent swimming forward in the tank. If fish did not move at all during the 5 minutes period, then 300s was recorded as **NewLat** for further statistical analysis (Farwell and McLaughlin, 2009). To cause minimal disturbance to fish, observers stood stationary 1 m away from the tank.

Test 3. Confinement test

Fish were individually placed for five minutes in a plastic tank with reduced dimensions (25 x 14 x 8 cm for juveniles and 56 x 36 x 30 cm for breeders) that simulated a confinement situation. Tests were adapted from Brelin et al., 2005; Ruiz-Gomez et al., 2008; Kittilsen et al., 200 and validated in chapter 2. Two parameters were registered for juveniles and breeders: **i)** the latency time to move, **ConLat**, considered as the first moment that fish started to move and **ii)** the total activity time, **ConAct**, restricted to active locomotion in the confinement container. If fish did not move during the test, then 300s was recorded as **ConLat** for further statistical analysis (Farwell and McLaughlin, 2009). Observers stood stationary 1 m away from the container to not disturb fish.

Test 4. Risk taking test

This test was performed on juveniles and breeders with the same behavioural criteria, one month after finalizing individual tests. This test aimed to determine fish capacity to cross from a known area, or safe zone, to an unknown area, or risky zone (adapted from Carter et al., 2013; Herrera et al., 2014; Ferrari et al., 2015). The safe zone was isolated from light (2 and 3 lux at the surface for juveniles and breeders, respectively) and the bottom covered with sand, to provide a comfortable and secure space for fish. On the contrary, the risky zone were more illuminated (15 and 11 lux at the surface for juveniles and breeders, respectively) and devoid of sand. For juveniles, a 500 L tank was divided into two equal zones by a rigid plastic screen and a window (5 cm high x 20 cm width) was located at the bottom of the screen, with a door allowing fish to cross between both areas. For breeders, the test was realized in a 16 m^3 tank, divided into two equal areas by a wooden screen. A window (30 cm width x 15 cm tall) was placed at the base of the screen with a door that could be opened to allow fish to pass from an area to another. The windows in the divisions were placed at the centre of a reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that was employed to read the tag numbers of fish that passed through the window. Additionally, two submersibles cameras (square black and white CCD camera, model F60B/NIR580-50G Korea Technology and Communications Co. Ltd., Korea supplied in waterproof housing by Praentesis S.L., Barcelona) were installed 10 cm below the water surface in both safe and risky zone, to corroborate information obtained from the antenna.

Before starting the test, both juveniles and breeders were submitted to a 24 hours acclimation in the safe zone, by keeping windows closed until the beginning of the test, which started at 10:00 hours and lasted 24 hours. Juveniles were tested by groups of 15 individuals and breeders by groups of 10 individuals, to avoid stress induced by high stocking densities. Fish that successfully crossed from the safe to the risky zone were defined as proactive, while fish that did not cross were labelled as reactive, considering criteria given by Wilson et al. (1993), Wilson and Godin (2009), Tudorache et al. (2013) and Mittelbach et al. (2014). The latency time of each organism to cross from one area to another was recorded. A maximum time of 1440 min was assigned to fish that did not cross during the 24 h period of the test.

2.4. Glucocorticoids analysis

Two blood samples were obtained from each juvenile and breeders for quantifying cortisol, glucose and lactate levels. To avoid coagulation of blood after its extraction, needles and syringes were coated with heparin and tubes and 10 µl heparin (5%, 25.000 UI; HOSPIRA) and 15 µl aprotinin (from bovine lung; 0.9% NaCl, 0.9% benzyl alcohol and 1.7 mg of protein; SIGMA) was mixed with the blood in the eppendorf. Blood samples were centrifuged (M23i, ThermoScientific) at 3000 G and 4ºC during 15 min and plasma supernatant was removed and stored in triplicates at −80ºC prior to analysis. Cortisol levels was measured by means of a competitive conjugated binding ligand with a commercial ELISA kit (Range of detection: 0-800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany), whereas glucose and lactate concentrations were measured by means of commercial enzymatic colorimetric kits (SPINREACT, Gerona, Spain). Cortisol, glucose and lactate absorptions were read by a spectrophotometer (Infinite M-200; TECAN, Switzerland) at 23ºC and 505 nm.

2.5. Statistical analysis

All statistical analyses were performed using SPSS 20.0 software for Windows. Values were presented as means ± standard error mean (S.E.M.). Statistical differences were established when $P < 0.05$ for all analysis. Normality of data was checked through a Kolmogorov Smirnov test with Lilliefors correction and homogeneity of variances was checked through a Levene´s test. The repeatability intra- and inter-individual consistency was assessed by performing a General Linear Model with Repeated Measures analyses of variance (GLM-RM), with a Wilk´s lambda criterion and Fisher´s exact test, incorporating general behavioural responses of juveniles and breeders for new environment, confinement and glucocorticoids levels from runs 1 to 3. GLM-RM analyses were performed separately for the restraining test for juveniles and breeders, since total activity and escape attempts variables were measured differently in both groups (in the air and inside the water). Intra-individual repeatability was assessed by means of a GLM. Significant differences in the behavioural response of each individual among the different runs indicated a high intra-individual variability, while no significant differences pointed out a low intra-individual variability and the repeatability of a behavioural trait. A test-retest analysis of reliability-consistency, with an Alpha Cronbach´s (αC), Fisher tests and Intra-class correlation coefficient (ICC), was performed

to confirm the previous results and to examine intra- and inter-individual responses of juveniles and breeders over time and for the each individual tests and blood parameters. A αC value over 0.7 and P-values below 0.05 for the behavioural responses of juveniles and breeders among the three runs indicated high inter- and intra-behavioural correlation and consistency.

Three principal components analysis (PCA) were successively performed on: i) NetAct and NetEsc from the restraining test (runs 1 to 3); ii) NewLat and NewAct from the new environment test (runs 1 and 2) and iii) ConLat and ConAct from the confinement test (runs 1 to 3). For each PCA, the variable that explained the highest variance and showed eigenvalue over 1 (Kaiser-Guttman criterion) was the most representative of each test performed and was retained to represent the composite behaviour of each individual, also called individual Principal Component Score (PCSj) for each test. Thus, the variables selected for juveniles were: NetEscA (eigenvalue = 4.43, variance = 73.9%, defined as: *restraining-PCSj*), NewLat (eigenvalue = 2.85, variance = 71.2%, defined as: *confinement-PCSj*) and ConLat (eigenvalue = 4.36, variance = 72.8%, defined as: *environment-PCSj*), while for breeders, they were: NetEscW (eigenvalue = 3.04, variance = 50.8%, defined as: *restraining-PCSb*), NewLat (eigenvalue = 2.53, variance = 63.4%, defined as: environment-PCSb) and ConLat (eigenvalue = 2.86, variance = 48.0%, defined as: *confinement-PCSb*). Indeed, significant positive correlations were observed between NetEscA and NewLat (R = 0.288, P = 0.037) and between NewLat and ConLat (R = 0.377, P = 0.029) in juveniles and between NetEscW and ConAct (R = 0.302, P = 0.028) and between NewLat and ConLat (R = 0.394, P = 0.022) in breeders. Lastly, the correlation coefficient between blood parameter, fish morphometric and each PCS for juveniles and for breeders were analysed with a Pearson´s analysis.

Two general linear model analyses were performed: i) to compare the three PCS of juveniles with and without gametogenesis, and ii) to compare the three PCS of fish that crossed and that did not cross in the risk taking test. Additionally, a Chi-square test, with a Phi and Cramer´s nominal analysis, was performed in the risk taking test to evaluate whether the proportion of fish that crossed in run 1 was similar to the proportion of fish that crossed in run 2, for juveniles and breeders. Then, risk taking of juveniles in the risk taking test was compared between proportions of fish with and without gametogenesis, by means of a Chi-square test.

3. Results

3.1. Consistency of behavioural responses of juveniles

Senegalese sole juveniles showed similar behavioural responses amongst runs 1 to 3, in the restraining, new environment and confinement tests (Table 1). Indeed, these variations were not significantly different, which confirmed a high intra-individual repeatability in these three tests (GLM-RM, Table 2). Indeed, the Alpha-Cronbach´s reliability test confirmed the intra-individual consistency with significant intra-class correlation (ICC) over time with values ranging from α = 0.878, ICC = 0.706, $F_{60, 120} = 8.21$, $P = 0.001$ to α = 0.989, ICC = 0.978, $F_{60, 120} = 93.52$, $P < 0.001$, for ConLat and NewLat, respectively (Table 3), while Pearson's analysis also demonstrated a high correlation degree between tests or across context (ranging from the lowest of $R = 0.466$, P = 0.019 to the highest of $R = 0.939$, $P = 0.001$ for ConLat and ConAct, respectively, Table 4). However, juveniles varied in the production of glucocorticoids concentrations, since their significant variations from a run to another and were different (Table 2), lactate concentration was not consistent over time (Table 3) and glucose concentrations were not significantly correlated over time (Table 4).

Table 1. Behavioural responses of sole juveniles and breeders over time.

Tests	Variables	Juveniles			Breeders		
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Restraining	NetActA	10.2 ± 1.0	11.0 ± 0.8	12.0 ± 1.0	na	na	na
	NetEscA	25.0 ± 2.2	23.8 ± 2.1	26.2 ± 1.9	na	na	na
	NetActW	na	na	na	18.1 ± 2.2	15.4 ± 1.7	17.4 ± 1.7
	NetEscW	na	na	na	5.4 ± 0.9	6.6 ± 1.2	7.2 ± 1.0
New environment	NewLat	140.0 ± 16.2	na	134.3 ± 15.5	109.5 ± 16.8	na	93.6 ± 14.2
	NewAct	12.7 ± 2.3	na	17.0 ± 2.4	26.1 ± 4.6	na	28.6 ± 4.6
Confinement	ConLat	126.2 ± 17.0	112.4 ± 16.7	107.8 ± 15.2	112.4 ± 16.3	72.2 ± 14.8	86.5 ± 13.5
	ConAct	36.6 ± 6.1	37.0 ± 6.0	41.5 ± 6.3	24.2 ± 4.7	25.2 ± 3.8	28.0 ± 3.7
Risk taking	Cross	24	na	18	17	na	19
	Not cross	37	na	43	42	na	40
Blood parameters	Cortisol (ng/ml)	58.0 ± 8.1	na	79.6 ± 8.3	20.6 ± 7.2	na	16.8 ± 5.2
	Glucose (mmol/l)	4.3 ± 0.4	na	6.2 ± 0.4	4.7 ± 0.3	na	8.5 ± 0.9
	Lactate (mmol/l)	19.7 ± 0.7	na	26.8 ± 0.7	6.6 ± 0.8	na	10.6 ± 1.1

na = not applicable

Table 2. Parameters of the GLM repeated measures examining intra and inter-individual variability of behavioural responses of Senegalese sole juveniles and breeders across the different tests and over time. **λ** = Wilk´s lambda value, **d.f.** = degrees of freedom, **F** = Fisher value, **P** = significance level. P-values > 0.05 in bold indicated high intra-and inter-individual repeatability

Tests	Variables	Juveniles				Breeders				Juvenile - Breeders			
		λ	d.f.	F	P	λ	d.f.	F	P	λ	d.f.	F	P
Restraining	NetActA	**0.748**	**2, 59**	**1.69**	**0.194**	na	na	na	na	na	na	na	na
	NetEscA	**0.944**	**2, 59**	**1.71**	**0.184**	na	na	na	na	na	na	na	na
	NetActW	na	na	na	na	**0.973**	**2, 57**	**0.77**	**0.464**	na	na	na	na
	NetEscW	na	na	na	na	**0.946**	**2, 57**	**1.16**	**0.208**	na	na	na	na
New environment	NewLat	**0.959**	**2, 59**	**2.55**	**0.115**	**0.962**	**2, 57**	**2.31**	**0.134**	0.969	2, 117	3.81	0.048
	NewAct	**0.789**	**2, 59**	**6.02**	**0.175**	**0.993**	**2, 57**	**0.436**	**0.512**	**0.976**	**2, 117**	**2.96**	**0.088**
Confinement	ConLat	**0.959**	**2, 59**	**1.25**	**0.292**	**0.907**	**2, 57**	**2.92**	**0.062**	0.934	2, 117	4.11	0.019
	ConAct	**0.901**	**2, 59**	**2.90**	**0.69**	**0.962**	**2, 57**	**2.11**	**0.335**	0.938	2, 117	3.85	0.024
Blood parameters	Cortisol	0.640	2, 59	33.75	0.001	**0.997**	**2, 57**	**0.19**	**0.664**	0.971	2, 117	64.11	0.000
	Glucose	0.538	2, 59	51.58	0.000	**0.966**	**2, 57**	**2.06**	**0.161**	0.648	**2, 117**	**3.48**	**0.065**
	Lactate	0.483	2, 59	64.16	0.000	**0.966**	**2, 57**	**2.03**	**0.159**	0.730	2, 117	43.62	0.004

Table 3. Parameters of the test-retest reliability analysis examining intra and inter-individual variabilities of behavioural responses of Senegalese sole juveniles and breeders across the different tests and over time. **α** = Alpha Cronbach´s value, **ICC** = within intraclass correlation, **d.f.** = degrees of freedom, **F** = Fisher value, **P** = significance level. P-values < 0.05 in bold indicated high intra-and inter-individual consistency

Tests	Variables	Juveniles					Breeders					Juvenile-Breeders				
		α	ICC	d.f.	F	P	α	ICC	d.f.	F	P	α	ICC	d.f.	F	P
Restraining	NetActA	**0.959**	**0.872**	**60, 120**	**64.16**	**0.000**	na	na	na	na	na	na	na	na	na	na
	NetEscA	**0.942**	**0.844**	**60, 120**	**17.37**	**0.000**	na	na	na	na	na	na	na	na	na	na
	NetActW	na	na	na	na	na	**0.785**	**0.548**	**58, 116**	**4.64**	**0.000**	na	na	na	na	na
	NetEscW	na	na	na	na	na	**0.704**	**0.285**	**58, 116**	**2.19**	**0.047**	na	na	na	na	na
New environment	NewLat	**0.989**	**0.978**	**60, 120**	**93.52**	**0.000**	**0.871**	**0.768**	**58, 58**	**7.76**	**0.000**	0.938	0.880	119, 119	2.25	0.059
	NewAct	**0.948**	**0.879**	**60, 120**	**19.13**	**0.000**	**0.794**	**0.661**	**58, 58**	**4.85**	**0.009**	**0.840**	**0.721**	**119, 119**	**16.06**	**0.000**
Confinement	ConLat	**0.878**	**0.706**	**60, 120**	**8.21**	**0.001**	**0.705**	**0.313**	**58, 116**	**2.40**	**0.046**	0.678	0.224	119, 238	4.82	0.054
	ConAct	**0.985**	**0.954**	**60, 120**	**67.10**	**0.000**	**0.792**	**0.561**	**58, 116**	**4.79**	**0.000**	**0.942**	**0.822**	**119, 238**	**17.15**	**0.000**
Blood parameters	Cortisol	**0.946**	**0.851**	**60, 120**	**18.51**	**0.000**	0.017	0.009	58, 58	1.01	0.474	0.616	0.129	119, 238	8.46	0.063
	Glucose	**0.881**	**0.669**	**60, 120**	**8.34**	**0.001**	**0.992**	**0.885**	**58, 58**	**92.28**	**0.000**	0.498	0.216	119, 119	4.52	0.051
	Lactate	0.311	0.100	60, 120	1.45	0.076	**0.987**	**0.687**	**58, 58**	**77.08**	**0.000**	0.837	0.620	119, 119	3.13	0.059

Table 4. Pearson´s correlations among runs 1 to 3 for Senegalese sole juveniles and breeders.

Tests	Variables	Values	Juveniles			Breeders		
			run 1 vs run 2	run 1 vs run 3	run 2 vs run 3	run 1 vs run 2	run 1 vs run 3	run 2 vs run 3
Restraining	NetActA	R	**0.788**	**0.757**	**0.817**	na	na	na
		P	**0.001**	**0.001**	**0.001**	na	na	na
	NetEscA	R	**0.739**	**0.662**	**0.754**	na	na	na
		P	**0.001**	**0.004**	**0.001**	na	na	na
	NetActW	R	na	na	Na	**0.422**	**0.653**	**0.437**
		P	na	na	Na	**0.025**	**0.001**	**0.019**
	NetEscW	R	na	na	Na	**0.285**	**0.458**	0.161
		P	na	na	Na	**0.035**	**0.021**	0.223
New environment	NewLat	R	na	**0.931**	Na	na	**0.738**	na
		P	na	**0.001**	Na	na	**0.001**	na
	NewAct	R	na	**0.812**	Na	na	**0.658**	na
		P	na	**0.001**	Na	na	**0.001**	na
Confinement	ConLat	R	**0.551**	**0.542**	**0.466**	0.042	**0.702**	0.201
		P	**0.009**	**0.011**	**0.019**	0.762	**0.001**	0.127
	ConAct	R	**0.939**	**0.897**	**0.910**	**0.403**	**0.805**	**0.431**
		P	**0.001**	**0.001**	**0.001**	**0.018**	**0.001**	**0.001**
Blood parameters	Cortisol (ng/ml)	R	na	**0.806**	Na	na	0.009	na
		P	na	**0.001**	Na	na	0.946	na
	Glucose (mmol/l)	R	na	0.034	Na	na	**0.457**	na
		P	na	0.785	Na	na	**0.002**	na
	Lactate (mmol/l)	R	na	**0.619**	Na	na	0.234	na
		P	na	**0.008**	Na	na	0.071	na

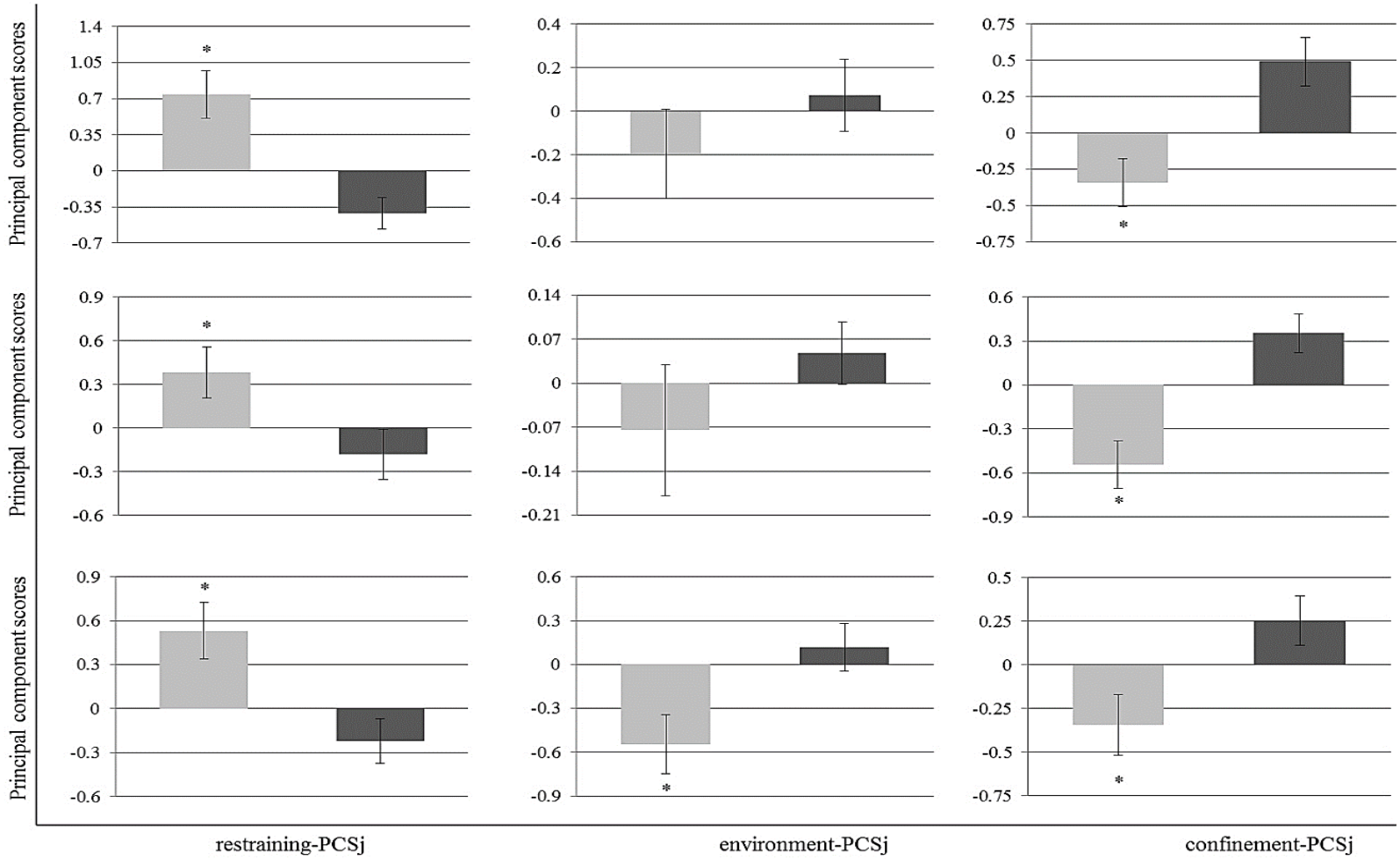
na= not applicable

Table 5. Morphometric parameters, behavioural responses and glucocorticoids blood concentrations of Senegalese sole juveniles grouped by risk taking and gametogenesis (runs 1 and 3).

Variables	Gonadal development		Risk taking_run 1		Risk taking_run 3	
	Gametogenesis	No gametogenesis	Crossed	Not crossed	Crossed	Not crossed
Weight (g)	290.0 ± 25.4^{A}	189.4 ± 20.4^{B}	46.2 ± 2.8	45.5 ± 2.4	239.7 ± 27.2	216.2 ± 21.6
Length (cm)	27.3 ± 0.8^{A}	23.5 ± 0.7^{B}	15.0 ± 0.3	15.4 ± 0.2	25.1 ± 0.8	24.2 ± 0.8
restraining-PCSj	0.74 ± 0.23^{A}	-0.41 ± 0.15^{B}	0.38 ± 0.17^{A}	-0.18 ± 0.11^{B}	0.53 ± 0.19^{A}	-0.22 ± 0.15^{B}
environment-PCSj	-0.19 ± 0.20	0.07 ± 0.16	-0.07 ± 0.10	0.35 ± 0.13	-0.54 ± 0.20^{A}	0.25 ± 0.14^{B}
confinement-PCSj	-0.34 ± 0.16^{A}	0.49 ± 0.16^{B}	-0.54 ± 0.16^{A}	0.04 ± 0.04^{B}	-0.34 ± 0.17^{A}	0.11 ± 0.16^{B}
Cortisol (ng/ml)	35.70 ± 10.5^{A}	70.60 ± 10.70^{B}	26.84 ± 4.90^{A}	78.29 ± 11.90^{B}	32.60 ± 7.25^{A}	68.70 ± 10.72^{B}
Glucose (mmol/l)	4.41 ± 1.0	4.11 ± 0.31	4.63 ± 0.90	4.04 ± 0.33	5.0 ± 1.21	3.98 ± 0.29
Lactate (mmol/l)	19.70 ± 1.2	19.74 ± 0.81	20.80 ± 1.16	19.00 ± 0.80	20.92 ± 1.32	19.20 ± 0.76

In the first risk taking test (run 1), a total of 24 fish crossed and 37 did not cross, while in the second test (run 3) 18 fish crossed and 43 did not cross. From the total number of fish that crossed in run 1, 14 fish repeated in run 3 indicating intra-individual consistency. The Chi-square test showed no significant differences (X^2 = 1.38, *df* = 2, *P* = 0.501) between the proportion of fish that crossed and those that did not cross between runs 1 and 3. Juveniles that successfully crossed showed significant higher weight (GLM, $F_{1, 54}$ = 3.33, *P* = 0.024) and length (GLM, $F_{1, 54}$ = 2.01, *P* = 0.047) than fish that did not crossed only in run 3 (Table 5). Moreover, juveniles that successfully crossed presented significantly higher scores for restraining-PCSj (GLM, $F_{1, 54}$ = 5.14 and *P* = 0.027 in run 1 and $F_{1, 54}$ = 3.08, *P* = 0.033 in run 3, Figure 1) and lower scores for confinement-PCSj (GLM, $F_{1, 54}$ = 10.87 and *P* = 0.002 for run 1 and $F_{1, 54}$ = 3.66 and *P* = 0.029 for run 3, Figure 1) than juveniles that did not crossed, in both runs. However, they presented significantly lower scores for environment-PCSj in run 3 (GLM, $F_{1, 54}$ = 4.57, *P* = 0.025) and tended to show lower scores for environment-PCSj in run 1(GLM, $F_{1, 54}$ = 0.65, *P* = 0.440) than fish that did not crossed only in run 3 (Figure 1). Hence, the overall behavioural pattern of juveniles that took higher risk were characterized by higher activity and lower cortisol production, contrary to behaviour of fish that did not cross, and this patterns were distinctive of two coping styles strategies.

Figure 1. PCS of juveniles grouped by gametogenesis (first row, light grey = gametogenesis, dark grey = no gametogenesis), risk taking run 1 (second row, light grey = crossed, dark grey = not crossed) and risk taking run 2 (third row, light grey = crossed, dark grey = not crossed). * indicates significant differences.



Twenty-two of sixty-nine juveniles were observed with gametogenesis (11 females and 11 males). Four of the 11 females were found in stage 1 and seven in stage 2, while nine of the eleven males presented 20% of motile sperm cells and two showed 10% of motile sperm cells. In addition, juveniles with gametogenesis were significantly heavier and larger (GLM, $F_{1, 54} = 4.25$, $P = 0.008$ and $F_{1, 54} = 3.58$, $P = 0.022$, respectively) than juveniles without gametogenesis (Table 5). The Principal Component Score of juveniles with gametogenesis were significantly higher than fish without gametogenesis for restraining-PCSj (GLM, $F_{1, 54} = 3.93$, $P = 0.038$) and lower in confinement-PCSj (GLM, $F_{1, 54} = 4.27$, $P = 0.026$), but they did not differ for environment-PCSj (GLM, $F_{1, 54} = 0.38$, $P = 0.538$) (Figure 1). Moreover, fish with gametogenesis showed significantly lower cortisol levels (half less) in run 1 (GLM, $F_{1, 54} = 2.67$, $P = 0.042$) and in run 3 than fish without gametogenesis (Table 5). Interestingly, 18 fish of 22 with gametogenesis (81.2 %) crossed in the risk taking test (in both runs 1 and 3) and none of the fish without gametogenesis crossed in the risk taking test. The Chi-square test detected significant differences in fish disposition to take risk between the proportion of individuals with and without gametogenesis ($X^2 = 13.21$, $df = 1$, $P = 0.021$). These results suggested that behavioural patterns of fish with gonadal development were consistent with proactive strategies, since their higher *restraining-PCSj* (higher escapes attempts), lower *environment-PCSj* and *confinement-PCSj* scores (lower latency to move) and higher risk taking predisposition. No significant correlations (Pearson, $P > 0.05$) were detected between the three PCS, morphometric parameters and blood parameters, neither for fish with gametogenesis, not for fish without gametogenesis.

3.2. Intra-individual behavioural responses of breeders

Similarly to juveniles, from run 1 to 3, breeders tended to show increasing total activity time and number of escapes attempts in the restraining test, decreasing latency time and increasing total activity time in new environment and confinement tests, reduced production of cortisol and increasing productions of glucose and lactate (Table 1). Nonetheless, these variations were not significantly different, and the GLM-RM (Table 2), the Alpha-Cronbach´s reliability test confirmed the significantly high intra-individual repeatability and high the intra-class consistency (ICC) over time with values ranging from $\alpha = 0.704$, ICC = 0.285, $F_{58, 116} = 2.19$, $P = 0.047$ to $\alpha = 0.987$, ICC = 0.687, $F58_{, 116}$

= 77.08, $P < 0.001$, for NewEscW and Lactate, respectively (Table 3) and the high degree of correlation across the three tests performed (from the lowest of $R = 0.285$, $P = 0.035$ to highest of $R = 0.805$, $P = 0.001$ for NetEscW and ConLact, respectively, Table 4). Besides, glucocorticoids blood concentrations were repeatable (Table 2), but cortisol concentration was not consistent over time (Table 3) and cortisol and lactate concentrations were not significantly correlated over time (Table 4).

Seventeen fish of a total of 59 breeders crossed from the safe to risky area in the risk taking test (run 1), while 20 breeders crossed in the second risk taking test (run 3). Thirteen fish were identified as the same fish to cross in both runs. In both tests, breeders that crossed tended to be heavier than fish that did not cross, but this difference was not statistically different (GLM, $F_{1, 55} = 1.18$ and $P = 0.067$ in run 1 and $F_{1, 55} = 0.818$ and $P = 0.111$ in run 3) (Table 6). The proportion of fish that successfully crossed in run 1 was significantly similar to the proportion of fish that crossed in run 3 ($X^2 = 5.99$, $df = 2$, $P = 0.059$). Breeders that successfully crossed presented significantly higher scores for *restraining-PCSb* (GLM, $F_{1, 55} = 3.56$ and $P = 0.036$ in run 1 and $F_{2, 55} = 3.25$ and $P = 0.042$ in run 3) and lower scores for *environment-PCSb* (GLM, $F_{1, 55} = 3.18$ and $P = 0.047$ in run 1 and GLM, $F_{2, 55} = 3.90$, $P = 0.026$ in run 3), but no significant differences were detected for *confinement-PCSb* neither in run 1 (GLM, $F_{1, 54} = 1.29$, $P = 0.259$), nor in run 3 (GLM, $F_{1, 54} = 1.48$, $P = 0.236$) (Figure 2).

Fish that successfully crossed showed significant lower cortisol concentrations than fish that did not cross (GLM, $F_{1, 55} = 4.13$ and $P = 0.014$ in run 1 and $F_{2, 55} = 7.11$ and $P = 0.001$ in run 3) (Table 6). Thus, the behavioural characteristics of fish that took risk were comparable to proactive behaviours and breeders that did not cross with reactive behaviours, since their differences in their principal components scores and cortisol levels.

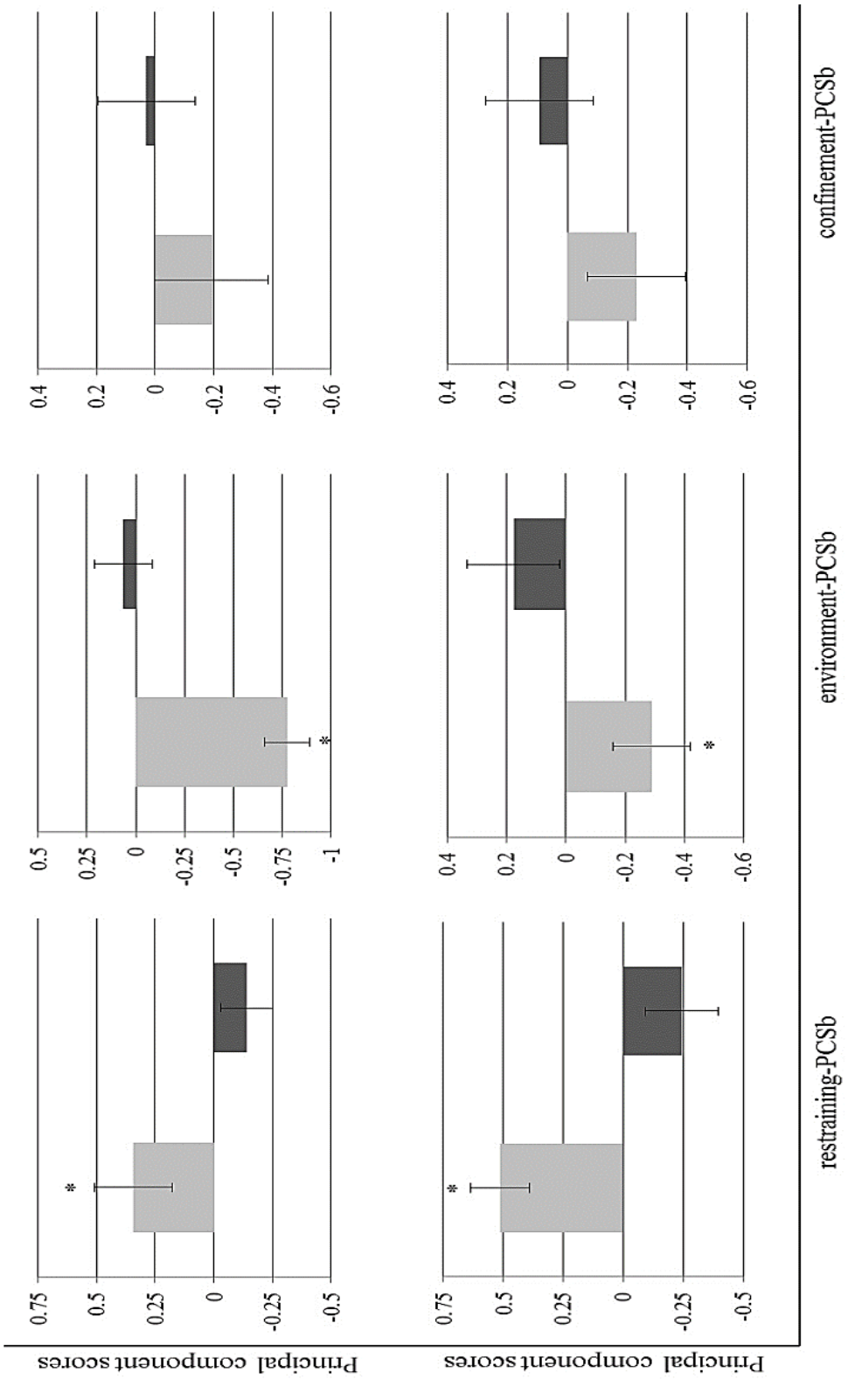


Figure 2. PCS of breeders that crossed (light grey) and those that did not cross (dark grey) in the risk taking run 1 (first row) and run 2 (second row).

* indicates significant differences.

3.3. Comparison of inter-individual behavioural responses between juveniles and breeders

The behavioural responses of juveniles and breeders for individuals and group tests performed were variable between groups and overtime. The repeated measures analysis showed that juveniles significantly differed in their behavioural responses from breeders in the new environment (NewLat), in the confinement (ConLat and ConAct) and in glucocorticoids blood production (cortisol and lactate concentrations) (Table 2). Indeed, the αC reliability-consistency test-retest showed that most of the behavioural responses of juveniles were not consistent with those of the breeders (NewLat, ConLat, cortisol, glucose and lactate concentrations) (Table 3). The only parameter which resulted significantly repeatable (Table 2) and consistent (Table 3) between juveniles and breeders over time was NewAct. Regarding the risk taking test, significant differences were detected between the proportion of juveniles and the proportion of breeders that crossed from the safe to the risky area (X^2 = 35.90, *df* = 2, *P* = 0.009). These results demonstrated that juveniles and breeders exhibited different behavioural responses to most of the coping style tests to which they were exposed.

Table 6. Morphometric parameters, behavioural responses and glucocorticoids blood concentrations of Senegalese sole breeders grouped by risk taking (runs 1 and 3).

Variables	Risk taking_run 1		Risk taking_run 3	
	Crossed	**Not crossed**	**Crossed**	**Not crossed**
Weight (g)	1303 ± 111.4	1211 ± 63.5	1232 ± 91.7	1171 ± 59.2
restraining-PCSb	0.38 ± 0.17^{A}	-0.18 ± 0.11^{B}	0.53 ± 0.19^{A}	-0.22 ± 0.15^{B}
environment-PCSb	-0.07 ± 0.10^{A}	0.35 ± 0.13^{B}	-0.54 ± 0.20^{A}	0.25 ± 0.14^{B}
confinement-PCSb	-0.54 ± 0.16	0.04 ± 0.04	-0.34 ± 0.17	0.11 ± 0.16
Cortisol (ng/ml)	26.84 ± 4.90^{B}	78.29 ± 11.90^{A}	32.60 ± 7.25^{B}	68.70 ± 10.72^{A}
Glucose (mmol/l)	4.63 ± 0.90	4.04 ± 0.33	5.0 ± 1.21	3.98 ± 0.29
Lactate (mmol/l)	20.80 ± 1.16	19.00 ± 0.80	20.92 ± 1.32	19.20 ± 0.76

4. Discussion

The purpose of this paper was to examine the behavioural responses of Senegalese sole juveniles and breeders and, particularly, to evaluate the intra-and inter-individual behavioural repeatability, consistency and correlation over time by conducting three individual, one grouping tests and looking at three physiological parameters after stress. Overall, significant inter-individual behavioural variations were demonstrated between juveniles and breeders over time, when considering all the individual tests performed and their responses in the risk taking test confirmed these differences. However, the most striking result was the significant degree of intra- and inter-individual repeatability and consistency over time and context for the three individual tests within juveniles' group and within breeders' group. These main findings were in agreement with the features associated to stress coping styles personalities, considering three basic aspects: i) variation in individual behaviour, ii) consistency or repeatability over time, and iii) cross-contexts correlations (Koolhaas et al., 1999; Wolf and Weissing, 2010; Wolf and McNamara, 2012; Sih et al., 2015).

4.1. Intra-individual behavioural repeatability, consistency and correlation in Senegalese sole juveniles and breeders

Senegalese sole juveniles and breeders not only showed intra-individual consistency which was repeatable and consistent over time, but also the variation between individuals was high ranging from proactive to reactive in their risk taking predisposition and therefore constituted coping style traits. Firstly, the GLM repeated measure analysis showed that Senegalese sole juveniles and breeders, taken by separately, exhibited significantly high degree of intra-individual repeatability in the restraining (NetActA and NetEscA; NetActW and NetEscW), new environment (NewLat and NewAct) and confinement (ConLat and ConAct) tests. Secondly, the test-retest of reliability confirmed the significantly degree of consistency (high intraclass correlation) of juveniles and breeders, taken by separately, in their individual behavioural responses to restraining, new environment and confinement tests. Regarding glucocorticoids blood production, juveniles did not show intra-individual repeatability, while breeders presented a high intra-individual consistency. However, juveniles and breeders showed high correlations over time and between tests. Considering all the results together, responses exhibited by each individual (from juveniles and breeders groups) were consistently repeated in each

coping style test and over time. Further, most of individuals (juveniles and breeders) were confirmed to present and to maintain the expression of two extreme behavioural traits (proactive/reactive) in response to the different tests performed and over time. In other words, individuals presenting a high number of escapes attempts (proactive) in run 1 also showed a high number of escapes in the successive runs (2 and 3). Interestingly, when the repeatability and consistency degree was compared between the two groups of distinct developmental stage, juveniles and breeders, only NewAct and glucose levels were observed to be repeatable and consistent within and between both groups, while the rest of the examined parameters were not repeatable and lacked of consistency. Therefore, juveniles and breeders were hypothesized to present different types of response to the different tests performed, possibly due to the influence of genetic, age, and life experience on fish (Dingemanse et al., 2002, Bell and Stamps, 2004; Réale et al., 2007; Bell et al., 2009).

Only a few studies evaluated fish behaviour over long time periods, like in the present study. Nonetheless, repeatability and consistency displayed by juveniles and breeders in their activity in restraining, new environment and confinement tests performed over three and two years, respectively, were consistent with the results of those studies that evaluated activity in response to similar tests over short time periods, such as in swordtail *Xiphophorus nigrensis* (Cummings and Mollaghan, 2006), bluegill sunfish *Lepomis macrochirus* (Wilson and Godin, 2009), gilthead sea bream *Sparus aurata* (Castanheira et al., 2013) and sheepshead swordtail *X. birchmanni* (Boulton et al., 2015), and over long time periods, such as in lion-head cichlid *Steatocranus casuarius* (Budaev et al., 1999), cichlid *Neolamprologus pulcher* (Chervet et al., 2011), mosquito fish *Gambussia holbrooki* (Biro and Adriaenssens, 2013) and European sea bass *Dicentrarchus labrax* (Ferrari et al., 2015). However, correlation coefficients reported in those studies for intra-individual variability over time in all those fish species were inferior to those reported in the present work for both Senegalese sole juveniles and breeders groups. Additionally, some authors manifested that the of intra-individual consistency decreased over time to show more variation, while in Senegalese sole, repeatability and consistency were maintained over time, and even more, tended to increase (*e.g.* activity in restraining, new environment and in confinement). Three possible explanations for the increasing repeatability and consistency observed in Senegalese sole juveniles and breeders over time in the present work: a) a high habituation of fish to tests and instruments, meaning

that sole got used to tests themselves and, hence, tests did not represented the same challenge for fish, b) a high flexibility of individuals in their response to the different tests performed, and c) the age of individuals, since behaviour tended to become rigid and follow more set patterns over time (Dall et al., 2004; Uher, 2008; Bell et al., 2009). Nonetheless, the previous assumptions should be confirmed by performing more studies on this fish species, by including modifications in the experimental procedures, by changing the type of tests and by modifying the sampling intervals (short and long time periods).

The Pearson correlations analysis showed high correlations in restraining, new environment and confinement tests for Senegalese sole juveniles and breeders. However, it was observed that correlations in breeders were lower when comparing data/results between runs 1 and 2 and between runs 2 and 3 than between runs 2 and 3. Regarding the low correlations observed in breeders between run 1 and 2 and between runs 2 and 3 than between runs 2 and 3, it might be attributed to the season in which tests were performed in run 2 (June). At this time of the year, Senegalese sole were recovering from their breeding season. Thus, it is possible that energy and metabolism were not in their optimal levels and then induced lower activity in fish (Careau and Garland, 2012). Other possibility is that maturity status and hormones (*e.g.* testosterone, 17-estradiol, etc.) influenced the Senegalese sole breeder's behaviour, as had been observed in other fish species, such as stickleback, African cichlid fish and Siamese fighting fish (Bell, 2004; Huffman et al., 2013; Hebert et al., 2014), whom observed changes in risk taking ability, aggression and reproductive dysfunctions. Regarding glucocorticoids blood levels, significant correlations over time were observed for cortisol and lactate concentrations in juveniles and only for glucose concentrations in breeders. The present results were similar to other studies that analysed overall correlations over time (Castanheira et al., 2013; Ferrari et al., 2015). Lastly, the low repeatability, consistency and correlation of glucocorticoids blood concentrations in breeders over time may be attributed to, physical manipulation, fish age and size and the nutritional status of individuals or simply because of individual differences in comportment. These factors have commonly been observed to regulate glucocorticoids blood production in fish (Øverli et al., 2007; Ruiz-Gomez et al., 2011; Cook et al., 2012).

4.2. Behavioural patterns of fish with and without gametogenesis

A key result of the present investigation was to observe that juveniles with gametogenesis (determined following criteria of Anguis and Cañavate, 2005) presented higher scores in the restraining test and lower scores in the confinement test, showed lower cortisol blood levels in both runs (1 and 3) and exhibited higher disposition to take risk. Indeed, this group presented significantly higher weight and length than fish with no gametogenesis. These observations suggested that fish with proactive behaviours enter puberty and gametogenesis earlier than fish with reactive traits that were not observed to enter gametogenesis during the study. These results were similar to those reported by Bell and Stamps (2004) and Edenbrow and Croft (2011), whom documented the significant influence of behavioural traits on first sexual maturity in sticklebacks (*Gasterosteus aculeatus*) and mangrove killifish (*Kryptolebias marmoratus*) respectively. Indeed, results were in agreement with studies that evaluated relationships between coping styles, growth, activity and physiological changes over time (Brodin, 2008; Wilson and Godin, 2009; Edenbrow and Croft, 2011). One probable explanation about these individual behavioural differences between fish with and without gonadal development might rely on their metabolic rates and requirements, which were possibly higher in fish with gametogenesis than in fish without gametogenesis. Higher metabolisms have been generally hypothesized to be translated into higher activity, aggression and boldness in contexts related to domination or to risk taking. Further, individuals with higher metabolic rate have higher possibilities for food acquisition and thereby for energy gain that involved greater growth rates, improved physiological development and faster reproduction (Biro and Stamps, 2008, 2010; Huntingford et al., 2010; Careau and Garland, 2012). In addition, Réale et al. (2010) proposed that positive feedbacks favour either a fast lifestyle (*e.g.* high metabolisms) associated with bold, aggressive, risky behaviour and rapid reproduction, or a slow lifestyle (*e.g.* low metabolisms) with cautious behaviour and delayed reproduction. Another possible explanation for these behavioural differences between fish with and without gametogenesis is the influence of hormones on Senegalese sole behaviour. Sex hormones (*e.g.* testosterone), produced during gametogenesis, have been documented to influence the aggressiveness, a trait that tends to be correlated with coping styles (Koolhaas et al., 2010; Conrad et al., 2011; Sih et al., 2015) in other fish species (Chang et al., 2012; Huffman et al., 2013). Therefore, it would be recommendable to perform more studies focusing on this aspect to corroborate the link

between gonadal development, hormones and behavioural traits during fish ontogeny, since it would provide important information for both aquaculture research and production sectors.

4.3. Inter-individual behavioural variations in Senegalese sole juveniles and breeders based on the risk taking test

Overall, fish that successfully crossed presented significantly higher scores in the restraining (juveniles and breeders), in the new environment (breeders) and in the confinement (juveniles) tests and produced lower cortisol (juveniles and breeders) than fish that did not cross. Together, these observations demonstrated inter-individual differences between fish that successfully crossed and did not cross in the risk taking test. Further, fish showed behavioural patterns consistent with proactive stress coping styles, because of their higher number of escapes attempts (*restraining-PCS*), their lower overall latency time to move (*environment-PCS* and *confinement-PCS*) and lower cortisol production, while behavioural patterns of fish that did not cross in the risk taking test were related to reactive coping styles, for both juveniles and breeders. Several studies in different taxa have established that differences in activity, risk predisposition and glucocorticoids blood concentrations were trustworthy indicators of stress coping styles traits and, therefore, were in line with the arguments considered in the present study (rodents: Koolhaas et al., 1999; birds: Dingemanse et al., 2002; fish: Wilson et al., 1993; Sneddon, 2003, Øverli et al., 2007; Martins et al., 2012; Castanheira et al., 2015). Thus, the differences in behaviours observed in the present test may be interpreted as an adaptive strategy of individuals to confront stressful situations. In agreement with this assumption, different models have suggested that each individual possesses different tactical responses to changes of environmental conditions, and such adaptations result from the long-term effect of natural selection and from their motivational state (Wilson et al., 1993; Budaev and Zworykin, 2002; Réale et al., 2010).

Another parameter proving that inter-individual behavioural responses of juveniles and breeders were consistent with coping styles were the significant cross-contexts relationships founded between their principal component scores. Juveniles showed significant positive correlations between restraining-PCSj and confinement-PCSj and between environment-PCSj and confinement-PCSj. In breeders, environment-PCSb was

significantly and positively correlated with restraining-PCSb and with confinement-PCSb. This suggested that the number of escapes attempts in the restraining test was related with the total activity time in the new environment and the confinement tests, revealing the existence of behavioural syndromes in both populations (Sih et al., 2004b). Moreover, the Principal Component Scores exhibited two axis of personalities defined as "fearfulness-avoidance" axe, linked to fish agitation, anxiety and propensity to escape during the restraining test (*restraining-PCS*) and the "activity and exploratory" axe, linked to fish exploratory behaviour and propensity to seek stimulus (*environment-PCS* and *confinement-PCS*) to novel situations. These two behavioural axes were comparable to other studies performed in fish species (Budaev et al., 1999; Millot et al., 2009; Wilson and Godin, 2009; Conrad et al., 2011; Castanheira et al., 2013).

5. Conclusions

The present work provided new outcomes on Senegalese sole stress coping styles. Juveniles and breeders showed consistent intra-individual consistency in behaviours across the different tests (context) and over time. Within these consistent behavioural responses were stress coping styles that were consistent with proactive and reactive personalities. Furthermore, this study is one of the first demonstrating the significant high degree of intra-individual repeatability, consistency and correlation in the behaviours of Senegalese sole juveniles and breeders across different individual-based and group-based coping style tests and over three and two years, respectively. The value of this result within the groups (juveniles and breeders) was not reduced by the lack of inter-individual repeatability and consistency between juveniles and breeders. For the first time, significant behavioural differences were established between juveniles with and without gametogenesis. The significant and strong degree of repeatability, consistency and correlation of behavioural traits in Senegalese sole juveniles and breeders observed in the present study confirmed that the set of individual-based (restraining, new environment and confinement) and group-based (risk taking) tests were suitable and robust to measure temperament in this fish species, as described previously for this species (Ibarra-Zatarain et al., in chapter 2). Further, this study indicates the value to select animals with certain kinds of behavioural traits at juvenile and adult developmental stages, thanks to their higher predictability over long time periods (Sih et al., 2015). Nonetheless, more studies are needed to confirm these results in Senegalese sole and in other fish species.

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Chapter 4

Exploring the relationship between stress coping styles and reproductive success, sex and origin in Senegalese sole (*Solea senengalensis*) breeders held in captivity

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Abstract

Individuals commonly adopt different behavioural patterns defined as proactive and reactive when are confronted with threatening situations. Such behavioural adaptations, labelled coping styles, have suggested to impact the reproductive success and differ between sex (female/male) and origin (wild/hatchery). Hence, the present study investigated whether or not the reproductive success of Senegalese sole (*Solea senegalensis*) is linked to coping styles and to establish the behavioural differences in sole depending on sex and origin. A total of 198 breeders, from two institutions, were submitted to three individual tests (restraining, new environment and confinement) and one grouping test (risk taking). In addition, a blood sample to quantify cortisol glucose and lactate levels was obtained from each individual after ending the individual tests. Senegalese sole showed individual differences in behaviour across the different coping style tests that were consistent with proactive and reactive coping styles traits. However, the most striking result was to demonstrate, by two different statistical approaches, that reproduction, sex and origin of Senegalese sole was not biased to any specific coping style behaviour. Indeed, the behavioural responses were similar and consistent between fish grouped by reproductive success, sex (only in one approach) and origin. This study presented information that contrast with different studies. However, suggest that maintaining both coping styles strategies are fundamental to make sustainable this non-aggressive species.

***Keywords**: Solea senegalensis, coping styles, fitness,*

1. Introduction

Individuals within a same population when confronted with threatening situations commonly adopt different behavioural patterns, which have been labelled as animal personalities (Dall et al., 2004), behavioural syndromes (Sih et al., 2004), or stress coping styles (Koolhaas et al., 1999); stress coping style is the preferred term for the present study. Stress coping styles have been observed in different taxa of animals (birds: Van Oers et al., 2005; mammals: Réale et al., 2009; fish: Castanheira et al., 2015) and tend to vary in between two axis: proactiveness and reactiveness. In contrast to reactive individuals, proactive organisms are consistent with bold personalities, are highly active, motivated to take risk and to explore unfamiliar environments, are aggressive and have lower post-stress glucocorticoids levels and higher sympathetic adrenal-medullar system activity (Koolhaas et al., 1999; Coopens et al., 2010; Sørensen et al., 2013; Mittelbach et al., 2014). Nonetheless, proactive fish are less flexible to environmental changes and tend to adopt routines in novel situations (Sih et al., 2004; Réale et al., 2009). On the other hand, reactive fish usually pay more attention to external stimuli and possess a higher capacity to adjust behaviour to novel situations (Koolhaas, et al., 1999; Sih et al., 2004; Ruiz-Gomez et al., 2011). Moreover, such differences in behaviour have been recognized to influence the overall fitness of fishes (Mittelbach et al., 2014; Castanheira et al., 2015).

Some hypotheses have suggested that coping styles impact the reproductive success and may significantly differ between sex (female/male) and origin (wild/hatchery) of individuals (see review of Huntingford and Adams, 2005; Schuett et al., 2010). For instance, Royle et al. (2005) demonstrated that proactive and aggressive swordfish (*Xiphophorus helleri*) males had higher reproductive success than reactive males. Ariyomo and Watt (2012) also observed that proactive males of zebrafish (*Danio rerio*) fertilized higher number of eggs than reactive males. Nonetheless, the relationship between proactiveness and reproduction success was not observed in mosquitofish (Wilson et al., 2010) and Sih and Watters (2005) found that proactive and aggressive organisms had a reduced reproductive capacity compared to reactive fish. Regarding fish origin, Huntingford and Adams (2005) demonstrated that fish born and reared in hatchery usually tended to be more proactive, or bolder, and to take higher risk when foraging than wild individuals in rearing conditions. Considering behavioural differences depending on fish sex, King et al. (2013) demonstrated that stickleback's males (*Gasterosteus*

aculeatus) were significantly more active and took more risks than females. However, in specific situations wild fish and females have been suggested to be able to modify their behavioural responses to become more proactive than males and hatchery-reared individuals (Øverli et al., 2006; Adriaenssens and Johnsson, 2011). Thus, these studies indicate that a) different stress coping styles can be associated with reproductive success, sex and origin, and b) fish behaviour adapts to both short term (*e.g.* seasonal effects) and long term (*e.g.* rearing conditions) environmental situations, in order to improve the individuals opportunity to survive and reproduce in a broader variety of conditions and/or situations (Dall et al., 2004; Sih et al., 2015). Additionally, models have been established that fish show high individual flexibility in their behavioural responses to stressful situations, which depend not only on social factors, but also on fish motivational state in particular contexts and on the immediate environmental stimuli (Budaev and Zworykin, 2002; Sih and Watters, 2005; Coopens et al., 2010).

Therefore, the aim of the present study was to investigate whether or not the reproductive success of Senegalese sole (*Solea senegalensis*) is linked to any specific coping styles pattern and to establish possible behavioural differences in sole depending on fish sex (female / male) and origin (wild / hatchery). Senegalese sole is a high-value flatfish species, commonly reared in intensive aquaculture production systems (Morais et al., 2014) in Southern European regions. However, the inability if the first generation of males (G1) to court females and to fertilize eggs in one of the most important bottlenecks that still restrict it production (Morais et al., 2014). In accordance with Carazo (2013) this reproductive atrophy possibly relies on the fact that G1 males may present a kind of behavioural reproductive problem. Thus, the results of the present investigation will aim to demonstrate if the dysfunctions of reproduction, particularly of G1 males, are biased or not to different stress coping styles behaviours.

2. Materials and methods

2.1. Ethic statement

All experimental procedures on fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee

of the Research & Technology Food & Agriculture centre (IRTA, Sant Carles de la Rápita, Spain).

2.2. Fish maintenance

A total of 198 Senegalese sole breeders, from which 59 corresponded to IRTA (Sant Carles de la Rápita, Spain) and 139 to the Spanish Oceanographic Institute IEO (Santander, Spain), were used in the present study. All individuals were PIT-tagged (ID-100 Unique, Trovan-Zeus, Madrid, Spain) for identification.

Breeders from IRTA presented a mean weight of 1189 ± 50 g were housed in four 13 m^3 rectangular tanks located in a greenhouse structure. Water was supplied to the tanks with a recirculation system (IRTAmar®) to maintain a simulated natural temperature (9 - 19ºC: winter to summer) and oxygen (5 - 6 mg/L) levels. Photoperiod was natural ranging from light dark (L:D) 14: 10 in the summer to LD10:14 in the winter for breeders and juveniles. Water temperature was 19°C and oxygen concentration was 6.0 mg/L during experimental period. Fish were hand-fed *ab libitum* every morning (10:00 h) according to the following regime: on Monday and Sunday balanced feed (LE-7 ELITE line, Skretting Co.), on Wednesday cooked mussels (Sariego Intermares, Spain), and on Friday, marine polychaetes (Topsy-Baits, Holland). One hour after feeding, uneaten food was removed from tanks to maintain optimal physicochemical water conditions.

Breeders from IEO presented a mean weight of 1357 ± 28g and were housed in four 15 m^3 rectangular tanks located in a greenhouse structure. The tanks were flow through with simulated natural temperature (11-22ºC) and constant photoperiod LD16:8. Water temperature was 18ºC and oxygen level was 7 mg/L during the study period. The diet was similar to that used at IRTA, fish being fed *ab libitum* every morning according to the following regime: on Monday and Friday cooked mussels (*Mytilus sp*.), and on Tuesday, Wednesday, Thursday and Saturday fresh squid (*Loligo sp.*).

2.3. Stress coping styles tests

Coping styles tests were performed between 10:00 – 16:00 h in both locations. In IRTA, the assays were performed the first week of October, while IEO tests were performed in the second week of the same month. In addition, tanks, nets and other instruments used to

characterize the stress coping styles behaviour were similar in both sites. All tests were performed out of the breeding season in order to reduce the influence of maturity status on fish behavioural responses. The percentage of reproductive success of sole breeders was estimated by microsatellite analyses (GENEAQUA, Lugo, Spain) of progeny from all spawns that produced larvae.

2.3.1. Restraining test

This test consisted in capturing a fish with a net and maintaining it out of the water while two variables were examined for 90 seconds: the Total Activity Time **NetAct** (duration of fish movement in the net) and the Number of Escapes Attempts **NetEsc** (number of contortions or strong movements made by fish to escape) (Figure 1A). These variables and the experimental design used in the present test were selected and modified in accordance to criteria defined by Ramsay et al. (2009), Castanheira et al. (2013) and validated for Senegalese sole in chapter 2.

2.3.2. Reaction to novel environment

This test aimed to evaluate fish reaction to a novel environment. Fish were placed in a 110 x 110 x 90 cm (width x length x depth) plastic tank that simulates a novel environment (Figure 1B). During a 5 minutes period, two behavioural parameters were evaluated: the First Activity time **NewLat** and the Total Activity time **NewAct**. Methodology was modified from previous criteria described by Wilson and Godin (2009), Huntingford et al. (2010) and validated for Senegalese sole in chapter 2. The definition of activity was restricted to active locomotion or swimming. It fish did not move during the 5 minutes period, then 300 s was recorded and used for statistical analysis (Farwell and McLaughlin, 2009).

2.3.3. Reaction to confinement

Immediately after the novel environment test, fish were submitted to a confinement test. The confinement situation was simulated by placing fish, individually, in a small plastic tray 56 x 36 x 30 cm (width x length x depth) (Figure 1C). During a 5 minutes period, two behavioural parameters were evaluated: the First Activity Time **ConLat** and the Total Activity Time **ConAct** following methodologies described by Brelin et al. (2005), Backström et al. (2011) and validated for Senegalese sole in chapter 2. Similarly to the novel environment test, activity was restricted to active locomotion against the wall of the

confinement tank. If fish failed to move during the 5 minutes period, then 300 s was recorded for statistical analysis (Farwell and McLaughlin, 2009).

2.3.4. Risk taking

This test was performed one month after finalizing the 3 individual tests, in order to allow fish to recover. The test aimed to determine fish capacity to cross from a known area (safe zone) to an unknown area (risky zone) (Figure 1D). The test was realized in a 16 m^3 tank (6 m length x 3 m width x 0.9 m depth), divided into two equal volumes by a wood barrier. The safe zone was isolated from light (2 lux) and covered with sand, to provide a comfortable and secure space for fish. On the contrary, the risky area was more illuminated (11 lux on the surface of water; OSRAM DULUX 48 and 150W) and the bottom of the tank was devoid of sand. A window (30 cm width x 15 cm tall) was opened at the base of the wooden barrier separating the safe from the risky zone, with a door allowing to close or to open fish crossing way from an area to another. This window was at the centre of a PIT (passive integrated transducer) tag reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain), which was positioned to read the tag number of fish that passed through the window toward the risky zone.

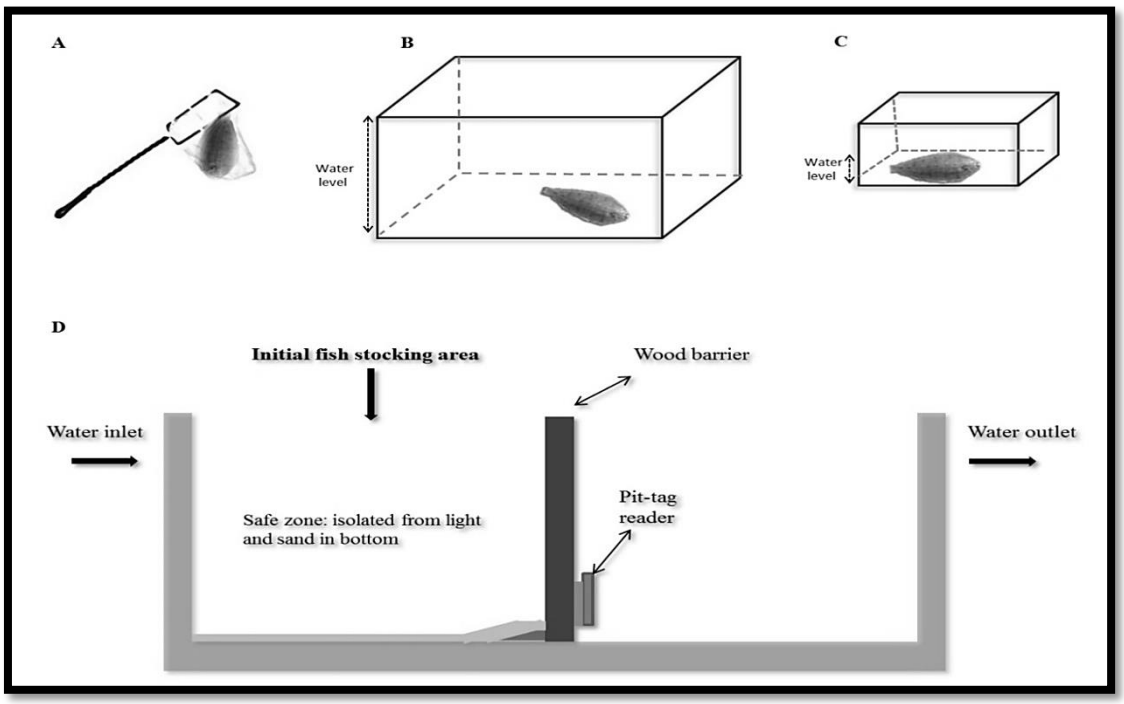

Figure 1. Description of equipment used to perform the coping styles tests on Senegalese sole breeders. **A**= Netting the fish; **B=** Novel environment test; **C=** Confinement test; **D=** Risk taking test

Before the beginning of the test, breeders were submitted to a 24 hours acclimation in the safe zone, by keeping the window closed until the beginning of the test, which lasted 24 hours. Breeders were tested in groups of 10 individuals to avoid inducing stress due to high stocking densities. Fish that successfully crossed from the safe zone to the risky zone were opposed to those fish that did not cross, following recommendations of Budaev (1997), Frost et al. (2007), Wilson and Godin (2009), Huntingford et al. (2010), Tudorache et al. (2013) and Herrera et al. (2014). The latency time of each organism to cross from one area to another was recorded. A maximum time of 1440 min was assigned to fish that did not cross during the 24 h period of the test.

2.4. Cortisol, lactate and glucose quantifications

Blood samples (0.5 ml) were extracted from the caudal vein of anesthetized fish (MS-222; Argent, USA, 100 ppm) to measure cortisol, lactate and glucose concentrations. Blood extraction was performed approximately 40 minutes after completing individual tests. To avoid blood coagulation, a solution of 10 µl sodium heparin (5%, 25.000 UI; HOSPIRA) and 15 µl aprotinin (from bovine lung; 0.9% NaCl, 0.9% benzyl alcohol and 1.7 mg of protein; SIGMA) was placed inside the eppendorfs, while syringes and needles were coated with heparin. Blood samples were centrifuged (ThermoScientific centrifuge, M23i; Thermo rotor AM 2.18; 24 x 1.5 ml) at 3000 G and 4ºC during 15 min and plasma supernatant was removed and stored in triplicates at −80ºC prior to analysis (Martins et al., 2011). Cortisol level was measured with a commercial ELISA kit (Range of detection: 0-800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany), by means of a competitive reaction with a conjugated binding ligand, whereas glucose and lactate concentrations were measured by means of commercial enzymatic colorimetric kits (SPINREACT, Gerona, Spain). Cortisol, glucose and lactate absorptions were read by a spectrophotometer (Infinite M-200; TECAN, Switzerland) at 23ºC and 505 nm.

2.5. Data analysis

Statistical analyses were performed using PASW 20 software for Windows. Values are presented as means ± standard deviation. Statistical differences were established when $P < 0.05$ for all analysis. Normality of data was checked through a Kolmogorov Smirnov test with Lilliefors correction. Two approaches were used to analyse coping styles in Senegalese sole. Firstly, the variables measured in each individual coping style test

(NetAct and NetEsc for the restraining test, NewAct and NewLat for the new environment test and ConAct and ConLat for the confinement test) were entered into three successive PCA (one per test). Then, the three Principal Component Scores resulting from these PCA´s (hereafter defined as *restraining-PCS1*, *new environment-PCS2* and *confinement-PCS3*) were used as single composite score that represented the individual behaviour of sole for each individual test (Budaev, 1997; Wilson and Godin, 2009). Secondly, the six variables from all tests and glucocorticoids levels (cortisol, glucose and lactate) were entered in one PCA and two components were generated (defined as *PC1-global* and *PC2-global*) following criteria defined in chapter 2. All PCA were performed with a Bartlett's test of sphericity, a Kaiser-Meyer-Olkin test and varimax rotation. Correlations between *restraining-PCS1*, *new environment-PCS2* and *confinement-PCS3*, glucocorticoids levels and fish morphometric variables (weight and length) were evaluated by means of Pearson analysis, as well as correlations between *PC1-global* and *PC2-global*, glucocorticoids levels and fish morphometric variables. Correlations between coping styles variables of fish grouped by reproductive success, sex, origin, institutes were analysed by Point-biserial correlations, an extension of Pearson analysis for dichotomous variables.

Next, two General Multivariate Linear Models (GLMM) were performed: i) on *restraining-PCS1*, *new environment-PCS2*, *confinement-PCS3* and cortisol, glucose and lactate concentrations and ii) on *PC1-global* and *PC2-global*, in order to identify possible significant differences between fish with different reproductive success (spawned/not spawned), between sex (female/male), origin (wild/hatchery), institutes (IRTA/IEO) and results obtained in the risk taking test (crossed/not crossed). Kolmogorov-Smirnov test (KS-test), with Fisher´s *Z*-test, was performed to analyse the behavioural distributions of breeders grouped by reproductive success, sex, origin and groups. A logistic regression analysis, with a Fisher exact test, was performed to establish if the latency time to move in the new environment (NewLat) and in confinement (ConLat) tests were correlated with the fish that crossed and did not cross (yes / no variables) in the risky area. A Chi-square test (X^2-test) was executed to establish significant differences in the proportion of fish that crossed in the risk taking test versus those that did not cross, between the fish separated by reproduction success, sex and origin.

3. Results

3.1. General observations between the individual and grouping tests

Overall, Senegalese sole (*n* = 198) showed a high behavioural variability in the restraining (total activity min = 0 to max = 93.4 sec, CV = 93.4%; escape attempts min = 0 to max = 93.4, CV = 134.7%), new environment (latency min = 1 to max = 300 sec, CV = 143.4%; total activity min = 0 to max = 227 sec) and confinement (latency min = 1 to max = 300 sec, CV = 143.4%; total activity min = 0 to max = 132 sec) tests (Table 1), indicating the existence of a broad range of coping styles behaviours from Senegalese sole breeders that exhibited high activity and latency to move characteristic of fight-flight behaviours associated with proactive coping styles to low activity and high latency to move characteristic of freezing-hiding behaviours associated with reactive coping styles.

Table 1. Overall responses of Senegalese sole (*n* = 198) for the restraining, new environment and confinement tests. SD = standard deviation; CV = coefficient of variation (CV = SD / mean * 100); Min = minimum; Max = maximun

Variable	Mean	SD	CV	Min	Max	Distribution
NetActW	19,00	17,74	93,39	0	80,00	positive skew
NetEscW	5,33	7,18	134,75	0	49,00	positive skew
NewLat	70,21	100,72	143,46	1,00	300,00	bimodal
NewAct	21,92	28,60	130,50	0	227,00	skew
ConLat	54,54	111,19	203,88	1,00	300,00	bimodal
ConAct	20,22	26,86	132,81	0	132,00	skew

The first approach showed that NetEsc, NewLat and ConLat were the variables that explained the highest variance in the 3 successive PCA, 72.58% of the *restraining-PCS1*, 69.27% of the *new environment-PCS2* 62.26% of the *confinement-PCS3*, respectively and presented eigenvalues larger than 1. Moreover, the Pearson´s correlation analysis showed that *restraining-PCS1* was significantly and negatively correlated with *new environment-PCS2* (R = -0.301, P < 0.001) and with *confinement-PCS3* (R = -0.341, P < 0.001), suggesting that those fish with more escapes attempts (higher scores) started to explore the new environment and resumed activity in confinement faster (lower scores). Besides,

new environment-PCS2 was positively correlated with *confinement-PCS3* (R = 0.412, P < 0.001). These correlations further demonstrated the existence of proactive and reactive stress coping styles as for the restraining and the exploration in Senegalese sole adults. Whilst the second approach (considering all variables together) showed that NetAct and NewAct explained the higher variance (42.8%). However, these two factors were not significantly correlated (Pearson, P > 0.05).

In the risk taking test, a total of 29 individuals (IRTA = 17, IEO = 12) crossed from the safe to the risk zone and 169 did not cross (IRTA = 42, IEO = 127). Sole that successfully crossed presented significant higher *restraining-PCS1* (GLMM, F_{173} = 3.71, P = 0.040) than fish that did not cross, indicating higher escape attempts. However, no statistical differences were detected between both groups of fish for *new environment-PCS2* (GLMM, F_{173} = 0.143, P = 0.521), *confinement-PCS3* (GLMM, F_{173} = 1.15, P = 0.285), cortisol (GLMM, F_{173} = 0.416, P = 0.520), glucose (GLMM, F_{173} = 1.91, P = 0.169) and lactate (GLMM, F_{173} = 0.934, P = 0.335) concentrations (Figure 2). When considering components from the second approach, no statistical differences were observed between soles that crossed and those that did not cross, neither for *PC1-global* (F_{173} = 0.2.878, P = 0.092) nor for *PC2-global* (F_{173} = 0.063, P = 0.802). The proportion of sole that crossed was not significant different from those fish that did not cross, when clustering fish by reproductive success (X^2 = 0.742, F_1 = 0.779, P = 0.268), sex (X^2 = 1.584, F_1 = 1.573, P = 0.146) or origin (X^2 = 3.063, F_1 = 3.110, P = 0.065). However, the proportion of fish from IRTA that successfully crossed was significantly higher than the proportion of fish from IEO (X^2 = 13.496, F_1 = 12.366, P = 0.001) (Figure 3). The linear regression predicted that latency time to cross was statistically correlated with *restraining-PCS1* (R = 0.254, F_{196} = 3.947, P = 0.048), with *new environment-PCS2* (R = 0.321, F_{196} = 1.158, P = 0.031) and *confinement-PCS3* (R = 0.535, F_{196} = 8.432, P = 0.001). No significant correlations (Pearson, P > 0.05) were detected between fish that crossed and did not cross with the latency time to move in the new environment and confinement tests.

3.2. Morphometric differences of breeders

Senegalese sole that spawned showed significant higher length than sole that did not spawn (F_{196} = 23.4, P = 0.001), females had higher weight than males (F_{196} = 14.85, P =

0.001), and hatchery breeders had higher weight and length (F_{196} = 7.21, P = 0.008 and F_{196} = 103.21, P = 0.001, respectively) than wild breeders (Table 2).

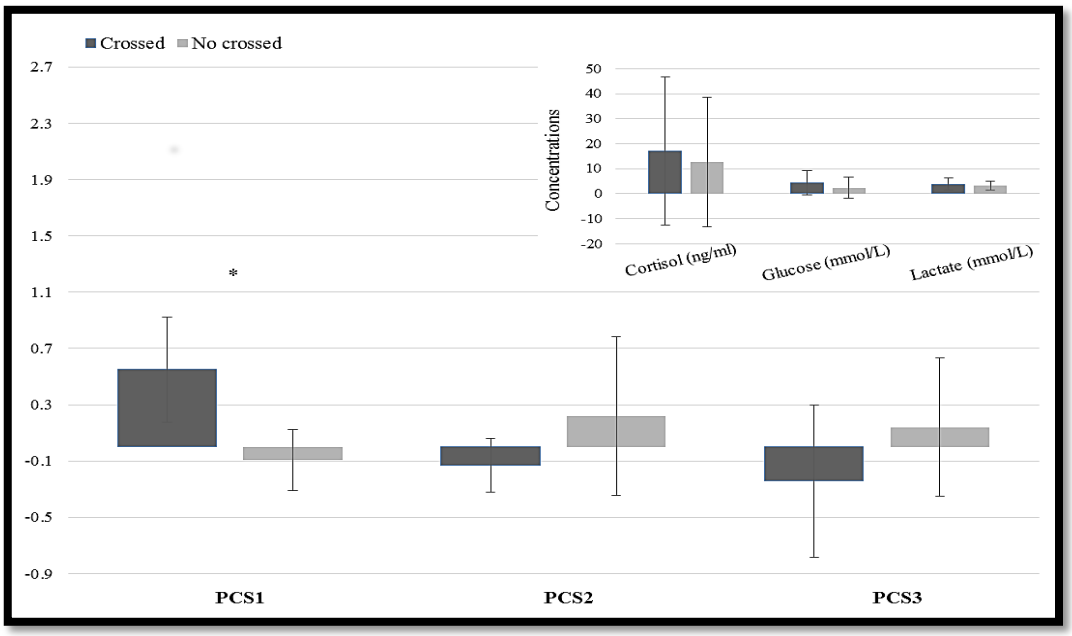

Figure 2. PCS and glucocorticoids concentrations differences between the fish that successfully crossed and those that did not cross. * indicates significant differences

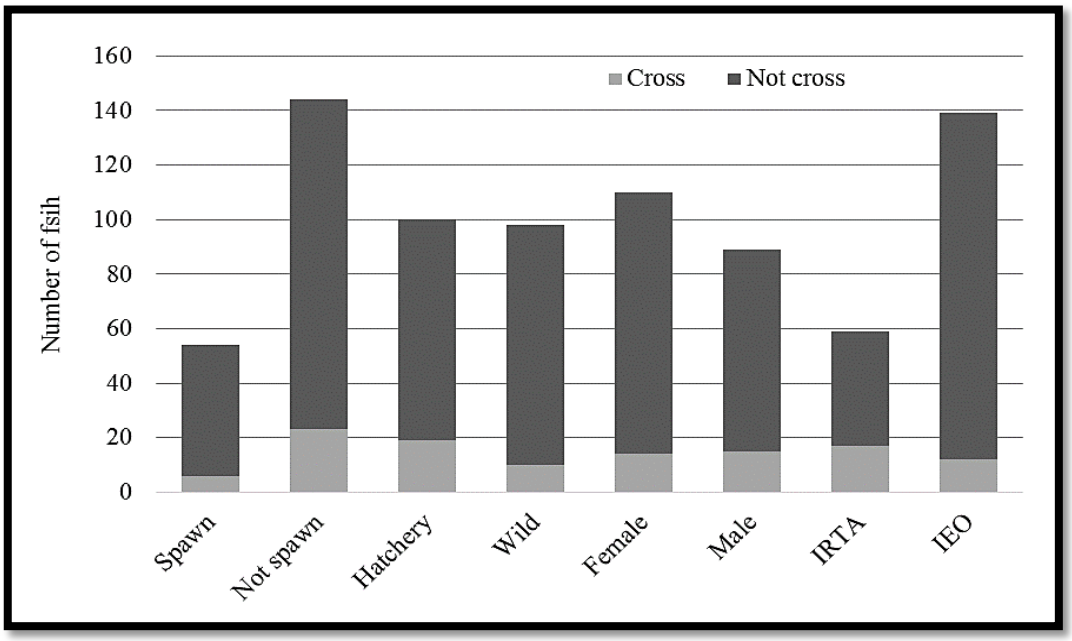

Figure 3. Number of fish that successfully crossed (light grey) and did not cross (dark grey) in the risk taking test, grouped by reproductive success, origin, sex, and institute. * Indicates significant differences

Table 2. Comparison between sexes, origin and spawning success of fish growth, variables for test 1-3 and biochemical quantifications (means ± sem). Numbers in parenthesis correspond to the number of animals in each group. Majuscule superscript letters designated significant differences between fish weight and length (resulted from GLMM). Cortisol is expressed in ng/ml, glucose and lactate in mmol/l

Tests	Variable	Female (110)	Male (88)	Hatchery (100)	Wild (98)	Spawn (54)	No spawn (144)	IRTA (59)	IEO (139)
Morphology	Weight	1391 ± 37^{A}	1200 ± 29^{B}	1240.2 ± 28.6^{A}	1374.8 ± 41.3^{B}	1350.0 ± 55	1290.7 ± 28.1	1190 ± 50	1357 ± 29
	Length	47.0 ± 0.6	47.2 ± 0.5	44.0 ± 0.4^{A}	50.4 ± 0.5^{B}	50.1 ± 0.7^{A}	46.0 ± 0.4^{B}	45.8 ± 0.6	47.7 ± 0.5
Netting the fish	NetEsc	17 ± 1.5	21.6 ± 2.1	17.3 ± 1.8	20.7 ± 1.2	20.7 ±2.1	18.3 ± 1.5	16.4 ± 1.7	20.1 ± 1.6
	NetAct	4.6 ± 0.6	6.2 ± 0.9	5.7 ± 0.8	5.0 ± 0.6	4.6 ± 0.8	5.6 ± 0.6	7.4 ± 1.1	4.5 ± 0.5
New environment	NewLat	73.3 ± 11.0	66.4 ± 11.6	73.3 ± 11.1	67.0 ± 11.3	69.2 ± 15.0	70.5 ± 9.3	98.3 ± 14.5	58.3 ± 9.3
	NewAct	19.2 ± 2.2	25.1 ± 3.2	24.3 ± 2.9	19.4 ±2.5	21.1 ± 3.7	22.2 ± 2.2	26.4 ± 3.9	20.2 ± 2.1
Confinement	ConLat	58.9 ± 10.0	49.1 ± 10.3	53.0 ± 10.0	56.1 ± 10.4	50.4 ± 13.2	56.1 ± 8.5	66.1 ± 12.2	49.6 ± 8.8
	ConAct	21.4 ± 3.0	18.7 ± 2.8	22.8 ± 2.8	17.5 ± 2.9	17.0 ± 3.1	21.5 ± 2.5	27.9 ± 3.7	16.9 ± 2.4
Risk taking	Cross	14	15	19	10	6	23		
	Not cross	96	74	81	88	48	121		
Blood analysis	Cortisol	11.8 ± 2.7	15.1 ± 3.0	15.2 ± 2.6	11.3 ± 3.0	12.2 ± 4.4	13.6 ± 2.2	16.7 ± 5.2	11.7 ± 1.8
	Glucose	3.1 ± 0.2	3.2 ± 0.2	3.3 ± 0.2	3.0 ± 0.2	3.1 ± 0.2	3.1 ±0.1	4.7 ± 0.3^{A}	2.5 ± 0.1^{B}
	Lactate	2.7 ± 0.4	2.6 ± 0.5	3.0 ± 0.5	2.3 ± 0.4	3.2 ± 0.7	2.5 ±0.3	7.0 ± 0.8^{A}	0.8 ± 0.1^{B}

3.3. Comparison of sole behaviours by reproductive success, sex and origin

a) Reproduction

Sole reproduction was not linked to any specific coping style strategy, since the general behavioural responses of fish that successfully spawned (n = 54) were similar to those that did not spawn (n = 144) in the three individual tests (Table 2). Further, no statistical differences were detected in the component scores obtained in the first approach between fish that successfully spawned and those that did not spawn (GLMM, *restraining-PCS1* F_{173} = 1.45, P = 0.230; *new environment-PCS2* F_{173} = 0.593, P = 0.442; *confinement-PCS3* F_{173} = 0.483, P = 0.490) neither in the components scores obtained in the second approach (GLMM, *PC1-global* F_{184} = 0.282, P = 0.596 and *PC2-global* F_{184} = 0.193, P = 0.661). Moreover, fish that successfully spawned and those that did not spawn showed significantly similar distributions, whatever the approach (KS-test approach one, *restraining-PCS1* P = 0.425, *new environment-PCS2* P = 0.598 and *confinement-PCS3* P = 0.822; KS-test for approach two, *PC1-global* P = 0.493 and *PC2-global* P = 0.982). In addition, blood parameters were similar in fish of both groups (cortisol F_{173} = 0.001, P = 0.999, glucose F_{173} = 0.021, P = 0.884 and lactate F_{173} = 0.011, P = 0.916). These results suggested that reproduction of sole is not related to coping styles (approach one Figure 4A; approach two Figure 5A; Table 3).

b) Sex

Males (n = 88) showed similar behavioural responses to females (n = 110) in the individual tests (Table 2). However, when comparing their PCS from the first approach, only *restraining-PCS1* was statistically different between males and females (F_{173} = 4.33, P = 0.039), indicating that males did more escape attempts than females, whilst *new environment-PCS2* (F_{173} = 0.013, P = 0.909) and *confinement-PCS3* (F_{173} = 0.267, P = 0.267) were not statistically different between both sexes (Figure 4B). Indeed, the KS-test showed that these two last PCs presented similar distributions (P = 0.790 and P = 0.837, respectively) in both groups. The second approach (Figure 5B) showed no statistical differences between males and females (PC1-global F_{184} = 0.029, P = 0.864 and PC2-global F_{184} = 0.070, P = 0.792) and between their distributions (KS-test, PC1-global P = 0.646 and PC2-global P = 0.287). Blood parameters were neither significantly different between males and females (cortisol F_{173} = 2.09, P = 0.150, glucose F_{173} = 0.606, P = 0.437 and lactate F_{173} = 2.35, P = 0.127). Therefore all tests and approaches (Table 3)

indicated no differences in stress coping styles between males and females with the exception of the restraining test that indicated males were more proactive than females.

c) Origen

In the first approach (Table 3), coping styles of hatchery breeders (n = 100) were observed to not statistically differ from those of wild breeders (n = 98) for *restraining-PCS1* (F_{173} = 3.61, P = 0.060), *new environment-PCS2* (F_{173} = 1.37, P = 0.243) and *confinement-PCS3* (F_{173} = 0.220, P = 0.883) (Figure 4C). Moreover, both groups presented highly similar distributions for the three PCs (KS-test, PCS1 P = 0.501, PCS2 P = 0.268 and PCS3 P = 0.311). The second approach (Table 3) showed similar results to the first approach, with no statistically differences and similar distributions between hatchery and wild fish (PC1-global F_{184} = 0.003, P = 0.959 and PC2-global F_{184} = 0.863, P = 0.354; KS-test P = 0.870 and P = 0.483, respectively) (Figure 5C). Therefore, the origin of the fish did not appear to be linked to proactive or reactive coping styles behaviour.

d) Institutions

Lastly, when breeders from IEO and IRTA were compared, the IEO breeders (n = 139) exhibited significantly higher scores for *restraining-PCS1* (F_{173} = 5.21, P = 0.024) (Figure 4D) and produced less glucose and lactate levels (F_{173} = 53.91, P < 0.001; F_{173} = 49.74, P < 0.001, respectively) than IRTA breeders (n = 59). However, the *new environment-PCS2, confinement-PCS3* and cortisol were not significant differently (F_{173} = 0.712, P = 0.400, F_{173} = 0.257, P = 0.613 and F_{173} = 0.812, P = 0.369, respectively). The KS-test also showed different behavioural distributions between both groups for *restraining-PCS1 (*P = 0.041) and *confinement-PCS3* (P = 0.049). The second approach (Figure 5D) showed significant differences between fish from IEO and IRTA and between their distributions in *PC2-global* (GLMM, F_{173} = 6.178, P = 0.010; KS-test P = 0.001), but not in *PC1-global* (GLMM, F_{173} = 1.969, P = 0.162; KS-test P = 0.002). Hence, IEO breeders and IRTA showed different behavioural responses when were analyzed with the two statistical approaches (Table 3). No significant correlations (Pearson analysis, P > 0.05) were found between fish PCs (from both approaches) and glucocorticoids levels and fish morphometric (weight and length), neither between reproductive success, sex, origin and institutes (Point-biserial analysis, P > 0.05).

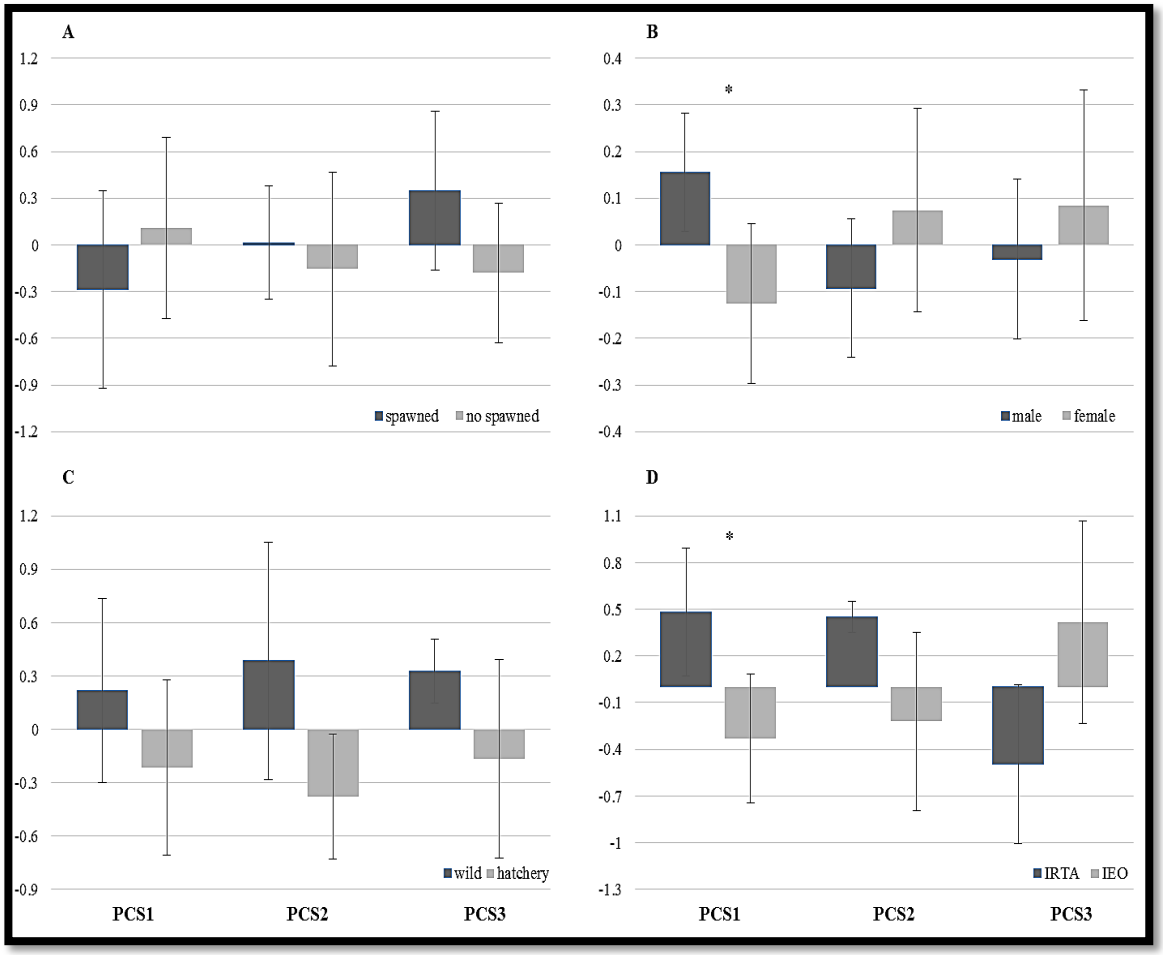

Figure 4. Comparison of principal component scores of sole breeders calculated in first approach for restraining (PCS1), new environment (PCS2) and confinement (PCS3). Graphics split by sex (**A**), origin (**B**), spawning success (**C**) and group (**D**). ***** indicates significant differences between groups of fish within a PCS.

Table 3. Association of stress coping style from different classifications with groups separated by different characteristics of the fish, reproductive success, sex, origin and holding centre. "Yes" indicates an association was found and "no" indicates no association was found.

Coping style classification	Reproductive success	Sex	Origin	Institution	Risk
restraining-PCS1	no	yes	no	yes	no
new environment-PCS2	no	no	no	no	no
Confinement-PCS3	no	no	no	no	no
PC1-global	no	no	no	yes	no
PC2-global	no	no	no	no	yes

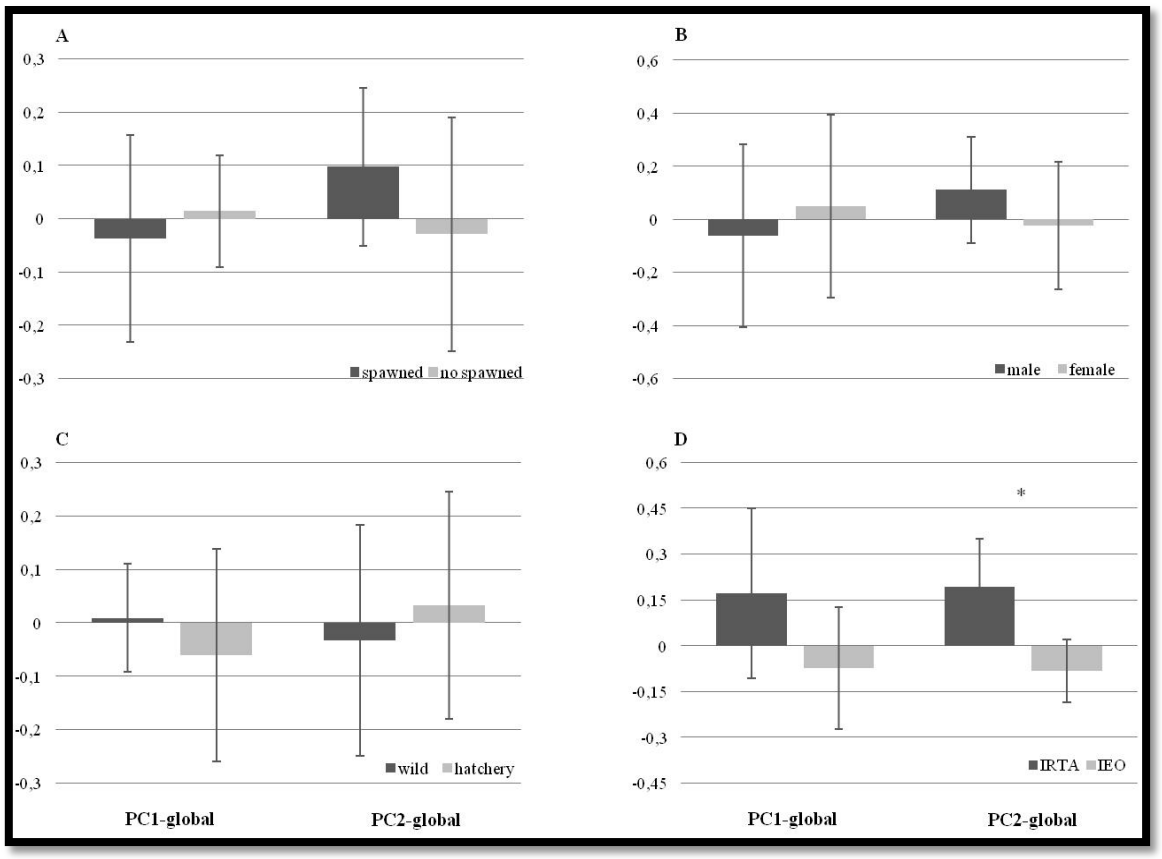

Figure 5. Comparison of principal component scores of sole breeders calculated from the second approach considering all six variables and glucocorticoids levels. Graphics split by sex (**A**), origin (**B**), spawning success (**C**) and group (**D**). ***** indicates significant differences between groups of fish within a PCS.

4. Discussion

Senegalese sole breeders exhibited considerable individual differences in their responses to the three individual stress tests performed in this study. These behavioural variations were consistent with the coping style theory, incorporating aspects of activity, exploration in novel situations, risk taking and physiological factors (Koolhaas et al., 1999; Coopens et al., 2010; Huntingford et al., 2010; Castanheira et al., 2015). In addition, Senegalese sole showed high behavioural consistency across the different contexts evaluated with the first statistical approach confirming the existence of behavioural syndromes in adult specimens of this species (Sih et al., 2004; Carter et al., 2013). However, no correlations were observed when components loaded from the second approach were analysed. Hence, individuals presenting an overall high activity, low latency to explore novel situations and low glucocorticoids levels were defined as proactive, whilst reactive sole were those with minor activity, high latency to start exploration in a new environment and high

glucocorticoids levels. These coping style criteria to distinguish proactive and reactive traits are in line with previous studies performed in several other fish species that used the same behavioural parameters (Brelin et al., 2005; Farwell and McLaughlin, 2009; Castanheira et al., 2015) and in other taxa, such as, birds (Van Oers et al., 2005), crustaceans (Fürtbauer, 2015) and mammals (Réale et al., 2010).

Regarding the principal component scores from approach one, NetEsc (*restraining-PCS1*), NewLat (*new environment-PCS2*) and ConLat (*confinement*-PCS3) explained the highest individual variance within each test performed. In addition, *restraining-PCS1* was significantly and negatively correlated with NewLat and with ConLat, while the two last cited variables were significantly and positively correlated. Indicating that those fish with higher escapes attempts resumed activity in new environment and confinement faster and that exploration was similar in different contexts. The second approach (global) showed that NetAct (*PC1-global*) and NewAct (*PC2-global*) variables explained the highest variance, however, these variables were not significantly correlated. Further, the loaded axes in PCA one (NetEsc, NewLat and ConLat) and PCA two (NetAct and NewAct) represented two behavioural dimensions of Senegalese sole defined as "fear-reaction" and "exploration-activity" behaviours, which are in agreement with other studies (Budaev 1997; Réale et al., 2007; Millot et al., 2009; Wilson and Godin, 2009; Castanheira et al., 2013). Therefore, the behavioural tests although not in strict agreement identified similar stress coping styles for the individual breeders and enabled the comparison of these classifications of stress coping styles to be compared amongst different groups drawn from characteristics such as reproductive success, sex, origin and centre.

Reproductive success, sex and origin were not apparently related to any specific coping styles, defined as proactive or reactive. Indeed, a high cross-context consistency was observed between groups (grouped by reproductive success and origin) within a same individual test. Indeed, both approaches showed similar results when comparing Senegalese sole grouped by reproductive success (no differences in both PCA), origin (no differences in both PCA) and institutions (differences in both PCA), but diverged when comparing males and females. Therefore, both approaches were suggested to be suitable to analyse Senegalese sole behaviours, but results might be interpreted carefully because of the differences between males and females and stocks in different centres.

The importance of comparative analyses for understanding the effect of behavioural traits on reproduction has been previously documented (Schuett et al., 2010). In the present work, the reproduction of Senegalese sole was demonstrated to not be markedly inclined toward proactive or reactive coping styles, but was considered more as a combination of both behavioural characteristics. Fish with high reproductive success presented similar coping style responses to fish with low reproductive success in all trials performed (individual and group tests) and the GLMM and X^2 analysis did not show any statistical differences in overall approaches, cortisol, glucose and lactate concentrations between these two groups of fish. Nevertheless, overall results were not in agreement with previous studies which reported that fish with high reproductive success or with high spawning participation tended to present proactive coping styles, while reactive fish were associated with little reproductive success or spawning participation (swordfish Royle et al., 2005; guppies, *Poecilia reticulata*, Goding and Dugatkin, 1996, Godin and Hair, 2009; pink salmon, *Oncorhynchus gorbuscha*, Cook et al., 2011; zebrafish, *Danio rerio*, Ariyomo and Watt, 2012). Interestingly, these studies have used fish aggressiveness as a coping style predictor, which is recognized to positively influence mate preference, dominance and reproductive success (Øverli et al., 2007; Conrad et al., 2011). Under rearing conditions, Senegalese sole is considered as a non-aggressive fish species (Silva et al., 2010), but a social one, and this point might in part explain the lack of correlation between reproductive success and stress coping styles behaviours in this species. Social animals tend to present some forms of cooperation to make their reproduction successful that include synchronised behaviours to counteract stressful situations, attract more females, create coalitions and shares reproduction. These combined aspects of a cooperative strategy among individuals may enhance the reproductive fitness and, ultimately, the offspring quality and survival (Wey et al., 2013; Díaz-Muñoz et al., 2015). Another reasonable hypothesis to explain the absence of relationship between reproductive patterns and coping styles observed in Senegalese sole in the present study may be the effect of the natural selection. According to Smith and Blumstein (2008) and to Schuett et al. (2010), natural selection is a mechanism that contributes to the maintenance of individual behavioural variations among animals within a same species. These authors suggested that if individuals vary in their preferences for selecting a mate (*e.g.* size, colour and behaviour), then sexual selection may be able to preserve the different behavioural strategies or coping styles within a population over the time. The conservation of both coping strategies likely allows species to improve the ability of

individuals to adapt their behavioural responses to a variety of environmental conditions. In the case where only one behaviour would be conserved, reproductive potential of individuals could be mismatched (Réale et al., 2010, Wolf and Weissing, 2010).

Regarding behavioural differences between sexes, models and previous studies have suggested that male's behaviour is closer to proactiveness, while female's behaviour are usually associated to reactiveness (Godin and Dugatkin, 1996; Candolin, 1999; Harris et al., 2010; Ariyomo and Watt, 2012, King et al., 2013; Mamuneas et al., 2014). These studies have based their interpretations on the observation that males had higher overall activity, foraged more in risky situations, resumed activity earlier than females after a stressful situation and made faster decisions towards food reward in unknown contexts. In the present study, males were overall more active and took more risk than females, however, the only significant difference observed between sexes was the higher total number of escape attempts (first approach = *restraining-PCS1*) in males than in females. Nevertheless, it is important to notice that females were significantly heavier than males that possibly influenced the ability of females to escape from the net, although no significant correlations were observed between morphometric parameters and coping style responses nor between morphometric parameters and principal component scores. Hence, the differences observed in escape responses between females and males might probably be due to a divergent phenotypic distinction that created differences in escape abilities, as observed in other fish species (Ramsay et al., 2009; Harris et al., 2010; Castanheira et al., 2013). If consider the significant differences between males and females in the *restraining-PCS1*, is possible to assume that males were prone to proactive coping styles than females; however, the majority of statistical analyses performed (*restraining-PCS1*, *new environment-PCS2*, *confinement*-PCS3, PC1-global and *PC2-global*) showed no differences between sexes in stress coping styles. A straightforward ecological and evolutional explanation for such behavioural differences between females and males was considered by Schuett et al. (2010). This author proposed that behaviour differs between sexes because "*the resulting competition for access to reproduction leads usually to greater variance in males than of females*". Thus, males are expected to tend to maximize their fitness by taking higher risks and forage more to increase their opportunities to reproduce and to provide their genetic characteristics to fry, whereas females give advantage to a longer life-span to maximize their reproductive opportunities,

thence, they reduced foraging and risk taking (Piyapong et al., 2009; Harris et al., 2010; Schuett et al. 2010; King et al., 2013).

It has been hypothesized that fish domestication may have profoundly affected their morphology, behaviour and life history (Huntingford, 2004; Robinson and Rowland, 2005). In the present study, hatchery and wild breeders showed similar behavioural responses and no significant differences were detected between their PCs (from approach one and two) and their glucocorticoids levels. In addition, morphometric parameters were not significantly correlated with stress coping responses. The lack of significant behavioural differences between hatchery-reared and wild Senegalese sole may be attributed to life experience of individuals, to fish adaptations of their rearing environment or perhaps, to the limited number of tests performed (Huntingford, 2004; Adriaenssens and Johnson, 2011). Nonetheless, hatchery breeders slightly tended to present a higher activity in individual tests (restraining "NetAct", new environment "NewAct" and confinement "ContAct") and in their risk taking capacity in comparison of wild individuals. Therefore, this low, but detectable, variability in behaviours between wild and hatchery-reared fish might be considered as the first consequence of domestication and genetic changes, which played a fundamental role on fish personality modelling (Dingemanse et al., 2012). Similar observations and tendencies, in overall activity and risk taking to those observed in the present study have been reported in other fish species, such as zebrafish *Danio rerio* (Robinson and Rowland, 2005), rainbow trout *Oncorhynchus mykiss* (Biro et al., 2004, 2006), brown trout *Salmo trutta* (Adriaenssens and Johnson, 2011), seabass *Dicentrarchus labrax* (Benhaïm et al., 2013), Atlantic salmon *Salmo salar* (Metcalfe et al., 2003). In addition, Huntingford and Adams (2005) reviewed that hatchery-reared salmonids regularly tended to be proactive, more aggressive and took higher risk when foraging than wild specimens. In captivity, fish are involved into a constant selection for improving growth, promoting disease resistance and increasing overall performance and cognition (Huntingford, 2004; Huntingford and Adams, 2005; Benhaïm et al., 2013). Nevertheless, it is worth to consider that these slightly behavioural differences in activity and in risk taking between wild and hatchery-reared fish can be the reflection of a pre-existing genetic variation between both strains, which are innate and independent of domestication, but related to different coping style strategies.

Regarding the differences in *restraining*-PCS1 scores and glucose and lactate levels and *PC2-global* scores, observed between IRTA and IEO breeders, they might be interpreted as behavioural adaptations of each group to their rearing conditions, handling, life experience and origin of fish populations, being in agreement with previous studies that observed similar behavioural variations among different fish populations (Wilson et al., 1993; Harris et al., 2010; Dingemanse et al., 2012).

5. Conclusions

Altogether these results demonstrated that Senegalese sole presented different behavioural responses or coping styles to the tests performed. However, the key results were to demonstrate that proactive or reactive patterns were not significantly related to reproductive success, sex and origin, as it has been observed in previous studies on other taxa. Both coping styles strategies appeared to be fundamental to make sustainable this non-aggressive species. Proactive and reactive behaviours seem to be essential for animals to optimize their reproduction and survival. Lastly, the behavioural dysfunction of G1 males were confirmed to not be related to their coping style behaviours. The results of the present study are particularly beneficial for research and production sectors in aquaculture, to understand the breeding participation and to optimize selection lines of breeders stocks.

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Chapter 5

Dietary fatty acid composition significantly influenced the proactive-reactive behaviour of Senegalese sole (*Solea senegalensis*) post-larvae

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Abstract

Few studies have examined the influence of diet on larval proactive-reactive behavioural dimension of stress coping style responses. The present study evaluated the influence of using different vegetables oils (Linseed; Soybean; Olive) and fish oil (Cod liver) for *Artemia* metanauplii nutritional enrichment on the proactive-reactive behavioural responses of Senegalese sole (*Solea senegalensis*) post-larvae (40 days post hatch). Forty-two Senegalese sole larvae from each of the four replicate tanks per treatment were tested. Two tests were performed: a new environment individual-based test, which evaluate the larvae latency time to move, total activity time and total distance moved; and a risk group-based test, which consisted in evaluating the larval capacity to cross from a "comfort" zone to a "risk" zone. In the group-based test, proactive, intermediate and reactive individuals were identified depending on the time taken to cross between two zones. Larvae fed with *Artemia* metanauplii enriched with the cod liver oil emulsion were significantly (P = 0.01) larger and in the individual-based test presented significantly higher total activity time (P = 0.08) and total distance moved (P = 0.01) than larvae from the other dietary treatments. No significant correlations (P > 0.05) were observed between larvae total length and latency time to move, total activity time or total distance moved across all treatments or within any dietary treatment. In the group-based test, fish fed with *Artemia* enriched with the cod liver oil emulsion presented a significantly higher proportion of proactive larvae (P = 0.02) and the lower proportion of reactive larvae. The present study showed for the first time that i) Senegalese sole presented a defined proactive-reactive behaviour from early ontogenesis, and ii) dietary fatty acid composition significantly influenced the proactive-reactive behavioural dimension of stress coping style of sole larvae. The current study has practical implications that open the possibility to produce organisms that have behavioural styles that could ultimately result in improved aquaculture productivity.

***Keywords**: Solea senegalensis, stress, personality, nutrition, dietary lipids, fish oil*

1. Introduction

Animals including fish when confronted with a threatening or stressful situation have been recognised to have two different behavioural responses, respectively named as proactive and reactive (Koolhaas et al. 1999; Øverli et al., 2007). So-called proactive fish have been characterised to have fight-flight behavioural response and were observed to explore unfamiliar environments and take risks (Bell, 2005; Brelin et al., 2005; Koolhaas et al., 2007; Castanheira et al., 2015). By contrast, reactive fish have been characterised to freeze or hide and generally have lower activity, avoid risk and tend to stay immobile when submitted to novel environments (Brelin et al., 2005; Koolhaas et al., 2007; Toms et al., 2010; Castanheira et al., 2015).

Physiologically, proactive fish were characterised by a low hypothalamus-pituitary-adrenal/interrenal (HPA/HPI) axis activity, leading to low post-stress levels of glucocorticoids, in contrast to reactive fish, which were characterised by a higher HPA/HPI response and levels of glucocorticoids (Koolhaas et al., 2010; Conrad et al., 2011). Together the behavioural and physiological dimensions combined with a consistency in responses over time or contexts have been described as the stress coping style of an organism (Koolhaas et al. 1999; Conrad et al., 2011; Castanheira et al., 2015). A diverse range of behavioural tests have been used to identify the behavioural dimension of stress coping style responses in teleost fish, such as reaction to confinement (Brelin et al., 2005), feeding motivation after being transferred into a novel environment (Mota-Silva et al., 2010), inter-individual aggression (Ruiz-Gomez and Huntingford, 2012), predatory situations (Archard et al., 2012) and group based tests (Bell, 2005; Wilson and Godin, 2009; Castanheira et al., 2013).

The performance of fish with different stress coping styles has been reported for different fish species. In a captive or aquaculture type environment proactive organisms were generally found to present higher growth (Mas-Muñoz et al., 2011), lower disease susceptibility (Mackenzie et al., 2009), lower latency time to recover and feed after a disturbance (Øverli et al., 2007) and were more disposed to follow routines ignoring novel changes in the environment (Ruiz-Gomez et al., 2011), which are characteristics that would favour aquaculture production. Thus, studying the behaviour during larval stages of fish species reared in aquaculture might be of great interest, in order to establish

the period of development of behavioural characteristics, factors that influence the development and the consequences of presenting different behavioural traits on growth, performance and development.

Among various factors assumed to influence larval survival, growth and quality, the importance of early larval nutrition, especially dietary lipids and essential fatty acids (EFA), have been highlighted in many studies (Izquierdo et al., 2000; Sargent et al., 2002). Diverse studies have addressed the effect of diets on fish larvae behaviour (*e.g.* swimming speed, escape reaction, etc.) in several fish species such as gilthead seabream (*Sparus aurata*) (Benítez-Santana et al., 2007), black sea bass (*Centropristis striata*) (Rezeck et al., 2010) and pikeperch (*Sander lucioperca*) (Lund et al., 2013). Moreover, different authors have indicated that vegetable oils and/or fish oils, used to improve the nutrition of live preys, may influence the fish growth, fatty acid body composition, gene expression and neuronal activity (Montero et al., 2003; Sales and Glencross, 2010; Benítez-Dorta et al., 2012; Benítez-Santana et al., 2014). Studies on the effects of vegetable oils on all life stages (larval, juvenile: pre-ongrowing and adult: ongrowing and breeders) of aquaculture species are required as the replacement of fish oils with vegetable oils is increasing rapidly to increase the sustainability of the aquaculture industry (Sargent et al., 2002; Naylor et al., 2009). Nonetheless, the question of how these dietary nutrients and the sources might have an impact on larvae behavioural dimension of stress coping style has, to our knowledge, received little attention.

Senegalese sole (*Solea senegalensis*) is a flatfish species that has great interest and potential for aquaculture diversification in Europe. One of the advantages of this species for aquaculture is that compared to other marine species larval rearing is not complicated and larvae possess high growth rate and survival (Morais et al., 2014). Furthermore, sole have been found to be particularly resilient to handling stress during the larval and early post-larval stages compared to other cultured species (Rønnestad et al., 2001). However, this species present high size variation and high mortality rates have been observed at the weaning period (Morais et al., 2014). Therefore, Senegalese sole larvae are a particularly interesting fish model in which to study the behavioural dimension of stress coping styles in relation to fatty acid nutrition since it has been demonstrated that an inappropriate fatty acid profile affected growth and muscle formation (Benitez-Dorta et al., 2012), digestive

system maturation (Boglino et al., 2012) and glucocorticoids regulation (Martins et al., 2013).

In view of these arguments, the present study aimed to determine whether i) Senegalese sole larvae exhibit proactive-reactive behaviours in standardised novel environment and risk tests and ii) whether dietary fatty acid composition from vegetable oils and fish oils, used as rotifers and *Artemia* enrichments, can influence the behaviour of sole larvae. Results will provide novel information related to Senegalese sole larvae behaviour at early life stages and how diets could influence physical fitness and behaviour. In addition, results may be valuable for the aquaculture industry in order to produce larvae with a specific behavioural characteristic.

2. Materials and methods

2.1 Ethic statement

All the experimentation on fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee of IRTA.

2.2. Experimental animals and housing

Senegalese sole larvae, supplied by a commercial farm (Stolt Sea Farm S.A., Galicia, Spain), were housed in sixteen 100 L tanks at a density of 100 larvae L^{-1}, in four replicate tanks per treatment. Tanks were connected through a recirculation system (IRTAmar®) and 50% of total water was renewed daily. Water parameters, such as temperature, salinity and dissolved oxygen were maintained at 16.5 ± 0.5 ºC, 35 ppm and 7.5 mg L^{-1}, respectively. Photoperiod was adjusted to follow a light-dark cycle of 16 L: 8 D hours.

2.3. Experimental emulsions, live feed enrichment and feeding protocol

Four experimental emulsions were prepared with different oils following the methodology described by Villalta et al. (2005) and used to enrich rotifers and *Artemia* metanauplii. The four oils used were: cod liver oil from *Gadus morhua*, cod (Sigma-Aldrich Co., St Louis, MO, Germany); linseed oil, linseed (Biolasi Products Naturals, S.L., Guipùzcoa, Spain); soybean oil, soybean (Huilerie Emile Noël, S.A.S., Pont Saint

Esprit, France); and olive oil, olive (Borges Pont, Lleida, Spain). Oils were emulsified in warm distilled water (50º C) with soy lecithin (Dietetica Rosa, S.A. Barcelona, Spain) and *α*- tocopherol (Sigma-Aldrich Co., St Louis, MO, Germany), using an Ultra-turrax T25 homogenizer at high speed for 60-90 s. Emulsions were kept refrigerated at 4 ºC in the absence of air until used for enriching the rotifers and *Artemia* metanauplii.

Senegalese sole larvae were fed twice a day, from 2 to 8 days post-hatching (dph) with rotifers (*Brachionus plicatilis*) enriched with the oil emulsions at a density of 10 rotifers mL^{-1}. Freshly enriched *Artemia* metanauplii (EG type; INVE, Belgium) were introduced in tanks at 6 dph until 24 dph, in quantities ranging from 0.5 to 6 metanauplii mL^{-1}, adjusted based upon the increase of weight of the larvae, with the daily food ration being calculated as described by Cañavate et al. (2006). As larvae metamorphosed and became benthonic, live *Artemia* metanauplii were gradually substituted with frozen *Artemia* metanauplii. From 19 to 24 dph and from 25 dph larvae were fed exclusively with frozen *Artemia* at a density of 6 metanauplii mL^{-1} until 30 dph and then 12 metanauplii mL^{-1} until the end of the experiment (40 dph).

Rotifers (*Brachionus plicatilis*) were cultured as described in Boglino et al. (2012). *Artemia* metanauplii were hatched in standard conditions and metanauplii enrichment was performed in 20 L conical containers at 150 metanauplii mL^{-1} for 16 h at 28 °C with oxygen (≥ 5 mg L^{-1}), and using 0.6 g of each emulsion L^{-1}. Subsequently, enriched *Artemia* metanauplii were washed with UV-treated filtered sea water and disinfected with hydrogen peroxide (8000 ppm) for 5 min. Then washed for 15 min period in 150 µm plankton nets with UV-treated filtered sea water. A batch of *Artemia* was frozen and kept at -20 ºC until being given to post-metamorphosed larvae.

2.4. Lipid and fatty acid analysis

For biochemical analysis of the larvae, pools of 50 post-larvae were taken per tank at 37 dph. Sampled larvae were euthanized with MS-222, washed with distilled water and immediately frozen at -20 ºC. Total lipids were extracted in chloroform: methanol (2:1, v:v) using the method described by Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow, followed by vacuum desiccation overnight. Total lipids were stored in chloroform: methanol (2:1, 20 mg mL^{-1}) containing

0.01% butylated hydroxytoluene (BHT) at -20 °C prior to analysis. Methyl esters were extracted twice using isohexane: diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo Electron-TraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK). A two-stage thermal gradient was used, from 50 °C (injection temperature) to 150 °C, after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}. Helium (1.2 ml min^{-1} constant flow rate) was used as the carrier gas and on-column injection and flame ionization detection was performed at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified using an internal standard, 21:0 fatty acid, added prior to transmethylation using a Chrom-card for Windows (TraceGC, Thermo Finnigan, Italy).

The fatty acid composition of the *Artemia* metanauplii varied in relation to the enrichment with the four emulsions. *Artemia* enriched with the cod emulsion presented the highest proportions of total saturated fatty acids (SFA), mainly 18:0. The amount of total monounsaturated fatty acids (MUFA), mainly oleic acid (OA, 18:1n-9), was the highest in the *Artemia* enriched with the olive emulsion and intermediary in those enriched with the cod emulsion. *Artemia* enriched with the soybean emulsion had the most elevated contents of total n-6 polyunsaturated fatty acids (n-6 PUFA), mainly linoleic acid (LA, 18:2n-6). Arachidonic acid (ARA) levels were relatively stable among the dietary treatments, and ranged from 1.2 (olive diet) to 1.5 (cod and soybean diets). The content of n-3 PUFA was the highest in the *Artemia* enriched with the linseed emulsion, mainly due to linolenic acid (LNA, 18:3n-3) contents. Finally, levels of n-3 LC-PUFA, EPA and DHA, were highest in *Artemia* enriched with the cod emulsion.

The fatty acid composition of the sole larvae fed with the four different dietary treatments reflected the fatty acid pattern observed in the enriched *Artemia* metanauplii (Table 1). Sole larvae fed with the cod emulsion presented the highest proportions of total saturated fatty acids (SFA), EPA and DHA. Respectively, EPA and DHA in larvae fed the cod diet was 4.9 and 7.2 compared to ranges of 0.9 - 1.2 and 2.3 - 2.7 in larvae fed with the other diets.

Table 1. Fatty acid composition of sole post-larvae fed with *artemia* metanauplii enriched with different experimental emulsions.

	LSO §	**CLO ¤**	**SBO †**	**OO ‡**
Formulation (mg g^{-1})				
Cod liver oil ¤	0	528	0	0
Linseed oil §	528	0	0	0
Soybean oil †	0	0	528	0
Olive oil ‡	0	0	0	528
Supplements [e]	52	52	52	52
Distilled water	420	420	420	420
Fatty acid composition (%TFA)				
Total saturated	16.5 ± 2.2	19.7 ± 1.2	17.9 ± 0.7	15.6 ± 0.7
18:1n-9 (OA)	22.3 ± 0.7	22.6 ± 0.4	21.2 ± 0.5	35.8 ± 0.8
Total monounsaturated (MUFA)	30.0 ± 1.0	35.4 ± 0.6	29.0 ± 0.7	45.6 ± 1.3
18:2n-6 (LA)	14.9 ± 0.2	7.8 ± 0.7	22.9 ± 0.5	10.1 ± 0.3
20:4n-6 (ARA)	3.9 ± 0.3	3.0 ± 0.1	3.2 ± 0.1	2.7 ± 0.4
Total n-6 PUFA	19.8 ± 0.7	11.7 ± 1.0	27.4 ± 0.3	14.2 ± 0.7
18:3n-3 (LNA)	25.2 ± 3.6	13.1 ± 1.1	15.1 ± 1.5	14.3 ± 0.8
20:5n-3 (EPA)	0.9 ± 0.2	4.9 ± 0.2	1.0 ± 0.2	1.2 ± 0.2
22:6n-3 (DHA)	2.3 ± 0.6	7.2 ± 0.2	2.7 ± 0.5	3.1 ± 1.2
Total n-3 PUFA	31.6 ± 3.5	30.7 ± 1.2	22.4 ± 0.7	22.4 ± 0.9
Total PUFA	51.4 ± 3.5	42.4 ± 1.9	49.8 ± 1.0	36.6 ± 1.2
MUFA/PUFA	0.6	0.8	0.6	1.2
n-3/n-6	1.6	2.6	0.8	1.6
DHA/EPA	2.6	1.5	2.7	2.6
ARA/DHA	1.7	0.4	1.2	0.9
ARA/EPA	4.3	0.6	3.2	2.3

¤ CLO: Cod liver oil

§ LSO: Linseed oil

† SBO: Soybean oil

‡ OO: Olive oil

[e] Supplements: Soy lecithin, 4 g; Vit. E 1,2 g.

The amount of total monounsaturated fatty acids (MUFA), mainly oleic acid (OA, 18:1n-9), was the highest (35.8) in the sole larvae fed with the olive emulsion and lower ranging from 21.6 – 22.6 in larvae fed with the other diets. Sole larvae fed with the soybean diet had the most elevated contents of total n-6 polyunsaturated fatty acids (n-6 PUFA), mainly linoleic acid (LA, 18:2n-6) with a level of 27.4 compared to 11.7 - 19.8 with other diets. ARA levels were relatively stable among the dietary treatments, and ranged from 2.7 (olive diet) to 3.9 (linseed diet). The content of n-3 PUFA was the highest in the sole fed with the linseed emulsion, mainly due to linolenic acid (LNA, 18:3n-3) contents.

2.5. Proactive-reactive behavioural characterization

2.5.1. Individual-based test

Forty-two Senegalese sole larvae (aged 40 dph) from each of the four replicate tanks per treatment were tested. Larvae were captured in groups of six from the experimental tank and placed in a 1L beaker. After a standardised 1 minute in the beaker to ensure all larvae had a similar pre-test treatment each larva was individually introduced into a separate plastic transparent petri plates (six dishes of 9 cm of diameter, see Figure 1A) used to perform the individual test. Larva were always placed in the same position (close to the edge) in the petri plate, which was gridded (squares equal to $1cm^2$, Figure 1A). Petri plates were filled with 15 ml of sea water from the holding tanks and renewed for each sampled larvae. A digital camera (Casio-Exilim, model EX-ZS100, Japan) was fixed 40 cm above the petri plates (Figure 1B) to video record larval activity for 5 minutes. The observer stood 1 m away from the working area, in order to cause minimal disturbance to larvae, following the criteria described by Øverli et al. (2002). Once video recording were achieved, three behavioural responses were registered for each larvae, being: i) the Latency Time to Move defined as the time (in seconds) of first forward movement since the beginning of the test (adapted from Bell, 2005; Archard et al., 2012); ii) the Total Activity Time considered as the total swimming activity time (in seconds) of the larvae in the new environment (adapted from Millot et al., 2009; Benhaïm et al., 2012); and iii) the Total Distance Moved determined by counting the total number of squares crossed by the larvae during the experimental time (adapted from Millot et al., 2009; Benhaïm et al., 2012). Larvae total length was measured as well, with a Vernier scale (0.001 error; model CD-20PK; Mitutoyo, Japan Corp).

2.5.2. Group-based risk test

In the group-based risk test, a different pool of larvae than the one used in the individual-based test was screened in a risk taking experience. Experimental methodology was adapted from previous studies performed in stickleback, *Gasterosteus aculeatus* (Bell, 2005; Ruiz-Gomez and Huntingford, 2012), gilthead sea bream (Castanheira et al., 2013), common carp, *Cyprinus carpio* (Huntingford et al., 2010) and bluegill sunfish, *Lepomis macrochirus* (Wilson and Godin, 2009). Four group risk tests were made per treatment, one test per treatment replica. The test consisted in placing 30 individuals in a rectangular tank (80 cm length × 241 10.5 cm depth × 13 cm width) divided into two areas, in order to determine the capacity of larvae to cross from a known area defined as a "comfort zone" to an unknown area defined as a "risk zone" (Figure 1C). The experimental tank was divided in two equal volumes with a black plastic barrier, including a 1.5 cm width-length window at the base of the barrier, through which fish were able to pass (Figure 1C). During the acclimation period, each group of fish (n = 30) was retained for 1h in the comfort zone, by keeping the window closed until the beginning of the test. The comfort zone was completely isolated from light by a black plastic cover, while the risk area was illuminated by a fluorescent white light (OSRAM DULUX, 48W, 450 lux in surface) placed 25 cm above the water surface. A gentle water flow (2 l h^{-1}) and aeration were provided during the period of the test and similar water parameters than those in the housing tanks (temperature of 16.5 ± 0.5 ºC, salinity of 35 ppm and dissolved oxygen of 7.5 mg L^{-1}) were maintained. A digital camera (Casio-Exilim, model EX-ZS100, Japan) was fixed on the risk zone to video record the larvae that successfully crossed from comfort zone to the risk zone.

After the 1 hour acclimation the door was opened and the behaviour registered during 1 hour with the door open. Larval behaviour was established following the criteria of exploration activity, defining proactive larvae as those that crossed from the comfort to the risk zone in the first 20 minutes, intermediate larvae as those that took to cross between 21 and 40 min and reactive larvae as those that crossed more than 41 minutes after the beginning of the test or as those that did not cross (Wilson and Godin, 2009; Huntingford et al., 2010; Ruiz-Gomez and Huntingford, 2012; Castanheira et al., 2013). The time larvae crossed from the comfort zone to the risk zone was registered from the video recording. No larvae were observed to return to the comfort zone during the short

experimental period. The video analysis was corroborated with real time counts that were made every 10 minutes of the risk zones.

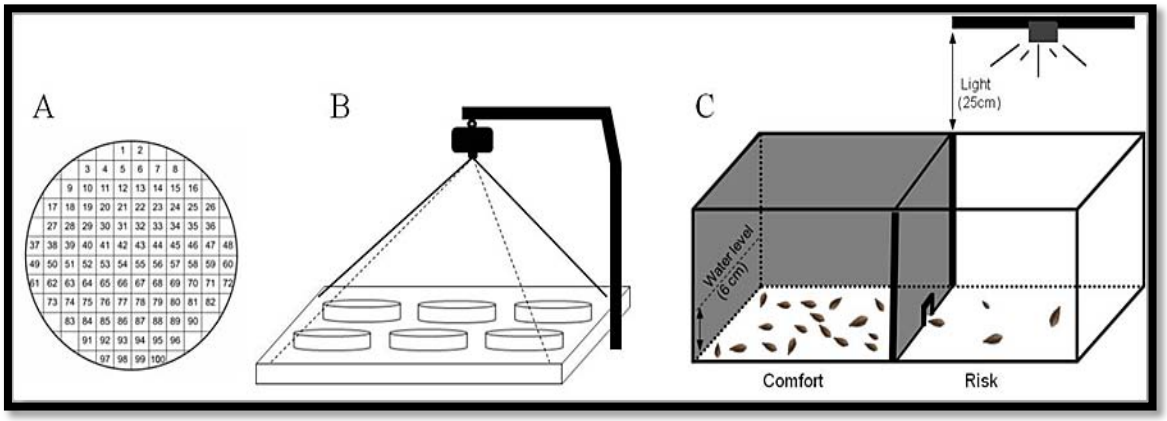

Figure 1. Equipment for determining performance and behavioural personality of Senegalese sole (*Solea senegalensis*) larvae. **A**= Petri plate gridded (1 cm^2) to measure larvae performance; **B**= Platform and camera installed to analyse individual performance; and **C**= Tank for grouping test (Comfort zone=covered; Risk zone= illuminated).

2.6. Statistics

Results were expressed as means ± standard error S.E. (n = 168 for the individual tests and TL). All data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test). Individual-based test means (n = 42) from each replica within each treatment were compared with a one-way Analysis of Variance (ANOVA) and as no differences were observed the data from replicas was combined. Two Multivariate Analysis of Variance (MANOVA) were performed, first among treatments on larval activity parameters (n = 42 x 4 = 168) of the individual-based test and the *post-hoc* Tukey HSD test was performed when significant differences were found and second to detect possible interferences among the position of the petri plates (Fig. 1A) on the individuals responses of sole larvae (length, latency to move, total activity time and total distance moved). No statistical differences were observed among the location of the petri plates (Fig. 1B) for the larvae length (F_5 = 1.29, P = 0.30), the latency time to move (F_5 = 0.65, P = 0.65), total activity time (F_5 = 0.88, P = 0.49) and total distance moved (F_5 = 0.63, P = 0.67) to discount that there was an effect on behaviour of petri dish position of the experimental setup. A Pearson correlation was performed in order to determine possible

correlations between variables including total length. A rank analysis, with N-tiles frequencies subcommand, was performed to examine the possible effect of size of larvae on the latency time to move, the total activity and the total distance moved. Individuals from all dietary treatments taken together were separated into size ranges: small from 0.5 to 0.8 mm, medium from 0.9 to 1.0 mm and large from 1.1 to 1.9 mm. The numbers of individuals in the medium size range were 49, 64, 67 and 76 respectively for Cod, Linseed, Olive and Soybean dietary treatments. A one-way ANOVA was performed to compare the behavioural parameters amongst the treatments for the selected larvae that were all in the same medium size range. Groups of 30 larvae (n = 30) from each replica (4 replicas) in each treatment (4 treatments) were used in each group test (total 4 x 4 = 16 group tests).

A Chi square (χ^2) test was performed on data from the grouped-based tests, in order to determine the differences in the proportions of proactive, intermediate and reactive larvae. First, a Chi squared test was made to compare the data from replicas within each treatment (3 x 4 matrix). No differences were observed amongst replicas within treatments. The replicate data was combined (n = 30 x 4 = 120) and dietary treatments were compared (3 x 4 matrix). A value of $P < 0.05$ was accepted as statistically significant for all statistical tests realized on data from the individual and grouping tests. All the statistical analysis was conducted using SPSS statistics V.17 (IBM Inc., Chicago, Illinois, USA).

3. Results

3.1. Individual behaviour characteristics

Feeding Senegalese sole larvae with *Artemia* metanauplii enriched with the four different emulsions significantly affected growth and individual activity performance when introduced into a new environment (Figure 2). Larvae fed with *Artemia* metanauplii enriched with the cod emulsion presented a significant higher total length (Figure 2A; $F_{1.24} = 8.6$, $P = 0.01$; 1.13 ± 0.03 cm) than larvae fed *Artemia* enriched with linseed, olive and soybean emulsions (1.01 ± 0.03 cm; 0.92 ± 0.03 cm and 0.93 ± 0.02 cm, respectively). The activity of larvae in the new petri dish environment in all treatments ranged from larvae that that did not move to larvae that moved immediately (low latency

time) with a high level of activity (distance moved and time of activity) during the five minutes.

No significant difference ($F_{1.24}$ = 2.3, P = 0.079) was found in latency time to move among larvae fed the different dietary treatments (Figure 2B), although larvae fed with *Artemia* enriched with the cod emulsion tended to have a lower latency time to move (36.6 ± 3.4 s) and those on the olive treatment had a slightly higher latency time (47.5 ± 2.4 s). Larvae from the cod group had significantly higher total activity time (Figure 2C; $F_{1.24}$ = 4.1, P = 0.08; 50.4 ± 3.7 s) than those from the linseed and olive groups (36.3 ± 3 s and 37.6 ± 2.7 s, respectively), while larvae fed the soybean diet displayed an intermediate and non-significantly different total activity time value (39.6 ± 3.6 s).

Similarly, larvae fed with *Artemia* metanauplii enriched with the cod emulsion showed a significantly higher total distance moved (Figure 2D; $F_{1.24}$ = 12.8, P = 0.01; 51.47 ± 3.4 squares) than those fed the three other diets and larvae fed with *Artemia* enriched with the soybean emulsion presented a significantly lower total distance moved ($F_{1.24}$ = 4.4, P = 0.06; 25.3 ± 2.3 squares) than the olive group (39.9 ± 3.2 squares), while those fed the linseed diet showed an intermediate value of total distance moved (32.4 ± 3 squares).

When larvae were selected by medium size (0.9 - 1.0 mm, medium of all treatments) from each treatment group and compared the same pattern of differences was found amongst treatment groups. The 0.9 - 1.0 mm larvae from the Cod treatment presented significantly lower latency time to move (F_{252}= 3.603, P = 0.014), significantly higher total activity time (F_{252} = 5.926, P = 0.001) and significantly higher total distance moved (F_{252} = 20.414, P < 0.001) than 0.9-1.0 mm larvae from some or all of the other treatments. In addition, no significant correlations (P > 0.05) were observed between larval total length and latency time to move, total activity time or total distance moved across all treatments or within any dietary treatment. The correlation coefficients were low ranging from the highest of R = 0.27, P = 0.08 observed between total length and total activity time in the cod treatment to the lowest between total length and total distance moved in the linseed treatment (R = 0.08, P = 0.62).

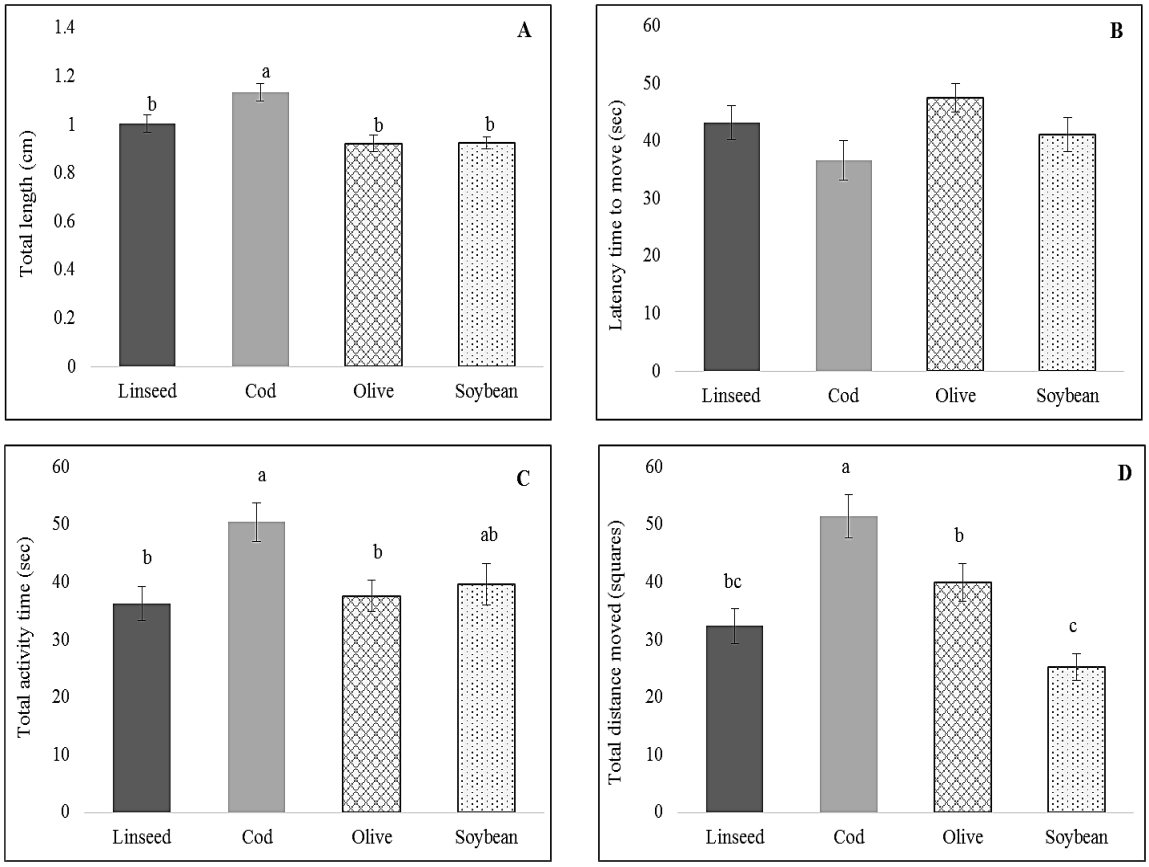

Figure 2. Size and behavioural performance of Senegalese sole (*Solea senegalensis*) larvae fed with *Artemia* metanauplii enriched with one of four different oil emulsions: linseed oil; cod liver oil; olive oil and soybean oil. **A**) total length; **B**) latency time to move; **C**) total activity time and **D**) total distance moved. n = 168 per treatment. Different letters indicate significant differences between treatments.

3.2. Risk taking by group

All treatments had larvae that passed through the door to the risk zone soon after opening (proactive) and larvae that did not cross from the comfort zone to the risk zone (reactive). Larvae from the cod treatment presented a significant higher proportion (χ^2 = 24.27, df = 6, P = 0.02) of proactive and intermediate organisms (14 and 7, respectively) and the lowest number of reactive larvae (9) (Figure 3). On the contrary, most of the larvae fed the soybean and olive diets did not cross from the comfort to the risk zone (21 and 25, respectively), suggesting predominantly reactive behaviour. Larvae fed with *Artemia* metanauplii enriched with the linseed emulsion presented intermediary values of

proactive (9), intermediate (3) and reactive larvae (18) (Figure 3). Larvae crossed only once from comfort zone to risk zone.

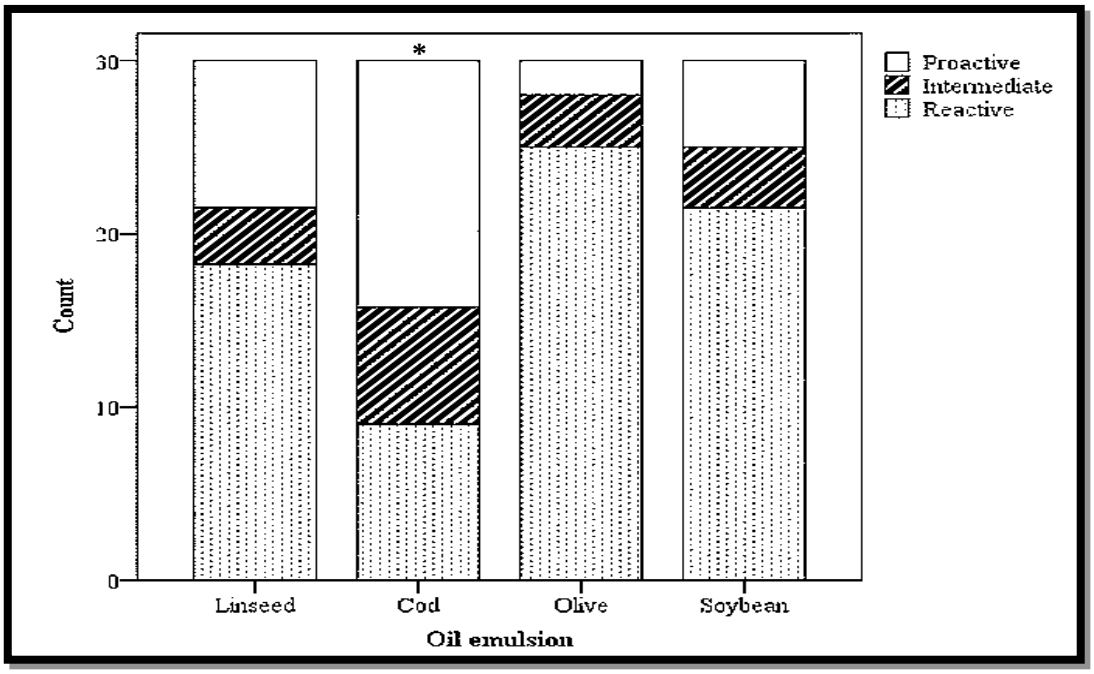

Figure 3. Number of proactive, intermediate and reactive Senegalese sole (*Solea senegalensis*) larvae fed with *Artemia* metanauplii enriched with one of four different oil emulsions: linseed oil; cod liver oil; olive oil and soybean oil (χ^2, P = 0.001). n = 120 per treatment. ***** Indicates proportions were significantly different from expected values

4. Discussion

This is one of the first studies that investigated the effect of diets on the proactive-reactive behavioural dimension of stress coping style during the larval development period. The tests employed in the present study enabled the characterization, for the first time, of proactive-reactive behaviour of Senegalese sole post-larvae at 40 dph. The activity of sole post-larvae in the new environment individual-based tests and latency to enter an unknown risk area in the risk group-based test enabled the identification of proactive and reactive behavioural traits. In agreement with the classification used in other studies, proactive larvae had low latency to move into a risk zone or in a new environment and were more active in a new environment, whilst reactive larvae had higher latency to move into a risk zone or in a new environment and were less active in a new environment. All dietary treatments had this range of behaviours, although the levels of activity in a new environment and proportions of proactive, intermediate and reactive larvae significantly

changed depending on diet. The identified proactive - reactive behaviours as determined by activity in the individual-based tests did not appear to be related to the size of the larvae as the same pattern of differences were found in the behaviours of similarly sized (0.9-1.0 mm) larvae that were selected from each treatment group and there was no correlation between larvae length and individual-based test activity variables latency time to move, total activity or total distance moved. The activity in new environment and the risk taking tests used in the present study to differentiate the proactive and reactive behaviours have been both previously reported as relevant measurements to determine proactive and reactive traits in a wide range of fish species (Toms et al., 2010; Castanheira et al., 2013) including Senegalese sole (Mota-Silva et al., 2010; Martins et al., 2011). This behavioural dimension of stress coping styles were described in the damselfly *Lestes congener* (Brodin, 2008), common carp (Huntingford et al., 2010), rainbow trout *Oncorhynchus mykiss* (Ruiz-Gomez et al., 2011) and three-spined stickleback (Ruiz-Gomez and Huntingford, 2012). Altogether, these studies, which are in line with the present results, have provided a solid base of evidence for the existence of proactive-reactive coping style responses in those fish species and demonstrated that proactive individuals, when in novel environments, resumed activity earlier, tended to take risks and spent more time in movement than reactive individuals.

The results from the present study have clearly shown that diets differing in fatty acid profile significantly influenced the proactive-reactive behavioural dimension of stress coping style of Senegalese sole larvae. To our knowledge, there is no information on the early interactions between dietary fatty acid composition and the stress coping style of Senegalese sole larvae, or any other fish larvae. Sole larvae fed with *Artemia* metanauplii enriched with the fish oil emulsion showed significantly higher activity time and swam longer distances in the new environment individual-based test and a significantly higher proportion of larvae took the risk to cross to and explore an unknown environment in the risk group-based test compared to larvae fed with *Artemia* enriched with the vegetable oil emulsions (linseed oil, olive oil and soybean oil). In contrast, larvae from the latter groups were less active in the individual-based test, more cautious in the reaction to novel situations and mostly stayed sheltered in the safe zone with a significantly lower proportion crossing to an unknown environment (risk group-based test). Therefore, the fish oil enrichment (cod liver oil) appeared to promote a proactive explorative behaviour, while vegetable oils (linseed oil, olive oil and soybean oil) resulted in a reactive

behaviour in Senegalese sole larvae. Possible explanations for these significant dietary effects on proactive – reactive behaviour could be a direct effect of nutritional status on physiology, metabolic rate and/or development that resulted in retarded growth or differences in physical condition that affect activity and personality of the larvae.

Cod liver oil diet contained higher amounts of SFA and of the EFAs, EPA and DHA (15.2; 4.7 and 2.6% TFA, respectively) than the other diets. In addition, it had the second highest level of MUFA, after the olive treatment. Therefore, overall, this diet possibly supplied higher levels of important energy substrates, as well as of critical structural components of bio-membranes and precursors of essential metabolites, resulting in a more balanced diet for fish larval and metamorphosing stages with fast growth associated with high requirements for development and organogenesis (Conceição, 1997; Morais et al., 2005). This difference in nutrition may also explain the higher growth of sole fed the cod diet. Other studies have shown that red sea bream *Pagrus major* (Nakayama et al., 2003), gilthead seabream (Benítez-Santana et al., 2007) and pikeperch (Lund et al., 2012) fed with higher inclusions of PUFA, particularly DHA, in diets improved fish growth, activity, swimming speed, escape ability and reduced mortality after a stress confinement situation. Similarly, Liu et al. (2002) and Atalah et al. (2011) suggested that increasing the dietary amount of EPA and ARA supplied to gilthead seabream and European sea bass improved resistance to handling stress in comparison to larvae fed with diets containing poor levels of these ingredients. However, in the present study, the poor correlation between larval length and activity and the same pattern of behavioural differences amongst fish of the same size from the different treatments provides compelling evidence that the diet effect on the behavioural dimension of stress coping style was more profound than just increasing energy reserves and growth to increase activity and hence the proportion of proactive larvae.

Possibly one of the most relevant roles of DHA and EPA related to this study is its crucial importance for neurogenesis, being that DHA and EPA a major components of cell membranes in the eyes and brain of fish (Bell and Dick, 1991; Bell et al., 1996). Studies have demonstrated that providing an adequate DHA and EPA proportions during early development of fish might improve the brain development, the central nervous system, the neuromasts formation (*i.e.* sensory cells associated to lateral line), the activation of mauthner cells (*i.e.* neurons responsible for escape responses) optical tectum, cerebellum,

vision, the antipredator behaviour, as others (Bell et al., 1995; Ishizaki et al., 2001; Nakayama et al., 2003; Benítez-Santana et al., 2007, 2014). Moreover, the same authors observed that larvae fed with higher inclusions of DHA and EPA showed higher ability to swim, formed schooling patterns and improved anti predator escape capacity. Therefore, higher dietary levels of LC-PUFA, particularly of DHA and EPA, as provided by the cod diet in the present study, could have had a positive effect on the ontogenic development of the neural system and sensorial organs which in turn influenced the fish brain development or neurogenesis, cognition, swimming activity, learning capacity and explorative behaviour of larvae's. These assumptions are in agreement with the studies performed by Øverli et al. (2007), Sørensen et al. (2007) and Koolhaas et al. (2010) whom confirmed that behavioural stress coping styles of fish at different ages are associated with neurogenesis and neural plasticity. In reference to the vegetable emulsions, these three diets led to similar coping style responses in sole larvae. A possible explanation of this is that vegetable oils presented lower DHA/EPA ratio, higher ARA/DHA and ARA/EPA ratios and unbalanced ratios of MUFA/PUFA and n-3/n-6 PUFAs. Therefore, it is possible to suggest that energy, physical fitness and enzymatic material of larvae was similar within the three vegetable emulsions, but lower in comparison with larvae fed fish oil emulsion (Sargent et al., 2002; Villalta et al., 2005; Benítez-Dorta et al., 2012; Boglino et al., 2012). However, further systematic studies should be performed in order to explore the relation between nutrition, neurogenesis and coping styles, since it is not entirely clear how these aspects can be involved in fish larval behaviour.

Lastly, it should be mentioned that stress coping style is defined as a coherent set of behavioural and physiological stress responses that is characteristic to a certain group of individual (Koolhaas et al., 1999). In the present study, diets influenced the behaviour of 40 DAH Senegalese sole larvae in a coherent way. The dietary treatments had a coherent influence on larval behaviour as no differences were found in the behavioural parameters amongst the four replicas within each dietary treatment indicating that the behavioural patterns in the replicas were highly consistent between individuals that had received the same treatment. Stress coping style has been shown to be determined or perhaps influenced by genetic and environmental factors (Dingemanse and Réale, 2005; Koolhaas et al., 2007). It has also been hypothesized that individual´s behaviour may change in accordance with developmental stage or age (Groothuis and Trillmich, 2011). From an

environmental point of view fish behaviour may depend on environmental stimuli (*i.e.* predators, density, etc.), food availability, social structure or motivational state and negative experiences (Koolhaas et al., 2007; Ruiz-Gomez et al., 2011; Frost et al., 2013). The present study highlighted that nutrition was an important environmental factor in determining or influencing behavioural development of the proactive-reactive behavioural dimension of stress coping style of sole larvae in different contexts.

The implication of understanding fish stress coping styles is of major importance, not only from an evolutionary perspective but also in practical disciplines, such as neurosciences (Benítez-Santana et al., 2014), disease susceptibility (MacKenzie et al., 2009) and especially aquaculture production (Øverli et al., 2002). In this context, the characterization of Senegalese sole larvae proactive - reactive behavioural dimension of stress coping style may have important practical applications for the aquaculture industry for the selection or production of larvae with specific behavioural characteristic that may have benefits for welfare protocols, selective genetic programs, improved growth, stress resistance and/or survival. For instance, Øverli et al. (2002) and Pottinger (2006) found that selected lines of proactive rainbow trout juveniles presented higher levels of locomotor activity and growth rates. Mackenzie et al. (2009) indicated that coping style responses in common carp were related to susceptibility to diseases in inflammatory challenges. Hansen et al. (2009) showed that proactive Atlantic cod, *Gadus morhua*, individuals presented a higher learning ability compared to reactive fish. In the present study, proactive larvae (fed with cod oil) were more active, showed higher length and take more risk than reactive larvae (fed with vegetable oils).

5. Conclusions

The present study indicated that proactive-reactive behavioural dimension of stress coping style developed early during ontogenesis and, furthermore, that the proportion of the different proactive-reactive behaviour was modulated by diets. These findings are of great interest to certain sectors such the aquaculture, since it may offer the possibility to produce a higher proportion of organisms that have similar behavioural styles that may result in improved growth, welfare, stress resistance, fitness and thus increment aquaculture productivity.

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Chapter 6

Exploring coping style behaviours in Gilthead seabream (*Sparus aurata*) breeders reared in captivity and their implications on fitness

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In review

Abstract

Stress coping styles establish behavioural differences between individuals depending on the context over a period of time. Moreover, coping style include potential effects on survival, growth and reproduction. Therefore the aim of the present study was to characterize coping styles in gilthead seabream (*Sparus aurata*), evaluate their relation with spawning success and determine the intra-individual repeatability over time. Twenty-two breeders were submitted to three individual-based and one group-based tests and were blood sampled. Tests were performed in June and September. Seabream presented both extreme coping style responses: proactive and reactive. In contrast to reactive seabreams, proactive ones were significantly more active, took higher risk and showed lower glucocorticoids levels. Seabream spawning pattern was significantly correlated to behaviour. Further, fish with high spawning participation tended to be characterized by proactive coping strategy. Additionally, males were observed to be significantly more active than females. Lastly, seabream showed a high behavioural repeatability in two of the tests over the time. The present study demonstrated, for the first time, the implications of coping styles on gilthead seabream fitness.

Keywords: *behavioural traits, intra-individual repeatability, reproduction, mate choice coping, physiology*

1. Introduction

Stress coping styles establish behavioural differences between individuals depending on the context over a period of time and across contexts (Koolhaas et al., 1999). Models have suggested two distinct reactions to stressful situations labelled as proactive and reactive. Proactive individuals actively challenge stressors, take risk, show high levels of activity and produce low post-stress glucocorticoids levels and their behavioural profiles are consistent with boldness, while reactive individuals are shy, show low activity, avoid risk and show high post-stress glucocorticoids levels (Koolhaas et al., 1999, 2007; Coopens et al., 2010).

Ecological and biological consequences of presenting such different stress coping style behaviours include potential effects on survival, growth, disease resistance, reproduction and population dynamics (*i.e.* social interactions, dominance and aggressiveness) (Huntingford, 2004; Sih and Bell, 2008; Mittelbach et al., 2014). For instance, the existence of distinct coping styles behaviours that have an impact on fitness has been reported in several fish species (Sih and Bell, 2008; Wilson and Godin, 2009; Toms et al., 2010; Raoult et al., 2012; Tudorache et al., 2013). Thus, proactive and reactive behaviours might play important roles in fish fitness and performance and, hence, their study may result interesting for fields such as aquaculture, ecology and in the analysis of controlling mechanisms with gene expression or neurosciences (Huntingford, 2004; Koolhaas et al., 2007; Sørensen et al., 2013).

The present study evaluated the coping style behaviours of gilthead seabream (*Sparus aurata*) breeders, a model of fish species in aquaculture research and a highly produced fish species in the European aquaculture industry (Pavlidis and Mylonas, 2011). According to Castanheira et al. (2013), juveniles of this species present defined coping styles, but to date, it has not been evaluated in adults. Therefore, the aim of the present study was to i) characterize coping style behaviours in gilthead seabream adults, ii) link them with spawning participation and iii) confirm the behavioural repeatability over time.

2. Material and methods

A total of 22 pit-tagged gilthead seabream breeders were tested. Coping style tests, performed in two trials (June and September), consisted in three individual tests: **a)**

restraining (120 s) to evaluate i) the first activity time in net (NetFirstAct), ii) the total activity time in net (NetTotAct) and iii) the total number of escape attempts (NetEsc); **b)** confinement (5 min) to evaluate **i)** the first activity time in confinement (ConLat) and **ii)** the total activity time in confinement (ConAct); **c)** time to induce 5 levels anaesthesia (Table 1).

Table 1. Description of the different anesthesia levels in fish and recognition on behavior (*Schoettger and Julin, 1967).

Anaesthesia levels	Characteristics
1	Partial loss of reaction to external stimuli
2	Partial loss of equilibrium without reaction to stimulus
3a	Fish usually turn-over but maintain swimming ability
3b	Swimming activity stops but react to pressure in extremities
4	Loss of reflex, no reaction to strong external stimuli

Blood was sampled from each fish to evaluate cortisol, glucose and lactate concentrations after completing individual tests in June. A risk taking test was performed for 24 h one month after trial 1 (in July) to evaluate fish disposition to cross from a safe to a risky area. Tests were adapted from other studies (Wilson and Godin, 2009; Toms et al., 2010; Raoult et al., 2012; Castanheira et al., 2013; Tudorache et al., 2013; Ferrari et al., 2015). Microsatellite analyses were performed to identify breeders spawning participation. To this aim, a single multiplex of 13 microsatellite markers (STRs) was developed and validated on a group of known families analysed in a previous work (Chavanne et al., 2012). The STRs were chosen from the literature (Franch et al., 2006; Parati et al., 2010) based on their polymorphism and characteristics to be amplified in a single tube (Table 2). The methods of DNA extraction from fin-clips of parents and larvae, of fragment analysis for the genotyping and the PCR conditions (annealing temperature, Ta: 57°C) were as described by Parati et al. (2010). Parentage assignments were established by the specific allocation software wHDP (Parati et al., 2010; Galli et al., 2011). Breeders spawning participation was considered high above 20% paternity and low below 20%.

Table 2. Genetic characteristics and description of the 13 microsatellite loci composing the single multiplex used for parentage allocation of larvae. **A** = number of alleles; **He** = expected heterozygosity; **Ho** = observed heterozygosity; **Fis (W&C)** = fixation index estimated by Weir and Cockerham approach; **PE** = Probability of Exclusion (Combined exclusion probabilities over the 13 loci of 1 - 424x10-6). All loci were in Hardy-Weinberg equilibrium (P > 0.05).

Locus	Forward Primers (5′–3′)	Reverse Primers (5′–3′)	Dye	Repeat motif	A	Size Range (bp)	He	Ho	Fis (W&C)	PE	Gen Bank Acc. no
SA2-B09	AACGTTCTCAAAATAAAACCAGGC	TGTGGGTGTAATTAATGCTTGGA	6-FAM	GT	13	118-170	0,88	0,78	-0,120	0.701	DQ435030
BId-04-F	TCCGTGACTCTGTCTCACCA	TCTTTGTCCGCATGTTTCAC	VIC	CA	8	104-120	0,74	0,68	-0,047	0,417	DQ851276
Bd-71-F	AGAGCTGTGGAGGTGAAGGA	CAGAGCTGCACAAACAAGGT	NED	TG	20	208-252	0,93	0.85	-0,091	0.786	DQ851271
C77b	CGATGGAACTACCCACCTC	GCGACCATGAACCTGAAA	PET	AC	3	222-226	0,46	0,42	-0,169	0,239	DQ851297
C90b	CAGAGTCAAGCAGCGGATAA	GGACGGAGGAGAAAACCAG	VIC	AC	19	188-226	0,91	0,89	-0,169	0.73	DQ851299
CId-21-F	CAAGTCACACACACGCACAC	ACAACGAAGGACGACAAAGG	NED	CA	7	102-120	0,54	0.54	-0,899	0.301	DQ851305
CId-29-T	GTCGGATTTTCGCATGTTGT	CTGCAGGGAGGAAACAAGAC	NED	TG	7	144-162	0,78	0,67	-0,008	0,545	DQ851308
CId-35-H	TGGGGTTTAGCTGTCAATCC	GCGGCTATGCCTACAACTTC	PET	CA	5	201-213	0,66	0,65	0,121	0,317	DQ851311
Dt23	CACACAACACACGATTACAGCA	CCAAAAACAGCGATTTGTCC	PET	GATA	21	246-324	0,91	0,90	0,124	0,719	DQ851346
SA5-E05	AGCCCAAAGCCCCGG	CCCTCGTCCTCCTCCCTTTA	6-FAM	CA	11	140-180	0,87	0,80	-0.076	0.505	DQ435040
BMap20	AGAGCTCACTGTGACGATCAG	CAGGATCCCATCCTTACACA	6-FAM	CA	10	228-256	0,75	0,69	-0,108	0,543	DQ851286
G1	ACGGTCATTTCTGAGGTTGC	GGCAGAGAGTTATGGTCCACTT	6-FAM	TAA	7	286-300	0,69	0,66	-0,084	0.338	AF195646
SA2-F05	TGGAGAAGGTGTTGGCTTTCA	CCCTCTACCTGGTCAATAGTTAAAGACT	PET	Di	7	106 - 119	0,66	0,62	0,066	0.447	DQ435044

The variables from restraining, confinement and anaesthesia tests from both trials were reduced into three principal component scores (PCS1, PCS2 and PCS3), after performing 3 successive principal component analysis (PCA). A general lineal multivariate model (GLMM) was performed on data to identify for differences between the PCS 1 - 3 and blood parameters and spawning participation, risk taking and sex. A hierarchical linear regression (HLR) was performed to evaluate correlations among PCS 1 – 3 in breeders. The intra-individual repeatability, between trials 1 and 2 (June and September) were assessed for the three individual tests by a two-way repeated measure ANOVA and complete with a Pearson correlations analysis. Results were expressed as mean ± sem.

3. Results

A total of 2698 larvae were analysed, approximately 200 per spawn. The spawns were analysed as follows: one spawn per week was selected over the entire spawning season from January to April and six spawns were analysed from consecutive days in the middle of the spawning season (Table 2). Gilthead seabream breeders showed consistent individual differences in behaviour (Table 3). Breeders that crossed from the safe to risky area, in the risk taking test (n = 10), showed significant higher activity in restraining (F_{14} = 5.66, P = 0.022) and confinement (F_{14} = 5.66, P = 0.022) tests and had lower cortisol (F_{14} = 8.75, P = 0.019) and glucose (F_{14} = 25.25, P = 0.001) levels than fish that did not cross (n = 12) (Figure 1). These results confirmed the existence of both coping styles.

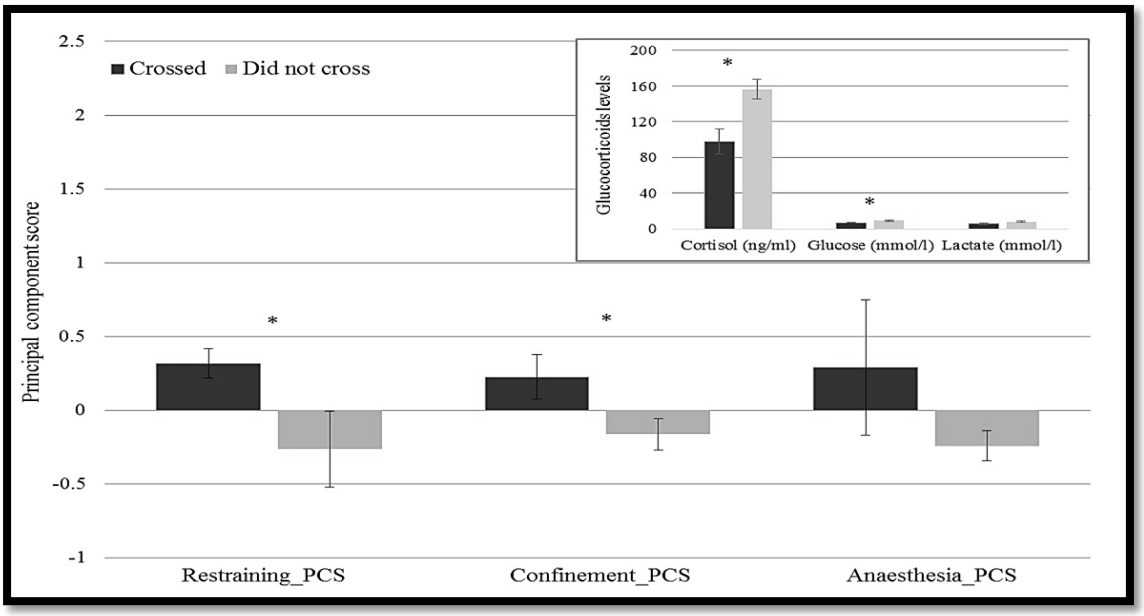

Figure 1. Principal component scores of seabream that crossed and those that did not cross in the risk test. * Indicates significant differences

Table 3. General coping style of breeders in trial 1 and 2. Min, max and coefficient of variation (CV) were calculated from both trials. CV was calculated to represent the inter-individual variation (CV = standard deviation / mean * 100). na = no applied

Tests	Variables	Trial 1 (June)	Trial 2 (September)	Min	Max	CV (%)	Repeated ANOVA			
							Wilk´s λ	F	df	P
Restraining	NetFirstAct	51,4 ± 10,6	43,5 ± 9,1	1	120	20.7	0.868	1.700	21	0.032
	NetTotAct	16,3 ± 2,1	16,3 2,3	1	52	13.5	**0.992**	**0.161**	**21**	**0.693**
	NetEsc	14,5 ± 2,2	16,8 ± 2,5	1	46	15.1	**0.979**	**0.443**	**21**	**0.443**
Confinement	ConLat	41,8 ± 10,8	35,3 ± 11,6	1	219	29.0	**0.757**	**0.728**	**21**	**0.594**
	ConAct	131 ± 34,4	123,2 ± 31,1	4	667	25.7	**0.972**	**0.609**	**21**	**0.444**
Anaesthesia	ANE 1	28,9 ± 1,51	31,6 ± 2,3	18	59	6.2	0.609	1.302	21	0.011
	ANE 2	59,2 ± 3,2	52,7 ± 3,1	31	91	5.6	0.725	0.987	21	0.037
	ANE 3a	108,6 ± 7,8	104,3 ± 5,1	50	189	11.8	**0.959**	**0.038**	**21**	**0.847**
	ANE 3b	182,3 ± 16,5	126,3 ± 6,7	81	368	7.5	0.968	12.98	21	0.002
	ANE 4	211 ± 16,1	166,5 ± 12,0	102	379	7.4	0.874	14.012	21	0.001
Blood analysis	Cortisol (ng/mL)	129,8 ± 11,9	na	47	249	9.3	na	na	na	na
	Glucose (mmol/L)	8,01 ± 0,4	na	3	12	5.0	na	na	na	na
	Lactate (mmol/L)	6,9 ± 0,5	na	3	12	7.2	na	na	na	na

Breeders with high spawning participation (n = 8) showed significant higher scores in restraining PCS(F_{14} = 7.91, P = 0.020) and confinement PCS (F_{14} = 22.18, P = 0.001) and lower cortisol (F_{14} = 4.81, P = 0.037) and lactate (F_{14} = 5.63, P = 0.033) levels than fish with low spawning participation (n = 14), indicating that the behaviour of fish with high spawning participation were considerably consistent with a proactive traits (Table 4, Figure 2). The PCS of restraining and confinement tests (PCS1 and 2) were significantly correlated (R^2 = 0.423, P = 0.037), demonstrating the existence of behavioural syndromes in seabreams.

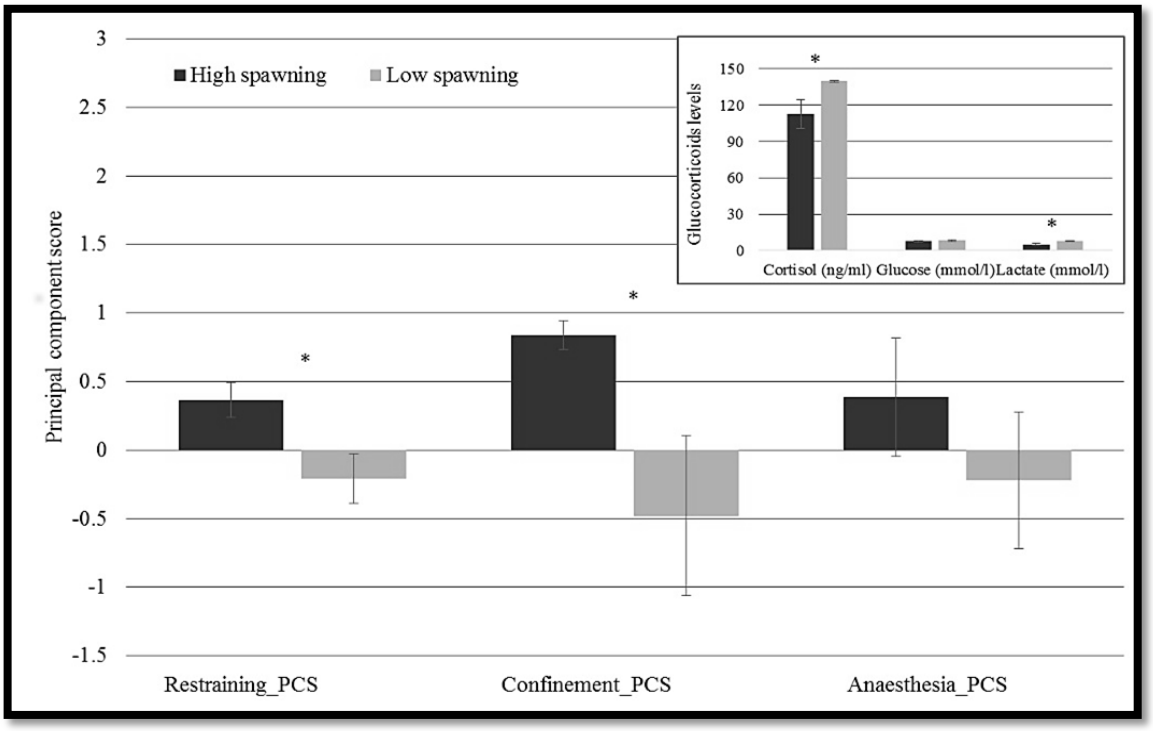

Figure 2. Differences between behaviour of breeders with high and low spawning participation by comparing their PCS. * Indicates significant differences.

Additionally, males (n = 10) showed significantly higher scores for restraining (F_{14} = 13.60, P = 0.002) and confinement (F_{14} = 6.82, P = 0.027) tests and produced lower cortisol (F_{14} = 9.45, P = 0.008) levels than females (n = 12), indicating that males were more prone to proactiveness than females (Figure 3).

Table 4. Coping style responses averaged from trial 1 and 2 of breeders with high and low spawning participation.

Tests	Variables	High part. (*n* = 8)	Low part. (*n* = 14)
Restraining	NetFirstAct	30,1 ± 13,4	57,4 ± 13
	NetTotAct	20,3 ± 4,4	14,1 ± 2,2
	NetEsc	18,3 ± 4,1	14,2 ± 3
Confinement	ConLat	11,7 ± 3,1	53,9 ± 16,3
	ConAct	223,5 ± 72	72,1 ± 21,4
Anaesthesia	ANE 1	29,4 ± 3,5	30,7 ± 2,3
	ANE 2	54,6 ± 5,4	56,7 ± 4
	ANE 3a	106,4 ± 12	93,1 ± 7,5
	ANE 3b	179,5 ± 23,5	140,0 ± 11,3
	ANE 4	223,5 ± 29,8	168,8 ± 11,5
Blood analysis	Cortisol (ng/mL)	109,5 ± 13,7^{A}	135,7 ± 12^{B}
	Glucose (mmol/L)	7,6 ± 0,8	8,1 ± 0,5
	Lactate (mmol/L)	5,7 ± 0,5^{A}	7,5 ± 0,6^{B}

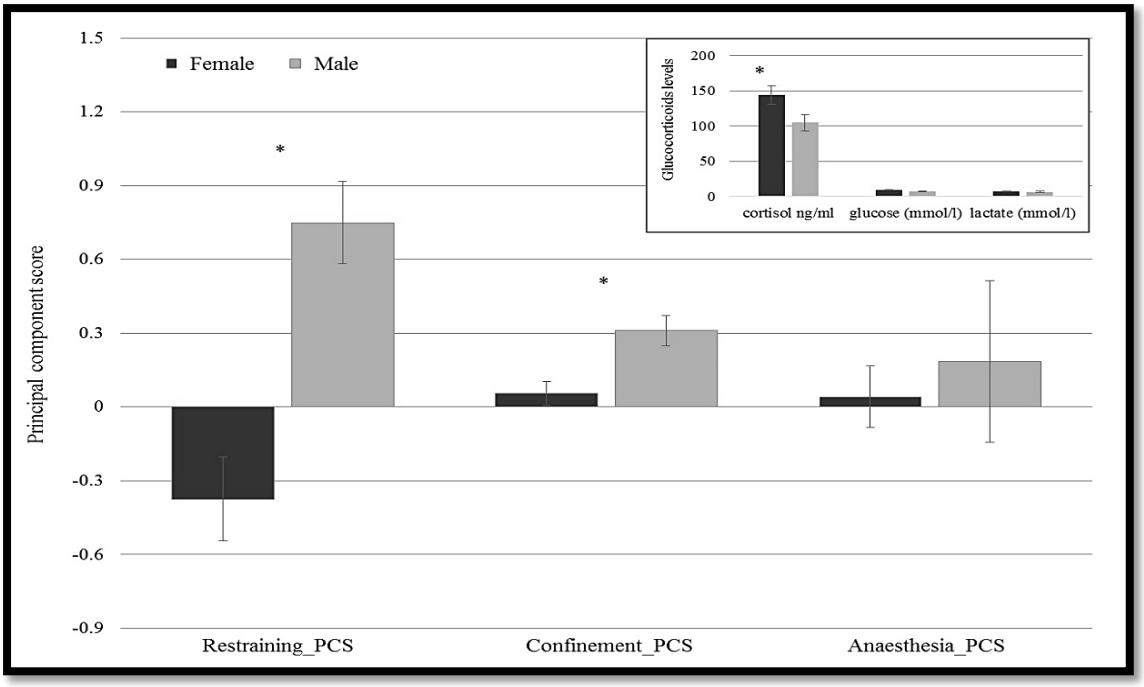

Figure 3. Principal component scores and glucocorticoids differences in females and males of gilthead seabream. * Indicates significant differences

Finally, seabream showed a high intra-individual repeatability (Table 3), with high correlations in NetFirstAct, NetTotAct and NetEsc (restraining), ConLat and ConAct (confinement) and ANE3 (anaesthesia) between trial 1 and 2 (Figure 4).

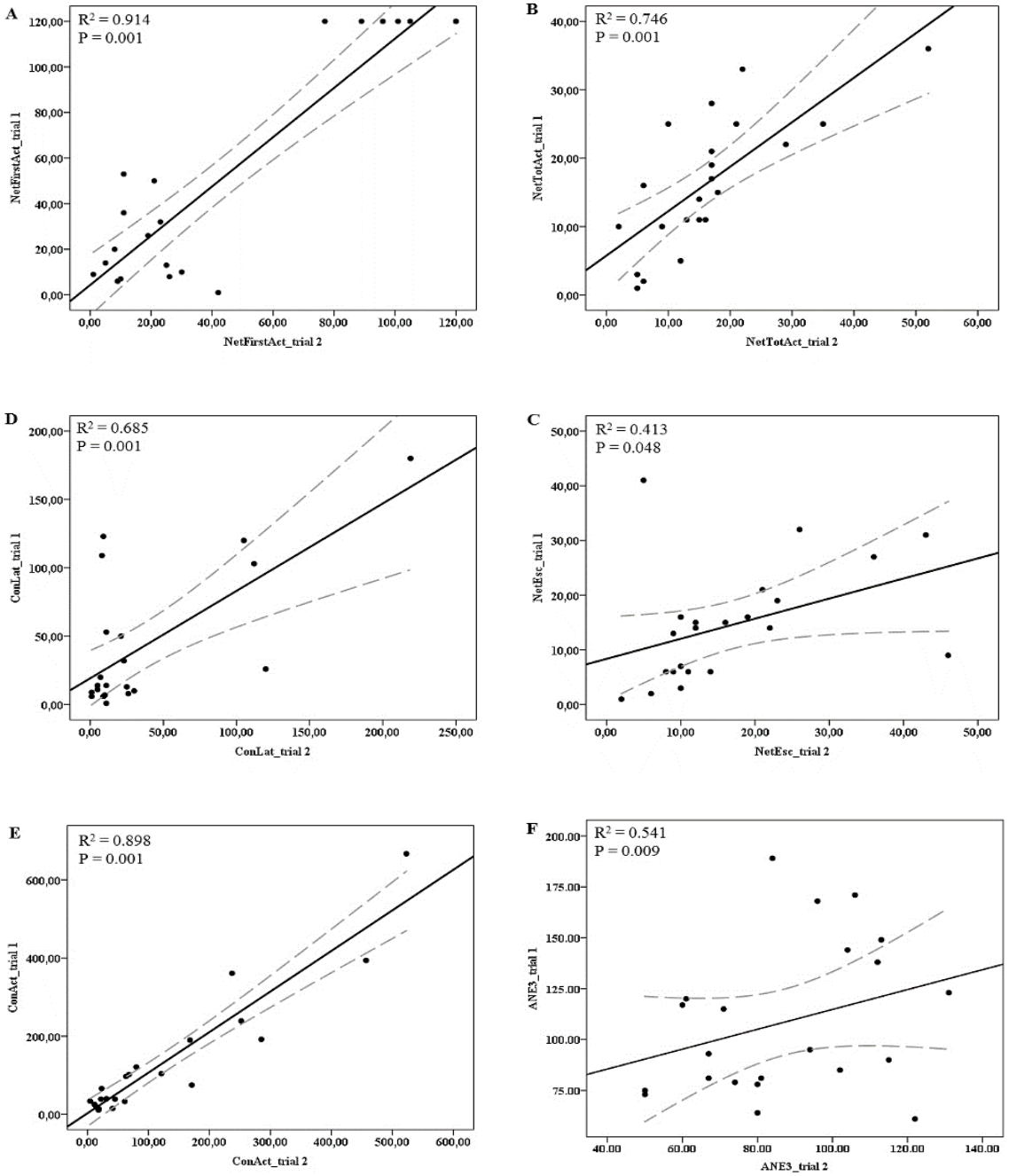

Figure 4. Significant correlations of gilthead seabream along the time (90 days). A = NetFirstAct, B = NetTotAct, C = NetEsc, D = ConLat, E = ConAct, and F = ANE3.

4. Discussion

Gilthead seabream breeders presented two constant behavioural responses in the different tests performed. Individual tests identified fish with high and low activity and induced different physiological changes, such patterns being consistent with proactive and reactive traits (Koolhaas et al., 1999; Réale et al., 2007; Toms et al., 2010). In addition, the risk taking test confirmed the behavioural differences between coping styles of fish that crossed, defined as proactive, and those that did not cross defined as reactive (Raoult et al., 2012; Castanheira et al., 2013; Herrera et al., 2014). The behavioural characteristics of proactive and reactive seabream were in agreement with those previously reported in the same fish species and in other species (Wilson and Godin, 2009; Toms et al., 2010; Castanheira et al., 2013; King et al., 2013; Herrera et al., 2014). Hence, individual behavioural differences observed in the different tests might represent non-adaptive variations and might have been caused by differences in many factors, such as neuroendocrine profiles, fitness, curiosity, dominance, motivation or genetic, as has been hypothesized in previous studies (Koolhaas et al., 1999; Wolf and Wessing, 2012; Mittelbach et al., 2014). However, this assumption should be further explored in this fish species.

Behavioural differences between breeders with high and low spawning participation and between males and females had been previously observed in different fish and mammal's species (Harris et al., 2010; Ariyomo and Watt, 2012; King et al., 2013). Theory predicts that behavioural differences between spawning success and sexes are expected *a priori*, since males and females have different life stories priorities, requirements and mating strategies. Hence, highly active or proactive individuals, females and males, are thought to be favoured for reproduction, since being proactive is an indicator of good genetic heritage and improved growth (Biro and Stamps, 2008; Sih and Bell, 2008). In relation to behavioural differences between sexes, hypotheses suggested that males possess higher physical fitness, consistent with proactiveness that allow them to forage more and to reproduce with a higher number of females during their life, while females spend less time foraging, being consistent with reactiveness, but maximize their physical fitness by increasing longevity to produce more offspring's (Biro and Stamps, 2008; Sih and Bell, 2008; King et al., 2013). However, according to Biro and Stamps (2008) and Mittelbach et al. (2014), the conservation of both coping styles strategies in fish is fundamental

because each trait represents different adaptive solutions to complex situations and promotes the cooperation between individuals. For example, some individuals are consistently risk-prone whereas others are consistently risk-averse. In this context, the present study demonstrated that proactive and reactive fish successfully contributed, but in different proportions, to spawning, being in agreement with the previous hypotheses. Other studies have indicated that a stable environment with low predation such as an aquaculture environment would favour a proactive strategy (Huntingford, 2004; Sih and Bell, 2008; Wolf and Wessing, 2012).

Lastly, seabream showed significant cross-context correlations between PCS 1 and 2, intra-individual behavioural repeatability and consistency in the restraining, confinement and anaesthesia (ANE3) tests over time. Therefore, coping style of seabream was suggested to be context-specific and predictable (Koolhaas et al., 2007; Wilson and Stevens, 2005). These results were similar to those reported in the same species and in other fish species such as the bluegill sunfish *Lepomis macrochirus* and seabass *Dicentrarchus labrax* (Wilson and Godin, 2009; Castanheira et al., 2013; Ferrari et al., 2015). Hence, the behavioural consistency over time and across contexts observed in the present work respond to the definition of stress coping styles (Koolhaas et al., 1999, 2007; Coopens et al., 2010) and reinforced their existence in this fish species.

5. Conclusions

Altogether, the present study confirmed for the first time that seabream breeders presented: i) defined proactive and reactive behaviours, ii) significant correlations between coping styles, blood parameters and spawning participation, iii) significant differences between males and females behaviour, with males presenting higher activity, reacting faster to novel situations and producing lower glucocorticoids levels than females and, iv) high behavioural consistency over time. We suggest to perform more analyses and to multiply contexts to reinforce the results of the present study.

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Chapter 7

Mating behaviour and gamete release in gilthead seabream (*Sparus aurata*, Linnaeus 1758) held in captivity

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Abstract

The present study aimed to describe the reproductive behaviour of gilthead seabream (*Sparus aurata*) in captivity. Twenty-four mature gilthead seabream, divided in two tanks, were utilized for the present study. Reproductive behaviour was recorded using submersibles cameras. A total of 67 spawning events were analysed. The mean duration time that gilthead seabream spent spawning was 54 ± 4 min per day, during which mean number of individual spawning events was 5.6 ± 0.2. The mean volume of eggs produced by both broodstocks was 405 ± 13.4 ml with a fertilization rate of 91.6 ± 0.4 %. The reproductive behaviour began with a schooling behaviour and then forming light aggregations. From an aggregation or an encounter while swimming freely a female initiated a spawning rush followed by one or more males to gametes liberation. The spawning rush was brief, 1.6 ± 0.5 seconds, over an approximately 1.7 ± 0.2 m distance from the tank bottom to the water surface. Pair spawning, between a single female and male, was the most common (71.6%). Group spawning was less common and involved a single female spawning with two males (22.5%) or three males (4.9%). Spawning rushes involving more than one female were not observed.

Keywords*: mate selection, pair group mass spawning, rush, aggregation*

1. Introduction

Gilthead seabream (*Sparus aurata*), from the Sparidae family, is one of the most extensively farmed fish species in the Mediterranean region. During the last two decades, many studies have described aspects of the biology of the species, including reproduction and genetics (Holland et al., 1998; Almansa et al., 1999; Meiri et al., 2004; Rosi et al., 2006; Arabaci et al., 2010 and Mylonas et al., 2011). Gilthead seabream is a protandrous hermaphrodite species with an asynchronous ovarian development (Zohar et al., 1995). Broodstock held in captivity under natural conditions typically start vitellogenesis in September-November, spawning begins during December-January and lasts for 3 – 5 months with daily spawning, leading to an annual fecundity of 2,000,000 eggs kg^{-1} (diameter < 1 mm) with a fertilization rate of 80-85% (Barbaro et al., 1997; Arabaci et al., 2010 and Mylonas et al., 2011). However, the reproductive behaviour of gilthead seabream has not been reported, despite an increasing need to understand the factors that influence a breeder's participation in spawning in order to control the families produced from a broodstock (Gorshkov et al., 1997, Brown et al., 2005; Porta et al., 2009 and Chavanne et al., 2012).

Reproductive behaviour has been described in some species of the family Sparidae, kept in captivity, including silver seabream (*Chrysophrys auratus*) (Smith, 1986 and Mylonas et al., 2011), santer seabream (*Cheimerius nufar*) (Buxton and Garratt, 1990 and Garratt, 1991), roman seabream (*Chrysoblephus laticeps*) (Buxton, 1990), silver bream (*Rhabdosargus sarba*) (Leu, 1994) and southern black bream (*Acanthopagrus butcheri*) (Mylonas et al., 2011). Although there was variation among species a general similarity was observed (see review in Mylonas et al., 2011). Spawning was usually early morning (dawn) or early evening (dusk) (06:00 and 19:00, respectively). Courtship consisted of males pursuing and nudging females, a tight circling swimming behaviour to form aggregations before spawning, which consisted of a spawning rush usually either to perform pair spawning involving a single pair (a male and a female) or group spawning a single female followed by multiple males (Smith, 1986; Buxton and Garratt, 1990; Garratt, 1991; Leu, 1994 and Mylonas et al., 2011).

A number of studies have examined gilthead seabream parental contribution to spawning events as there is a need to genetically improve cultured gilthead seabream to obtain

desirable traits such as faster growth that will reduce production costs (Gorshkov et al., 1997; Brown et al., 2005; Porta et al., 2009; Chavanne et al., 2012 and Duncan et al., 2013). Gilthead seabream spawning success was low when held in pairs (22% success) or groups of 15 females with a single male (44% success) and gilthead seabream were difficult to strip spawn for artificial fertilisation (Gorshkov et al., 1997). Different authors have concluded that large groups of breeders were required for successful spawning of gilthead seabream (Gorshkov et al., 1997 and Duncan et al., 2013) and Sparidae in general (Pankhurst, 1998 and Mylonas et al., 2011). Parental assignment of progeny using microsatellites identified that although large broodstocks produce large volume spawns to which many breeders contributed the participation of breeders was variable and a proportion of breeders did not participate in spawning (Brown et al., 2005; Porta et al., 2009 and Chavanne et al., 2012). Consequently, the effective spawning population size was reduced compared to the actual number of breeders in the broodstock, inbreeding was higher than expected and the families obtained were not predictable. Brown et al. (2005) and Chavanne et al. (2012) referred to gilthead seabream spawning behaviour as mass-spawning, which has been defined as "*spawning that consists of the great majority to all of an aggregation spawning simultaneously, as a single unit*" (Domeier and Colin 1997).

Therefore, there is a need to study the spawning behaviour of gilthead seabream to increase the understanding of spawning in Sparidae and to enable geneticists and broodstock managers to understand the parental contributions obtained for genetic improvement programmes. The aim of the present study was to investigate and describe the particularities of reproductive behaviour of the gilthead seabream in rearing conditions.

2. Materials and methods

2.1 Ethic statement

All the experimentation on fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee of IRTA.

2.2 Fish maintenance

Twenty four mature gilthead seabream (*Sparus aurata*) with a mean weight of 2.59 ± 0.15 kg and a length of 49 ± 4 cm were used for this study. Fish were pit-tagged for identification and divided among two 16.2 m^3 rectangular (6 x 3 x 0.9 m) fibreglass tanks (identified ahead as C1 and C2). Sex ratio per tank was 7 females and 5 males; a ratio biased to females is commonly used in the industry. Females were larger and older (mean weight: 2.91 ± 0.12 kg) than males (mean weight: 2.27 ± 0.17 kg), and this morphological difference was established as the main criteria to distinguish males from females in the video recordings.

Tanks were located outside in a green house structure covered with shade netting. Photoperiod was adjusted to follow the natural seasonal cycle by using two halogen white lights installed inside of each tank. Lights turned on-off in tanks with a photocell sensor. Water temperature and oxygen were maintained between 18 – 19°C and 5 - 6 mg/L, respectively. Fish were fed, *ad-libitum*, daily in the mornings (between 0900-1000 hours) with a commercial extruded balanced diet (Vitalis CAL-9, Skretting, Burgos, Spain).

2.4 Video and observations of the reproductive behaviour

Fish behaviour was recorded with four submersible black and white cameras (F60B/NIR580-50G model, Korea Technology Co. Ltd, supplied by Praentesis S.L., Barcelona) connected to a recorder (DVR- 0404HB model, Dahua Technology Co. Ltd, supplied by Praentesis S.L., Barcelona). Cameras were installed in each tank 5 cm under the water surface and adjusted to achieve a field of vision that covered more than 95% of the area and water column of the tanks.

The video recording was completed on different dates for both tanks as only one video recording system was available. Tank C1 behaviour was recorded from 10th to 24th January and from the 1st to the 4th February 2012; subsequently, tank C2 was recorded from the 5th to 14th February and from 30th of May to 7th of June 2012. The video recording program was daily starting at 0800 until 1300 hours in both tanks. This schedule was determined in relation to egg collection, generally collectors were observed to be empty at 0800 hours and after collection at 1300 hours no more eggs were collected until the following day after 0800 hours.

Focal animal observations of spawning behaviour and behaviour in general were made from the recorded videos following recommendations published by Altman (1974). A total of 67 spawning events were analysed. In tank C1, spawning observations corresponded to days 12th, 13th, 16th and 18th January and 1st - 2nd February, whilst in tank C2, observations corresponded to days 05th, 09th - 12th February and 30th May. The following types of behaviours and observations were described from the videos: **i)** pre-spawning interactions between individuals or in a group, **ii)** the behaviour directly associated with gamete liberation, **iii)** fish aggregation patterns and duration, **iv)** number of fish participating in each spawn (pair or group spawning) and the sex proportion per spawn, **v)** the frequency, duration and position of fish in tank when spawning and **vi)** estimation of the average distance (estimated from known distances between reference points in the tank) of the spawning rush. These parameters were selected in accordance to previous work realized on Sparidae species (Smith, 1986; Buxton and Garratt, 1990; Garratt, 1991; Leu, 1994 and Mylonas et al., 2011) and in particular terminology defined by Domeier and Colin (1997) was used to describe behaviours and actions. These included the following definitions of types of spawning from Domeier and Colin (1997) "*Pair spawning: spawning by a single male and single female. Group spawning: rush consisting of more than two fish, often many individuals. The group usually consists of a single female and multiple males. Mass spawning: a form of group spawning that consists of the great majority to all of an aggregation spawning simultaneously, as a single unit"*.

2.5 Eggs collection and evaluation

Egg collection was daily between 1130 - 1200 hours from both tanks. A 2 L measuring cylinder was used to measure the total volume of spawned eggs and the fertilization rate was determined by counting fertilized and unfertilized eggs from a sample of 50 eggs. Fertilized eggs were identified by observing cellular divisions, while unfertilized eggs did not present any cellular divisions. Likewise, the developmental stage of the embryonic phase of eggs was analyzed and established with accordance to Kamaci et al. (2005), in order to corroborate the estimates of spawning time obtained from videos with the developmental stage of eggs.

2.6 Statistics

All data were expressed in mean ± S.E.M. Student´s t- test was performed to compare different behavioural patterns between the two broodstocks (tank C1 and C2), such as the total number of aggregations prior a spawning, spawning duration, frequency of spawns per day, the distance displaced to spawn and the sex proportion per spawn. Pearson correlation test was performed between the number of daily events of gamete release and the volume of eggs collected. All the statistical analyses were conducted using SPSS software (Chicago, IL, USA) and a significant difference was considered when $P < 0.05$.

3. Results

3.1 Observations and description of the sea bream reproductive behaviour.

Based on the video-observations, the gilthead seabream reproductive behaviour was divided into two phases: the pre-spawning and the spawning behaviour. It was noted that seabream in the present study had a tendency to spawn daily in both tanks with close to all eggs being spawned between 0800 and 1100 hours. However, a small number of spawns were outside of these hours.

3.2 Pre-spawning behaviour

- ***Resting behaviour***

Resting behaviour was observed when lights in both tanks were turned on (on average activated by photocell sensor at 0830 hours). This behaviour was characterized with fish totally dispersed, without interactions and disaggregated around the tanks, and fish swam alone or in small groups around the tank without any specific direction or preference (Figure 1, Table 1).

- ***Schooling behaviour***

On average 42 ± 8 minutes after the lights were turned on, fish behaviour changed and fish started to form groups and swim together following one behind another from one side of the tank to the other. However, fish did not present any specific direction or preference, but always were swimming near the bottom of the tank. It was also observed that some fish maintained a reduced distance in relation to other fish and this included some fish touching or sneaking after each other (Figure 1, Table 1). This behaviour pattern was observed daily in both tanks for approximately ten minutes and prior to the aggregation

behaviour. However, no particular leading fish or inter-individual dominance between fish could be observed amongst the individuals of both tanks.

3.3 Spawning behaviour

- ***Aggregations and courtship behaviour***

Aggregations and courtship behaviour commenced when the whole group of breeders started to form aggregations near the bottom of the tanks (Figure 1, Table 1, Suppl. Video S1), being comparable to a "loose ball" and occasionally aggregations became tighter as the fish swam closer together; nonetheless, in the majority of the observations a "tight ball" of fish was not formed. Also, during this stage, males were observed to become slightly darker and occasionally males were rubbing and nudging (Figure 1, Table 1) some females close to the genital pore. The change in colour of males in addition to differences in size between males and females was also used to identify males. Territorial dominance or aggression amongst fish of the same or different sex was not observed.

In parallel to the aggregation behaviour, fish (males and females) initiated the courtship behaviour, which was mostly brief, and started when one of the females increased swimming speed at the bottom of the tank and slightly separate from the rest of the group, although on repeated occasions this was punctuated by immobile periods of the female in mid-water column and periods of circling aggregation behaviour as described above. After 10 -15 seconds of this behaviour, the female with one or more males was observed to dramatically increase swimming speed to initiate the spawning rush (Figure 1, Table 1). Aggregation and courtship behaviour ranged from 5 to 70 min to average 21 ± 4 min in both tanks.

- ***Spawning rush.***

The spawning rush was observed to follow when either i) the female started a circling behaviour followed by the male(s), which again produced the "loose ball" aggregation behaviour described previously, but the female would then exit from the group at speed or ii) after an apparently coincidental brief encounter with a male, the female dramatically increased swimming speed. This dramatic increase in swimming speed by the female was in all cases from low in the water column close to the bottom in a diagonal line towards the water surface. The female swam rapidly away from other fish followed by one or

more males (Figure 1 and 2; Table 1, Suppl. Video S1). Gamete release, egg and sperm, were synchronous at speed during the rush and in the top half of the water column.

The rush lasted in average 1.6 ± 0.5 seconds in both tanks (Figure 1). During this phase, the female was mostly swimming in a head-down position exposing her abdomen to males, which were always positioned beneath her and with the lead male snout close to or touching the abdomen and oviduct area of the female (Figure 2). In addition, males were observed to swim with open mouths at the moment of gametes liberation. Once gametes were released, the spawners separated and returned to swim around the tank to subsequently reintegrate into the group (Figure 1, Table 1) until the initiation of another spawning event.

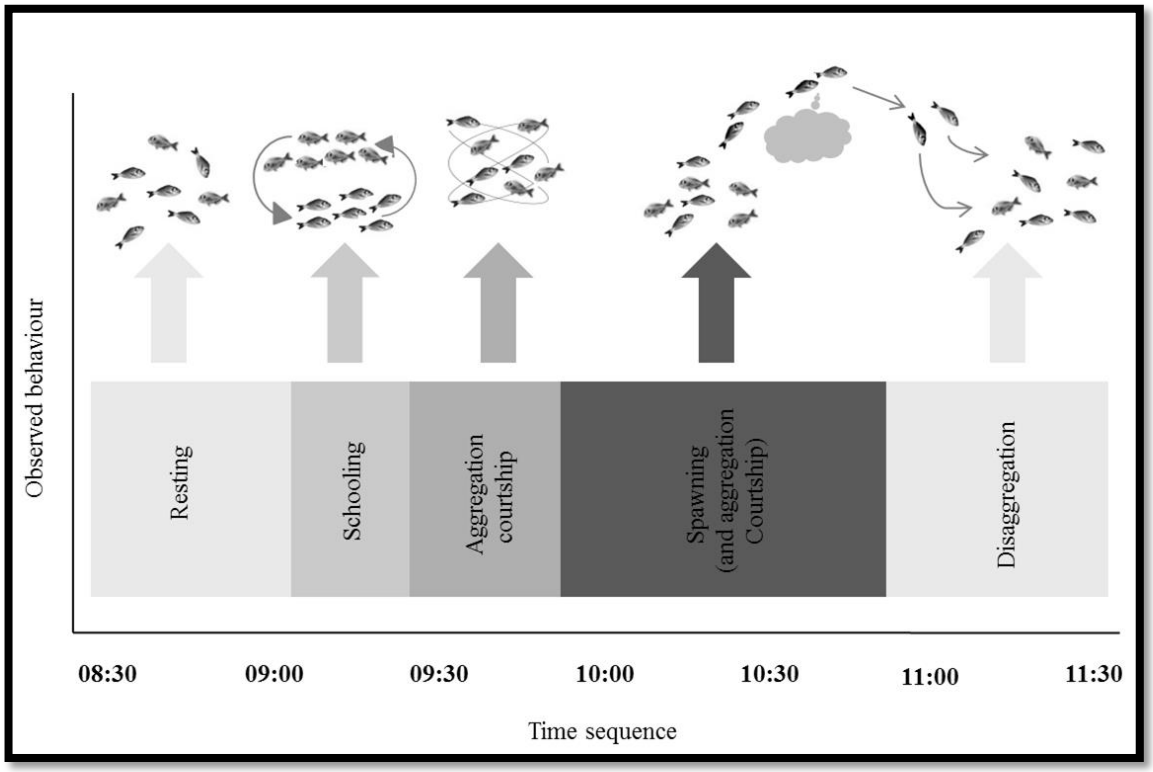

Figure 1. Average time periods of the different behavioural patterns observed in the gilthead seabream during the pre-spawning and the spawning events.

Spawning rushes involving more than one female were not observed in the present study. The presence of a second and a third male was observed on repeated occasions, although these second and third males were always behind the lead male during the spawning rush.

- ***Disggregation.***

The spawning behaviour (from the formation of the aggregation to the end of the spawning rush) ended when the group of fish disaggregated and returned to a resting behaviour, with fish dispersed, swimming in all directions and biting the floor as if they were looking for food (Figure 1). In addition all the fish in the group presented a similar colour and no dark males were observed, which also appeared to indicate the end of the spawning behaviour.

Table 1. Description of courtship and spawning behaviour for marine fish adapted for gilthead seabream (*Sparus aurata*) (modified from Erisma and Allen, 2006).

Behaviour	Description
Resting	Fish are disaggregate around the tank
Schooling	Breeders form groups, start to swim together with uniform movements
Rub-Nudge	Male approach gravid female and makes physical contact, with mouth, in the lower abdomen near the genital pore
Aggregation-courtship	Males swim near the females forming like a tight ball and made several contacts, this pattern commonly preceded the spawning rush of fish
Spawning rush	Female and a male separates from group and swim rapidly in a straight line while close together, male directly behind (at times touching) the female and oriented in the same manner. Rushes vary in direction from diagonally vertical (most common) to horizontal (rare); rush ends with synchronized gamete liberation, after which the fish separated

3.4 Spawning pattern in both broodstocks

- ***Aggregations and courtship behaviour.***

From the 67 spawning events observed, 35 corresponded to the breeders in tank C1 and 32 to the breeders in tank C2. The spawning rush initiated from a fish aggregation behaviour in 38 of the spawns (25 in tank C1 and 13 in tank C2,) and 29 events occurred without an aggregation immediately prior to spawning (10 corresponded to tank C1 and 19 in tank C2) (Table 2). Thirty four spawns occurred after the aggregation and courtship behaviour (described above, female increased swimming speed and momentarily froze) between a male and a female (20 in tank C1 and 14 in tank C2) and 33 occurred without this courtship behaviour immediately prior to the spawning rush (14 in tank C1 and 19 in

tank C2). No significant differences were observed between the two broodstocks groups in the number of spawns recorded with aggregations and without aggregations or courtships (Table 2).

Figure 2. Video captures of the gilthead seabream spawning rush. P shows two examples of a pair spawning with female followed by the male; G shows two examples of group spawning. The upper photo shows a female followed by two males and the lower photo a female followed by three males.

- ***Spawning duration and frequency of spawns per day.***

The duration and frequency of the spawning activity in the two broodstocks, kept in tank C1 and C2, were not significantly different. Fish spawning activity lasted in average 50 ± 4 and 57 ± 5 min in tank C1 and tank C2 (Table 2), respectively. The average numbers or frequency of spawns per day recorded were 5.83 ± 0.21 in tank C1 and 5.33 ± 0.32 in tank C2 (Table 2); and after completing the spawning rush breeders, from both tanks, returned back to the group.

- ***Distance of spawning rush and preferred area to spawn.***

The approximate distance displaced by broodstock in tank C1 (1.8 ± 0.2 m) to liberate the gametes during the spawning rush was no different from broodstock held in tank C2 (1.6

± 0.3 m) (Table 2). Also, on the 67 recorded spawning events it was observed that seabream spawned 39.8% in the water inlet area of the tanks, 37.4% in the middle part and, finally, 22.7% spawns occurred in the water outlet area of the tanks. It was also observed that seabream spawned in the majority of occasions near the water surface.

Table 2. Means values of different spawning patterns observed in the gilthead seabream.

Spawning parameter	Tank C1	Tank C2
Number of spawn with aggregations	25	13
Number of spawn without aggregations	10	19
Number of spawn with courtships	20	14
Number of spawn without courtships	14	19
Mean spawning activity per day (min)	50 ± 4	57 ± 5
Number of spawns per day	5.83 ± 0.21	5.33 ± 0.32
Mean distance displaced (m) per rush	1.8 ± 0.2	1.6 ± 0.3
Mean eggs volume (ml) spawned	343 ± 12	467 ± 15
Mean fertilisation rate (%)	95 ± 0.2	88 ± 0.5

- ***Sex proportion per spawn.***

All spawns were with the attendance of only one female with one or more males. A total of 50 pair spawns were recorded with one female and one male (27 corresponded to tank C1 and 23 in the tank C2). Group spawning was also observed, 13 spawning events were observed with one female and two males (8 in tank C1 and 5 in tank C2) and 4 spawns were with the presence of one female with three males (all 4 corresponded to broodstock held in tank C2) (Figure 3). No significant differences were observed between the proportion of pair and group spawning in the two broodstocks. The spawning by two females at the same time was not observed, no spawns were observed that involved more than one female and two or more females were not observed to spawn at same time in different spawning rushes in different areas of the tank. Mass spawning, including all individuals spawning as a single unit, was not observed during the present study.

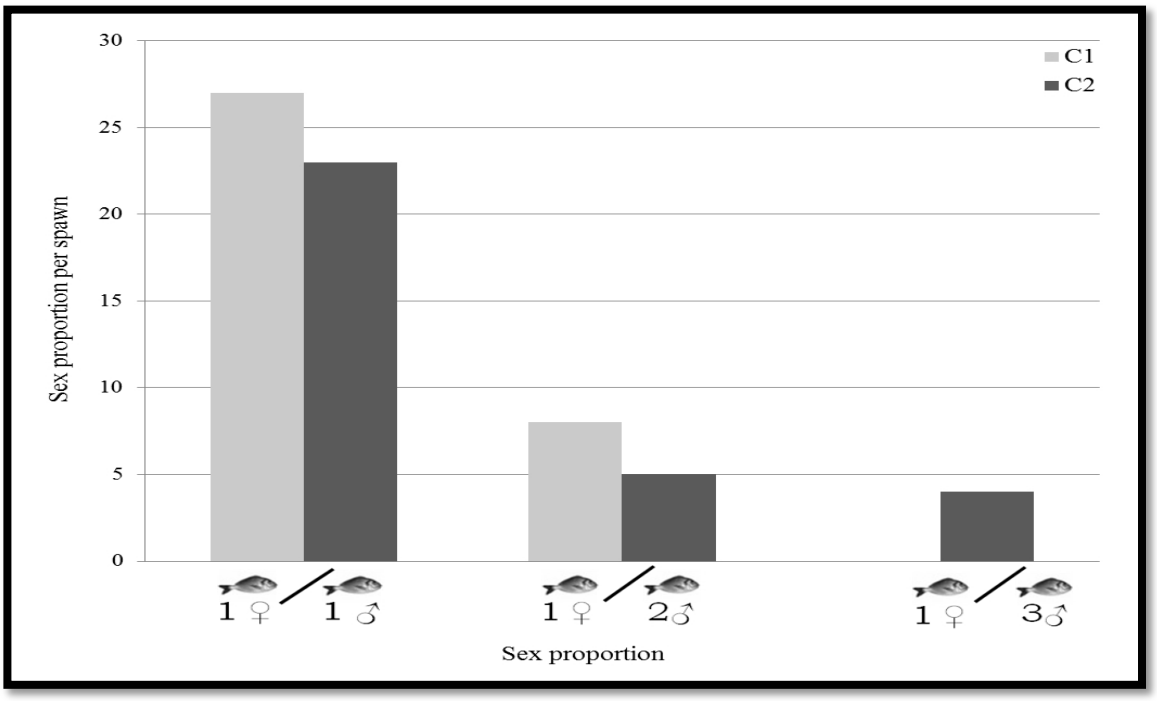

Figure 3. Sex proportion per spawning in the gilthead seabream (*Sparus aurata*) held incaptivity.

- ***Eggs volume, fertilization rate and developmental stage.***

Regular daily spawning began in both tanks in early January and spawning finished in June. The peak period of spawning in both tanks extended late January to mid-April and during the period 24 Jan to 15 April the mean volume of daily floating eggs were 434 ± 193 mL from tank C1 and 273 ± 155 mL from tank C2. On the days selected to analyse the spawning behaviour the breeders in tank C1 spawned a daily mean of 343 ± 12 mL and in tank C2 spawned 467 ± 15 mL (Table 2). No correlation was found between the number of spawning events per day and the volume of eggs collected (R = 0.2323, P > 0.221). Eggs collected from tank C1 presented a fertilization rate of 95 ± 0.2%, while in tank C2 the mean fertilization rate was 88 ± 0.5% (Table 2). The embryonic phase of development of the collected eggs were mainly between 2 - 32 cell division (phase 1A to 1E as defined by Kamaci *et al*., 2005); nonetheless, on occasions it was observed some eggs to be in morula or gastrula phase (1F and 1G) and these developmental phases corresponded to the timing of the observed spawning events.

4. Discussion

The present study described, for the first time, the reproductive behaviour of gilthead seabream (*Sparus aurata*) held in captivity. The reproductive behaviour was similar to that described for other Sparidae species (Smith, 1986; Buxton, 1990; Buxton and Garratt, 1990; Garratt, 1991; Leu, 1994 and Mylonas et al., 2011). Gilthead seabream were observed to form defined aggregations prior to the spawning event and females were observed to make a spawning rush with one or more males that finished with gamete liberation.

In accordance with Domeier and Colin (1997) the aggregation behaviour performed by fish was defined as a group of conspecific fish that gathered for the purpose of spawning, with fish densities or numbers significantly higher than those found in the area during the non reproductive period. In the present study, gilthead seabream aggregations were well defined, included the participation of all the stock and were clearly associated with spawning. The courtship behaviour of gilthead seabream was mostly brief and characterized by rapid forward swimming by females followed by one or more males. In addition, males displayed two characteristics: a colour change to become slightly darker and nudging and rubbing the female´s bellies close to the oviduct. The formation of aggregations and the courtship (changes in swimming speed, colour changes and nudging) appeared to offer the opportunity for mate selection and brought all the available individuals together for mate selection. Dichromatism (ability to take on one of two different colours patterns separately) was suggested to be a motivational factor for females to select males with better physical condition and social status (Kodric-Brown, 1998; Okumura et al., 2002; Kline et al., 2011). The action of rubbing and nudging was hypothesized to help males to perceive female pheromones, trigger the ovulation and induce the oocytes liberation (Domeier and Colin, 1997; Bond, 1996; Heyman, et al., 2005 and Stacey and Sorensen, 2008). In the present study, obvious behaviours associated with gaining dominance were not observed between males or males and females. However, a passive process of selection between males and females can be suggested as both observations of behavioural and morphological aspects appeared to offer opportunities for females to accept or reject advances from males. These indications that presented opportunities consisted of: a) spawning was often in a pair indicating the pair could select each other, b) aggregations brought all the fish together for close contact to

aid selection and spawning was often soon after an aggregation, c) males followed females perhaps seeking selection, d) males nudged females to possibly stimulate selection, e) females were observed to swim away from advances from males and f) males changed colour changing appearance to perhaps aid selection by the female. Aggregations and/or courtship behaviours similar to the present study have been described in other species of Sparidae including silver seabream (*Chrysophrys auratus*) (Smith, 1986; Mylonas et al., 2011), santer seabream (*Cheimerius nufar*) (Buxton and Garratt, 1990; Garratt, 1991), roman seabream (*Chrysoblephus laticeps*) (Buxton, 1990), silver bream (*Rhabdosargus sarba*) (Leu, 1994) and southern black bream (*Acanthopagrus butcheri*) (Mylonas et al., 2011) and non-Sparidae such as the spotted sand bass (*Paralabrax maculatofasciatus*) (Miller and Allen, 2006), yellowtail amberjack (*Seriola lalandi*) (Moran et al., 2007), dusky grouper (*Epinephelus marginatus*) (Zabala et al., 1997), cubera snapper (*Lutjanus cyanopterus*) (Heyman et al., 2005) and white seabass (*Atractoscion nobilis*) (Aalbers and Drawbridge, 2008).

However, in the present study, aggregations were not always observed immediately prior to gilthead seabream spawning and no inter-individual dominances were observed. Liberation of gametes was observed both in gilthead seabream coming from an aggregation (with or without courtship) and fish that had not participated in aggregation (or courtship) behaviour immediately prior to spawning. However, the importance of these social interactions (aggregations and courtship) during the spawning period should not be lessened by these observations. Gilthead seabream spawning success was low when held in pairs (Gorshkov et al., 1997; personal observation N. Duncan) or groups of 15 females with a single male (Gorshkov et al., 1997). Holding gilthead seabream in pairs or 15 females with a single male would be too few fish or the wrong sex ratios to enable the social interactions (aggregations and courtship) observed in the present study and this may explain the poor spawning success observed in gilthead seabream held in pairs or small groups (Gorshkov et al., 1997; Duncan et al., 2013). Various authors have suggested large groups of breeders were required for successful spawning of gilthead seabream (Gorshkov et al., 1997; Duncan et al., 2013) and Sparidae in general (Pankhurst, 1998; Mylonas et al., 2011).

In the present study, gilthead seabream made a spawning rush with a preference to rush and spawn as a pair and 71.6% of total spawns were observed to be between a single

female and male. However, gilthead seabream were also observed to group spawn when one female spawned with several males: two (22.5%) or three males (4.9%). Species from the Sparidae family all presented a spawning rush and different species presented pair or group or both types of spawning. The silver seabream (Smith, 1986; Mylonas et al., 2011) and santer seabream (Buxton and Garratt, 1990; Garratt, 1991), like the gilthead seabream presented both pair and group spawning. However, silver seabream were predominantly group spawners with one female being followed by many males (Smith, 1986; Mylonas et al., 2011), but pair spawning was observed on one occasion (Smith, 1986). Santer seabream pair spawned (Buxton and Garratt, 1990; Garratt, 1991) and the dominant male was aggressive towards other males, however, on occasions a "streaker" or "sneaker" male was observed to successfully participate in spawns by keeping to the opposite side of the female to the dominate male (Garratt, 1991). In the present study, no evidence of sneaker males was observed in gilthead seabream, although, when group spawning was observed there was always a lead male closest to the female followed by a second and less frequently a third male. The roman seabream (Buxton, 1990) and silver bream (Leu, 1994) were only observed to pair spawn and the southern black bream was only observed to group spawn (Mylonas et al., 2011). To date no Sparidae species has been observed to mass spawn and the observed pair and / or group spawning preceded by social interactions related to mate selection were characteristic of gilthead seabream and other Sparidae species.

Domeier and Colin (1997) defined a mass spawning as "*a form of group spawning that consists of the great majority to all of an aggregation spawning simultaneously, as a single unit*". Studies on parental assignment of progeny (Brown et al., 2005; Chavanne et al., 2012) have referred to gilthead seabream spawning behaviour as mass spawning. However, the present study found that gilthead seabream only participated in pair and group spawning in agreement with other studies on Sparidae species. Nevertheless, all these observations were made on fish held in captivity and no reports have been published on the reproductive behaviour of wild populations of Sparidae. Although to date no study on a Sparidae species has observed mass spawning this spawning type cannot be discounted as a possible spawning behaviour in Sparidae and gilthead seabream. Mass spawning reproductive behaviour has been documented in several marine fish species such as the *Lutjanus cyanopterus* (Heyman et al., 2005) and the *Dermatolepis dermatolepis* (Erisma et al., 2009). Both species were observed in natural conditions and

fish were described to release a massive cloud of gametes into the water column that made observation difficult. Females were, however, observed to exit from the mass spawning aggregations with accompanying males in examples of simultaneous group spawning. Therefore, the group spawning observed in Sparidae and the gilthead seabream could form part of mass spawning in different conditions. Domeier and Colin (1997) in an extensive review of aggregations and spawning type observed that species change spawning type in relation to the situation, with pair spawning more common in the absence of an aggregation and group spawning more common in aggregations and mass spawning was observed in some species to involve many incidents of simultaneous group spawning (as mentioned above). However, caution should be used in referring to a species such as gilthead seabream as mass spawning when only pair and group spawning has been observed.

Parental assignment of progeny also identified that the participation of gilthead seabream breeders was variable with a proportion of breeders that did not participate in spawning (Brown et al., 2005; Porta et al., 2009; Chavanne et al., 2012) and this variation or dominance by certain fish was particularly clear amongst male breeders (Brown et al., 2005). A similar situation was observed in the parental assignment of male cod breeders to progeny (Bekkevold et al., 2002) and this coupled with observations of cod reproductive behaviour (Brawn, 1961; Hutchings et al., 1999) suggested that cod males had reproductive hierarchies that explained the dominance of progeny by certain males (Bekkevold et al., 2002). A similar coupling of the present study on gilthead seabream spawning behaviour with studies on parental assignment of gilthead seabream progeny (Brown et al., 2005; Porta et al., 2009; Chavanne et al., 2012) also suggested the hypothesis that gilthead seabream had reproductive hierarchies that resulted in the dominance of progeny by certain breeders particularly amongst males. Chavanne et al. (2012) concluded that further research was required to understand the spawning kinetics of gilthead seabream. The present study, highlights that such studies need to also focus on spawning behaviour to understand why certain fish dominate spawning in relation to the spawning environment considering both physical (tank design, size) and social (characteristics of individuals, sex ratios, density) aspects. This, the first description of gilthead seabream spawning behaviour provides an important bases for these studies and for the first time researchers and broodstock managers can have a clear idea of the

spawning behaviour when considering physical and social manipulations to increase parental contribution for breeding programs.

In the present study, the spawning activity took place midmorning, which was actually initiated 42 ± 8 min after the lights switched on and can be considered similar to previous studies that established that gilthead seabream and others sparid fish such as silver seabream (*Sparus sarba*), Pacific seabream (*Acanthopagrus pacificus*), yellowfin bream (*Acanthopagrus australis*), red seabream (*Pagrus major*) and black bream (*Acanthopagrus butcheri*) tend to spawn at sunset or early in the morning (Pollock, 1982; Matsuyama et al., 1988; Mihelakakis and Kitajima, 1995; Haddy and Pankhurst, 1998; Meseguer et al., 2008; Sheaves and Molony, 2013). In the present study, spawning was successfully and regularly obtained and presented a prolonged spawning season (up to 5 months). Spawning was close to every day in both tanks. These observations were characteristic of this species, and in accordance with Zohar et al. (1995), Barbaro et al. (1997) and Arabaci et al. (2010).

5. Conclusions

The present study demonstrated that gilthead seabream spawning behaviour was similar to other sparids. In most occasions, spawns were observed to initiate in the morning hours and presented the characteristic to be associated with aggregation behaviour, followed by the spawning rush performed by a single female pursued by a male or, less common, by two or three males. Aggregation and courtship behaviour appeared to be an essential part of the spawning behaviour probably related to mate selection, highlighting the need to have a group of breeders and not single pairs. These findings described for the first time the characteristics of gilthead seabream reproductive behaviour and that many spawning events during a short space of time were involved in the production of a “spawn”. Altogether the study provided valuable information that may explain the uneven participation of breeders in studies that determined paternity of progeny with microsatellites and provides a solid basis for future work to increase parental contributions to breeding programs.

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Chapter **8**

General discussion and conclusions

1. Stress coping styles in Senegalese sole and gilthead seabream

The study of animal behaviour has become an interesting and fascinating field of research, since several studies have demonstrated that characters of animals are different between individuals within a same population. Moreover, behavioural strategies are associated with many ecological and biological relevant features (for specific details read Conrad et al., 2011; Mittelbach et al., 2014; Castanheira et al., 2015). Considering this variation in individual behaviour related to different strategies, **Chapters 2 to 6** contributed to the identification and characterization of stress coping styles behaviours in Senegalese sole (larvae, juveniles and breeders) and gilthead seabream (breeders) by considering three basic features associated with animal personalities: i) variation in behaviour among individuals of a same group or population (inter-individual consistency), ii) correlation between intra-individual behavioural traits across different contexts (intra-individual consistency across context), and iii) repeatability, consistency and correlation of intra-individual behaviour over different periods of time (intra-individual consistency across time) (Koolhaas et al., 1999 and 2007; Wolf and Weissing, 2010; Wolf and McNamara, 2012; Sih et al., 2015).

1.1. Tests performed to characterize coping styles in Senegalese sole and gilthead seabream.

On one hand, gilthead seabream breeders were submitted to a series of three individual-based tests (restraining, confinement and anaesthesia) and one group-based test (risk taking) that have been recognized as standardized coping styles tests in round fish species, since they represent and cover different behavioural contexts (Burns et al., 2008; Carter et al., 2013; Castanheira et al., 2015). On the other hand, Senegalese sole coping styles were assessed by means of three individual-based tests (restraining, new environment and confinement), which were selected from six individual-based tests, and one group-based test (risk taking test) that were chosen from two group-based tests. The three individual-based tests, selected in **Chapter 2**, were demonstrated for first time to successfully characterize coping styles behaviours of juveniles and breeders of Senegalese sole. Firstly, these three tests revealed intra-individual consistency over context in behaviours, since the behavioural responses at the same time of some individuals were, higher escapes attempts, lower latency to explore new contexts and higher total activity time in the tests restraining, new environment and confinement

(proactive) whilst the responses of other specimens were the opposite responses (low activity, low escape attempts and high latency) in all tests (reactive). Interestingly, behavioural responses of juveniles and breeders across these three tests were significantly similar and cross-context correlated, thus suggesting similar coping styles strategies or responses to stress (proactive vs. reactive) between Senegalese sole juveniles and breeders. Secondly, when these tests were reduced by a principal component analysis, results demonstrated two personality axis, categorized as **fearfulness-reactivity** (*e.g.* represented as fish reaction to aversive situations that resembled fear) and **exploration-activity** (*e.g.* represented as fish activity that resembled curiosity) behaviours. Indeed, both traits have been recognized to represent an important part of animal's personalities (Budaev, 1997; Budaev, 1998; Réale et al., 2007; Conrad et al., 2011; Castanheira et al., 2013). Thirdly, the selected tests (restraining, new environment and confinement) meet some basic criteria that could result attractive for the aquaculture industry, since they are easy to perform, consume relatively little time to be executed, do not require significant modifications of the rearing system, are logistically simple to execute, are simple in comparison to other tests that require complicated systems or analysis and can be applied on a large number of fish in a short time with immediate results. The study described in **Chapter 2** provided valuable information to the field of ethology, by demonstrating that individuals significantly differed in their patterns of habituations, reactions, motivations, which are reliable estimators of behavioural traits or stress coping styles (Burns, 2008; Beckman and Biro, 2010; Biro, 2012).

It is important to mention that coping styles characterization of Senegalese sole and gilthead seabream were performed by individual-based and group-based tests. Research on fish species is considering more and more often the evaluation of both individual and group behaviours to describe fish characters, since fish are often living in shoals and, thus, are considered as sociable. This specific behavioural trait related to the ecology of some fish species has been observed to influence some behavioural traits of fish (see Réale et al., 2007; Magnhansen and Bunnefeld, 2009; Huntingford et al., 2010; Castanheira et al., 2013). Hence, when evaluating behaviour in animals, it is important to consider that individual tests may not be representative of the overall behavioural characteristics of a population and may produce pseudoreplicacion of results. Moreover, the behavioural differences have been suggested to be altered depending on the size of the group (Hemelrijk and Wantia, 2005; Sumpter et al., 2008). Therefore, both types of

evaluations were considered in the present Thesis, in order to take into account confusing effects of social interactions on personality, since both Senegalese sole and gilthead seabream live communally in their natural environment.

1.2. Behavioural differences in Senegalese sole (larvae, juveniles and breeders) and gilthead seabream (breeders).

In both fish species, two groups of individuals were identified, with different behavioural responses that matched with proactive and reactive traits, respectively (fully described in **Chapters 3** to **6**). As initially expected, the proactive and reactive fish consistently differed in total activity, latency time to explore, glucocorticoids production and risk taking predisposition. Hence, those organisms that presented high levels of total activity, high willingness to explore, high risk taking predisposition and low glucocorticoids concentrations were characterized as proactive fish, while those fish with low total activity, low exploration and risk taking willingness and high glucocorticoids concentrations were labelled as reactive fish (Koolhaas et al., 1999, 2007; Øverli et al., 2007; Coopeens et al., 2010; Conrad et al., 2011). This characterization is in agreement with a number of studies performed on different fish species, such as cichlids, sticklebacks, salmonids, trout, sunfishes, killifish and other fish species (see Conrad et al., 2011; Castanheira et al., 2015; Sih et al., 2015) that considered the same individual differences in activity, exploration, risk taking and glucocorticoids production. Hence, reasonable explanations for the individual differences of coping styles observed in Senegalese sole and gilthead seabream might rely on fish genetic, flexibility, motivational state and on the immediate environmental stimuli (Øverli et al., 2007; Réale et al., 2007; Conrad et al., 2011; Sih et al., 2015). This means that one individual may consider a stressful situation as a highly traumatic situation, while another one may react to the same stressful situation in a more relaxed way.

In addition, the current investigation documented the existence of behavioural syndromes or temperaments in Senegalese sole and gilthead seabream (Sih et al., 2004; Réale et al., 2007). This assumption was deduced from the positive cross-context correlations observed between the principal component scores of restraining, new environment and confinement tests in both fish species. For example, **Chapter 4** showed that Senegalese sole juveniles and breeders with high number of escapes attempts and/or with high total

activity in the restraining test were also more active in the new environment and confinement tests. Many studies in other species recorded correlations between behaviours in different contexts (*e.g.* boldness with sociability or exploration with activity) and their results were in agreement with the present study (see Bell and Stamps, 2004; White et al., 2013; Ferrari et al., 2015). The reason why behavioural traits were consistent across different contexts is possibly due to their important ecological and evolutionary implications for the generation of trade-offs (Bell and Stamps, 2004). In other words, the concept of behavioural syndrome proposes that a behavioural trait presented by an individual in a specific context is coupled with similar behavioural traits in other contexts (Sih et al., 2004).

1.3. Repeatability and consistency of coping styles in Senegalese sole juveniles and breeders and gilthead seabream breeders.

Accumulating evidences from a wide variety of studies on behavioural traits suggested that some individuals are consistently more aggressive, more exploratory, or more proactive than other individuals and these differences in behaviours are often heritable (Van Oers et al., 2005; St-Cyr and Aubin-Horth, 2009). Up to now, still relatively little work has been completed to investigate the repeatability of behavioural strategies through different developmental stages or over different period of time (Cumming and Mollaghan, 2006; Chervet et al., 2011; Biro and Adriaenssens, 2013; Ferrari et al., 2015). Therefore, in the present thesis (**Chapter 3** and **6**) Senegalese sole juveniles and breeders and gilthead seabream breeders were demonstrated to show repeatable, consistent and cross-context correlated behaviours over time (90 days in seabream, 3 years in sole juveniles and 2 years in sole breeders). Indeed, the degrees of repeatability, consistency and correlation observed in both fish species were higher in comparison to studies evaluating repeatability and consistency in other fish species (Wilson and Godin, 2009; McGhee and Travis, 2010; Castanheira et al., 2013; Ferrari et al., 2015). This higher behavioural repeatability and consistency in Senegalese sole and gilthead seabream may be explained by the influence of the controlled rearing conditions on fish behaviour, which induced high levels of habituations and, possibly, at some point the tests might not represented a real challenge for fish. Nevertheless, this assumption should be confirmed by performing new tests favouring new behavioural challenges for this species (*e.g.* simulate predation, dominance, etc.). Interestingly, when inter-individual responses between Senegalese sole

juveniles and breeders were analysed more in detail, only two behavioural parameters were consistently repeated over time: total activity times in confinement and in a new environment. Therefore, it was concluded that Senegalese sole at different developmental stages differed in their behavioural responses. Regarding glucocorticoids, results demonstrated that blood parameters were consistently repeated and correlated in breeders, while in juveniles were correlated over time, but not intra-individual repeated (**chapter 3; section 3.1. and 3.2**). Such differences between distinct developmental stages of the same fish species may be interpreted as the result of fish adaptation, changes in fish life experience and metabolism over time and developmental stages. Firstly, juveniles are confronted with adaptation processes which are more important than breeders in order to survive, grow and reproduce, while breeders' behaviour is already established or "fixed" from their previous life experience and thus present more stability over time (Groothuis and Trillmich, 2011). Secondly, it has been argued that personalities are linked to life history traits and factors such as predation, novel environment, group linkage and reproduction may influence fish behaviour and fitness (Wolf and Weissing, 2012), Thirdly, possible differences in metabolism between juveniles and adults may lead to differences in behaviour, since a higher metabolism induces a higher foraging activity to find food and growth for juveniles than for breeders (Biro and Stamps, 2010). The present thesis confirmed, in **Chapters 3** and **6**, which Senegalese sole juveniles and breeders and gilthead seabream breeders behaviours were repeatable and consistent over time and correlated across different contexts. Indeed, the present results confirmed the existence of coping styles in both fish species, in accordance with the definition of Koolhaas et al. (1999). Further, these observations are in line with the hypotheses proposed by Conrad et al. (2011), whom stated that coping styles might have higher impact on individuals' life if personality traits are repeatable over certain periods of time.

Moreover, in **Chapter 3,** it was observed that Senegalese sole juveniles that started gametogenesis were demonstrated to present behaviours compatible with proactive coping strategies, while behaviours of juveniles without gametogenesis were more similar to reactiveness. Only a few studies have investigated whether coping styles match with early sexual maturation in fish and have suggested that fish presenting advanced physiological parameters tended to proactiveness, being in line with the findings of the present study (Biro and Stamps, 2004; Brelin, 2008; Edenbrow and Croft, 2011). A hypothesis to explain the present observations may be that sole juveniles with

gametogenesis probably had higher metabolic rate and, hence, high energy requirement for high growth ("fast life") than fish with no gonadal development ("slow life") and their proactive behaviour strove for optimal growth and development. Indeed, these proactive fish that matured earlier were significantly larger than reactive fish that did not mature. According to Réale et al. (2010), such differences might be interpreted as different pace-of-syndrome, which illustrate the potential integration of behavioural and physiological traits of individuals within a same population along two continuums, being "slow" and "fast" life strategies (Figure 1). For instance, proactiveness is positively correlated to high growth rates and accelerated sexual maturity in Atlantic silverside (*Menidia menidia*; Walsh et al. 2006).

This study highlighted a highly significant degree of intra-individual repeatability, consistency and correlation of behaviours of Senegalese sole juveniles and breeders and gilthead seabream breeders across different individual-based and group-based coping style tests and over time. This investigation may contribute to the selection of animals with certain kinds of behavioural traits at juvenile and adult developmental stages, thanks to their higher predictability over long time periods.

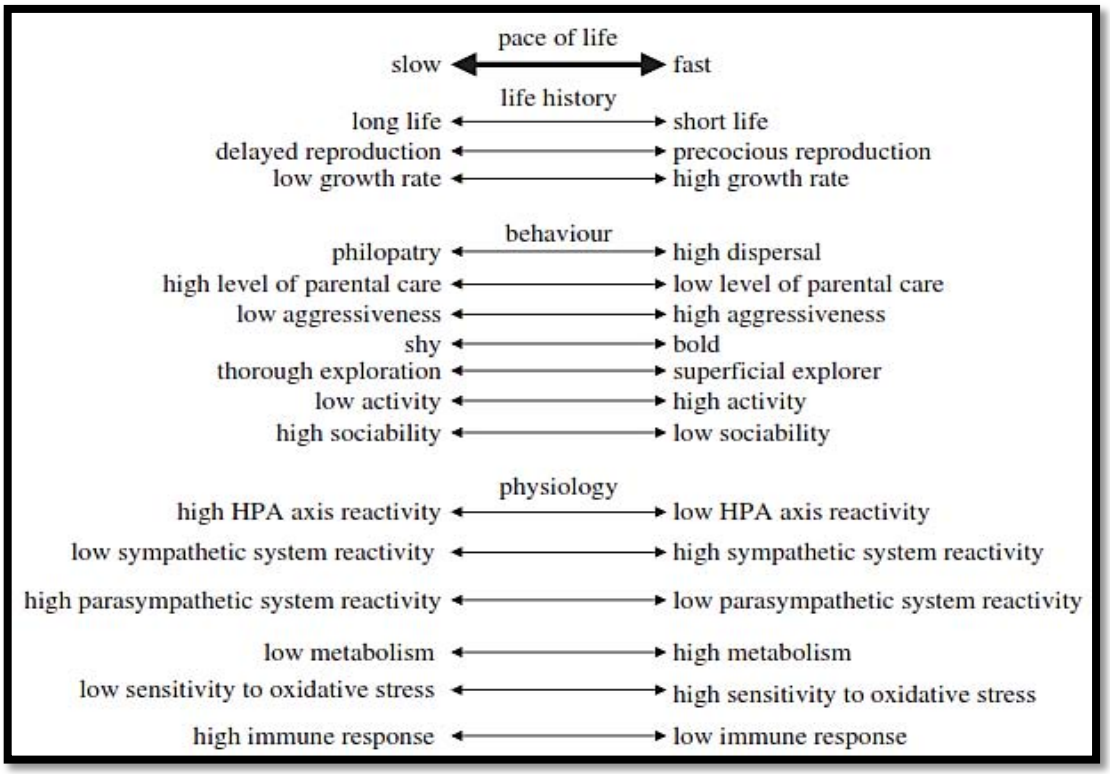

Figure 1. Implications of presenting a fast (proactive) or low (reactive) life span in individuals of the same population (developed by Réale et al., 2010).

1.4. Impact of stress coping styles on reproductive success, sex and origin of Senegalese sole breeders and gilthead seabream breeders.

Several authors have demonstrated that coping styles behaviours were significantly related to reproductive success, sex, origin, and other physiological factors in many fish species (Piyapong et al., 2010; Schuett et al., 2010; King et al., 2013). The behavioural responses of Senegalese sole breeders reared in IRTA and IEO (**Chapter 4**) and of gilthead seabream breeders reared in IRTA (**Chapter 6**) were analysed in relation to their reproductive success (whether they contributed to spawns or not), sex (males or females) and origin (hatchery-reared fish or wild, only for Senegalese sole). Significant differences were observed between the behavioural tactics used by each fish species. On one hand, gilthead seabream showed high relationships between coping styles and reproductive success and between coping styles and sex. Concretely, when grouped by either seabream breeders that presented a higher participation in spawning events or males, these groups showed higher overall activity in restraining and confinement tests, resumed activity earlier in the confinement test, took higher risk in the risk taking test and exhibited lower glucocorticoids levels than fish with a low spawning participation and than females, respectively. On the contrary, two statistical approaches established on behavioural data from Senegalese sole breeders showed that reproductive success, sex and origin were not significantly biased to any specific stress response (proactive/reactive) in this species. Further, breeders with different reproductive success, sex and origin were documented to present similar behavioural tendencies. However, breeders from IRTA were observed to show different behavioural tendencies than breeders from IEO (see details of the analysis in **Chapter 4**).

Considering those results for both fish species, behavioural responses of gilthead seabream were more in agreement with those theories postulating that reproductive success and sexual dimorphism are strongly influenced by personality or coping styles than those of Senegalese sole. Actually, some authors demonstrated and confirmed that proactive behaviours were indicative of improved genetic selection, higher growth and resistance to diseases and enhanced aptitudes to survive in unknown contexts in several animal taxa (Biro and Stamps, 2008; Sih and Bell, 2008; Schuett et al., 2010). Consequently, both females and males with proactive behaviours seem to be favoured to reproduce compared with both females and males with reactive coping styles, as it has

been observed in zebrafish (Ariyomo and Watt, 2012), guppies (Piyapong et al., 2010) and sticklebacks (King et al., 2013). However, it was also demonstrated that highly proactive individuals might have reduced opportunities to reproduce, because of high aggression being shown by proactive fish towards potential mates (Sih and Watters, 2005; Smith and Blumstein, 2008). Regarding behavioural variations between gilthead seabream sexes, some authors have proposed that females and males significantly differ in their behaviours because of selection pressures. In other words, females and males are submitted to different environmental pressures and present distinct behavioural tactics in response to stress (*e.g.* aggression, dominance, foraging, reproduction efforts, etc.) leading them to exhibit different behavioural strategies (Biro and Stamps, 2008).

Contrary to gilthead seabream, reproductive success, sex and origin were not significantly related to proactive and reactive behaviours in Senegalese sole breeders. These results led to formulate the following hypothesis: behaviours in Senegalese sole may be influenced by group, since this is a social fish species with high interactions between individuals (Ibarra-Zatarain and Duncan, personal observations). Social animals generally tend to present some forms of cooperation to gain resources and make their reproduction more successful by developing synchronised behaviours and forming coalitions to improve males fitness to attract a higher number of females (Wey et al., 2018; Budaev and Brown, 2011; Díaz-Muñoz et al., 2014). Indeed, it is highlighted for a long time that variability of physiological traits among individuals within a population, such as growth rate and reproduction, is able to increase the stability, persistence and resistance to extinction of the population (Wilson et al., 1993; Dall et al., 2004; Budaev and Brown, 2011). Regarding the effect of the origin of Senegalese sole breeders on their behavioural characteristics, results indicated that hatchery-reared breeders did not differ from wild breeders in their behavioural responses across the different coping styles tests performed. Therefore, in the present study, hatchery-reared and wild breeders were assumed to present similar coping styles responses, being in disagreement with other studies on brown trout, European seabass (*Dicentrarchus labrax*) and other species that demonstrated cultured fish usually develop proactive strategies, while wild individuals are prone to reactiveness (Metcalfe et al., 2003; Huntingford, 2004; Huntingford and Adams, 2005; Adriaenssens and Johnson, 2011; Benhaïm et al., 2012). Three hypotheses may be proposed to explain these similarities of behaviours between hatchery-reared and wild Senegalese sole: i) the time fish spent in captivity (fish may have acquired a strong

adaptation to confinement situations, as individuals were used to human manipulation, and may not consider coping styles tests as challenging as was expected), ii) the influence of the group (fish may have learn from their co-specifics, providing them higher abilities to rapidly adapt their behaviour in different stressful situations), and iii) the genetic of organisms were similar (hatchery-reared breeders were from the first generation born in captivity from wild parents, so their genetic profiles were probably still highly similar to their wild counterparts). Nonetheless, more studies should be performed in this field in order to reinforce the results of the present investigation to generate understandable bases justifying these assumptions.

1.5. Coping styles behaviours in Senegalese sole at early ontogenesis and the influence of the diet.

Among various factors assumed to influence larval survival, growth, quality and ultimately, behaviours, the importance of early larval nutrition, especially dietary lipids and essential fatty acids (EFA), have been highlighted in many studies (Sargent et al., 2002; Lund et al., 2012). In **Chapter 5,** coping styles of Senegalese sole post-larvae (40 days post-hatch) were characterised for four different groups that each received a different enriched diet of *Artemia*. Senegalese sole larvae at early ontogenesis showed several behavioural strategies that were relevant to coping styles and differs depending on the dietary treatment. Indeed, larvae fed with *Artemia* metanauplii enriched with the cod liver emulsion were significantly larger and presented significantly higher total activity time and total distance moved in the individual-based test than larvae fed with *Artemia* metanauplii enriched with vegetable emulsions (soybean, olive and linseed oils), being a higher proportion of behaviours consistent with proactive coping style, while the vegetable enrichments induced a higher proportion of behaviours consistent with reactive traits. Only few studies have investigated the effect of diets on coping styles of fish larvae, but those available demonstrated that diets significantly influenced fish responses to stress (*e.g.* escape ability, cortisol production) at different developmental stages (Benítez-Santana et al., 2007; Lund et al., 2012), being in line with the results of the present investigation.

Besides, the fatty acid composition of larvae fed with *Artemia* metanauplii enriched with the cod liver oil showed higher amounts of essential fatty acids, such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids, which are essential nutrients

required for suitable organogenesis, especially for brain, eyes and nervous system development, and represent the main energy substrates for metabolism, growth, and development. According to Øverli et al. (2007) and Careau and Garland (2012), a proper organogenesis together with increased levels of energy, which may be reached by the diet, result in different behavioural traits. Therefore, these nutritional differences among dietary treatments were suggested to possibly explain the variability of proactive and reactive behaviours in Senegalese sole post-larvae, since larval nutritional status may affect metabolic rates, physiology, growth and development, resulting in variations in physical condition, activity and ultimately, in personality of the larvae.

The present study showed that Senegalese sole post-larvae presented defined proactive-reactive behaviours, and that dietary fatty acid composition significantly influenced stress coping styles in sole larvae. Current results might have practical implications that open the possibility to select and produce organisms with certain kinds of behavioural styles in order to improve aquaculture productivity.

2. Gilthead seabream spawning behaviour

Spawning refers to the process of releasing eggs and sperm in fish. The process of spawning typically involves females releasing ova (unfertilized eggs) into the water, often in large quantities, while male(s) simultaneously or sequentially releases spermatozoa (milt) to fertilize the eggs. There are many variations in the way spawning occurs, depending on anatomical differences between sexes, on how both sexes are related to each other, on where and how spawning is performed, and on whether or how eggs are subsequently guarded or incubated (Duncan et al., 2013). **Chapter 7** described the spawning behaviour of gilthead seabream in rearing conditions. The key results of this study of seabream breeders were the observation of two defined patterns prior to spawning: a) the pre-spawning behaviour, which was characterized by a resting behaviour (*e.g.* fish totally dispersed around the tank) and a schooling behaviour (*e.g.* fish swam together as a group around the tank), and b) the spawning behaviour itself, in which fish formed aggregations and courtships (*e.g.* fish formed aggregations as a “tights balls” and males started to swim close to females), executed the spawning rush (*e.g.* couple swam together toward the water surface to liberate gametes) and ended with a disaggregation (*e.g.* fish separated and came back to a resting behaviour). Aggregation and courtship

behaviours, inherently related to the spawning process, appeared to represent the opportunity for mate choice, since all the individuals were brought together for mate selection and reproduction (Domeier and Colin, 1997; Domeier, 2012). Alternatively, males (generally smaller than females) tended to change their colour and were observed to rub and nudge females' bellies during courtship. This ability, to take on one or different colour patterns separately (dichromatism), have been suggested to be a motivational factor for females to select males with improved physical condition, social status and possibly genetic improvement (Kodric-Brown, 1998; Kline et al., 2011; Colihueque and Araneda, 2014). The pre-spawning and spawning behaviours (with their specific patterns) observed in gilthead seabream were analogous to those described in other Sparidae species (Garratt, 1991; Leu, 1994; Mylonas et al., 2011) and non-Sparidae, such as the cod *Gadus callaris* (Brawn, 1961), cubera snapper *Lutjanus cyanopterus* (Heyman et al., 2005), Atlantic salmon *Salmo salar* (Webb and Hawkins, 1989) and leopard grouper *Mycteroperca rosacea* (Erisman et al., 2007).

Additionally, gilthead seabream breeders showed a tendency to spawn in a pair (71.6 %) and group spawning was less common (28.4%). Pair spawning seems to be a common tactic used by a number of fishes, in rearing conditions and in wild populations, in order to insure fertilization and to maximize reproductive investment (Brawn, 1961, Donaldson, 1989; Leu, 1994; Heyman et al., 2005; Mylonas et al., 2011). Another criteria that probably influenced this pair spawning pattern was the density of breeders stocked per tank. According to McCormick et al. (2010), low densities reduced levels of aggression between males, and improve the probability for females to choose a male. Though, females and males can modify their spawning modes (pair or group spawning) depending on environmental situations and food availability. Alternatively, during spawning seabream did not show obvious behaviours associated with gaining dominance or aggression between males or between males and females. However, a passive process of selection between males and females may be proposed as observations of both behavioural (*e.g.* chasing, aggregating, nudging) and morphological (*e.g.* dichromatism, size, fitness) aspects appeared to offer opportunities for females to accept or reject advances from males. These opportunities consisted of: a) spawning was often performed in pair indicating that the pair was able to select each other, b) aggregations brought all fish together for a closer contact to aid selection and spawning often occurred just after an aggregation, c) males followed females probably in order to select a mate, d) males

nudged females possibly in order to enhance the selection, and e) males changed colour, thus changing appearance possibly in order to be selected by females.

Parental assignment of progeny identified that the involvement in spawning of gilthead seabream breeders was diverse with a proportion of breeders that never participated in spawning (Brown et al., 2005; Porta et al., 2009; Chavanne et al., 2012), thus showing possible hierarchies of a group of males on others (Brown et al., 2005). A similar situation was observed in the parental assignment of progeny in males of cod breeders (Bekkevold et al., 2002) and these results coupled with observations on cod reproductive behaviour (Hutchings et al., 1999) suggested that cod males presented reproductive hierarchies that explained the dominance of progeny by certain males (Bekkevold et al., 2002). Therefore, behaviours or personalities, such as coping styles, may be considered to play some specific roles in mate selection and reproduction in this fish species (discussed in section **1.4.**), as it has been postulated in a number of other fish species, such as guppies (Godin and Dugatkin, 1996), salmon (Cook et al., 2011), zebrafish (Ariyomo and Watt, 2012) and Siamese fighting fish (Dzieweczynski et al., 2014). Indeed, the operational sex ratios, defined as the total number of sexually active males divided by the total number of sexually active adults of both sexes (reviewed in Kvarnemo and Ahnesjo 1996), is known to affect the mating behaviour (Kvarnemo and Ahnesjo 1996; Magellan and Magurran, 2007). When one sex becomes a limiting resource for the other, members of the available sex are compelled to modify their behaviour for mates of the limiting sex to have access to reproduction and are therefore under a greater sexual selection (Emlen and Oring 1977). Nevertheless, these assumptions might be tested on gilthead seabream breeders in order to improve the present results and improve the knowledge of the reproductive behaviour of this valuable fish species.

The present findings described for the first time the characteristics of gilthead seabream reproductive behaviour and established that many spawning events during a short period of time were involved in a spawn, in its broadest sense. Understanding the spawning behaviour of this species from an ecological point of view may be valuable for the management and conservation of wild populations, since the consumption of this important fishery species is increasing in different countries and this is accelerating the reduction of fishery stocks. For the aquaculture industry, this study provided valuable information and probable explanations for the absence of participation of some breeders

in the reproduction (paternity of progeny with microsatellites) and a solid basis for future works to improve parental contributions in new breeding programs.

3. Senegalese sole key observations

One of the main objectives of the present thesis was to evaluate if SCS influenced the reproductive success in Senegalese sole, and particularly to demonstrate if G1 males showed distinct SCS from wild counterparts that may explain why G1 males do not exhibit reproductive behaviour. General results demonstrated that Senegalese sole that successfully spawned presented similar behavioural styles to those fish that did not spawn (**chapter 4**). In other words, fish that spawned and did not spawn were not predisposed to any proactive nor reactive SCS. Similarly, stress coping style of hatchery breeders was comparable to wild breeders, indicating that both fish groups used the same coping strategies to face stressful situations. Therefore, it seemed that reproductive dysfunction of G1 males was not completely influenced by SCS as it had been initially hypothesized. Different possible explanations were proposed for this issue (**chapter 4**). However, two interesting approaches, which might be further considered and evaluated, were the *aggression* and the *sociability*. According to Budaev (1998, 1999), Smith and Blumstein (2008) and Conrad et al. (2011), both factors are axis of personality that are highly linked to fish reproduction and fitness. Senegalese sole is considered as a non-aggressive and a sociable species with high interactions between individuals. Hence, performing new tests that consider *aggression* and *sociability* factors might increase the knowledge on the relationship between SCS and reproduction for this species. Remarkably, Senegalese sole at puberty (fish starting gametogenesis) were observed to present personalities consistent with proactive traits, while those fish that did not start gonadal development yet showed traits consistent with reactive behaviours (**chapter 3**). This finding have demonstrated that (a) G1 males started gametogenesis and produced sperm in captivity and (b) coping styles are of great importance at this age, since proactive fish were those with motile sperm. Therefore, it is highly recommendable performing new studies that include the establishment of selected lines (based in their coping styles), since it may represent an alternative to explains the reproductive dysfunction of G1 males breeders.

This was one of the first studies that demonstrated that SCS differed in Senegalese sole at early stages of gonadal maturation and opened a “new area” of investigation that may help to find an explanation for the reproduction failure of this species. The overall

findings of the present Thesis provided solid bases to characterize and understand Senegalese sole behaviour. In addition, current results may be of great interest for aquaculture industry, since Senegalese sole SCS were described at early ontogenesis, at puberty and at adult stage.

4. Conclusions

- Senegalese sole (larvae, juveniles and breeders) and gilthead seabream (breeders) showed defined proactive and reactive coping styles behaviours. Further, proactive fish were characterized by some or all of the following charateristics higher activity, lower latency to explore novel contexts, higher risk disposition and lower glucocorticoids levels in comparison to reactive individuals.

- Three individual-based coping styles tests were demonstrated to be reliable and to easily characterize Senegalese sole juveniles and breeders stress coping styles behaviours: restraining, new environment and confinement tests. These tests have been demonstrated to be easy to achieve in aquaculture farms by non-specialized staff or laypeople and can be performed on a large number of fish in a relative short time period.

- Coping styles of both Senegalese sole (larvae, juveniles and breeders) and gilthead seabream (breeders) presented a high degree of predictability, since their behavioural responses, observed across some different tests (context) and/or over time, were significantly and highly repeatable, consistent and correlated.

- Reproductive success (spawn / not spawn), sex (males / females) and origin (hatchery / wild) of Senegalese sole were not biased to proactive or reactive stress coping styles.

- In gilthead seabream, proactive coping styles were associated with the reproductive success and coping styles of males were consistent with proactive behaviours and females were consistent with reactive strategies.

- Senegalese sole juveniles that reached gametogenesis presented behaviours consistent with proactive strategies, while juveniles without gametogenesis tended to be consistent with reactive coping styles.

- G1 male reproductive dyfuncion appeared to not be influenced by stress coping style, since behaviours were similar wild breeders in general and wild breeders that spawned.

- In comparison to vegetables oils, used as live prey enrichments, cod liver oil induced higher number of proactive individuals in Senegalese sole post-larvae.

- Gilthead seabream presented a mating pattern characterized by two distinctive behaviours, defined as pre-spawning and spawning behaviours, including formation of coordinated swimming behaviours (schooling) until the release of gametes into the water (spawning rush).

- Spawns in gilthead seabream commonly involved one female and one male (pair-spawning: 71.6%) and less commonly one female and multiple males (group-spawning: 28.4%), suggesting the proclivity to monogamy.

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Annexes

1. List of accepted publications (Peer-reviewed journals)

Ibarra-Zatarain Z and Duncan N, 2015. Mating behaviour and gamete release in gilthead seabream (*Sparus aurata*, Linnaeus 1758) held in captivity. Span. J. Agric. Res. 13, e04-001, 11 pages

Ibarra-Zatarain Z, Morais S, Bonacic K, Campoverde C and Duncan N, 2015. Dietary fatty acid composition significantly influenced the proactive-reactive behaviour of Senegalese sole (*Solea senegalensis*) post-larvae. App. Anim. Behav. Sci. doi: 10.1016/j.applanim.2015.08.007

2. Ongoing publications (Peer-reviewed journals)

Ibarra-Zatarain Z, Parati K, Cenadelli S, Duncan N. Exploring coping style behaviours in gilthead seabream (*Sparus aurata*) breeders reared in captivity and their implications on fitness. In review

Ibarra-Zatarain Z, Fatsini E, Rey S, Chereguini O, Martin I, Rasines I, Alcaraz C and Duncan N. Characterization of stress coping style in Senegalese sole (*Solea senegalensis*) juveniles and breeders for aquaculture. In review

Ibarra-Zatarain Z, Fatsini E, Chereguini O, Martin I, Rasines I and Duncan N. Exploring the relationship between stress coping styles and reproductive success, sex and origin in Senegalese sole (*Solea senegalensis*) breeders held in captivity. In process

Ibarra-Zatarain Z, Fatsini E and Duncan N. Senegalese sole (*Solea senegalensis*) show repeatable and consistent behavioural responses across different contexts and over considerable long time periods. In process

Participation in professional meetings

1. **Ibarra-Zatarain, Z** and Duncan, N. Spawning behaviour of gilthead seabream (*Sparus aurata*) reared in captivity. The Physiology of Fish Behaviour Symposium. Norwich, United Kingdom. 9th – 13th July, 2012. Oral presentation

2. Duncan, N, **Ibarra-Zatarain, Z**, Valles, R. and Fatsini, E. Does the variation in reproductive success of marine fish held in captivity need tob e considering when assessing gamete quality? 4th International Workshop on Biology of Fish Gametes. Faro, Portugal. 17th – 20th September, 2013. Oral presentation

3. **Ibarra-Zatarain Z**, Fatsini E, Rey S, Chereguini O, Rasines I and Duncan N. Preliminary results on the influence of stress coping styles on spawning success in Senegalese sole (*Solea senegalensis*) broodstock held in captivity. 4th International Workshop on Biology of Fish Gametes. Faro, Portugal. 17th – 20th September, 2013. Poster

4. Fatsini E, **Ibarra-Zatarain Z** and Duncan N. Análisis morfológico de las rosetas olfativas (dorsal y ventral) del lenguado senegalés (*Solea senegalensis*). XIV Congreso Nacional de Acuicultura. Gijón, España. 23rd – 25th September, 2013. Poster

5. **Ibarra-Zatarain Z**, Fatsini E, Rey S, Chereguini O, Rasines I. and Duncan N. Descripción y selección de pruebas para identificar los tipos de afrontamiento al estrés de reproductores del lenguado senegalés (*Solea senegalensis*). XIV Congreso Nacional de Acuicultura. Gijón, España. 23rd – 25th September, 2013. Oral presentation

6. Fatsini E, **Ibarra-Zatarain Z** and Duncan, N. Observations of the courtship of mixed wild and captivity breed Senegalese sole (*Solea senegalensis*) broodstock. 10th International Symposium on Reproductive Physiology of Fish. Olhao, Portugal. 25th – 30th May 2014. Poster

7. **Ibarra-Zatarain Z**, Fatsini E, Chereguini O, Rasines I, Martin I, Rey S and Duncan N. Study of behavioural profiles in Senegalese sole (*Solea senegalensis*) broodstock to characterise stress coping styles in relation to reproductive success. 10th International Symposium on Reproductive Physiology of Fish. Olhao, Portugal. 25th – 30th May 2014. Oral presentation

8. **Ibarra-Zatarain Z**, Morais S, Bonacic K, Campoverde C and Duncan N. Dietary fatty acid composition influences the stress coping style behaviour of Senegalese ole (*Solea senegalensis*) pos-larvae and has implications for aquaculture development. XI Conference of Ecology and Behaviour Conference. Toulouse, France. 18th – 21th May, 2015. Oral presentation

9. **Ibarra-Zatarain Z**, Parati K, Cedanelli S and Duncan N. Los estilos de afrontamiento al éstres y su vinculo con el éxito reproductivo en la dorada (*Sparus aurata*). XV Congreso Nacional de Acuicultura. Huelva, España. 13th – 16th October, 2015. Oral presentation

Zohar´s semblance

Zohar Ibarra Zatarain was born in Mazatlán, México in 1979. In 2001 he obtained a bachelor degree in Marine Biology focus in Aquaculture at the Facultad de Ciencias del Mar (FACIMAR), Mazatlán, México. In 2006, Zohar completed his Master in Science degree in Aquaculture at the Centro de Investigación en Alimentación y Desarrollo (CIAD, Unidad Mazatlán, México). At professional level, Zohar worked as seafood quality inspector (SGS, Société Générale de Surveillance, Mèxico), later he worked for the Mexican Commission of Aquaculture and Fisheries (CONAPESCA) as Aquaculture project evaluator and participated in the creation and establishment of sanitary aquatic norms for México. From 2010 to 2011, Zohar was general manager in a private marine finsfish hatchery (Alevines de México, S.A.) and his duties consisted in maintenance of broodstock, larval rearing, assisting live feed production and commercialization of fingerlings. In addition, Zohar assessored two commercial aquaculture companies located in México with the design and construction of installations, which were funded by the Mexican office of aquaculture. As scientific level, Zohar have 3 publications in peer-reviewed journals, 3 divulgative publications, 6 manuscipt in review (peer-review journals) and co-autored a book chapter. Further, Zohar have participated in more than 12 international aquaculture meetings and have collaborated in 6 research projects (Mèxico and Europe). At the end of 2011, he enrolled to UAB and IRTA to start his PhD in aquaculture.


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