Universitat Autònoma de Barcelona

Reproductive behaviour, dominance and in vitro fertilisation in Senegalese sole (Solea senegalensis)

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Doctoral Thesis

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Barcelona 2020

Universitat Autònoma de Barcelona

CONACYT Consejo Nacional de Ciencia y Tecnología

IRTA RECERCA I TECNOLOGIA AGROALIMENTÀRIES

Generalitat de Catalunya

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Doctoral Thesis

Comportamiento reproductivo, dominancia y fertilización *in vitro* en el lenguado senegalés (*Solea senegalensis*)

Tesis Doctoral

Memoria presentada por:

Wendy Ángela González López

Para optar por el grado de:

Doctora en Acuicultura por la Universitat Autónoma de Barcelona

Director de Tesis: Neil John Duncan, IRTA, Sant Carles de la Rápita

Tutora: Nerea Roher, Universitat Autónoma de Barcelona

Tesis adscrita al Departamento de Biología Animal, Biología Vegetal y Ecología

Barcelona 2020

GOBIERNO DE ESPAÑA MINISTERIO DE CIENCIA E INNOVACIÓN

INIA Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

Acknowledgements

Al final de este ciclo y la culminación de esta meta personal, sólo me queda agradecer a todos aquellos que contribuyeron durante este tiempo para que fuera posible la realización de este proyecto profesional.

Dr. Neil Duncan, thank you very much for the opportunity to participate in your project, for your infinite patience, your advice and professional experience so that I could successfully conclude this thesis. I have learned a lot along the way and the experiences I have had during this time are of great value to me. Again THANK YOU for so many considerations to me!!

También quiero agradecer a los directivos y personal administrativo del IRTA Sant Carles de la Rápita por el apoyo para trabajar en esta institución y ayuda en los trámites administrativos durante mi estancia. Mil gracias al personal de mantenimiento, David, Iván, Xavi Ingla por el apoyo para la gestión de material para mis experimentos y la paciencia para realizar cada petición hecha. También muchas gracias al personal técnico, Magda Monllao (gracias por el compromiso y entusiasmo en tu trabajo), José Luis Celades (gracias por tanta ayuda en los experimentos y cuidar a los pequeñines), Sandra Molas por tu buen humor y paciencia para ayudar en lo que se te requería profesionalmente. A los técnicos de laboratorio que tantas veces me dieron consejos, información y ayuda para realizar de la mejor manera mi trabajo, Marta Sastre (MIL GRACIAS, siempre tan entusiasta y presta para ayudar y enseñar), Olga Bellot por tu paciencia para enseñar técnicas histológicas y solucionar todas las peticiones hechas, a Laura, Nuria, Edgar y demás personas que de una u otra forma colaboraron en mi proyecto. Un agradecimiento a los estudiantes y personal investigador de intercambio que realizaron sus prácticas en este proyecto, colaborando en tareas diarias como colecta e incubación de huevos obtenidos en los stocks de peces, alimentación de peces, obtención de datos y apoyo técnico para análisis de videos, identificación celular, análisis de muestras y diversas tareas a lo largo de experimentos, Noemi Cota Mamani (investigadora de Intercambio de Peru), Carlos Marrero (estudiante de máster en acuicultura), Lourenço Pombo Cardoso (estudiante de grado), Mario Villalta Vega y Alex Rullo Reverté, así como muchos otros estudiantes de la IES Alfacs

Escola d'Aqüicultura (http://aquicultura.insalfacs.cat/) que en su paso por el IRTA mostraron dedicación y entusiasmo en las tareas encomendadas.
De manera personal, mi agradecimiento a mis compañeros doctorandos, que entre risas, momentos chuscos y también de dificultad hicieron que esta estancia en el doctorado fuera más agradable, haciéndome sentir integrada con ustedes, Elvira Fatsini por tus consejos y ayuda en la tesis, Ana Toldrá, Sandra Leonardo, Joana, Angels, Greta, Riki, Toño, Vicky, Faiz, Javi, María y claro no podía olvidarme de mi “Little sister”, Sandra Ramos tantas aventuras juntas y vivencias personales y profesionales, cómo agradecerte tu gran apoyo!!! También mi agradecimiento infinito a la Dra. Ana Roque por el apoyo personal y ánimos para finalizar la tesis.

Thank to Dr. José Beirão and Nord University and Erasmus+ Programme Key Action 1 fellowship, for the support to carry out my stay in Bodø and for the protein analysis. I would like to acknowledge the support for the WOLFSTORE project (AF0078) funded by MABIT program Norway. José, thank for your advices to this thesis, for giving me the opportunity to participate in your project and learn new knowledge. Also thank you for the initiative to make visible so many hours of work, sampling and achievements in the aquaculture of Wolffish. Thanks to the technical staff at Mørkvedbukta Research Station (Heidi, Dalia, Ingvild) and the technical support of Deepti Patel in the laboratory. Thanks to Willy Sandaa and the AMINOR AS staff for the access to broodstock and the facilities to set up the mini-lab needed to conduct these experiments. It was a great experience in the Arctic winter.

Mi agradecimiento al Dr. Joan Cerdá por las facilidades otorgadas en su laboratorio para realizar diversas labores de mi proyecto y al Dr. Francois Chauvigné por las múltiples veces que brindo consejos e información para realizar mi trabajo. Además quisiera dar las gracias al Dr. Ignacio Giménez por las ideas y sugerencias aportadas a mi trabajo para la gestión de gametos. Agradezco a Julien Peris Martin and IMV-Technologies Company por el suministro de las soluciones Stor Fish® and Marine Freeze® solutions utilizadas en los experimentos.
También quiero agradecer el apoyo brindado por el Consejo Nacional de Ciencia y Tecnología (CONACYT), México por la beca otorgada para

realización de mis estudios y al Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria por los fondos apoyados al Proyecto INIA -FEDER (RTA2014-00048).

Mi agradecimiento a tantos amigos encontrados en estos lares, sin importar nacionalidad o idioma compartimos grandes momentos, Toni Ferreres, Sirwe, Evita, Carlos, Paloma, Nikunj, Nicoló, André, Maude y tantas personas más que enriquecieron mi quehacer diario. Asimismo mis amigos desde México que a pesar del tiempo y kilometros que nos separan, siempre me brindan su alegría y ánimo, Julieta, Alberto, Vicky, Dianela, Sheila, Joanne, Fernando Torres y una lista interminable de buenos amigos.
Finalmente, mi mayor agradecimiento es a mi familia, mi madre, hermanos, a Kate y Kimberly que siempre han apoyado mis más locos sueños y proyectos de vida, aún en la distancia siempre me acompañan.

Wendy Ángela González López

Para mi Madre,

y mis hijas

Index

Abstract

The Senegalese sole (*Solea senegalensis)* is an emerging aquaculture species in Europe. However, in captivity Senegalese sole present a reproductive failure, which results in the lack of fertile spawning from cultured breeders that were hatched and reared in captivity. Although the cultured breeders produce viable gametes, the dysfunction causes the loss of reproductive behaviour or courtship in cultured males and, therefore, the eggs released by females are not fertilized. However, viable egg production is obtained from wild breeders held in captivity, but the exclusive production of eggs from wild breeders is not sustainable in the long term. In addition, parental analysis of hatched larvae from wild breeders has determined that few wild breeders participate in spawning, which resulted in a loss of genetic variability in subsequent generations. Two approaches exist to solve this bottleneck: achieving natural spontaneous spawning in cultured breeders with a solution to the behavioural dysfunction in cultured males or the use of artificial fertilization to bypass the behavioural dysfunction. Therefore, the present thesis has been focused on: **a)** experiments to solve the dysfunction in the reproductive behaviour of cultured breeders, **b)** the influence of dominance behaviour on reproductive success, **c)** methods to preserve the sperm quality needed to achieve successful *in vitro* fertilisation procedures in Senegalese sole and Spotted wolffish *(Anarhichas minor)* and **d)** determine the sperm to egg ratio require for *in vitro* fertilisation in Senegalese sole. The reproductive behaviour and spawning success of Senegalese sole breeders from different origins (wild and cultured fish) was evaluated in the first section of this thesis (Chapter 2), which presents the last year of a four year experiment. Behaviour and spawning were recorded for three experimental groups, two groups of mixed wild and cultured fish (Groups M1 and M2) and one control (only cultured fish). The behaviour denominated as “Follow” that has been shown to be part of the courtship and identified as an indicator of spawning was evaluated and participating fish identified. No fertile spawns were registered from the control group. An increase in the participation of cultured breeders in the reproductive behaviour and spawns was observed in the mixed groups. A cultured male breeder had parental contribution, with two wild females and one cultured female. The effect of the cohabitation of cultured and wild Senegalese sole

breeders promoted the reproductive behaviour and spawning in cultured breeders. The participation of cultured males appeared to be associated with social activity and a learning process by observation of conspecifics. Understanding the mechanism of development of reproductive behaviour and acquisition of skills for mating would help to solve the reproductive problem. Therefore, the effect of different social conditions that offered different social learning opportunities was examined (Chapter 3). Four groups of cultured breeders were set up, which prior to the experiment had different social conditions from the juvenile stage until puberty. Two replicate groups (W1 and W2) were reared prior to the experiment with spawning wild breeders. A positive control group (CP) was reared prior to the experiment with cultured breeders that liberated unfertilised eggs. A negative control (CN) was reared prior to the experiment in isolation as a single year class and had no contact with adult breeders during rearing. In Group W1, eight fertile spawns were obtained from a single couple of breeders, from a total 38 spawns during the first year of study. The fertile spawns had a mean fertilisation rate of 28.0 ± 13.8 % and mean rate of hatching larvae of 15.0 ± 10.4 %. Fertile spawns were not registered in other groups and in the subsequent two years no group including group W1 produced fertile spawns. It appears that although the learning process has taken place in cultured breeders, the retention and recognition of what had been learned, may be limited to a short period of time. However, the low involvement of breeders in the reproduction process and fertile spawning that was observed in both mixed origin (cultured and wild) groups (M1 and M2) and in cultured breeders (Group W1) appeared to have a possible relationship to reproductive and social dominance. Therefore, the relationship of dominance behaviour was explored under two aspects: the reproductive success in breeders (males and females) and reproductive potential in sole males. Breeders from different origin (wild and cultured) were classified as dominant and subordinate through a dyadic test and were related to the reproductive success of each fish (Chapter 4). A higher proportion of socially dominant breeders ($P = 0.025$) participated in the fertile spawns in comparison to subordinate fish. The social status appeared to be a limitation to achieve reproductive success. Also, the hierarchies that develop in a broodstock may lead to control of resources such as territory by dominant individuals. In sole, mainly males appear to be subjected to competition and

difficulty to achieve reproductive success, due to both social structure and the complex reproductive behaviour. The social context of males within a group may influence in physiological changes and reduce reproductive potential. Therefore, cultured males were classified as dominant and subordinate by a dyadic test and dominance was related to sperm quality, hormone concentrations and testicular cell morphology (Chapter 5). The dominance status was related to behavioural and physiological differentiation between categories of male breeders. Dominant males had significantly higher sperm motility (P=0.034) and Gonadosomatic Index (P=0.040) compared to subordinate fish. However, in spite of the social constraint on the subordinate fish, there appeared to be no suppression in reproductive potential. Sperm and germ cell production at all stages of development were found in the testes irrelevant of social position. Despite of the advances obtained to understand reproductive behaviour, to achieve the control of reproduction and solve the reproductive failure in cultured sole breeders, the increase in spawning participation was insufficient to provide reliable supplies of viable eggs. Hence, an alternative to solving the reproductive dysfunction in cultured breeders and obtain fertilized eggs through *in vitro* fertilisation appeared to be necessary. However, the low volumes of poor quality contaminated sperm in *S. senegalensis* have complicated the development of this procedure. The first step was to examine, short-term cold storage methods and diluent solutions to preserve sperm quality to fertilize the eggs (Chapter 6). Diluent solutions such as modified Leibovitz and Marine Freeze® helped to maintain sperm motility after 24 hours of storage and counteracted the effect of urine contamination compared to undiluted samples. The difficulty of collecting a high sperm volume in sole requires optimizing the effectiveness of sperm for a successful fertilisation. Therefore, appropriate sperm to egg ratios for the use of limited sperm volume for the successful experimental and massive *in vitro* fertilisation in Senegalese sole (*S. senegalensis*) was examined (Chapter 7). Sperm was collected from cultured males, diluted in modified Leibovitz and used fresh to fertilise the eggs. Cultured females were induced to ovulate with a 5 µg kg^{-1} single injection of gonadotropin releasing hormone and eggs were stripped 41:57 ± 1:46 h after the injection and fertilisations were performed. A non-linear regression, an exponential rise to a maximum ($R = 0.93$, $P < 0.0001$) described

the number of motile sperm required to fertilise a viable egg and 1617 motile sperm were sufficient to fertilise 99 ± 12% (± 95% confidence interval) of viable eggs. Similar, spz egg^{-1} ratios of 592 ± 611 motile spz egg^{-1} were used in massive *in vitro* fertilisations to fertilise 190,512 ± 38,471 egg and obtain 70 ± 14 % hatch. The low spz egg^{-1} ratio required for maximum fertilisation was consistent with the reproductive behaviour and strategies of the species. In addition to the reproductive challenge in Senegalese sole, spotted wolffish *(A. minor)* is another species that presents a reproductive dysfunction (Chapter 8). The reproduction in captivity is dependent on *in vitro* fertilisation. Wolffish sperm is motile on stripping and currently it is not possible to immobilize and reactivate. Thus, the possibility to store spotted wolffish sperm by refrigeration was assessed between diluted (Smith and Ryan solution) and undiluted sperm. Sperm samples were collected by stripping into a pipette and extracted directly from testes. Factors such as sperm contamination by urine and energy stores (ATP) that may limit the duration of the motility period were assessed. Diluted sperm maintained a higher percentage of motile cells during the storage time. The urea concentration was similar in sperm samples collected by stripping (17.10 ± 1.98 mg/dL) and from the testes (12.59 ± 2.37 mg/dL). The ATP concentration (initial value 5.65 ± 0.86 nmol/10^9) remained stable ($p= 0.099$) during 30 h after sperm collection. Sperm refrigeration protocols may extend the period of mobility that usually lasts between 1 and 2 days.

This thesis has advanced understanding of the reproductive dysfunction in Senegalese sole cultured breeders. Participation in reproductive behaviour and spontaneous fertile spawning by cultured breeders appeared to be increased with opportunities for social learning of reproductive behaviour, but suppressed by social status amongst breeders. Further studies are required to obtain sufficient spontaneous fertile spawning from cultured broodstock for aquaculture. The alternative solution, *in vitro* fertilisation was developed and demonstrated to be a method that would ensure mass production of larvae for the aquaculture industry.

Chapter 1

General Introduction

Chapter 1. General Introduction

1.1 Overview of aquaculture in the world

Aquaculture is a fast growing sector due to the global demand for aquatic products. The overexploitation of valuable species by fisheries has contributed to the increase in aquaculture activity to supply these products. According to FAO (2018) data, aquaculture provides about 50% of the world's aquatic food supply and during the last three decades the production has increased continuously at a rate of 2.56% per year. More than 600 species are cultivated worldwide in both coastal and inland areas and are produced from farms with basic infrastructure to large multinational companies. The aquaculture production, including fish, molluscs, crustaceans, echinoderms, amphibians, reptiles and aquatic plants, reached 110.2 million tons in 2018. However, of the total diversity of species, finfish farming is the most diverse group, representing 84.2% of the total species compared to other groups such as molluscs or crustaceans. The increase in finfish production and diversification has been based on advances in the technological development of cultured species. The domestication of species from diverse aquatic environments and the control in the production have contributed to accelerate growth. In addition, the optimization of the different production systems (intensive, extensive, others), food innovation and maintaining the health of reared species have been improved to achieve efficient production levels. Therefore, these advances have led to aquaculture being considered a profitable activity.

Furthermore, aquaculture activity represents a source of employment (19.3 million directly and about 6.5 million indirectly) and economic development in many regions in the world. In 2016, aquaculture production in 37 countries was higher than that of wild-caught fish. These countries represent about half of the world's population. Thus, aquaculture contributes significantly to improving the food security and nutrition of the population. In regions of the world, Asia was the larger producer of aquatic products by aquaculture (91.9 %), followed by production in America (3.2 %), Europe (2.7 %), Africa (2.0 %) and finally Oceania with 0.2 % (APROMAR 2019) (Figure 1). In Europe, aquaculture

production in 2017 was 1,353,201 tons and among the main species cultured are mussels (*Mytilus spp*) with a production of 493,844 tons. Other species being cultured are Atlantic salmon (*Salmo salar*) with 209,180 tons and rainbow trout (*Oncorhynchus mykiss*) with 185,316 tons (APROMAR 2019). While in Spain, the aquaculture production reached 348.395 tons and the most important species produced are rainbow trout, with 18.856 tons, European mussels, with 273.600 tons, gilthead sea bream (*Sparus aurata*), with 14.930 tons, European seabass (*Dicentrarchus labrax*), with 22.460 tons and turbot (*Scophthalmus maximus*) 7.450 tons (APROMAR 2019).

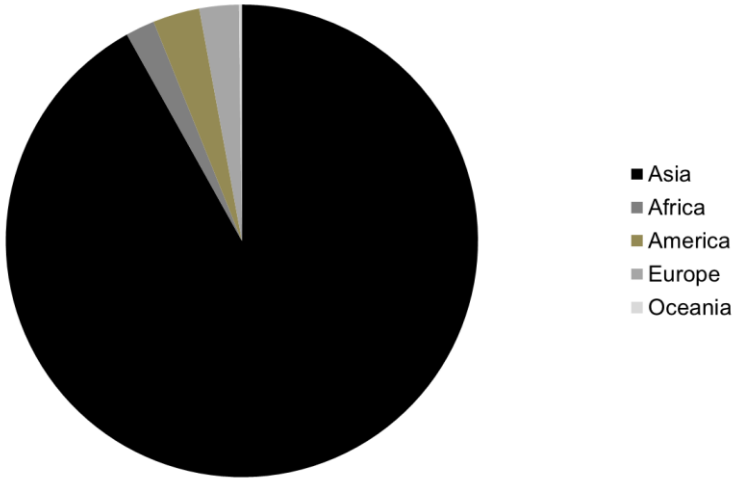

Figure 1. Distribution of aquaculture production in the five continents. (Graphic modified from APROMAR 2019).

However, the production of other emergent species has been increasing and strengthened by the development and implementation of innovative aquaculture techniques. In the diversification of species, the cultivation of flatfish has been favoured. Among the species of commercial importance and special interest is the Senegalese sole (*Solea senegalensis*) (Anguis and Cañavate, 2005), which has been studied in this thesis. The good growth rate, relatively easy larval rearing (Dinis *et al*., 1999; Fernández-Díaz *et al*., 2001), tolerance to handling, the consumer acceptance and economic return from the market, has demonstrated the species potential for aquaculture and promoted an interest to achieve a commercial viability (Howell and Dinis, 2019). Demand for Senegalese sole in the market has increased in the last decade and the global

aquaculture production reached 1,616 tons in 2018, of which 774 tons was produced in Spain (APROMAR 2019).

1.2 Characteristics and biology of Senegalese sole (*Solea senegalensis*)

The Senegalese sole (*S. senegalensis*) (Kaup 1858) (Figure 2), is a marine flatfish with benthonic habits that may also be found in estuarine environments. This species belongs to the order Pleuronectiformes and the family Soleidae. The natural distribution of this species includes from northeast Atlantic coast, northwest Africa and western Mediterranean Sea (Howell and Dinis, 2019). The sole has an oval and asymmetric body with eyes on the right side and the interradial area of the pectoral fin is black to give a stripped appearance, which is a distinctive feature of this species with respect to common sole (*Solea solea*) (Ben-Tuvia 1990). Their usual diet in the natural environment is based in polychaetes, small crustaceans and molluscs.

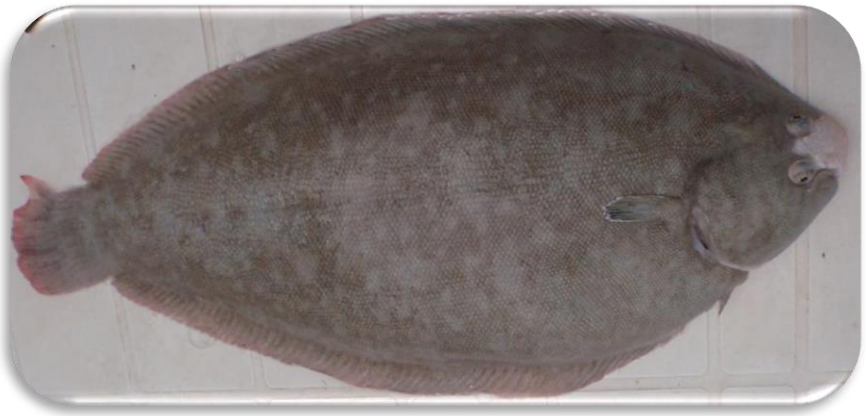

Figure 2. Senegalese sole breeder. (Photo credit: W. A. González-López, 2019)

The Senegalese sole is a gonochoric species; individuals reach sexual maturity at 3 years with a size of about 32 cm and no apparent differential sexual characteristics between females and males. The main reproductive season is during spring (March to June) with temperatures between 8 and 22℃ and natural photoperiod, and an additional period in autumn (October to November). The type of fertilisation between breeders is external and from fertilised eggs, pelagic larvae hatch after 42 h of incubation with a mean size of 2.4 ± 0.1 mm total length (Dinis *et al*., 1999; Imsland *et al*., 2003; Anguis and Cañavate, 2005). Larval rearing was reported to be relatively easy to perform and larvae

were fed on a diet of freshly hatched Artemia nauplii and rotifers. However, growth heterogeneity has been observed in juveniles, affecting the mean growth rates (Imsland *et al*., 2003; Salas-Leitón et al. 2011). Some diseases caused by bacteria and viruses have affected Senegalese sole culture. *Photobacterium damsela ssp. Piscicida* is the causative agent of pasteurellosis and darkening of the skin, swelling in the abdominal cavity, paleness of kidney and spleen and massive mortalities are the typical features of the disease. While vibriosis caused by *Vibrio harveyi* and *V. parahaemolyticus*, produce surface ulcers and moderate mortalities. Change in colouration, erratic swimming and irregular behaviour were attributed to the birnavirus (Imsland *et al*., 2003).

1.3 Reproduction in captivity

The Reproduction of Senegalese sole in captivity has been controlled in southern European including Spain and Portugal since the 1980´s (Dinis et al., 1999). The culture has been based on the natural spawning of broodstocks caught from the wild environment and acclimated in tanks with a thin layer of sand on the bottom (Anguis and Cañavate, 2005; Martin *et al*., 2014). Natural spawning are obtained with temperatures between 16.5 ± 0.5 to 22 ± 1.0℃ and constant salinities between 30 to 35 ppt; changes in the temperature appear to stimulate the spawns in captive wild breeders. The fecundity of this species has been calculated to be over 100,000 eggs kg^{-1} day^{-1} and variations registered may be due to fluctuations in the temperature. A high percentage of larvae have been hatched (72.1 ± 26.5%), achieving successful management of captive wild broodstocks (Dinis *et al*., 1999; Imsland *et al*., 2003; Anguis and Cañavate, 2005). Currently, the increase in the industrial culture of sole has led to the improvement of culture systems with a bio-economic model. Recirculation systems with controlled environmental conditions have been installed to optimize water quality and productivity. However, in spite of the advances in farming techniques, commercial growth for Senegalese sole has been limited due to the reproductive failure of the cultured breeders (fish hatched and raised in captivity) (Carazo 2013), as well as large fluctuations in fecundities from wild broodstocks (Martín *et al*., 2014). In addition to the proper management of breeders, the success of aquaculture production of a species requires full

reproductive control in captivity. Only by achieving this critical point of reproductive control, is it possible to provide good quality of gametes and offspring used in the hatcheries, that will ensure the feasibility of commercial production of the specie (Zohar and Mylonas, 2001; Duncan *et al*., 2013).

1.4 Reproductive failure in Senegalese sole.

Reproductive dysfunctions have been observed in captive wild fish and fish hatched and reared in captivity (Zohar and Mylonas, 2001), as is the case of Senegalese sole. The captive environment provides different conditions compared to the natural environment and may cause negative consequences on development, maturation and spawning in breeders (Zohar and Mylonas, 2001; Duncan *et al*., 2013). Zohar and Mylonas (2001) described three different types of reproductive dysfunction that may be found in broodstock: 1) the process in vitellogenesis and spermatogenesis fails completely, as have been observed in *Mugil cephalus*; 2) absence of the final oocyte maturation process (FOM), in the females the oocytes are arrested, become atretic and are reabsorbed, for example in meagre (*Argyrosomus regius*) and 3) the absence of spawning after ovulation or maturation of gametes and absence of successful reproductive behaviour such as in Senegalese sole. However, the manual extraction of Senegalese sole gametes has been performed and through *in vitro* fertilisation fertilised eggs were obtained (Rasines *et al*., 2012). However, this approach has been frustrated as the sperm production from males is low and poor quality (Cabrita, *et al*, 2006) and the period after ovulation for the extraction of viable gametes was short (Rasines *et al*., 2012; 2013). Ovulated eggs need to be fertilised in the correct moment before factors such as water temperature influence in the overripening of the eggs, limiting their viability to weeks, hours or even minutes after ovulation (Zohar and Mylonas, 2001).

The reproductive dysfunction that causes this failure was identified in Senegalese sole males of cultured origin, which do not fertilize the eggs released by females of either wild or cultured origin (Mañanós *et al.,* 2007; Carazo 2013; Martin 2016; Martin *et al*., 2019). Therefore, absence of successful spawns, often of low fecundity, have been obtained from groups composed of only cultured breeders (Guzmán *et al.* 2008; Carazo 2013; Martin

et al., 2019). Several aspects such as reproductive physiology, nutrition and genetics have been considered to explain the reproductive failure from cultured breeders in comparison to wild breeders that reproduce in captivity. Guzmán *et al.* (2008), studied through three consecutive years, spawning performance of cultured broodstocks and analysed the Vitellogenin (VTG) protein and sex steroid profiles in cultured breeders. The profiles of VTG were correlated with sexual steroids and spawning performance of cultured broodstock and appeared normal, however, despite of these profiles all spawns were infertile. Moreover, the influence of nutrition was considered as a possible influence in the reproductive dysfunction exhibited by cultured Senegalese sole. The levels of essential fatty acids, other lipids, prostaglandins (PG) and steroids were assessed (Norambuena *et al.*, 2012a; 2012b). Differences were observed in lipids and fatty acids in cultured breeders compared to wild broodstock; in addition, wild fish showed higher levels of PGs. Hence it was concluded that a nutritional imbalance may contribute to significant differences in reproductive physiology and the reproductive dysfunction (Norambuena *et al.*, 2012a; 2012b).

Another physiological aspect that has been examined is that an endocrine dysfunction during reproductive development of cultured breeders could result in the loss of capacity to participate in viable spawns. A reproductive endocrine dysfunction could explain lower sperm volume and quality in cultured males compared to wild males (Cabrita *et al*., 2006). Riesco *et al.* (2019) evaluated the sperm quality, expression of molecular markers related to reproductive success (sperm mRNAs and miRs) and the dopaminergic pathway through brain dopamine mRNA markers and tyrosine hydroxylase (Th) protein levels, in both wild and cultured males. The dopaminergic pathway is involved in the inhibition of gonadotrophin production and contributes to the sexual motivation and regulation of male reproduction. The differences in Th protein expression exhibited a downregulation in cultured males, which may be related to the dysfunction in the reward pathway in the sole and consequently to reproductive aspects. While, differences in the miRNAs expression could be one of the vehicles for the transmission of determined features to the offspring, such as

the reproductive dysfunction. However, further studies would be needed to clarify the relationship between these results and the reproductive dysfunction.

Attempts made to solve the reproductive dysfunction in cultured breeders and achieve control of reproduction were focused on hormonal therapies using treatments based on the application of gonadotropin-releasing hormone agonistic (GnRHa), human chorionic gonadotropin (hCG) and 11 ketoandrostenedione (OA). Gonadotropin-releasing hormone agonistic (GnRHa), were applied to cultured breeders to induce spermiation and spawning. Although ovulation and egg release were obtained in the females (Agulleiro *et al*., 2006; Guzmán *et al.*, 2009), the treatment was ineffective with the males, the sperm production was not increased and eggs were not fertilised (Agulleiro *et al*., 2006). Other treatments in cultured breeders based on GnRHa and OA (Agulleiro *et al.,* 2007) or GnRHa and hCG (Guzmán *et al*., 2011), had a positive effect on spermatogenesis and plasma concentration of sex steroids. In the treatment with GnRHa and hCG, plasma testosterone (T), 11-ketotestosterone (11-KT) levels and the gonadosomatic index (GSI) and sperm motility were stimulated in the males, whilst fecundity in females was increased. However only one fertile spawn was obtained using the hormone induction, with hCG applied to males and GnRHa to females.

The lack of fertile spawning has been related to the behavioural function of first-generation cultured males. The study of mechanisms that control reproductive behaviour in sole males may provide solutions to the dysfunction in reproductive behaviour identified in culture males. The complex process of the courtship in Senegalese sole was described in reproductively successful wild breeders by Carazo *et al*. (2016). The behavioural differences between wild and cultured breeders were examined (Mañanos et al 2007; Martín *et al*. 2019). Breeders from different origins were formed in the following different groups to analyse the reproductive behaviour: a) only wild males and females; b) only cultured males and females and c) mixed groups: cultured males and wild female and on the other hand, cultured females and wild males. The increase of locomotor activity preceded a set of behaviours performed by males to encourage females to mate to provide the opportunity for males to fertilise the released eggs. The behaviours in the courtship were characterized following various steps, starting

by “Follow” behaviour, which is a type of procession, where a swimming fish (leader) was chased by other fish (followers). In the next step, Rest the Head behaviour, was determined as a behaviour performed by males to females; where the male place its head on the body of another fish, usually the ovulating female. Another similar behaviour described was Guardian, which was performed by a male to “protect” a female from another male. These behaviours were performed by the males to achieve that the females swam from the bottom to the surface so that mating could take place. Finally, the Couple behaviour was performed in the water column, the male and the female swam together and the male placed himself under the female to fertilise the eggs that were released. However, in all groups formed by cultured males, all these steps in the courtship behaviour were not observed and consequently eggs were not fertilised. The locomotor activity was low and Couple behaviour was not performed. Breeder groups consisting of cultured males and cultured or wild females did not provide fertilised eggs. However, viable egg production from the group of wild males and cultured females was similar to the egg production obtained from wild broodstocks (Martin *et al*., 2014; 2019). This demonstrated that cultured females had the capacity to produce large quantities of fertilised viable eggs. While cultured males did not appear to have this capacity to execute the courtship behaviour and fertilise the eggs. Therefore, the poor quality inviable egg production obtained from cultured broodstocks was attributed to reproductive dysfunction in the cultured males. Additionally, treatments to stimulate the reproductive behaviour based on hormonal induction with GnRHa, hCG and PGF2α were applied in cultured sole breeders (Carazo 2013). Treatments resulted in an enhancement in the sperm quality in males and egg production in the females; however, these did not solve the reproductive failure in cultured breeders. Differences between reared fish and wild fish have been observed in other species such as Atlantic salmon (Fleming *et al.* 1996) or rainbow trout in terms of reproductive success (Chilcote, 2003). Huntingford (2004) noted remarkable behavioural differences between reared fish and wild fish related to aspects such as feeding and reproductive behaviour. It would appear that the opportunities and conditions to achieve the entire reproductive process successfully have been reduced, due to the breeder

origin, reproductive behaviour and mate selection (Carazo 2013; Carazo *et al.,* 2016; Fatsini 2017).

Therefore, the solution to the reproductive dysfunction in Senegalese sole is still unclear. To solve this bottleneck, two approaches were proposed: achieving natural spontaneous spawning in cultured breeders with a solution to the behavioural dysfunction in cultured males or the use of artificial fertilization to bypass the behavioural dysfunction.

2. Thesis objectives and overview

The main objective of this thesis was to provide a solution to the reproductive bottleneck in cultured broodstocks that make aquaculture practices with the species unsustainable and without the possibility to implement programs for genetic improvement. The two approaches addressed, were to either solve or bypass the reproductive dysfunction. In the first approach, the present thesis aimed at providing solutions to the reproductive dysfunction and continued a behavioural approach (Carazo 2013; Fatsini 2017). Therefore, the controlling mechanisms of reproductive behaviour were examined to solve the reproductive dysfunction based on behavioural strategies and alternative methods to control the reproduction in Senegalese sole cultured breeders. Reproductive behaviour and spawning were analysed, considering the effect of cohabitation with wild breeders and different holding conditions presented from the juvenile stage until puberty in cultured breeders. In addition, the influence of dominance relationships between the breeders on the reproductive success was assessed. The effect of social context on development of fish and reproductive ability and alternatives to solve the reproductive dysfunction were explored, focusing on two behavioural aspects: **a)** mechanisms to increase the **reproductive behaviour** of cultured breeders and solve the reproductive dysfunction, **b)** the effect of **dominance behaviour** on reproductive success.

In the alternative approach, the objective was to develop artificial fertilisation procedures to obtain fertilised eggs. In this approach, sperm quality preservation was tested for two species: Senegalese sole and spotted wolffish (*Anarhichas minor*) and the appropriate proportion of sperm and eggs was examined in experimental and mass *in vitro* fertilization in Senegalese sole to,

altogether, provide methods to preserve sperm quality and achieve ***in vitro*** **fertilisation**.

- **Chapter 2,** presented the last year (2016) of an experiment conducted over four years and initiated by Fatsini (2017). The aims of the chapter were: a) Assess the effect that the presence of successfully spawning wild breeders has on cultured breeders held in the same experimental groups, b) To study sperm production in wild breeders that successfully spawned and cultured breeders that did not successfully spawn. Therefore, the effect of cohabitation of wild and cultured Senegalese sole breeders on the reproductive behaviour and spawning of cultured breeders was assessed. Behaviour, sperm quality and spawning were recorded for three experimental groups, two groups of mixed wild and cultured fish (Groups M1 and M2) and one control (only cultured fish).

- **Chapter 3,** examined the effect of different social conditions that offered different social learning opportunities on reproductive behaviour and successful spawning in cultured breeders during three reproductive seasons. The aim of the chapter was to examine the reproductive behaviour and successful spawning in relation to different social holding conditions from the juvenile stage until puberty. Behaviour and spawning were evaluated in four experimental groups of cultured breeders with different social conditions from the juvenile stage until puberty. Prior to the experiment, two groups (W1 and W2) had been reared with wild breeders that spawned successfully, a positive control group (CP), had been reared with cultured breeders that spawned unfertile eggs, and a negative control group (CN), had been reared in isolation.

- **Chapter 4,** examined the effect of dominance behaviour on the reproductive success in wild and cultured breeders. Dominance categories were characterized in sole juveniles by Fatsini *et al.* (2017), based on feeding response and place preference and associated to a set

of behaviours. The aim of chapter 4, was to examine behaviour, preferred space (territory), and feeding response to determine hierarchical status amongst the breeders. Once determined, the aim was to examine the relationships between hierarchical status and the reproductive success determined by the breeders participation in spawning and the “Follow” behaviour. Dominance relationship was determined through a dyadic test between pairs of breeders. Behaviours, preferred space (territory), and feeding response were examined to determine social status (dominant and subordinate fish). The hierarchical categories were explored in relation to reproductive success defined by participation in fertile spawns and the “Follow” behaviour.

- **Chapter 5,** analysed the effect of social hierarchy on the reproductive potential (sperm quality, hormone concentrations and germ cells production) of Senegalese sole males. The aim of the chapter was to examine the relation between social hierarchy and the reproductive potential of cultured males. The sole males are subjected to sexual and social competition to mate with females. Differences in social position in fish (dominant and subordinate) could influence in processes such as spermatogenesis, spermiation and mechanisms that control male sexual behaviour. Social dominance relationship was determined through a dyadic test between pairs of males through behaviours and preferred space (territory) parameters. Social status was explored in relation to sperm quality, hormone concentrations, testes size and testicular cell development.

- **Chapter 6,** described the anatomy of the urinary and male reproductive systems, the relationship between urine contamination and sperm collection and examined short-term cold storage methods to preserve quality of sperm diluted in different solutions. The aim of the chapter was to: (a) describe the anatomy of the urinary and male reproductive system to understand why Senegalese sole sperm is usually contaminated; (b)

describe the characteristics of Senegalese sole sperm in relation to urine contamination; (c) examine the use of a range of extender solutions for chilled short-term storage to maintain the sperm quality parameters, motility and velocity. Sperm samples were collected from cultured male breeders and stored diluted and undiluted (control) for twenty-four hours. Urine contamination was analysed for all samples and contrasted with sperm quality parameters.

- **Chapter 7,** evaluated the sperm to egg ratios for *in vitro* fertilisation in Senegalese sole. The chapter, aimed to determine the spermatozoa to egg ratio required for *in vitro* fertilisation on an experimental scale and then apply the ratio on a commercial scale in massive *in vitro* fertilisations. An additional aim was, to determine the viability of ovulated eggs stored at room temperature. Low sperm volume produced by sole males, has thwarted the application of *in vitro* fertilisation methods. Sperm to egg ratios need to be known in order to optimize the use of collected sperm and ensure high levels of fertilisation in Senegalese sole.

- **Chapter 8,** evaluated methods for the short-term refrigerated storage for spotted wolffish sperm and factors limiting the period of sperm motility. The chaptered aimed, to test methods for the short-term refrigerated storage of the spotted wolffish sperm, examining the potential negative effect of urine contamination and the importance of the energy reserves on sperm quality and short-term refrigerated storage. The reproduction of spotted wolffish in captive depends on *in vitro* fertilization and development of short-term refrigerated storage would represent an easy and inexpensive tool to assist in artificial fertilization programs in fish farms.

3. References

Agulleiro, M. J., Anguis, V., Canavate, J. P., Martinez-Rodriguez, G., Mylonas, C. C., Cerda, J., 2006. Induction of spawning of captive-reared Senegal sole (Solea senegalensis) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257(1-4): 511-524.

Agulleiro, M.J., Scott, A.P., Duncan, N., Mylonas, C.C., Cerdà, J., 2007. Treatment of GnRHaimplanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 147, 885-892.

Anguis, V., Cañavate, J.P., 2005. Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. Aquaculture 243, 133-145.

APROMAR, 2019. La acuicultura en España 2019. Asociación Empresarial de Productores de Cultivos Marino Chiclana (Cádiz). http://www.apromar.es/content/informes-anuales

Ben-Tuvia, A., 1990. A taxonomic reappraisal of the Atlanto-Mediterranean soles *Solea solea*, *S. senegalensis* and *S. lascaris*. Journal of Fish Biology 36, 947-960.

Cabrita, E., Soares, F., Dinis M. T., 2006. Characterization of Senegalese sole, Solea senegalensis, male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975.

Cabrita, E., Riesco, M. F., Mañanós, E.L., 2019. Sperm physiology and artificial fertilization. In: The Biology of Sole. Editors, José A. Muñoz Cueto, Evaristo L. Mañanós Sánchez, F. Javier Sánchez Vázquez. CRC Press. Boca Raton Florida. Pp 142- 68.

Carazo, I. 2013. Comportamiento reproductivo y fisiología del lenguado senegalés (Solea senegalensis) en cautividad. Ph. D. Thesis, Universidad de Barcelona (Spain).

Carazo, I., Chereguini, O., Martín, I., Huntingford, F., Duncan, N. 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (Solea senegalensis). Spanish Journal of Agricultural Research 14, 13pp. https://dx.doi.org/10.5424/sjar/2016144-9108

Chilcote, M. W., 2003. Relationship between natural productivity and the frequency of wild fish in mixed spawning populations of wild and hatchery steelhead (*Oncorhynchus mykiis*) Canadian Journal of Fisheries and Aquatic Sciences, 60, 9; ProQuest pg. 105

Dinis, M. T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of Solea senegalensis in Spain and in Portugal. Aquaculture 176: 27–38.

Duncan, N. J., Sonesson, A. K., Chavanne, H., 2013. Principles of finfish broodstock management in aquaculture: control of reproduction and genetic improvement. In Advances in Aquaculture Hatchery Technology Woodhead Publishing Series in Food Science, Technology and Nutrition Pages 23-75. Woodhead Publishing Limited. DOI: 10.15333/9780857097460.1.23

Fatsini, E., 2017. Reproduction, olfaction and Dominance behaviour in Senegalese sole (*Solea senegalensis*). PhD Thesis. Polytechnic University of Valencia. 203 pp.

Fatsini, E., González, W., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

FAO. 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome.

Fernández-Díaz, C., Yúfera, M., Cañavate, J., P., Moyano, F., J., Alarcón, F., J., Díaz, J., 2001. Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory. Journal of Fish Biology 58: 1086–1097. doi:10.1006/jfbi.2000.1514.

Fleming, I. A., Jonsson, B., Gross, M. R., Lamberg, A., 1996. An Experimental Study of the Reproductive Behaviour and Success of Farmed and Wild Atlantic Salmon (*Salmo salar*). Journal of Applied Ecology, Vol. 33, No. 4, pp. 893-905

Guzman, J. M., Norberg, B., Ramos, J., Mylonas, C. C., Mañanós, E. L., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). General and Comparative Endocrinology 156: 285–297.

Guzman, J. M., Ramos, J., Mylonas, C. C., Mañanós, E. L., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. Aquaculture 291(3-4): 200-209.

Guzman, J. M., Ramos, J., Mylonas, C. C., Mañanós, E. L., 2011. Comparative effects of human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa) treatments on the stimulation of male Senegalese sole (*Solea senegalensis*) reproduction. Aquaculture 316: 121-128. doi:10.1016/j.aquaculture.2011.03.014

Huntingford, F. A., 2004. Implications of domestication and rearing conditions for the behaviour of cultivated fishes. Journal of Fish Biology 65 (Supplement A): 122–142. doi:10.1111/j.1095-8649.2004.00562.

Imsland, A.K., Foss, A., Conceiçao, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. sengalensis*. Reviews Fish Biology and Fisheries 13, 379-407

Mañanós, E., Ferreiro, I., Bolón, D., Guzmán, J.M., Mylonas, C.C., Riaza, A., 2007. Different responses of Senegalese sole *Solea senegalensis* broodstock to a hormonal spawning induction therapy, depending on their wild or captive-reared origin, Aquaculture Europe 2007, Istanbul, Turkey, pp. 330-331.

Martín, I., Rasines, I., Gómez, M., Rodriguez, C., Martinez, P., Chereguini, O. 2014. Evolution of egg production and parental contribution in Senegalese sole,

Solea senegalensis, during four consecutive spawning seasons. Aquaculture 424-425, 45-52. https://doi.org/10.1016/j.aquaculture.2013.12.042 537

Martín, I.E., 2016. Advances in the Reproductive Biology and Zootechnics of the Senegalese Sole (*Solea senegalensis* Kaup, 1858). Universidad de Cantabria, Cantabria, Departamento de Ciencias y Técnicas del Agua y del Medio Ambiente, pp. 197.

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Spanish Journal of Agricultural Research, [S.l.], v. 17, n. 4, p. e0608. ISSN 2171-9292 doi:http://dx.doi.org/10.5424/sjar/2019174-14953.

Norambuena, F., Estévez, A., Bell, G., Carazo, I., Duncan, N., 2012a. Proximate and fatty acids composition in muscle, liver and gonads of wild versus cultured broodstock of Senegalese sole (*Solea senegalensis*). Aquaculture 356-357, 176-185.

Norambuena, F., Mackenzie, S., Bell, J.G., Callol, A., Estevez, A., Duncan, N., 2012b. Prostaglandin (F and E, 2- and 3-series) production and cyclooxygenase (COX-2) gene expression of wild and cultured broodstock of Senegalese sole (Solea senegalensis). General and Comparative Endocrinology 177, 256-262.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326-329: 129–135.

Rasines, I. 2013. Reproducción en cautividad del lenguado senegalés (*Solea senegalensis*): inducción hormonal a la ovulación y obtención de huevos para la fecundación artificial. Tesis Doctoral. Universidad de Oviedo. Pp. 183.

Riesco, M. F., Valcarce, D. G., Martínez-Vázquez, J. M., Martín, I., Calderón-García, A. A., González-Nunez, V., Robles V., 2019. Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question. Reproduction, Fertility and Development. https://doi.org/10.1071/RD18453

Salas-Leiton, E., Anguis, V., Rodríguez-Rúa, J.,P., Cañavate, J.,P., 2011. High stocking density and food restriction have minimum impacto n size dispersal of cultured Senegalese sole (*Solea senegalensis* Kaup 1858) juveniles. Evidence for individual growth being regulated by population structure. Aquacultural Engineering 45:43-50.

Zohar, Y., Mylonas, C.C., 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197: 99

Chapter 2

The cohabitation of cultured *Solea senegalensis* breeders with wild breeders promotes the reproductive behaviour and spawning in cultured breeders.

Chapter 2 The cohabitation of cultured *Solea senegalensis* breeders with wild breeders promotes the reproductive behaviour and spawning in cultured breeders.

1. Introduction

The culture of Senegalese sole has made a substantial advance in the last decade, through the optimization of intensive culture systems (Howell and Dinis 2019). In spite of this advance, the production of Senegalese sole is currently based on the spawning of wild broodstock through natural spontaneous spawning (Morais *et al.,* 2016). However, wild breeders exhibit fidelity patterns and reproductive dominance that appeared to be responsible for the low participation of wild breeders in the spawning (Martin *et al*., 2014; Fatsini *et al*., 2020). Studies of parental contribution from a broodstock determined that between 11 % and 38.9 % of breeders were responsible for all the offspring during a reproductive season and that as high as 61.7 % of spawns were the result of a single couple (Martín *et al*., 2014; Fatsini *et al*., 2020). The low percentage of involvement of wild breeders in successful spawning has been shown to cause a loss in the genetic variability in the offspring, which could lead to inbreeding and have negative consequences in the sole production (Porta *et al*., 2006).

Nevertheless, the main problem facing the Senegalese sole industry is the reproductive dysfunction in breeders hatched and reared in captivity (cultured breeders) (Carazo 2013; Martin *et al*., 2019). This dysfunction, causes the lack of reproductive control, characterized by obtaining non-fertile spawning from cultured breeders, which make Senegalese sole production unsustainable (Carazo 2013; Martín *et al.,* 2019). Several kinds of reproductive dysfunctions can be attributed to stress caused by captive conditions and the loss of the natural environment that stimulates spawning (Zohar and Mylonas, 2001). Species of flatfish such as turbot and the Atlantic halibut, have been inhibited to spontaneously spawn in captivity, due to stress caused by housing conditions (Gibson, 2005). In the case of Senegalese sole, under similar culture conditions wild and cultured broodstock do not spawn similarly, as wild fish produce fertile

spawns compared to infertile spawns from cultured fish. Therefore, the environmental conditions in captivity during the spawning season appear not to be the cause of the reproductive dysfunction and studies should focus on other aspects to determine the origin of this problem (Duncan *et al*., 2019). The cultured sole breeders have been shown to become sexually mature and have functional gametes (Rasines *et al*., 2012). Despite of the cultured males that produce a reduced volume of sperm and variable sperm quality (Cabrita *et al*., 2006) successful artificial fertilization has been achieved (Rasines *et al*., 2012; 2013). Cultured females were hormonally induced with GnRHa and the eggs were manually extracted and fertilised with cryopreserved sperm from cultured males (Rasines *et al*., 2012; 2013). However, this method has not been described for use on an industrial scale.

To overcome the reproductive dysfunction and to achieve natural spawning, several treatments based on hormonal therapies have been carried out with cultured breeders (Agulleiro *et al*., 2006; Guzmán *et al*., 2009; Guzmán *et al*., 2011; Carazo, 2013). Gonadotropin-Releasing Hormone analogue (GnRHa) induced an increase in the release of non-fertile eggs (Agulleiro *et al.*, 2006; Guzmán *et al.*, 2009). While in cultured breeders induced with human Chorionic Gonadotropin (hCG) and GnRHa a single fertile spawn was collected (Guzmán *et al.*, 2011). Carazo (2013), performed treatments to sole breeders, based on GnRHa and PGF2α, but no fertile spawns were obtained. Moreover, Martín *et al*. (2019) housed mixed groups (cultured and wild Sole) without carrying out hormonal therapy, collecting fertile spawns only from groups with wild males. These attempts did not find solutions for the reproductive failure in cultured breeders.

A series of studies have defined that the dysfunction in *Solea senegalensis* is a behavioural reproductive dysfunction attributed specifically to the cultured males (Carazo 2013; Carazo *et al*., 2016; Martin 2016; Fatsini 2017; Martín *et al.,* 2019). Carazo *et al*. (2016) described the courtship process and mating choice carried out by wild breeders to achieve the successful spawning of fertilised eggs. This process started with an increase of locomotor activity and specific behaviours such as the Follow behaviour, where two or more fish are involved swimming in a procession and which has been linked to successful spawning.

During the courtship, coordinated pre-spawning behaviours were performed between males and females (always by a single pair) and the male encouraged a female to swim from bottom towards the surface to achieve spawning success. Although, a failed spawning rate of 5.6 fold higher than successful spawning has been recorded, suggesting that the courtship process could be complex to perform, involving only a few breeders. However, it was observed that cultured males did not perform all of the behaviours related to the courtship and hence no fertile spawns were obtained (Carazo 2013; Martin *et al.*, 2019). Therefore, the reproductive failure in cultured breeders appears to be a behavioural issue and in particular a behavioural reproductive dysfunction in cultured male sole (Carazo 2013; Martin *et al*., 2019).

Studies initiated by Fatsini (2017), set out to determine whether exposing cultured breeders to the reproductive behaviour of wild breeders would increase the participation of cultured breeders in courtship and successful spawning. A study initiated in October 2012 recorded the reproductive behaviour and spawning of mixed wild and cultured stocks (wild males and females mixed with cultured males and females) during four reproductive seasons (from March to June in each year from 2013 to 2016). The first three years (2013 to 2015) were reported by Fatsini (2017) and the final year (2016) in the present chapter (also see the complete study in Fatsini *et al*., 2020). Fatsini (2017), found an increase in participation in reproductive behaviour and a small increase in successful spawning of cultured breeders during three reproductive seasons (2013-2015). In consequence, the presence of wild breeders that perform the full courtship to give fertile spawns, appeared to have an effect on the reproductive success and reproductive behaviour in cultured breeders, which may provide a solution for the reproductive dysfunction found in cultured males.

This chapter, presents the last year (2016) of the experiment, the reproductive behaviour and spawning success of Senegalese sole breeders from different origins (wild and cultured fish). The analysis includes parental contribution of hatched larvae and the identification of wild and cultured breeders that participated in the “Follow” behaviour, related with the fertile spawning. In addition, the sperm quality in the male breeders was also evaluated. Therefore, the aims of the present study were: a) Assess the effect that the presence of

successfully spawning wild breeders has on cultured breeders held in the same experimental groups, b) To study sperm production in wild breeders that successfully spawned and cultured breeders that did not successfully spawn.

2. Methods

Three experimental groups were tested: two groups of wild and cultured Senegalese sole breeders and a control group with just cultured breeders. The behaviour and reproductive parameters were studied during the reproductive season from March to June, 2016. The cultured Senegalese sole used in the mix groups and control, were the offspring obtained from wild broodstocks captured in the Atlantic zone; while the wild breeders used in the groups were captured from the zone of the Ebro Delta.

2.1 Broodstock management

Senegalese sole (*Solea senegalensis*), wild and culture origin (G1) broodstocks, were Pit-tagged (ID-100 Unique, Trovan-Zeus, Madrid, Spain) for their later identification. The broodstocks were kept in the facilities in IRTA Sant Carles de la Rápita, (Catalonia, and Spain), in tanks (14 m^3) with sand covering half of the bottom of each tank that was connected to a recirculation system (IRTAmar®). A natural temperature cycle in a range from 9 to 20 ºC was used, however, the temperature was controlled weekly during the spawning period upon reaching 18 °C in order to stimulate the spawning. From Monday to Thursday tanks were maintained at 16 ± 1 ºC and from Thursday to Monday at 18 ± 1 ºC (Martín et al., 2014 and Fatsini, 2017). Photoperiod was natural (9 - 14 hours light) and during the spawning season red light night illumination was used, adjusted to 5 lux at surface to enable the recording and observation of behaviours during the night (Carazo *et al*., 2013). The fish were fed with 0.75% of wet feed based on polychaetes (Topsy-Baits, Holland) and mussels (Sariego Intermares, Spain) and 0.55% dry feed (pellets Repro-Vitalis, LE-7 mm ELITE, Skretting Co.) of the total biomass, four days a week.

2.2 Experimental design

The breeders were set up in three tanks (Table 1), grouped according to different treatments:

1.- Two experimental groups (M1 and M2), which were formed with breeders from different origins, wild and cultured (G1).

2.- One Control group (C) constituted of only cultured breeders. The breeders placed in this group belonged to the same stock as cultured breeders in groups M1 and M2 that had never successfully spawned.

Table 1. Summary of Senegalese sole (*Solea senegalensis*) breeders distribution in different Groups: Groups M1 and M2, formed with wild and cultured breeders and Group Control formed with cultured breeders. The number of individuals per tank (N), weight (mean ± SEM) of the fish, stocking density, origin of breeders and sex (male, female).

Treatments	**N**	**Weight (g)**	**Stocking density (Kg/m³)**	**Origin**	**Sex**
Group Control (cultured breeders G1)	10	1143,50 ± 185,51	0,81	Culture	5 Males 5 Females
Group M1 (Mix of wild and cultured (G1) breeders)	17	1365,18 ± 514,67	1,65	Culture	5 Males 5 Females
				Wild	2 Males 5 Females
Group M2 (Mix of wild and cultured (G1) breeders)	18	1180,06 ± 488,01	1,45	Culture	5 Males 5 Females
				Wild	3 Males 5 Females

All the tanks were left undisturbed during the spawning seasons, from March to June of each year. All spawns were collected and spawning data registered. The paternity of larvae hatched from viable spawns was determined through microsatellites. Lastly, each group of fish (treatments) was video recorded during the night for later behavioural analysis, as described by Carazo *et al.* (2016).

In group M2, a wild male identified in previous years as the dominant father of offspring (Fatsini 2017), died in the first week that spawns were recorded. There were no mortalities during 2016 in Groups M1 and Control.

2.3 Egg collection and Quality

Egg collectors were placed in the surface water outlet of each tank and were checked every day during the spawning season at 9:00 am. The eggs were collected and assessed for the following parameters: total volume of eggs (mL), floating (viable) and non-floating (dead) volume of eggs (mL), weight of floating eggs (g), total number of floating eggs, fertilisation rate (%), hatching rate (%) and Total number of larvae.

Fertilisation and developmental stage was determined by visually examining 50+ eggs using a binocular microscope at the time of collection and at 24 h after egg collection.

Developmental stage was classified using a guide supplied by Rasines and Martin (personal communication). The number of floating eggs was calculated using the weight of eggs and counting the number of eggs in a known weighed subsample of eggs by triplicate. The relative fecundity was calculated according to the total number of eggs divided by the total weight of females in each tank (kg). The floating portion of eggs was placed in an incubator (~30 L) at natural temperature and photoperiod that was supplied with an open water flow. The larvae hatched after 36-48 h incubation. The hatching rate was determined by counting the number of eggs and larvae from three subsamples (100 mL) taken from the homogeneously mixed incubator. Larvae samples from each viable spawn was individually preserved in Eppendorf's with 96% ethanol for later analysis.

2.4 Sperm quality

2.4.1 Samples collection

At end of reproductive season (May 2016), individual males from all experimental groups were anesthetized with 60 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma-Aldrich, Spain) and weighed. Semen

samples were obtained by stripping, gentle abdominal pressure was applied from the testis towards the urogenital pore and sperm were collected with a 1 mL syringe. The volume collected was recorded and the sperm were placed in Eppendorf tubes over crushed ice.

2.4.2 Cell concentration

To determine cell concentration (spermatozoa mL-1), fresh sperm was diluted 1:500 in 10% formalin and 10 µL of this dilution was placed into a Thoma cell counting chamber. After 10 minutes to allow the spermatozoa to sediment in the counting chamber, the samples were observed with a x100 magnification using a Olympus BH microscope that was connected to GigE digital camera (model: DMK 22BUC03 Monochrome, The Imagin source, Bremen, Germany). Images of three different fields from each sample were taken with IC Capture Software (www.theimagingsource.com). The number of cells in each field captured was counted with the image processor ImageJ software (http://imagej.nih.gov/ij/). The mean was calculated from the three counts to determine the cell concentration of spermatozoa per mL^{-1} of collected semen and spermatozoa (spz) per kg of male bodyweight.

2.4.3 Motility parameters

Motility percentage and Average Path Velocity (VAP, µm/s), were measured from sperm previously diluted with modified Leibovitz (Fauvel *et al.,* 2012) that was activated and sperm paths video recorded. The sperm was activated by gently mixing 1 µL of diluted sperm with 19 µL of natural seawater containing bovine serum albumin (BSA), prepared at 30%. One microliter of activated sperm was placed in a counting chamber ISAS R2C10 (Proiser R+D, S.L. Paterna, Spain). The sperm paths were recorded until the motility ceased, using the IC Capture software and GigE digital camera (described above) connected to the microscope Olympus BH with a x200 magnification. The videos obtained (AVI format) were processed with Virtual Dub 1.10.4 software (http://www.virtualdub.org/) to convert the video into image sequences in format *.jpeg. The files of image sequences were imported to ImageJ software and the sperm kinetics parameters were assessed at 20 seconds post-activation, during two seconds, using a computer-assisted sperm analysis (CASA) ImageJ plugin

(http://rsb.info.nih.gov/ij/plugins/). The settings to analyse the videos were set as follows: brightness and contrast, -87 to -93/156 to 171; threshold, 0/138 to 142; minimum sperm size (pixels), 10; maximum sperm size (pixels), 500; minimum track length (frames), 15; maximum sperm velocity between frames (pixels), 20; frame rate, 30; microns/1000 pixels, 305; Print motion, 1; the additional settings were not modified. All samples were recorded and analysed by triplicate.

2.5 Paternity analysis

Hatched larvae (n=10), were collected from every spawn obtained and preserve individually in 96% ethanol within an Eppendorf (1 mL) and were sent to GENEAQUA (Faculty of Veterinary, Lugo, Spain) to identify the paternity of each larvae. Previously, the identification of parents was carried out by genotyping each broodstock through specific microsatellites for sole. The analysis started using four microsatellites and when the paternity of larvae was not determined by possible kinship with more than two parents, two microsatellites more were added making an analysis with six microsatellites.

2.6 Behavioural analysis

A square black and white CCD digital camera (model F60B/N80-50G, KT&C Co. Ltd., Korea Technology and Communications, Korea, supplied in waterproof housing by Praesentis S.L. Barcelona, Spain) connected to a digital video recorder (model DVR-0804HB, supplied by Praesentis S.L.) was placed just below the water's surface in the corner of each tank to video record the activity and the behaviours of each study group. Daily, during the spawning season, the fish activity was recorded for six hours (17:00 to 23:00 h) for all tanks (Carazo, 2013; Fatsini, 2017).

2.6.1 Locomotor activity

During the spawning season, the activity (locomotor activity) of the fish during the period of video recording was determined. Five days with spawning and five days without spawning were randomly chosen and the activity was analysed. The activity was determined by dividing the field of vision in each video in half into two parts with a line in the middle and the movements were recorded when

a fish crossed the line. The mean number of movements counted in each experimental group was divided by the number of fish in the tank to account for the slight differences in fish numbers between tanks (Fatsini, 2017). From this work, the profile of activity was described and the period of the day when the fish had higher activity (locomotor activity) was determined.

2.6.2 Behaviours

During the peak of locomotor activity (19:00-20:00 h), different behaviours related to the courtship were counted. These behaviours have been previously described by Carazo *et al.,* 2016 and were the following:

a) Rest the Head: an individual rests it´s head on some part of the body of the other fish.

b) Guardian: Frequently a male rests the head on the head of another fish (female) to isolate the fish from another male.

c) Follow: two or more individuals swim in a procession following each other: the following fish perform the same movements and path as the leading fish.

d) Couple: a male and female swim to the surface, the male keeping under the female with their bodies and genital pores pressed closely together for the release and fertilisation of gametes.

The hour of peak activity was divided into 5 minute sections and the behaviours were counted at the end of each 5 minute section. The behaviours were counted in the peak hour of the same five nights with spawning that were analysed for locomotor activity.

2.6.3 Identification of individuals in “Follow” behaviours

From the videos recorded, the participation of breeders (wild or cultured) in the “Follow” behaviour related to the courtship was determined in groups M1 and M2. In order to identify the origin of the breeders that participated, “Follow” behaviours (n = 30) were chosen at random from videos recorded in days with spawning during the reproductive season. The fish involved in the “Follow”

behaviour, were identified by three different observers from a guide of photos and videos, previously taken of each breeder. The guide of photos and videos, highlighted specific features such as caudal fin pattern, fish shape or swimming style, it was possible to identify the fish with a 80% reliability. The observations by the three observers were compared and the identity of each breeder was determined.

2.7 Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). The locomotor activity was assessed following the method used by Carazo *et al*. (2016). The mean of sperm quality parameters (cell concentration, motility percentage, VCL and VAP) and behavioural counts (mean number of movements for activity and mean counts of different behaviours exhibited) were compared amongst experimental groups using a one-way ANOVA. Multiple comparisons amongst groups were completed with a Games-Howell post-hoc test. The spawning quality was evaluated calculating the percentage of fertilisation and hatching of spawning among the treatments. A value of $P<0.05$ was considered to be statistically different. Statistical analysis was carried out using SPSS Statistic 23 for Windows (SPSS Inc. Chicago, IL, USA).

3. Results

3.1. Spawns Quality

During the reproductive season 2016, spawns were obtained from all experimental groups. However, in the Group Control fewer spawns were obtained and none were fertilized, in comparison with the Groups M1 and M2 (Figure 1). The Group M1 had the highest number of spawns and 61.3 % of the spawns were fertile. Whilst in the Group M2, only 21.4 % of spawns were fertile. Significant differences ($P<0.05$) were found in the mean volume of floating eggs and mean weight of floating eggs, amongst the Groups. The highest total volume of floating eggs was found in Group M1 compared to the lowest in the Control Group, which was different from Group M1 ($P=0.001$) and M2 ($P=0.005$), while the volume was similar between Groups M1 and M2 ($P=0.993$) (Figure 2). Likewise the mean weight of floating eggs, was significantly higher in

the Groups M1 (P=0.004) and M2 (P=0.003) compared to the Control Group, whilst there was no difference between Groups M1 and M2 (P=0.894) (Table 2). There were no significant differences amongst the groups for the parameters, mean total volume of eggs (Figure 3) and mean egg production per kg of female (Figure 4). Similar fertilization (P=0.170) and hatching rate (P=0.378) were obtained between Groups M1 and M2, whilst no fertile spawns were obtained from the Control Group (Table 2). In addition, over the entire spawning season compared to groups M1 and M2, the Control Group had the lowest values of mean weight of floating eggs, total volume of eggs (mL), total volume of floating eggs (mL), total volume of non-floating eggs (mL) and total eggs production per kg female in (Table 2).

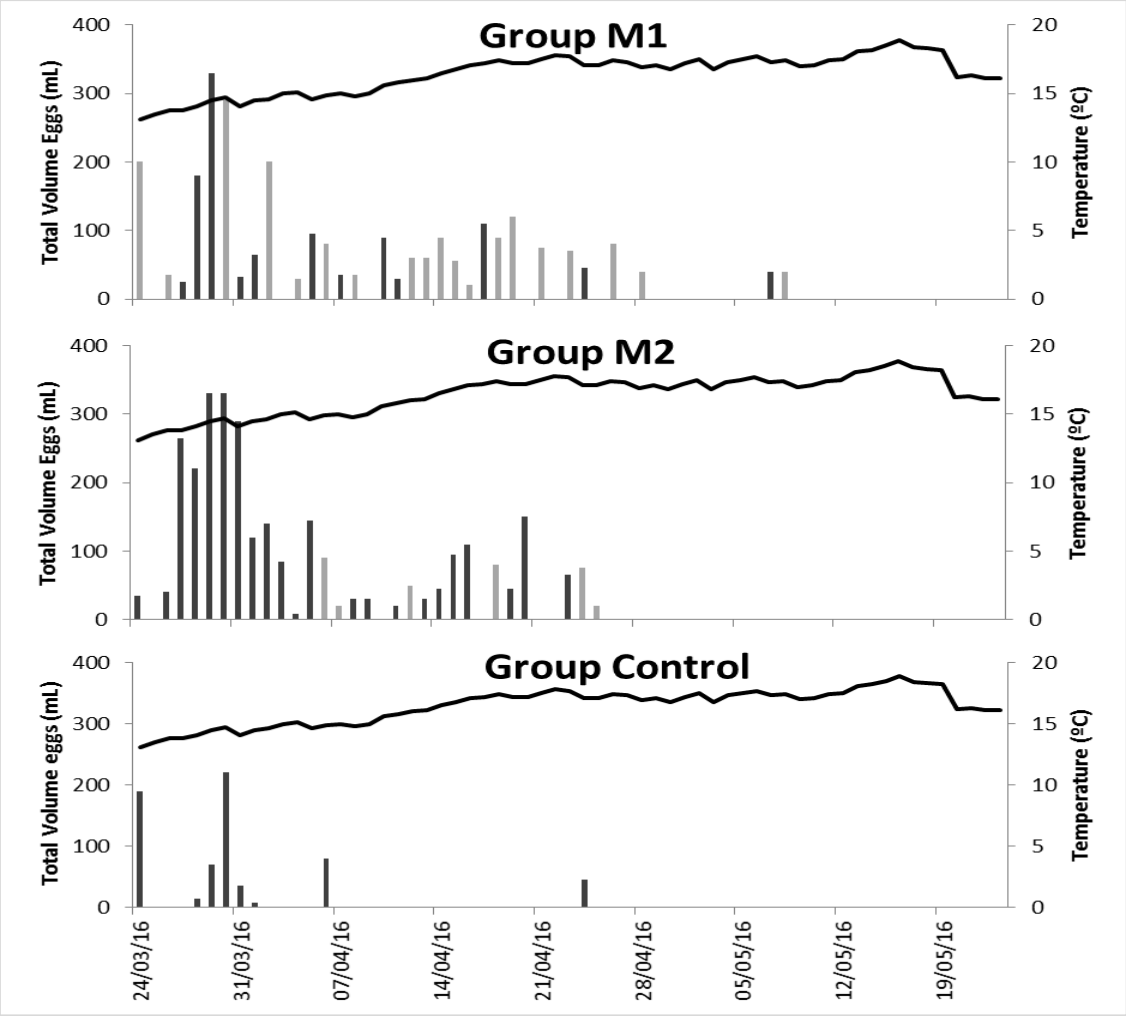

Figure 1. The 2016 spawning season for the treatments: Group M1 and Group M2, formed with *Solea senegalensis* breeders from different origins, wild and cultured breeders and Group Control formed with culture breeders. The grey bars represent fertile spawning and the black bars are non-fertile spawning.

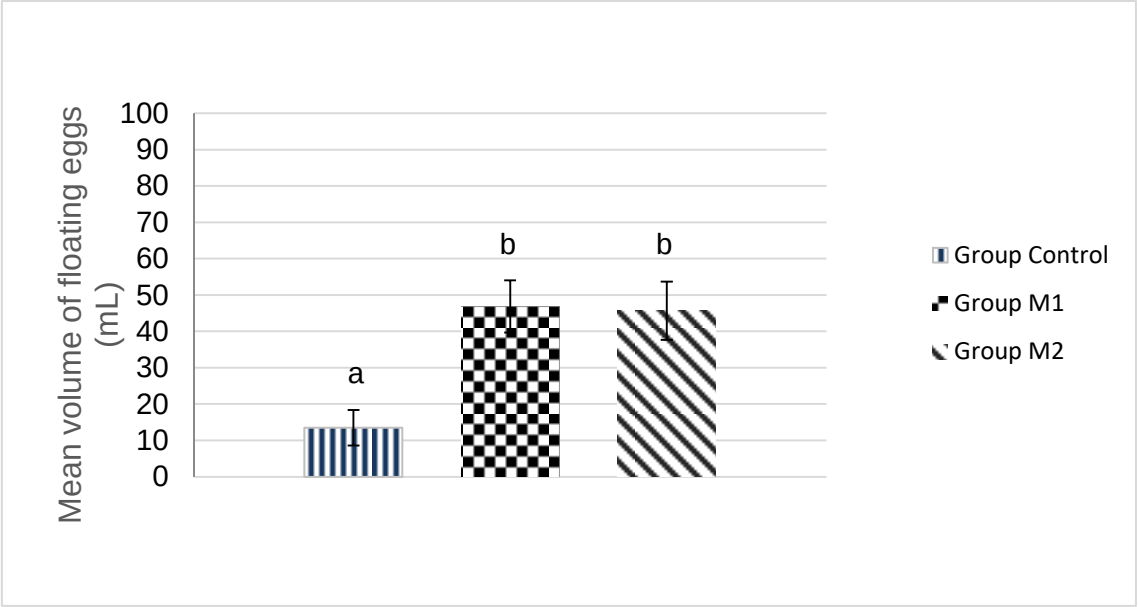

Figure 2. Mean volume of floating eggs from *Solea senegalensis*, during the 2016 season from three groups: Group Control (only cultured fish), Group M1 (mixed cultured and wild breeders) and Group M2 (mixed cultured and wild breeders). The different letters above the bars indicates significant differences amongst the different experimental groups.

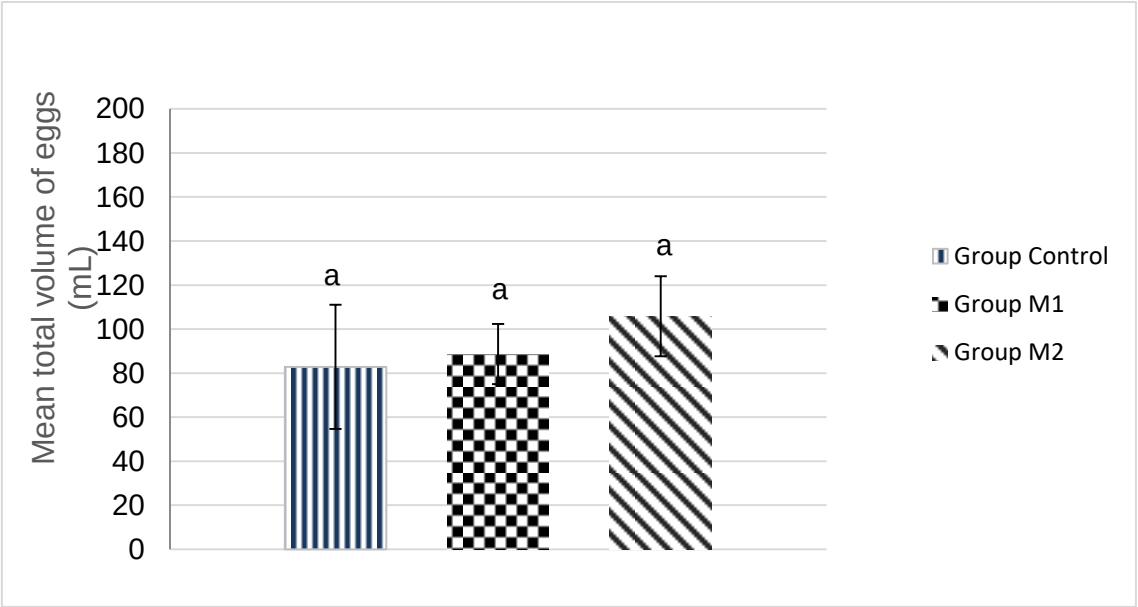

Figure 3. Mean total volume of eggs from sole (*Solea senegalensis*) during the 2016 season from three groups: Group Control (only cultured fish), Group M1 (mixed cultured and wild breeders) and Group M2 (mixed cultured and wild breeders).

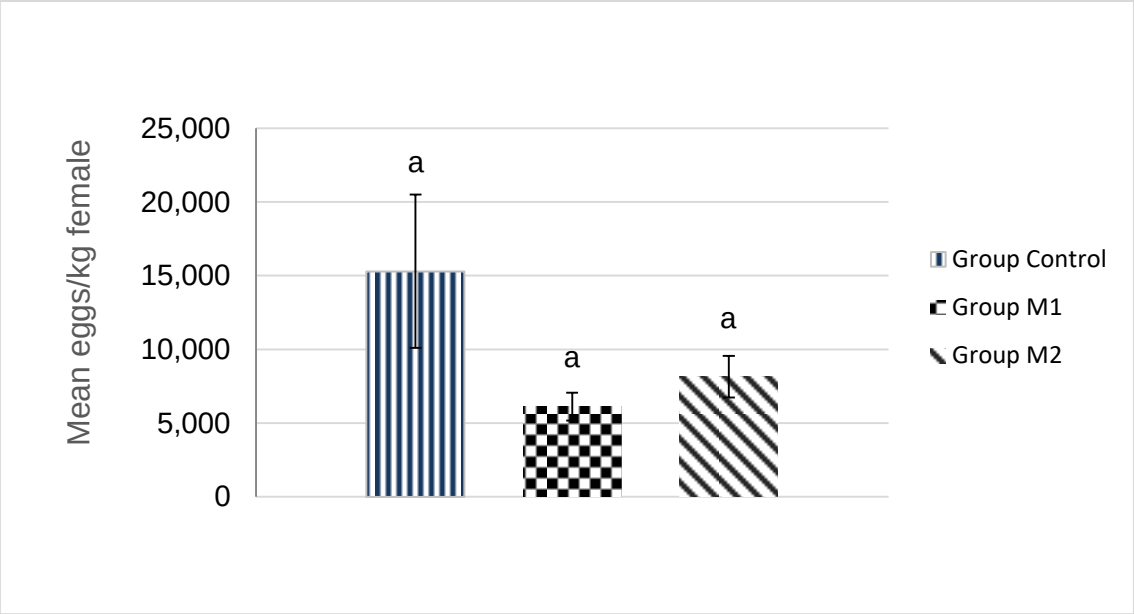

Figure 4. Mean eggs/kg of sole (*Solea senegalensis*) females during the 2016 season from three groups: Group Control (only cultured fish), Group M1 (mixed cultured and wild breeders) and Group M2 (mixed cultured and wild breeders).

3.2 Sperm quality

Overall, there were significant differences in sperm quality amongst the three groups that were assessed at the end of the breeding season (2016) (Table 3). The total time of motility, was significantly higher (P=0.02) in the Control Group compared to Group M1. The percentage motility, was significantly different amongst the three experimental groups (P<0.001), the lowest percentage was recorded in the Group M1 and highest in the Control Group. In the velocity values, Group M1 exhibited significantly higher (P<0.05) velocities compared to lower velocities found in Control Group and Group M1. There were no differences in the cell concentration, spz per kg^{-1} fish and spz per mL^{-1}, amongst the groups.

There were no significant differences between cultured and wild males (belonging to the groups M1 and M2) for the sperm quality parameters, motility percentage (P=0.40), VCL (P=0.08) and VAP (P=0.10), cell concentration: spz per kg^{-1} fish (P=0.817) and spz per mL^{-1} (P=0.545) and sperm volume collected (P=0.101) (Table 4).

Table 2. Summary of *Solea senegalensis* spawn quality for 2016 for the treatment groups: Group Control (formed with cultured breeders), Groups M1 and M2 (formed with cultured and wild breeders). The parameters, number of spawns (N= total number of spawns and H= number of fertile spawns), mean weight of floating eggs (g), total volume of eggs (mL), total volume of floating eggs and non-floating eggs (mL), relative fecundity for the season – total of number of eggs per kg of female, mean fertilisation rate (mean ± SEM) of fertile spawns, mean hatching rate (mean ± SEM) of fertile spawns and total number of hatched larvae (the letters next to value indicate significant differences).

Treatments	Number of Spawns / N-H	Mean Weight of Floating eggs (g)	Total volume of eggs (mL)	Total Volume of floating/non floating eggs (mL)	Total Eggs production (eggs/kg female)	Mean Fertilization rate (%)	Mean Hatching rate (%)	Total Number of hatched larvae
Group Control (Cultured breeders G1)	8-0	13.71 ± 5.99a	663	108 - 555	122.400	0.00 a	0.00 a	0.00
Group M1 (Mix of wild and cultured (G1) breeders)	31-19	47.98 ± 7.88b	2748	1453 -1295	189.638	55.00 ± 8.81b	23.20 ± 9.44b	105232
Group M2 (Mix of wild and cultured (G1) breeders)	28-5	53.58 ± 9.56b	2963	1278 - 1685	228.118	21.50 ± 5.10b	6.27 ± 9.47b	9634

Table 3. Sperm quality parameters of *Solea senegalensis*, in the three experimental groups (Control, formed with cultured breeders and M1 and M2 formed with a mix of cultured and wild breeders). Total time of sperm motility (s), percentage sperm motility, curvy linear velocity VCL (µm/s), average path velocity VAP (µm/s), cell concentration - spermatozoa per mL (spz mL^{-1}) and spermatozoa per kg of body weight (spz kg^{-1}). All values are mean ± SEM. Different letters represent significant differences (P<0.05) between groups.

Treatments	Total time motility (s)	% Motility	VCL	VAP	Cell concentration spz per kg^{-1} fish	Cell concentration spz per mL^{-1}
Group Control	103,33 ± 15,45 [a]	22,66 ± 2,27 [a]	66,37 ± 2,45 [a]	51,79 ± 2,52 [a]	3,31E+08 ± 8,79E+07 [a]	1,83E+09 ± 3,82E+08 [a]
Group M1	74,80 ± 2,15 [b]	8,88 ± 0,79 [b]	81,51 ± 5,72 [b]	77,79 ± 5,56 [b]	6,76E+08 ± 2,22E+08 [a]	1,99E+09 ± 3,13E+08 [a]
Group M2	80,87 ± 3,33 [ab]	15,49 ± 1,46 [c]	69,13 ± 2,49 [a]	58,39 ± 2,37 [a]	9,75E+08 ± 1,93E+08 [a]	2,79E+09 ± 5,33E+08 [a]

Table 4. Comparison of sperm quality parameters between cultured (n=8) and wild (n=3) *Solea senegalensis* male breeders (from both Groups M1 and M2 formed by mixed of cultured and wild breeders). Sperm volume (µL), percentage sperm motility, curvy linear velocity VCL (µm/s), average path velocity VAP (µm/s), cell concentration - spermatozoa per mL (spz mL^{-1}) and spermatozoa per kg of body weight (spz kg^{-1}). All values are mean ± SEM.

Breeders origin	Sperm Volume (µL)	% Motility	VCL	VAP	Cell concentration spz per kg^{-1} fish	Cell concentration spz per mL^{-1}
Cultured males	339,45 ± 68,51 [a]	19,35 ± 3,47 [a]	91,15 ± 8,82 [a]	84,07 ± 9,75 [a]	8,71E+08 ± 1,83E+08 [a]	2,37E+09 ± 3,48E+08 [a]
Wild males	148,67 ± 44,745 [a]	13,89 ± 3,27 [a]	57,92 ± 14,11 [a]	51,91 ± 11,54 [a]	5,63E+08 ± 2,19E+08 [a]	2,19E+09 ± 6,83E+08 [a]

3.3 Paternity analysis

A total of 190 larvae were collected for paternity analysis from Group M1 from the 19 fertile spawns obtained, of which, 86.84 % were identified as coming from a single wild pair of breeders and 13.16 % were not identified (Figure 5). In the Group M2 a total 36 larvae were collected and analysed from five fertile spawns, 86.11 % were identified as offspring from four breeder couples, while 13.89 % was not identified. One pair was formed by a wild female and a wild male (figure 6). The male (MW2) died at the beginning of reproductive season and just participated in one fertile spawn. This male (MW2) had dominated spawning during previous years (Fatsini 2017). The other three families were from spawns between one cultured male and three different females, one cultured female and two wild females. The pair composed of two cultured breeders, participated in one fertile spawning; while, the pairs formed with two wild females and the culture male participated in three fertile spawns. The unidentified larvae were due to poor quality of the DNA from the sample or genetic similarity between different breeders.

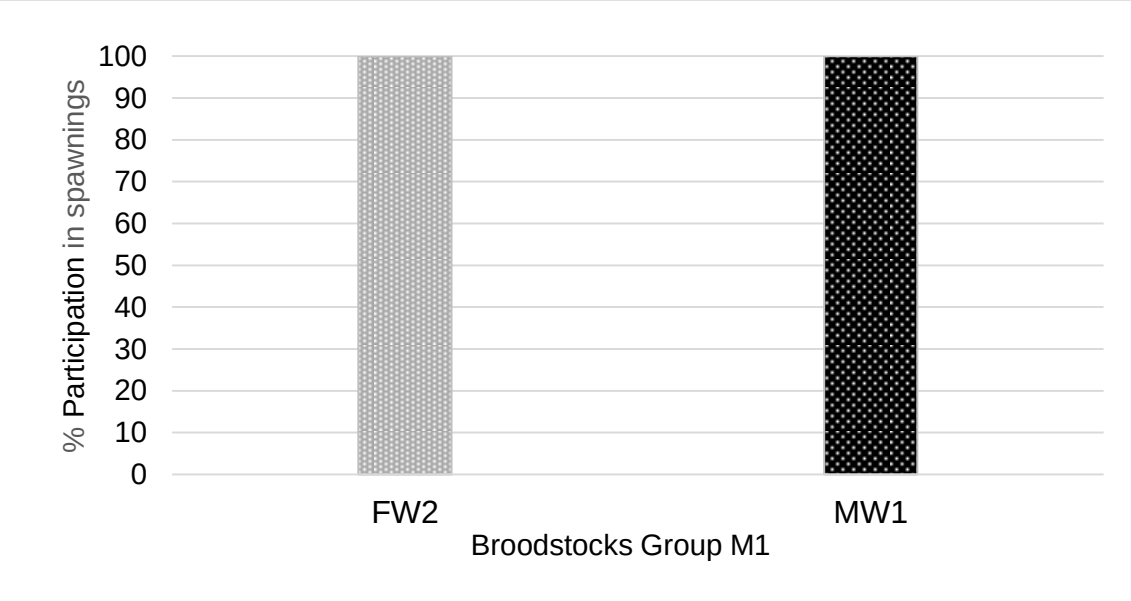

Figure 5. Participation of *Solea senegalensis* breeders in the fertile spawns obtained from Group M1 (Group Mixed of cultured and wild breeders), in accordance with paternity analysis during the 2016 reproductive season. Each bar represents a single breeder, F = female, M = male and W = wild breeder.

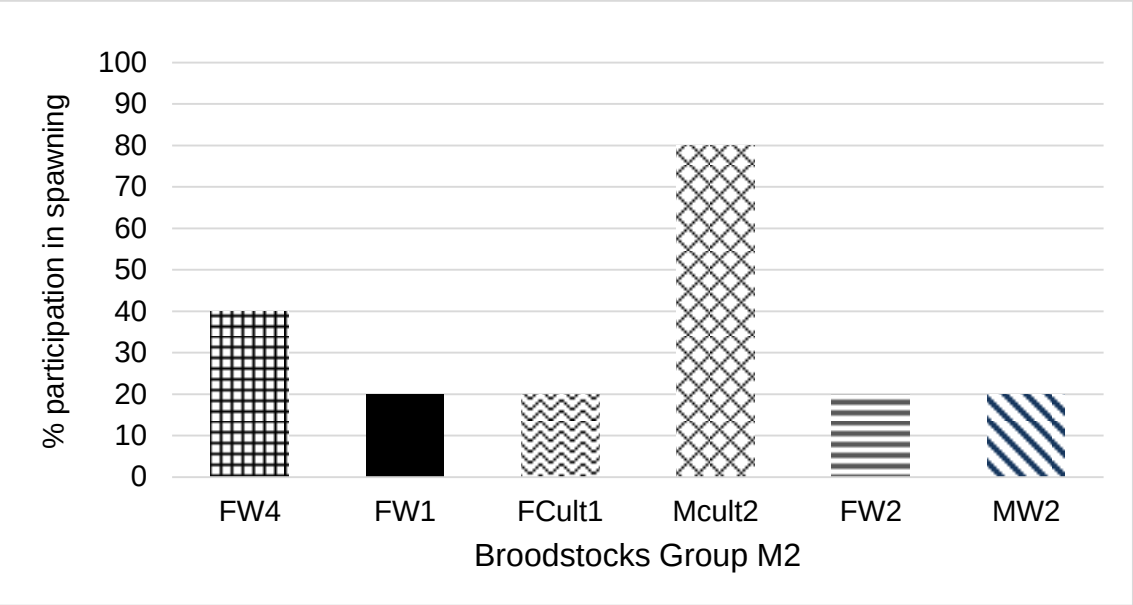

Figure 6. Participation of *Solea senegalensis* breeders in the fertile spawns obtained from Group M2 (Group Mixed of cultured and wild breeders), in accordance with paternity analysis during the reproductive season. Each bar represents a single, F = female, M = male, W = wild breeder and Cult = cultured breeder.

3.4 Behavioural analysis

3.4.1 Locomotor activity

The breeders were observed to increase the number of movements or activity to a peak between 19:00 and 20:00 after which the activity gradually decreased (Figure 7). The activity in the fish was more pronounced on spawning days, compared to days without spawning.

3.4.2 Behaviours related to the courtship, during the peak of locomotor activity.

Behaviours of Rest the head (RTH) and “Follow”, were recorded in all experimental groups (Figure 8). The Couple behaviour was only observed in the Group M1 and the Guardian behaviour was only observed in the Groups M1 and M2. The RTH behaviour, showed significantly higher (P=0,021) number of counts in the Group M1 compared with the control Group. The Guardian behaviour was significantly lower (P=0.001) in Group M2 than Group M1. There were no significant differences in the counts of the Follow behaviour amongst the groups. Only Group M1 exhibited all behaviours related to the courtship and

Group M1 behavioural counts for each behaviour were highest of the three groups.

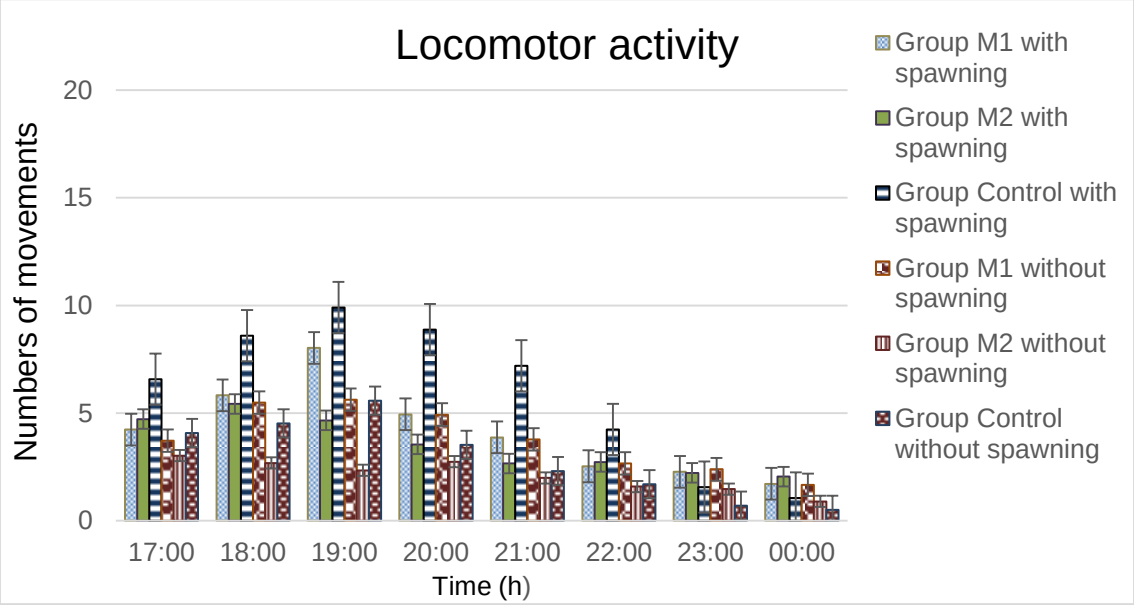

Figure 7. Mean Locomotor activity per hour (n=5) (number of times that a fish crossed the line in middle of the field observed) of *Solea senegalensis* breeders during five days with spawning and five days without spawning in the Groups Control (Group formed just by cultured breeders), M1 and M2 (Groups formed by mixed of cultured and wild breeders). Data was shown as mean ± SEM.

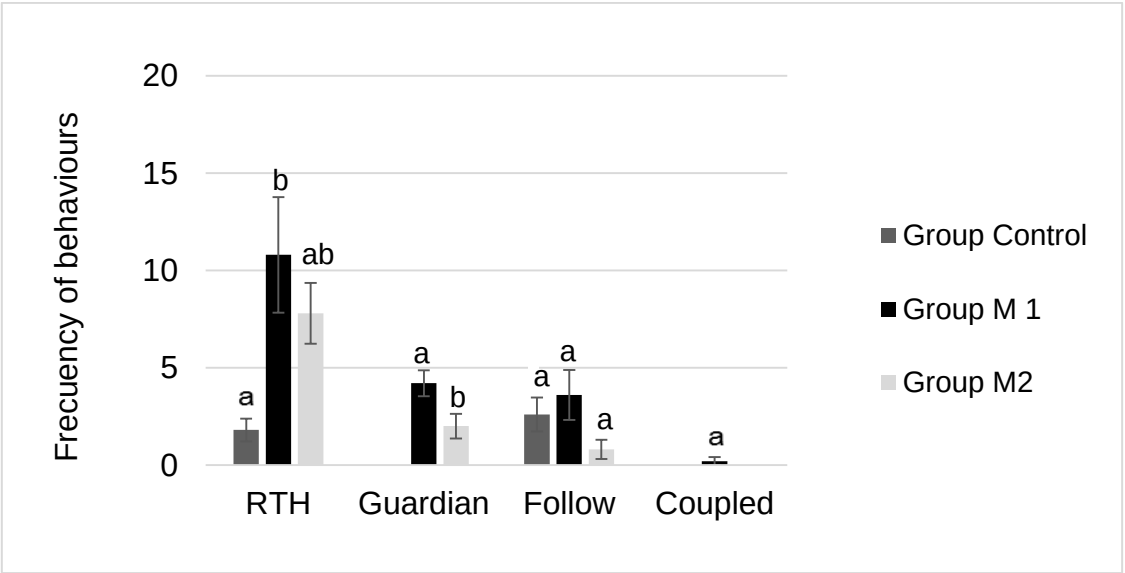

Figure 8. Mean counts of behaviours (Rest the head, Follow, Guardian and Coupled) observed during the peak hour of activity (19:00 to 20:00) in *Solea senegalensis* experimental groups (Control formed just by cultured breeders, M1 and M2 formed by mixed cultured and wild breeders). The groups were evaluated during days with spawning (n = 5). Different letters above the bars represent significant differences between groups.

3.4.3 Identification of individuals in “Follow” behaviours

The Follow Behaviour was analysed in the Groups M1 (Figure 9) and M2 (Figure 10), which had breeders of both origins (wild and cultured). Overall in both Groups (M1 and M2), 85.71 % of males participated in the Follow behaviour and 30 % of females participated. The mean participation of all breeders was 10.66 ± 4.47 % and ranged from 64.10 % (Mcul2 in Group M1) to fish with 0 % participation. The position of individuals in the “Follow” behaviour (Leader or Follower), was different between males and females. The females (cultured and wild) were observed predominantly in the position of leader (95.80 %) during the “Follow” behaviour. In contrast, the “Follower” position was mainly observed in 83.33 % of males, where wild males performed 76.25 % of “Follower” behaviours and cultured males 47.00 % as “Follower”. In spite that the wild males showed a high participation in “Follow” behaviour, the involvement of cultured males in this behaviour was evident in both Groups (M1 and M2). The five cultured males belonging to Group M1, had a participation in “Follow” behaviour from 7.69 % to 64.10 %, whilst in the Group M2, three cultured males participated in the Follow behaviour between 2.86 % and 60.00 %.

In addition, the participation of male breeders in “Follow” behaviour was associated with participation in fertile spawning during the reproductive season. The males from Groups M1 and M2 that contributed as parents according to paternity analysis were observed to also have a high percentage of participation in “Follow” behaviour and mainly in the “follower” position. In the Group M1, the wild male, MW1 that dominated 100% of the spawning had a 41.0 % participation in the “Follow” behaviours (Figure 9). In the Group M2, a cultured male, Mcul2 that dominated 80% of the spawning had a 48.6 % participation in the “Follow” behaviours (Figure 10).

In the case of the Group Control, the “Follow” behaviour was not analysed as all individuals were cultured breeders, participation in the “Follow” behaviour was less frequent than in the others groups and no fertile spawns were obtained.

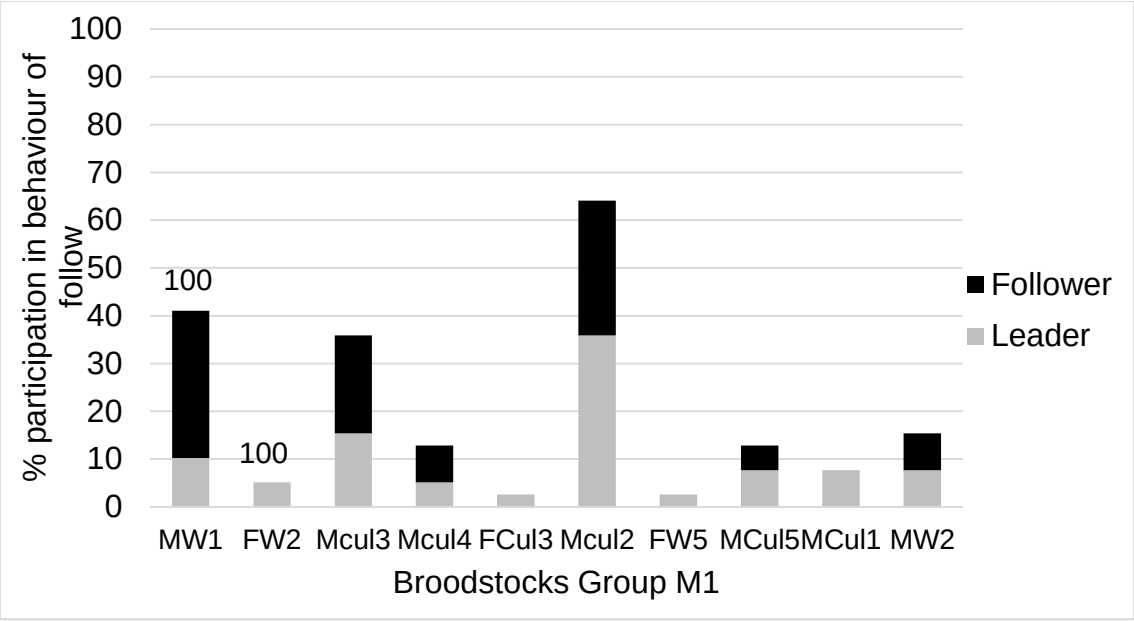

Figure 9. Individual *Solea senegalensis* breeders in Group M1 that were identified to be involved in the "Follow" behaviour in spawning period. The different section in the bars, represents the percentage an individual was the leader (grey), followed by the other individuals and the percentage an individual was a follower (black) and followed the lead fish. M = male, F = female, W = wild breeders, Cul = cultured breeders. The value above the bars indicates the percentage of parental contribution in the spawning of each breeder in accordance to the paternity analysis.

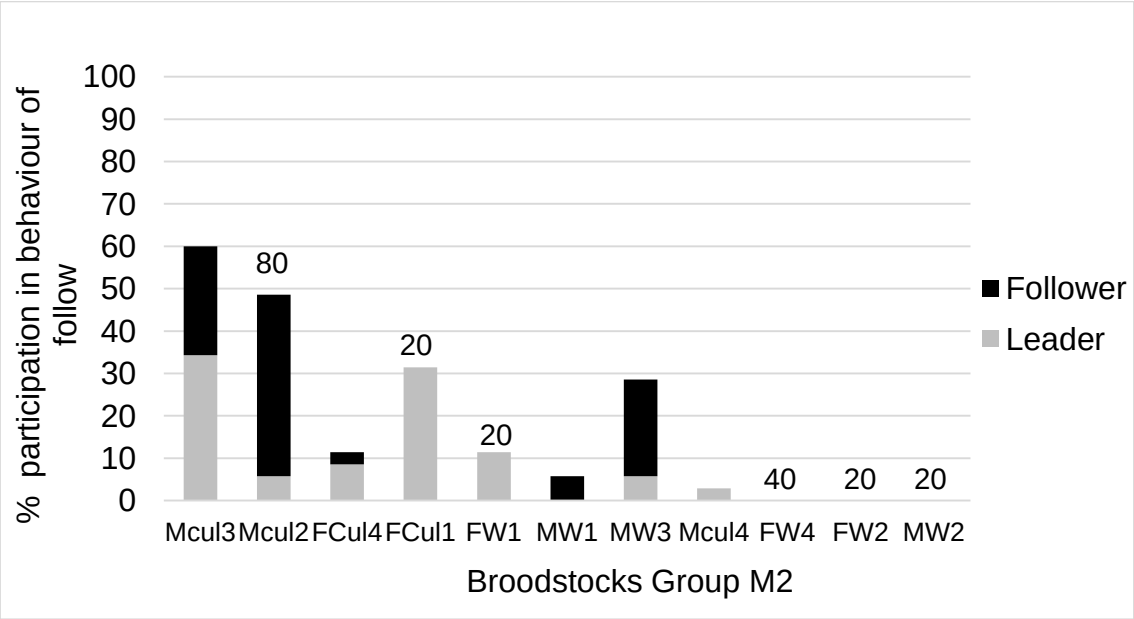

Figure 10. Individual *Solea senegalensis* breeders in Group M2 that were identified to be involved in the "Follow" behaviour in period with spawning. The different section in the bars, represents the percentage an individual was the leader (grey), followed by the other individuals and follower (black) as an individual following to others fish. M = male, F = female, W = wild breeders, Cul = cultured breeders. The value above the bars indicates the percentage of parental contribution in the spawning of each breeder in accordance to the paternity analysis.

4. Discussion

During the entire period of the study (2013 to 2016, see Fatsini et al., 2020), the participation in the courtship behaviour and the spawning of cultured sole breeders increased in the presence of wild breeders that successfully spawned. In this last experimental year (2016), fertile spawns were recorded from the Groups M1 and M2, where cultured breeders and wild breeders cohabited together. In comparison, the Group Control produced fewer spawns that were not fertile. In Group M2, a couple of cultured breeders was identified as the parents of a fertile spawn. The cultured male, also participated in spawns with two wild females to father three more fertile spawns. In the Group M1 all spawns recorded were from a single couple of wild breeders. The fertile spawn obtained from cultured breeders, was the second season that the couple participated in spawning. The previous reproductive season was in 2014 when the same couple had parental contribution in one fertile spawn (Fatsini 2017). In addition, the high participation in behaviours defined as precursors of success spawning (Carazo *et al*., 2016), such as Follow behaviour, was also observed in the cultured breeders to both groups (M1 and M2). The results obtained in the last year and from 2013 to 2015 reproductive seasons (Fatsini 2017), exhibited an increase in the reproductive behaviour of cultured breeders during the period held with wild breeders. Unlike the Group Control (only cultured breeders), where the spawning were not successful during the same period. Some previous treatments with hormone therapies (Agulleiro *et al.,* 2006; Guzmán *et al*., 2008; Carazo 2013), have failed to promote reproductive success in cultured males. Therefore, the stimuli from wild breeders that successfully spawn, to encourage the reproductive behaviour in cultured males, could be an alternative to solve the reproductive dysfunction problem in cultured breeders.

The reproductive success in *Solea senegalensis*, is based on a complex process of courtship and spawning, which involves an increase of locomotor activity and a set of behaviours. The males protect the females from other males and encourage the females to swim from the bottom to release eggs for fertilization (Carazo *et al*., 2016). The presence or absence of courtship behaviours, would indicate the possibility of successful spawning. In the present

study, the behaviours described in the courtship (Carazo *et al*., 2016), such as Rest the Head, Guardian, Follow and Couple behaviours were recorded. Similar reproductive behaviours have been described in other species of flatfish, such as *Engyprosopon grandisquama*, where approach, protection and following females by the males are part of the courtship process (Gibson 2005). The courtship behaviours were higher and at times significantly higher in the Groups M1 and M2 with mixed origin breeders (wild and cultured). In the Group Control, the frequency of behaviours was lower and Guardian and Coupled behaviours were not observed. These behaviours (Guardian and Couple) have relevance as the male protects the female from other males and the coupled swim is to fertilize the eggs release by females. Therefore, the reproductive failure in the cultured breeders held in the Group Control was clear, both by obtaining a few unviable eggs released and the absence of certain critical courtship behaviours during the reproductive period. Similar results were registered in other studies (Carazo 2013; Fatsini, 2017; Martín *et al.,* 2019), where stocks consisting entirely of cultured breeders (males and females), produced only non-fertile spawns. The same patterns in the behaviours from each group, were recorded in the previous reproductive seasons (2013-2015) in the study by Fatsini (2017).

Furthermore the “Follow” behaviour, involved in the first courtship phase and identified as predictors of fertile spawning, has been attributed principally to males (Carazo *et al*., 2016; Martin *et al*., 2019). Initially, the males participated in chases around the tank, both in leader as well as “follower” position, which increased the locomotor activity. The locomotor activity and the behaviours performed by Senegalese sole have been related to the biological rhythms and changes in photoperiod (hours of light–dark) (Bayarri *et al*., 2004; Carazo *et al*., 2013). The peak hour of locomotor activity was recorded from 19:00 to 20:00, coincident with twilight activity of this species (Bayarri *et al*., 2004; Carazo 2013). The females are involved in a lesser extent, however, usually participate as leader fish and were followed by males (Carazo 2013; Carazo *et al*., 2016). During the “Follow” behaviour, the following of leader females may be related to the release of fluids, such as urine or intestinal fluid to stimulate the olfactory system of males (Fatsini *et al*., 2017). The interchange of chemical cues contained in the fluids, would increase the olfactory sensitivity of males, sending

a pheromone message to perform courtship and spawning. However, the reception of these cues might be different depending the origin of male breeders (Fatsini *et al*., 2017). A higher participation in the “Follow” behaviour was shown from cultured breeders in Groups M1 and M2. In the Group M1, all cultured males had participation in the “Follow” behaviour, although the highest participation was by a wild male (MW1), which had parental contribution in all spawns. Whilst in the Group M2, the higher participation in the “Follow” behaviour and mainly in the Follower position was observed in the cultured male (MCult2), which had the parental contribution with a culture female and two wild females. This participation, was gradually increased in the cultured males, in previous years of the study (from 2013 to 2015), reported by Fatsini (2017) until this last year (2016). The participation in courtship behaviours of cultured breeders in the Groups M1 and M2, indicated an effect after a cohabitation period with wild breeders that successfully spawn. Therefore, even though early stage experiences may influence the cognitive ability and social development of fish, the individuals retain neural plasticity to adapt their physiology and behaviour according to recent interactions (Ebbesson and Braithwaite, 2012). The performance of reproductive behaviours in cultured breeders, is linked to a learning process carried out by observation, where cultured breeders observed and learned from wild breeders, the behaviours necessary to spawn.

The ability to learn through the process of observation of conspecifics has been described in fish in relation to several types of behaviour (Brown and Laland, 2003). Takahashi *et al.* (2014), observed that jack (*Pseudocaranx dentex*) juveniles, increased the feed efficiency after observation of the behaviours performed from trained experienced conspecifics. De Gasperin and Macías (2014), found that courtship behaviour in *Girardinichthys multiradiatus*, had been affected during development and early social experience, however, this behaviour appeared to be flexible throughout life and could be adjusted by learning from new experiences. Guppie females (*Poecilia reticulata*) replicated the mate choice behaviour that was learnt from a model female courting with a male (Dungatkin *et al*., 1992). Guevara-Fiore (2012), demonstrated that young male guppies, learned to perform the courtship during their early experience, by observing other males courting, to difference of males that grew up in isolation.

The results obtained by Guevara- Fiore (2012), indicate a similar behavioural pattern to that found in the present study. In the present study, observational learning process, supports the change in the reproductive behaviour observed in cultured *Solea senegalensis* breeders during a period of cohabitation with the experienced wild males. Therefore, the experience with males that performed the courtship, would be transmitted, influencing in the development of sexual behaviour of inexperienced young males (Freeberg 2000). Thus, the courtship may be a socially learned behaviour, as a mechanism to ensure the reproductive success (Galef and Laland, 2005).

Furthermore, the mate selection system may also be a learning process on previous experiences or by observation of conspecifics (Dungatkin *et al*., 1992). Mainly, the females have preference functions to accept or reject the males that perform the courtship (Ramsey *et al*., 2014). Some cues chemical, physical or behavioural may be used to choose potential partners (Auld *et al*., 2019). Dungatkin and Godin (1993) found that in female guppies, the decision to choose a mate was based on copying another female's choice for a specific male. In whitebelly damselfish (*Amblyglyphidodon leucogaster*), the mate choice copying, increases the fitness of chosen partner and provides to the males higher likelihood to find other females (Verzijden *et al*., 2012). The role of females in the mate choice would be related to a social system that avoids assessing males and chooses males that have already succeeded in mating (Dungatkin and Godin 1993). Therefore, males that have not had previous experience in the mate process would have variable reproductive success (Dungatkin 1992). These statements could elucidate why no others pairs are formed within the groups and why the females choose specific males to mate. Consequently, the participation of total male breeders might also be limited by the mate choice system from females.

In addition, despite of the involvement of cultured breeders in courtship behaviour, few breeders exhibited parental contribution regarding to the hatched larvae. In Group M1, all offspring were assigned to a wild couple. In the Group M2, a spawning was obtained from a wild female (FW2) coupled with a wild male (MW2) and afterwards a cultured male (MCult2) was paired with two wild females and one cultured female. A reduced parental contribution in *S.*

senegalensis, was also observed by Porta *et al.* (2006), where a few breeders were responsible for hatched larvae, causing the loss of genetic variability. Moreover, Martin *et al*. (2014), observed reproductive dominance within *S. senegalensis* broodstock and fidelity between the couples formed during the reproductive seasons. The formation of dominant monogamous couples, would explain the lack of participation of others breeders in the spawning and the scarce parental contribution. In contrast to others flatfish, such as winter flounder, a single female spawning with various males without the exhibition of agonistic behaviours amongst breeders (Stoner *et al*. 1999). However, even though the reproductive dominance was observed amongst breeders, the social opportunity to ascend in the reproductive status was observed in the Group M2. The cultured male (MCult2) mated with three females (two wild females and one cultured female), due to the absence by mortality of the dominant wild male (MW2) in previous seasons (Fatsini 2017). This behaviour has been observed in other species as African cichlid fish, where the reproductively supressed males that perceive an opportunity to mate, adopt the dominant role to gain reproductive success (Maruska and Fernald 2010). However, the few spawning obtained from the cultured male, appears not to be sufficient to determine total reproductive success in cultured males. Nevertheless, these spontaneous fertile spawns have shown that cultured males have the reproductive capacity to participate in the entire process of mating and reproduction to produce viable larvae and that as previously demonstrated through artificial fertilization (Rasines *et al*., 2012; 2013) the cultured males produce viable sperm. The sperm quality assessed between cultured and wild males in the present study, exhibited no significant differences in the cell concentration, motility percentage and velocity parameters. It should be mentioned that although the “n” was low and that there was no difference should be concluded with caution, all mean sperm quality parameters were actually higher in cultured males. This is in contrast to that obtained by Cabrita *et al*. (2006), who found a lower cell concentration and motility percentage in the cultured males spermatozoa compared to wild males. However Riesco *et al.* (2019), did not find differences in viability and total motility in sperm samples analyzed from wild and cultured males except in the velocity (VCL). While the differences found amongst Group M1 and Groups M2 and Control in motility percentage as velocity (VCL and

VAP), perhaps could have been influenced by the mix of breeders. Some differences in sperm quality recorded amongst studies might be due to the method to collect the sample (Gwo, 2009) or broodstock management practices (Bobbe and Labbé, 2010).

5. Conclusions

The increased participation of the cultured breeders, in courtship behaviour and fertile spawning, indicated the positive effect on cultured Sole males of a period of cohabitation with spawning wild Sole breeders. The participation from cultured breeders in the Follow behaviour, was related to fertile spawning obtained from a cultured male (Group M2), with parental contribution in larvae hatched from four spawns. While in comparison, the Group Control did not provide fertile spawns and the courtship process was not complete, as has been described as the reproductive dysfunction in cultured males (Carazo 2013; Martin et al., 2019). The outcomes in the Mixed Groups, M1 and M2, indicate that there was a learning process, where cultured breeders observed wild breeders with reproductive success. However, the reproductive success of cultured males may have also been influenced by mate choice behaviour and reproductive dominance. The behavioural study in the reproduction of *S. senegalensis*, has open a relevant behavioural mechanism that offers potential to solve the reproductive dysfunction in the cultured males. However, reproductive success remains unpredictable and deficient to obtain sufficient spontaneous fertile spawning from cultured breeders for the aquaculture industry. Therefore, further research is required to focus on other physiological and behavioural aspects of this species that are not yet known.

6. References

Agulleiro, M. J., Anguis, V., Canavate, J. P., Martinez-Rodriguez, G., Mylonas, C. C., Cerda, J., 2006. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257(1-4): 511-524.

APROMAR, 2019. La acuicultura en España 2019. Asociación Empresarial de Productores de Cultivos Marino Chiclana (Cádiz). http://www.apromar.es/content/informes-anuales

Auld, H., L., Noakes, D., L., G., Banks, M., A., 2019. Advancing mate choice studies in salmonids. Rev Fish Biol Fisheries, 29:249–276. https://doi.org/10.1007/s11160-019-09551-5

Bayarri, M.J., Munoz-Cueto, J.A., Lopez-Olmeda, J.F., Vera, L.M., Rol de Lama, M.A., Madrid, J.A., Sanchez-Vazquez, F.J., 2004. Daily locomotor activity and melatonin rhythms in Senegal sole (*Solea senegalensis*). Physiol. Behav. 81, 577–583.

Bobe, J., Labbé C., 2010. Egg and sperm quality in fish. General and Comparative Endocrinology 165: 535–548.

Brown, C., Laland, K. N., 2003. Social learning in fishes: A review. Fish and Fisheries 4: 280- 288.

Cabrita, E., Soares, F., Dinis M. T., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975.

Carazo, I. 2013. Comportamiento reproductivo y fisiología del lenguado senegalés (*Solea senegalensis*) en cautividad. Ph. D. Thesis, Universidad de Barcelona (Spain).

Carazo, I., Norambuena, F., Oliveira, C., Sanchez-Vazquez, F. J., Duncan, N. J., 2013. The effect of night illumination, red and infrared light, on locomotor activity, behaviour and melatonin of Senegalese sole (*Solea senegalensis*) broodstock. Physiology & Behavior 118: 201-207.

Carazo, I., Chereguini, O., Martín, I., Huntingford, F., Duncan, N. 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*). Spanish Journal of Agricultural Research 14, 13pp. https://dx.doi.org/10.5424/sjar/2016144-9108

De Gasperin, O., Macías, G. C., 2014. Congenital predispositions and early social experience determine the courtship patterns of males of the Amarillo fish. Behav Ecol Sociobiol. 68:639–648 DOI 10.1007/s00265-013-1678-3

Dinis, M. T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176: 27–38.

Dugatkin, L.A., 1992. Sexual selection and imitation: females copy the mate choice of others. Am. Nat. 139, 1384-1489.

Dugatkin, L.A., Godin, L., A., 1993. Female mate copying in the guppy (Poecilia reticulata): age-dependent effects. Behavioral Ecology Vol. 4 No. 4: 289- 292.

Duncan, N., Carazo, I., Chereguini, O., Mañanós, E. 2019. Mating Behaviour. In: Muñoz-Cueto, J.A., Mañanós-Sánchez, E.L., Sánchez- Vázquez, F.J. (Eds). The Biology of Sole. Boca Raton, FL: CRC Press Taylor and Francis Group. Pp. 169-184.

Ebbesson, L. O. E., Braithwaite, V. A., 2012. Environmental effects on fish neural plasticity and cognition. Journal of Fish Biology 81, 2151–2174 doi:10.1111/j.1095-8649.2012.03486.x

Fatsini, E., 2017. Reproduction, olfaction and Dominance behaviour in Senegalese sole (*Solea senegalensis*). PhD Thesis. Polytechnic University of Valencia. 203 pp.

Fatsini, E., Carazo, I., Chauvigne, F., Manchado, M., Cerda, J., Hubbard, P. C., Duncan, N. J., 2017. Olfactory sensitivity of the marine flatfish *Solea senegalensis* to conspecific body fluids. J. Exp. Biol. 220, 2057-2065.

Fatsini, E., González, W., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

Fernández-Díaz, C., Yúfera, M., Cañavate, J., P., Moyano, F., J., Alarcón, F., J., Díaz, J., 2001. Growth and physiological changes during metamorphosis of

Senegal sole reared in the laboratory. Journal of Fish Biology 58: 1086–1097. doi:10.1006/jfbi.2000.1514.
Freeberg, T., M., 2000. Culture and courtship in vertebrates: a review of social learning and transmission of courtship systems and mating patterns. Behavioural Processes 51, 177–192.

Galef, B., G., Laland, K., N., 2005. Social Learning in Animals: Empirical Studies and Theoretical Models. BioScience, Vol. 55 No. 6, 489-500.

Gibson, R., 2005. Flatfishes: Biology and Exploitation: Blackwell Science Ltd. a Blackwell Publishing Company. 391 pp.

Guevara-Fiore, P., 2012. Early social experience significantly affects sexual behaviour in male guppies. Animal Behaviour 84: 191-195. doi:10.1016/j.anbehav.2012.04.031

Guzman, J. M., Ramos, J., Mylonas, C. C., Mañanós, E. L., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. Aquaculture 291(3-4): 200-209.

Guzman, J. M., Ramos, J., Mylonas, C. C., Mañanós, E. L., 2011. Comparative effects of human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa) treatments on the stimulation of male Senegalese sole (*Solea senegalensis*) reproduction. Aquaculture 316: 121-128. doi:10.1016/j.aquaculture.2011.03.014

Gwo, J. C., 2009. Methods for sperm collection. In Cabrita, E., Robles, V., Herraez, P (Eds.), Methods in Reproductive Aquaculture. Boca Raton, FL: CRC Press Taylor and Francis Group, pp.81-89.

Howell, B.R., Dinis, M.T. 2019. An overview of Soleid (Pleuronectiformes) Fisheries and Aquaculture. In: The Biology of Sole. Editors, José A. Muñoz Cueto, Evaristo L. Mañanós Sánchez, F. Javier Sánchez Vázquez. CRC Press. Boca Raton Florida. Pp 3-23.

Martín, I., Rasines, I., Gómez, M., Rodriguez, C., Martinez, P., Chereguini, O. 2014. Evolution of egg production and parental contribution in Senegalese sole,

Solea senegalensis, during four consecutive spawning seasons. Aquaculture 424-425, 45-52. https://doi.org/10.1016/j.aquaculture.2013.12.042 537

Martín, I.E., 2016. Advances in the Reproductive Biology and Zootechnics of the

Senegalese Sole (*Solea senegalensis* Kaup, 1858). Universidad de Cantabria, Cantabria, Departamento de Ciencias y Técnicas del Agua y del Medio Ambiente, pp. 197.

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Spanish Journal of Agricultural Research, [S.l.], v. 17, n. 4, p. e0608. ISSN 2171-9292 doi:http://dx.doi.org/10.5424/sjar/2019174-14953.

Maruska, K.P., Fernald, R. D. 2010. Behavioral and physiological plasticity: Rapid changes during social ascent in an African Cichlid fish. Hormones and Behaviour 58: 230-240.

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M. and Dinis, M. T. 2016, New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture 8, 227–263. https://doi.org/10.1111/raq.12091

Porta, J., Porta, J.M., Martínez-Rodríguez, G., Alvareza, M.C., 2006. Genetic structure and genetic relatedness of a hatchery stock of Senegal sole (*Solea senegalensis*) inferred by microsatellites. Aquaculture 251: 46– 55.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326-329: 129–135.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2013. Artificial fertilisation of cultured Senegalese sole (*Solea senegalensis*):

effects of the time of day of hormonal treatment on inducing ovulation. Aquaculture 392–395: 94–97.

Ramsey, M., E., Vu, W., Cummings, M., E., 2014. Testing synaptic plasticity in dynamic mate choice decisions: N-methyl D-aspartate receptor blockade disrupts female preference. Proceedings Real Society B 281: 20140047. http://dx.doi.org/10.1098/rspb.2014.0047

Riesco, M. F., Valcarce, D. G., Martínez-Vázquez, J. M., Martín, I., Calderón-García, A. A., González-Nunez, V., Robles V., 2019. Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question. Reproduction, Fertility and Development. https://doi.org/10.1071/RD18453

Stoner, A.W., Bejda, A.J., Manderson, J. P., Phelan, B. A., Stehlik, L. L., Pessutti, J.P., 1999. Behaviour of winter flounder, *Pseudopleuronectes americanus*, during the reproductive season: laboratory and field observations on spawning, feeding, and locomotion. Fish. Bull. 97: 999–1016.

Takahashi, K., Masuda, R., Yamashita, Y., 2014. What to copy: the key factor of observational learning in striped jack (*Pseudocaranx dentex*) juveniles. Anim Cogn. 17:495–501 DOI 10.1007/s10071-013-0686-z

Verzijden, M., N., Cate, C., Servedio, M., R., Kozak, G., M., Boughman, J., W., Svensson, E., I., 2012. The impact of learning on sexual selection and speciation. Trends in Ecology and Evolution, Vol. 27, No. 9, 511- 519. http://dx.doi.org/10.1016/j.tree.2012.05.007

Zohar, Y., Mylonas, C.C., 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197: 99–136.

Chapter 3

Reproductive behaviour and fertile spawning in cultured *Solea senegalensis* broodstocks that prior to puberty had different social learning opportunities.

Chapter 3 Reproductive behaviour and fertile spawning in cultured *Solea senegalensis* broodstocks that prior to puberty had different social learning opportunities.

1. Introduction

The reproductive failure presented in *Solea senegalensis* cultured breeders represents a problem for the species reproductive control in captivity. This problem has been attributed to the lack of reproductive behaviour in cultured males to court with females to mate (Carazo 2013; Martin *et al*., 2019). Unlike wild male breeders, which perform the courtship properly, achieving synchronisation with the females to obtain fertile spawns (Carazo *et al*., 2016). However, the lack of reproductive behaviour in cultured males, could involve processes associated to social learning. The breeders would be influenced by social learning to participate in courtship and spawning (Bennett and Laland, 2005; Fatsini *et al*., 2020; Chapter 2). Therefore, the study of the mechanisms that control reproductive behaviour in Sole males may provide solutions to the dysfunction in reproductive behaviour identified in culture males.

The reproductive differences between wild and cultured breeders, could be a consequence to several processes occurring during domestication. Hence, the early rearing and breeding conditions may be a cause for the development of reproductive dysfunction (Huntingford, 2004). The cultured rearing environment provides different experiences to the cultured males compared to those experienced by wild broodstock. Experiences during the early development stage could lead to different cues and specific learning that alter behaviours at later developmental stages (Wells, 2007; Huntingford, 2004). Thereby, the changes that occurred in these early development stages, may bring modifications at later stages. These alterations may be inherited to modifying behaviour in just a few generations (Huntingford, 2004; Batenson, 2017). Fish reared in the hatchery have been observed to develop novel strategies, mechanisms or solutions, to the demand or situations to which they are exposed. For example, fish reared in captivity, increased the efficiency to access to new food (prey) after exposure to a learning experience as ‘observer’ of conspecifics (Huntingford, 2004). Moreover, the intensive interaction with

conspecifics, owing to high density or tank confinement, could serve as an element of learning, helping to reduce the error in the response to new situations (Huntingford, 2004; Guevara-Fiore, 2012). Cogliati *et al*. (2019), tested the spatial learning ability to exit a maze, in juveniles of chinook *Oncorhynchus tshawytscha* salmon reared in different environments. The experience demonstrated that the hatchery fish were motivated to learn, due to the rearing density. The stimulation of fish of the same species that had completed the test, resulted in a positive reinforcement for test fish. This last point, demonstrates how the fish bred in captivity can acquire new knowledge or information by observing. This learning may be carried out by interaction with conspecifics, their environment or particular incidence occurred, which is called social learning (Brown and Laland, 2003). The social learning, in addition to involving the acquisition of knowledge or information, facilitate the adaptability to achieve a certain task or behaviour that was previously unknown to the individuals (Brown and Laland, 2003).

Social learning has been studied in various taxa included fish species, such as guppies (Guevara-Fiore, 2012) and fish of the Acanthuridae family. A variety of aspects of learning have been included, such as food choice, response to predators or matchmaking. This learning has been performed by having contact or seeing the behaviour from experienced or wild conspecifics (Brown and Laland, 2003). Usually, these learning experiences are obtained from early life stages, allowing for the adequate development of individuals. A relevant aspect, where the social learning plays an important role is the mate choice. The opportunity to mate is an evolutionary goal, driven by a strong competition between conspecifics and hence a wide variety of strategies are used to achieve successful mating. An essential aspect of male development may be the learning of courtship behaviour in order to stimulate females to mate with the correct sexual behaviour. These mating strategies and courtship behaviour may need to be learnt by immature males through interactions with mature males that perform the courtship with females. This interaction can be by observing the behaviour of conspecifics or also previous recognition of females by interactions (Guevara-Fiore, 2012; Brown and Laland, 2003).

Therefore, the lack of reproductive behaviour of cultured males could be related to social learning and the loss of opportunities to learning reproductive behaviour. In the study carried out by Fatsini *et al*. (2020), which include the results obtained in the last year of study and reported in the Chapter 2 of this thesis, was observed the effect of the presence of spawning wild breeders on the reproductive success and behaviour of cultured breeders. Wild broodstocks (males and females) that have had successful spawns in captivity were established with cultured breeders (males and females), which had no previous experience in the reproduction, in two different groups (M1 and M2). These groups with different reproductive capacities were compared during four consecutive reproductive seasons (from 2013 to 2016). Also, a Control group formed only with cultured breeders (males and females), which have never spawned fertilised eggs was recorded and compared. The spawns collected and behaviour performed by the fish, were analysed in the three groups and paternity analysis of the larvae obtained was determined using the specific microsatellites for sole. The analysis of parental contribution indicated the participation of cultured breeders in the Group M2, during two reproductive seasons (2014 and 2016). Where a cultured male participated to fertilize spawns with both wild and cultured females. In addition during the last year of the study, 2016, the wild dominant male died (Group M2, Chapter 2) and it appeared this enabled a cultured male to participate in all fertile spawns. The participation in the courtship process and in fertile spawns was increased significantly when the cultured breeders were housed together with wild breeders. While in the Control group, no fertile spawning were obtained and the complete sequence of reproductive behaviour was not observed. The findings observed, suggests a clear participation of cultured males, associated with social activity and a learning process; the mechanism of development of social behaviour by contact with experienced wild conspecifics, would help to the acquisition of skills to mating and solve the reproductive problem (Duncan *et al.*, 2019).

Therefore in the present study, after a period of cohabitation with wild breeders, stocks of cultured sole breeders, were organized as different experimental groups without the presence of wild breeders (See description below W1 and

W2) as well as Control groups (See below CP and CN) and were tested during three reproductive seasons, in order to examine the reproductive behaviour and successful spawning considering the different holding conditions presented from the juvenile stage until puberty in each stock.

2. Methods

The experiment was carried out during three years (from December 2015 to December 2018), testing four groups of cultured Senegalese sole breeders, during each annual reproductive season.

2.1 Broodstock management

The experimental conditions, handling, temperature regime, photoperiod and feeding of the breeders in the experimental groups in the present study, were similar to those described in chapter 2, section 2.1.

2.2 Experimental design

The breeders (Table 1) were set up in four tanks from November - December 2015, grouped according to different treatments:

1.- Groups W1 and W2, were two replicate tanks of cultured male and female breeders that were reared prior to the experiment, from December 2012 to December 2015, with spawning wild breeders. The fish were juveniles that had approximately 100 g when stocked with the wild breeders. Over the three years prior to the experiment the fish grow to 619.53 ± 60.85 g (Group W1) and 644.34 ± 62.63 g (Group W2) and attained puberty. The juveniles were from the same stock as the fish in groups CP and CN.

2.- Group CP, was a positive control of cultured male and female breeders that were reared prior to the experiment, from December 2012 to December 2015 with a cultured breeders that liberate unfertilised eggs. The fish were juveniles that had approximately 100 g when stocked with the cultured breeders. Over the three years prior to the experiment the fish grow to 619.69 ± 69.08 g and attained puberty. The juveniles were from the same stock as the fish in groups W1, W2 and CN.

3. - Group CN, was a negative control of cultured male and female breeders that were reared prior to the experiment in isolation as a single year class and had no contact with adult breeders during rearing. The fish were from the same stock as the fish in groups W1, W2 and CP. In the three years prior to the initiation of the experiment, the fish grow from juveniles of approximately 100 g to 424.04 ± 45.89 g and attained puberty.

All the tanks were left undisturbed during the spawning seasons, from March to June of each year. The same parameters referred to in Chapter 2 were determined: spawning data, paternity of larvae and reproductive behaviour. During the study, there was a reduction in the fish stock in each tank caused by mortality of fish from disease (Table 1). During 2018, disease killed all but four fish in the Group W2. The four surviving fish were transferred to the Group W1, a replicate of the same treatment.

2.3 Collection and Spawns Quality

The evaluation of egg quality and preservation of larvae were carried out as described in chapter 2, section 2.3 during three reproductive seasons (2016-2018). The parameters measured were the following: number of total spawns, mean weight of floating eggs (g), total volume of eggs (mL), total volume of floating eggs (mL), total volume of sinking eggs (mL), relative fecundity - eggs production per kg female (g), mean fertilisation rate (%), mean hatching rate (%) and total number of hatched larvae.

2.4 Sperm quality

2.4.1 Samples collection

Sperm samples (n=45), were collected under the same method described in the Chapter 2, section 2.4.1, from all males included in the four experimental groups. The sampling was carried out once at the end of the first reproductive season (May 2016).

Table 1. Summary of the distribution of Senegalese sole (*Solea senegalensis*) breeders in different Groups: Groups W1 and W2 that were reared prior to puberty with wild breeders, Group CP that were reared prior to puberty with cultured breeders and Group CN that were reared with no contact with breeders indicating, the number of individuals per tank (N), stock density, sex (M = male, F = female) and weight (mean ± SEM) of the fish.

Treatments	Year	N	Stock density (kg/m³)	Sex	Weight (g)
Cultured Fish reared with wild breeders (W1)	2016	27	1,30	M=13 F=14	674,99 ± 65,87
	2017	27	1,84	M=13 F=14	954,44 ± 84,56
	2018	24	1,57	M=12 F=12	920,58 ± 90,11
Cultured Fish reared with wild breeders (W2)	2016	26	1,36	M=11 F=15	734,74 ± 67,75
	2017	26	1,91	M=11 F=15	1031,35 ± 79,37
	2018	-	-	-	-
Cultured Fish reared with F1 breeders (CP)	2016	27	1,55	M=9 F=18	804,87 ± 68,98
	2017	26	2,09	M=9 F=17	1129,11 ± 74,25
	2018	25	2,03	M=9 F=16	1136,96 ± 76,85
Cultured Fish reared isolated (CN)	2016	27	0,90	M=14 F=13	486,09 ± 35,31
	2017	20	1,12	M=12 F=8	788,32 ± 60,94
	2018	19	1,08	M=12 F=7	798,13 ± 60,94

2.4.2 Sperm quality parameters

Cell concentration of sperm samples was determined as described in Chapter 2, section 2.4.2. The sperm samples collected from males in the four experimental groups were assessed to determine total time of motility (s) and motility percentage. Sperm samples were analysed immediately within 10 minutes after collection. A 1 µL subsample from each sperm sample was placed

on the slide and activated by mixing with 19 µL of natural seawater. After activation, the movement of sperm was observed under the microscope Leica with the objective 10 x, the initial, within the first 15 seconds after activation, percentage motility was estimated in units of ten and motility was timed until it ceased (no forward movement) and the total time of sperm motility was recorded. The activations were performed in triplicate for each sperm sample. The motility analysis was carried out by the same person to avoid subjective errors.

2.5 Paternity analysis

The genetic analysis to identify paternity of the larvae hatched, was as described in Chapter 2, section 2.5.

2.6 Behavioural analysis

The behavioural analysis in the four experimental groups was performed as described in the Chapter 2 section 2.6. The Locomotor Activity analysis in Sole breeders was determined as in section 2.6.1 and the frequency of behaviours (RTH, Guardian, Follow and Couple) during the peak of Locomotor activity was determined in the same way as in section 2.6.2., by randomly choosing three days where were obtained fertile spawning previously analysed.

2.7 Statistical analysis

All data were presented as mean ± one standard error of the mean (SEM). The locomotor activity was assessed following the method used by Carazo *et al*. (2016). The mean of sperm volume, cell concentration and motility percentage and counts of different behaviours were compared through a one way ANOVA ($p < 0.05$) and a Games-Howell post-hoc test was applied to determine significant differences among treatments. The spawning quality was presented as the mean percentage of fertilisation and hatching of spawning among the treatments. Statistical analysis was carried out using SPSS Statistic 23 for Windows (SPSS Inc. Chicago, IL, USA).

3. Results

3.1 Spawning quality

During the three years, spawning was obtained from all tanks (Table 2). However, the only year in which fertilised eggs were obtained was the 2016 season. The fertilised eggs were collected from Group W1, a replica treatment where the cultured fish had been reared prior to puberty with spawning wild breeders (Figure 1). The eight fertilized spawns collected, accounted for 21.1 % of the total number of spawns (38) obtained throughout the season (Table 2).

Successful spawns were obtained mainly in the third week of April and one in mid-May. The mean floating eggs portion from the eight spawns collected was 98,550 ± 15,468 with a fertilization rate of 28.02 ± 13.80 %, a hatching rate of 15.04 ± 10.40 % and the mean number of larvae obtained per spawn was 7,683 ± 5,947 and the total number of larvae from all eight spawns was 61,468.

The assignation of parents to the hatched larvae was completed using 6 microsatellites. A total of 70 larvae from 8 different spawning were collected from Group W1 and analysed. 64.3% of larvae were assigned to one family or a single couple of breeders, while 34.3 % of larvae were not assigned to a single family, but inconclusively to more than three parents. This was due to the similarity in genetic identity as the potential parents had high levels of kinship among the breeders. Lastly, 1.4 % of larvae were discarded owing to the poor quality of the genetic material.

Table 2. Summary of spawn quality for each year and each treatment, Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared isolated. The treatment in each tank, year, number of spawns (N= total number of spawns and H= number of hatched spawns), total weight of floating eggs (g), total volume of eggs (mL), total volume of floating eggs (mL), total number of eggs produced per kg of female. The fertilisation rate (mean ± SEM) and hatching rate (mean ± SEM) were calculated for the eight fertile spawns.

Treatments (tanks)	Year	Spawning number/ N - H	Weight Floating eggs (g)	Total Volume eggs (mL)	Floating volume eggs (mL)	Eggs Production /female (kg)	Mean Fertilisation rate %	Mean Hatching rate %
F1 Fish reared with wild broodstock (W1)	2016	38-8	1164,90	3458	1558	107.618	28.02 ± 13.80	15.04 ± 10.40
	2017	34-0	565,88	2150	785	50.651	0,00	0,00
	2018	35-0	760,49	3190	1015	72.010	0,00	0,00
F1 Fish reared with wild broodstock (W2)	2016	22-0	395,68	1146	576	36.275	0,00	0,00
	2017	34-0	747,64	2315	935	51.716	0,00	0,00
	2018	-	-	-	-	-	-	-
F1 Fish reared with F1 broodstock (CP)	2016	13-0	274,31	715	405	17.702	0,00	0,00
	2017	5-0	202,20	435	275	12.394	0,00	0,00
	2018	15-0	349,15	1220	410	22.363	0,00	0,00
F1 Fish reared isolated (CN)	2016	21-0	474,63	1093	683	68.461	0,00	0,00
	2017	15-0	239,56	570	330	49.471	0,00	0,00
	2018	23-0	389,32	1570	500	80.002	0,00	0,00

The breeders identified as parents of hatched larvae, were a male with a weight of 818 g and longitude of 33.5 cm. The male had the following sperm quality parameters: a similar sperm volume (245 µL) to the mean obtained to the Group W1 (Table 3). The motility percentage (90 %) and total time of motility were higher than the average and the cells concentration both per kg (4.09E+08 spz/kg) as per mL (1.37E+09 spz/mL), were lower than the mean recorded in the Group W1. The female had a weight of 929 g, a longitude of 33.5 cm and was classified in stage 4, according to the abdominal swelling and visual ovary size, which goes along the entire length of the visceral cavity (García-López *et al*., 2006). The other three groups (W2, CP and CN) compared to Group W1, had less spawns, lower volume and number of eggs and no fertilisation.

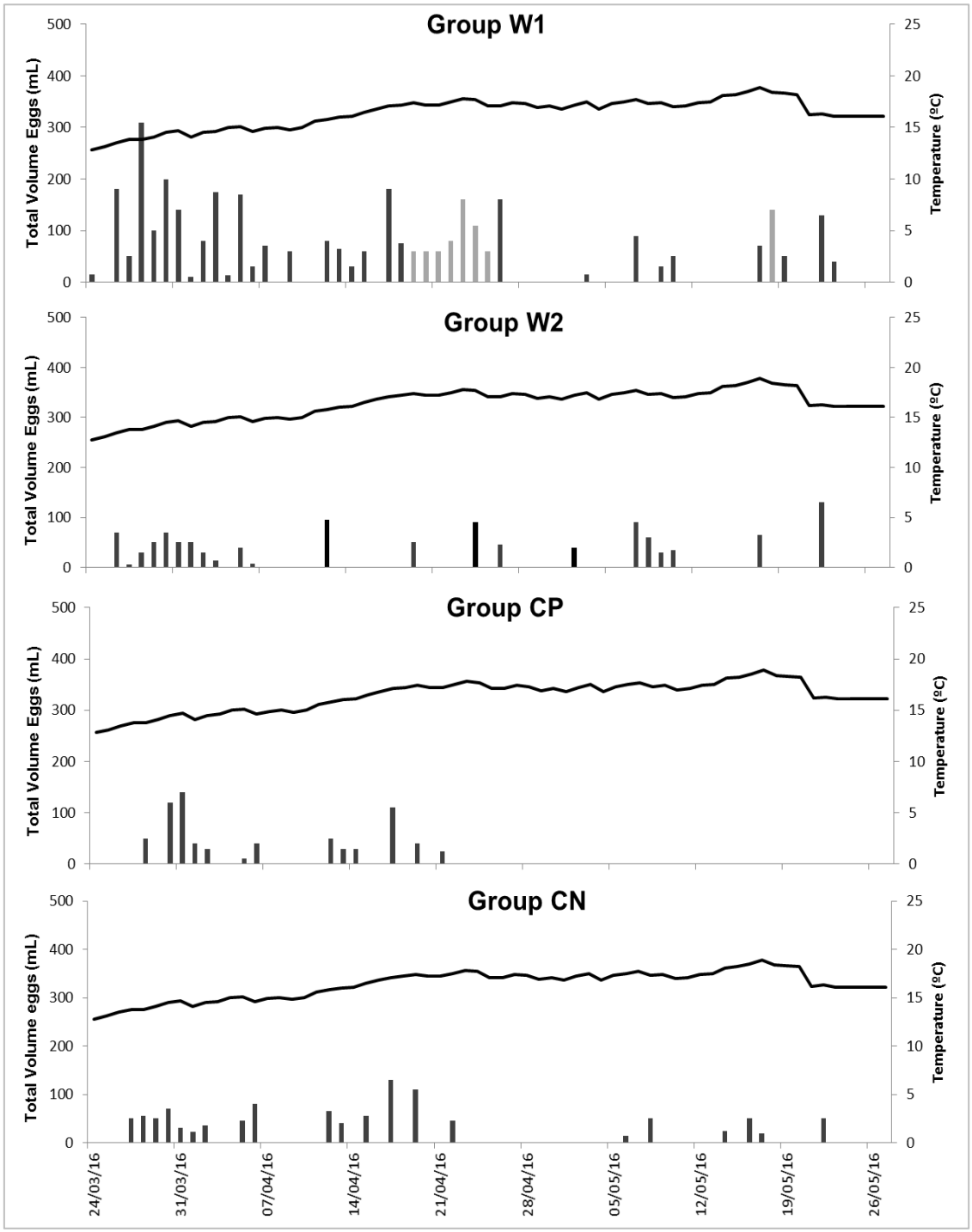

Figure 1. Total sole (*Solea senegalensis*) egg volume collected each day and temperature during the 2016 spawning season for all treatments: Group W1, cultured fish reared before puberty with wild breeders; Group W2, cultured fish reared before puberty with wild breeders; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared in isolation. The grey bars represent fertile spawning and the black bars are non-fertile spawning.

In addition, a considerable variation in egg production was found amongst years and treatments. In the 2016 season, the highest total volume of eggs (mL) was obtained from Group W1 with a significant difference ($P<0.001$) compared to the Groups W2, CP, and CN (Fig. 4). With regard to floating eggs (mL) in the 2016 season, significant differences were found amongst Groups W1 and W2, CP and CN ($P<0.001$). The highest volume was recorded in Group W1 and the lowest in the Group CP, while Groups W2 and CN had similar volume (Fig. 5). Also, significant difference was found in the weight of floating eggs (g) amongst the experimental groups ($P=0.001$). A highest weight was in the Group W1 and lowest in the Group CP, while the Groups W2 and CN were similar to both Groups W1 and CP (Fig. 6).

Although no more fertilised spawns were obtained during 2017 a total of 88 spawns were obtained from all tanks (Figure 2). In this season, the total eggs volume (mL) was significantly different ($P<0.001$) amongst Groups. The total eggs volume in the Groups W1 and W2 was similar, but higher than in the Groups CP and CN. The lowest total volume of eggs was recorded in the Group CP (Fig. 4). The floating eggs volume (mL) in the Groups W1 and W2 were similar, but were found different with regard to the groups CP ($P= 0.008$ and $P=0.002$) and CN ($P= 0.004$ and $P=0.001$ respectively). The highest volume of floating eggs (mL) was observed in the Group W2 and the lowest volume in the Group CP (Fig. 5). In addition, the weight of floating eggs (g) amongst the experimental groups did not exhibit significant differences ($P=0.335$) (Fig. 6).

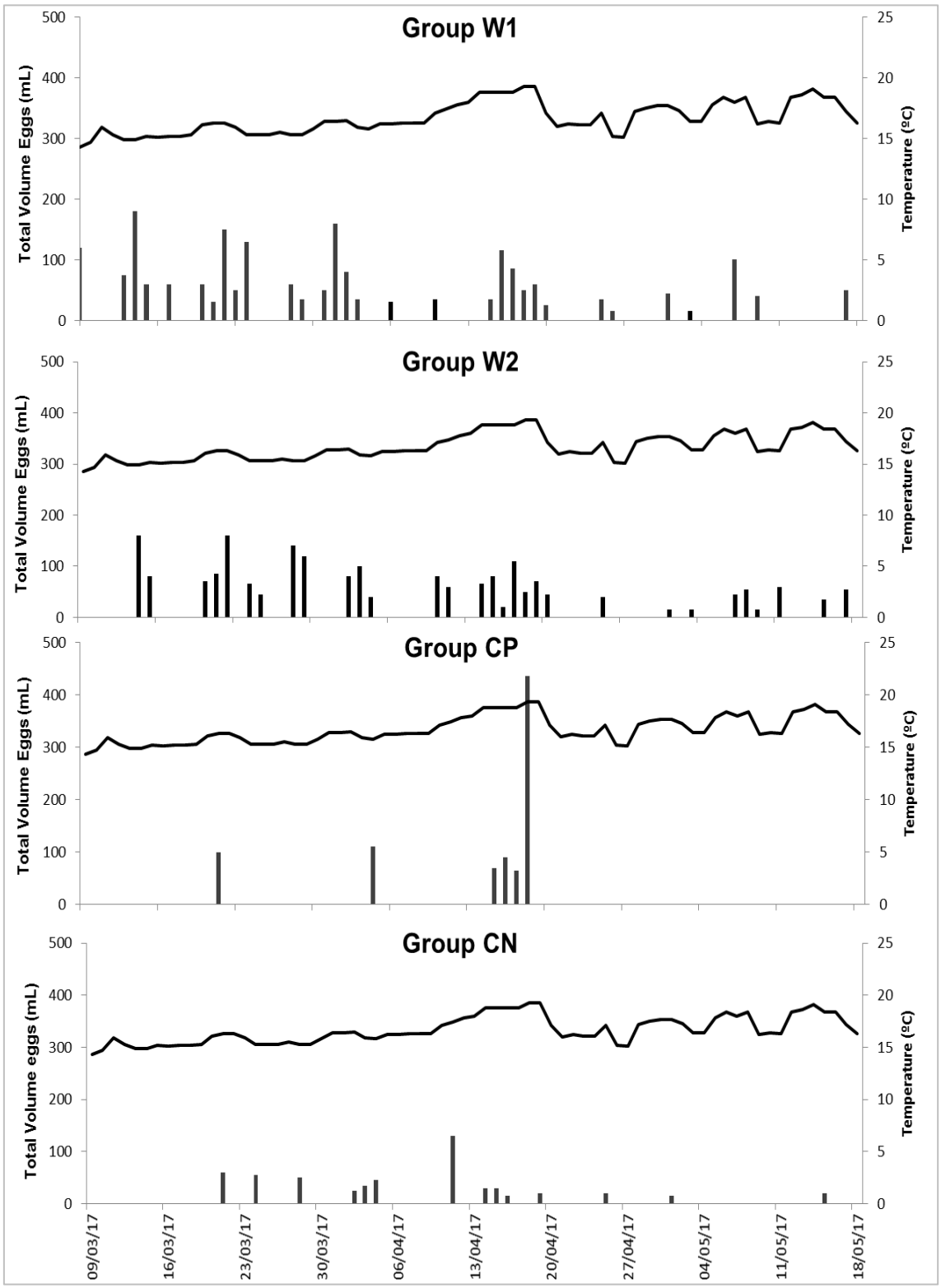

Figure 2. Total sole (*Solea senegalensis*) egg volume collected each day and temperature during the 2017 spawning season for all treatments: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared in isolation. No fertilised spawns were collected.

In the season 2018, a total of 73 spawns were collected (Figure 3). The total volume of eggs (mL) and volume of floating eggs, were significantly higher in Group W1 compared to Group CP ($P<0.05$), while Group CN was intermediate and similar to Groups W1 and CP (Fig. 4 and 5). The weight of floating eggs (g) collected was similar amongst groups ($P=0.253$) (Fig. 6).

Generally, 2016 had the most spawning and 2018 the lowest, while spawning in Group W1 decreased slightly, in Group W2 increased slightly and in groups CP and CN varied with little change (Table 2). Similarly, total volume of eggs (mL), the weight of floating eggs (g) and the floating eggs volume (mL), had some variations during the reproductive seasons (2016-2018). The Groups W1 and W2, did not show significant differences over time, in the total volume of eggs (mL) (Fig. 4) or in the weight of floating eggs (g) (Fig. 6). However, in Group W1, the floating volume of eggs (mL) (Fig. 5), exhibited significant differences between the 2016 and 2017 seasons ($P=0.025$). The higher volume of floating eggs was recorded in 2016. In Group W2, the volumes of floating eggs (mL) were similar over all reproductive seasons (Fig. 5). Also, in the Group CP no significant differences were found in the total volume of eggs (mL) (Fig. 4) or in the floating eggs volume (mL) (Fig 5) and weight floating eggs (g) (Fig. 6) over the years. In the Group CN, the total volume of eggs was significantly higher in the 2018 than in 2017 season where the lowest volume was observed ($P=0.005$) and 2016 was intermediate and similar to 2018 and 2017 seasons (Fig. 4). In group CN, the volume of floating eggs (mL) (Fig. 5) and the weight of floating eggs (g) (Fig. 6), did not show significant differences amongst the years.

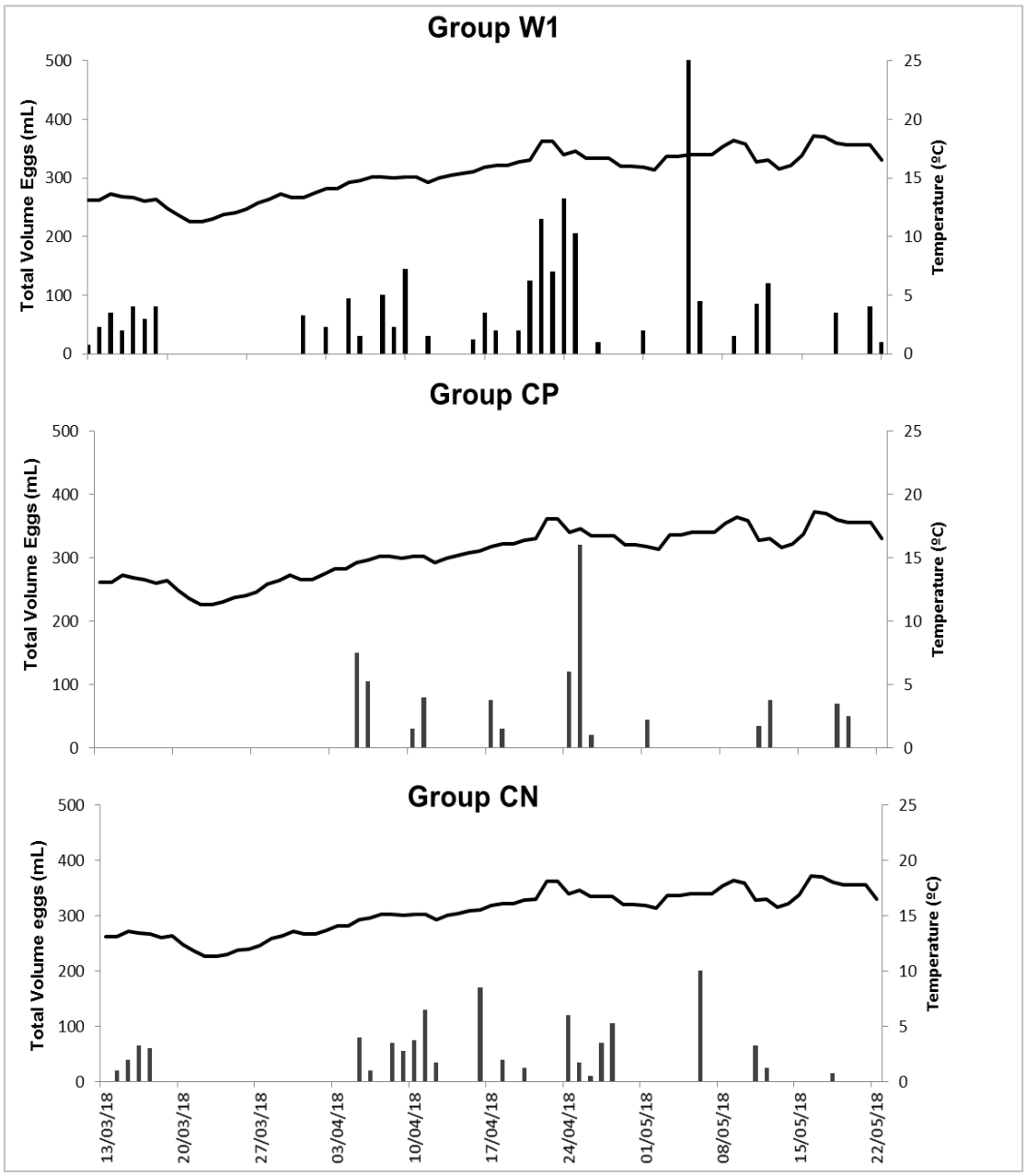

Figure 3. Total sole (*Solea senegalensis*) egg volume collected each day and temperature during the 2018 spawning season for all treatments: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, F1 fish reared in isolation. No fertilised spawns were collected.

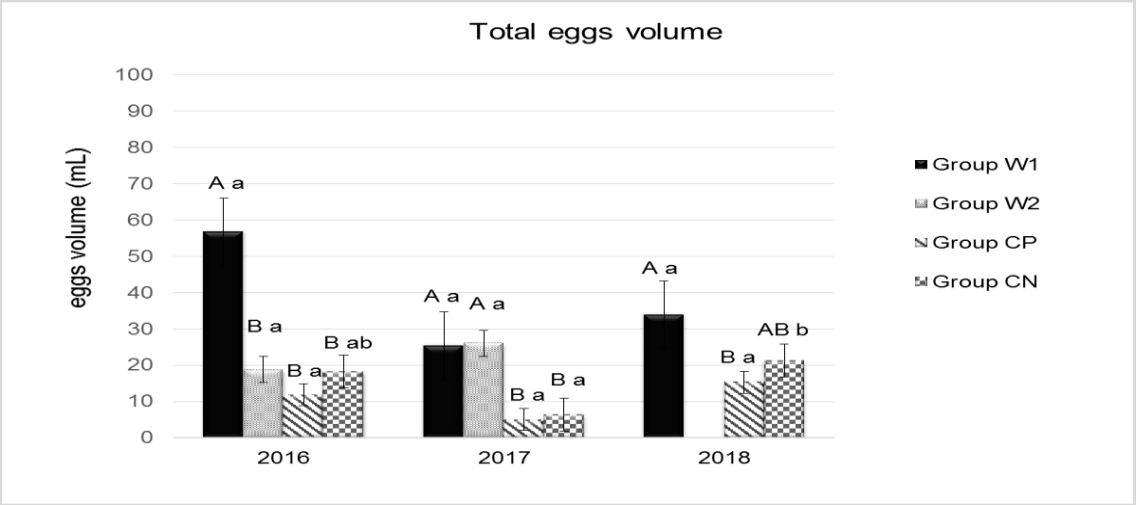

Figure 4. Mean total volume of eggs for sole (*Solea senegalensis*) of spawns collected during three seasons from four groups: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared in isolation were performed. The different uppercase letters above the bars indicates significant differences amongst the different experimental groups within each year. The different lowercase letter above the bars indicates significant differences of eggs volume of each group over time.

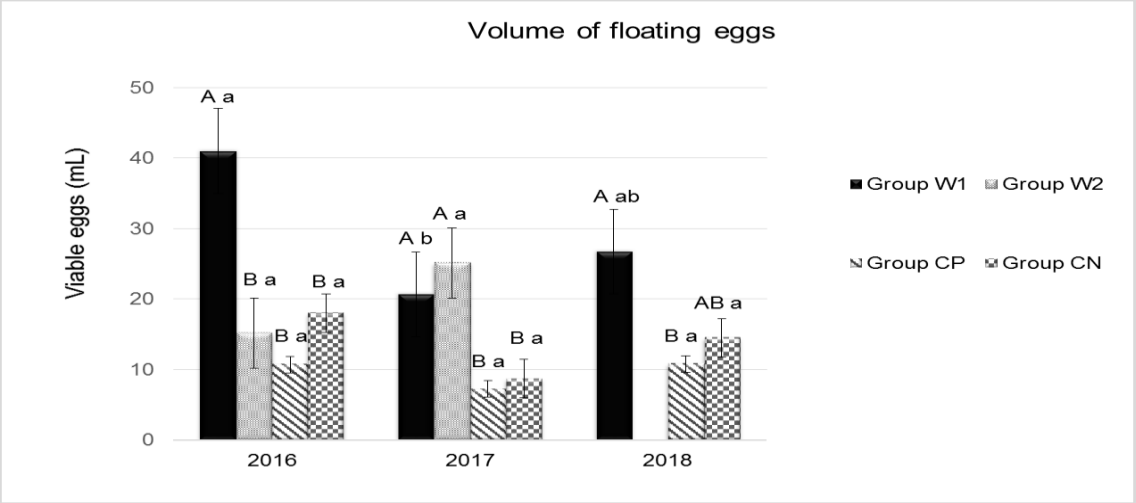

Figure 5. Mean of Volume of floating eggs of *Solea senegalensis*, obtained in the spawning during three seasons from four groups: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared in isolation were performed. The different uppercase letters above the bars indicates significant differences amongst the different experimental groups within each year. The different lowercase letter above the bars indicates significant differences of eggs volume of each group over time.

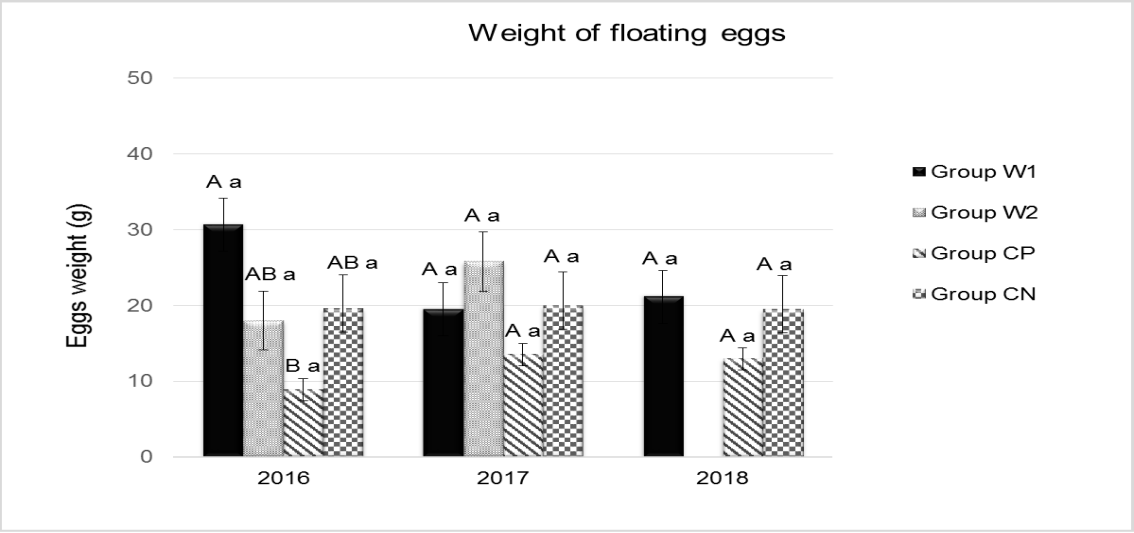

Figure 6. Mean of weight of floating eggs of *Solea senegalensis*, obtained in the spawning during three seasons from four groups: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared in isolation were performed. The different uppercase letters above the bars indicates significant differences amongst the different experimental groups within each year. The different lowercase letter above the bars indicates significant differences of eggs weight of each group over time.

3.3 Sperm quality

Sperm quality was measured in 2016 (Table 3). The sperm volume collected in the males from the four experimental groups (W1, W2, CP and CN) was similar in all groups (P=0,499).The percentage motility (P=0,582), total time of sperm motility (P=0,264) and cell concentration per kilo of male (spz per kg fish) (P=0,342) did not show significant differences among the groups. However, the cell concentration per mL of semen collected (spz per mL^{-1}), was significantly lower in Group W1 compared to Group CN (P = 0,041), but Groups W2 (P=0,496) and CP (P=0,420) were similar with all groups.

3.4 Behavioural Analysis

Both the locomotor activity and the behaviours related to the courtship were only analysed for the 2016 season, because this was the only season in which fertile spawns were obtained.

Table 3. Sperm quality parameters of *Solea senegalensis*, in the four experimental groups (W1, W2, CP and CN) during the reproductive season 2016: Total time of sperm motility (s), sperm motility percentage, cell concentration spermatozoa, (per kg of body weight (spz kg^{-1}) and (spz mL^{-1}). All values were referred as mean ± SEM. Different letters represent significant differences between groups.

Treatments	Sperm Volume (mL)	Total time motility (s)	% Motility	Cell concentration spz per kg^{-1} fish	Cell concentration spz per mL^{-1}
Group W1	0,21 ± 0,06^{a}	88,31 ± 10,12^{a}	65,38 ± 6,28^{a}	6,90E+08 ± 2,11E+08 ^{a}	1,52E+09 ± 2,63E+08 ^{a}
Group W2	0,22 ± 0,05^{a}	97,2 ± 11,53^{a}	71,00 ± 7,16^{a}	7,98E+08 ± 1,09E+08 ^{a}	1,97E+09 ± 1,74E+08 ^{ab}
Group CP	0,16 ± 0,01^{a}	105,33 ± 12,16^{a}	73,33 ± 7,55^{a}	5,93E+08 ± 1,04E+08 ^{a}	2,13E+09 ± 2,87E+08 ^{ab}
Group CN	0,14 ± 0,01^{a}	116,46 ± 10,12^{a}	77,69 ± 6,28^{a}	9,80E+08 ± 1,26E+08 ^{a}	3,24E+09 ± 5,25E+08 ^{b}

3.4.1 Locomotor activity.

The peak of locomotor activity was observed from the 19:00 to 20:00 h on days with spawns. Activity on days with spawning was highest in Group W1 (reared with wild broodstock 1), intermediate in groups W2 and CP and lowest in group CN, although significant differences were not found amongst groups. The locomotor activity in days without spawning was low and close to zero (Figure 7).

3.4.2 Behaviours related to the courtship, during the peak of locomotor activity.

The behaviours related to courtship were recorded and analysed during the peak of locomotor activity. The behaviours most observed were Rest the head (RTH) and “Follow”, mainly in the Group W1 fish that were reared before puberty with wild fish. In addition, the couple swimming behaviour was only observed in Group W1. The RTH behaviour was significantly less frequent in the group W2 compared to Group CP, while other groups were intermediate. There were no differences in, Guardian and Follow behaviours amongst treatments; despite not showing differences amongst groups, Group W1 showed the highest numbers of behaviours in comparison with others groups (figure 8).

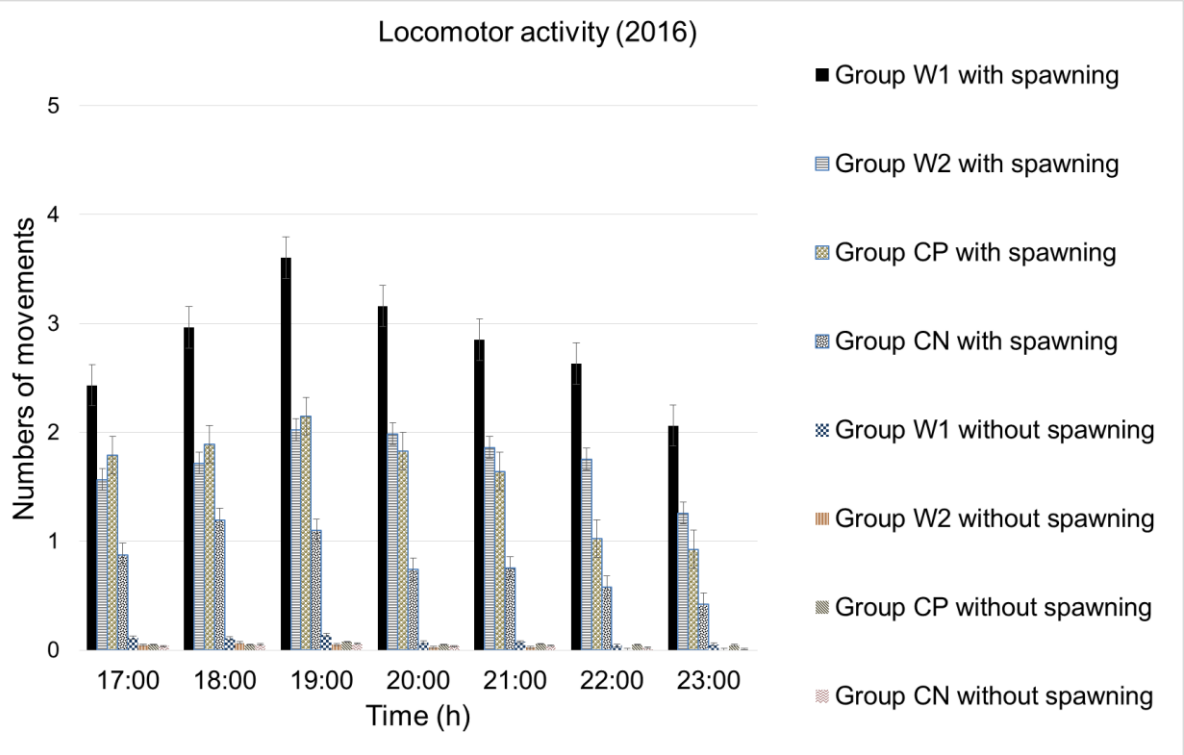

Figure 7. Locomotor activity. The mean number of movements per fish (*Solea senegalensis*) per each hour analysed (from 17:00 to 23:00 hours), on five days with spawning and five days without spawning in each treatment: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared in isolation. The peak of highest activity was observed from the 19:00 to 20:00 h.

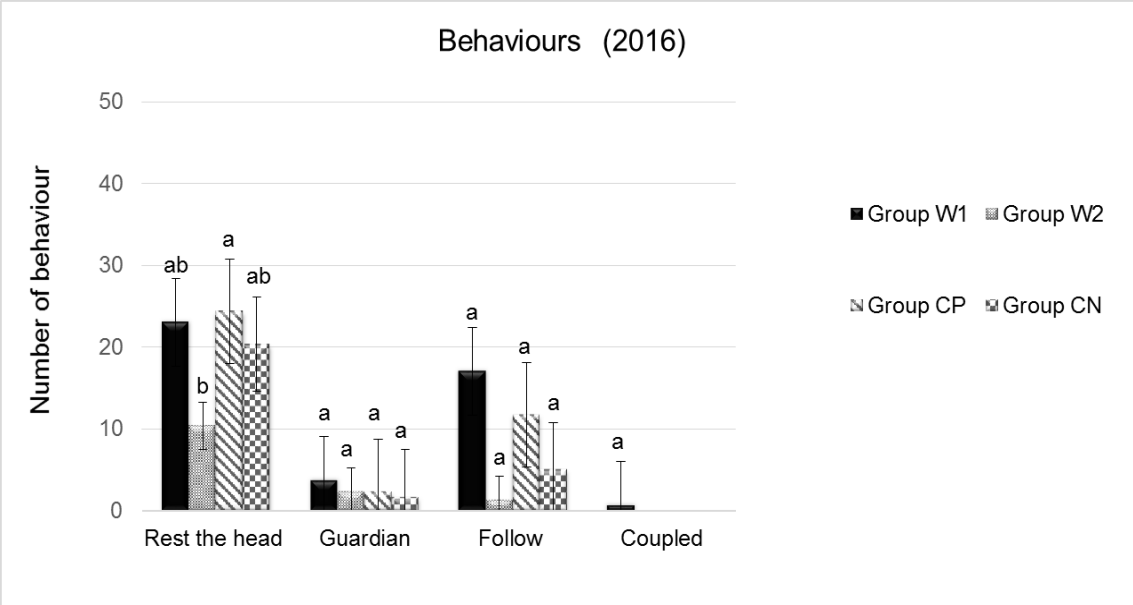

Figure 8. Mean number of behaviours related to the courtship (Rest the head, Guardian, Follow and Coupled Behaviours) observed during the peak of locomotor activity of *Solea senegalensis*. The behaviours were accounted during three days with spawns for each treatment: Group W1, cultured fish reared before puberty with wild breeders 1; Group W2, cultured fish reared before puberty with wild breeders 2; Group CP, cultured fish reared before puberty with cultured breeders; and Group CN, cultured fish reared isolated. The different letter above the bars indicates significant differences amongst groups for each behaviour.

4. Discussion

The reproductive dysfunction that affects spawning behaviour in Senegalese sole cultured breeders reared in captivity is a challenge to the aquaculture industry. Successfully fertilized eggs are currently obtained from wild broodstock (Martin *et al*., 2014), while in the case of cultured broodstock egg production is still incipient or null through natural spontaneous spawning (Mañanós *et al*., 2019). This situation of poor reproductive control remains despite of approaches to solve this problem that have examined various aspects such as, hormone control to promote spawning (Agulleiro *et al*., 2006; Guzmán *et al*., 2008; Guzmán *et al*., 2011); analysis of molecular markers in endocrine patterns resulting in sperm disorders (Riesco *et al.,* 2019), or the determination of factors affecting reproductive behaviour and mate selection (Carazo *et a*l., 2016; Martín *et al*., 2019).

In the present study, four groups of cultured breeders were evaluated to describe the reproductive success over three spawning seasons. The spawning was variable amongst the different groups of culture breeders during the three years sampled. Similar results with a high variation in the spawns were found with stocks of just cultured breeders (Guzmán *et al*. 2008), stocks mixed by genders from either wild or cultured origin (Mañanós *et al*., 2007; Martin *et al*., 2019) and stocks of both genders of wild and cultured origin (Fatsini *et al*. 2020; Chapter 2). However, in spite of the spawning obtained from the four groups in the present study during the three reproductive seasons, only a very low percentage (28.02 ± 13.80 % in group W1 in 2016) was fertilized and hatched (15.04 ± 10.40 %). A total of eight fertilised spawns that produced larvae were obtained from Group W1 in the first year of experimentation. These eight spawns were from a total of 38 from Group W1 and all other spawns from the other groups and the following years were not fertile.

In addition to the fertile spawns obtained in the present work, two other studies have obtained fertile spawns from cultured breeders (Guzmán *et al*., 2011; Fatsini *et al.,* 2020; Chapter 2). A single fertile spawn was obtained during the reproductive season from cultured breeders of *Solea senegalensis,* induced by hormonal therapy (Guzmán *et al*., 2011). However, although a spontaneous spawning was obtained under natural conditions of temperature and photoperiod, the breeders were hormonally induced, using human chorionic gonadotropin (hCG) in cultured males and GnRHa-implants for cultured females and after 33 days of treatment one fertile spawn was collected. In the study by Fatsini *et al.* (2020), which include the results of chapter 2 of this thesis, fertile spawns were recorded in two reproductive seasons, from a breeder pair consisting only of cultured fish, which were stimulated by the presence of wild breeders that successfully spawned. Therefore, the results achieved in the present study, would be the first to obtain fertile spawns without hormonal induction under natural conditions and in groups formed only by cultured breeders after a period of cohabitation with wild breeders. However, although these two studies (Fatsini *et al*., 2020; Chapter 2; present Chapter 3) represent an increase in spawning compared to hormonally treated

cultured breeders (Guzmán et al., 2011) and other studies on cultured stocks (Guzmán *et al*. 2008¸ Carazo, 2013; Martín *et al*., 2019), the level of fertile spawning was still low (seven spawns involving a cultured male in Fatsini *et al*., 2020 and eight spawns in the present Chapter 3).

In wild breeders, a low participation of individuals has been observed in the reproductive process. A few breeders participate in the courtship and hence in the spawning, causing a significant loss of genetic variability in the offspring (Porta *et al.,* 2006; Martin *et al.,* 2014; Fatsini *et al*., 2020). Unlike other flatfish species, such as *Pseudopleuronectes americanus,* with a high genetic diversity in the offspring, where multiple males fertilize the eggs released by one female (Stoner *et al.,* 1999). Porta *et al.* (2006) found that 75 % of individuals in a progeny of Senegalese sole, were related genetically as full-sibs or half-sibs. Therefore, a negative effect in the reduction of genetic variability could to contribute to the poor or lack of reproductive participation of hatchery cultured breeders. Thus, the low genetic variability (Porta *et al.,* 2006) and genetic changes in the development, could affect the reproductive process in cultured breeders (Riesco *et al.,* 2019; Duncan *et al.,* 2019).

Furthermore, the spawns in the group W1 and the result of parental contribution, showed little involvement in spawning of the breeders with only one pair as parents of all the offspring, representing 7.40 % of the total potential breeders for reproduction in the group. In addition, although the sperm volume was low in the cultured male breeders, with a similar volume collected in other studies (Cabrita *et al*., 2006; Beirão *et al*., 2009) the sperm was activated in all samples, which means that the sperm was viable. The analysis of sperm quality, in males from each group of broodstock, found no significant differences between groups in sperm quality, except in the cell concentration (spermatozoa per mL^{-1}) parameter, which was actually higher in groups with less spawning and no fertilised eggs. This suggests that the mate selection system, the participation in the reproduction and overall the reproductive behaviour in cultured breeders, have still unknown implications. Carazo *et al.* (2016) described the reproductive behaviour in Senegalese sole, as a

complex process, where the males must be able to encourage the females to swim from the bottom towards the surface of water, in order to fertilize the eggs that the females release. The reproductive dysfunction in cultured males has been described as an inability to complete the courtship behaviour to fertilise the eggs released by females (Carazo 2013; Martin *et al*., 2019). However, the loss of behaviours to fertilise eggs is not specific of *Solea senegalensis*, since this problem has been observed in others flatfishes, such as Paralichthyidae species (Smith *et al*., 1999; Watanabe and Carroll, 2001; Bambill *et al*., 2006), as well as *Scophthalmus máximus, L.* (Devauchelle *et al*., 1988), which has also been related to failure in the courtship and spawning behaviour.

In some animals, the lack of behaviour considered innate, has been attributed to the changes in the conditions and environment that organisms were exposed to during early stages of development (Bennett and Laland, 2005; Cogliati *et al.,* 2019). It has been suggested that this could be the case for sole reared in captivity, without the previous experience and learning of complex courtship and spawning, which may be available to the wild breeders (Duncan *et al.,* 2019). In this way, the particular development of the organisms would be associated with cues experienced by each individual and the learning opportunities (Wells, 2007; Cogliati *et al.,* 2019). Therefore, the interaction from early developmental stages of cultured sole with conspecifics that perform the courtship process and successful spawning would be necessary to stimulate the learning of the reproductive behaviour under a social context and behavioural observation from these conspecifics (Duncan *et al*., 2019). The study by Fatsini *et al*. (2020) and Chapter 2, carried out during four years, found that *S. senegalensis* cultured male breeders were involved in behaviours related to the courtship from the first year of study, that involvement increased significantly during the four years of the study and in the second and fourth year a cultured male spawned with a cultured female. A slightly higher participation was observed in the last year of study (Fatsini *et al*., 2020; Chapter 2), when the dominant reproductive male died and the cultured male ascended to reproductive dominance within the broodstock and participated again in the fertile spawns with both wild and cultured females. The cultured breeders

had been held during all the experiment with wild males that performed the courtship process and consequently produced fertile spawns. Therefore, the presence of spawning wild fish may exhibit two effects on the cultured males, behavioural learning by cultured males from the presence of experienced wild males and dominance by wild males to suppress the participation of cultured males in spawning.

The fertile spawns obtained from experimental group W1, during the first year of study (2016), were related with higher locomotor activity and an increase in the behaviours, RTH and “Follow”. These behaviours, have been identified as behaviours performed in the courtship, resulting in the successful fertilisation of eggs. In the present study, the behaviours, RTH and “Follow”, were in comparison to Group W1 that had fertilised spawns, less frequent in the others groups where no fertilised spawns were obtained. These behaviours, may be influenced by the observational learning obtained from the successful spawning behaviour of wild fish, during the juvenile stage of the cultured breeders (Fatsini *et al*., 2020; Chapter 2), compared with experimental groups (CP and CN). However, the lack of fertile spawning in the following years, in the groups (W1 and W2) that were exposed to wild breeders prior to puberty, suggests the cultured breeders did not have the capacity to maintain the behavioural learning over longer time periods to achieve successful courtship behaviour and fertile spawns. It appeared that although two breeders retained the information for one year (from one spawning season to the next) after a further year no breeders retained the information necesary to spawn successfully. Although a substantial volume of eggs were collected during the reproductive seasons 2017 and 2018, mainly from Groups W1 and W2, these eggs were not fertilized. This response could be related to the fact that, although the learning process has taken place in reared fish, there were factors that can condition the information and skills acquired by individuals. In many animals, a physiological response on behavioural learning may be motivated by the activation of cellular and molecular mechanisms, as well as, linked to stimulus as a chemical cue or repetition of processes or activities learned (Warburton, 2003; Bekinschtein *et al.,* 2018). Furthermore, the retention and recognition of what has been learned,

based on behavioural observation, has been associated with the process of memory (memory window) that allow to recognize process, decision-making and executing functions (Warburton, 2003; Brown *et al.,* 2013; Bloch *et al.,* 2019). Gonzalo *et al*. (2009), examined the retention of conditioned response to a predator through chemical cues, in Iberian green frog tadpoles, after a period of learning, finding that up to 12 days the response was significant, but after this period the response declined over time, indicating an apparent forgetting of chemical cues learned.

In teleost fish, the ability to learn and the capacity to retain what has been learned has also been shown (Brown and Laland 2003). However, in the case of hatchery fish, the retention of learned information may be limited to a short period of time (Brown *et al.,* 2013). Bloch *et al.* (2019), found that adult zebrafish, were able to learn and perform tasks related to matching-to-sample through of visual stimulus by the colour discrimination, however, although the fish made the tasks after repeated learning sessions, the fish were capable of retaining approximately 70 % of the information, but they were not able to perform the task after long periods more than forty-eight days. Brown *et al*. (2013), tested the response learned between salmon juveniles reared in hatchery and wild fish, to recognize predators post conditioning, where the hatchery reared fish showed a lack of or weak response and did not retain the learned response after 9 days post conditioning. In flatfish, Arai *et al*. (2007), evaluated the response to predation of flounder juveniles (*Paralichthys olivaceus*) with and without experience of the predation stimuli. A higher response to avoiding a predator was obtained in the fish subjected to a learning experience and observational learning of exposed/observational predator, compared to the fish without learning stimuli. However after 48 hours, the response was not significant, indicating that there is a limitation in their memory capacity of the conditioning.

In fish, the lack of retention of what was learned over a long time, after an observational process of learning, could be due to the probable limitation in memory capacity (Hazlett, 2007). Despite that there may be a degradation of

memories, the original learning would be associated to conditioned stimulus, through mechanisms that involve the reinforcement, in order to retain and/or reactivate the experience learned (Hazlett, 2007). Therefore, the learning would be highly associated to the frequency and intensity of reinforcement and on stimulus as responses such as chemical cues (Warburton, 2003). However, to generate long-term memories, could interfere with the learning of new information as well as the expiration of information previously retained (Ingraham *et al*., 2016). In mammal species, the competition by recognition and retrieval of particular memories or to demand for specific behaviour can trigger the forgetting, to contain distracting memories that interfere with them (Bekinschtein *et al*., 2018). In animals it is important to establish memories on aspects relevant to survival, but also, require the capability to adjust them depending on their circumstances. Hence, individuals would be able to activate the forgetting as a strategy to ensure flexible, adaptive behaviour according to a new learning process (Ingraham *et al*., 2016; Bekinschtein *et al*., 2018).

5. Conclusions

The results obtained in the present work, revealed the plasticity and potential of *Solea senegalensis* to learn, through observational learning with conspecific wild fish that successfully perform courtship and spawning behaviour. However, the lack of consistency in the reproductive behaviour and spawning has shown a weak response overtime, of what was learned, which could be associated to a failure in the memory process or maladaptive behaviour. To achieve memories that are maintained over a long time period may need a reinforcement system, through cue or stimuli that condition the response and allow reactivating or reacquiring of the behaviour. In addition, the loss of this visual stimuli or cue from wild breeders, could have acted as a mechanism of inhibition or forgetting with respect to the information learned to achieve a successful courtship. Although, the results here exhibited, drive towards insight to a possible solution to the reproductive dysfunction problem in *Solea senegalensis* cultured breeders, through behavioural learning, there are still required further studies to completely solve the lack of

reproductive behaviour and provide the quantities of eggs required by the aquaculture industry.

6. References

Agulleiro, M. J., Anguis, V., Canavate, J. P., Martinez-Rodriguez, G., Mylonas, C. C., Cerda, J., 2006. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257(1-4): 511-524.

Arai, T., Tominaga, O., Seikai, T., Masuda, R., 2007. Observational learning improves predator avoidance in hatchery-reared Japanese flounder *Paralichthys olivaceus* juveniles. Journal of Sea Research 58: 59–64.

Bambill, G.A., Masakazu, O., Radonic, M., López, A.V., Müler, M.I., Boccanfuso, J.J., Bianca, F.A., 2006. Broodstock management and induced spawning of flounder (*Paralichthys orbignyanus*) (Valenciennes, 1893) under a closed recirculated system. Revista de Biología Marina y Oceanográfica 41, 45–55.

Bateson P. 2017 Adaptability and evolution. Interface Focus 7: 20160126. http://dx.doi.org/10.1098/rsfs.2016.0126

Beirão, J., Soares, F., Herraez, M. P., Dinis M. T., Cabrita E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72(9): 1251-1261.

Bekinschtein, P., Weisstaub, N.V., Gallo, F., Renner, M., Anderson, M.C., 2018. A retrieval-specific mechanism of adaptive forgetting in the mammalian brain Nature Communications 9:4660. DOI: 10.1038/s41467-018-07128-7

Bennett G, G., Jr., Laland, K. N., 2005. Social Learning in Animals: Empirical Studies and Theoretical Models. Bioscience 55 (6) ProQuest pg. 489.

Bloch, S., Froc, C., Pontiggia, A., Yamamoto, K., 2019. Existence of working memory in teleosts: Establishment of the delayed matching-to-sample task in adult zebrafish. Behavioural Brain Research 370: 111924. https://doi.org/10.1016/j.bbr.2019.111924

Brown, C., Laland, K. N., 2003. Social learning in fishes: A review. Fish and Fisheries 4: 280- 288.

Brown, G.E., Ferrari, M.C.O., Chivers, D.P., 2013. Adaptive Forgetting: Why Predator Recognition Training Might Not Enhance Post stocking Survival. Fisheries (38): 1, 16-25.

Carazo, I. 2013. Comportamiento reproductivo y fisiología del lenguado senegalés (*Solea senegalensis*) en cautividad. Ph. D. Thesis, Universidad de Barcelona (Spain).

Carazo, I., Chereguini, O., Martín, I., Huntingford, F., Duncan, N. 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*). Spanish Journal of Agricultural Research 14, 13pp. https://dx.doi.org/10.5424/sjar/2016144-9108

Cabrita, E., Soares, F., Dinis M. T., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975.

Cogliati, K. M., Unrein, J. R., Schreck, C. B., Noakes, D.L.G., 2019. Rearing environment affects spatial learning in juvenile Chinook salmon *Oncorhynchus tshawytscha*. Journal of Fish Biology, 95:870–880. DOI: 10.1111/jfb.14083

Devauchelle, N., Alexandre, J.C., Le Corre, N., Letty, Y., 1988. Spawning of turbot (*Scophthalmus maximus*) in captivity. Aquaculture, 69: 159-184.

Duncan, N., Carazo, I., Chereguini, O., Mañanós, E. 2019. Mating Behaviour. In: Muñoz-Cueto, J.A., Mañanós-Sánchez, E.L., Sánchez- Vázquez, F.J. (Eds). The

Biology of Sole. Boca Raton, FL: CRC Press Taylor and Francis Group. Pp. 169-184.

Fatsini, E., González, Wendy., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

Gonzalo, A., López, P., Martín, J., 2009. Learning, memorizing and apparent forgetting of chemical cues from new predators by Iberian green frog tadpoles. Anim Cogn 12:745–750. DOI 10.1007/s10071-009-0232-1

García-López, A., Anguis, V., Couto, E., Canario, A. V. M., Cañavate, J. P., Sarasquete, C., Martínez-Rodríguez, G. 2006. Non-invasive assessment of reproductive status and cycle of sex steroid levels in a captive wild broodstock of Senegalese sole *Solea senegalensis* (Kaup). Aquaculture, 254:583–593.

Guevara-Fiore, P., 2012. Early social experience significantly affects sexual behaviour in male guppies. Animal Behaviour 84: 191-195. doi:10.1016/j.anbehav.2012.04.031

Guzman, J. M., Norberg, B., Ramos, J., Mylonas, C. C., Mañanós, E. L., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). General and Comparative Endocrinology 156: 285–297.

Guzman, J. M., Ramos, J., Mylonas, C. C., Mananos, E. L., 2011. Comparative effects of human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone agonist (GnRHa) treatments on the stimulation of male Senegalese sole (Solea senegalensis) reproduction. Aquaculture 316: 121-128. doi:10.1016/j.aquaculture.2011.03.014

Hazlett, B.A., 2007. Conditioned reinforcement in the crayfish *Orconectes rusticus.* Behaviour, Vol. 144, No. 7, pp. 847-859.

Huntingford, F. A., 2004. Implications of domestication and rearing conditions for the behaviour of cultivated fishes. Journal of Fish Biology 65 (Supplement A): 122–142. doi:10.1111/j.1095-8649.2004.00562.

Ingraham, E., Anderson, N.D., Hurd, P.L., Hamilton, T.J., 2016. Twelve-Day Reinforcement-Based Memory Retention in African Cichlids (*Labidochromis caeruleus*). Front. Behav.Neurosci.10:157.doi: 10.3389/fnbeh.2016.00157

Mañanós, E., Ferreiro, I., Bolón, D., Guzmán, J.M., Mylonas, C.C., Riaza, A., 2007. Different responses of Senegalese sole *Solea senegalensis* broodstock to a hormonal spawning induction therapy, depending on their wild or captive-reared origin, Aquaculture Europe 2007, Istanbul, Turkey, pp. 330-331.

Mañanós-Sánchez, E.L., Duncan, N., Sarasquete-Reiriz, C., García-López, A., Guzmán, J.M., Muñoz-Cueto, J.A., 2019. Reproductive Physiology and Broodstock management of Soles. In: Muñoz-Cueto, J.A., Mañanós-Sánchez, E.L., Sánchez-Vázquez, F.J. (Eds). The Biology of Sole. Boca Raton, FL: CRC Press Taylor and Francis Group. Pp. 90-141.

Martín, I., Rasines, I., Gómez, M., Rodriguez, C., Martinez, P., Chereguini, O. 2014. Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons. Aquaculture 424-425, 45-52. https://doi.org/10.1016/j.aquaculture.2013.12.042 537

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Spanish Journal of Agricultural Research, Vol 17, No 4. p. e0608, jan. 2020. ISSN 2171-9292. doi: http://dx.doi.org/10.5424/sjar/2019174-14953.

Porta, J., Porta, J.M., Martínez-Rodríguez, G., Alvareza, M.C., 2006. Genetic structure and genetic relatedness of a hatchery stock of Senegal sole (*Solea senegalensis*) inferred by microsatellites. Aquaculture 251 : 46– 55.

Riesco, M. F., Valcarce, D. G., Martínez-Vázquez, J. M., Martín, I., Calderón-García, A. A., González-Nunez, V., Robles V., 2019. Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question. Reproduction, Fertility and Development. https://doi.org/10.1071/RD18453

Smith, T.I.J., McVey, D.C., Jenkins, W.E., Denson, M.R., Heyward, L.D., Sullivan, C.V., Berlinsky, D.L., 1999. Broodstock management and spawning of southern flounder, *Paralichthys lethostigma*. Aquaculture 176, 87–99.

Stoner, A.W., Bejda, A.J., Manderson, J. P., Phelan, B. A., Stehlik, L. L., Pessutti, J.P.,1999. Behaviour of winter flounder, *Pseudopleuronectes americanus*, during the reproductive season: laboratory and field observations on spawning, feeding, and locomotion. Fish. Bull. 97: 999–1016.

Watanabe, W.O., Carroll, P.M., 2001. Progress in controlled breeding of summer flounder, *Paralichthys dentatus*, and southern flounder, *P. lethostigma.* J. Appl. Aquacult. 11, 89–111.

Warburton, k., 2003. Learning of foraging skills by fish. Fish and Fisheries 4 (3): 203-215.

Wells, J.C.K. 2007. Environmental Quality, Developmental Plasticity and the Thrifty Phenotype: A Review of Evolutionary Models. Evolutionary Bioinformatics 3: 109–120

Chapter 4

Dominance behaviour related to reproductive success in *Solea senegalensis* cultured and wild broodstocks.

Chapter 4. Dominance behaviour related to reproductive success in *Solea senegalensis* cultured and wild broodstocks.

1. Introduction

Dominant relationships are established in many animal species within the context of social groups (Fernald, 2014). These relationships influence the formation of hierarchies amongst individuals through interactions communicated by a variety of signals. The responses to the interactions are integrated to determine the hierarchical position of each individual in the social system (Fernald, 2014; Dey *et al*., 2015; Maytin and Ligocki, 2019). However, the social interactions in a population can have deep effects on the health, growth, physiology, behaviour and reproductive capacities of individuals (Maruska and Fernald, 2010).

The competition for resources such as food, territory and reproductive opportunity may lead to dominance relationships amongst individuals of a population (Dey *et al*., 2015; Maytin and Ligocki, 2019). In the case of fish stocks held in captivity, dominance relationships are usually established through dyadic encounters amongst members of the stock (Chase *et al*., 2003). The outcomes from encounters may influence in future interactions with other individuals or within a group (Maytin and Ligocki, 2019). Physical encounters and behavioural traits would determine the position of the fish within the hierarchy (Colléter and Brown 2011). In aggressive species, such as some cichlids, *Astatotilapia burtoni* (O'Connell *et al*., 2013) or *Amatitlania siquia* (Laubu *et al*., 2017), patterns of social behaviour were clearly defined and related to the hierarchical status (Renn *et al*., 2016). Therefore, individuals have been differentiated by not only behaviour, but also hierarchical status and the roles individuals play within the group (Fernald 2014). However, the interactions and hierarchical status may create disadvantages and lags amongst individuals in their social environment (Maytin *et al*., 2019). In species considered non-aggressive, such as flatfish *Solea senegalensis* that have benthic habits (Gibson 2005; Fatsini *et al*., 2017), the dominant patterns may be less obvious (Salas- Leitón *et al.,* 2008). However, the social hierarchy in *Solea senegalensis*,

has shown effects on the growth factors in the individuals (Salas-Leitón *et al.,* 2011). Growth dispersion amongst individuals in the fish stocks has usually been attributed to the formation of hierarchies (Salas-Leitón *et al.,* 2010). Even though the study by Salas-Leitón *et al*. (2010), exhibited that the difference in size amongst sole individuals was the result of hierarchies. However, the social structure established amongst individuals, may contribute to other adverse effects on fish development and their survival (Bolasina *et al*., 2006). The increase in the production of Senegalese sole in recent years (Morais *et al*., 2016) leads to consider the effects that social interactions may have on their commercial rearing (Salas-Leitón *et al.,* 2011). The advances in Senegalese sole culture, have allowed the industry to optimize intensive production systems to hold high stocking densities of fish in captivity (Howell and Dinis 2019). Therefore, the social relationships maintained by sole should be taken into consideration for the arrangement of the fish stocks (Salas-Leitón *et al*., 2010; 2011).

Furthermore, the definition of dominance patterns presented by Sole could provide insight how the social hierarchy may be related to other developmental processes. In Senegalese sole juvenile individuals, the dominance pattern was characterized by Fatsini *et al*. (2017) defining social status (dominant and subordinate fish) through dyadic and group testing. The feeding response and place preference (territory) parameters were used to determine dominance status. In addition, specific behaviours, such as Approaches, Rest the head, Swimming above another, Displacement and Burying were compared between fish classified as dominant and subordinate. A relation between the hierarchical system established by feeding and spatial dominance was associated to the behaviours evaluated. These observations show that the dominance status play a role in the control to access to space and feeding. Therefore, these limitations could affect the rearing and interfere in the development of fish in respect to the social status of each fish (dominant and subordinate). These hierarchical systems may also be a factor that has implications on the potential of fish culture in intensive systems (Fatsini *et al*., 2017; Maytin and Ligocki, 2019). Therefore, the identification of dominance

patterns through association with behavioural factors may improve the management of sole during rearing (Fatsini *et al*., 2017).

The determination of dominance patterns indicates aspects that may explain the why fish are involved with their conspecifics within the social structure over time (Maytin and Ligocki*,* 2019). However, the dominant behaviour could be conducted through complex social relationships, particularly, when the fish pass through different biological stages and the interactions amongst fish may be uncertain (Oliveira and Almada, 1996). In species with an established social system, a challenging stage for individuals is the reproductive stage (Dey *et al.,* 2015). Social status may determine reproductive success where dominant individuals participate successfully to reproduce and subordinate individuals may be excluded (Dey *et al*., 2015). Although S*. senegalensis* wild breeders, held in captivity successfully reproduced, few individuals were involved in the spawning (Martín *et al*., 2014; Martín *et al*., 2019; Fatsini *et al*., 2020). The scarce participation in spawning, exhibited a reproductive dominance amongst the breeders (Martín *et al*., 2014; Fatsini *et al.,* 2020). In addition, fidelity patterns were determined between the couples identified as parents of the hatched larvae (Martín *et al*., 2014; Martín *et al*., 2019; Fatsini *et al*., 2020). In addition, breeders hatched and reared in captivity (cultured breeders), exhibit a reproductive behavioural dysfunction, which represents a bottleneck for the control of reproduction of this species (Carazo 2013; Martin *et al*., 2019). The lack of reproductive success has been identified as the failure to perform the complex courtship in cultured males (Carazo 2013; Martin *et al.,* 2019). It would appear that successful courtship requires the execution of specific courtship behaviours to obtain fertile spawns (Carazo *et al*., 2016). One of the behaviours that have been identified prior to fertile spawning was the “Follow” behaviour, in which the fish displayed by swimming in a procession (Carazo *et al*., 2016; Fatsini *et al*., 2020). Thus, participation in the “Follow” behaviour by the Sole breeders may be related to spawning success (Fatsini *et al*., 2020). However, both factors, reproductive dominance and the lack of courtship behaviour could be the factors that inhibit the reproductive success of the cultured breeders (Martín *et al*.,

2014; Carazo *et al*., 2016; Fatsini *et al*., 2020). Therefore, exploration of dominance patterns and behaviours related to courtship in Sole breeders could predict the reproductive success.

Although in Senegalese sole juveniles dominance patterns have been described (Fatsini *et al*., 2017), these social interactions in breeders have not been analysed. Therefore, the hierarchical status (dominant and subordinate) may be determined between the breeders and the influence of this social context examined (Dey *et al*., 2015). The social hierarchies based in behavioural patterns that dominate resources such as territory and feed, could be examined to determine possible dominance profiles in the breeders related with reproductive dominance. This knowledge of dominance relationships in Sole adults may facilitate the adjustment of breeding groups, helping to minimize the effects that dominance hierarchies may have on reproductive success of individuals and a broodstock. Optimisation of breeders would improve their reproductive success and offer a possibility to reducing the reproductive dysfunction in cultured males.

In the present study, the behaviour, preferred space (territory), and feeding response was examined in S. senegalensis breeders to determine hierarchical status amongst the breeders. In addition, the relationships between hierarchical status and the reproductive success determined by the breeders participation in spawning and the “Follow” behaviour were explored.

2. Methods

2.1 Animal rearing conditions and experimental tanks

Two Senegalese sole broodstocks, Group 1 and Group 2, were used in the present study (Table 1). In the Group 1 (n= 24), breeders were from different origins (cultured and wild) that had been held together during the previous reproductive seasons (2013-2016) during which spawns were collected. These individuals were part of experimental Groups reported in the chapter 2 determined as Group M1 (n=16) and M2 (n=8). In the Group 2 (n= 24), the fish were of cultured origin, which

have never had reproductive success. The fish of Group 2, were part of the experiment performed in the Chapter 3, in the Group Control Negative (CN). A total of 48 adult individuals (females and males) with a mean weight of 954,89 ± 72,35 g and mean size of 38,16 ± 1,12 cm, were kept in captivity in the facilities in IRTA Sant Carles de la Rápita, (Catalonia, Spain), held in tanks (14 m^3) connected to a recirculation system (IRTAmar®). The broodstocks were held under natural environmental conditions with a simulated natural temperature cycle (9-20 ºC) and a natural photoperiod (9-14 hours light). The fish were fed with 0.75% of wet feed (polychaetes and mussels) and 0.55% dry feed of total biomass, four days a week. The breeders were previously tagged with a passive integrated transponder (PIT) tags (ID-100A, Unique Tovan®-Zeus; Euro Inversiones S.L., Madrid, Spain); in addition, photos were taken of each individual, in order to be identified later.

The trial was performed in experimental tanks with a capacity of 400 L, connected to a recirculation system (IRTAmar®). Inside each tank, a sandy area (15 X 20 x 2 cm) was made with white tiles and filled with sand, which could only be occupied by one fish. In addition, two dark grey plastic divides were used to create two areas of physical separation (wall) within the tank. The temperature during all trial was controlled (16 ºC) and red lights were used to record and observe the behaviours during the night (Carazo *et al*., 2013).

Table 1. Breeders distribution in different experimental groups used in dyadic test. Total number of fish (N) and number of fish distributed by sex and origin in each group.

Group	N	Sex and origin
1	24	Cultured females= 7 Cultured males= 7 Wild females= 6 Wild males= 4
2	24	Cultured females= 11 Cultured males= 13

2.2 Video recording

The activity of the fish were video recorded during 24 hours through a digital camera (Square black and white CCD camera, model F60B/N80-50G, KT&C Co. Ltd., Korea Technology and Communications, Korea, supplied in waterproof housing by Praesentis S.L. Barcelona, Spain), which was placed above each tank so that the field of view covered the whole tank. The camera was connected to a digital video recorder (model DVR-0804HB, supplied by Praesentis S.L.).

2.2.1 Video Analysis

The videos were analysed to determine the time each fish was in the sandy area. In addition, behavioural interactions between the pair of fish were classified and counted. The behaviours were classified as indicated in the Behavioural Trial section and described in the ethogram of behaviours (Table 2). The behaviours were analysed during two periods, which were two hours at the beginning of the trial and for the last two hours at the end of the trail. The Feeding response of each fish was also registered.

2.3 Behavioural Trial

According to the study realized by Fatsini *et al.* (2017), a dominance test was carried out in both experimental groups, during October – November (year 2016) after the last reproductive season reported in the Chapter 2. Couples were formed within each experimental Group (1 and 2), without mixing individuals between groups. The breeders were tested through a single dyadic test during 24 hours. The status dominance between pair of fish was explored using the behavioural parameters, place preference and feeding order. In the present study, the individuals tested by couple, had a similar size to avoid possible interference of fish size in the outcomes (~ 10 % weight difference between fish).

2.3.1 Dyadic test

Each pair of fish was placed within the experimental tank at 18:00 hours. The fish remained isolated by the separating wall (dark grey plastic divides), during a night of acclimation (18:00-08:00 h) (Figure 1). The following morning (8:00 h), the wall was removed and each fish had the opportunity to access the sandy area and be in contact with each other during 24 hours when the test was finished.

Figure 1. Experimental tank set up used during dyadic test. In first image (left), dark grey plastic create a dividing wall for the isolation of the fish. In second image, the wall was removed for fish interaction.

The following parameters were assessed:

a) Behavioural parameters

The behaviours assessed were: “Approaches”, “Swimming above another” (SAA), “Rest the head” (RTH), “Displacement” and “Burying” (Table 2). The dominance status, based on the behavioural parameters was determined as indicated by Fatsini *et al*. (2017), where within each test the frequency of behaviours performed by each fish towards the other fish were registered. The fish with a higher number of behaviours were classified as dominant and the fish with the lower number as subordinated.

b) Preferred space

The parameters related to preferred space were recorded: “Total time” in the preferred space (TT), “Initial time” the time during the first two hour of the test that

each fish first occupied the sand area (TI), “Final time” the time during the last two hour of the test that each fish occupied the sand area (TF). In addition, the order to occupy the sand area was recorded to indicate which fish was “first” or “last” to occupy the sand area (Table 2).

c) Feeding Response

Two hours after removing the wall and allowing interaction between each pair of fish, feed at approximately 1% of the total biomass of the pair of fish was delivered manually in the same place in each tank. The order of feeding of each fish was recorded as first, second or did not feed (Table 2).

2.4 Reproductive success

In the Group 1 (n=24), the individuals participation in the “Follow” behaviour and in spawning during four reproductive seasons (from 2013 to 2016) was previously registered. The results of reproductive seasons have been published by Fatsini *et al.* (2020). The reproductive success was determined by the parental contribution of fish to the larvae obtained from fertilised spawns. The parents of the larvae were identified with microsatellite analysis (description in Chapter 2, section 2.5). The participation of fish in the Follow behaviour was also registered by studying and identifying fish in videos. The description of Follow behaviour and how it was evaluated is indicated in the Chapter 2, section 2.6.3 of this thesis.

2.5 Steroid analysis

The blood samples were taken at end of the experiment from each fish (n=48), by puncture of the caudal vein with heparin coated syringes. The blood samples were centrifuged at 4°C for 15 minutes at 3000 rpm and plasma was removed for later analysis. From supernatant plasma, 3.5 µl (in males) and 100 µl (in females) was taken and diluted in 100 µl of IEA buffer (0.1M K2HPO4/KH2PO4, 1.54mM sodium azide, 0.4M NaCl, 1mM EDTA, and 0.1%BSA, pH 7.4), then the samples were processed twice in methanol to obtain free steroids and the pellet generated, was

reconstituted again with 250 µl of IEA buffer. The levels of the steroids 11-Ketotestosterona (KT) in males and Estradiol (E2) in females were determined using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, Cat. 501890 and 582751 respectively) (Chauvigné *et al*., 2017).

Table 2. Ethogram of parameters and behaviours recorded in *Solea senegalensis* broodstocks, based on the study by Fatsini *et al*. (2017).

Parameters and behaviours evaluated	Definition
Approaches	A fish swims near to another fish with no contact.
Swimming above another (SAA)	A fish swims over another individual.
Rest the head (RTH)	An individual supports its head on some part of the body of another fish.
Displacement	A fish moves another fish from its place or position by a physical action.
Burying	Movement performed by a fish characterized by a vigorous beats of the head and a wave of muscular contraction in the substrate, with which the fish buries itself.
Feeding response	Fish consume or ingest the food dispensed and what individual ate in first position.
Initial time (TI)	The time spent in the sand space by each Sole in the first 2 hours at the start of the test. The parameter was measured in minutes.
Total time (TT)	The total time each Sole spent in the sand space during all the test. The parameter was measured in minutes.
Final time (TF)	The time spent in the sand space by each Sole in the last 2 hours at end of the test. The parameter was measured in minutes.
Order position in the sand	Order in which each sole occupied the space in the sand at the start and end of the test. “First” position was the Sole that was located in the sand first at beginning the test and “Last” was determined by the Sole that was in the sand space at end of the test. The two fish could not be in the sand at the same time. If a fish was on top of the other fish in the sand, the lower fish in the sand was counted as being in the sand.

2.6 Statistics analysis

The results are presented as mean ± 1 standard error of the mean (SEM). The results in behavioural parameters (Approaches, SAA, RTH, Displacement and Burying) and preferred space parameters (TI, TF and TT) were compared by a Student's t-test between the groups (1 and 2) (data no showed). The comparison was made to corroborate that there were no significant differences in results of parameters assessed between the two groups and before combining the two data sets for the Principal Component Analysis (PCA). A PCA was used to group parameters that exhibited similar variation and represented the variation across the data set. The coefficient of variation (CV % = SD/ mean 100) were calculated for the behaviours evaluated for both categories of fish (dominant and subordinate). The preferred space parameters were compared by a paired Student's t-test in a single analysis between dominant or subordinate status individuals. Also a Pearson´s correlation was made amongst parameters: behavioural, preferred space, Feeding response and Steroid values. A Chi-squared analysis was used to compare proportions of dominant and subordinate fish that participated in spawning and "Follow" behaviours. Three parameters, behavioural counts (dominate fish had higher counts), last position (dominant fish occupied the preferred space at the end of the test) and Feeding response (dominate fish fed first) were used to define dominance status (dominant and subordinate) of breeders. The observed values were compared with calculated expected values that considered that dominance status determined by the parameters, behavioural, last position or feeding response had no effect on participation in spawning or the "Follow" behaviour. The data used to represent participation was nominal data (yes / no) that indicated: Yes = participation in spawning / reproductive success, No = no participation in spawning / no reproductive success or Yes = participation in the "Follow" behaviour, No = no participation in the "Follow" behaviour. Statistical analysis was carried out using SPSS Statistic 20 for Windows (SPSS Inc. Chicago, IL, USA).

3. Results

3.1 Dominance Classification

No differences were found in the data sets from Groups 1 and 2 for the parameters assessed, behaviours, feeding and preferred space. Therefore, the data sets were combined to explore similarities in variation between the different parameters. The PCA (KMO 0.694, Bartlett's test (P< 0.000) and X^2 176.163), grouped three principal components that described 61.17 % of total variance (Table 3). Principal component one (PC1) grouped the parameters related to preferred space: TI (time spent in the sand area during the first two hours at start of the test), TT (Total time that each fish spent in the sand area during the test), "first" position (first fish that entered the sand area at the start of the test) and the Burying behaviour. The second principal component (PC2) grouped: Approaches, SAA, RTH and Displacement behaviours. The third component grouped "Last" position (the fish was the last in the sand area at end the test) and TF (time in the sand area during the last 2 hours of the test). The Feeding response (order of feeding, first, second / did not feed) was poorly associated to each component. Therefore, the two parameters, behavioural counts and place preference were considered as parameters to classify the dominance status between paired fish.

3.2 Behavioural parameters

In the behavioural counts, Approach, SAA, RTH and Displacement behaviours were assessed. Counts for the Burying behaviour were not included as burying was not grouped in PC2. The fish were classified as dominant and subordinate fish according to the total number of behaviours counted for each fish in the pairs. The fish with the higher count was classified as the dominant fish and the fish with the lower count as subordinate. In the dyadic tests, the mean values in the behaviours showed highest variability amongst the fish classified as subordinate: Approaches (2.71± 0.57), CV (104.14 %) SAA (1.79± 0.35), CV (97.34 %) and RTH (2.00 ± 0.39), CV (97.80 %), compared to variation in dominant fish: Approaches (4.92 ±0.430), CV (42.80 %), SAA (4.00 ± 0.64), CV (79.06 %) and RTH (3.96 ± 0.69),

CV (86.07 %). However, variation was similar in the behaviour Displacement between subordinate (0.67± 0.25), CV (185.92 %) and dominant fish (0.79 ± 0.19), CV (117.67 %). Moreover, correlations were observed between the behaviours, SAA behaviour was highly correlated (R=0.702; P=0.000) with RTH behaviour and weak correlations were observed between, Approaches behaviour and SAA (R=0.488; P=0.016) and Approaches behaviour with Displacement behaviour (R=0.484; P=0.017).

Table 3. Proportion of variables descriptors to define Dominance behaviour in males *Solea senegalensis* and used in the Principal Component Analysis.

	Component		
	1	2	3
Initial time	.870	-.131	.140
Total time	.844	-.156	.325
Burying	.742	-.311	.208
First position (sand)	.724	.198	-.435
Feeding response	-.224	-.223	-.159
Swimmming Above Another	-.037	.820	-.004
Rest the head	.041	.785	-.105
Approaches	-.236	.749	.027
Displacement	-.096	.407	-.176
Last position (sand)	.034	-.174	.816
Final time	.294	.031	.784

3.3 Preferred space

The dominant fish classified by behavioural counts appeared to also dominant the preferred sand space. Dominant fish (69.56 %), compared to subordinate fish (30.43 %), were more often in the sand at the end of the test (last) and the

proportions were significantly different (P=0.009) compared to the expected value (even distribution or no effect of dominance) (Fig. 2D). However, there was no significant difference in the proportion of dominate and subordinate fish that occupied the sand “first” position at the beginning of the test. Also, no significant differences were observed between the proportions of both classified fish (dominant and subordinate) during the total time (minutes) spent in the sand area: TI (31.94 ± 7.24), CV (105.82 %), TT (323.50 ± 57.97), CV (85.01 %) and TF (26.08 ± 7.80), CV (151.15 %) than the subordinate fish: TI (27.42 ± 6.55), CV (123.35 %), TT (301.13 ±64.02), CV (107.28 %) and TF (21.71 ± 4.16), CV (87.44 %) (Fig. 2A, 2B, 2C).

3.4 Feeding Response

The proportion of dominant fish classified by the behavioural parameters, that ate in first position was lower (37.50 %) than in the subordinate fish (62.50 %). However the difference was not significant (P=0.083). The Feeding variable was poorly associated to the three PC that represented the dominance classifying behaviours (Approaches, SAA, RTH, Displacement and Burying), the preferred space parameters (TI, TF and TT) and the position in the sand (First or Last).

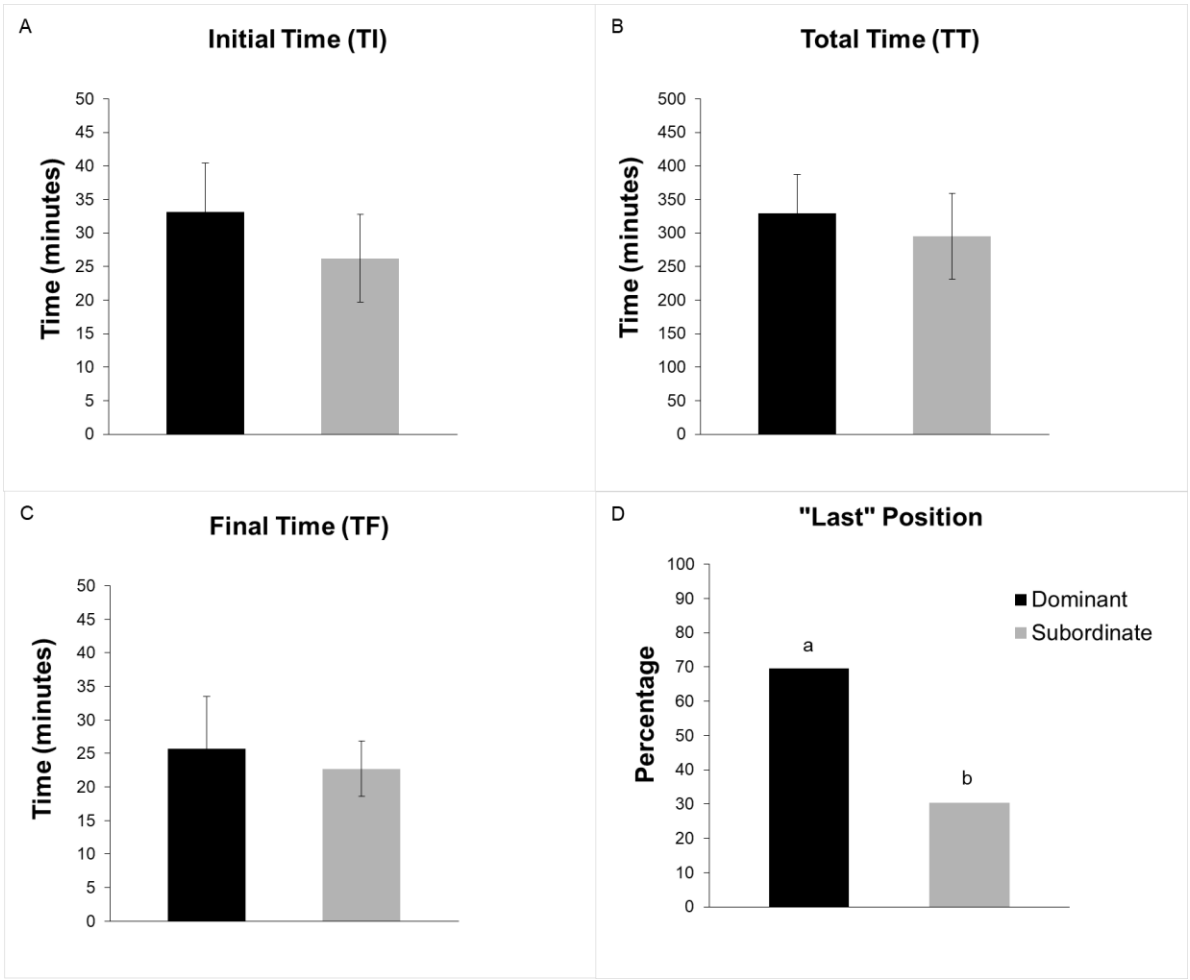

Figure 2. Parameters related to the preferred sand space for dominant and subordinate *Solea senegalensis* breeders. The position of dominance, dominant and subordinate, was determined by behavioural counts. (A) Mean time (minutes) that dominant or subordinate fish remained in the sand during the first two hours at the start of test (TI). (B) Total time dominant or subordinate fish remained in the sand during the 24 hours test (minutes). (C) Mean time (minutes) that dominant or subordinate fish remained in the sand during the last two hours of the test (TF). (D) Proportion of Dominant and Subordinate fish occupied the “Last” position. Different letters indicated significant differences ($P<0.05$).

3.5 Reproductive success

The proportions of dominant and subordinate fish (classified by the behaviours) that did or did not participate in spawning was significantly different ($P = 0.025$) from the expected values that considered that dominance determined by

behavioural counts had no effect on spawning (Table 3). Six (85%) of the seven fish that participated in spawning were dominant in the behaviours, whilst 11 (65%) of the 17 fish that did not spawn were subordinate in the behaviours. However, no significant differences were found between the proportion of dominant and subordinate fish that participated in the “Follow” behaviour and observed and expected proportions were exactly the same (Table 5).

The proportions of dominant and subordinate fish (defined by last in the sand) that spawned and did not spawn were not significantly different ($P = 0.476$) from expected values considering that dominance determined by last position had no effect on spawning (Table 4). Likewise, the proportions of subordinate and dominant fish (defined by last in the sand being classified as dominant) that participated in the “Follow” behaviour was not significantly different ($P = 0.219$) from expected values (Table 5). Similarly, the proportions of dominant and subordinate fish defined by “Feeding response” (first fish to feed classified as dominant) that participated in spawning or “Follow” behaviours were not significantly different ($P > 0.05$) from expected proportions (Table 4 and 5).

In addition, fish that participated in spawning and courtship exhibited significantly ($P<0.05$) more “approaches” and “displacement” behaviours compared to fish that did not spawn or participate in "Follow" behaviour. However, there were no differences in the proportions of dominate fish for the behavioural counts for “RTH” or “swimming”. The Follow behaviour was correlated with RTH behaviour ($R=0.652$; $P=0.000$).

3.5 Steroid analysis

Overall, the steroid levels measured, showed a mean of 3.68 ± 0.46 ng mL^{-1} of 11 KT in plasma and E2 plasma levels of 0.55 ± 0.16 ng mL^{-1}. The fish classified by dominance using the behavioural parameters, showed that the males had higher 11 KT concentration in fish determined as dominant (4.09 ± 0.725 ng mL^{-1}), which was not significantly different compared to the subordinate males (3.19 ± 0.752 ng

mL⁻¹) (Fig. 3). Similar results were found for females, the E2 concentration, was higher in dominant females (0.76 ± 0.426 ng mL⁻¹), but was not significantly different compared to subordinate females (0.29 ± 0.069 ng mL⁻¹) (Fig. 3). In addition, there was a significant correlation between 11 KT concentration and Rest the Head (RTH) behaviour (R=0.516; P=0.010), indicating that males that more often RTH had higher levels of 11 KT. However, no other correlations were found between steroid levels and behavioural parameters.

Table 4. Number of dominant and subordinate *S. senegalensis* breeders that participated in fertile spawning or did not participate in spawning. Three parameters, behavioural counts (dominate fish had higher counts), last position (dominant fish occupied the preferred space at the end of the test) and feeding response (dominate fish fed first) were used to define dominance status. The results of Chi-square analysis compared the observed values in the table with expected values considering that dominance determined by the parameters, behavioural, last position or feeding response had no effect on spawning participation. An asterisk * indicates a significate difference between observed and expected proportions P < 0.05.

Parameters used to classify dominance	**Dominance Status**	**Fish that participated in Spawning YES**	**Fish that did not participate in Spawning NO**	**Total count of individuals**	**Chi ² Results**
Behavioural	Dominant	6	6	12	$X^2 = 5.042$, gl.= 1, P = 0.025*
	Subordinate	1	11	12	
Last Position	Dominant	4	7	11	$X^2 = 0.509$, gl.= 1, P = 0.476
	Subordinate	3	10	13	
Feeding response	Dominant	1	8	9	$X^2 = 2.272$, gl.= 1, P = 0.132
	Subordinate	6	9	15	

Table 5. Number of dominant and subordinate *S. senegalensis* breeders that participated or did not participate in the "Follow" behaviour. Three parameters, behavioural counts (dominate fish had higher counts), last position (dominant fish occupied the preferred space at the end of the test) and feeding response (dominate fish fed first) were used to define dominance status. The results of Chi-square analysis compared the observed values in the table with expected values considering that dominance determined by the parameters, behavioural, last position or feeding response had no effect on participation in the "Follow" behaviour.

Parameters used to classify dominance	Dominance Status	Participation in the Follow behaviour YES	No participation in the Follow behaviour NO	Total count of individuals	Chi ^{2} Results
Behavioural	Dominant	6	6	12	$X^2 = 0.000$, gl.= 1, P = 1.000
	Subordinate	6	6	12	
Last Position	Dominant	7	4	11	$X^2 = 1.510$, gl.= 1, P = 0.219
	Subordinate	5	8	13	
Feeding response	Dominant	4	5	9	$X^2 = 0.178$, gl.= 1, P = 0.673
	Subordinate	8	7	15	

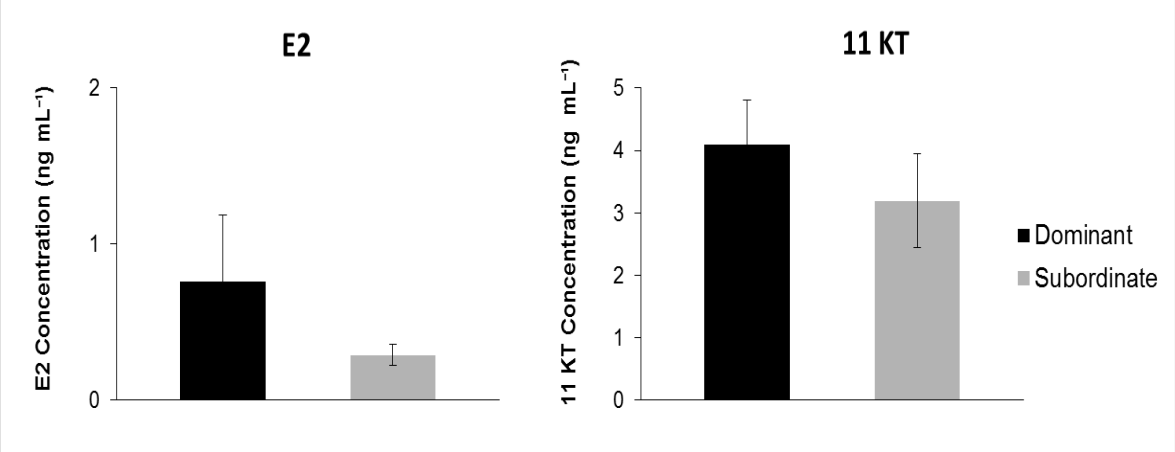

Figure 3. Mean plasma concentrations (ng mL-1) of 11 KT and E2 in *Solea senegalensis* according to the dominance hierarchy (Dominant and Subordinate fish) determined by behavioural counts (fish with higher counts classified as dominant). Steroid levels of 11 KT were measured in males and E2 in females.

4. Discussion

In the present study, dominance status in *S. senegalensis* breeders was evaluated by a dyadic test applied to individuals from two broodstock Groups (1 and 2). The dominance status was explored, evaluated and classified through three parameters: a) Behavioural, b) Preferred space and c) Feeding response. The behavioural parameters and preferred space exhibited similar tendencies to determine a similar dominance status to individuals, whilst Feeding response appeared not to be related. Therefore, within each fish pair in the dyadic tests, dominant and subordinate individuals were classified with regard to behavioural parameters. The behaviours grouped to represent dominance status were Approaches, SAA, RTH and Displacement, with a correlation amongst them. Fish classified as dominant using these behaviours (fish with highest counts classified as dominant), spent more time in the “Last” position. This parameter exhibited significant differences with a higher percentage of dominant fish, compared to subordinate fish occupying the preferred space. The dominance status by behaviours was associated with reproductive success, which was defined by obtaining fertile spawns from breeders. The fish classified as dominant by behavioural parameters, was significantly related with the fish that had more fertile spawns during four previous reproductive seasons. From the behaviours evaluated, Rest the Head behaviour was correlated with the participation of breeders in the “Follow” Behaviour and with 11 KT concentrations. However, the steroids concentration did not show significant differences between dominant and subordinate fish. Therefore, there appeared to be some connection amongst social dominance (evaluated from behaviours), place preference and reproductive parameters, which could be indicative of reproductive success or dominance within a group.

Although in aggressive species, agonistic behaviours may be observed between individuals, in Sole this interaction between individuals may not be evident (Fatsini *et al*., 2017). However the relationship between the social behaviours and preferred space parameters, gives a guideline to define social status in sole

breeders. Since the preferred space (sand area) is a reliable parameter linked to the benthic habits where the sole develops most of the daily activities (Gibson 2005). Using the behavioural parameters, the fish were classified according to the frequency of behaviours and in the dyadic test the fish with a higher count of behaviours was classified to be dominate over the subordinate fish that had a lower count of behaviours. This method to determine hierarchical status, through several types of social behaviours performed amongst individuals, has been used in others studies. Liljedal and Folstad (2003) constituted hierarchical structure between *Salvelinus alpinus* males classifying dominant and subordinate individuals through behaviours. While Kagawa *et al.* (2013), determined social rank in *Oryzias latipes*. In the present study, strong correlations amongst behaviours realized by Sole broodstocks were found, in specific between SAA and RTH behaviours. These behaviours grouped to represent social status, were also observed and associated to dominance in *S. senegalensis* juveniles (Fatsini *et al*., 2017). Where the dominant fish performed a higher number of behaviours compared to the subordinate fish. Although the dominance hierarchy characterized by Fatsini *et al.* (2017) was evaluated by the feeding response. However, Fatsini *et al.* (2017), also classified social status using place preference (sandy area), which gave similar results in the “Last” position. Thus, the behavioural parameters and the dominance found in relation to preferred space were consistent result in both juveniles and adults. Therefore, the dynamic interactions between behaviours and space preference amongst individuals appear to be related to dominance relationships in two life stages of *Senegalese sole*, as has been described in other species (Dey *et al.*, 2015).

The preference of dominant fish to remain in the sand area, would avoid the access to resources such as territory to another individuals. The sandy space represents a natural environment to Senegalese sole and flat fish in general, which provides both feeding and protected areas (Gibson 2005). The control of a space with predictable resources has been observed in species such as cichlids (Grand and Grant 1994). The dominant fish became more aggressive and competitive towards conspecifics to defend the territory and access to shelter. In the present

study, dominant fish spent more time in the sand area in each period assessed (TI, TT and TF). In addition, the Last position in the sand, occupied by the dominant fish was significantly higher compared with the subordinate sole. Similar to sole breeders, this pattern of spatial dominance was also observed in juvenile Sole (Fatsini *et al.* 2017). This pattern has been related to the activities that sole perform during the day. Overall, the sole remains sedentary at the bottom, however, the period of locomotor activity increases during the afternoon from 15:00 to 23:00 (Carazo *et al*., 2016; Fatsini *et al.,* 2020, chapters 2 and 3), whilst during the morning the activity decrease. During this resting period, individuals bury themselves in sand, avoiding predators or exposure to adverse environmental conditions (Gibson 2005). Therefore, the dominance in the sand area at the end of the test (08:00 a.m.) revealed the monopoly of a desirable living space. In addition, the defence of territories could be associated to other purposes, such as feeding (Grand and Grant 1994).

The social dominance identified in sole, based on behaviours and appropriation of preferential space could identify other kind of dominance. In the present study, the dominance status classified by behaviours was related with the reproductive success. The reproductive success was determined in the breeders with parental contribution identified from larvae hatched. The dominant fish by behaviours were coincident with the breeders that made a parental contribution to offspring during four reproductive seasons (Fatsini *et al.,* 2020; chapter 2). In comparison, the subordinate breeders exhibited a scarce participation in fertile spawning and hatched larvae. The reproductive dominance has also been observed in zebrafish held in captivity (Paull *et al*., 2010). Where the breeders had implications in the reproductive success according to their dominance status. The fish determined to have a higher range of dominance occupied a privileged position, monopolizing the access to females. The dominant fish also exhibited more aggressive behaviour as a strategy to maintain the social hierarchy. In comparison, the subordinate individuals were stragglers without access to resources in the aquarium or participation in mating with females (Paull *et al.,* 2010). Similar, in Japanese minnows (*Pseudorasbora parva)*, the breeders with higher dominance rank had

reproductive success (Maekawa *et al*., 1996). However, the relationship between reproductive seasons associated to parental contribution was not related with parameters in the preferred space. Unlike other species such as *Oreochromis mossambicus*, dominant males compete for large territories, in order to attract females and achieve reproductive success (Oliveira and Almada, 1998). Similarly, dominant male *Colisa lalia* and *Astatotilapia burtoni*, retain the territory to have the opportunity to mate with the females, protecting them from other males and building nests (Hayakawa and Kobayashi, 2012; Alcazar *et al.* 2016). In *S. senegalensis*, the beginning of courtship was performed on the bottom, where the male approached and rested its head on the female (Carazo *et al.,* 2016). Later the male encouraged the female to swim from the bottom to mate and release gametes into the water column. While, the preferred space determined by the sandy area is mainly used to rest, foraging and shelter as mentioned above (Gibson 2005).

However, the social dominance observed in sole, could be driven by the capacity to perform complex courting behaviours, mainly in males. Where the males must be able to encourage the females to start the reproductive behaviour. The correct execution of behaviours from males, will secure the reproductive success and fertile spawning (Carazo *et al*., 2016). Therefore, the interactions amongst breeders may increase during the reproductive season, with a competition amongst the male breeders (Carazo *et al*., 2016; Martin *et al.,* 2019). The displaying of dominance behaviour previous to reproductive season could highlight breeders that are socially dominant and hence potentially successful in reproduction (Dey *et al*., 2015). The social dominance exhibited in individuals, may be a way to access to the choice of partner between dominant breeders (Magellan *et al*., 2009). This pattern was observed in medaka fish (*Oryzias latipes*) where the females have mate preference to dominant males in relation to other males (Yokoi *et al.,* 2016). These social interactions would be part of mate choice process (Laubu *et al*., 2017). The acceptance or rejection by the females could be based on the pattern of males behavioural, where the females choose males of higher dominance rank, ensuring reproductive advantages and to their offspring

(Maekawa *et al*., 1996; Laubu *et al*., 2017). Therefore, reproductive dominance would have social behavioural component (Maekawa *et al*., 1996) and the position of individuals in the social dominance hierarchy may influence in the reproductive success (Colléter and Brown 2011). In the case of dominant males, territoriality, courtship and reproductive experiences would be involved in the reproductive success (Pfenning *et al.,* 2012) and dominance relationship may also affect the pair formation. In specific Senegalese sole has demonstrated a strong trend to form monogamous couples influenced by a reproductive dominance (Martin *et al*., 2014; Fatsini *et al*., 2020) and related to social dominance. However, these factors may be a limitation to achieving the formation of multiple pairs with successful spawns within a captive stock. Therefore, identification of dominance behaviour in breeders might contribute to the management and improvement of stock conformation to minimize the effect of dominance on reproductive success of individuals.

However, the participation in Follow behaviour was not linked to social hierarchy. This fact may be due to the number and type of individuals involved in “Follow” behaviour, unlike the fish that participate in fertile spawning. The trend between fish classified as dominant and subordinate was not clear regarding to “Follow” behaviour. Although this behaviour has been associated to the courtship and observed in periods with spawning, as indicative to deduct reproductive success (Carazo *et al.,* 2016; Fatsini *et al*., 2020; chapter 2). However, Follow behaviour was correlated with Rest the Head, which is behaviour observed with the courtship. The RTH behaviour performed by a dominant male, imply the protecting or dominating the female from other males in addition to acceptance or mate selection by the female (Carazo *et al*., 2016). Nevertheless, the execution of behaviours such the Rest the Head has been observed to be a common behaviour made by sole outside of reproductive season (Duncan *et al.,* 2019).

In the present study, the feeding response in breeders was not related to hierarchical status. While in sole juveniles, dominance behaviour was determined by feeding response, classifying as dominant to fish that ate first and subordinate

that ate in second or did not eat (Fatsini *et al.,* 2017). The discrepancy in the parameters used to classify dominance status in the Sole breeders and juveniles could be due to various factors. The possibility of stress in breeders caused by experimental set up conditions and interactions with conspecifics could modify the feeding response. Experimental tanks (400 L) were used for larger fish, different from the tanks in which fish are usually kept (10,000 L). On the contrary the juveniles fish, due to smaller size were better acclimated to experimental tanks. In swordtail fish (*Xiphophorus helleri*), alterations in tank design with a smaller surface area caused a decrease in the feeding time in dominant individuals. The reduction in energy expenditure or improved foraging efficiency could have caused this effect in fish (Magellan *et al.,* 2012). A decrease in food intake was observed in Arctic charr due to intensified agonistic interactions that suppress foraging (Brown *et al.,* 1992). The exploration between reproductive success and feeding in breeders, showed a low response regarding the dominant fish participating in the spawning. Therefore, it did not represent an influence parameter for reproduction. However, dominance patterns have been reported to cause disadvantage to obtain feed amongst individuals and size disparity in *S. senegalensis* (Salas-Leitón *et al*., 2010). In other species, such as Atlantic salmon (*Salmo salar*) juveniles, injures to the fins were observed and were attributed to food competition, where dominant fish competed aggressively biting the fins of subordinate fish (MacLean *et al*., 2000). Likewise Magnuson (1962) observed that in juvenile stages of medaka (*Oryzias latipes*), competition and aggressive behaviour from dominant individuals was exhibited for food supply.

Overall the 11 KT and E2 total concentrations measured in males and females respectively, was similar to what was reported by Chauvigné *et al*. (2016). The 11 KT and E2 concentrations, did not exhibit significant differences between dominant and subordinate individuals. Similar results were reported by Scaia *et al*. (2018), who did not find significant difference in sex steroids concentrations in cichlid fish as response to dyadic encounters. The importance of steroids lies in the fact that these hormones have an important role in the maturation of gonads, development of secondary sex characters and the control of reproductive behaviour (Oliveira *et*

al., 2001). The concentrations of sex steroids can be influenced during reproductive episodes and according to mating tactics (Oliveira *et al*., 2001; 2002). However, in the present study, the steroid concentration corresponds to a value outside the main reproductive season. Although the response to the interactions between the broodstocks, showed a correlation of the steroids concentration with Rest the Head behaviour. Therefore, the higher expression of social behaviour has an impact on the circulation and increase of sex steroids (Oliveira *et al.,* 2002). During the breeding season, agonistic interactions between peacock blenny (*Salaria pavo*) males were linked to endocrine response. This response was an adaptation in the fish to adjust the social behaviour according to hierarchical level. The competence between fish increases androgen levels during the establishment of the social hierarchy (Oliveira *et al.,* 2001). The relationship between sex steroids and behaviour in Senegalese sole would result from the acquisition of dominance status between breeders.

5. Conclusions

Dominance behaviour was similarly classified by behavioural and preferred space parameters in *Solea senegalensis* breeders. Behaviours such as Rest the head, Swimming above another, Approaches and Displacement were used to classify dominant and subordinate status by the interactions amongst individuals. This behavioural social status appeared to influence competition and acquisition of resources such as space and reproductive success. Dominant fish by behavioural parameters had higher participation in fertile spawning compared to subordinate breeders. This relationship between social dominance and reproductive dominance could establish guidelines for the proper management of sole breeders. Considering hierarchical factors could be a useful tool to increase the reproductive success and attempt to predict potential successful breeders through behavioural parameters.

6. References

Alcazar, R., M., Becker, L., Hilliard, A., T., Kent K., R., Fernald, R., D., 2016. Two types of dominant male cichlid fish: behavioural and hormonal characteristics. Biology Open 5, 1061-1071 doi:10.1242/bio.017640

Bolasina, S., Tagawa, M., Yamashita, Y., Tanaka, M., 2006. Effect of stocking density on growth, digestive enzyme activity and cortisol level in larvae and juveniles of Japanese flounder, *Paralichthys olivaceus*. Aquaculture 259: 42-443.

Brown, G. E., Brown, J. A., Srivasta, R. K., 1992. The effect of stocking density on the behaviour of Arctic charr (Salvelinus alpinus L.). Journal of Fish Biology 41: 955-963. DOI: 10.1111/j.1095-8649.1992.tb02722.x

Carazo, I. 2013. Comportamiento reproductivo y fisiología del lenguado senegalés (*Solea senegalensis*) en cautividad. Ph. D. Thesis, Universidad de Barcelona (Spain).

Carazo I, Norambuena F, Oliveira C, Sanchez-Vazquez FJ, Duncan NJ, 2013. The effect of night illumination, red and infrared light, on locomotor activity, behaviour and melatonin of Senegalese sole (*Solea senegalensis*) broodstock.PhysiolBehav 118: 201-207. http://dx.doi.org/10.1016/j.physbeh. 2013.05.032.

Carazo, I., Chereguini, O., Martin, I., Huntingford, F., Duncan, N.J. 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*). Span J Agric Res. 14(4): e0401.

Chauvigné, F., Fatsini, E., Duncan, N., Ollé, J., Zanuy, S., Gómez, A., Cerdà, J., 2016. Plasma levels of follicle-stimulating and luteinizing hormones during the reproductive cycle of wild and cultured Senegalese sole (*Solea senegalensis*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 191, 35–43.

Chauvigné, F., Ollé, J., González, W., Duncan, N., Giménez, I., Cerdà, J. 2017. Toward developing recombinant gonadotropin-based hormone therapies for increasing fertility in the flatfish Senegalese sole. PLoSONE 12(3): e0174387. https://doi.org/10.1371/journal.pone.0174387

Chase, I., D., Tovey, C., Murch, P., 2003. Two's company, three's acrowd: differences in dominance relationships in isolated versus socially embedded pairs of fish. Behaviour 140:1193-1217.

Colléter, M., Brown, C. 2011. Personality traits predict hierarchy rank in male rainbowfish social groups. Animal Behaviour, 81(6), 1231-1237.

Dey, C., J., Tan, Q.Y. J., O'connor, C., Reddon, M., A., R., Caldwell, J., R., Balshine, S.,2015. Dominance network structure across reproductive contexts in the cooperatively breeding cichlid fish *Neolamprologus pulcher* . Current Zoology 61 (1): 45–54

Duncan, N., Carazo, I., Chereguini, O., Mañanós, E. 2019. Mating Behaviour. In: Muñoz-Cueto, J.A., Mañanós-Sánchez, E.L., Sánchez- Vázquez, F.J. (Eds). The Biology of Sole. Boca Raton, FL: CRC Press Taylor and Francis Group. Pp. 169-184.

Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Mackenzie, S., Duncan, N.J. 2017. Dominance behaviour in a non-aggressive flatfish, Senegalese sole (*Solea senegalensis*) and brain mRNA abundance of selected transcripts. PLoSONE 12 (9): e0184283.https://doi.org/10.1371/journal. pone.0184283

Fatsini, E., González, Wendy., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

Fernald, R., D., 2014. Communication about social status. Current opinion in Neurobiology 28:1-4.

Gibson, R., 2005. Flatfishes: Biology and Exploitation: Blackwell Science Ltd. a Blackwell Publishing Company. 391 pp.

Grand, T.,C., Grant, J.,W.,A., 1994Spatial predictability of food influences its monopolization and defence by juvenile convict cichlids. Anim Behav.; 47: 91–100.

Hayakawa, Y., Kobayashi, M., 2012. Influences of Establishment and Maintenance of Territory on Reproductive Activity in the Male Dwarf Gourami *Colisa lalia*. Zoological Science, 29(3):141-146. DOI: http://dx.doi.org/10.2108/zsj.29.141

Howell, B.R., Dinis, M.T. 2019. An overview of Soleid (Pleuronectiformes) Fisheries and Aquaculture. In: The Biology of Sole. Editors, José A. Muñoz Cueto, Evaristo L. Mañanós Sánchez, F. Javier Sánchez Vázquez. CRC Press. Boca Raton Florida. Pp 3-23.

Kagawa, N. 2013. Social rank-dependent expression of arginine vasotocin in distinct preoptic regions in male *Oryzias latipes*. Journal of Fish Biology 82, 354–363. doi:10.1111/j.1095-8649.2012.03490.x.

Laubu, C., Schweitzer, C., Motreuil, S., Louâpre, P., Dechaume-Moncharmont F., X., 2017. Mate choice based on behavioural type: do convict cichlids prefer similar partners? Animal Behaviour 126: 281-291.

Liljedal, S., Folstad, I. 2003. Milt quality, parasites, and immune function in dominant and subordinate Arctic charrCan. J. Zool. 81: 221–227. Doi: 10.1139/Z02-244.

MacLean, A., Metcalfe, N.,B., Mitchell, D.,L., 2000. Alternative competitive strategies in juvenile Atlantic salmon (*Salmo salar*): evidence from fin damage. Aquaculture; 184: 291–302.

Maekawa, K., Iguchi, K., Katano, O., 1996. Reproductive success in male Japanese minnows, *Pseudorasbora parva*: observations under experimental conditions. Ichthyol. Res., 43 (3): 257-266.

Magellan, K., and Kaiser, H., 2010. Male Aggression and Mating Opportunity in a Poeciliid Fish. African Zoology, 45(1): 18-23. https://doi.org/10.3377/004.045.0115

Magellan, K., Johnson, A., Williamson, L., Richardson, M., Watt, W., Kaiser, H., 2012. Alteration of tank dimensions reduces male aggression in the swordtail. J. Appl. Ichthyol. 28: 91–9. doi: 10.1111/j.1439-0426.2011.01803.x

Magnuson, J.,J., 1962. An analysis of aggressive behavior, growth, and competition for food and space in medaka (*Oryzias latipes* (Pisces, Cyprinodontidae)). Canadian Journal of zoology. Vol. 40: 313-363.

Martín, I., Rasines, I., Gómez, M., Rodriguez, C., Martinez, P., Chereguini, O. 2014. Evolution of egg production and parental contribution in Senegalese sole, Solea senegalensis, during four consecutive spawning seasons. Aquaculture 424-425, 45-52. https://doi.org/10.1016/j.aquaculture.2013.12.042

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Gómez, M., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Span. J. Agric. Res. 17 (4), e0401.

Maruska, K.P., Fernald, R. D. 2010. Behavioral and physiological plasticity: Rapid changes during social ascent in an African Cichlid fish. Hormones and Behavior 58: 230-240.

Maytin, A., K., Ligocki, I., Y., 2019. Dominance hierarchy establishment in the invasive round goby, *Neogobius melanostomus*. Behavioural Processes 158: 41-46. https://doi.org/10.1016/j.beproc.2018.10.024

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M. and Dinis, M. T. 2016, New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture6, 1-54137.https://doi.org/10.1111/raq.12091

O'Connell LA, Fontenot MR, Hofmann HA. 2013. Neurochemical profiling of dopaminergic neurons in the forebrain of a cichlid fish, *Astatotilapia burtoni.* J Chem Neuroanat.; 47: 106–15. https://doi.org/10. 1016/j.jchemneu.2012.12.007 PMID: 23295359

Oliveira, R.,F., Almada, V., C., 1998. Mating tactics and male-male courtship in the lek breeding chiclid *Oreochromis mossambicus*. Journal of Fish Biology 52, 1115–1129.

Oliveira, R.,F., Almada, V., C., Gonçalves, E., J., Forsgren, E., Canario, A., V.,M., 2001. Androgen levels and social interactions in breeding males of the peacock blenny. Journal of Fish Biology 58**:** 897–908 doi:10.1006/jfbi.2000.
1501.

Oliveira, R., F., Hirschenhausera, K., Carneiroa, L.,A., Canario, A., V.,M., 2002. Social modulation of androgen levels in male teleost fish. Comparative Biochemistry and Physiology Part B 132: 203–215

Paull, G.,C., Filby, A.,L., Giddins, H., G., Coe, T., S., Hamilton, P., B., Tyler, C., R., 2010. Dominance Hierarchies in Zebrafish (Danio rerio) and Their Relationship with Reproductive Success. Zebrafish Volume 7, Number 1:109-117. DOI: 10.1089=zeb.2009.0618

Renn, S.,C.,P., O'Rourke, C., F., Aubin-Horth, N., Fraser, E., J., Hofmann, H., A., 2016. Dissecting the Transcriptional Patterns of Social Dominance across Teleosts. Integrative and Comparative Biology, volume 56, number 6, pp. 1250–1265. doi:10.1093/icb/icw118

Salas-Leiton E., Anguis, V., Manchado, M., Cañavate, J.,P., 2008. Growth, feeding and oxygen consumption of Senegalese sole (*Solea senegalensis*) juveniles stocked at different densities. Aquaculture 285: 84–9.

Salas-Leiton, E., Anguis, V., Rodríguez-Rúa, J.,P., 2010. Stocking homogeneus size gruops does not improve growth performance of Senegalese sole (*Solea*

senegalensis Kaup 1858) juveniles: Individual growth related to fish size. Aquacultural Engineering 43:108-113. doi:10.1016/j.aquaeng.2010.08.001

Salas-Leiton, E., Anguis, V., Rodríguez-Rúa, J.,P., Cañavate, J.,P., 2011. High stocking density and food restriction have minimum impacto n size dispersal of cultured Senegalese sole (*Solea senegalensis* Kaup 1858) juveniles. Evidence for individual growth being regulated by population structure. Aquacultural Engineering 45:43-50.

Scaia, M., F., Morandini, L., Noguera, C., A., Trudeauc, V., L., Somozad, G., M., Pandolfi, M., 2018. Can estrogens be considered as key elements of the challenge hypothesis? The case of intrasexual aggression in a cichlid fish. Physiology & Behaviour 194: 481–490.

Yokoi, S., Ansai, S., Kinoshita, M., Naruse, K., Kamei, Y., Young, L., J., Okuyama, T., Takeuchi, H., 2016. Mate-guarding behaviour enhances male reproductive success via familiarization with mating partners in medaka fish. Frontiers in Zoology 13:21 DOI 10.1186/s12983-016-0152-2

Chapter 5

Effect of dominance status on sperm quality, hormone levels and testicular cell composition in *Solea senegalensis*

Chapter 5. Effect of dominance status on sperm quality, hormone levels and testicular cell composition in *Solea senegalensis.*

1. Introduction

Social interactions amongst individuals are generated due to the competition for several resources such as food, space or opportunities to reproduce (Martin and Bateson, 1993). These asymmetric interactions between individuals exhibit complex behaviours that shape social structures and ranking within the group, determining dominant and subordinate individuals (Maruska and Fernald, 2013). However, the position of individuals in the ranking may drive to changes in the behaviour and physiological aspects in the animals (Scaia *et al*., 2020). Overall, agonistic behaviours have been observed in males of several species, such as *Cichlasoma dimerus*, *Danio rerio*, *Oreochromis niloticus* (Ramallo *et al*., 2015; Teles and Oliveira 2016; Pfenning *et al*., 2012). Dominant males typically express aggressive behaviour towards their opponents, defending a territory, accessing food or to mate with females. This implies that the counterpart (subordinate) has to adjust its behaviour with respect to their social status (Kawaga *et al*., 2013; Teles and Oliveira 2016). The establishment of hierarchies seems relevant when broodstocks are kept in captivity, as happens in the aquaculture industry. The possibilities of fleeing from an opponent and/or assuming a role in a social status are limited, which could be highly oppressive in a portion of stock. These conditions would cause some individuals to be left behind because of their condition and thus affect the health, welfare and reproductive potential of breeders (Øverli *et al.,* 1999; 2004). However, the effects of the social position in the individuals within a hierarchy may be varied.

In fish, such as *Solea senegalensis*, hierarchical relationships have been described in different aspects based on social behaviour. Salas-Leitón *et al*. (2010) determined that the establishment of social hierarchies caused disparity in growth as result of the high interaction amongst the sole juveniles. Dominance behaviour was determined in sole juveniles, by Fatsini *et al.* (2017), through the feeding

response and territory parameters. Social categories and dominant and subordinate individuals were identified and associated to behaviours such as Rest the head (RTH), Approaches and Swimming Above Another (SAA). The dominant fish controlled more time the area determined as territory and exhibited higher frequency in the associated behaviours than subordinate fish. While in sole breeders, reproductive dominance has been recorded in several studies (Martin *et al.* 2014; 2019, Fatsini *et al*., 2020; chapter 2). A few breeders participated in mating and fertile spawns, according to the results of parental genetic analysis (Porta *et al*., 2006; Martin *et al.*, 2014; Fatsini *et al*., 2020), where couples maintained fidelity over reproductive seasons (Martín *et al.,* 2014; 2019; Fatsini *et al.*, 2020). In specific, the reproductive success of sole breeders held in captivity has been limited. Although wild breeders normally reproduce, cultured breeders have a reproductive dysfunction attributed to the lack of reproductive behaviour in males (Carazo *et al.,* 2016; Martín *et al*., 2019). In addition to the reproductive dysfunction, the social domination observed by dominant individuals appeared to limit opportunities and decreased the possibility that other fish participated in the reproduction (Fatsini *et al*., 2020). Thus, within the social context, dominance behaviour in sole breeders may have control over reproductive success (Chapter 4). The relationship between social behaviour and reproductive success has been described in species of cichlids such as *Astatotilapia burtoni* and *Oreochromis mossambicus* (Scaia *et al*., 2020). Where dominant territorial males courted with the females, while subordinate males developed a submissive behaviour. In sole, males mainly are more subject to competition and difficulty to achieve reproductive success, due to either social structure and / or the complex reproductive behaviour. While the females (wild and cultured), seem to have no problems to reproduce (Martín *et al.*, 2019). Nevertheless, the participation of the females is conditioned to the execution of a complex courtship performed by the males to encourage them to mate (Carazo *et al*., 2016; Martín *et al*., 2019). Some behaviours such as “Follow” or “Guardian” have been described as part of courtship and exhibit a way of competition and access to females (Carazo *et al*., 2016; Fatsini *et al*., 2020). Besides, the hierarchical dominance system could be involved in the mate choice

process (Jennions and Petrie 1997). Competition between males may identify behavioural patterns and features through which females may have mating preferences, detect differences between males and choose by preference dominant males (Maruska and Fernald 2011; Bierbach *et al*., 2014). Therefore, the social hierarchy in the males (dominance and subordinate) could mean that some individuals have higher reproduction opportunities than others (Maruska and Fernald 2013). Since socially dominant fish have higher reproductive success than subordinate fish (Chapter 4; Maruska and Fernald 2013). However, these subordinate fish may have a certain role in reproduction. In some fish as *Neolamprologus pulcher*, subordinates and subdominants fish are involved in the care of the offspring, although sometimes subdominant may agree to participate in the reproduction (Bender *et al*., 2006).

Nevertheless, the hierarchical structures are not immovable or permanent. The dynamics interactions amongst the fish, may lead to the subordinate fish to opportunities for advancement in the social rank (Maruska and Fernald 2010). This adjustment in social status may improve the condition in the physiological ability to reproduce (Maruska 2014). In cichlid fishes, hierarchies may be linear amongst individuals and an anticipated reproductive response would be adapted according to the possibility of a change in social rank (Alonso *et al*., 2012). Subordinate African cichlids fish, maintain the reproductive potential despite social suppression and within few hours after a social change may be able to produce viable sperm (Kustan *et al.,* 2012). While in Arctic charr (*Salvelinus alpinus*) investment in the sperm quality was identified to adjust to mating opportunities (Rudolfsen *et al*., 2006). In addition, subordinate fish could adopt other strategies to compensate for reproductive disadvantages (Kustan *et al.*, 2012).

In *S. senegalensis*, the complex reproductive behaviour mentioned above and the social hierarchical system has influence in the reproductive success (Carazo *et al.,* 2016; Fatsini *et al*., 2020; chapter 4). The dominance condition of males appears to contribute in the participation in the courtship and fertile spawning of this species. The participation in the parental contribution showed a dominance of wild males

regarding to cultured breeders (Fatsini *et al*., 2020). However, over several reproductive seasons the participation of cultured breeders in the courtship increased and a cultured male became reproductively dominant after the dominant wild male died. Despite the participation of a cultured male no other cultured male took part in fertile spawning. Perhaps the cultured breeder could have maintained reproductive dominance towards other cultured males. Dominance relationships between male sole breeders and possible effects on reproductive ability or physiological changes that may occur between dominant and subordinate individuals have not been explored. In other species, such as *Cichlasoma dimerus* and African cichlid *Astatotilapia burtoni*, the subordinate fish might undergo physiological changes and be suppressed reproductively due to social domination by dominant fish (Maruska 2014; Scaia *et al.,* 2020). The differences between both categories (dominant and subordinate) concerned not only phenotypical features, but also regulate cellular, molecular mechanisms and androgen response related to reproduction (Maruska 2014). Therefore, process such as spermatogenesis, spermiation and activation of male sexual behaviour could be influenced by the social context and position of each individual within the dominance hierarchy (Gonçalves and Oliveira, 2010). However, in *S. senegalensis*, sperm quality has been described, highlighting the scarce and variable sperm volume produced in reference to wild and cultured breeders (Cabrita *et al*., 2006). The male reproductive system is characterized by the small size of the testes containing two regions, cortex and medulla, in which are found the sperm ducts that storage the sperm (García-López *et al*., 2006). Although an effect due to dominance status as has been reported in cichlids fish would be the increase of testes size (Maruska 2014). In addition, the possible differences in sperm produced at molecular level between wild and cultured breeders, has been evaluated as cause of reproductive dysfunction in cultured breeders (Riesco *et al*., 2019). However, the social dominance could be a factor affecting sperm quality, fluctuations of androgens or spermatogenesis development between male breeders. Examine social factors involved in the reproduction, could improve the understanding of the lack of reproductive success in some male breeders.

The present study analysed the social behaviour in males of *Solea senegalensis* in relation to the effect on the reproductive potential of the fish. Social status was determined through a dyadic test and categories were identified as dominant and subordinate between male breeders. Social behaviour was evaluated and related to sperm quality, hormone concentrations and testicular cell production, in breeders according to the social hierarchy between fish.

2. Methods

2.1 Animal rearing conditions and experimental tanks

The Senegalese sole cultured broodstock (n=24) with a mean weight of 450.35 ± 17.41 g, were used in the present study. The breeders were kept in the facilities in IRTA Sant Carles de la Rápita, (Catalonia, and Spain), under the same conditions described in the chapter 4, section 2.1. The experimental tanks used to carried out the trial, had similar characteristics used in the study performed by Fatsini *et al*. (2017) and in the Chapter 4, section 2.1 of this thesis (Figure 1). All the individuals were placed inside tanks similar to the experimental tanks, but without incorporating sand area, to be acclimated for two weeks until the test. The experimental area was isolated to avoid any disturbance to the fish. Once the acclimation period was over, each pair of fish was moved to the experimental tank. Each couple tested had a similar size to avoid possible interference of fish size in the outcomes. The breeders were previously identified for later identification similar to the method used in Chapter 4 section 2.1.

2.2 Video recording

The activity of the fish was video recorded during 24 hours for five days. The recording was made similar that in the test in Section 2.2 of Chapter 4, using the same equipment.

Figure 1. Design of the experimental tank (400 L) used in the dyadic test. Two dark grey plastic divisions to keep the fish separate at the beginning of the test. A sand area (15 X 20 x 2 cm) created inside the tank to evaluate the preferred space test.

2.2.1 Video Analysis

The videos were analysed during the 24 hours each day. The behavioural interactions of each fish to the other fish were classified and counted each day, during the five days. These behaviours were classified as Approaches, Swimming above another (SAA), Rest the head (RTH), Displacement and Burying, according to section 2.2.1 in the Chapter 4 of this thesis. During each day, the behaviours were registered to each fish in three periods: a) initial time (09:00-11:00 h), b) peak of activity, PA (18:00- 20:00 h) and final time in the next day, TF (06:00- 08:00 h). In addition, the time each fish spent in the sand area was recorded in the same periods. The Feeding response was observed in the initial time to each day between each pair of fish.

2.3 Behavioural Trial

The test was carried out in early spring season and the breeders were tested through a dyadic test during five days. The dominance status was evaluated for each couple using the parameters, Behaviours: Approaches, Swimming above another (SAA), Rest the head (RTH), Displacement, Burying; Feeding and Place preference: Initial time (TI) Time in peak of locomotor activity, Total time (TT), Final time (TF) (Table 1) and described fully in the section 2.3, Chapter 4. At the end of the test, fish were anaesthetized with 60 mg L^{-1} tricaine methanesulfonate (MS-

222; Sigma-Aldrich, Spain). The fish were weighed, blood samples were taken to later analysis and sperm samples were collected to assess the sperm quality parameters for each fish. In addition, six fish classified as dominant, and the six fish classified as subordinate were euthanized with an overdose of MS-222, in order to be dissected the testes, which were fixed in formalin solution for histological analysis.

2.3.1 Dyadic test

Each pair of fish was placed within the experimental tank at 18:00 hours. The fish remained isolated by a separating wall (dark grey plastic divides) (Figure 1), during a night of acclimation (18:00-09:00 h). The following morning (9:00 h), the wall was removed and each fish had the opportunity to access the sandy area and be in contact with each other. During five days, the interactions between fish, time in the sand area and Feeding response were registered. The following parameters were assessed as possible indicator of dominance:

a) Behavioural parameters

The evaluation of behavioural parameters (Table 1) was performed with the same method used in the Chapter 4, section 2.3.1, subsection (a).

b) Preferred space

The parameters (Table 1) assessed and related to preferred space were: a) “Total time” in the preferred space (TT), b) “Initial time” the time during the first two hour of the test that each fish first occupied the sand area (TI), c) “Time in peak of locomotor activity” during one hour and d) “Final time” the time during the last two hour of the test that each fish occupied the sand area (TF). In addition, the order to occupy the sand area was recorded to indicate which fish was “first” or “last” to occupy the sand area.

c) Feeding Response

The Feeding response (Table 1) was evaluated as in the Chapter 4, section 2.3.1, subsection (c), at the same time during five days in the test.

Table 1. Ethogram of parameters and behaviours recorded in *Solea senegalensis* broodstocks, based on the study by Fatsini *et al*. (2017).

Parameters and behaviours evaluated	Definition
Approaches	A fish swims near to another fish with no contact.
Swimming above another (SAA)	A fish swims over another individual.
Rest the head (RTH)	An individual supports its head on some part of the body of another fish.
Displacement	A fish moves another fish from its place or position by a physical action.
Burying	Movement performed by a fish characterized by a vigorous beats of the head and a wave of muscular contraction in the substrate, with which the fish buries itself.
Feeding	Fish consume or ingest the food dispensed and what individual ate in first position.
Initial time (TI)	The time spent in the sand space by each Sole in the first 2 hours at the start of the test. The parameter was measured in minutes.
Time in peak of locomotor activity	The time spent in the sand space by each Sole in the hour of higher locomotor activity (19:00 to 20:00 hours) during the day.
Total time (TT)	The total time each Sole spent in the sand space during all the test. The parameter was measured in minutes.
Final time (TF)	The time spent in the sand space by each Sole in the last 2 hours at end of the test. The parameter was measured in minutes.
Order position in the sand	Order in which each sole occupied the space in the sand at the starting and end of the test. “First” position was the Sole that was located in the sand first at beginning of the test and “Last” was determined by the Sole that was in the sand space at end of the test. The two fish could not be in the sand at the same time. If a fish was on top of the other fish in the sand, the lower fish in the sand was counted as being in the sand.

2.4 Steroid analysis

The steroid analysis (11-Ketotestosterone) performed in the blood samples taken from each fish, was made following the manufactures procedure and as carried out in the Chapter 4, section 2.5.

2.5 Sperm quality

The sperm samples from each fish were analysed to determine their quality according to the following parameters: cell concentration, percentage of motile cells (% sperm motility), Velocity Curvilinear (VCL, μm s^{-1}) and Velocity Average Path (VAP, μm s^{-1}). The collection and the analysis of samples were realized as described in the methods of Chapter 2, sections 2.4.1, 2.4.2 and 2.4.3.

2.6 Histological analysis

The testes were removed at the end of the five days test, weighed, and the gonadosomatic index (IG) was calculated. The upper testis was fixed by immersion in 10% formalin solution for 24 hours at room temperature and later dehydrated in ascending concentrations of ethanol, clarified with xylene and embedded in paraplast, sectioned at 3 μm and stained with eosin / hematoxilin.

From the sections of testes, photomicrographs were taken with a camera Olympus DP70 connected to the microscope (Leica Mod. DMLB) and observed with a 400x magnification. From sections of the testis of each dominant and subordinate individual, ten randomly selected seminiferous tubules were examined and the tubule area was measured using the Soft Imaging System analySIS 5.1 programme. The testicular cells were classified according to the types of germ cells found and as described by García-López *et al*. (2006). The number of spermatogonia, spermatocytes, spermatids and spermatozoa in the medular and cortical section of the testis, was counted and normalized to the total area of the seminiferous tubules analysed.

2.7 Statistics analysis

The data were calculated and expressed as the mean ± one standard error of the mean (SEM) in the behavioural parameter and preferred space (TI, TF and TT), recorded during five days for each fish. The First and Last position, as well as Feeding response was determined for each day (5 days) for each fish and out come with the highest frequency was used to represent the response of each fish. A Principal Component Analysis (PCA) was conducted to group parameters that both exhibited similar variation and represented the variation across the data set. The components resulting were depicted from an eigenvalue >1 based on the Kaiser Selection criterion and Bartlett's test of sphericity with Varimax rotation was applied. The coefficient of variation (CV % =SD/mean *100) was calculated to compare the variability between dominant and subordinate individuals in the behavioural parameters and preferred space (TI, TF and TT). A Pearson´s correlation was applied to determine the relationships between the parameters related to preferred space, Feeding response, sperm quality parameters, hormonal analysis (11KT), testes weight and gonadosomatic index. A paired Student's t-test was realized to compare the means of preferred space parameters (TI, TF and TT), sperm quality parameters, steroid hormones (11 KT), gonadosomatic index (GSI) and composition of testicular cells type between dominant and subordinate individuals. A Chi-squared analysis was used to compare proportions assessed between dominant and subordinate individuals in the parameters: Feeding response, First and Last position in the sand. The data used to analyse the proportions were nominal data (yes / no): Yes = in social dominance / feeding response, No = no in social dominance / feeding response; Yes = in social dominance / first position, No = no in social dominance / first position; Yes = in social dominance/ last position, No = no in social dominance/ last position. Statistical analysis was carried out using SPSS Statistic 20 for Windows (SPSS Inc. Chicago, IL, USA) and Sigma Plot 12.0. Systat Software Inc.

3. Results

3.1 Dominance Classification in Breeders.

In the present study, a dyadic test was carried out during five days in order to determine dominance status amongst male Senegalese sole breeders. A Principal Component Analysis (PCA) was carried out to group the variables based on the variables assessed (behaviours, place preference and feeding order) and explain the variability of data. The PCA (KMO =0.657, P= 0.000 and X2= 362.947), described 60.28 % of the variability in the data (Table 1). Three Components were grouped as follows: the first component (PC1) was related to the behaviours Approaches, RTH, SSA and Displacement. The second component (PC2) grouped variables related to preferred space, Initial time, Final time, Time in peak of locomotor activity and Total time on the sandy area and burying behaviour. The third component (PC3) was related to the first and last position in relation to the preferred sand area. However, the parameter of feeding response had a poor and negative relation to the three components indicating that feeding was not representative of the variation in the data set. Therefore, the behavioural parameters and preferred space were used to determine dominance status between each pair of fish. The dominance ranking was done at the end of the five days evaluated on each fish pair.

3.2 Behavioural parameters

The behavioural parameters (approaches, SAA, RTH and displacement) grouped in the Principal component one, were used to classify the dominance status between the breeders. The burying behaviour was not grouped in the component relating to behaviours, therefore was not considered. On each day, the number of behaviours carried out from one individual to another between each pair of fish was counted. A previous dominance classification was carried out daily according to the higher or lower frequency of the behaviours performed by each fish during five days. The fish with a higher number of behaviours were classified as dominant and the fish with the lower number as subordinated. At the end of the five days

evaluated, the final dominance designation in each fish was determined. Each dominance category in fish was assigned according to the higher number of times what was found during the five days.

Table 1. Proportion of variables descriptors to define Dominance behaviour in males *Solea senegalensis* and used in the Principal Component Analysis.

	Component		
	1	2	3
RTH	.906	.030	-.084
SAA	.905	-.058	-.051
Approach	.852	-.150	-.029
Displacement	.757	.058	.215
Total Time (sand)	-.071	.912	-.031
Peak of activity (sand)	-.104	.776	.157
Final time (sand)	-.045	.685	-.326
Initial time (sand)	-.091	.598	.084
Burying	.169	.534	-.204
First position	.000	-.100	.762
Last position	-.181	-.409	.530
Feeding	-.195	-.296	-.480

The dominant individuals showed less variability (approach= 9.67 ± 1.11 counts; CV= 84.17 %; SAA= 6.66 ± 1.57 counts; CV= 142.05 %; RTH= 13.07 ± 2.66 counts; CV=105.18 %; however the displacement behaviour, exhibited a higher frequency (1.11± 0.35 counts) but with a highest variability (CV= 232.82 %), in contrast with the subordinate males had lower counts and variation (approaches= 6.62 ± 0.86 counts; CV= 94.63 %; SAA= 1.98 ± 0.42 counts; CV= 157.60%; RTH= 5.64 ± 0.83 counts; CV= 107.32 %; and displacement= 0.71± 0.15 counts; CV= 158.02 %). The behavioural parameters were strongly correlated with each other (Table 2). Likewise, a strong correlation was found in SSA, RTH and Displacement regarding to testis weigh (Table 2). In addition these three behaviours were also correlated with Cell concentration (Table 2) and RTH and Displacement were weakly correlated to motility percentage (Table 2). The spermatocyte cells found in the medulla area in the testis were also correlated with SAA and RTH. While, negative correlations were found between the behaviours (Approaches, SSA, RTH and displacement) and spermatozoa cells contained in the sperm ducts.

Table 2. Correlations between behavioural parameters, sperm quality parameters (percentage of sperm motility and cell concentration), testis weight, GSI and testicular cell types. Asterisks (*) indicate the level of significance in each correlation: * Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).

Parameters		Approaches	SAA	RTH	Displacement
Approaches	Pearson Correlation	1	0.782**	0.797**	0.727**
	Sig.		0.000	0.000	0.000
SSA	Pearson Correlation	0.782**	1	0.969**	0.892**
	Sig.	0.000		0.000	0.000
RTH	Pearson Correlation	0.797**	0.969**	1	0.921**
	Sig.	0.000	0.000		0.000
Displacement	Pearson Correlation	0.727**	0.892**	0.921**	1
	Sig.	0.000	0.000	0.000	
Testis weight	Pearson Correlation		0.722**	0.752**	0.691*
	Sig.		0.008	0.005	0.013
GSI	Pearson Correlation	0.565*	0.316	0.385	0.331
	Sig.	0.044	0.293	0.194	0.269
Cell concentration	Pearson Correlation	0.298	0.495*	0.515*	0.541**
	Sig.	0.157	0.014	0.010	0.006
Motility percentage	Pearson Correlation	0.371	0.398	0.481*	0.447*
	Sig.	0.074	0.054	0.017	0.029
Spermatocyte cells (medulla area)	Pearson Correlation	0.254	0.635*	0.636*	0.588
	Sig.	0.452	0.036	0.036	0.057
Spermatozoa cells (sperm ducts)	Pearson Correlation	-0.604*	-0.755**	-0.756**	-0.663*
	Sig.	0.049	0.007	0.006	0.026

3.3 Preferred space

For the test of place preference, the time fish stayed in the sand area displayed less variability in dominant fish than in subordinate (dominance determined by behavioural counts). The values of parameters related to place preference of dominant fish as determined by behavioural counts were the following: Initial time (TI)= 25.91 ± 3.59 min; CV= 48.04 %; peak of activity (PA)= 52.48 ± 8.98 min; CV= 59.29 %; Final time (TF)= 47.32 ± 7.55 min; CV= 55.29 %; Total time (TT)= 104.68 ± 15.10 min; CV= 49.98 %. Whilst the subordinate fish had higher variability: Initial time (TI)= 30.03 ± 5.93 min; CV= 68.40 %; peak of activity (PA)= 39.92 ± 9.55 min; CV= 68.40 %; Final time (TF)= 42.69 ± 7.04 min; CV= 57.16 %; Total time (TT)= 95.30 ± 18.18 min; CV= 68.62 %. However, in all periods that the fish were in the sand, no significant differences were exhibited between the dominant and subordinate fish.

In the Last position, the dominant fish (64.70 %) had a higher percentage than subordinates (35.30 %) (Figure 2). The proportion of dominant and subordinate (dominance by behaviours) fish that were in the last position at the end of the test showed significant differences (P= 0.014) with respect to the expected values, estimating that the dominance determined by behavioural counts had no effect on which fish occupied the last position (Table 3). Nine (75%) of the twelve fish that were in the last position were dominant in the behaviours and similar percentage (75 %) of the twelve fish that were not in the last position were subordinate (n=9). While the proportion of dominant and subordinate fish (dominance by behaviours) that were in the first position did not exhibit significant differences (P= 0.201) between the expected and observed proportions (Table 3). The means of initial time (26.12 ± 4.07), time during the peak of activity (52.48 ± 8.98 min), final time (47.32 ± 7.55 min) and total time (104.68 ± 15.10 min) spent on the sand area, by dominants fish were not significantly different to what was observed for subordinates (30.18 ± 4.98 min; 39.92 ± 9.55 min; 42.69 ± 7.04 min; 95.30 ± 18.18 min respectively).

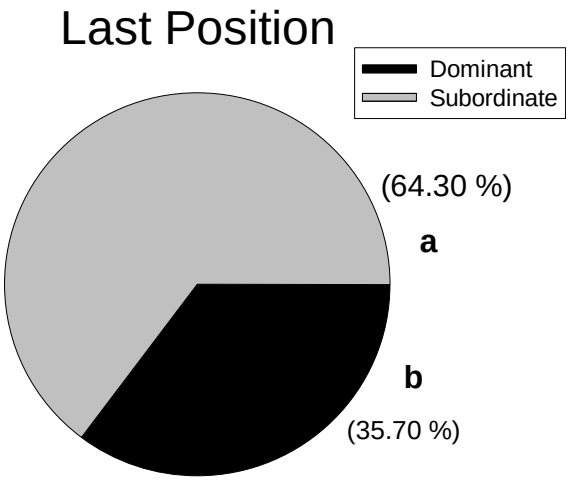

Figure 2. Proportion of dominant and subordinate fish in the last position. Different letters next to each proportion indicate significant differences.

Table 3. Number of dominant and subordinate males of *S. senegalensis* that were in First and Last position or were not in First and Last position. Behavioural counts (dominate fish had higher counts), was used to define dominance status. The results of Chi-square analysis compared the observed values in the table with expected values considering that dominance determined by behavioural counts, had no effect to stay in last or first position. An asterisk * indicates a significate difference between observed and expected proportions $P < 0.05$.

Parameters evaluated	**Dominance status by behaviours**		**Total count of individuals**	**Chi ^{2} Results**
	Dominant	Subordinate		
Fish that were in Last position: YES	9	3	12	$X^2 = 6.000$, gl.= 1, P = 0.014*
Fish that were not in Last position: NO	3	9	12	
Fish that were in First position: YES	7	4	11	$X^2 = 1.636$, gl.= 1, P = 0.201
Fish that were not in First position: NO	4	7	11	

3.4 Feeding Response

Regarding to the feeding order, 50.00 % of fish that were classified as dominant with the behavioural counts ate first and equally 50.00 % of the subordinate fish ate first (Figure 3). No significant differences were found between the proportion of dominant and subordinate fish according to dominance defined by behavioural parameters that fed in first position (P = 1), determining that the expected and observed values were similar (Table 4).

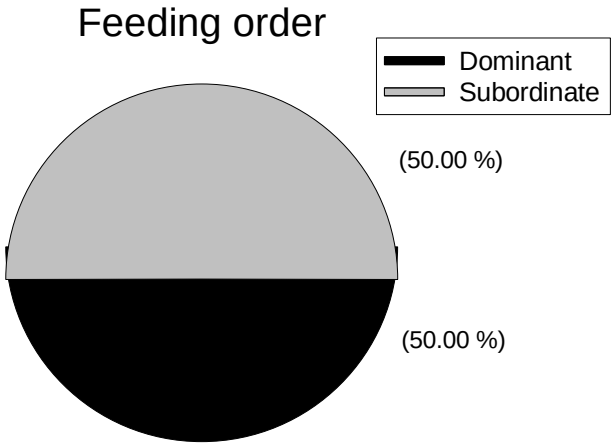

Figure 3. Proportion of dominant and subordinate fish that ate first in feeding order.

Table 4. Number of dominant and subordinate males of *S. senegalensis* that ate first and did not eat first in feeding order. Behavioural counts (dominate fish had higher counts), was used to define dominance status. The results of Chi-square analysis compared the observed values in the table with expected values considering that dominance determined by behavioural counts, had no effect on feeding order.

Parameters evaluated	**Dominance status by behaviours**		**Total count of individuals**	**Chi ^{2} Results**
	Dominant	Subordinate		
Fish feeding in first position: YES	6	6	12	$X^2 = 0.000$, gl.= 1, P = 1.000
Fish that did not feed in first position: NO	6	6	12	

3.5 Sperm quality and dominance status

The sperm collected had a mean volume of 376.25 ± 35.00 µL, where subordinate males released a volume of 371.67 ± 41.32 µL and dominant males 380.83 ± 41.32 µL, resulting no significant differences. The motility percentage of spermatozoa was low overall (8.25 ± 1.36 %). However, motility in dominant males (10.73 ± 2.22 %) was almost twice the percentage from subordinate males (5.77 ± 1.30 %), exhibiting significant difference between the groups (P=0.034) (Figure 4A). In addition, motility percentage parameter was strongly correlated to testes weight (R=0.822; P=0.001) and GSI (R=0.653; P=0.021).

The cell concentration of spermatozoa had a mean 1.57 x 10^9 ± 3.41 x 10^8 spz mL^{-1} overall. In dominant males, the cell concentration was 1.41 ± 1.71 x 10^9 spz mL^{-1} than in subordinates 1.73 ± 1.69 x 10^9 spz mL^{-1}. Spermatozoa per kg of body weight (mean 1.18 x 10^9 ± 2.25 x 10^8 spz kg^{-1}) was also compared between dominant and subordinate males (1.19 x 10^9 ± 3.31 x 10^8 spz kg^{-1} and 1.16 x 10^9 ± 3.19 x 10^8 spz kg^{-1}). However did not exhibit any significant difference between both categories (P=0.651 and P=0.950 respectively) (Figure 4B).
Furthermore, sperm velocity parameters between dominant and subordinate fish were not found to be significantly different in VCL (P=0.562) (Figure 4C) and VAP (P=0.229) (Figure 4D). In subordinate fish the mean was recorded in VLC was 95.32 ± 5.55 µm s^{-1} and VAP was 80.55 ± 5.80 µm s^{-1}, while in dominants VLC was 89.99 ± 7.14 µm s^{-1} and VAP, 69.17 ± 7.14 µm s^{-1}.

3.6 Hormonal Analysis

The 11 KT concentration in plasma in dominant males was 15.06 ± 3.37 ng ml^{-1} and subordinate males (10.29 ± 2.50 ng mL^{-1}) (Figure 5A), no significant differences were found between the fish groups (P= 0.098). A correlation was found between the 11 KT concentration and the testes weight (R= 0.727; P= 0.007) and GSI (R=0.604; P= 0.037).

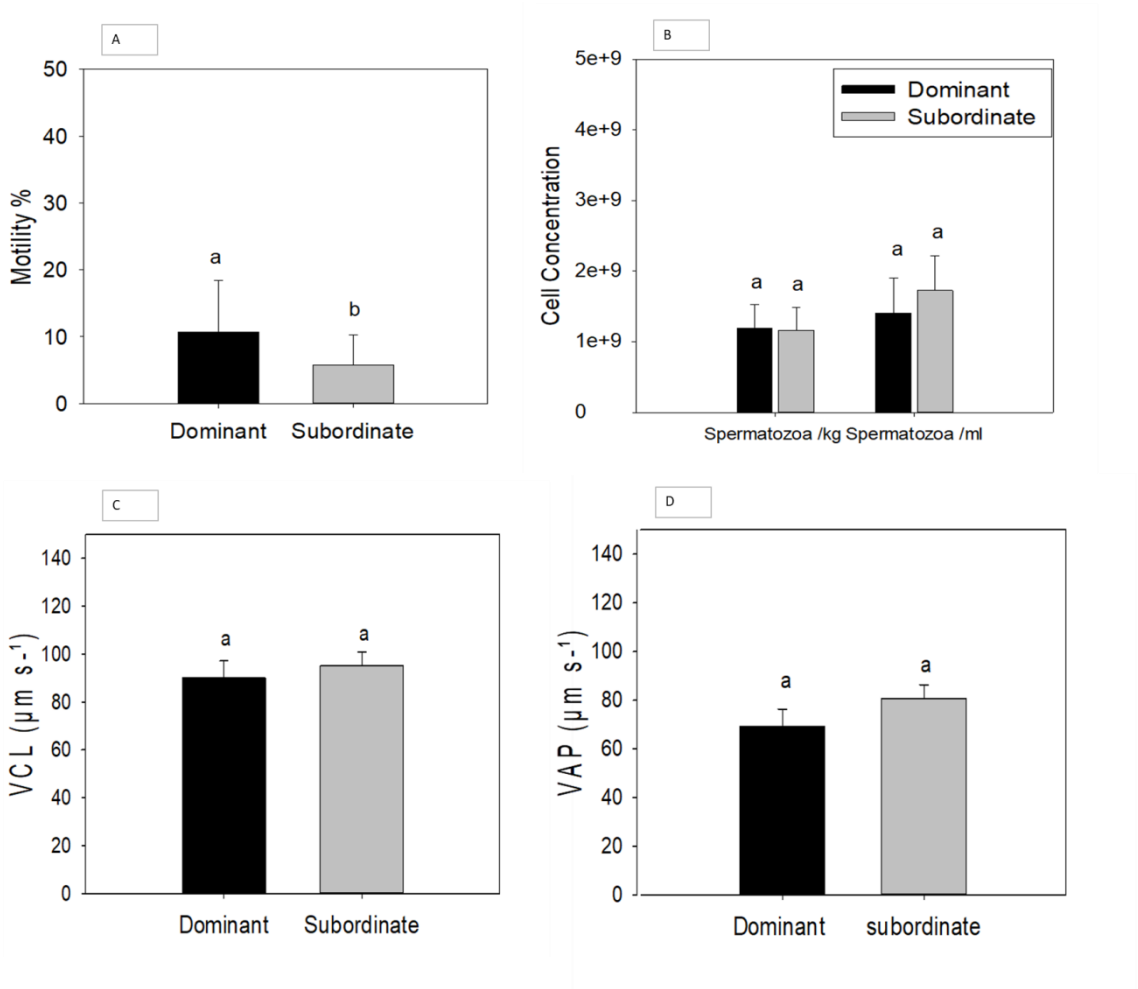

Figure 4. Mean percentage motility in dominant and subordinate fish. B. Mean cell concentration in dominant and subordinate males. Mean spermatozoa number per kg and spermatozoa number per mL. C. Mean Curvilinear Velocity (VCL) and D. Average Path Velocity (VAP) between Dominant and Subordinate males. Different letters above the bars show significant differences between the groups.

3.7 Composition of testicular cells type

The dominant males had heavier testes than subordinate males (0.47 ± 0.06 g and 0.38 ± 0.03 g respectively) (Figure 5B), but there was no significant difference (P= 0.212). The mean overall of GSI value was 0.09 ± 0.01 %. The comparison of mean values between dominant and subordinate males, was significant (P=0.040);

the GSI in dominant males was higher (0.11 ± 0.01 %) than in the subordinate males (0.08 ± 0.01 %) (Figure 5C).

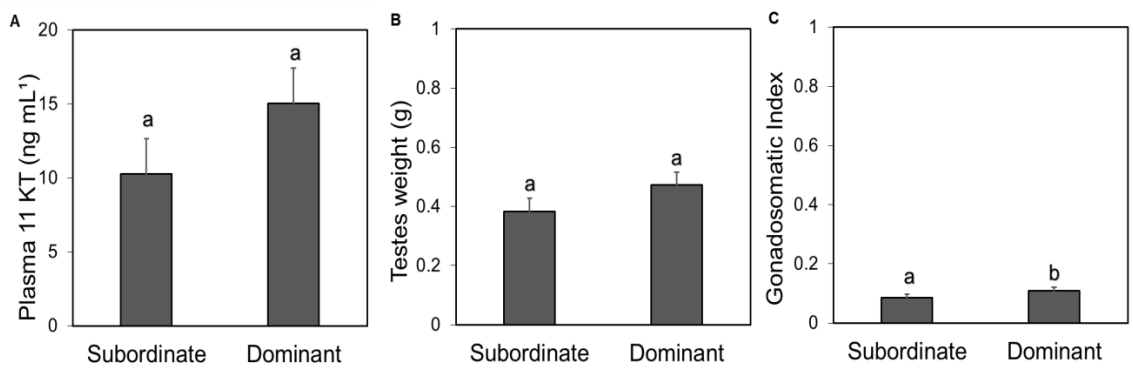

Figure 5. Comparative values between dominant and subordinate males of mean 11- KT levels (A), mean Testes weight (B) and mean Gonadosomatic Index (C). The data are expressed in mean ± SEM and the bars with different superscript above are significantly different (P<0.05).

Testes development in all samples was determined in the stage III according to the classification realized by García-López *et al*. (2006). The cortical area contained cells in all developmental stages: spermatogonia, spermatocytes, spermatids and spermatozoa (Figure 6A). However, the most abundant cells were spermatids (48.49%), while of the least abundant was spermatocytes (9.79%). In the medulla of the testis, the tubules contained spermatocytes, spermatids and mainly spermatozoa that were the most abundant (67.63%) of all the three cell types (Figure 6B). The spermatids and spermatozoa cells were observed in the sperm ducts (Figure 6C).

The comparison in cell composition in the cortex between dominant and subordinate males did not exhibit significant differences in the cells number of spermatogonia (P=0.645), spermatocytes (P=0.416) and spermatids (P=0.499). However significant difference in the number of spermatozoa (P=0.027) between the dominant and subordinate males was found (Figure 7A). Regarding to the type of cells observed in the medulla area, there was no significant difference between dominant and subordinate males for any type of germ cell (spermatocytes, spermatids and spermatozoa) (Figure 7B). In the sperm ducts similar numbers of

spermatids and spermatozoa were found in dominant compared to subordinate males (Figure 8). While that number of spermatid cells found in sperm duct were correlated to 11 KT concentration (R=0.766; P=0.006).

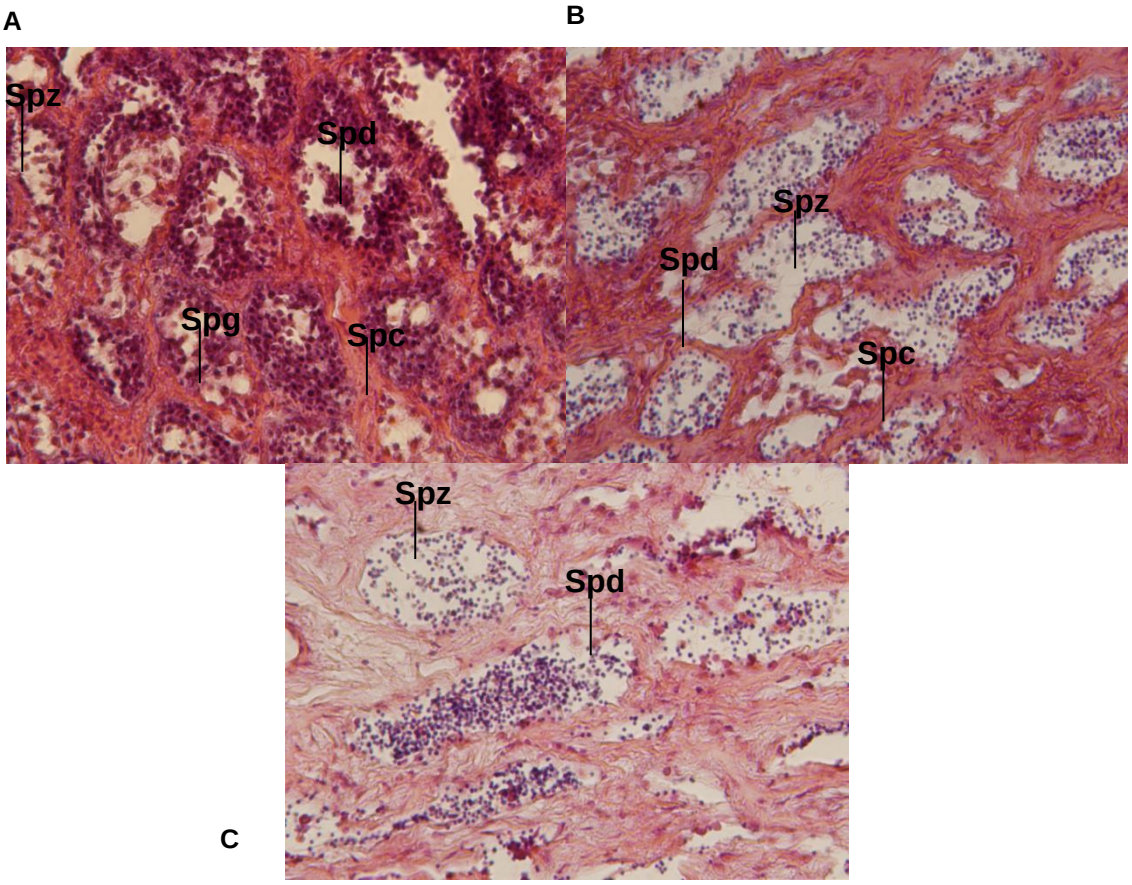

Figure 6. Representative histological sections of testes development in males of Senegalese sole (*S. senegalensis*). Different types of germ cells found in testes areas: Spg, spermatogonia; Spc, spermatocyte; Spd, spermatid; Spz, spermatozoa. A) Germ cells in cortical area, B) germ cells in Medulla area and C) germ cells in sperm ducts. Scale bars, 50μm.

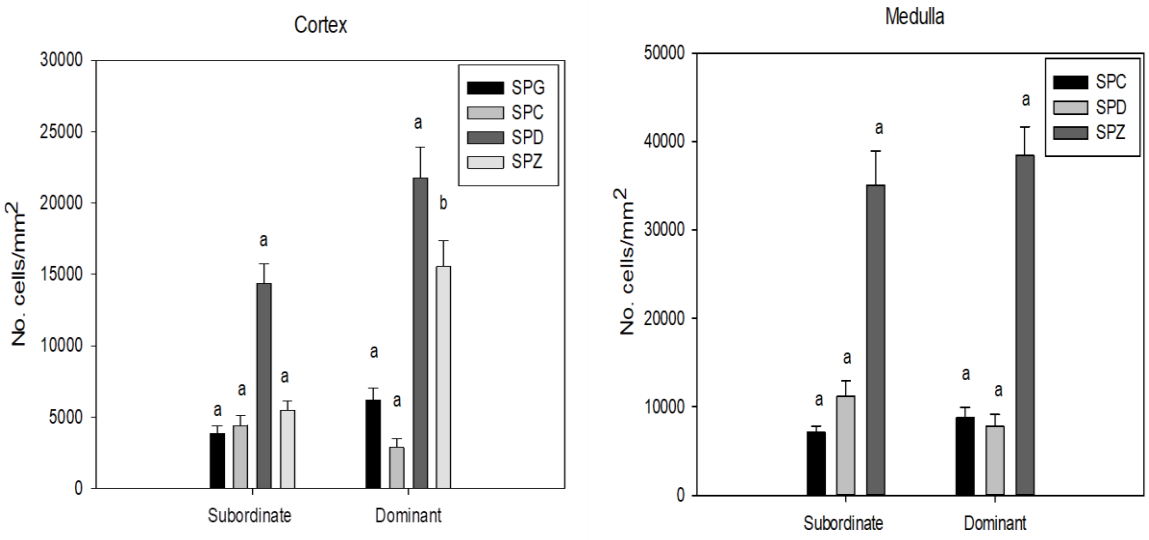

Figure 7. Mean number of germ cells, spermatogonia (SPG), spermatocytes (SPC), spermatids (SPD) and spermatozoa (SPZ), in the cortex and medulla region per unit area of tubules (n=10) in the testis according to social status subordinate and dominant as determined by behavioural counts. A) Mean number of germ cells by area of tubule in the cortex of the testis in males dominant and subordinate; B) Mean number of germ cells by area of the tubule in the medulla region of the testis in dominant and subordinate males. The data are expressed in mean ± SEM and the bars of the same cell type with different superscript were significantly different (P<0.05).

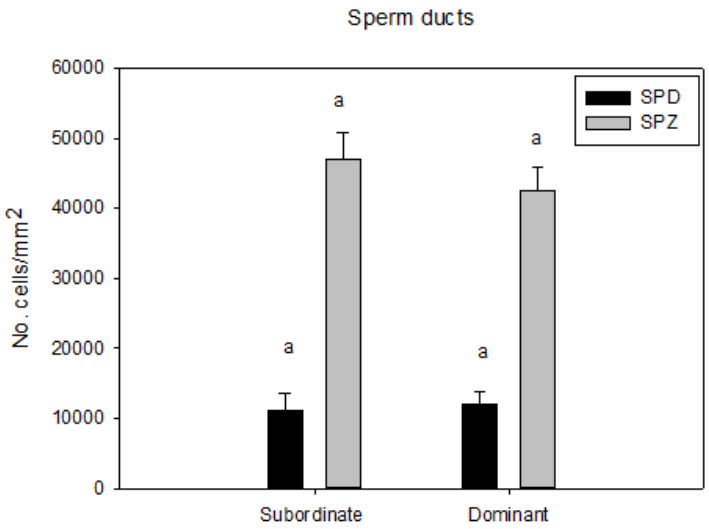

Figure 8. Mean number of spermatids and spermatozoa cells in the sperm ducts contained in the area of duct lumen in testis from dominant and subordinate males. No differences significant were found between groups. The data are expressed in mean ± SEM.

4. Discussion

In the present study, dominance status in *S. senegalensis* males was determined by a dyadic test, classifying dominant and subordinate individuals. The relationship between social hierarchy and sperm quality, hormonal concentration and germ cell composition was evaluated according to category determined between each pair of males. The exploration of the hierarchy in social dominance was performed through three parameters: a) Behavioural, b) Preferred space and c) Feeding response. However, only behavioural parameters and preferred space showed comparable patterns that appeared to be related to the hierarchical status between males. While feeding response did not show variation in relation to the other parameters and did not appear to exhibit association to hierarchical classification. The behaviours: Approaches, SAA, RTH and Displacement were grouped together and showed a strong correlation with each other, whilst burying was not grouped in this parameter set. The behavioural parameters correlated were taken to define hierarchical category between each pair of fish. The fish with highest number of behavioural counts over the five day test were classified as dominant. The hierarchical classification by behavioural counts had some agreement with place preference as dominant fish by behaviours remained in the last position at end of the test with a higher proportion of individuals in comparison to subordinate fish.

The behaviours were correlated with testes weight, GSI and cell concentration. While a weak correlation was obtained between behavioural parameters and sperm motility percentage. The dominant males exhibited higher sperm motility percentage, GSI and higher number of spermatozoa cells contained in the cortex area of testes. In addition, the number of spermatocyte cells found in medulla was correlated with behaviours, RTH and SAA, which were related to social status. However in contrast negative correlations were obtained between the four behaviours and spermatozoa cells contained in the sperm ducts. Lastly, the subordinate males exhibited similar values to dominant males in some parameters in the preferred space, feeding response, cell concentration, sperm velocity, 11 KT concentration and weight testis. The cell composition in the cortex region (SPG,

SPC and SPD), in the medulla region (SPC, SPD and SPZ) and the cells in sperm ducts were also similar between both categories of males. Therefore, it seems that the hierarchical status does not imply a total suppression in the reproductive potential in subordinate fish. However the social hierarchy could influence in the control of resources such as territory and elements in the sperm quality and gonadal development.

In sole males the social status was classified by a pattern of behaviours, which were strongly associated with each other. The behaviours used in the present study, were also associated to dominance behaviour in Senegalese sole juveniles (Fatsini *et al*., 2017). Dominance behaviour in sole juveniles was characterized based on feeding response and monopolization of territory (sand area); dominant and subordinate categories were identified and associated to behaviours (Fatsini *et al*., 2017). The hierarchical classification between each fish pair was made according to the frequency of the behaviours during the dyadic test. The fish with a higher count of behaviours was classified to be dominant over the subordinate fish that had a lower count of behaviours. In species, such as Arctic charr (Liljedal and Folstad, 2003), or neotropical cichlid fish (Ramallo *et al.*, 2015), the social dominant fish had a higher number of interactions towards the subordinate fish. While, only a low percentage of subordinate fish were reciprocal to cope to behaviours received. This social competition was also reflected in the control of resources, such as territory. A higher proportion of dominant male soles (by behaviours) in comparison to subordinate males, remained in the sand area occupying the last position. A relevant feature that has been marked in *S. senegalensis* as a positioning for a space associated to its benthic habits and to rest during the early morning hours (Gibson 2005; Fatsini *et al*., 2017; Chapter 4). The sandy bottom is the environment that provides protection and shelter place. Therefore, competition for a limited resource as territory may emphasize dominance behaviour between individuals. In others species, such as African cichlids *(*Kustan *et al.,* 2012; Pfenning, *et al*., 2012; Maruska, 2014) or rainbow trout (Liley and Kroon 1995), the dominant individuals maintain the control to the access to the territories respect to subordinate fish. These spaces usually were

used for feeding, nest, parental care or protection and shelter against predators (Ramallo *et al*., 2015).

However, the social context of individuals within a group also influence in the reproduction (Dey *et al*., 2015; Fatsini *et al.,* 2020; Chapter 4). Due to that socially dominant individuals not only have control over territory or food, but also over the access to mate with the females (Maruska and Fernald 2013; Fatsini *et al*., 2017; Chapter 4). In sole breeders reproductive dominance was identified (Martín *et al*., 2014; Fatsini *et al*., 2020). Mainly, during the reproduction season males are exposed to competition for mating with females (Carazo *et al*., 2016; Fatsini *et al*., 2020). While females often seem to choose the socially dominant males, which would leave subordinate fish at a disadvantage (Fatsini *et al*., 2020; Bierbach *et al*., 2014). Therefore, inside the social environment of soles, dominant and subordinate individuals may reach a different reproductive success (Chapter 4). The lack of participation of some individuals in reproduction, would be related to social disadvantage associated with physiological changes and reduction in reproductive potential (Maruska and Fernald, 2013; Scaia *et al*., 2020). Therefore, social competence can influence adjustments and changes in reproductive structures in males.

In present study, behaviours that define social status showed correlations with parameters linked to testes features (weight and GSI) as well as sperm motility, concentration and cell testicular development. The reproduction process is closely linked to the social environment and gonadal development (Pfenning *et al.*, 2012). Thus, implications in the sperm quality and reproductive success could be driven for changes in gonadal structure and social dynamics (Kustan *et al.,* 2012; Pfenning *et al.*, 2012). Dominant sole males, exhibited differences in respect to subordinate fish in sperm motility percentage, GSI and spermatozoa number contained in cortex area in the testes. The investment in the gonadal development and sperm quality (motility percentage and sperm velocity), work as a mechanism to adjust the reproduction potential between categories of breeders (Maruska 2014). Sperm motility percentage has often been a measure used to determine the

sperm quality (Kime *et al*., 2001). While sperm velocity (VCL) is a relevant parameter indicator to determine reproductive success, linked to the high rate of fertilization of the eggs (Rudolfsen *et al*., 2006; Gallego *et al*., 2013). The sperm quality may reduce the reproductive success in the males (Beirão *et al*., 2009), hence the male efficiency in the sperm production represents advantage in the reproduction (Cabrita *et al.,* 2006; Maruska 2014). The sperm produced in sole have been determined with low and variable sperm volume and poor quality and particularly to males reared in captivity (Cabrita *et al.*, 2006). Therefore, the identification of males with higher sperm quality could be a factor to improve the reproduction in this species. The higher sperm motility percentage obtained in dominant males, suggest a response to the hierarchical position between the males (Liljedal and Folstad, 2003; Rudolfsen *et al*., 2006). However, similar velocity values (VCL) and sperm volume was measured between both categories of sole males. In African cichlids fish this sperm quality pattern was also observed. The dominant territorial males showed a higher sperm quality pattern (motility percentage) compared to subordinate males, but similar velocity values (Kustan et al., 2012). However, the sperm motility may be affected by several factors such as the composition of seminal plasma, contamination with external substances (water, urine or faeces) or alteration in osmolality and pH (Cabrita *et al*., 2008; Fauvel *et al.,* 2010; Cabrita et al., 2019). Moreover, GSI has been described as a tool for measuring the sexual maturity according to gonadal development and it may be used as evidence of changes in endocrine function (Pait and Neilson 2002). Overall GSI determined in sole males was similar result obtained by Chauvigné *et al.* (2014) and García López *et al*. (2006). The adverse effect of social status on the gonadal development would be related to individuals that remain in lower dominance level, while in dominant males the GSI was higher (Alonso *et al.*, 2012). In some species cichlids such as *A. burtoni* or *C. dimerus* social cues through of aggressive interactions performed from dominant fish to subordinate fish, may influence the brain, sex steroids response and reproductive system (Maruska and Fernald, 2013; Ramallo *et al*., 2015). The information received through social interactions between the individuals send a signal to the social regions in the brain.

This signal is transmitted to the reproductive axis and ends up influencing the testes. The activation of brain regions regulates the neuro-molecular response and influence in the reproductive axis, where the gonadotropin-releasing hormone 1 (GnRH1) neurons are located. These GnRH1 integrate the information extracted from external and internal stimuli by impacting on the pituitary gland to modulate the reproduction (Maruska and Fernald, 2011). However, the physiological characteristics may be reversed, depending on the changes in social context and adjustments to the hierarchical level amongst individuals (Scaia *et al*., 2020).

Nevertheless, behind of physiological adjustments and changes between dominant and subordinate males, a dynamic system regulates behaviour-endocrine response, through the circulation and hormonal release (Ramallo *et al.,* 2015; Teles and Oliveira 2016). Although the increase in androgens levels such as 11 KT is expected as a response to an acute social challenge (Rudolfsen *et al*., 2006; Teles and Oliveira 2016), 11 KT concentrations were similar between dominant and subordinate sole. Overall the 11 KT values were similar to what was noted by García-López *et al.* (2006) and slightly lower to what was found by Chauvigné *et al.* (2016) for sole in the same season. Also, no correlations were determined with behaviours used to define dominance status. However, the relationships between 11 KT concentrations and testes weight as well as GSI reveal the influence on the size and development of the tests. Different phases of spermatogenesis and sperm production would be stimulated by androgenic sex hormone which modulates the effect on germ cell proliferation and development (Rudolfsen *et al*., 2006; Schulz *et al*., 2010). This could be consistent with the cell germ proliferation found in the testes between both categories of males and suggests that spermatogenic activity is not suppressed in subordinate males. Germ cells (SPG, SPC and SPD) found within the cortical region were similar between dominant and subordinate fish, however, SPZ were higher in dominant than subordinate males but germ cells number was similar between both categories of fish. The SPZ contained in the cortex region show the capacity in dominant males to generate a higher amount of germ cells (García-López et al., 2005), although germ cells stored in the medulla region did not exhibit differences. In *Astatotilapia burtoni* males, differences in the

percentage of mature sperm in testes were not observed in regard to the social status (Maruska and Fernald 2011). The authors suggested that the subordinate males that were able to retain viable sperm had probably previously been dominant.

Despite the social constraint on the subordinate fish, there appears to be no suppression in reproductive potential. In a hierarchical system, dominant males are expected to perform breeding behaviour and subordinate males are expected to adopt a reserved position. However, although the subordinate males have a disfavoured role in respect to the breeding opportunities, this condition may be compensated by others strategies (Kawaga *et al*., 2013; Maruska and Fernald, 2013). The results indicated an investment in sperm production even though the mating opportunities are limited and the gonadal development is lower (Pfennig *et al*., 2012). Socially subordinate males, may maintain sperm production in the testes, although life span can be short, compared to sperm production in dominant fish (Kustan *et al*., 2012). While dominant males often have the ability to mate and they may be able to reduce the amount of sperm stored, preserving energy and gametes. This strategy allows them to maintain long-term mating opportunities (Liljedal and Folstad, 2003). The subordinate males also exhibited germ cells at all stage of development, indicating that the spermatogenesis process is continued, even in a state of social submission. The similarity found in germ cells between dominant and subordinate males, has been related to the concentration of androgens, that promote the proliferation of cells from early stages (Maruska and Fernald, 2011; Kustan *et al.,* 2012). This has also been found in cichlid species, where the subordinate males had cells proliferation in all stages, in order to maintain their reproductive potential in case the fish ascended in the social ranking (Maruska and Fernald, 2011; Kustan *et al.*, 2012; Ramallo *et al*., 2015). The ascent in the hierarchy of reproductive dominance was observed in *S. senegalensis,* after removal of the reproductively dominant wild male, a cultured male occupied the highest hierarchical position and participated in fertile spawning (Fatsini *et al.,* 2020; Chapter 2).

5. Conclusions

In *S. senegalensis* broodstock, the dominance behaviour was determined through social behaviours. The dominant males by behaviours controlled the last position in the preference place, considered a feature related to sole that dominate a preferred space. The influence of dominance behaviour may have a relative effect on sperm quality and cell testicular composition. However, the dominance behaviours and hierarchical position did not appear to influence physiological response in the production of 11-KT reproductive steroid. The spermatogenesis process appeared to on the whole be continued in fish determined as subordinate, carrying out an adaptive mechanism according to its social context.

6. References

Alonso, F., Honji, R. M., Moreira, R. G., Pandolfi, M. 2002. Dominance hierarchies and social status ascent opportunity: Anticipatory behavioral and physiological adjustments in a Neotropical cichlid fish. Physiology & Behavior, Volume 106, Issue 5, Pages 612-618. Doi.org/10.1016/j.physbeh.2012.04.003.

Beirão, J., Soares, F., Herraez, M. P., Dinis M. T., Cabrita E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72(9): 1251-1261.

Bender, N, Heg, D., Hamilton, I. M., Bachar, Z., Taborsky, M., Oliveira, R. F., 2006. The relationship between social status, behaviour, growth and steroids in male helpers and breeders of a cooperatively breeding cichlid. Hormones and Behaviour Volume 50, Issue 2: 173-182. https://doi.org/10.1016/j.yhbeh. 2006.02.009

Bierbach, D., Oster, S., Jourdan, J., Arias-Rodriguez, L., Krause, Wilson, A. J., M., Plath, M., 2014. Social network analysis resolves temporal dynamics of male dominance relationships. Behav Ecol Sociobiol 68:935–945.DOI 10.1007/s00265-014-1706-y

Carazo, I., Chereguini, O., Martin, I., Huntingford, F., Duncan, N.J. 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*). Span J Agric Res. 14(4): e0401.

Cabrita, E., Soares, F., Dinis M. T., 2006. Characterization of Senegalese sole, *Solea senegalensis,* male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975.

Cabrita, E., Martínez-Pastor F., Soares, F., Dinis, M.T., 2008. Motility activation and subpopulation analysis in *Solea senegalensis* spermatozoa. Cybium, 32(2) suppl.: 185-186.

Cabrita, E., Riesco, M. F., Mañanós, E.L., 2019. Sperm physiology and artificial fertilization. In: The Biology of Sole. Editors, José A. Muñoz Cueto, Evaristo L. Mañanós Sánchez, F. Javier Sánchez Vázquez. CRC Press. Boca Raton Florida. Pp 142- 68.

Chauvigné, F., Zapater, C., Crespo, D., Planas, J., Cerdà, J. 2014. Fsh and Lh direct conserved and specific pathways during flatfish semicystic spermatogenesis. Journal of molecular endocrinology 53(2): 175-190. DOI: 10.1530/JME-14-0087.

Chauvigné, F., Fatsini, E., Duncan, N., Ollé, J., Zanuy, S., Gómez, A., Cerdà, J., 2016. Plasma levels of follicle-stimulating and luteinizing hormones during the reproductive cycle of wild and cultured Senegalese sole (*Solea senegalensis*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 191, 35–43.

Dey, C., J., Tan, Q.Y. J., O'connor, C., Reddon, M., A., R., Caldwell, J., R., Balshine, S., 2015. Dominance network structure across reproductive contexts in the cooperatively breeding cichlid fish *Neolamprologus pulcher* . Current Zoology 61 (1): 45–54

Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Mackenzie, S., Duncan, N.J. 2017. Dominance behaviourin a non-aggressive flatfish, Senegalese sole (*Solea senegalensis*) and brain mRNA abundance of selected transcripts. PLoSONE 12 (9): e0184283.https://doi.org/10.1371/journal. pone.0184283.

Fatsini, E., González, Wendy., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. J. Appl. Ichthyol. 26 : 636–643.

Gallego, V., Pérez, L., Asturiano, J.F., Yoshiba, M., 2013. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture, 416-417: 238-243.

Garcia-Lopez, A., Martinez-Rodriguez, G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole, *Solea senegalensis* (Kaup): Anatomy, histology and histochemistry. Histology and Histopathology 20(4): 1179-1189.

García-López, A., Fernández-Pasquier, V., Couto, E., Canario, A.V.M., Sarasquete, C., Martínez-Rodríguez, G. 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. General and Comparative Endocrinology, Volume 147, Issue 3, Pages 343-351.

Gibson, R., 2005. Flatfishes: Biology and Exploitation: Blackwell Science Ltd. a Blackwell Publishing Company. 391 pp.

Gonçalves, D.M. and Oliveira, R.F. 2010. Hormones and Sexual Behavior of Teleost Fishes. In Hormones and Reproduction of Vertebrates. Fishes. Editors Norris, D.O and Lopez, K.H. Academy Press. Volume 1. Pp. 119-147.

Jennions, M. D., Petrie, M., 1997. Variation in mate choice and mating preferences: a review of causes and consequences. Biol. Rev. 72, pp. 283–327.

Kagawa, N. 2013. Social rank-dependent expression of arginine vasotocin in distinct preoptic regions in male *Oryzias latipes*. Journal of Fish Biology 82, 354–363. doi:10.1111/j.1095-8649.2012.03490.x.

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F., 2001. Computer-assisted sperm analysis CASA as a tool for monitoring sperm quality in fish. Comparative Biochemistry and Physiology Part C 130: 425 – 433.

Kustan, J.M., Maruska, K.P., Fernald, R.D. 2012. Subordinate male cichlids retain reproductive competence during social suppresion. Proceedings of the Royal Society. 279: 434-443.

Liley, N.R., Kroon, F.J. 1995. Male dominance, plasma hormone concentrations, and availability of milt in male rainbow trout (*Oncorhynchus mykiss*). Can. J. Zool. 73: 826–836.

Liljedal, S., Folstad, I. 2003. Milt quality, parasites, and immune function in dominant and subordinate Arctic charrCan. J. Zool. 81: 221–227. Doi: 10.1139/Z02-244.

Martín, I., Rasines, I., Gómez, M., Rodriguez, C., Martinez, P., Chereguini, O. 2014. Evolution of egg production and parental contribution in Senegalese sole, Solea senegalensis, during four consecutive spawning seasons. Aquaculture 424-425, 45-52. https://doi.org/10.1016/j.aquaculture.2013.12.042

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Gómez, M., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Span. J. Agric. Res. 17 (4), e0401.

Martin, P., Batenson, P., 1993. Measuring Behaviour. Cambridge University Press. UK. 222 pp.

Maruska, K.P., Fernald, R. D. 2010. Behavioral and physiological plasticity: Rapid changes during social ascent in an African Cichlid fish. Hormones and Behavior 58: 230-240.

Maruska, K.P., Fernald, R. D. 2011. Social regulation of Gene expression in the Hypothalamic-Pituitary-Gonadal Axis. Physiology 26: 412-423.Doi: 10.1152/physiol.00032.2011.

Maruska, K.P., Fernald, R. D. 2011. Plasticity of the Reproductive Axis caused by social status change in an African Cichlid fish: II. Testicular Gene Expression and Spermatogenesis. Endocrinology 152 (1): 291-302.

Maruska, K.P., Fernald, R. D. 2013. Social regulation of male reproductive plasticity in an African Cichlid fish. Integrative and Comparative Biology, volume 53, number 6, pp. 938–950.

Maruska, K.P. 2014. Social regulation of reproduction in male cichlid fishes. General and Comparative Endocrinology. 207: 2-12.

Øverli, Ø., Harris, C.A., Winberg, S. 1999. Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. Brain Behav Evol. 54(5): 263–75. PMID: 10640786.

Øverli, Ø., Korzan, W., Höglund, E., Winberg, S., Bollig, H., Watt, M., Forster, G.L., Barton, B.A., ØVerli, E., Renner, K., Summers, C. H. 2004. Stress coping style predicts aggression and social dominance in rainbow trout. Hormones and Behavior 45(4):235-41. DOI: 10.1016/j.yhbeh.2003.12.002.

Pfennig, F., Kurth, T., Meißner, S., Standke, A., Hoppe, M., Zieschang, F., Reitmayer, C., Göbel, K., Gutzeit, H.O. 2012. The social status of the male Nile tilapia (*Oreochromis niloticus*) influences testis structure and gene expression. Reproduction 143:71-84.

Pait, A. S., Nelson, J. O., 2002. Endocrine Disruption in Fish: An Assessment of Recent Research and Results. NOAA Tech. Memo. NOS NCCOS CCMA 149. Silver Spring, MD: NOAA, NOS, Center for Coastal Monitoring and Assessment 55 pp.

Ramallo, M.R., Birba, A., Honji, R.M., Morandini, L., Moreira, R.G., Somoza, G.M., Pandolfi, M. 2015. A multidisciplinary study on social status and the relationship between inter-individual variation in hormone levels and agonistic behaviour in a Neotropical cichlid fish. Hormones and Behaviour 69:139-151.

Salas-Leiton, E., Anguis, V., Rodríguez-Rúa, J.,P., 2010. Stocking homogeneus size gruops does not improve growth performance of Senegalese sole (*Solea senegalensis* Kaup 1858) juveniles: Individual growth related to fish size. Aquacultural Engineering 43:108-113. doi:10.1016/j.aquaeng.2010.08.001

Scaia, M., F., Morandini, L., Noguera, C., A., Trudeauc, V., L., Somozad, G., M., Pandolfi, M., 2018. Can estrogens be considered as key elements of the challenge hypothesis? The case of intrasexual aggression in a cichlid fish. Physiology & Behaviour 194: 481–490.

Schulz, R.W., de Franca L.R., Lareyre J.J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., (2010). Spermatogenesis in fish. Gen Comp Endocrinol 165:390–411. DOI:10.1016/j.ygcen.2009.02.013

Riesco, M. F., Valcarce, D. G., Martínez-Vázquez, J. M., Martín, I., Calderón-García, A. A., González-Nunez, V., Robles V., 2019. Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question. Reproduction, Fertility and Development. https://doi.org/10.1071/RD18453

Rudolfsen, G., Figenschou, L., Folstad, I., Tveiten, H., Figenschou, M. 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc Biol Sci. 273(1584): 325–332. https://doi.org/10.1098/rspb.2005.3305.

Teles, M.C, Cardoso, S.D., Oliveira, R.F. 2016. Social Plasticity Relies on Different Neuroplasticity Mechanisms across the Brain Social Decision-Making Network in Zebrafish. Front. Behav. Neurosci. 10:16. doi: 10.3389/fnbeh.2016.00016.

Teles, M.C, Oliveira, R.F. 2016. Androgen response to social competition in a shoaling fish. Hormones and Behavior 78:8-12. http://dx.doi.org/10.1016/j.yhbeh.2015.10.009

Chapter 6

Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage.

Chapter 6 Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage.

1. Introduction

Senegalese sole (*Solea senegalensis*) is a marine flatfish of important commercial value that is emerging as an aquaculture species. In five years, aquaculture production of Senegalese sole has increased from 95t in 2012 to 1818t in 2017 (FAO 2019). Nevertheless, the control of Senegalese sole reproduction in captivity has not been fully successful as hatchery reared males have a reproductive behavioural dysfunction and do not fertilize the eggs released by females (Guzman *et al*., 2009; Carazo 2013; Martin 2016; Martin *et al*., 2019). Currently, sole production is based on wild broodstocks that spawn spontaneously in captivity and, therefore, the industry relies on the capture of wild breeders, which is unsustainable (Morais *et al*., 2016). A possible solution to this problem has been the development of artificial fertilisation methods using gametes stripped from mature cultured Senegalese sole (Liu *et al*., 2008; Rasines *et al*., 2012; 2013). However, the development and application of artificial fertilisation protocols at industrial scale has been frustrated by the low volumes of sperm, poor sperm quality and high variability in sperm quality among individuals (Cabrita *et al*., 2006; 2011; Beirão *et al*., 2009; 2011; Chauvigné *et al*., 2016; 2017). Therefore, solutions are required to address these problems.

Low sperm volumes are probably related to the small testes size, the semi-cystic spermatozoa development and the spawning behaviour. Males have two small testes and low gonadal somatic index (Gracía-López *et al*., 2005), which produce low volumes of sperm. Spermatogenesis in sole is semi-cystic, which is different to the cystic development observed in most aquaculture species and which may be another factor implicated in low sperm production (García-López *et al*., 2005, Mylonas *et al*., 2017). This low sperm production may be related to low sperm requirements considering the mating behaviour of Senegalese sole (Carazo *et al*., 2016). During spawning, males hold the urogenital pore in close proximity to the

oviduct and sperm are introduced to the eggs at the point of release from the oviduct, which probably reduces the requirement for large numbers of sperm to achieve a successful fertilisation. Initial attempts to increase sperm volume with hormones doubled sperm production (Agulleiro *et al*., 2006; 2007; Guzman *et al*., 2011), however, recent studies with species-specific recombinant gonadotropins have increased sperm production by four times (Chauvigné *et al*., 2017; 2018).

A second aspect that affects both sperm volume and quality is the contamination with urine. In Senegalese sole, the spermatic ducts and the urinary system share the same urogenital pore (Gracía-López *et al*., 2005), thus it is difficult to avoid contamination with urine when sperm is collected. In other species, urine contamination has been determined by measuring urea in the seminal plasma (Dreanno *et al*., 1998) and contamination by urine or the presence of urea has been shown to negatively affect the quality of sperm in various species (Król *et al*., 2018; Cabrita *et al*., 2001; Rurangwa *et al*., 2004). The urine contamination changes the environment of the spermatozoa by altering aspects of the seminal plasma such as osmolality and pH (Cosson *et al*., 2008). Urine induced changes in osmolality and ion content, may cause the activation of spermatozoa during the collection of sperm. In freshwater fish the hypo-osmotic urine may reduce the seminal plasma osmolality to activate the spermatozoa (Alavi *et al*., 2007), whilst in marine fish the variable, but similar iso-osmotic urine (Fauvel *et al*., 2012) may change ion balance or even vary the osmolality of the seminal plasma to also activate the spermatozoa (Cosson *et al*., 2008; Valdebenito *et al*., 2009). This early activation reduces the percentage of motile spermatozoa, spermatozoa swimming speed and, therefore, the ability of the sperm to fertilize eggs (Poupard *et al*., 1998; Rurangwa *et al*., 2004; Linhart *et al*., 2003; Alavi *et al*., 2006; Cejko *et al*. 2010). In addition, urine contamination has caused a decrease in pH (acidification) (Ciereszko *et al*., 2010; Fauvel *et al*., 2012), which has been observed to also reduce motility (Nynca *et al*., 2012). Therefore, sperm samples contaminated with urine are usually discarded (Dreanno *et al*., 1998; Poupard *et al*., 1998; Król *et al*., 2018) and most studies with Senegalese sole only use what was considered by appearance to be only sperm and samples that appeared to be contaminated were

not used (Agulleiro *et al.,* 2006; Cabrita *et al*., 2006; 2011; Beirão *et al*., 2008; 2009; 2015; Martinez-Pastor *et al*., 2008; Valcarce *et al*., 2016; Riesco *et al*., 2017; 2019; Fernandez *et al*., 2019). To date, no studies have examined the effect of urine contamination on the quality of Senegalese sole sperm.

Extender solutions have been used to preserve contaminated sperm and maintain sperm quality. These extender treatments have been developed to prevent the activation and damage of the spermatozoa by urine contamination (Rodina *et al*., 2004; Sarosiek *et al*., 2012; Gallego *et al*., 2013; Beirão *et al*., 2019). Generally, the sperm is diluted with the extender solution that lengthens the storage period and maintains sperm quality parameters. Extender solutions have been made from a combination of ions, antioxidants, amino acids, sugars and antibiotics and are species-specific. Extenders solutions have become an essential aspect for sperm conservation (short or long term storage), which ensures the availability of sperm for artificial fertilisation (Rodina *et al*., 2004; Bobe and Labbé 2009; Cabrita *et al*., 2010; Gallego *et al*., 2013; Beirão *et al*., 2019). Cryopreservation protocols have been studied for Senegalese sole (Rasines *et al*., 2012; Valcarce and Robles 2016; Riesco *et al*., 2017) and used also to have availability of sperm for artificial fertilisation (Rasines *et al*., 2012; 2013). These cryopreservation protocols used only what was considered uncontaminated sperm. Short term chilled storage of sperm using extenders have the possibility to work with contaminated sperm and are also useful for artificial fertilisation protocols (Bobe and Labbé 2009; Beirão *et al*., 2019; Ramos-Júdez *et al*., 2019). In addition, short term chilled storage of sperm is easier, cheaper and a more practical method to preserve sperm in the hatcheries. However, no studies have been published on the use of extender solutions for the short-term storage of Senegalese sole sperm.

The aim of the present study was to: (a) describe the anatomy of the urinary and male reproductive system to understand why Senegalese sole sperm is usually contaminated; (b) describe the characteristics of Senegalese sole sperm in relation to urine contamination; (c) examine the use of a range of extender solutions for

chilled short-term storage to maintain the sperm quality parameters, motility and velocity.

2. Methods

2.1 Animals and sample collection

The Senegalese sole broodstock used in the present study was kept in the facilities in IRTA Sant Carles de la Rápita (Catalonia, Spain). The broodstock was kept in two tanks (14 m^3) connected to a recirculation system (IRTAmar®) with a controlled natural temperature cycle (9-20 ºC) and under natural photoperiod (9-14 hours light). The fish were fed with 0.75% of wet feed (polychaetes and mussels) and 0.55% dry feed (balance diet) of total biomass, four days a week.

Trials were carried out during the two natural periods of reproduction of the sole, in autumn and in spring. Individual males (mean weight = 559 ± 193 g) were chosen randomly and anesthetized with 60 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma-Aldrich, Spain) and weighed. Semen samples were obtained by applying gentle abdominal pressure towards the urogenital pore and collected with a 1 mL syringe. First, the testes were located by touch and gently massaged and then, the sperm duct was gently stripped from the testes towards the urogenital pore. This testes massage followed by sperm duct stripping was repeated to obtain the sperm sample. The volume collected was recorded and the sperm was placed in Eppendorf tubes above crushed ice.

The structure of the sole male reproductive and urinary system was examined in nine specimens. Males were sacrificed with an overdose of MS-222 (120 mg L−1). The reproductive and urinary system was dissected and the morphology and organization of both systems was examined and described. The length of seminal ducts and testis size were measured with a Vernier calliper and the testes weighed. The sperm ducts were fixed in Bouin´s solution, dehydrated in a series of alcohol baths, embedded in paraffin, cut into 5 µm sections and stained with H&E (Hematoxylin and eosin) for histological examination.

The broodstock was handled (routine management and experimentation) in agreement with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA, http://www.felasa.eu/).

2.2 Assessment of sperm parameters

When collected, each sperm sample obtained was described according to the features such as tonality (sample colour: yellow, whitish yellow or whitish), transparency (translucent or opaque feature of the sample) and consistency (viscosity or fluidity of the sample) (Fauvel *et al*., 1999; 2012). All samples were divided into three sub-samples, the first subsample (100 µL) was used to assess the sperm quality in the short-time storage and diluents, the second subsample (20 µL) was used to measure the pH and cell concentration and the third sub-sample (80 µL) was centrifuged to perform different analysis. All samples were stored at 4 °C until assessment. During storage, the Eppendorf tubes were kept open for gas exchange. The following parameters: pH, cell concentration, osmolality and protein concentration were measured for each sample.

The pH was measured with a Hach electrode and CyberScan Instruments (Eutech Ins. pH510). To determine cell concentration (spermatozoa mL^{-1}), fresh sperm was diluted 1:500 in 10% formalin and 10 µL of this dilution was placed into a Thoma cell counting chamber that was left 10 minutes for spermatozoa to sediment. The sedimented sample was observed under the microscope Olympus BH with a 10x objective and a picture taken with a GigE digital camera (model: DMK 22BUC03 Monochrome, The Imaginsource, Bremen, Germany). Images of three different fields from each sample were taken with IC Capture Software (www.theimagingsource.com). The number of cells were counted with the image processor; ImageJ software (http://imagej.nih.gov/ij/); and processed by analysing the particles in each captured field. The mean from the triplicate measures was used to calculate the mean cell concentration. Seminal plasma was obtained by taking the supernatant after a sperm sub-sample was centrifuged (15 min, 4 ° C and 3000 rpm). To determinate the osmolality (mOsmol kg^{-1}), 10 µL of seminal plasma was put into Vapor Pressure Osmometer 5520 (Wescor, USA) and each

sample was measured in triplicate. The protein concentration was measured in seminal plasma through Invitrogen Qubit 4 (Qubit Fluorometric Quantification. Thermo Fisher Scientific); 2 µL of seminal plasma were diluted in buffer solution mixed with the protein reagent (protein Assay kit. Thermo Fisher Scientific) and incubated for 15 min at room temperature before quantification of proteins in a Qubit fluorometer. The principle of the method is the fluorescence from the binding of fluorescent dyes to proteins is quantified with a Qubit Fluorometer, previously calibrated with standard solutions.

2.3 Evaluation of sperm quality

In all trials, the spermatozoa were activated and their paths recorded, until the motion ceased, using the IC Capture software and GigE digital camera (described above) connected to the microscope Olympus BH with a 20x objective. For sperm activation, either 1 µL of diluted sperm (extender trails, see below) was added to 20 µL of natural seawater with bovine serum albumin (BSA) prepared at 30% or 1 µL of undiluted sperm (control) added to 60 µL of seawater with BSA and gently mixed. One microliter of activated sperm was placed in a counting chamber ISAS R2C10 (Proiser *R+D, S.L.* Paterna, Spain) and the sperm motility was recorded. The videos obtained (AVI format) were processed with Virtual Dub 1.10.4 software (http://www.virtualdub.org/) to convert the video into image sequences in format *.jpeg. The files of image sequences were imported to ImageJ software and the sperm kinetics parameters were assessed at 15 seconds post-activation, using a computer-assisted sperm analysis (CASA) ImageJ plugin (http://rsb.info.nih.gov/ij/plugins/). The settings to analyse the videos were set as follows: brightness and contrast, -10 to 15/224 to 238; threshold, 0/198 to 202; minimum sperm size (pixels), 10; maximum sperm size (pixels), 400; minimum track length (frames), 10; maximum sperm velocity between frames (pixels), 30; frame rate, 30; microns/1000 pixels, 303; Print motion, 1; the additional settings were not modified. The parameters assessed during 2 seconds were the percentage of motile cells (% sperm motility), Curvilinear Velocity (VCL, µm/s) and Average Path Velocity (VAP, µm/s). Each sample was analysed in triplicate.

2.4 Urine Contamination

To determine the urine contamination, the urea concentration was measured, in the seminal plasma, using a urea kit (Urea-LQ urease –GLDH. Kinetic. Liquid, Spinreact, Sant Esteve de Bas, Spain). The principle of the method is two simultaneous enzymatic reactions, which are dependent on urea content, that is read through absorbance at 340 nm and the urea concentration is calculated and expressed in units of mmol L^{-1}.

In addition, urine samples from females (n=3) were collected to compare the urea concentration, pH and osmolality between urine and seminal plasma. Samples were obtained from female fish in order to avoid contamination with sperm. After collection, the urine was kept on ice until the analysis. The urea concentration was measured with the same method as seminal plasma.

2.5 Extender trials

Samples that had motility lower than 10% were not used in this analysis. The samples were evaluated at 0, 3, 6 and 24 hours after being collected. Portions of each sample were diluted in the different extenders (see composition table 1) at a 1:3 dilution, ratio semen (20 µL): extender (40 µL) and one portion was conserved without adding extender solution as a control sample. At each time interval (0, 3, 6 and 24 hours) spermatozoa from each sample were activated and evaluated as described above.

In the first trial during the autumn, 12 samples were used and four extenders tested: modified Leibovitz (Fauvel *et al.*, 2012), Ringer (Chereguini *et al*., 1997; Rasines *et al.*, 2012), NAM (Fauvel *et al.*, 1999) and Sucrose (Cabrita *et al.*, 2006). The second trial was performed during the spring when ten samples were used and two extenders solutions tested: modified Leibovitz (Fauvel *et al*., 2012), and Stor Fish® (Haffray and Labbé, 2008). In the third trial, the extenders solutions of modified Leibovitz (Fauvel *et al.*, 2012) and Marine Freeze® (IMV Technologies)

were tested during the autumn on six sperm samples. The procedures were the same in all trials.

All extenders osmolality and pH values where adjusted to fish semen parameters. Initially, the extenders medium had an osmolality range between 200 and 310 mOsmol kg^{-1} which was adjusted to 300 mOsmol kg^{-1} in order to avoid early activation of spermatozoa (Nynca *et al.*, 2012; Król *et al.*, 2018). A NaCl (5 M) solution was added to increase the osmolality and distilled water to decrease. With respect to pH, the range was between 7.7 and 8.06 among the different extenders and pH was adjusted to 8.0. An HCl (1 M) solution was added to lower the pH and NaOH (0.5 M) to increase the pH.

Table 1. Composition of different extender solutions per litre.

Composition	**Ringer**	**Leibovitz**	**NAM**	**Sucrose**	**Stor Fish ®**	**Marine Freeze ®**
Leibovitz L-15**		14.8 g				
NaCl	2.165 g		1.875 g			
KCl	1.000 g		0.05 g			
MgCl			0.615 g			
$CaCl_2$	0.099 g		0.195 g			
NaH_2CO_3	0.067 g		0.84 g			
Glucose			0.04 g			
Sucrose				51.35 g		
BSA***		20 mg mL^{-1}	10 mg			Yes*
Glutamine		300 µg mL^{-1}				Yes*
Sodium pyruvate		6 mg mL^{-1}				
Gentamycin		1 mg mL^{-1}			0.5 g	Yes*
Ultra-pure water	1 L	1 L	1 L	1 L	Yes*	Yes*
Biological buffer					Yes*	Yes*
Salts					Yes*	Yes*

*Manufacture only indicated what was present and quantities were not specified.

**Leibovitz L-15 medium, Sigma-Aldrich, Spain (product code: L-4386)

***Bovine Serum Albumine

2.7 Statistical analysis

The data was expressed as mean ± standard deviation (SD). All analyses were performed at a significance level at $P < 0.05$. Pearson´s correlation test was used to determine the existence of a correlation between urine contamination and the parameters analysed, as predictors of semen quality. The samples classified according to appearance (colour, transparency and consistency) were compared through a multivariate General linear model to determine if there were differences in quality parameters. In addition, a Principal Component Analysis (PCA) was used in order to examine linear correlation amongst parameters and to obtain principal components using the Kaiser criterion, where the components PC1 and PC2, were chosen. A Clusters analysis was performed on the variables of sperm quality and seminal plasma characteristics, in order to classify the samples into groups with homogeneous features. The samples were clustered into three groups using Ward's method established on Euclidean distances. The means of different parameters of the three clusters were compared with a one-way analysis of variance (ANOVA) and a Games-Howell post-hoc test was applied to determine significant differences between clusters. The effect of short-time storage and extenders on sperm motility parameters were assessed by a Repeated Measures Designs and a Bonferroni test with multiple comparisons between the means. Statistical analysis was carried out using SPSS Statistic 20 for Windows (SPSS Inc. Chicago, IL, USA).

3. Results

During three sampling periods, a total of 49 cultured male sole were examined to obtain sperm samples for the study. From these 49 males, a total of 32 (65.3%) samples were obtained with the characteristics required for the study. The rejected males either had no sperm (n=3) or low volumes with low initial motilities that were not sufficient for all the proposed analysis (n=14). Although these 17 males were rejected, 13 did have motile sperm and, therefore, 45 (91.8%) from 49 randomly selected males had motile sperm. The initial values of sperm quality parameters

exhibited high variation amongst the 32 males used in the study and in particular spermatozoa concentration followed by motility, urea and protein concentration were highly variable (table 2).

Table 2**.** The initial values of sperm quality parameters. The values were measured from sperm samples: sperm volume (µL), sperm motility percentage, VCL (µm/s), VAP (µm/s), duration sperm activity (s), cell concentration (spz mL^{-1}) and spermatozoa per kg of body weight (spz kg^{-1}) and from seminal plasma: pH, osmolality (mOsmol kg^{-1}), Urea concentration (mmol L^{-1}) and Protein concentration (µg mL). All values were referred as mean ± SD.

Parameter	Mean ± SD.	Minimum	Maximum	Coefficient of variation
Sperm volume (µL)	361.40 ± 173.40	130	700	48%
Initial sperm motility (%)	29.02 ± 20.42	4.54	77	70%
VCL (µm/s)	144.84 ± 64.51	57.35	277.81	45%
VAP (µm/s)	117.49 ± 64.89	42.07	255.30	55%
Duration sperm activity (s)	143.95 ± 5.33	85	240	4%
Cell conc. (spz mL^{-1})	1.48 ± 2.92 x 10^9	1.25 x10^8	1.38 x10^{10}	197%
Spermatozoa per kg (spz kg^{-1})	2.81 ± 5.21 x 10^9	1.82 x 10^8	2.45 x 10^{10}	185%
pH	6.91 ± 0.38	6.21	7.59	5%
Osmolality (mOsmol kg^{-1})	360.67 ± 138.46	185	713	38%
Urea conc. (mmol L^{-1})	2.58 ± 1.60	0.41	7.99	62%
Protein conc. (µg mL)	13.21 ± 8.14	3.45	24.30	62%

3.1 Morphology of male reproductive and urinary systems

As previously described by García-López *et al*. (2005), the male reproductive system of Senegalese sole is located in the abdominal cavity and is formed by two

asymmetric testicular lobes. The abdominal cavity is divided, in the posterior region, into upper (ocular side) and lower (blind side) cavities by a central skeletal dividing wall. The testes are located close to the anterior edge of the skeletal division on either side of the division (Figure 1). The largest testis is located on the upper ocular side of the division and the smallest testis, on the lower blind side. The upper testis is adhered to the upper side of the skeletal division and the lower testis is adhered to the lower (blind side) wall of the abdominal cavity. The urinary bladder is located anteriorly to the skeletal division and extends along the anterior edge of the division from the position of the testes to where the skeletal division connects with the abdominal cavity wall. The urinary bladder continues along the abdominal wall and ends where the urinary duct emerges and enters the abdominal wall. The urinary bladder appeared to be full of urine in all the males examined. From each testis, the spermatic duct emerges and travels along the length of the urinary bladder to the point where the urinary duct emerges from the urinary bladder and enters the wall of the abdominal cavity. The spermatic duct from the upper testis is adhered to the upper ocular side of the urinary bladder and the spermatic duct from the lower testis is adhered to the lower blind side of the urinary bladder. All three ducts, two spermatic ducts and the urinary duct enter the abdominal wall at the same point as separate ducts. Within the abdominal wall, the ducts combine and emerge on the outside of the fish as a single urogenital pore (Figure 1). The mean length of the spermatic ducts, from testicles to the urogenital pore, was 3.60 ± 0.91 cm in individuals with a weight of 791.3 ± 376.5 g and a length of 37.3 ± 6.3 cm. The spermatic ducts were entirely full of spermatozoa (Fig. 2A, 2B, 2C) as shown in a longitudinal section from a middle section between the testis and abdominal wall (Fig. 2A) and a cross section made close to the testis (Fig. 2B).

3.2 Contamination with urine and sperm quality

The sperm samples showed signs of contamination by urine, owing to the tonality or colour (yellow, whitish yellow or whitish), yellow samples had the appearance of sperm mixed with a lot of urine, samples described as whitish yellow had the

appearance of sperm mixed with smaller amounts of urine and samples described as whitish had the appearance of sperm with little or no urine contamination. Transparency (transparent or opaque) and consistency (viscous or fluid) also exhibited variation, but did not seem related to sperm concentration.

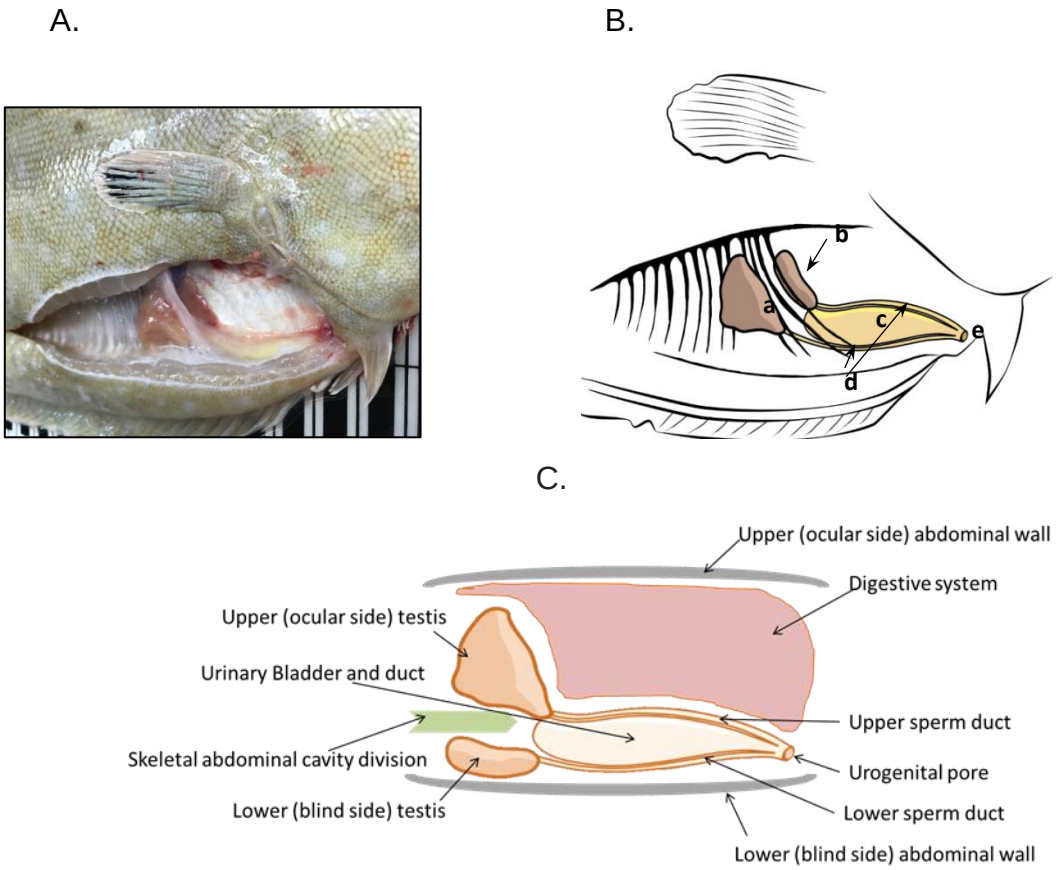

Figure 1. Male reproductive system in Senegalese sole (*Solea senegalensis*)*;* 1A. Photograph of dissected sole showing testes and urinary system. 1B. Diagram from photograph showing, a, upper ocular testicular lobe; b, lower, blind side, testicular lobe; c, urinary bladder; d, spermatic ducts; e, urogenital pore. 1C Diagram of cross section to show the position of testes, sperm ducts and urinary system.

A total of 51.1 % of samples had a yellow tonality, 22.2% had whitish yellow and 26.7% had whitish tonality; whilst 65.7% of samples showed opacity and 34.3% were transparent; regarding consistency, 45.2% were fluent and 54.8% were viscous. The samples described based on the tonality (yellow, whitish yellow or whitish) showed significant differences amongst mean sperm motility (P=0.001),

urea concentration (P=0.04) and osmolality (P=0.011) (table 3). The whitish samples had significantly higher sperm motility and urea concentration and osmolality were similar compared to yellow samples. Cell concentration was similar irrespective of sample colour (P=0.772) (table 3). The samples classified by different features of transparency and consistency did not have any differences indicating that these features did not differentiate between sperm quality or seminal fluid characteristics.

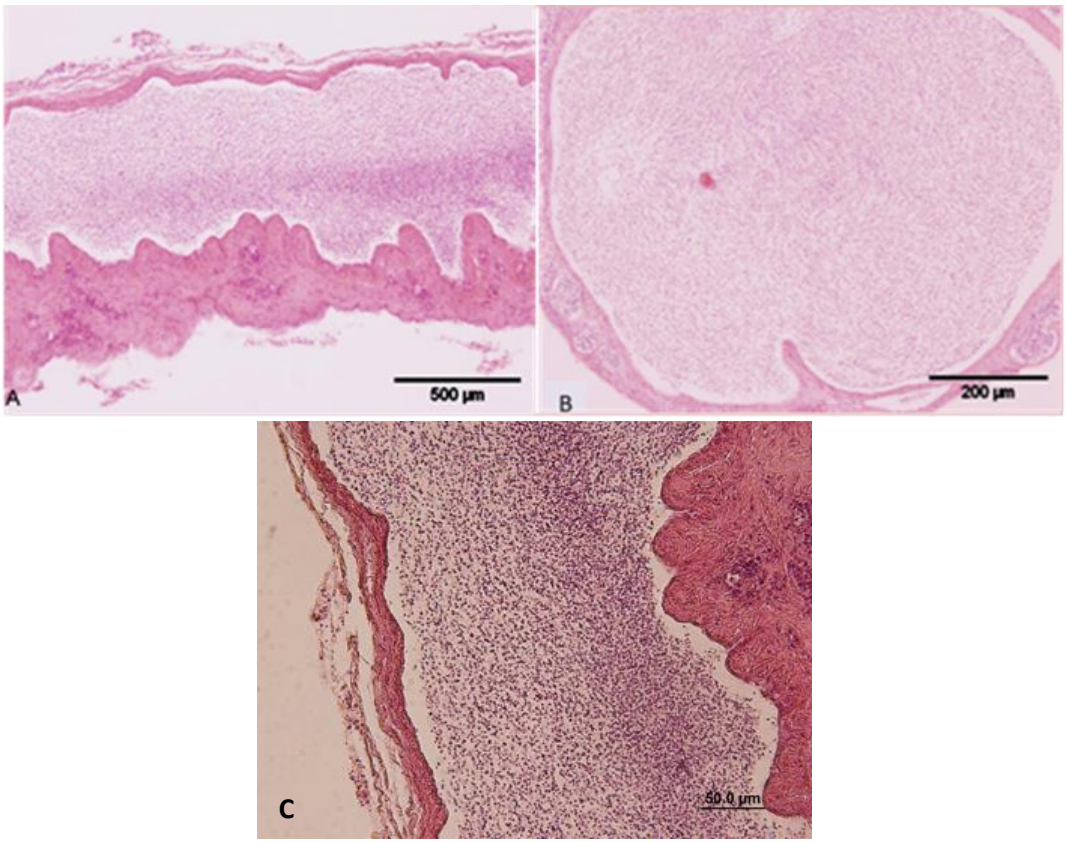

Figure 2. Longitudinal mid-section of spermatic duct (A), transverse section of spermatic duct close to testis (B) and longitudinal mid-section of spermatic duct (C) of Senegalese sole (*Solea senegalensis*) showing the ducts were full of spermatozoa.

The level of urea concentration contained in seminal plasma samples ranged between 0.41 and 7.99 mmol L^{-1}. The urea concentration and osmolality of the seminal plasma had a significant positive correlation (R= 0.513; P< 0.004) (Fig. 3). However, no correlation was found between urea concentration and others

parameters. In addition, the following parameters were analysed in female urine samples: pH, osmolality and urea concentration in order to compare with seminal plasma; where the urea concentration and pH showed a significant difference between the samples (table 4).

Table 3. Comparative values of sperm motility percentage, osmolality (mOsmol kg^{-1}), Urea concentration (mmol L^{-1}) and cell concentration (spz mL^{-1}) amongst the samples described based on the tonality (yellow, whitish yellow or whitish). All values were referred as mean ± SD. Different letters indicate significant differences (P<0.05).

Parameter	Whitish samples	Whitish yellow samples	Yellow samples
Sperm motility (%)	45.75 ± 20.18^{a}	30.83 ± 31.16^{ab}	17.76 ± 9.81^{b}
Urea conc. (mmol L^{-1})	1.95 ± 1.16^{a}	3.83 ± 1.25^{b}	2.94 ± 0.94^{ab}
Osmolality (mOsmol kg^{-1})	311.59 ± 59.64^{a}	464.66 ± 104.75^{b}	380.30 ± 46.84^{ab}
Cell conc. (spz mL^{-1})	1.85 ± 3.98 x10^{9} ^{a}	0.36 ± 0.32 x 10^{9}^{a}	1.51 ± 2.49 x 10^{9} ^{a}
Spz per kg (spz kg^{-1})	1.41 ± 0.83 x 10^{9} ^{a}	0.12 ± 0.58 x 10^{9} ^{a}	1.19 ± 0.58 x 10^{9} ^{a}

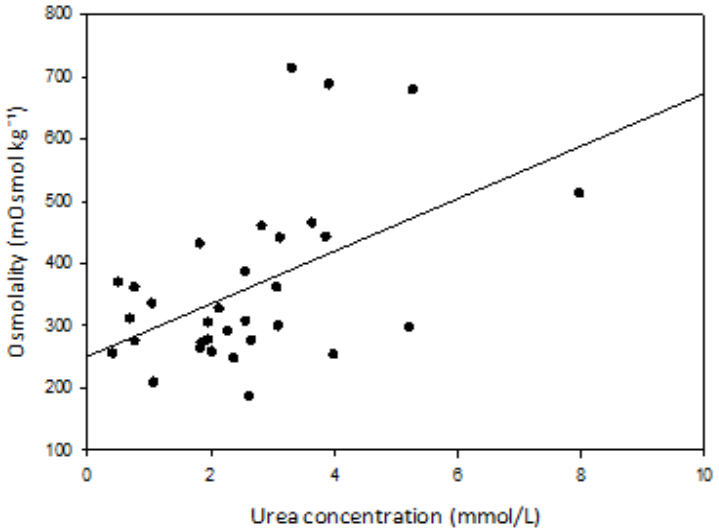

Figure 3. Positive correlation (R=0.513; P<0.004) between osmolality (mOsmol kg^{-1}) and urea concentration (mmol/L) in seminal plasma from Senegalese sole (*Solea senegalensis*).

Table 4. Mean and standard deviation of urea concentration, pH and osmolality in urine from females (n=3) and seminal plasma from males (n=32). Different letters indicate significant differences (P<0.05).

Samples	Urea (mmol L^{-1})	pH	Osmolality (mOsmol kg^{-1})
Urine	7.60 ± 3.17[a]	6.23 ± 0.27[a]	289.44 ± 31.18[a]
Seminal fluid	2.58 ± 1.60[b]	6.91 ± 0.38[b]	360.77 ± 138.46[a]

3.3 PCA and Cluster analysis

The PCA defined two components, describing 54.36 % of the variability in the data. Velocity parameters were related in the first component (PC1), together with protein concentration, pH and cell concentration that were negative values; the second component (PC2) was loaded positively to urea concentration and osmolality, whilst motility was included as a negative value (Table 5) (Fig. 4A).

The samples were grouped through cluster analysis and three groups were obtained. Each clustered group was characterized according to the variables of seminal plasma, cell concentration and kinetic parameters that described the sperm quality (Fig. 4B). The cluster formation had a significant interaction amongst groups (P=0.005). Significant differences were found amongst the means of the groups for the following parameters: urea concentration (P=0.002), osmolality (P=0.000), VAP (P=0.000), VCL (P=0.000) and pH (P=0.036), whilst no differences were found for cell concentration, protein concentration, and percentage motility (Fig. 5).

In general terms, group 1 had lower levels of sperm quality and higher levels of urine contamination, group 2 had intermediate values (between groups 1 and 3) and group 3 represented the samples with higher sperm quality and lower levels of urine contamination. Therefore, group 1 had significantly higher levels of urea and osmolality compared to groups 2 and 3 and a lower pH (acidification) compared to group 3 (Fig. 5). While group 3 had significantly higher levels of sperm velocity

(VAP and VCL) and higher (not significant) percentage motility than groups 1 and 2 (Fig. 5).

Table 5. Proportion of variables descriptors to sperm quality used in the Principal Component Analysis.

Parameters	Component	
	1	2
VCL (μm/seg)	0.846	-0.0675
VAP (μm/seg)	0.840	-0.153
Protein concentration (μg ml^{-1})	-0.693	0.134
pH	-0.567	-0.329
Cell concentration (x10^{9} spermatozoa ml^{-1})	-0.495	-0.067
Urea concentration (mmol L^{-1})	-0.229	0.830
Osmolality (mOsmol kg^{-1})	0.361	0.825
Motility %	0.283	-0.289

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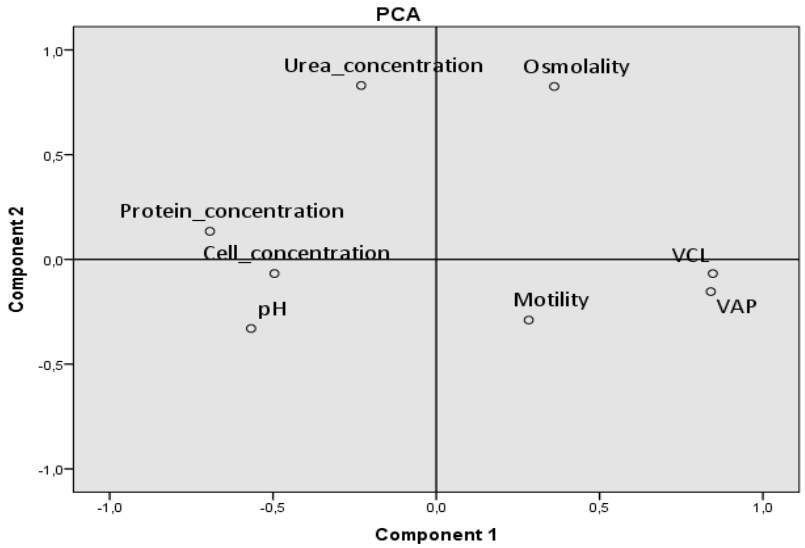

Figure 4A. Distribution of variables, descriptors of sperm quality and seminal plasma from Senegalese sole (*Solea senegalensis*) for the two principal components

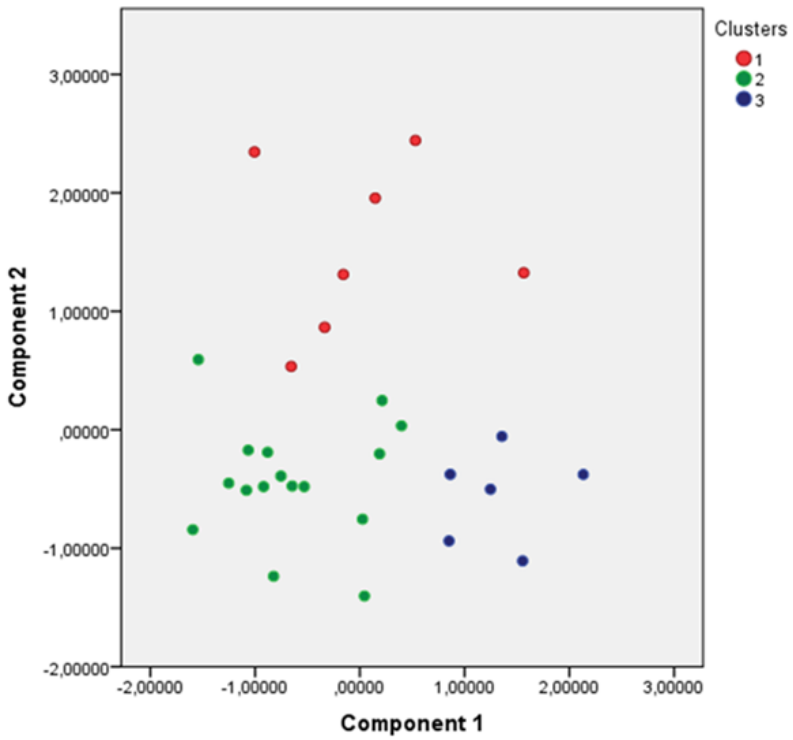

Figure 4B. Clusters obtained from Principal Component Analysis that formed three groups 1 (red), 2 (green) and 3 (blue) based on the parameters of sperm quality and seminal plasma from Senegalese sole (*Solea senegalensis*).

3.4 Short-term storage

In all three short-term sperm storage trials, there were no differences in sperm quality parameters, percentage motility and velocity (VCL and VAP) when the samples were collected and diluted in the different extenders (T = 0) and mean percentage motility ranged between 24.73 ± 14.14 % (Leibovitz, trail 3) and 38.89 ± 25.32 % (NAM, trail 1). Significant ($P<0.05$) differences were found, for motility and velocity parameters (VCL and VAP), for groups over time and amongst groups within some time points (Figs. 6, 7 and 8). There were also significant interactions between the different extender solutions and storage time for motility ($P<0.05$), VCL ($P<0.05$) and VAP ($P<0.05$) with the exception of VAP ($P=0.102$) in trail 2 and VCL ($P=0.525$) in trail 3.

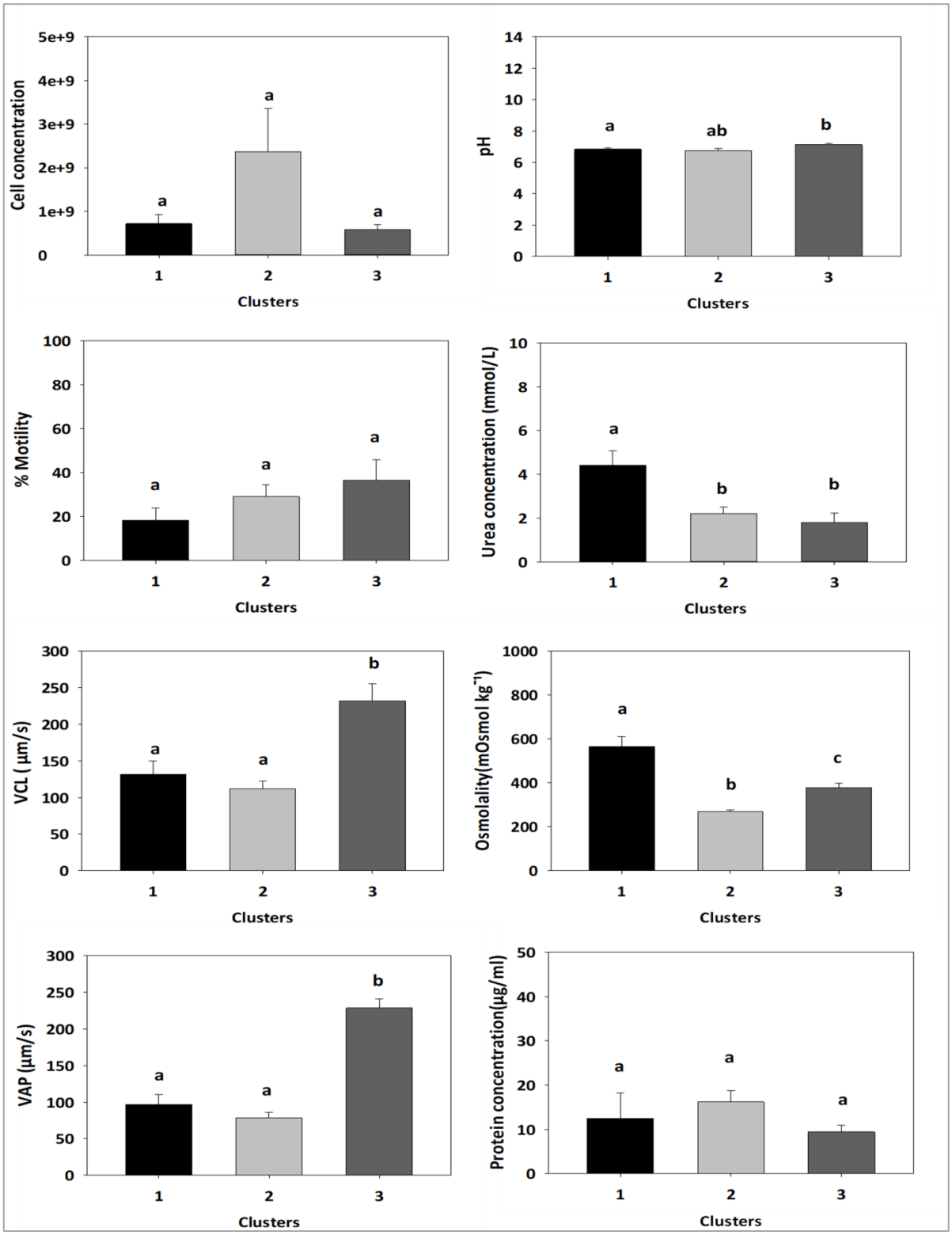

Figure 5. Mean value of clusters obtained from parameters of sperm quality and seminal plasma from Senegalese sole (*Solea senegalensis*). Different letters above each bar indicate significant differences (P<0.05) amongst groups.

In trial 1 (n=12), the rate of decrease in kinetic parameters in relation to storage time was different amongst the groups. The control (P=0.026) and Sucrose (P=0.005) groups had declined significantly three hours after collection. The Ringer group had declined significantly (P=0.038) six hours after collection. The NAM (P=0.012) and Leibovitz (P=0.038) groups did not decline significantly until 24 hours after collection. A similar trend was observed in relation to sperm velocities parameters. Velocities (VCL and VAP) declined significantly (P<0.05) in groups control and Sucrose six hours after collection, in Ringers and NAM 24 hours after collection and values were similar at all time points for the Leibovitz group. The comparison of the motility among all extenders revealed differences after six hours of storage when motility was significantly higher for sperm stored in modified Leibovitz compared to Sucrose (P<0.005) (Fig. 6). After 24 hours of storage, samples diluted with Leibovitz extender maintained a significantly (P<0.005) higher percentage motility, VAP, and VCL (Fig. 6) compared to controls and Sucrose. The motility of sperm stored in NAM and Ringer was intermediate with no significant differences compared to controls and other extenders. In the second trial (n=10), after three hours of storage, a significant (P=0.016) decrease in motility was observed in control samples that were significantly lower than samples in Leibovitz and Stor Fish® (Fig. 7).

After six hours of storage, a significant (P= 0.049) decrease in motility was observed in samples diluted with Stor Fish®. After 24 hours of storage, a significant (P=0.01) decrease in motility was observed in samples diluted with Leibovitz. At 24 hours, the sperm samples stored with Leibovitz showed significantly (P<0.05) higher motility rate, VCL and VAP (Fig. 7), compared to the control samples and the samples stored in Stor Fish®. In relation to the velocity parameters, the VCL exhibited a significant decrease at 24 hours of storage in control samples, (P=0.004) and samples diluted in Stor Fish® (P=0.006). However, in samples diluted in Leibovitz, the only significant (P=0.022) difference was between three hours and 24 hours of storage. Likewise, the VAP values decreased after 24 hours of storage for all samples, control (P=0.005), Stor Fish® (P= 0.001) and Leibovitz (P=0.003).

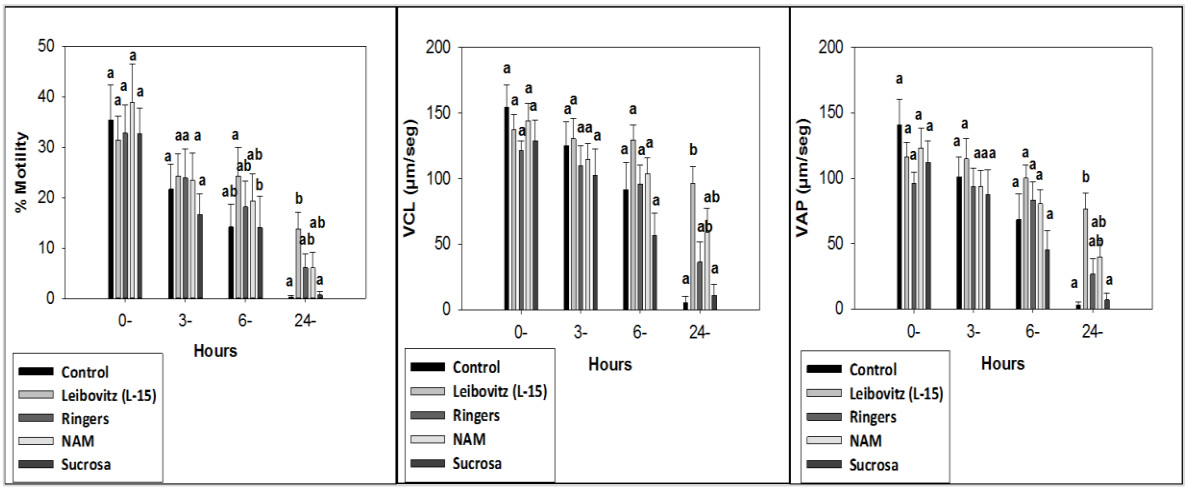

Figure 6. Effect on percentage motility, VCL and VAP of storage time on Senegalese sole (*Solea senegalensis*) control sperm samples and sperm samples diluted in the extenders Leibovitz, Ringer, NAM and Sucrose. Different letters above each bar indicate significant differences (P<0.05) among treatments within the sample time.

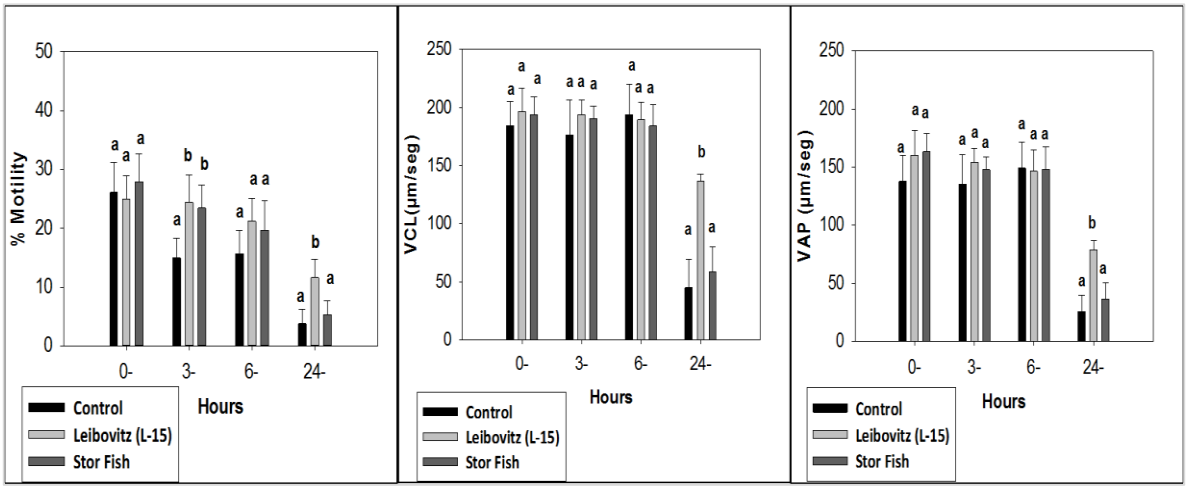

Figure 7. Effect on percentage motility, VCL and VAP of storage time on Senegalese sole (*Solea senegalensis*) control sperm samples and sperm samples diluted in the extender, Leibovitz and Stor Fish®. Different letters above each bar indicate significant differences (P<0.05) among treatments within a sample time.

In the third trial (n = 6), the control samples (P=0.014) and samples stored in Leibovitz (P=0.012) did not decline significantly until 24 hours after collection. Samples stored in Marine Freeze®, did not exhibit a significant decline in motility

and maintained similar values during the 24 hours of storage. After 3 hours of storage, the samples diluted in Leibovitz solution had significantly (P=0.006) lower motility compared to samples stored in Marine Freeze®. However, after 24 hours of storage, the motility of samples stored in Marine Freeze® were significantly (P=0.008) higher than control samples (Fig. 8) and samples in Leibovitz were not different from control or Marine Freeze®. The velocity parameters (VCL and VAP) did not exhibit significant differences over time or amongst groups within time points (Fig. 8).

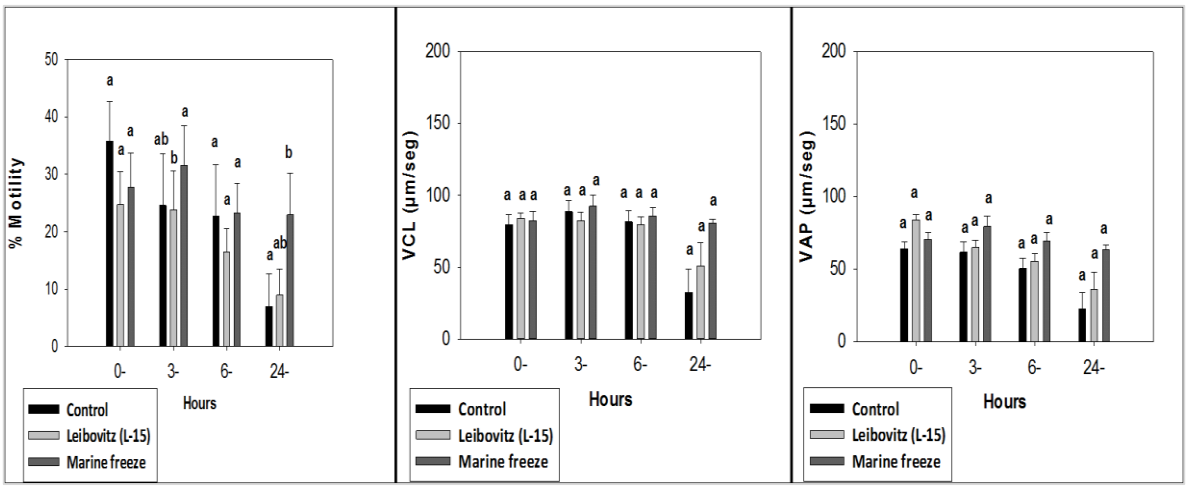

Figure 8. Effect on percentage motility, VCL and VAP of storage time on Senegalese sole (*Solea senegalensis*) control sperm samples and sperm samples diluted in the extenders, Leibovitz and Marine Freeze®. Different letters above each bar indicate significant differences (P<0.05) among treatments within the sample time.

4. Discussion

All sperm samples used in the present study contained concentrations of urea that indicated the samples were contaminated by urine. Although urea is a natural metabolite found in most body fluids and tissues, the concentration is normally low as the toxic urea is removed, concentrated in urine and expelled. Urea concentration in uncontaminated sperm samples was 0.01 µmol L^{-1} in testicular sperm from rainbow trout (*Oncorhynchus mykiss*) (Billard and Menezo, 1984) and

48 µmol L^{-1} in sperm collected from the sperm ducts of Walleye (*Stizostedion vitreum*) (Gregory 1970), which are > 50 times lower than the mean of the samples (2.58 ± 1.60 mmol L^{-1}, table 3) obtained in the present study. Therefore, urea has been used and demonstrated to be an indicator of urine contamination in the present study as in other studies in marine fish (Dreanno *et al*., 1998) and other taxa (Althouse *et al*., 1989).

The description of the anatomy of the urinary and male reproductive systems clearly indicates why samples contained urine contamination. The spermatozoa are located in the testes lumen and the sperm ducts and sperm must be collected from the common urogenital pore (Garcia-Lopez *et al*., 2005). The present study demonstrated that sperm was obtained by applying gentle pressure, through the abdominal wall (lower blind side) or the abdominal wall and digestive system (upper ocular side), to the testes and along the sperm ducts towards the urogenital pore. However, the sperm ducts pass along the upper and lower side of the urinary bladder and, therefore, pressure applied to the sperm ducts was also applied to the urinary bladder to extract spermatozoa mixed with urine.

The mean urea concentration obtained in seminal plasma of Senegalese sole in the present study was similar to that obtained in turbot (*Psetta máxima),* where the samples were collected by a similar method (Dreanno *et al*., 1998). Dreanno *et al*. (1998) described two methods to extract sperm and found that emptying the urinary bladder before collection of sperm, which was impossible in Senegalese sole (see above), did not avoid concentrations of urea that indicated urine contamination. Various studies in other species have shown that urine contamination negatively influenced sperm quality, duration of motility, efficiency of movement after being activated and fertilisation ability in fresh water fish (Rurangwa *et al*., 2004; Rodina *et al*; 2004; Alavi *et al*., 2006; 2007; Sarosiek *et al*. 2016; Sadegui *et al*., 2017; Król *et al*. 2018) and marine fish (Dreanno *et al*., 1998; Linhart *et al*., 1999; Fauvel *et al*. 2012). Although the reduced sperm quality and even mechanisms affected were similar in fresh water and marine fish, the causes appear to be different, as for fresh water fish a decrease in osmolality and ions

activates sperm and urine is hypo-osmotic (Król *et al*., 2018; Cejko *et al*. 2010; Linhart *et al*., 2003; Nynca *et al*., 2012; Poupard *et al*., 1998; Rurangwa *et al*., 2004) compared to marine fish where an increase in osmolality and ions activates sperm and urine is isosmotic (Cosson *et al*., 2008; Valdebenito *et al*., 2009). Therefore, in fresh water fish the premature activation of spz and reduced motility has been attributed to an osmotic shock when urine contamination lowers the osmolality (Perchec *et al*., 1995), whilst in marine fish although changes in osmolality have not been completely discounted, changes in ion balance, pH and ATP stores have been implicated in the premature activation of spz and reduced motility (Dreanno *et al*., 1998; Fauvel *et al*. 2012). In marine fish, urine contamination appeared to vary the composition of seminal plasma, decreasing significantly Na+, Cl^-, pH and intracellular ATP, which in turn modified the spz integrity to reduce motility percentage and spz velocity (Dreanno *et al*., 1998, Fauvel *et al*., 2012). In the present study, a significant positive correlation was obtained, between the urea concentration and the osmolality in seminal plasma and although not correlated, associations (PCA and cluster analysis) were found. Samples with significantly lower urine concentration, lower osmolality, higher pH and higher sperm quality (motility and velocities VAP and VCL) were clustered together. Therefore, as observed in other marine fish, in the present study, urine contamination appeared to reduce sperm quality probably due to an increase in osmolality and an associated decrease in pH (acidification).

The detrimental effect of urine on sperm quality reduces the possibility to use the sperm after a period of storage (Ciereszko *et al*., 2010; Sarosiek *et al*., 2012). An essential part of artificial fertilisation procedures is the storage of sperm for a short to long period to have sperm available when females ovulate and this has been achieved using extenders for short or long term storage (Chereguini *et al*., 1997; Dreanno *et al*., 1998; Rurangwa *et al*., 2004; Bobe and Labbe 2009; Cejko *et al*., 2010; Wang *et al.*, 2016; Beirão *et al*., 2019; Ramos-Júdez *et al*., 2019). Methods for the short term storage of sperm control the temperature and may also dilute the sperm in extenders to provide suitable conditions that maintain sperm quality during storage (Ciereszko *et al*., 2010; Fauvel *et al*., 2012; Gallego *et al*., 2013;

Sadegui *et al*., 2017; Santos *et al*., 2018). Usually, cold storage of sperm (around 4 ºC), has been successfully used in order to lower metabolism and avoid damage to the sperm (Chereguini *et al*., 1997; Favuel *et al*., 2012; Santos *et al*., 2018). A temperature of 4ºC was used in the present work, however, chilled storage alone was not successful for sperm storage and the motility of the spz decayed within three-six hours after collection as has been observed in other species where extenders were required (Chereguini *et al*., 1997; Rodina *et al*., 2004; Berríos *et al*., 2010; Fauvel *et al*., 2012; Gallego *et al*., 2013; Santos *et al*., 2018). On the contrary, sperm samples that were diluted in immobilising solutions showed an increase in the storage time, reducing the loss of sperm quality and in addition, counteracted the negative effects of others factors such as urine contamination (Dreanno *et al*., 1998; Rodina *et al*., 2004; Bobe and Labbé, 2008; Fauvel *et al*., 2012, Gallego *et al*., 2013; Król *et al*., 2018).

In the trials in the present study, all sperm samples diluted in extenders with the exception of Sucrose solution prolonged sperm quality parameters during storage. Sucrose solution was ineffective and the decline in sperm quality parameters was similar to control samples. Samples in Ringer and Stor Fish® had decreased significantly six hours after collection and in NAM 24 hours after collection. On the contrary to sole, Cherenguini *et al*. (1997) found that the Ringer extender was suitable for short term storage of turbot (*Scophthalmus maximus*) sperm. Stor Fish®, has been successfully used for sperm storage in various species (Haffray and Labbé, 2008), including the Patagonia blenny (*Eleginops maclovinus*) (Contreras *et al*., 2017) and a range of salmonids, Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Merino *et al*., 2016; Risopatrón *et al*., 2017). However, the present study found that for sole sperm, Stor Fish® was not suitable for short term sperm storage. In the marine species, meagre (*Argyrosomus regius*), NAM was also found to be a poor extender for sperm storage (Santos *et al*., 2018).

Leibovitz and Marine Freeze® had significantly higher sperm quality parameters than control samples 24 hours after collection and while samples in Leibovitz

declined significantly 24 hours after collection, samples in Marine Freeze® did not decline during 24 hours. Similarly, Fauvel *et al*. (2012) described that sperm samples from sea bass (*Dicentrarchus labrax*) that were diluted with cell culture medium Leibovitz L15 as an extender solution had improved motility when activated 24 hours after collection. The modified Leibovitz solution contained elements that had positive effects on the spz by providing a stable osmolality (different salts), stable pH, energy (pyruvate), aminoacids (glutamine), a shield for the plasma membrane (BSA) and an antibiotic was added to prevent bacterial growth (Bobe and Labbé, 2008; Niksirat *et al.*, 2011; Gallego *et al*., 2013). Marine Freeze®, according to the manufacturers (IMV Technologies) description, contains similar elements and had a similar effect as Leibovitz for sperm storage. Leibovitz and Marine Freeze® were the most successful in inhibiting the loss of motility and mitigating the detrimental effects of urine contamination.

Another factor that plays a role in short term storage in an extender is the dilution ratio that determines the reduction in sperm concentration, dilutes the urine contamination and influences the osmolality and pH control (Bobe and Labbé, 2008). In the present study, a dilution ratio of 1:3 was used after preliminary tests on different dilutions ratios. The same ratio has been successfully used with Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*) and rainbow smelt (*Osmerus mordax*) (Bobe and Labbé, 2008), while dilution ratios 1:4 and 1:9 were used for meagre (*Argyrosomus regius*) (Santos *et al*., 2018; Ramos-Júdez *et al*., 2019) and 1:5 for European seabass (Fauvel *et al*., 2012). However, some species may be sensitive to the dilution ratio and components of an extender and for this reason many studies on sperm storage have developed specific extenders for each species, trying to approximate extender composition to the species seminal fluid and secure osmotic balance between the extender solution and sperm (Bobe and Labbé, 2008; Gallego *et al*., 2013; Beirão *et al*., 2019). In the case of Senegalese sole sperm, the use of diluents is a tool that can help to maintain sperm quality during storage and improved tailor-made extenders may further improve storage.

Currently, Senegalese sole aquaculture production is based on wild broodstocks and the development of artificial fertilisation methods has been frustrated by the low volumes of poor quality sperm (Cabrita, *et al*., 2006; 2011; Beirão *et al*., 2009; Rasines *et al*., 2012; 2013; Chauvigné *et al*., 2016; 2017). However, a contributing factor to these low sperm volumes may be that aquaculture technicians working with sperm and most published studies to date only use sperm samples that were considered subjectively by appearance to be uncontaminated sperm (Agulleiro *et al*., 2006; Cabrita *et al*., 2006; 2011; Beirão *et al*., 2008; 2009; 2015; Martinez-Pastor *et al*., 2008; Valcarce *et al*., 2016; Riesco *et al*., 2017; 2019; Fernandez *et al*., 2019) and contaminated samples were discarded. In the present study a subjective assessment was made to determine differences between samples that by appearance were considered uncontaminated (whitish) or contaminated (yellow). All samples grouped by colour (whitish, whitish yellow and yellow) contained high spz densities and exhibited motility. Whitish (uncontaminated) samples had significantly higher motility, but similar spz densities, urea concentration and osmolality as yellow (contaminated) samples. The mean motility of the whitish samples (45.75 ± 20.18 %) was similar to the mean motility reported in other studies working with uncontaminated samples from Senegalese sole that ranged from 20-30 % (seasonal baseline values in Cabrita *et al*., 2011) to ~80 % (Cabrita *et al*., 2006; Riesco *et al*., 2019). The yellow samples had a motility of 17.76 ± 9.81%, which was similar to the lowest motilities reported in other studies (Cabrita *et al*., 2008; 2011). The mean spz densities from yellow and whitish samples were similar to lower densities reported for uncontaminated sperm, which ranged from 1.0×10^9 spz mL^{-1} (0.7 to 1.2×10^9 spz mL^{-1} in cultured males in Cabrita *et al*., 2006) to 6.84×10^9 spz mL^{-1} (Fernandez *et al*., 2019). By weight densities in the present study, were four to 100-fold higher than densities per kg that have been reported, which ranged from 0.01 to 0.3×10^9 spz kg^{-1} (Cabrita *et al*., 2006; Agulleiro *et al*., 2006; 2007; Beirão *et al*., 2011). The sperm densities per kg in the present study were similar to densities reported by Chauvigné *et al*. (2017; 2018), who used similar methods to obtain all the sperm and assess the sperm production capacity of males. Therefore, the subjective analysis in the

present study and comparisons of motility and spz densities within the present study and with other studies indicate that uncontaminated samples may actually be contaminated, that only collecting whitish sperm samples (or uncontaminated samples) will exclude or discard samples with high densities of sperm that had a degree of motility and underestimate spz densities per kg of male.

Cryopreservation protocols have been studied for Senegalese sole (Rasines *et al*., 2012; Valcarce and Robles, 2016; Riesco *et al*., 2017) and used to have availability of sperm for artificial fertilisation (Rasines *et al*., 2012; 2013).These cryopreservation protocols used only what was considered uncontaminated sperm. The present study found that only 26.7% of males had sperm that appeared to be uncontaminated (whitish samples) and therefore, few males appear to have the sperm quality required for methods that need uncontaminated sperm. The use of only uncontaminated sperm may make methods difficult or impossible to implement in the industry as it will be difficult to obtain enough sperm for large scale fertilised egg production or to have enough males to form sufficient families for a breeding program. The present study has demonstrated that contaminated sperm samples and short term chilled storage in extenders to mitigate the negative effects of urine contamination may represent a viable sperm management system that can be used by the sole aquaculture industry. In the present study, 91.8% of males had motile sperm and 65.3% had adequate samples for the present study. However, further work is need to improve sperm management using short term chilled storage for the sole culture industry.

5. Conclusions

The morphology of the urogenital system of Senegalese sole contributes greatly to the contamination by urine observed in the sperm samples collected by the stripping method. The proximity of the seminal ducts and the urinary bladder, makes it difficult or impossible to obtain sperm without urine contamination. Although, the colouration of the sperm sample may help identify samples with

improved motility, all samples (yellow, whitish yellow and whitish) contained large numbers of motile spz and discarding samples that have a yellow colouration will discard large quantities of sperm. The effect of urine contamination, measured as urea, induced a reduction in sperm quality which may have been caused by a decrease in pH (acidification) and an increase in osmolality, which are known to activate sole sperm and reduce quality in marine fish. Urea contamination was positively correlated with the osmolality values in the seminal plasma. The tests carried out with extender solutions revealed that samples diluted with modified Leibovitz and Marine Freeze® extenders had significantly higher motility after 24 hours compared to control samples. In particular, the use of extender solutions is relevant to help to cushion the effect of urine contamination when the sperm is required for artificial fertilisation. However, although the present work is promising giving important insights for sperm management in sole, further work is required to determine the most suitable compounds to elaborate extenders that can further offset the negative effects of urine contamination as well as work to improve the methods to collect the sperm.

6. References

Agulleiro, M. J., Anguis, V., Canavate, J. P., Martinez-Rodriguez, G., Mylonas, C. C., Cerda, J., 2006. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257(1-4): 511-524.

Agulleiro, M. J., Scott, A. P., Duncan, N., Mylonas, C. C., Cerda, J., 2007. Treatment of GnRHa-implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 147(4): 885-892.

Alavi, S. M., Cosson, J., 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 30(1): 1-14.

Alavi, S. M. H., Rodina, M., Policar, T., Kozak, P., Psenicka, M., Linhart, O. 2007. Semen of Perca fluviatilis L.: sperm volume and density, seminal plasma indices and effects of dilution ratio, ions and osmolality on sperm motility. Theriogenology 68:276–83.

Althouse, G.C., Seager, S.W., Varner, D.D., Webb, G.W., 1989. Diagnostic aids for the detection of urine in the equine ejaculate. Theriogenology 31 (6): 1141–1148.

Beirão, J., Cabrita, E., Soares, F., Herraez, M. P., Dinis, M. T., 2008. Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: comet analysis and Annexin V–Fluorescein staining. Journal of Applied Ichthyology 24:508–13.

Beirão, J., Soares, F., Herraez, M. P., Dinis M. T., Cabrita E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72(9): 1251-1261.

Beirão, J., Soares, F., Herraez, M. P., Dinis M. T., Cabrita E., 2011.Changes in *Solea senegalensis* sperm quality throughout the year. Animal reproduction science 126 (1-2):122-129. DOI: 10.1016/j.anireprosci.2011.04.009

Beirão, J., Soares, F., Pousão-Ferreira, P., Diogo, P., Dias, J., Dinis, M. T., 2015. The effect of enriched diets on *Solea senegalensis* sperm quality. Aquaculture 435:187–94. DOI:10.1016/j.aquaculture.2014.09.025.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J. K., Peixoto, S., Robeck T. R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133: 161-178.

Berríos, O., Valdebenito, I., Treulén, F., Ubilla, A., 2010. Almacenamiento en frío de espermatozoides de trucha arcoiris (*Oncorhynchus mykiss*): Efectos en la motilidad, superóxido intracelular, integridad de la membrana plasmática y potencial de membrana mitocondrial. Archivos de medicina veterinaria 42(3):179-186.

Billard, R., Menezo, Y., 1984. The amino acid composition of rainbow trout_*Salmo gairdneri*.seminal fluid and blood plasma: a comparison with carp_*Cyprinus carpio*.. Aquaculture 41, 341–349.

Bobe, J., Labbé C., 2009. Chilled storage of sperm and eggs, in Cabrita, E., Robles, V., Herraez, P (Eds.), Methods in Reproductive Aquaculture. Boca Raton, FL: CRC Press Taylor and Francis Group, pp. 219-231.

Cabrita, E., Robles, V., Alvarez R., Herraez, M. P., 2001. Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. Aquaculture 201(3-4): 301-314.

Cabrita, E., Soares, F., Dinis M. T., 2006. Characterization of Senegalese sole, *Solea senegalensis,* male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975.

Cabrita, E., Martínez-Pastor F., Soares, F., Dinis, M.T., 2008. Motility activation and subpopulation analysis in *Solea senegalensis* spermatozoa. *Cybium, 32(2) suppl.: 185-186.*

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M. P., 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology 26(5): 623-635.

Cabrita, E., Soares, F., Beirão, J., Garcia-Lopez, A., Martinez-Rodriguez, G., Dinis, M.T., 2011. Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. Theriogenology 75(1): 1-9.

Carazo, I., Norambuena, F., Oliveira, C., Sanchez-Vazquez, F. J., Duncan, N. J., 2013. The effect of night illumination, red and infrared light, on locomotor activity, behaviour and melatonin of Senegalese sole (*Solea senegalensis*) broodstock. Physiology & Behavior 118: 201-207.

Carazo, I., Chereguini, O., Martin, I., Huntingford, F., Duncan, N., 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*). Spanish Journal of Agricultural Research 14(4): 13.

Cejko, B. I., Zarski, D., Targonska, K., Krejszeff, S., Kucharczyk D., Glogowski, J., 2010. Osmolality of seminal plasma as an indicator of milt contamination with urine

based on the example of the tench. *Tinca tinca* (L.). Polish Journal of Natural Sciences 25(3): 287-298.

Chauvigne, F., Fatsini, E., Duncan, N., Olle, J., Zanuy, S., Gomez, A., Cerda, J., 2016. Plasma levels of follicle-stimulating and luteinizing hormones during the reproductive cycle of wild and cultured Senegalese sole (*Solea Senegalensis*). Comparative Biochemistry and Physiology a Molecular & Integrative Physiology 191: 35-43.

Chauvigne, F., Olle, J., Gonzalez, W., Duncan, N., Gimenez, I., Cerda, J., 2017. Toward developing recombinant gonadotropin-based hormone therapies for increasing fertility in the flatfish Senegalese sole. Plos One 12(3): 27.

Chauvigne, F., Gonzalez, W., Ramos, S., Ducat, C., Duncan, N., Gimenez, I., Cerda, J., 2018. Seasonal-and dose-dependent effects of recombinant gonadotropins on sperm production and quality in the flatfish *Solea senegalensis*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 225: 59-64.

Chereguini, O., Cal, R. M., Dreanno, C., deBaulny, B.O., Suquet M., Maisse, G., 1997. Short-term storage and cryopreservation of turbot (*Scophthalmus maximus*) sperm. Aquatic Living Resources 10(4): 251-255.

Ciereszko, A., Dietrich, G. J., Dietrich, M. A., Nynca, J., Kuźmiński, H., Dobosz S., Grudniewska J., 2010. Effects of pH on sperm motility in several Salmoniformes species: *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Salmo trutta*, *Salmo salar* and *Thymallus thymallus*. Journal of Applied Ichthyology 26(5): 665-667.

Contreras, P., Ulloa, P., Merino, O., Valdebenito, I., Figueroa, E., Farías J., Risopatrón, J., 2017. Effect of short-term storage on sperm function in Patagonian blenny (*Eleginops maclovinus*) sperm. Aquaculture 481: 58-63.

Cosson, J., Groison, A. L, Suquet, M., Fauvel, C., Dreanno, C., Billard, R. 2008. Studying sperm motility in marine fish: an overview on the state of the art. Journal of Applied Ichthyology 24:460–86.

Dreanno, C., Suquet, M., Desbruyeres, E., Cosson, J., Le Delliou, H., Billard, R., 1998. Effect of urine on semen quality in turbot (*Psetta maxima*). Aquaculture 169(3-4): 247-262.

FAO. 2019. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2017 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2019. www.fao.org/fishery/statistics/software/fishstatj/en

Fauvel, C., Savoye, O., Dreanno, C., Cosson J., Suquet, M., 1999. Characteristics of sperm of captive seabass in relation to its fertilization potential. Journal of Fish Biology 54(2): 356-369.

Fauvel, C., Boryshpolets, S., Cosson, J., Wilson Leedy, J. G., Labbé, C., Haffray P., Suquet M., 2012. Improvement of chilled seabass sperm conservation using a cell culture medium. Journal of Applied Ichthyology 28(6): 961-966.

Fernandez, I., Fernandes, J. M. O., Roberto, V. P., Kopp, M., Oliveira, C., Riesco, M. F., Dias, J., Cox, C. J., Cancela, M. L., Cabrita, E., Gavaia, P., 2019. Circulating small non-coding RNAs provide new insights into vitamin K nutrition and reproductive physiology in teleost fish. Biochimica Et Biophysica Acta-General Subjects 1863(1): 39-51.

Gallego, V., Pérez, L., Asturiano, J. F., Yoshida, M., 2013. Study of pufferfish (*Takifugu niphobles*) sperm: Development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca2+ and K+ in the initiation of sperm motility. Aquaculture 414-415: 82-91.

Garcia-Lopez, A., Martinez-Rodriguez, G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole, *Solea senegalensis* (Kaup): Anatomy, histology and histochemistry. Histology and Histopathology 20(4): 1179-1189.

Gregory, R.W., 1970. Physical and chemical properties of Walleye sperm and seminal plasma. Transactions of the American Fisheries Society 3, 518–525.

Guzman, J. M., Ramos, J., Mylonas, C. C., Mananos, E. L., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. Aquaculture 291(3-4): 200-209.

Guzman, J. M., Cal, R., García-López, A., Chereguini, O., Kight, K., Olmedo, M., Sarasquete, C., Mylonas, C. C., Peleteiro, J.B., Zohar, Y., Mananos, E. L., 2011. Effect of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis of

Senegalese sole (*Solea senegalensis*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 158 (2), pp. 235-245.

Haffray, P., Labbé, C., IMV Technologies, Maisse, G., 2008. Fish sperm cryopreservation in France: from laboratory studies to application in selective breeding programs. Cybium 32(2): 127-129.

Krol, J., Zarski, D., Bernath, G., Palinska-Zarska, K., Krejszeff, S., Dlugonski, A., Horvath A., 2018. Effect of urine contamination on semen quality variables in Eurasian perch *Perca fluviatilis L*. Animal Reproduction Science 197: 240-246.

Linhart, O., Mims, S. D., Gomelsky, B., Hiott, A. E., Shelton, W. L., Cosson, J., Rodina, M., Gela D., Bastl, J., 2003. Ionic composition and osmolality of paddlefish (*Polyodon spathula*, Acipenseriformes) seminal fluid. Aquaculture International 11(4): 357-368.

Linhart, O., Walford, J., Sivaloganathan, B., Lam, T. J., 1999. Effects of osmolality and ions on the motility of stripped and testicular sperm of freshwater- and seawater-acclimated tilapia, *Oreochromis mossambicus*. Journal of Fish Biology 55(6): 1344-1358.

Liu, X., Lian, J., Wang Y., Zhang F., Yu, H., Ma, A., Liu, S., Zhai, J., 2008. Large scale artificial reproduction and rearing of Senegal sole, *Solea senegalensis* Kaup. Marine Fisheries Research 29 (2): 10-16.

Martin, I. E., 2016. Advances in the reproductive biology and zootechnics of the Senegalense sole (*Solea senegalensis* Kaup, 1858). Ph.D. Thesis, Universidad de Cantabria (Spain).

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Gómez, M., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Spanish Journal of Agricultural Research, In press.

Martinez-Pastor, F., Cabrita, E., Soares, F., Anel, L., Dinis, M. T., 2008. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 135(4): 449-459.

Merino, O., Figueroa, E., Cheuqueman, C., Valdebenito, I., Isachenko, V., Isachenko, E., Sanchez, R., Farias, J., Risopatron, J., 2017. Short-term storage of salmonids semen in a sodium alginate-based extender. Andrologia 49: e12661. https://doi.org/10.1111/and.12661

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E. C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M., Dinis, M. T., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture 8(3): 227-263.

Mylonas, C. C., Duncan, N. J., Asturiano, J. F., 2017. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 472: 21-44.

Niksirat, H., Sarvi, K., Abdoli, A., Hajirezaee, S., 2011. Short-Term Storage of Semen of Rainbow Trout: Interactions of Time, Antibiotic, and Activator. Journal of Applied Aquaculture 23(4): 358-366.

Nynca, J., Dietrich, G. J., Kuźmiński, H., Dobosz, S., Ciereszko, A., 2012. Motility activation of rainbow trout spermatozoa at pH 6.5 is directly related to contamination of milt with urine. Aquaculture 330-333: 185-188.

Perchec, G., Cosson, J., André, F., Billard, R., 1995a. Degradation of the quality of carp sperm by urine contamination during stripping. Aquaculture 129: 135.

Poupard, G. P., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160(3-4): 317-328.

Ramos-Júdez, S., González, W., Dutto, G., Mylonas, C. C., Fauvel, C., Duncan, N., 2019. Gamete quality and management for *in vitro* fertilisation in meagre (*Argyrosomus regius*). Aquaculture 509: 227–235.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326-329: 129–135.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanos, E., Chereguini, O., 2013. Artificial fertilisation of cultured Senegalese sole (*Solea senegalensis*): effects of the time of day of hormonal treatment on inducing ovulation. Aquaculture 392–395: 94–97.

Riesco, M.F., Oliveira C., Soares F., Gavaia, P.J., Dinis, M.T., Cabrita, E., 2017. *Solea senegalensis* sperm cryopreservation: New insights on sperm quality. PLoS ONE 12(10): e0186542. https://doi. org/10.1371/journal.pone.0186542

Riesco, M. F., Valcarce, D. G., Martínez-Vázquez, J. M., Martín, I., Calderón-García, A. A., González-Nunez, V., Robles V., 2019. Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question. Reproduction, Fertility and Development. https://doi.org/10.1071/RD18453

Risopatron, J., Merino, O., Cheuqueman, C., Figueroa, E., Sanchez, R., Farias, J. G., Valdebenito, I., 2018. Effect of the age of broodstock males on sperm function during cold storage in the trout (*Oncorhynchus mykiss*). Andrología 50:e12857.

Rodina, M., Cosson, J., Gela, D., Linhart, O., 2004. Kurokura solution as immobilizing medium for spermatozoa of tench (*Tinca tinca L*.). Aquaculture International 12(1): 119-131.

Rurangwa, E., Kime, D. E., Ollevier, F., Nash J. P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234(1-4): 1-28.

Saedeghi, S., Nunez, J., Soler, C., Silvestre, M. A., 2017. Effect of the Activation Media with Different Osmolality and Cool Storage on Spermatozoa Motility Parameters over Time in Zebrafish, *Danio rerio*. Turkish Journal of Fisheries and Aquatic Sciences 17: 111-120. DOI: 10.4194/1303-2712-v17_1_13

Santos, M., Soares, F., Moreira, M., Beirão, J., 2018. Evaluation of different extenders for the cold storage of meagre (*Argyrosomus regius*) semen. Aquaculture Research 49(8): 2723-2731.

Sarosiek, B., Cejko, B. I., Glogowski, J., Targońska, K., Żarski, D., Kowalski, R. K., Kucharczyk D., 2012. Spermatozoa motility and short-term sperm storage of colourful orfe (*Leuciscus idus aberr orfus*). Italian Journal of Animal Science 11(3): 270-274.

Sarosiek, B., Dryl, K., Krejszeff, S., Zarski, D., 2016. Characterization of pikeperch (Sander lucioperca) milt collected with a syringe and a catheter. Aquaculture 450: 14-16.

Valcarce, D.G., Robles, V., 2016 .Effect of captivity and cryopreservation on ROS production in *Solea senegalensis* spermatozoa. Reproduction 152: 439–446. DOI: 10.1530/REP-16-0270.

Valcarce, D.G., Herráez, M.P., Chereguini, O., Rodríguez, C., Robles, V., 2016. Selection of nonapoptotic sperm by magnetic-activated cell sorting in Senegalese sole (*Solea senegalensis*).Theriogenology 86 1195–1202.

Valdebenito, I., Fletcher, C., Vera, V., Fernández, J., 2009. Physical-chemical factors that regulate spermatic motility in fish: basic applied aspects. A review. Archivos de Medicina Veterinaria 41: 97-106.

Wang, X., Shi, X., Liu, Y., Yu, D., Guan, S., Liu, Q., Li, J., 2016. Effects of chilled storage and cryopreservation on sperm characteristics, antioxidant enzyme activities, and lipid peroxidation in Pacific cod *Gadus microcephalus*. Chinese Journal of Oceanology and Limnology 34(4): 763-771.

Chapter 7

Low sperm to egg ratio required for the successful experimental and massive *in vitro* fertilisation in Senegalese sole (*Solea senegalensis*).

Chapter 7. Low sperm to egg ratio required for the successful experimental and massive *in vitro* fertilisation in Senegalese sole (*Solea senegalensis*).

1. Introduction

Senegalese sole (*Solea senegalensis*) appears to be one of the most promising emerging aquaculture species in Europe. Sole production from land based farms in Spain, Portugal, France and Iceland has increased rapidly to 1,700 t in 2019 (APROMAR, 2019). This increase is driven by good market prices, high market demand and successful culture practices (Morais *et al.,* 2016) that permit cost effective production despite of the need for high levels of investment in culture infrastructure.

However, the production cycle is not fully controlled and at present relies on the capture of wild broodstock that spawn sufficient eggs to achieve targeted aquaculture productions (Anguis and Cañavate, 2005; Martín *et al.*, 2014). The progeny of these wild broodstock and in particular the males exhibit a reproductive behavioural dysfunction and do not participate in the courtship to fertilise eggs (Duncan *et al.*, 2019; Fatsini *et al.,* 2020; Guzmán *et al.*, 2008; Martín *et al.*, 2020). Consequentially, cultured broodstocks that were reared entirely in captivity produce unfertilised, inviable eggs (Duncan *et al.*, 2019; Guzmán *et al.*, 2008). Differences between wild and cultured breeders have suggested that the reproductive dysfunction has a base in a combination of endocrine reproductive control (Guzmán *et al*., 2011; Riesco *et al.,* 2019), social conditions during rearing (Fatsini *et al.,* 2020; Martín *et al.,* 2020), broodstock nutrition (Norambuena *et al.*, 2013a, 2013b, 2012b, 2012c, 2012a) and olfactory capacity (Fatsini *et al*., 2017). However, these insights into the dysfunction have yielded few and insufficient increases in the spawning of viable eggs from cultured broodstocks (Fatsini *et al.*, 2020; Guzmán *et al.*, 2011) that do not meet industry needs for good quality eggs.

In flatfish culture, *in vitro* fertilisation methods are commonly used to obtain fertilised eggs in the quantities required (Mañanós *et al*., 2008). *In vitro* fertilisation enables aquaculturists to bypass behavioural reproductive dysfunctions of the type observed in cultured male Senegalese sole. However, Senegalese sole males produce small quantities of poor quality sperm that has frustrated the application of *in vitro* fertilisation methods (Beirão *et al*., 2011, 2009; Cabrita *et al*., 2011, 2006; González-López *et al.*, 2020). Rasines et al. (2013, 2012), has described *in vitro* fertilisation procedures for Senegalese sole on an experimental scale. Female sole were induced to ovulate with gonadotropin releasing hormone agonist (GnRHa) and batches of 1 mL of stripped eggs were fertilised with 30 µL of cryopreserved sperm from cultured males. Similarly, Liu *et al.* (2008) described *in vitro* fertilisation of Senegalese sole eggs on an industrial scale. Again, eggs were obtained from GnRHa induced cultured females and all the eggs from each female were fertilised *in vitro* with the sperm from three to four cultured males. However, few details were given on the amount of sperm or eggs used and the sperm to egg ratio was not detailed.

When sperm is limiting it is of critical importance to know the sperm to egg ratio to plan *in vitro* fertilisation procedures. Sperm to egg ratios for *in vitro* fertilisation of fish eggs show considerable variation ranging from $\times 10^3$ to $\times 10^6$ (Beirão et al., 2019). Most fish species require in excess of 1×10^4 spermatozoa (spz) egg^{-1}, for example African catfish (*Clarias gariepinus*) required 1.5×10^4 spz egg^{-1} (Rurangwa *et al.*, 1998), European seabass (*Dicentrarchus labrax*) 6.6×10^4 spz egg^{-1} (Fauvel *et al.*, 1999), meagre (*Argyrosomus regius*) 1.5×10^5 spz egg^{-1} (Ramos-Júdez *et al.*, 2019) and wolffish (*Anarhichas minor*) 5×10^5 spz egg^{-1} (Beirão and Ottesen, 2018). It would appear that flatfish have lower spz requirements as winter flounder (*Pseudopleuronectes americanus*) required 3.4×10^4 spz egg^{-1} (Butts *et al.,* 2012) and turbot (*Scophthalmus maximus*) required just 3000 to 6000 spz egg^{-1} (Chereguini *et al.,* 1999; Suquet *et al.*, 1995). This variation in spz requirements per egg has been discussed in relation to gamete characteristics of both sperm and eggs (Ramos-Júdez *et al*., 2019; Sanches *et al*., 2016; Suquet *et al*., 1995), chemical and physical guidance of sperm to the

micropyle (Yanagimachi *et al*., 2017) and sperm competition and reproductive behaviour (Baker *et al.*, 2019; Stockley *et al*., 1997; Suquet *et al*., 2012).

The present study, aimed to determine the sperm to egg ratio required for *in vitro* fertilisation in Senegalese sole. The aim was to determine the sperm to egg ratio on an experimental scale and then apply the ratio on a commercial scale in massive *in vitro* fertilisations. An additional aim was to determine the viability of ovulated eggs stored at room temperature.

2. Methods

All Senegalese sole broodstock used were cultured fish that had been hatched and reared entirely in captivity. Fish were maintained in 10,000 L tanks in IRTA Sant Carles de la Rápita (Catalonia, Spain). Prior to experiments fish were held in surface sea water (~35 ppt, >5 $mg.L^{-1}$ O2) and a controlled natural temperature cycle (9-20 ºC) using recirculation systems (IRTAmar®). Tanks were covered with shade netting and photoperiod was natural with natural light. The fish were fed four days a week with either unfrozen polychaetes and mussels (0.75 % of biomass) or 5 mm pelleted Broodfeedlean broodstock diet (0.55% of biomass) (Sparos, Olhao, Portugal). During experiments conducted from April to June, fish were held in the same conditions with the exceptions that water temperature was maintained at a constant 16 ± 1 ºC and fish were not feed 24 hours before any manipulation.

Females used had an average weight of 1.53 ± 0.28 kg and males had a weight of 1.05 ± 0.25 kg. The fish were handled (routine husbandry and experimentation) in accordance with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA, http://www.felasa.eu/). For all handling and sampling, fish were anesthetised with 60 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma-Aldrich, Spain).

Viable eggs were obtained by inducing ovulation. Ovarian biopsies were taken from females with swollen ovaries and the diameters of 20 oocytes were measured (x40 Leica compound microscope). Females were selected that had mean oocyte

diameter larger than 600 µm. The females were administered 5 µg kg^{-1} of GnRHa (Sigma code L4513, Sigma, Spain) at approximately 18:00 – 19:00. The females were held with constant temperature (16 ± 1 ºC) and total darkness until ovulation. Females were checked for ovulation every 2-3 hours starting from 40 h after the administration of GnRHa and all eggs were stripped from the ovulated females.

Sperm was obtained from males by repeatedly, gently massaging the testes and applying pressure along the full length of the sperm ducts to the urogenital pore. All sperm with urine contamination was collected in a 1 mL syringe (Gonzalez *et al*., 2019). The volume collected was measured with the syringe to an accuracy of 10 µL and the sperm was transferred to a 1.5 mL Eppendorf and immediately diluted with modified Leibovitz (Gonzalez *et al*., 2019) using the dilution required for the experiment (see below). The sperm motility was initially observed (x 100 Leica) by activating 1 µL of diluted sperm with 19 µL of clean seawater. Sperm samples with low or no motility were rejected. All sperm samples were stored over ice or at 4ºC (refrigerated) until analysis or used to fertilise eggs.

Sperm quality parameters were also determined using ImageJ software and the CASA plugin (Gonzalez *et al*., 2019). The spermatozoa (spz) concentration (spz mL^{-1}) was measured using a Thoma cell counting chamber. A 10 µL sample of sperm was diluted 1:500 in 10% formalin and 10 µL of the dilution was pipetted into the counting chamber. After 10 minutes for spz to sediment, the camber was observed using a microscope (x100 magnification with Olympus BH microscope), photographed (IC Capture software and GigE digital camera model: DMK 22BUC03 Monochrome, The Imaginsource, Bremen, Germany) and the number of spz counted (ImageJ software, http://imagej.nih.gov/ij/).

To measure motility and related parameters spz were activated and tracks were video recorded until the motion ceased. Spermatozoa were activated by mixing 1 µL of diluted sperm (1:4 with leibovitz) with 20 µL of seawater with 30% bovine serum albumin (BSA). One µL of activated sperm was pipetted into an ISAS R2C10 counting chamber (Proiser *R+D, S.L.* Paterna, Spain) previously mounted and focused on the microscope (200x magnification Olympus BH). Spermatozoa

tracks were recorded (IC Capture software and GigE digital camera) and the videos (AVI format) were converted into image sequences (jpeg format using Virtual Dub 1.10.4 software http://www.virtualdub.org/). The image sequences were analysed using ImageJ software with the computer-assisted sperm analysis (CASA) plugin (ImageJ http://rsb.info.nih.gov/ij/plugins/). CASA analyse was with the settings: brightness and contrast, -10 to 15/224 to 238; threshold, 0/198 to 202; minimum sperm size (pixels), 10; maximum sperm size (pixels), 400; minimum track length (frames), 10; maximum sperm velocity between frames (pixels), 30; frame rate, 30; microns/1000 pixels, 303; Print motion, 1; the additional settings were not modified. The parameters, percentage of motile cells (% sperm motility), Curvilinear Velocity (VCL, µm/s) and Average Path Velocity (VAP, µm/s), were assessed during 2 seconds, 15 seconds (unless otherwise stated) after the sperm was activated. All sperm samples were analysed in triplicate.

Sperm to egg ratio experiment

Five different females and five different males were used during this experiment. Immediately after an ovulated female was encountered, sperm was collected and checked to find a male with 300+ µL of motile sperm. The sperm was serially diluted with modified Leibovitz (Gonzalez *et al*., 2019) to achieve eight dilutions: 1:4; 1:19; 1:79; 1:319; 1:959; 1:2879; 1:5759; 1:11519. A sample of the first dilution (1:4) was used to determine the spz concentration (see above) and percentage motility of spz (ImageJ CASA, see above). The spz concentration in the dilution 1:4 was used to calculate the spz concentration in each dilution and sperm motility to calculate the concentration of motile spz. The eggs and diluted sperm were used to make three triplicate fertilisations for each serial dilution. Fertilisations were made in a 100 mL beaker by pipetting in close sequence, 0.5 mL of eggs, 20 µL of diluted sperm and 5 mL clean seawater. A 1 mL pipette with a cut tip was used to pipette eggs, a 100 µL pipette with a cut tip was used to pipette diluted sperm and a 5 mL pipette was used to pipette seawater. The eggs, sperm and seawater were gently mixed by rocking and swirling the beaker and after 3-5 minutes, the volume of seawater was topped up to 100 mL. The beakers of fertilised eggs were transferred

to a 16 ºC incubator. After 24 hours the eggs from each beaker were concentrated in a sieve and placed in a 10 mL Bogorov camber and 50+ eggs were randomly examined using a binocular microscope (Olympus) to determine the number of eggs developing with an embryo or dead not developing. In addition, the number of eggs in an entire 0.5 mL sample was counted in triplicate for each female.

Commercial scale *in vitro* fertilisation experiment

Seven different females and seven different males were used during this experiment. Immediately after an ovulated female was encountered, sperm was collected and checked to find a male with 150+ µL of motile sperm. The sperm was immediately diluted 1:4 in Leibovitz and a sample of 50 µL of diluted sperm was taken to determine spz concentration (as above) and percentage motility (ImageJ CASA, see above). All the eggs were stripped from the female into a clean, dry 1 L jug and the volume of eggs was measured with an accuracy of 10 mL. Three samples of 0.5 mL of eggs were taken and counted. All remaining sperm obtained from the male was added to the eggs followed by a volume of seawater that was equal to the volume of eggs. The eggs, sperm and seawater were gently swirled in the jug to mix the contents. After 2-3 minutes the jug was topped up to 1 L with seawater. The eggs were then divided in two or three and each part was placed in a 30 L incubator with the same conditions as the broodstock holding tanks. The number of eggs in each incubator was estimated by mixing the incubator homogenously and taking three 100 mL samples and counting the eggs in each sample. The eggs were left two days to hatch and the number of hatched larvae in each incubator was estimated as above for the eggs. The hatch rate was calculated from the number of eggs stocked and number of larvae hatched and the mean was calculated for the replica incubators used for each female - male pair.

Viability of eggs at room temperature experiment

Three different females and three different males were used during this experiment. Immediately after an ovulated female was encountered, sperm was collected and checked to find a male with 100+ µL of motile sperm. The sperm was

immediately diluted 1:4 in Leibovitz. All the eggs were stripped from the female into a clean, dry 1 L jug. The eggs were covered and stored at room temperature inside a building (out of sun light). As soon as possible after the gametes were stripped the first fertilisation was completed as described above (in egg-sperm ratios section) by mixing 0.5 mL of eggs with 0.2 µL of diluted sperm and 5 mL clean seawater in a 100 mL beaker. The time the first fertilisation was made after the eggs were stripped was recorded. Further fertilisations were completed at 30 to 60 minute intervals and the time after the eggs were stripped was noted. Fertilisations were completed in duplicate or triplicate. As described above, the beakers of fertilised eggs were transferred to a 16 ºC incubator and after 24 hours the percentage of developing eggs was determined for each fertilisation.

Statistics

All means are with one standard deviation unless otherwise stated. For the spz egg^{-1} ratio experiment, the percentage of viable eggs fertilised was calculated by dividing the actual fertilisation rate by the mean maximum fertilisation rate for each female. The number of motile sperm per egg was calculated by multiplying the volume of diluted sperm added by the spermatozoa concentration and the percentage motility and dividing the total by the number of eggs in 0.5 mL. A non-linear regression based on an equation for an exponential rise to a maximum with double, five parameters described the variation of percentage of viable eggs fertilised in relation to number of motile sperm per egg. For the viability of eggs at room temperature experiment the percentage of viable eggs fertilised was calculated as above. A non-linear regression based on an equation for a four parameter logistic curve described the variation of percentage of viable eggs fertilised in relation to time the eggs were stored at room temperature after the eggs were stripped. All statistical comparisons and regressions were made using Sigma Plot 12 (Systat Software, Inc., San Jose, CA 95110. USA).

3. Results

A total of 46 males were checked to obtain 28 (60.9 %) males with the required sperm quantity and quality for the experiments. All males were only used once. A total of 20 females were selected by ovarian swelling and oocyte diameter determined. Five females were rejected as the ovaries contained ovulated ova and two as the ovaries had solid cysts. A total of 13 females had oocytes >600 µm and were induced with GnRHa from which eight (62%) females ovulated good quality eggs that were used in the different experiments. The eggs from some females were used for more than one experiment. The mean latency time from injection with GnRHa to the ovulation was 41:57 ± 1:46 h and mean maximum fertilisation was 82.6 ± 9.2%. The mean fecundity was 130,789 ± 36,723 eggs $fish^{-1}$ or 87,174 ± 24,378 egg kg^{-1} of female body weight.

Sperm to egg ratio experiment

The percentage of viable eggs fertilised in relation to number of motile sperm per egg showed a rapid increase from zero that was represented by a non-linear regression based on an equation for an exponential rise to a maximum with double, five parameters ($R = 0.93$, $P < 0.0001$) (Fig. 1). The non-linear regression described that as low as 326 motile spz per egg fertilised 79 ± 15% (± 95% CI - confidence interval) of viable eggs, that 649 motile sperm fertilise 90 ± 13% (± 95% CI) of viable eggs and 1617 motile spz fertilise 99 ± 12% (± 95% CI) of viable eggs.

Commercial scale *in vitro* fertilisation experiment

The seven commercial scale *in vitro* fertilisations gave a mean percentage hatch of 70 ± 14 % to produce a mean of 131,540 ± 34,448 larvae per fertilisation. The sperm from a single selected cultured male with a volume of 145 ± 50 µL and total spz count of $8 \pm 6.8 \times 10^8$ was sufficient to fertilise large numbers of eggs (> 100,000) and produce large numbers of larvae (> 100,000). The mean number of spz per egg used for the commercial fertilisations was 2,981 ± 2,932 spz egg^{-1} or 592 ± 611 motile spz egg^{-1}.

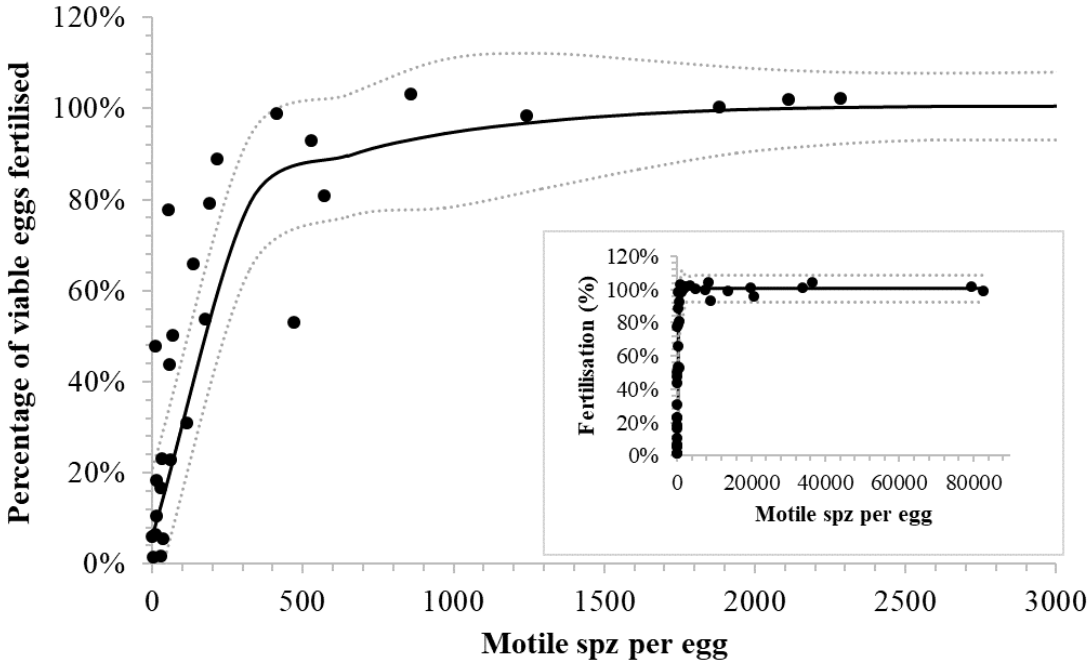

Figure 1. The percentage of viable eggs fertilised in relation to the number of motile spermatozoa (spz) per viable egg for Senegalese sole (*Solea senegalensis*). The insert figure shows the entire data set up to over 80,000 spz per egg and the large figure shows a close up of the data up to 3,000 motile spz per egg. The continuous line shows a non-linear regression based on an equation for an exponential rise to a maximum with double, five parameters ($R = 0.93$, $P < 0.0001$) that represents the variation in percentage of viable eggs fertilised in relation to number of motile sperm per egg. The dotted lines indicate 95% confidence intervals for the non-linear regression.

Viability of eggs at room temperature experiment

The percentage of viable eggs fertilised decreased gradually from after being stripped (Fig. 2). The non-linear regression based on an equation for a four parameter logistic curve ($R = 0.80$, $P = 0.008$) represented the variation in percentage of viable eggs fertilised in relation to time the eggs from the three females were stored at room temperature. The non-linear regression indicated that after 30 minutes, fertilisation had decreased to 81 ± 26% (± 95% CI), after an hour to 57 ± 20% (± 95% CI) and after two hours 32 ± 19% (± 95% CI) fertilisation of viable eggs.

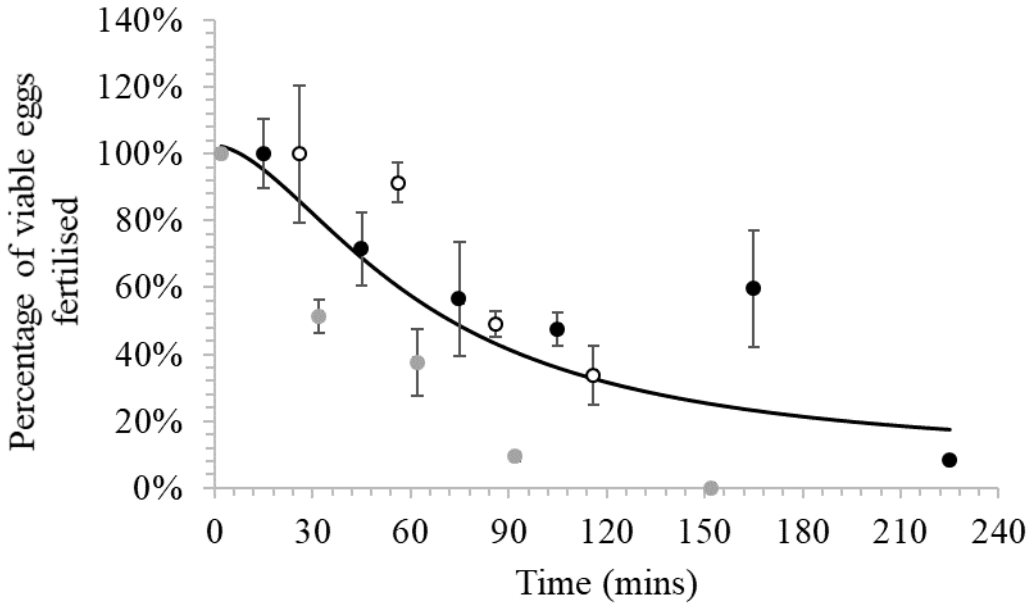

Figure 2. The percentage of viable eggs fertilised in relation to time eggs were stored at room temperature for three Senegalese sole (*Solea senegalensis*) females. Different dots represent different females. The line shows a non-linear regression based on an equation for a four parameter logistic curve (R = 0.08, P = 0.008) that represents the variation in percentage of viable eggs fertilised in relation to time the eggs from the three females were stored.

4. Discussion

The present study demonstrated that low numbers of spz egg^{-1} were required to ensure high levels of fertilisation in Senegalese sole. An exponential rise to a maximum (R = 0.93, P < 0.0001) described the number of motile sperm required to fertilise a viable egg and 1617 motile sperm were sufficient to fertilise 99 ± 12% (± 95% CI) of viable eggs. Logically, the spz egg^{-1} ratio required is related to the success of individual sperm to fertilise an egg. The success of spz will depend on factors that hinder or aid the spz to reach the micropyle of the egg. The environment used for *in vitro* fertilisation has been shown to affect the spz egg^{-1} ratio. For example, the volume or space provided for fertilisation affected the spz egg^{-1} ratio, as larger volumes increased the space to be travelled to fertilise the egg and hence increased the number of spz required (Bombardelli *et al.*, 2013;

Chereguini *et al.*, 1999; Sanches *et al.,* 2016). Similarly, increasing concentrations of toxic agents such as mercury reduced the fertilisation capacity of the spz and hence increased the number of spz required (Rurangwa et al., 1998). Variation in the fertilisation environment complicates the comparison of different studies within and amongst species. However, generally the differences were not great, for example in turbot two ratios have been reported of 3000 – 4000 spz egg^{-1} (Chereguini *et al.,* 1999) and 6000 spz egg^{-1} (Suquet *et al.,* 1995). When ratio differences were larger the cause may be explained by differences in the protocols, for example in Atlantic cod (*Gadus morhua*) fresh sperm gave a ratio of 1×10^5 spz egg^{-1} (Butts *et al.,* 2009) while cryopreserved sperm gave 3×10^5 spz egg^{-1} (Babiak *et al*., 2012). Alternately, ratios may appear to be different, as one study did not test the range of ratios necessary to encounter the lower limit of the spz egg^{-1} ratio. Therefore, the large variation observed amongst species in spz egg^{-1} ratios from $\times 10^3$ to $\times 10^6$ (Beirão *et al.*, 2019) are probably related to other factors, such as differences in species-specific gamete characteristics.

Within species, variations in gamete quality or characteristics have been shown to affect the spz egg^{-1} ratio. Obviously, percentage motility affects the ratio, but velocity has also been shown to affect the ratio in walleye (*Sander vitreus*) (Casselman *et al.*, 2006) and pufferfish (*Takifugu niphobles*) (Gallego *et al.,* 2013b). Spermatozoa with higher mean swimming speeds (and also more range as time of activity is limited) had lower spz egg^{-1} ratios to fertilise high percentages of eggs. The characteristics of the unfertilised egg have also been implicated in fertilisation success. Fish eggs have been shown to have chemical and physical properties that guide the spz to the micropyle and these mechanisms vary amongst species (Yanagimachi *et al.,* 2017). Flounder (black flounder - *Pleuronectes obscurus* and barfin flounder - *Verasper moseri*) (Yanagimachi *et al.*, 2017) and salmon (rainbow trout - *Oncorhynchus mykiss* and coho salmon - *Ochorhynchus kisurch*) (Yanagimachi *et al.,* 1992), were shown to have a glycoprotein substance that guided the spz to the micropyle and when the glycoprotein was removed fertilisation although still possible was less efficient. These differences in ability of

eggs to guide the sperm to the micropyle (Yanagimachi *et al.,* 2017) would presumably influence differences in fertilisation success amongst species.

These species-specific differences in gamete characteristics have evolved to enable the species to achieve maximum levels of fertilisation in their natural spawning environment. The natural spawning environment is both related to the environment inhabited by the species and the species reproductive strategies and behaviour. Different species have very varied strategies and behaviours that can range from mass spawning in aggregations in open water (Domeier and Colin, 1995; Ibarra-Zatarain and Duncan, 2015) to spawning between two fish in an enclosed space or close to the seabed (Carazo *et al.,* 2016; Tatarenkov *et al.,* 2006). A comparison of sperm characteristics between Pacific oysters, turbot and European seabass suggested that distance between spawning individuals and swimming speed when spawning, may be related to sperm swimming speed and distance (Suquet *et al.,* 2012). The spawning behaviour and number of individuals involved will also influence the degree of sperm competition that gametes must negotiate to achieve fertilisation. Sperm competition has been shown to influence fertilisation success and the number of spz that a species produces (Parker and Pizzari, 2010; Stockley *et al.,* 1997). Monogamy and the absence of sperm competition was demonstrated to reduce testes size across different taxa and monogamous fish species had significantly smaller testes compared to polyandrous species (group spawning of males with a female) (Baker *et al.*, 2019).

Senegalese sole spawn as a monogamous pair with no involvement by other individuals (Carazo *et al.*, 2016) and spawning pairs show a degree fidelity during and between spawning seasons (Fatsini *et al.,* 2020; Martín *et al.,* 2014). Therefore, Senegalese sole fertilisation does not involve sperm competition. In addition, the two sexes swim in synchrony with the genital pores held close together (Carazo *et al.,* 2016). The male urogenital duct is slightly raised and the female oviduct forms a kind of well when eggs are being stripped (personal observations), which together with the closeness of the fish during gamete liberation suggests that the male and female place the spz next to the eggs in very

close proximity. The strategies and behaviour of Senegalese sole indicate that the species requires low production of sperm numbers and a low spz egg^{-1} ratio for fertilisation. The present study has described that a low spz egg^{-1} ratio was required and it is widely known that Senegalese sole males have small testes and produce small amounts of sperm (García-López *et al*., 2005; González-López *et al.*, 2020).

In the present study, the decline in quality of stripped Senegalese sole eggs stored at room temperature was gradual and continuous. Egg quality appeared to decline during the first 30 min of storage with no plateau period of good egg quality, which indicated the sooner eggs were fertilised after stripping the higher the viability of the eggs. Other species had similar rapid declines in egg quality, but with a period of 1 h in curimata (*Prochilodus marggravii*) (Rizzo *et al*., 2003) and 50 min in meagre (Ramos-Júdez *et al*., 2019) before quality declined. There is considerable variation across species and some species have very different egg storage capacities, for example eggs from a Cyprinidae species kutum (*Rutilus frisii*) (Samarin *et al.*, 2011) maintained good quality during eight hours of storage and salmonid eggs can be stored successfully for 4-5 days (Bromage, 1992).

The present study used a GnRHa dose of 5 µg kg^{-1} to induce the ovulation of eggs compared to 25 µg kg^{-1} used by Rasines *et al*. (2013, 2012). These studies had similar holding conditions and temperature (16 ºC) and obtained very similar timing of ovulation with means close to 42 h (range of 39 to 44 h). Egg quality appeared to be higher in the present study, but differences in methods and particular sperm storage and usage make comparisons inappropriate. Different doses of GnRHa have been compared to induce spontaneous liberations of eggs in Senegalese sole. Agulleiro *et al*. (2006), tested the injection of GnRHa doses of one, five and 30 µg kg^{-1}. The dose of 5 µg kg^{-1} produced the most eggs and the dose of 30 µg kg^{-1} produced no liberations of eggs. Guzman *et al*. (2009) compared injections of 5 and 25 µg kg^{-1} of GnRHa, but found no differences in number of eggs released between GnRHa injected and untreated control fish. However, the number of oocytes advancing to hydration in the females treated with 5 µg kg^{-1} appeared to be

higher than in females treated with 25 µg kg^{-1} and controls. The present study combined with studies on GnRHa induced spontaneous liberations of eggs would indicate that the lower dose of 5 µg kg^{-1} of GnRHa provided similar ovulation timing and egg quality as 25 µg kg^{-1} and perhaps suggests that better results may be obtained with lower doses.

The massive *in vitro* fertilisations with in excess of 160,000 eggs and the sperm from a single male gave a mean fertilisation of 70 ± 14 %. The success of massive *in vitro* fertilisations, combined with indications of egg viability during storage, together provide a protocol that can be used on an industrial scale to provide eggs from cultured breeders for hatchery production. Obtaining viable eggs from cultured breeders has been a bottleneck in the sole aquaculture industry, which meant the industry was unsustainable and unable to implement breeding programs. Breeding programs are an essential part of an aquaculture business plan that enable companies to improve growth and product quality. However, ideally, reproduction must be controlled to enable the selection and production of viable gametes from any animal that has the desired production traits. In the present study, females were initially selected by ovarian swelling and it is unclear what percentage of females would be available for selected over a reproductive season. Of the females that were GnRHa induced 62% ovulated with good quality eggs. Therefore, studies are needed to identify the number of females available for GnRHa induction and to improve the success rate of GnRHa inductions. In the present study 61% of the males checked for sperm had the required quantity and quality needed for the experiments and *in vitro* fertilisations. This can already be improved as therapies with recombinant gonadotropins exist that both increase the sperm production and quality (Chauvigné et al., 2018, 2017). These recombinant gonadotropin therapies, significantly increased sperm production by up to seven times and significantly increased the sperm quality parameters, percentage motility, progressivity and velocity of spz.

5. Conclusions

In conclusion, Senegalese sole require a low spz egg^{-1} ratio to achieve high percentages of fertilisation both on an experimental scale and in massive commercial scale *in vitro* fertilisations. The low spz egg^{-1} ratio required for maximum fertilisation was consistent with the reproductive behaviour and strategies of the species. The methods described in the present study will enable the Senegalese sole aquaculture industry to operate sustainably and establish breeding programs.

6. References

Agulleiro, M.J., Anguis, V., Cañavate, J.P., Martínez-Rodríguez, G., Mylonas, C.C., Cerdà, J., 2006. Induction of spawning of captive-reared Senegal sole (Solea senegalensis) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 257, 511–524. https://doi.org/10.1016/j.aquaculture.2006.02.001

Anguis, V., Cañavate, J.P., 2005. Spawning of captive Senegal sole (Solea senegalensis) under a naturally fluctuating temperature regime. Aquaculture 243, 133–145. https://doi.org/10.1016/j.aquaculture.2004.09.026

APROMAR, 2019. La acuicultura en España.

Babiak, I., Marschhäuser, V., Ottesen, O., Rudolfsen, G., Eggen, B., Babiak, J., 2012. Effects of extender, storage and sperm-to-egg ratio on cryopreservation success of Atlantic cod (*Gadus morhua* L.) sperm. J. Appl. Ichthyol. 28, 941–947. https://doi.org/10.1111/jai.12063

Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Chilled storage of semen from Atlantic halibut, Hippoglossus hippoglossus L. Theriogenology 66, 2025–2035. https://doi.org/10.1016/j.theriogenology.2006.06.003

Baker, J., Humphries, S., Ferguson-Gow, H., Venditti, C., 2019. Rapid decreases in relative testes mass among monogamous birds but not in other vertebrates 10.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133, 161–178. https://doi.org/10.1016/j.theriogenology.2019.05.004

Beirão, J., Ottesen, O.H., 2018. Optimization of a fertilization protocol for spotted wolffish (Anarhichas minor). Aquaculture 484, 133–138. https://doi.org/10.1016/j.aquaculture.2017.11.004

Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2011. Changes in Solea senegalensis sperm quality throughout the year. Anim. Reprod. Sci. 126, 122–129. https://doi.org/10.1016/j.anireprosci.2011.04.009

Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2009. Sperm quality evaluation in Solea senegalensis during the reproductive season at cellular level. Theriogenology 72, 1251–1261. https://doi.org/10.1016/j.theriogenology.2009.07.021

Bombardelli, R.A., Sanches, E.A., Baggio, D.M., Sykora, R.M., Souza, B.E. de, Tessaro, L., Piana, P.A., 2013. Effects of the spermatozoa: oocyte ratio, water volume and water temperature on artificial fertilization and sperm activation of cascudo-preto. Rev. Bras. Zootec. 42, 1–6. https://doi.org/10.1590/S1516-35982013000100001

Bromage, N., 1992. Propagation and stock improvement, in: Shepherd, J., Bromage, N. (Eds.), Intensive Fish Farming. pp. 103–153.

Butts, I., Roustaian, P., Litvak, M., 2012. Fertilization strategies for winter flounder: effects of spermatozoa density and the duration of gamete receptivity. Aquat. Biol. 16, 115–124. https://doi.org/10.3354/ab00439

Butts, I.A.E., Trippel, E.A., Litvak, M.K., 2009. The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod Gadus morhua L. Aquaculture 286, 89–94. https://doi.org/10.1016/j.aquaculture.2008.09.005

Cabrita, E., Soares, F., Beirão, J., García-López, A., Martínez-Rodríguez, G., Dinis, M.T., 2011. Endocrine and milt response of Senegalese sole, Solea senegalensis, males maintained in captivity. Theriogenology 75, 1–9. https://doi.org/10.1016/j.theriogenology.2010.07.003

Cabrita, E., Soares, F., Dinis, M.T., 2006. Characterization of Senegalese sole, Solea senegalensis, male broodstock in terms of sperm production and quality. Aquaculture 261, 967–975. https://doi.org/10.1016/j.aquaculture.2006.08.020

Carazo, I., Chereguini, O., Martín, I., Huntingford, F., Duncan, N., 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (Solea senegalensis). Span. J. Agric. Res. 14, e0401. https://doi.org/10.5424/sjar/2016144-9108

Casselman, S.J., Schulte-Hostedde, A.I., Montgomerie, R., 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci. 63, 2119–2125. https://doi.org/10.1139/f06-108

Cejko, B.I., Żarski, D., Targońska, K., Krejszeff, S., Kucharczyk, D., Glogowski, J., 2010. Osmolality of Seminal Plasma as an Indicator of Milt Contamination with Urine Based on the Example of the Tench *Tinca tinca* (L.). Pol. J. Nat. Sci. 25, 287–298. https://doi.org/10.2478/v10020-010-0026-6

Chauvigné, F., González, W., Ramos, S., Ducat, C., Duncan, N., Giménez, I., Cerdà, J., 2018. Seasonal-and dose-dependent effects of recombinant gonadotropins on sperm production and quality in the flatfish Solea senegalensis. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 225, 59–64. https://doi.org/10.1016/j.cbpa.2018.06.022

Chauvigné, F., Ollé, J., González, W., Duncan, N., Giménez, I., Cerdà, J., 2017. Toward developing recombinant gonadotropin-based hormone therapies for

increasing fertility in the flatfish Senegalese sole. PLOS ONE 12, e0174387. https://doi.org/10.1371/journal.pone.0174387

Chereguini, O., de la Banda, I.G., Rasines, I., Fernandez, A., 1999. Artificial fertilization in turbot, Scophthalmus maximus (L.): different methods and determination of the optimal sperm-egg ratio. Aquac. Res. 30, 319–324. https://doi.org/10.1046/j.1365-2109.1999.00326.x

Domeier, M.L., Colin, P.L., 1995. Tropical reef fish spawning aggregations: defined and reviewed 29.

Duncan, N., Carazo, I., Chereguini, O., Mañanós, E., 2019. Mating Behaviour, in: Munoz-Cueto, J., Mañanós-Sánchez, E., Sánchez-Vázquez, J. (Eds.), Biology of Sole. CRC Press, Boca Raton.

Fatsini, E., Carazo, I., Chauvigné, F., Manchado, M., Cerdà, J., Hubbard, P.C., Duncan, N.J., 2017. Olfactory sensitivity of the marine flatfish *Solea senegalensis* to conspecific body fluids. J. Exp. Biol. 220, 2057–2065. https://doi.org/10.1242/jeb.150318

Fatsini, E., González, W., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J., 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519, 734922. https://doi.org/10.1016/j.aquaculture.2020.734922

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., Suquet, M., 1999. Characteristics of sperm of captive seabass in relation to its fertilization potential. J. Fish Biol. 54, 356–369.

Gallego, V., Carneiro, P.C.F., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Soler, C., Pérez, L., Asturiano, J.F., 2013a. Standardization of European eel (Anguilla anguilla) sperm motility evaluation by CASA software. Theriogenology 79, 1034–1040. https://doi.org/10.1016/j.theriogenology.2013.01.019

Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2013b. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (Takifugu niphobles). Aquaculture 416–417, 238–243. https://doi.org/10.1016/j.aquaculture.2013.08.035

García-López, Á., Martínez-Rodríguez, G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole Solea senegalensis (Kaup): Anatomy, histology and histochemistry 11.

González-López, W.Á., Ramos-Júdez, S., Giménez, I., Duncan, N.J., 2020. Sperm contamination by urine in Senegalese sole (Solea senegalensis) and the use of extender solutions for short-term chilled storage. Aquaculture 516, 734649. https://doi.org/10.1016/j.aquaculture.2019.734649

Guzmán, J.M., Cal, R., García-López, Á., Chereguini, O., Kight, K., Olmedo, M., Sarasquete, C., Mylonas, C.C., Peleteiro, J.B., Zohar, Y., Mañanós, E.L., 2011. Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis of Senegalese sole (Solea senegalensis). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 158, 235–245. https://doi.org/10.1016/j.cbpa.2010.11.016

Guzmán, J.M., Norberg, B., Ramos, J., Mylonas, C.C., Mañanós, E.L., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (Solea senegalensis). Gen. Comp. Endocrinol. 156, 285–297. https://doi.org/10.1016/j.ygcen.2008.02.002

Guzmán, J.M., Ramos, J., Mylonas, C.C., Mañanós, E.L., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (Solea senegalensis) treated with different GnRHa-delivery systems. Aquaculture 291, 200–209. https://doi.org/10.1016/j.aquaculture.2009.03.024

Ibarra-Zatarain, Z., Duncan, N., 2015. Mating behaviour and gamete release in gilthead seabream (Sparus aurata, Linnaeus 1758) held in captivity. Span. J. Agric. Res. 13, e0401. https://doi.org/10.5424/sjar/2015131-6750

Linhart, O., Rodina, M., Bastl, J., Cosson, J., 2003. Urinary bladder, ionic composition of seminal fluid and urine with characterization of sperm motility in tench (Tinca tinca L.). J. Appl. Ichthyol. 19, 177–181. https://doi.org/10.1046/j.1439-0426.2003.00470.x

Liu, Xin-fu, Liu, Xue-zhou, Lian, J., Wang, Y., Zhang, F., Yu, H., Ma, A., Liu, S., Zhai, J., 2008. Large scale artificial reproduction and rearing of Senegal sole, Solea senegalensis Kaup. Mar. Fish. Res. 29, 10–16.

Mañanós, E., Duncan, N., Mylonas, C., 2008. Reproduction and Control of Ovulation, Spermiation and Spawning in Cultured Fish, in: Cabrita, E., Robles, V., Herráez, P. (Eds.), Methods in Reproductive Aquaculture, Marine Biology. CRC Press, pp. 3–80. https://doi.org/10.1201/9780849380549.sec1

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernández, R., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2020. Reproductive performance of captive Senegalese sole, Solea senegalensis, according to the origin (wild or cultured) and gender. Span. J. Agric. Res. 17, e0608. https://doi.org/10.5424/sjar/2019174-14953

Martín, I., Rasines, I., Gómez, M., Rodríguez, C., Martínez, P., Chereguini, O., 2014. Evolution of egg production and parental contribution in Senegalese sole, Solea senegalensis, during four consecutive spawning seasons. Aquaculture 424–425, 45–52. https://doi.org/10.1016/j.aquaculture.2013.12.042

Morais, S., Aragão, C., Cabrita, E., Conceição, L.E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L.M.P., Yúfera, M., Dinis, M.T., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Rev. Aquac. 8, 227–263. https://doi.org/10.1111/raq.12091

Norambuena, F., Estevez, A., Bell, G., Carazo, I., Duncan, N., 2012a. Proximate and fatty acid compositions in muscle, liver and gonads of wild versus cultured broodstock of Senegalese sole (Solea senegalensis). Aquaculture 356–357, 176–185. https://doi.org/10.1016/j.aquaculture.2012.05.018

Norambuena, F., Estévez, A., Mañanós, E., Bell, J.G., Carazo, I., Duncan, N., 2013a. Effects of graded levels of arachidonic acid on the reproductive physiology of Senegalese sole (Solea senegalensis): Fatty acid composition, prostaglandins and steroid levels in the blood of broodstock bred in captivity. Gen. Comp. Endocrinol. 191, 92–101. https://doi.org/10.1016/j.ygcen.2013.06.006

Norambuena, F., Estévez, A., Sánchez-Vázquez, F.J., Carazo, I., Duncan, N., 2012b. Self-selection of diets with different contents of arachidonic acid by Senegalese sole (Solea senegalensis) broodstock. Aquaculture 364–365, 198–205. https://doi.org/10.1016/j.aquaculture.2012.08.016

Norambuena, F., Mackenzie, S., Bell, J.G., Callol, A., Estévez, A., Duncan, N., 2012c. Prostaglandin (F and E, 2- and 3-series) production and cyclooxygenase (COX-2) gene expression of wild and cultured broodstock of senegalese sole (Solea senegalensis). Gen. Comp. Endocrinol. 177, 256–262. https://doi.org/10.1016/j.ygcen.2012.04.009

Norambuena, F., Morais, S., Estévez, A., Bell, J.G., Tocher, D.R., Navarro, J.C., Cerdà, J., Duncan, N., 2013b. Dietary modulation of arachidonic acid metabolism in senegalese sole (Solea Senegalensis) broodstock reared in captivity. Aquaculture 372–375, 80–88. https://doi.org/10.1016/j.aquaculture.2012.10.035

Parker, G.A., Pizzari, T., 2010. Sperm competition and ejaculate economics. Biol. Rev. no-no. https://doi.org/10.1111/j.1469-185X.2010.00140.x

Perchec Poupard, G., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160, 317–328. https://doi.org/10.1016/S0044-8486(97)00301-3

Ramos-Júdez, S., González, W., Dutto, G., Mylonas, C.C., Fauvel, C., Duncan, N., 2019. Gamete quality and management for in vitro fertilisation in meagre (Argyrosomus regius). Aquaculture 509, 227–235. https://doi.org/10.1016/j.aquaculture.2019.05.033

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2013. Artificial fertilisation of cultured Senegalese sole (Solea senegalensis): Effects of the time of day of hormonal treatment on inducing ovulation. Aquaculture 392–395, 94–97. https://doi.org/10.1016/j.aquaculture.2013.02.011

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (Solea senegalensis): Hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326–329, 129–135. https://doi.org/10.1016/j.aquaculture.2011.11.021

Riesco, M.F., Valcarce, D.G., Martínez-Vázquez, J.M., Martín, I., Calderón-García, A.Á., Gonzalez-Nunez, V., Robles, V., 2019. Male reproductive dysfunction in Solea senegalensis: new insights into an unsolved question. Reprod. Fertil. Dev. 31, 1104. https://doi.org/10.1071/RD18453

Rizzo, E., Godinho, H.P., Sato, Y., 2003. Short-term storage of oocytes from the neotropical teleost fish Prochilodus marggravii. Theriogenology 60, 1059–1070. https://doi.org/10.1016/S0093-691X(03)00108-0

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E., Ollevier, F., 1998. The minimum effective spermatozoa : egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in Clarias gariepinus. J. Fish Biol. 53, 402–413. https://doi.org/10.1111/j.1095-8649.1998.tb00989.x

Samarin, A.M., Amiri, B.M., Soltani, M., Mohammad, R., Kamali, A., Naghavi, M.R., 2011. Effects of storage duration and storage temperature on viability of stored ova of kutum (Rutilus frisii kutum) in ovarian fluid 6.

Sanches, E.A., Caneppele, D., Okawara, R.Y., Damasceno, Z., Bombardelli, R.A., Romagosa, E., 2016. Inseminating dose and water volume applied to the artificial fertilization of Steindachneridion parahybae (Steindachner, 1877) (Siluriformes: Pimelodidae): Brazilian endangered fish. Neotropical Ichthyol. 8.

Sarosiek, B., Dryl, K., Krejszeff, S., Żarski, D., 2016. Characterization of pikeperch (Sander lucioperca) milt collected with a syringe and a catheter. Aquaculture 450, 14–16. https://doi.org/10.1016/j.aquaculture.2015.06.040

Stockley, P., Gage, M.J.G., Parker, G.A., work(s):, and A.P.M.R., 1997. Sperm Competition in Fishes: The Evolution of Testis Size and Ejaculate Characteristics. Am. Nat. 149, 933–954.

Suquet, M., Billard, R., Cosson, J., Normant, Y., Fauvel, C., 1995. Artificial insemination in turbot (Scophthalmus maximus): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture 133, 83–90. https://doi.org/10.1016/0044-8486(94)00395-5

Suquet, M., Cosson, J., Donval, A., Labbé, C., Boulais, M., Haffray, P., Bernard, I., Fauvel, C., 2012. Marathon vs sprint racers: an adaptation of sperm characteristics to the reproductive strategy of Pacific oyster, turbot and seabass. J. Appl. Ichthyol. 28, 956–960. https://doi.org/10.1111/jai.12061

Tatarenkov, A., Barreto, F., Winkelman, D.L., Avise, J.C., 2006. Genetic Monogamy in the Channel Catfish, Ictalurus Punctatus, a Species with Uniparental Nest Guarding. Copeia 2006, 735–741. https://doi.org/10.1643/0045-8511(2006)6[735:GMITCC]2.0.CO;2

Yanagimachi, R., Cherr, G.N., Pillai, M.C., Baldwin, J.D., 1992. Factors Controlling Sperm Entry into the Micropyles of Salmonid and Herring Eggs. (fish/sperm/egg/micropyle/fertilization). Dev. Growth Differ. 34, 447–461. https://doi.org/10.1111/j.1440-169X.1992.00447.x

Yanagimachi, R., Harumi, T., Matsubara, H., Yan, W., Yuan, S., Hirohashi, N., Iida, T., Yamaha, E., Arai, K., Matsubara, T., Andoh, T., Vines, C., Cherr, G.N., 2017.

Chemical and physical guidance of fish spermatozoa into the egg through the micropyle. Biol. Reprod. 96, 780–799. https://doi.org/10.1093/biolre/iox015

Chapter 8

Parameters relevant to cold storage of spotted wolffish sperm (*Anarhichas minor*)

Chapter 8. Parameters relevant to cold storage of spotted wolffish sperm (*Anarhichas minor*)

1. Introduction

The spotted wolfish, has emerged as a potential species for marine cold-water aquaculture in Canada, Iceland, Sweden and Norway. This aquaculture potential is based on good growth rates, high fillet yield, few disease problems, relatively high market price and consumer acceptance (Falk-Petersen *et al.* 1999; François et al. 2002; Foss *et al*. 2004). In Norway, the species is already reared on a commercial scale, but production volumes are low and quite unstable. The main reason for this instability has been attributed to problems to provide sufficient good quality eggs. In captivity, the spotted wolffish reproductive behaviour is disrupted and fertilization must be conducted *in vitro* (Falk-Petersen et al. 1999; Beirão and Ottesen 2018). However, males produced relatively low sperm volume with a low concentration and motility compared with other marine fish (Le Francois and Archer 2007; Beirão and Ottesen 2018). In addition, the sperm that is already motile at stripping remains motile from several hours up to two days, but is inactivated by contact with seawater (Kime and Tveiten 2002). Thus, sperm storage procedures, through cryopreservation, have been suggested as methods to ensure the availability of good quality sperm when required (Le Francois *et al.* 2008; Gunnarsson *et al*. 2009; Santana *et al*. 2020).

Cryopreservation protocols require access to liquid nitrogen, specific material and chemicals, trained personnel and in small hatcheries, as is the case for spotted wolffish, are logistically difficult to implement. In addition, cryopreservation procedures are often used for long-term sperm storage (Shaliutina et al., 2013). Short-term storage of sperm by refrigeration, on the other hand, is a simple and inexpensive procedure relatively easy to implement in the hatchery environment (Bobe and Labbé 2008; Contreras et al. 2019). Sperm refrigeration protocols have been tested in several species and can be a useful tool to assist in artificial

fertilization programs, synchronization in availability of gametes and transport of gametes between different facilities (Contreras et al. 2019; Beirão *et al.* 2019). Short-term storage is especially relevant when the sperm volume obtained is limited (Bobbe and Labbe 2008). Thus, in this study we test the possibility of sperm refrigeration as an option to secure spotted wolffish sperm availability for a few days.

Whilst sperm refrigeration is an attractive option for spotted wolffish, it presents challenges as the sperm is already motile at stripping and it is currently not possible to immobilize and reactivate wolffish sperm. Therefore, sperm refrigeration protocols must provide the conditions to ensure the sperm motility period extends over the duration of the required storage period. Furthermore, spotted wolffish sperm is collected by stripping (Le Francois and Archer 2007), which increases the risk of contamination of semen with urine, as observed in other fish species (Sarosiek *et al.* 2016; Król *et al.* 2018). As an example, in both pikeperch (*Sander lucioperca*) and turbot (*Psetta maxima),* a higher urine contamination was exhibited in the sperm collected by stripping compared with sperm collected with a catheter (Dreanno *et al.,* 1998; Sarosiek *et al*., 2016). This contamination with urine is usually linked to a reduction in sperm quality and storability (Król *et al.* 2018; González-López *et al.* 2019). A decrease in the percentage of sperm motility and sperm velocity parameters (VCL and VSL) in pikeperch was observed due to urine contamination (Sarosiek et al., 2016). In turbot higher urine concentration caused a delay in the motility initiation (Dreanno et al., 1998). Damage to sperm motility rate in sperm samples from Senegalese sole (*S. senegalensis*) and Eurasian perch (*Perch fluviatilis*) stored for twenty-four hours was related to the urine concentration in sperm (Król *et al.* 2018; González-López *et al.* 2019).

In the present study, two experiments were conducted in order to test methods for the short-term refrigerated storage of spotted wolffish sperm. In the first experiment, the degree of urine contamination in relation to a stripping method to avoid urine and short-term refrigerated storage of the sperm was evaluated. In the

second experiment, the importance of the energy reserves on sperm quality and short-term refrigerated storage was assessed.

2. Methods

Samples collection

This study was carried on with the licence (No A08, 017) from the Norwegian Food Safety Authority (Mattilsynet) attributed to the Faculty of Bioscience and Aquaculture, Nord University, to perform experiments on animals. Sperm samples were obtained from the spotted wolffish producer AMINOR AS (Halsa, Nordland, Norway) during the reproductive season (January-March 2019). Ten year old adult breeders were kept all year round in 1,600 L rectangular tanks, water depth 0.4 m, with an open flow through system under natural temperature and photoperiod conditions (66°74′N, 13°51′E). Oxygen measurements of the outgoing water were kept over 80 %. The fish were fed with pellets (Vitalis CAL and Vitalis REPRO, Skretting).

Individuals were anesthetized with 500 ppm tricaine methanesulfonate (MS-222, Sigma-Aldrich) for five minutes. The sperm collection procedure follows Beirão and Ottesen (2018). After the release of most urine by pressing the abdominal area, a gentle massage was applied from the lateral region of the abdomen where the testes and the sperm ducts are located towards the urogenital pore to obtain sperm samples. The sperm samples (1.0 mL for experiment 1 and 1.5 mL for experiment 2, see below) were collected in a plastic pasteur pipette attached to the urogenital pore. The samples were kept in a refrigerator (2 °C) until analysis. Urine samples (2 mL) were obtained by applying pressure to the urinary bladder. The urine samples were collected into a 15 mL falcon tube and stored at - 20°C until analysis of the urea concentration.

Sperm quality assessment

a) Motility parameters

Percentage of motile cells and curvilinear velocity (VCL) (µm/s) were evaluated. Initially, 1µL of the sperm samples was diluted 1:10 in solution previously developed by Smith and Ryan (2010) for internal fertilizing fish (207 mM NaCl, 1.3 mM $CaCl_2$, 0.41 mM $MgSO_4$, 5.4mM KCl, 0.49mM $MgCl_2$ and 10 mM Trizma) with the addition of 1% BSA and the pH was adjusted to 7.5. A preliminary trial demonstrated that the solution developed by Smith and Ryan (2010) was an adequate extender for refrigerated wolffish sperm storage compared with the solution previously developed by Kime and Tveiten (2002). A 2 µL drop of the diluted sample was placed between a coverslip and a slide and videos were recorded with the CASA system SCA 6.2 (Microptic, Barcelona, Spain). Images were recorded using a digital camera (Basler acA1300-200uc, Ahrensburg, Germany) attached to an optical phase-contrast microscope (Nikon Eclipse Ci, Tokyo, Japan) with 10 × negative phase contrast objective, with a stage temperature controller set to 6ºC (Linkam T95-PE, Tadworth, United Kingdom). Samples were analysed in triplicate.

b) Cell concentration

The cell concentration in each sperm sample was calculated from counts of diluted sperm sample. A sample of 10 µL of fresh sperm was diluted 1:500 with Smith and Ryan (2010) extender and 10 µL of this dilution was placed into a Neubauer counting chamber. The sample was observed under the phase-contrast microscope with a 10 × objective. For each sample the count was made in triplicate.

c) pH and Osmolality

The pH was determined in the seminal plasma by the colorimetric method using pH indicator strips (Hydrion, Sigma-Aldrich), with a detection range between 5 to 9 pH. The osmolality (mOsm/kg) was determined from 10 µL seminal plasma using the

freezing point depression osmometer (Fiske One-Ten, Fiske® Associates) and each sample was measured in duplicate.

d) ATP and Glucose concentration

A 100 µL sample of semen was used to determine the ATP concentration. Initially, 100 µL of EDTA solution with TCA (4%) was added to 100 µL semen and mixed with a vortex mixer before being centrifuged (10,000 g for 10 min at 4°C). From this centrifuged suspension 100 µL of supernatant were collected and added to 500 µL Sorensen buffer (adjusted pH 7.8) before being stored in a 1.5 mL centrifuge tube at -20°C for further analysis. The ATP concentration was determined by using the Adenosine 5´-triphosphate (ATP) Bioluminescent Assay kit (FLAA, Sigma-Aldrich) in accordance to the manufacturer's instructions. The samples luminescence was read in a FLUOstart Optima plate reader (BMG LABTECH, Ortenberg, Germany) in a 96-well white plate. The ATP concentration was calculated and expressed in nmol per 10^9 cell.

The glucose concentration (µg/mL), was assessed with the Glucose (GO) Assay kit (GAGO-20, Sigma-Aldrich) following the manufacturer's instructions. Seminal plasma samples diluted 5× were processed with the kit and the absorbance was read in a 96-well transparent plate at 540 nm in the FLUOstar Optima plate reader (BMG LABTECH).

e) Protein analysis

The total protein concentration (mg/mL) was evaluated using the Invitrogen Qubit® Protein Assay Kit and Qubit™ Fluorometer (Thermo Fisher Scientific). One dimensional gel electrophoresis was used to separate the proteins in the seminal plasma according to molecular weight. The sample for the gel electrophoresis was prepared by diluting (1:1) the seminal plasma in 0.7% NaCl solution. The diluted sample (20 µl) was mixed with equal amount of 1 × Laemmli buffer (Bio-Rad, USA). The mixture was vortexed for 30 sec and incubated for 5 minutes at 95°C; after incubation, 30 µl of each sample was loaded in the gel (12.5% polyacrylamide

gel) together with the protein ladder (Precision Plus Protein™ Kaleidoscope prestained protein standards, Bio-Rad, USA). The gel was ran at 200 V until the dye front reached the bottom of the gel (~ 30min). After the electrophoresis, the bands were stained using Coomassie Brilliant Blue R-250 (Bio-Rad, USA) for 2h. After 2h, the destaining solution (40% methanol and 10% glacial acetic acid) was used on the gel until the background was clear (~ 2h). The gel was then rinsed in miliQ water and image was documented using ChemiDoc™ MP imaging system, Bio-Rad, USA. The analysis of the bands was performed using Image Lab Software (Bio-Rad).

f) Urea concentration

Urea concentration was measured in both seminal plasma and urine samples with the Urea assay kit (KA 1652, Abnova, Denmark) following the manufacturer's instructions. The samples, processed with the kits reagents, were placed into a 96-well plate and incubated for 20 minutes at room temperature. The plate was read in the FLUOstar Optima plate reader (BMG LABTECH) at 520 nm. The readings were transformed and expressed in units of mg/dL.

Experiment 1: Stripping method, urine contamination and short-term refrigerated storage of sperm

To evaluate the degree of urine contamination on sperm quality and the effect on storability, sperm and urine samples (n = 9) were collected from different males. The sperm samples were divided in four aliquots. The first aliquot, with 15 µL, was used to assess cell concentration and percentage of motility and VCL within 10 minutes of sperm collection. The second aliquot, with 100 µL, was diluted (D) 1:2 (final volume 300 µL) in the solution developed by Smith and Ryan (2010). The third aliquot, with 300 µL, was undiluted (UD). Both the second (D) and third aliquot (UD) were stored in an incubator at 2 °C to assess the percentage of motile cells and VCL at different times after collection (5, 10, 20, 30, 40 and 50 h). The fourth aliquot, with 400 µL, was centrifuged for 10 minutes at 300 g and room

temperature to collect the seminal plasma. In addition, seminal plasma was obtained from sperm obtained directly from the testes of two sacrificed males. The seminal plasma was stored at - 20 °C until its analysis in the laboratory for pH, osmolality, urea and total protein.

Experiment 2: Effect of energy reserves on short-term refrigerated storage of sperm

To assess the importance of ATP and glucose reserves on sperm storability, sperm samples (n = 8) were separated in five aliquots. The first aliquot of 15 µL was used to assess cell concentration and percentage of motility and VCL within 10 minutes of sperm collection. The second aliquot of 300 µL was diluted (D) 1:1 (final volume 600 µL) in the Smith and Ryan (2010) solution. The third aliquot of 600 µL was undiluted (UD). Both the second and the third aliquots were stored in an incubator at 2 °C and assess at different times (5, 10, 20 and 30 h) for percentage of motility, VCL, ATP and glucose. Glucose was only measured in UD samples. The fourth aliquot of 100 µL of fresh sperm was processed to analyse the ATP content. The fifth aliquot of 450 µL was centrifuged for 10 min at 500 g and 4 °C and the seminal plasma was collected and placed in 1.5 mL centrifuge tubes and frozen at -20 °C until analysis of pH, osmolality, glucose, total protein and proteins based on their molecular weight.

Statistical analysis

The data were analysed with IBM SPSS Statistics 20 and expressed as mean ± one standard error of the mean (SEM). The data were analysed for normality using the Shapiro-Wilks test. In both trials, correlations between percentage of motility, VCL and the different parameters measured were analysed with a Pearson´s correlation. For each experiment (1 and 2) two General Linear Models (repeated measures) were performed to detect possible differences between D and UD

treatments, one for the percentage of motility and the other for VCL, during the storage time. An addition General Linear Model (repeated measures) was performed to evaluate the ATP parameter between D and UD treatments and over time. Urea concentration measured in urine and seminal plasma collected from the testes and by stripping were compared through the One-way ANOVA and a Tukey post-hoc. Differences were considered significant for $p<0.05$.

3. Results

Experiment 1: Stripping method, urine contamination and short-term refrigerated storage of sperm

In the first experiment, the initial sperm motility was 36.4 ± 7.89 % and VCL was 20.11 ± 1.14 μm/s, with a range between 7.35 to 70.81 % and from 12.63 to 25.22 μm/s respectively. For the UD samples, there was a significant decrease ($p=0.042$) during the storage time, from 0 to 50 h, in the percentage of motile cells, whilst D samples did not show any difference ($p=0.065$) between 0 and 50 h (Fig. 1A). However, at 50 h after storage, there was no significant difference ($p=0.434$) between the percentage of motility in D (6.88 ± 1.76 %) and in UD (4.39 ± 1.08 %). For the VCL, there were no significant differences for D and UD over time or between the two treatments at 50 h ($P\geq0.063$) (Fig. 1B).

The urea concentration was significantly higher in the urine (110.18 ± 9.83 mg/dL) compared with the stripped sperm seminal plasma (17.10 ± 1.98 mg/dL). No differences in urea concentration were observed between the seminal plasma of stripped sperm and the seminal plasma from testis sperm (Fig. 2). The urea concentration in the stripped samples was correlated with percentage of motile cells at 50 h after storage ($R=0.752$; $p=0.031$). The urine samples pH was 5.61 ± 0.13 and the osmolality 299.89 ± 4.94 mOsm/kg. Neither the seminal plasma pH 6.37 ± 0.06 nor osmolality 319.56 ± 9.94 mOsm/kg was correlated with the sperm motility parameters. Whilst, the total protein concentration (0.76 ± 0.16 mg/ml) was negatively correlated with VCL at 5, 20, 30 and 40 h of storage in the diluted

samples (R=-0.690, p=0.040; R=-0.774, p=0.024; R=-0.802, p=0.009; R= -0.755, p=0.030 respectively).

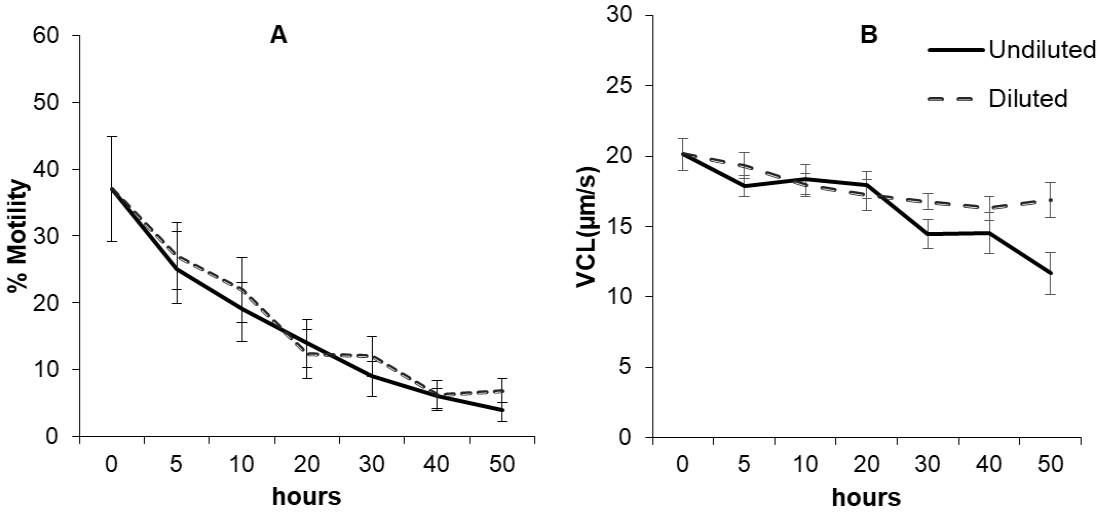

Figure 1 A) Percentage of sperm motility and B) curvilinear velocity (VCL) (µm/s) between diluted (D) and undiluted (UD) spotted wolffish sperm samples during refrigerated storage in the experiment 1. Values represent the mean ± SEM (n = 9)

Experiment 2: Effect of energy reserves on short-term refrigerated storage of sperm

The initial sperm quality values (44.56 ± 3.85 % motile cells, VCL 23.55 ± 1.04 µm/s, seminal plasma pH 6.11 ± 0.12 and osmolality 314.25 ± 4.37 mOsm/ kg) observed in the Experiment 2 were similar to Experiment 1. However, significant differences were found for the percentage of motile cells between the D (20.83 ±5.02%) and UD (15.79± 4.05%) treatments ($p< 0.05$) at 30 h of storage (Fig. 3A). On the other hand, as in Experiment 1, the sperm velocity (VCL) was not significantly different between the diluted sperm samples (21.21 ± 0.95 µm/s) and the undiluted samples (20.68 ± 1.40 µm/s) at 30h (Fig. 3B).

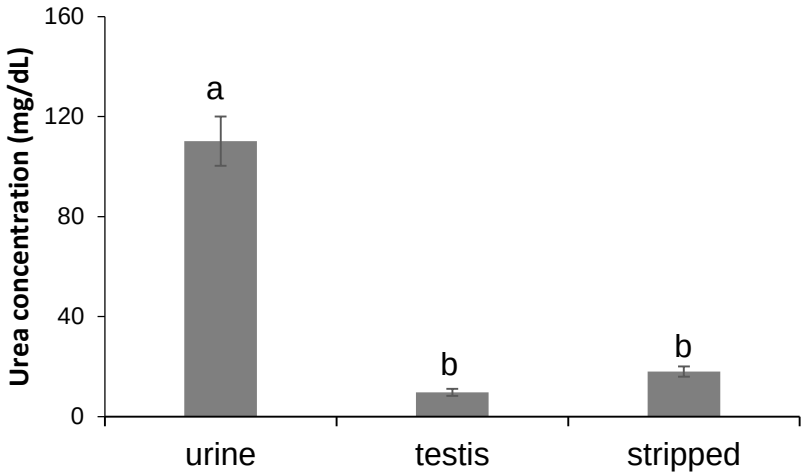

Figure 2 Differences in urea concentrations (mg/dL) in spotted wolffish pure urine samples and seminal plasma obtained from testes sperm and by stripping. Different letters stand for significant differences between group of samples as detected with a one-way ANOVA ($p<0.05$). Values represent the mean ± SEM.

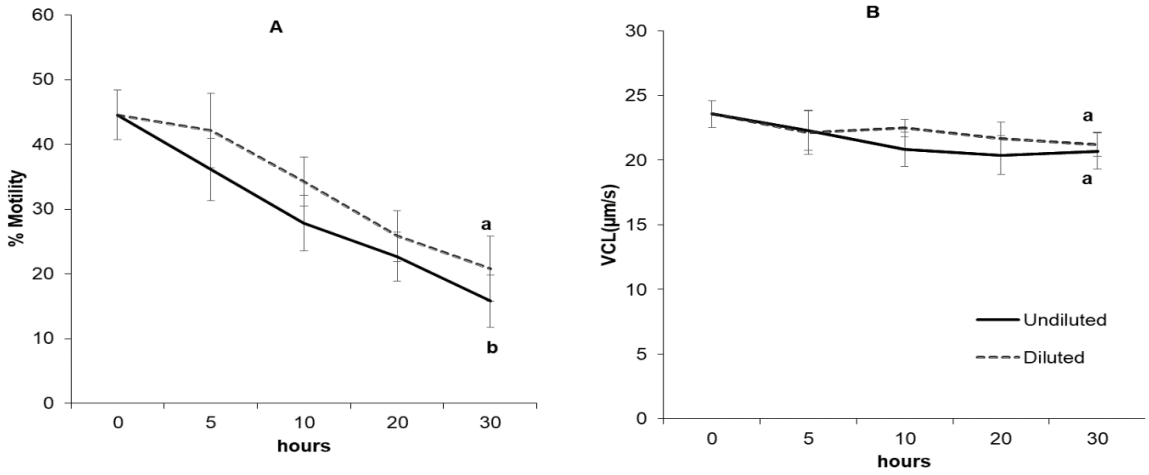

Figure 3 A) Percentage of sperm motility and B) curvilinear velocity (VCL) (µm/s) between diluted (D) and undiluted (UD) spotted wolffish sperm samples during the refrigerated storage in the experiment 2. Values represent the mean ± SEM (n = 8). Different lowercase letters indicate significant differences between samples (D) and (UD) at the end of short-term sperm storage.

The initial ATP values (5.65 ± 0.86 nmol/10^9) were not significantly affect (p= 0.099) by the storage period and at 30 h the values were similar (p= 0.329) in both D (3.88 ± 1.35 nmol/10^9) and UD (4.76 ± 1.08 nmol/10^9) samples (Fig. 4). Likewise, the pH (initial value 6.11 ± 0.12; final value 6.15 ± 0.20) and glucose (initial value

9.13 ± 2.32 µg/mL; final value 9.51 ± 4.99 µg/mL) values also remained stable (p= 0.981 and p=0.732 respectively) during the storage time (Fig. 5A and 5B). While osmolality parameter had a significant increase (p= 0.039) through the storage period (initial value 314.52 ± 4.37 mOsm/kg; final value 330.62 ± 2.82 mOsm/kg). In addition, no correlation was observed between ATP or the glucose and the sperm motility parameters (percentage of motile cells and VCL).

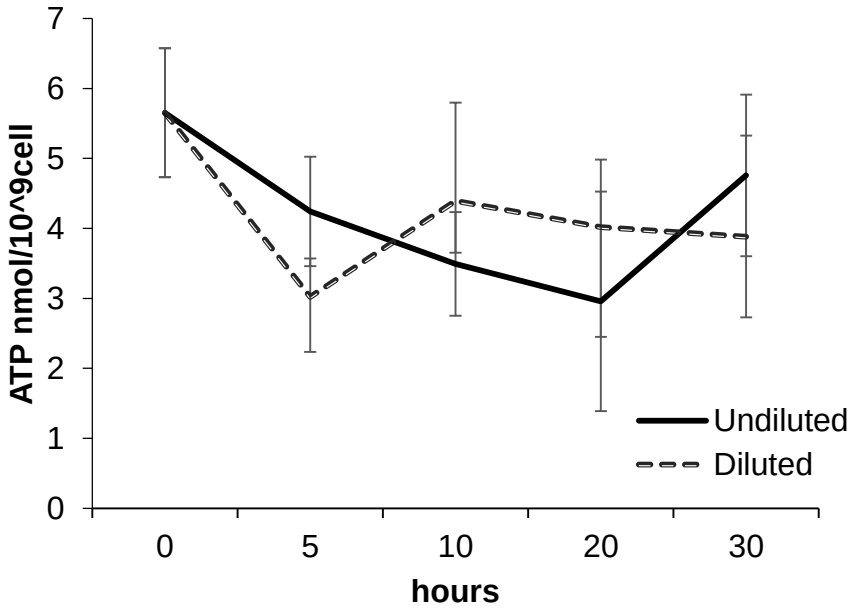

Figure 4 Mean ATP values observed (nmol per 10^9 cells) in diluted (D) and undiluted (UD) spotted wolffish sperm samples during the refrigerated storage in experiment 2. Values represent the mean ± SEM (n = 8).

The protein concentration was similar to the first experiment (0.76 ± 0.23 mg/ml). Fourteen protein bands were detected. The bands with molecular weights of 80, 70, 60, 20 and 15 kDa each represented more than 10% of the protein amount detected in the seminal plasma (Table 1). The 150 kDa protein band, although absent in three of the seminal plasma samples, had negative correlation with both percentage of motility (R=-0.966; p=0.007) and VCL (R=-0.964; p=0.008) at the time 0 h. The 80 kDa protein band was also negatively correlated with VCL at 30 h (R=-0.777; p=0.023). The remaining protein bands with higher percentage of protein were not correlated with either the percentage of motile sperm or VCL.

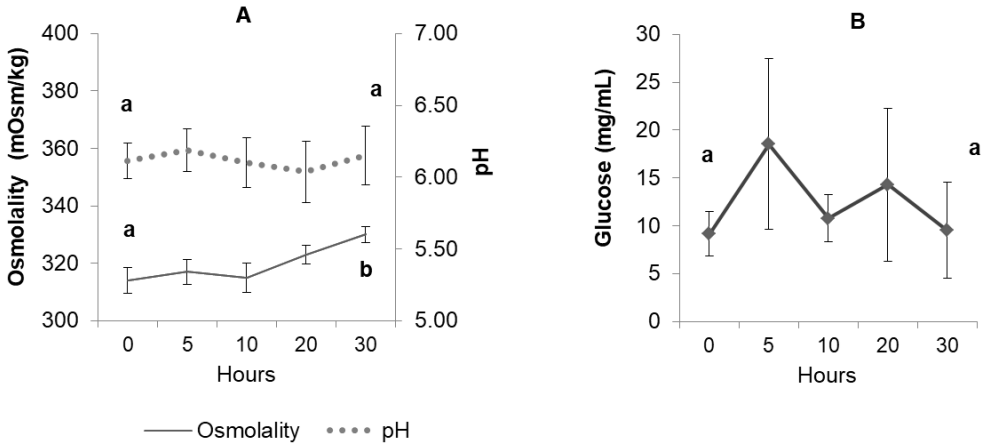

Figure 5 Osmolality (mOsm/kg), pH (a) and glucose (mg/mL) values (b) in seminal plasma of undiluted (UD) spotted wolffish sperm during the refrigerated storage time in experiment 2. Values represent the mean ± SEM (n = 8). Different lowercase letters indicate significant differences between the initial value and the final value at the end of short-term sperm storage.

Table 1. Protein bands according to the molecular weight found in seminal plasma of spotted wolffish. Values in the right column represent the mean percentage (n = 8).

Proteins (kDa)	Mean (%)
80	14.86
70	20.80
60	26.56
20	11.73
15	18.08

4. Discussion

Refrigeration of sperm could be a more practical approach for storage of spotted wolffish sperm for short periods (days) compared to the logistically complex and

expensive sperm cryopreservation protocols currently used. In both short-term storage experiments, after 30 h of refrigerated storage, the samples maintained percentage of sperm motilities between 10 and 20 % and sperm velocities close to 20 µm/s. These sperm quality parameters exhibited similar values to the cryopreservation method used in spotted wolffish (Santana *et al.* 2020). After cryopreservation, Santana *et al.* (2020), depending on the spermatozoa: egg ratio could obtain values above 80% fertilization. This indicates that the refrigeration method described in the present study can be used to store sperm for 1-2 days, diluted sperm samples retained higher percentage of motile sperm throughout the storage period.

After the storage time, the diluted sperm percentage of motility was only significantly higher in the second experiment. Nonetheless, in the first experiment, diluted samples retain similar percentage of motile cells through the storage, while undiluted had a significant decrease. The large range of initial samples quality in the first experiment could partly explain the difficulty to detect significant differences in storability in terms of percentage of motile sperm. Most authors report an improvement in samples storability by the use of an extender solution (Gallego *et al*. 2013; Santos et al. 2018; González-López *et al.* 2019). The use of these extender solutions is beneficial for several reasons such as reduces the sperm density and thus improves the oxygen supply; controls pH through the use of buffers such as Tris in our case; prevents the dehydration of the cells and decreases the harmful effect of urine contamination (reviewed by Contreras *et al.* 2019; Beirão *et al.* 2019). However, in most cases reported the extender resemble the seminal plasma and is designed to keep the sperm in quiescent immotile state until activation by contact with fresh (freshwater species) or seawater (marine species) (Contreras *et al.,* 2019), which is not the case of spotted wolffish. Few exceptions exist for internal fertilizing species, such as guppy *Poecilia reticulata* (Sun *et al.,* 2010), where the sperm was continuously motile in the extender solution. However, in these livebearers aquarium fish, such as the guppy (Sun *et al.,* 2010) or the green swordtail *Xiphophorus helleri* (Yang *et al.* 2006), it was

possible to recover sperm motility after inhibition with at low or high osmolality. In spotted wolffish preliminary trials indicate that it is not possible to recover motility after exposing to higher or low osmolality inhibiting solution (data not shown).

Several authors have correlated the urea levels with contamination by urine and decrease sperm quality (Dreanno *et al.* 1998; Fauvel *et al.* 2012; Król *et al.* 2018; González-López *et al*. 2019). Nonetheless, in our study, the level of urea concentration in seminal plasma was similar both in the samples obtained from testis or by stripping, and significantly lower than in urine. In addition, at the time of collection (0 h) there was no correlation between urea concentration and sperm quality parameters indicating that the low concentrations of urea in the present study did not affect sperm quality. Other studies have found that relatively low urine contamination does not necessarily affect the sperm quality, as was observed in Atlantic halibut *Hippoglossus hippoglossus* (Babiak *et al*. 2006) and European seabass *Dicentrarchus labrax* (Fauvel *et al.* 2012). In addition, seminal plasma pH and osmolality values are usually affected by contamination with urea. Which does not seem to be the case of our study, the urine pH and osmolalilty values were lower than the seminal plasma values (5.61 ± 0.13 vs 6.37 ± 0.06 for pH and 299.89 ± 4.94 mOsm/kg vs 319.56 ± 9.94 mOsm/kg for urine and seminal plasma respectively), and neither of them was correlated with the urea concentration in the seminal plasma. Whereas, the osmolality values were similar to the ones previously reported by Kime and Tveiten (2002) of 310-330 mOsm/kg, the seminal plasma pH obtained in our study was higher (pH of 4.8-7.7 in Kime and Tveiten 2002). This could indicate that part of the samples collected by Kime and Tveiten (2002) had some urine contamination. According to the methods described, these authors collected the sperm by direct pressure in the belly whereas we applied the sperm collection method recommended by Beirão and Ottesen (2018). In this case the urine is cleared before, and only after the sperm is collected. Thus, the procedures of sperm collection used in this work should be enough to avoid semen pollution with urine in spotted wolffish. However, the level of urea was positively correlated with percentage of motility assessed at 50 h after storage in undiluted samples, which indicates that the urea measured in the seminal plasma is more

related with the normal sperm protein metabolism. It would appear that higher numbers of motile sperm for a long time increased the urea concentration compared to samples with lower numbers of motile sperm.

Unexpectedly the ATP concentration remained stable along the storage time even though the percentage of motile sperm dropped from 44.56 ± 3.85 % to 20.83 ±5.02 % in diluted sperm and 15.79± 4.05 % in undiluted sperm. To our knowledge, all studies so far that have looked at ATP during sperm storage by refrigeration have observed a decrease in its concentration along the time (e.g, meagre *Argyrosomus regius* (Santos *et al*. 2018), rainbow trout *Oncorhynchus mykiss* (Bencic *et al.* 1999)). However, in most studies sperm is kept in a quiescent immotile state, whereas the sperm of spotted wolffish is motile on stripping. Thus, different conditions for refrigerated sperm storage need to be considered. Moreover, the initial ATP concentration of 5.65 ± 0.86 nmol/10^9 is relatively low compared with other fish species, usually in the range of 4 – 24 nmol/10^8 (revised by Dzyuba *et al.* 2017). In most species with external fertilization, the sperm activation occurs by an osmotic or ionic change, the motility period is very short (1-2 min) and at high velocity (> 100 µm/s), which leads to rapid decrease in ATP concentration (Dzyuba *et al.* 2017; Kowalski and Cejko 2019). Indeed, the intracellular ATP content is usually related with duration of the sperm motility period and sperm velocity, and the completion of the motility period is partly caused by low intracellular ATP (Dreanno *et al*. 1999; Dzyuba *et al.* 2017). In contrast, spotted wolffish is characterized by low velocity and long period of sperm motility as observed in the present study and reported by Kime and Tveiten (2002). In addition, the spermatozoa are characterized by a large midpiece with high number of mitochondria, as observed in the closely related common wolffish *Anarhichas lupus* (Pavlov *et al*. 1997). Thus, the low velocity, and thus low intracellular ATP consumption, can probably be compensated rapidly enough by mitochondrial synthesis of ATP via respiration for the 1 to 2 days period of motility usually observed.

Similar to the ATP also the seminal plasma glucose values remained constant during the storage time (30h). The initial glucose concentration obtained in this work (9.13 ± 2.32 µg/mL) was higher than the values observed in other marine species, such as gilthead seabream *Sparus aurata* 6.13 ± 4.68 µg/mL, total value for semen with cells, (Lahnsteiner *et al.* 2010) or turbot *Scophthalmus maximus* 0.92±0.20 µg/mL (Dreanno *et al.* 1998). As discussed, by Yang and Tiersch (2009) different species use different sources of energy for sperm motility. Whereas in some species sperm cells are able to use exogenous sources as carbohydrates, in other species the sperm cells apparently use exclusively endogenous sources of energy. As an example, both in African catfish *Clarias gariepinus* (Zietara *et al.* 2004) and in abant trout *Salmo trutta abanticus* (Hatipoglu and Akcay 2010) the addition of energetic substrates, such as glucose, helped maintain the ATP levels and improved motility during the storage time. On the other hand, in medaka *Oryzias latipes* the sperm motility is not affected by the presence or absence of glucose in the medium (Yang and Tiersch 2009). The fact that spotted wolffish seminal plasma glucose levels remain constant during the 30 h storage period does not mean it cannot use exogenous sources for the maintenance of the ATP levels, since it could be using other sources. Thus, this question should be further explored to aid in the development of refrigerated storage protocols for the sperm of this species.

The protein concentration in both experiments, compared with other marine species, was lower than in Atlantic halibut (6.4 to 19.4 mg/mL) (Mommens et al. 2008), but in similar range to Atlantic cod *Gadus morhua* (0.78 to 1.05 mg/mL) (Butts *et al.* 2011). The proteins contained in the seminal plasma are mostly involved in sperm protection, but each of them has different roles such as sperm maturation, cell death, respiration, lipid metabolism, energy production and motility (reviewed by Ciereszko *et al.* 2016). Indeed, most authors link the increase in specific proteins with improved sperm quality. For example, in European eel *Anguilla anguilla* samples with proteins with molecular weight less than 50 kDa were linked to increase in the sperm motility (Peñaranda *et al.* 2010). However, in

our work most of the correlations we observed between the proteins (total and protein bands) and sperm quality parameters were negative correlations. Spotted wolffish do not release sperm naturally in captivity, and thus the sperm obtained by stripping could be overripe and these proteins be related with cell death. However, in order to make such conclusion a more detailed proteomic study will be needed using for example markers for proteolytic activity (e.g. Mommens *et al.* 2008) or a 2-D gel analysis (e.g. Zilli *et al.* 2014) for protein identification. Nonetheless, the analysis of the proteins in the present experiment opens the possibility to use proteomic indicators for sperm selection.

5. Conclusions

In this work we present important data that will help in the future development of a refrigerated storage protocol for spotted wolffish sperm. First, wolffish sperm can be stored for 1-2 days, the dilution of the sperm in a medium improves its storability, and thus the composition of this medium should be further explored. Secondly, using the reported sperm collection method (Beirão and Ottesen 2018) urine contamination was avoided and hence did not affect the sperm storability. Finally, the sperm motility period is not limited by the ATP levels, that remain stable at least for the first 30h, however the energy source used to maintain these ATP levels is not clear.

6. References

Babiak, I., Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L. II: Effect of spermiation advancement, catheterization of semen, and production-scale application. Theriogenology 66:2036–2046. https://doi.org/10.1016/j.theriogenology.2006.06.002

Beirão, J., Boulais, M., Gallego, V., O'Brien, J. K., Peixoto, S., Robeck T. R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133:161–178. https://doi.org/10.1016/j.theriogenology.2019.05.004

Beirão, J., Ottesen, O.,H., 2018. Optimization of a fertilization protocol for spotted wolffish (*Anarhichas minor*). Aquaculture 484:133–138. https://doi.org/10.1016/j.aquaculture.2017.11.004

Bencic, D., C., Krisfalusi, M., Cloud, J., G., Ingermann, R., L., 1999. Maintenance of steelhead trout (*Oncorhynchus mykiss*) sperm at different in vitro oxygen tensions alters ATP levels and cell functional characteristics. Fish Physiol Biochem 21:193–200. https://doi.org/10.1023/A:1007880426488

Bobe, J., Labbé, C., 2008. Chilled storage of sperm and eggs. In: Cabrita E, Robles V, Herráez P (Eds.), Methods in reproductive aquaculture: marine and freshwater species, CRC Press, Boca Raton (2009), pp. 219-236

Butts, I., A., E., Trippel, E., A., Ciereszko, A., 2011. Seminal plasma biochemistry and spermatozoa characteristics of Atlantic cod (*Gadus morhua* L.) of wild and cultivated origin. Comp Biochem Physiol, Part A Mol Integr Physiol 159:16–24. https://doi.org/10.1016/j.cbpa.2011.01.014

Ciereszko, A., Dietrich, M., A., Nynca, J., 2016. Fish semen proteomics — New opportunities in fish reproductive research. Aquaculture. https://doi.org/10.1016/j.aquaculture.2016.03.005

Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farías, J., G., Valdebenito, I., Risopatrón, J., 2019. Effects of short-term storage on sperm function in fish semen: a review. Reviews in Aquaculture 0: https://doi.org/10.1111/raq.12387

Dreanno, C., Cosson, J., Suquet, M., et al 1999. Nucleotide content, oxydative phosphorylation, morphology, and fertilizing capacity of turbot (*Psetta maxima*) spermatozoa during the motility period. Mol Reprod Dev 53:230–243.

https://doi.org/10.1002/(SICI)1098-2795(199906)53:2<230::AID-MRD12>3.0.CO;2-H

Dreanno, C., Suquet, M., Desbruyères, E., E., Cosson, J., Le Delliou, H., Billard, R., 1998. Effect of urine on semen quality in turbot (*Psetta maxima*). Aquaculture 169:247–262. https://doi.org/10.1016/S0044-8486(98)00262-2

Dzyuba, B., Bondarenko, O., Fedorov, P., Gazo, I., Prokopchuk, G., Cosson, J., 2017. Energetics of fish spermatozoa: The proven and the possible. Aquaculture 472:60–72. https://doi.org/10.1016/j.aquaculture.2016.05.038

Falk-Petersen, I., B., Hansen, T., K., Fieler, R., Sunde, L., M., 1999. Cultivation of the spotted wolf fish *Anarhichas minor* (Olafsen) - a new candidate for cold-water fish farming. Aquac Res 30:711–718. https://doi.org/10.1046/j.1365-2109.1999.00392.x

Fauvel, C., Boryshpolets, S., Cosson, J., Suquet, M., 2012. Improvement of chilled seabass sperm conservation using a cell culture medium. Journal of Applied Ichthyology 28:961–966. https://doi.org/10.1111/jai.12071

Foss, A., Imsland, A., K., Falk-Petersen, I., B., Oiestad, V., 2004. A review of the culture potential of spotted wolffish *Anarhichas minor* Olafsen. Rev Fish Biol Fish 14:277–294. https://doi.org/10.1007/s11160-004-8360-9

Gallego, V., Pérez, L., Asturiano, J., F., Yoshida, M., 2013. Study of pufferfish (*Takifugu niphobles*) sperm: Development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca2+ and K+ in the initiation of sperm motility. Aquaculture 414–415:82–91. https://doi.org/10.1016/j.aquaculture.2013.07.046

González-López, W., Á., Ramos-Júdez, S., Giménez, I., Duncan, N., J., 2019. Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage. Aquaculture 734649. https://doi.org/10.1016/j.aquaculture.2019.734649

Gunnarsson, S., Sigurdsson, S., Thorarensen, H., Imsland, A., K., 2009. Cryopreservation of sperm from spotted wolffish. Aquac Int 17:385–389. https://doi.org/10.1007/s10499-008-9210-x

Hatipoglu, T., Akcay, E., 2010. Fertilizing ability of short-term preserved spermatozoa Abant trout (*Salmo trutta abanticus* T, 1954). Ank Univ Vet Fak Derg 57:33–38

Kime, D., E., Tveiten, H., 2002. Unusual motility characteristics of sperm of the spotted wolffish. J Fish Biol 61:1549–1559. https://doi.org/10.1006/jfbi.2002.2174

Kowalski, R., K., Cejko, B., I., 2019. Sperm quality in fish: Determinants and affecting factors. Theriogenology 135:94-108. 10.1016/j.theriogenology.2019.06.009

Król, J., Żarski, D., Bernáth, G., Palinska-Zarska, K., Krejszeff, S., Dlugonski, A., Horvath A., 2018. Effect of urine contamination on semen quality variables in Eurasian perch *Perca fluviatilis* L. Animal Reproduction Science 197:240–246. https://doi.org/10.1016/j.anireprosci.2018.08.034

Lahnsteiner, F., Mansour, N., Caberlotto, S., 2010. Composition and metabolism of carbohydrates and lipids in *Sparus aurata* semen and its relation to viability expressed as sperm motility when activated. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 157:39–45. https://doi.org/10.1016/j.cbpb.2010.04.016

Le Francois, N., Lamarre, S., Tveiten, H., Blier, P., U., Bailey, J., 2008. Sperm cryoconservation in *Anarhichas* sp., endangered cold-water aquaculture species with internal fertilization. Aquaculture International 16:273–279. https://doi.org/10.1007/s10499-007-9137-7

Le Francois, N., R., Archer, B., 2007. L'élevage du loup tacheté: reproduction et incubation. Direction de l'innovation et des technologies, Ministère de l'agriculture, des pêcheries et de l'alimentation du Québec.

http://www.mapaq.gouv.qc.ca/fr/publications/guidetechnique007_dit.pdf,Accessed date: September 2019.

Le François, N., R., Lemieux, H., Blier, P., U., 2002. Biological and technical evaluation of the potential of marine and anadromous fish species for cold-water mariculture. Aquaculture Research 33:95–108. https://doi.org/10.1046/j.1365-2109.2002.00652.x

Mommens, M., Wojtczak, M., Ciereszko, A., Babiak, I., 2008. Seminal plasma proteins of Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Physiol Biochem 34:349–355. https://doi.org/10.1007/s10695-007-9194-x

Pavlov, D., A., Knudsen, P., Emelyanova, N., G., Moksness, E., 1997. Spermatozoon ultrastructure and sperm production in wolffish (*Anarhichas lupus*), a species with internal fertilization. Aquat Living Resour 10:187–194. https://doi.org/10.1051/alr:1997020

Peñaranda, D., S., Marco-Jiménez, F., Pérez, L., Gallego, V., Mazzeo, I., Jover, M., Asturiano, J., F., 2010. Protein profile study in European eel (*Anguilla anguilla*) seminal plasma and its correlation with sperm quality. Journal of Applied Ichthyology 26:746–752. https://doi.org/10.1111/j.1439-0426.2010.01540.x

Santana, J., Cabrita, E., Eggen, B., Beirão, J., 2020. Step by step optimization of a sperm cryopreservation protocol for spotted wolffish (Anarhichas minor Olafsen1772).Theriogenology,149:16-24.https://doi.org/10.1016/j.therigenology. 2020.03.019.

Santos, M., Soares, F., Moreira, M., Beirão, J., 2018. Evaluation of different extenders for the cold storage of meagre (*Argyrosomus regius*) semen. Aquaculture Research 49:2723–2731. https://doi.org/10.1111/are.13733

Sarosiek, B., Dryl, K., Krejszeff, S., Żarski, D., 2016. Characterization of pikeperch (*Sander lucioperca*) milt collected with a syringe and a catheter. Aquaculture 450:14–16. https://doi.org/10.1016/j.aquaculture.2015.06.040

Shaliutina, A., Hulak, M., Gazo, I., Linhartova, P., Linhart, O., 2013. Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (Acipenser gueldenstaedtii) and Siberian (Acipenser baerii) sturgeon sperm. Animal Reproduction Science 139:127-135.

Smith, C., C., Ryan, M., J., 2010. Evolution of sperm quality but not quantity in the internally fertilized fish *Xiphophorus nigrensis*. Journal of Evolutionary Biology 23:1759–1771. https://doi.org/10.1111/j.1420-9101.2010.02041.x

Sun, C., Huang, C., Su, X., Zhao, X., Dong, Q., 2010. Optimization of handling and refrigerated storage of guppy *Poecilia reticulata* sperm. Journal of Fish Biology 77:54–66. https://doi.org/10.1111/j.1095-8649.2010.02658.x

Yang, H., Tiersch, T., R., 2009. Sperm motility initiation and duration in a euryhaline fish, medaka (*Oryzias latipes*). Theriogenology 72:386–392. https://doi.org/10.1016/j.theriogenology.2009.03.007

Yang, H., P., Hazelwood, L., Walter, R., B., Tiersch, T., R., 2006. Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*. Cryobiology 52:209–218. https://doi.org/10.1016/j.cryobiol.2005.11.002

Zietara, M., S., Slominska, E., Swierczynski, J., Rurangwa, E., Ollevier, F., Skorkowski, E., F., 2004. ATP content and adenine nucleotide catabolism in African catfish spermatozoa stored in various energetic substrates. Fish Physiol Biochem 30:119–127. https://doi.org/10.1007/s10695-005-2493-1

Zilli, L., Beirão, J., Schiavone, R., Herraez, M., P., Gnoni, A., Vilella, S., 2014. Comparative proteome analysis of cryopreserved flagella and head plasma membrane proteins from sea bream spermatozoa: effect of antifreeze proteins. PLoS ONE 9:e99992. https://doi.org/10.1371/journal.pone.0099992

Chapter 9

General discussion

The reproductive dysfunction presented in Senegalese sole cultured breeders has been a bottleneck to close the reproductive cycle in captivity and achieve sustainable culture. Although wild breeders produce fertilised eggs, the spawns from cultured breeders are not viable and do not provide larvae. This failure has been attributed to a lack of reproductive behaviour in cultured males (Carazo *et al*., 2016; Martin *et al*., 2019). Intrinsic behavioural factors in the reproductive process, reproductive dominance and social relationships into the broodstock may be linked to the reproductive failure (Carazo *et al.,* 2016; Martin *et al*., 2014; Fatsini 2017). Hence, the understanding of mechanisms for controlling of reproductive behaviour and social aspects in the rearing conditions, transmission of learning and behaviour amongst breeders (Huntingford, 2004; Guevara-Fiore, 2012; De gasperin and Macías, 2014), could provide possible solutions to the reproductive dysfunction. Alternatively, solutions that avoid the reproductive dysfunction in Senegalese sole may be explored. Procedures based in gamete management and *in vitro* fertilisation may ensure the mass production of viable eggs and sustainable sole farming by avoiding or bypassing the dysfunction. Therefore, in the present thesis, different options were examined to both solve and bypass the reproductive dysfunction in cultured breeders. **Chapters 2** and **3**, assessed the involvement of wild and cultured breeders in the reproductive behaviour and spawning and the effect of different social holding conditions on reproductive ability in cultured breeders. **Chapters 4** and **5**, examined the effect of dominance behaviour on reproductive success in wild and cultured breeders and reproductive potential in cultured males. **Chapters 6** and **7**, evaluated methods to preserve the quality and viability of sperm to perform *in vitro* fertilisation in Senegalese sole and determining the proper sperm to egg ratio to achieve high fertilisation rates. Finally, another specie was examined, the spotted wolffish (*Anarhichas minor*) that present a reproductive dysfunction and specific sperm characteristics, leading to the need to use *in vitro* fertilisation procedures to obtain fertilised eggs. **Chapter 8** analysed limiting factors in the preservation of sperm quality of spotted wolffish in a

refrigerated storage method and dilution solutions to maximize the time sperm are viable.

1.1 Reproductive behaviour and success in cultured breeders

Two experiments were conducted to evaluate the participation in the reproductive behaviour and the spawning in cultured breeders of Senegalese sole. In the first experiment (**Chapter 2**), three groups were formed: two mixed (wild and cultured breeders) and one control group (only cultured breeders). The chapter presented the last year (2016) of the experiment that was started in 2012 by Fatsini (2017) and the complete study was published by Fatsini *et al.* (2020). The participation in the courtship behaviour and the spawning of cultured sole breeders increased with the presence of wild breeders that successfully spawned (Mixed Groups). Fertile spawns were only recorded from the two mixed groups; while the Control Group produced fewer spawns that were not fertile. According to genetic analysis, the parental contribution was assigned to a cultured male that spawned with a cultured female and two wild females, held in a Mixed Group. The cultured couple spawned during two years, one year reported by Fatsini (2017) and a second year in Chapter 2**.** Significantly, more behaviours related to the courtship process described by Carazo (2016) were observed in the Mixed Groups. The second experiment, examined four groups that experienced different social conditions and learning opportunities during the juvenile stage until puberty (**Chapter 3**). Two replicate groups (W1 and W2) were reared prior to the experiment with spawning wild breeders. A positive control group (CP) was reared prior to the experiment with cultured breeders that liberated unfertilised eggs. A negative control (CN) was reared prior to the experiment in isolation as a single year class and had no contact with adult breeders during rearing. The observations performed during three reproductive seasons, showed in the first year, higher frequency of behaviours related to courtship in the Group W1, in comparison with other groups where the coupled swimming behaviour was not exhibited. Eight fertile spawns were recorded from Group W1, in the first year, while in other groups and following years no fertile spawns were obtained including Group W1. Cultured breeders appear to learn

reproductive behaviours and participate in fertile spawning, influenced by cohabitation with successful wild breeders either during the spawning season or prior to the spawning season before puberty. The behavioural development in cultured sole appeared to be related to the social learning opportunities experienced during the interaction between cultured and wild breeders in the two experiments. However, cultured soles do not seem to retain the learning of courtship behaviour and consequently spawning over time. Although cultured breeders learned and participated in courtship behaviour, both the mixed group (Chapter 2) and Group W1 (Chapter 3) exhibited low participation in spawning according to the analysis of parental contribution.

1.2 Reproductive behaviour and social learning

The reproductive behaviour could be considered innate; however, the early fish development conditions may influence cognitive ability, causing the loss of behavioural characteristics (Galef and Laland, 2005). The influence of genetic background and breeding environment on the general development of sensory systems may lead to generate differences in behaviour and learning in cultured fish (Huntingford 2004). In addition, previous experience and learning of complex courtship and spawning behaviours in cultured breeders may not be available as for wild fish in the natural environment. Differences in the reproductive behaviour and fertilisation success were found between cultured and wild Atlantic salmon (*Salmo salar*). The decline in reproductive success in cultured fish was related to artificial breeding and rearing conditions, which prevented the natural development of reproductive skills with respect to wild breeders (Fleming *et al.,* 1996). Similarly, in Amarillo fish (*Girardinichthys multiradiatus*), the courtship pattern in males was affected by ontogenetic factors and early social experience. However, this behaviour may be modified through learning from new experiences (De Gasperin and Macías 2014). In Senegalese sole, the reproductive behaviour has been described as a complex process, where the males play an important role to encourage the females to mate (Carazo *et al.*, 2016). The spawning is carried out by a single pair, after the male has gained acceptance from the female and the

approach to swim together to the surface to release and fertilise the eggs. However, differences in behaviour patterns amongst males have been associated with their reproductive success and as a determining factor in the mate choice by the females (Meager *et al*., 2018). Unlike wild breeders that perform the courtship, the lack of reproductive behaviour and spawning in cultured breeders could be linked to the loss of stimulation and learning of behaviour during the early development. Therefore, social learning from successful breeders would enable the development and acquisition of mating skills (Brown and Laland 2003; Huntingford 2004; Duncan *et al.,* 2019). Social learning involves information that is learned socially through observation or imitation of other experienced individuals (Brown and Laland 2003). The experience gained and knowledge transmitted from experienced males that successfully performed courtship would assist in the development of reproductive behaviour (Freeberg 2000) and would solve the reproductive dysfunction. In species such as Atlantic Cod (*Gadus morhua*), the development of behaviour was determined by learning process and conditioned by experience previously obtained (Meager *et al*., 2018). While in individuals of *Poecilia reticulata* and *P. picta*, the initial recognition and mate choice with preference for conspecifics, was a learned process by imitation or observation from adult wild fish (Dungatkin *et al*., 1992; Magurran and Ramnarine, 2004). Information transmitted from others may provide a useful guide to solve challenges, makes it possible to avoid the error and give solutions in inexperienced individuals (Dungatkin *et al*., 1992; Dungatkin and Godin 1993; Galef and Laland 2005). In this way, the social learning of courtship and mate choice will provide the ability to ensure the reproductive success (Galef and Laland, 2005). Therefore, the increase registered in courtship and spawning participation in cultured sole breeders could be attributed to the effect of social learning during the period of cohabitation with successful wild breeders (**Chapter 2**).

1.3 Social learning and information retention

The social transmission of information between the fish groups could be linked to learning opportunities during the development in early life stages. The behaviour

development and acquisition of information in the individuals may be favoured by certain environments and different experiences obtained (Dungatkin 2007). In some species, such as *Poecilia reticulata*, the transmission of social information and conformation of behaviour was tested with respect to the mate choice responses (Dungatkin 2007). Different juvenile fish groups were exposed to different social environments during their early development: a) interacting with males and females that are sexually receptive, b) kept with adult fish that did not choose a mate, and c) interacting with other juvenile fish. Only juvenile fish that were kept with adult fish that were sexually receptive developed the same mate choice pattern as adults. While in other groups the social transmission for the development of mate choice behaviour failed (Dungatkin 2007). Experiences in early development stages can influence the display of reproductive behaviour in males, which is necessary for successful reproduction (Guevara-Fiore *et al.,* 2012). In immature male guppies, a similar development of courtship behaviour was learned during the early juvenile stage by observing other males courting, unlike males that grew up in isolation (Guevara-Fiore *et al.,* 2012). Therefore, the differences in the behaviour formation in the fish were related to the social learning experienced during the developmental period in juvenile fish. In Senegalese sole, the reproductive behaviour development and participation in spawning of cultured breeders was observed in the Group W1, which had interaction with wild adult fish during the juvenile stage (**Chapter 3**). This behaviour appeared to be related with the experiences obtained by social transmission during the interaction period with successful wild fish. However, the experiment had a duration of three reproductive seasons and although during the first reproductive season reproductive behaviour was performed and eight fertile spawns were obtained, no fertile spawning was obtained in the second and third seasons. The loss of the sequence of fertile spawning in this group, suggested that, although the social learning and development of reproductive behaviour was acquired from spawning wild breeders, it does not appear to have been retained by cultured breeders over time. Recognition of learned information may be conditioned by response to a stimulus and associated with a memory process (Warburton, 2003; Bloch *et al.,* 2019). A

probable restriction in memory capacity, could affect the response to what was learned, limited to a short period of time (Hazlett, 2007; Brown *et al.,* 2013). In juvenile flatfish (*Paralichthys olivaceus*) flounder, the response to predation was learned from experienced conspecifics through observation; however the response was limited after 48 hours and this was attributed to memory capacity (Arai *et al*., 2007). The degradation of memories would cause the lack of recognition of processes and execution of decisions and functions (Warburton, 2003; Brown *et al.,* 2013; Bloch *et al.,* 2019). Perhaps, a reinforcement process with stimulus and cues would have a motivation to reactivate the learning obtained previously (Warburton, 2003; Hazlett, 2007). However, an apparent forgetting could be a strategy to ensure the learning of new experiences, updated to the recent social context (Bekinschtein *et al*., 2018). The expiration of information previously retained would help to adjust the memory capability, generating opportunities for storage of information required (Brown *et al.,* 2013). Advances in the process of developing reproductive behaviour in cultured breeders by learning from successful wild breeders highlights a potential solution to the reproductive dysfunction. However, apparent lack of retention of what was learned and low participation in spawns determined in breeders, could be mechanisms that limit the reproductive success in cultured breeders.

1.4 Participation in courtship and spawning.

The participation of breeders (wild or cultured) in the spawning in both experiments (Chapter 2 and Chapter 3), was low. This situation may be associated with fidelity between couples and reproductive dominance amongst breeders (Martin *et al.,* 2014; 2019). Senegalese sole is a species with monogamous spawning (Carazo et al., 2016; Duncan *et al*., 2019), unlike other species such as winter flounder where various males may be implied in the spawn with a single female, without agonistic behaviour (see in Gibson 2005). The formation of couples could have a criteria based on the display of dominance behaviour and the ability to execute the courtship behaviour (Carazo *et al.,* 2016; Duncan *et al*., 2019). A high participation was observed in the courtship and behaviours such as “Follow”, which has been

determined as an indicator of successful spawning (Fatsini *et al.,* 2020). The "Follow" behaviour is a type of procession and non-aggressive competition, mainly performed by males in the follower position and females in the leader position (Carazo *et al*., 2016; Fatsini *et al.,* 2020). In addition, the "Follow" behaviour could indicate a type of dominance. The participation and position in the "Follow" behaviour (leader or follower) of males was correlated with participation in spawning (Fatsini *et al*., 2020). However, in spite of this high participation in reproductive behaviours and a significant increase in the complex reproductive behaviour by inexperienced cultured breeders, participation in spawning was low. In mixed groups (Chapter 2), wild breeders were identified as dominant in spawning and the participation in spawns by cultured breeders was low (Chapter 2 and 3). However, the participation of cultured breeders in spawning after the death of a reproductively dominant wild male indicated the limitation in the spawning participation of cultured males may be due to a dominance effect of reproductive wild males. This mechanism seems to be contrary to cultured breeders developing reproductive behaviour as reproductive dominance minimises the achievement of fertile spawns (Fatsini *et al.,* 2020). While all spawns obtained in the Group W1 (cultured breeder in Chapter 3) showed the reproductive dominance by one couple. This suggests that hierarchies in reproduction are formed within groups, not related to breeders origin, but probably to the ability to execute courtship behaviour or other factors not yet described.

1.5 Dominance behaviour and reproductive success.

Control of limited resources such as food, territory and reproductive opportunity may lead to the development of behavioural patterns related to the establishment of social hierarchies within a population (Dey *et al*., 2015; Maytin and Ligocki, 2019). The dominance relationships, may determine the social position between individuals according to subsequent encounters between pairs within the stock (Chase *et al*., 2003). In juvenile sole, social dominance behaviour has been characterized with regard to feeding response and territory (Fatsini *et al*., 2017). However, the influence of dominance hierarchies in sole adults has not yet been

explored. In Senegalese sole broodstock, dominance hierarchies within groups may have influence in breeders in several aspects. Two experiments were carried out to assess the influence of dominance behaviour on: 1) reproductive success and participation in “Follow” behaviour in male and female breeders from wild and cultured origin (**Chapter 4**), and 2) reproductive potential in cultured males and the relation with sperm quality, hormone concentrations, testes size and testicular cell development (**Chapter 5**). In both experiments, dyadic tests were conducted to determine hierarchical categories between the fish pairs. Dominant and subordinate fish were determined by behavioural parameters, which had previously been associated to dominance in juvenile Senegalese sole (Fatsini *et al*. 2017). In both experiments, the behaviourally dominant fish coincided with the fish that dominated the preferred spatial parameter, last position. In the first experiment, dominance status classified by behaviours was related with the reproductive success (Chapter 4), defined by the participation in fertile spawns obtained during four reproductive seasons (Fatsini *et al.,* 2020; chapter 2). A higher proportion of dominant fish participated in fertile spawns in comparison with the subordinate fish. While a similar proportion of dominant and subordinate breeders participated in “Follow” behaviours. In the second experiment, dominant males had higher sperm motility, Gonadosomatic index and number of spermatozoa cells in the cortex area of testes compared to subordinate males (Chapter 5). However, the subordinate males maintained their reproductive potential, developing germ cells at all stages of maturation and similar sperm velocity parameters to dominant males, despite submission in social behavioural. In addition, the plasma levels of sex steroids were similar in subordinate and dominant fish and perhaps stimulated similarly the proliferation of germ cells and, therefore, ability to modulate sexual behaviour (Rudolfsen *et al*., 2006; Schulz *et al*., 2010).

The effect of dominant behaviour on individuals may impact on physical, physiological and behavioural aspects (Oliveira and Almada, 1996; Dey *et al*., 2015; Scaia *et al*., 2020). In species such as *Astatotilapia burtoni* and *Oreochromis mossambicus,* the social regulation had effects on behaviour (aggressive or submissive) and changes in colouration, size and reproductive success (Maruska

2014; Scaia *et al*., 2020). While in Senegalese sole juveniles, hierarchies caused growth heterogeneity amongst the individuals of a stock (Salas-Leitón *et al.,* 2011). In sole adults, dominance behaviour was related with the reproductive success (Chapter 4). Socially ranked dominant individuals have been previously described to participate successfully in reproduction while subordinate individuals were excluded (Dey *et al*., 2015). The social component such as the hierarchical position amongst breeders would be relevant to deduce the reproductive success (Colléter and Brown 2011; Pfenning *et al.,* 2012). In species such as medaka fish (*Oryzias latipes*) the dominance in the reproduction was linked to social dominance (Yokoi *et al.,* 2016). Socially dominant males have higher access to females as females tend to choose the dominant males for mating, securing the success in the offspring (Maekawa *et al*., 1996; Yokoi *et al.,* 2016; Laubu *et al*., 2017). Therefore, the mate choice and successful spawning were related to previous social interactions between individuals that established social position (Maekawa *et al*., 1996; Laubu *et al*., 2017). Moreover, in Senegalese sole the participation in mate choice and successful spawning was related to the ability of males to perform the reproductive behaviour (Fatsini *et al*., 2020; Chapter 2). Complex reproductive behaviour can challenge males to encourage females to mate. Therefore, the dominant males could have more access to females and be able to properly execute the courtship. However, in spite of the males ability to perform the courtship, the poor participation of breeders in spawning may be linked to the reproductive dominance and influenced by the social hierarchies (Martín *et al*., 2014; Martín *et al*., 2019; Fatsini *et al*., 2020; Chapter 2 and 3). As described above, few spawning couples were formed and fidelity between the pair often remained through reproductive seasons (Martín *et al*., 2014; Martín *et al*., 2019; Fatsini *et al*., 2020). The influence of dominance behaviour appears to limit participation in spawning. Furthermore, although “Follow” behaviour has been identified as indicative of reproductive success (Fatsini *et al*., 2020), it was not related with social dominance or territorial dominance (preferred space) (**Chapter 4)**. However, a correlation between the “Follow” and Rest the Head (RTH) behaviour was found. The RTH behaviour was associated or used to define social

dominance (Fatsini et al., 2017; Chapters 4 and 5) and was defined as a reproductive behaviour performed by males to approach and gain acceptance with females during the courtship (Carazo *et al.,* 2016).

Moreover, the dominance behaviour could be a factor that influences in the reproductive dysfunction, causing a failure both to development reproductive behaviour and controlling physiological changes in cultured breeders. In particular, the males are subject to higher competition and display of mating behaviours with the females (Maruska and Fernald 2011; Bierbach *et al*., 2014). However, under the dominance factor, the response to display the reproductive behaviour could implicate changes caused by the social categorical position in each individual (dominant and subordinate). Adjustment in the reproduction by social hierarchy could influence in physical and physiological adaptations linked to reproductive behaviour in fish (Maruska 2014). Social interactions between breeders transmit signals that regulate the neuro-molecular response and influence physiological changes such as the testes development. Socially disadvantaged fish could exhibit higher impacts on reproductive function, limiting the reproductive opportunities (Maruska and Fernald, 2011). In the species such as *Cichlasoma dimerus* and African cichlid *Astatotilapia burtoni*, subordinate fish might undergo reproductive suppression due to social domination by dominant fish (Maruska 2014; Scaia *et al.,* 2020). However, an investment in sperm production may offer mating opportunities, in a social rank adjustment (Pfennig *et al*., 2012; Kustan *et al.*, 2012; Maruska 2014). This adaptive mechanism may be perceived in the breeders and respond physiologically to the regulation of social position (Alonso *et al.,* 2012). In Senegalese sole, the opportunity to access mating was provided by the removal of a reproductively dominant wild male and the ascent of a cultured male in the ranking, to achieve fertile spawning was observed (Chapter 2). Subordinate males maintained a similar sperm production capacity as dominate fish (Chapter 5) and cultured individuals that did not participate in spawning had similar or better sperm quality compared to wild males that participated in spawning (Chapter 2). Although some effects on reproductive potential in cultured male breeders may be related to the social dominance (Chapter 5), physiological characteristics and reproductive

capacity appear to be maintained to response to the reproductive likelihood. Therefore, the social and reproductive dominance could be a limiting to the reproductive success of sole breeders, which would partly explain the scarce parental contribution in Groups mixed (Chapter 2) and Group W1 (Chapter 3).

The reproductive dysfunction related to the lack of courtship behaviour in cultured breeders has been a bottleneck to control their reproduction. Behavioural aspects had influence on spawning success in culture sole breeders. The presence of spawning wild breeders increased the participation in the courtship and obtaining fertile spawning in cultured sole breeders (Fatsini *et al.,* 2020; Chapter 2). However, a low participation of breeders in parental contribution was evident in all groups where fertile spawning were recorded (Chapters 2 and 3). This limited participation was attributed to the establishment of dominance hierarchies within broodstock. Social and reproductive dominance (Chapter 4) were shown to influence reproductive success. Dominance behaviour of spawning breeders suppressed the participation in fertile spawning in cultured breeders, while the cohabitation with spawning breeders aids development of reproductive behaviour in cultured breeders. Both aspects appear to have the opposite influence on reproductive success of cultured breeders. Further studies are required on these two aspects in the same broodstocks to determine how these opposing influences can be controlled to achieve successful spawning of cultured broodstock.

1.6 Methods to preserve sperm quality and *in vitro* fertilisation.

The behavioural approaches have explored how to solve the reproductive dysfunction; however, a clear solution to the reproductive dysfunction was not obtained. Therefore, an alternative approach to this reproductive failure is developing artificial fertilisation procedures to obtain fertilised eggs, using gametes stripped from mature cultured Senegalese sole (Liu *et al*., 2008; Rasines *et al*., 2012; 2013).

In aquaculture, *In vitro* fertilisation represents an option to obtain fertile eggs and to establish breeding programs (Beirão *et al.,* 2019) for several captive species affected by reproductive dysfunctions. However optimizing the protocols and critical parameters of gamete preservation are necessary to ensure that viable gametes are available at the same time to obtain high fertilisation and hatching success. Senegalese sole and spotted wolffish *(A. minor)*, are two species that present reproductive dysfunctions in captivity and instability in the control of reproduction to obtain fertile eggs (Carazo et al., 2016; Beirão and Ottesen 2018). Although both species produce viable gametes, spontaneous spawns are not performed. Gamete synchronization from both sexes is often difficult to achieve, hence management for the preservation of gametes is crucial for fertilisation (Falk-Petersen et al. 1999; Beirão and Ottesen 2018). In Senegalese sole, low volume and quality of sperm (Cabrita *et al.,* 2006; Beirão *et al.,* 2009) complicate this procedure. The scarce sperm production has been related to the small size of testes with semi-cystic spermatozoa development and reproductive strategy (García-López *et al*., 2005; Carazo *et al*., 2016). While in spotted wolffish, sperm is motile on stripping and currently it is not possible to immobilize and reactivate (Kime and Tveiten 2002). Therefore, a longer duration of sperm motility is required to synchronize availability of sperm with the ovulation of females. Sperm preservation protocols have been developed to maintain the viability of gametes to short and long term. Cryopreservation protocols have been described to ensure the availability of sperm (Le Francois *et al*. 2008; Gunnarsson *et al*. 2009; Rasines *et al*., 2012; Santana et al. 2020). In flatfish such as turbot (*Scophthalmus maximus*) cryopreservation protocols have been developed to maintain maximum gamete quality and obtain a high rate of fertilised eggs from artificial fertilisation procedures (Chereguini *et al.,* 1997). While in Atlantic halibut (*Hippoglossus hippoglossus*), cryopreservation protocols were applied on a commercial scale ensuring reliable sperm volume (Ding *et al.,* 2011). However, it could be a complex procedure that requires special equipment and trained personnel to carry out and samples visibly contaminated by urine, faeces or blood would be discarded (Cabrita *et al*., 2006). Therefore, a simple and inexpensive procedure is needed to be used in hatcheries

during short-term storage. Cold storage methods are used as a procedure relatively easy to implement in the hatchery environment (Bobe and Labbé 2008; Contreras et al. 2019). However, the sperm conditions may limit the duration of the required storage period. Collecting methods, contamination in sperm samples and an improper sperm handling may cause negative effect in sperm quality and decreasing the ability of the sperm to fertilize eggs (Bobe and Labbé 2008;). Therefore, short-term cold storage procedures and use of diluent solutions to preserve the sperm quality post-collection and prevent the damage of the spermatozoa were tested in two experiments. In Senegalese sole, sperm samples collected by stripping from cultured breeders were diluted in five different solutions and undiluted samples were stored during 24 hours and sperm quality parameters were recorded (**Chapter 6**). In spotted wolffish diluted and undiluted sperm samples were assessed in the sperm quality parameters until sperm motility ceased and variation in ATP values were measured during the test (**Chapter 8**). Possible urine contamination in sperm was explored in both experiments to determine its effect on sperm quality. In both experiments the diluted sperm samples maintained higher sperm motility in short term cold storage in comparison with undiluted samples. The elements of diluent solutions provide stability to sperm components and antimicrobial action inhibiting the loss of motility and prolong the viability of sperm over time (Bobe and Labbé, 2008). Also, reduce the sperm density and thus improves the oxygen supply, controls pH through the and decreases the harmful effect of urine contamination (reviewed by Contreras *et al*. 2019; Beirão *et al.* 2019). In sperm samples of Senegalese sole, urine contamination showed adverse effects on sperm quality. Changes were registered in osmolality and pH, which prematurely triggered sperm activation causing a decrease in sperm motility and velocity parameters needed to fertilise the eggs. The closeness between the reproductive and urinary systems often causes sperm contamination by urine when the sperm is collected. Similar effects of urine contamination on sperm quality were recorded in species such as turbot (*Scophthalmus maxima*) (Dreanno *et al.,* 1998), tilapia (*Oreochromis mossambicus*) (Linhart *et al.,* 1999), rainbow trout (*Oncorhynchus mykkis*) (Nynca

et al., 2012) and Eurasian perch (*Perca fluviatilis L*.) (Król *et al.,* 2018). In freshwater fish, the urine contamination cause a negative effect on osmolality values by prematurely activating sperm motility (Linhart *et al*., 2003; Król et al., 2018), while in marine fish, the variation in seminal plasma composition and intracellular ATP, reduce motility percentage and sperm velocity (Dreanno *et al*., 1998, Fauvel *et al*., 2012). The decrease in sperm quality prohibits proper storage either short or long term and reduced the possibility to achieve success *in vitro* fertilisation (Ciereszko *et al*., 2010; Beirão *et al*., 2019). In spotted wolffish, the sperm motility values recorded at end of test in diluted samples was similar to those reported by Santana et al. (2020), during the sperm cryopreservation process. In short-term refrigerated sperm storage the viability of sperm samples lasted between 1 and 2 days. Urine contamination and energy reserves were not limiting factors in extending the period of sperm motility in sperm refrigeration protocols. The samples did not show negative effects by urine contamination and ATP values remained stable during 30h after sperm collection and osmolality and pH values were in the range reported by Kime and Tveiten (2002). Therefore, short-term sperm refrigerated storage may be a useful method to preserve sperm quality in both species and provide an easy and inexpensive tool to assist in artificial fertilisation programs in fish farms.

The difficulty of collecting enough uncontaminated sperm in Senegalese sole may be a limit to obtain enough sperm to complete large-scale commercial *in vitro* fertilisation. Viable sperm management is needed to provide the sole aquaculture industry a sustainable alternative for obtaining fertilised eggs from cultured breeders. Although *In vitro* fertilisation procedures for Senegalese sole has been performed on an experimental scale (Rasines et al., 2013, 2012) and on an industrial scale (Liu et al. 2008) the spermatozoa to egg ratio required has not been established. Consequentially, it has not been possible to develop protocols to manage the low sperm volumes collected and plan *in vitro* fertilisation procedures to achieve efficient large-scale fertilisation of eggs. Therefore, appropriate sperm to egg ratios for the use of limited sperm volume for the successful experimental and massive *in vitro* fertilisation in Senegalese sole (*S. senegalensis*) was examined

(**Chapter 7**). A low spz egg^{-1} ratio was required to achieve high percentage of fertilisation and larval hatching, which was coherent and related to reproductive strategies in Senegalese sole. A mean fertilisation of 70 ± 14 % was recorded in the massive *in vitro* fertilisations performed with mean motile spermatozoa to egg ratio of 592 ± 611 spz egg^{-1} for in excess of 160,000 eggs and using the sperm from a single male. The *in vitro* fertilisation procedure is an alternative option for solving the reproductive behaviour problem in captive species in the aquaculture industry to obtain viable eggs from cultured broodstock. The improved gamete management and optimization of using low volumes of sperm applied to two species, spotted wolffish and Senegalese sole has facilitated the development of protocols for the large-scale application of *in vitro* fertilisation procedures for these species. These procedures allow the control in reproduction and implementation of breeding programs that will improve growth and product quality.

The advances registered in this thesis, have increased the understanding of the reproductive dysfunction in Senegalese sole cultured breeders. The increase in the participation in reproductive behaviour and spontaneous fertile spawning by cultured breeders appeared to be linked to the opportunities for development of reproductive behaviour through social learning with experienced conspecifics. However, the reproductive behaviour may be suppressed by social status amongst breeders. Further studies are required to obtain sufficient spontaneous fertile spawning from cultured Senegalese sole broodstock for aquaculture. An alternative solution, *in vitro* fertilisation was developed, as a method that would ensure mass production from cultured breeders of larvae for the aquaculture industry.

2. References

Alonso, F., Honji, R. M., Moreira, R. G., Pandolfi, M. 2002. Dominance hierarchies and social status ascent opportunity: Anticipatory behavioral and physiological adjustments in a Neotropical cichlid fish. Physiology & Behavior, Volume 106, Issue 5, Pages 612-618. Doi.org/10.1016/j.physbeh.2012.04.003.

Arai, T., Tominaga, O., Seikai, T., Masuda, R., 2007. Observational learning improves predator avoidance in hatchery-reared Japanese flounder *Paralichthys olivaceus* juveniles. Journal of Sea Research 58: 59–64.

Baynes, S., M., Howell, B., R., Beard, T., W., Hallam, J., D., 1994. A description of spawning behaviour of captive dover sole, *Solea solea* (L.). Netherlands Journal of Sea Research, Volume 32, Issues 3–4, Pages 271-275 doi.org/10.1016/0077-7579(94)90004-3.

Beirão, J., Cabrita, E., Soares, F., Herraez, M. P., Dinis, M. T., 2008. Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: comet analysis and Annexin V–Fluorescein staining. Journal of Applied Ichthyology 24:508–13.

Beirão, J., Soares, F., Herraez, M. P., Dinis M. T., Cabrita E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72(9): 1251-1261.

Beirão J, Ottesen OH (2018) Optimization of a fertilization protocol for spotted wolffish (*Anarhichas minor*).Aquaculture 484:133–138. https://doi.org/10.1016/j.aquaculture.2017.11.004

Beirão, J., Boulais, M., Gallego, V., O'Brien, J. K., Peixoto, S., Robeck T. R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133: 161-178.

Bekinschtein, P., Weisstaub, N.V., Gallo, F., Renner, M., Anderson, M.C., 2018. A retrieval-specific mechanism of adaptive forgetting in the mammalian brain Nature Communications 9:4660. DOI: 10.1038/s41467-018-07128-7

Bierbach, D., Oster, S., Jourdan, J., Arias-Rodriguez, L., Krause, Wilson, A. J., M., Plath, M., 2014. Social network analysis resolves temporal dynamics of male dominance relationships. Behav Ecol Sociobiol 68:935–945.DOI 10.1007/s00265-014-1706-y

Bobe, J., Labbé C., 2009. Chilled storage of sperm and eggs, in Cabrita, E., Robles, V., Herraez, P (Eds.), Methods in Reproductive Aquaculture. Boca Raton, FL: CRC Press Taylor and Francis Group, pp. 219-231.

Bloch, S., Froc, C., Pontiggia, A., Yamamoto, K., 2019. Existence of working memory in teleosts: Establishment of the delayed matching-to-sample task in adult zebrafish. Behavioural Brain Research 370: 111924. https://doi.org/10.1016/j.bbr.2019.111924

Bombardelli, R.,A., Sanches, E.,A., Baggio, D.,M., Sykora, R.,M., Souza, B.,E. de, Tessaro, L., Piana, P.,A., 2013. Effects of the spermatozoa: oocyte ratio, water volume and water temperature on artificial fertilization and sperm activation of cascudo-preto. Rev. Bras. Zootec. 42, 1–6. https://doi.org/10.1590/S1516-35982013000100001

Brown, C., Laland, K. N., 2003. Social learning in fishes: A review. Fish and Fisheries 4: 280- 288.

Brown, G.E., Ferrari, M.C.O., Chivers, D.P., 2013. Adaptive Forgetting: Why Predator Recognition Training Might Not Enhance Post stocking Survival. Fisheries (38): 1, 16-25.

Cabrita, E., Soares, F., Dinis M. T., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975.

Cabrita, E., Soares, F., Beirão, J., Garcia-Lopez, A., Martinez-Rodriguez, G., Dinis, M.T., 2011. Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. Theriogenology 75(1): 1-9.

Carazo, I., Chereguini, O., Martín, I., Huntingford, F., Duncan, N. 2016. Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*). Spanish Journal of Agricultural Research 14, 13pp. https://dx.doi.org/10.5424/sjar/2016144-9108

Ciereszko, A., Dietrich, G. J., Dietrich, M. A., Nynca, J., Kuźmiński, H., Dobosz S., Grudniewska J., 2010. Effects of pH on sperm motility in several Salmoniformes species: *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Salmo trutta*, *Salmo salar* and *Thymallus thymallus*. Journal of Applied Ichthyology 26(5): 665-667.

Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farías, J., G., Valdebenito, I., Risopatrón, J., 2019. Effects of short-term storage on sperm function in fish semen: a review. Reviews in Aquaculture 0: https://doi.org/10.1111/raq.12387

Chase, I., D., Tovey, C., Murch, P., 2003. Two's company, three's acrowd: differences in dominance relationships in isolated versus socially embedded pairs of fish. Behaviour 140:1193-1217.

Chereguini, O., Cal, R. M., Dreanno, C., deBaulny, B.O., Suquet M., Maisse, G., 1997. Short-term storage and cryopreservation of turbot (*Scophthalmus maximus*) sperm. Aquatic Living Resources 10(4): 251-255.

Chereguini, O., de la Banda, I.G., Rasines, I., Fernandez, A., 1999. Artificial fertilization in turbot, Scophthalmus maximus (L.): different methods and determination of the optimal sperm-egg ratio. Aquac. Res. 30, 319–324. https://doi.org/10.1046/j.1365-2109.1999.00326.x

Colléter, M., Brown, C. 2011. Personality traits predict hierarchy rank in male rainbowfish social groups. Animal Behaviour, 81(6), 1231-1237.

De Gasperin, O., Macías, G. C., 2014. Congenital predispositions and early social experience determine the courtship patterns of males of the Amarillo fish. Behav Ecol Sociobiol. 68:639–648 DOI 10.1007/s00265-013-1678-3

Dey, C., J., Tan, Q.Y. J., O'connor, C., Reddon, M., A., R., Caldwell, J., R., Balshine, S., 2015. Dominance network structure across reproductive contexts in the cooperatively breeding cichlid fish *Neolamprologus pulcher* . Current Zoology 61 (1): 45–54

Ding, F., Lall, S.P., Li, J., Lei, J., Rommens, M., Milley, J. E., 2011. Cryopreservation of sperm from Atlantic halibut (*Hippoglossus hippoglossus, L*.) for commercial application. Cryobiology, Volume 63, Issue 1, Pages 56-60.

Dreanno, C., Suquet, M., Desbruyeres, E., Cosson, J., Le Delliou, H., Billard, R., 1998. Effect of urine on semen quality in turbot (*Psetta maxima*). Aquaculture 169(3-4): 247-262.

Dugatkin, L.A., 1992. Sexual selection and imitation: females copy the mate choice of others. Am. Nat. 139, 1384-1489.

Dugatkin, L.A., Godin, L., A., 1993. Female mate copying in the guppy (Poecilia reticulata): age-dependent effects. Behavioral Ecology Vol. 4 No. 4: 289- 292.

Dugatkin, L.A., 2007. Developmental environment, cultural transmission, and mate choice copying. Naturwissenschaften 94:651–656. DOI 10.1007/s00114-007-0238-y.

Duncan, N., Carazo, I., Chereguini, O., Mañanós, E. 2019. Mating Behaviour. In: Muñoz-Cueto, J.A., Mañanós-Sánchez, E.L., Sánchez- Vázquez, F.J. (Eds). The Biology of Sole. Boca Raton, FL: CRC Press Taylor and Francis Group. Pp. 169-184.

Falk-Petersen IB, Hansen TK, Fieler R, Sunde LM (1999) Cultivation of the spotted wolf fish *Anarhichas minor* (Olafsen) - a new candidate for cold-water fish farming. Aquac Res 30:711–718. https://doi.org/10.1046/j.1365-2109.1999.00392.x

Fatsini, E., 2017. Reproduction, olfaction and Dominance behaviour in Senegalese sole (*Solea senegalensis*). PhD Thesis. Polytechnic University of Valencia. 203 pp.

Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Mackenzie, S., Duncan, N.J. 2017. Dominance behaviour in a non-aggressive flatfish, Senegalese sole (*Solea senegalensis*) and brain mRNA abundance of selected transcripts. PLoSONE 12 (9): e0184283.https://doi.org/10.1371/journal. pone.0184283

Fatsini, E., González, W., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

Fleming, I. A., Jonsson, B., Gross, M. R., Lamberg, A. 1996. An Experimental Study of the Reproductive Behaviour and Success of Farmed and Wild Atlantic Salmon (*Salmo salar*). Journal of Applied Ecology, Vol. 33, No. 4, pp. 893-905.

Freeberg, T., M., 2000. Culture and courtship in vertebrates: a review of social learning and transmission of courtship systems and mating patterns. Behavioural Processes 51, 177–192.

Galef, B., G., Laland, K., N., 2005. Social Learning in Animals: Empirical Studies and Theoretical Models. BioScience, Vol. 55 No. 6, 489-500.

Gallego, V., Pérez, L., Asturiano, J.F., Yoshiba, M., 2013. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture, 416-417: 238-243.

Garcia-Lopez, A., Martinez-Rodriguez, G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole, *Solea senegalensis* (Kaup): Anatomy, histology and histochemistry. Histology and Histopathology 20(4): 1179-1189.

Gibson, R., 2005. Flatfishes: Biology and Exploitation: Blackwell Science Ltd. a Blackwell Publishing Company. 391 pp.

Guevara-Fiore, P., 2012. Early social experience significantly affects sexual behaviour in male guppies. Animal Behaviour 84: 191-195. doi:10.1016/j.anbehav.2012.04.031

Gunnarsson S, Sigurdsson S, Thorarensen H, Imsland AK (2009) Cryopreservation of sperm from spotted wolffish. Aquac Int 17:385–389. https://doi.org/10.1007/s10499-008-9210-x

Hazlett, B.A., 2007. Conditioned reinforcement in the crayfish *Orconectes rusticus.* Behaviour, Vol. 144, No. 7, pp. 847-859.

Huntingford, F. A., 2004. Implications of domestication and rearing conditions for the behaviour of cultivated fishes. Journal of Fish Biology 65 (Supplement A): 122–142. doi:10.1111/j.1095-8649.2004.00562.

Krol, J., Zarski, D., Bernath, G., Palinska-Zarska, K., Krejszeff, S., Dlugonski, A., Horvath A., 2018**.** Effect of urine contamination on semen quality variables in Eurasian perch *Perca fluviatilis L*. Animal Reproduction Science 197: 240-246.

Kustan, J.M., Maruska, K.P., Fernald, R.D. 2012. Subordinate male cichlids retain reproductive competence during social suppresion. Proceedings of the Royal Society. 279: 434-443.

Laubu, C., Schweitzer, C., Motreuil, S., Louâpre, P., Dechaume-Moncharmont F., X., 2017. Mate choice based on behavioural type: do convict cichlids prefer similar partners? Animal Behaviour 126: 281-291.

Le Francois N, Lamarre S, Tveiten H, et al (2008) Sperm cryoconservation in *Anarhichas* sp., endangered cold-water aquaculture species with internal fertilization. Aquaculture International 16:273–279. https://doi.org/10.1007/s10499-007-9137-7

Linhart, O., Walford, J., Sivaloganathan, B., Lam, T. J., 1999. Effects of osmolality and ions on the motility of stripped and testicular sperm of freshwater- and seawater-acclimated tilapia, *Oreochromis mossambicus*. Journal of Fish Biology 55(6): 1344-1358.

Liu, X., Lian, J., Wang Y., Zhang F., Yu, H., Ma, A., Liu, S., Zhai, J., 2008. Large scale artificial reproduction and rearing of Senegal sole, *Solea senegalensis* Kaup. Marine Fisheries Research 29 (2): 10-16.

Maekawa, K., Iguchi, K., Katano, O., 1996. Reproductive success in male Japanese minnows, *Pseudorasbora parva*: observations under experimental conditions. Ichthyol. Res., 43 (3): 257-266.

Magurran, A.E, Ramnarine, I.W., 2004. Learned mate recognition and reproductive isolation in guppies. Animal Behaviour, 67, 1077e1082 doi: 10.1016/j.anbehav.2003.10.010

Manabe, H., Ide, M., Shinomiya, A., 2000. Mating system of the lefteye flounder,*Engyprosopon grandisquama*. Ichthyological Research volumen 47, pages 69–74.

Martín, I., Rasines, I., Gómez, M., Rodriguez, C., Martinez, P., Chereguini, O. 2014. Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons. Aquaculture 424-425, 45-52. https://doi.org/10.1016/j.aquaculture.2013.12.042 537

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernandez, R., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Spanish Journal of Agricultural Research, [S.l.], v. 17, n. 4, p. e0608. ISSN 2171-9292 doi:http://dx.doi.org/10.5424/sjar/2019174-14953.

Martinez-Pastor, F., Cabrita, E., Soares, F., Anel, L., Dinis, M. T., 2008. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 135(4): 449-459.

Maruska, K.P., Fernald, R. D. 2011. Social regulation of Gene expression in the Hypothalamic-Pituitary-Gonadal Axis. Physiology 26: 412-423.Doi: 10.1152/physiol.00032.2011.

Maruska, K.P. 2014. Social regulation of reproduction in male cichlid fishes. General and Comparative Endocrinology. 207: 2-12.

Maytin, A., K., Ligocki, I., Y., 2019. Dominance hierarchy establishment in the invasive round goby, *Neogobius melanostomus*. Behavioural Processes 158: 41-46. https://doi.org/10.1016/j.beproc.2018.10.024

Meager, J., J., Ferno, A., Skjæraasen, J. E., 2018. The behavioural diversity of Atlantic cod: insights into variability within and between individuals. Rev Fish Biol Fisheries 28:153–176 https://doi.org/10.1007/s11160-017-9505-y.

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M. and Dinis, M. T. 2016, New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture 8, 227–263. https://doi.org/10.1111/raq.12091

Moyer, J., T., Yogo, Y., Zaiser, M., J., Tsukahara, H., 1985. Spawning Behavior and Social Organization of the Flounder Crossorhombus kobensis (Bothidae)at Miyake-jima,Japan. Japanese Journal of Ichthyology Vol.32,No.3. DOI: 10.11369/jji1950.32.363

Nynca, J., Dietrich, G. J., Kuźmiński, H., Dobosz, S., Ciereszko, A., 2012. Motility activation of rainbow trout spermatozoa at pH 6.5 is directly related to contamination of milt with urine. Aquaculture 330-333: 185-188.

Oliveira, R. F., Almada, V. C. 2007. On the (In)stability of Dominance Hierarchies in the Cichiid Fish Oreochromis mossambicus. AGGRESSIVE BEHAVIOR Volume 22, pages 37-45.

Parker, G.A., Pizzari, T., 2010. Sperm competition and ejaculate economics. Biol. Rev. no-no. https://doi.org/10.1111/j.1469-185X.2010.00140.x

Pfennig, F., Kurth, T., Meißner, S., Standke, A., Hoppe, M., Zieschang, F., Reitmayer, C., Göbel, K., Gutzeit, H.O. 2012. The social status of the male Nile tilapia (*Oreochromis niloticus*) influences testis structure and gene expression. Reproduction 143:71-84.

Poupard, G. P., Paxion, C., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1998. Initiation of carp spermatozoa motility and early ATP reduction after milt contamination by urine. Aquaculture 160(3-4): 317-328.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326-329: 129–135.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2013. Artificial fertilisation of cultured Senegalese sole (*Solea senegalensis*): effects of the time of day of hormonal treatment on inducing ovulation. Aquaculture 392–395: 94–97.

Riesco, M.F., Oliveira C., Soares F., Gavaia, P.J., Dinis, M.T., Cabrita, E., 2017. *Solea senegalensis* sperm cryopreservation: New insights on sperm quality. PLoS ONE 12(10): e0186542. https://doi. org/10.1371/journal.pone.0186542

Riesco, M. F., Valcarce, D. G., Martínez-Vázquez, J. M., Martín, I., Calderón-García, A. A., González-Nunez, V., Robles V., 2019. Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question. Reproduction, Fertility and Development. https://doi.org/10.1071/RD18453

Rudolfsen, G., Figenschou, L., Folstad, I., Tveiten, H., Figenschou, M. 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc Biol Sci. 273(1584): 325–332. https://doi.org/10.1098/rspb.2005.3305.

Rurangwa, E., Kime, D. E., Ollevier, F., Nash J. P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234(1-4): 1-28.

Salas-Leiton, E., Anguis, V., Rodríguez-Rúa, J.,P., Cañavate, J.,P., 2011. High stocking density and food restriction have minimum impacto n size dispersal of cultured Senegalese sole (*Solea senegalensis* Kaup 1858) juveniles. Evidence for individual growth being regulated by population structure. Aquacultural Engineering 45:43-50.

Sanches, E.A., Caneppele, D., Okawara, R.Y., Damasceno, Z., Bombardelli, R.A., Romagosa, E., 2016. Inseminating dose and water volume applied to the artificial

fertilization of Steindachneridion parahybae (Steindachner, 1877) (Siluriformes: Pimelodidae): Brazilian endangered fish. Neotropical Ichthyol. 8.

Scaia, M., F., Morandini, L., Noguera, C., A., Trudeauc, V., L., Somozad, G., M., Pandolfi, M., 2018. Can estrogens be considered as key elements of the challenge hypothesis? The case of intrasexual aggression in a cichlid fish. Physiology & Behaviour 194: 481–490.

Schulz, R.W., de Franca L.R., Lareyre J.J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., (2010). Spermatogenesis in fish. Gen Comp Endocrinol 165:390–411. DOI:10.1016/j.ygcen.2009.02.013

Stockley, P., Gage, M.J.G., Parker, G.A., work(s):, and A.P.M.R., 1997. Sperm Competition in Fishes: The Evolution of Testis Size and Ejaculate Characteristics. Am. Nat. 149, 933–954.

Yanagimachi, R., Harumi, T., Matsubara, H., Yan, W., Yuan, S., Hirohashi, N., Iida, T., Yamaha, E., Arai, K., Matsubara, T., Andoh, T., Vines, C., Cherr, G.N., 2017. Chemical and physical guidance of fish spermatozoa into the egg through the micropyle†,‡. Biol. Reprod. 96, 780–799. https://doi.org/10.1093/biolre/iox015

Yokoi, S., Ansai, S., Kinoshita, M., Naruse, K., Kamei, Y., Young, L., J., Okuyama, T., Takeuchi, H., 2016. Mate-guarding behaviour enhances male reproductive success via familiarization with mating partners in medaka fish. Frontiers in Zoology 13:21 DOI 10.1186/s12983-016-0152-2

Valcarce, D.G., Robles, V., 2016 .Effect of captivity and cryopreservation on ROS production in *Solea senegalensis* spermatozoa. Reproduction 152: 439–446. DOI: 10.1530/REP-16-0270.

Warburton, K., 2003. Learning of foraging skills by fish. Fish and Fisheries 4 (3): 203-215.

Final conclusions

Final Conclusions

- The participation in the reproductive behaviour and obtaining spontaneous fertile spawning by cultured breeders was increased in the presence of spawning wild Senegalese sole breeders. The process of developing reproductive behaviour in cultured breeders was associated with social interaction between both types of breeders.

- The development of reproductive behaviour suggests a link to the opportunities for social learning during the early stages and/or the spawning season. However, the sequence in the development of behaviour could be conditioned by the ability to retain the information acquired.

- Low parental contribution in fertile spawning was observed in all breeders and especially cultured breeders. Differences and changes in spawning participation suggested the effect of reproductive dominance amongst breeders reduced the participation in spawning.

- Social dominance appears to be related with behaviours such as Approaches, Swimming Above Another, Rest the Head and Displacement, which were strongly associated with each other. The socially dominant breeders were concordant with the fish that remained more time in the “last” position in the preferred space (sand).

- Social dominance had a significant effect on reproductive success. The socially dominant breeders exhibited higher involvement in the fertile spawning than socially subordinate fish.

- The influence of dominance behaviour had a relative effect on sperm quality and germ cells composition. Socially dominant fish had higher sperm motility, gonadosomatic index and the number of spermatozoa in the cortex section. Socially subordinate fish exhibited a functional spermatogenesis process and produced viable sperm despite social suppression.

- The morphology of the urogenital system of Senegalese sole increases the possibility of urine contamination in sperm samples collected by the stripping method.

- The urine contamination reduces the sperm quality in Senegalese sole. Urine lowered the pH (acidification) and increased the osmolality, which appeared activate sperm motility prematurely.

- All sperm samples irrelevant of different levels of urine contamination had similar cell concentration.

- Short-term cold storage and the use of diluent solutions helped to preserve the sperm quality. In Senegalese sole, Leibovitz (L-15) and Marine freeze® solutions maintained sperm viability for 24 hours and mitigated the effect of possible contamination on collected sperm to be used in fertilisations. In spotted wolffish, Smith and Ryan diluent solution improved the storability of sperm for 1-2 days, helping to maintain the sperm motility.

- In wolfish, the sperm motility period was not limited by the ATP levels, which remained stable during the storage time and the method of sperm collection used was efficient to prevent urine contamination of samples.

- In Senegalese sole, a low spermatozoa egg^{-1} ratio gave high percentages of fertilisation. According to the non-linear regression, 1617 motile spz fertilised 99 ± 12% of viable eggs. The *in vitro* fertilisations on both an experimental scale and a massive commercial scale, allow the control of reproduction to obtain high numbers of hatching larvae.

Annex 1

List of accepted publications

González-López, W.Á., Ramos-Júdez, S., Giménez, I., Duncan, N.J., 2020. Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage. Aquaculture 516, 734649. https://doi.org/10.1016/j.aquaculture.2019.734649

Fatsini, E., González, W., Ibarra-Zatarain, Z., Napuchi, J., Duncan, N.J. 2020. The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics. Aquaculture 519 (734922): In press. https://doi.org/10.1016/j.aquaculture.2020.734922

González-López, W.A., Patel, D.M., Duncan, N., Beirão, J., 2020. Is it possible to store spotted wolffish (*Anarhichas minor*) sperm by refrigeration? Fish Physiol. Biochem. https://doi.org/10.1007/s10695-020-00820-w

Participation in congresses and meetings

1. **Gonzalez -López, W.A.,** Fatsini, E., Ramos, S., Duncan, N. La presencia de reproductores salvajes de lenguado senegalés (*Solea senegalensis*; Kaup 1858) aumenta la participación en el cortejo y el éxito reproductivo de los lenguados reproductores de cultivo.XVI Congreso Nacional de Acuicultura. Zaragoza, España. 3th – 5th October 2017. Oral presentation.
2. **Gonzalez -López, W.A.,** Fatsini, E., Ramos, S., Duncan, N. Gestión de los reproductores basada en su comportamiento para aumentar la producción de gametos y el éxito reproductivo en el lenguado senegalés (*Solea senegalensis*) cultivado y salvaje. III Jornada científica del Departamento de Biología animal, Biología vegetal y Ecología, UAB. Barcelona 8th June 2017. Oral presentation.

3. **Gonzalez -López, W.A.,** Fatsini, E., Duncan, N. Dominance behaviours and spawning in Senegalese sole (*Solea senegalensis*) broodstocks. 11th International Symposium on Reproductive Physiology of Fish (ISRPF - 2018). Manaus, Amazonas, Brazil, 3th – 8th June 2018. Poster presentation.
4. **Gonzalez -López, W.A.,** Fatsini, E., Duncan, N. Do cultured male Senegalese sole (*Solea senegalensis*) have to learn reproductive behaviour? 11th International Symposium on Reproductive Physiology of Fish (ISRPF - 2018). Manaus, Amazonas, Brazil, 3th – 8th June 2018. Poster presentation.
5. **Gonzalez -López, W.A.,** Ramos, S., Marrero, C., Giménez, I., Duncan, N. Efecto de la contaminación por orina en el esperma de *Solea senegalensis* y la utilización de medios extensores en su almacenamiento a corto plazo en frío. XVII Congreso Nacional de Acuicultura. Cartagena, España. 7th – 10th May 2019. Oral presentation.
6. **Gonzalez -López, W.A.,** Ramos, S., Giménez, I., Duncan, N. Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage. PhD student Annual Seminar IRTA. 5th November 2019. Oral presentation.


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