# REPRODUCTION, OLFACTION AND DOMINANCE BEHAVIOUR IN SENEGALESE SOLE (*Solea senegalensis*)

Ph.D. thesis

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# REPRODUCCIÓN, OLFATO Y COMPORTAMINETO DE DOMINANCIA EN EL LENAGUADO SENEGALÉS (*Solea senegalensis*)

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# REPRODUCCIÓ, OLFACTE I COMPORTAMENT DE DOMINÀNCIA EN EL LLENGUADO SENEGALÈS (*Solea senegalensis*)

*Doctoral Thesis*

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# Research Contributions:

## Published articles

- "Transcriptomic profiles of the upper olfactory rosette in cultured and wild Senegalese sole (*Solea senegalensis*) males". 2016. Fatsini, E., Bautista, R., Manchado, M. and Duncan, N. J. *Comparative Biochemistry and Physiology*, *Part D* 20, 125-135.

- "Plasma levels of follicle-stimulating and luteinizing hormones during the reproductive cycle of wild and cultured Senegalese sole (*Solea senegalensis*)". 2016. Chauvigné, F., Fatsini, E., Duncan, N., Olle, J., Zanuy, S., Gomez, A., Cerdà, J. *Comparative Biochemistry and Physiology Part A. Molecular Integrative Physiology* 191, 35-43.

## Presentations in conferences

- "Análisis Morfológico de las rosetas olfativas (dorsal y ventral) del lenguado senegalés (*Solea senegalensis*)" Fatsini, E., Ibarra, Z. and Duncan, N. In the *XIV Congreso Nacional de Acuicultura*. Gijón (Spain), September 23 – 25, 2013 (Poster).

- "Dominance in juvenile Senegalese sole (*Solea senegalensis*)". 2014. Fatsini, E., Ibarra, Z., Rey-Planellas, S. and Duncan, N. In *Scottish Conference for Animal Behaviour (SCAB)*, Edinburgh (Scotland), 29th of March 2014. (Poster).

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

- "Parámetros de dominancia en el lenguado Senegalés (*Solea senegalensis*) relacionados con la alimentación y el territorio". 2015. Fatsini, E. Ibarra-Zatarain, Z., Rey, S. and Duncan, N. J. In the *XV Congreso Nacional y I Congreso Ibérico de Acuicultura 2015*, Huelva (Spain), October 13 – 16, 2015. (Oral presentation).

- "Olfactory sensitivity of the Senegalese sole (*Solea senegalensis*) to conspecific urine, and its potential role in chemical communication". 2016. Fatsini, E., Carazo, I., Chauvigné, F., Manchado, M., Cerdà, J., Hubbard, P. C., and Duncan, N. J. 2016. In *Society for Experimental Biology (SEB) annual meeting*, Brighton, (U. K.) July 3-7, 2016. (Oral presentation).

- "Determination of dominance in Senegalese sole (*Solea senegalensis*) related to feeding and territory". 2016. Fatsini, E., Rey, S. Ibarra-Zatarain, Z. and Duncan, N. In *Scottish Conference for Animal Behaviour (SCAB)*, Stirling (Scotland) in 2nd of April of 2016. (Oral presentation).

RESEARCH CONTRIBUTIONS

- "The enigmatic life of Senegalese sole (*Solea senegalensis*) in captivity". 2016. Fatsini, E. In *Aquaculture Europe 2016*, Edinburgh (Scotland), September 20 – 23, 2016. (Oral Snap Presentation).

- "Linking social status with brain gene expression in Senegalese sole (*Solea senegalensis*)". 2016. Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Mackenzie, S. and Duncan, N. J. In *Aquaculture Europe 2016*, Edinburgh (Scotland), September 20 – 23, 2016. (Oral presentation).

*A mi familia*

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# Summary

Senegalese sole (*Solea senegalensis*) is a flatfish species with increasing importance for the aquaculture industry due to its good performance (survival and growth) in captivity and high market price. However, one of the principal bottlenecks to the expansion of the species culture is the reproductive behavioural dysfunction in cultured males (born and reared in captivity), which complicates establishing a closed cycle in captivity. The reproductive behavioural dysfunction is exhibited by the cultured males that do not participate in the courtship and consequently most eggs obtained from cultured broodstocks are unfertilised. Therefore, Senegalese sole production relies on wild males, which is unsustainable in the long term. With the aim to understand and look for solutions to this bottleneck, the present thesis focuses on different aspects of behaviour related to reproduction and the olfactory system that may control the reproductive behaviour.

The effect of the presence of spawning wild Senegalese sole breeders on the reproductive behaviour and reproductive success of cohabiting cultured breeders was examined. Two groups formed with wild and cultured animals and one control group formed with only cultured sole were compared during three consecutive spawning seasons. Fertilised spawns were not obtained from the control group. However, fertilised spawns were obtained from the mixed-origin groups, principally from the wild fish. In the experimental group cultured males were observed to participate in the courtship in "Follow" behaviours, which are behaviours associated with competition among the fish (principally males) chasing each other. One cultured male fertilised spawns in 2014. Over the three years the participation of culture males (and females) increased, which suggested a learning process in this species for behaviours associated to reproduction.

To establish if culture males and females had the olfactory structure for chemical communication the structure of the olfactory rosettes upper (UOR) and lower (LOR) of wild ($n$ = 10) and cultured ($n$ = 10) Senegalese sole juveniles were compared. This report was the first description of the olfactory rosettes of Senegalese sole and the structure was similar to other fish species above all flatfish. No significant differences in tissue structure, cell types and cellular distribution pattern (olfactory sensory neurons) were observed between cultured and wild specimens, however, differences were found between UOR and LOR in number of lamellae and amount of goblet cells in the ridge region of the lamella, which were more frequent in LOR.

To determine if cultured males and females had the olfactory functionality to communicate chemically, transcriptomic profiles using RNA-seq of the UOR in cultured ($n$ = 3) and wild ($n$ = 3) Senegalese sole mature males were characterized and compared. A total of 2,387 transcripts were differentially expressed between cultured and wild mature males. Transcripts of some olfactory receptors (OR, TAAR and V2R-like) and other transcripts associated with the control of reproduction (brain aromatase and Tachykinin) demonstrated clear differences in functionalities between origins. Furthermore, cultured males presented higher expression of genes related to goblet cells

and mucin production that controls inherent and adaptive immune responses. Many of these changes could be explained by different nutritional status and diet preference.
To establish the form of chemical communication, the olfactory sensitivity of cultured Senegalese sole, juveniles ($n$ = 12) and adults ($n$ = 12) to urine and ovarian fluid from mature conspecific (wild and cultured) was evaluated using electro-olfactogram (EOG). Urine was confirmed to be a potent olfactory stimulus for both conspecific stages, juvenile and adult, inducing large-amplitude, concentration-dependent EOG responses, with thresholds of detection at a dilution of $1{:}10^6$. Significant differences in the amplitude of perception to urine in relation to sex and maturity of both the donor and the receiver indicated that urine may play a role in reproduction by communicating sex and maturity status. In addition to this, urine from mature females evoked a small, but significant increase in plasmas levels of luteinizing hormone (LH) in mature males, further demonstrating that urine-released odorants play a role in reproduction in the Senegalese sole. However, perhaps contrary to expectations, the olfactory potency of urine from wild females was significantly lower than urine from cultured females.

To examine dominance in Senegalese sole, two different groups of Senegalese sole juveniles (early; $n$ = 74 and late; $n$ = 34) were used to conduct dyadic dominance tests (feeding response and place preference test) and group tests (4 groups; $n$ = 6). In addition, transcripts related to dominance in other species were tested to differentiate between dominant and subordinate individuals. This was the first study related to dominance behaviour in this species, which could be very relevant to the low participation in the parental contribution during the spawning season. Dyadic tests found that dominance existed in relation to feeding and space (restricted area with sand) and observed that dominant animals exhibited a higher frequency than subordinate animals of the behaviours resting the head on an individual, approaching and swimming above another individual. Additionally, dominant sole dominated the sand at the end of the test and occupied the sand area at the end of the test. Moreover, these behaviours (resting the head, approaching and swimming above another) that were associated with dominance were corroborated in group-test, where two index (rest the head Index and position before feeding) determined the two dominance categories in the same group. Two transcripts related to neurogenesis (*nrd2*) and neuroplasticity (*c-fos*) were differentially expressed between dominant and subordinate sole juveniles demonstrating the different transcriptomic activity between dominant and subordinate sole.

In some species SCS have been related to reproductive exit and gene expression. In the present study the stress coping styles (SCS) (proactive, intermediate and reactive) in Senegalese sole juveniles ($n$ = 30) was determined in relation to several genes associated with coping styles in other species were tested to find a marker. There were four transcripts which linked behavioural SCS categories with brain gene expression, *gapdh-2* (metabolism), *pparß* (lipid metabolism and feeding behaviour), *igh-Ia* (growth and feeding behaviour*)* and *per1* (circadian rhythm and feeding behaviour).

The present thesis has demonstrated that the presence of spawning wild sole increased the participation of cultured cohabitating sole in courtship behaviour and spawning, which suggested a learning aspect to reproductive behaviours. The olfactory

system appears to have importance in these behaviours and courtship with the capacity to increase LH plasma levels and differentiate between sex and maturity. In addition, similar behaviours (to courtship) were identified to be related to dominance in feeding and space. Together these advances strengthen the importance of these research lines as areas that can give a solution to the reproductive dysfunction that can enable the aquaculture industry to close the species life cycle in captivity to make Senegalese sole culture sustainable.

# Resumen

El lenguado Senegalés (*Solea senegalensis*) es una especie de pez plano que está en constante crecimiento de producción dentro de la industria de la acuicultura debido a su buena capacidad de supervivencia y crecimiento en cautividad, también por poseer un elevado precio en el mercado. En cambio, uno de los principales problemas de expansión del cultivo de esta especie es la disfunción reproductiva asociada al comportamiento complicando el cierre del ciclo de vida del lenguado en cautividad. La disfunción reproductiva se observa en los machos de cultivo (nacidos y criados en cautividad) que no participan en el cortejo, de forma que la mayoría de los huevos obtenidos de lenguados de cultivo están sin fertilizar. Consecuentemente, la producción del lenguado Senegalés incurre en los machos salvajes (procedentes de la naturaleza) siendo insostenible a largo plazo. Esta tesis tiene como objetivo principal entender y buscar posibles soluciones a este problema enfocándose en diferentes aspectos relacionados con la reproducción y el sistema olfativo que podría controlar el comportamiento reproductivo.

Para analizar el efecto de la presencia de lenguados salvajes que se reproducen en el comportamiento y el éxito reproductivo de los lenguados de cultivo, que no se reproducen, se compararon dos grupos formados por lenguados salvajes y de cultivo y un grupo control formado solo con lenguados de cultivo durante tres temporadas consecutivas de puesta. No se obtuvo puestas fecundadas del grupo control. En cambio, sí que se obtuvo puestas fecundadas de los dos grupos mixtos, principalmente de los individuos salvajes. Los machos de cultivo que estaban conviviendo con los salvajes participaron de forma activa en los comportamientos de “Persecución” asociados al cortejo, que son comportamientos relacionados con la competición (sobre todo entre los machos) persiguiéndose entre ellos. Un macho de cultivo fertilizó dos puestas en el año 2014. Durante los tres años, la participación de los machos (y hembras) de cultivo aumentó de forma progresiva indicando un posible proceso de aprendizaje de los comportamientos reproductivos.

Para establecer un posible problema en la comunicación química a nivel estructural, se diseccionaron las rosetas olfativas del lado ocular (UOR) y la del lado ciego (LOR) de diez lenguados juveniles salvajes y diez lenguados de cultivo. Este estudio es el primero en reportar la descripción histológica detallada de las rosetas olfativas del lenguado Senegalés siendo la estructura similar a otras especies descritas, sobre todo a las de peces planos. No se hallaron diferencias significativas en la estructura tisular, tipos de células y distribución celular (neuronas sensoriales olfativas) entre lenguados de diferente origen, en cambio, se encontraron diferencias entre la UOR y la LOR en número total de lamelas y en cantidad de células goblet concentradas en la parte apical de las lamelas siendo más frecuente en la LOR.

Para determinar si los machos y las hembras de lenguado Senegalés poseían la capacidad olfativa de comunicar químicamente, se realizó un perfil transcriptómico de la UOR usando RNA-seq en tres machos reproductores de cultivo y tres machos salvajes, todos maduros, para después poder comparar dichos perfiles de expresión. Se

diferenciaron de forma significativa un total de 2.387 transcritos entre los machos de cultivo y salvajes estando en el mismo grado de madurez. Los transcritos de algunas receptores olfativos (OR, TAAR y V2R-like) y otros transcritos asociados con el control de reproducción (aromatasa cerebral y Tachykinina) demostraron claras diferencias en la funcionalidad de la UOR entre los lenguados de diferentes orígenes. Además, los machos de cultivo presentaron mayor expresión en genes relacionados con las células goblet y producción de mucinas que controlan las respuestas inmunes innata y adaptativa. Muchos de estos cambios se podrían explicar con la diferencia en el estatus nutricional y la preferencia en la dieta.

Para determinar la forma de comunicación química, se evaluó la sensibilidad olfativa a la orina y al fluido ovárico procedentes de conspecíficos maduros de diferente origen (salvajes y de cultivo) de doce lenguados juveniles y doce lenguados adultos, todos de cultivo, usando electro-olfatograma (EOG). La orina se confirmó como un potente estimulante olfativo para ambos estadios, juveniles y adultos, la cual indujo una respuesta amplia en el EOG dependiente de la concentración con niveles de detección a una dilución $1{:}10^6$. Se encontraron diferencias significativas en la amplitud de percepción de la orina en relación al sexo y al estado de madurez tanto del emisor como del receptor indicando que la orina podría estar implicada en la reproducción comunicando el sexo y el estado de madurez. Además, la orina procedente de hembras maduras produjo un pequeño, pero significativo incremento en plasma de la hormona Luteinizante (LH) en machos maduros, demostrando que los olores liberados con la orina juegan un papel importante en la reproducción del lenguado senegalés. Sin embargo, la potencia olfativa de la orina procedente de hembras salvajes fue significativamente menor que la orina de hembras de cultivo, contrario a nuestras expectativas.

Para examinar la dominancia en el lenguado senegalés, dos grupos de diferentes estadios de juveniles (tempranos; $n$ = 74 y tardíos; $n$ = 34) fueron utilizados para realizar dos pruebas de dominancia diádica (respuesta a la alimentación y preferencia de lugar o espacio) y una prueba de dominancia en grupo (4 grupos; $n$ = 6). Asimismo, se examinaron diferentes transcritos relacionados con la dominancia en otras especies para distinguir entre individuos dominantes y subordinados. Este estudio ha sido el primero en reportar la dominancia como comportamiento en esta especie, la cual podría ser relevante debido a la baja contribución parental durante la época de puesta. Las pruebas diádicas encontraron la existencia de la dominancia relacionada con la alimentación y el territorio (zona restringida con arena) y se observó que los animales dominantes exhibieron mayor frecuencia de los comportamientos apoyar la cabeza en un individuo, acercarse y nadar por encima de otro individuo que los animales subordinados, además de dominar la zona con arena en el tiempo final de la prueba y ocupar la arena al momento de terminar la prueba. Igualmente, estos comportamientos se corroboraron en la prueba grupal, donde dos índices (índice de apoyar la cabeza e índice de posición antes de la alimentación) determinaron las dos categorías de dominancia en el mismo grupo. Dos transcritos relacionados con la neurogénesis (*nrd2*) y con la neuroplasticidad (*c-fos*) se expresaron significativamente diferente entre juveniles tempranos de diferente

estatus demostrando que hay diferencias en la actividad transcriptómica entre lenguados dominantes y subordinados.

Los estilos de afrontamiento al estrés (SCS) han sido relacionados con el éxito reproductivo y la expresión génica en algunas especies de peces. En este estudio se determinaron los diferentes SCS (proactivos, intermedios y reactivos) en treinta lenguados juveniles y además se determinó la asociación de dichos estilos con la expresión génica de algunos genes relacionados con SCS en otras especies con la finalidad de encontrar un biomarcador. Se encontraron cuatro transcritos que asociaron los diferentes perfiles de comportamiento con la expresión génica en el cerebro en esta especie, *gapdh-2* (metabolismo), *pparβ* (metabolismo lipídico y comportamiento en la alimentación), *igh-Ia* (crecimiento y comportamiento en la alimentación) y *per1* (ritmos circadianos y comportamiento en la alimentación) siendo todos ellos genes importantes a nivel biológico y funcional.

Esta tesis ha demostrado que la presencia de lenguados salvajes que se reproducen incrementa la participación en el cortejo y puestas de los lenguados de cultivo, sugiriendo la existencia de un proceso de aprendizaje de los comportamientos reproductivos. El sistema olfativo parece tener un papel importante en esos comportamientos reproductivos e implicación en el cortejo con la capacidad de acrecentar los niveles de LH en plasma y diferenciar entre sexo y estado de madurez. Además, se identificaron comportamientos similares al cortejo relacionados con la dominancia en la alimentación y el espacio. Conjuntamente estos avances fortalecen la importancia de estas líneas de investigación como áreas que pueden dar solución a la disfunción reproductiva pudiendo cerrar el ciclo de vida del lenguado senegalés en cautividad para hacer la producción de dicha especie sostenible en la industria de la acuicultura.

# Resum

El llenguado Senegalès (*Solea senegalensis*) es una espècie de peix pla que està en constant creixement de producció dins de la industria de l'aqüicultura degut a la seua capacitat de supervivència i creixement en captivitat, a més de posseir un elevat preu al mercat. En canvi, un dels principals problemes d'expansió del cultiu d'aquesta espècie és la disfunció reproductiva associada al comportament que complica tancar el cicle de vida del llenguado en captivitat. La disfunció reproductiva s'observa als mascles de cultiu (nascuts i criats en captivitat) que no participen al seguici, de forma que la majoria dels ous obtinguts de llenguados de cultiu es troben sense fertilitzar. Conseqüentment, la producció del llenguado Senegalès depèn dels mascles salvatges (procedents de la natura) el qual es insostenible a llarg termini. Esta tesi té com objectiu principal entendre i buscar possibles solucions a aquest problema enfocant-se en diferents aspectes relacionats amb la reproducció i el sistema olfactiu que podria controlar el comportament reproductiu.

Per analitzar l'efecte de la presència de llenguados salvatges que es reprodueixen en el comportament i el èxit reproductiu dels llenguados de cultiu, que no es reprodueixen, es van comparar dos grups formats per llenguados salvatges i de cultiu amb un grup control format només amb llenguados de cultiu durant tres temporades consecutives de posta. No es van obtenir postes fecundades del grup control. En canvi, sí que es van obtenir postes fecundades dels dos grups mixtos, principalment del individus salvatges. Els mascles de cultiu que estaven convivint amb els llenguados salvatges van participar de modo actiu en els comportaments de "Persecució" associats al seguici, que són comportaments relacionats amb la competició (sobre tot entre els mascles) perseguint-se entre ells. Un mascle de cultiu va fertilitzar dos postes en el any 2014. Durant els tres anys, la participació dels mascles (i femelles) de cultiu va augmentar de forma progressiva indicant un possible procés d'aprenentatge dels comportaments reproductius.

Per establir un possible problema en la comunicació química a nivell estructural, es van diseccionar les rosetes olfactives del costat ocular (UOR) i del costat cec (LOR) de deu llenguados salvatges juvenils i deu llenguados de cultiu juvenils. Aquest estudi és el primer en reportar la descripció histològica detallada de les rosetes olfactives del llenguado Senegalès sent la estructura similar a la d'altres espècies descrites, sobre tot especies de peixos plans. No es van obtenir diferències significatives en la estructura tissular, tipus de cèl·lules i distribució cel·lular, en canvi, es van obtenir diferències entre la UOR i la LOR en número total de lamel·les i en quantitat de cèl·lules goblet concentrades en la part apical de les lamel·les que són més freqüents en la LOR.

Per a determinar si els mascles i femelles de llenguado Senegalès posseïen la capacitat olfactiva de comunicar químicament, es va realitzar un perfil transcriptomic de la UOR utilitzant RNA-seq en tres mascles reproductors de cultiu i tres mascles reproductors salvatges, tots madurs, per a després poder comparar els nomenats perfils d'expressió. Es van diferenciar de forma significativa 2.387 transcrits entre mascles de cultiu i salvatges els quals estaven al mateix grau de maduresa. Els transcrits d'alguns

receptors olfactius (OR, TAAR i V2R-like) i altres transcrits associats amb el control de reproducció (aromatasa cerebral i Tachykinina) van demostrar clares diferències en la funcionalitat de la UOR entre els llenguados de diferent origen. A més a més, els mascles de cultiu van presentar major expressió en gens relacionats amb les cèl·lules goblet i producció de mucines que controlen les respostes immunes innata i adaptativa. Molts d'aquests canvis es podrien explicar amb la diferència en el estatus nutricional i la preferència de la dieta.

Per a determinar la forma de comunicació química, es va avaluar la sensibilitat olfactiva de l'orina i el fluid ovàric procedents de conspecifics madurs de diferent origen (salvatges i de cultiu) de dotze llenguados juvenils i dotze llenguados adults, tots de cultiu, utilitzant electró-olfactogram (EOG). L'orina es va confirmar com un potent estimulant olfactiu per a ambos estadis, juvenils i adults, la qual va induir una resposta amplia en el EOG depenent de la concentració amb nivells de detecció a una dilució $1{:}10^6$. Es van trobar diferències significatives en la amplitud de la percepció de l'orina en relació al sexe i a l'estat de maduresa tant de l'emissor com de receptor indicant que l'orina podria estar implicada en la reproducció comunicant el sexe i l'estat de maduresa. A més, l'orina procedent de femelles madures va produir un petit, però significant increment en plasma de la hormona Luteïnitzant (LH) en mascles madurs, demostrant que els productes alliberats amb l'orina juguen un paper important en la reproducció del llenguado Senegalès. Però, la potència olfactiva de l'orina procedent de femelles salvatges va ser significativament menor que l'orina de femelles de cultiu, contràriament a les nostres expectatives.

Per a examinar la dominància en el llenguado Senegalès, dos grups de diferents estadis de juvenils (primerencs; $n = 74$ i tardans; $n = 34$) van ser utilitzats per a realitzar dos probes de dominància diàdica (resposta a l'alimentació i preferència de lloc o espai) i una proba de dominància en grup (4 grups; $n = 6$). Així mateix, es van examinar diferents transcrits relacionats amb la dominància en altres especies per a distingir entre individus dominants i subordinats. Aquest estudi ha sigut el primer en reportar l'existència de la dominància com a comportament en aquesta espècie, la qual podria ser rellevant degut a la baixa contribució parental durant l'època de posta. Les probes diàdiques van trobar l'existència de la dominància relacionada amb l'alimentació i el territori (zona restringida amb sorra) i es va observar que els animals dominants exhibien major freqüència dels comportaments recolzar el cap en un individu, apropar-se i nadar per damunt d'un altre individu que els animals subordinats, a més de dominar la zona amb sorra en el temps final de la proba i ocupar la sorra al moment de terminar la proba. Igualment, aquests comportaments es van corroborar en la proba grupal, on dos índex (índex de recolzar el cap e índex de posició abans de l'alimentació) van determinar les dues categories de dominància en el mateix grup. Dos transcrits relacionats amb la neurogénesis (*nrd2*) i la neuroplasticidad (*c-fos*) es van expressar significativament diferent entre juvenils primerencs de diferent estatus social demostrant que hi ha diferències en l'activitat transcriptómica entre llenguados dominants i subordinats.

Els estils d'afrontament a l'estrès (SCS) han sigut relacionats amb l'èxit reproductiu i l'expressió gènica en algunes especies de peixos. En aquest estudi es van

determinar els diferents SCS (proactius, intermedis i reactius) en trenta llenguados juvenils i a més es va determinar l'associació d'aquests estils amb l'expressió gènica d'alguns gens relacionats amb SCS en altres especies amb la finalitat de trobar un biomarcador. Es van trobar quatre transcrits que van associar els diferents perfils de comportament amb l'expressió gènica del cervell en aquesta espècie, *gapdh-2* (metabolisme), *pparß* (metabolisme lipídic i comportament en l'alimentació), *igh-Ia* (creixement i comportament en l'alimentació) y *per1* (ritmes circadians i comportament en l'alimentació) sent tots ells gens importants a nivell biològic i funcional.

Aquesta tesi ha demostrat que la presència de llenguados salvatges que es reprodueixen incrementa la participació en el seguici i postes dels llenguados de cultiu, suggerint l'existència d'un procés d'aprenentatge dels comportaments reproductius. El sistema olfactiu pareix tindre un paper important en aquests comportaments reproductius e implicació en el seguici amb la capacitat d'incrementar els nivells de LH en plasma i diferenciar entre sexe i maduresa. A més a més, es van identificar comportaments similars als del seguici relacionats amb la dominància en l'alimentació i el territori. Conjuntament aquests avanços enforteixen la importància d'aquestes línies d'investigació com àrees que puguin donar solució a la disfunció reproductiva podent tancar el cicle de vida del llenguado Senegalès en captivitat per a fer la producció d'aquesta espècie sostenible en l'industria de l'aqüicultura.

# Section 1: Background

# Chapter 1

# General Introduction

# General Introduction

*Aquaculture*

Aquaculture was defined by FAO as *"the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated. For statistical purposes, aquatic organisms which are harvested by an individual or corporate body which has owned them throughout their rearing period contribute to aquaculture, while aquatic organisms which are exploitable by the public as a common property resource, with or without appropriate licences, are the harvest of fisheries."*

Nowadays, aquaculture is an important source of food that contributes to meeting the significant increase in demand for seafood, due to the fast growth in the population of the world, which is predicted to reach 9.7 billion in 2050 (FAO, 2016). World per capita fish supply is continuously increasing (20 kg / person in 2014) due to the increase in aquaculture, which provides half of all fish supply for human consumption and in addition is reducing the fishing pressure on fish stocks that have been over exploited with decades of overfishing.

Currently, 600 aquatic species (fish, crustaceans, molluscs amongst other) are cultivated worldwide using different production systems (intensive, extensive, etc.) in all water environments (seawater, freshwater and brackish waters). Asia is the continent with the highest production from aquaculture and that registered 62.6 million tonnes of production in 2013 combining inland aquaculture and mariculture (FAO, 2015). In 2013, Europe produced 1.3 million tonnes from aquaculture, which represented 18.8 % of the total world production. Aquaculture production in the European Union (EU) was valued at 4,178 million Euros in 2014 and the sector has a significant number of employees that increased by 1.9 % from 2014 to 2015 to directly employ 19,913 people in Spain (APROMAR, 2016). Nowadays, there are 35 species produced in intensive production systems in the EU. Spain is the country with the highest aquaculture production volume in the EU with 282,242 tonnes in 2014, of which 59,533 tonnes were fish species. There are six marine fish species that are produced in Spain. In 2015, production was 21,324 tonnes of sea bass (*Dicentrarchus labrax*), 16,231 tonnes of gilthead seabream (*Sparus aurata*), 7,715 tonnes of turbot (*Psetta maxima*), 1,642 tonnes of meagre (*Argyrosomus regius*), 664 tonnes of Senegalese sole (*Solea senegalensis*), which is the species studied in this thesis, and 320 tonnes of tuna (*Thunnus thynnus*) (APROMAR, 2016).

**Senegalese sole (*Solea senegalensis*)**

This thesis focuses on the study of Senegalese sole (*Solea senegalensis* Kaup, 1858) (Fig. 1) and specifically in some factors to improve the culture of this species.

Senegalese sole is a common flatfish species considered as a promising species for the aquaculture industry in Europe, due to its good growth rate and high market price (12.25 € $kg^{-1}$ was paid per cultured sole in MercaMadrid in 2013). Additionally, the economical perspective for the expansion of this species is good according to the economic analysis by Bjorndal and Guillen (2014). This species belongs to Type: Vertebrata; Subtype: Gnathosmata; Superclass: Pisces; Class: Osteichthyes; Order: Heterosomata (Pleuronectiformes); Suborder: Soleoidei; Family: Soleidae; Subfamily: Soleinae; Genre: Solea; Species: Senegalensis.

**Figure 1.** Senegalese sole (*Solea senegalensis*) juvenile. (Photo credit: E. Fatsini, 2014). The surrounded fin with black strips rather than all black can be used to distinguish this species from the common sole (*Solea solea*), which is a related species with an overlapping, but more northerly geographic distribution.

Senegalese sole is naturally distributed from Senegal, Africa to La Rochelle, France and in the Western Mediterranean Sea (Ben-Tuvia, 1990). Senegalese sole is industrially cultured in the Mediterranean and Atlantic coasts, being the species of fish that have more recently been incorporated into the production of large-scale aquaculture in Spain. Aquaculture production has been increasing over recent years (20.5 % from 2014 to 2015) (Fig. 2). The main farms are located in Galicia (58.4 %), Andalusia (37.2 %) and Canary Island (4.4 %). APROMAR (2016) predicts that Senegalese sole culture in Europe will be among the main five species for 2030 (sea bass, sea bream, meagre, turbot and sole).

In the last 70 years, the culture conditions of Senegalese sole have been studied to improve the cultivation of this flatfish species. Until recent years, Senegalese sole culture was extensive in earthen ponds along the south coasts of Portugal and Spain (Morais et al., 2014). However, currently the tendency is for intensive production using recirculation systems in tanks installed in different sort of facilities (indoor or outdoor), using balanced feed and controlled environmental conditions. The recirculation systems have improved the sole farming as the environmental control of especially water temperature and nitrogen residues appears to have increased growth and survival (Imsland et al., 2003).

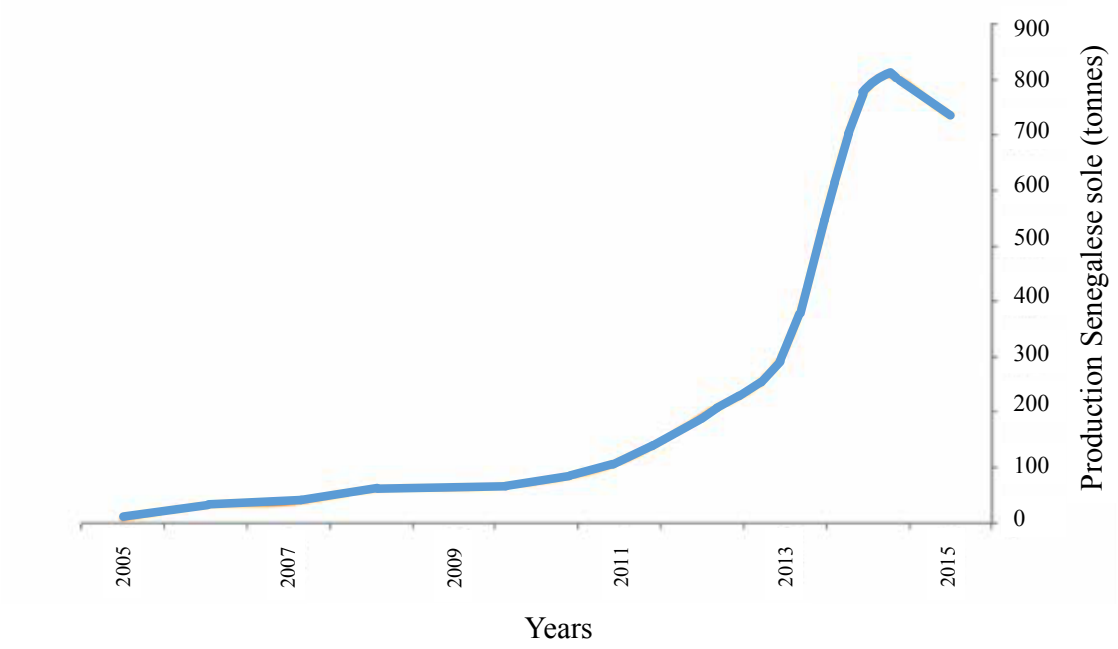


**Figure 2.** Evolution of Senegalese sole (*Solea senegalensis*) production in Spain (2005-2015). Modified from APROMAR 2016.

Currently, these controlled culture Senegalese sole conditions are: natural photoperiod and thermoperiod, however, high temperatures are avoided and temperatures below 20 °C are maintained as temperatures above 22 °C increase the probability of disease problems (Cañavate, 2005). Sole juveniles are euryhaline and the salinity depends on the culture area, however, the normal range used is from 25 to 39 ppt (Arjona et al., 2009). Sole have a strict bottom-feeding behaviour. The feeding regime changes depending on the stage of development and the farms. In most farms, sole juveniles are fed over the entire 24 h using automatic feeders (Morais et al., 2014). Densities around 30 kg $m^{-2}$ have been used with no effects on growth (Salas-Leiton et al., 2008), however, Senegalese sole exhibits large size disparity under culture conditions and the cause of this size disparity is unknown.

However, there are two principal problems facing Senegalese sole culture, diseases and reproductive dysfunction in individuals born and reared in captivity. In the case of pathologies, microbiological diseases and poor nutrition due to a deficiency of understanding on digestive, physiology and nutritional necessities, have caused high mortalities during larval weaning and quality (Morais et al., 2014). The reproductive dysfunction in relation with reproductive behaviour in individuals born and reared in captivity prevents closing the life cycle in the culture of Senegalese sole. The causes of this dysfunction remain unknown, however, this problem has been studied from several points of view, such as, nutrition, physiology, epigenetics and behaviour (Morais et al., 2014). This thesis continues with the aim of finding the possible causes of this reproductive dysfunction by studying behaviour and mechanisms involved in the control of behaviour.

### *Reproduction*

Senegalese sole is gonochoric, and females present higher growth rates and mature later (at age +3) than males. Commercial culture of this species has been based on the spawning of captive wild broodstocks (Dinis et al., 1999; Anguis and Cañavate, 2005; Martín et al., 2014; Morais et al., 2014). The individuals are caught from nature and transported to aquaculture or research installations. These animals need a period of quarantine and acclimation to captive conditions before entering into a broodstock (Dinis et al., 1999; Imsland et al., 2003).

The principal spawning season is registered in spring, from March to June, and in some conditions, there exist another additional spawning period, which is considered less important, in autumn, from October to November (Howell et al., 2003; Anguis and Cañavate, 2005). As in other fish species, both temperature and photoperiod are implicated in the regulation of the timing of spawning in Senegalese sole. In the case of the studied species, natural photoperiod has been usually applied for successful spawning events (Dinis et al., 1999; Howell et al., 2003; Anguis and Cañavate, 2005). Moreover, spawning has been associated with the temperature cycle (Dinis et al., 1999; Anguis and Cañavate, 2005) obtaining the highest fecundities between 16 and 21°C (Anguis and Cañavate, 2005). Using temperature control, fluctuating temperatures between 16 and 18 °C, the natural spawning period has been extended from March to November, although with variation in egg production (Martín et al., 2014). Microsatellite analysis to determine parentage contribution demonstrated that just 8.7 – 51.7 % of all the breeders in a broodstock participated in spawning (Porta et al., 2006; Martín et al., 2014), which causes a sub-estimation of relative fecundity when 100 % involvement of breeders is assumed. Fecundities have been quantified over 100,000 eggs $kg^{-1}$ $day^{-1}$, however, this quantity might fluctuate with temperature (Anguis and Cañavate, 2005).

As mentioned before one of the major bottlenecks in the culture of Senegalese sole is the complete absence of spawning of viable eggs from cultured breeders that were hatched and reared in captivity. Several studies have reported that cultured breeders produce few spontaneous spawns of eggs that were of low quality and unfertilized (Howell et al., 2011; Carazo, 2013; Duncan et al., 2013; Morais et al., 2014). In general terms some researchers had hypothesised that the absence of successfully spawning was related to dysfunctions of physiological, nutritional or genetic aspects during early stages of rearing (Agulleiro et al., 2007; Howell et al., 2009; Rasines et al., 2012; Morais et al., 2014). Nutrition, was an approach tested which could be implicated in cultured Senegalese sole reproductive dysfunction. Norambuena et al. (2012a) found differences in proximate, lipid and fatty acids composition between wild and cultured breeders and cultured breeders showed lower levels of 2-series prostaglandins and higher levels of 3-series PGs in comparison to wild sole. These differences in body composition and nutritional state appeared to affect different pathways for the production of prostaglandins and several steroids may give rise to the

differences in reproductive physiology (Norambuena et al., 2012b). However, the root of the reproductive issue may not be related to just a nutritional adjustment (Morais et al., 2014).

Another approach widely investigated was the control of gamete production (ovulation and spermiation) and spawning using hormone therapies in this species (Agulleiro et al., 2006; Guzmán et al., 2008; Guzmán et al., 2009b; Rasines et al., 2012; 2013). These hormone therapies have been applied in Senegalese sole males and females to induce spawning. For example, Agulleiro et al., (2006, 2007) and Guzmán et al., (2009) used GnRHa (Gonadotropin-Releasing Hormone analogue) in cultured sole acquiring large increases in spontaneous spawning, however, the eggs were also not fertilized. Another study applying hCG (human Chorionic Gonadotropin) and different concentrations of GnRHa in cultured males obtained positive results in terms of sperm production, however, the eggs were again not fertilised (Agulleiro et al., 2006; Guzmán et al., 2011), just one spawn was obtained from cultured animals after applying double GnRHa treatment in cultured females and hCG in cultured males. These studies suggest that hormonal dysfunctions in the control of gametogenesis are not the cause of the reproduction failure in cultured breeders, due to this species achieves gametogenesis and produces viable gametes (Guzmán et al., 2009a; Morais et al., 2014). Although unsuccessful in inducing spontaneous spawning, the application of GnRHa treatments to cultured sole females has been used to obtain ovulated eggs for artificial fertilization (Chereguini, 2006; Chereguini et al., 2007; Rasines et al., 2012; 2013). The results of this strategy were positive obtaining viable eggs by abdominal pressure after hormone treatment of females and the eggs were fertilised with fresh and cryopreserved sperm from cultured males. However, this strategy is complicated due to the low sperm volume obtained from Senegalese sole (Beirao et al., 2009; Cabrita et al., 2011). Therefore, artificial fertilisation clearly demonstrated that cultured sole produce viable gametes and has offered the first reliable (but difficult to apply on an industrial scale) method to ensure fertilised eggs from cultured sole. However, artificial fertilization does not clarify the causes of the reproductive dysfunction in cultured breeders.

There was a significant advance in our understanding of the cause of the reproductive dysfunction from two studies examining cohabitation of breeders from different origins, wild and cultured, with hormone manipulation (Mañanos et al 2007) and without hormone manipulation (Carazo 2013; Martin 2016). Mañanós et al. (2007) conducted two experiments simultaneously. In experiment 1, six different groups were distributed in six tanks, two groups were formed only by cultured males and females (one group was treated with hormonal therapy and the other group was untreated), two other groups were formed by wild females and males (one group was treated with hormonal therapy and the other group was untreated) and the last two groups were formed by wild females and cultured males (one group was treated with hormonal therapy and the other group was untreated). In experiment 2, three groups were used, one group was formed by only cultured fish, males and females, another group was formed by wild females and cultured males and the last group was established with cultured females and wild males, in this case cultured breeders were hormonally treated

and wild breeders remained untreated. All groups hormonal therapies were the same, females with GnRHa slow-release implants and males with injections of hCG. Regarding experiment 1, treatment of the cultured broodstock increased number of spawns and egg production, in comparison to untreated group, however, the majority of the spawns were unfertilised, except one from the cultured treated group. In the case of wild broodstock, treated and untreated groups spawned spontaneously, increasing number of spawns in treated group but with little effect over total production. Treatment of the mixed broodstock increased number of spawns and total egg production, however, no fertilization was obtained. Regarding experiment 2, fertilised spawns were observed from the broodstock having treated cultured females and untreated wild males, but not in the other way, treated cultured males with untreated wild females from which only unfertilised eggs were obtained. This study, reported for the first time the critical reproductive dysfunction was associated with the captive male breeders. Carazo corroborated those results and made several studies observing the reproductive behaviour, including the first description of the reproductive behaviour of the species (Carazo, 2013; Carazo et al., 2016; Martín, 2016). The reproductive behaviour was observed using lightning and video recording that did not affect, activity, behaviour or melatonin (Carazo et al., 2013). Carazo (2013) observed different groups of Senegalese sole breeders from different origins during spawning season to describe the reproductive behaviour in the different groups established at different times from 2008 to 2010. These groups were two groups formed by only wild males and females, one group formed by pure cultured males and female breeders, and two mixed-origin groups (one of them formed of cultured males and wild females, and another one formed of cultured females and wild males). The courtship was described in wild-captive Senegalese sole groups that produced fertilised spawns (Carazo et al., 2016). Wild Senegalese sole exhibited a complex courtship, which was a series of behaviours that increased locomotor activity. The behaviours described were divided in three steps (Carazo, 2013; Carazo et al., 2016).

- Step 1 was characterized by a period of high activity in the tank due to the "Follow" behaviours, where several breeders (usually males) were chasing each other swimming around the tank without aggressive interactions in a kind of procession. Two types "Follow" behaviours were classified, a) "Followed": one fish was followed by other fish being the leader of the procession and b) "Follower": fish that actively followed the leader. The females in this step could be involved in "Follow" behaviours being less common or stayed quiet on the bottom.
- Step 2 was distinguished by the "Rest the Head" behaviours performed by the males towards the females. These behaviours were frequently observed and were defined as "an individual rested its head on another fish" (Carazo et al., 2016). This behaviour was also categorized by where the head was placed or rested on the body part of the lower fish (head, back, ovary or tail). A behaviour included in "Rest the Head" just described in Senegalese sole is

the "Guardian" behaviour defined as "a situation when a fish actively guarded another fish from the advances of a third fish". The "Guardian" behaviour usually was performed by males protecting females from other males (Carazo et al., 2016). The main objective of the male in the "Rest the Head" step 2 was trying to elicit that the female swam from the bottom to initiate the swim to the surface together. This behaviour was denominated "Coupled" swim and was considered the next step.

- Step 3 was characterized by the swimming behaviour previously commented "Coupled" swim. The "Coupled" behaviour was described as the female swam from the bottom and began to swim to the surface pushed by the male, the male was placed under the female and the pair swam together to the surface performing wave movements. The finality of this behaviour was to release the gametes in the surface, at the moment the gametes were released the couple broke apart and returned to the bottom.

The "Coupled" swim could be interrupted during these steps. These situations were termed failure and three types of coupled swim failure were observed (*more details in* Carazo et al. 2016).

When the behaviour of the tanks of only cultured breeders were examined the behaviour on both nights, with egg release (unfertilised) and without egg release, were similar to wild fish on a night without spawning. There were no "Follow" or "Coupled" behaviours and activity was low. This was the first study to describe that the reproductive dysfunction in cultured sole was a behavioural problem as there was no courtship and this also demonstrated for the first time that the eggs released from cultured broodstocks were actually not fertilised. An almost identical situation was observed in the mixed origin group of wild females with cultured males. There were no "Follow" or "Coupled" behaviours, activity was low and no fertilised spawns were obtained. However, as found by Mañanós et al. (2007) the mixed origin group of cultured females with wild males produced fertilised spawns and observation of the courtship on the nights with successful spawning found that the cultured females completed the full courtship with wild males including the "Follow" and "Coupled" behaviours. Therefore, these studies (Carazo 2013) confirmed that the reproductive dysfunction was associated with the captive male breeders and demonstrated for the first time that it was actually a behavioural reproductive dysfunction that prevented the males from participating in the courtship to fertilise the eggs of the females (cultured or wild females).

One of the possibilities tested to induce the reproductive behaviour in this species was hormonal induction (Carazo, 2013). However, the results of these studies only corroborated that GnRHa, hCG and PGF2α treatments in cultured females improved the maturity stage and in cultured males these hormone therapies also had a positive effect in the sperm quality. However, the behavioural activity of those animals was very low, showing that the role of these hormone therapies in reproductive behaviour did not offer a solution for the reproductive dysfunction.

Currently, the reasons for this failure remain uncertain. Therefore, the present thesis focused on continuing this behavioural approach and examining mechanisms that control reproductive behaviour and that may, therefore, be implicated in the reproductive behavioural dysfunction presented in cultured Senegalese sole reproduction. For this purpose, three broad approaches were examined: 1) the effect of the presence of spawning wild breeders on the performance in the courtship and reproductive success of cultured breeders, 2) the involvement of chemical communication by carrying out deeper studies in the olfactory system and finally, 3) the existence of behaviours, dominance and stress coping styles and associated gene expression that may be related to reproduction of this flatfish species.

### *Chemical communication, Olfaction and Physiology*

Chemical communication is the most primordial system and a common system to exchange information between organisms. The message is transferred *via* chemicals liberated to the environment by an emitting organism to be received by the receiver organism. These chemical emissions have become specialised as hormones, neurotransmitters, or other products with highly specific activities. These compounds or substances that have highly specific purposes on the receiver are called pheromones. These compounds are not accumulated like hormones, but are synthesised and released and additionally these substances usually have a short half-life (Sorensen and Stacey, 2004). In mammals, the reproductive hormones have two main functions, the synchronization of gametogenesis and control of sexual interactions among individual conspecifics (Pfaff, 2005). When the pheromone stimulus is perceived by the receiver the brain of the receiver responds by inducing physiological changes that synchronise maturational development and stimulate reproductive behavioural (Johnston, 1983). The olfactory system and taste are the senses that perceive and detect the chemical stimulus from the environment (Hagino-Yamagishi et al., 2004). Fish are not an exception in the use and perception of chemical substances through these two chemosensory pathways and actually the characteristics of living in an aquatic ecosystem with the water medium providing advantages to disperse chemical signals combined with difficulties such as the deprivation of visual cues, the olfaction or olfactory system provides an important communication system for fish (Sorensen and Stacey, 2004). Olfaction has evolved to increase the survival of aquatic species by using chemical signals to find food, to guide migration (homing), to recognise relatives (imprinting) find mates and control reproductive behaviour. In the case of homing, several species have been widely studied to understand the migration process, above all in salmonid species such as Atlantic salmon (*Salmo salar*) (Stabell, 1984), brown trout (*Salmo trutta*) (Armstrong and Herbert, 1997), among others.

In the case of reproduction, the olfactory system is linked to fish reproduction at different levels. The reproduction cycle in fish is regulated by the endocrine system, where hormones play an important role, along the brain-pituitary-gonad (BPG) axis,

which is also called the reproductive axis. The principal hormones involved are gonadotropin releasing hormones (GnRHs), the pituitary gonadotropins (GTHs), Follicle-Stimulating Hormone (FSH) and Luteinizing-Hormone (LH) and sex steroids (Testosterone, 11-ketotestosterone, estradiol-17*ß* in females, 17α, 20*ß*-dihydroxy-4-pregnen-one - 17, 20*ß*-P or MIS), which all have important roles in the endocrine control in reproduction. The signal to stimulate the emission of these GTHs initiates in the brain, through the activation of the GnRH system at the moment the brain processes the external signals that indicate the correct conditions for maturation to progress. The gonadotropins, FSH and LH, after being secreted, act on the gonad to stimulate the synthesis of sex steroids (Testosterone and 11-ketotestosterone in males and estradiol-17*ß* in females), the last effectors of gonadal development (Mañanós et al., 2008). The relationship between olfaction and reproduction has been demonstrated in salmonid species, for example an amino acid, L-kynurenine, was detected using electro-olfactogram (EOG) and high performance liquid chromatography (HPLC) as a sex pheromone in the urine of ovulated female in masu salmon (*Oncorhynchus masou*) (Yambe et al., 2006). Another clear example was the implication of F-series prostaglandins as priming pheromones in mature males in Atlantic salmon parr using EOG (Moore and Waring, 1996). Mozambique tilapia (*Oreochromis mossambicus*) is another example of that relationship (Keller-Costa, 2014). However, the most studied species of the connection between olfaction and reproduction is the goldfish (*Carassius auratus*). Female goldfish release a gonadal steroid 17α, 20*ß*-dihydroxy-4-pregnen-one (17, 20*ß*-P) into the water before ovulation. The 17, 20*ß*-P acts as a pheromone and is detected by the male olfactory system that through the brain induces an increase in blood GTH and consequentially an increase in sperm volume (Sorensen et al., 1990). In this case, the medial olfactory tract (MOT) was the mechanism responsible of those responses in males, so at the moment the pheromone (17, 20*ß*-P) was perceived by the males, the signal was transduced to the brain by the MOTs (Dulka and Stacey, 1991). The hormone, 17, 20*ß*-P or maturation inducing steroid (MIS) was also found to induce maturation in a marine flatfish species, plaice (*Pleuronectes platessa*), which accumulated MIS in urine indicating that the species may use urine as a vehicle to release steroid pheromones that were synthesized by the gonads (Canario and Scott, 1989). Actually a wide range of substances have been suggested (but few have been demonstrated) as reproductive pheromones that are excreted by fish to the environment such as bile salts (Hara, 1994; Zhang et al., 2001; Li et al., 2002; Huertas et al., 2007), steroid molecules (Barata et al., 2008) and prostaglandins (Velez et al., 2009; Hubbard, 2014) which might be released with different fluids (urine, faeces, mucus) acting as messengers to conspecifics.

The olfactory system varies depending on the fish species and the habitat where they live. The most common and important structure in the olfactory system present in fish is the olfactory rosette, located in the olfactory chamber. The olfactory rosette is connected with the environment through the nostrils and receives chemical stimuli through the olfactory epithelium located in the lamellae. The olfactory epithelium contains a non-sensory cell group formed by supporting, basal, goblet and ciliated non-

sensory cells and a sensory cell group formed by olfactory sensory neurons (OSNs) (ciliated, microvillous, crypt and kappe neurons). The non-sensory cell group maintains the structure and function of the olfactory rosette and in particularly of the sensory cells. The sensory cell group receives the chemical information from the environment and then this information is directly transmitted to the brain through the olfactory nerve (Zeiske et al., 1992; Hansen and Zielinski, 2005). The OSNs are able to detect an immense spectrum of odours that express specifically a subset of olfactory receptors (known as "one neuron–one receptor" rule) (Miyasaka et al., 2013) whose axons converge in the olfactory bulb to establish a discrete and precise map of olfactory receptor expression (Buck et al., 2000; Miyasaka et al., 2013). Nevertheless, the number of lamellae, the shape of the olfactory rosette and the distribution of the OSNs differs amongst fish species. The structure of the olfactory rosettes has been described in several fish species such as zebrafish (Hansen and Zeiske, 1998), sturgeons (*Acipenser ruthenus* and *Acipenser baerii*) (Zeiske et al., 2003), European eel (*Anguilla anguilla*) (Atta, 2013) and in flatfish such as winter flounder (*Pseudopleuronectes americanus*) (Prasada Rao and Finger, 1984) and turbot (*Psetta maxima*) (Doldán et al., 2011).

Senegalese sole, resides in benthic habitats that often have low light irradiance and high sediment loads and it would be probable that olfactory cues are important to the species. Flatfish species have a unique peculiarity compared to other teleost, which is the asymmetry with both eyes on the upper side and a nostril / olfactory rosette on the upper and lower side (Kasumyan, 2004). Nevertheless, the eyes are symmetrical in larvae that go through a complex metamorphosis (Ribeiro et al., 1999; Fernandez-Diaz et al., 2001). During the metamorphosis the left eye migrates to the right upper side and the olfactory system also suffers a transformation and one olfactory rosette is located in the upper (right) side with the nostril opening into the water column and the other olfactory rosette is located in the lower (blind) side which is often buried in the substrate. This metamorphosis has lead to several problems in aquaculture and was associated with skeletal deformities (Gavaia et al., 2002; Engrola et al., 2005) and incorrect pigmentation (Soares, 2002; Villalta et al., 2005). However, these problems were also found in sole in the wild environment, but in a lower proportion. Gavaia et al. (2009) found wild Senegalese sole presented less skeletal deformities (19 %) than those larvae that were reared in captivity (79 %), which indicated these differences were produced by a selective mortality of wild deformed fish in nature and/or that aquaculture-related rearing conditions were involved in the development of skeletal deformities in sole. These types of deformities during the metamorphosis could have an impact on the olfactory system that may differentiate wild from cultured soles affecting the functionality in the olfactory rosettes. In the same manner, the genes expressed in the olfactory rosettes could be differentially expressed between wild and cultured Senegalese sole and these sort of differences between origins at morphological and molecular level have been reported in other species such as red sea bream (*Pagrus major*) (Mana and Kawamura, 2002), black sea bream (*Acanthopagrus schlegeli*) (Mana and Kawamura, 2002) and blue catfish (*Ictalurus furcatus*) (Li et al., 2014).

Further studies demonstrated that the olfactory asymmetry in Senegalese sole was associated and probably related to different functionality of the olfactory rosettes (Velez et al., 2005) and olfactory signal transduction (Velez et al., 2013). Food-related odorants such as, L-phenylalanine and 1-Methyl-L-tryptophan gave significantly higher electro-olfactogram (EOG) responses in the lower olfactory epithelium (Velez et al., 2007b; Velez et al., 2011) in comparison to the upper olfactory epithelium which in turn had significantly higher EOG responses to conspecific-derived odorants such as taurocholic acid (Velez et al., 2007a; Velez et al., 2009). For this reason, Carazo (2013) evaluated the different products excreted by Senegalese sole to examine the olfactory sensitivity of those products using EOG between conspecific in the upper olfactory rosette. For that purpose, five different groups of fish were sampled to collect water samples, mucus, urine and faeces (donor). A total of five groups were sampled, two groups were formed by mature cultured Senegalese sole, one group formed by immature wild Senegalese sole, another group formed by mature wild sole which obtained spontaneous spawns and the last group formed by mature cultured fish from the second generation (produced with *in vitro* fertilisation). Four cultured females were hormonally treated, two of them with prostaglandins and the other two with 17, 20*β*-P, different water samples (12 samples in total per female) were collected before and after the induction. The same process was performed with two cultured females without induction as control. Water and urine samples were collected in the inlet and outlet of the tanks, the different samples were concentrated using sep-pack fractioning the sample according to its molecular weight (C-18 and C-2). The EOG response in immature Senegalese sole (receivers) was recorded to the different samples. The different water samples, did not register any significant signal in the different EOG performed. Similarly, the mucus samples did not register any significant signal in the different EOG. Nevertheless, the different dilutions of urine and faeces were differentially perceived by the receivers, which could differentiate between urine and faeces from mature and immature conspecifics and could differentiate between males and females. Additionally, the urine C-18 eluate retained the major olfactory activity and presented the same response as untreated urine differentiating between maturity stages. These results revealed that urine and faeces were potent conspecific fluids for Senegalese sole. The olfactory sensitivity was significantly different in relation to maturity status and sex of the donor.

This thesis examines the differences in the olfactory system between wild and cultured Senegalese sole at different levels, morphological and molecular. In addition, this thesis evaluates the effect of urine from mature sole of different origin (wild and cultured) and ovarian fluid on different conspecifics with different maturity stages. Lastly, the effect of urine on the reproductive axis, more specifically, on the reproductive hormone LH was examined in adult sole.

***Behavioural analyses***

Behavioural patterns of animals have been studied for several decades in respect to different aspects such as physiological determinants, adaptive conditions, ultimate causation and the behaviour of healthy animals (Hart, 1988). Additionally, behavioural ecology was included as a scientific discipline to comprehend the development of animal behaviour and understand the interactions between individuals and the consequences of these interactions in the population (Gross, 1994). Fish have a wide role inside of this discipline in several topics, including mate choice, territoriality, fighting and diet selection among others (Huntigford, 1993). The knowledge of the general ecology and evolution in the different behavioural traits might increase the possibilities to optimize the cultivation of a species under rearing conditions.

## Dominance and stress coping styles

According to Qvarnstrom and Forsgren (1998) in contests of mate selection: "*Dominance could be defined as success in contests. Dominant individuals exclude at least some of their rivals from access to limited resources, such as food, resting sites or mates*".

The status in a hierarchy might be crucial to the access to food, shelter and sexual partners (Noakes, 1978). Social status is directly linked with welfare and physiology in fish, being a very important point under captive conditions, as intensive production can affect the welfare of a population (Fox et al., 1997; Filby et al., 2010). Hierarchies have been observed in many fish species both in wild and captive conditions. Furthermore, social rank has been associated with reproduction, including the synchrony of sexual maturation and gonadal condition (Desjardins and Fernald, 2008). The low participation in parental contribution in captive Senegalese sole during the spawning season (Porta et al., 2006; Martín et al., 2014) suggests there may be a social hierarchy in the population that influences the participation of individuals in successful spawning. However, Senegalese sole are considered a non-aggressive species (Salas-Leiton et al., 2008) and nothing is known about the social dominance parameters by which this species is governed.

Dominance hierarchies within a group or population are often established through dyadic interactions where the winning depends on the relative fighting capability of the adversaries (Chase et al., 2003). Therefore, individuals that win a contest or are successful in agonistic behaviours achieve dominant status and then the probability of winning the subsequent contests is increased, in the same way when an individual loses a contest it increases the probability to lose the next contest. The agonistic behaviour could include aggression, threats, displays and fighting indicating competition over resources, such food, places or mates (Chase et al., 2003). The decisions of two animals to enter a conflict are defined by the interplay between costs and benefits as contests have high costs considering energetic expenditure, possibility of injury and increased susceptibility to predators (Abbott and Dill, 1985; Puckett and Dill, 1985). The contests between dominant and subordinate individuals are very usual, however, the conditions between them are not always equal (Chase et al., 2003).

According to Huntingford (1993), the inequality between individuals could be categorized into three types of connections:

1) Resource-holding potential: animals that are capable to protect resources often win without much physical interaction.
2) Resource value: animals more invested in a resource are likely to invest more in the fight despite of the possibility for suffering greater costs.
3) Intruder retreats: when participants are of equal fighting aptitude and competing for a certain place, the resident of the territory is likely to end as the winner because he values the territory more.

Therefore, the individual who arises victorious is rewarded with the dominant status, demonstrating physical superiority. Nevertheless, this winning incurs costs as well as benefits. In mammals and insects, the benefits have been listed as reproductive and foraging success. Intriguingly, fish are not an exception and several fish species have been used to determine mate choice and study the progression of different displays of parental care strategies (Huntingford, 1993). In the case of Senegalese sole, the non-aggressive competition "Follow" behaviours among males during the courtship (Carazo et al., 2016) could lead to the possibility that females choose the male that wins that competition.

Dominance is related to agonistic behaviour, which is any social behaviour associated with fighting. The term has broader meaning than aggressive behaviour because it includes threats, displays, retreats, placation, and conciliation (Scott and Fredericson, 1951). Aggression is considered essential in social fish species such as zebrafish (*Danio rerio*) (Filby et al., 2010), Nile tilapia (*Oerochromis niloticus*) (Cardoso and Volpato, 1997), rainbow trout (*Oncorhynchus mykiss*) (Andersson and Höglund, 2012), three-spined sticklebacks (*Gasterosteus aculeatus*) (Ruiz-Gomez and Huntingford, 2012) among others. The ranking of each individual in a hierarchy can be determined by conscientiously watching the outcome of the initial fights. However, each social fish species has its own strategy to display dominance (Reebs, 2008). The strategy to determine and display dominance is related to the species ecology and this must be considered when a species is submitted to captive conditions as the intensive conditions in aquaculture could influence behavioural adaptations to the environment (Huntingford, 2004). There are some species that do not display the same aggressive behaviour, or at least not so marked as mentioned above due to the costs that this supposes (Reebs, 2008). Senegalese sole is considered a non-aggressive species, which do not display aggressive interactions between conspecifics with different stocking densities, high or low (Salas-Leiton et al., 2008). However, the absence of aggressive interactions does not mean that threatening or competitive interactions are absent from the population (Reebs, 2008). Therefore, Senegalese sole dominance could be governed by other pathways than aggressive interactions towards the conspecific.

Territoriality during feeding is very common at two different levels, first, defending a specific territory and secondly, the possibility to obtain a limited resource

very appreciated by all individuals which form the population. In some salmonids, the animals with different rank present different feeding and aggressive strategies. For example, coho salmon (*Oncorhynchus kisutch*) juveniles defend feeding territories in streams. This feeding territoriality is an important part of foraging where the fish compete for a restricted number of feeding places (Puckett and Dill, 1985) and the success in competition for areas with access to food determine the organisms feeding efficiency and in consequence the fitness. Øverli et al. (1998) observed in Arctic charr (*Salvelinus alpinus*) that dominant fish presented high food intake and swimming activity levels, besides, subordinate fish showed inhibition in food intake in presence of dominant fish. In general, aggressiveness is the key for this competition. Paull et al. (2010) observed in males and females of zebrafish that those with the higher positions in the hierarchy according to territoriality, feeding and spawning site, achieved higher reproductive success. Salas-Leiton et al. (2010) suggested that the growth dispersion onto a Senegalese sole population could be affected by the social hierarchy, however, as commented before, nothing is known about dominance in this species. Feeding in flatfish species has been particularly studied due to the metamorphosis performed by these animals, for example, the feeding regime is the parameter which determine the growth and locomotor activity in greenback flounder (*Rhombosolea tapirina*) (Chen and Purser, 2001) and feeding is connected directly with general activity and growth in Senegalese sole larvae (Fernandez-Diaz et al., 2001). In addition, animals that acquire the dominant position in early life stages in fitness-related traits might expand this social status to reproductive success (English et al., 2013).

Moreover, dominance behaviour underlies neurobiological mechanisms and previous studies have demonstrated that some fish species present different gene expression profiles in some genes related to dominance (monoaminergic/neurotransmitter activity) depending on the dominance category (dominant/subordinate) (Winberg and Nilsson, 1993). The effect of social status on brain monoamine metabolite expression levels has been observed in Arctic charr, where higher concentrations of dopamine were found in the telencephalon of the dominant individuals (Winberg et al., 1991). In some cases, serotonergic activity is related to food intake, for example in rainbow trout, feeding hierarchies have been demonstrated in groups with abundant fish, besides, individual food consumption seems to provide an index of individual social status (McCarthy et al., 1992), so subordinate fish showed higher levels of serotonin decreasing the aggressive episodes and controlling the food intake. This situation was also observed in Arctic charr (Øverli et al., 1998). This difference in gene profiles between dominant and subordinate could be very useful to obtain a dominance marker for a tool selection in Senegalese sole.

Furthermore, in many animal species, social defeat is a potent stressor that can alter the physiology and behaviour. This stress could have behavioural effects such as appetite privation, decrease aggression and might reduce reproductive behaviour (Perret, 1992; Blanchard et al., 2001). Different behavioural-physiological profiles are defined as stress coping styles (Koolhaas et al., 1999), proactive animals (bold, active, impulsive and present lower levels of glucocorticoids) and reactive animals (shy,

passive, less active, immobility and higher levels of glucocorticoids). For example, Øverli et al. (2004) found that different stress coping styles categories influence in social rank and levels of aggressive behaviour in rainbow trout. Another study performed in the same species found dominant fish presented more aggressive, higher activity, bold and larger body size than subordinate fish, so activity-boldness syndrome was linked to dominance and high levels of competition (Colléter and Brown, 2011). Brain gene expression has been assessed to different stress coping styles (proactive, reactive and, in some cases, intermediates) in several fish species. For example, Rey et al. (2013) found different individual variation in personality and transcriptomic profiles in zebrafish, and this difference in expression was related to behavioural traits. Rey et al. (2016) demonstrated in common carp (*Cyprinus carpio*) that after an immune challenge, proactive fish presented different mRNA expression in some genes compared to reactive fish, highlighting that screening for stress-coping styles previous to trials in adaptive physiology can confuse the understanding of data obtained in individual variation. Recently, several stress coping styles tests were shown (restraining, confinement and exploratory tests) to successfully characterise the stress coping styles of Senegalese sole larvae, juveniles and breeders and were used to identify the presence of proactive and reactive individuals (Ibarra-Zatarain, 2015; Ibarra-Zatarain et al., 2015; Ibarra-Zatarain et al., 2016). Nevertheless, Senegalese sole breeders with different reproductive success and from different origins (wild and cultured) presented similar profiles of coping styles (Ibarra-Zatarain, 2015). Therefore, stress coping style did not appear to be associated with reproductive success and did not offer an explanation for the existence of the reproductive dysfunction in cultured breeders (Ibarra-Zatarain, 2015). However, a proactive stress coping style was associated with higher growth and earlier puberty (Ibarra-Zatarain, 2015) and a possible gene marker associated to coping styles would be valuable selection tool for fish farms.

This thesis aimed to examine dominance behaviour from a behavioural-molecular point of view to characterize dominant and subordinate Senegalese sole juveniles including and identifying associated behaviour and gene expression. Additionally, dominance behaviour and gene expression was examined in association to coping styles.

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# Section 2: Thesis overview

# Thesis overview

## Section 1: Background

*Chapter 1*, comprised a general introduction justifying Senegalese sole problematic in culture development and the different sub-objectives of this thesis.

## Section 2: Thesis overview

*Thesis overview* summarized the structure of this thesis (the current section). Description of the different aims and explanation of each section. Thesis overview would be an extension of the general introduction.

The main goal of this thesis was to understand the reproductive social dysfunction present in Senegalese sole breeders, especially in males. For this purpose, three sub-objectives were established: (1) to examine the effect of the presence of the spawning wild Senegalese sole breeders in the courtship and reproductive success of cultured breeders, (2) to describe the olfactory system at different levels and (3) to establish dominance parameters in a behavioural-molecular approach. Therefore, this thesis is divided into three main sub-objectives (section 3, 4 and 5). In each section, physiological and / or molecular analyses are included to provide information from gene right through to social behavioural parameters.

## Section 3: Reproductive behaviour and spawning

*Chapter 2*, examined the effect of the presence of successfully spawning wild Senegalese sole breeders on the courtship and reproductive success of cultured Senegalese sole breeders by observing the evolution of courtship and reproductive success during three consecutive spawning seasons. The experiment compared a control group that was formed of only cultured breeders that had not had any contact with wild animals with two groups formed of wild and cultured breeders. Several behavioural parameters (activity and behaviours from the courtship) were compared amongst the mixed groups and control group. Different reproductive parameters from spawns were evaluated (volume of floating and non-floating eggs, fertilized, hatching and survival rates) and paternity analysis was used to identify parents involved in fertilised spawns. In addition, FSH and LH were measured to observe the differences between sole that reproduced and that did not reproduce. The main results of the present study were observed behavioural differences among groups, with the first report of the participation of cultured males in the courtship, which resulted in two isolated spawns from cultured breeders.

**Section 4: Olfaction**

*Chapter 3* describes the structure and morphology of the olfactory rosettes (upper and lower) from male and female Senegalese sole juveniles. The analysis was made using five different histological stains on the olfactory rosettes from wild and cultured Senegalese sole. The main results of the present study were to observe that both origins (wild and cultured) presented similar structure of the olfactory rosettes. This study described for the first time the different cell composition in the olfactory rosettes of the Senegalese sole. (Part of the results of this Chapter were published in the journal *"Comparative Biochemistry and Physiology- Part D"*).

*Chapter 4* investigated the genomic organization in the upper olfactory rosette in mature Senegalese sole males and the differences of this profile between origin, wild or cultured. The upper olfactory rosette from three males of each origin (wild and cultured) were sampled during the spawning season and analysed by RNA-seq to determine the transcriptomic and functional association of the upper olfactory rosette (associated with the conspecific' chemical communication). The results of this study showed interesting different expression of transcripts related to olfaction, reproduction, nutrient sensing and immune system between origin, which could provide new clues about the abnormal sexual behaviour in this species. This study was the first report of the analysis of this structure using RNA-seq in Senegalese sole. (Published in the journal *"Comparative Biochemistry and Physiology- Part D"*).

*Chapter 5* evaluated the olfactory sensitivity of the Senegalese sole to urine from mature conspecifics collected during one complete spawning period (March to May). The study was performed by recording electro-olfactogram (EOG) in juveniles and adult cultured Senegalese sole females and males. In addition, LH was measured after stimulating the olfactory system with urine at different exposition times in adult males to observe the differences in plasma levels of this gonadotropin. The results of this study demonstrated that males of both stages presented higher perception to urine than females and urine was an important vehicle of chemical communication. In addition, mature female urine evoked a slight but significant increasing of the LH plasma levels in mature adult males and these levels were maintained until 30 minutes after of the olfactory stimulus was applied. These results showed the possible association between urine and reproduction. (In review in the "*Journal Experimental Biology*")

**Section 5: Behavioural analysis**

*Chapter 6* established dominance parameters in early and late cultured Senegalese sole juveniles in different dominance tests in pairs and in groups. The different parameters were established regarding different limited resources (food and territory). In addition, several transcripts were tested to check the differences between dominant and subordinates at molecular level. The main results of the present study

were that the dominance parameters were in concordance between food and territory tests. A number of behaviours (approaches, Rest the Head and Swimming above another) related to dominance were identified that could be used as an indicator of dominance in this species. Additionally, social status established by those tests also exhibited two mRNA that were differently expressed between categories (dominant and subordinate). This was the first study that reported dominance parameters in pair-wise interactions in this species and the linkage between social status and brain gene expression. (In review in the Journal "*PLOS one"*)

*Chapter 7* focused on gene expression in relation to stress coping styles to determine a possible behavioural biomarker in early juvenile cultured Senegalese sole. Juveniles were submitted to different individual and grouping "stress coping style" (SCS) tests according to Ibarra-Zatarain et al., (2016). After the animals were categorized as proactive, intermediate and reactive, molecular analysis was applied to test different important genes related to different SCS categories. The results of this study demonstrated that coping style influenced in the expression of some transcripts tested. This study was the first report linking behavioural traits and brain gene expression in this species.

**Section 6: Final discussion**

*Chapter 8* summarized and discussed the main outcomes of the present thesis.

**Section 7: Conclusions**

*Chapter 9* remarked the main points from each chapter to provide the main concluding arguments.

**Section 8: Future Research**

*Future research* section explained several lines of research which might be applied in the future to go on with the development efforts and establish a breeding program based on cultured Senegalese sole broodstock.

# Section 3: Reproductive behaviour and spawning

# Chapter 2

## The effect of the presence of spawning wild Senegalese sole breeders on the courtship and reproductive success of cultured Senegalese sole breeders

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Reproductive Behaviour
Social learning
Spawns
Origins

**Abstract**

The main objective of this study was to determine the effect of the presence of spawning wild Senegalese sole breeders on the courtship and reproduction of cultured breeders. The life cycle of Senegalese sole is not closed in captivity due to a reproductive dysfunction related to the lack of the participation in the courtship of cultured male breeders. Three experimental groups were formed: Control group ($n$ = 10) formed by only cultured sole; B1 group constituted by mixed-origin sole (10 cultured and 9 wild breeders); B2 group composed by mixed-origin sole (10 cultured and 8 wild breeders). All cultured breeders were from the same stock that had never successfully reproduced, whilst the wild broodstock had spawned viable eggs in captivity. All groups were held in the same captive conditions and behaviour and spawning were recorded for each group over three spawning seasons from 2013 to 2015. Behaviour was studied with video recordings to compare locomotor activity and courtship behaviours including the "Follow" behaviours where sole swim after each other in a procession. All spawns were collected and the parameters registered were: floating and non-floating eggs, fertilisation, hatching and survival rates. In addition, parental analysis was made of larvae from viable spawns. Lastly, steroids, LH and FSH plasma levels were measured using ELISA to observe differences between wild sole that reproduced and wild breeders that did not reproduce. No viable spawns were obtained from the control group and locomotor activity and courtship behaviours counts were significantly lower than in the experimental mixed-origin groups (B1 and B2). In the experimental groups, cultured males participated actively in the "Follow" behaviours with the courting wild sole and this participation increased significantly over the three years of the experiment. Two spawns were naturally obtained from one cultured male, which fertilized eggs from one cultured female. The same cultured female coupled with a wild male. There were no differences in gonadotropins and sex steroid plasma levels between breeders that reproduced and that did not reproduce. This is the first report of cultured male breeders participating in reproductive behaviour, which could be associated with social learning processes where cultured males copied the spawning behaviour of wild Senegalese sole breeders.

## Introduction

During recent decades, the marine finfish aquaculture industry in Spain has mainly produced six species: gilt-head seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*), turbot (*Psetta maxima*), meagre (*Argyrosomus regius*), tuna (*Thunnus thynnus*) and Senegalese sole (*Solea senegalensis*). The aquaculture producers organisation, APROMAR (2015), stated that Senegalese sole had good characteristics for aquaculture, additionally, indicating that this species had in recent years the highest economic return of all aquaculture marine fish species that were commercialised in Spain. The good characteristics of this species for aquaculture are good growth rate, larval survival and high capacity for adjusting to intensive production. However, there exist some issues with the cultivation of Senegalese sole that have to be improved to achieve an optimum industrial production (Morais et al., 2014). Nowadays, after years of research, the two main problems that have limited the Senegalese sole production are a) high frequency of bacterial infections (Morais et al., 2014) which have led to high mortalities (Padrós et al., 2003; Toranzo et al., 2003) and b) a reproductive dysfunction in cultured breeders that were bred and reared in captivity. The bacterial infections have been shown to be the reflection of the poor understanding of the physiological necessities of this species (Cañavate, 2005). The reproductive dysfunction, means Senegalese sole production has been based on the spawning of wild-origin breeders (Dinis et al., 1999; Anguis and Cañavate, 2005; Martín et al., 2014) a practice that is unsustainable in a long-term period. This situation is the consequence of the absence of spontaneous spawning of viable eggs from cultured breeders. The spawns from cultured broodstock have been infrequent and eggs did not hatch (Agulleiro et al., 2007; Guzmán et al., 2008; Howell et al., 2009; Norambuena et al., 2012; Rasines et al., 2012). Carazo (2013) observed that the eggs from cultured breeders were not fertilized. All these observations and studies have led to the hypothesis that some dysfunction in an attribute (developmental, nutritional, social and genetic) in the larval or juvenile stage might be responsible for the failure of cultured sole spawning (Howell et al., 2011).

One of the first approaches applied for trying to solve this reproductive dysfunction was the control of gamete production and spawning using hormone therapies (Agulleiro et al., 2006; Guzmán et al., 2008; Guzmán et al., 2009; Rasines et al., 2012; 2013). Several hormone therapies have been applied in Senegalese sole males and females to induce spawning. For example, Agulleiro et al. (2007) and Guzmán et al. (2009) applied Gonadotropin-Releasing Hormone analogue (GnRHa) implants and injections in cultured Senegalese sole obtaining spontaneous spawning but the eggs were not fertilized. The same researchers (Agulleiro et al., 2006; Guzmán et al., 2011) also applied human Chorionic Gonadotropin (hCG) and different doses of GnRHa in cultured males obtaining significantly more production of sperm, however, the spawned eggs were still mainly unfertilized with just one spawn obtained from cultured animals after applying double GnRHa treatment in cultured females and hCG in cultured males (Guzmán et al., 2011). These results suggested that hormonal issues in the control of gametogenesis were not the reason of the reproductive dysfunction in cultured breeders

(Guzmán et al., 2009; Morais et al., 2014). Currently, the application of GnRHa treatments in females is used in artificial fertilization with cultured sole breeders (Chereguini, 2006; Chereguini et al., 2007; Rasines et al., 2012; 2013). The cultured females were induced to ovulate with GnRHa and the viable eggs were extracted by abdominal pressure and were fertilized with fresh and cryopreserved sperm from cultured males (Rasines et al., 2012; 2013). This practice demonstrated that cultured breeders produce viable gametes and is considered a good approach in Senegalese sole aquaculture, like in turbot. However, the artificial fertilization method is complicated due to the low sperm volume found in Senegalese sole (Beirao et al., 2009; Cabrita et al., 2011) and, therefore, difficult to implement on a commercial scale.

The knowledge of the cause of this reproductive dysfunction increased with a series of experiments that were performed to examine broodstocks with breeders of mixed origin (wild and cultured) which were cohabiting, with hormone manipulation (Mañanós et al., 2007) and without hormonal treatment (Carazo, 2013; Martín, 2016). In the case of the experiments performed by Mañanós et al. (2007), different groups were established, one formed by cultured males and females breeders, another group established by wild females and cultured males and another group formed by cultured females and wild males. In this experiment only the cultured animals were hormonally treated. The hormonal therapies were the same for all groups, females with GnRHa slow-release implants and males with injections of hCG. Fertilised spawns were observed from the broodstock having treated cultured females and untreated wild males, but not in the other way, treated cultured males with untreated wild females, which only produced unfertilised eggs. This study, reported for the first time the critical reproductive dysfunction was associated with the captive male breeders.

Carazo (2013), corroborated those results and by observing the reproductive behaviour advanced the understanding of reproductive dysfunction. As a step towards these advances an ethogram of reproductive behaviour of captive-wild Senegalese sole was described for the first time by Carazo et al. (2016) using digital video recording and lightning, which did not disturb the normal activity, behaviour or plasma levels of melatonin, (Carazo et al., 2013). Carazo (2013) conducted different observational trials establishing different groups of Senegalese sole breeders from 2008 to 2010. These groups were two groups formed of only wild males and females, one group formed of only cultured male and female breeders, and two mixed-origin groups (one formed of cultured males and wild females, and of cultured females and wild males). The successful courtship was first described in the wild Senegalese sole groups that produced fertilised spawns. The courtship was characterised as a complex serial of behaviours, which resulted in an increase in activity. The description highlighted how males performed, competed and attracted the attention of females. The females appeared to select the males, accepting or rejecting the attention of the males. The courtship could be described into three steps: step 1 (~1-2 hours) where the females stay quiet and males swam following each other around the tank in a distinctive “Follow” behaviour; step 2 (~10-15 mins) which consisted of “Rest the Head” behaviours, where principally males rest their head on the female and moved over the female to stimulate

the female to initiate the swim from the bottom to the surface in a coupled swim; step 3 (~1-3 mins) was the "Coupled" swim to release gametes, where the two fish swam in synchrony with the male under the female apparently push his dorsal side against the females lateral side. The coupled swim was from the bottom to the surface, where the gametes were released. Carazo (2013) compared the behaviours of wild fish on a night with spawning with the behaviours on a night without spawning and observed a significant decrease in activity and the absence of the "Follow" and "Coupled" behaviours on a night without spawning. Therefore, the courtship behaviour was fully described and in particular the complex behaviour required by males to achieve selection and successful spawning with the females was highlighted.

When the behaviour of the tanks of only cultured breeders were examined the behaviour on both nights with egg release (unfertilised) and without egg release were similar to wild fish on a night without spawning. There were no "Follow" or "Coupled" behaviours and activity was low. This was the first study to describe that the reproductive dysfunction in cultured sole was a behavioural problem as there was no courtship and this also demonstrated for the first time that the eggs released from cultured broodstocks were actually not fertilised. An almost identical situation was observed in the mixed origin group of wild females with cultured males. There were no "Follow" or "Coupled" behaviours and activity was low and no fertilised spawns were obtained. However, as found by Mañanós et al. (2007) the mixed origin group of cultured females with wild males produced fertilised spawns and observation of the courtship on the nights with successful spawning found that the cultured females completed the full courtship with wild males including the "Follow" and "Coupled" behaviours. Therefore, these studies (Carazo 2013) confirmed that the reproductive dysfunction was associated with the captive male breeders and demonstrated for the first time that it was actually a behavioural reproductive dysfunction that prevented the males from participating in the courtship to spawn fertilise the eggs of the females (cultured or wild females).

Furthermore, Carazo (2013) applied hormone therapies to try to trigger the reproductive behaviour in cultured Senegalese sole males. Hormonal treatments, GnRHa, hCG and PGF2α, in cultured females improved the maturity stage and spontaneous spawning were obtained without fertilization. In the case of cultured males, hormone therapies also had a positive effect in the sperm quality, however, the behavioural activity of the animals that were induced was low, showing that the role of these hormone therapies in reproductive behaviour and the environment did not offer a solution for the reproductive dysfunction.

It should also be mentioned that even in wild broodstocks there is a low participation in reproduction of this species. Parental analysis with microsatellites indicated that spawning was dominated by a few wild breeders, only 8.5 - 51.7 % of the broodstock (Porta et al., 2006; Carazo, 2013; Martín et al., 2014; Carazo et al., 2016), which leads to an underestimation of eggs production of breeders. In consequence, the spawning failure was shown to be due a behavioural reproductive dysfunction associated with cultured males showing that the environmental parameters (Anguis and

Cañavate, 2005), sperm quality (Cabrita et al., 2006) and hormonal treatment (Agulleiro et al., 2006; Rasines et al., 2013) did not offer a possible solution and were not the triggers to promote natural spawning.

Therefore, the aim of the present study was to observe the effect of the presence of spawning wild breeders in reproductive success and behaviour of cultured breeders. For this purpose, the spawning success and behaviour was analysed in two different mixed (wild and cultured) groups of Senegalese sole and compared with a control group (pure cultured breeders) during three consecutive spawning seasons. Furthermore, mate selection was determined by microsatellite parentage analysis based on the results of parental assignation of larvae collected during the study period. Moreover, levels of LH, FSH and sex steroids related to reproduction were measured using ELISA to determine if there were differences between sole with different reproductive success. Understanding the evolution of these broodstocks during these years may help to improve the management of this species under aquaculture conditions and give a better understanding of the reproductive dysfunction of cultured Senegalese sole.

## Material and Methods

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

### *Broodstocks and management*

The experiment had a duration of three years from October 2012 until October 2015, which included three spawning periods from March to June of each year.

Senegalese sole breeders (*Solea senegalensis*) ($n$ = 47; 1123.5 ± 61.1 g) from different origins (wild and cultured) were placed in three different tanks forming three different experimental groups. All individuals were Pit-tagged (ID-100 Unique, Trovan-Zeus, Madrid, Spain) and photographed for future identification. All experimental groups of breeders were located in IRTA Sant Carles de la Ràpita (Spain) in fibre-glass tanks of 10 $m^3$ (2.5 m x 5.6 m x 1.4 m) included in a recirculation system (IRTAmar®). The temperature regimen was controlled to simulate a natural cycle that ranged from 9-20ºC and during the spawning season after a natural rise to 18 ºC a weekly temperature cycle was used to stimulate spawning (Monday to Thursday at 16 ± 1 °C and Thursday to Monday at 18 ± 1 °C as described in Martín et al., 2014). Photoperiod was natural (artificial lightning) ranging from Light: Dark (LD) 9:15 to LD 15:9 with approximately LD 12:12 to 14:10 during the spawning season. Daytime lighting was delivered with fluorescent lighting (50 lux at surface) during the entire year. During the spawning season, red night lighting was used that allowed recording and observation of the sole behaviour. Red light was from fluorescent illuminations covered with a red filter that were adjusted to approximately 5 lux at the water surface. Carazo et al. (2013)

demonstrated that this illumination system did not affect sole behaviour, locomotor activity or plasma melatonin levels. Half of the bottom of each tank was covered by sand. Breeders were fed *ad libitum* with approximately 1 % of the total biomass five days a week at 09:30 h. The diet consisted on fresh feed (cooked mussels (Sariego Intermares, Spain), marine polychaetes (Topsy-Baits, Holland) and balanced feed (Repro-Vitalis, LE-7 mm ELITE, Skretting Co.).

*Experimental groups and experimental design*

Breeders were distributed in three experimental groups (Table 1): control, B1 and B2. Control group constituted of only cultured breeders ($n$ = 10; 5 males and 5 females). The other two groups were formed by breeders from different origin (wild and cultured): group B1 ($n$ = 19) was established by 10 cultured breeders (5 males and 5 females) and 9 wild breeders (3 males and 6 females); group B2 ($n$ = 18) was formed by 10 cultured breeders (5 males and 5 females) and 8 wild breeders (3 males and 5 females). All cultured breeders used for this study were from the same stock that had never successfully spawned. On the other hand, wild breeders used for this experiment had spawned in captivity, however, the individual identities of spawners were unknown. All groups were monitored in the same manner: video recording was used to perform the behavioural analyses, and regarding the spawns, the collection, incubation and parental analyses of larvae were exactly performed for every group (*see details below*). Moreover, all groups were formed since 2012 and approximately 30 cultured Senegalese sole juveniles provided by Stolt Sea Farm in May 2012 were cohabiting with breeders in each group at the moment the tanks were established. An egg collector was placed at the outlet of the tank of each group each year during the spawning season, March to June.

*Behavioural analyses*

Digital cameras (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) were used to film the fish behaviour during the spawning season. Two cameras were placed in the control tank, and three cameras were located in B1 and B2 tanks, eight cameras in total; four cameras were connected to a digital video recorder (model DVR-Camtronics-UCDI-DV4150-1500, supplied by Praesentis S.L.) and the other four were connected to another video recorder (model XMOTION-304H by Praesentis, S.L). In 2013 and 2014, 14 h recordings were made during the period of study (17:00 to 07:00). After the videos were analysed from 2013-2014 the recording period in 2015 was reduced to 10 h (14:00 to 00:00). The cameras were situated just below the water surface angled downwards. In the case of control tank with one of the cameras the field of view was almost the complete length of the tank and with the other camera the sand part of half the tank was observed. In the case of the other tanks, another angled was added with another camera, from the middle

of the tank to the water outlet, with this camera the view of the tank was completely covered. The cameras positions enabled 96 % of the entire water column of the tank to be filmed and recorded. All the tanks (control, B1 and B2) were studied from 25$^{th}$ of March to 3$^{rd}$ of June of each year (Senegalese sole spawning period in IRTA).

**Table 1.** Broodstocks distribution and characteristics of the different tanks monitored during three consecutive spawning seasons, 2013-2015. Columns indicate: Tank name, "N" (number of breeders in the tank), weight (Mean ± SD), stock density, origin, sex, previous cohabiting with spawning wild sole in IRTA's facilities.

| Tank | N | Weight (g) | Stock density ($kg/m^3$) | Origin | Sex | Previous cohabiting with spawning wild in IRTA's facilities |
|---|---|---|---|---|---|---|
| **Control** | 10 | 1165.3 ± 195.5 | 1.04 | Cultured | 5 Males<br>5 Females | NO |
| **B1** | 19 | 1146.9 ± 490.4 | 2.1 | Cultured | 5 Males<br>5 Females | NO |
| | | | | Wild | 3 Males<br>6 Females | NO |
| **B2** | 18 | 1037.2 ± 456.3 | 1.5 | Cultured | 5 Males<br>5 Females | NO |
| | | | | Wild | 3 Males<br>5 Females | NO |

*Activity*

The locomotor activity during the spawning season was measured counting the movements of the sole on 5 randomly selected days with spawning events and 5 random days without spawning events. Locomotor activity was assessed by putting a line across the middle of the screen dividing the field of vision of the camera (and the tank) in two and the number of times a fish crossed the line was counted for every hour recorded (In 2013 and 2014 the recording period was 14 hours from 17:00 to 7:00, however, in 2015 the period was 10 hours, from 14:00 to 00:00) (Carazo et al., 2013). To compare the locomotor activity among experimental groups the mean of every hour for the 5 days and each tank was divided by the number of breeders in the experimental groups.

*Behaviours registered during the peak of activity (courtship)*

A behavioural analysis was made by counting specific pre-defined behaviours (Carazo et al., 2016), "Rest the head", "Follow", "Guardian" and "Coupled" (*described below*). These behaviours have been previously implicated in the different steps of the courtship (Carazo et al., 2016). Those behaviours were counted during the peak hour (19:00-20:00) of locomotor activity.

- Rest the head: a sole resting the head on some part of the body of another sole.
- Follow: a sole following ("Follower") the lead ("Followed") fish. The "Follower" sole copy almost exactly the movements of the lead "Followed" fish. The "Follow" behaviours can last several minutes.
- Guardian: a sole (usually a male) rests the head on another fish (usually a female) and actively guards the sole from a third sole (another male).
- Coupled: a pair, male and female swim together, the dorsal side of the male pressed against the ventral side of the female, to the surface to release gametes. Gamete release might be visible in the recordings as an opaque cloud in the water (surface).

The peak hour of activity was sectioned in 5 min frames to count the behaviours registered in two different cameras having almost the complete vision of the tank. This analysis was made for the same 5 days with spawning events that were analysed for the locomotor activity for each tank and each year.

*Identification of individuals in "Follow" behaviours*

To determine the origin (wild or cultured) of the breeders involved in the behaviours termed as "Follow", the fish participating in "Follow" behaviours in groups B1 and B2 were identified. For this purpose, "Follow" behaviours ($n$ = 30) were randomly selected and analysed by three different observers that watched the videos and using a photo-video identification catalogue identified which fish were involved in the behaviours. The photo-video identification catalogue consisted of photos and short

video recordings of movements (swimming display) of each fish in each tank. To examine the frequency of the participation of cultured breeders over years in both experimental groups, B1 and B2, the number of cultured sole involved in "Follow" behaviours analysed before were counted in every "Follow" behaviour ($n$ = 30) for each year (2013 - 2015).

*Collection and evaluation of spawns*

The egg collectors were checked for eggs each morning at 08:00. Spawned eggs were collected and the following parameters were determined: total volume of eggs (ml), volume of floating and non-floating (inviable) eggs (ml), stage of development (to determine 50 eggs were analysed) and percentage fertilisation of floating eggs which was calculated with a sample of floating eggs placed in a graduated Petri's dish and the total number of developing eggs (with embryo) divided by the total number of eggs, after counting the number of eggs in three replicates of 100 mL subsamples. The daily fecundity was calculated as the total number of eggs related to the total weight of the females in the tank in kg. The total number of eggs was calculated by multiplying the total volume of collected eggs by 1080 (Anguis and Cañavate, 2005). Once the spawns were evaluated, the floating part was incubated in approximately 30 L incubators with open flow water and natural conditions (temperature and photoperiod). The larvae hatched after 36 - 48 h of incubation, depending on the water temperature (13 – 23 °C in open flow) and the embryonic phase at which the eggs were collected. Hatching rate was determined as the total number of larvae hatched divided by the total number of floating eggs, after counting the number of larvae in three 100 mL subsamples. Survival rate was determined as the number of larvae alive after 24 hours hatched, counted in three 100 mL subsamples, divided by the total number of larvae hatched. Larvae obtained were held in the incubators until 5 day post hatch (DPH), when larvae were collected to proceed with the paternity analysis.

*Paternity analyses*

Ten larvae obtained from every spawn were placed individually in 1.5 mL Eppendorf filled with 96 % ethanol and were sent to GENEAQUA (Facultad de Veteranía de la Universidad de Lugo, Lugo, Spain) to determine the paternity of the larvae. For this analysis, all breeders from all tanks were genotyped using the specific microsatellites for sole.

*LH and FSH from wild Senegalese sole breeders*

Blood samples were extracted from sole breeders which reproduced ($n$ = 6 females; $n$ = 4 males) and did not ($n$ = 4 females; $n$ = 2 males) in 17$^{th}$ May of 2015 to measure LH, FSH and steroids. For this purpose, blood samples were centrifuge at 3000 G and 4 °C during 15 min and plasma was collected and stored at -20 °C for further

analysis.

Plasma LH and FSH levels were quantified using ELISAs as previously explained for Senegalese sole Chauvigné et al. (2015) for FSH and Chauvigné et al. (2016) for LH.

*Steroid measurement*

Levels of the different steroids measured (T, E2 and 11-KT) in plasma were quantified in duplicate by commercial enzyme immunosorbent assay (EIA; Cayman Chemical Company) as previously described (Agulleiro et al., 2006; Chauvigné et al., 2012).

*Statistical analyses*

All the results were presented with means ± standard error (Mean ± S.E.M), and in some cases mean ± standard deviation (Mean ± SD), if indicated. Data were analysed with the Kolmogorov-Smirnov test and found to have a normal distribution. The analysis of the locomotor activity was made according to the description of daily activity profiles by Bayarri et al. (2004) and Carazo et al (2013; 2016). The "Follow" behaviour was transformed to percentage for better evaluation among groups and years. In addition, the number of cultured breeders participating in "Follow" behaviours amongst the years were compared with One-way ANOVA ($P < 0.05$) for both experimental groups. Behaviours quantity (number of times fish were observed displaying behaviours) for different tanks and different years were compared with One-way ANOVA ($P < 0.05$) with Dunnett's *post hoc* test to compare the differences with the control versus mixed tanks. Each behaviour was represented in frequency (number of times the behaviour was displayed during the hour of observation) and a mean (± S.E.M) for calculated for the 5 days. The comparison of LH, FSH and steroids in May 2015 between sole that reproduced in 2015 and sole that did not reproduce was carried out with a Student's t-test ($P < 0.05$) with Welch's correction. In all cases, data were logarithmic-transformed when needed. The statistical analysis was performed with SPSS Statistics 19.0 software (IBM Co., Hong Kong).

## Results

*Behavioural analysis (courtship)*

*Activity*

The locomotor activity of the Senegalese sole breeders showed a circadian rhythm associated with spawning in each group presenting repetition during the recording period for each year (Fig. 1). In 2015, the recording period was shorter than the other years starting at 14:00 and ended at 00:00. The reason of this modification was decided after analysing the two first years, 2013 and 2014, and the research group

resolute to find out the first increasing activity in the different groups adding hours' analysis previous 17:00. The locomotor activity seemed to increase before 14:00, however, the hours before were not recorded because the workers might disturb the activity of the tanks, due to several people worked around the tanks from 8:00 to 14:00 every day.

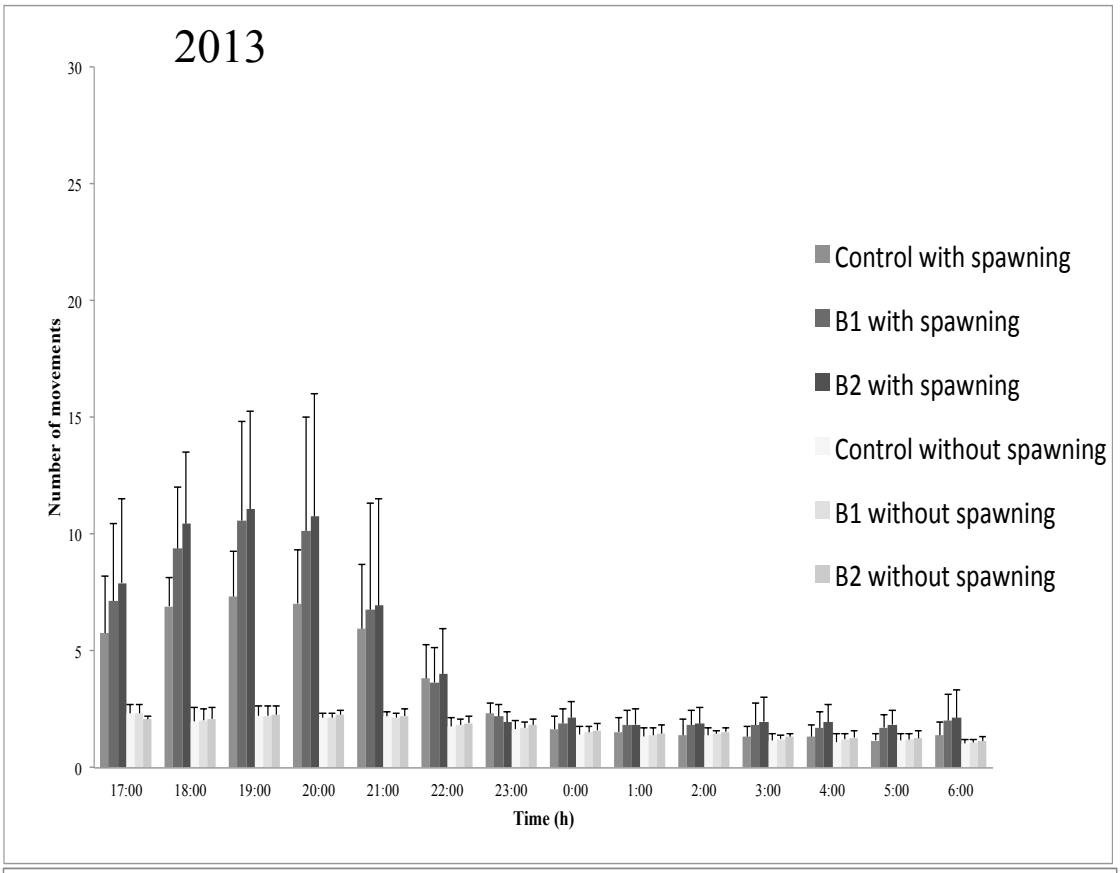
The activity was higher in the days with spawning events than days without spawning events for all the tanks and all years. In general, the locomotor activity was increasing in all tanks over the years, however, the increase was not significant. During periods with spawning the activity began to rise during the afternoon achieving the maximum at 19:00 and the minimum from 2:00 to 7:00. Thus, the peak hour of activity was registered from 19:00 to 20:00 in all the tanks and each year activity decreased after 20:00. Activity during periods without spawning was 2 times less during the peak hour of locomotor activity compared to days with spawning. On days without spawning the peak with less pronounced or there was no peak.

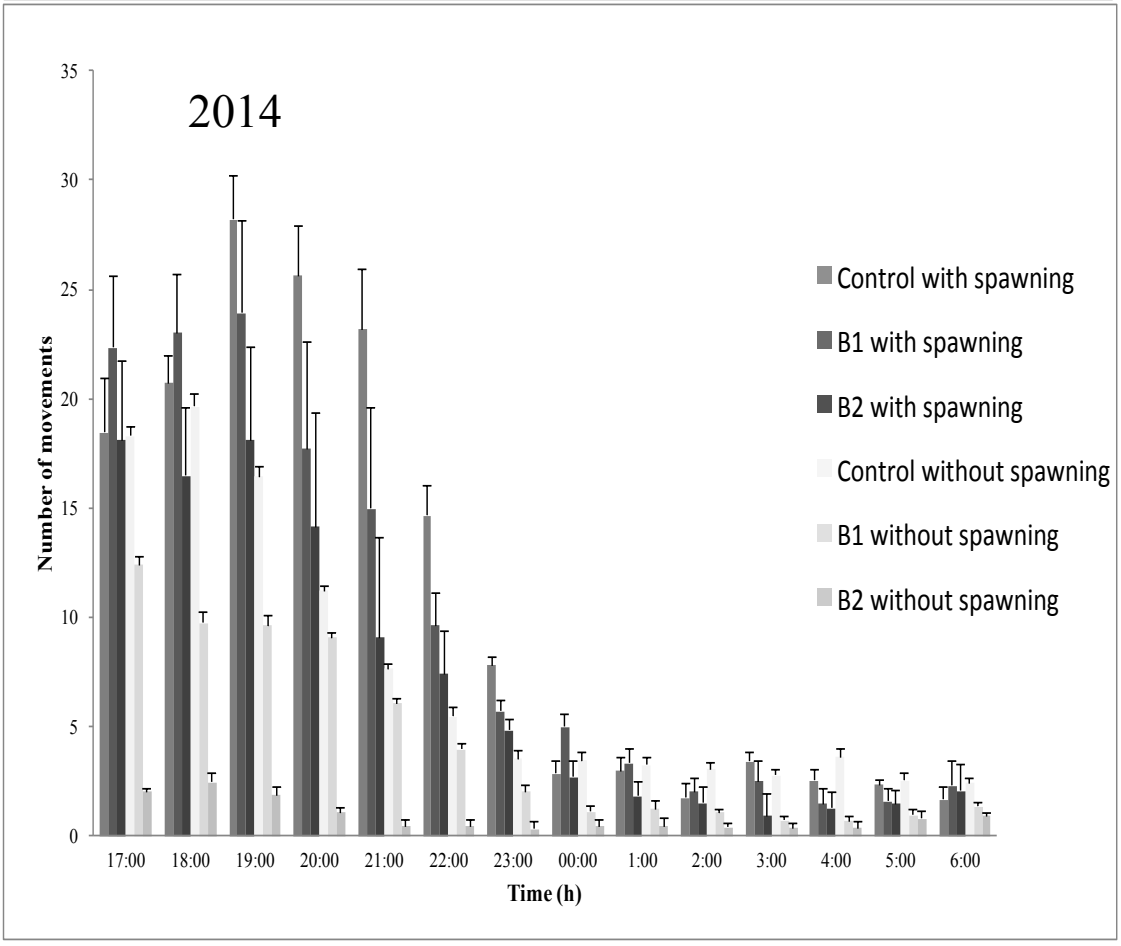
*Behaviours registered during the peak of activity (courtship)*

The behaviours registered were: "Rest the head", "Follow", "Guardian" and "Coupled". In **2013**, there were significantly less "Rest the head" behaviours in the control group (12.8 ± 9.1; $F_{2,14} = 3.907$; $P = 0.045$; Fig. 2A) compared to groups B1 (19.3 ± 16.1; $P = 0.03$; Fig. 2A) and B2 (29.2 ± 9.4; $P = 0.04$; Fig. 2A). In addition, the control group (1.3 ± 0.3; $F_{2,14} = 7.718$; $P = 0.06$; Fig. 2A) exhibited significantly lower levels of the "Follow" behaviours in comparison with B1 (2.7 ± 3.4; $P = 0.03$; Fig. 2A) and B2 (5.8 ± 5.0; $P = 0.034$; Fig. 2A), the groups with a mixed origin of breeders. Nonetheless, there were not differences among B1 and B2 groups for the other behaviours analysed, "Guardian" and "Coupled" swim ("Guardian" $F_{2,14} = 0.746$; $P = 0.492$; "Coupled" $F_{2,14} = 0.906$; $P = 0.427$; Fig. 2B). In **2014**, the behavioural counts of "Rest the head" were significantly different among the three groups groups. The control group was significantly lower than B2, which was significantly lower than B1 (Control = 2.0 ± 0.7; $F_{2,12} = 50.374$; $P < 0.01$; B1 = 45.7 ± 4.0; $P = 0.049$ and B2 = 24.5 ± 3.9; $P < 0.01$; Fig. 2A). For the "Follow" behaviour group B2 (6.5 ± 1.2; $F_{2,12} = 1.096$; $P = 0.36$) was intermediate and did not present differences in comparison to groups control and B2, whilst the control group was significantly lower than group B1 (Control = 2.0 ± 0.7; $P = 0.04$; B1 = 14.3 ± 2.3; $P = 0.03$; Fig. 2A). "Guardian" behaviour was just registered in the tank B2 (0.5 ± 0.3) and "Gamete release" was not registered at all in any group, B1 and B2 (Fig. 2B). In **2015**, there were not differences among experimental groups in the behaviour "Rest the head" (Control = 14.8 ± 2.0; $F_{2,12} = 1.972$; $P = 0.182$; B1 = 23.1 ± 3.6; $P = 0.883$; B2 = 21.2 ± 3.4; $P = 0.277$; Fig. 2A). However, for the "Follow" behaviour group B1 (5.5 ± 1.3; $F_{2, 12} = 10.364$; $P = 0.002$; Fig. 2A) presented significantly more "Follow" behaviours than the other experimental group, B2, that were not significantly different (Control = 1.7 ± 0.7; $P = 0.003$; B2 = 2.1 ± 1.1; $P = 0.004$; Fig. 2A). "Guardian" behaviour was lower in the control (0.0 ± 0.0; $F_{2, 12} = 7.281$; $P = 0.009$; Fig. 2B) compared to the other groups (B1 = 0.2 ± 0.01; $P =$

0.024 and B2 = 1.2 ± 0.4; $P$ = 0.007; Fig. 2B). “Gamete release” behaviour was not registered during the peak hour of locomotor activity.

Regarding the comparison among years, 2014 presented higher “Rest the head” ($F_{2, 44}$ = 10.630; $P$ < 0.01; Fig. 2A) and “Follow” behaviour ($F_{2, 44}$ = 2.994; $P$ = 0.06) than 2013 and 2015. No differences were found in the other behaviours registered among the three-year period studied (Fig. 2B).


2013
Number of movements
Time (h)
Control with spawning
B1 with spawning
B2 with spawning
Control without spawning
B1 without spawning
B2 without spawning


2014
Number of movements
Time (h)
Control with spawning
B1 with spawning
B2 with spawning
Control without spawning
B1 without spawning
B2 without spawning

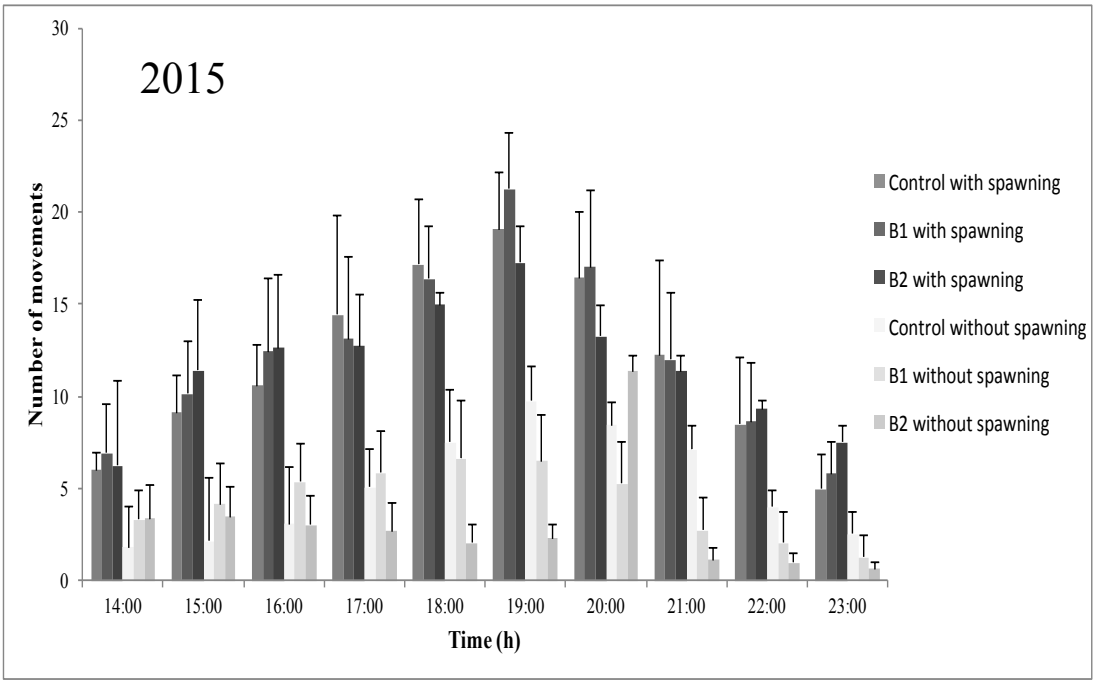



**Figure 1.** Mean tank activity (number of times an individual crossed a line in middle of the field of view of the camera which covered the entire length of the tank) of Senegalese sole (*Solea senegalensis*) breeders during the different periods that included spawning ($n$ = 5) and periods without spawning ($n$ = 5) for each year and each experimental group studied (Control, B1 and B2). In the case of 2013 and 2014 the activity was recorded during 14 hours from 17 h to 7 h. In 2015 the activity was recorded during 10 hours from 14 h to 00 h. Data was shown in Mean ± SEM.

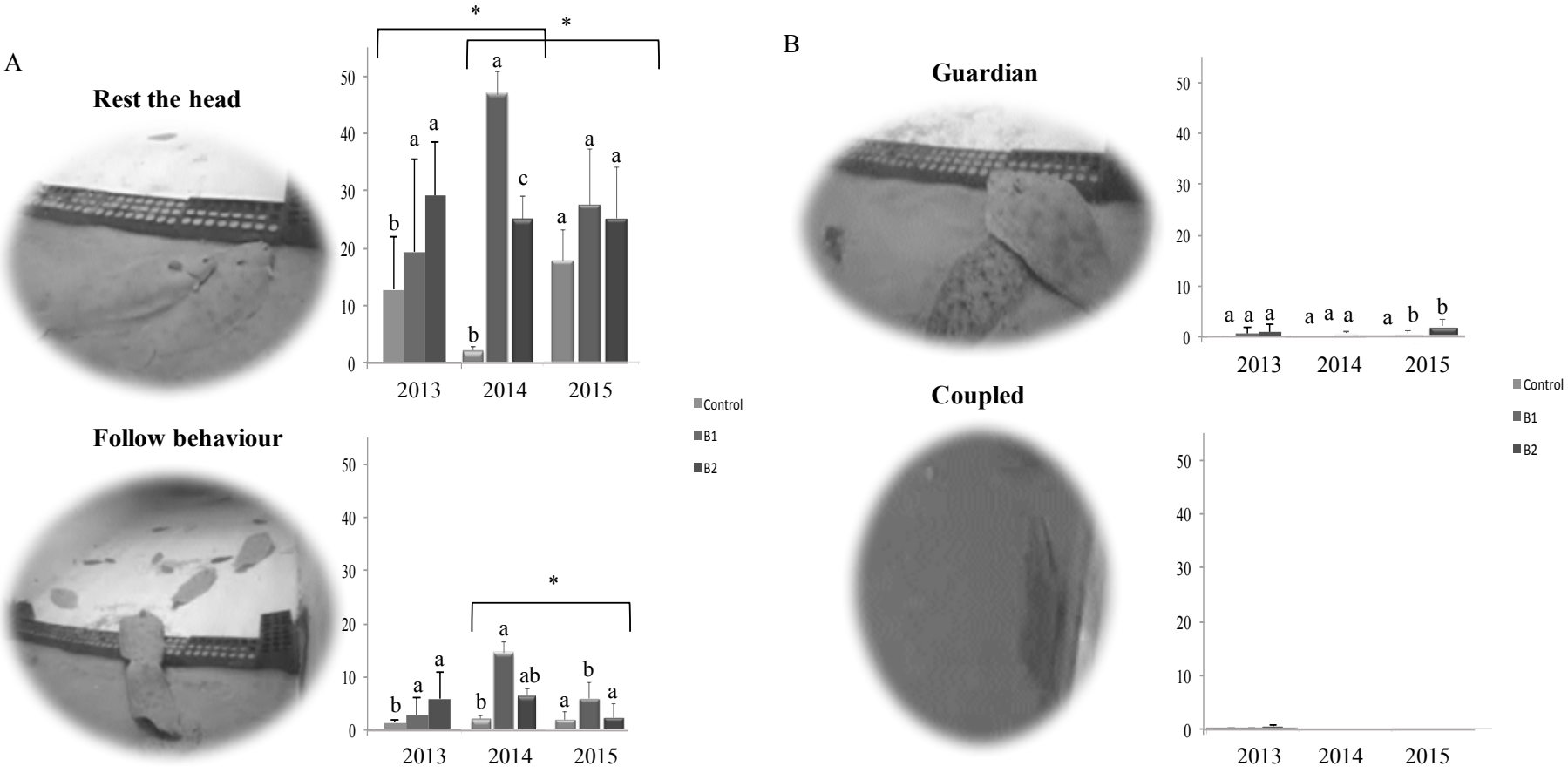


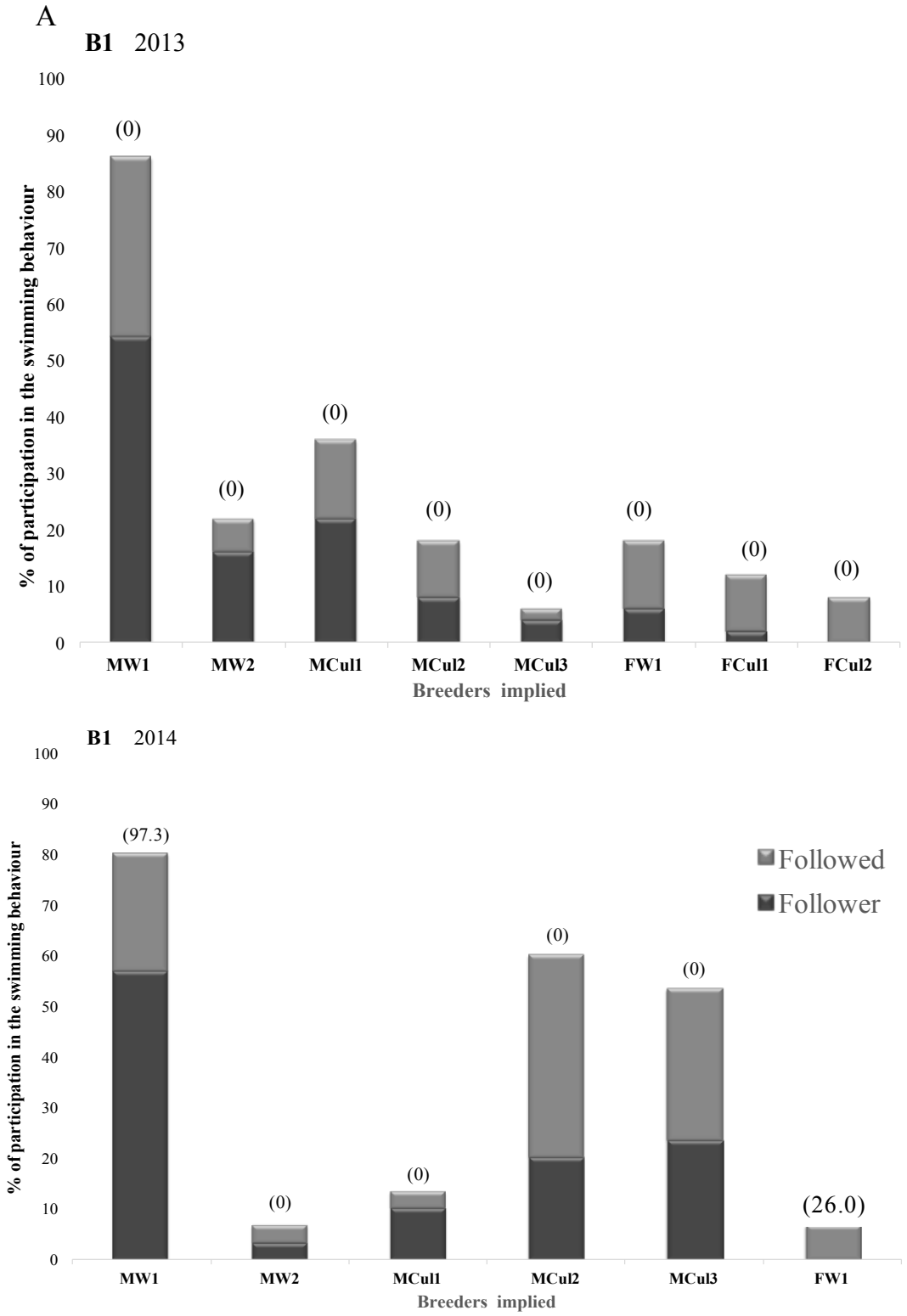

**Figure 2.** Video captures and mean frequency of behaviours of Senegalese sole (*Solea senegalensis*) breeders observed during the peak hour of activity (19:00 to 20:00) in periods with spawning ($n$ = 5) comparing each experimental group (Control, B1 and B2) and each year (2013-2015). **A** Depicts the two behaviours, Rest the head and Swim Follow. **B** Depicts the two behaviours, Guardian and Coupled swim to release gametes. Different letters indicate significant differences among experimental groups during a particular year. Data was shown in Mean ± SEM. (*) indicates significant differences between years within the brackets (One-way ANOVA Dunnett' *Post hoc*; $P < 0.05$).

*"Follow" individual identification*

The control group was not analysed for this behavioural part because (a) all fish were of cultured origin and obviously all fish involved in any behaviour were of cultured origin (b) previously the swimming behaviours "Follow and Followed" had not been observed in cultured breeders (c) there was no reproductive success in the control group. In addition, the swimming behaviours "Follow and Followed" were lower in the control group compared to the mixed groups, B1 and B2.

Frequent "Follow" behaviours were observed in the peak hour of the locomotor activity in both mixed groups, B1 and B2. In the tank B1 (Fig. 3A) cultured males gained importance in the "Follow" behaviour in 2015, which was the last year analysed, however, wild males seemed to dominate the "Follower" position (45-55 %) in each year. On the other hand, the "Follow" profile was different over the years in the experimental group B2 (Fig. 4A). In both tanks, the evolution of the participation of the cultured animals was increasing significantly over the years. In the group B1, 2013 presented significantly the lowest participation from cultured sole than in 2014 ($F_{2, 87}$ = 3.462; $P < 0.05$; Fig. 3B) and in 2015 ($F_{2, 87}$ = 6.275; $P < 0.001$; Fig. 3B), however, no differences were found between 2014 and 2015 ($F_{2, 87}$ = 2.813; $P = 0.06$; Fig. 3B). In the group B2, no differences were found between 2013 and 2014 in participation of cultured breeders ($F_{2, 87}$ = 1.220; $P = 0.181$; Fig. 4B), however differences were found between 2013 and 2014 ($F_{2, 87}$ = 6.099; $P < 0.01$; Fig. 4B) and between 2014 and 2015 ($F_{2, 87}$ = 4.879; $P < 0.05$; Fig. 4B). The participation was the highest registered in both groups in 2015. Cultured males participated in the "Follow" behaviours since 2013. Wild males dominated the "Follower" position in 2013 and 2014, however, that position was more controlled by the cultured males in the group B2 in 2015, at least in the "Follow" analysed. In case of the group B1, wild males dominated the "Follower" position in the three years studied. In general, females from both origins (wild and cultured) occupied the "Followed" position in both mixed groups. In ~1.5 % of those "Follow" behaviours, females started the persecution and several males followed her.

**Figure 3A.** Frequency (% of total) of the "Follow" behaviour ($n$ = 30, per experimental group) of Senegalese sole (*Solea senegalensis*) breeders noted during peak hour of activity (19:00-20:00) in periods with spawning identifying the breeders implied in the swimming behaviour for the B1 for the three-year spawning period. M = male, F = female, W = wild breeders, Cul = cultured breeders. The dark section of the bar corresponds to the percentage by which the sole was occupying the last positions of the "Follow" behaviour (Follower) and the clear section of the bar resembles to the percentage by which sole was followed by other sole (Followed). The number between brackets above the bars denotes the parental contribution in % of each sole.



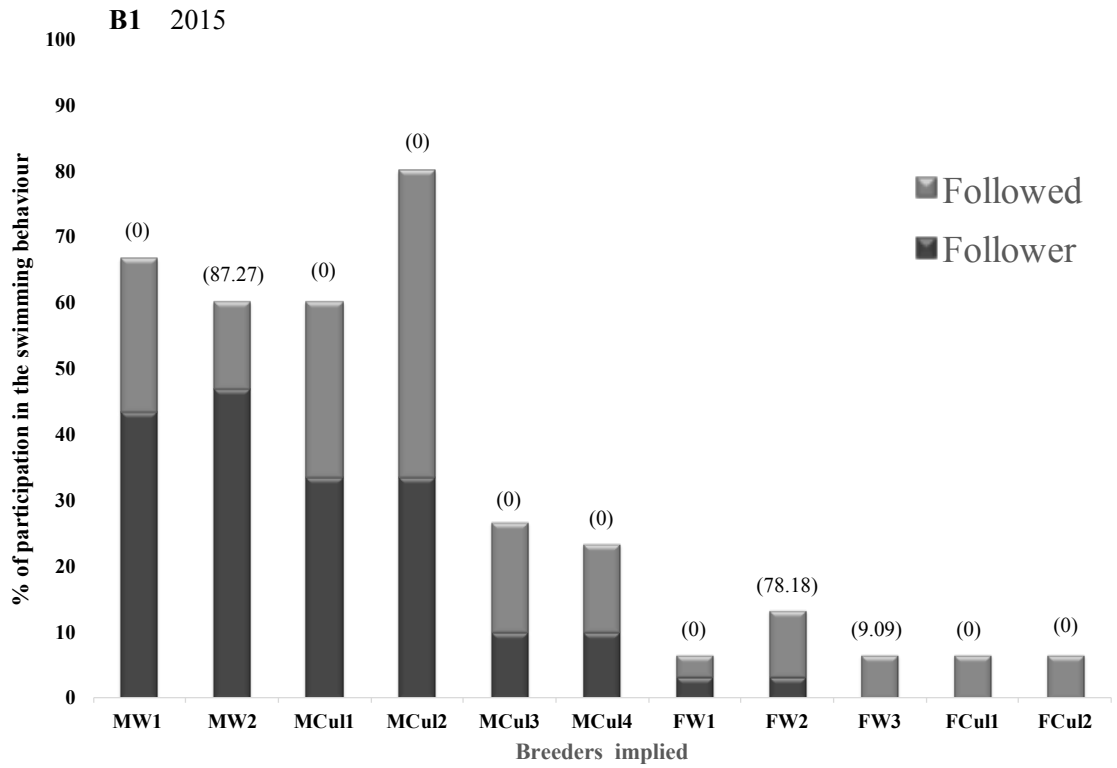



**Figure 3B.** Follow behaviour in cultured Senegalese sole where the number of cultured sole were noted in every Follow behaviour ($n$ = 30 per experimental group, $n$ = 60 total) and each year. Data are shown in Mean ± SEM. Different letter denoted significant differences (One-Way ANOVA; $P < 0.05$).

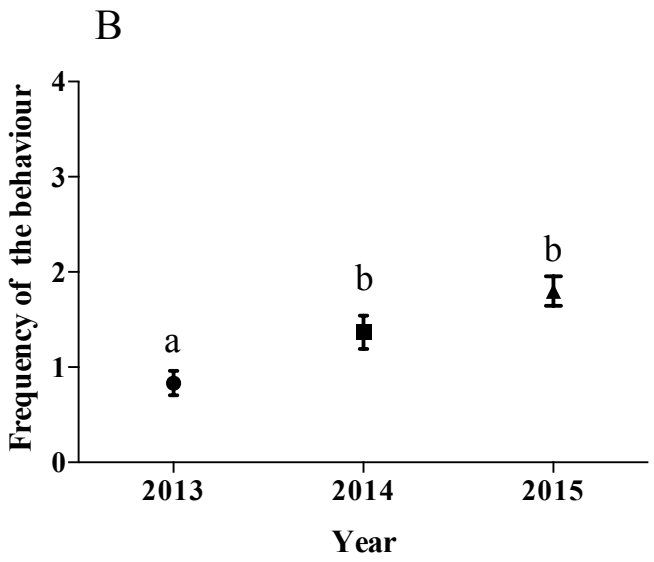
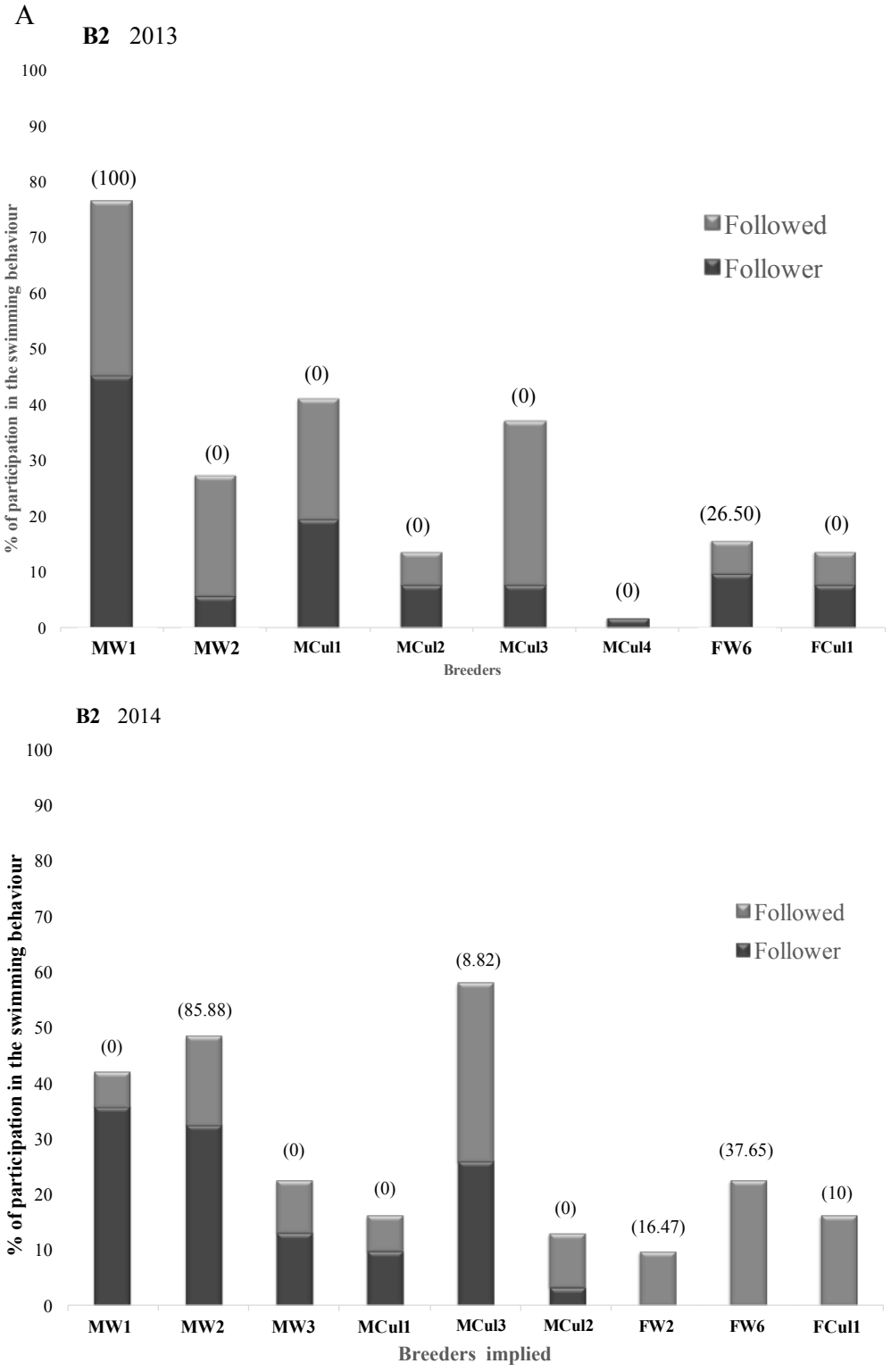


**Figure 4A.** Frequency (% of total) of the "Follow" behaviour (*n* =30 per experimental group) of Senegalese sole (*Solea senegalensis*) breeders noted during peak hour of activity (19:00-20:00) in periods with spawning identifying the breeders implied in the swimming behaviour for the B2 for the three-year spawning period. M = male, F = female, W = wild breeders, Cul = cultured breeders. The dark section of the bar corresponds to the percentage by which the sole was occupying the last positions of the "Follow" behaviour (Follower) and the clear section of the bar resembles to the percentage by which sole was followed by other sole (Followed). The number between brackets above the bars denotes the parental contribution in % of every sole.



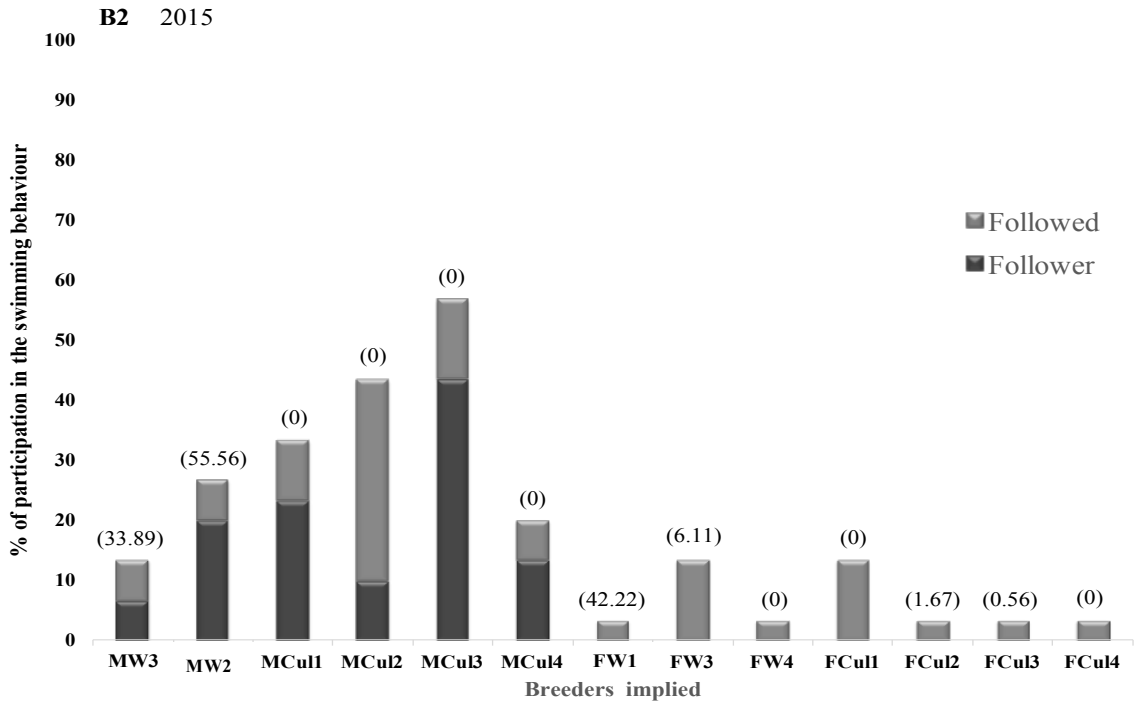
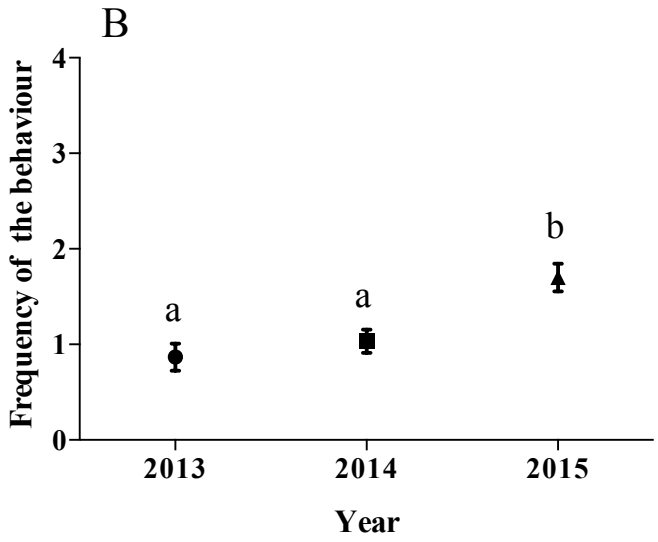



**Figure 4B.** Follow behaviour in cultured Senegalese sole where the number of cultured sole were noted in every Follow behaviour ($n$ = 30 per experimental group, $n$ = 60 total) and each year. Data are shown in Mean ± SEM. Different letter denoted significant differences (One-Way ANOVA; $P < 0.05$).



*Spawns*

The spawning fecundity showed a large variation in relation to experimental group and year (Fig. 5; Table 2). Over the three-year total fecundity from the three tanks ranged from 796 to 64036 eggs per kg of females per year, with the lowest fecundity being in 2014 for all the tanks. The average volume of eggs collected in the three tanks was 2486.7 ± 1059.1 mL in 2013, 1405.0 ± 517.7 mL in 2014 and 3798.7 ± 876.0 mL in 2015. In the mixed-groups, B1 and B2 the number of spawns increased during the period of the study and fertilised spawns were obtained from both groups. No fertilized spawns were collected from the control tank, however, the control tank also presented an increasing number of spawns over the years. In 2013, a total of 74 spawns were collected, however, only 20 of those were fertilized and all corresponded to tank B2, the average number of larvae hatched was 29631 ± 3853. In 2014, a total of 105 spawns were collected, and 33 were fertilized. In 2014, the fertilised spawns were from both mixed origin tanks (B1 and B2), 15 fertilised spawns from B1 with an average number of hatched larvae of 17404 ± 3913 and 18 from B2 with an average number of hatched larvae of 17786 ± 4052. In 2015, a total of 130 spawns were collected, however, the same number, 33 spawns (as in 2014) were fertilized and hatched, of which 13 were collected from B1 with an average number of hatched larvae of 6977 ± 1962 and 20 from B2 with an average number of hatched larvae of 9475 ± 3065.

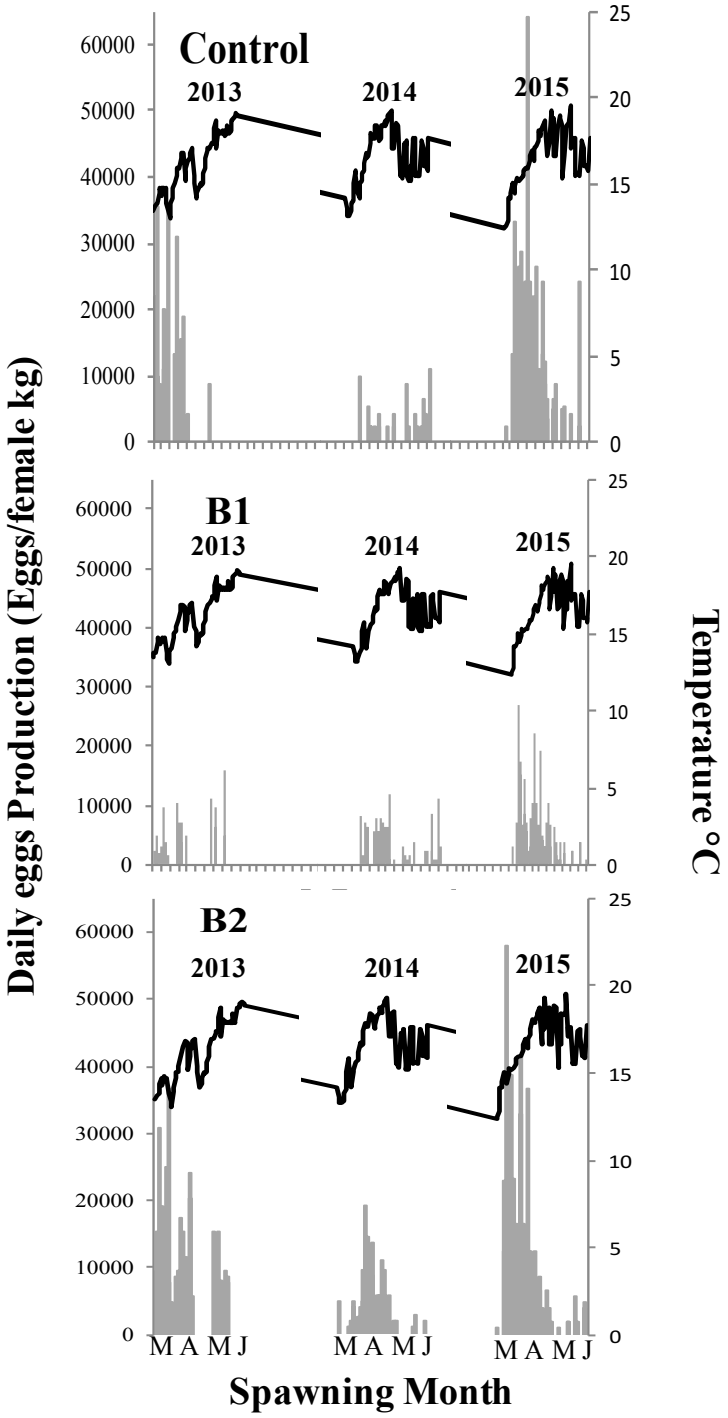



**Figure 5**. Daily egg production of Senegalese sole (*Solea senegalensis*) and temperature regime of the three experimental groups (Control, B1 and B2) during each spawning period over three years.

**Table 2:** Summary of the broodstock production parameters for each year and each experimental group. Tank, year, total, floating and non-floating egg volume, egg production per kg of female in the tank, spawns (N = Total number of spawns; H = Number of spawns that hatched), fertilization rate (mean ± S.E.M), hatching rate (mean ± S.E.M) and survival rate (mean ± S.E.M) are denoted.

| Tank | Year | Total egg volume (ml) | Floating egg volume (ml) | Inviable egg volume (ml) | Egg Production (eggs/kg female) | Spawns (N-H) | Fertilization rate (%) | Hatching rate (%) | Survival rate (%) |
|---|---|---|---|---|---|---|---|---|---|
| **Control** | 2013 | 1255 | 580 | 675 | 277.121 | 18-0 | 0.0 | 0.0 | 0.0 |
| | 2014 | 555 | 120 | 435 | 122.551 | 25-0 | 0.0 | 0.0 | 0.0 |
| | 2015 | 2339 | 944 | 1395 | 516.483 | 37-0 | 0.0 | 0.0 | 0.0 |
| **B1** | 2013 | 1410 | 895 | 515 | 112.209 | 19-0 | 0.00 | 0.00 | 0.00 |
| | 2014 | 2050 | 750 | 1300 | 163.141 | 38-15 | 50.5 ± 7.6 | 29.4 ± 5.7 | 57.9 ± 6.6 |
| | 2015 | 3851 | 1301 | 2550 | 306.468 | 46-13 | 33.8 ± 5.2 | 10.1 ± 3.6 | 29.9 ± 1.6 |
| **B2** | 2013 | 4595 | 3000 | 1595 | 443.287 | 37-20 | 73.0 ± 4.8 | 30.0 ± 0.1 | 41.1 ± 2.4 |
| | 2014 | 2230 | 805 | 1425 | 215.131 | 42-18 | 63.6 ± 4.9 | 36.3 ± 6.5 | 36.3 ± 6.5 |
| | 2015 | 5489 | 1799 | 3690 | 529.532 | 47-20 | 32.8 ± 4.7 | 22.7 ± 9.2 | 69.2 ± 5.4 |
| **Total** | 2013 | 7260 | 4475 | 2785 | 832.617 | 74-20 | - | - | - |
| | 2014 | 4835 | 1555 | 3280 | 500.823 | 105-33 | - | - | - |
| | 2105 | 11679 | 4044 | 7635 | 1.352.483 | 130-33 | - | - | - |

*Paternity analysis*

In **2013**, no parental contribution was obtained from B1 group. The mixed group B2 was the only group that had fertilized spawns. The larvae were assigned to five breeders, which represented a participation of 27.8 % of the breeders in the total broodstock in the spawning (Figs. 7A and 4A). All the spawns (20 = 200 larvae) were associated with one wild father (100 %) and four wild mothers. The female with the major participation had 26.50 % and the lowest 10.50 %. In **2014**, the group B1 had, for the first time, fertilised spawns with a total of 21.1 % of the breeders in the broodstock making a contribution. For this group (Figs. 6A and 3A), one wild male was assigned as the father of all larvae analysed (150 larvae) with 97.33 % of contribution. Three wild females were assigned as the mothers of the spawns analysed ranging from a female with the highest participation of 62.67 % to a female with the lowest of 8.67 %. There were 2.67 % of the larvae without parentage assignation due to the impossibility of DNA extraction. Group B2 (Figs. 7B and 4A) had a total contribution from 33.3 % of the breeders in the broodstock, which included the same repeated couples from 2013. The same wild male was assigned as the father of the majority of the larvae analysed (180 larvae) with an 85.88 % of the participation. The same three wild females were also assigned as mothers with 37.65 %, 15.29 % and 16.47 % of contribution. The remaining larvae were assigned to a cultured couple, which reproduced for the first time, the cultured male had a contribution of 8.82 % and the cultured female had a contribution of 10.00 %. In this tank, 5.29 % of the larvae were without assignation due to the poor quality of the extracted DNA. In **2015**, 15.80 % of the total breeders in group B1 participated in the fertilised spawns (Figs. 6B and 3A). In this tank females that were assigned as mothers of the 96 larvae of the total number of larvae collected (110) were the same females which spawned in 2014, however, the father was another wild male with the 87.27 % of contribution. In the case of the tank B2 (Figs. 7C and 4A), 38.90 % of the total breeders participated in the spawning. There were two wild males that contributed as parents for the first time in this experimental group being the fathers of the 172 larvae of the 180 analysed. The other wild male, which contributed in 2013 and 2014, contributed with 6.11 % in 2015. Wild females assigned as the mothers of the larvae were the same females that reproduced in 2013 and 2014, however, there were two cultured females which participated for the first time in paternity. Analysing the figures, more participation was observed over the years, with some fidelity between couples. A number of larvae (12.73 % in the group B1, and 4.44 % in B2) were not clearly assigned to a parental pair, but more than two possible breeders and were represented as not assigned.

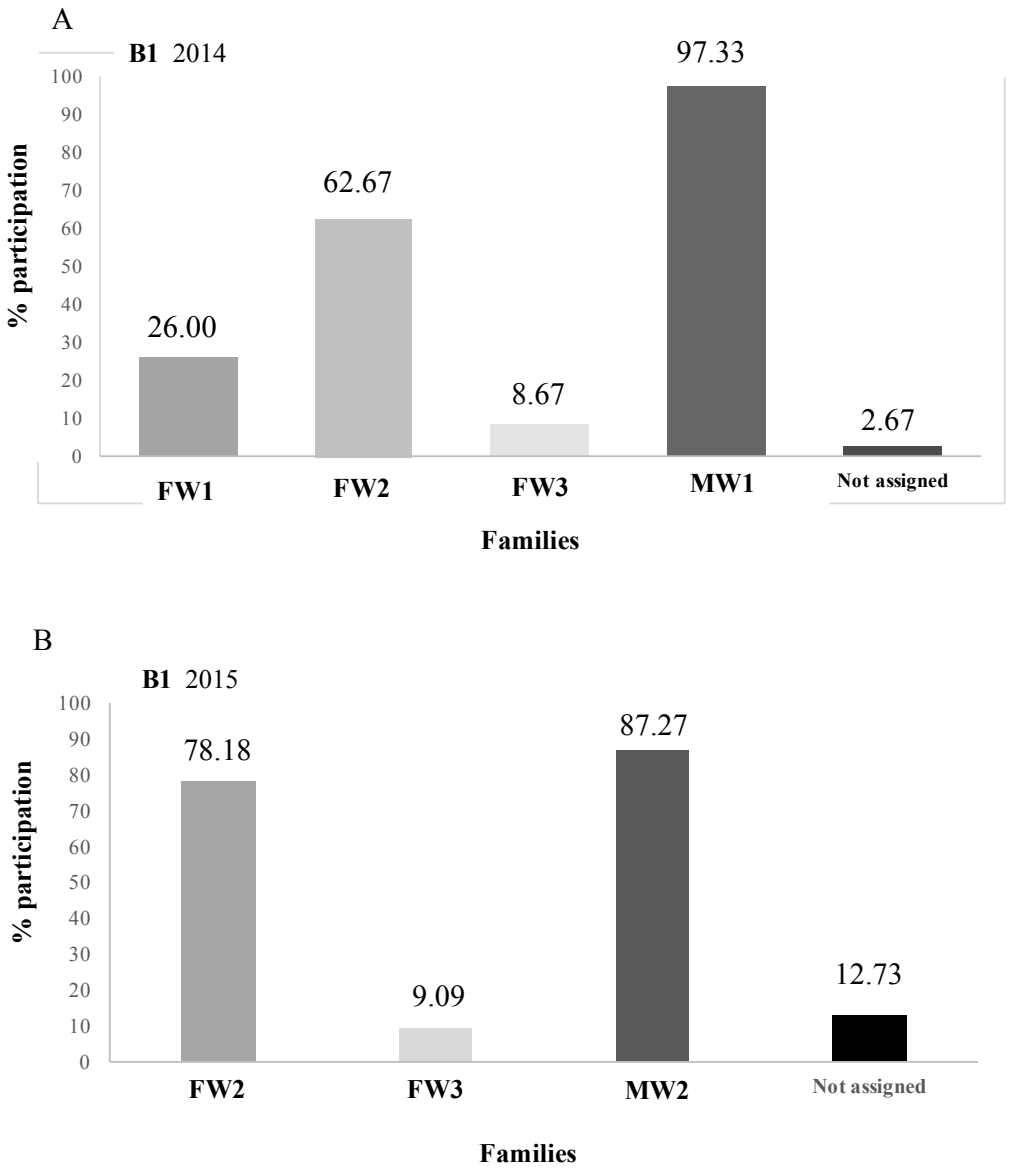



**Figure 6.** Percentage of offspring from Senegalese sole breeders (*Solea senegalensis*) **A** from B1 group (*n* = 150 larvae analysed from a total of 15 spawns) involved in the spawning season of 2014 and **B** in the spawning season of 2015 (*n* = 110 larvae analysed from a total of 11 spawns). M = male, F = female, W = wild breeders, Cult = cultured breeders.

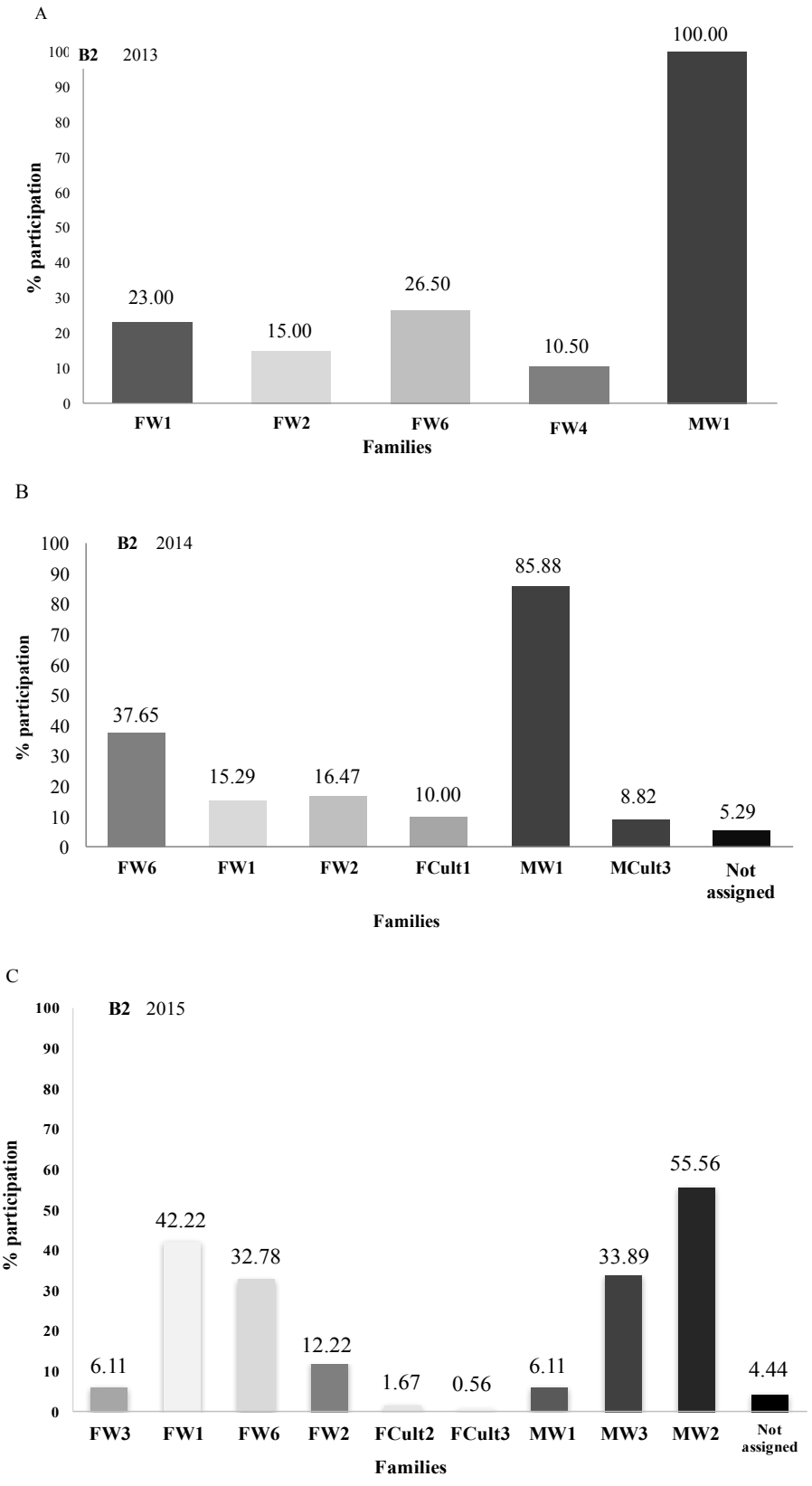



**Figure 7.** Percentage of offspring from Senegalese sole (*Solea senegalensis*) breeders from B2 group implied in the **A** spawning season of 2013 (*n* = 200 larvae analysed from a total of 20 spawns); **B** the spawning season of 2014 (*n* = 180 larvae analysed from a total of 18 spawns); **C** the spawning season of 2015 (*n* = 180 larvae analysed from a total of 18 spawns). M = male, F = female, W = wild breeders, Cult = cultured breeders.

*LH and FSH analysis related to reproductive contribution*

There were 6 wild females and 4 wild males (joining the results of both tanks, B1 and B2) which reproduced and 4 wild females and 2 wild males which did not reproduce in 2015. The plasma levels FSH, LH, T and E2 were not different between females that reproduced and did not reproduce (Table 3). Furthermore, plasma levels of FSH, LH, T and 11-KT were not different between males that reproduced and that did not reproduce (Table 3). However, differences were detected in plasma levels of LH between females and males that reproduced ($t = 3.558$; $df = 6.910$; $P = 0.0094$).

**Table 3:** Plasma levels of gonadotropins and sex steroids (mean ± S.E.M) in wild Senegalese sole that reproduced and that did not reproduce during the spawning period in May 2015. Data in the same row with different letters are significantly different ($P < 0.05$).

| | **Reproduced** | | **Did not Reproduce** | |
|---|---|---|---|---|
| | ***Female*** | ***Male*** | ***Female*** | ***Male*** |
| *n* | 6 | 4 | 4 | 2 |
| Fsh (ng/ml) | 9.46 ± 3.21 | 8.54 ± 2.12 | 7.03 ± 2.06 | 11.49 ± 2.33 |
| Lh (ng/ml) | 105.28 ± 10.00a | 65.88 ± 4.73b | 115.47 ± 26.42ab | 64.76 ± 9.28ab |
| T (ng/ml) | 0.29 ± 0.03 | 0.45 ± 0.08 | 0.61 ± 0.17 | 0.64 ± 0.04 |
| E2 (ng/ml) | 0.72 ± 0.10 | | 1.17 ± 0.26 | |
| 11-KT (ng/ml) | | 4.33 ± 1.99 | | 7.85 ± 2.30 |

## Discussion

This is the first study, which reports the participation of the cultured males in reproductive behaviour. During the first year of the experiment cultured males were observed to participate in the "Follow" behaviours. The participation in reproductive behaviour of cultured breeders appear to lead to the contribution to paternity from one cultured male in 2 spawns with a cultured female, which also spawned with a wild male. This demonstrates the effect of cohabitation with spawning wild Senegalese sole on the behaviour and reproductive success of cultured breeders. In the entire period of the study, the control group did not present fertilised spawns and the behaviours associated with courtship that were registered during the spawning season were significantly lower than those observed in the experimental groups B1 and B2 that housed cultured breeders with wild breeders that successfully spawned.

The locomotor activity in all groups exhibited a circadian pattern in each of the three years studied. The peak hour of activity was registered from 19:00 to 20:00, coinciding with dusk, in the three-year period, from 2013 to 2015. The activity started to increase at 14:00 and after the peak hour the activity decreased to basal levels at 00:00. These results coincided for the three groups (control, B1 and B2), however, in control group, the activity was slightly lower than the mixed-groups, B1 and B2. In all tanks activity was lower on days without spawning events compared to days with spawning events. Carazo et al. (2016), working with a different Senegalese sole broodstocks located in the north of Spain obtained similar locomotor activity profiles

that demonstrated, the peak hour activity in days with and without spawning events coincided with that found in the present study. Midnight was the hour when activity returned to minimum basal levels (*see* Fig. 2), which were homogeneous until 7:00 when video recording of activity was terminated. Carazo et al. (2016) registered similar profiles of activity and identified that the time of successful spawns was from 17:00 to 00:00 and the majority of spawns (8 of the 12 spawns) were observed from 17:00 to 20:00, which were the peak hours of activity. Oliveira et al. (2009), perceived that the spawning time of Senegalese sole was during the first part of the night (19:00 to 23:00), which was similar to Carazo et al. (2016) and the present study. In the case of Senegalese sole there appears to be a clear association between the increase in activity and the spawning process, with the increase in activity and spawning events being registered near to sunset and during the first hours of the night in captivity.

The principal behaviour that appears to be responsible for this increase in activity was the "Follow" behaviours. The "Follow" behaviours were identified as the main behaviours involved in step 1 of the courtship (Carazo et al., 2016) and were defined as several swimming actions associated with a competition but without aggressive connotations due to, Senegalese sole have been considered as a non-aggressive species (Salas-Leiton et al., 2008). This conduct has only been observed as part of the spawning season and this step involves several individuals, usually males following males, but females are also sometimes involved, usually occupying the "Followed" lead position (*see* Fig. 3 and 4). In our study, the "Follow" behaviour was the second most observed behaviour in the peak hour of activity in the days with spawning events, demonstrating the increase in activity in the tank was due to the presence of this behaviour. The "Follow" behaviour precedes the spawning and fish that successfully spawned were involved in "Follow" behaviours before 10 out the 12 spawns in the study by Carazo et al. (2016). Carazo (2013) observed that cultured males did not participate in this (and any) step of the courtship. Nevertheless, in the present study, the "Follow" behaviours were also observed in the control tank, formed by cultured breeders, during the three spawning seasons, however, the frequency was much lower than that observed in the mixed origin tanks, B1 and B2. Considering the results obtained, cultured males breeders displayed "Follow" behaviours in both mixed groups, B1 and B2, however, these animals usually were in the "Followed" position. Females, from both origins, were usually in the "Followed" or leader position. Females, when involved, were observed to often start the "Follow" behaviours by starting to swimming and one or several males then started to follow the female, in this case chemical communication could be involved indicating that the female might be excreting or releasing some body fluids to communicate readiness for spawning. The cultured males were also usually in the leader position ("Followed"), similar to females, while wild males were following cultured males or the females ("Follower" positions) (*see* Fig. 3 and 4). Therefore, it appeared that dominate males that participated in spawning dominated the "Follower" position during the "Follow" behaviours.

The participation of cultured breeders in the "Follow" behaviours in both mixed-groups increased significantly over years from 2013 through to 2015. It would appear

that during the experimental period (2013 to 2015) the cohabitation of cultured breeders with wild breeders that completed courtship and spawning facilitated the participation of cultured breeders and particularly males in the "Follow" behaviours and in the courtship in general. This increasing participation could be associated with social learning like in other animal species. There are many processes through which social learning may occur, however in this case, the process could be associated with social transmission of learning (Thorpe, 1963; Kieffer and Colgan, 1992; Brown and Laland, 2003), where the knowledge is acquired by observing other animals. From the moment cultured breeders were in the presence of spawning wild sole, above all cultured males, started to perform the courtship. Therefore, cultured Senegalese sole males might have obtained new behavioural patterns through the observation of spawning wild males. This process is called observational learning or contextual imitation, which can be defined as "an animal copies a specific technique used by a conspecific that is interacting with a specific set of features in the environment" (Lefebvre and Palameta, 1988; Brown and Laland, 2003). For example, Mazeroll and Montgomery (1995) reported in brown surgeonfish (*Acanthurus nigrofuscus*) that the Followers fish in local migrations imitated perfectly the route of leaders and even more the same postural changes. In this example, the social learning is associated with migration, however, swimming behaviours are also implied in this process. Moreover, Brown (2001) demonstrated that chemical cues are important in learning and demonstrated the association between the chemical cues with and experience acquired in relation to predation and danger. Another process of social learning which could be involved in Senegalese sole reproductive behaviour is mate choice copying. This process has been considered because of the low parental contribution which have been observed in this species in the present study and previous studies conducted in Senegalese sole (Porta et al., 2006; Martín et al., 2014; Carazo et al., 2016). Mate choice copying can be defined as "an individual selecting a partner because others of the same sex have selected that individual as a partner previously" (Gibson and Hoglund, 1992). For example, Dugatkin (1992) showed using guppies (*Poecilia reticulata*) that one female considered as observer, chose the same male (there were two males in the same aquarium which were not able to make physical contact) that the model female considered demonstrator had chosen. This behaviour has been observed in several fish species such as mollies (*Poecilia latipina*) (Schlupp et al., 1994) and gobies (*Pomatoschistus microps*) (Reynolds and Jones, 1999). In our study, paternity analysis was performed for all viable spawns that were only obtained from mixed origin tanks in the three-year period. The results were consistent over the years in terms of contribution, however, the group B2 obtained higher numbers of spawns than B1. Moreover, there was a couple which spawned formed of only cultured breeders in 2014 and the same cultured female spawned with one wild male, which had reproduced with another wild female. This is not the first report of cultured Senegalese sole participating in the spawning season, Guzmán et al. (2011) observed 1 fertilised spawn of a total of 60 spawns after GnRHa implants applied into the females and hCG treatment in males, however, the two spawns obtained in the present study were naturally achieved. Furthermore, Martín et al. (2014)

found a fidelity of mating couples over years, which this situation has been also observed in our study showing the importance of the mate choice in this species. Intriguingly, the males with higher parental contribution were the males that displayed more frequently "Follow" behaviours, specifically in the "Follower" position, which could be an indicative of spawning success.

The "Rest the head" behaviours were identified as the main behaviour involved in step 2 of the Senegalese sole courtship (Carazo et al., 2016). In the present study, this behaviour was the most repetitive behaviour in the peak hour of activity during days with spawning events for all groups and all years, being less displayed by the control tank. The aim of this behaviour from male towards female in the courtship appears to be to pursue the female in a process of mate selection and start the couple swimming to the surface to release the gametes. The number of "Rest the head" behaviours was significantly different among the three groups in 2014, which was also the year with the lowest fecundity for the two mixed groups and the highest fertilization and hatching rates. In addition, it was the year that a pair of two cultured breeders obtained two successful spawns. Similar "Rest the head" behaviours have been described in largescale flounder courtship (Manabe et al., 2000) where males touch the eyed side of the female and is a common behaviour detailed for bothid species studied in the natural habitat (*reviewed in* Gibson, 2005). The "Guardian" behaviour, which was also identified as a behaviour involved in step 2 of the courtship (Carazo et al., 2016), describes how a male protects or shields a female from the approaches of other competitors, usually other males. In the present study, the frequency of this behaviour was low during the peak hour of activity. This behaviour has not been described before for other flatfish species, however, this does not mean that this behaviour occurs or not in other species considering that "Rest the head" has been described before for other flatfish species (Gibson, 2005). The "Coupled" swim (step 3) was the last behaviour analysed in this study during the peak hour of activity. In case that the reproduction is successful, gamete release was the end of the "Coupled" behaviour. There are three types of failure of the "Coupled" swim defined for when the "Coupled" swim does not achieve gamete release (Carazo et al., 2016): Type 1, the female swam away at the moment the coupled swims starts; type 2, when the couple was broken up by the interaction from a second male and type 3, the couple was broken up by the impact with the tank wall or another item. The "Coupled" swim behaviour was observed only twice in one group in 2013 during the peak hour activity. Nevertheless, the analyses of all behaviours were done in 5-minutes sections (one hour = 12 frames) from two different cameras which contributed to observe the same moments in two different views to corroborate the analysis, therefore, some of these behaviours could be implicit in the other frames which did not observe.

The spawning period in the present study was from the end of March to the beginning of June without the secondary spawning period, being the dates consistent during the three-year period of the study. Nevertheless, previous studies on captive broodstocks found that the spawning period could be extended to 6 months including a secondary spawning period in autumn (Anguis and Cañavate, 2005) and to 8 months of

continued production due to thermoperiod manipulation (Martín et al. 2014). It would appear that the holding conditions and particularly the temperature regime in the present study gave a spawning period of 2-3 months from March to June.

In relation to the egg volume, fertilization, hatching and survival rates, there were not significant differences between mixed tanks, B1 and B2. Nevertheless, B2 obtained higher number of spawns and larvae than B1. In general, the fertilization rates were similar to those reported by Anguis and Cañavate (2005), however, were lower than those reported by Martín et al. (2014). However, the present study had lower fertilization, hatching and survival rates that other studies working with wild-origin Senegalese sole breeders adapted to captivity (Anguis and Cañavate, 2005; Martín et al., 2014; Carazo et al., 2016). Differences in egg quality between centres can have an infinite number of possible origins, that include water quality, nutrition, genetic origin of breeders and husbandry procedures of breeders and / or eggs. The mixed-origin broodstocks might be another reason for these lower hatching rates. For example, Chilcote (2003) found that a mixed population (equal individuals of cultured and wild fish) of steelhead (*Oncorhynchus mykiss*) was less productive than populations of only wild fish. It is probable that a complex interaction of these different factors reduced the egg quality in the present study.

The different reproductive processes in fish are mainly regulated by the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are involved in gametogenesis (oogenesis in females and spermiogenesis in males) (Lubzens et al., 2010; Chauvigné et al., 2012). The FSH plasma levels in this study for wild males and females (4 – 24 ng/ml) were in the same range as that observed for rainbow trout (*Oncorhynchus mykiss*) (5 – 30 ng/ml) (Prat et al., 1996; Breton et al., 1998), Nile tilapia (*Oreochromis niloticus*) (2 - 10 ng/ml) (Aizen et al., 2007), European seabass (15 – 40 ng/ml) (Moles et al., 2012) and levels measured in the Senegalese sole broodstock in a previous study (Chauvigné et al., 2015). The LH plasma levels, measured in the same animals in the same period, presented more variability, 61 – 147 ng/ml for females and 56 – 74 ng/ml for males in May. In general, these values were higher than those measured in Nile tilapia (3 – 12 ng/ml) (Aizen et al., 2007), however, European seabass (1 – 70 ng/ml) (Mazon et al., 2013), Indian catfish (*Clarias batrachus*) (10 – 120 ng/ml) (Sarkar and Nath, 2012) had similar levels. In this study, the FSH and LH plasma levels comparing wild sole that reproduced and that did not reproduce were similar. The plasma levels of LH differentially observed between males and females is completely normal due to, as explained before, this values entered inside of the usual range for males and females in this species. This suggests that all wild Senegalese sole breeders were more or less in the same stage of reproduction and prepare for spawning. Hence, the participation in reproductive events does not seem to be a relationship with the gonadotropins levels in the wild population.

In conclusion, this is the first report of cultured breeders participating in the courtship and reproduction. This participation was stimulated by the presence of spawning wild Senegalese sole breeders. Cultured Senegalese sole male breeders

participated in the "Follow" behaviour in mixed-origin groups and this participation increased significantly over the years of the study. The cultured males participated in the "Follower" position, however, wild male breeders dominated the "Follower" position while cultured males dominated the "Followed" position. The "Follower" position was associated with dominance in spawning, whilst the "Followed" position was associated with female participation and males with low participation in spawning. The control group formed by only cultured animals exhibited a similar spawning behaviour than mixed-origin broodstocks, however, the frequency of behaviours was much lower than that observed in the both mixed groups. There were no differences in gonadotropins and sex steroid plasma levels between wild breeders, males and females that reproduced and that did not reproduce showing that the parental contribution might not be related to the gonadotropins plasma levels. Different processes of social learning, such as, observational conditioning, imitating and mate choice copying could be involved in the increase of the participation in the courtship of the cultured male breeders suggesting that the behavioural reproductive dysfunction in male cultured sole could be solved by rearing cultured sole in the presence of successfully spawning Senegalese sole.

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# Section 4: Olfaction

# Chapter 3

## Histomorphology and histochemical description of the olfactory rosettes from wild and cultured Senegalese sole (*Solea senegalensis*) Kaup 1858

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**Abstract**

Wild Senegalese sole *Solea senegalensis* males can reproduce naturally in captive conditions, while cultured males from the first generation have a behavioural dysfunction having a low participation in the courtship without spawning, which is a problem for this species production. The olfactory system plays an important role in reproduction, and could be one of the reasons for the dysfunction of cultured males. The present study described in-depth the structure of both olfactory rosettes (upper and lower) in Senegalese sole. Ten Senegalese sole juveniles from each origin, wild and cultured, were euthanatized and both olfactory rosettes were dissected and processed to compare the structure by histomorphology and histochemical. Five stains (Haematoxylin & Eosin, Haematoxylin & V. O. F, Alcian Blue, P.A.S and Silver Staining) were used to distinguish the different cells, function and distribution in the olfactory rosette. Wild and cultured juveniles presented similar structure and cell distribution. The olfactory rosette was ovoid in shape and consisted of about 19-21 lamellae in the upper olfactory rosette (UOR) and 17-19 in the lower olfactory rosette (LOR). These lamellae originated from the mid-line called raphe and olfactory epithelium was within lamellae. The olfactory epithelium consisted of non-sensory cells: supporting, ciliated non-sensory, goblet and basal cells. Sensory cells were spread out by whole lamella, however, the different types of olfactory receptors neurons could not be distinguished by histology procedures. To the best of our knowledge, this work is the first to describe the structure and organization of the olfactory system of Senegalese sole, additionally, the lack of differences between the structures of cultured and wild individuals may demonstrate that the failure from cultured males is not related to the morphology of the olfactory system.

## Introduction

The olfaction is a sense that exists in all vertebrates including fish and for which the phylogeny has been defined (Taniguchi and Taniguchi, 2014). Olfaction has evolved to increase the survival of species by using chemical signals to find food, to guide migration (homing) and control reproductive behaviour. In addition, the olfactory system has been demonstrated to have a role in the control of reproduction in several fish species such as goldfish (*Carassius auratus*) (Sorensen et al., 1990), Atlantic salmon (*Salmo salar*) (Moore and Waring, 1996), brown trout (*Salmo trutta*) (Moore, 2002), Indian major carp (*Catla catla*) (Bhute and Baile, 2007; Biju et al., 2003), Crucian carp (*Carassius carassius*) (Hamdani and Døving, 2006), masu salmon (*Oncorhynchus masou*) (Yambe et al., 2006) and Mozambique tilapia (*Oreochromis mossambicus*) (Keller-Costa, 2014). For instance, Sorensen (1992) suggested sex hormones and their metabolites to function as sex pheromones that control the reproductive-physiology and behaviour of the goldfish through the olfactory system.

During larval developmental, the olfactory placode or olfactory precursor differentiates into the olfactory epithelium (OE) to conform the main structure of the olfactory system, the olfactory rosette. This rosette is a structure of outward organised and symmetrical lamellae projecting to both sides of the raphe (midline of the olfactory rosette). In fish, the olfactory rosette is usually located into the olfactory cavity, which links the OE with the aquatic environment to perceive the different stimuli. For this purpose, the OE is composed by sensory and non-sensory cells. Related to sensory group, four different sorts of cells compose this cluster: ciliated, microvillous, crypt and kappe neurons, in general named olfactory sensory neurons (OSN) (Ahuja et al., 2014; Biechl et al., 2016; Hamdani el and Doving, 2007; Hansen and Zeiske, 1998), which transduce the signals to the brain. Concerning non-sensory group three types of cells, supporting and basal cells, considered sustentacular, and all these cells are surrounded by ciliated nonsensory and mucus cells (goblet cells) (Hamdani and Døving, 2007; Hansen and Zielinski, 2005; Yamamoto et al., 2004). The morphology of olfactory rosette and the olfactory lamella vary among species, but the olfactory epithelium usually posses similar cellular structure across different species; receptor or sensory, support, mucous and basal cells (Hansen and Zeiske, 1998; Hansen and Zielinski, 2005; Saito et al., 2004; Yamamoto et al., 2004).

The olfactory system of benthic fish plays a major role in behaviour and feeding due to low light levels and high sediment loads present in benthic environments, which may reduce the visual cues. Flatfish in addition to this benthic life characteristic have a unique asymmetric olfactory system (Kasumyan, 2004). These distinctive fish often lye half-buried in the substrate with the eyes and upper nostril above the substrate and the lower nostril buried. Vélez et al., (2013) found by electro-olfactogram (EOG) that there were differences in both the olfactory receptors and the signal transduction pathways between the upper and lower olfactory epithelia of Senegalese sole (*Solea senegalensis*) (Velez et al., 2005; Velez et al., 2013; Velez et al., 2007a; Velez et al., 2007b; Velez et al., 2011; Velez et al., 2009a; Velez et al., 2009b). For example, food-related odorants

(e.g. L-phenylalanine and 1-Methyl-L-tryptophan) were detected with a significantly higher EOG response by the lower epithelium compared to the upper epithelium, while conspecific-derived odorants (e.g. taurocholic acid) were detected with a significantly higher EOG response by the upper epithelium.

Senegalese sole has been identified as one of the most interesting and promising species for aquaculture development and diversification on the Atlantic coast of Southern Europe and the Mediterranean Sea. However, Senegalese sole cultivation depends on the spawning of wild-origin broodstocks (Morais et al., 2014), due to the failed spawning of fertilised eggs from males reared in captivity, which do not participate in the complex courtship during the spawning season (Agulleiro et al., 2007; Carazo, 2013; Carazo et al., 2011; Carazo et al., 2013; García-López et al., 2006; Morais et al., 2014). This species performs a complex metamorphosis, which resulted in the both eyes on the upper side (right) of the head in juvenile and adult stages. Before the metamorphosis, the eyes are symmetrical in the larval stage, and during metamorphosis the left eye migrates to the right side (Fernandez-Diaz et al., 2001; Ribeiro et al., 1999). However, some problems have been reported in aquaculture due to this adaptation to benthic life. These issues include incorrect pigmentation (Soares, 2002; Villalta et al., 2005) and high incidence of skeletal deformities (Engrola et al., 2005; Gavaia et al., 2002). Gavaia et al., (2009) found wild Senegalese sole presented less skeletal deformities (19 %) than those larvae that were reared in captivity (79 %), which indicated these differences were caused by a selective mortality of wild deformed fish in nature and/or the effect of aquaculture-related rearing conditions in the development of skeletal deformities in sole. These type of efforts during the metamorphosis could have an impact on the olfactory system that may differentiate wild from cultured soles.

Therefore, bringing together this information exhibits that upper and lower olfactory epithelia in Senegalese sole have distinct roles and go through a metamorphosis that has been shown to compromise aspects of development in cultured sole. The aim of this study was to compare the morphology of both rosettes, upper (UOR) and lower (LOR), by histology and make an in-depth description of olfactory epithelium from both wild and cultured Senegalese sole to determine whether structural differences exist in relation to fish origin.

## Material and Methods

The present study has been carried out in accordance with EC Directive 86/ 609/EEC for animal experiments and Spanish regulations on animal welfare. All procedures were accepted the by Animal Ethics Committee of IRTA.

### *Experimental animals and sampling procedures*

Twenty early juvenile Senegalese sole (11 females and 9 males), ten specimens of each origin were euthanatized in May of 2013. Cultured fish (weight 80-100 g, and total length 21-25 cm) were provided by Stolt Sea Farm (Santiago de Compostela, Spain) and held at IRTA's facilities (Tarragona, Spain) for > 1 year until sacrifice. During this period, the sole were fed dry feed (Skretting, Burgos, Spain). Environmental conditions were: Temperature between 9 and 24 °C, salinity 35 - 37 ppt, oxygen 5 – 6 ppm, photoperiod 9 – 15h light cycle depending on the season. Wild sole (weight 70–95 g, total length 20–23 cm) were caught alive by local fishermen using trammel nets and sacrificed and sampled on the same day. All animals were euthanatized with an overdose of anaesthetic MS-222 (Tricain metanosulfonate; Acros-Organic, New Jersey, USA). Both olfactory rosettes were carefully dissected and placed in a histology cassette immersed in 4% buffered formalin for further histological analysis.

*Histological procedures*

The olfactory rosettes (UOR and LOR) were dehydrated through a series of ethanol baths, infiltrated and embedded in paraffin blocks in different orientations. The blocks were cut with a microtome (Leica RM2155, Barcelona, Spain) with sections from 2 to 8 µm and stained with five different stains: Harris' Haematoxylin & Eosin (H/E), Haematoxylin & V.O.F. of Gutiérrez (H/VOF), Schiff Reactive and Periodic Acid Staining (PAS), Alcian Blue pH 2.5 (AB) to detect mucopolysaccharides and Silver Staining to observe neuronal tissue using Bio-Optica Milano s.p.a kit. The samples were examined under a light microscope (Leica BMLB, Barcelona, Spain) and images taken with a camera (Olympus DP70, Barcelona, Spain) connected to the same microscope. Goblet cells were counted to analyse the differences between wild and cultured structures. For each fish ($n$ = 5 fish per group: cultured and wild) goblet cells were counted in a lamella from each of three regions distinguished in the lamella (ridge, middle and ventral). Goblet cells were counted in this way in three randomly selected histological sections ($n$ = 9 lamellae per fish). The sections used were stained with AB pH 2.5 and examined at a 40X magnification.

*Statistical analysis*

All data were checked for normal distribution with the Shapiro - Wilks test. Counts of goblet cells in the rosettes between cultured and wild fish were compared using Student's t-test ($P < 0.05$ level of significance) carried out by SPSS 19.0 (IBM Statistics).

## Results

*General morphology of the olfactory organ in Senegalese sole*

Both wild and cultured Senegalese sole juvenile had two olfactory rosettes located in the same position, one was located on the upper part of the head, named upper (right) (UOR) and one located in the lower part of the head, named lower (left)

(LOR). The UOR was located inside of a nasal chamber, which was connected to the external water environment by an anterior and a posterior nostril. The nostrils protruded from the fish, where the anterior nostril was larger than the posterior (Fig. 1A). The LOR was also located inside of a nasal chamber, which was also connected to the external water environment by an anterior nostril and a posterior nostril. In this case, the nostrils also protruded from the fish, with the anterior nostril not protruding as much as the upper anterior nostril, but the walls were thicker (Fig. 1A'). The lower posterior nostril was also smaller than the lower anterior nostril. The connection between the lower anterior and posterior nostrils was larger than the upper and extended ventro-caudally to place the lower posterior nostril further away from the anterior nostril compared to the upper nostrils that were in relatively close proximity (Fig 1A').

All sole exhibited an oval shaped UOR and LOR with bilateral symmetrical lamellae on both sides of the mid-line raphe (Fig. 1B and 1B'). The rosettes size was approximately ~3 and ~ 2 mm in length and width, respectively containing a total number of 19 - 21 lamellae in the UOR and 17 - 19 lamellae in the LOR. No differences in external morphology, cell types and cell distribution pattern (OSNs) were identified between wild and cultured specimens. Goblet cells of different lamella regions (ridge, middle and ventral; Fig. 1B') were counted to compare UOR and LOR from wild and cultured animals (Table 1). Nevertheless, UOR and LOR were considered different regarding the number of goblet cells in the ridge region in both origins, wild and cultured (Table 1).

**Table 1.** Frequency of the goblet cells in the three regions of a lamella (ridge, middle and basal) was analysed in the wild and cultured groups of Senegalese sole (*Solea senegalensis*) ($n$ = 5 per group) in each olfactory rosette (UOR: Upper olfactory rosette; LOR: Lower olfactory rosette). Data was shown Mean ± SD and different letters denote significant differences (Student's t-test; $P < 0.05$).

| | **Wild** | **Cultured** |
|---|---|---|
| **UOR** | | |
| Ridge | $36 \pm 3^{a}$ | $34 \pm 4^{a}$ |
| Middle | $21 \pm 1^{a}$ | $20 \pm 2^{a}$ |
| Ventral | $4 \pm 1^{a}$ | $3 \pm 1^{a}$ |
| **LOR** | | |
| Ridge | $44 \pm 3^{b}$ | $49 \pm 1^{b}$ |
| Middle | $20 \pm 1^{a}$ | $20 \pm 3^{a}$ |
| Ventral | $4 \pm 1^{a}$ | $4 \pm 1^{a}$ |

The surface of the mid-line raphe was covered by connective tissue and melanin centres (Fig. 1B and 1C'). The lamellae of the olfactory rosette contained the sensory epithelium, which originated from raphe, was broader towards the rostral end and narrower towards the proximal end. The lamellae ran parallel away from the raphe on both sides and were attached at the base to the nasal chamber, joining the sides of the

nasal chamber opposite from the central raphe. Grooves were covered by goblet cells, besides these cells were observed between the lamellae predominantly in the lower olfactory rosette at the ridge of the lamellae. The lamellae had different lengths depending on the position in the rosette; the smallest size was 500 μm at the extremities of the raphe and the central lamellae were the largest with 1300 μm (Fig. 1B and 1B'). No secondary folds in the lamellae were observed in Senegalese sole juvenile.

*Structural description of the olfactory epithelium from UOR and LOR*

The thickness of the olfactory epithelium was ~ 50 μm and the epithelium contained both non-sensory and sensory cell groups did not differ among cultured and wild sole. The OE comprised four types of non-sensory cell (supporting, ciliated non-sensory, goblet and basal cells) and the olfactory sensory neurons (OSNs) forming the sensory cell group. These two cell groups were in close proximity and distributed throughout the lamellae closely packed. Starting with the non-sensory group, supporting cells were P.A.S. negative and were not stained by AB pH 2.5 or by Silver staining. The nuclei are basophilic stained in purple by Haematoxylin (Fig. 1C and 1C'). Based on the structure and the location, these cells were cylindrical and distributed throughout the olfactory epithelium positioned around the OSNs (Fig. 1C and 1C'). The supporting cells were the unstained white spaces, surrounding the OSNs, stained in black (Fig. 1E'). One supporting cell was observed to surround more than one OSN and these sustentacular cells did not seem to have a pattern of allocation in the epithelium due to the location of the supporting cells nuclei depended on the distribution of the OSNs. Ciliated non-sensory cells were similar to supporting cells in shape (cylindrical), so were P.A.S. negative and were not stained by AB pH 2.5 or by Silver staining with a nuclei moderately basophilic. These cells were differentiated from supporting cells because of the presence of kinocilia that extended to the lumen of the olfactory cavity. The kinocilia were observed on the surface around the lamella at a magnification of 100X (*not shown*).

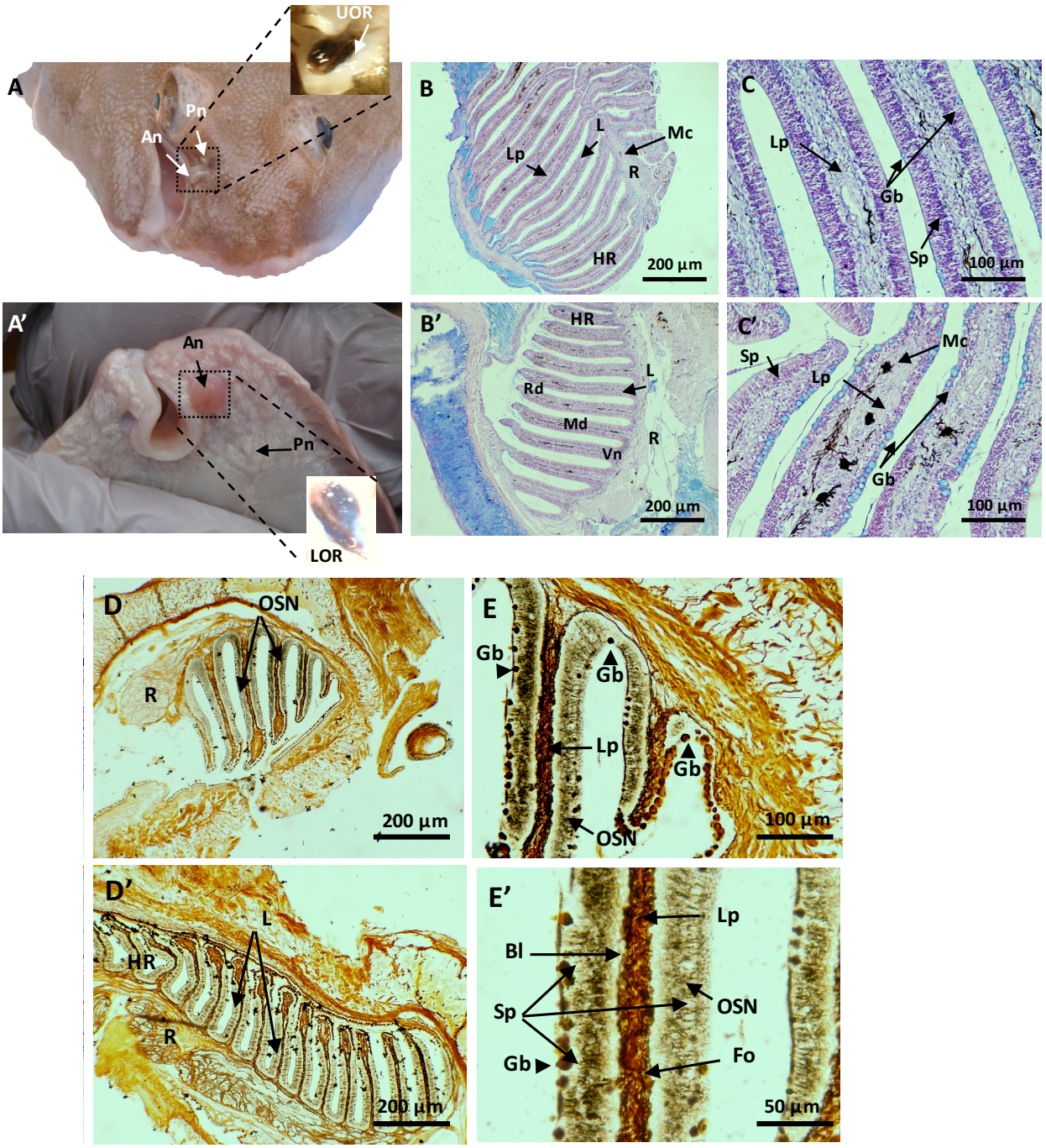


**Figure 1.** Senegalese sole (*Solea senegalensis*) UOR (A to E) and LOR (A' to E') morphology and histological analysis. **A** and **A'** localization of the upper and lower olfactory rosettes next to the eye and mouth. **B** and **B'** upper and lower olfactory rosettes. AB pH 2.5 staining. **C** and **C'** some upper and lower lamellae details showing the olfactory epithelium and the different cells. AB pH 2.5 staining. **D** and **D'** upper and lower olfactory rosettes Silver staining with OSNs stained in black. **E** and **E'** some upper and lower lamellae details showing the olfactory epithelium and the different cells. Silver staining. (An) Anterior nostril; (Pn) Posterior nostril; (OR) Olfactory rosette; (HR) Hemi-rosette; (L) Lamella; (R) Raphe; (Lp) Lamina propria; (Mc) Melanin centres; (Rd) Ridge; (Md) Middle; (Vn) Ventral; (Sp) Supporting cells; (Gb) Goblet cells; (OSN) Olfactory sensory neurons; (Bl) Basal layer; (Fo) Fila olfactoria.

Another cell type observed was the goblet cells which were P.A.S. positive, stained moderately by AB pH 2.5 and not stained by HE. Goblet cells were located only

in the surface, next to the lumen, and were surrounded by ciliated non-sensory cells and supporting cells. Goblet cells were distributed throughout the whole lamella, but the concentration varied in the different regions of the lamella (ridge, middle and ventral) (Fig. 1C, 1C', 1E and 1E'). These cells were oval in shape and the nuclei lied basally with triangle shape. Goblet cells were filled with large mucus granules for secretion onto the lumen of the olfactory cavity, which was proved by the AB pH 2.5 staining. A protuberance was observed from some goblet cells that was the connexion between lumen and goblet cell that can be distinguished by histology (Fig. 1E'). Basal cells possessed nucleus slightly basophilic and large in relation to the total basal cell size. These cells were small and located between the ventral part of supporting and ciliated non-sensory cells and lamina propria to conform the basal lamina and the fila olfactoria, which is a layer formed by the OSNs axons and penetrates into the basal lamina in bundles (Fig. 1E'). The lamina propria was located between the two olfactory epithelia or at the centre of the lamella, below to the basal lamina. The lamina propria was filled with connective tissue, capillaries and fat cells (Fig. 1B, 1C, 1E and 1E'). In some sections the lamina propria also contained pigment cells, macrophages, and granulocytes (Fig. 1C').

In relation with the sensory cell types, the silver staining showed the distribution of the different types of OSN in both rosettes UOR (Fig. 1D and 1E) and LOR (Fig. 1D' and 1E'). The OSN were distributed throughout the whole lamella, but it was difficult to distinguish what type each cell was with the histological techniques employed in the present study (Fig. 1E').

## Discussion

At structural level, no significant differences in the structure of UOR and LOR between cultured and wild sole were observed. The lamella number, cell composition (including sensory and non-sensory cells) and the distribution pattern in a continuous and uniform manner through the lamellae were similar between both origins. This OE structure agrees with those previously described in several other teleosts such as zebrafish (Hansen and Zeiske, 1998), eel (*Anguilla anguilla*) (Atta, 2013), catfish (*Ictalurus punctatus*) (Morita and Finger, 1998), carp (Hamdani and Døving, 2006; Kumari, 2008) and particularly flatfish such as winter flounder (*Pseudopleuronectes americanus*) (Prasada Rao and Finger, 1984), common sole (*Solea solea*) and plaice (*Pleuronectes platessa*) (Harvey, 1996). The morphology of the olfactory organ was almost identical to the morphology described in common sole and other flatfish (Harvey, 1996). Senegalese sole presented one accessory nasal sac, which connected the two olfactory rosettes and to the buccal cavity. Webb (1993) described the channels connexion with the environment in common sole as a pair of sensory nasal sacs fused medially as a single, median accessory nasal sac overlaying the roof of the mouth, coinciding with the description of Senegalese sole in the present study. This indicated that the general morphology had not been affected or changed by any problems associated with the metamorphosis in cultured Senegalese sole.

The main difference found between the two olfactory rosettes was the number of lamellae and the number of the goblet cells in the ridge region in the lamella (*discussed below*). This coincided with the general description for flatfish species such as the winter flounder (Prasada Rao and Finger, 1984), common sole and plaice (Harvey, 1996), where lower side usually differs in size and number of lamellae in the olfactory rosette. The number of lamellae in the olfactory organ at the upper side was around 1.5 times greater than the lower side (Harvey, 1996). Nevertheless, this is contrary to fish with symmetrical body shape that have the same number of lamellae in both olfactory rosettes (Kasumyan, 2004). The different number of lamellae in the upper and lower olfactory rosette is probably related to differences in the environment and differences in ability to detect different chemicals. The fewer number of lamellae in the lower olfactory rosette could indicate that upper olfactory rosette plays a bigger role for the different necessities, given the olfactory rosette is in direct contact with the water environment and may receive more chemical stimuli than the lower olfactory rosette which would be buried in the sand. The upper olfactory rosette in Senegalese sole was demonstrated to be specialized in sensing conspecific-derived odorants, but also detected some amino acids (e.g. L-cysteine), whilst the lower rosette was related to food- odorants (Velez et al., 2013). The length and shape of lamellae vary according to their position in the rosette. The anterior lamellae were the smallest with their length gradually increasing towards the mid-region of the rosette. The olfactory epithelium in both cultured and wild Senegalese sole juveniles were fully developed and functional, however, the number of lamellae might increase with the age to expand the surface area to enable the larger organisms to sense the chemical cues necessary in the olfactory area as this has been observed in other fish species such as zebrafish (Hansen and Zeiske, 1998), catfish (Morita and Finger, 1998) and Indian carp (Bhute and Baile, 2007). The basic function of olfactory lamellae and secondary folds is to increase the area of the olfactory epithelium bearing the OSNs that perceive chemical stimuli (Kasumyan, 2004).

The olfactory epithelium of Senegalese sole was composed basically by non-sensory cells (supporting, ciliated non-sensory, goblet and basal cells) and sensory cells (OSNs) that were distributed relatively evenly throughout the epithelium in continuous pattern with no areas where either cell type was more or less dominant. This composition was also found in other flatfish such as turbot (*Psetta maxima*) (Doldán et al., 2011) and barfin flounder (*Verasper moseri*) (Yamamoto et al., 2004). Supporting cells have been described to perform the function of support and protect the OSNs whereas ciliated non-sensory cells with their kinocilia are responsible for the ventilation of the olfactory cavity in areas located between near lamellae of the olfactory rosette (Kasumyan, 2004). Goblet cells secrete mucus, which forms a layer covering the surface of the OE. Kasumyan (2004) suggested that this layer of mucous or glycocalix might play an important role in chemoreception. This would be an explanation to why goblet cells were found in upper part of the layer of lamellae. Moreover, LOR presented more frequent goblet cells than UOR in the ridge of the lamella. The location of the rosette could be an explanation, due to the mucus secreted by the goblet cells also has a

protective function of flagella and microvilli of OSNs, and the direct contact with the sand could damage the rosette at the moment the nostrils start to work driven particles by the water movement (Kasumyan, 2004). Basal cells are considered totipotent cells, due to these cells are available for growth and regeneration of several type of cells. Depending on the necessity of the olfactory epithelium to restore, supporting, and mucous, having limited longevity, develop and differentiate from basal cells. On the other hand, OSNs are renewed every 7-10 days. Old cells of the epithelium are secreted into the nasal chamber or are phagocytised by lymphatic cells (Kasumyan, 2004).

The distribution of OSNs may differ in different fish species, for example two areas perfectly separated on the lamella (sensory area covered by OSNs and non-sensory area covered by non-sensory cells) were found in zebrafish *Danio rerio* (Hansen and Zeiske, 1998). However, the distribution of the OSNs was continuous over the surface of lamellae and no differences were appreciated in the distribution across both epitheliums of cultured and wild Senegalese sole. This distribution was in concordance with the olfactory epithelium in common sole and plaice (Harvey, 1996) as both flatfish species presented continuous sensory epithelium. Four kinds of OSNs have been described in fish (Hamdani and Døving, 2006; Hansen and Zeiske, 1998; Hansen and Zielinski, 2005; Laberge and Hara, 2001; Morita and Finger, 1998). Two of these OSN (ciliated and microvillous cells) are ontogenetically and morphologically different and exist in vertebrates, while crypt cells are relatively new and are unique olfactory neurons in fish (Saito et al., 2004). Two of these sensory cells have been found in common sole (Harvey, 1996), microvillous and ciliated cells whereas crypt cells were not observed. Nevertheless, different types of OSNs were not distinguished in the present study due to the technique used, however, it could be expected that Senegalese sole own the same sensory cells that were observed in common sole as fish belonging to the same family, which usually have olfactory rosettes of the same or similar type (Yamamoto, 1982; Zeiske et al., 1992).

In conclusion, the structure of the olfactory system, the olfactory epithelium and the cell types observed in juvenile Senegalese sole were similar to other flatfish fish species and in particular to common sole (relative species). There were no differences between the structures of cultured and wild juveniles indicating that cultured juveniles metamorphosed correctly and that structural differences do not offer a possible explanation for the behavioural dysfunction observed in cultured males, which do not participate in the courtship behaviour (Carazo, 2013; Carazo et al., 2011). However, differences were found between rosettes, UOR and LOR, in the total number of lamellae and the amount of goblet cells in the ridge region of lamella. Thus, this work constitutes a basis for further research to determine if the olfactory system has a role in the Senegalese sole reproductive dysfunction. Further research could focus on capacity of chemical communication using EOGs, gene expression and the ultrastructural of both epitheliums to distinguish the OSNs implicated in the olfaction using transmission (TEM) and scanning (SEM) electron microscopy.

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# Chapter 4

# Transcriptomic profiles of the upper olfactory rosette in cultured and wild Senegalese sole (*Solea senegalensis*) males

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**Abstract**

The aims of this study were the characterization of the upper olfactory epithelium of cultured and wild Senegalese sole mature males at transcriptomic level (using RNA-Seq). Deep transcriptomic analysis showed 2,387 transcripts were differentially expressed between cultured and wild groups. A detailed analysis identified the differentially expressed transcripts included some olfactory receptors (OR, TAAR and V2R-like) and transcripts related with the control of reproduction such as the brain aromatase cytochrome P450 and tachykinin-3. Also a wide set of genes related with lipid sensing, metabolism and transport were differentially expressed and these transcripts were often down-regulated in cultured fish. Furthermore, cultured males presented a higher expression of genes related with goblet cells and mucin production that modulates innate and adaptive immune responses. All these changes in gene expression could be explained by different nutritional status and diet preference. The different expression of transcripts related to olfaction, reproduction, nutrient sensing and immune system demonstrate distinct differences in functionalities between cultured and wild soles providing new clues about the sexual dysfunction in this species.

## Introduction

The olfactory system provides a key sense in fish necessary for food detection, reproduction, social interactions and signalling threats as well as to cope with environmental challenges. The olfactory rosette, which is the main structure of the olfactory system, is located into the olfactory chamber, which connects with the exterior environment through the nostrils that control the water flow through the olfactory cavity (Hansen and Zielinski, 2005; Zeiske et al., 1992). At cellular level, the olfactory epithelium (OE) is a complex tissue that contains both sensory and non-sensory cell groups. The former cell groups consist of three main cell types, the receptor cells or olfactory sensory neurons (OSNs), the supporting (sustentacular) cells and the progenitor basal cell. These sensory cells are surrounded by ciliated nonsensory and mucous cells (goblet cells). Moreover, the OSNs comprise four different cell types: ciliated, microvillous, crypt and kappe neurons (Ahuja et al., 2014; Biechl et al., 2016; Hamdani el and Doving, 2007; Hansen and Zeiske, 1998; Hara, 1994; Yamamoto et al., 2004). These OSNs express a vast repertoire of olfactory receptors accounting for more than 1000 and approximately 300 encoding different genes in mice and zebrafish, respectively. The OSNs are able to detect an immense spectrum of odours that express specifically a subset of olfactory receptors (known as "one neuron–one receptor" rule) (Miyasaka et al., 2013) whose axons converge in the olfactory bulb to establish a discrete and precise map of olfactory receptor expression (Buck, 2000; Miyasaka et al., 2013). Until now, four main olfactory receptor families were reported: the odorant receptors (ORs) and trace amine-associated receptors (TAARs), mainly expressed in ciliated OSNs, and the vomeronasal receptors V1Rs and V2Rs, mainly expressed in microvillous OSNs and crypt cells (Alioto and Ngai, 2005, 2006; Biechl et al., 2016; Kermen et al., 2013; Miyasaka et al., 2013; Oka et al., 2012). Therefore, the olfactory system is considered as an extremely complex and specialized organ that through the detection of an immense range of odour cues controls social interactions, environmental adaptation and life cycles modulating in this way feeding regulation, alarm responses, migration and homing behaviours, kin imprinting and reproduction (Biechl et al., 2016; Hamdani and Doving, 2007; Hansen and Zielinski, 2005; Hinz et al., 2013).

In benthic flatfish, olfaction is a particularly important and well developed sense due to low light irradiance and high load of sediments at the bottom where this fish inhabits. To overcome these environmental factors, flatfish have developed highly specialized olfactory sensing mechanisms. These specializations are established during the larval metamorphosis through major morphological and functional changes in the olfactory organs. In Senegalese sole (*Solea senegalensis*), early larval pelagic stages are fully dependent on light for feeding, but close to metamorphosis (9-10 days post-hatched), larvae switch to nocturnal activity and become less dependent on visual stimuli to feed (Blanco-Vives et al., 2012; Cañavate et al., 2006). Moreover, flatfish asymmetry is associated with an odour-specialization between the upper (UOR; eyed-side of the body) and lower olfactory rosettes (LOR; blind side). Structural studies in common sole and plaice determined that UOR developed more rapidly and contained

more lamellae than the LOR (Harvey, 1996; Kasumyan, 2004). Moreover, functional studies using electro-olfactogram (EOG) in Senegalese sole demonstrated a functional asymmetry of the olfactory system with the upper OE more specialized in intra-specific chemical communication and the lower OE in prey location and identification. These differences in odour sensitivity between the UOR (open water column) and LOR (sediments and water close to the bottom) were canalized by different olfactory receptors and transduction mechanisms confirming the specialization of this organ in this species (Velez et al., 2013; Velez et al., 2007a; Velez et al., 2007b; Velez et al., 2011).

Senegalese sole has been identified as one of the most interesting and promising species for aquaculture diversification in Southern Europe due to the high market price and high growth rates. As a consequence, a considerable research effort to optimize rearing methodologies at industrial level has been completed in the last three decades (Morais et al., 2016). However, Senegalese sole reproduction in captivity depends on wild-origin broodstocks since hatchery reared or cultured sole fail systematically to release fertilised eggs (Agulleiro et al., 2007; Carazo, 2013; Carazo et al., 2011; García-López et al., 2006) in spite of having the capacity to produce viable gametes suitable for *in vitro* fertilization (Agulleiro et al., 2007; Carazo, 2013; Carazo et al., 2011; García-López et al., 2006; Rasines et al., 2012). Hormonal therapies were unsuccessful to correct this dysfunction and behavioural studies have demonstrated that the lack of proper courtship behaviour in cultured males appears to be behind this problem (Carazo et al., 2011; Guzmán et al., 2011). The olfactory system represents a key organ that controls hormonal communication for reproductive priming and/or synchronization courtship behaviour in several teleosts (Moore, 2002; Moore and Waring, 1996; Sorensen, 1992; Sorensen et al., 1990). Hence, to increase the knowledge and identified new clues about the reproductive problem in cultured male, the aims of this study were the characterization of the upper OE in wild and cultured males at transcriptomic level by using RNA-Seq deep sequencing. The functional analysis of differentially expressed transcripts and validation of results by qPCR offer a new set of molecular markers to solve the sexual dysfunction of cultured soles.

## Material and Methods

The present study has been performed in accordance with EC Directive 86/ 609/EEC for animal experiments and Spanish regulations on animal welfare. All procedures were accepted the by Animal Ethics Committee of IRTA.

### *Animal rearing and sample processing*

Thirty adult Senegalese soles were captured from Ebro's delta by using trammel nets in the period from October to December of 2013. Fish were immediately transported to IRTA's facilities and acclimated for around one month in quarantine facilities. The soles were then kept in 2,000 L fiberglass tank under natural

environmental conditions (as above with natural temperature and photoperiod) and fed with fresh food (cooked mussels (Sariego Intermares, Spain) and marine polychaetes (Topsy-Baits, Holland). Cultured soles (12 specimens) were obtained from IFAPA centre El Toruño (Cádiz, Spain) in April 2014 and transported to IRTA's facilities where they were maintained in a similar 2,000 L fiberglass tank under the same natural environmental conditions. Two weeks later, ten cultured males (IFAPA-Toruño) and ten wild males (Ebro's delta) were moved together to a 2,000 L circular fiberglass tank. The tank was connected to a flow-through water system and the physicochemical parameters (temperature, salinity and oxygen) were registered daily to monitor the quality of the water and avoid the environmental influence in chemical communication amongst animals. The males were selected according to similar sperm quality characteristics that indicated an advanced stage of maturity with motile sperm production. Two mature wild females from IRTA' broodstock were also added to the tank in order to stimulate chemical sensing. The selected females had swollen ovaries indicating an advanced stage of maturity. The tank was covered with a black shade net to provide a reduced light intensity. Fish were fed every day with a mixed diet composed of dry-feed (Repro-Vitalis, Skretting Co.), cooked mussels (Sariego Intermares, Spain) and marine polychaetes (Topsy-Baits, Holland). Fish were fed with the same regime as explained above. After 1 month of cohabitation, four cultured (499.3 ± 10.6 g) and four wild males (158.1 ± 11.2 g) were randomly chosen and euthanized by decapitation to avoid interference of anaesthesia overdose on olfactory gene expression as previously described in channel catfish (*Ictalurus punctatus*) (Lewis et al., 1985). The UOR was rapidly dissected, weighted, frozen in dry ice and preserved at -80ºC for further molecular analysis. Also, sperm quality was evaluated for each individual (% motile cells and time post-activation) and one testis preserved in buffered 4 % formalin for histology analysis.

*Histological procedures*

The testis samples were dehydrated through a series of ethanol baths, infiltrated and embedded in paraffin blocks. Transversal portions were cut at 3 μm thick and stained by H/E. To analyse males' maturity, six random pictures were taken from testis cortex with a camera (Olympus DP70) at 63X optic vision and the different germ cells (spermatogonia (Spg), spermatocytes (Spc) spermatids (Spd) and spermatozoa (Spz)) were counted. Maturity stages (Stage I-II, III and IV) were defined as previously described (García-López et al., 2006; García-López et al., 2005).

*RNA isolation, RNA-seq libraries preparation and sequencing analysis*

For RNA isolation, the UOR samples were homogenized using the Mini BeadBeater (Biospect products). Total RNA (~45-50 mg) was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen) following the manufacturer's protocol. Total RNA was treated twice with DNase I using the RNase-Free DNase kit (Qiagen) for 30

min in order to avoid amplification of genomic DNA. RNA sample quality was checked on an agarose gel, and quantification was determined spectrophotometrically using the Nanodrop ND-2000. RNA integrity was further investigated using the Bioanalyzer 2100 (Agilent Technologies) before RNA-seq libraries preparation. Illumina libraries were constructed at the Centre Nacional d'Anàlisi Genòmica (Barcelona, Spain) as previously described in Hachero-Cruzado et al. (2014). For RNA-seq analysis, Illumina short-reads were pre-processed using SeqTrimNext pipeline (Falgueras et al., 2010) available at the Andalusian Bioinformatics Platform (University of Málaga, Spain) using the specific configuration parameters for illumina data. Clean reads were mapped onto the reference and full transcriptomes v4.1 of *S. senegalensis* available at SoleaDB (Benzekri et al., 2014) using Bowtie2 (Langmead and Salzberg, 2012). These transcriptomes offer complementary information during statistical analysis since the reference transcriptome has a low level of transcript redundancy whereas the full transcriptome possesses the highest level of transcript representation. Total number of transcript counts was extracted using Sam2count.py (http://github.com/vsbuffalo/sam2counts). Transcripts with no mapped sequences were removed from the statistical procedures. Lastly, differential gene expression analysis was carried out using edgeR as implemented in Robina (Lohse et al., 2012), NOISeq (Tarazona et al., 2015) and TCC (Sun et al., 2013). Only significant data after Benjamin-Hochberg method for multiple testing correction with a *P*-value cut-off 0.05 were considered. Enrichment Functional analysis was carried using the ClueGO Cytoscape plug-in (Bindea et al., 2009) and the orthology annotations available in the SoleaDB (Benzekri et al., 2014). RNA-seq data has been deposited in the Sequence Read Archive (SRA) database with bioproject number PRJNA319182.

*qPCR validation*

To validate RNA-*seq* data, thirteen differentially expressed transcripts (DET) by different statistical methods (Edge-R, NOISeq or TCC) and using the full and reference transcriptomes were selected to validate their expression levels by RT-*q*PCR. These set of transcripts included; odorant receptor *or52b2l,* the V2R-like receptors *CPpr9* and *v2rh13* associated with fish olfaction, brain aromatase *cyp19b* with reproduction (*cyp19mUTR* for primers located in the 3'-UTR and *cyp19bm* for primers located in mature coding sequence), apolipoprotein D1 (*apoD1)* and endothelial lipase (*lipg*) with lipid metabolism, the purinoceptors *p2xr1, p2xr5* and *p2yr13* with nutrient sensing, fibronectin Type III domain containing 4 (*fndc4*) with immune system, *calcr* with calcium metabolism, cystatin B (*cstb*) with neural development and otospiralin (*otos*) with sensory organs (Table 1). Total RNA (1 µg) from each sample was reverse-transcribed with the iScript™ cDNA Synthesis kit (Bio-Rad) following the manufacturer's protocol. The *q*PCR assays were performed in duplicate on a CFX96™ Real-Time System (Bio-Rad). Real-time reactions were carried out in a 10 µL volume containing cDNA generated from 10 ng of original RNA template, 300 nM each of specific forward and reverse primers (Table 1), and 5 µL of iQ™ SYBR Green

Supermix (Bio-Rad). The amplification protocol used was as follows: initial 7 min denaturation and enzyme activation at 95 ºC, 40 cycles of 95 ºC for 15 s and 70 ºC for 30 s. In case of *cyp19UTR* and *cyp19bm* melting temperature was 60 ºC for 30 s.

Data were normalized using the geometric mean of three reference genes: glyceraldehyde-3-phosphate dehydrogenase (*gapdh2*), beta actin (*actb1*) and ubiquitin (*ub52*), which have been previously demonstrate to be suitable as reference genes in sole (Infante et al., 2008; Manchado et al., 2007). Relative mRNA expression was determined using the $2^{(\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001).

*Statistical analysis*

All data were checked for normal distribution with the Kolmogorov-Smirnov test as well as for homogeneity of variances with the Levene's test. Counts of germ cells in the testis between cultured and wild fish were compared using Student's t-tests (level of significance $P < 0.05$). To determine differences in transcript amounts between wild and cultured soles as determined by qPCR, Student's t-tests were also applied using Statistix 9.

## Results

*Sperm quality and histological analysis of testis*

Prior to the characterization of structure and expression profiles of UOR in wild and cultured specimens, the maturation status of testis as a key indicator of sexual and hormonal capacity was determined. The wild animals were smaller than cultured soles ($P<0.05$) (Table 2). However, no significant differences in terms of % motile cells, time post-activation and germ cell percentages were observed between wild and cultured sole groups (Table 2). Testis developmental stage corresponded to stage III according to García-López et al. (2006) in both fish groups.

**Table 2.** Biometric data, sperm quality evaluation and histological characterization of males analysed in the wild and cultured groups of Senegalese sole (*Solea senegalensis*) (n=4 per group). Different letters denote significant differences (Student's t-test; $P < 0.05$).

| | **Wild** | **Cultured** |
|---|---|---|
| *Biometric data* | | |
| Weight (g) | 157.1±19.5[a] | 499.3±18.3[b] |
| Length (cm) | 29.5±0.5 [a] | 35.2±1.3[a] |
| *Sperm quality* | | |
| % motile cells | 76.7±23.3[a] | 91.7±14.4[a] |
| Total time motile (s) | 83.0±8.9[a] | 82.8±6.5[a] |
| *Histological maturity evaluation* | | |
| % Spermatogonia | 0.7±0.3[a] | 1.0±0.5[a] |
| % Spermatocytes | 5.4±0.4[a] | 2.8±0.6[a] |
| % Spermatids | 77.0±4.5[a] | 78.7±6.6[a] |

| | | |
|---|---|---|
| % Spermatozoa | 16.9±4.9[a] | 17.5±5.6[a] |

**Table 1.** Primers used in this study to validate RNA-seq data. Gene, mapping (Ref = DET Reference and F = DET Full transcriptomic data; Statistical methods representing the data were shown between parenthesis), gene name, accession number (SoleaDBv4.1) and primer sequence are indicated.

| Gene | Mapping | Gene name | Size | Acc. Number | Primers (5' 3') |
|---|---|---|---|---|---|
| Calcitonin Receptor | Ref(2)/F(3) | *calcr* | 109 | unigene34587 | GCCAGCGGGATCACACGCGTCCCAA<br>TTGGTGAACCCATTGCCCGTCCTCT |
| Pheromone receptor CPpr9 | Ref(1)/F(3) | *CPpr9* | 76 | unigene279002 | ATGAGTCAGCAATTCAATGGAGCACAGGGT<br>CCTGGACGACAGCGCTCACTGCAAAC |
| Cystatin-B | Ref(1)/F(3) | *cstb* | 82 | unigene327535 | AGCGACGTAGAGGCCAAAGTGGGAAAG<br>TGGTTCCGGCCACAATCTGCGACGTAT |
| Otospiralin | Ref(1)/F(1) | *otos* | 98 | unigene3991 | CGCCGGGTTCGTCCTCTGCCTCATC<br>AGTAGGGCATAGCTGCTGGTTCGTCGT |
| P2X purinoceptor 5* | Ref(1)/F(1) | *p2xr5* | 104 | unigene9573 | AGAGGCTGGGACACTGTGCTGAGAGTA<br>TTCTCCATGGCCAGCCACCACCATCTT |
| P2X purinoceptor 1 | F(1) | *p2xr1* | 134 | unigene232746 | ACGATCACGTCATACCGTACCCTCCTTT<br>GTATGATGCATCGACGCCTTCCACCAGGTT |
| P2Y purinoceptor 13 | F(1) | *p2yr13* | 78 | unigene56110 | TGTTTCGGAGACGACTGACAGCCACTTT<br>ATGTTACCGTGGGAGATTCTGGGGTACCT |
| P450 aromatase | Ref(3)/F(3) | *cyp19bUTR* | 107 | unigene689047 | TTCATCCCTCGCAACACACAACCTCAA<br>CTGGCACAAAGAAAGTAAGGAAAATACGAT |
| P450 aromatase mature | Ref(3)/F(3) | *cyp19bm* | 88 | unigene689047 | ACATGGCAAGCTGTTCTTATCAAGCCTGAC<br>GCAGTTCTTGGGCTGCTCTCTTGTGCTTA |
| Endothelial lipase | Ref(3)/F(3) | *lipg* | 119 | unigene16871 | ACTTTCAACAACCGATGCGCCGTCACC<br>CCCCATTGTGTGCTGATCAATCCTCCT |
| Vomeronasal 2 receptor h13 | Ref(2)/F(1) | *v2rh13** | 102 | unigene469981 | ACTCCCCGACAACTTCAACGAGGCCAAA<br>TCCAGGAGAACTGACGTAGGCGGGAACA |
| Apolipoprotein D1 | Ref(2)/F(1) | *apoD1* | 111 | unigene22901_split_0 | GGGACGTACTGGATCCTGACCACGGAC<br>CGAGCGGCTGAGGACCCAGGCAAA |
| Fibronectin type III domain containing 4 | Ref(3)/F(3) | *fndc4* | 104 | unigene8879 | CATCTCACAGCAGAGGCAGGACGGCTT<br>TGCGTGTTCTCGTCCAGGTCCCACAG |
| Odorant receptor 52 B-like | F(1) | *or52b2l* | 113 | unigene470621 | CCACGCACCTCATCGTCTTCCTCCT<br>AGTGGACAGCCCTATGAATCGCCTCAA |

*RNA-seq and functional analysis*

To investigate the differences in the UOR expression patterns between wild and cultured sole, a wide transcriptomic analysis using RNA-seq was carried out. As indicated above, all sole included in the analysis had the same stage of maturity (stage III testis) and similar weight for UOR.

Total mean number of input raw reads were 50.8 million for each Illumina library achieving ~ 98% of useful paired-reads and ~ 0.7% of single reads for gene expression analysis. To assess differential gene expression, pre-processed Illumina short-reads were firstly mapped onto the *S. senegalensis* reference and full transcriptomes *v*4.1 available at SoleaDB (Benzekri et al., 2014) and DET were identified by using three distinct statistical methods (DESeq implemented in Robina, TCC and NOISeq). Percentage of reads mapped onto the reference transcriptome ranged between 72.6 and 76.5% and between 91.0-93.5% for full transcriptome. As depicted in Fig. 1A, a total of 778, 414 and 481 DET were identified using the reference transcriptome after Robina, TCC and NOISeq analyses, respectively that corresponded to 1,003 distinct DET. For full transcriptome, the number of DET increased to 1,655, 770 and 1,503 with Robina, TCC and NOISeq analyses, respectively that corresponded to 2,387 distinct transcripts. A comparison of DET across both transcriptomes revealed a 53% matching between reference and full transcriptomes (Fig. 1B). The complete set of DET using the reference and full transcriptomes by statistical method is indicated in supplementary file 1 (*published in* Fatsini et al., 2016).

To gain a better knowledge of meaningful biological pathways differentially expressed between cultured and wild fish, a Gene Ontology enrichment procedure was carried out. To improve data mining and enhance the robustness of the analysis to identify mostly represented categories, only those DET statistically significant at least by two statistical methods (DESeq, TCC and/or NOISeq) were considered. Cultured sole showed a lower expression of "inorganic and anion transport", "steroid metabolic process" and "regulation of embryonic development" categories (Fig. 1C and D). In contrast, biological process annotation identified an up-regulation of transcripts over-represented in oligodendrocyte differentiation (including 7 related categories), vesicle organization, response to hypoxia and oxygen transport (including 3 related categories), positive regulation of cellular component biogenesis (including 7 related categories), cell redox homeostasis, translational elongation and negative regulation of catalytic activity.

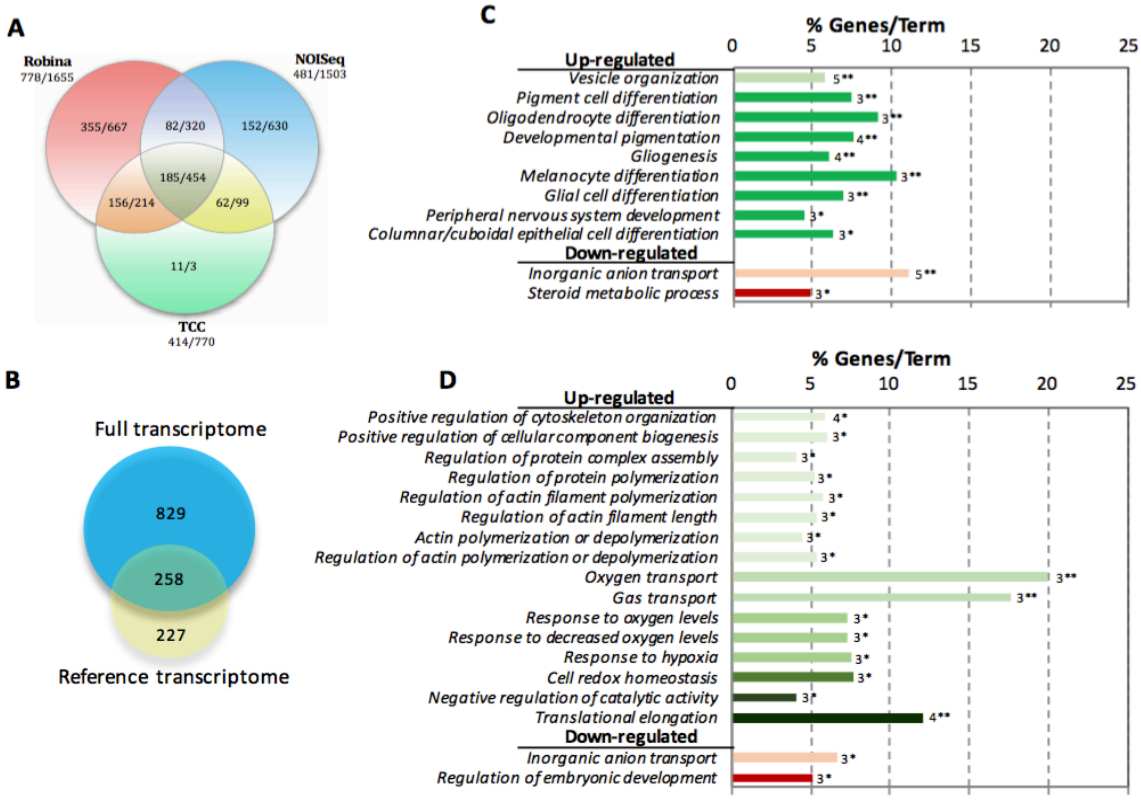


**Figure 1.** Venn diagrams DET and Gene Ontology (GO) categories significantly enriched for DET obtained from the UOR of cultured and wild Senegalese sole (*Solea senegalensis*). **A** Venn diagram showing shared and unique reads among the 3 statistical methods: Robina, TCC and NOIseq based on Reference/Full DET. **B** Venn diagram illustrating overlap of reads between DET Full and Reference. **C** Reactome categories showing genes-term pathways differentiating up-regulated than down-regulated genes. **D** Biological process at level 6 illustrating gene-terms differentiating up-regulated than down-regulated pathways. Hierarchical related categories are indicated in the same colour. The number of genes in each category are also shown.

A detailed analysis of DET (Table 3 and supp. file 1 (*published in* Fatsini et al., 2016) identified ten olfactory receptors: four OR receptors (*or52n5l*, *or52p1l*, *or52b2l*, *or51e2l*), two TAAR receptors (*taar10b* and *taar11*) and three V2R-like receptors (*casrl, v2rh13* and *CPpr9*). Interestingly, some genes related with neuroendocrine control of reproduction were over-expressed in cultured sole including the brain aromatase cytochrome P450 and tachykinin-3. Also, a wide set of genes related with lipid sensing (*cd36*), metabolism (*srebf2, fads2, fas, lipg, lpl, ldlr*) and transport (*fabp2, fabp6, apoE2, apoD1)* was differentially expressed, most of them down-regulated in cultured fish (*apoE2*, *fads2*, *fabp6*, *lipg*, *lpl*, *ldlr*).

In addition to olfactory-, lipid- and reproductive-related transcripts, cultured fish showed a higher expression of genes related with goblet cell differentiation and mucin production (*agr2*, *agr3*, *foxd3*, *foxe3*, *muc2l*, *spdef*, *gcnt3*) and reduced expression of cytoskeletal-related transcripts including type I and II cytokeratins (*krt13*, *krt15*, *krt8*), collagen type IV and laminin 3. Moreover, several DET involved in the innate (including cytokines, chemokines, antimicrobial peptides and antiviral defence) and adaptive (immunoglobulins-related genes) immune system were identified. Some other

DET were related with the defence mechanism against toxic substances (*pxr*), appetite control (*cck* and purinoceptors (*p2xr1*, *p2xr5* and *p2yr13*), ion homeostasis (calcitonin receptor), growth (*igfbp1a* and *igfbp3*) and thyroid metabolism (*dio3*) (Table 3).

**Table 3.** Selected differentially expressed transcripts in UOR of cultured and wild Senegalese sole (*Solea senegalensis*) after RNA-seq analysis. Transcript ID (SoleaDBv4.1), gene description, gene name, log2 fold-change (logFC) and mapping are indicated (Ref = DET Reference and F = DET Full transcriptomic data; Statistical methods significantly different were showed between parenthesis). Up-regulation shows higher expression in cultured males and down-regulation shows higher expression in wild than cultured males.

| Transcript_ID | Gene description | Gene name | logFC | Mapping |
|---|---|---|---|---|
| *Olfactory-related genes* | | | | |
| unigene396561 | Olfactory receptor 52N5-like | *or52n5l* | 1.9 | Ref(3)/F(3) |
| unigene385268 | Olfactory receptor 52P1-like | *or52p1l* | 1 | F(1) |
| unigene470621 | Olfactory receptor 52B2-like | *or52b2l* | -1.3 | F(1) |
| unigene463589 | Olfactory receptor 51E2-like | *or51e2l* | -1 | Ref(1) |
| unigene649764 | Trace amine-associated receptor10b | *taar10b* | -1.5 | Ref(2) |
| unigene633774 | Trace amine-associated receptor 11 | *taar11* | -1.5 | Ref(2) |
| unigene241070 | Extracellular calcium-sensing receptor-like | *casrl* | -0.9 | F(1) |
| unigene469981 | Vomeronasal 2 receptor h13 | *v2rh13* | -0.9 | Ref(2)/F(1) |
| unigene279002 | Pheromone receptor CPpr9 | *CPpr9* | -1.4 | Ref(1)/F(3) |
| unigene65990 | Otospiralin | *otos* | 0.8 | Ref(1)/F(1) |
| *Reproduction-related genes* | | | | |
| unigene29904 | Tachykinin-3 | *tac3* | 1.2 | Ref(2)/F(1) |
| unigene689047 | Brain aromatase cytochrome P450 | *cyp19b* | 1.3 | Ref(3)/F(3) |
| *Lipid sensing and metabolism* | | | | |
| unigene546426 | Apolipoprotein E2 | *apoE2* | -0.9 | Ref(2) |
| unigene22901_split_0 | Apolipoprotein D1 | *apoD1* | 0.7 | Ref(2)/F(1) |
| unigene19903 | CD36 | *cd36* | 1.8 | Ref(3)/F(1) |
| unigene29090 | Fatty acyl delta-6 desaturase | *fads2* | -1.1 | Ref(3)/F(1) |
| unigene571808 | Intestinal fatty acid binding protein 2 | *fabp2* | 2 | Ref(3)/F(3) |
| unigene31641 | Fatty acid binding protein 6 | *fabp6* | -1.8 | Ref(2) |
| unigene571036 | Fatty acid synthase | *fas* | -0.8 | Ref(1)/F(1) |
| unigene16871 | Endothelial lipase | *lipg* | -1.6 | Ref(3)/F(3) |
| unigene21966 | Lipoprotein lipase | *lpl* | -1.5 | Ref(3)/F(1) |
| unigene461067 | Low density lipoprotein receptor | *ldlr* | -0.9 | Ref(2)/F(1) |
| unigene28404 | Cholesterol sensing sterol regulatory element-binding protein 2-like | *srebf2* | -0.8 | Ref(2)/F(1) |
| *Goblet cells-related genes* | | | | |
| unigene225209 | Anterior gradient protein 2 | *agr2* | 3.7 | Ref(3) |
| unigene535952 | Anterior gradient homolog 3 | *agr3* | 3.6 | Ref(3)/F(3) |
| unigene232169 | Forkhead box D3 | *foxd3* | 1.3 | Ref(2)/F(1) |
| unigene5927 | Forkhead box E1 | *foxe3* | 1.2 | Ref(3)/F(3) |
| unigene52046 | Mucin 2-like | *muc2l* | 1.7 | Ref(3)/F(3) |
| unigene42701 | SAM-pointed domain containing Ets-like | *spdef* | 1.3 | Ref(3)/F(3) |
| unigene23680 | Glucosaminyl transferase 3, mucin type-like | *gcnt3* | 1.2 | Ref(1) |
| *Structural-related genes* | | | | |
| unigene282346 | Keratin13, type I | *krt13* | -1.7 | Ref(3)/F(1) |
| unigene70559 | Keratin 15, type I | *krt15* | -1.5 | Ref(3)/F(3) |
| unigene562578 | Keratin 8, type II | *krt8* | -2.2 | Ref(3)/F(3) |
| unigene280213 | Keratin 8-like, type II | *krt8l* | 1.2 | F(3) |
| unigene94047 | Laminin alpha 3 | *lama3* | -1.4 | Ref(3)/F(3) |
| unigene2368 | Collagen, type IV | *col4* | -1.4 | Ref(1)/F(1) |
| *Immune system-related genes* | | | | |
| unigene44683 | Interferon-inducible protein 56 | *ifi56* | -0.7 | Ref(1)/F(1) |
| unigene22503 | Interferon-stimulated gene 15 | *isg15* | -1 | F(1) |
| unigene69612 | CC motif chemokine 25 | *ccl25* | -0.8 | Ref(2)/F(1) |
| unigene18346 | Interleukin 8 | *il8* | -1.7 | Ref(2) |
| unigene45283 | Pleurocidin-like peptide | *plcl* | -1.4 | Ref(1)/F(1) |
| unigene281157 | Interferon-induced GTP-binding protein Mx | *mx* | 0.8 | Ref(2) |
| unigene416010 | Lipopolysaccharide-induced tumor necrosis | *litaf* | 0.9 | F(1) |
| unigene39697 | CC chemokine CK8 | *ck8* | 1.9 | Ref(2)/F(3) |
| unigene13182 | IgGFc-binding protein-like | *fcgbp* | 1.8 | Ref(3)/F(3) |
| unigene20687 | NF-kappa-B inhibitor zeta | *nfkbiz* | 0.9 | Ref(1)/F(2) |
| unigene8879 | Fibronectin type III domain containing 4 | *fndc4* | -4.9 | Ref(3)/F(3) |

*qPCR validation*

To validate RNA-seq data, a set of 13 DET obtained by distinct statistical methods using the reference or full transcriptomes were selected. For brain aromatase, two primer pairs that amplified the 3'-UTR and mature forms were used. Ten genes from the 13 transcripts exhibited significant differences in expression between cultured and wild sole (Figs. 2 and 3) and confirmed DET in the RNA-seq data. Three transcripts (*cstb* $P = 0.249$; *v2rh13* $P = 0.119$; and *p2xr5* $P = 0.065$) exhibited not significant differences although the fold-change values of transcript levels were similar to those observed in the RNA-seq data set. Correlation between RNA-seq expression and qPCR fold-change was $r^2=0.90$ (Fig. 4). Data significance showed no association with the statistical method used or transcriptome indicating that the whole set of data used in the analysis explained the differences between cultured and wild sole.

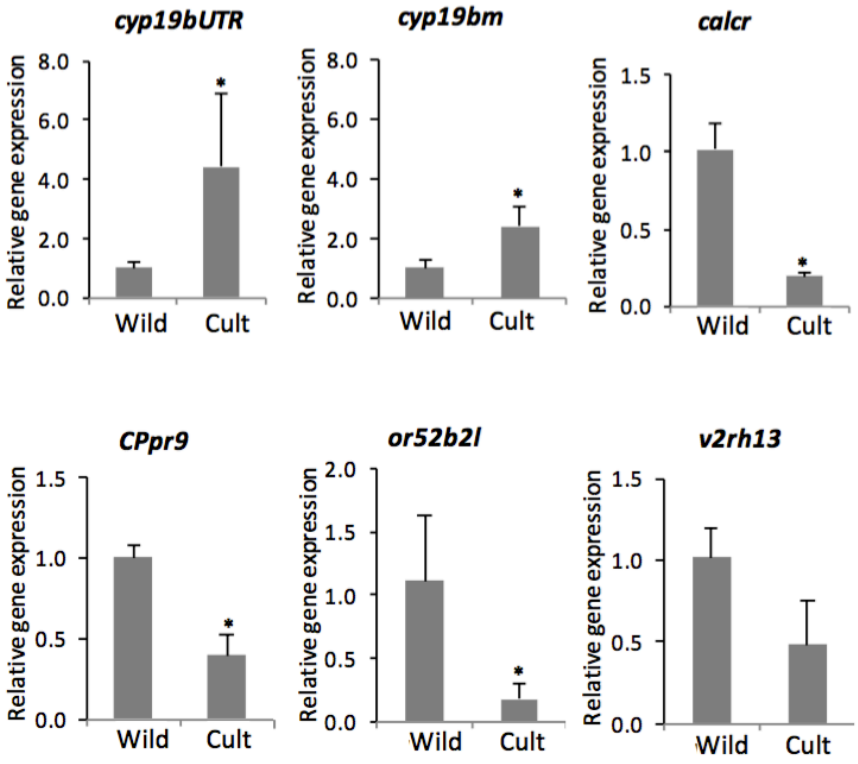


**Figure 2.** qPCR validation of the expression of a set of 5 genes in the UOR of cultured and wild Senegalese sole (*Solea senegalensis*). The 5 genes were obtained by distinct statistical methods using the reference or full-transcriptomes. *cyp19bUTR* and *cyp19bm* related to reproduction; *calcr* related to calcium metabolism; *or52b2l*, *v2rh13* and *CPpr9* related to fish olfaction. (*) Significantly different between wild (Wild) and cultured (Cult) sole (Student's t-test; $P < 0.05$).

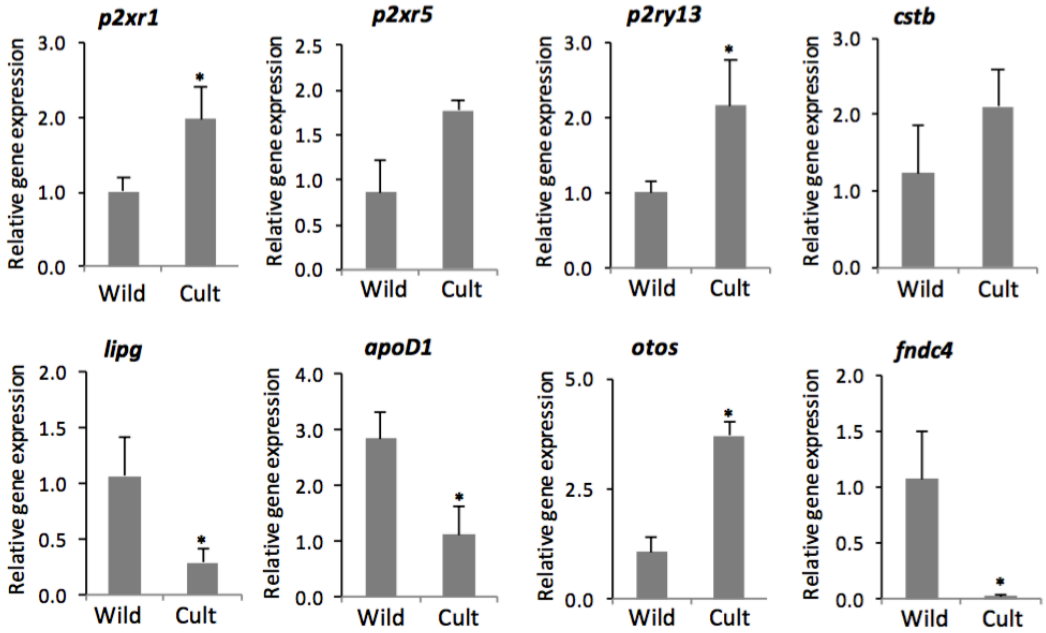


**Figure 3.** qPCR validation of the expression of a set of 8 genes in the UOR of cultured and wild Senegalese sole (*Solea senegalensis*). The 8 genes were obtained by distinct statistical methods using the reference or full-transcriptomes. *p2xr1*, *p2xr5* and *p2yr13* related to nutrient sensing; *cstb* related to neural development; *lipg* and *apoD1* related to lipid metabolism; *otos* related to sensory organs and *fndc4* related to immune system. (*) Significantly different between wild (Wild) and cultured (Cult) sole (Student's t-test; $P < 0.05$).

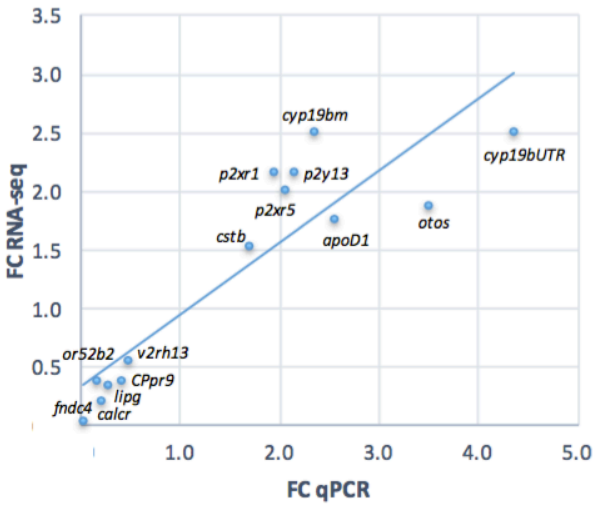


**Figure 5.** Correlation between RNA-seq expression and qPCR fold-change in expression. A set of 13 genes differentially expressed between the UOR of cultured and wild Senegalese sole (*Solea senegalensis*) ($r^2$=0.90).

## Discussion

The control of reproduction in cultured specimens of Senegalese sole remains a major research challenge for the aquaculture industry since programming of larval and

fry production in hatcheries and the design of genetic breeding programs are fully dependent on wild fish, a situation that is not sustainable in a long-term. Although some studies identified impaired production and secretion of androgens and LH in cultured soles (Chauvigné et al., 2016; Guzmán et al., 2009), hormonal therapies resulted unsuccessful to obtain fertilised spawns (Agulleiro et al., 2006). Further research identified the lack of proper courtship behaviour as the major factor responsible for the absence of fertilized spawning in cultured broodstocks suggesting a failure in social communication (Carazo, 2013). Thus, this study focuses on the characterization of expression profiles of the OE in wild and cultured male sole. All animals selected for the RNA-seq analysis were in the same testes maturity stage according to sperm quality and histological analysis (late spermatogenesis stage III; (García-López et al., 2006) with a similar UOR size at the moment of sampling.

Senegalese sole, like other flatfish, have a highly specialized olfactory sensing mechanisms that are established during the larval metamorphosis through major morphological and functional changes in the olfactory organs (*see* Introduction). As it has been seen in the anterior Chapter, it would appear that structural differences do not offer a possible explanation for the reproductive behavioural dysfunction (Carazo, 2013; Carazo et al., 2011) and functional differences in the OE should be considered.

The goblet cells are an important nonsensory cells in the UOR responsible for mucus production and secretion onto the olfactory chamber. This mucus barrier not only protects flagella and microvilli of OSNs against mechanical damage conducted by particles driven by the water but also modulates the detection of external cues and chemosensory responses (Kasumyan, 2004). Previous studies have demonstrated that the distribution of these cells in the OE is species-specific. In Senegalese sole, they appeared scattered in the sensory and nonsensory areas but with a higher concentration at the edge of the lamellae close to the raphe (*see* Chapter 3). A similar distribution was found in the Indian major carp (*Labeo rohita*) (Bhute and Baile, 2007), European eel (*Anguilla anguilla*) (Atta, 2013) and African butterflyfish (*Pantodon buchholtzi*) (Hansen and Zielinski, 2005). Contrarily, goblet cells appear concentrated in the nonsensory area perfectly separated from OSNs in zebrafish (*Danio rerio*) (Hansen and Zeiske, 1998) and featherback fish (*Notopteurs notopterus*) (Patle and Baile, 2014).

Mucus production in the goblet cells is highly modulated by factors including metals, nutritional status, genetic background and microbiological challenges. Metals such as copper or cadmium increase the number of goblet cells and mucus secretion imposing severe behavioural and physiological alterations due to smelling interference (Julliard et al., 1993; Tierney et al., 2010; Williams and Gallagher, 2013). Also, starving conditions and diets based on vegetable oils decrease the number of intestinal goblet cells and expression of mucus-related genes that in turn increase susceptibility to disease outbreaks (Estensoro et al., 2012; Li et al., 2014; Perez-Sanchez et al., 2013). Moreover, genetic families susceptible to disease can also display a higher expression of genes related with mucus production in gill and skin mucosa (Peatman et al., 2013). In our study, a set of 8 up-regulated DET (*agr2*, *agr3*, *muc2l*, *foxd3 foxe3*, *spdef*, *gcnt3*, *fcgbp*) related with mucus production and goblet cell differentiation (Chen et al., 2012;

Zheng et al., 2006) was observed in cultured sole. Although it is difficult to assign a single factor as responsible for this expression pattern, the differences in diet preference between wild (fresh food) and cultured sole (dry feed) and the coincidence between the set of DET and those mucus-related genes differentially expressed in surface mucosa in channel catfish after a fasting period (Li et al., 2014) indicate the nutritional status as a plausible explanation. Moreover, these changes in goblet cell differentiation and functionality seem to be associated to small tissue remodelling as observed by differential expression of genes related with *positive regulation of cytoskeleton organization* and *positive regulation of cellular component biogenesis* categories. Changes in transcript levels of type I (*krt13* and *krt15*) and type II (*krt8*) keratins, actin-related genes, collagen (*col4*) and laminin (*lama3*) suggest changes in supporting and basal cells, neurite outgrowth and olfactory extracellular matrix (Plendl and Sinowatz, 1998; Suzuki and Takeda, 1991).

Olfactory system controls nutrient sensing and feed intake. Previous studies in Senegalese sole demonstrated a functional asymmetry of LOR and UOR responding preferably, although not exclusively, to food-related odorants (i.e. amino acids) and conspecific-derived odorants (i.e. bile salts), respectively (Velez et al., 2005; Velez et al., 2007a; Velez et al., 2009). These separate sensibilities are possible due to the action of distinct olfactory receptors and transduction mechanisms (Velez et al., 2013). In our study, we found differentially expressed four OR, two TAAR and three V2R-like receptors. The former are expressed mainly in ciliated sensory neurons whereas the later in microvillous OSNs and crypt cells under the model "one neuron-one receptor" providing specialized OSN subsets that detect target odorants (Alioto and Ngai, 2005, 2006; Biechl et al., 2016; Kermen et al., 2013; Miyasaka et al., 2013). The wide repertoire of olfactory receptors able to sense several stimuli including amino acids, bile salts, nucleotides or pheromones makes difficult to establish an unequivocally action of each olfactory receptor (*reviewed in:* (Alioto and Ngai, 2006; Hansen and Reutter, 2004; Luu et al., 2004; Naito et al., 1998). Nevertheless, the differential expression of some transcripts related with nucleotide sensing and appetite control such as the purinoceptors *p2xr5*, *p2xr1* and *p2yr13* (Kittner et al., 2004), the anorexic hormone cholecystokinin (*cck*), (Morley, 2001) and orexigenic neuropeptide *agrp* (Oyama et al., 2010) as well as other several transcripts related with lipid sensing (*cd36*), transport and metabolism (*see* Table 3) (Efeyan et al., 2015) indicate that UOR is participating in nutrient sensing as well as modulation of appetite. Although under experimental design both cultured and wild specimens were offered the same diet (a mix of fresh food and dry pellets), we cannot exclude a selective attraction for each food type according to previous life-style (wild-fresh food and cultured-dry feed). Interesting, *fabp6*, a molecule able to bind bile acids (Alves-Costa et al., 2008), was down-regulated in UOR of cultured fish indicating changes in the sensibility to these conspecific-derived odorants (Velez et al., 2009).

Although differences in feed intake could explain partially the differences in the transcriptional profiles observed between wild and cultured sole, the role of specific olfactory receptors in social communication and reproduction should be considered

particularly relevant due to the conspecific odour-specialization of UOR in sole (Velez et al., 2013). In mammals, the vomeronasal receptors play a key role in pheromone detection action on gonadotropin-releasing hormone (GnRH)-secreting neurons to control some peripheral and central aspects of reproduction (Hagino-Yamagishi, 2008). Moreover, V2Rs are co-expressed with a family of MHC class I molecules for mate recognition in mice (Leinders-Zufall et al., 2004). Although V2Rs have been mostly associated with the detection of food cues in fish (Alioto and Ngai, 2006), these receptors located in microvillous or crypt-type OSNs have also been related with conspecific odours and pheromone detection to control reproduction and kin imprinting in some species (Biechl et al., 2016; Cao et al., 1998; Hansen et al., 2004; Hansen and Reutter, 2004). Similar to mammals, MHC peptide located on the cell surface of OE are involved in the neural process of kin imprinting in zebrafish (Gerlach et al., 2008; Hinz et al., 2013). In this study, some transcripts encoding MHC class I and II were differently expressed between wild and cultured animals suggesting differences in the recognition pattern mechanisms. Interesting, the neprilysin, a membrane-bound neutral peptidase that flank the genomic olfactory receptors gene clusters in some teleosts, was also down-regulated in cultured soles. This enzyme has been related with the digestion and release of peptides involved in signalling reproduction and/or genetic identity (Johnstone et al., 2009). All these data suggest that some DET could be involved in social interactions and arise the hypothesis if kin recognition could represent a selective barrier for sole reproduction. In fact, the reproductive behaviour of sole is associated to severe dominance hierarchies during mating as evident from the skewed genetic structure of offspring for a given breeding population (Martín et al., 2014; Porta et al., 2006). Hence, demonstration of kin imprinting and the function of MHC in the OE should be a priority in this species.

Intriguingly, the *steroid metabolism* pathway appeared down-regulated in cultured males. A reduced expression of *apoeb*, *fabp6* and *srebp2*, related with cholesterol metabolism, transport and sensing, and *cyb5r2*, a flavoprotein that catalyses the transfer of electrons from NADH to cytochrome *b*5 that in turn modulate the activities of an important steroidogenic enzyme, cytochrome P450 17 alpha-hydroxylase/17,20-lyase (Bhatt et al., 2016), were identified. Moreover, cultured soles exhibited a higher expression of brain aromatase (*cyp19b*) responsible for oestrogen biosynthesis from androgens. In the zebrafish, aromatase was suggested to play a role in male reproductive behaviour since this gene was strongly expressed in the olfactory bulb of males and triggered its expression with female spawning (Goto-Kazeto et al., 2004). Moreover, endler guppy (*Poecilia reticulata*) males treated with an aromatase inhibitor did not display proper courtship behaviour due to the lack of detection for olfactory cues associated with ovulation in receptive females (Hallgren et al., 2006). Also, Schlinger et al. (1999) found in midshipman (*Porichthys notatus*) that the aromatase activity reduced in the brain of active males with proper territoriality/courtship behaviour when compared with females or passive males that did not perform the acoustical courtship. Accordingly, with those studies, the different expression patterns observed for aromatase in the OE could affect social interactions in

cultured and wild males. Also, a higher expression of tachykinin-3 (*tac3*) was also observed in cultured soles. Tachykinins are detected in olfactory bulbs of goldfish with significant differences between males and females after feeding (Peyon et al., 2000). This gene was also activated during puberty and after estradiol treatment in zebrafish and it has been related with FSH and LH release in tilapia (Biran et al., 2014). Further research is necessary to identify if this gene is related with the impaired LH secretion observed in cultured sole during spawning and suggested as an underlying cause for the low reproductive performance of cultured males (Chauvigné et al., 2016).

The nasopharynx-associated lymphoid tissue has been reported as a first line of immune defence effective to fight against water-carried pathogens and to control microorganism proliferation. Although the sole in the present study cohabited under the same environmental conditions during the experimental period, they exhibited a set of DET involved in innate immunity (antimicrobial peptides, chemokines, cytokines, and interferon-related genes), adaptive immunity (several immunoglobulin-related genes) and antiviral genes (Mx). Small differences in mucus production and goblet cells activity as observed in this study could modify the interaction between microbial communities and surface colonization. Hence, several molecules of innate and adaptive immune system need to be activated to eliminate pathogens (Tacchi et al., 2014). Also, vascularization is very high in the olfactory systems of vertebrates for recruitment of lymphocytes and macrophages. The significant up-regulation of *Oxygen and gas transport* as well as *response to oxygen levels categories* and some other genes belonging to the *renin-angiotensin system* (RAS) (Armesto et al., 2015) suggests differences in blood supply to cover the specific homeostatic demands in the OE.

In conclusion, expression profiles in the UOR of cultured and wild sole have been characterized. The identification of several transcripts related with mucus production and goblet cells differentiation, nutrient sensing and feed intake could be the consequence of nutritional status and diet preference. Moreover, the differential expression of some transcripts related with olfaction including OR, TAAR and V2R-like receptors as well as reproduction and kin imprinting suggest new mechanisms behind the sexual dysfunction of cultured males. The high number DET related with innate and adaptive immune-related genes demonstrate the important role of surface mucosa to control microorganism proliferation.

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# Chapter 5

# Olfactory sensitivity of the marine flatfish *Solea senegalensis* to conspecific body fluids

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## Abstract

Chemical communication is better understood in freshwater than marine fish. The Senegalese sole (*Solea senegalensis*) is a marine flatfish wherein one of the bottlenecks in aquaculture is the poor reproductive performance of cultured males. The current study assessed whether chemical communication plays a role in reproduction in this species. Urine, and ovarian fluid were collected from adult fish, wild-caught and cultured, during the spawning season (March to May), and tested for olfactory potency using the electro-olfactogram (EOG). The effect of exposure of the olfactory system to adult female urine on circulating luteinizing hormone (LH) levels was also tested in males. Urine proved to be potent olfactory stimuli for both juvenile and adult conspecifics, evoking large-amplitude, concentration-dependent EOG responses, with thresholds of detection around $1{:}10^6$. However, the amplitude of response to urine depended on the sex and state of maturity of both the donor and the receiver. Furthermore, the olfactory potency of urine differed between wild-caught and cultured fish; however, contrary to expectations, urine from wild-caught females was less potent than that from cultured females. Urine from mature females evoked a slight, but significant, increase in circulating [LH] levels in mature males 30 minutes after exposure. Taken together, these results strongly suggest that urine-released odorants play a role in reproduction in Senegalese sole, and that a fault in this system may contribute to poor reproductive success in cultured fish.

## Introduction

Many teleosts use pheromones to regulate reproductive physiology and behaviour, in a variety of different ways (Stacey, 2015; Wyatt, 2014). However, the chemical identity and exact biological roles of such pheromones have been clearly demonstrated only in a few fresh-water species such as the goldfish (*Carassius auratus*; Dulka et al., 1987; Sorensen et al., 1988), masu salmon (*Oncorhynchus masou*; Yambe et al., 2006) and Mozambique tilapia (*Oreochromis mossambicus*; Keller-Costa et al., 2014). Marine fish have received much less attention (Hubbard, 2015); the black goby (*Gobius niger*) is the only example wherein the chemical identity of a pheromone is known (Colombo et al., 1980). Urine is a common vehicle for pheromones (Wyatt, 2014); however, urine production is much lower in marine fish than freshwater fish, so other body fluids, such as intestinal fluid (Hubbard et al., 2003) or mucus (Huertas et al., 2007), may be involved.

Flatfish (order Pleuronectiformes) are almost exclusively marine; in addition, their anatomy and lifestyle suggest functional asymmetry in the olfactory system (Doldán et al., 2011; Kasumyan, 2004; Prasada Rao and Finger, 1984). Flatfish often remain half-buried in the substrate with the eyes and upper nostril in contact with the water column, and the lower nostril buried and in direct contact with interstitial water. This is especially true in soles (family Soleidae) as the left olfactory epithelium does not migrate at all during metamorphosis. Given that - in teleosts - it is the olfactory system that mediates chemical communication (fish have no vomero-nasal or accessory olfactory organ), it is possible that the upper (eyed side) olfactory epithelium is relatively specialized for chemical communication, and the lower (blind side) epithelium for prey detection. In Senegalese sole (*Solea senegalensis* Kaup, 1858), for example, higher sensitivity for conspecific-derived odorants was found in the upper (right) olfactory epithelium, and for prey-derived odorants in the lower (left) epithelium (Velez et al., 2005; Velez et al., 2007; Velez et al., 2011; Velez et al., 2009). This asymmetry may not be confined to differential receptor expression, but could extend to transduction pathways (Velez et al., 2013) and neuronal processing in the olfactory bulb and above. Thus, this species may prove to be a valuable model for the comparison of neural detection and processing of two different functional classes of odorants; sex pheromones and kairomones (prey-related odorants in this case).

Furthermore, the European aquaculture industry has recently invested in the culture of Senegalese sole due to growing confidence in culture techniques and its high market price. However, reproductive bottlenecks hinder its culture; reproduction relies on wild-caught broodstock (Dinis et al., 1999), as breeders reared in captivity only spawn unviable eggs (Agulleiro et al., 2006; Guzmán et al., 2009). In particular, it appears that cultured males do not complete courtship behaviour (Carazo, 2013; Mañanós et al., 2007; Martín, 2016). Spawning performance of mixed (wild-caught and cultured) broodstocks shows that cultured females and wild-caught males produced viable eggs, whereas wild-caught females and cultured males do not. Moreover, plasma levels of luteinizing hormone (LH), a pituitary hormone that regulates spermiogenesis

and spermiation in teleosts (Chauvigné et al., 2014b; Schulz et al., 2010), are lower in cultured males than wild-caught males (Chauvigné et al., 2016). Therefore, cultured males may have a reproductive dysfunction; our working hypothesis is that the fault may lie in the chemical communication system.

The aim of the current study was, firstly, to establish which body fluids may be involved as vehicles for odorants involved in chemical communication during reproduction (urine or ovarian fluid) in Senegalese sole and, secondly, whether there may be differences in the strength of such signals between wild-caught and cultured fish. Finally, we examined a possible endocrine function of exposure to urine from mature females on the circulating levels of LH in mature males.

## Material and Methods

*Experimental animals*

Fish care and experimentation complied with the guidelines of the Portuguese legislation for the use of laboratory animals under a "Group-1" licence issued by the Veterinary General Directorate of the Ministry of Agriculture, Rural Development and Fisheries of Portugal. All EOGs were recorded from the upper (right) olfactory rosette of captivity-bred Senegalese sole (hereafter 'sole'). The fish from which samples were collected were kept in IRTA under natural photoperiod and temperature and fed a mixture of dry and fresh diet; dry consisted of balanced feed (Skretting, Stavenger, Norway), and fresh of mussels (*Mytilus edulis*), squid (*Loligo gahi*) and polychaetes (*Nereis virens*). Three developmental stages - juvenile, adult-immature and mature - were used; juvenile fish had undergone metamorphosis but not passed puberty, adult-immature were fish that had past puberty but were not mature when sampled, and mature were adult fish that had the presence of sperm or a swollen ovary with vitellogenic oocytes.

*Conspecific urine and ovarian fluid*

For sampling conspecific fluids, the fish were removed from the experimental tank and placed with eyes covered on a table. The sole were anaesthetised (45 mg. $l^{-1}$ MS222; 3-aminobenzoic acid ethyl ester; SigmaAldrichQuímica, Sintra, Spain), and light pressure applied to the abdomen in the area of the urinary bladder; extruded urine was collected with a syringe and kept on ice. These samples were centrifuged and either supernatant was collected as separate samples or an equal volume of the supernatant taken from each sample to form a pool. All samples were stored at - 20ºC until use.

Urine was collected during the spawning season (March to May) from mature males and females. The collection took place on the 17$^{th}$ of each month. Urine samples were collected from 13 cultured females (1096 ± 384 g) and 11 cultured males (877 ± 295 g) under natural photoperiod and temperature. During sampling on the 17 May, one

female was found to have ovulated. The ova and fluid were collected and centrifuged, and the supernatant taken off and stored at -20 ° C. In the case of the wild-caught fish, urine samples were collected only on 17 May 2015, in order not to disturb the fish during spawning. Urine samples from wild-caught sole were collected from 10 females (1500 ± 564 g) and three males (660 ± 239 g).

*Recording the electro-olfactogram (EOG)*

Samples were used to record EOG on cultured sole juveniles. Twelve juvenile sole, 6 females and 6 males (48 ± 3 g; 156 ± 6 mm), were used for recording EOGs. Prior to EOG recordings, the sole were maintained in the field station Ramalhete (Universidade do Algarve) under natural photoperiod and temperature. Two weeks before EOG recording the fish were acclimated from a salinity of 35 ppt to 12 ppt in four daily steps. After acclimation, the sole were maintained at 12 ppt and feeding and behaviour were normal.

Six adult females (624 ± 65 g; 348 ± 11 mm; GSI 1.05 ± 0.05 %) and six adult males (513 ± 100 g; 326 ± 22 mm; GSI 0.04 ± 0.005 %) were also used. Females were in resting stage of maturity and the most advanced oocyte stage observed was cortical alveolus with no vitellogenic oocytes present. Males were mature and motile sperm was obtained from the testes. Adult sole were obtained from IFAPA- Toruño (Cádiz, Spain) and moved to Ramalhete where they were acclimated for a month before EOG recording. Adult fish were maintained under natural photoperiod and temperature and acclimated and maintained at a salinity of 12 ppt as described above.

The method for EOG recording in sole has been previously described in detail (Velez et al., 2005). Briefly, the EOG recording was carried out at 12 ppt to reduce the electrical shunting effect of seawater; the amplitudes of EOGs recorded from marine fish in full seawater are considerably smaller than those of freshwater fish (Silver et al., 1976). The sole were anaesthetized by immersion in water (12 ppt) containing 100 mg. $l^{-1}$ MS222 buffered with 200 mg.$l^{-1}$ $NaHCO_3$, and immobilised with 6 mg.$kg^{-1}$ gallamine triethiodide (SigmaAldrich) injected intramuscularly. Once anaesthetized and immobilised, the fish were wrapped in a damp cloth and placed in a Perspex box with aerated water (containing anaesthetic) pumped over the gills (approximately 100 ml.100 g body-weight$^{-1}$.min$^{-1}$) via a tube inserted into the mouth. The upper olfactory rosette was exposed by cutting the overlying skin and musculature. Stimulus delivery to the olfactory epithelium was similar to that previously described (Hubbard et al., 2002) but the outlet of the stimulus-delivery tube was pulled into a finer point (~0.5 mm) to increase the velocity of the water-flow onto the olfactory epithelium; this prevented the build-up of mucus on the olfactory epithelium, a continuous problem in this species. The electrodes were pulled from borosilicate glass tubes, filled with 3 M NaCl in 1% agar and bridged to solid-state electronics via an Ag/AgCl pellet. The amplifier was a Grass AC/DC strain gauge (CP122; Astro-Med, West Warwick, RI, USA) with low-pass filter set at 30Hz. The recording electrode was placed at a position that resulted in the largest response to the standard stimulus ($10^{-3}$ M L-cysteine); this was usually

between the two lamellae near the middle of the raphe. The reference electrode was touching slightly the skin of the head nearby. The fish was grounded with an Ag/AgCl pellet electrode placed under the head. The recordings were digitized (DigiData 1322A, Axon Instruments, now Molecular Devices, LLC, Sunnyvale, CA, USA) and stored on a PC running Axoscope software (Molecular Devices). All stimuli were dissolved directly in charcoal-filtered seawater of 12 ppt. At least 1 minute was allowed to elapse between successive stimuli. The stimuli were applied in a varied order, but for concentration-response-curves of pooled urine and intestinal fluid were presented in order of increasing concentration. Individual urine samples (1:1000) were tested on three juvenile males and three juvenile females, and three adult males and three adult females, and the arithmetic mean of these three normalised responses was used in subsequent analysis. At the end of the experiment the fish were sacrificed with an overdose of MS222 and the GSI (gonad weight/body weight x 100) and wet mounts of the gonads were fixed and processed for histology.

*Effect of Female Urine on Circulating LH*

Five cultured males (532 ± 67 g; 339 ± 2 mm with GSI 0.043 ± 0.004 %) were used to assess the effect of olfactory exposure to mature female urine on circulating luteinizing hormone (LH) concentration. A sperm quality test was applied to ensure the males were mature and fluent. The fish were anaesthetized, immobilised, with the upper olfactory epithelium exposed and olfactory stimulus was delivered as described for EOG recording; however, electrodes were not placed and no EOG was recorded. The stimulus was pooled mature female urine ($n$ = 4), diluted 1:1000, and delivered to the upper olfactory rosette of the fish. The stimulus was given in 4-second pulses; every 10 seconds for 3 minutes (18 pulses in total). Three blood samples were taken (*via* the caudal vein into heparinised syringes); after the fish was set up in the experimental apparatus, as described, and immediately before the stimulus was applied (0 minutes), at three minutes (immediately after the olfactory stimulation had ended) and at 30 minutes after the first sample. At the end of the experiment the fish were euthanatized with an overdose of MS222, and sex and GSI checked. The blood samples were immediately centrifuged and the plasma collected and frozen (-20ºC) until assayed for LH using a specific enzyme-linked immunosorbent assay (ELISA) as described by Chauvigné *et al.* (2016).

*Data treatment*

EOG amplitudes were measured in millivolts. This was then blank subtracted (EOG amplitude response to the same water used to dilute the stimuli). Data were normalised to the amplitude of the response to $10^{-3}$ M L-cysteine (responses to $10^{-3}$ M L-cysteine were recorded every 10 - 15 minutes). A normality test and one- or two-way repeated-measures ANOVA (as appropriate) followed by Student-Newman-Keuls or Dunnett's *post hoc* test (circulating [LH]) (SigmaPlot 12, Systat Software, Germany)

were applied. Data are shown as mean ± SEM, and a significance level of 0.05 was used throughout with two-tailed tests.

## Results

Individual urine samples taken from mature males and females in April (the month with the highest number of spawn events) evoked larger amplitude EOGs in juvenile conspecifics than samples taken from the same fish in either March or May (Figs. 1A and 1B). However, in juvenile males (Fig. 1A), urine from mature males and females was equipotent, whereas in juvenile females (Fig. 1B), the urine from mature males was more potent than that from females, suggesting that juvenile males can detect substances in the urine of mature females that juvenile females cannot. When the same individual samples were tested on adult fish, however, the peak of olfactory potency in April was less apparent (Figs. 1C and 1D); adult males gave stronger EOG responses to those samples taken in March from mature males than the juvenile males (compare Fig. 1A with 1C), but the clear difference in olfactory potency between mature male and female urine when tested on adult females was maintained.

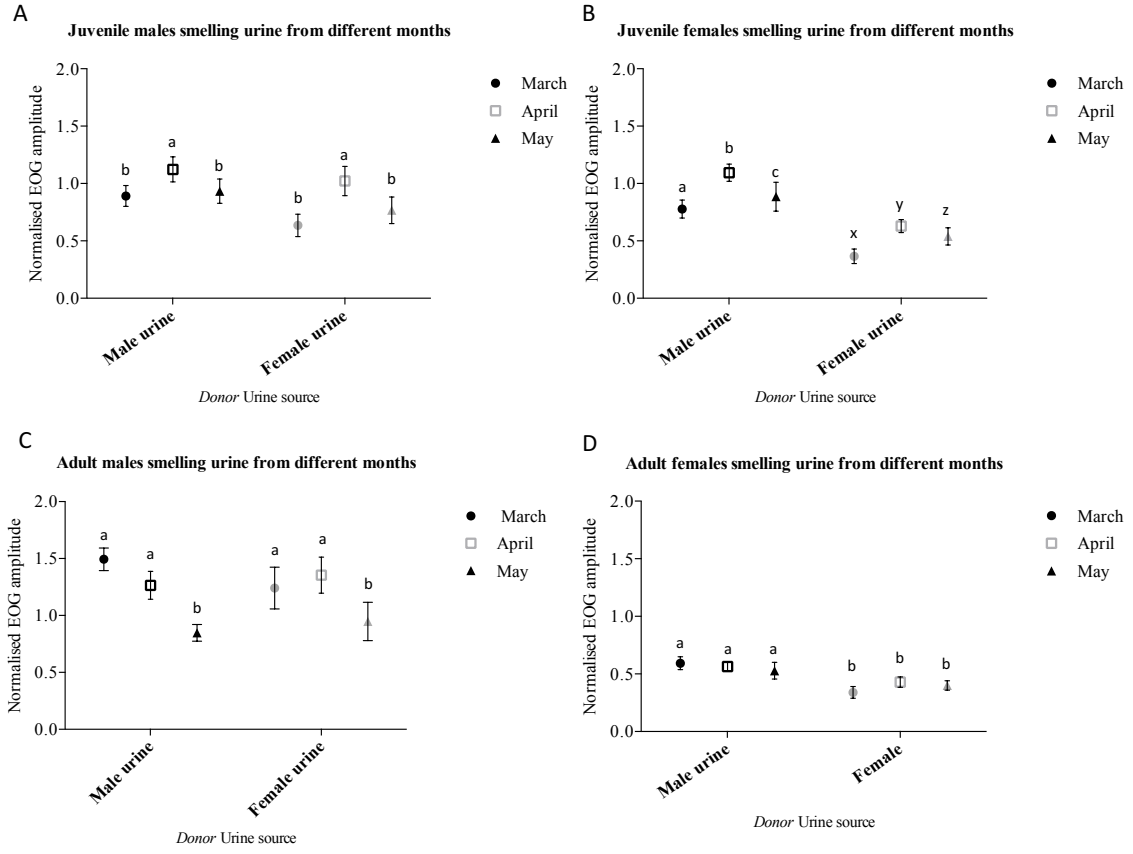


**Figure 1.** Olfactory potency of conspecific urine over the spawning season. Normalised EOG responses to individual urine samples (1:1000 dilution; mean of three independent EOGs) from adult sole over three months of the spawning season March to May (the fish spawned more frequently during April). **A** shows the responses of juvenile males, **B** shows the responses of juvenile females, **C** shows the responses of adult-immature males and **D** shows the responses of adult-immature females (all to the same urine samples). Different letters denote statistical differences ($P < 0.05$; Two-way repeated-measures ANOVA followed by the Student-Newman-Keuls *post hoc* test, $n$ =11 (males) or $n$ =13 (females). Note that juvenile fish give larger responses to urine taken from adult fish during April (coinciding with spawning), but that male urine evokes larger responses in females than female urine, irrespective of the month. Also,

adult fish do not show such a markedly higher response to samples taken during April; however, the larger response to male urine than female urine is maintained in adult females. Interestingly, adult males respond to something in both male and female urine from March that the juvenile males do not.

Urine from mature male and mature female sole consistently evoked larger amplitude EOGs in juvenile males than females (Fig. 2A), but the difference was only statistically significant in the case of juvenile females (*see* Fig. 1A). However, this difference in sensitivity was much less apparent when the same samples were tested on adult sole (Fig. 2B). This change with state of maturity or age of the receiver was apparently due to a greater variability of EOG response amplitude of the adult females compared to juvenile females; this suggests that the process of maturation modulates olfactory sensitivity of females to certain odorants in both mature male and female urine. Together, these results strongly suggest that the olfactory potency of urine depends not only on the sex and state of maturity of the *donor*, but also the sex and state of maturity (or age) of the *receiver*.

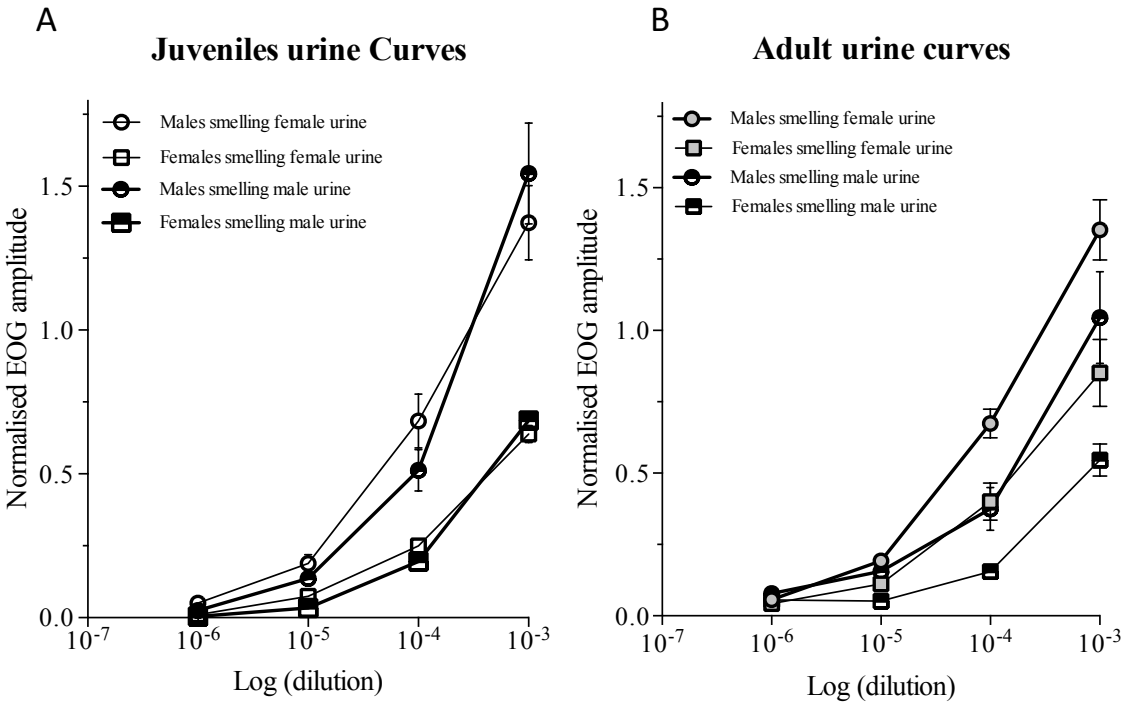


**Figure 2.** Olfactory responses recorded in Senegalese sole cultured males and females that were stimulated with mature cultured female or male urine. Semi-logarithmic plots of pooled normalised EOG amplitude (means ± SEM) recorded in **A** juvenile females and males smelling dilutions of male urine ($n$ = 5); ($P$ = 0.05) and female urine ($n$ = 5); ($P$ = 0.065) and **B** adult-immature females and males smelling male urine ($n$ = 6); ($P$ = 0.065) and female urine ($n$ = 6); ($P$ = 0.065). Repeated-measures ANOVA followed by Dunnett's test statistical analysis ($P < 0.05$ level of significance) was performed to compare responses from (juvenile and adult-immature) males and females smelling the same urine source.

In adult males, exposure of the olfactory epithelium to urine (1: 1,000) from a mature female (the same urine as in Fig. 2B for males) caused an increase in circulating plasma [LH] after three minutes that was maintained for at least 30 minutes (Fig. 3).

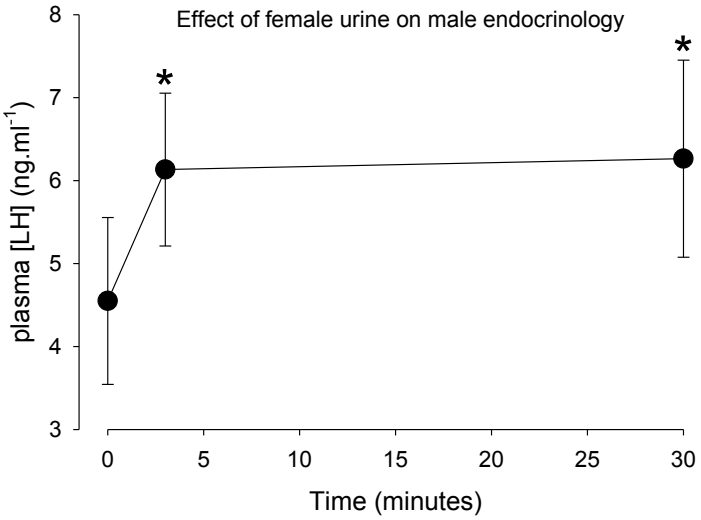


**Figure 3.** Effect of exposure to mature female urine on circulating [LH] in adult male sole. The olfactory epithelium of mature male sole was exposed to mature female urine (1:1,000) in pulses over three minutes; LH was measured in blood plasma taken immediately prior to exposure, immediately after exposure and 30 minutes after the start of the exposure. * $P < 0.05$; repeated-measures ANOVA followed by Dunnett's test.

Clear differences were seen in the olfactory potency of urine samples taken from wild-caught fish compared to those from cultured fish (Fig. 4A). However, contrary to expectations, urine from wild-caught fish was *less* potent than that from cultured fish. Juvenile males and females perceived significantly different the urine from females of different origin. Similar results were obtained from adult-immature fish of both sexes in the case of potency, however, in the case of perception, only the females perceived differentially the male and female urine from different origin (Fig. 4B). It should be emphasised that the wild-caught fish were kept in the same conditions and fed the same diet as cultured fish for at least four years before taking the urine samples.

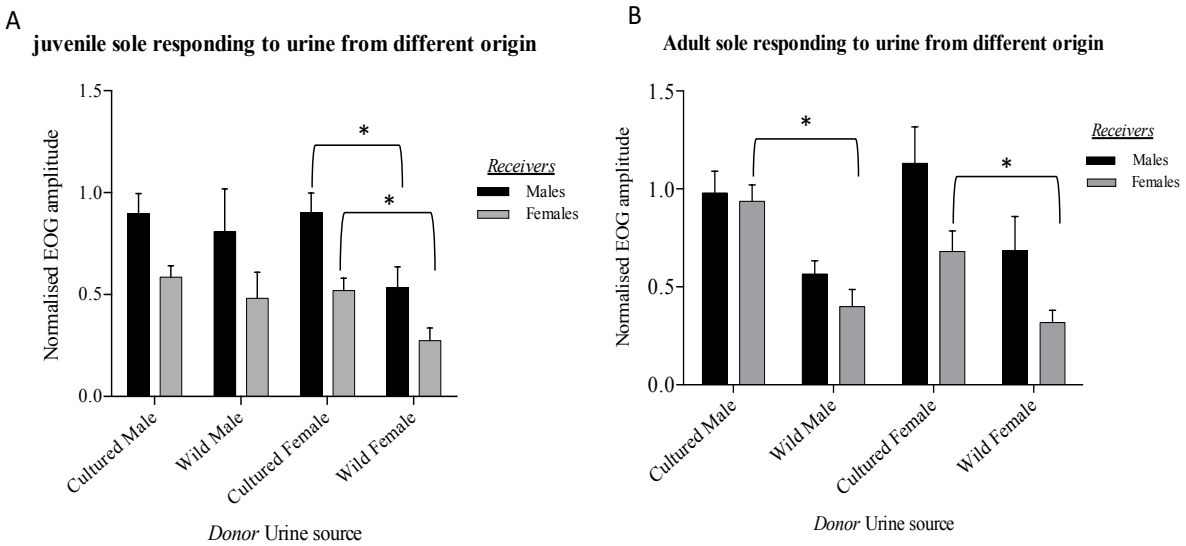


**Figure 4.** Olfactory responses recorded in Senegalese sole cultured males and females that were stimulated with urine from different origins. hatchery female ($n$ = 10) and male ($n$ = 10) urine; wild-caught female ($n$ = 10) and male ($n$ = 3) urine sampled in May during the spawning season. Normalised EOG amplitude (means ± SEM) in response to urine used to stimulate **A** juvenile males and females and **B** mature males and adult-immature females. A t-Student test (*$P < 0.05$ level of significance) was

applied to compare responses from (juvenile and adult) males and females smelling the urine from different origins.

Juvenile and adult sole of both sexes gave similar responses to the sample of ovarian fluid (Fig. 5); no differences were noted between adult and juvenile fish.

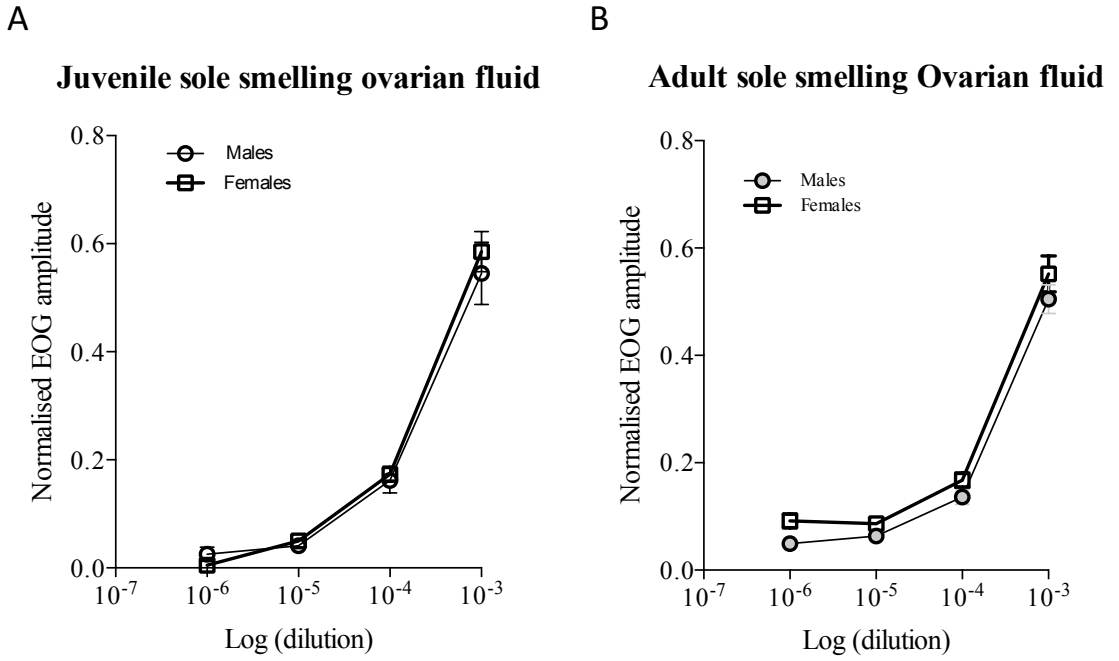


**Figure 5.** Olfactory sensitivity to conspecific ovarian fluid. Semi-logarithmic plot of normalised amplitude of EOG response to dilutions of ovarian fluid in juvenile **A** and adult-immature **B** of both sexes. No statistical differences were seen between responses from juvenile males and females ($P$ = 0.710; two-way repeated-measures ANOVA) nor adult males and females ($P$ = 0.595; Two-way repeated-measures ANOVA). No differences were seen between responses of juvenile and adults of either sex (*data not shown*).

## Discussion

The current study shows clearly that, in the sole, conspecific urine is a potent olfactory stimulus, with thresholds of detection around $1{:}10^6$ (urine); the olfactory potency of urine depends on both the sex and maturity of the donor. This is consistent with, indeed suggestive of, a role for urine in chemical communication during reproduction. Urine has been identified as a vehicle for reproductive pheromones in various freshwater teleosts, such as the goldfish (Appelt and Sorensen, 2007), masu salmon (Yambe et al., 2006) and Mozambique tilapia (Keller-Costa et al., 2014). In the goldfish, the female pre-ovulatory pheromonal steroid, 17α,20β-dihydroxy-4-pregnen-3-one, stimulates spermatogenesis and milt production in males *via* increased plasma gonadotropin concentrations (Dulka et al., 1987; Stacey et al., 1989). The current study suggests that a similar role may be played by urine from mature females in the sole; a brief exposure of the olfactory system of mature males to female urine provoked a sustained (at least 30 minutes) increase in plasma [LH]. This is therefore likely to have similar effects on spermatogenesis and milt production in the sole (Chauvigné et al., 2014a; Chauvigné et al., 2014b). Given the difference in urinary odorants between mature and immature males, it is possible that females use this chemical information in

mate-choice, similar to MHC-related odorants in sticklebacks (Aeschlimann et al., 2003; Milinski et al., 2005; Milinski et al., 2010) or the anal glands in blennies (Barata et al., 2008).

Furthermore, olfactory sensitivity to at least some of the urinary odorants also depends on sex and maturity of the receiver; for example, mature adult males have higher sensitivity to (a) component(s) in female urine than females themselves. Thus, the process of sexual maturation may influence the expression of olfactory receptor genes in the olfactory epithelium in the sole, as has been shown in the eel (Churcher et al., 2015), and in cyprinids olfactory sensitivity is increased by androgens (Belanger et al., 2010; Cardwell et al., 1995; Ghosal and Sorensen, 2016). It is likely, therefore, that during sexual maturation, increased circulating sex steroid evoke higher expression of pheromone receptors in the olfactory epithelium. To clarify this issue, the urinary pheromones must be identified, as must their respective olfactory receptors, as has recently been achieved for prostaglandin $F_2\alpha$ in the zebrafish (Yabuki et al., 2016) and initial studies have begun on gene expression in the olfactory of the Senegalese sole (Fatsini et al., 2016). This is also important for the comparison of wild-caught and cultured males (*see below*).

The ovarian fluid proved less potent than urine, and the response depended neither on the sex nor the maturity of the receiver is, perhaps, counter-intuitive. However, although salmonids have been shown to have a similar olfactory sensitivity to 'urogenital fluid' (assumed to be mostly ovarian fluid; Kitamura and Ogata, 1989), it does not have the same attraction to males as urine from mature females (Olsén et al., 2002), although it may have some attractive properties (Emanuel and Dodson, 1979). Sole spawn in pairs (Baynes et al., 1994; Carazo et al., 2016) with little opportunity for other males or females to participate once the swimming assent has started to liberate gametes and this may be related to the lack of involvement of ovarian fluid in communication. Together, these findings suggest that ovarian fluid is less important than urine in chemical communication in fish, although it may enhance sperm motility in the sole (Diogo et al., 2010) as in other teleosts (for example, see Elofsson et al., 2006; Rosengrave et al., 2009).

One of the aims of the current study was to investigate whether the poor reproductive performance of cultured males could be due, at least in part, to a fault in the reproductive pheromonal system. However, contrary to expectations, the urine from wild-caught sole was *less* potent than that of cultured fish. Thus, it seems unlikely (although possible) that wild-caught fish are releasing less of an important pheromonal component than cultured fish; our original hypothesis. Nevertheless, this finding clearly allows for the possibility that environmental or social cues, found in the natural environment but not in captivity (or *vice versa*), are important for future production of pheromonal cues during spawning. It is also possible that such cues also modulate receptor expression, particularly pheromone receptors, in the olfactory epithelium in a similar manner to that of sex steroids mentioned above. Or that the neural processing of the pheromonal message is faulty in cultured fish. Given the apparent complexity of both the pheromonal message, and the respective olfactory receptors in the olfactory

epithelium, to address this hypothesis we need to identify the active components in both male and female urine, their biological roles, and their receptors in the olfactory epithelium, then make a full comparison between wild-caught and cultured males.

In conclusion, the current study has shown that conspecific urine, but not ovarian fluid, may play roles in chemical communication during reproduction in the sole. Moreover, olfactory sensitivity to at least some of the compounds involved depends on both the sex and state of maturity of the receiver, suggesting regulation at the level of the olfactory epithelium. One physiological role of mature female urine may be to increase spermatogenesis and milt production in males *via* stimulation of the release of LH from the pituitary gland, as has been previously shown for 17α,20β-dihydroxy-4-pregnen-3-one in the goldfish. Deficiencies in the pheromonal system may contribute to the poor reproductive performance of cultured males; however, much work remains to identify exactly where these deficiencies may lay.

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# Section 5: Behavioural analyses

# Chapter 6

# Dominance in Senegalese sole (*Solea senegalensis*) linked to brain gene expression

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## Abstract

Dominance in the context of animal behaviour is defined as the preferential access to resources of one individual over another and is related to a higher social status relative to one or more individuals usually from the same species. The aim of the present study was to characterise dominance on those terms in early and late Senegalese sole (*Solea senegalensis*) juvenile stages by 1) describing the different behaviours associated with dominance in a defined non-aggressive species, and 2) linking the two dominance categories (dominant and subordinate) with the brain gene expression. Early juveniles (n=74, 37 pairs in 37 tests) were subjected to a dyadic dominance test related to feeding and euthanatized to determine the brain mRNA expression profile for ten different genes related to dominance and aggressiveness. Late juveniles were subjected to two individual dyadic dominance tests ($n$ = 34, 17 pairs in 17 tests), related to feeding and territoriality and one group test (4 groups / tests of $n$ = 6) combining both dyadic tests. Senegalese sole feeding first were categorized as dominant and sole feeding second or not feeding as subordinate. Three behaviours (i. Resting the head on another fish, ii. Approaching another fish, iii. Swimming above another fish) were associated with dominance of feeding and two parameters (i. total time occupying the preferred area during the last 2 hours of the test, ii. Organism occupying the preferred area when the test ended) were representative of dominance in the place preference test. In all tests, dominate fish compared to subordinate fish displayed a significantly higher number of the behaviours rest the head, approaches and swim above another. In the place preference test dominate sole dominated the sand at the end of the test and in the group test dominate sole both ate first and dominated the area close to the feed delivery point before feed was delivered. The mRNA expression levels of genes related to neurogenesis (*nrd2*) and neuroplasticity (*c-fos*) were expressed significantly higher in dominate sole compared to subordinate sole. This study demonstrated for the first time the existence of individual dominance categories at early developmental stages by using reliable tests (dyadic and group), identified behaviours related to dominance and also the relationship between dominance and gene expression in Senegalese sole. The identification of dominance and behavioural parameters that indicate dominance has relevance to both ecology and aquaculture offering the potential to initiate studies to improve culture.

## Introduction

Behavioural studies in fish have been used as a model background for the field of perceptive ethology/evolutionary psychology. Associating several cognitive activities (memory, kin recognition, learning, among others) with morphology, ecology and a variety of behavioural parameters could provide a general vision for cognitive ethology (Griffin, 1984; Cheney and Seyfarth, 1991; Barkow, 1992; Dugatkin and Wilson, 1993). Moreover, the study of flatfish behaviour has attracted the attention of researchers due to their ecology, life history and adaptations. However, when considered in comparison with other pelagic fish species flatfish behaviour has received comparatively little attention. General aspects in the behavioural catalogue of flatfish have been described including feeding behaviour, locomotion, mimicry and spawning behaviour (*review in* Gibson 2005). However, dominance behaviour in general for flatfish has not been described. The understanding of their behavioural patterns would be very useful to comprehend the biology and ecology of the species and to help their management in aquaculture therefore decreasing pressure on natural populations.

Flatfish are a large group of fish distributed widely in all oceans that have the following common characteristics: both eyes are on the same side of the head, flattened shape, the ability to mimic the bottom and bury themselves in the sediment. These diverse characteristics and eye migration in the larva, called metamorphosis, make this group of fish distinctive and the focus of many biological studies particularly during development. The variation in size and habitat means that flatfish have a wide range of ecological niches with different physiological and behavioural adaptations to benthic life. The nature of the sediment/substrate varies between species and stages (Allen and Baltz, 1997; Phelan et al., 2001). One particularly important flatfish species in the Mediterranean Sea and the Southern European Atlantic coasts is the Senegalese sole (*Solea senegalensis*), an economically high value species, which is commercially extracted and cultured.

Currently, the farming of Senegalese sole relies on wild captures of breeding adults. Cultured males (born and reared in captivity) present dysfunctional behaviour and as a consequence did not complete the reproductive courtship to fertilise eggs (Martín, 2016; Carazo et al., 2011; Carazo, 2013). Therefore, aquaculture of this species is not sustainable and may not be viable in the long term (Morais et al., 2014). In order to understand the behavioural dysfunction to develop the production of this species, the reproductive behaviour has been described (ethogram) (Carazo et al., 2016) and stress coping styles (personalities) have been described for individual fish and compared between groups with different origins and reproductive success (Silva et al., 2010; Ibarra-Zatarain, 2015; Ibarra-Zatarain et al., 2016). However, further studies are needed to understand inter-individual interactions in captivity of this species.

Under aquaculture conditions Senegalese sole has a significant dispersion in size during weaning and this has been attributed to their hierarchical distribution in the tank environment (Salas-Leiton et al., 2008; Salas-Leiton et al., 2010). Interestingly wild pairs (male and female broodstock) that reproduce in captivity show intra- and inter-annual fidelity resulting in producing few families dominating the offspring (Martín et

al., 2014). It has been reported that only a few individuals (8 - 40 %) in the population contribute to offspring (Porta et al., 2006; Martín et al., 2014). Social hierarchical structure is essential to achieve reproductive success in some fish species, for example in the African cichlid (*Astatotilapia burtoni*) only alpha males procreate while the subordinate male's reproductive system is suppressed and these males wait for a chance to take over a territory (Kustan et al., 2012). In the case of coho salmon (*Onchorhynchus kisutch*), two types of males are found in the population, jacks and hooknose. Hooknose males are dominant while jacks are sneakers and their contribution to offspring is limited (Berglund, 1997). One behavioural pattern related to dominance, which can be defined as success in competitions over limited resources such as food, specific (preferred) areas, shelter, mates, spawning places and offspring (Noakes, 1978). Thus, in general, dominant organisms can have better access to food and shelter, lower rates of predation and higher mating success (Grant, 1997) than subordinate organisms. Conversely, subordinate organisms in comparison can suffer chronic stress, immune depression and reduced disease resistance (Øverli et al., 1999). Aggressive behaviour is related with social hierarchies and competition, which is considered an essential behaviour in fish such as zebrafish (*Danio rerio*), Nile tilapia (*Oreochromis niloticus*) or rainbow trout (*Oncorhynchus mykiss*) among others (Cardoso and Volpato, 1997; Grant, 1997; Andersson and Höglund, 2012; Ruiz-Gomez and Huntingford, 2012). In fish, social hierarchies are often categorised by agonistic behaviours that are habitually registered through feeding contests or territoriality. Several variables could be measured to assess these social hierarchies and one of them is feeding behaviour (Øverli et al., 2004a) which has been associated with physiological indicators of stress (Øverli et al., 2002a; Øverli et al., 2007), aggression and mating success (Korzan et al., 2006). The place preference test (PPT) is one of the tests used to analyse territorial dominance. This test has been employed in experiments with mammals (Griffin and Speck, 2004). Currently, PPT is also used for fish (Delicio et al., 2006) with an active implication in reproduction. For example, Nile tilapia (*Oreochromis niloticus*) is a tropical fish species that build nests and defends a territory to attract females. Thus, individuals choose between different cubicles depending on the stimulus motivating the visit, shelter or reproduction. The knowledge of the natural preference and the competition for different compartments is essential to avoid a misleading interpretation of results (Serra et al., 1999; Delicio et al., 2006). In addition, animals which acquire the dominant position in early life stages (larvae and juvenile) in fitness-related traits might expand this social status to reproductive success (English et al., 2013).

In addition to behavioural studies several works have reported upon the underpinning molecular mechanisms. Genes related to neuroendocrine mechanisms have been associated to social dominance in some fish species (Øverli et al., 1999; Winberg et al., 2001; Øverli et al., 2004b; Teles et al., 2013). For example, in the zebrafish differential expression of several genes in the brain (Dopamine *β*-hydroxylase, catecholamine and arginine vasopressin) was reported between dominant and subordinate males and these differences may reflect social rank (Pavlidis et al., 2011). In addition, there are some genes, such as, brain-derived neurotrophic factor (*bdnf*), c-FOS (*c-fos*) and neurogenic differentiation (*neurod*), which have been associated with

social status as social plasticity relies on neural plasticity and neural regeneration in zebrafish (Teles et al., 2016). The monoamine neurotransmitter serotonin (5-Hydroxytryptamine; *5HT*) has also been associated with the control of several behavioural aspects including aggression among other genes such as tryptophan hydroxylase 1 (*tph1*) and solute carrier family 6 member 13 (*slc6a13*), which have been also related to aggression and impulsivity (Ho et al., 1998; Øverli et al., 1999; Lesch and Merschdorf, 2000; Koolhaas et al., 2007; Silva et al., 2014) and feed intake (De Pedro et al., 1998). Thus, the behavioural phenotype was the result of the modulation of the inter-related function at different levels of biological regulation, demonstrating the behaviour could be linked with gene expression (Rey et al., 2013).

In this study we provide insight into dominance and social interactions of captive Senegalese sole aiming to define dominance behaviour by relating behavioural patterns to feeding response and territory as well as defining brain gene expression associated to these behavioural responses.

## Material and Methods

*Ethics statement*

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

*Animal rearing conditions*

Sole were provided by Stolt Sea Farm (Santiago de Compostela, Spain), different fish from the same batch were used for two successive years to conduct different behavioural tests. Fish were transported from A Coruña, where the facilities of Stolt Sea Farm are located, with a specialized transport for aquatic live animals. Fish were maintained at the Research Centre facilities of IRTA, in St. Carles de la Ràpita, North East Spain and distributed in two 10 $m^3$ fiberglass tanks with natural photoperiod (using artificial lighting) and seasonal simulated external temperatures (40°62'82.42", 0°66'09.37"/ 9-32 °C) maintained within the range of 9 - 20 ºC with a recirculation system (IRTAMar®) that recirculated +400% and renewed 10% of the water daily. Sole were fed *ad libitum* five days per week with balanced feed (LE-3mm ELITE, Skretting, Co.). Animals were moved to a 400 L fiberglass tank and acclimated for one week before dyadic tests started. All fish were tagged with a passive integrated transponder (PIT) tag (ID-100A, Unique Trovan-Zeuss; Madrid, Spain) and photo identified individually. Individual markings in the photographs were matched with the corresponding PIT Tag for later identification in the video images. The acclimation and experimental tanks were in a recirculation system to control the temperature and water quality in order to reduce environmental variation. During the tests the temperature was maintained at 15 °C with a recirculation system (IRTAMar® described above). The

animals were fasted for 48 hours prior to the experiment.

*Behavioural studies*

*Preliminary dyadic test*

Preliminary dyadic test was performed with ten early juvenile sole (when fish weight was approximately 100 g) according to Huntingford et al. (1993) to decide which behavioural test was appropriate to characterise dominance behaviour in Senegalese sole. Animals were provided with different experimental cues which were visual (animals were separated by a transparent screen through which the animals could see each other), chemical (animals were kept apart by an opaque screen with holes through which the sole could exchange chemical cues) and isolated (sole were separated by an opaque screen without the possibility to see and smell each other). The different tests were performed at different daylight hours (morning or night). When preliminary results were analysed Senegalese sole did not show differences in behaviour due to the different experimental setups, and all fish resumed feeding and ate normally. Therefore, visual and chemical cues between separated fish did not appear to affect feeding. Considering this, the isolated approach was selected to provide a condition where the animals had equal status before testing for dominance over a limited resource, which is an approach that has been previously used in fish (Øverli et al., 2002b; Øverli et al., 2004a).

*Dominance tests*

Three different behavioural tests (two in pairs and one in group) were performed for Senegalese sole juveniles to test for dominance. Only one of the behavioural tests, feeding response in a dyadic test, was applied to early juveniles ($n$ = 74; 100.4 ± 10.6 g). All three tests were applied to late juveniles ($n$ = 34; 287.0 ± 30.4 g) and the resting time between tests was 15 days to allow for full recovery, return to basal conditions and avoid learning and conditioning processes (*see* Fig. 1 for set-up and time line of the experiments). In all dyadic tests the fish were size-matched and paired (< 10 % of weight and size between animals).

**Dyadic tests**

1) Feeding response after a dyadic test

Two size matched sole were kept apart by a opaque/grey polyvinyl chloride (PVC) wall from 19:00 to 8:00, being isolated overnight. The wall was removed next morning at 08:00 and their behaviours were recorded for the first two hours. After two hours, fish were fed and the individual that ate first was classified as dominant. The individual that ate afterwards or did not eat at all was classified as subordinate. Behaviours were individually registered and their frequency of occurrence was recorded by video observation. The behaviours recorded were: “Approaches”, “Swimming above another” (SAA), “Rest the head” (RTH), “Displacement”, “Burying” and finally order

of feeding “Feeding” that was used to define dominance (categorical variable) (Table 1: ethogram of behaviours).

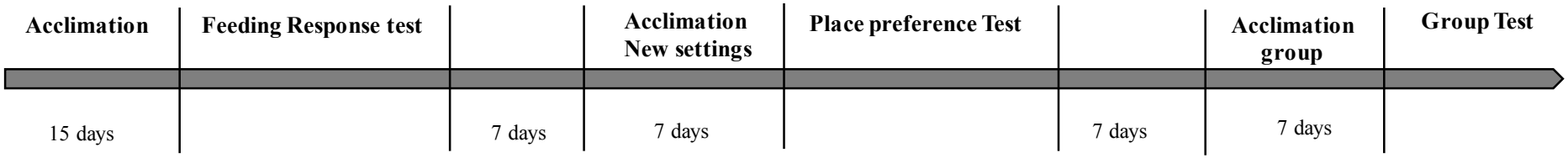


**Figure 1.** Chronogram showing the experimental design of the different dominance behavioural tests conducted on late Senegalese sole juveniles.

2) Competitive behaviour for a preferred area, place preference test (PPT)

For the place preference test (PPT) the settings of the tank had to be modified. The modifications were: to create a false substrate on the tank by placing twenty white tiling pieces (measuring 24 x 11.5 x 1.5 cm each and the same colour as the bottom of the tank) and leaving one piece out to provide a space that was filled with sand. The new sandy area created was the standard size of one fish and so just one fish could comfortably occupy this area. Sole individuals used for this test had an extra week of acclimation to those novel objects: the sand and the tiling. The purpose of this test was to create a preferred area with sand (in nature, sole live buried in sand), which just one fish could occupy and, therefore, dominate that area (*see* Fig. 2 for set-up details). Previous studies have shown that sole prefer sandy areas (Hampel et al., 2008). The experimental tank was set up with PVC opaque/grey divisions that separated the two fish from each other and from a third area that contained the sand. The same pairs of sole that were previously used in the feeding test were also used in the PPT test and this enabled the PPT behaviour of the sole to be analysed in relation to the dominant and subordinate status observed in the feeding test. Fish were physically isolated and were introduced into the tank at 19:00 and left overnight. The dividers were removed the following morning at 08:00 allowing the fish to see each other and access the restricted preferred area (sand). The behaviour of the fish and in relation to the preferred sandy area was recorded continuously for 24 hours for further video analysis of recorded behaviours (Table 1). Low intensity red lighting that did not affect behaviour or other physiological parameters was used for night recordings (Carazo et al., 2013). The variables registered in this test were measured in minutes and regarding to the preferred area (sand). The variables were "Total time" in the preferred area (TT), "Initial time" to occupy the sand (Ti), "Final time" to occupy the sand (Tf) and order: "first" or "last" (categorical variable) (Table 1).

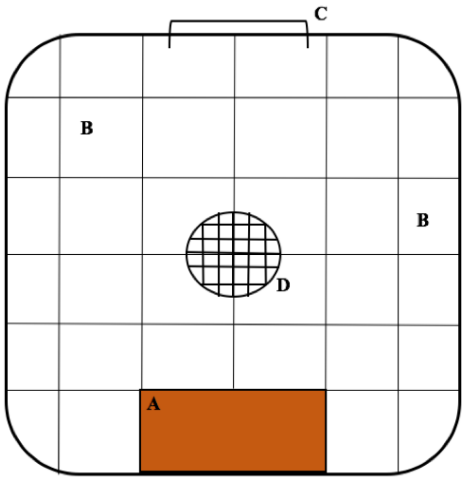


**Figure 2.** Experimental tank set up used in the place preference test (sand) in pairs. (A) Preferred area (sand), (B) white tiles forming a false bottom characterized the novel conditions, (C) Water inlet, (D) Water outlet.

**Group test**

3) Dominance in groups

Four groups of 6 fish (24 fish in total) were randomly selected and placed in 400 L tanks (four tanks in total) for two weeks after the place preference test was performed. The fish were kept in four groups of six for one week of acclimation and to allow that hierarchies were established. After this period of acclimation, each group of fish was recorded to analyse the social interactions in the group. The different behaviours (Table 1) were recorded for 2 hours (every 5 minutes analysing a total of 24 frames) before fish were fed. The test was performed for four days and the same behaviours for each of the groups of fish were recorded to test for consistency and repeatability among days. Fish were visually individually identified. An automatic feeder was placed in a corner of each tank and feed was delivered directly to the bottom of the tank through an 18 mm PVC tube to provide a single point of feed delivery (Fig. 3). This point feeder set-up is known to trigger territoriality and feeding competition among the individuals and that dominant fish, at higher positions in the social hierarchical rank, tend to monopolise it (Rubenstein, 1981; Grand and Grant, 1994; Grant et al., 2002; Reebs, 2008). The exact physical position of the fish in relation to the point of feeding was recorded before and after feeding events by order. Preferred area was ranked regarding to the distance from the feeding area (territoriality). The behaviours registered were related to both tests performed in pairs. Behaviours for the "feeding response test" were "Rest the head" (RTH) and "Swimming above another" (SAA) (Table 1.). The behaviours were registered in counts and different indexes were calculated (actions amongst animals). The variables registered for the place preference test (PPT) were the different fish positions within the tank in relation to the feeder (*see* Fig. 3 for set-up details): "Position before feeding" (POSITB) and "Position after feeding" (POSITA) were noted for every individual sole. In addition, the "Feeding order" (compare to 'pecking order') for every day of the experimental period was registered to check for consistency over time (Table 1).

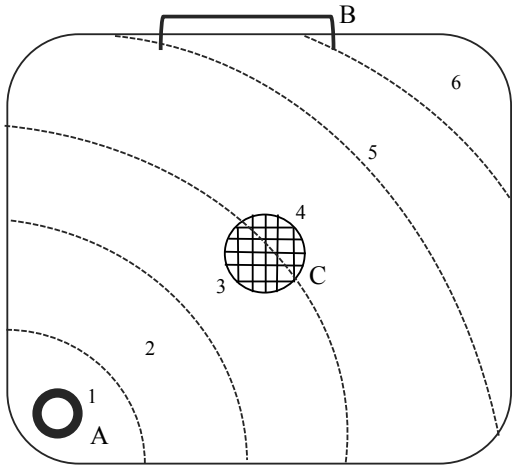

**Figure 3.** Tank set up used in Group experiments to examine feeding response and Place preference test. (A) PVC tube to deliver the food, (B) Water Inlet (C) Water Outlet. Different position areas (1-6) were shown by dotted lines.

**Table 1.** Ethogram of different behaviours registered (number of each behaviour performed were counted and registered) for the three dominance tests performed (1 = "Feeding response", 2 = "Place preference" and 3 = "Group").

| Behaviours, parameters and Index | Acronym | Test | Description |
|---|---|---|---|
| Approaches | | 1 | A fish approaches another fish without making physical contact. |
| Swimming above another | SAA | 1 | A fish swims near and above another fish. |
| Rest the head | RTH | 1 | A fish rests the head on another fish. This behaviour is performed resting the head on different parts of the body. |
| Displacement | | 1 | A fish displaces another fish making contact, for example, swimming directly towards the another fish to make direct contact. |
| Burying | | 1 | A fish makes a wave type movement of the body and lateral fins starting from the head to the tail that in substrate would bury the animal. This behaviour has been associated with fear or escape, burying to rest and to reject other fish. |
| Feeding | | 1 | A fish eats the pellets provided registered as "Yes" or "No". |
| Initial time or latency | Ti | 2 | The total time that each fish remains in the preferred area (sand) during the first 2 hours (minutes) of the experiment. |
| Total time | TT | 2 | The total time of each animal remains in the sand during the 24 hours test (minutes) |
| Final time | Tf | 2 | The total time that each fish remains in the preferred area (sand) during the last 2 hours (minutes) of the test. |
| Order position | | 2 | Order that the fish were observed in the preferred sand area at the beginning and end of the experiment, "First" was the animal which entered the sand first. "last" was the animal in the sand when the the test finished after 24 hours. The same fish could have both positions. |
| Feeding order | | 3 | Order which fish ate in the group test. Fish that ate first was 1, second fish to eat was 2 etc. |
| Position "before feeding" | POSITB | 3 | Ranked position of the fish (1-6) in relation to the feed delivery point 5 minutes before feed was delivered. Fish closest to the feeding point was ranked 1, second closest was ranked 2 etc. |
| Position "after feeding" | POSITA | 3 | Ranked position of the fish (1-6) in relation to the feed delivery point 5 minutes after feed was delivered. Fish closest to the feeding point was ranked 1, second closest was ranked 2 etc. |
| Rest the head index | RTH Index | 3 | The number of times that a fish rests the head on another fish minus the number of times other fish rested the head on the fish under consideration. |
| Swimming above another index | SAA Index | 3 | The number of times that a fish swims closely above another fish minus the number of times other fish swam above the fish under consideration. |
| Position "before feeding" index | POSITB Index | 3 | Mean position of each fish before feed was delivered over the 4 days of the group test, Index = (*position on day 1*) + (*position on day 2*) + (*position on day 3*) + (*position on day 4*))/4. See POSITB for definition of position. |
| Position "after feeding" index | POSITA Index | 3 | Mean position of each fish after feed was delivered over the 4 days of the group test, Index = (*position on day 1*) + (*position on day 2*) + (*position on day 3*) + (*position on day 4*))/4. See POSITA for definition of position. |
| Feeding index | | 3 | The mean of the feeding order registered each of the 4 days for each individual. |

*RNA isolation, Complementary DNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction Assay*

Thirty of the seventy-four early juveniles were randomly chosen using randomise numbers associated to fish, fifteen fish from each category (dominant/subordinate) and were sacrificed with an overdose of MS222 (tricaine methanesulfonate; Acros-Organic, New Jersey, USA). Whole brains were extracted, frozen in dry ice and stored at -80 ºC for molecular analysis. The RNA was extracted using TRI Reagent RNA Isolation Reagent following manufacturer's instructions (SigmaAldrich). The cDNA was synthesised using 1 µg of total RNA and oligo dT (20) in 20 µl reactions and the SuperScript® III First-Strand Synthesis SuperMix 50 rxn kit following the manufacturer's protocol (Invitrogen, Life technologies, USA). Primers were designed using Primer 3 (Rozen and Skaletsky, 2000) in 3UTR region. Before performing the qPCR, primers were validated by conventional PCR using a cDNA pool from several samples randomly chosen to analyse the primers. A MyTaq$^{TM}$ HS Mix (Bioline) was used to run the conventional PCR with the following conditions: initial activation step at 98 °C for 1 min, followed by 35 cycles: denaturation at 95 °C for 10s, annealing at Tm (58 - 60 °C) gradient conditions for 15s and extension at 72 °C for 15s. Primer efficiency was evaluated by serial dilutions to ensure that it was close to 100 % performing *real time* PCR. Target genes involved in neuroplasticity, neurogenesis and brain activation (*bdnf*, *c-fos* and *nrd2*), dopaminergic responses (*nr4a2* and *etv5)* and aggression (*5-HT*, *tph1b*, *avpl* and *slc6a13*, *MR*) were analysed by quantitative PCR (*q*PCR) (see primer design in Table 2). The *q*PCR was run using a Biometra Optical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of Luminaris Color HiGreen *q*PCR Master Mix (Thermo Scientific), 1 µl of the primer corresponding to the analysed gene (10 pmol), 3 µl of RNA/DNA water free and 5 µl of cDNA in its corresponding dilution. Furthermore, amplifications were carried out with a systematic negative control (NTC; no template control) containing no cDNA. Standard amplification conditions contained an UDG pre-treatment at 50 °C for 2 min, an initial activation step at 95 °C for 10 min, followed by 35 cycles: 15s at 95 °C, 30s at the annealing Tm and 30s at 72 °C. Results were normalised using three housekeeping genes ubiquitin (*ubi52*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh2*) and elongase factor 1 alpha (*eef1a*) and applying a geometric average (Vandesompele et al., 2002). Relative mRNA expression for each gene was determined using the Pfaffl (2001) method.

**Table 2**. Primers used in this study as possible dominance biomarkers. Gene, gene name, size accession number (SoleaDBv4.1) and primer sequence are indicated.

| Gene | Gene name | Amplicón size | Acc. Number | Primers (5' 3') |
|---|---|---|---|---|
| c-FOS | *c-fos* | 175 | unigene4094 | F- CTGGAGTTCATTCTGGCTGC<br>R- TTGAGGTGAATGTTGGCTGC |
| Brain-derived neurotrophic factor | *BDNF* | 154 | unigene54354 | F- ACTCGTTTGAAACATCCGGC<br>R- CAGACAGGGTGAGTGGAGAA |
| Neurogenic differentation factor 2 | *nrd2* | 396 | unigene1444 | F- TTATCAGTGTGCGCGTCTGT<br>R- TTCAGTTCGTCGTACACGGG |
| ETS translocation variant 5 | *etv5* | 165 | unigene42532 | F- CACTCTGATGCCAACGTTCA<br>R- CAGCGACAAGAACACGGAG |
| Nuclear Receptor Subfamily 4, Group A, Member 2 | *nr4a2* | 187 | unigene55326 | F- TCTCCCGAGTTTCAGCACTT<br>R- CCCAGAGTGAGCCATCATTT |
| 5-hydroxytryptamine receptor 1A | *5-HT* | 180 | Unigene35339 | F- GCTGGCTGCCCTTTTTCATC<br>R- CCGCATGTGGTTATTGCCTG |
| Arginine Vasopressin-induced protein 1 | *avplr1* | 153 | Unigene17371 | F- TGTTGTCGACCACTCACTCA<br>R- TGAAAGGTTGTGCGTGTCTG |
| Tryptophan hydroxylase 1 | *tph1* | 218 | Unigene62116 | F- GGAAGCTGCGAGCATATGGA<br>R- GAAGGGACGCTTGATGTTCT |
| Solute carrier family 6 member 13 | *slc6a13* | 166 | Unigene3332 | F- GTTAACTGCCTGTCCCGTCA<br>R- ACCGTGTAGTGTGAACGAGG |
| Mineralcorticoid receptor | *MR* | 204 | Unigene4626 | F- GCACTCCACATGCACTCAAA<br>R- CCTTTGCCCTGTAGTCTTGC |

*Statistical analysis*

Behaviour

All means are presented as mean ± standard error (SEM) and $P < 0.05$ was used to establish significant differences. Statistical analyses were performed using SPSS Statistics 19.0 software (IBM Co., Hong Kong) and GraphPad Prism 6 software (GraphPad Software, Inc.).

In the case of paired tests (early and late juveniles), the coefficient of variation (CV % = SD/mean*100) that represents the inter-individual sole variability were calculated for each category (dominant and subordinate) and compared. Data was tested for normality by means of Shapiro-Wilks test. If the data were not normal a $Log_{10}$ transformation was applied. To reduce variables and define behaviours that best represented dominance, a Principal Component Analysis (PCA) with adequacy of Kaiser-Meyer-Olkin test and Bartlett's test of spherity and Varimax rotation was applied. In the case of early juveniles, a Spearman's correlation analysis was run for the feeding response test between the variable "Feeding" (if the animals ate or not) and those variables which were representative in the PCA run for this test. The reason for this correlations analysis was to see the relationship between the representative variables and the fact that the animals eat or not. Afterwards, Student's t-test was performed to compare mean behavioural counts between dominant and subordinate fish.

For the group test Kendall's concordance coefficient (0.43 fair concordance) was calculated for each behaviour (RTH, SAA, POSITA and POSITB) to check the concordance among the 4 days for the fish in each group. Afterwards, K means cluster was applied with those variables chosen by Kendall's concordance coefficient for all groups. As there was concordance in the behaviours over the 4 days, different Indexes were calculated (*see* Table 1) for the variables: "Rest the head" and "Swimming above another" POSITB and POSITA. Thus, Student's t-test was applied to check the differences between dominants and subordinates ($P < 0.05$ level of significance).

*q*- rtPCR

The outliers of the different categories (dominant/subordinate) of the corrected ratio of every mRNA were extracted using the Tukey's test formula ($k = 1.5$). The data was transformed to $Log_{10}$ ($var + 1$) and Student's t-test was applied to observed if the mRNA was differently expressed between dominant and subordinate individuals. The threshold was considered at 0.3 related to a pooled control animals simulating population, where values under that threshold indicated down-regulation.

## Results

*Senegalese sole behavioural responses in dominance tests*

Senegalese sole were classified as dominant or subordinate according to their feeding response (in dyadic and group feeding tests), the first fish to feed were classified as dominant and fish that fed second or did not feed at all were classified as

subordinate. The Place Preference Test (PPT), used the same pairs that were previously classified as dominant and subordinate in the dyadic feeding test. The occupation of the preferred sand area by the dominant and subordinate sole, as defined in the feeding test, was examined. The two groups of dominant or subordinate individuals presented different behavioural interactions.

In general, subordinate individuals showed more variability in responses than dominant fish indicative of more inter-individual variation. Dominant early juveniles ($n$ = 36 of 74) displayed less variability within the behaviours (Approaches = 9.6 ± 1.4 counts; CV = 86.5 %; SAA = 5.8 ± 1.2 counts; CV = 119.1%; RTH = 4.5 ± 0.6 counts; CV = 76.5 %; Burying = 4.4 ± 0.5 counts; CV = 65.8 %; Displacement = 1.0 ± 0.2 counts; CV = 150.3 %) than subordinates ($n$ = 38 of 74) (Approaches = 5.5 ± 1.0 counts; CV = 109.2 %; SAA = 2.5 ± 0.6 counts; CV = 141.1 %; RTH = 2.6 ± 0.5 counts; CV = 105.4 %; Burying = 4.6 ± 0.5 counts; CV = 66.0 %; Displacement = 0.5 ± 0.2 counts; CV = 187.6 %). Whilst, the two behaviours, "Burying" and "Displacement", presented a similar variability for both dominant and subordinate fish. The trend in late juveniles was similar to that observed in early juveniles, with similar levels of variation in the behaviours and dominant late juveniles ($n$ = 17 of 34) also displayed less variability (Approaches = 18.9 ± 2.6 counts; CV = 56.4 %; SAA = 27.3 ± 9.5 counts; CV = 144.3 %; RTH = 9.1 ± 1.4 counts; CV = 65.9 %) than subordinates ($n$ = 17 of 34) (Approaches = 12.1 ± 2.5 counts; CV = 83.8 %; SAA = 12.3 ± 3.6 counts; CV = 120.3 %; RTH = 4.1 ± 0.9 counts; CV = 91.5 %). In the place preference test, dominant late juveniles spent more time in the preferred sand area at the end of the test and had less variability (Initial time (Ti) = 25.5 ± 9.2 min; CV = 149.6 %; Total time (TT) = 377.1 ± 71.1 min; CV = 77.7 %; Final time (Tf) = 60.7 ± 11.4 min; CV = 77.8 %) than subordinates (Ti = 33.6 ± 11.7 min; CV = 144.2 %; TT = 302.9 ± 93.9 min; CV = 127.8 %; Tf = 38.4 ± 11.1 min; CV = 119.6 %).

*Dominance parameters selection*

In early juveniles, three behaviours, "Approaches", "Swimming above another" (SAA) and "Rest the head" (RTH) (Table 1) were grouped together (PCA, KMO (0.667), Bartlett's test ($P < 0.001$) and $X^2$ (133.523); Fig. 4) and formed a principal component that represented 51% of the variance of the data.

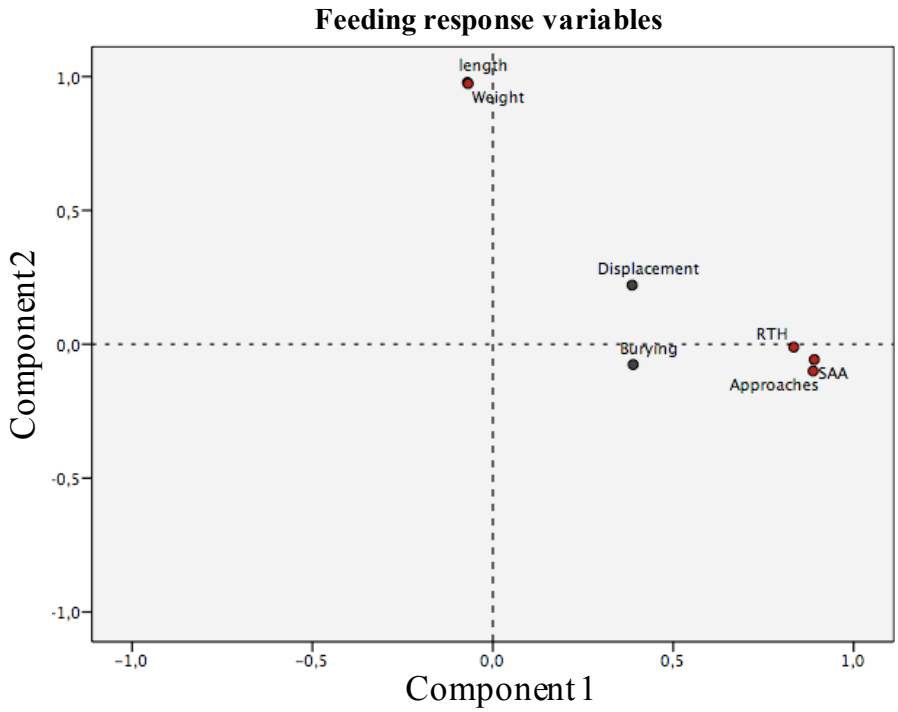


**Figure 4.** Principal Component Analysis of the different behaviours registered during the "Feeding response test" in pairs. The three variables "Approaches, SAA and RTH" were grouped together and explained the 53% of the variance of the data. KMO (0.667), Bartlett's test ($P < 0.001$) and $X^2$ (133.523) (SPSS 19.0 IBM Statistics).

These three behaviours were weakly correlated to feeding response (Approaches: $r_s$= 0.372, $P$ = 0.001; RTH: $r_s$=0.358, $P < 0.005$; SAA: $r_s$= 0.4, $P < 0.001$; Fig. 5) or to dominate and subordinate animals, when the animals were classified as dominant (animals that fed first) or subordinate (animals that fed second).

**Correlations**

| | | | Feeding |
|---|---|---|---|
| Rho de Spearman | Approaches | Coeficiente de correlación | ,372** |
| | | Sig. (bilateral) | ,001 |
| | | N | 74 |
| | RTH | Coeficiente de correlación | ,358** |
| | | Sig. (bilateral) | ,002 |
| | | N | 74 |
| | LogSAA | Coeficiente de correlación | ,400** |
| | | Sig. (bilateral) | ,000 |
| | | N | 74 |
| | Feeding | Coeficiente de correlación | 1,000 |
| | | Sig. (bilateral) | . |
| | | N | 74 |

**Figure 5.** Spearman's correlations from the three variables "Approaches, SAA and RTH" with Feeding. (**) correlation was significant $P < 0.001$ (2-tailed) (SPSS 19.0 IBM Statistics).

Consequently, the three behaviours were significantly different between dominant and subordinates (Approaches: $t = 2.675$, $df = 72$, $P < 0.01$; RTH: $t = 2.814$, $df = 72$, $P < 0.01$; SAA: $t = 2.877$, $df = 72$, $P < 0.01$; Fig. 6A). Dominant sole displayed significantly higher more approaches, resting the head and swimming above another than subordinate sole.

The association between behaviours and dominance was similar for the late-juveniles. The PCA of all the variables from the feeding response and place preference tests that were applied to late juveniles extracted three components that explained 72% of the variance of the data (KMO (0.6), Bartlett's test ($P < 0.001$) and $X^2$ (116.806); Fig. 7). The three behaviours feeding response, Approaches, RTH and SAA were grouped together as the first principal component (PC1). These three behaviours were also grouped together for the early juvenile sole establishing that the feeding response test applied to two different groups of sole of different ages and size gave similar consistent results. The second (PC2) and third components (PC3) were both related to behaviours from the place preference test. The PC2 was formed by the variables; total time each fish occupied the preferred sand area during the last two hours of the test (Final time (Tf)) and which fish was in the sand when the test finished at 24 hours (last position). PC3 consisted of; the time each fish first remained in the sand during the first two hours (Initial time (Ti)), total time each fish was in the sand during the entire 24-hour test (Total time (TT)) and which fish was first to enter the sand area (first position (First)) (*see* Table 1).

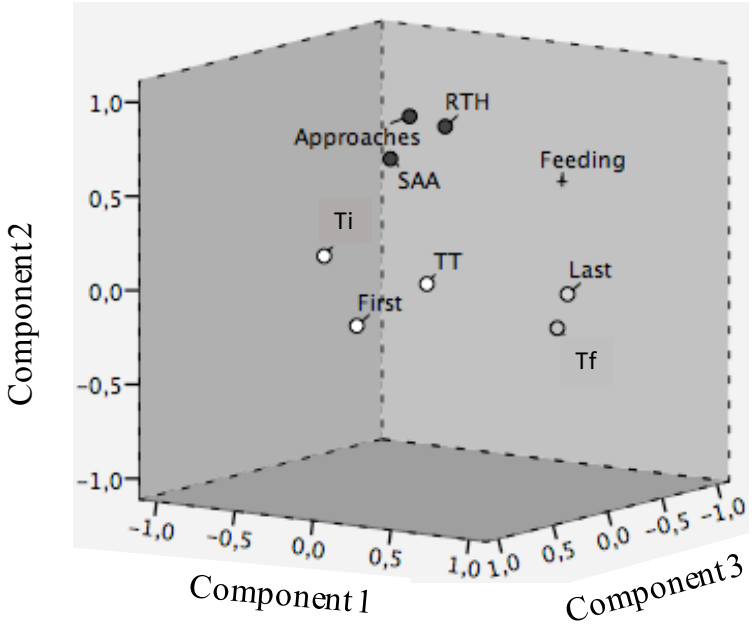


**Figure 7.** Principal Component Analysis of the different behaviours registered during the "Feeding dominance test" and "Place Preference test" (sand) in pairs for late juveniles. The three variables "Approaches, SAA and RTH" and the "TF and last" explained the 56 % of the variance of the data in two different components. KMO (0.6), Bartlett's test ($P < 0.001$) and $X^2$ (116.806) (SPSS 19.0 IBM Statistics).

The behaviours Approaches ($t = 2.036$, $df = 31.69$, $P = 0.05$) and RTH ($t = 2.894$, $df = 26.30$, $P = 0.008$) were significantly higher in dominants than subordinates

in late juveniles. However, SAA ($t$ = 1.083, $df$ = 30.94, $P$ = 0.2874) was not different between dominants and subordinates (Fig. 6B).

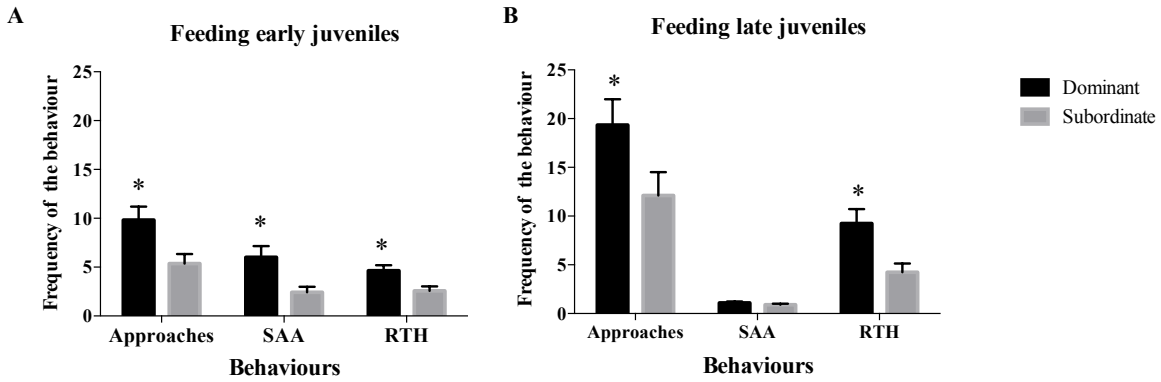


**Figure 6.** Frequency (number of movements were counted during the 2-hour test) that dominant and subordinate **A** early juveniles and **B** late juveniles Senegalese sole exhibited the three behaviours Approaches, Swimming above another (SAA) and Rest the head (RTH). Data was shown in Mean ± SEM. An * indicates a significant difference ($P$ < 0.05) between dominant and subordinate for the behaviour.

The variables which formed the second component were significantly different between dominant and subordinate fish classified by feeding response test, thus sole classified as dominant (in the feeding test) spent more time in the preferred sandy area during the last two hours of the test (Tf: $t$ = 2.186, $df$ = 16, $P$ = 0.044; Fig. 8A) and dominated the area when the test finished after 24 hours ($X^2$ = 5.674, $P$ = 0.017; Fig. 8B).

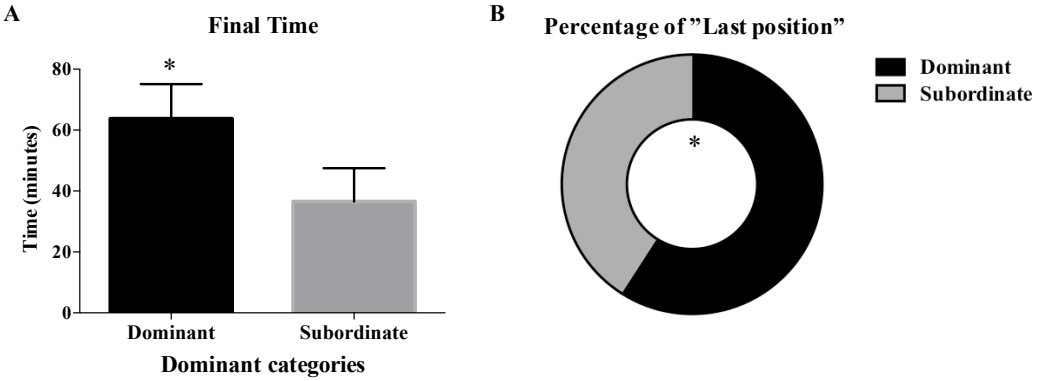


**Figure 8.** Place preference test in late juvenile sole. **A** Time in minutes during the last two hours of the test that dominant and subordinate Senegalese sole were in the preferred sand area (Tf). **B** The proportion of dominant and subordinate fish that were in the preferred sand area when the test finished (last). An * indicates a significant difference ($P$ < 0.05) between dominant and subordinate fish for the behaviour.

In summary, the dominant fish that ate first spent more time in the preferred area over the last two hours of the test and monopolised the sand, showing that the final position is a better indication of dominant status. All results were analysed from size-matched pairs and, therefore, variability due to unequal size distribution was avoided. Thus, dominance parameters were observed in each pair representing pairs of similar weight and length.

In the group analysis, rest the head index (RTH Index), swimming above another index (SAA Index), position of the fish in relation to the feed delivery tube before (POSITB) feed was delivered and Feeding Order (which fish fed 1st, 2nd...6th) (Table 1) showed agreement (0.43) according to Kendall's concordance coefficient (W) among the 4 days for each group (Table 3). The animals in the different groups were analysed as a single population and the Kendall's concordance coefficient demonstrated that the inter-groups Index were consistent. In addition, k means cluster classified the animals in two clusters that represented dominant and subordinate animals.

**Table 3.** Classification of the different variables in groups according to Kendall's concordance coefficient (W) for every group. ((*) level of significance).

| Group | Index | Kendall's concordance coefficient (W) | p-value |
|---|---|---|---|
| 1 | RTH | 0.6415* | P < 0.01 |
| | SAA | 0.4303* | P < 0.05 |
| | POSIT B | 0.4300* | P < 0.05 |
| | POSIT A | 0.2799 | P > 0.05 |
| | Feeding Order | 0.5214* | P < 0.05 |
| 2 | RTH | 0.4305* | P < 0.05 |
| | SAA | 0.4389* | P < 0.05 |
| | POSIT B | 0.4456* | P < 0.05 |
| | POSIT A | 0.3558 | P > 0.05 |
| | Feeding Order | 0.4643* | P < 0.05 |
| 3 | RTH | 0.4855* | P < 0.05 |
| | SAA | 0.4873* | P < 0.05 |
| | POSIT B | 0.4356* | P < 0.05 |
| | POSIT A | 0.3507 | P > 0.05 |
| | Feeding Order | 0.5643* | P < 0.05 |
| 4 | RTH | 0.5342* | P < 0.05 |
| | SAA | 0.4816* | P < 0.05 |
| | POSIT B | 0.5349* | P < 0.05 |
| | POSIT A | 0.0789 | P > 0.05 |
| | Feeding Order | 0.4714* | P < 0.05 |

The two clusters grouped the same animals according to feeding RTH, SAA and POSITB Index indicating concordance of these behaviours in the different groups. Therefore, 12 fish were classified as dominant and 12 as subordinates. Student's t-test applied in those index to compare the significant differences between dominants and subordinates for different behaviours showed that RTH Index ($t = 2.659$, $df = 10.46$, $P < 0.05$; Fig. 9A) and POSITB Index ($t = 4.873$, $df = 21.54$, $P < 0.001$; Fig. 9C) were significantly different between dominant and subordinate groups. However, the SAA Index ($t = 1.231$, $df = 19.35$, $P = 0.2330$; Fig. 9B) was not different between dominant and subordinate fish. To summarize our findings, the fish considered dominant more often occupied positions closer to the feed delivery tube even before the food was provided (Place preference test was covered with those positions) and rested the head (RTH) on other fish more often than the subordinate fish. The group analysis was performed with the four groups as a population consisting of individuals of different sizes (simulating the cohabitation in nature). However, no relationship was established between fish size and the repetition of the different dominance parameters observed. For

example, in one of the groups, the weight range was from 195.7 to 310.6 g and the two smallest animals presented more actions in RTH and POSITB and presented more consistent in their data than the largest individual throughout the experimental period.

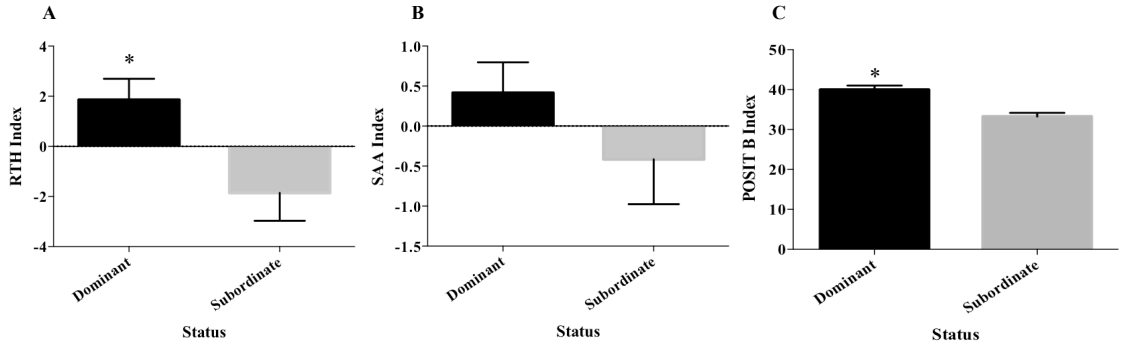


**Figure 9.** Mean behavioural indices for dominant and subordinate late juveniles sole in the Group test for the variables selected by the Kendall's Concordance Coefficient: **A** Rest the head Index (RTH Index). **B** Swimming above Index (SAA Index). **C** Position before feeding (POSIT Index). An * indicates significant difference ($P < 0.05$) between dominant and subordinate fish for the behavioural index.

*Behaviour observed*

The feeding behaviour of the Senegalese sole was similar to that observed for other flatfish species with a defined "predation cycle": searching, encountering, capturing and ingesting the food or modal action patterns (MAP) (Campbell, 1996; Gibson, 2005). In the present study, Senegalese sole showed MAP associated with feeding behaviour at the moment food was introduced into the experimental tank. Therefore, the sole considered as dominant interacted first with the food and blocked other interactions. Furthermore, individuals that ate and were considered as dominant sole, interacted with the food in the first 10 minutes of food delivery starting with a "searching" MAP that consisted in slow creeping movements over the bottom, moving the head from side to side in short actions while slowly approaching the food. At the moment an individual detected the smell of food the fish orientated and swam rapidly straight to it and started to defend the food and the area in which the food was delivered (*direct observation*).

*Brain gene expression in relation to dominance*

Only two of the ten (Table 2) genes tested exhibited significant differences in gene expression between dominant and subordinate sole. Both genes, *c-fos* ($t = 2.014$, $df = 20.13$, $P < 0.05$; Fig. 10A) and *nrd2* ($t = 1.861$, $df = 27.15$, $P < 0.05$; Fig. 10B), were down regulated in both dominant and subordinate fish in relation to pooled controls that represent the entire range for the population. This down-regulation was more pronounced in dominant sole than in subordinate individuals. The *nr4a2* mRNA presented marginally different expression levels ($t = 1.987$, $df = 22.56$, $P = 0.0592$).

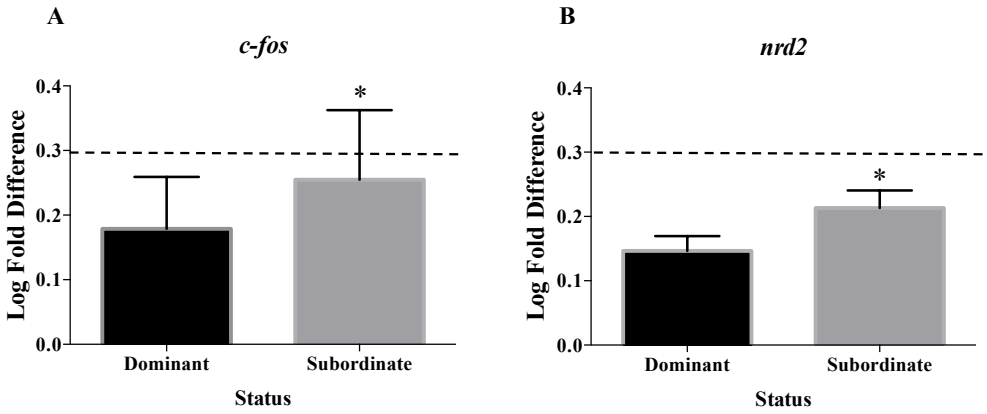


**Figure 10.** Gene expression of dominant and subordinate early juvenile sole. Expression for two genes **A** *c-fos* and **B** *nrd2* shown as mean ± SEM in Log (*var*+1) transformation. Dashed line at 0.3 indicates the value of a pooled control simulating the population, values under 0.3 indicate down-regulation. An * denotes a significant difference ($P < 0.05$) between dominant and subordinate fish for the gene expression.

## Discussion

*Feeding response test*

The present study has shown that Senegalese sole can be categorized for different social status (dominant and subordinate) when: (a) observed in pairs and groups, (b) in relation to both feeding response and place preference, and (c) at different juvenile stages, from early juvenile, when fish weight is approximately 100 g, to late juvenile when it is almost at an adult stage, before puberty (at approximately 300 g). Previous studies in rainbow trout demonstrated that after a period of isolation the fish which ate first won the subsequent contest, showed more aggressive behaviour and were subsequently categorized as dominant individuals (Øverli et al., 2004a). This is in agreement with the present study where both animals were isolated before starting the behavioural test and the sole that ate first was also the dominant individual in place preference test. Aggression in feeding behaviour is the most common method used to establish hierarchies in different species with dominant fish often being the larger individuals (MacLean et al., 2000). Salas-Leiton et al. (2008) demonstrated that juvenile Senegalese sole did not show any aggressive behaviour at different stocking densities when fed according to the biomass. Furthermore, Salas-Leiton et al. (2010) suggested that fish size distribution and variation in captivity could be the consequence of hierarchical structure. However, in the present study the repetition of dominance parameters specifically “Rest the head” (RTH), “Swimming above another” (SAA) and “Approaches”, did not identify larger body size as a factor in dominant fish. Although, weight differences were small between pairs, the weight varied among animals in groups, where the social rank was stable during the experimental period and those behaviours were correlated with social status. The different behaviours used during this experimental period were described before by Carazo et al. (2016) in an ethogram for wild Senegalese sole breeders observed in captivity. Rest the head (RTH) could occur in any part of the body (head, back, tail and whole fish), in addition this behaviour is

displayed in the complex courtship behaviour (spawning behaviour) performed for this species (Carazo et al., 2016). Similar behaviours where the head was repeatedly touched during courtship has been observed in other flatfish species such as the wide-eyed flounder (*Bothus podas*) (Carvalho et al., 2003) and the largescale flounder (*Engyprosopon grandisquama*) (Manabe et al., 2000) among others. RTH was discerned to have an important implication in the spawning behaviour together with "Approaches" (without physical contact), where males have to approach the females to perform one part of the courtship. Apart from spawning behaviour, "Approaches" have been associated with feeding behaviour in flatfish (*discussed below*) which could be displayed differently depending on the type of prey (Gibson, 2005). On the other hand, swimming behaviours (SAA) lose relevance from early to late juvenile stages. This could be due to the change in the level of activity during the developmental stages in swimming behaviour, where the first larval stages are pelagic and when they move to benthic life where swimming activity is significantly reduced. Furthermore, previous studies have shown that benthic larvae of Senegalese sole have more activity than juveniles, and that juveniles present more activity than adult sole under certain conditions (Ibarra-Zatarain, 2015). Moreover, individuals considered as dominant fish in group analyses (which ate in the first positions) were observed to more frequently rest the head (RTH) on other animals compared to subordinates and in turn subordinate animals received more RTH from dominant animals. Thus, RTH was associated with dominance in all tests and the RTH behaviour appeared to be a strong indicator of dominance both in paired and grouped tests.

The feeding behaviour observed during the test was similar to that described in the common sole (*Solea solea*) and other species such as turbot where visual and olfactory cues are important for searching for feed (Holmes and Gibson, 1983; 1986; Livingston, 1987). Performance of the different parts of the predation cycle (search, encounter, capture and ingestion) can vary between the species. In *Solea solea* feeding behaviour and boldness, associated in some fish species with aggression and social status such as in cichlid fish (Clement et al., 2005), were related to feed intake using isolation and group tests (Mas-Munoz et al., 2011) explaining variability in growth where proactive fish appear to have better feeding tactics in captivity. Feeding order in fish could be similar to the 'pecking order' found in other farmed species (poultry, pigs, cows, etc.), pecking order is established at a younger age, and remains stable during life determining the social status and hierarchies. In addition, pecking order has been associated with different activities such as feeding, drinking, and mating (Guhl, 1956; 1968). This could be the explanation for the consistent feeding behaviour in groups among the four days, considering that the individual behaviour can affect the variability of the group in captivity.

*Place preference test*

Place preference test consisted of two areas as choice possibilities, where the animal associates one of those areas as preferred for a particular purpose (hide, shelter, mating, among others) (Mattioli et al., 1998; Coelho et al., 2001). In our study, there

was a clear preference for the area with substrate by all experimental sole. However, the objective of this test was to observe the behaviour of paired Senegalese sole in response to a preferred area (sand) simulating a limited resource. Our experimental design incorporated an acclimation period prior the dominance test in order to avoid the effect of novelty. Preference by dominant sole regarding territory was identified taking the different times of every individual dominating the substrate. Therefore, dominant sole spent more time at the end of the test (last two hours of test) and were more often present in the sand at the end of the 24-hour test (last position). As a consequence, subordinate fish spent less time at the end of the test and entered the sand earlier in the test or did not enter at all. This was in agreement with other fish species with different biology and ecology such as Mozambique tilapia (*Oreochromis mossambicus*) where substrate is important in different contexts (Galhardo et al., 2009). Another example of the use of substrate is the Nile tilapia (*Oreochromis niloticus*), where animals were isolated and individuals could choose different compartments where the gravel-enriched compartment was the most visited (Delicio et al., 2006). The movement performed by sole when in contact with the sand was burying. This behaviour was described by Carazo et al., 2016 for Senegalese sole as a fear/escape action and in some cases performed to displace another sole (when the burying is displayed under or on top of another sole). Kruuk (1963) described the same behaviour for common sole having several functions, such as burying to help mimic the sediment to initiate a resting period and to avoid currents. Camouflage enables flatfish to avoid predators and remain hidden to prey. The conduct consisted in a continuous movement of the head against the sediment with the whole body and lateral fins making waves, so the sediment wafts and falls back onto the fish body surface which was described by Kruuk (1963) for common sole and Gibson (2005) for flatfish in general. However, this behaviour was not extracted as a representative parameter in "feeding response test" in early juveniles. Previous studies demonstrated that habitat preference increased the level of territorial protection or dominance displayed by brown trout (*Salmo trutta*) (Johnsson et al., 2000). In contests dominant animals defended and displayed more aggression in order to dominate their preferred territory. In the present study, when both sole coincided in the sand the sole in the upper position normally the dominant individual rested the head (whatever part of the body) on the sole in the bottom position. This behaviour would not be considered as an aggression as the other animal was not injured, but it could be a harassment tactic. So, the rest the head (RTH) parameter extracted in the feeding response test was also associated with RTH behaviour in the place preference test. However, the situation of both animals coinciding in the sand area was not usually observed in fact only in two of the seventeen pairs studied. In the case of the group test the place preference test was analysed according to the position regarding the feeder supplier area. Intriguingly, the POSITB Index corroborated that animals which dominated that area were the animals that ate in the first positions, being stable during the experimental period. This situation is commonly observed in cultivated fish due to domestication, which depending on the fish species is performed in a different manner achieving different tactics according to the food delivery (Huntingford, 2004).

*Linking behaviour and gene expression*

The brain gene expression obtained in this study to our knowledge is the first observation that transcriptional activity could achieve different relationship to dominance categories of a complex behaviour in Senegalese sole. Several sets of genes related to different behavioural processes were analysed in the present study. However, just two mRNA transcripts related to neurogenesis (*nrd2*) and neuroplasticity (*c-fos*) were differently expressed between dominant and subordinate sole and one dopaminergic gene (*nr4a2*) was marginally different. These three transcripts presented down-regulation in both categories (dominant and subordinate) compared to control group (*see* Fig. 10 for *nrd2* and *c-fos*). In some cases, the presence of down-regulation in some genes in both dominance categories, dominant and subordinate, results in the adaptive response to slight social stress in comparison to the undisturbed animals (Berton et al., 1999). This could be the explanation of why in our study animals of both dominance categories showed down-regulation, therefore, dominant and subordinate sole would present the same pattern of social stress being more accused in dominant sole.

In several animal models for social studies such as rodents, social subordination has been associated to reduced neurogenesis (Blanchard et al., 2001). The mRNA transcript related to neurogenesis was *nrd2* that is essential for the survival of specific populations of neurons and neuronal differentiation in mice (Olson et al., 2001). Previous studies in dyadic rainbow trout contests, performed to analyse dominance, demonstrated that subordinate fish were persistently stressed and exhibited reduced forebrain cell production. Furthermore, in our study social interaction reduced cell proliferation in both categories whereas in subordinate trout the down-regulation was more pronounced (Sorensen et al. 2007). Neuroplasticity has also been associated with social interactions in fish where the profile of immediate early genes (IEG), including *c-fos*, in zebrafish exhibited acute changes in the pattern of expression due to different social status (Teles et al., 2015; Teles et al., 2016). Another example is found in tilapia (*Astatotilapia burtoni*); males of this species have the ability to switch between subordinate and dominant (when the dominant male is removed) and *vice versa* (when a larger dominant male is introduced in the tank) in the same territory, this behavioural trait can be also observed at molecular level when the IEG *c-fos* increased in all cells implicated in the social network for ascending males, presenting adaptive phenotypic changes depending on the situation (Cardoso et al., 2015). In the present study, the expression pattern was distinct where dominant and subordinate sole presented lower *c-fos* mRNA expression than a representative population pool corroborating that neuroplasticity was also associated with social behaviour in this species. Dopaminergic activity (*nr4a2*) was lower in both categories (dominant and subordinate) in comparison with control group. This result is in agreement with a previous study where fish were subjected to two different stressors and dopaminergic activity was lower than control fish (non-stressed sole) (Weber et al., 2015). Furthermore, in the same study levels of serotonin were similar in all fish tested in comparison to control fish. In our study, serotonergic activity (*5-HT*) jointly with other aggression-related genes such as *tph1b*,

*avpl* and *slc6a13*, *MR* were not differentially expressed between dominant and subordinate sole as reported in other studies (Filby et al., 2012; Teles et al., 2013). However, as previously mentioned Senegalese sole is considered a non-aggressive species, so may be possible that the correlation between aggression and social interactions might not be present in this species. In addition, these genes are implicated in the stress response to contests or social stress where many salmonid species increase the stress-related biomarkers (high plasma levels of cortisol and agonistic behaviour) and in consequence dopaminergic and serotonergic activity in the brain (Øverli et al., 1999). However, these results (no differences in serotonergic activity and low dopaminergic activity) suggest the social interactions in contests might be assimilated as low-intensity stress in Senegalese sole.

In conclusion, this is the first study using dyadic contests in a flatfish species to determine social status (dominant/subordinate) when resources are limited. This study reports upon individual differences in dominance behaviour in Senegalese sole for the first time. This flatfish is considered as a non-aggressive species, according to different dominance parameters related to feeding response and territory. Those dominance behaviours were consistent in the different juvenile stages (early and late juveniles) and additionally, those parameters were reliable when applied to sole in groups. Both dominant and subordinate Senegalese sole juvenile exhibited specific and significantly different expression of two brain mRNAs that are associated to neurogenesis and neuroplasticity. These results are highly relevant for the fish farming industry of Senegalese sole in order to improve culture conditions by understanding the behavioural profile of these animals. This essential knowledge of hierarchical distribution in the population will be linked to methods to ensure future reproductive success.

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# Chapter 7

# Linking stress coping styles with brain gene expression in Senegalese sole (*Solea senegalensis*)

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*Key Words:* Flatfish
Intermediate category
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Proactive
Reactive

## Abstract

The aim of the present study was to link stress coping styles (SCS) with brain gene expression in Senegalese sole (*Solea senegalensis*) juveniles and observe the relationship between SCS and dominance behaviour in this species. Senegalese sole is a marine flatfish species with high importance in the industry of aquaculture. However, improvements are needed and variation in behavioural traits such as stress coping styles have been linked to variation in performance in the aquaculture environment. A total of 50 juveniles were subjected to three individual behavioural tests (Restraining, New environment and Confinement) and one group-test (Risk-taking). Moreover, 30 fish of those 50 were previously (two weeks prior) subjected to a dyadic dominance behaviour test related to feeding response, which classified 14 fish as dominant and 16 as subordinate. The fish were classified in three SCS categories applying a hierarchical cluster according to the variable "Total activity" which was measuring the total activity time the fish was active in each test. The fish were classified as 10 proactive, 10 intermediate and 30 reactive individual sole. The expression of six mRNA related to metabolism (*gapdh-2*), feeding behaviour (*per1*, *igf-Ia*, *pparß*) and stress response (*crh-BP* and *hsp90aa*) were measured in 30 juveniles (10 samples per SCS category) using *q*PCR to observe differences in brain gene expression among SCS categories. Four genes were differentially expressed among SCS categories, *gapdh-2*, showed up-regulation in proactive and intermediate sole while reactive fish showed down-regulation. In the case of the other mRNA, *pparß*, *igf-Ia* and *per1*, proactive and reactive showed up-regulation and intermediate down-regulation. These results all together demonstrated the relationship between the behavioural individual variation and the fluctuation in brain gene expression for the first time in Senegalese sole. Additionally, no apparent relationship between dominance and behavioural traits was observed in Senegalese sole juveniles.

## Introduction

Senegalese sole is a marine flatfish species in which the European aquaculture industry has been investing during the last decades due to high market demand for human consumption and high market price (Howell et al., 2011). Despite of the commercial significance there remain some issues in the culture of this species such as variable growth, malformations, pathologies and reproduction (Morais et al., 2014). The study of the different behavioural traits in Senegalese sole may identify individuals with improved performance in culture conditions that could improve aquaculture production.

Individuals exhibit different individual responses or stress coping styles (SCS) when subjected to a stressful or risky situation. When a set of behaviours that show variation across coping styles traits are correlated to show intra-individual consistency and the different behavioural responses also show intra-individual consistency across time and in different context (predation, confinement, environmental variations, among others) (Coppens et al., 2010; Braithwaite et al., 2011), the set of behaviours is considered a behavioural syndrome (Sih et al., 2004; Reale et al., 2007; Wolf and Weissing, 2012). One behavioural syndrome is proactive-reactive stress coping style axis (Koolhaas et al., 1999). Following this axis approach, proactive animals are considered more active, aggressive, which grow faster and have better mating choices, but show lower flexibility to variations in the natural environment than reactive animals (Koolhaas et al., 1999; Sih et al., 2004; Wilson and Godin, 2009; Coppens et al., 2010). On the other hand, reactive individuals present a more flexible adaptive response to changing unpredictable environments and only engaging in risky behaviour when required (Brelin et al., 2005; Koolhaas et al., 2007; Toms et al., 2010; Castanheira et al., 2015; Wolf & Weissing 2012). Moreover, the proactive versus reactive as stress coping styles extremes have been reinforced by the fact that the phenotypical dissimilarity appears to have a genetic basis (Koolhaas et al., 1999). Additionally, proactive animals were proposed to display a fast lifestyle (Reale et al., 2010), due to the positive correlation between proactivity and competitive capacity, therefore the expectance of the positive relationship between proactive profile and social status. Previous studies have demonstrated that dominant animals tend to display proactive traits (David et al., 2011) such as exploration (Verbeek et al., 1996; Favati et al., 2014) and higher activity inspecting a novel object or territory (Colléter and Brown, 2011; Dahlbom et al., 2011).

The study of individual differences in animal behaviour is recognised as an important field in social studies related to ecology and evolution in animals (Morgan and Dall, 2015). Such behavioural studies have been considered an essential tool that may be used to explain individual variation (Reale et al., 2007; Wolf and Weissing, 2012). The knowledge of coping styles in fish has high importance, not only from an evolutionary perspective but also for aquaculture, in order to increase productivity and to aid genetic breeding programs achieve improved survival, growth and enhanced immune responses (Huntingford, 2004). For example, some researchers demonstrated that proactive fish tended to possess a higher immune reaction (MacKenzie et al., 2009; Rey et al., 2016), higher growth rates (Millot et al., 2009), and higher reproductive success (Godin and Dugatkin, 1996), however, showed lower anti-predatory responses

than reactive fish (Huntingford et al., 2010). Physiologically, proactive fish have a lower activity at hypothalamus-pituitary-adrenal/interrenal (HPI) level, which affects the stress response to different stressors, presenting lower post-stress levels of glucocorticoids, which may be broadly classified to affect two major categories, immunological and metabolic response (Koolhaas et al., 2010; Braithwaite et al., 2011). Different SCS classifications (proactive, reactive) related to behavioural and physiological SCS have been defined in a wide range of fish species such as, rainbow trout (*Oncorhynchus mykiss*) (Øverli et al., 2002; Øverli et al., 2006), brown trout (*Salmo trutta*) (Kristiansen, 1999; Brelin et al., 2005), zebrafish (*Danio rerio*) (Rey et al., 2013; Tudorache et al., 2013; Tudorache et al., 2015), African catfish (*Clarias gariepinus*) (Martins et al., 2005; 2006) and include flatfish, halibut (*Hippoglossus hippoglossus*) (Kristiansen et al., 2004; Kristiansen and Fernö, 2007), olive flounder (*Paralichthys olivaceus*) (Rupia et al., 2016) and Senegalese sole (*Solea senegalensis*) (Silva et al., 2010; Ibarra-Zatarain et al., 2015; Ibarra-Zatarain et al., 2016).

Furthermore, all those factors are linked to a genetic basis for the expression of behavioural and physiological components of individual coping categories, proactive and reactive (Driscoll et al., 1998; Øverli et al., 2007; Koolhaas et al., 2010). For example, the scale of the cortisol response to stress is an individual characteristic which is stable over time in rainbow trout, with high degree of heritability (Pottinger and Carrick, 1999). Genetic selection was used to select certain behavioural or physiological features to produce genetic strains of animals that possessed stable characteristics that were useful to practices like aquaculture. However, the transcriptome is dynamic and there exists variations in responses of the transcripome to the same situation, including SCS considered as behavioural traits. This approach has been used to address or provide possible answers to various ecological, evolutionary and environmental questions (Goetz and MacKenzie, 2008). The information of these mRNAs differentially expressed between diverse SCS groups could be used for the interpretation of biological responses to resolve variation, knowing that those variations might be adaptive or genetically fixed within the population (MacKenzie et al., 2009). As commented before, the behavioural features are connected to physiological characteristics and related to this physiological association, recent molecular measurements have been associated with behavioural coping styles in response to fluctuating environmental conditions and after an inflammatory challenge in several fish species (Koolhaas et al., 2007; Øverli et al., 2007; MacKenzie et al., 2009; Huntingford et al., 2010; Rey et al., 2013; Rey et al., 2016).

In the specific case of Senegalese sole, Silva et al. (2010) found differences in feeding behaviour (latency) and cortisol levels in juveniles, where proactive individuals displayed shorter feeding latency and lower undisturbed cortisol levels than reactive sole. This association between coping style and physiological condition was corroborated in Senegalese sole juvenile and breeder stages by Ibarra-Zatarain et al. (2016) who conducted individual and group coping styles tests to categorized the fish in different categories, proactive or reactive and proved the existence of two principal axes "fearfulness-reactivity" and "activity-exploration" in this species. Thus, these tests were indicative of the physiological axis of stress coping style where cortisol production was

significantly lower in proactive fish in both Senegalese sole juvenile and breeder stages. Reactive sole presented higher cortisol concentration in plasma and proactive sole presented significantly lower levels of cortisol. Nevertheless, Ibarra-Zatarain (2015) did not find differences in SCS between breeders that reproduced and that did not reproduce and found that both groups had similar SCS behavioural profiles. However, proactive sole juveniles had higher growth rates and earlier puberty than reactive sole. The control of growth and puberty are interesting characteristics for aquaculture. In this context, coping styles were assessed in a population of early Senegalese sole juveniles and linked with mRNA brain expression of several genes with biological importance (metabolic rate, lipid metabolism, growth, circadian rhythmicity and stress responses) that were identified in Senegalese sole. The physiological, behavioural and molecular characteristics may provide markers that could be used for a selection breeding plan in order to obtain a line with desirable behavioural traits for Senegalese sole in captivity.

## Material and Methods

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

1. *Animal rearing conditions*

Fish used for this experiment were provided by Stolt Sea Farm (Santiago de Compostela, Spain) and were transported from La Coruña to IRTA's facilities in March of 2012. Fish were maintained in the Research Centre facilities of IRTA, in St. Carles de la Ràpita, North East Spain and were held in 10 $m^3$ fiberglass tanks with natural photoperiod (40°62'82.42", 0°66'09.37, using artificial lighting) and controlled natural temperatures (9-20°C) with a recirculation system (IRTAMar®) that recirculated +400% and renewed 10% of the water daily. Sole were fed *ad libitum* five days per week with balanced feed (LE-3mm ELITE, Skretting, Co.). Fifty early juvenile Senegalese sole (121.4 ± 8.1 g) were randomly selected to conduct the behavioural tests in November of 2013. In addition, thirty of those early Senegalese sole juveniles that were randomly chosen had been previously (two weeks prior SCS tests) subjected to a dominance test related to feeding response (**explained in Chapter 6**). The classification of these 30 animals in terms of SCS (proactive and reactive) and dominance (dominant and subordinate) was compared to examine the association between SCS and dominance categories. Animals were moved and acclimated to two 400 L fiberglass tanks a week before tests started. The acclimation tank was connected to a recirculation system (IRTAMar®), described above) to maintain a constant temperature of 15 ºC in order to avoid the environmental influences in the different behaviours among individuals. All fish were PIT (Passive Integrated Transponder) tagged (ID-100A, Unique Trovan-Zeuss; Madrid, Spain) for identification.

2. *Behavioural assays*

The tests applied were selected as appropriate SCS tests by Ibarra-Zatarain et al. (2016) who demonstrated that one risk-taking in groups test and three individual tests (restraining, new environment and confinement) screened Senegalese sole juveniles into two different coping styles (proactive and reactive), and those tests were the most representative to explain the individual variance. In addition, the existence of two behavioural axes "fearfulness-reactivity" and "activity-exploration" were demonstrated and the tests also separated individuals in relation to the physiological axis of stress coping style.

*2.1. In groups testing*

The first test performed was "*Risk taking"* in groups. The objective of this test was to determine the fish willingness to cross from a well-known "safe" area to an unfamiliar area (risky zone). This has been established as a standardised test to screen for SCS in fish and other animals (Smith et al., 1992; van Oers et al., 2004; Huntingford et al., 2010). A 400 L fiberglass tank was divided into two equal zones by a polyvinyl chloride (PVC) wall. The wall had a small window (5 cm high x 20 cm width) at the bottom to allow fish to cross between both areas. The window was at the centre of a PIT (passive integrated transducer) tag reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that read the tag number of the fish which crossed through the window to the unfamiliar zone. (Fig. 1A). The known sheltered area simulated natural conditions for the species, the area was isolated from light (2 lux on the surface) and covered by sand. On the other hand, the risky or unknown area was provided with more light (15 lux (OSRAM DULUX 48W on the surface) and the bottom was lacking substrate. Before beginning the test, the fish were acclimated 24 hours in the well-known sheltered zone keeping the window closed until the beginning of the test. The duration of the test was 24 hours and the risk taking test was also filmed and recorded to validate the results registered by the antenna. The test was performed for two groups of 25 fish.

*2.2.Individual testing*

The other stress coping style tests were performed individually in a series - when in relation to the risk test (*see* Fig. 2 for experimental design and time line of the behavioural tests).

The first test performed was "*Restraining*" (REST), which was evaluated by holding individual fish in a net inside the water for 90 seconds (Fig. 1B). The net used had the following characteristics: 54 x 60 cm rectangular shape, white colour with 6 mm mesh. The parameters registered in this test were a) latency to first movement or first activity b) the total number of escape attempts described as the number of body contortions (elevation of the whole body from the net) and finally c) the total activity time that fish was moving inside the net.

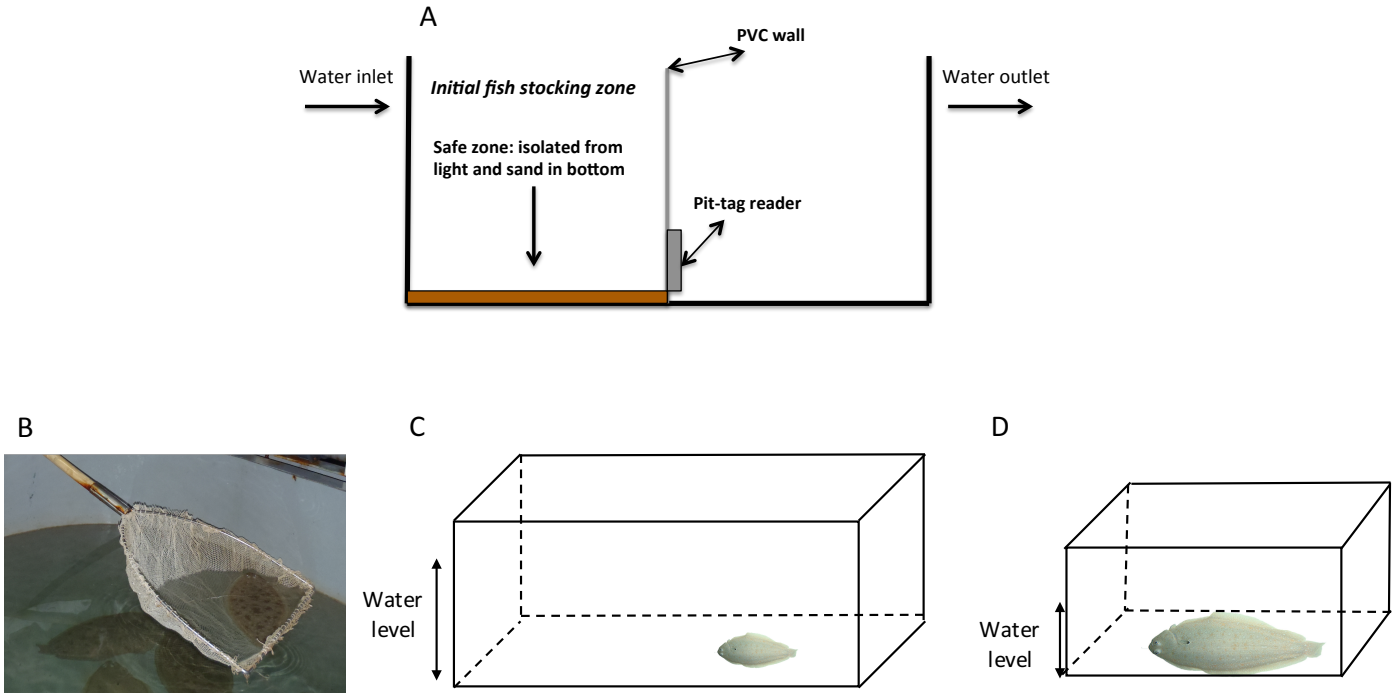


**Figure 1.** Description of material and indications to perform the coping styles tests on Senegalese sole juveniles. **A** Risk-taking test. **B** Restraining test. **C** New environment test. **D** Confinement test.

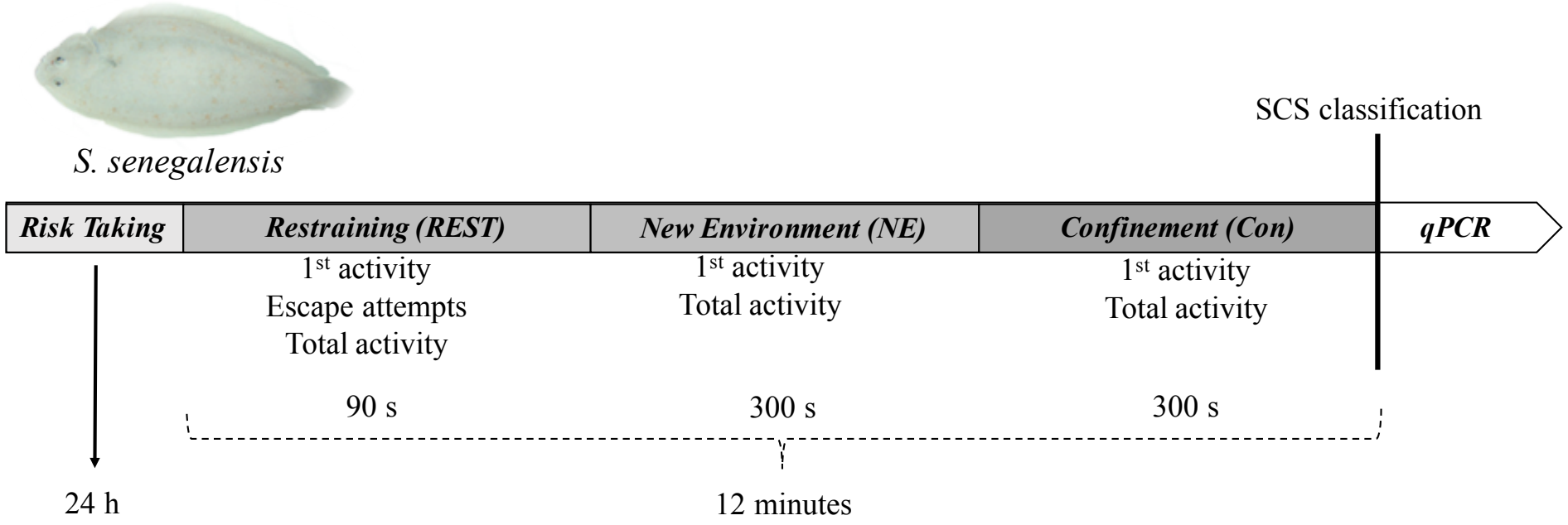


**Figure 2.** Chronogram illustrating the experimental design of the different stress coping styles (SCS) tests performed by early Senegalese sole juveniles. First activity (1st act) and total activity.

The next test performed, was *"New environment*" test (NE); each fish was individually placed in a plastic tank that was considered as a new environment. The tank dimensions for this test were 56.5 x 36.5 x 30 cm, rectangular shape and grey colour (Fig. 1C). The duration of the test was 300 seconds, during which two parameters were measured: a) the latency time or time of first activity when the fish started to explore the new environment and b) the total activity time, which was the total time the fish spent exploring, swimming forward in the tank.

The last test performed was "*Confinement"* test (CON); each fish was individually placed in a plastic tank that simulated a confinement situation. The tank dimensions were 25 x 14 x 8 cm, rectangular shape and white colour (Fig. 1D). The duration of the test was 300 seconds, during which two parameters were measured: a) the latency time or time of first activity when the fish started to move in the tank and b) the total activity time referring to the total time the fish was moving. For the last two test (new environment and confinement situation test), if fish did not move at all during the period of the test, 300 s was noted for statistical analysis. Therefore, the 50 sole were categorised into 3 coping style categories, proactive, intermediate and reactive. At the end of the last test the animals were euthanatized with an overdose of MS222 (tricaine methanesulfonate; Acros-Organic, New Jersey, USA). Afterwards, brains were dissected and frozen in dry ice and stored at -80º C for further molecular analysis.

*Quantitative real time PCR*

The differential expression of brain target genes for stress coping behaviour (Table 1) was measured in brains from thirty sole, ten fish randomly selected from each "stress coping style" category (proactive / intermediate / reactive). The candidate genes selected for this study were chosen for their biological role that can be associated with stress coping style behaviour, such as, metabolic rate, lipid metabolism, growth, circadian rhythmicity and stress responses. The genes were analysed by quantitative PCR (*q*PCR) (Table 2). Data were normalised using 18*S* as a housekeeping gene. Relative mRNA expression for each gene was determined using the method $(1 + E_T)^{(\Delta Ct)} / (1 + E_R)^{(\Delta Ct)}$ (Pfaffl, 2001). For this purpose, RNA was extracted using TRI Reagent RNA Isolation Reagent following manufacturer's instructions (SigmaAldrich). The complementary DNA was synthesised using 1 µg of total RNA and oligo dT (20) in 20 µl reactions and the SuperScript® III First-Strand Synthesis SuperMix 50 rxn kit following the manufacturer's protocol (Invitrogen, Life technologies, USA). Target genes were chosen according to some related genes to stress coping styles in zebrafish (Rey et al., 2013). Primers used were specific for Senegalese sole and published in different bibliographic references. Before performing the *q*PCR, primers were validated by conventional PCR using a cDNA pool from several samples randomly chosen. HS X My taq Mix (Bioline) was used to perform the conventional PCR with the following conditions: initial activation step at 98 °C for 1 min, followed by 35 cycles: denaturation at 95 °C for 10s, annealing at Tm (58-60 °C) for 15s and extension at 72 °C for 15s. Primers efficiency was evaluated by serial dilutions from 10 to 10,000. The *Q*PCR was run using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen,

Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific), 1 µl of the primer corresponding to the analysed gene (10 pmol), 3 µl of RNA/DNA water free and 5 µl of cDNA at the validated dilution. Furthermore, amplifications were carried out with a systematic negative control (NTC; no template control) containing no cDNA. Standard amplification conditions contained an uracil DNA glycosylase (UDG) pre-treatment at 50 °C for 2 min, an initial activation step at 95 °C for 10 min, followed by 35 cycles: 15s at 95 °C, 30s at the annealing Tm and 30s at 72 °C.

*Statistical analyses*

Behaviour

Statistical analyses were performed using SPSS Statistics 20.0 (IBM®) and GraphPad Prism 6 software (GraphPad Software, Inc.). A hierarchical cluster was applied to classify the fifty sole into different SCS categories (proactive, intermediate and reactive) according to the variable "total activity" time (in seconds) of all tests conducted (*pers. com. Ibarra*) (Fig. 3). A coefficient of variation (CV % = SD/mean*100) was calculated for each category (proactive, intermediate and reactive) representing the inter-individual sole variability in the population studied. The SCS categories were not distributed normally (Shapiro-Wilks) in all tests and a Kruskal-Wallis non-parametric test was performed to analyse the significant differences among personalities in the different tests which were non-normally distributed. However, when data possessed normality the statistical test performed was One-way ANOVA. Significance was set at $P$-value < 0.05 for all cases.

*q*- rtPCR

Results were expressed as mean ± S.E.M (Standard error of the mean) and analyses were performed using SPSS software and GraphPad Prism 6 software. Outliers of the corrected ratio for every mRNA on the different groups (proactive, intermediate and reactive) were extracted using the Tukey's test formula ($k$ = 1.5). All data sets analysed were normally distributed (Shapiro-Wilks), although logarithmic transformation was performed when needed. Comparisons of the mRNA transcripts among proactive, intermediate and reactive groups were made using One-way ANOVA, followed by Tukey's *post-hoc* test. A $P$-value < 0.05 indicated a statistically significant difference in all tests performed.

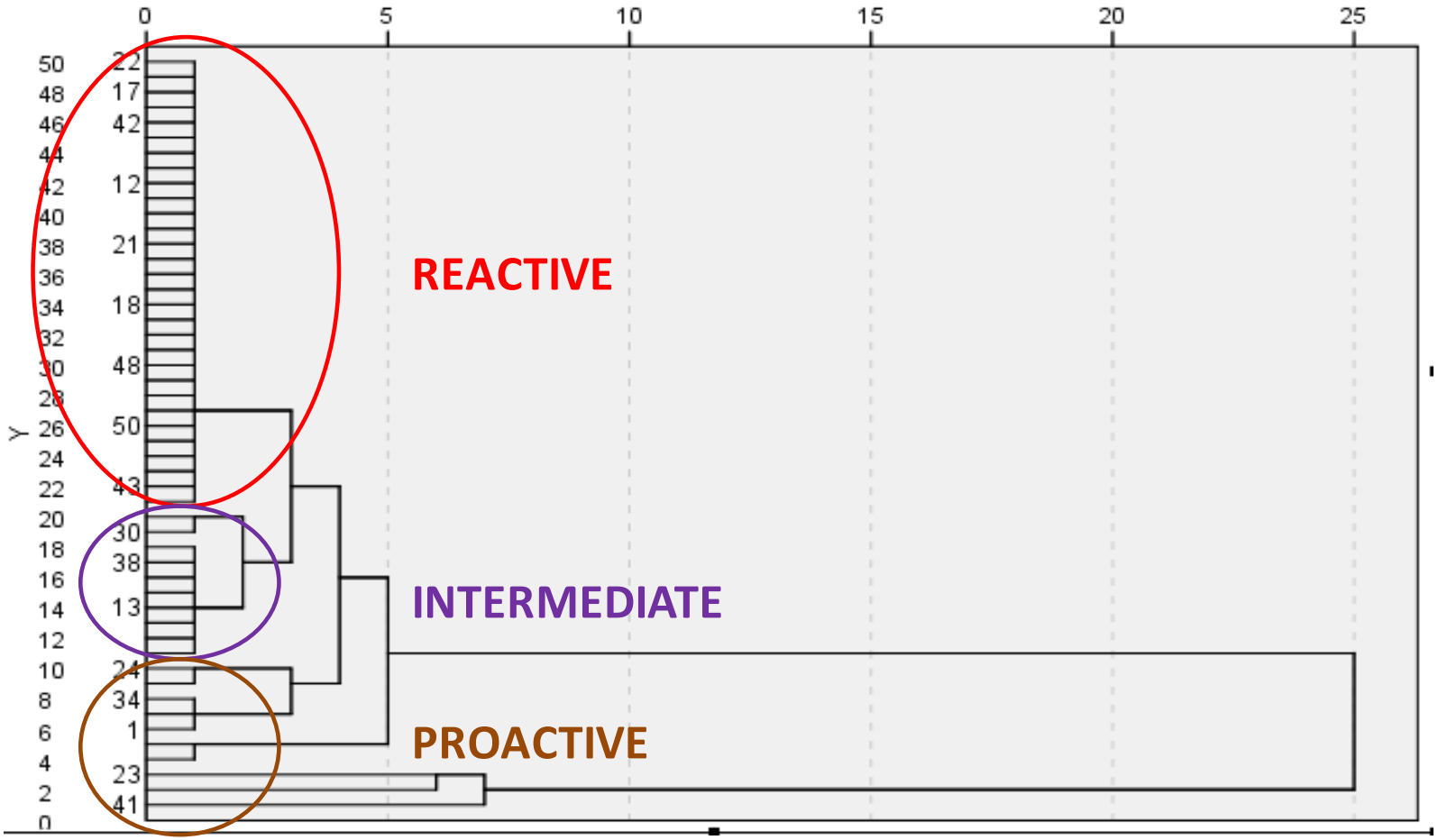


**Figure 3.** Hierarchical cluster applied to classify the fifty sole in three different SCS categories (Proactive, Intermediate and Reactive). The different groups presented significant differences among them in all test intra and inter-group (One-Way ANOVA).

**Table 1.** Primers used in this study as possible stress coping style biomarkers in Senegalese sole (*Solea senegalensis*). Gene, gene name, amplicon size, accession number and primer sequence are indicated.

| Gene | Gene name | Amplicon size | Accession Number | Primers (5' 3') |
|---|---|---|---|---|
| **Glyceraldehyde-3-phosphate dehydrogenases 2** | *gapdh-2* | 107 | AB291587 | F- AGCCACCGTGTCGCCGACCT<br>R- AAAAGAGGAGATGGTGGGGGGTGGT |
| **Period** | *per1* | 141 | FM180505.1 | F- ACATCACCTCCGAATACACC<br>R- ACACAGACCCCTGAAGACAC |
| **Heat shock protein 90, alpha (cytosolic) class A** | *hsp90aa* | 105 | AB367526.1 | F- GACCAAGCCTATCTGGACCCGCAAC<br>R- TTGACAGCCAGGTGGTCCTCCCAGT |
| **Insuline-like Growth factors** | *igf-Ia* | 144 | AB248825.1 | F- GCACAAGGCGGACAAGGGCACA<br>R- CGAGGGCACCGAAGAGACCTTTACCTG |
| **Peroxisome proliferator-activated receptor** | *pparβ* | 75 | JX424080 | F- GCTCTGGAGCTGGATGATAGTG<br>R- CAGCCCGGGACGATCTC |
| **Specific hypothalamic corticotropin-releasing hormone (CRH) binding protein** | *crh-BP* | | FR745428 | F- GGCAATGGCATAGACACCTC<br>R- CACTGGACACCAGCCTCAC |

**Table 2.** Genes (including abbreviations and known major functions) used in this study. (*) showed differential expression among categories (Proactive, Intermediate and Reactive) in early Senegalese sole juveniles.

| Gene | Gene name | Functions | Reference |
|---|---|---|---|
| Glyceraldehyde-3-phosphate dehydrogenase * | *gapdh-2* | Metabolic pathway, membrane fusion, phosphotransferase activity, nuclear RNA export, DNA replication and repair, apoptosis, age-related neurodegenerative disease, viral pathogenesis | Fothergill-Gilmore and Michels, 1993<br>Sirover, 1999<br>Manchado et al., 2007 |
| Peroxisome proliferator-activated receptor * | *pparß* | Nuclear receptor for vitamin A and fatty acids, mitochondrial biogenesis, energy metabolism | Michalik et al., 2002<br>Leaver et al., 2005<br>Darias et al., 2012 |
| Insuline-like growth factor * | *igf-Ia* | Somatic growth, metabolism, development, cell differentiation, reproduction, osmoregulation and immune response | Reinecke et al., 2005<br>Salas-Leiton et al., 2010<br>Darias et al., 2012<br>Campos et al., 2013 |
| Period * | *Per1* | Circadian rhythmicity, feeding behaviour, locomotor activity, reproductive strategies | Martín-Robles et al., 2012<br>Reppert and Weaver, 2002<br>Stokkan et al., 2001<br>Migaud et al., 2007 |
| Heat shock protein 90 alpha class | *hsp90aa* | Cellular stress, cellular protection, cellular homeostasis | Reinecke et al., 2005<br>Feder and Hofmann, 1999<br>Iwama et al., 1998<br>Salas-Leiton et al., 2010<br>Manchado et al., 2008<br>Campos et al., 2013 |
| Specific hypothalamic corticotropin-releasing hormone binding protein | *crh-BP* | Stress response (antagonist of CRH) | Wunderink et al., 2011<br>Huising et al., 2004<br>McClennen et al., 1998 |

## Results

Senegalese sole individuals presented a wide range of responses to the different tests performed indicative of inter-individual behavioural differences. The variability of the individual tests for the parameter total activity was similar for the tests "Restraining" (REST; CV = 123.9 %) and "New Environment" (NE; CV = 132.7 %). However, the "Confinement" test presented the highest variability for this variable (CON; CV = 213.9 %). According to the other variables measured as first activity, REST had more variability than total activity, CV = 315.8 % and the lowest variability data in this behavioural test was exhibited by the parameter escape attempts, CV = 88.0 %. On the other hand, NE and CON showed similar variability of the data for the first activity (CV = 90.7 % and 120.7 % respectively).

The different variables (total activity, first activity and escape attempts) were analysed by One-way ANOVA or Kruskal-Wallis to see if there were significant differences among the SCS (proactive, intermediate and reactive) after the classification with the hierarchical cluster. Total activity (Fig. 4) in the "New environment" (NE; K-W = 26.13; $P < 0.001$; Fig. 4B) and "Confinement" (CON; K-W = 25.46; $P < 0.001$; Fig. 4C) were differentially displayed among SCS categories. In the case of NE, intermediate (Total activity = 34.5 s; CV = 19.5 %; $P < 0.001$) and proactive juveniles (Total activity = 16.2 s; CV= 122.0 %; $P < 0.05$) showed significantly higher total activity than reactive (Total activity = 3.1 s; CV = 178.0 %), but not between proactive and intermediate. In the case of CON, significant differences were found between proactive (Total activity = 55.5 s; CV = 75.6 %) and intermediate (Total activity = 3.8 s; CV = 108.0 %; $P = 0.001$) and reactive (Total activity = 2.1 s; CV = 147.1 %; $P < 0.001$), but not between intermediate and reactive. In the case of the "Restraining" test, REST, marginal differences were found among groups (K-W = 5.491; $P = 0.0642$; Fig. 4A) and there were no significant differences among proactive (Total activity = 14.1 s; CV = 122.7 %), intermediate (Total activity = 13.8 s; CV = 96.7 %) and reactive (Total activity = 4.9 s; CV = 55.3 %).

Regarding first activity (Fig. 5) the situation was similar to the total activity, so the "New Environment" (NE; $F_{2, 47} = 7.822$; $P = 0.0012$; Fig. 5B) and "Confinement" (CON; $F_{2, 47} = 3.387$; $P = 0.0423$; Fig. 5C) tests presented differences among SCS categories. In case of the NE, intermediate (first activity = 38.6 s; CV = 167.0 %; $P < 0.001$) presented significantly lower latencies than reactive sole juveniles (first activity = 203.6 s; CV = 65.4 %), however, proactive animals (first activity = 105.9 s; CV = 117 %; $P > 0.05$) presented no significant differences in comparison to intermediate and reactive sole. "Confinement" test, CON, showed clearly differences between proactive (first activity = 27.4 s; CV = 225.5 %; $P < 0.001$) and reactive latencies (first activity = 150.5 s; CV = 96.2 %), however, intermediate sole (first activity = 95 s; CV = 149.0 %; $P > 0.05$) did not present differences in latencies with the extremes. In the case of REST, no differences were found among coping styles (K-W = 2.366; P = 0.3064; Fig. 4A), where proactive animals (first activity = 10.8 s; CV = 258.1 %), intermediate (first activity = 1.9 s; CV = 149.8 %) and reactive (first activity = 8.2 s; CV = 278.2 %; $P > 0.05$) showed similar latencies profile.

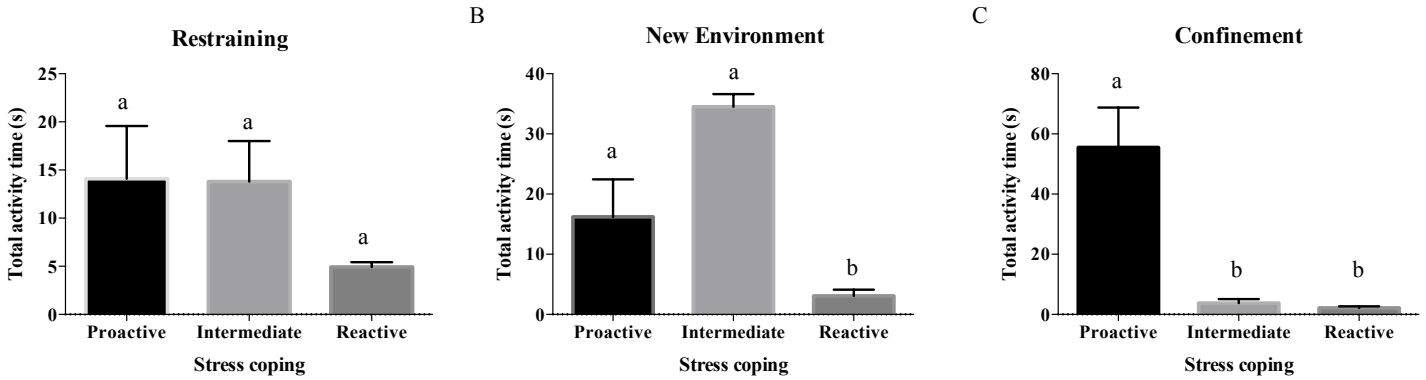


**Figure 4.** Stress coping styles tests regarding Total activity variable in seconds. **A** Restraining **B** New environment and **C** Confinement compared among the different stress coping styles categories (proactive, intermediate and reactive) classified according to total activity measurement. Data was shown in Mean ± SEM. Different letters indicate significant differences (Kruskal-Wallis $P < 0.05$ level of significance).

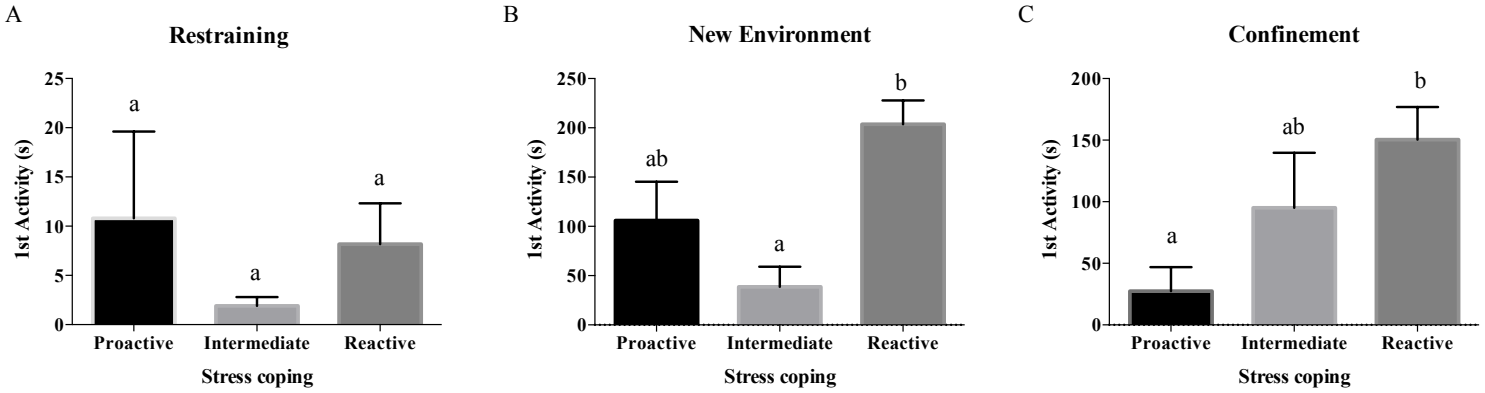


**Figure 5.** Stress coping styles tests regarding First activity parameter in seconds. **A** Restraining, **B** New environment and **C** Confinement compared among the different stress coping styles categories (proactive, intermediate and reactive) classified according to first activity measurement. Data was shown in Mean ± SEM. Different letters indicate significant differences (Kruskal-Wallis and One-Way ANOVA $P < 0.05$ level of significance).

Escape attempts (Fig. 6) was measured in the "Restraining" test and, no significant differences were found among stress coping styles groups (K-W = 0.4959; $P > 0.05$), proactive juveniles (escape attempts = 3.2; CV = 98.9 %), intermediate (escape attempts = 3.7; CV = 85.5 %) and reactive (escape attempts = 3.0; CV = 82.6 %; $P > 0.05$).

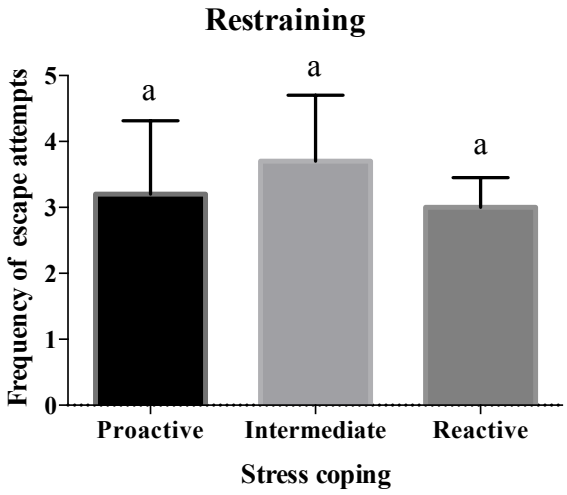


**Figure 6.** Restraining test regarding Escape attempts variable in frequency compared among the different stress coping styles' categories (proactive, intermediate and reactive) classified according to total activity measurement. Data was shown in Mean ± SEM. No differences were found among groups (Kruskal-Wallis $P > 0.05$).

Analysing the group-test, the "risk-taking" test, eleven of fifty juveniles (22 %) crossed from the well-known to the unfamiliar area, 6 of them coincided with proactive classification, 4 with intermediate and 1 was classified as reactive by the cluster. According to the results, the classification of the stress coping style groups was considered appropriate to continue with the statistical analysis of the brain gene expression.

Brain gene expression was analysed in ten samples of every category (proactive, intermediate and reactive) (Fig. 7). In the case of reactive group, the ten fish considered as the most reactive (the last ten fish in the list of the hierarchical cluster) were used to balance the number among categories. According to the brain gene expression in sole juveniles, four of the six mRNAs tested were significantly different expressed among coping styles' categories. In the case of glyceraldehyde-3-phosphate dehydrogenases 2 (*gapdh-2*) proactive and intermediate individuals (up-regulated) exhibited significantly higher expression than reactive individuals (down-regulated) ($F_{2, 27} = 8.173$; $P = 0.0017$; Fig. 7A). The other transcripts presented similar expression profile for the extremes categories (proactive and reactive), which were up-regulated and, expression was significantly different compared to intermediate (down-regulated): Period 1 (*per1*) (K-W = 14.43; $P = 0.0007$; Fig. 7B), Insuline-like Growth factor (*igf-Ia*) ($F_{2, 27} = 4.606$; $P = 0.0190$; Fig. 7C) and Peroxisome proliferator-activated receptor (*pparβ*) ($F_{2, 25} = 7.554$; $P = 0.0027$; Fig. 7D). The other two genes did not present significant differences

in expression, Specific hypothalamic corticotropin-releasing hormone (CRH) binding protein (*crh-BP*) ($F_{2, 24} = 0.4842$; $P = 0.6221$) and Heat shock protein 90, alpha (cytosolic) class (*hsp90aa*) ($F_{2, 27} = 2.346$; $P = 0.1150$).

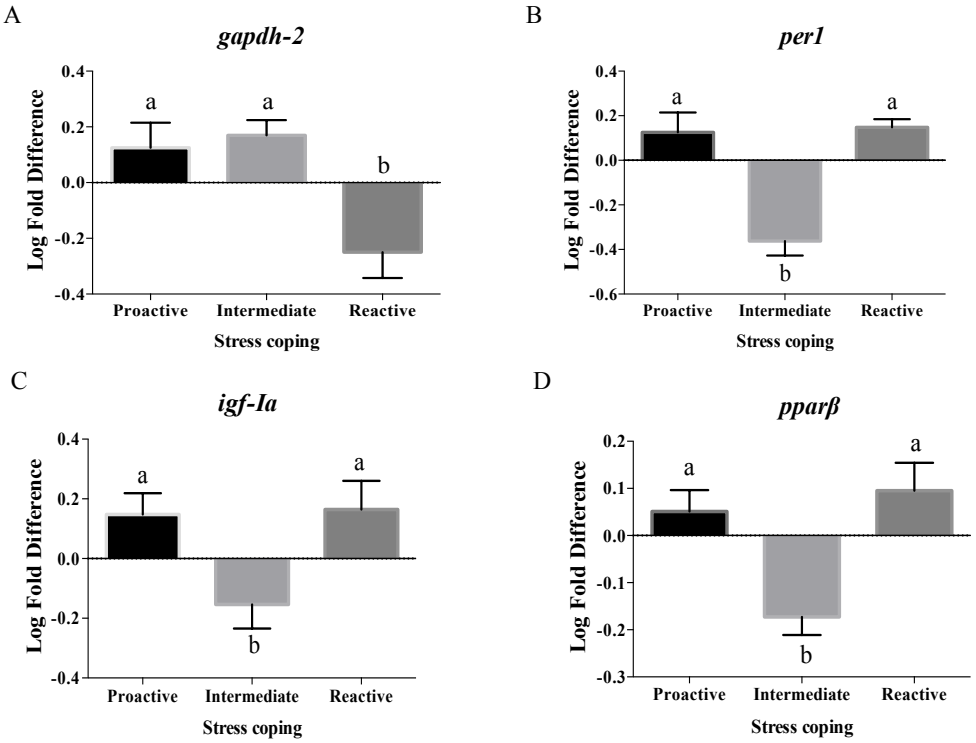


**Figure 7.** Brain gene expression of different genes which were differentially expressed among groups (proactive, intermediate and reactive). **A** *gapdh-2*, **B** *per1*, **C** *igh-Ia* and **D** *pparβ*. Data was transformed to $Log_{10}$ and was shown in Mean ± SEM. Different letters indicate significant differences in expression (One-Way ANOVA $P < 0.05$ level of significance).

Contrary to expectations the data regarding the association between SCS categories (proactive, intermediate and reactive) and dominance behaviour (dominant and subordinate) related to feeding response in early Senegalese sole juveniles did not show a relationship. In the case of the 14 dominant fish, 3 individuals were proactive, 4 intermediate and 7 reactive, and in the case of 16 subordinates, 4 were proactive, 4 intermediate and 8 reactive (Fig. 8).

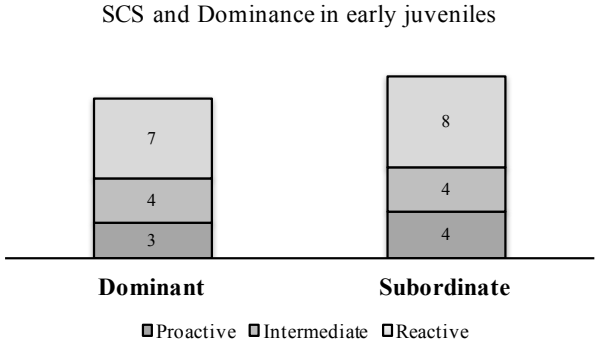


**Figure 8.** Association between stress coping styles (SCS) and dominance behaviour related to feeding response in early Senegalese sole juveniles. The individuals which performed the dominance behavioural test classified in dominant and subordinate were categorized according to the SCS categories (proactive, intermediate and reactive).

## Discussion

Linking the phenotype variation with genotype within a population in reaction to an environmental change is essential to the understanding the effect of a change on the biology of an organism. In this study, the phenotype variation was evaluated with behavioural tests to define coping styles. Coping styles have been assessed more recently in Senegalese sole by Ibarra-Zatarain et al. (2016) finding differences in fish with diverse activity, latencies and physiological characteristics as cortisol. Additionally, differences in coping styles were assessed in larvae depending on their dietary fatty acids (Ibarra-Zatarain et al., 2015), showing the importance of nutrition. Ibarra-Zatarain et al. (2016), demonstrated that the three behavioural tests applied in this study, "Restraining" test (REST), "New environment" (NE) as exploratory test, and "Confinement" (CON) as confinement and the "risk-taking" test (group-test) can be used to characterise stress coping styles (from proactive to reactive) in Senegalese sole juveniles and breeders. In addition, the three individual tests were quick to perform and attractive for the aquaculture industry. In general, populations have been classified into just two SCS categories (proactive and reactive) or three SCS categories (proactive, intermediate and reactive). For example, Oortmerssen and Busser (1989) observed in a natural feral mice population a bimodal distribution of SCS variables, presenting only proactive and reactive profiles. However, this distribution changed when the experiment was performed under laboratory conditions, where another coping style category was found, the intermediate, probably due to the low natural selection pressure in captive conditions. Those experiments suggested the existence of at least two coping styles, proactive and reactive, and depending on the situation/condition a third coping style could appear, the intermediate (Koolhaas et al., 1999). This model, with proactive, reactive and intermediate coping styles, has been observed in a widespread variety of animal species, including fish (Carter and Bransden, 2001; Øverli et al., 2006; Martins et al., 2011). This is in concordance with the present study, where the three behavioural tests proposed by Ibarra-Zatarain et al. (2016) gave three different categories of coping styles (proactive, intermediate and reactive) according to diverse behaviours when

confronted with the stress in the test. Therefore, proactive sole presented lower latencies and higher activity than reactive and intermediate, implicating higher explorative behaviour and different response to stressful circumstances. Previous studies have demonstrated that the same behavioural tests classify animals according to their personality in diverse fish species, such as stickleback (Bell, 2005), gilt-head seabream (Castanheira et al., 2013) and zebrafish (Tudorache et al., 2015).

The understanding of the different interactions between the environmental changes and the capability of the individuals to react to them is an enigmatic question. Gene expression is considered a complicated analysis in terms of variability which could be influenced by several alterations including environmental factors. The interpretation of such interactions with the different variations between individuals inter and intra-populations have remarkable potential for evolution, unravelling the patterns of gene expression and phenotypic variation (Whitehead and Crawford, 2006). In our study, those interactions were considered according to the different coping styles profiles (proactive, intermediate and reactive) where Senegalese sole provided different levels of mRNAs under the same environmental undisturbed conditions without exposing the fish to a challenge. These results were in concordance to previous studies, for example, MacKenzie et al. (2009) found differences in gene expression between proactive and reactive common carp (*Cyprinus carpio*) when those animals were under the same environmental circumstances. In that report, coping styles were included in the analysis reducing the unexplained variation and increasing the interpretation of the experimental data. Another example, Oleksiak et al. (2005) evaluated individual gene expression in the hearts of males of common mummichog (*Fundulus heteroclitus*), the cardiac metabolism using different energy substrates differed between individuals in a population, suggesting that this alteration was biologically relevant. However, the differences in gene expression was not very large between groups of individuals. This state was similar in our study, where differences in gene expression among different coping styles (proactive, intermediate, reactive) were generally minor. The brain gene expression profile was carried out by *q*-rtPCR in 6 mRNAs (*gapdh2*, *Per1*, *igf-Ia*, *pparß*, *hsp90aa* and *crh-BP*) where significant differences were found in 4 mRNAs (*gapdh2*, *pparß, igf-Ia* and *Per1*) suggesting that there exist variations in gene expression among Senegalese sole coping styles. The different mRNAs chosen for this study were related to metabolism, stress responses and biologic conditions specifics for Senegalese sole which could provide important information in terms of cultivation (*see* Table 2). Differences in metabolism have been linked with changes in coping styles in some species (Biro and Stamps, 2008; Martins et al., 2011). In relation to the stress coping style biomarkers, the literature is scarce, however some studies have been performed in fish, such as, zebrafish (Rey et al., 2013), common carp (MacKenzie et al., 2009; Rey et al., 2016), Nile tilapia (*Oreochromis niloticus*) (Vera Cruz and Brown, 2007), rainbow trout (Thomson et al., 2011) associating physiological and brain gene expression characteristics with behavioural phenotype.

Differences in whole-brain gene expression among categories (proactive, intermediate and reactive) represent the first evidence in Senegalese sole correlating stress responsiveness with molecular responses. All these genes have high importance

in Senegalese sole at biological level and have been widely studied in this species by several researchers (*see* Table 2), besides, have been associated with stress coping styles in other fish species (Rey et al., 2013). One of the genes differentially expressed was Glyceraldehyde-3-phosphate dehydrogenases (*gapdh*), which is habitually used as a housekeeping gene to normalize variations and different technical errors in quantitative rt-PCR. However, *gapdh* expression quantities may vary among tissues, development, or during different physiological processes including different behavioural traits (MacKenzie et al., 2009; Rey et al., 2013). Moreover, *gapdh* was discarded as a suitable housekeeping gene for Senegalese sole (Infante et al., 2008). The metabolic function might be compromised by acute and chronic stress, explaining why *gapdh-2*, which has been demonstrated to be the *gapdh* isoform more expressed in brain in Senegalese sole (Manchado et al., 2007), was up-regulated in proactive sole relative to reactive fish (down-regulated). MacKenzie et al. (2009) made similar observations with common carp, where *gapdh* presented up-regulation in proactive fish and down-regulation in reactive animals demonstrating differences between coping styles and basic metabolism.

The other three genes (*ppar*β, *igf-Ia* and *per1*) differentially expressed among coping style categories in this study, presented similar expression profiles in proactive and reactive animals that were up-regulated and intermediate animals presented down-regulation, and these genes are associated with feeding behaviour and nutrition. In general, intermediate animals present more plasticity than the extremes coping styles categories, proactive and reactive (Dingemanse et al., 2010). According to these results in brain gene expression, intermediate sole presented different profiles depending on the behavioural test performed (*see* Figs. 3, 4 and 5), consequently, the intermediate sole appeared similar to proactive or reactive animals depending on the stressor test. The first gene differentially expressed with this profile and associated with nutrition was peroxisome proliferator-activated receptor (*pparβ*) which is implicated in the skeletal, brain and skin functions in mammals (Lee et al., 2003; Giaginis et al., 2007) and in addition, this nuclear receptor has been associated with the early step towards adipogenesis. Moreover, *pparβ* is a target gene for fatty acids and vitamin A. The expression of *pparβ* is influenced by nutrition in fish such as gilthead seabream (Fernandez et al., 2011) and sea bass (Vagner et al., 2009) acting as regulators of lipid and lipoprotein metabolism and associated with feeding behaviour. The second gene associated with nutrition analysed in this study was Insuline-like growth factor I (*igf-I*), which shows a central role in postnatal growth in mammals (Baxter, 1994). In addition, *igf-I* mRNA profile in hepatic and non-hepatic tissues are dependent to the growth hormone (GH), which is synthesized in the pituitary gland and secreted into the blood circulation under the regulation of different factors such as neuronal, hormonal and nutritional. Nevertheless, GH might not control the relative expression of *igf-I* in non-hepatic tissues in fish. Duan (1998) demonstrated that IGF-I protein is highly conserved between fish and mammals and is found in all development stages in fish. Besides, nutritional status has a deep effect on *igf-I* expression in fish. The third gene associated with feeding behaviour was Period 1 (*Per1*), which is one of the clock genes that

control the circadian rhythm. The *Period* (*Per1, per2 and per3*) genes are negative regulators, which inhibit the CLOCK and BMAL1 activators (Reppert and Weaver, 2002). This mechanism is cyclic, where the expression of clock genes is approximately daily. PER are expressed in the daylight (diurnal) and CLOCK and BMAL1 in the night (nocturnal). In previous studies performed in this species, *Per1* was expressed in almost all tissues in Senegalese sole; however, the tissues that presented more expression in this species were gills, liver, ovary and cerebellum (Martin-Robles et al., 2012). Fish have a feeding schedule when held under captive conditions like in aquaculture and feeding can work as a strong synchronizer of circadian rhythms in several animals, increasing the locomotor activity some hours before the food is provided, which is called food anticipatory activity (Mistlberger, 2009). This activity can affect the expression of the clock genes, for example in zebrafish was observed that the animals exposed to different lights and different feeding schedules, including random feeding presented different *Per1* expression profiles (Lopez-Olmeda et al., 2010). In the random feeding regime, the animals did not present food anticipatory activity and *Per1* expression rhythm disappeared demonstrating the importance of feeding behaviour in the circadian rhythmicity. In the present study, sole were fasted 24 hours prior the behavioural tests and according to their feeding regime all sole used for the experiment should present more or less the same expression profile, however, only proactive and reactive presented up-regulation in these three genes and intermediate sole showed down-regulation, so the different expression among categories in those genes might be explained just by the behavioural screening prior to molecular analysis.

Intriguingly, both stress-related genes (*hsp90aa* and *crh-BP*) tested in this study were not differentially expressed among coping styles' categories. Heat shock protein 90, alpha (cytosolic) class (*hsp90aa*) presented more or less the same profile than the last three genes explained, that means, proactive and reactive groups were up-regulated and intermediate group was down-regulated, however, corticotropin releasing hormone binding protein (*crh-BP*) presented down-regulation in the three groups, but the variability intra- and inter-group resulted higher than the other genes. This could be explained if the different tests did not exert a highly stressful situation to the animals that were tested. The HSP90 gene has been associated with nutritional stress in early stages in fish (Cara et al., 2005) and as a protection against different stressors such as infections, heat shock, etc. (Basu et al., 2002). In previous studies performed with Senegalese sole revealed that *hsp90aa* was activated at the moment that sole were under a heat shock treatment, however, no significant differences were found after a cold shock treatment. Nevertheless, in our study, all animals used for the experiment were under the same conditions both prior to and during the experiment without any treatment, so the change in the regulation of *hsp90aa* transcript was caused by the variability between individuals. Wunderink et al. (2011) found that CRH-BP levels were not affected at different stocking densities (chronic stress response) in Senegalese sole and in addition, the *crh-BP* expression was improved in both densities when animals were moved to hypersaline seawater (acute stress response) proposing that *crh-BP* worked as a modulator of the acute stress reaction. Therefore, in the present study,

the down-regulation in all groups could be explained that in the moment the fish were sampled at the end of the tests the fish and the genes that were measured were not reacting to an acute stress.

Apart from the linkage between SCS and brain gene expression, no association was found between stress coping styles and dominance behaviour in early Senegalese sole juveniles. This is in agreement to previous studies that showed no association between coping styles and dominance rank (Boogert et al., 2006; Kurvers et al., 2009; Funghi et al., 2015). For example, Devost et al. (2016) demonstrated that proactive-reactive axis in black-capped chickadees (*Poecile atricapillus*) based on exploratory, activity and neophilic tests had no correlation with dominance in this bird species. Another example is the study performed by Riebli et al. (2012) in breeding cichlid (*Neolamprologus pulcher*), where aggressive propensity, as a behavioural syndrome, was studied to observe the influence in competition to obtain mates and shelters. This syndrome was marginally related to dominance and animals that had few effects of aggressive character obtained higher dominant position and better territories. Nevertheless, the association between dominance and behavioural traits has been observed in previous studies performed in other fish species such as rainbow trout (Øverli et al., 2004) and Nile tilapia (Silva et al., 2014). Øverli et al. (2004) observed in rainbow trout that the winners in contests for social status could be predicted from the appetite inhibition after transfer to a new environment (stress coping style test). This association was found using aggression as behavioural characteristic to determine social rank and aggression may have a closer relationship to SCS. Different measure or behaviours of a behavioural syndrome such as coping styles might not be stated equally in all challenging situations (Koolhaas et al., 1999), and the same theory could be applied for the social status (Reebs, 2008) showing the importance of behavioural strategy or syndrome to show dominance and coping styles. The present study was performed with a non-aggressive species where the dominance was perceived by feeding behaviour **(Chapter 6)** and coping styles were extracted by proactivity aspects as latencies and total activities, which were more discriminative than those focused on reactivity facets (Koolhaas et al., 1999). Moreover, the linkage between coping styles and dominance could appear in the breeder stage after the life history traits of these animals develop in competition and cohabitation (Ruiz-Gomez and Huntingford, 2012). Besides, it is relevant to consider that individual tests may not be illustrative of the general behavioural characteristics of a population and may generate pseudo replication of results when behaviour is evaluated in animals (Castanheira et al., 2013). This was the reason why grouped-tests were applied in both behavioural studies, coping styles and dominance (Chapter 6), since Senegalese sole live communally in their natural habitat.

In conclusion, Senegalese sole were categorised into three different stress coping styles groups, proactive, intermediate and reactive. The brain gene expression of 6 genes was examined among these coping style groups. One gene, *gapdh-2* was differentially expressed between proactive and reactive coping styles, indicating it was a

possible marker gene for coping style in Senegalese sole. Coping style and molecular expression appear to be linked in this species with clear differential expression between coping style groups, in addition to similar expression in some genes, which together indicates the complexity and the potential to explain mechanism controlling behavioural traits and increase our understanding of the molecular context of adaptive variation among individuals within and between populations. In addition, no evident association was found between coping styles and dominance behaviour in this species. However, more studies are needed to understand the different stress coping style classification in the brain gene expression to find an appropriate coping styles' biomarker in Senegalese sole and facilitate in that case the understanding of the complex biology of this species in captivity.

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# Section 6: Final discussion

# Chapter 8

# Final Discussion

# Final discussion

Senegalese sole farming is not sustainable and implementation of genetic breeding programs is complicated due to the failure of behavioural aspects in cultured males related to reproduction (Morais et al., 2014). Cultured males do not perform the reproductive behaviour and, therefore, do not fertilise the eggs (Carazo, 2013). Little is known about the exact cause of the behavioural failure or possible controlling mechanisms such as chemical communication. The relationship between olfaction, behaviour and reproduction has been demonstrated in a few species (Hubbard, 2014) and the association of these aspects with the ecology and biology has improved aquaculture (Huntingford, 1993; Hara, 1994; Conrad et al., 2011) to highlight that research in these areas could be useful to solve reproductive behavioural problems and improve Senegalese sole farming. Consequently, the present thesis (Chapters 2 to 7) contributed to why Senegalese sole life cycle is not currently completely close in captive conditions from different perspectives. Specifically, Chapter 2 focused on the effect on cultured breeders behaviour of cohabitating with spawning wild breeders, Chapters 3, 4 and 5 analysed different aspects of the chemical communication through olfaction and Chapters 6 and 7 contribute to the identification and characterization of dominance behaviours as well as behavioural gene markers.

## 1. Different processes of social Learning in Senegalese sole

With respect to the effect of the presence of spawning wild breeders on the courtship and reproductive success of cultured breeders (**Chapter 2**), two spawns were obtained in 2014 from a cultured couple and the same cultured female spawned with a wild male. Moreover, cultured breeders cohabitating with wild breeders exhibited significantly higher participation in courtship behaviours compared to the control group and the participation of cohabitating cultured breeders in the courtship significantly increased during the study from 2013 to 2015. The different behaviours analysed during the years of cohabitation were “Rest the head”, similar behaviour was detected during the courtship in other flatfish species such as wide-eyed flounder (Carvalho et al., 2003), largescale flounder (Manabe et al., 2000); “Follow” behaviours that have been observed in the spawning behaviour of winter flounder (Stoner et al., 1999); “Guardian”, which has only been described for Senegalese sole (Carazo et al., 2016) and “Coupled” swim which was also described for wild-captive common sole (Baynes et al., 1994). However, the most common behaviours were “Rest the head” and “Follow” coinciding with the step 1 and 2 of the Senegalese sole’ courtship (Carazo et al., 2016). One important finding of this study was the participation of cultured male breeders in the “Follow” behaviour in the mixed-origin groups. This was the first study that observed cultured male breeders participating in the “Follow” behaviours. The participation in that behaviour increased significantly in cultured animals over the study period. This increase may indicate a process of social learning that develops or strengthens instinctive behaviours. However, wild sole breeders continued dominating

the “Follower” position and cultured breeders participated predominantly as “Followed”. The transfer of knowledge relies on social learning, which entrains the acquisition of new behaviour or information of an individual about their situation (environment) via observation or interaction with other animals (Brown and Laland, 2003; Laland et al., 2003). There exist different social learning processes in animals including stimulus and local enhancement, imitation, emulation, response facilitation and the social enhancement of food preferences (Thorpe, 1963). Social learning has a clear involvement in the courtship of some animals. For example, Freeberg (2004) demonstrated in brown-headed cowbirds (*Molothrus ater*) that when in captivity the birds that were captured in the same area preferred to pair together for mating. This “mate choice” was made from learned courtship behaviour where males sing different patterns of songs to court the female (Freeberg, 1996). The experiment was developed by Freeberg (1996) who captured young males in one location that were maintained in an aviary with adults from different location and those young males developed songs patterns of their birdcage comrades rather than from their natal community. At the same time, females held in an aviary with adult males from a different location exhibited a clear priority for songs patterns of their cage companions rather than others, and consequently mating preferentially with those males singing similar patterns, moreover, this courtship behaviour might be transmitted across generations (Galef and Laland, 2005). In case of fish, researchers from a wide range of subjects are finding that learning plays a vital role in the evolution of fish behaviour (Laland et al., 2003). Brown and Laland (2003) reviewed social learning in fish and described how social learning was used to improve behaviours in: foraging, antipredator behaviour, migration, orientation and mate choice. These categories utilised different forms of social learning such as: observational conditioning and imitation. These two types of social learning, observational conditioning and imitation, have relevance to the present study were cultured Senegalese sole appeared to learn courtship behaviours from wild breeders. Observational conditioning was defined by Brown and Laland (2003) as “the response of a demonstrator to a stimulus that elicits a matching response from the observer, that simultaneously perceives the original stimulus and effectively learns the behaviour is an appropriate response to the stimulus” and imitation was defined by the same authors as “learning a procedure of particular body movements through observation of others”. Senegalese sole performs a complex courtship which entrains several behaviours that were divided into three steps described by Carazo et al. (2016). The “Follow” behaviours were the principal behaviours in the first step of courtship and had clear possibilities for social learning. Two types of “Follow” behaviours were categorised: the “Follower” behaviour a fish following the lead fish and the “Followed” behaviour which was the lead fish that was followed. The following fish copy nearly exactly the manoeuvres of the lead “Followed” fish imitating and possibly learning the behaviour. This behaviour can last for a long period (Carazo et al., 2016), during which observers can use observational conditioning of the behaviour itself as well as imitating the movements of wild breeders to learn the “Follow” behaviours. Therefore, there exist clear opportunities for the cultured sole cohabitating with wild sole to socially learn the courtship behaviours from the wild sole.

However, courtship behaviours are considered innate or instinctive in some animal species and, therefore, a learning process would not be required. Innate behaviour was defined as "an inborn tendency to behave in a way characteristic of a species" (Marler, 2004). For example, the courtship of fly males, *Drosophila melanogaster*, is considered a fixed-action pattern (instinct) which involves multiple sensory inputs and motor activity outputs (Demir and Dickson, 2005); brown-headed cowbirds (*explained above*), these animals can change their song patterns depending on the location where they are, however, these birds sing their courtship songs naturally (Freeberg, 2004). In some cases, there are some behaviours which are instinctive but depend on experience process to emerge (Campbell and Reece, 2002). For example, foraging behaviour in fish has an innate component, but the experience is also needed to become fully developed (Huntingford, 2004) and learning is essential for fish skills influencing on genotype, phenotype and experience during ontogeny (Bell, 2008). Rodewald et al. (2011) performed an intriguing study with Atlantic salmon (*Salmo salar*) where cultured salmon presented lower foraging rates on natural prey and impaired skill to avoid natural predators when these animals were released to the wild, in consequence cultured salmon showed lower survival in comparison to wild conspecifics. However, this situation changed at the moment that the environment was enriched in the installations and, even more, when cultured salmon cohabited with wild salmon, consequently, cultured salmon which were reared in those conditions presented higher feeding rates and started to forage earlier on natural prey and it appeared that this improvement was associated with learning from the environment and wild individuals (Rodewald et al., 2011). Analysing the results of the present study with Senegalese sole, the relationship between social learning, specifically observational conditioning and imitation, and the participation in the courtship and reproductive success in Senegalese sole could be established being the reproductive behaviour considered as a possible maturational behaviour which they have to learn and obtain some expertise. Moreover, the presence of the sand in the tanks as an enrichment environment could be another aspect that enriched the environment and aide to stimulate the courtship. It has been demonstrated that environmental enrichment encourages learning in fish and enrichment in early life develops behavioural flexibility, including learning (Strand et al., 2010).

Another interesting aspect to discuss is that cultured common sole (*Solea solea*) a closely related species does not present the same problems as Senegalese sole to reproduce in captivity (Palstra et al., 2015). In this case common sole could have an adaptive social learning advantaging compared to Senegalese sole. Previous studies demonstrated that there are differences in social learning between species, even closely related species. Laland (2006) reported that nine-spined sticklebacks were able to learn foraging in a feeding territory by observing other fish, however, this situation was not observed for three-spined stickleback. This difference is thought to be an adaptive specialization in social learning.

Another possible consideration would be the reproductive strategy involved in mate selection, i.e. Does the strategy behind mate selection in Senegalese sole males affect the capacity to make a mate selection? For example, some fish species such as the guppy (*Poecilia reticulata*) copy mate selection. Females choose to mate with males

which had been chosen before by other females. Dugatkin (1992) separated two guppy males in the same aquarium with a model female (demonstrator) residing near one of the males, then after the model female was removed, one female considered observer was liberated in the middle of the aquarium freely to choose the place of swimming and this observer female swam more time in the area closest to the male that had been near to the demonstrator female. In this context, Senegalese sole presented a clear mate choice by both the male and female during the reproductive behaviour. Senegalese sole only spawn as a pair and this gives the opportunity for the male and female to choose each other. The parental contribution to families has shown that the pairs usually show fidelity (reproducing) over years, so the mate selection was considered an important parameter included in the reproductive behaviour. In the present study, the wild females prefer wild males, which were the sole that usually reproduced. However, there were spawning events performed by the same male with other females that were not his partner, but the parental contribution was much lower with those females. Nevertheless, the females which participated in those events, were females which actively reproduced.

Cultured Sengalese sole showed different processes of social learning related to reproductive behaviour. Observational conditioning and imitating can explain the increasing participation in "Follow" behaviours of cultured sole which could be developed as part of a learning process. Moreover, a possible mate copying could appear in those cultured sole which tried to court the females to form a couple to reproduce. Therefore, this study demonstrated the importance in the processes of social learning for cultured Senegalese sole breeders in the presence and observing spawning wild breeders to increase participation in the courtship and reproduction.

## 2. Chemical communication:

An approach used throughout this thesis to identify and solve the reproductive behavioural problem was to compare different levels of possible controlling mechanisms between wild and cultured Senegalese sole breeders, given that captive-wild Senegalese sole male breeders can reproduce whereas cultured Senegalese sole males cannot. Additionally, neuroendocrine and sensory systems development could be affected by the general environmental experiences (*reviewed* in Huntingford, 2004). Consequently, the olfactory system as a sensory system from both wild and cultured sole was studied and compared in **Chapters 3, 4 and 5** to evaluate a possible deficiency in chemical communication in cultured animals.

Senegalese sole, like other flatfish, have highly specialized olfactory sensing mechanisms that are established during larval metamorphosis to achieve specific anatomical, physiological and functional adaptations of the species (Fernandez-Diaz et al., 2001). During the metamorphosis in captivity, different developmental problems have been reported in this species. Wild Senegalese sole larvae presented less skeletal deformities than those reared in captivity showing a higher incidence of deformities in captivity or a natural selection in the wild environment, which did not appear in aquaculture (Gavaia et al., 2009). In consequence, these issues during the structural

development including the metamorphosis process could have an impact on the asymmetrical olfactory system that may differentiate the olfactory system and chemical communication between wild and cultured sole. In **Chapter 3**, the main structure of the olfactory system, the upper and lower olfactory rosettes, was histologically analysed from wild and cultured juveniles of Senegalese sole. At the structural level, no differences were observed in the structure of the olfactory epithelium (OE) between wild and cultured sole. Both the upper and lower olfactory rosettes from each origin (wild and cultured) had similar external morphology, size, cell types (goblet, support and basal cells) and cell distribution pattern regarding olfactory sensory neurons (OSNs). The pattern of OSNs can be different among species, but in the case of the Senegalese sole the pattern of OSNs was continuous and uniform throughout the lamellae in both origins, wild and cultured sole. The structure of the olfactory rosette was in concordance with other teleost species such as zebrafish (Hansen and Zeiske, 1998), European eel (Atta, 2013) and catfish (Morita and Finger, 1998) among others and also similar to other flatfish species such as winter flounder (Prasada Rao and Finger, 1984), common sole and plaice (Harvey, 1996). The number of lamellae in the upper olfactory rosette (UOR) was greater than the lower side in fish from both origins (wild and cultured), which is a common characteristic of flatfish species (Harvey, 1996; Kasumyan, 2004). Consequently, this Chapter indicated that the structure of the upper and lower OE in cultured Senegalese sole was not different from wild sole and that the structural development of the OE during cultured juvenile metamorphosis was apparently appropriate at morphological level. It would appear that structural differences did not offer a possible explanation for the reproductive behavioural dysfunction associated with cultured males (Carazo et al., 2011). However, functional differences in the OE between wild and cultured mature males should be considered. Four main olfactory receptor families have been reported: olfactory receptors (ORs), trace amine-associated receptors (TAARs) and two vomeronasal receptors (V1Rs and V2Rs) with every family with different functions expressed in a different OSNs (ciliated, microvillous and crypt cells). Hence, olfactory system is a very complex organ specialized for the detection of an enormous range of odour signals which could control social interactions, environmental adaptation and feeding regulation, amongst other aspects (Hamdani el and Doving, 2007; Biechl et al., 2016). The expression profiles of the OE in wild and cultured male sole in a mature stage (spermatogenesis stage III according to Garcia-Lopez et al. (2005) were evaluated in **Chapter 4** after an appropriate stimulation from cohabiting mature females. For this purpose, deep RNA sequencing analysis was carried out in the upper olfactory rosette (UOR) from both origins. The UOR was analysed as it has been related to the chemical information exchange between conspecifics in Senegalese sole (Velez et al., 2005; Velez et al., 2007). In this study, several differences were found at different functional levels. The identification of several transcripts related to mucus production and goblet cells as well as nutrient sensing and feed intake demonstrated that wild males presented different nutritional status and diet preference (fresh vs dry food) from cultured males. Differences were also found in the expression of immune-related genes which showed the important role of the surface mucosa to control microorganism propagation.

Additionally, some transcripts associated with olfaction (OR, TAAR and V2R-like receptors) together with reproduction and kin imprinting were differentially expressed between origin. Such differences between distinct origin may be interpreted as the result of fish adaptation from wild to captive conditions. **Chapter 5** showed the different olfactory sensitivity of Senegalese sole males and females to urine from mature conspecifics by performing electro-olfactogram (EOG) recordings. This study showed that juveniles and adult males presented higher olfactory responses to urine than females, and adult males presented higher responses than juveniles, suggesting that the olfactory potency depends on the sex and maturity of the donor and also on the receiver. However, ovarian fluid proved to be less potent than urine. Moreover, the different responses indicate that olfaction could be used to identify sex and maturity status and suggested a role for urine in chemical communication during reproduction. Urine has been identified as a powerful vehicle for reproductive pheromones in other fish species such as goldfish, where the females release a pheromonal steroid in the urine that stimulates several changes of different aspects: behavioural changes related to the courtship and aggressive behaviours, physiological changes related to spermatogenesis such as gonadotropins plasma levels and milt production (Dulka et al., 1987; Stacey et al., 1989; Sorensen et al., 1990). A similar relationship between urine and gonadotropins plasma concentrations was also found in Senegalese sole in the present study. Urine that was pooled from mature females was used to stimulate the olfactory epithelium of mature males and this triggered an increase of LH plasma levels in the mature males. Additionally, the urine collected from wild mature males and females showed lower responses in the cultured juveniles males and females EOG recordings than the urine collected from cultured sole. This result was unexpected, and perhaps difficult to explained. However, the analysis was performed in May, when the spawning season in IRTA's facilities was almost finished. During the spawning season wild fish successfully reproduced and may have exhausted the signalling products contained in the urine, compared with cultured sole that may have had higher concentrations of signalling products as these fish had a lower participation in courtship and spawning. However, further work is required in order to analyse the urine composition to determine the signally products and compare these between wild and cultured sole.

Altogether the present studies comparing the olfactory system between wild and cultured breeders found there was no difference in the structure of the olfactory rosettes indicating that cultured fish have the structure to communicate chemically. However, the transcriptome of the olfactory rosettes had clear differentiation between wild and cultured males indicating that there exist differences in the communication within these two groups which could be associated to behavioural differences. Analysis of the sensitivity of the olfactory rosettes with the EOG, demonstrated that chemical communication has relevance to reproduction enabling the sole to distinguish between, sex and maturity status. The chemical products in the urine of female sole signalled the increase in males of circulating LH, which has been demonstrated in other male fish to initiate spermiation and spawning. Clearly, the olfactory system has importance in the

reproductive process of Senegalese sole and work should focus of identifying the control of male reproductive behaviours in both wild and cultured breeders.

### 2.1. Olfaction associated with reproductive behaviour and dominance behaviour in Senegalese sole

The involvement of the olfactory system in courtship or reproductive behaviour as has been previously observed in several animals; for example, vomeronasal system is directly involved in reproductive behaviour releasing chemical cues with urine in rats (Powers and Winans, 1975). The relationship between olfaction and reproductive behaviour has also been assessed and demonstrated in several fish species and probably exits in most species (Hubbard, 2014). Fish use chemical cues through olfaction to find a companion ready for spawning, regulate the physiological responses, and for synchronization between the population, such as goldfish, masu salmon, zebrafish, brown trout, rainbow trout, Mozambique tilapia, among others (Kasumyan, 2004). Notably, preceding studies have demonstrated that the implication of chemical signalization on behaviour in males is more relevant than in females (*reviewed in* Kasumyan (2004). For instance, a sexual pheromone released by females of the goldfish during ovulation, triggers pre-spawning behaviour of males (Sorensen, 1992). Hence, the complex courtship and the different behaviours performed by the males of Senegalese sole (**Chapter 2**) suggest the implication of the olfaction on this behavioural process, which might bring out a possible deficiency in the chemical communication of cultured males of this species. There are two different steps in Senegalese sole courtship in which olfaction could be directly implied for mating selection (Carazo et al., 2016). One is the "Follow" behaviour which was classified as the first step in the courtship. This behaviour is mainly displayed by males chasing or following other males, but also females. However, when females were involved in "Follow" behaviours they were usually in the "Followed" position (leader), which in a point of view of chemical communication might be very interesting as these females could release the different urine products that males could detect and this may have stimulated the males to follow the females. Additionally, this thesis has demonstrated that Senegalese sole males perceive better the olfactory signals than females and that mature males present higher sensitivity to mature male urine in March, coinciding with the first month of the spawning season, than immature males (**Chapter 5**). Therefore, according to the observation that during the spawning season, the activity of the tank increased on days with spawning as a result of the appearance of the "Follow" behaviours (**Chapter 2**). This increase in behaviour might be related to the higher response of mature males to urine from mature females, which could be the trigger to participate in this behaviour. However, wild males control the Follower position, above all during the first year of the cohabitation experiment. In other fish species the lack of participation in the courtship or reproductive behaviour of some males was associated with an overexpression of brain aromatase (*cyp19b*) (Schindler et al., 1999; Hallgren et al., 2006) being the aromatase responsible for oestrogen biosynthesis from androgens. An overexpression of aromatase was observed in the upper olfactory rosette (UOR) of Senegalese sole

cultured males **(Chapter 4)**. Consequently, the lack of participation in the different steps of the courtship by cultured Senegalese sole males could be related to the overexpression of *cyp19b* in the UOR. Another explanation could be related to dominance behaviour which could be implicated in the participation in the "Follow" behaviours. There are a variety of aspects such as chemical signals, colouration, size, aggressive behaviour, among others could influence the development and maintenance of hierarchical organization (Moore and Bergman, 2005). The chemical signals form part of those factors, influencing the effect that the winner has on establishing a social structure (Bergman et al., 2003). Previous studies in other social animals have demonstrated that chemical cues in aquatic environments are used to communicate social status and consequently modify aggressive behaviour, which is the main component of dominance. Bergman and Moore (2005) demonstrated that the different dominance categories (dominant and subordinate) changed in crayfish (*Orconectes rusticus*) after exposer to different odours that were previously established as social odours that communicate hierarchical status. Urine was the vehicle used for this species to communicate the hierarchical status. In this sense, previous studies have shown that urine from dominant individuals contains signals that can be perceived by an adversary and were used to establish a hierarchical ranking with conspecifics (Zulandt-Schneider et al., 1999; Zulandt-Schneider et al., 2001; Bergman et al., 2003). Crayfish is not the only example of the use of urine to establish the dominance rank, indicate the maturity status and mark a territory. In the case of mammals, there is a clear relationship between olfaction and territory, for example, Dunbar (1977) observed that male dogs visited more frequently those urinary signals from estrous females than those signals released by non-estrous females. Even more, male dogs presented fewer visits to urine-signals liberated by other males, being urine and faeces the vehicles that females use to share their maturity status. In the case of fish, dominance rank was assessed using urination during aggressive male-male contests in Mozambique tilapia. Keller-Costa et al. (2012) observed that urine played a key role in chemical communication in this species as differences were found between dominant and subordinate males in the olfactory potency, being more potent in dominant males. Moreover the reproductive status could change depending on the social rank in males, where dominant males were the males with the highest reproductive events. In addition, there existed a morphological modification between males from different dominance category (dominant or subordinate), where dominant males present heavier and thicker urinary bladders than subordinate males or females, demonstrating the importance of urine to signal social status. All these characteristics were accompanied by an increase plasma levels of 11-KT and aggressive behaviour from dominant fish. This exchange of information (social rank and motivation) modulate aggression in the different contest between males. As has been observed in other species, Senegalese sole may use chemical communication to establish dominance and mate selection. The present study has shown that sole use chemical communication and have clear opportunities during the courtship to communicate. Although, not demonstrated such communication would appear logical and can indicate how sole communicate to both select or reject a mate.

## 3. Behavioural analyses:

Previous studies in several species have demonstrated that cultured fish differed from wild conspecifics and that hypothetically behavioural differences occur in three modes, 1) different experiences, 2) different mortality and survival of behavioural phenotypes within a single generation and 3) selection for inherited behavioural traits over several generations (Huntingford, 2004). Besides, taking into account the different results obtained in the previous Chapters and the clear dominance regarding wild males of Senegalese sole in the reproductive behaviour, including the parental analysis, **Chapters 6 and 7** summarise different behavioural aspects which could affect mate selection as it has been demonstrated in other fish species.

### 3.1.The existence of dominance behaviour in Senegalese sole

Previous studies associate the dominance status with reproductive success in different social animals such as chimpanzees (Pusey et al., 1997), where high-ranking females established and preserved access to better foraging regions reducing stress from aggression and this may have helped the dominate females achieve increased levels of reproductive success. There are several primate species in which the hierarchy is based on dominant females and males, where dominant females obtain better conditions than subordinate in several aspects, such as food and water resources (Fedigan, 1983). Continuing with primates, female preferences for sex mates seem to have little to do with the male social status, however, Ellis (1995) suggested that males with the highest and middle rank have at least a reproductive advantage over the lowest ranking males. Primates are not the only social animals which exhibit relationship between dominance and reproductive success. This also occurs in some insects such as ants (Franks and Scovell, 1983). Therefore, many animal species exhibit a situation where dominant individuals achieve greater reproductive success in relation to dominance of access to food resources, food territories and mating selection. Fish are not an exception, Paull et al. (2010) observed that social status developed into a greater reproductive success in males of zebrafish, and that dominant females achieved more offspring with dominant males than subordinates, resulting in some benefits for both sexes in this species.

However, nothing has been previously reported about dominance in Senegalese sole. Therefore, the different behaviours associated with dominance in juveniles in this flatfish species were determined (**Chapter 6**). Different stages (early and late juveniles) were used in order to observe the consistency in dyadic dominance test related to feeding response and territory, despite it is known that dominance in adults is built by early life outcomes (English et al., 2013; Holekamp and Strauss, 2016). Additionally, group test confirmed those dominance behaviours in late juveniles. Three behaviours were determined as the most representative of dominance of feeding in early juveniles, “Approaches”, “Rest the head” and “Swimming above another”. Dominant sole, which ate first displayed more frequently these behaviours towards the subordinate sole. Two of these behaviours, “Approaches” and “Rest the head”, were confirmed in late juveniles that were classified as dominant (ate first) in the feeding response test. In the

territory tests, two variables related to final time remaining in the sand ("Final time") and the order to enter and dominate the preferred area (limited space covered with sand) were classified as the most representative for dominance test related to territory or place preference test (PTT). These behaviours were associated with dominance feeding response test showing that the dominant sole, which ate first and attempted in more occasions to approach, and rest the head in the subordinate sole, also spend more time in the preferred sand space in the last two hours of the PPT and were the individual occupying the preferred sand area when the test finished after 24 hours. Intriguingly, in the dominance group test, which joined both dominance test related to feeding response and Position in relation to the food delivery pipe before feeding (POSITB) also found that dominant fish ate first and occupied the favoured positions closest to the feed delivery pipe and dominate fish displayed more frequently the behavioural indicators (rest the head, approaches and swim above another) towards the subordinate sole. Notably, feeding behaviour was also observed in Senegalese sole, which was similar to that described in other flatfish species following the modal action patterns (MAP) defined as "predation cycle" (Gibson, 2005). These type of dominance tests (feeding response and PPT) have been previously applied to other fish species such as rainbow trout (Øverli et al., 2004), Nile tilapia (Delicio et al., 2006; Silva et al., 2014) or brown trout (Heggenes, 1988), among others. Aggression behaviour is the reason of the establishment of the different hierarchy in most of the salmonid species (MacLean et al., 2000) and zebrafish (Oliveira et al., 2011). However, previous studies demonstrated that Senegalese sole is considered a non-aggressive species (Salas-Leiton et al., 2008).

Furthermore, dominance status is facilitated by neuroendocrine levels (Holekamp and Strauss, 2016). In the present study, two mRNAs related to neurogenesis (*nrd2*) and neuroplasticity (*c-fos*) were differentially expressed between dominant and subordinate (**Chapter 6**). Curiously, dominant and subordinate sole presented similar expression profiles in genes related to aggression behaviour, which were differentially expressed in other teleost species such as zebrafish (Teles et al., 2016) and tilapia (Fitzpatrick et al., 2005; Silva et al., 2014). This characteristic was also observed in Senegalese sole juveniles by Weber et al. (2015), where after different exposure to several stressors some neurotransmitters presented similar expression profile. This situation could be explained because both dominance categories showed the same stress status. Consequently, dominant Senegalese sole juveniles that were first to obtain food and occupied a preferred sandy area also presented different transcriptional activity compared to subordinate sole.

These studies in dominance behaviour have demonstrated the existence of dominance behaviour in Senegalese sole juveniles (early and late) in pairs and groups, moreover some of the behaviours obtained in these studies coincided with those described in the courtship, which was also demonstrated to be dominated by certain fish that dominated the production of progeny.

### 3.2. Stress coping styles associated with brain gene expression in Senegalese sole

Stress coping styles have been shown to play an important role in the population ecology, such as growth, health, resistance to diseases, welfare and reproduction. Senegalese sole have shown different coping styles categories using different factors such as activity, latencies in feeding motivation, latency to first activities in different tests and physiological factors related to stress (Silva et al., 2010; Ibarra-Zatarain et al., 2016). Nevertheless, Ibarra-Zatarain (2015) demonstrated that reproductive success was not related to coping styles in this species. However, in the same study Ibarra-Zatarain (2015) demonstrated that proactive Senegalese sole juveniles exhibited higher growth rates and earlier puberty than reactive sole. Therefore, genetic markers could be useful for a future breeding program to select individuals with desired traits and optimize Senegalese sole cultivation. In **Chapter 7** six genes related to coping styles which were differentially expressed in other fish species in relation to stress coping styles were tested in Senegalese sole juveniles after performing four stress coping styles tests to characterise individuals as proactive, intermediate or reactive (Ibarra-Zatarain et al. 2016). Four of those genes were differentially expressed among coping styles categories (proactive, intermediate and reactive). However, only one gene related to metabolism (*gapdh-2*) presented differences between the extremes of coping style, proactive and reactive. The other 3 genes (*pparß, igf-Ia* and *per1*) related to nutrition, feeding behaviour and daily rhythmicity presented differences between intermediate individuals and the extremes, proactive and reactive. The animals were held in exactly the same conditions in all behavioural tests without exception, therefore, the changes in expression were related to the individual variation in terms of behaviour and behavioural strategies that each sole expressed when faced with the stressful situation.

In the present study no relationship was found between stress coping style and dominance in Senegalese sole juveniles. In other animals, dominance behaviour or social status is associated with coping styles (David et al., 2011; Martins et al., 2011; Favati et al., 2014). Previous studies in several animal species, have shown that coping styles are related to fitness in several aspects, such as growth, fecundity and survival (Wilson et al., 1993; Smith and Blumstein, 2008). For example, David et al. (2011) found a clear relationship between personality and dominance in zebra finches (*Taeniotygia guttata*), where proactive animal stayed in high-rank positions. In the case of fish species, Colléter and Brown (2011) established that the behavioural traits (aggression, activity and boldness) were associated with the position in hierarchy in rainbowfish (*Melanotaenia duboulayi*) males. Another example was rainbow trout, where juvenile individuals from different lineage showed that coping styles could influence social rank in pairs (Øverli et al., 2004). However, the relationship between coping styles and dominance could not be established in Senegalese sole juveniles due to the variability obtained in the data, where dominant animals were not always the proactive sole. Nevertheless, the studies mentioned before established the hierarchy through aggression behaviour as the behavioural trait (aggression syndrome) (Sih et al., 2004). As demonstrated in several studies, Senegalese sole is considered a non-aggressive, social species, with social interactions between fish (Salas-Leiton et al., 2008). Moreover, Senegalese sole juveniles presented more variability when confronted to adaptive activities than breeders (Ibarra-Zatarain et al., 2016), whilst the behaviour of

breeders was more stable over time possibly due to the previous life experience and the participation in factors such as predation or reproduction among others, which could have an effect on fitness as has been demonstrated in other fish species (Groothuis and Trillmich, 2011; Wolf and McNamara, 2012). Hence, the linkage between coping styles and dominance could appear in the breeder stage after the life history traits of these animals develop in competition and cohabitation (Ruiz-Gomez and Huntingford, 2012). Moreover, it is relevant to consider that individual tests may not be illustrative of the general behavioural characteristics of a population and may generate pseudo replication of results when behaviour is evaluated in animals (Castanheira et al., 2013). This was the reason why grouped-tests were applied in both behavioural studies, coping styles and dominance, since Senegalese sole live communally in their natural habitat. Furthermore, it could exist the possibility that definitely Senegalese sole did not present linkage between coping styles and dominance behaviour, as previous studies demonstrated in other animal species such as starlings (*Sturnus vulgaris*) (Boogert et al., 2006), common waxbill (*Estrilda astrild*) (Funghi et al., 2015), barnacle geese (*Branta leucopsis*) (Kurvers et al., 2009), among others. For example, a recent study carried out by Devost et al. (2016) demonstrated that coping styles in black-capped chickadees (*Poecile atricapillus*) based on exploratory, activity and neophilic tests, were not correlated with the dominance rank in the wild. Another example performed by Riebli et al. (2012) with a cichlid fish species (*Neolamprologus pulcher*) did not observe the relationship between coping styles based on aggressive propensity and competition for dominance, mates and territories. This variation in the association of stress coping styles with dominance across different species will depend on the strategies employed by the species. Aggressive species appear to have a stronger relationship between proactivity and dominance whilst the relationship appears to be weaker in social non-aggressive species such as Senegalese sole.

This study demonstrated the existence of the individual variation in the transcriptome related to behavioural traits, which could be applied in aquaculture as a potent tool for future selection program in Senegalese sole.

**Reproductive behaviour, chemical communication and dominance in Senegalese sole**

To sum up, this thesis has improved the understanding of why cultured Senegalese sole males do not perform the courtship and identifies research lines that may lead to a solution to this behavioural reproductive dysfunction, which would enable Senegalese sole farming to control the reproduction of cultured breeders. First of all, this thesis demonstrated that the presence of spawning wild Senegalese sole breeders appeared to stimulate the cultured breeders to learn the process of reproductive behaviours with the finality of reproducing. Moreover, olfaction plays an important role in reproduction, urine was the vehicle through which sole communicated sex, maturity status and induced elevated LH circulation and different upper olfactory rosette gene

expression related to reproduction, immunological, metabolism and nutrition was observed between wild and cultured males. In addition, this thesis established for the first time that Senegalese sole juveniles showed a clear hierarchy (dominance behaviour) related to feeding and territory and behaviours related to dominance coincided with behaviours in the courtship. Moreover, a relationship between coping styles and brain gene expression was established showing individual variation in behaviour was linked with the expression of several genes with important biological functions in Senegalese sole. However, coping styles and dominance were not associated in Senegalese sole juveniles due to the variability obtained in the data, where dominant animals were not always the proactive sole.

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# Section 7: Conclusions

# Chapter 9

# Conclusions

# Conclusions

- The presence of spawning wild Senegalese sole breeders increased the participation of cultured sole breeders in courtship behaviours and reproductive success. Social learning from wild sole was suggested to be the mechanism that enabled cultured sole to increase courtship behaviour.

- Olfactory rosettes from wild and cultured Senegalese sole juveniles did not present differences in structure showing that metamorphosis in cultured sole was correctly performed and that structural differences do not offer a justification for the behavioural dysfunction observed in cultured males.

- Mature cultured Senegalese sole males had different transcriptomic profile in the upper olfactory rosette (UOR) in comparison to mature wild Senegalese sole males. The expression of transcripts related to mucus production and goblet cells differentiation, nutrient sensing and feed intake, and above all transcripts associated with reproduction and olfaction showed the clear difference in the olfactory perception between cultured and wild Senegalese sole males.

- Urine from mature males and females of Senegalese sole gave different EOG responses in cultured Senegalese sole (juveniles / adult) in relation of sex and stage of maturity of both *donor* and *receiver* indicating that sole can distinguish between sex and maturity through olfaction. These results suggested the importance of urine as a possible vehicle for chemical communication of Senegalese sole. Moreover, urine seems to play an important role in the reproduction of Senegalese sole.

- Dominance parameters and behaviours were established in Senegalese sole (early / late) juveniles using two dyadic tests and one group test related to feeding response and place preference. Dominant sole were found to more frequently rest the head and approach subordinate sole and occupied the preferred territories such as an area with sand and positions closest to the feed delivery pipe. Furthermore, two mRNA *c-fos*, related to neuroplasticity and *nrd2* associated with neurogenesis showed down-regulation in dominant and subordinate sole compared to the control group showing the different transcriptional expression between dominance categories.

- Three categories of stress coping styles (proactive, intermediate and reactive) were determined in early Senegalese sole juveniles applying three individual tests and one group test. Four transcripts, one related to metabolism (*gapdh-2*) and three related to feeding behaviour (*pparß, igf-Ia* and *per1*) were differentially expressed among proactive, intermediate and reactive individuals. Moreover, there was no relationship between stress coping styles and dominance behaviour in early Senegalese sole juveniles.

# Section 8: Future Research

# Future Research

The knowledge gained in this thesis is important for future development of sustainable rearing methods for Senegalese sole and should be continued to provide a solution to obtain natural spawns with cultured sole, born and reared in captivity. In addition, the studies on chemical communication and dominance behaviour provide the bases to work towards understanding the mechanisms that both control reproduction and may be involved in the reproductive dysfunction in Senegalese sole. Therefore, it is suggested that future research in social learning, chemical communication and dominance should be aimed at the following aspects:

Social learning:

- Discern whether Senegalese sole juveniles (early life stages) that cohabite with mixed-broodstocks learn the courtship behaviours and increase reproductive success.
- Examine the relationship between the transcriptome and social learning to obtain a marker. For example, *c-fos* that was significantly expressed in dominant fish and is also associated in social learning.

Chemical communication:

- Determine and compare the olfactory sensitivity in wild Senegalese sole (juveniles and adult fish) with cultured specimens.
- Analyse RNA-seq in the lower olfactory rosette (LOR) of wild and cultured mature Senegalese sole males to obtain a global vision of the olfactory gene expression.
- Establish the female and male urine composition by chemistry techniques like HPLC and chromatography to determine the products involved in chemical communication.

Dominance behaviour:

- Establish dominance behaviour related to feeding and territory in Senegalese sole breeders and determine the consistency with behaviours identified in juveniles.
- Examine the relationship between dominance behaviours and reproductive success in Senegalese sole breeders.


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