Universidad de Cantabria

Departamento de Ciencias y Técnicas
del Agua y del Medio Ambiente

TESIS DOCTORAL

Avances en la biología reproductiva y zootecnia del lenguado senegalés (*Solea senegalensis* Kaup, 1858).

Ph.D. THESIS

Advances in the reproductive biology and zootechnics of the senegalese sole (*Solea senegalensis* Kaup, 1858).

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Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons. Ignacio Martín, Inmaculada Rasines, Marcos Gómez, Cristina Rodríguez, Paulino Martínez, Olvido Chereguini. 2014. ***Aquaculture*** **Vol. 424-425, 45-52.** http://dx.doi.org/10.1016/j.aquaculture.2013.12.042

Is it possible to transport *Solea senegalensis* specimens without water?. Ignacio Martín, Jose Ramón Gutierrez, Juan Antonio Martos-Sitcha, Inmaculada Rasines, Cristina Rodríguez, Juan Miguel Mancera, Olvido Chereguini. ***Aquaculture Europe 2014*****. (San Sebastián)**

Surface disinfection of Senegalese sole (*Solea senegalensis*, Kaup 1858) eggs using iodine. Ignacio Martín, Marcos Gómez, Cristina Rodríguez, Inmaculada Rasines, Gloria Gradillas, Olvido Chereguini. 2014. ***Aquaculture Research*** **1-8.** doi:10.1111/are.12632

Reproductive responses of captive Senegalese sole, *Solea senegalensis*, according to the type of feeding and the origin (wild or cultivated) of each gender. Ignacio Martín, Inmaculada Rasines, Cristina Rodríguez, Olvido Chereguini. ***Aquaculture*** (Under Consideration)

Resumen

Resumen

De acuerdo con la normativa de estudios de doctorado de la Universidad de Cantabria en relación a los requerimientos exigidos para aquellas tesis redactadas en un idioma diferente al español, aprobada por Junta de Gobierno de 12 de marzo de 1999 y actualizada a 17 de diciembre de 2012, a continuación se presenta un resumen "suficientemente extenso" del documento original redactado en inglés.

1 Capítulo 1. Introducción

1.1 Exposición de motivos

Los recursos naturales en el planeta tierra son limitados, con una población creciente y que se estima rondará los 9.5 billones en 2050 (UN, 2014), los sistemas de producción de alimento suponen la principal solución para abastecer a la población humana. En este sentido, la acuicultura es una actividad en desarrollo que además de suponer una fuente de alimento, explota un espacio para su desarrollo que en la mayor parte de los casos no compite con otros usos del suelo. Esto implica un gran aumento potencial del espacio para producir alimento, ya que el 70% de la superficie terrestre es agua. Además, la acuicultura marina presenta la ventaja añadida de consumir poca agua dulce para su desarrollo, lo que supone la protección de otro de los grandes recursos limitantes para la humanidad.

Se entiende por Acuicultura: *el cultivo de organismos acuáticos, incluyendo peces, moluscos, crustáceos y plantas acuáticas, lo cual implica la intervención del hombre en el proceso de cría para aumentarla producción, en operaciones como la siembra, la alimentación y la protección de depredadores, etc. Estos serán, a lo largo de toda la*

fase de cría o de cultivo y hasta el momento de su recogida inclusive, propiedad de una persona física o jurídica. Es decir, esta producción implica la propiedad individual o corporativa del organismo cultivado, según la definición de la FAO y de la Comisión Europea (www.fao.org).

La acuicultura comenzó hace 4000 años en China, pero solo hace 50 años que representa una actividad socioeconómica relevante, con más de 12 millones de empleos en todo el mundo. En la actualidad presenta los mismos niveles de producción en toneladas que la pesca extractiva, y si se tienen en cuenta las cantidades destinadas a consumo humano, la acuicultura supera las toneladas producidas. Además, el descenso en las capturas por sobreexplotación, la reducción de tallas de algunas poblaciones, el exceso de subvención de la pesca en muchos países, etc., hace de la acuicultura una alternativa que debe desarrollarse paralelamente a una escrupulosa gestión de las pesquerías para conseguir alcanzar un uso sostenible de los recursos marinos.

Actualmente se explotan unas 600 especies acuáticas en todo el mundo en una gran variedad de sistemas productivos y niveles de tecnificación, utilizando aguas marinas, dulces y salobres. Entre los organismos comestibles cultivados figuran peces, crustáceos, moluscos, anfibios (ranas), reptiles acuáticos (excepto cocodrilos) y otros animales acuáticos (por ejemplo equinodermos, tunicados o cnidarios). Según los datos de la FAO (2014), la producción procedente de la acuicultura se destina casi en su totalidad al consumo humano.

Una de las líneas de trabajo en acuicultura ha sido la diversificación, dirigida a aumentar el número de especies cultivadas en función de las característica de las distintas zonas y en función también de los hábitos de consumo de los distintos mercados a los que vayan dirigidas las especies cultivadas. Actualmente esta línea está siendo muy discutida en los foros profesionales científicos debido al excesivo número

de especies candidatas a dicha diversificación aparecidas en los últimos años. Empieza a cuestionarse si el gran esfuerzo necesario para consolidar una especie a nivel industrial (adaptación a la cautividad, adaptación a la alimentación artificial, reproducción en cautividad, puesta a punto de los protocolos de cultivo larvario, optimización de las tasas de crecimiento y optimización del uso del alimento, así como hacer frente a los problemas de patologías específicos de las especies adaptadas), se justifica para todas las especies propuestas. Parece más razonable aunar esfuerzos en terminar de consolidar especies que se encuentran en la fase final que comenzar con una especie nueva. En este sentido, el lenguado senegalés lleva casi 3 décadas ocupando una posición estratégica para la diversificación, pero ha sido en los últimos 5 años cuando ha dado el salto definitivo a la producción industrial. A pesar de ello continúa presentando limitaciones para ser un cultivo totalmente sostenible.

Las dos principales limitaciones que han retrasado tanto la implantación de esta especie en la industria acuícola, son las patologías y la disfunción reproductiva de los ejemplares nacidos en cautividad (G1, G2,…). Las patologías, frecuentes en los estadios de post-larva y en menor medida en juveniles en fase de engorde, pueden desencadenar grandes episodios de mortalidad (Padrós *et al.*, 2003; Toranzo *et al.*, 2003; Arijo *et al.*, 2005) y son en gran medida consecuencia de la falta de conocimiento de los requerimientos fisiológicos de la especie (Cañavate, 2005). En cuanto a la disfunción reproductiva de los ejemplares nacidos en cautividad, aun hoy se desconocen la causa o causas de dicha disfunción, pero se ha avanzado mucho en el conocimiento de los factores que pueden estar relacionados con esta alteración (Morais et al, 2014). Esta problemática se ha abordado desde numerosos puntos de vista tales como la alimentación, el comportamiento, la epigenética, o la fisiología de la especie.

En la actualidad, y después de años investigando el problema, parece que la falta de puestas naturales en los ejemplares nacidos en cautividad radica en los machos de la

especie, y aunque los motivos no están claros, la ausencia de comportamiento reproductivo podría estar detrás del problema (Carazo, 2013; Morais *et al.*, 2014)

1.2 Ámbito de estudio

Esta tesis se centra en el estudio del lenguado senegalés, *Solea senegalensis*, Kaup 1858, y más concretamente, de esta especie en cautividad. Esta especie pertenece al Tipo: Vertebrata; Subtipo: Gnathosmata; Superclase: Pisces; Clase: Osteichthyes; Orden: Heterosomata (Pleuronectiformes); Suborden: Soleoidei; Familia: Soleidae; Subfamilia: Soleinae; Género: *Solea*; Especie: *Senegalensis*.

Se trata de peces planos, con cuerpo ovalado y asimétrico (ojos en el lado derecho). La membrana interradial de la aleta pectoral en el lado de los ojos es de color negro, esta característica le distingue de lenguado común (*Solea solea*). Se distribuye en el Atlántico, desde el Golfo de Vizcaya hasta las costas de Senegal, y es menos frecuente en el Mediterráneo occidental. Es una especie bentónica, predominantemente del litoral marino, que viven en fondos arenosos y/o fangosos, en zonas costeras hasta 100 m de profundidad. Se alimenta básicamente de invertebrados bentónicos, tales como larvas de poliquetos, moluscos bivalvos y crustáceos pequeños. Alcanza la madurez sexual con tallas alrededor de 30 cm. El desove ocurre entre los meses de marzo y junio (www.fishbase.org).

Un estudio taxonómico detallado realizado por Adam Ben-Tuvia (1990) para clarificar la situación taxonómica de las principales especies del género *Solea* de la región Atlántico-Mediterránea, concluyó las siguientes características para *S. senegalensis*: *Presenta 44-46 vértebras. Últimos radios de aletas dorsal y anal unidas por una membrana que baja hasta la base de la aleta caudal. Rama supratemporal de la línea lateral formando un arco. Fosa nasal anterior en el lado ciego no engrosada, su diámetro es alrededor de la mitad que la de lado ocular, situada cerca del margen frontal de la cabeza, esta distancia es ligeramente mayor que la separación entre la*

ventana de la nariz y la hendidura bucal, (proporción 1:1-1.4). Longitud de escamas sobre el tubo nasal en el lado ocular mucho menor que la longitud del tubo. Aleta pectoral del lado ocular con mancha negra sobre toda la parte media y distal de la aleta, la aleta del lado ciego es blanquecina.

2 Capítulo 2. Objetivos

Los objetivos de esta tesis están dirigidos a 1) ampliar el conocimiento sobre aspectos de la biología reproductiva de la especie en cautividad, 2) tratar de conocer porque los ejemplares nacidos en cautividad no se reproducen de manera natural, y 3) aportar mejoras a la zootecnia de la especie.

Para el primer objetivo se realizó un estudio detallado de las puestas naturales de un stock de reproductores salvajes compuesto por 77 individuos estabulados en tres tanques de 14 m^3, sometidos a manipulación de termoperiodo para inducir la maduración sexual y las puestas. Dicho estudio tuvo una duración de cuatro años. Durante este periodo se realizó un estudio de parentesco de todas las puestas fecundadas de los tres tanques con el fin de determinar que ejemplares del stock participaban en las puestas, en qué proporción y si existía alguna pauta en dichos cruces.

Para el segundo objetivo se llevaron a cabo dos experiencias, la primera consistió en un estudio equivalente al realizado en salvajes, pero con ejemplares nacidos en cautividad de primera generación alimentados con dos dietas distintas, para valorar la influencia del alimento en el éxito reproductivo de los ejemplares cultivados de esta especie. El stock utilizado para este estudio estuvo compuesto por 32 ejemplares distribuidos 2 tanques de 7 m^3 uno de los cuales se alimento con pienso comercial de reproductores y otro con alimento natural (mejillón, calamar y poliqueto). La segunda

experiencia consistió en estabular hembras salvajes con machos cultivados por un lado, y hembras cultivadas con machos salvajes por otro, para determinar así, si las disfunciones reproductivas de los ejemplares cultivados afectaban por igual a ambos sexos o si se trataba de un problema específico de alguno de los sexos.

En cuanto al tercer objetivo, dirigido a mejorar el manejo de la especie en cautividad, se abordaron 2 aspectos distintos. Por un lado la desinfección de huevos fecundados, y por otro el transporte de ejemplares vivos de esta especie.

En relación a la desinfección de huevos, se realizaron experimentos con Iodo, para determinar la dosis letal de esta sustancia para los huevos fecundados de esta especie. Se realizó un análisis de la eficacia desinfectiva de este método y se valoró como afectaba el uso de este compuesto en las tasas de eclosión y supervivencia larvaria de la especie. Además se llevó a cabo un análisis a nivel específico de la flora bacteriana presente en puestas naturales de la especie en nuestras instalaciones.

En cuanto al transporte de ejemplares vivos se realizó un estudio para determinar el tiempo máximo que esta especie sobrevive fuera del agua en condiciones de humedad ambiental saturada, evaluar los niveles de estrés originados por el transporte en estas condiciones, y valorar el tiempo de primera ingesta tras dicho transporte.

3 Capitulo 3. Evolución de la producción de huevos y contribución parental en Lenguado senegalés, *Solea senegalensis*, durante cuatro estaciones de puesta.

3.1 Justificación

El sentido de este capítulo de tesis fue llevar a cabo una descripción lo más detallada y completa posible de la reproducción natural de esta especie en cautividad. Como ya se ha comentado en otros momentos de esta tesis, esta especie presenta un desorden reproductivo en los ejemplares nacidos en cautividad que continua sin resolverse, a pesar de los numerosos esfuerzos llevados a cabo en esta línea por numerosos centros de investigación y universidades, sobre todo de España y Portugal.

En esta línea, este capítulo recoge información de las puestas naturales registradas y valoradas entre 2007 y 2010, ambos incluidos, así como los resultados individuales de apareamiento derivados del estudio de asignación parental de una muestra de larvas de cada puesta recogida en ese periodo.

3.2 Material y métodos

Para este estudio se utilizó un stock salvaje de lenguado senegalés, adaptado a cautividad desde el 2002 (N = 77 ejemplares), estabulado en 3 tanques de 14 m^3 (B1, B2 y B3). El peso medio ± SE de las hembras y los machos al inicio del estudio (Enero de 2007) fue 1677 ± 105 g y 966 ± 68 g en el tanque B1 (N= 23), 1970 ± 65 g y 1179 ± 91 g en el tanque B2 (N= 25), y 1948 ± 86 g y 1190 ± 55 g en el tanque B3 (N= 29), respectivamente. La alimentación fue natural a base de mejillón, chipirón y gusana, 6 días a la semana y la ración diaria se ajustó al 1% de la biomasa del tanque. Mensualmente se realizaron muestreos de peso y longitud individual, y se determinó el grado de maduración de las hembras.

Los tanques estuvieron en el interior de una nave industrial, en circuito abierto, con una tasa de renovación del agua de 1,7 m^3/hora y aireación constante moderada. La

salinidad media registrada durante el estudio fue de 34,6 ± 0,87 PSU. Se utilizó un fotoperiodo artificial de 16 horas de luz y 8 de oscuridad durante todo el año. La intensidad de la luz en los tanques se redujo mediante el uso de mallas de sombreado sobre los tanques que permitían una intensidad máxima de luz en la superficie del agua de 50 lux.

Para inducir la maduración sexual de los ejemplares y las puestas, a partir de mediados de febrero comenzó a manipularse el termoperiodo. La temperatura se incrementó 0,5º C por semana hasta alcanzar los 16º C, y a partir de esa temperatura se realizaron fluctuaciones de +2 y -2ºC cada 3 y 4 días respectivamente. La manipulación del termoperiodo finalizó a mediados/finales de junio, al igualarse la temperatura ambiental del agua de mar con la temperatura del agua manipulada. Las puestas del verano y otoño se obtuvieron por las fluctuaciones naturales de la zona. La calidad de las puestas se valoró diariamente: volumen total (mL); volumen flotante y no flotante (mL); tasa de fecundación y eclosión. La tasa de fecundación se determinó, a partir de una muestra de la fracción flotante de 200 µL en placa Bogorov, como el nº de huevos fecundados por nº total de huevos, contando un mínimo de 200 huevos. A continuación la fracción flotante se llevó a incubadores de 70 litros con agua de mar filtrada (1µm) a 18º C y con una renovación de 1,5 L/min. Tras 24 y 48 h de incubación, se determinó la tasa de eclosión como el nº total de larvas nacidas por el nº total de huevos flotantes, contando como mínimo tres submuestras de 100 mL. A partir de los datos obtenidos se calculó la fecundidad relativa diaria, como el nº de huevos obtenidos por Kg de hembra para cada puesta natural.

Para el análisis genético de la descendencia, previamente hubo que realizar un estudio de parentesco de los reproductores y una redistribución de los ejemplares, con el doble objetivo, por un lado de reducir al máximo los problemas asociados a la consanguinidad, y por otro a favorecer al máximo el éxito a la hora de asignar el parentesco a cada larva estudiada. Para este objetivo se tomaron muestras de la aleta

caudal de todos los ejemplares y se realizo la extracción de ADN usando el método descrito por Walsh *et al.*, (1991), usando resina sintética Chelex 100. La caracterización del stock se realizó mediante la amplificación de los siguientes loci microsatélites: F13-7, Smax-02, SseGATA38 y CA13, mediante PCR multiplex. Usando la información obtenida, se estimo el coeficiente de parentesco (r) por parejas de todos los ejemplares usando el estimador de Wang (Wang, 2000.). A partir de estos resultados, los ejemplares se distribuyeron en tres tanques atendiendo a que no presentaran coeficientes de parentesco molecular superiores a 0,25, lo que correspondería con un valor medio de medios hermanos. Con los reproductores estabulados en base a estos criterios, el segundo paso fue llevar a cabo la asignación parental de las larvas nacidas durante el periodo de estudio. Para ello, de todas las puestas registradas con un volumen flotante mayor o igual a 20 mL, e incubadas como se describió anteriormente, se analizaron 10 larvas utilizando los mismo microsatélites y métodos descritos para los reproductores. Durante este periodo se asignaron un total de 3630 larvas de 363 puestas.

Los peces siempre se manejaron (gestión de rutina y experimentación) de acuerdo con la Directiva de la Unión Europea (CEE, 1986) para la protección de los animales utilizados para experimentación y otros fines científicos.

Los datos obtenidos fueron analizados mediante un análisis de la varianza (ANOVA) previo análisis de la normalidad (Kolmogorov-Smirnov) y homogeneidad de varianzas (Levene), y cuando hubo significación ($p<0,05$) se aplicó el test de Tukey para comparar las diferencias entre grupos. Algunos datos se normalizaron mediante transformación logarítmica o angular; y cuando los datos no fueron normales, se analizaron mediante análisis de la varianza no paramétrico Kruskal-Wallis.

3.3 Resultados:

3.3.1 Puestas y calidad de los huevos.

La fecundidad relativa diaria de los tanques (B1, B2 y B3) a lo largo de los cuatro años de estudio y el termoperiodo al que fueron sometidos están representados en la Figura 3.1. Como se muestra en dicha figura el periodo de puesta duró entre 6 y 8 meses dependiendo del tanque y año, y se encontró una gran variabilidad entre las puestas (de 734 a 34874 huevos por Kg de hembra). No se encontraron diferencias significativas en la fecundidad relativa media ni en la tasa de fecundación entre los distintos años de estudio (Tabla 3.1.). Si hubo diferencias en la proporción de huevos flotantes, superior en 2008, y en la tasa de eclosión, significativamente menor en 2007. El número total de larvas nacidas y el volumen flotante de huevos presentó los valores más bajos en 2007, seguido de dos años de producción regular (2008 y 2009), en 2010 se produjo un descenso respecto de los años anteriores.

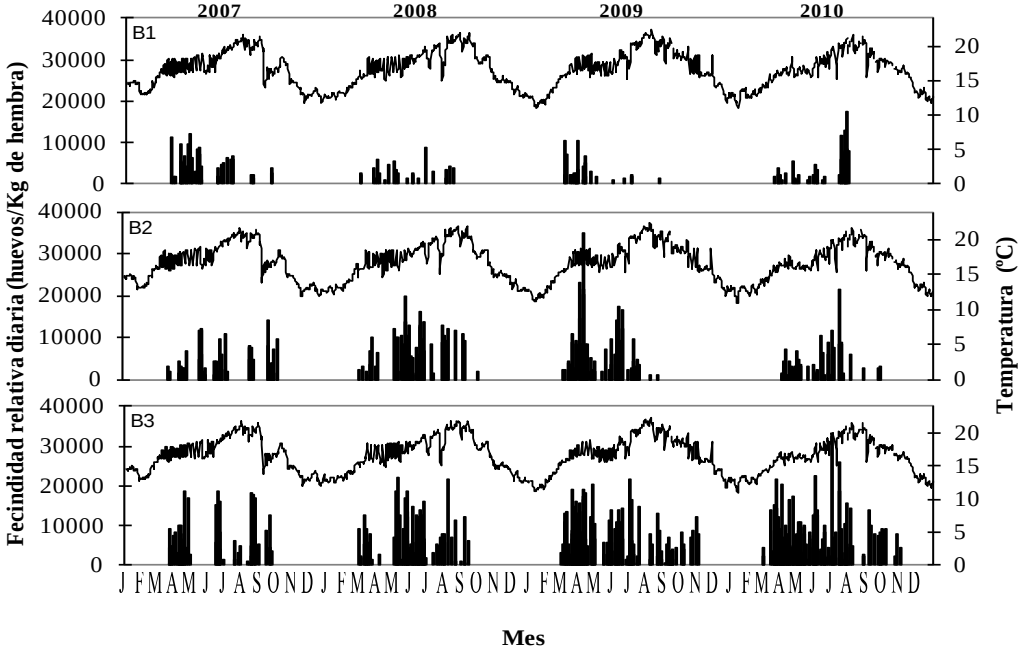

Figura 3.1. Fecundidad relativa diaria y régimen de temperatura de los tres tanques de reproductores a los largo del periodo de estudio.

Tabla 3.1. Resumen de los parámetros productivos anuales del stock de reproductores. Las letras en superíndice indican diferencias significativas en un parámetro entre distintos años. MRFec: significa fecundidad relativa media.

Año	Volumen total (mL)	Volumen flotante (mL)	Porcentaje de volumen flotante (%)	MRFec (10^3 huevos / kg de hembra ± SE)	Numero de puestas	Tasa de fecundación ± SE	Tasa de eclosión ± SE	Larvas producidas
2007	16710	7115	35,05±2,49^{b}	5,72±0,39	124	77,35±2,42	49,45±4,83^{b}	3694961
2008	23755	13242	45,68±2,64^{a}	6,68±0,41	143	78,46±2,93	65,22±2,99^{a}	8812167
2009	30999	13432	37,32±2,12^{b}	6,97±0,41	185	68,03±3,18	64,28±2,91^{a}	8457883
2010	23793	9653	35,00±2,04^{b}	6,44±0,39	181	71,70±3,07	63,15±3,59^{a}	5909869
Total	**95257**	**43442**	**38,1±1,16**	**6,51±0,2**	**633**	**73,28±1,54**	**61,56±1,76**	**26874880**

Se encontraron diferencias significativas en los parámetros de calidad de los huevos entre los tres tanques (ANOVA $p<0.05$). Como muestra la Tabla 3.2., la fecundidad relativa media, la tasa de fecundación y de eclosión fue en todos los casos mayor en el tanque B3. Entre el tanque B1 y B2 solo se encontraron diferencias significativas en la fecundidad relativa, que fue mayor en el B2. El 75% de las larvas nacidas durante el periodo de estudio fueron resultado de puestas del tanque B3.

Tabla 3.2. Resumen de los parámetros productivos de los distintos tanques del stock de reproductores. Las letras en superíndice indican diferencias significativas en un parámetro entre distintos tanques. MRFec: significa fecundidad relativa media.

Tanque	Volumen total (mL)	Volumen flotante (mL)	Porcentaje de volumen flotante (%)	MRFec (10^3 huevos / kg de hembra ± SE)	Número de puestas	Tasa de fecundación ± SE	Tasa de eclosión ± SE	Larvas producidas
B1	8819	3975	37,50±0,13^{a,b}	4,26±0,30^{c}	108	58,43±4,81^{b}	45,08±6,03^{b}	1323256
B2	24085	9407	31,44±2,02^{b}	6,06±0,36^{b}	194	66,04±3,15^{b}	56,68±3,22^{b}	5799625
B3	62355	30060	42,20±1,53^{a}	7,51±0,30^{a}	331	80,64±1,67^{a}	66,25±2,14^{a}	19752006
Total	**95257**	**43442**	**38,1±1,16**	**6,51±0,2**	**633**	**73,28±1,54**	**61,56±1,76**	**26874880**

Con los resultados de las 2 hembras más productivas del stock (hembra 14 y 35), se llevo a cabo un estudio individual del diámetro de los huevos de sus puestas. Los resultados muestran una correlación moderada negativa del diámetro respecto del tiempo, -0,362 (p =0,000) para la hembra 14 y -0,426 (p=0,000) para la 35,

observándose por tanto una tendencia a la disminución del diámetro a lo largo del periodo de puesta. Además, el estudio de regresión de los datos reveló un ajuste a un modelo cúbico, indicando recuperaciones parciales del diámetro.

3.3.2 Análisis genético y contribución parental.

La diversidad genética del stock completo de reproductores (ejemplares del B1, B2 y B3 analizados conjuntamente) encontrada a partir del estudio de 4 loci microsatélites fue muy alta, con 18 alelos por locus y una heterocigosidad esperada y observada de 0,875 y 0,866 de media, respectivamente. Las frecuencias estimadas de alelos nulos del stock se situaron por debajo de 0,05 para todos los loci, en consonancia con resultados de estudios anteriores (Castro *et al.*, 2006), lo que apoya la utilidad de este set de marcadores para asignación parental de la descendencia. Como se esperaba, los valores de parentesco por parejas del stock de reproductores fueron muy bajos en la mayoría de los casos, ya que se trataba de un stock salvaje no emparentado, y solo el 2,7% de las parejas resulto emparentada a nivel de hermanos. Con esta situación de partida, el potencial teórico de inferencia de la paternidad fue muy alto, siendo la probabilidad de exclusión Excl1 0,975 y Excl2 0,996 (Tabla 3.3.). Además, este potencial fue aún mayor debido a las condiciones de cultivo en nuestro estudio, ya que los reproductores se subdividieron en tres tanques, por lo que el número de cruces potenciales se redujo. En este escenario, las 3630 larvas analizadas a lo largo de las cuatro temporadas de puesta fueron asignadas a sus progenitores con plena confianza.

Tabla 3.3. Estimadores de diversidad genética, probabilidades de exclusión y frecuencias de alelos nulos para los 4 loci microsatelites. k: número de alelos, H(O): heterocigosidad observada, H(E): heterocigosidad esperada, PIC: información del contenido polimórfico, Excl(1): exclusión de un progenitor, Excl(2): exclusión de dos progenitores. Todos los cálculos se llevaron a cabo con el software CERVUS 2.0.

Locus	k	N	H(O)	H(E)	PIC	Excl(1)	Excl(2)
CA13	14	80	0,863	0,866	0,846	0,566	0,724
F13-7	19	80	0,85	0,884	0,868	0,617	0,764
M2	16	80	0,863	0,841	0,82	0,525	0,691
Sse38	23	80	0,887	0,909	0,896	0,68	0,809
Media/Total	**18**	**80**	**0,866**	**0,875**	**0,858**	**0,975**	**0,996**

A partir de los resultados genéticos se encontró que las hembras activas representaban una pequeña proporción del total de hembras estabuladas en cada tanque (entre el 8 y el 57%). Algunas de estas hembras mantuvieron la dominancia a lo largo del periodo de estudio (Tabla 3.4.). Los machos mostraron un patrón similar, entre un 10 y un 60% de los ejemplares fueron activos, y las dominancias se mantuvieron también durante el periodo de estudio. En la Tabla 3.4. se pueden observar patrones de reproducción y fidelidad entre las parejas activas a lo largo de las distintas temporadas de puesta. Todos los tanques mostraron parejas dominantes (H_M), como por ejemplo la 14_48, 35_57, 36_60, 36_57, 37_60 o 37_58 con al menos tres años de fidelidad.

Atendiendo al número de reproductores implicados en cada puesta, la Tabla 3.5. muestra que la mayoría de puestas (61,7%) fueron resultado del cruce de parejas únicas, el 31,5% de las puestas resultó del cruce de varias parejas, el 3,9% del cruce de una hembra con varios machos, y el 2,9% del cruce de varias hembras con un solo macho.

Tabla 3.4. Resumen de las parejas registradas en cada tanque y año a través de la asignación parental de las larvas nacidas durante el estudio. En cada pareja se muestra el numero de puestas (N) y el volumen de huevos derivados de estas puestas. Durante el estudio murieron los siguientes ejemplares: B1(**17** en Oct-09; **4** en May-10), B2 (**26** en Jul-08; **13** en Dec-08; **3** en Jul-10) y B3 (**56** en Jul-08; **54** en Dec-09).

	2007				2008				2009				2010			
Tanque	Hembra	Macho	N	Volumen	Hembra	Macho	N	Volumen	Hembra	Macho	N	Volumen	Hembra	Macho	N	Volumen
	17-	**71**	9	615	**17-**	**71**	6	336	**10-**	**71**	1	120	**10-**	**74**	4	185
	17-	**66**	9	210	**4-**	**71**	1	11					**10-**	**67**	2	108
B1					**10-**	**67**	1	22					**15-**	**71**	2	176
													15-	**67**	1	64
													12-	**71**	3	45
													4-	**70**	1	15
	14-	**48**	5	498	**14-**	**23**	23	3211	**14-**	**48**	17	1358	**14-**	**48**	9	397
	14-	**79**	1	110	**14-**	**43**	4	423	**14-**	**43**	2	183	**14-**	**79**	5	153
	26-	**23**	1	32	**41-**	**21**	2	99	**14-**	**79**	4	142	**14-**	**43**	2	56
	26-	**18**	1	24	**41-**	**43**	1	17	**14-**	**62**	2	13	**41-**	**48**	4	126
					7-	**53**	1	48	**3-**	**24**	1	160	**7-**	**18**	1	6
B2					**7-**	**48**	1	20	**41-**	**21**	2	127				
					7-	**43**	1	17	**41-**	**48**	2	77				
					13-	**79**	1	28	**41-**	**43**	2	46				
					42-	**48**	1	17	**7-**	**24**	1	60				
					25-	**48**	1	9	**7-**	**62**	1	60				
									7-	**43**	1	22				
	35-	**57**	11	838	**35-**	**57**	25	2307	**35-**	**57**	55	4844	**35-**	**57**	63	5076
	35-	**56**	4	264	**35-**	**56**	12	690	**35-**	**58**	1	49	**35-**	**58**	1	32
	36-	**60**	6	621	**35-**	**60**	3	169	**35-**	**60**	1	30	**35-**	**80**	2	22
	36-	**57**	4	439	**35-**	**58**	1	24	**36-**	**60**	1	1653	**36-**	**60**	18	1355
	37-	**56**	7	449	**36-**	**60**	14	1589	**36-**	**57**	1	224	**36-**	**58**	2	30
	37-	**57**	3	274	**36-**	**80**	1	64	**36-**	**58**	1	24	**37-**	**60**	4	296
	37-	**49**	3	236	**36-**	**57**	1	28	**36-**	**80**	1	12	**37-**	**58**	2	48
	37-	**60**	3	171	**36-**	**46**	1	16	**37-**	**60**	8	593	**38-**	**58**	1	38
	37-	**58**	2	134	**37-**	**56**	17	1132	**37-**	**80**	5	440				
	37-	**80**	1	14	**37-**	**60**	2	187	**37-**	**49**	2	220				
	54-	**60**	1	8	**37-**	**57**	1	125	**37-**	**58**	5	119				
B3					**37-**	**58**	1	63	**37-**	**57**	3	68				
					37-	**80**	1	40	**38-**	**58**	2	230				
					37-	**45**	1	16	**38-**	**60**	4	142				
					38-	**57**	1	56	**38-**	**49**	1	100				
					38-	**56**	1	16	**38-**	**80**	1	16				
					38-	**80**	1	16	**54-**	**58**	1	32				
					54-	**51**	2	46	**54-**	**60**	1	26				
					54-	**56**	1	28	**33-**	**46**	1	22				
					27-	**56**	1	40								
					27-	**57**	1	33								
					39-	**56**	1	19								
					34-	**45**	1	4								

Tabla 3.5. Clasificación de las puestas de cada tanque en función de estar producidas por: un macho y una hembra, varios machos y hembras, varios machos y una hembra o varias hembras y un macho. Los resultados se presentan como tanto por ciento respecto al número total de puestas.

	Tanque			
Ejemplares que intervinieron en las puestas	**B1**	**B2**	**B3**	**Total**
Un macho y una hembra	77,78	71,23	56,40	**61,74**
Varios machos y hembras	18,52	15,07	38,86	**31,51**
Varios machos y una hembra	3,70	5,48	3,32	**3,86**
Varias hembras y un macho	0,00	8,22	1,42	**2,89**

La Figura 3.2. muestra la fecundidad relativa diaria y el termoperiodo de las hembras más productivas, que formaban parte de las parejas dominantes (14, 35, 36 y 37).

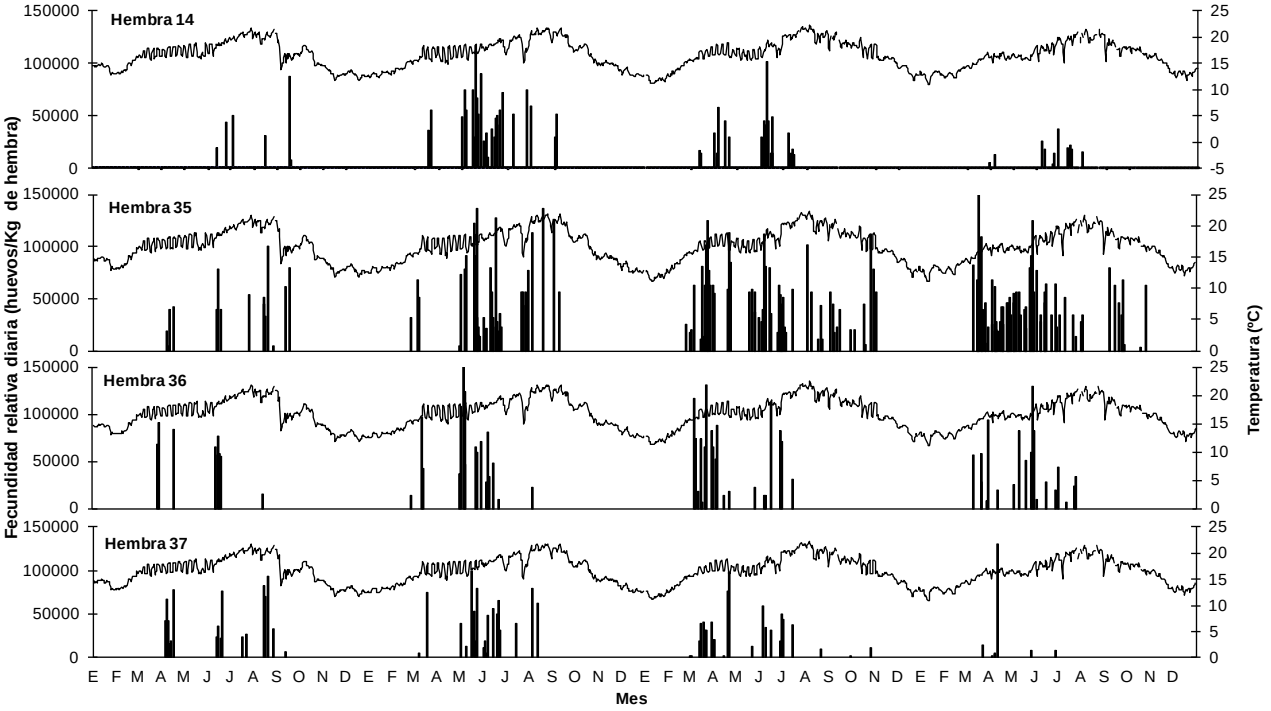

Figura 3.2. Fecundidad relativa diaria y régimen de temperatura de cuatro hembras dominantes durante los cuatro años de estudio. La línea continua representa el termoperiodo y las barras representan la fecundidad relativa diaria.

La Tabla 3.6. muestra los patrones reproductivos de estas hembras cada año. Los periodos de puesta variaron entre 96 y 252 días, y el número de puestas por hembra entre 6 y 63. La frecuencia media de puesta del conjunto de estas hembras fue de 7,3 ± 0,6 días variando individualmente entre 3,7 y 19,8 días. El mínimo intervalo entre puestas consecutivas de una misma hembra fue de 1 día, y el máximo de 56 días.

Tabla 3.6. Resumen de parámetros reproductivos de las hembras más productivas. La duración del periodo, el intervalo medio de puesta y la diferencia máxima y mínima entre puestas están expresados en días.

Hembra	Año	Periodo de puesta		Duración del periodo	Nº puestas	Intervalo medio de puesta ± SE	Mínima diferencia entre puestas	Máxima diferencia entre puestas
14	2007	12/06/2007	19/09/2007	99	6	19,8±7,9	2	43
	2008	18/03/2008	04/09/2008	170	26	6,8±2	1	41
	2009	11/03/2009	14/07/2009	125	21	6,3±2,3	1	42
	2010	30/03/2010	31/07/2010	123	13	10,3±3,2	2	37
35	2007	08/04/2007	18/09/2007	163	15	11,6±4,4	1	56
	2008	26/02/2008	09/09/2008	196	36	5,6±1,7	1	53
	2009	24/02/2009	03/11/2009	252	56	4,6±0,7	1	23
	2010	11/03/2010	25/10/2010	228	63	3,7±0,7	1	34
36	2007	28/03/2007	14/08/2007	139	9	17,4±8,6	1	55
	2008	26/02/2008	04/08/2008	160	17	10±3,7	1	47
	2009	07/03/2009	14/07/2009	129	20	6,8±1,8	1	34
	2010	11/03/2010	24/07/2010	135	19	7,5±1,3	1	21
37	2007	06/04/2007	12/09/2007	159	17	9,9±3,7	1	56
	2008	07/03/2008	11/08/2008	157	20	8,3±2,4	1	43
	2009	01/03/2009	26/10/2009	239	23	10,9±2,5	2	38
	2010	24/03/2010	28/06/2010	96	7	16±7,5	1	44

3.4 Discusión:

En términos de la fecundidad relativa, los resultados obtenidos en este estudio fueron menores que los obtenidos por Anguis y Cañavate en 2005, y equivalentes a los obtenidos por Dinis en 1986. Este trabajo pone de manifiesto la relativa importancia de este parámetro, que si bien resulta útil a nivel de comparación con trabajos anteriores, aporta escasa información real de lo que sucede en el tanque de cultivo

debido a la baja proporción de hembras que realmente contribuyen a la producción de huevos dentro del stock (siempre menor del 60% en este estudio). Las tasas de fecundación fueron mayores que las publicadas por los autores citados, y las tasas de eclosión mostraron valores similares.

Un resultado destacable de este trabajo es la ampliación del periodo de puesta de esta especie descrito por otros autores tanto en cautividad como en medio natural. El periodo descrito en condiciones de cultivo comprende de 4 a 6 meses en primavera-verano con alguna puesta aislada durante el otoño (Andrade, 1990; Anguis y Cañavate, 2005; Dinis, 1986). En medio natural el periodo descrito es aún menor, 3 meses según Vinagre *et al.* (2013). En este estudio, y sometidos a las condiciones de cultivo detalladas en la metodología se han registrado hasta 8,4 meses de actividad reproductiva regular (tanque B3 en 2009).

En relación a la respuesta reproductiva a nivel de tanque, se encontraron diferencias significativas entre los distintos tanques con parámetros productivos más de 10 veces mayores en algunos tanques. Este hecho destaca la importancia individual en los resultados globales de un stock de reproductores. Los resultados obtenidos en este trabajo, similares a los obtenidos por Anguís y Cañavate (2005), muestran que el porcentaje de machos realmente implicados en las puestas de los tanques varió entre el 10% y el 60%, con una media de participación del 47,1%. A pesar de estos porcentajes relativamente altos, tras el análisis de la descendencia de todas las puestas analizadas, podemos confirmar que más del 50% de la producción de un tanque es a menudo resultado de una única pareja. Estos resultados coinciden con los obtenidos por Mira *et al.* (2010) en *S. senegalensis* y los obtenidos por Blonk *et al.* (2009) o Guarniero *et al.* (2010) en *S. solea*.

Respecto a la contribución parental a la descendencia en *S. senegalensis*, a diferencia de otras especies como el bacalao (*Gadus morhua*), o la dorada (*Sparus aurata*) con

puestas masivas multimaternales y multipaternales (Herlin *et al.*, 2008; Chavanne *et al.*, 2012), la mayoría de las puestas fueron el resultado del cruce de un único macho con una única hembra (61,74%), lo que destaca de nuevo la importancia individual en la biología reproductiva de esta especie. Estos resultados coinciden con los patrones de comportamiento reproductivo descritos por Carazo (2013), quien describe en el cortejo de esta especie como los dos individuos que se reproducen nadan muy cerca y coordinadamente. El otro gran grupo de puestas (31,5%) asignadas a varios machos y varias hembras, basándonos en los resultados de Carazo (2013), se atribuye a varias parejas que habrían realizado varios encuentros aislados más que una puesta colectiva de varios machos y hembras.

En el estudio de los estadíos de maduración de las hembras más prolíficas de stock, los resultados coinciden con los obtenidos por García-López *et al.* (2006a), registrándose los estadíos máximos en los meses de invierno y los mínimos en los de verano. Estos resultados son consecuentes con el patrón de puesta asincrónico descrito por Rodriguez (1984).

El estudio de asignación parental ha permitido aumentar el conocimiento sobre los ritmos de ovulación de las hembras. Los resultados obtenidos indican que las hembras de esta especie son capaces de ovular hasta 6 días consecutivos (hembra 35 en 2008 y 2009). Además se ha definido una frecuencia media de ovulación de 7,3 ± 0,6 días, lo que puede ser de aplicación la hora de desarrollar protocolos de inducción hormonal o previsiones de producción.

En resumen, los resultados obtenidos en este estudio, y en especial los patrones de fidelidad descritos así como las respuestas reproductivas individuales detalladas, contribuyen a un mayor entendimiento de la biología reproductiva de esta especien en cautividad, y muestran la necesidad de continuar estudiando otros factores para mejorar la respuesta reproductiva tales como la proporción macho-hembra óptima, el

número mínimo de reproductores necesarios para maximizar la producción, o determinar el papel de los ejemplares que no intervienen en la reproducción.

4 Capitulo 4. Respuestas reproductivas en lenguado senegalés, *Solea senegalensis*, en relación con el tipo de alimentación, y el origen (cultivado o salvaje) de cada género.

4.1 Justificación

Las disfunciones reproductivas de ejemplares cultivados (G1) de lenguado senegalés en cautividad han sido ampliamente mencionadas en la literatura científica, pero no ha sido igualmente descritas .Existen datos de puestas natural de ejemplares nacidos en cautividad, y de ejemplares cultivados sometidos a diferentes terapias hormonales (Agulleiro *et al.*, 2006; Mañanos *et al.*, 2007; Martín *et al.*, 2007; Anguis *et al.,* 2007; Guzmán *et al.*, 2008 y 2009), pero no se conoce con detalle la evolución de la producción de un stock cultivado de S*olea senegalensis* a lo largo de varias temporadas de puestas, a pesar de ser un problema claramente identificado (Cañavate, 2005; Howell *et al.*, 2009; Morais *et al.*, 2014). El estudio de la reproducción natural de los ejemplares nacidos en cautividad de esta especie, persigue, reducir la dependencia de los ejemplares salvajes para poder conseguir un control total de la reproducción de esta especie en cautividad y conocer cuál o cuáles son los motivos que impiden la obtención de puestas naturales fecundadas de los ejemplares G1.

Desde el principio de la identificación de la disfunción reproductiva del lenguado se apuntó a la alimentación como un posible responsable, ya que con mucha frecuencia los ejemplares salvajes adaptados a la cautividad se alimentan con alimento natural (calamar, gusana o mejillón) y los ejemplares cultivados se alimentan desde el nacimiento hasta que se estabulan con fines reproductivos con piensos comerciales.

Numerosos estudios demuestran la relación entre la calidad del alimento y la calidad de los gametos (Springate *et al.,* 1985; Bennetau-Pelissero y Kaushik, 2001; Rungruangsak-Torrissen y Fosseidengen, 2007; Henrotte *et al.,* 2008). Otro punto de vista para esclarecer el origen de las disfunciones reproductivas de esta especie ha sido el estudio de su comportamiento en cautividad (Carazo et al. 2009, Carazo, 2013).

Este capítulo tuvo como objetivo, por un lado determinar la influencia de la alimentación en la respuesta reproductiva del lenguado senegalés cultivado (G1), y por otro lado tratar de determinar si la ausencia de puestas fecundadas afecta a ambos sexos por igual o no.

4.2 Material y Métodos

Para este estudio se utilizó un stock inicial de lenguado senegalés cultivado estabulado en la Planta de Cultivos Marinos de El Bocal (Santander). Para valorar la influencia de la dieta se utilizaron 32 ejemplares distribuidos en dos tanques de 7 m^3 (LP5 y LP6). El peso medio ± SE de las hembras y los machos al inicio del estudio en el tanque LP5 (N=16) fue de 1277,63 ± 95,32 g y 1,234 ± 76,48 g, y en el tanque LP6 (N=16) de 1576,63 ± 102,97 g y 1,499 ± 85,73 g respectivamente. La alimentación del tanque LP5 fue natural a base de mejillón, chipirón y gusana, 6 días a la semana y la ración diaria se ajustó al 1% de la biomasa del tanque. El tanque alimentado con dieta comercial, LP6, se alimentó 6 días a la semana con Vitalis Cal 9 Skretting S.L., y la cantidad de alimento se ajustó mensualmente al 0,5% de la biomasa del tanque.

Para valorar la influencia del origen, salvaje o cultivado, de cada género se utilizaron 32 ejemplares distribuidos en dos tanques de 7 m^3 (LP2 y LP3). En el tanque LP2 se estabularon conjuntamente hembras cultivadas con machos salvajes, y en el tanque LP3 hembras salvajes con machos cultivados. El peso medio ± SE de las hembras y los machos al inicio del estudio en el tanque LP2 (N=16) fue de 1606,13 ± 86,85 g y 1280,43 ± 87,74 g y en el tanque LP3 (N=16) de 1107,80 ± 178,11 g y 1101 ± 66,95 g

respectivamente. La alimentación fue natural a base de mejillón, chipirón y gusana, 6 días a la semana y la ración diaria se ajustó al 1% de la biomasa del tanque.

Todos los tanques estuvieron compuestos por reproductores con una relación macho: hembra de 1:1 y una densidad media en los tanques de 3 kg/m^2. Los tanques estuvieron en el interior de una nave industrial, en circuito abierto, con una tasa de renovación del agua de 1,7 m^3/hora y aireación constante moderada. La salinidad media registrada durante el estudio fue de 34,69 ± 0,77 PSU. Durante todo el año se utilizó un fotoperiodo artificial de 16 horas de luz y 8 de oscuridad. La intensidad de la luz en los tanques se redujo mediante el uso de mallas de sombreado sobre los tanques que permitían una intensidad máxima de luz en la superficie del agua de 50 lux. Los ejemplares que murieron durante el periodo de estudio, fueron sustituidos por otro de similares características (siempre con más de 4 años). Todos los individuos se muestrearon de talla y peso una vez al mes, después del muestreo los tanques recibieron un baño de peróxido de hidrógeno profiláctico (80 ppm durante una hora sin renovación).

Tanto la manipulación del termoperiodo para inducir la maduración de los ejemplares y obtener así las puestas naturales, como la valoración de las puestas obtenidas, se realizó como se ha descrito en el capitulo anterior.

Los peces siempre se manejaron (gestión de rutina y experimentación) de acuerdo con la Directiva de la Unión Europea (CEE, 1986) para la protección de los animales utilizados para experimentación y otros fines científicos.

Los datos obtenidos fueron analizados mediante un análisis de la varianza (ANOVA) previo análisis de la normalidad (Kolmogorov-Smirnov) y homogeneidad de varianzas (Levene), y cuando hubo significación ($p<0,05$) se aplicó el test de Tukey para comparar las diferencias entre grupos. Algunos datos se normalizaron mediante transformación

logarítmica o angular; y cuando los datos no fueron normales, se analizaron mediante análisis de la varianza no paramétrico Kruskal-Wallis.

4.3 Resultados

4.3.1 Respuesta reproductiva en función del tipo de alimento.

La evolución de las puestas, expresado como fecundidad relativa de cada tanque (LP5 y LP6) a lo largo de cuatro estaciones de puesta, así como el termoperiodo al que fueron sometidos los tanques se representa en la Figura 4.1. Como se muestra, el periodo de puesta del stock duró entre 56 y 195 días, dependiendo del tanque y el año. Se registró una gran variabilidad en la fecundidad relativa diaria de las puestas, este parámetro varió entre 1638 y 56400 huevos por Kg de hembra.

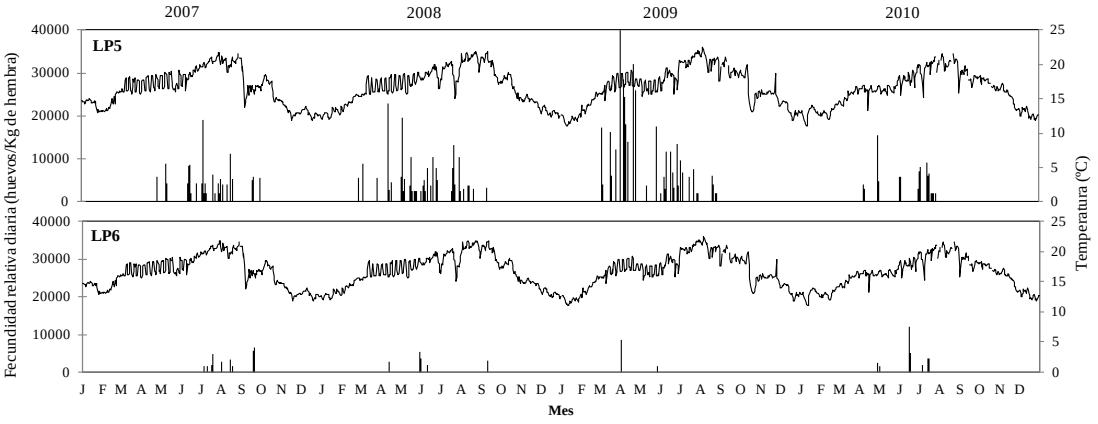

Figura 4.1. Fecundidad relativa diaria y régimen de temperatura de los dos tanques de reproductores con diferente alimentación (LP5 y 6) a los largo del periodo de estudio.

La Tabla 4.1. muestra un resumen de los parámetros productivos de stock con distintos tipos de alimentación. El volumen total, el volumen flotante y el número total de puestas anuales fueron siempre mayores en el tanque LP5. Solo se encontraron diferencias significativas en la fecundidad relativa media por tanque en 2007, y ésta fue más alta en el tanque LP5. No se encontraron diferencias significativas en el

porcentaje de volumen flotante entre tanques ningún año. En ningún caso se obtuvieron puestas fecundadas durante los cuatro años de estudio.

Estudiando todos los datos en conjunto (Tabla 4.2.), hubo diferencias significativas en la fecundidad relativa media entre los tanques, con valores mayores en el tanque de alimento natural (LP5). El volumen total y flotante fue seis veces mayor también en este tanque, como resultado del mayor número de puestas. La proporción de volumen flotante no fue significativamente diferente entre ambos tanques.

Tabla 4.1. Resumen de los parámetros productivos anuales del stock de reproductores con distinta alimentación. Las letras en superíndice indican diferencias significativas en un parámetro entre los tanques en un mismo año. MRFec: significa fecundidad relativa media.

Año	Tanque	Volumen total (mL)	Volumen flotante (mL)	Porcentaje de volume flotante (%)	MRFec (10^3 huevos /Kg de hembra ± SE)	Número de puestas
2007	LP5	1485	125	8,71±3,72	5,56±0,61[a]	30
	LP6	435	10	3,64±3,64	3,28±0,53[b]	11
2008	LP5	1843	43	2,76±1,35	5,81±0,67	42
	LP6	180	0	0±0	3,38±0,61	5
2009	LP5	2843	193	4,41±1,81	10,61±1,75	39
	LP6	120	60	30±30	5,10±3,40	2
2010	LP5	1007	87	5,56±2,65	5,24±0,81	19
	LP6	390	0	0±0	4,24±1,19	8
	Total	**8303**	**518**	**4,84±1,07**	**6,55±0,54**	**156**

Tabla 4.2. Resumen de los parámetros productivos de los tanques del stock de reproductores con distinta alimentación. Las letras en superíndice indican diferencias significativas en un parámetro entre distintos tanques. MRFec: significa fecundidad relativa media.

Tanque	Volumen total (mL)	Volumen flotante (mL)	Porcentaje de volume flotante (%)	MRFec (10^3 huevos /Kg de hembra ± SE)	Número de puestas
LP5	7178	448	5,04±1,17	7,11±0,62[a]	130
LP6	1125	70	3,85±2,72	3,74±0,48[b]	26
Total	**8303**	**518**	**4,84±1,07**	**6,55±0,54**	**156**

4.3.2 Respuesta reproductiva en relación al origen salvaje o cultivado de cada género.

la Figura 4.2. muestra la fecundidad relativa diaria de cada tanque (LP2 y LP3) a lo largo de dos estaciones de puesta, así como el termoperiodo al que estuvieron sometidos. El periodo de puesta del cada stock, obtenido por manipulación del termoperiodo, varió entre 1 y 258 días, dependiendo del tanque y año, además hubo una gran variabilidad en la fecundidad relativa diaria de las puestas, de 1756 a 25082 huevos por Kg de hembra.

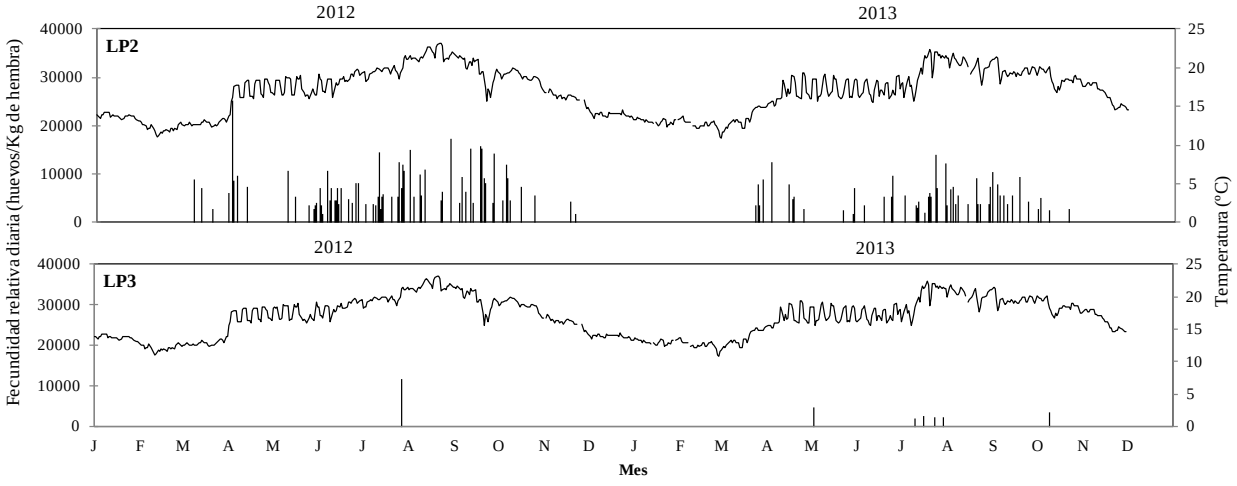

Figura 4.2. Fecundidad relativa diaria y régimen de temperatura de los dos tanques de reproductores con sexos de distinto origen (LP2 y 3) a los largo del periodo de estudio.

La Tabla 4.3. muestra un resumen de los parámetros productivos de los stock mixtos (machos salvajes con hembras cultivadas en el LP2 y machos cultivados con hembras salvajes en el LP3). El volumen total, el volumen flotante y el número total de puestas anuales fue siempre mayor en el tanque LP2. Solo se encontraron diferencias significativas en la fecundidad relativa media por tanque en 2013, siendo más alta en el tanque LP2. No se encontraron diferencias significativas en el porcentaje de volumen

flotante ningún año. Solo el tanque compuesto por machos salvajes y hembras cultivadas produjo puestas fecundadas que eclosionaron con éxito.

Tabla 4.3. Resumen de los parámetros productivos anuales del stock de reproductores mezclados de distinto origen (salvaje y cultivado). Las letras en superíndice indican diferencias significativas en un parámetro entre los tanques en un mismo año. MRFec: significa fecundidad relativa media.

Año	Tanque	Volumen total (mL)	Volumen flotante (mL)	Porcentaje de flotante (%)	MRFec (10^3 huevos/Kg de hembra kg ± SE)	Número de puestas	Tasa de Fecundación ± SE	Tasa de Eclosión ± SE	Larvas producidas
2012	LP2	5686	547	7,94±1,82	7,33±0,52	71	68,69±9,82	81,79±10,05	388794
	LP3	50	0	0	11,74	1	-	-	
2013	LP2	3151	216	6,84±1,67	5,69±0,40^a	50	61,79±11,72	65,67±34,33	42938
	LP3	157	27	18,81±8,03	2,86±0,39^b	6	-	-	
	Total	**9044**	**790**	**7,96±1,26**	**6,51±0,34**	**128**	**65,46±7,48**	**78,86±9,56**	**431732**

Estudiando todos los datos en conjunto (Tabla 4.4.), hubo diferencias significativas en la fecundidad relativa media entre los tanques, con valores mayores en el tanque LP2. El volumen total y flotante fue mucho mayor en el tanque LP2 que en el tanque LP3, como resultado de un mayor número de puestas. La proporción de volumen flotante no fue significativamente diferente entre ambos tanques.

Tabla 4.4. Resumen de los parámetros productivos de los distintos tanques del stock de reproductores mezclados de distinto origen (salvaje y cultivado). Las letras en superíndice indican diferencias significativas en un parámetro entre distintos tanques. MRFec: significa fecundidad relativa media.

Tanque	Volumen total (mL)	Volumen flotante (mL)	Porcentaje de flotante (%)	MRFec (10^3 huevos/Kg de hembra kg ± SE)	Número de puestas	Tasa de Fecundación ± SE	Tasa de Eclosión ± SE	Larvas
LP2	8837	763	7,49±1,27	6,65±0,35^a	121	65,46±7,48	78,86±9,56	431732
LP3	207	27	16,12±7,30	4,13±1,31^b	7	-	-	-
Total	**9044**	**790**	**7,96±1,26**	**6,51±0,34**	**128**	**65,46±7,48**	**78,86±9,56**	**431732**

4.4 Discusión

Como se ha mencionado anteriormente los problemas asociados a la reproducción del lenguado senegalés cultivado continúan sin resolver a nivel de puesta natural

fecundada. En este sentido, este trabajo describe la producción de huevos de cuatro tanques de reproductores, compuestos por 16 ejemplares nacidos en cautividad sometidos a diferentes condiciones experimentales.

Los resultados obtenidos con los distintos tipos de alimentación mostraron como el alimento influye en la producción mejorando los valores de fecundidad relativa diaria, número total de puestas, y volumen flotante y total de huevos, llegándose a obtener volúmenes totales de huevos del mismo orden a los obtenidos con ejemplares salvajes. También se vieron ampliados los periodos de puesta con el alimento natural, alcanzándose periodos de 8 meses, equivalente a los obtenidos en salvajes. Resultados equivalentes de optimización de la respuesta reproductiva se han descrito en trucha, *Oncorhynchus mykiss*, (Bromage, 1995), utilizando diferentes tasas de alimentación. Sin embargo, la alimentación no permite obtener los resultados de un stock salvaje adaptado a la cautividad (Anguis y Cañavate, 2005; Dinis *et al.,* 1999), y en ningún caso se obtuvieron puestas fecundadas.

El porcentaje de volumen flotante se ha utilizado comúnmente como un indicador de la calidad de la puesta en la acuicultura (Jia *et al.,* 2014, Aristizábal *et al.,* 2009). Este parámetro se ha relacionado directamente en algunas especies como *Sparidentex hasta*, con viabilidad de los huevos (Teng *et al.,* 1999). En este estudio, este parámetro fue de 4,84 ± 1,07 con los ejemplares cultivados (LP5 y LP6), bastante por debajo de los observados en individuos salvajes en condiciones similares (30-40%).

Parece que la alimentación puede suponer un factor clave para aumentar el rendimiento reproductivo y la productividad de un stock de reproductores, pero no parece ser responsable de las disfunciones reproductivas de esta especie, por lo menos con los tiempos de adaptación y suministro de los alimentos naturales ensayados en este estudio.

En cuanto a los resultados obtenidos estabulando conjuntamente individuos cultivados y salvajes de diferentes sexos, hay una clara relación entre el género y la falta de puestas fecundadas registradas en los tanques. Estos resultados concuerdan con los obtenidos por Mañanos *et al.*, (2007) y se corresponden también con los obtenidos por Carazo (2013) donde, con los mismos ejemplares de este estudio, se revela una ausencia de cortejo entre ejemplares cultivados, pero sí se describen comportamientos reproductivos entre hembras cultivadas y machos salvajes. La ausencia de comportamiento reproductivo parece ser un factor clave para resolver la ausencia de puestas fecundadas con ejemplares cultivados en esta especie.

Los resultados de este trabajo muestran que el principal problema de las puestas obtenidas con el grupo de machos salvajes y hembras cultivadas es la escasez de huevos fecundados en cada puesta, ya que el número de puestas anuales es similar al obtenido con salvajes, así como las tasas de fecundación y eclosión. Sin embargo, los volúmenes flotantes no alcanzaron el nivel de los reproductores salvajes. Los porcentajes de huevo flotante fueron alrededor del 8%, mientras que los valores medios obtenidos con ejemplares salvaje se sitúan alrededor del 35%. Este hecho afecta directamente el potencial de producción de larvas.

En conclusión, los resultados muestran que ambos, alimentación y origen del sexo, están relacionados con la respuesta reproductiva de *S. senegalensis* cultivado. En cuanto al alimento, se concluye que el régimen de alimentación con el que se consiguen puestas fecundadas en stocks salvajes, no resuelve la reproducción natural de los individuos cultivados de la especie en términos de fecundación, sin embargo algunos parámetros productivos (nº de puestas, volumen total y flotante o fecundidad relativa) si mejoran en comparación con una dieta comercial.

Además, los resultados obtenidos plantean la necesidad de continuar investigando diversos aspectos como la posible influencia de la alimentación en las primeras etapas

del desarrollo con el éxito reproductivo de la especie, las relaciones individuales establecidos en los tanques con machos salvajes y hembras cultivadas, o posibles formas de estimular o inducir las relaciones sociales (cortejo) en stocks de reproductores cultivados. En general, parece que la etología de los ejemplares nacidos en cautividad, y los mecanismos que inducen la puesta en marcha de la conducta reproductiva de esta especie, son líneas prometedores para resolver las disfunciones reproductivas de la especie.

5 Capitulo 5. Desinfección de la superficie de huevos de *Solea senegalensis* con Iodo.

5.1 Justificación

El trabajo incluido en este capítulo tiene como objetivo poner a punto la desinfección de huevos fecundados en esta especie. La desinfección de huevos es un procedimiento ensayado en numerosas especies acuícolas: trucha arcoiris, *Oncorhynchus mykiss* (Wagner *et al.*, 2010), perca americana, *Micropterus salmonides* (Wright y Snow, 1975), dorada, *Sparus aurata* (Escafre *et al.*, 2001), o rodaballo, *Scophthalmus maximus* (Salvesen y Vadstein, 1997), y persigue diferentes objetivos. En primer lugar tratar de eliminar cualquier patógeno que pudiera afectar la calidad de los huevos y evitar así descensos en las tasas de eclosión, o posibles mortalidades en las larvas recién nacidas. La desinfección de huevos trata también de evitar la transmisión vertical de enfermedades de padres a la descendencia a través de patógenos que pudieran quedar adheridos a la cubierta de los huevos e infectaran a la larva recién nacida al entrar en contacto con dicha cubierta. Otra de las posibles aplicaciones consiste en evitar la diseminación de patologías entre distintas instalaciones, comunidades, áreas, países, etc., ya que el intercambio de huevos entre instalaciones de una misma empresa, centros de investigación, etc., es una práctica bastante común.

El uso de iodo está muy extendido en la acuicultura, especialmente en acuicultura de salmónidos, se trata de un compuesto eficaz en la desinfección de virus, bacterias y hongos que se emplea rutinariamente en la desinfección de lotes de huevos a nivel comercial (MacFadden, 1969; Ross y Smith, 1972; Hirazawa *et al.*, 1999; Wagner *et al.*, 2008). El protocolo de administración de este producto debe ser puesto a punto para cada especie, debido a las distintas propiedades de los huevos de las distintas especies (tamaño, dureza del corion, permeabilidad de las membranas, sensibilidad a distintos químicos, etc.). Por este motivo, parámetros como la dosis, el tiempo de contacto, el ingrediente activo o el momento de desarrollo embrionario del huevo pueden afectar a la eficacia de desinfección, a las tasas de eclosión o al desarrollo embrionario.

5.2 Material y Métodos

5.2.1 Recolección de huevos

Para este trabajo se utilizaron puestas con volúmenes flotantes superiores a 30 mL y fecundaciones superiores al 75 %. Los huevos se desinfectaron en el estadío embrionario de mórula avanzada (12 horas postfecundación a 16-18ºC). El agua utilizada fue filtrada a 0,5 micras, pasada por ultravioleta y autoclavada. La relación huevo:desinfectante fue 1:100. El desinfectante utilizado fue un preparado comercial con base de Yodo.

5.2.2 Experimento 1. Determinación de la dosis que afecta la tasa de eclosión y supervivencia.

Para este experimento se distribuyeron 40 mL de huevos flotantes en 8 cilindros de PVC con malla de 350 µm en el fondo. Cada cilindro se introdujo 10 minutos en un vaso de vidrio estéril con las diferentes soluciones desinfectantes (0, 15, 25, 50, 75, 100, 150, 250 mgL^{-1} de Yodo). Durante los diez minutos de desinfección los huevos se agitaron moderadamente cada dos minutos para favorecer el contacto desinfectante-

huevo. Después, los huevos se aclararon 3 veces durante 5 minutos para eliminar los restos de desinfectante. Cada lote de huevos desinfectados se colocó en un recipiente con agua, ambos estériles. Posteriormente se llenaron tres placas de cultivo celular de 12X8 pocillos con 200 µL de agua de mar estéril en cada pocillo, y se introdujeron 12 huevos fecundados de cada tratamiento en cada fila de pocillos (un huevo por pocillo). Las placas se incubaron en oscuridad a 18ºC y se revisaron diariamente para determinar la tasa eclosión y la supervivencia acumulada de las larvas. A partir de los resultados de este experimento se determinaron las dosis de la siguiente experiencia.

5.2.3 Experimento 2. Evaluación de la capacidad desinfectante y tasas de eclosión en condiciones de cultivo.

Los huevos se desinfectaron como en el Experimento 1. Las concentraciones ensayadas fueron: 0, 15, 25, y 50 mgL^{-1} de Yodo. Tras la desinfección, unos 120 huevos de cada tratamiento se distribuyeron en 2 placas con agar sangre salino compuesto por (TSA 4%, citrato férrico 0.05%, esculina 0,1%, sangre ovina estéril desfibrinada 5% y cloruro sódico 2%) e incubaron en estufa a 20 ºC. El crecimiento bacteriano se valoró diariamente determinando el número de colonias (UFC) que aparecían. El cultivo bacteriano se mantuvo durante 72 horas. El resto de huevos de cada tratamiento se distribuyó en tres incubadores de 1 litro con renovación moderada para valorar la eclosión en condiciones de cultivo.

5.2.4 Experimento 3. Identificación de la flora bacteriana en huevos del stock de reproductores

En este experimento solo se valoró la dosis de 50 mgL^{-1} para confirmar los resultados anteriores y además se llevo a cabo una identificación bacteriana de los huevos para ver que especies estaban presentes en los huevos de nuestras instalaciones y si el desinfectante era selectivo para alguna especie. Para este propósito, se prepararon 4 placas petri con agar sangre salino para cada puesta (2 placas para huevos sin

desinfectar (Control), y otras dos para huevos desinfectados con 50 mgL^{-1}). Después de realizar una tinción Gram, un test de la catalasa con peróxido de hidrógeno al 3%, y un test de coagulasa con plasma de conejo, se realizó la identificación mediante el sistema Vitek® 2 que utiliza tarjetas con reactivos colorimétricos que son incubadas e interpretadas automáticamente.

5.3 Resultados

En el Experimento 1 no observaron diferencias significativas en relación a la finalización del desarrollo embrionario previo a la eclosión entre las dosis 0, 15, 25, 50, 75 y 100 mgL^{-1} ($p>0,05$). Si fue significativamente más baja a 150 y 250 mgL^{-1}. No hubo diferencias significativas en las tasas de eclosión entre el Control y los huevos desinfectados con 15, 25 o 50 mgL^{-1} de yodo, sin embargo fueron significativamente menores al Control a partir de la dosis de 75mgL^{-1}. (Figura 5.1.).

La supervivencia acumulada, fue más baja en los huevos tratados que en el Control ($p<0,05$), probablemente debido al escaso volumen de agua utilizado en la incubación en placa y los restos de yodo presentes en la superficie de los huevos (Figura 5.2.). No se encontraron diferencias significativas entre tratamientos ($p>0,05$).

La incubación en condiciones de cultivo del Experimento 2 no mostró diferencias significativas ($p>0,05$) en las tasas de eclosión a ninguna de las dosis, (Tabla 5.1.).

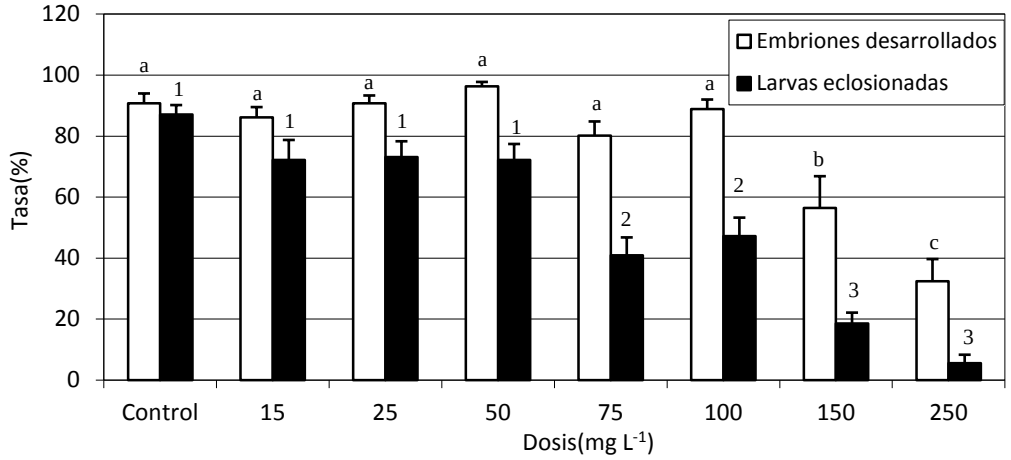

Figura 5.1. Finalización del desarrollo embrionario (estado IV; Laurence y Rogers, 1976) y tasas de eclosión a las distintas dosis ensayadas. El tiempo de contacto fue en todos los casos de 10 minutos. Las letras y los números sobre las barras de error indican diferencias significativas entre los distintos tratamientos en la finalización del desarrollo embrionario y la tasa de eclosión respectivamente.

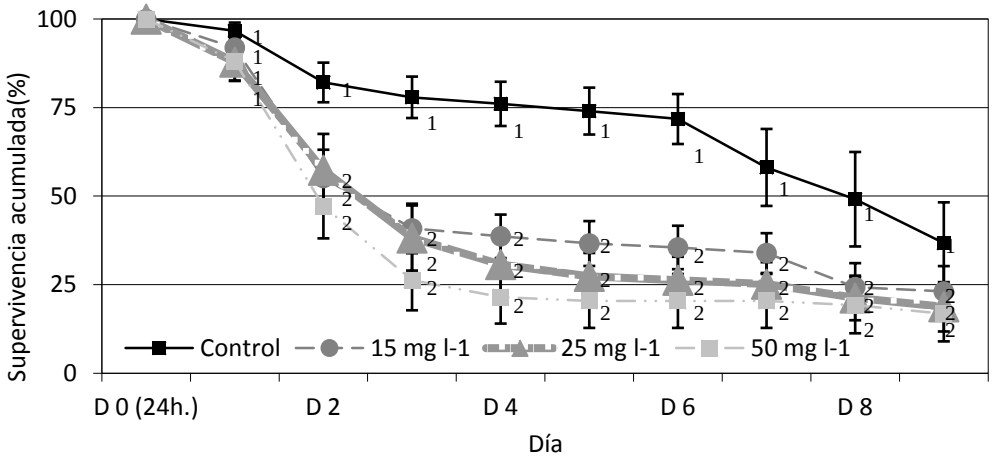

Figura 5.2. Evolución de la tasa de supervivencia acumulada de las dosis que no mostraron diferencias significativas en la tasa de eclosión (Control, 15, 25, 50 mg L^{-1} de Iodo activo durante 10 minutos). Los diferentes números indican diferencias significativas diarias entre las distintas concentraciones. D0 (24h.) significa 24 horas tras la eclosión.

Tabla 5.1. Media ± SE de la tasa de eclosión en condiciones de cultivo a 0, 15, 25, 50 mg L^{-1} de Iodo activo. El tiempo de contacto fue en todos los casos 10 minutos. No se encontraron diferencias significativas entre tratamientos.

Tratamiento	Tasa de eclosión (%)
Control	76,40 ± 4,06
15 mg L^{-1}	68,34 ± 5,58
25 mg L^{-1}	65,29 ± 5,52
50 mg L^{-1}	71,56 ± 4,37

En relación a la formación de colonias la Tabla 5.2. muestra las nuevas unidades formadoras de colonia (UFC) que aparecieron tras 24, 48 y 72 horas de incubación. El numero de UFCs fue menor en los huevos tratados 25 o 50 mg L^{-1} que en el Control, salvo a 72 horas, intervalo en el que no se detectaron diferencias entre estos tratamientos. No se detectaron diferencias en el número de UFC entre el Control y los huevos desinfectados con 15 mg L^{-1}. Las larvas de huevos incubados en incubadores de un litro con renovación moderada en vez de en placas de ELISA no mostraron ninguna diferencia en la tasa de supervivencia acumulada entre los tratamientos (15, 25 o 50 mg L^{-1}) y el Control ningún día tras la eclosión (ver Figura 5.3.).

Tabla 5.2. Número medio de UFC ± SE que aparecieron en los cultivos bacterianos tras 24, 48 y 72 horas de incubación. Letras y números superíndice indican deferencias significativas entre tratamientos cada 24 de incubación. El tiempo de contacto en todos los tratamientos fue 10 minutos.

	UFC / Placa Petri (60 huevos)			
Tratamientos	**24 h. postincubación**	**48 h. postincubación**	**72 h. postincubación**	**Total**
Control	$3,13 \pm 1,13^{1}$	$10,75 \pm 4,09^{x}$	3,38 ± 1,15	**$17,25 \pm 5,15^{a}$**
15 mg L^{-1}	$0,75 \pm 0,53^{1,2}$	$3,75 \pm 1,37^{x,y}$	2,13 ± 0,64	**$6,63 \pm 1,81^{a,b}$**
25 mg L^{-1}	0 ± 0^{2}	$3,88 \pm 2,50^{y}$	0,75 ± 0,31	**$4,63 \pm 2,42^{b,c}$**
50 mg L^{-1}	0 ± 0^{2}	$1,25 \pm 0,73^{y}$	0,5 ± 0,19	**$1,75 \pm 0,67^{c}$**

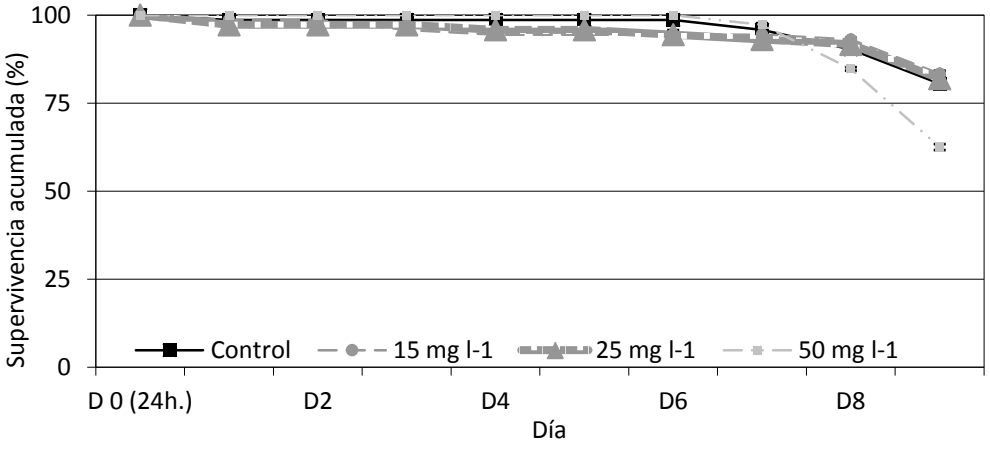

Figura 5.3. Evolución de la tasa de supervivencia acumulada tras la incubación de los huevos con renovación de agua de las dosis que no mostraron diferencias significativas en la tasa de eclosión (Control, 15, 25, 50 mg L^{-1} de Iodo activo durante 10 minutos). No hubo diferencias significativas entre tratamientos. D0 (24h.) significa 24 horas tras la eclosión.

En el Experimento 3, se identificaron las siguientes especies en las puestas estudiadas: *Staphylococcus spp coagulasa-negativo., Sphingomonas paucimobilis, Corynebacterium spp., Pseudomonas stutzeri, Aeromonas hydro/caviae* y *Vibrio alginolyticus*. En este experimento no hubo ningún crecimiento en las places con huevos desinfectados con 50 mgL^{-1}. La Tabla 5.3. muestra las especies aisladas en cada puesta. *Staphylococcus spp coagulasa-negativo* y *S. paucimobilis* aparecieron en las dos primeras puestas, *Corynebacterium spp*. apareció en la primera, y *P. Stutzeri* apareció en la segunda. A*. Hydro/caviae* y *V. Alginolyticus* solo aparecieron en la tercera puesta sin presencia de ninguna otra especie bacteriana.

Tabla 5.3. Especies bacterianas aisladas en tres puestas diferentes (1, 2 y 3) sin desinfectar (Control), y tras desinfectar con 50 mg L^{-1} de iodo. "-" significa que no hubo crecimiento.

	Control			50mg L^{-1}_10 min		
Especies	**1**	**2**	**3**	**1**	**2**	**3**
Coagulase-negative Staphylococcus spp.	+	+		-	-	-
Sphingomonas paucimobilis	+	+		-	-	-
Corynebacterium spp.	+			-	-	-
Pseudomonas stutzeri		+		-	-	-
Aeromonas hydro/caviae			+	-	-	-
Vibrio alginolyticus			+	-	-	-

5.4 Discusión

La elección de tiempos de contacto y dosis empleadas se basaron en estudios previos de otros autores, ver Tabla 5.4. Cada especie presenta un rango de concentraciones de desinfectante que no afectan al desarrollo embrionario de los huevos, pero es conveniente determinar qué valor de ese rango que presenta la máxima capacidad desinfectante. En el caso del *S. senegalensis*, y para el estadio de desarrollo utilizado, podemos establecer el rango de seguridad entre 0 y 50 mgL^{-1} con una exposición máxima de 10 minutos.

Tabla 5.4. Resumen de respuestas de huevos de otras especies de peces desinfectados con iodo. En las especies con la letra superíndice "a", la tasa de eclosión descendió hasta 0 a las dosis y tiempos de contacto mostrados. Las especies con una letra superíndice "b" se produjo una disminución significativa en la tasa de eclosión a las dosis y tiempos de contacto mostrados.

Especie	Dosis	Tiempo de contacto	Autor (año)
Micopterus salmonides[a]	200 ppm (Wescodyne)	15 min	Wright & Snow (1975)
Oreochromis mossambicus[a]	200ppm (Buffodine)	15 min	Subasinghe & Sommerville (1985)
Pleuronectes Platessa[a]	250 ppm (Buffodine)	10 min	Salvesen & Vadstein (1995)
Pargus major[a]	500 ppm (Povidon-iodide)	5 min	Hirazawa et al. (1999)
Diplodus sargus sargus[b]	25 ppm (PVP-iodine)	5 min	Katharios et al. (2007)
Osmerus mordax[b]	50 ppm (PVP-iodine)	15 min	Walker et al. (2010)
Pargus pargus[b]	100 ppm (PVP-iodine)	5 min	Katharios et al. (2007)

El desarrollo embrionario de los huevos parece es un factor clave a la hora de llevar a cabo las desinfecciones según diversos autores (Peck *et al.,* 2004; Tendencia, 2001; Hirazawa *et al.,* 1999; Douillet y Holt, 1994). En este estudio, la fase del desarrollo embrionario elegida para los ensayos tuvo en cuenta poder aplicarse con los dos métodos de reproducción de esta especie (natural y artificial). En el primer caso los huevos se recogen frecuentemente en este estadío a la mañana siguiente de la puesta natural a 16-18 ºC, ya que las puestas se producen generalmente a final de la tarde o principio de la noche (Carazo, 2013). En el caso de la reproducción artificial, los huevos obtenidos por stripping se fecundan posteriormente, y pueden ser incubados hasta alcanzar el estadío de desarrollo propuesto. Este estadío además previene posibles efectos adversos de la desinfección de huevos recién fecundados relacionados con la falta de endurecimiento del corion (Bergh y Jelmert 1996).

A diferencia de estudios con otras especies (Overton *et al.,* 2010; Khodabandeh y Abtahi, 2006; Treasurer *et al.,* 2005; Rach *et al.,* 1998), la desinfección con iodo no mejoró las tasas de eclosión en *S. senegalensis*. Esto está probablemente relacionado con el reducido tiempo que tardan los huevos de esta especien en eclosionar (24-48h a entre 17-20ºC), lo que reduce considerablemente el tiempo de contacto patógeno-huevo y limita la cantidad de bacterias totales que puede desarrollarse. Por este motivo, las razones de poner a punto el protocolo en esta especie están más relacionadas con aspectos de mejora del estatus sanitario de la especie y/o de las instalaciones. En este sentido los resultados cumplen los objetivos sin efectos adversos sobre el normal desarrollo embrionario de los huevos o la supervivencia larvaria hasta día 9.

La supervivencia larvaria de huevos desinfectados inicialmente mostró un descenso respecto a los Controles. Esta reducción probablemente fue debida al escaso volumen de agua utilizado en la incubación en placa de cultivo celular (200 µL por pocillo), y a las trazas de iodo que pudiera haber quedado en la superficie de los huevos a pesar de

los tres aclarados. Esta hipótesis se confirmó mediante la incubación, en placas de cultivo celular, de larvas, en vez de huevos, procedentes de puestas desinfectadas e incubadas con renovación de agua. El estudio de supervivencia en estas condiciones no mostró diferencias significativas entre los distintos tratamientos y el Control. Estos resultados indican que el cultivo en placas de cultivo celular, que es adecuado para experimentos de incubación de huevos o larvas (Panini *et al.,* 2001; Unuma *et al.,* 2004), debe realizarse con precaución cuando se realizan estudios con productos que puedan ser tóxicos a volúmenes tan bajos.

La carga bacteriana total se redujo con 25 y 50 mgL^{-1} de iodo. En ambos casos la reducción fue significativa, pero el nivel de reducción de UFCs con 50 mgl^{-1} fue alrededor del 90% mientras que con 25 mgL^{-1} la reducción fue del orden del 70%. No hubo diferencias entre los distintos tratamientos, pero la desinfección con 50 mgL^{-1} parece más adecuada porque a pesar de no ser significativa, la reducción fue en todos los casos mayor y no se detecto efecto adverso alguno con dicha dosis.

Las especies bacterianas aisladas e identificadas en este estudio no son patógenos que causen enfermedades graves en humanos salvo *Vibrio alginolyticus* y *Aeromonas hydrophila*. La primera se relaciona con enfermedades de peces marinos como la dorada *Sparus aurata* (Balebona *et al.,* 1998), el rodaballo *Scophthalmus maximus* o el mero *Epinephelus malabaricus* (Lee 1995). Esta bacteria también es responsable de infecciones humanas asociadas al contacto con agua marina o contaminación alimenticia (Blake *et al.,* 1980). En el lenguado senegalés, *V. alginolyticus* se ha identificado como patógeno secundario asociado a flexibacteriosis. *A. hydrophila* causa la enfermedad microbiana llamada "Motile Aeromonad Septicaemia" (MAS), y a pesar de estar más relacionada con brotes en peces de agua dulce, también se han encontrado y publicado casos en peces de aguas salobres y marinas (Das y Sahoo 2012). El resto de las especies aisladas no están directamente relacionadas con patologías de peces, pero como publicaron Barker *et al.* (1989), la carga bacteriana por

sí misma, en casos de excesivo crecimiento sobre la superficie de los huevos, puede afectar la evolución normal de los huevos por el consumo del oxigeno necesario para el desarrollo embrionario. En las desinfecciones realizadas en el apartado de identificación ($50 mgL^{-1}$), no hubo crecimiento bacteriano en ningún caso, mejorándose los resultados del Experimento 2.

En resumen, la desinfección con 50 mg L^{-1} de iodo durante 10 minutos, seguida de tres aclarados de 5 minutos con agua de mar, reduce significativamente, al menos la carga bacteriana en huevos de *Solea senegalensis,* sin afectar el desarrollo embrionario normal de los huevos, la tasa de eclosión y la supervivencia larvaria (9 días). Esta metodología es simple y fácil de poner en práctica a nivel industrial. Estos son los primeros resultados de desinfección de huevos de esta especie, y sería conveniente evaluar la eficacia del iodo para otros patógenos y los posibles efectos de desinfectar en distintos estadíos embrionarios.

6 Capitulo 6. Transporte en seco de ejemplares de *S. senegalensis*.

6.1 Justificación

El trabajo incluido en este capítulo tiene como objetivo determinar el tiempo máximo de emersión de ejemplares de *Solea senegalensis*, y el estrés generado por dicho proceso para su aplicación en el transporte de ejemplares vivos. El transporte de peces es una actividad industrial importante en acuicultura, no solo para trasladar ejemplares entre las distintas unidades de una empresa sino también para transportar los ejemplares hasta el punto de venta. Las mejoras en el transporte de ejemplares de talla comercial y/o reproductores, pueden repercutir en una mejor gestión de los recursos de las empresas y centros de investigación y puede contribuir a mejorar la

calidad del producto final. Una manera de mantener la calidad del pescado es retrasar los procesos de autolisis manteniendo los peces vivos tanto tiempo como sea posible.

El sistema más común de transportar peces es en agua, pero el transporte en seco de peces se ha llevado a cabo con éxito en algunas especies como el rodaballo (Caillens, 1996). El transporte con agua presenta algunos inconvenientes, como la necesidad de vehículos adaptados a dicho transporte, un alto coste e incluso problemas con el tratamiento de agua que se utiliza para el transporte en relación con la difusión de patologías. En cambio, el transporte en seco es fácil de realizar, económico y no genera problemas de residuos, lo que permite mover gran cantidad de individuos (Yin *et al.,* 1995), aunque no se puede utilizar con todas las especies.

6.2 Material y Métodos

El experimento se llevó a cabo con 56 individuos de *S. senegalensis* de 922,3 ± 29,5 g, y 39,8 ± 0,5 cm, estabulados en tanques de 1,125 m^3, en el circuito abierto y con una tasa de renovación del agua de 1,2 m^3/hora. Los ejemplares se dividieron aleatoriamente en 8 grupos de 7 individuos, se colocaron en cajas de poliestireno sobre bayetas húmedas (1 pez por caja) y se mantuvieron en una habitación a 15ºC. Al comienzo del experimento, se extrajo sangre a 7 individuos para establecer los niveles basales de los parámetros plasmáticos (cortisol, glucosa, lactato, triglicéridos, proteínas y amino ácidos). El resto de especímenes se muestrearon a las 2, 6, 12, 20, 28, 36, y 44 horas de emersión. Después del muestreo, cada grupo se introdujo en un tanque de recuperación durante 4 semanas con el fin de evaluar la mortalidad retardada. Para la determinación de los niveles de los parámetros plasmáticos, se extrajo sangre de la vena caudal usando jeringas de 1 mL heparinizadas en menos de tres minutos para evitar el aumento del cortisol circulante debido al estrés generado por el manejo. La sangre se transfirió a tubos Eppendorf con 10 µL de heparina y se centrifugó (3000 x g durante 15 min, 4 º C) para obtener la fracción de plasma, que

después se almacenó a -80 º C para su posterior análisis. Las concentraciones de glucosa, lactato y triglicéridos se midieron utilizando kits comerciales de Spinreact (Barcelona, España) adaptados a microplacas de 96 pocillos. Las concentraciones de proteína se midieron en una dilución de plasma 1:50 utilizando el método del ácido bicinconínico con el kit de proteína BCA (Pierce PO, Rockford, EE.UU.), con albúmina de suero bovino como estándar. Los niveles de aminoácidos libres se evaluaron colorimétricamente en una dilución de plasma 1:75 veces utilizando el método de la ninhidrina (Rosen, 1957). Todos los ensayos se realizaron en un lector de microplacas automático (340 PowerWave, BioTek Instrument Inc., Winooski, EE.UU.) controlado por software KCjunior ™. Los niveles de cortisol se midieron por inmuno-ensayo enzimático (EIA) utilizando placas de microtitulación (MaxiSorp ™, Nunc, Roskilde, Dinamarca).

Los peces siempre se manejaron (gestión de rutina y experimentación) de acuerdo con la Directiva de la Unión Europea (CEE, 1986) para la protección de los animales utilizados para experimentación y otros fines científicos.

Los datos obtenidos fueron analizados mediante un análisis de la varianza, y cuando había significación ($p<0,05$) se aplicó el test de Tukey para comparar entre grupos. Algunos datos se normalizaron mediante transformación logarítmica o angular; y cuando los datos no fueron normales, se analizaron mediante análisis de la varianza no paramétrico Kruskal-Wallis.

6.3 Resultados

Los datos de mortalidad se muestran en la Tabla 6.1. Solo se registraron bajas durante el periodo de emersión en el grupo de las 44 horas (66,7% de mortalidad). El resto de bajas se registraron en los tanques de recuperación. La mortalidad retardada fue del 42,9% y del 16,7% en los grupos de 36 y 44 horas, respectivamente, durante las primeras 24 horas después de la introducción de peces en tanques de recuperación. En el grupo de

20 horas sólo 1 murió un ejemplar (mortalidad 14,3%) quince días después de la prueba de emersión.

Tabla 6.1. Mortalidad directa y mortalidad retardada, tras 4 semanas de recuperación, en los grupos sometidos a distintas horas de emersión (0, 2, 6, 12, 20, 28, 36 y 44). Los valores se expresan en porcentaje respecto del total de ejemplares.

Tiempo de emersión (horas)	**0**	**2**	**6**	**12**	**20**	**28**	**36**	**44**
Tasa de moralidad (%)	0	0	0	0	0	0	0	66,6
Tasa de mortalidad retardada (%)	0	0	0	0	14,3	0	42,8	16,7
Tasa de mortalidad total (%)	**0**	**0**	**0**	**0**	**14,3**	**0**	**42,8**	**83,3**

La Figura 6.1. muestra los diferentes parámetros plasmáticos relacionados con el estrés (cortisol, glucosa y lactato) a las diferentes horas de emersión. A lo largo del tiempo de emersión se observó un aumento progresivo de los valores de cortisol, aunque no se encontraron diferencias significativas entre los grupos de 0, 2 y 6 horas. Los grupos con niveles significativamente más altos fueron los de 20, 28, 36 y 44 horas. Los niveles de glucosa fueron significativamente más altos en los grupos de 2 y 6 horas de emersión. El lactato mostró sus valores más altos en los grupos de 12 y 20 horas de emersión, siendo los valores más bajos en el grupo de 0 horas.

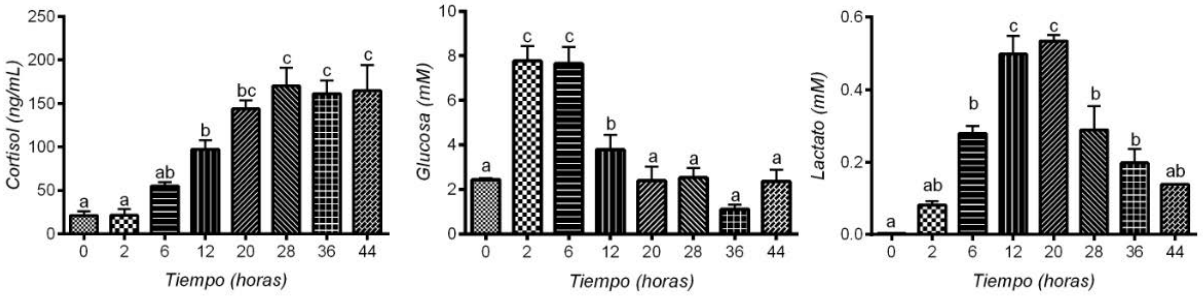

Figura 6.1. Niveles de los parámetros plasmáticos (cortisol, glucosa y lactato) a diferentes horas de emersión (0, 2, 6, 12, 20, 28, 36 y 44). Los superíndices indican diferencias significativas entre las distintas horas de emersión.

Los triglicéridos mostraron valores significativamente más altos en los grupos de 2 y 28 horas, sin diferencias significativas entre los otros grupos. Los niveles de proteína en plasma fueron significativamente mayores en los grupos de 20 y 44 horas. En cuanto a los aminoácidos, los valores más altos se encontraron a 0, 2 y 44 horas (Figura 6.2.).

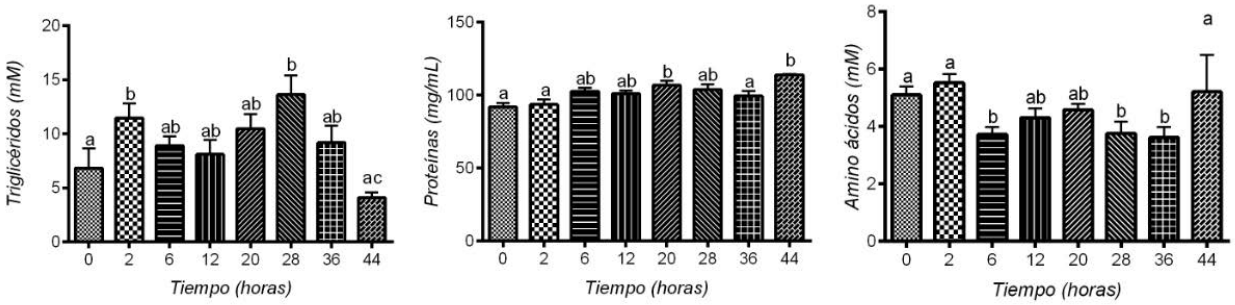

Figura 6.2. Niveles de los parámetros plasmáticos (triglicéridos, proteínas y amino ácidos) a diferentes horas de emersión (0, 2, 6, 12, 20, 28, 36 y 44). Los superíndices indican diferencias significativas entre las distintas horas de emersión.

6.4 Discusión

Este estudio tuvo como objetivo determinar el tiempo máximo de emersión de individuos de of *S. senegalensis* de en torno a un kilo de peso en cajas de poliestireno sobre bayetas húmedas, a 15 ºC, para su aplicación en transporte. Además, se realizó un análisis de parámetros plasmáticos; cortisol, glucosa, lactato, triglicéridos, aminoácidos y proteínas, para establecer el efecto de esta actividad en términos del estatus fisiológico y del bienestar animal.

De los resultados obtenidos en este estudio podemos establecer un rango de seguridad de 28 horas para el transporte en seco de *S. senegalensis*, sin mortalidad directa o retardada. La mortalidad observada en el grupo de 20 horas (1 solo ejemplar) no pudo asociarse directamente al ensayo, ya que se trató de un solo animal, y la mortalidad se registró 15 días después del experimento.

Respecto a los parámetros plasmáticos, los valores de cortisol obtenidos en este estudio para los grupos de 0 (Control), 2 y 6 horas son equivalentes a los obtenidos por Oliveira *et al.,* (2013) que con ejemplares de 413 ± 36 g en condiciones basales (sin estrés), obtuvo valores variando en un ciclo diario entre 5 y 36 ng/L. En base a estos resultados, podemos afirmar que un transporte en seco no generaría estrés hasta las 6 horas de emersión. Aunque comparando los valores obtenidos en este estudio con otros registrados en esta especie con ejemplares de menor tamaño, los valores mínimos descritos en este trabajo corresponderían con los individuos estresados (Wunderink *et al.,* 2011). Además, los valores obtenidos por Costas *et al.* (2011), también con ejemplares de menor peso, muestran como el pico de cortisol aparece entre los 5 minutos y las 2 horas después de la exposición a la fuente de estrés, periodo en el que nuestros animales no se muestrearon. El resto de parámetros relacionados con el estrés mostraron una evolución consecuente con la evolución del cortisol; a medida que aumentó el cortisol, se incrementaron los niveles de glucosa circulante para hacer frente al estrés, y a medida que la glucosa fue agotándose comenzaron a activarse las rutas metabólicas anaeróbicas que resultaron en un aumento de lactato.

Basándonos en los resultados obtenidos por Caillens (1996), los resultados obtenidos en este trabajo podrían mejorarse en relación al tiempo máximo de emersión sin mortalidad, bajando la temperatura durante el transporte.

En conclusión, el transporte sin agua en el lenguado senegalés es posible, aunque es necesario continuar estudiando aspectos como la temperatura óptima para el transporte, la carga máxima posible por caja, o el tamaño mínimo de los peces que puede soportar este tipo de transporte . Por otra parte, sería también necesario tener una buena descripción de los niveles basales de cortisol en esta especie a diferentes edades, con el fin de determinar cuándo aparece realmente el estrés.

7 Capitulo 7. Conclusiones y líneas futuras de investigación

El objetivo de esta tesis ha sido doble, por un lado contribuir al conocimiento de la biología de *S. senegalensis* en cautividad para intentar dar respuesta a la falta de puestas fecundadas obtenidas con ejemplares nacidos en cautividad. Por otro lado hacer avances en la zootecnia de esta especie mediante la adaptación de protocolos de desinfección de huevos y transporte de otras especies al lenguado senegalés. De acuerdo a estos objetivos se han llevado a cabo una serie de experimentaciones, y de los resultados obtenidos se establecen las siguientes conclusiones:

7.1. Conclusiones generales (desarrolladas por capítulos)

Evolución de la producción de huevos y contribución parental en lenguado senegalés, Solea senegalensis, durante cuatro estaciones de puesta.

- El bajo número de individuos que contribuyen a la producción total del stock (entre un 8,7% y un 51,7%) evidencian la importancia individual en la reproducción de esta especie.
- Las parejas que más contribuyen a la producción del stock, presentan patrones de fidelidad que se mantienen a lo largo de los sucesivos periodos de puesta.
- Las hembras de esta especie son capaces de ovular al menos 6 días consecutivos en las condiciones estudiadas. Además se ha establecido una frecuencia media de ovulación de 7,3 ± 0,6 días.
- Los huevos de *S. senegalensis* experimentan un descenso neto en el diámetro a lo largo del periodo de puesta, durante este periodo, se producen recuperaciones parciales del diámetro asociadas a paradas reproductivas, como pone de manifiesto el ajuste cúbico de la evolución del diámetro a lo largo del periodo de puesta.
- La manipulación del termoperiodo resulta eficaz para inducir puestas en lenguado senegalés salvaje, y permite ampliar el periodo de puesta descrito en la

literatura científica de 4-6 meses (dependiendo de los autores), a 8 meses y medio (Tanque B3, 2009).

Respuestas reproductivas en lenguado senegalés, Solea senegalensis, en relación con el tipo de alimentación, y el origen (cultivado o salvaje) de cada género.

- La alimentación natural mejora la respuesta reproductiva de los ejemplares cultivados frente a una dieta comercial a nivel de fecundidad relativa diaria, número de puestas producidas, y por tanto volúmenes de huevos obtenidos.
- La alimentación, adecuada para obtener puestas fecundadas en ejemplares salvajes adaptados a la cautividad, no resuelve la falta de fecundación en las puestas de ejemplares cultivados.
- La estabulación de hembras de cultivo con machos salvajes permite obtener puestas fecundadas con tasas de fecundación y eclosión similares a las obtenidas con ejemplares salvajes (65,46±7,48 % y 78,86±9,56 % respectivamente).
- Las puestas obtenidas con hembras salvajes y machos cultivados son menos numerosas y en ningún caso fecundadas.

Desinfección de la superficie de huevos de Solea senegalensis con Iodo.

- La desinfección con 50 mg L^{-1} de iodo seguido de tres lavados de 5 minutos con agua de mar, resulta efectiva para reducir la carga bacteriana en huevos de *S. senegalensis* sin afectar al desarrollo embrionario o la eclosión. La reducción obtenida fue de en torno al 90% en el número de UFC nuevas que aparecían a las 24, 48, 72 horas de incubación, y en el recuento bacteriano total.
- El método de incubación de huevos en placas de cultivo celular, efectivo para valorar la eclosión y supervivencia de huevos y larvas, ha de utilizarse con precaución en estudios con productos que puedan resultar tóxicos debido al bajo volumen de trabajo.

Transporte de ejemplares de S. senegalensis, sin necesidad de agua.

- Los resultados obtenidos, bajo las condiciones descritas y a 15ºC, permiten establecer un rango de 28 horas para el transporte sin agua de ejemplares de *S. senegalensis*, sin que la mortalidad directa o retardada se vea afectada.
- Además, este procedimiento no produce un incremento significativo de los niveles de cortisol hasta las 12 horas de emersión.

7.2. Líneas futuras de investigación

A pesar del conocimiento adquirido en la biología reproductiva de la especie en cautividad, y las mejoras de manejo propuestas para la desinfección de huevos y el transporte de ejemplares en seco, esta tesis no ha conseguido identificar el factor o factores clave para obtener puestas fecundadas en ejemplares nacidos en cautividad. Por este motivo, es necesario seguir investigando todos los posibles factores que puedan estar detrás de la falta de puestas fecundadas de los stocks cultivados. Además, los resultados obtenidos en la desinfección de huevos y el transporte, son los primeros para esta especie, y por tanto es necesario continuar estudiando los procesos que puedan servir para optimizar y/o mejorar la zootecnia de la especie.

En este sentido, algunas de las principales líneas de trabajo y aspectos que surgen a partir de los resultados obtenidos en esta tesis doctoral se detallan a continuación, divididas en las dos principales áreas de la tesis (Biología reproductiva y zootecnia):

Biología reproductiva

- Todos los factores que permitan optimizar el número de reproductores necesarios para una adecuada reproducción de la especie en cautividad, como por ejemplo; la relación de machos y hembras óptima, el número mínimo de

reproductores para maximizar la eficacia reproductiva del stock o determinar el papel de los ejemplares que no participan directamente en las puestas.

- La influencia de la alimentación en estadíos tempranos del cultivo y su relación con el éxito reproductivo.
- Las relaciones individuales que se establecen en los tanques de machos salvajes con hembras cultivadas.
- Los distintos mecanismos que puedan estimular o inducir el cortejo en ejemplares cultivados (terapias hormonales, feromonas, sistemas de aprendizaje, etc.)
- Profundizar en el control de la reproducción natural mediante manipulación de parámetros físico-químicos; protocolos de manipulación de la temperatura que permitan una mayor predictibilidad de las puestas, o definir el posible efecto de la luz para mejorar dicho control.
- La importancia del tipo de tanque, el tamaño mínimo, o volumen necesario para conseguir el mayor número posible de puestas fecundadas

Zootecnia

- La eficacia del iodo para eliminar virus, la eficacia de otros compuestos, o las consecuencias de desinfectar en distintos estadíos del desarrollo embrionario de *S. senegalensis*.
- Evaluar las diferencias a nivel de estrés producido, entre el transporte sin agua y el transporte convencional, determinar con precisión cuando aparece el estrés o cual es el tiempo necesario para recuperar de nuevo los niveles basales.
- Otros aspectos relacionados con las condiciones del transporte en seco; la influencia de anestésicos u otros compuestos para reducir el estrés durante el transporte, la temperatura más apropiada para llevar a cabo el transporte o los límites de talla y peso de los ejemplares para utilizarse en este tipo de transporte.

Summary

Summary

The Senegalese sole*, Solea senegalensis* is an interesting candidate for European aquaculture diversification (Imsland *et al.,* 2004; Howell *et al.,* 2009; Morais *et al.,* 2014). This species has not reached the production levels of other species despite the efforts invested by both the farming industry and research centers. However, both sectors maintain that developing a framework for the species is an important goal in the production sector (Howell *et al.,* 2009). Today, after more than 30 years of many teams, mainly from Spain and Portugal, researching the problem it appears that the lack of natural spawns in individuals born in captivity lies in the males of this species. Although the reasons are not are clear, it seems that the absence of reproductive behavior and its relationship to structural brain dysfunctions may be behind the problem. Fortunately, after the first artificial fertilizations in 2007 (Chereguini *et al.,* 2007) the recent advances in the artificial fertilization protocols developed for this species (Liu *et al.,* 2008; Rasines 2012, 2013 and 2014) bring about the possibility of its industrial culture non reliant on wild individuals. With this scenario, the study of the reproductive biology of the species is particularly interesting to achieve total control of reproduction in captivity for industrial purposes.

This thesis aims to deepen the knowledge of the reproductive biology of this species in captivity to try to determine the cause of the detected reproductive disorders of the cultivated breeders and to develop improvements in the management, namely the disinfection of fertilized eggs and the transport of specimens without need of water.

First, a detailed study of the production parameters of a wild stock of *S. senegalensis* divided into three 14m^3 tanks during four years to evaluate the quality and quantity of the natural spawns obtained by thermo-manipulation. After analyzing the results, it

was determined that there were significant differences in several of the studied productive parameters between the different tanks (proportion of floating volume, daily relative fecundity, fertilization and hatching rate). Parallel to this study, the larvae obtained were analyzed in all the spawns registered over the four years, to determine the breeders involved in each spawn. This study was conducted by analyzing four microsatellite loci previously validated for parental allocation in this species. The results of this part of the study reflected that only a low number of individuals were responsible for most of the spawns and the volume of eggs produced in each tank, and revealed that these dominant pairs maintained their relationships year after year.

Second, two experiments to try to explain the causes of the reproductive dysfunctions of this species in captivity were performed. The first one aimed to assess whether food could be behind the lack of fertilization of the natural spawns obtained from individuals born in captivity. The second aimed to determine whether the absence of fertilized spawns was an issue that affected both genders equally or not. The first experiment was carried out with cultivated specimens (G1) in 7 m^3 tanks. One of the tanks was fed with a commercial diet for breeders and the other with frozen natural food (mussels, worms, and squid). The hypothesis was to assess if a similar diet to that obtained by wild populations in the natural environment could include any element that was not present in commercial diets and that was a key factor in the quality of gametes or reproductive performance for a successful fertilization. The natural diet was significantly better in terms of daily relative fecundity, and noticeably better in terms of number of spawns and volumes obtained, but no fertilized spawn were recorded during the study period. The second experiment was conducted housing together wild males with cultivated females, and cultivated males with wild females, also in 7m^3 tanks. In this case, the goal was to determine whether reproductive disorders were a general problem of cultivated specimens or if, on the contrary, they affected males and females of the species differently. In this study, food was natural

and the cultivated individuals were previously adapted to this type of food. The results obtained in this experiment focus on the reproductive dysfunctions in cultivated males, as fertilized natural spawns were obtained with wild males and cultivated females. Moreover, this tank showed significant improvements in the number of spawns, relative fecundity and daily volumes obtained during the two years of study, compared with the wild females and cultured males tank.

The second part of this thesis aims to advance in the zootechnics of the species, particularly in the disinfection of fertilized eggs and the transport of specimens. Consequently, two separate experiments were performed. The first aimed to establish a specific protocol for disinfecting *S. senegalensis* eggs by adapting disinfection protocols intended for other species using iodine. For this purpose, a titration in iodine dosage was performed evaluating afterwards the effect on hatching and larval survival and the disinfectant capacity of the protocol. Increased doses were tested to determine the safety margins in the disinfection process. Disinfections at an experimental and commercial scale were tested, and the disinfectant success of the selected doses was assessed. Additionally, a specific determination of fungal and bacterial flora present was conducted in the eggs studied. After analyzing the results it was concluded that disinfection with 50 mg l^{-1} in iodine for 10 minutes followed by 3 rinses with sea water to remove traces in iodine is effective for removing most of the fungal and bacteria located on the egg surface without affecting the normal development of eggs of this species.

Finally and again related to improvements in the management, the possibility of transporting specimens of about 1 kg without water was studied, using a transport system previously used in turbot. The aim of this study was to assess the maximum time of emersion of this species to be used in transportation and in parallel, to assess the effect of this type of transport in terms of stress. Specimens of about 1 kg were placed individually in perforated polystyrene boxes on a wet cloth, and they were

sampled at different intervals (2, 6, 12, 20, 28, 36 and 44 hours of emersion) for the determination of the survival rate. At these intervals, 6 plasmatic parameters were also studied in plasma (cortisol, glucose, lactate, triglycerides, amino acids and proteins). After studying all data, a maximum time for this type of transport of 28 hours at 15 ºC, was established, without risk of mortality. When looking at stress, it was difficult to draw conclusions due to the lack of knowledge and the great variability of baseline levels, especially of cortisol, in this species. However, after 12 hours, cortisol levels increased significantly, reflecting objectively higher stress levels than previously observed.

The results of this thesis increase the knowledge on the reproductive biology of the *S. senegalensis* in captivity, and partially determine the cause of the reproductive disorders of the cultivated breeders, pointing at the males as the main responsible, while providing management tools that could improve the zootechnics of the species.

Chapter 1. Introduction

Chapter 1.

Introduction

1.1. Background and motivation for the research

The natural resources on planet earth are limited. With a growing population estimated to reach approximately 9.5 billion in 2050 according to the United Nations (UN) (2014), food production systems are one of the solutions to provide sustenance for the human population. To this effect, aquaculture is a developing activity that not only provides a source of food, but also exploits a space that, in most cases, does not compete with traditional land uses. This implies a great potential area for food production, since 70% of the Earth's surface is water. In addition, marine aquaculture has the added value of reducing the need for fresh water to carry out the activity, which is another important limited resource for humanity.

The FAO defines Aquaculture as "*the farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants, which implies human intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators, etc. These will, throughout the entire breeding or farming, and until and including harvesting owned by a natural or legal person. That is, this production implies individual or corporate ownership of the organism grown".*

Aquaculture began 4000 years ago in China, but it was only 50 years ago, that it started to represent an important socio-economic activity, creating over 12

million jobs worldwide. Currently it represents the same production levels in tons as that of capture fisheries, and taking into account the amounts destined for human consumption, aquaculture exceeds the tons produced (FAO 2014). The decline in catches due to overfishing, the size decrease of some populations or the excess of allowance of the fishing industry in many countries, all make aquaculture an alternative to be developed in parallel with scrupulous fishery management to help achieve a sustainable use of the marine resources. The global upwards trend of aquaculture development in total fish supply has remained uninterrupted. Farmed fish contributed a record 42.2 percent of the total 158 million tons of fish produced by capture fisheries (including those used for non food uses) and aquaculture in 2012 (Figure 1.1). This compares to just 13.4 percent in 1990 and 25.7 percent in 2000 (FAO, 2014).

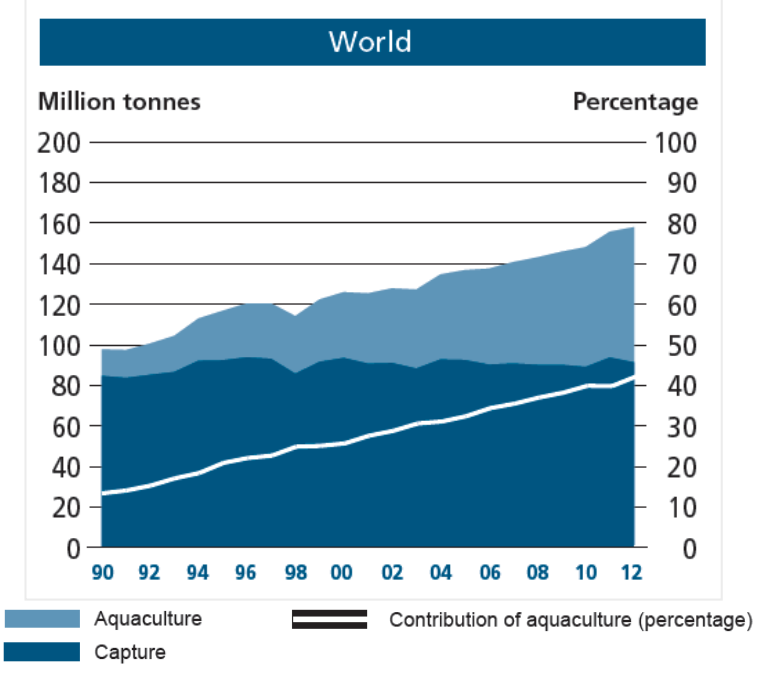

Figure1.1 Share of aquaculture out of total fish production. FAO. 2014

Around 600 aquatic species are currently being exploited worldwide in a variety of production systems and with different levels of technology using marine, fresh

and brackish water. Among the cultured food organisms, we find finfish, crustaceans, mollusks, amphibians (frogs), aquatic reptiles (excluding crocodiles), and other aquatic animals (such as echinoderms, tunicates and cnidarians), According to the latest FAO estimations (FAO, 2014), food fish aquaculture production rose by 5.8 percent to 70.5 million tons in the year 2013, and this production is intended almost entirely for human consumption.

One of the lines of work in aquaculture has been diversification, with the goal of increasing the number of cultivable species depending on the characteristic of the different areas and also adapting to the consumption habits of the markets to which these species are targeted. Currently this line is being widely discussed in professional scientific forums due to the excessive number of candidate species for such diversification which have appeared in recent years. Recently, the research community has begun to wonder whether the effort required to consolidate a species on an industrial scale (adaptation to captivity, adaptation to artificial feeding, captive breeding, development of larval rearing protocols, optimization of growth rates and optimization use of the food, as well as address the specific issues to face the pathologies that arise in the adapted species) is justified for all the proposed species. It seems more reasonable to combine efforts in consolidating species in the final zootechnical stages than to start from the beginning again with new species, in spite of these having great potential.

Flatfish are among the most popular and valuable human consumption species. They support valuable fisheries throughout the world, making them very attractive for aquaculture (Howell and Yamashita, 2005). In this sense, for the last three decades, the Senegalese sole has occupied a strategic position in the diversification discussions (Imsland *et al.,* 2004; Howell *et al.*, 2009; Morais *et al.*, 2014) mainly due to its economic value, but only in the last 5 years has it taken the final leap towards the industrial scale production. However, it continues to

present limitations for fully sustainable and profitable culture, and for example the production of sole in Spain is very far from the quantities of sea bass, seabream or turbot. However this production has multiplied fivefold in 8 years (Table 1.1), according to APROMAR in 2014. This organization envisages that the cultivation of this species in Europe will be among the main 5 species for 2030 (sea bass, sea bream, turbot, meagre and sole).

Table 1.1 Sole aquaculture production data for Spain in tones per different regions, production percentage of each region in 2013, and variation percentage between consecutive years. From APROMAR 2104.

LENGUADO	2005	2006	2007	2008	2009	2010	2011	2012	2013	2013%
Andalucía	20	55	24	10	10	4	0	0	13	3,8%
Canarias	0	0	6	15	28	30	31	24	30	8,7%
Galicia	20	0	30	30	150	170	79	170	300	87,5%
Murcia	20	25	0	0	0	0	0	0	0	0,0%
TOTAL	**60**	**80**	**60**	**55**	**188**	**204**	**110**	**194**	**343**	
Variación %	-20,0%	33,3%	-25,0%	-8,3%	241,8%	8,5%	-46,1%	76,4%	76,8%	

The two main constraints that have delayed the implementation of this species in the aquaculture industry are the diseases and reproductive dysfunction in individuals born in captivity (G1, G2, ...). Pathologies, common in post-larval stages and to a lesser extent in fattening juveniles, can trigger large mortality events (Toranzo *et al.*, 2003; Padrós *et al.*, 2003; Arijo *et al.*, 2005) and are largely the result of the lack of knowledge regarding the physiological requirements of the species (Cañavate, 2005). Concerning the reproductive dysfunction in individuals born in captivity, even today the cause or causes of this dysfunction are unknown, but progress has been made in understanding the factors that may be associated with this condition. This problem has been approached from many points of view including, among others, nutrition, behavior, physiology or epigenetics of the species (Morais *et al.*, 2014).

Today, after over 30 years of many teams researching the problem mainly in Spain and Portugal, it appears that the lack of natural spawns in individuals born in captivity lies in the males of this species. Although the reasons are not clear, it seems that the absence of reproductive behavior and its relationship to structural brain dysfunctions may be behind the problem. Fortunately, the first artificial fertilizations in 2007 (Chereguini *et al.*, 2007) and the recent advances in the artificial fertilization protocols developed for this species (Liu *et al.*, 2008; Rasines *et al.*, 2012, 2013; Rasines, 2014), open the possibility for its industrial culture without reliance on wild individuals.

1.2. Study species

This thesis focuses on the study of the Senegalese sole, *Solea senegalensis*, Kaup 1858, specifically on the study of this species in captivity (Figure 1.2). 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 1.2 Breeder of *S. senegalensis* born in the IEO Marine Culture Plant El Bocal, in Santander.

S. senegalensis are flatfish with an asymmetrical oval body (eyes on the right side). The interradial membrane of the pectoral fin on the side of the eye is black. This feature distinguishes it from the common sole (*Solea solea*), as it has a compact black stain on the back of the fin. It is distributed in the Atlantic from the Bay of Biscay to the coasts of Senegal and is less common in the western Mediterranean (Figure 1.3). It is a predominantly coastal marine benthic species, living in sandy and/or muddy bottoms in coastal areas, at depths of up to 100 m. It feeds mainly on benthic invertebrates such as polychaete larvae, bivalve mollusks and small crustaceans. Sexual maturity is reached when the size is around 30 cm. Spawning occurs between March and June (www.fishbase.org).

Figure 1.3 Distribution map of *S. senegalensis* adapted from www.fishbase.org.

A detailed taxonomic study performed by Ben-Tuvia, (1990) carried out to clarify the taxonomic status of the main species of the genus solea Atlantic-Mediterranean region, concluded the following features for *S. senegalensis*: "*Last ray of dorsal and anal fins joined by a low membrane to the base of the caudal fin. Supratemporal branch of lateral line present forming an arch row of scales along straight part of lateral line 119-124; anterior nostril on the blind side not enlarged,*

its diameter about half that of the eye; length of scales above nasal tube on ocular side much less than the length of the tube; anterior nostril on the blind side situated rather close to the front margin of head, the distance slightly greater than that separating the nostril from the mouth cleft, their ratio 1:1-1.4; gill rakers on first arch on ocular side in form of short knobs, six to seven on lower arm and two on upper; number of vertebrae 44-46; number of dorsal fin rays 46-89; number of anal fin rays 62-71. Colour: pectoral fin on the ocular side with a large dark blotch over the whole middle and distal part of the fin; blind side whitish".

1.3. Evolution of the research and cultivation of *S. senegalensis*

The first studies in *S. senegalensis* biology with aquaculture purposes were carried out in the mid-eighties in Cádiz (Spain) (Rodriguez and Pascual, 1982; Rodriguez, 1984) and in Portugal (Dinis, 1986; Dinis *et al.*, 1987). These experiments aimed to control the reproduction in captivity of the species and establish suitable culture conditions. Between the mid-eighties and mid-nineties, the industrial and scientific efforts focused on the cultivation of the sea bass and the sea bream, which caused market saturation and a decline in price (Rodriguez and Peleteiro, 2014). In this context, the need for a new competitive species reactivated the activity around sole. During these years, the efforts and results of the research community allowed the Senegalese sole to attract the interest of producers, and has been established as a strong candidate for Mediterranean aquaculture.

Since then, many studies have been carried out aimed at controlling the reproduction of this species in captivity (Anguis y Cañavate, 2005; Dinis *et al.*, 1999), controlling the previously identified diseases (Zorrilla *et al.,* 1999; Sarasquete *et al.*, 1993), and in summary, focused on developing the knowledge framework needed for the definitive industrial production. In these years, there

were significant the contributions that on the one hand reinforced sole's candidacy to diversify Mediterranean aquaculture, and on the other hand showed the main problems for the cultivation of this species: pathologies and reproductive dysfunctions in the captive born individuals. Important pathological events that produced uncontrollable mortality and an almost total lack of fertilized spawns in the generations born in captivity (Imsland *et al.,* 2004) delayed, until today, the definitive introduction of the species in the market.

The last years of work and research on the cultivation of this species have changed from a zootechnical perspective, to a more detailed study of the specific problems identified. Moreover, the important development of genetic and molecular tools has allowed new approaches to the study, and new ways to address these problems. The scientific production in recent years has grown exponentially due to the good economic situation of the countries involved in the development of the culture of this species, and also to the rise in the number of institutions that have participated (Universities, Regional and National research centers, private companies, training centers, foundations, etc ...).

The current situation of the Senegalese sole is still uncertain; however, there are companies mainly in Spain, France and Portugal, which are increasing the production on a yearly basis. The number of tones produced in Europe in the last decade has increased more than 7 times (Figure 1.4), and the new facilities that are being built for the culture of this species suggest a steady increase in the production (Rodríguez and Peleteiro, 2014).

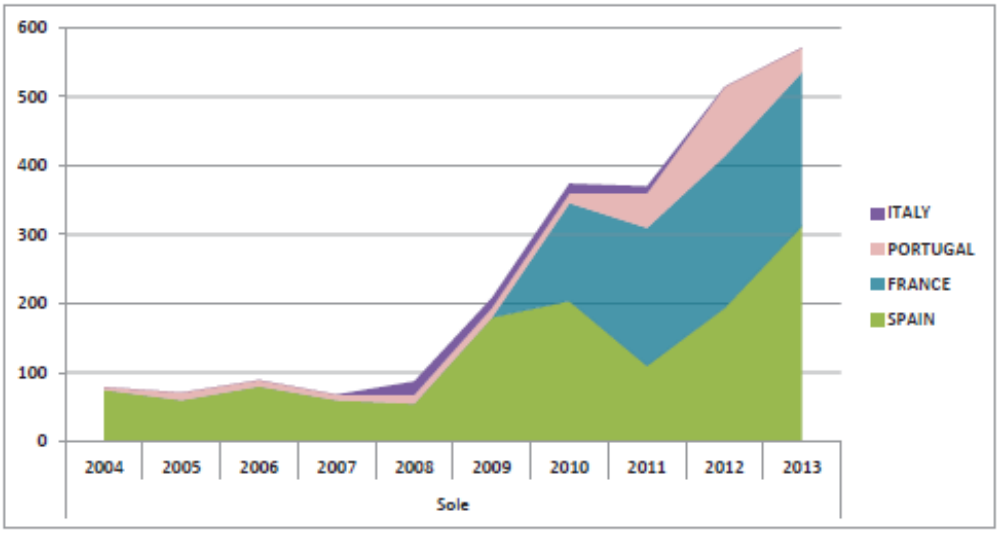

Figure 1.4 Sole Aquaculture production data in Europe in tones per different countries. Federation of European Aquaculture Producers (FEAP), Production Report, 2014.

1.4. Zootechnics of *S. senegalensis*

The cultivation of this species, like that of other marine aquaculture species, comprises four main stages: incubation, larval rearing, fattening and reproduction. In the case of sole, the fact that only wild individuals adapted to captivity spawn makes another phase necessary, which is, conditioning these wild breeders to captivity. Each phase has its own quirks and problems and almost every facility has different ways of addressing them, consequently, the process described below represents a general description of each phase, mainly based on Rodriguez and Peleteiro, (2014).

The incubation stage comprises the period between the collection of the spawn and the hatching of the eggs, and therefore the embryonic development of the individuals. In the case of Senegalese sole, this period is particularly short, lasting

between 24 and 48 hours at 18ºC (Figure 1.5). This depends on how much time has elapsed between spawning and egg collection (Figure 1.6).

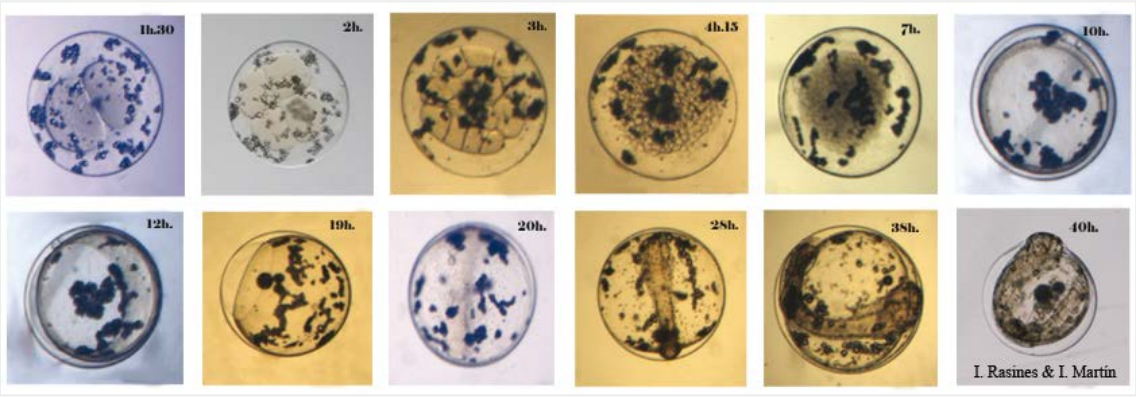

Figure 1.5 Embryonic development stages of *S. sensegalensis* eggs obtained through artificial fertilization at the IEO Marine Culture Plant El Bocal, in Santander.

In general, the eggs are incubated in 70-200 L incubators with filtered seawater (1 µm) at 18-20 ºC, with a water renewal rate of 1.5 L per minute, moderate aeration and a maximum density of 4500 eggs/L. Once the larvae hatch, they have to be transferred to larval rearing tanks within a 48 hour period.

Figure 1.6 Natural spawns collected in different experimental tanks at the IEO Marine Culture Plant El Bocal, in Santander.

The larval rearing begins after hatching. Larvae are pelagic and have a size of about 2.5 mm, are symmetrical, and are born with the anus and mouth shut. The optimum temperature is between 17 and 21 °C. Illumination typically includes light 24 hours a day until the larvae becomes pelagic, after which photoperiods of 16 hours of light and 8 of darkness are applied. The water renewal ranges from 5 to 10%h^{-1} at the beginning, up to renewals of 200%h^{-1}. The larval cultures of this species are performed at an average density of 20-40 larvae per liter. The use of phytoplankton (frequently genres *Chlorella* and *Isochrysis*) in the culture tanks during the first days is common. The larvae are fed with rotifers (*Brachiorus plicatis*), Artemia nauplii (*Artemia salina*) and microencapsulated feed. During the first 10 days, the larvae are fed with rotifer, which is then substituted for Artemia and the weaning is produced around 50-60 days after hatching. Modifications in feeding types are often carried out by cofeeding to avoid sudden changes and reduce the problems that the adaptation to the new food can cause. During this stage, the larvae, initially pelagic, suffer a metamorphosis, consisting of morphological (air bladder reabsorption, migration of an eye, ...) and physiological changes resulting in a benthic organism.

Fattening of this species includes the growth of the specimens to a commercial weight of approximately 300-400 gr. This stage is less complex technically but it is longer, usually taking around 18 months at 18-20 ºC, and consequently the great interest in reducing this phase from an industrial optimization point of view. The fattening stage consists of efficiently feeding the productions with extruded feed. Nowadays there are specific diets for this species on the market. At this stage, it is also important to prevent disease outbreaks in batches, since a pathological problem can cause significant economic losses. At the end of this stage, the individuals that present interesting parameters to be passed on to the following generations (usually good growth rates) are selected as future breeders.

The last stage, reproduction, consists of the control of the spawns, mainly through the manipulation of physico-chemical parameters, or the artificial fertilization, which occasionally implies male or female induction with hormonal methods. It is probably the most difficult stage to control because there are important differences between species. Most species, such as turbot or seabass and seabream, respond to the photothermal manipulation, but in the case of *S. senegalensis* the spawns are produced through temperature manipulation.

Figure 1.7 Artificial fertilizations at an experimental scale carried out at the IEO Marine Culture Plant El Bocal, in Santander.

Currently the reproduction of this species is being carried out by natural spawns of wild specimens adapted to captivity, or artificial fertilization of G1 individuals (Figure 1.7). Therefore, reproduction in this species requires the continuous adaptation of the wild breeders to captivity. Wild individuals quickly adapt to captivity, initially fed by annelids, mussels and squid. After a period of approximately 6-8 months, dry feed can also be added to their diet. These individuals can participate in the spawning process during their first year of captivity.

1.5. Reproductive biology in *S. senegalensis.*

The Senegalese sole is a teleost, dioecious, without differentiable external sexual characteristics, which first ripens around the third year of life. One of the earliest and most complete studies on the reproductive biology of *S. senegalensis* was conducted by Rodriguez (1984). In this work, in addition to morphological characteristics, geographical distribution, or biometric studies, several characteristics of the reproductive biology of this species in captivity were detailed.

First, Rodriguez revealed the great length of the reproductive cycle of the specimen and the important length of the spawning season, analyzing gonadal stages of females collected from the natural environment. Another aspect described was the discontinuous nature of their reproductive cycle; the ovary matures several batches of eggs per spawning season, allowing several spawns per female (multiple spawning). The iteroparity, i.e. the ability of an individual to participate in several reproductive cycles, was also reported in this species.

There is not much more scientific literature on the reproductive biology of this species in its natural environment, Dinis (1986), Sarasquete *et al.* (1993); Teixeira and Cabral (2010) or Vinagre *et al,* (2013) are the main examples. However, this research has greatly contributed to the understanding of the natural spawning periods in the different geographical areas, the evolution of the gonadal development during a spawning season, or the gonadal development stages in the natural environment.

With this baseline information, and the numerous studies conducted since the mid-eighties, an important amount of information on the reproductive biology of this species, especially in captivity, has been produced. The research in this field has been motivated initially by the economic potential of this species culture, and

later on by the reproductive dysfunctions described in G1 breeders. Of note is the pioneering study conducted by Dinis (1999) on the development of the culture of this species and by extension on its reproduction. This study described the first spawns in captivity, the spawning period duration (4-6 months), and the evolution of the eggs' diameter over a spawning season. Additionally, this research produced the first approaches to establish the appropriate conditions that allowed the reproduction in captivity (light, temperature, food, etc.).

One of the most important results in terms of control of the reproduction of this species in captivity where those obtained by Anguis and Cañavate (2005), these results allowed to define the relationship between weekly changes in temperature, occurring naturally in their facilities, with the onset of the reproductive activity, and determined that spawning was related to temperature increases of between 0.2 and 2.5°C. These results contributed greatly to the reproduction control in captivity through thermo-manipulation. The importance of this work is based on the possibility of recreating these temperature fluctuations artificially to induce spawning in any facility (Chereguini, 2006; Martin *et al.*, 2007).

Although it appears that the temperature is the main factor controlling the natural reproduction of this species in captivity, the influence of light in the reproduction of fish has been demonstrated in numerous species (Bromage, 1995). In this sense, several studies have been conducted showing the nocturnal character of the reproductive events of this species in captivity (Oliveira *et al.,* 2009), the inhibition of ripening under continuous regimes of light or temperature (García-López *et al.,* 2006b), or the influence of lunar cycles in the melatonin and sex steroids levels (Oliveira *et al.,* 2010). Unfortunately however, these works have not allowed, up to now, the establishment of a clear relationship between photoperiod and spawning in this species.

Currently, fertilized natural spawns are only obtained from wild individuals adapted to captivity or mixed broodstocks composed of wild males and cultured females (Mañanos *et al.*, 2007; Martín *et al.*, 2011). The natural reproduction of cultured individuals that reach sexual maturity only produce occasional spawns with small volumes of eggs that show low or no fertilization (Anguís *et al.*, 2007). Consequently, the integral culture cycle of the species cannot be considered completely closed just yet. Nowadays, work is underway to approach the problem of natural spawning from different perspectives, such as behavioral (Carazo *et al.*, 2011), see Figure 1.8, nutritional (Norambuena *et al.*, 2012a, 2012b), physiological (Guzman *et al.,* 2008, 2009) or epigenetical.

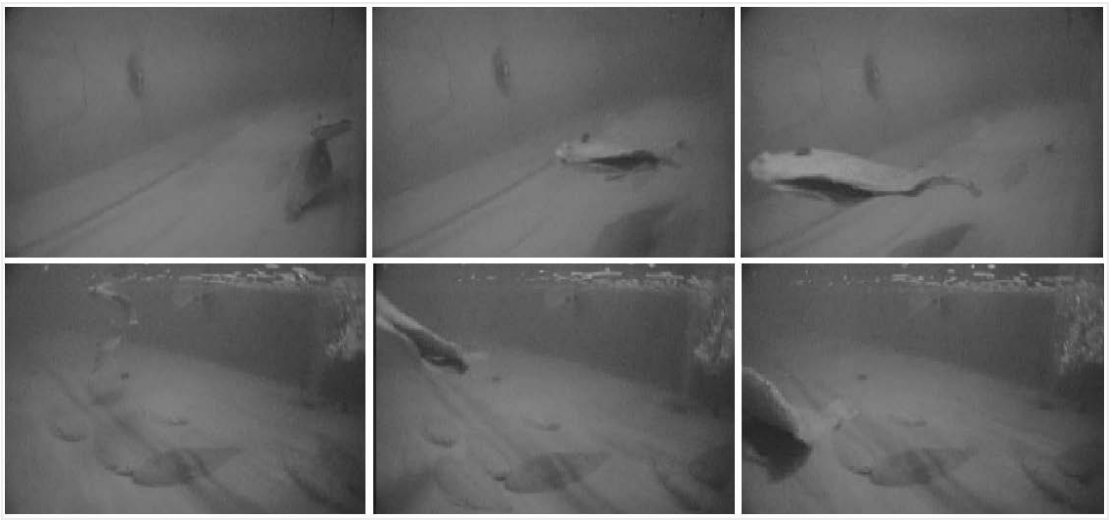

Figure 1.8 Frames captured by video recording the courtship of *S. senegalensis* held at the IEO Marine Culture Plant El Bocal, in Santander. Images captured by IRTA.

The control of ripening and ovulation induction via hormone therapy has been widely studied in this species (Agulleiro *et al.,* 2006; Guzmán *et al.,* 2008, 2009; Rasines *et al.,* 2012, 2013). In this sense, progress has been made in understanding the processes that control both neuroendocrine maturation and ovulation, and spawning in this species. There is currently a specific induction protocol developed

by Rasines, (2014) that allows a high level of control of the reproduction through artificial fertilization (Figure 1.9). In this sense, the reproduction cycle of this species in captivity could be considered closed since these protocols and the artificial fertilization make it almost possible to work on an industrial scale (Liu *et al.*, 2008).

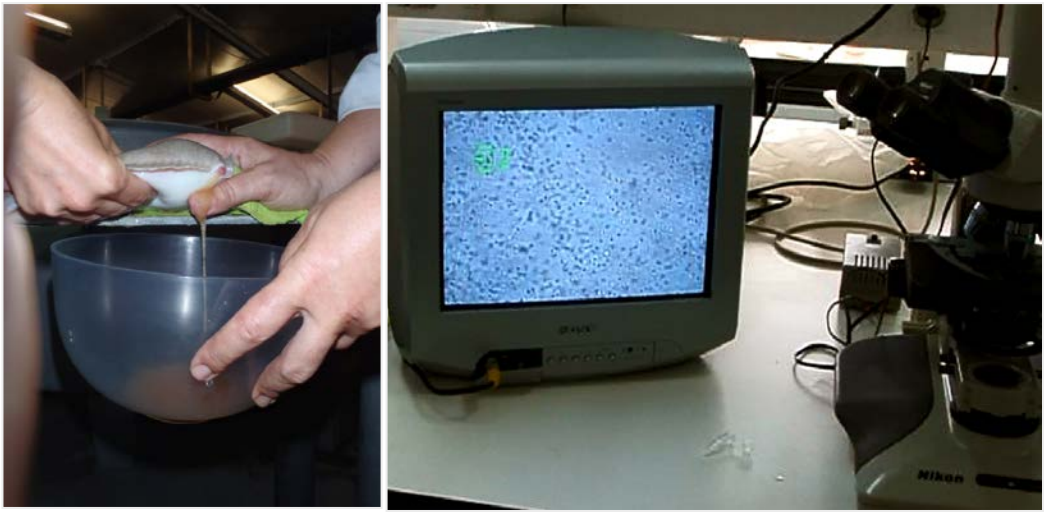

Figure 1.9 Egg stripping, and sperm video recording carried out with *S. senegalensis* individuals held at the IEO Marine Culture Plant El Bocal, in Santander.

However, and despite these advances, there are still some aspects that need to be optimized for a total and exclusive implementation of artificial fertilization systems industrially; on the one hand, the lack of reproducibility, especially at the level of fertilization and hatching rates obtained, and on the other, the need to improve the control of sperm production and management (Morais *et al.*, 2014).

Chapter 2. Objectives

Chapter 2

Objectives

The objectives of this thesis are: 1) to describe aspects of reproductive biology of the *Solea senegalensis* species in captivity, 2) to understand why individuals born in captivity do not breed naturally, and 3) to make improvements in the management of this species in captivity.

For the first objective, a detailed study of the natural spawns of a wild broodstock composed of 77 individuals housed in three 14 m^3 tanks subjected to thermo-manipulation to induce spawning was carried out. The study lasted four years. During this period, a study of all the mating relationships of the three tanks was performed to determine which individuals of the stock were involved in the spawns, what the individual contributions were, and how those relationships were produced.

For the second objective, two experiments were carried out. The first one consisted of a study equivalent to the one performed with wild individuals, conducted with captive breeders (G1). These were fed two different diets to assess the influence of this parameter on the reproductive success of the cultivated specimens of this species. This stock was composed of 32 breeders distributed in 2 7m^3 tanks, one of which was fed with commercial feed and the other with natural food (mussels, squid and worms). The second experiment, designed to assess possible causes of the reproductive dysfunctions of the G1 generation, evaluated the reproductive performance of mixed broodstocks: 1) wild caught males with G1 and G2 females, and 2) wild caught females with G1 and G2 males. The idea of this work was to determine whether the lack of

natural spawns from cultured individual affected both sexes equally, or if instead it was a problem specific to one of the sexes.

As for the third objective, aimed at improving the management of the species in captivity, two different aspects were addressed; on the one hand the disinfection of fertilized eggs, and on the other, the transport of live specimens of this species.

Egg disinfection is a routine process consolidated in most species in the industry, and its study has been included in this thesis to hone this technique for the Senegalese sole. There are four main advantages of disinfected eggs in a species in captivity. The elimination of all pathogens present on the surface of the eggs, which may affect the larvae after hatching. The elimination of vertical transmission of diseases (parent to offspring). The prevention of contamination between facilities (produced when the eggs are incubated at different facilities). Finally, the last advantage is the improvement of hatching rates, as found with other species. For this purpose, the disinfection of eggs with iodine, one of the most widely used disinfectants in aquaculture, was studied. First, the lethal dose of this compound for the disinfected eggs was determined. An analysis of the disinfection effectiveness of this method was also conducted. Then, the effects of the use of this compound in the hatching and larval survival of the species were evaluated, and finally, an analysis at a specific level of the bacterial flora was carried out in natural spawns of the soles reared in our facilities.

When considering live fish transport, there are several important factors to take into account: 1) broodstock management and ability to use these breeders at different facilities of a company, sending or sharing individuals between different facilities or institutions, and 2) keeping the product fresh for the consumer. To this effect, a technique for waterless transportation of turbot has been adapted to this species, not well detailed in the scientific literature. This technique has the great potential

advantage of being very simple to perform and potentially providing considerable economic and logistical savings compared with traditional water transport. A study was therefore conducted to determine the maximum time that this species survives out of water, in saturated humidity conditions, and to determine the stress levels caused by transportation in these conditions.

The specific objectives were:

1. Evaluate the production parameters of this species in captivity.
2. Determine the specific relations established in the breeding tanks.
3. Evaluate individual performances of the broodstock.
4. Determine the possible relationship between diet and reproductive dysfunction of the cultivated individuals.
5. Determine the possible relationship between the origin (wild or cultivated) of each gender and the reproductive dysfunction of the cultivated individuals.
6. Define a specific protocol for this species' egg disinfection using iodine.
7. Develop and evaluate the effects of waterless transport of individuals of this species.

Chapter 3. Evolution of egg production and parental contribution in Senegalese sole, Solea senegalensis, during four consecutive spawning seasons.

Chapter 3.

Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis,* during four consecutive spawning seasons.

3.1 Introduction

Currently, marine aquaculture in Europe is dominated by four species: seabass, *Dicentrarchus labrax*; gilt-head seabream, *Sparus aurata*; turbot, *Scophthalmus maximus*; and cod, *Gadus morhua*. Despite the growing demand from the productive sector to diversify the species produced through aquaculture, some species, such as Senegalese sole, which occupies a strategic position for such diversification over a period of 10 years (Imsland *et al.*, 2003), are not consistently represented in the fish farming companies (Howell *et al.*, 2009). Between 2006 and 2011, Senegalese sole showed the highest value of all the marine fish aquaculture species commercialised in Spain (APROMAR, 2012). In addition, Senegalese sole showed good performance for growth, larval survival and capacity for adapting to intensive culture, but there are specific issues with the culture of this species that limit its production on an industrial scale (Anguís *et al.*, 2007). The two main issues that have delayed the definitive introduction of Senegalese sole into industrial cultivation are reproductive dysfunction in cultured individuals and high incidence of bacteriological diseases. These diseases, which principally occur at the post-larval stage and, to a lesser extent, in the juvenile and growing phases, can lead to mortality (Padrós *et al.*, 2003; Toranzo *et al.*, 2003; Arijo *et al.*, 2005) and are a consequence of the poor understanding of the physiological requirements of the species (Cañavate, 2005). New reproduction

strategies have been tested over the past few years, including artificial fertilization with cultivated individuals from the first generation (Chereguini *et al.*, 2007; Liu *et al.*, 2008; Rasines *et al.*, 2012, 2013). Positive artificial fertilization and hatching rates have been obtained from females subjected to hormone therapy. The viable eggs, obtained by abdominal pressure, were fertilised with fresh and cryopreserved sperm. These advances reinforce the role of the Senegalese sole as an alternative for Mediterranean aquaculture. However, the relative complexity of the artificial fertilization method required more manpower than the natural spawns, and the lack of knowledge regarding the causes of reproductive disorders in cultured breeders necessitates the study of the natural reproduction of this species in captivity.

Currently, natural spawns are only obtained from wild individuals adapted to captivity or mixed broodstocks composed of wild males and cultured females (Mañanos *et al.*, 2007; Martín *et al.*, 2011). The cultured breeders that reach sexual maturity only produce occasional spawns with small volumes of eggs that show low or no fertilization. For this reason, the integral culture of the species still cannot be considered closed; therefore, further research is necessary to understand the reproductive biology of the species. Currently, work is underway to approach this problem from different perspectives, such as behavioural (Carazo *et al.*, 2011) or nutritional (Norambuena *et al.*, 2012a, 2012b).

The study of microsatellites for aquaculture species has increased enormously because of their usefulness for the identification and differentiation of stocks; for the control of inbreeding and kinship; and for the development of linkage maps, genetic selection programs or genetic manipulations related to polyploidy and gynogenesis (Martínez, 2005; Chistiakov *et al.*, 2006). In addition, parentage analysis based on genetic markers have led to an increase in the understanding of the reproductive biology of the species through the identification of the effective breeding population, the spawning dynamics or the individual spawning performances (Hutchings *et al.*, 1999; Cameron Brown *et*

al., 2005; Herlin *et al.*, 2008). In recent years, efficient and effective genetic tools for better management of Senegalese sole have been developed (Castro *et al.*, 2006; Porta *et al.*, 2006a, 2006b). In the Marine Aquaculture Plant of the Spanish Institute of Oceanography (I.E.O.), since 2003, non-hormone induced natural spawns have been obtained using wild specimens adapted to captivity (Chereguini, 2006; Martín *et al.*, 2007) by imitating the natural temperature fluctuations that occur in Spain's southern Atlantic region (Anguis and Cañavate, 2005). The aim of the present study is to evaluate the reproductive parameters of a Senegalese sole stock during four consecutive spawning seasons and to determine their mating relationships based on the results of parental allocation of larvae collected during the study period.

3.2 Material and Methods

3.2.1 Broodstock and management

The initial wild Senegalese sole stock from the I.E.O. Marine Cultivation Plant, originating from Huelva (Spain), was established in 2002. At the beginning of 2007, the initial wild sole stock (n= 75) was housed in three 14 m^3 tanks (B1, B2 and B3) avoiding allocation of highly inbred pairs in the same tank according to pair-wise relatedness estimation among individuals performed at the Genetics Department of the University of Santiago de Compostela (Spain) using a microsatellite tool (see sections 3.2.4 and 3.2.5). These three groups were composed of unrelated breeders with a male:female ratio of 1:1 and an average density in the tanks of 3 kg/m^2.

The tanks were located inside an industrial warehouse, in open flow circuit, with a water renovation rate of 1.7 m^3/hour and constant moderate aeration. The average salinity recorded during the study was 34.6±0.87 PSU. An artificial photoperiod of 16 hours of light, and 8 of darkness was used throughout the entire year. The light

intensity was reduced using mesh shading over the tanks that allowed a maximum light intensity at the water surface of 50 lux. The average total body weight ± SE of the females and the males at the beginning of the trial (January 2007) was 1677 ± 105 g and 966 ± 68 g for tank B1 (N = 23), 1970 ± 65 g and 1179 ± 91 g for tank B2 (N = 25), and 1948 ± 86 g and 1190 ± 55 g for tank B3 (N = 29), respectively. Each individual was tagged with a passive integrated transponder (Trovan®) placed in the dorsal area, allowing the specimens to be identified and monitored. All the individuals were weighed and measured (total length) once a month, and the gonadal maturity stage of the females was estimated by external examination of abdominal swelling according to the classification of Anguis and Cañavate (2005): stage 0 corresponds to externally undetectable development; stage I corresponds to ovary detection that can only be detected by touching the abdominal region of the females; stages II and III correspond to two levels (initial and intermediate) of externally visible abdominal swelling; and stage IV corresponds to maximum ovary development, characterised by high abdominal swelling and a slightly orange colour on the blind side. All tanks were treated with a prophylactic hydrogen peroxide bath after sampling (80 ppm for one hour without renovation).

The broodstock were fed with natural food composed of mussels, *Mytilus* spp., small squid, *Loligo* spp., and polychaete worms, *Nereis* spp., 6 days a week (Seabait Ltd., UK). In the pre-spawning and spawning period, they were fed with small squid 3 days a week (Tuesday, Thursday and Saturday) and mussels the other 3 days (Monday, Wednesday and Friday). In addition, frozen worms were added to their diet on Wednesday. For the rest of the year, they were fed with mussels 2 days a week (Monday and Friday) and small squid 4 days a week (Tuesday, Wednesday, Thursday and Saturday). The daily amount of food was adjusted to 1% of the total biomass of the fish in the tank, which was determined monthly.

The fish were always handled (routine management and experimentation) according to the European Union Directive (EEC, 1986) for the protection of Animals Used for Experimental and Other Scientific Purposes.

3.2.2 Thermoperiod manipulation

Beginning at the end of January each year, the thermoperiod was manipulated to induce the reproductive response of the broodstock. The initial environmental temperature was approximately 13 ºC. The temperature was increased in 0.5 ºC stepped intervals once each week. When the temperature reached 16 ºC, it was increased by 2 ºC to a maximum temperature of 18 ºC and decreased again to 16 ºC every four and three days, to imitate the natural fluctuations recorded in the Toruño (IFAPA, Cádiz) to induce natural spawning (Anguis and Cañavate, 2005). All of the temperature changes were performed by modifying the inlet water, and the variation in the tank was mitigated by the renovation rate (1.7 m^3/h). In the middle or end of June, when the environmental seawater temperature was equal to that of the artificially heated water, the thermoperiod was no longer manipulated. The natural spawns obtained from this time were due to the natural local fluctuations.

3.2.3 Collection and evaluation of spawns

Eggs obtained from natural spawns were collected daily from February to November in a 350 µm mesh net suspended into a 200 L tank located beneath the broodstock tank overflow outlet.

The spawns were placed in 1 L graduated cylinders to estimate the volume of floating and non-floating eggs. All of the spawns were recorded, but only those with a total volume that was greater than or equal to 20 mL were further used in this study.

To evaluate natural spawn quality, the total volume of eggs (mL), the floating and non-floating volumes (mL), the daily relative fecundity and the fertilization and hatching rates (%) were determined during all four years. The daily relative fecundity was calculated as the total number of eggs produced per day in a tank related to the total weight of the females in the tank. The total number of eggs was calculated by multiplying the total volume of spawning by 1080 (Anguis and Cañavate, 2005). To determine the fertilization rate, a sample of the floating eggs was placed in a Bogorov's plate with seawater; the rate of fertilization was determined by the number of fertilised eggs divided by the total number of eggs observed, counting a minimum of 200 eggs in each sample.

Once the spawns were evaluated, the floating fraction was incubated in 70 L incubators with filtered seawater (1 µm) at 18-20 ºC, with a water renewal rate of 1.5 L per minute, moderate aeration and a maximum density of 4500 eggs/L. The larvae hatched after 24-48 h of incubation, depending on the water temperature and the embryonic stage at which the eggs were collected. The 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 a minimum of three 100 mL subsamples. In 2009, the diameter of the eggs was also measured throughout the spawning season. A total of 100 eggs per spawn were measured using the software and hardware system AnalySIS®. The data from spawns where there was genetic evidence that only one female was involved were studied to evaluate the diameter of the eggs throughout the breeding season from single females without the interaction of other females.

3.2.4 Genetic analysis and parental assignment

The first stage of the study was to genetically characterise the broodstock at IEO Santander to estimate genetic diversity and the potential for paternity inference of a set of four highly polymorphic microsatellites (F13-7, Smax-02, SseGATA38 and CA13) previously validated in this species using a large set of families (Castro et al., 2006). For this purpose, caudal fin clips were obtained for each individual and preserved in absolute alcohol for DNA extraction using the synthetic resin Chelex®100 according to Walsh et al. (1991). All broods were genotyped for the four microsatellite loci included in a single multiplex PCR using an automated DNA sequencer ABI prism_3730 (Applied Biosystems). Allele sizes were scored against a GeneScan500 LIZ size standard using Gene Mapper 3.7 (Applied Biosystems).

In spite of the broodstock of the IEO Santander came from the wild and therefore, no relevant inbreeding should be expected, we took advantage of the genotyping performed with the microsatellite set to estimate kinship between all brood pairs using the relatedness coefficient (r). Pair-wise relatedness estimators have an important error to estimate kinship depending on the number of loci and the number of alleles per locus (Wang et al., 2002), however, we still considered this information relevant at least for detecting the highest related brood pairs to allocate them in different tanks to avoid inbreeding.

Once the parents were organised in different tanks, the second step was to perform the parental allocation of the larvae born during all four years of the study. To determine the parental allocation of the larvae, all spawns fertilised between February 2007 and October 2010 with floating egg volumes above 20 mL were incubated as described above. From the hatched larvae, 30 were washed with alcohol 96% and placed in eppendorf tubes with absolute alcohol for subsequent parentage analysis.

During this period, a total of 3630 larvae from 363 spawns were genetically characterised using the method described above.

3.2.5 Statistical analysis

All estimations were expressed as the mean ± standard error (SE). Data normality and homogeneity of variances were evaluated using the Kolmogorov–Smirnov and Levene tests, respectively. The non-normal variables were log- or angular-transformed either for the entire data set or for the ratios, respectively. Differences between means were examined using an ANOVA and Tukey´s test or the equivalent nonparametric Kruskal–Wallis and Mann–Whitney tests, with significance levels at $p<0.05$. Correlations between variables were analysed using the Pearson coefficient. A cubic regression model was used to adjust the evolution of egg diameter. All data were analysed using the statistical package SPSS 14.0.

Genetic diversity per locus in the broodstock was obtained by estimating the number of alleles, observed and expected heterozygosity, and polymorphic information content and then averaged over loci. The potential of the microsatellite set to allocate offspring from the IEO Santander broodstock was estimated by calculating the exclusion probability of detecting a false parent when no parent is known (Excl 1) and when one parent is known (Excl2). The frequency of null alleles was also estimated at each locus since frequencies above 5% may compromise the confidence for parental allocation (Marshall et al., 1998). All these estimations were conducted using the software CERVUS 2.0 (Marshall et al., 1998). Parental allocation of progenies was obtained using the exclusion method implemented in the FAP program (Taggart, 2006). Using genotype information, the relatedness coefficient (r) was estimated between all pairs of breeders using the Wang estimator (Wang, 2002), because it has demonstrated the best performances using simulated (Wang, 2002) and real

microsatellite data (Pino-Querido et al., 2009). Three broodstock tanks were established at the beginning of 2007 and stocked mostly with unrelated individuals (r = 0), but always showing molecular kinship coefficients r < 0.25, which corresponds to half-sib kinship, on average.

3.3 Results

3.3.1 Spawning and egg quality.

The evolution of spawns, expressed as the daily relative fecundity of each tank (B1, B2 and B3) over the four year period of the study as well as the thermoperiod to which they were exposed is represented in Figure 3.1. As shown, the spawning period of the stock subjected to a handling thermoperiod in our facilities lasted between 6 and 10 months, depending on the tank and the year, and there was a large variation of daily relative fecundity in the different spawns ranging between 734 and 34874 eggs per kg of females.

No differences were found in the mean relative fecundity or fertilization rate per year between the different years. There were differences in the proportion of floating eggs (highest in 2008) and in the hatching rate (significantly lowest in 2007). The total number of hatched larvae and the floating volume had the lowest values in 2007, followed by two regular and productive years (2008 and 2009) and a decline in 2010 (Table 3.1.).

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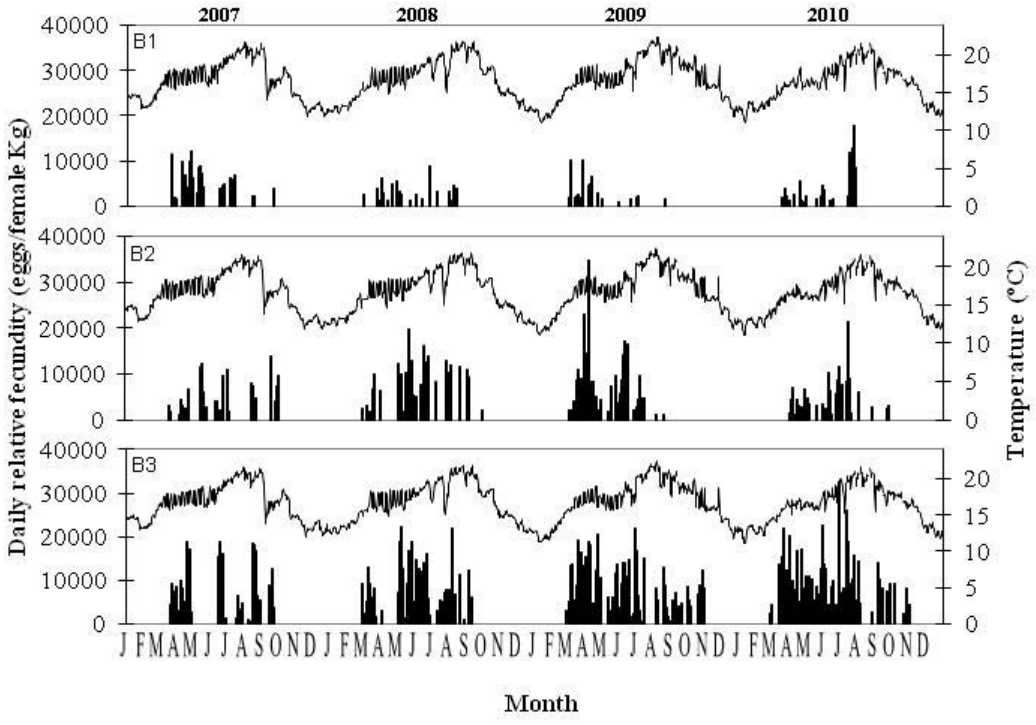


Figure 3.1. Daily relative fecundity and temperature regime of the three broodstock tanks over the four year study period.

Table 3.1. Summary of the broodstock production parameters for each year. Superscripted letters indicate significant differences in a parameter between different years. MRFec: mean average relative fecundity.

Year	Total Volume (mL)	Floating volume (mL)	Floating volume rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns	Fertilization rate ± SE	Hatching rate ± SE	Larvae
2007	16710	7115	35.05±2.49[b]	5.72±0.39	124	77.35±2.42	49.45±4.83[b]	3694961
2008	23755	13242	45.68±2.64[a]	6.68±0.41	143	78.46±2.93	65.22±2.99[a]	8812167
2009	30999	13432	37.32±2.12[b]	6.97±0.41	185	68.03±3.18	64.28±2.91[a]	8457883
2010	23793	9653	35.00±2.04[b]	6.44±0.39	181	71.70±3.07	63.15±3.59[a]	5909869
Total	**95257**	**43442**	**38.1±1.16**	**6.51±0.2**	**633**	**73.28±1.54**	**61.56±1.76**	**26874880**

There were significant differences in the quality parameters of the eggs between the different tanks (ANOVA $p<0.05$). The mean relative fecundity, fertilization rate and hatching rate were all higher in tank B3. Significant differences between tanks B1 and B2 were only found for relative fecundity, which was higher in tank B2. The final contribution to the larvae born between 2007 and 2010 was much higher in tank B3, which had almost 75% of the total production as a result of the higher number of spawns registered in this tank, as shown in Table 3.2.

Table 3.2. Summary of the broodstock production parameters for each tank. Superscripted letters indicate significant differences in a parameter between different tanks. MRFec: mean average relative fecundity.

Tank	Total Volume (mL)	Floating volume (mL)	Floating volume rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns	Fertilization rate ± SE	Hatching rate ± SE	Larvae
B1	8819	3975	$37.50\pm0.13^{a,b}$	4.26 ± 0.30^{a}	108	58.43 ± 4.81^{b}	45.08 ± 6.03^{b}	1323256
B2	24085	9407	31.44 ± 2.02^{b}	6.06 ± 0.36^{b}	194	66.04 ± 3.15^{b}	56.68 ± 3.22^{b}	5799625
B3	62355	30060	42.20 ± 1.53^{a}	7.51 ± 0.30^{c}	331	80.64 ± 1.67^{a}	66.25 ± 2.14^{a}	19752006
Total	**95257**	**43442**	**38.1±1.16**	**6.51±0.2**	**633**	**73.28±1.54**	**61.56±1.76**	**26874880**

The mean values of the quality parameters of the spawns were as follows: total volume of 150.48 ± 4.96 mL, floating volume of 68.62 ± 3.28 mL, fertilization rate of 73.28 ± 1.54 % and hatching rate of 61.56 ± 1.76%.

The two most prolific females were used to study of the diameter of eggs spawned by a single female; there were 12 spawns for female 14 and 19 spawns for female 35. The results in both cases showed a significant, moderately negative correlation, -0.362 ($p=0.000$) for female 14 and -0.426 ($p=0.000$) for the female 35, which indicates a significant reduction trend in the size of the eggs throughout the spawning period. Moreover, the regression analysis revealed a fit to a cubic model, indicating recoveries in egg size over the spawning period (Figure 3.2.).

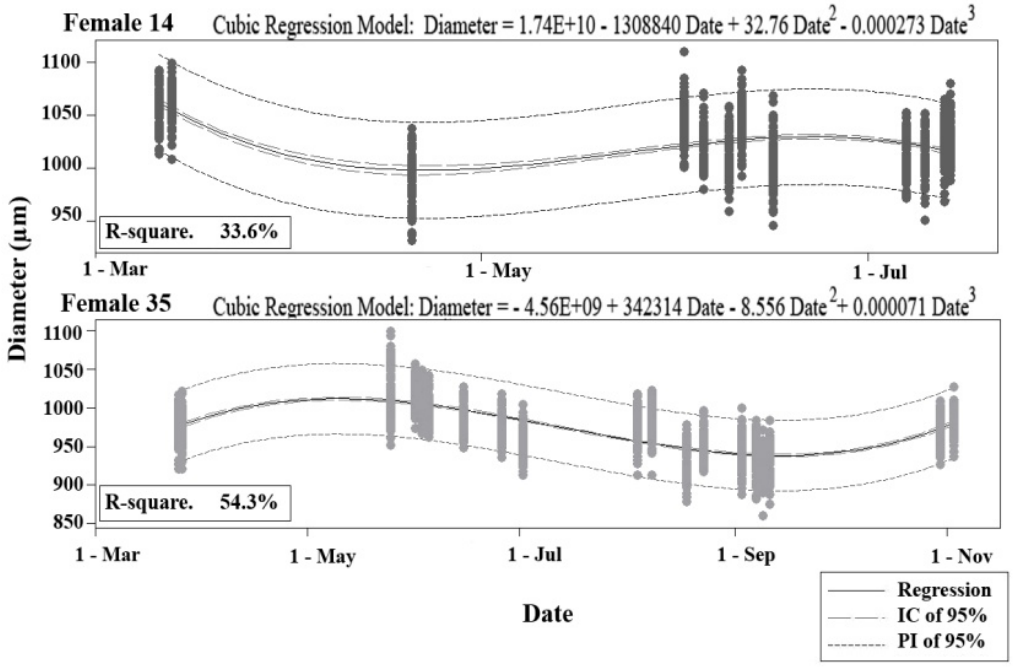


Figure 3.2. Change in the egg diameter of the natural spawns of females 14 and 35 throughout one spawning period and the cubic regression model associated with this evolution. IC indicates the confidence interval and PI indicates the prediction interval.

3.3.2 Genetic analysis and parental contribution

The genetic diversity found from studying the four microsatellite loci was very high, with 18 alleles per locus and expected and observed heterozygosity of 0.875 and 0.866 on average, respectively. Estimated frequencies of null alleles in the broodstock were below 0.05 for all loci in accordance with previous data (Castro et al., 2006), which support the usefulness of these set of markers for parental allocation. As expected pair-wise related values in the broodstock were very low at most cases, corresponding to unrelated individuals, and only 2.7% brood-pairs showed full-sib kinship and were allocated in different tanks. According to the high genetic diversity of the microsatellites, the theoretical potential for paternity inference in this broodstock was

also very high, the Excl1probability being 0.975 and Excl2 probability 0.996 (Table 3.3.). This potential was even higher because the breeding scenario was simpler in our study since breeders were subdivided in three tanks, so the number of potential crosses was reduced. Using this microsatellite tool and following the exclusion method implemented in the FAP package (Taggart, 2006), the 3630 larvae analysed along the four breeding seasons were allocated to a single couple with full confidence, other alternative couples always showing one or more mismatches.

Table 3.3. Estimates of genetic diversity, probabilities of exclusion and frequency of null alleles for the 4 microsatellite loci analysed. k: number of alleles, H(O): observed heterozygosity, H(E): expected heterozygosity, PIC: polymorphic information content, Excl(1): one parent exclusion, Excl(2): two parent exclusion. All calculations were performed with CERVUS 2.0 software.

Locus	k	N	H(O)	H(E)	PIC	Excl(1)	Excl(2)
CA13	14	80	0.863	0.866	0.846	0.566	0.724
F13-7	19	80	0.85	0.884	0.868	0.617	0.764
M2	16	80	0.863	0.841	0.82	0.525	0.691
Sse38	23	80	0.887	0.909	0.896	0.68	0.809
Mean/Total	**18**	**80**	**0.866**	**0.875**	**0.858**	**0.975**	**0.996**

Table 3.4. shows the matings that occurred each year in each tank, the number of spawns corresponding to each mating, and the estimated volume of eggs corresponding to each couple, based on the parental allocation results. From these results, it can be determined that the active females represented a small proportion of the total of females housed in each tank (between 8 and 57%) and that some of these females maintained their dominance throughout the study. The same situation occurred for the males; dominant males represented a small percentage of the total of males in the tanks (between 10 and 60%), and their dominance was maintained throughout the study. In Table 3.4., the patterns of fidelity can be observed. Dominant couples existed in all tanks. For example, couples 14-48, 35-57, 36-60, 36-57, 37-60 or 37-58 showed at least 3 years of fidelity.

Table 3.4. Summary of the mating pairs recorded in each tank and year through the parental allocation of larvae born during the study. In each pair, the number of spawns (N) and the volume of eggs derived from these spawns are shown. During the period of study, the following individuals died: B1(**17** in Oct-09; **4** in May-10), B2 (**26** in Jul-08; **13** in Dec-08; **3** in Jul-10) and B3 (**56** in Jul-08; **54** in Dec-09).

	2007				2008				2009				2010			
Tank	Female	Male	N	Vol.	Female	Male	N	Vol.	Female	Male	N	Vol.	Female	Male	N	Vol.
	17-	**71**	9	615	**17-**	**71**	6	336	**10-**	**71**	1	120	**10-**	**74**	4	185
	17-	**66**	9	210	**4-**	**71**	1	11					**10-**	**67**	2	108
					10-	**67**	1	22					**15-**	**71**	2	176
B1													**15-**	**67**	1	64
													12-	**71**	3	45
													4-	**70**	1	15
	14-	**48**	5	498	**14-**	**23**	23	3211	**14-**	**48**	17	1358	**14-**	**48**	9	397
	14-	**79**	1	110	**14-**	**43**	4	423	**14-**	**43**	2	183	**14-**	**79**	5	153
	26-	**23**	1	32	**41-**	**21**	2	99	**14-**	**79**	4	142	**14-**	**43**	2	56
	26-	**18**	1	24	**41-**	**43**	1	17	**14-**	**62**	2	13	**41-**	**48**	4	126
					7-	**53**	1	48	**3-**	**24**	1	160	**7-**	**18**	1	6
B2					**7-**	**48**	1	20	**41-**	**21**	2	127				
					7-	**43**	1	17	**41-**	**48**	2	77				
					13-	**79**	1	28	**41-**	**43**	2	46				
					42-	**48**	1	17	**7-**	**24**	1	60				
					25-	**48**	1	9	**7-**	**62**	1	60				
									7-	**43**	1	22				
	35-	**57**	11	838	**35-**	**57**	25	2307	**35-**	**57**	55	4844	**35-**	**57**	63	5076
	35-	**56**	4	264	**35-**	**56**	12	690	**35-**	**58**	1	49	**35-**	**58**	1	32
	36-	**60**	6	621	**35-**	**60**	3	169	**35-**	**60**	1	30	**35-**	**80**	2	22
	36-	**57**	4	439	**35-**	**58**	1	24	**36-**	**60**	1	1653	**36-**	**60**	18	1355
	37-	**56**	7	449	**36-**	**60**	14	1589	**36-**	**57**	1	224	**36-**	**58**	2	30
	37-	**57**	3	274	**36-**	**80**	1	64	**36-**	**58**	1	24	**37-**	**60**	4	296
	37-	**49**	3	236	**36-**	**57**	1	28	**36-**	**80**	1	12	**37-**	**58**	2	48
	37-	**60**	3	171	**36-**	**46**	1	16	**37-**	**60**	8	593	**38-**	**58**	1	38
	37-	**58**	2	134	**37-**	**56**	17	1132	**37-**	**80**	5	440				
	37-	**80**	1	14	**37-**	**60**	2	187	**37-**	**49**	2	220				
	54-	**60**	1	8	**37-**	**57**	1	125	**37-**	**58**	5	119				
B3					**37-**	**58**	1	63	**37-**	**57**	3	68				
					37-	**80**	1	40	**38-**	**58**	2	230				
					37-	**45**	1	16	**38-**	**60**	4	142				
					38-	**57**	1	56	**38-**	**49**	1	100				
					38-	**56**	1	16	**38-**	**80**	1	16				
					38-	**80**	1	16	**54-**	**58**	1	32				
					54-	**51**	2	46	**54-**	**60**	1	26				
					54-	**56**	1	28	**33-**	**46**	1	22				
					27-	**56**	1	40								
					27-	**57**	1	33								
					39-	**56**	1	19								
					34-	**45**	1	4								

Regarding the number of breeders involved in each spawn, Table 3.5. shows that most of the spawns (61.7%) were the result of only one couple, 31.5% of the spawns resulted from the cross of several couples, 3.9% were the result of crossing a single female with several males and 2.9% involved crossing a single male with several females.

Table 3.5. Classification of the tanks spawns as a result of crossing one female with one male, one female with several males, one male with several females or several females with several males. The results are represented as the percentage of the total spawns.

	Tank			
Individuals involved in the spawns	**B1**	**B2**	**B3**	**Total**
One male and one female	77.78	71.23	56.40	**61.74**
Multiple males and females	18.52	15.07	38.86	**31.51**
Multiple males and one female	3.70	5.48	3.32	**3.86**
Multiple females and one male	0.00	8.22	1.42	**2.89**

The results of the females that belonged to the dominant couples are summarised in Figure 3.3., which represents the daily relative fecundity of the spawns of each female (14, 35, 36 and 37) throughout the four breeding seasons, the thermoperiod, and the sexual maturation stage.

Table 3.6. shows the reproductive patterns of dominant females every year. The spawning periods ranged between 96 and 252 days, and the number of spawns per female per year ranged between 6 and 63. The mean frequency of spawning was 7.3 ± 0.6 days and ranged between 3.7 and 19.8 days among the different females. The minimum time between two consecutive spawns for a single female was, in most cases, 1 day, and the maximum time was 56 days.

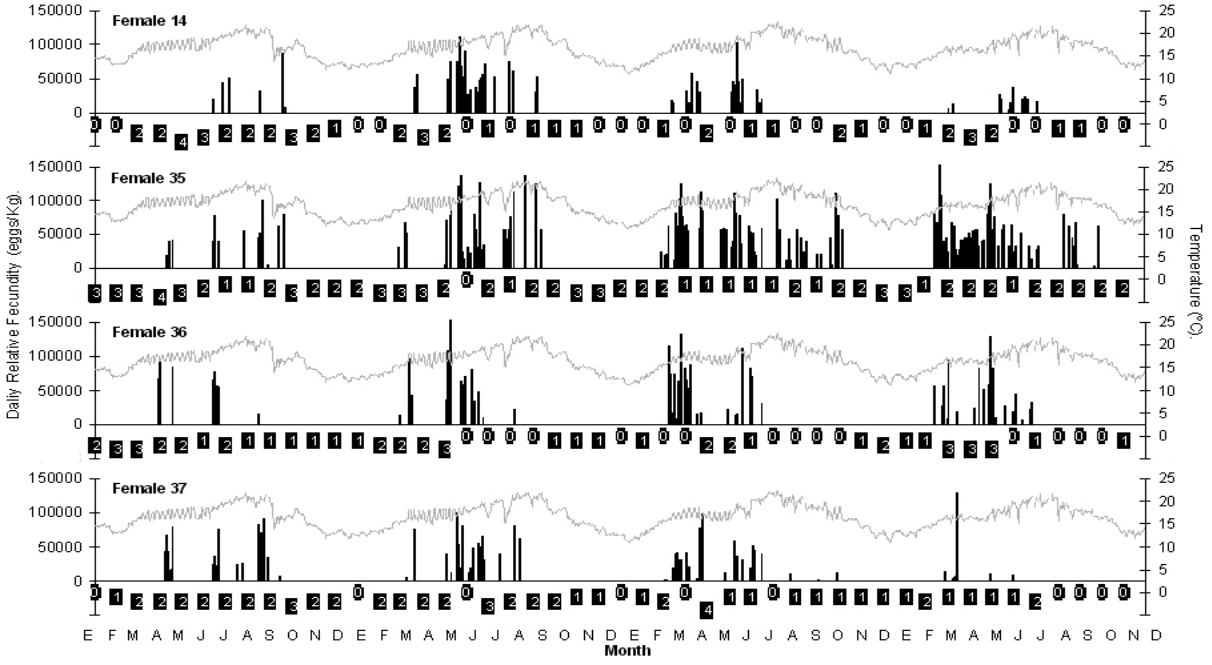


Figure 3.3. Daily relative fecundity, temperature regime and maturity stage of the four dominant females during the four year study period. A continuous line represents the thermoperiod, the bars represent the daily relative fecundity, and the numbers in a black box represent the maturity stages determined by monthly sampling.

Table 3.6. Annual summary of the individual spawning performance of the dominant females of the broodstock. The spawning period, mean spawning interval and maximum and minimum difference between spawns are expressed in days.

Female	Year	Spawning period		Sapawning period	Nº spawns	Mean spawning interval ± SE	Minimum difference between spawns	Maximum difference between spawns
14	2007	12/06/2007	19/09/2007	99	6	19.8±7.9	2	43
	2008	18/03/2008	04/09/2008	170	26	6.8±2	1	41
	2009	11/03/2009	14/07/2009	125	21	6.3±2.3	1	42
	2010	30/03/2010	31/07/2010	123	13	10.3±3.2	2	37
35	2007	08/04/2007	18/09/2007	163	15	11.6±4.4	1	56
	2008	26/02/2008	09/09/2008	196	36	5.6±1.7	1	53
	2009	24/02/2009	03/11/2009	252	56	4.6±0.7	1	23
	2010	11/03/2010	25/10/2010	228	63	3.7±0.7	1	34
36	2007	28/03/2007	14/08/2007	139	9	17.4±8.6	1	55
	2008	26/02/2008	04/08/2008	160	17	10±3.7	1	47
	2009	07/03/2009	14/07/2009	129	20	6.8±1.8	1	34
	2010	11/03/2010	24/07/2010	135	19	7.5±1.3	1	21
37	2007	06/04/2007	12/09/2007	159	17	9.9±3.7	1	56
	2008	07/03/2008	11/08/2008	157	20	8.3±2.4	1	43
	2009	01/03/2009	26/10/2009	239	23	10.9±2.5	2	38
	2010	24/03/2010	28/06/2010	96	7	16±7.5	1	44

3.4 Discussion

The problems associated with the reproduction of Senegalese sole in relation to natural spawning of cultured broodstock remain unresolved, and despite advances in artificial fertilization, a better knowledge of the reproductive biology of the species is necessary. In this regard, this study describes the egg production capacity of a wild stock comprised of 77 individuals adapted to captivity and distributed among three tanks. Furthermore, this study includes an evaluation of the quality of spawns obtained during four breeding seasons. Finally, this study provides an understanding of the mating relationships that occurred in the tanks through the parental allocation of a total of 3630 larvae collected in 363 spawns.

In terms of relative fecundity, the results obtained are lower than those obtained by Anguis and Cañavate (2005) and similar to those obtained by Dinis (1986). Although relative fecundity is an important parameter for comparison, our results suggest some caution is necessary because of the large variability in the number of females actually involved in the spawns of different broodstocks, which are always less than 60%. Fertilization rates are higher than those reported by Anguis and Cañavate (2005) and Dinis (1986), while hatching results showed similar values.

Another important result obtained in this study was the extension of the spawning period, usually described by other authors for this species as 4 to 6 months, with minor spawns in autumn in captivity (Dinis, 1986; Andrade, 1990; Anguis and Cañavate, 2005) or 3 months in the natural environment (Vinagre *et al.*, 2013). Under the conditions in the present study, thermoperiod manipulation was used to generate up to 8.4 months of regular production in tank B3 in 2009.

Other results, such as the decrease in the diameter of the eggs throughout the spawning season, were confirmed in this study. Furthermore, the study of individual female spawns, without the interaction of other females, demonstrates that the loss of

egg size is not only a general phenomenon of the stock (Dinis *et al.*, 1999) but also a general trend throughout the spawning period of a female. In addition, the results show that oocyte size is recovered after a period of reproductive inactivity. Thus, the cubic regression models described are useless for predicting the diameter of the eggs during a particular period in the spawning season because this factor depends on when each individual female initiates the reproductive activity, the intensity of these periods of activity, and the duration of the inactivity periods. However, these regression models can be useful as a tool, especially in artificial reproduction because they allow for the prediction of how long a period of inactivity may be necessary for a recovery of the oocytes diameter to occur.

Significant differences were found between the production performance of the three tanks, with productions rates of up to 10 times higher in some tanks. This fact highlights the individual component in the performance of a broodstock. The results of Anguis and Cañavate (2005) showed a ratio of running males that were stable throughout the period of study, approximately 60% of the specimens, with a peak in spring. The results of the present study were similar, showing that males who actually participated in the spawns varied between 10% (B1 tank, 2009) and 60% (tank B3, 2008), with an overall rate of 47.1% of participating males. Despite these rates, which are relatively high, after the analysis of the allocation of parental lineage was separated, we were able to ensure that more than 50% of the production of a specific tank was often the result of a single dominant couple, with insignificant contributions from other pairings. These results are similar to those obtained in Senegalese sole (Mira *et al.*, 2010) and *S. solea* (Blonk *et al.*, 2009; Guarniero *et al.*, 2010).

As for the parental contribution to the spawns, unlike other species, such as cod (*Gadus morhua*) or seabream (*Sparus aurata*), with massive multi maternal and multi paternal spawns (Herlin *et al.*, 2008; Chavanne *et al.*, 2012), in Senegalese sole, most of the spawns (61.74%) were the result of crossing a single female with a single male,

which again highlights the relevance of individuals in the reproductive biology of this species. These results are consistent with those obtained by Carazo (2013), who describes the courtship of this species, which involves two individuals that will mate by swimming very close and in coordination with one another. In the second largest group of spawns (31.5%), the larvae have been attributed to several couples, and based on the results by Carazo (2013), it is most likely that these spawns are the result of several individual crosses and not from a collective spawn.

The evolution of the sexual maturation stages of dominant females resulted in similar values to those obtained by García-López *et al.* (2006[a]), with maximum values obtained in the winter months and minimum values obtained during the summer. Additionally, these results are consistent with the asynchronous multispawn pattern described by Rodríguez (1984). The evolution of the most active female (35) during the spawning periods of 2009 and 2010 (February to September) shows that continuous reproductive activity prevents maturation above stage II.

Another result obtained in our study was the determination of the ovulation cycles of females from the results of parental allocation. These results indicate that the females of this species are able to ovulate for at least 6 consecutive days (female 35 in 2008 and 2009). In addition, an average ovulation frequency of 7.3 ± 0.6 days has been established, which may be helpful to design hormonal induction protocols or production provisions.

3.5 Conclusions

In conclusion, the results of a study of this length, especially the fidelity patterns described and the individual reproductive performances, contribute to a better understanding of the reproductive biology of this species in captivity and show the

necessity of studying other factors that enhance the reproductive performance of this species in captivity, such as the most suitable male:female ratio, the minimum number of breeders necessary to ensure the maximum efficiency of a broodstock, or the determination of the role of individuals that do not participate in spawns. Moreover, this study confirms the efficacy of thermoperiod manipulation to induce spawning in *S. Senegalensis*.

Chapter 4. Reproductive responses of captive Senegalese sole, Solea senegalensis, according to the type of feeding and the origin (wild or cultivated) of each gender.

Chapter 4.

Reproductive responses of captive Senegalese sole, *Solea senegalensis,* according to the type of feeding and the origin (wild or cultivated) of each gender.

4.1 Introduction

Reproductive dysfunctions of cultivated Senegalese sole have been widely mentioned in the literature, but not well detailed. There is information about natural spawns of cultivated individuals, and specimens subject to different hormonal therapies (Agulleiro *et al.*, 2006; Mañanos *et al.*, 2007; Martín *et al.*, 2007; Anguis *et al.* 2007; Guzmán *et al.*, 2008 and 2009). However, the evolution of the production of a cultivated broodstock of *Solea senegalensis* over several seasons has not been described, despite being a widely recognized problem (Cañavate, 2005; Howell *et al.*, 2009; Morais *et al.*, 2014).

The discovery of the reproductive dysfunction of captive sole targeted feeding as a possible influencer, since often wild individuals adapted to captivity were fed with natural food (squid, sea worms or mussels) and cultivated individuals were fed with commercial diets (dry feed) throughout their lifecycle. Numerous studies show the relationship between the quantity and quality of food, and the quality of the gametes (Springate *et al.*, 1985; Bennetau-Pelissero and Kaushik, 2001; Rungruangsak-Torrissen and Fosseidengen, 2007; Henrotte *et al.*, 2008).

Another approach to clarify the origin of the reproductive dysfunctions of this species in captivity has been the study of their reproductive behavior (Carazo, 2013). The work along these lines has led to a complete description of the stages of the courtship performed by wild individuals adapted to captivity. This courtship is briefly described as: *1) a period of intense activity between a potential female spawner, males and amongst males; 2) the female swims from the bottom of the tank accompanied by a male; 3) the female and male swim in synchrony at the water's surface with the genital ducts held closely together and liberate and fertilize the gametes* (Carazo *et al.*, 2011). Furthermore, the filming of cultivated specimens reveals an absence of reproductive behavior, and the spawns obtained are associated with the release of eggs by the females without the interaction of males and with the absence of a courtship.

Currently, all natural spawns of cultivated specimens exhibit low (rarely) or no fertilization rate, eliminating the possibility of a closed cycle in terms of natural reproduction in captivity. The entire industrial production, which according to the Marine Manufacturers Association (APROMAR) data provided in 2014 for the year 2013 amounting to nearly 343 tons in Spain, was produced through natural reproduction of wild individuals or artificial fertilization carried out with cultivated specimens.

The study of the natural reproduction of individuals born in captivity of this species aims, in the first place, to eliminate or at least reduce the dependence on wild individuals in order to fully control the reproduction of this species in captivity, and, secondly, to try to understand the reason or reasons that prevent the natural reproduction of individuals born in captivity.

The aim of this work is to determine the influence of diet on the reproductive response of cultured *S. senegalensis*, and to try to determine whether the absence of fertilized spawns is a matter that affects both genders equally or not.

4.2 Material and Methods

4.2.1 Broodstock and management

4.2.1.1 Feeding influence.

At the beginning of 2007, the initial cultured sole stock (n= 32) was housed in two 7 m^3 tanks (LP5 and LP6) for the feeding experiment. The experiment lasted 4 years. The average total body weight ± SE of the females and the males at the beginning of the trial in the LP5 tank was (N=16) 1277.63 ± 95.32 g and 1234 ± 76.48 g, and in the LP6 tank was (N=16) 1576.63 ± 102.97 g and 1499 ± 85.73 g respectively. The LP5 tank was fed with natural food composed of mussels, Mytilus spp., small squid, Loligo spp., and polychaete worms, Nereis spp. (Seabait Ltd., UK), 6 days a week. In the pre-spawning and spawning period, they were fed small squid 3 days a week (Tuesday, Thursday and Saturday) and mussels on the other 3 days (Monday, Wednesday and Friday). In addition, frozen worms were added to their diet every Wednesday. The rest of the year, they were fed mussels twice a week (Monday and Friday) and small squid 4 days a week (Tuesday, Wednesday, Thursday and Saturday). The daily amount of food was adjusted to 1% of the total biomass of the fish in the tank, which was determined monthly. The LP6 tank was fed with a commercial diet; Vitalis Cal 9 Skretting S.L. (6 days a week), and the amount of food was adjusted monthly to 0.5% of the total biomass of the fish in the tank.

4.2.1.2 Influence of the origin, wild o cultivated, of each gender.

At the beginning of 2012, the initial mixed sole stocks (n= 32) were housed in two 7 m^3 tanks (LP2 and LP3) for the gender experiment. The tank LP2 was composed of wild males, previously adapted to captivity, and cultivated females, and the tank LP3 was composed of cultivated males and wild females, previously adapted to captivity. The

duration of the experiment was 2 years. The average total body weight ± SE of females and males at the beginning of the trial was, in the LP2 tank (N=16) 1606.13 ± 86.85 g and 1280.43 ± 87.74 g, and in the LP6 tank (N=16) 1107.80 ± 178.11 g and 1101 ± 66.95 g respectively. The feeding regime of both tanks was the same as explained before for the LP5 tank (natural feeding), and cultivated individuals were adapted to natural food for 3 months prior to the experiment.

4.2.1.3 General housing conditions.

All the tanks were located inside an industrial warehouse, in open flow circuit, with a water renovation rate of 1.7 m^3/hour and constant moderate aeration. The average salinity ± SD recorded during the study was 34.69±0.77 PSU. An artificial photoperiod of 16 hours of light and 8 of darkness was used throughout the entire year. The light intensity was reduced using mesh shading over the tanks that allowed a maximum light intensity at the water surface of 50 lux. The four groups had a male:female ratio of 1:1 and an average density in the tanks of 3 kg/m2. When an individual died during the experiment, it was replaced with a specimen of similar origin and age (always over 4 years). All individuals were tagged with a passive integrated transponder (Trovan®) placed in the dorsal area, allowing the specimens to be identified and monitored. All individuals were weighed and measured (total length) once a month. All tanks were given a prophylactic hydrogen peroxide bath after sampling (80 ppm for one hour without water renewal).

The fish were at all times handled (routine management and experimentation) according to the European Union Directive (EEC, 1986) for the protection of Animals Used for Experimental and Other Scientific Purposes.

4.2.2 Thermoperiod manipulation

In the same manner as described in the previous chapter, beginning each year at the end of January, the thermoperiod was manipulated to induce the reproductive response of the broodstock. The initial environmental temperature was approximately 13 ºC. The temperature was increased in 0.5 ºC stepped intervals once per week. When the temperature reached 16 ºC, it was increased by 2 ºC to a maximum temperature of 18 ºC and decreased again to 16 ºC every four and three days, to imitate the natural fluctuations recorded in the Toruño (IFAPA, Cádiz) to induce natural spawning (Anguis and Cañavate, 2005). All temperature changes were made by modifying the inlet water, and the variation in the tank was mitigated by the renovation rate (1.7 m^3/h). In the middle or end of June, when the environmental seawater temperature was equal to that of the artificially heated water, the thermoperiod was no longer manipulated. The natural spawns obtained during this time were due to the natural local fluctuations.

4.2.3 Collection and evaluation of spawns

Eggs obtained from natural spawns were collected daily from February to November in a 350 µm mesh net suspended in a 200 L tank located beneath the broodstock tank overflow outlet.

The spawns were evaluated as described in the previous chapter. They were placed in 1 L graduated cylinders to estimate the volume of floating and non-floating eggs. All of the spawns were recorded, but only those with a total volume greater than or equal to 20 mL were used in this study.

To evaluate natural spawn quality, the total volume of eggs (mL), the floating and non-floating volumes (mL), the daily relative fecundity and the fertilization and hatching

rates (%) were determined throughout the entire test period. The daily relative fecundity was calculated as the total number of eggs produced per day in a tank related to the total weight of the females in the tank. The total number of eggs was calculated by multiplying the total volume of spawning by 1080 (Anguis and Cañavate, 2005). To determine the fertilization rate, a sample of the floating eggs was placed in a Bogorov's plate with seawater. The fertilization rate was determined by the number of fertilized eggs divided by the total number of eggs observed, counting a minimum of 200 eggs in each sample. Once the spawns were evaluated, the floating fraction was incubated in 70 L incubators with filtered seawater (1 µm) at 18-20 ºC, with a water renewal rate of 1.5 L per minute, moderate aeration and a maximum density of 4500 eggs/L. The larvae hatched after 24-48 hours of incubation, depending on the water temperature and the embryonic stage at which the eggs were collected. The 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 a minimum of three 100 mL subsamples.

4.2.4 Statistical analysis

All estimations were expressed as the mean ± standard error (SE). Data normality and homogeneity of variances were evaluated using the Kolmogorov–Smirnov and Levene tests, respectively. The non-normal variables were log- or angular-transformed either for the entire data set or for the ratios, respectively. Differences between means were examined using an ANOVA and Tukey´s test or the equivalent nonparametric Kruskal–Wallis and Mann–Whitney tests, with significance levels at $p<0.05$. All data was analyzed using the statistical package SPSS 14.0.

4.3 Results

4.3.1 Reproductive performance in relation to the type of feeding.

The evolution of spawns, expressed as the daily relative fecundity of each tank (LP5 and LP6) over the four year study period as well as the thermoperiod to which they were subjected is represented in Figure 4.1. The spawning period lasted between 56 and 195 days, depending on the tank and the year, and was always longer in the LP5 tank. There was a large variation of daily relative fecundity in the different spawns ranging between 1638 and 56400 eggs per kg of females.

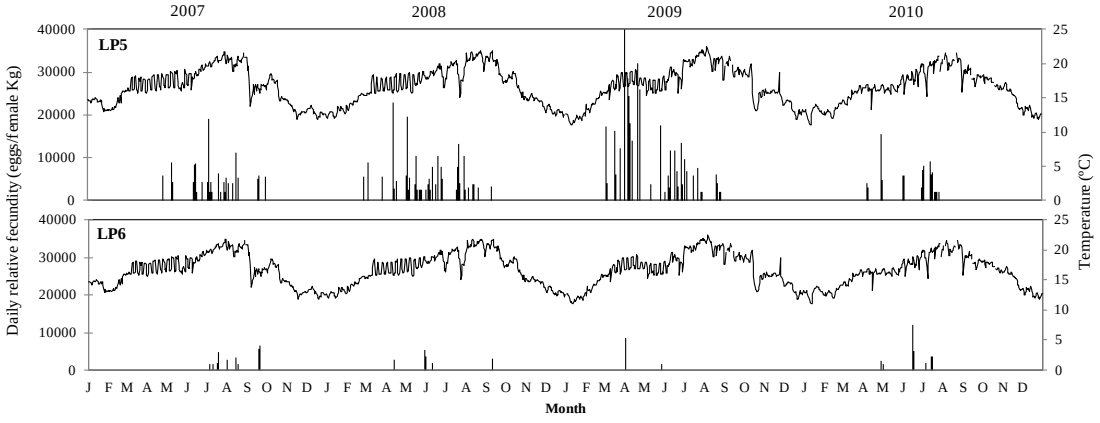


Figure 4.1. Daily relative fecundity and temperature regime of the broodstock with a different diet over the four year study period.

Table 4.1. shows a summary of the production parameters of the broodstock subjected to different feeding regimes during the four years of study. The total volume obtained, the total floating volume and the total number of annual spawns was always higher in the LP5 tank. Significant differences were found only in the mean relative fecundity per tank in 2007, being higher in LP5. There were no differences in the proportion of floating eggs any year. There was no fertilization during the four years of study in either tank.

Analyzing the entire data, there were significant differences in the mean relative fecundity between the different tanks, as shown in Table 4.2. This rate was higher in tank LP5. The total volume and the total floating volume produced was six times higher in the LP5 tank than in LP6 tank, which is directly related to the larger number of spawns produced in LP5. The floating volume rate did not present significant differences between tanks.

Table 4.1. Summary of the production parameters of the tanks with different diets for each year. Superscripted letters indicate significant differences in a parameter between different tanks each year. MRFec: mean average relative fecundity.

Year	Tank	Total Volume (mL)	Floating volume (mL)	Floating volume rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns
2007	LP5	1485	125	8.71±3.72	5.56±0.61[a]	30
	LP6	435	10	3.64±3.64	3.28±0.53[b]	11
2008	LP5	1843	43	2.76±1.35	5.81±0.67	42
	LP6	180	0	0±0	3.38±0.61	5
2009	LP5	2843	193	4.41±1.81	10.61±1.75	39
	LP6	120	60	30±30	5.10±3.40	2
2010	LP5	1007	87	5.56±2.65	5.24±0.81	19
	LP6	390	0	0±0	4.24±1.19	8
	Total	**8303**	**518**	**4.84±1.07**	**6.55±0.54**	**156**

Table 4.2. Summary of the production parameters of the tanks with different diets. Superscripted letters indicate significant differences in a parameter between different tanks. MRFec: mean average relative fecundity.

Tank	Total Volume (mL)	Floating volume (mL)	Floating volume rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns
LP5	7178	448	5.04±1.17	7.11±0.62[a]	130
LP6	1125	70	3.85±2.72	3.74±0.48[b]	26
Total	**8303**	**518**	**4.84±1.07**	**6.55±0.54**	**156**

4.3.2 Reproductive performance in relation to the wild or cultivated origin of each gender.

The evolution of spawns, expressed as the daily relative fecundity of each tank (LP2 and LP3) over the two-year period of the study as well as the thermoperiod to which the stock was subjected, is represented in Figure 4.2. As shown, the spawning period lasted between 1 and 258 days, depending on the tank and the year, and it was always longer in the LP2 tank. There was a large variation of daily relative fecundity in the different spawns ranging between 1756 and 25082 eggs per kg of females.

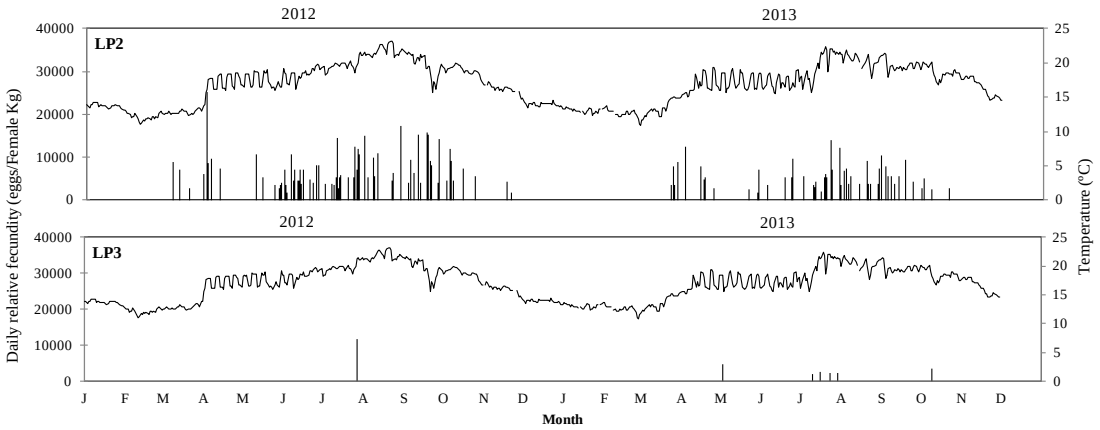


Figure 4.2. Daily relative fecundity and temperature regime of the cultured and wild mixed broodstock tanks over the two year study period.

Table 4.3. shows a summary of the production parameters of the mixed broodstock (wild males with cultured females in LP2 and wild females with cultured males in LP3). The total volume obtained, the total floating volume, and the total number of annual spawns were always higher in the LP2 tank. Significant differences were found only in the mean relative fecundity rate per tank in 2013. There were no differences in the proportion of floating eggs. Only the tank with wild males and cultured females produced fertilized spawns and viable larvae.

Table 4.3. Summary of the production parameters of the cultured and wild mixed broodstocks for each year. Superscripted letters indicate significant differences in a parameter between different tanks each year. MRFec: Mean average relative fecundity.

Year	Tank	Total Volume (mL)	Floating volume (mL)	Floating Volume rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns	Fertilization rate ± SE	Hatching rate ± SE	Larvae
2012	LP2	5686	547	7.94±1.82	7.33±0.52	71	68.69±9.82	81.79±10.05	388794
	LP3	50	0	0	11.74	1	-	-	
2013	LP2	3151	216	6.84±1.67	5.69±0.40[a]	50	61.79±11.72	65.67±34.33	42938
	LP3	157	27	18.81±8.03	2.86±0.39[b]	6	-	-	
	Total	**9044**	**790**	**7.96±1.26**	**6.51±0.34**	**128**	**65.46±7.48**	**78.86±9.56**	**431732**

Analyzing the complete data set, there were significant differences in the mean relative fecundity rate between the different tanks, as shown in Table 4.4., being greater in LP2. The total volume and the total floating volume produced were much higher in the LP2 tank than in LP3 tank, which is directly related to the greater number of spawns produced in LP2. The floating volume rate did not present significant differences between tanks.

Table 4.4. Summary of the production parameters of the cultured and wild mixed broodstocks. Superscripted letters indicate significant differences in a parameter between different tanks. MRFec: mean average relative fecundity.

Tank	Total Volume (mL)	Floating volume (mL)	Floating volume rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns	Fertilization rate ± SE	Hatching rate ± SE	Larvae
LP2	8837	763	7.49±1.27	6.65±0.35[a]	121	65.46±7.48	78.86±9.56	431732
LP3	207	27	16.12±7.30	4.13±1.31[b]	7	-	-	-
Total	**9044**	**790**	**7.96±1.26**	**6.51±0.34**	**128**	**65.46±7.48**	**78.86±9.56**	**431732**

4.4 Discussion

As previously mentioned, the lack of natural spawning of Senegalese sole cultured broodstocks remains unsolved, and a better knowledge of the reproductive biology of the species is necessary. In this regard, this work describes the egg production capacity of 4 cultivated stocks comprised of 16 individuals born in captivity. Each stock was subjected to different experimental conditions. Furthermore, this study includes the evaluation of the quality of spawns obtained through the different breeding seasons and experimental conditions.

The results obtained with the different types of food show how this variable does not provide results equivalent to those obtained with wild specimens adapted to captivity (Dinis *et al.*, 1999; Anguis and Cañavate, 2005), and in no case was fertilization obtained. The results obtained with natural food compared to the commercial diet are improved in terms of daily relative fecundity, total number of spawns, and total and floating volumes, reaching a total volume of eggs of the same order as that obtained with wild individuals in similar conditions. The spawning periods were also expanded in the tank with natural food comparing to the commercial diet, with periods of around 8 months, equivalent to those obtained with a wild broodstock. Similar results were obtained in rainbow trout, *Oncorhynchus mykiss*, (Bromage, 1995), using different feeding rates.

The percentage of floating eggs has been commonly used as an indicator of quality spawns in aquaculture (Aristizabal *et al.*, 2009; Jia *et al.*, 2014). This parameter has been directly related, in some species such as *Sparidentex hasta,* with egg viability (Teng *et al.*, 1999). In our study, this parameter was around 4-5% with both types of food, quite below those observed in wild individuals under similar conditions (30-40%).

It seems that food is a key factor to increase the reproductive performance and productivity of a broodstock, but it does not seem to be responsible for the

reproductive dysfunctions of this species, at least not influencing the times of adaptation and supply of the natural food tested.

As for the results obtained, housing together cultured and wild individuals of different genders, there is a clear relationship between gender and the lack of fertilized spawns recorded in the tanks. These results agree with those obtained by Mañanos *et al*. (2007) and are consistent with those obtained by Carazo (2013) using the same breeders of this study, where an almost complete absence of courtship with cultivated specimens was revealed. Reproductive courtship was found with cultivated females and wild males. The reproductive behavior seems to be a key factor in solving the absence of fertilized spawns with cultivated specimens in this species.

The results of this work show that the main problem of the spawns obtained with these groups (wild males and cultured females) is the scarcity of fertilized eggs collected in each spawn. The number of annual spawns is in the same order as those obtained in the wild, and fertilization and hatching rates are the same as well. The floating volumes however did not reach the level of the wild broodstocks. Average floating rates were around 8% volume in mixed stock, the values obtained with wild specimens are around 35%, and this fact directly affects the larval production potential.

Overall, it seems that ethology in captivity, and the mechanisms that induce the start up of the reproductive behavior of this species, are promising lines for solving reproductive dysfunctions of the species. Moreover, the influence of diet, despite the improvements in production, does not produce any positive results in terms of fertilized spawns. On the other hand, more improvements in the quality of gametes are not a decisive factor for natural spawning success, since the first artificial fertilization in this species demonstrated the viability of gametes of both sexes.

4.5 Conclusions

In conclusion, the results show that both food and gender are related to the reproductive response of cultured *S. senegalensis*. As for food, it is concluded that the feeding regime with which fertilized spawns are achieved in wild individuals, fails to enhance natural reproduction of the cultivated individuals of the species in terms of fertilization, although the production results are improved compared to a commercial diet.

Regarding the influence of gender, the results obtained highlight the lack of fertilized natural spawns in the tanks with cultured males of the species. Furthermore, these results support the need to continue the study of aspects such as the influence of feeding in early stages of the Senegalese sole culture on reproduction success, the individual relationships established in the tanks with wild males and cultured females, or possible ways to stimulate or induce social relations (courtship) in cultivated specimens.

Chapter 5. Surface disinfection of Senegalese sole, Solea senegalensis Kaup 1858, eggs using iodine.

Chapter 5.

Surface disinfection of Senegalese sole, *Solea senegalensis* Kaup 1858, eggs using iodine.

5.1 Introduction

The current state of marine fish farming in Europe is dominated by three species: seabass, *Dicentrarchus labrax*, gilthead sea bream, *Sparus aurata*, and turbot, *Scophthalmus maximus*. However, there is an increasing demand from the farming industry for new species to diversify the number of cultivated fish. *Solea senegalensis* is an attractive candidate for European aquaculture systems (Imsland *et al.*, 2004). This species has not reached the production levels of other species despite the efforts invested by both the farming industry and research centres. However, both sectors maintain that developing a framework for the species is an important goal in the production sector (Howell *et al.*, 2009).

As in other marine species, mortalities have been detected in early developmental stages. Some authors have reported that microorganisms could be responsible for these survival problems (Kusuda *et al.*, 1986; Barker *et al.*, 1989). Egg disinfection has been used to reduce bacterial, fungal or viral loads in other species, such as rainbow trout, *Oncorhynchus mykiss* (Wagner *et al.*, 2010), largemouth bass, *Micropterus salmonides* (Wright and Snow 1975), gilthead sea bream, *Sparus aurata* (Escafre *et al.*, 2001), and turbot, *Scophthalmus maximus* (Salvesen and Vadstein, 1997). Both the protocol and the product used must be adjusted for each species because some

parameters, such as the dosage, the contact time or the active ingredient, may affect egg development, hatching rates or the percentage of deformities in larvae.

The advantages of disinfection are not only related to the improvement of hatching rates and larval survival but also to the prevention of disease transmission between species and the spread of diseases among different facilities and different geographical areas (Overton *et al.*, 2010).

Many studies have reported the effectiveness of iodine in the disinfection of not only bacteria but also fungi and viruses (MacFadden, 1969; Ross and Smith, 1972; Hirazawa *et al.*, 1999; Wagner *et al.*, 2008). The aim of this study was to define a protocol for Senegalese sole egg disinfection with iodine, which minimises the bacterial load without affecting the hatching rate and larval survival.

5.2Materials and methods

5.2.1 Egg collection

Eggs were obtained from natural spawns of a wild stock of Senegalese sole maintained at the experimental facilities of the Spanish Institute of Oceanography in Santander, Spain. These spawns were collected daily in a 335 µm mesh net suspended in a 100 L tank located beneath the broodstock tank overflow outlet. The spawns were placed in 1 L graduated cylinders to estimate the volume of floating and non-floating eggs. The developmental stage and the fertilization rate were determined by microscopic observation of a subsample of the floating fraction (n=200 eggs). Only buoyant eggs from spawns with a floating volume over 30 mL and a fertilization rate above 75% were used in the experiments. All the egg batches used in this study (3 different batches in each experiment) were at the same developmental stage that corresponds

to the complete formation of a blastodermal cap, late I-early II (Laurence and Rogers 1976), as shown in Figure 5.1.

All the disinfectant solutions were prepared with filtered (0.5 µm), UV irradiated, autoclaved seawater. The solutions were prepared with yodipra® (5 mg active iodine mL^{-1}) to obtain a final volume of 0.5 L to achieve a 1:100 ratio of egg to water in the disinfection. The mean pH of the solutions was 7.96 ± 0.85.

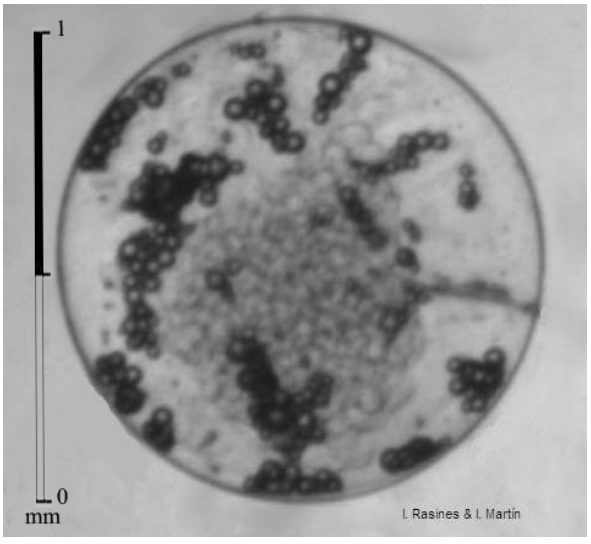


Figure 5.1. *S. senegalensis* egg in the developmental stage used for disinfection in this study. Authors: I. Rasines and I. Martín. The photograph was taken by the software and hardware system analySIS®.

5.2.2 Experiment 1. Effect of the dosage on hatching rate.

The three egg batches were collected as explained above and distributed in 8 PVC cylinders with a 350 µm mesh in the base of the cylinder. Floating eggs (5 mL or approximately 5000 eggs) were placed in each cylinder with a micropipette. Each cylinder was submerged on a sterile Pyrex glass with the different disinfectant solutions (control, 15, 25, 50, 75, 100, 150, 250 mg L^{-1} active iodine) for 10 minutes.

During the disinfectant process the eggs were moderately agitated every minute to enable all the eggs to come into contact with the disinfectant. Next, the eggs were washed 3 times with seawater for 5 minutes to remove the remains of the disinfectant. Each disinfected egg batch was placed in a different container with sterile seawater. After filling three 12x8 well microtiter plates with 200 µL of sterile seawater in each well, twelve fertilised eggs of each treatment group (control, 15, 25, 50, 75, 100, 150, 250 mg L^{-1} active iodine) were deposited into the well line of each plate. The plates were maintained in the dark at 18 ºC. The plates were checked daily to determine visible abnormalities in the embryonic development, the hatching rate and the cumulative survival rate of the hatched larvae. The hatching rate was determined as the total number of larvae hatched divided by the number of eggs incubated. The percentage of formed embryos was determined as the total number of eggs reaching stage IV; (Laurence & Rogers 1976), divided by the total number of eggs incubated. The doses that caused no reduction in the hatching rate in this experiment were used in the following experiments.

5.2.3 Experiment 2. Evaluation of the disinfectant capacity and hatching rate in culture conditions.

Three batches of eggs were disinfected as in Experiment 1. Only three concentrations were checked for this experiment (control, 15, 25, 50 mg L^{-1} active iodine). The seawater used for the three 5 minutes rinses was also sterilised to avoid the growth of new bacteria on the egg surface. Once the disinfection was completed, the eggs were incubated in 1 L truncated cone shape PVC incubators, 3 per treatment, to evaluate the hatching rate. Water was filtered at 1 µm and the renewal rate was 6 L h^{-1}. Before incubation, duplicates of 60 eggs were used for each concentration and placed on 2 NaCl blood agar Petri dishes (TSA 4%, ferric citrate 0.05%, esculin 0.1%, defibrinated

sterile sheep blood 5% and sodium chloride 2%) that were incubated in a heater at 20 ºC. The bacterial growth was checked daily to determine the number of new colony forming units (CFU) that appeared in the blood agar Petri dishes. The bacterial culture was maintained for 72 hours, which is the maximum time for egg hatching in normal culture conditions at 16-18 ºC. The survival study of Experiment 1 was repeated in culture conditions with continuous water renewal instead of incubating the disinfected eggs in the microtiter plates.

5.2.4 Experiment 3. Identification of bacterial flora in Senegalese sole eggs of the broodstock.

In this experiment, the 50 mg L^{-1} dose was tested again to confirm previous results as well as to perform an identification of the bacterial species appearing in the spawns of the broodstock in our facilities. For this purpose, three spawns were used and 5 mL of eggs were disinfected as in Experiment 1 and 2. Four NaCl blood agar Petri dishes were prepared from each spawn, two with 60 untreated eggs, and two with 60 disinfected eggs, per Petri dish. Samples were analysed for specific identification by the Vitek®2 system (Biomerieux, Madrid, Spain), which uses colorimetric reagent cards that are incubated and interpreted automatically, after performing a Gram stain, a catalase test with hydrogen peroxide 3% and a coagulase test with rabbit plasma.

5.2.5 Statistical analysis

All values are expressed as the mean ± standard error of the mean (SE). Data normality and homogeneity of variance were analysed using the Kolmogorov–Smirnov and Levene methods, respectively. The non-normal variables were transformed (logarithmic transformation for all data and angular transformation for the ratios).

Differences were examined using an ANOVA and Tukey's test or the equivalent nonparametric Kruskal–Wallis and Mann–Whitney tests with significance levels of $p<0.05$. All data were analysed with the statistical package SPSS 14.0.

5.3 Results

5.3.1 Experiment 1

The results showed that the two higher doses (150 and 250 mg L^{-1}) affected the normal embryonic development without formation of an advanced embryo (stage IV; Laurence and Rogers 1976). A significant reduction of the hatching rate appeared from 75 mg L^{-1}. There were no differences in the embryonic development and hatching rate between the control and the eggs treated with 15, 25 or 50 mg L^{-1} (Figure 5.2.).

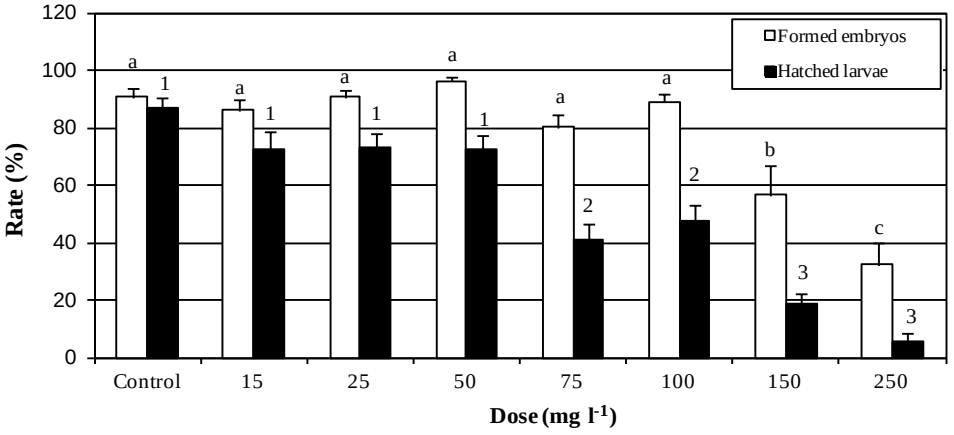


Figure 5.2. Finalisation of embryonic development (stage IV; Laurence and Rogers, 1976) and hatching rates of the different doses tested. The contact time in all cases was 10 minutes. The letters above the error bars indicate significant differences between treatments in the completion of embryonic development. The numbers on the error bars indicate significant differences between treatments in the hatching rate.

The cumulative survival of the hatched larvae, treated with 15, 25 and 50 mg L^{-1}, showed significant differences with the control, although no differences were found among the different treatments (15, 25 and 50 mg L^{-1}) (Figure 5.3.).

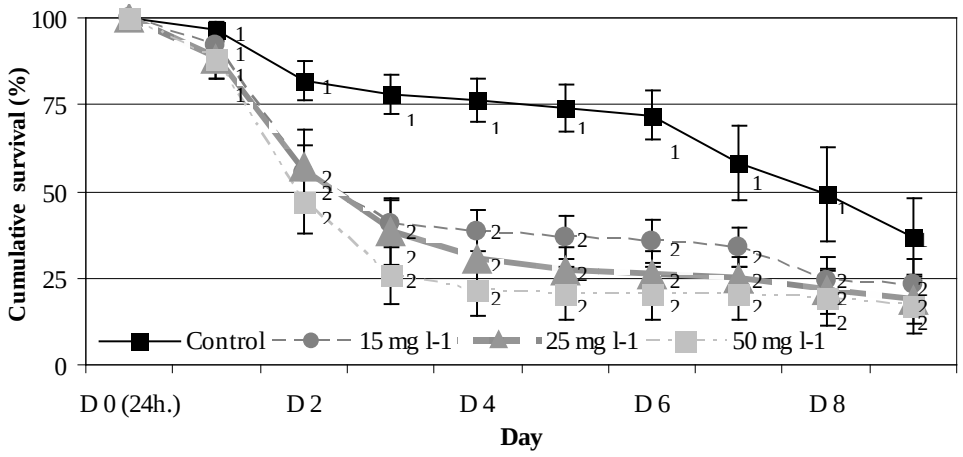


Figure 5.3. Evolution of the cumulative survival rate of the different doses that showed no significant differences in the hatching rate (control, 15, 25, 50 mg L^{-1} active iodine for 10 minutes). Different superscripts indicate significant daily differences among concentrations. D0 (24 h) indicates 24 hours after hatching.

5.3.2 Experiment 2

There were no differences in the hatching rate in culture conditions among treatments in the control, 15, 25 and 50 mg L^{-1} of iodine, as shown in Table 5.1.

Table 5.1. The mean ± SE of the hatching rates in culture conditions at 0, 15, 25, 50 mg L^{-1} active iodine. The contact time in all cases was 10 minutes. There were no differences among means.

Tratamiento	Tasa de eclosión (%)
Control	76.40 ± 4.06
15 mg L^{-1}	68.34 ± 5.58
25 mg L^{-1}	65.29 ± 5.52
50 mg L^{-1}	71.56 ± 4.37

Regarding bacterial growth, Table 5.2 shows the new colony forming units (CFUs) that appeared every 24 h (24, 48 and 72 h post incubation), as well as the cumulative number of CFUs (total count after 72 h). There were fewer CFUs in the eggs treated with 25 and 50 mg L^{-1} than in the control ($p<0.05$) in all counts (24, 48 h and total), except after 72 h of incubation, when there were no differences among treatments

($p>0.05$). There were no differences between the control eggs and those treated with 15 mg L^{-1}.

Table 5.2. The mean ± SE number of CFUs that appeared on the bacterial culture Petri dishes after incubation. Superscript letters and numbers indicate significant differences among treatments for each post incubation time period. The contact time in all treatments was 10 minutes.

	CFU / Petri dish (60 eggs)			
Treatment	**24 h. postincubation**	**48 h. postincubation**	**72 h. postincubation**	**Total**
Control	3.13 ± 1.13^{1}	10.75 ± 4.09^{x}	3.38 ± 1.15	**17.25 ± 5.15^{a}**
15 mg L^{-1}	$0.75 \pm 0.53^{1,2}$	$3.75 \pm 1.37^{x,y}$	2.13 ± 0.64	**$6.63 \pm 1.81^{a,b}$**
25 mg L^{-1}	0 ± 0^{2}	3.88 ± 2.50^{y}	0.75 ± 0.31	**$4.63 \pm 2.42^{b,c}$**
50 mg L^{-1}	0 ± 0^{2}	1.25 ± 0.73^{y}	0.5 ± 0.19	**1.75 ± 0.67^{c}**

Regarding survival rates, larvae obtained from eggs incubated with water renewal (6 L h^{-1}) instead of on microtiter plates did not show any difference in the cumulative survival rate between the control and 15, 25 and 50 mg L^{-1} at any point after hatching (see Figure 5.4.).

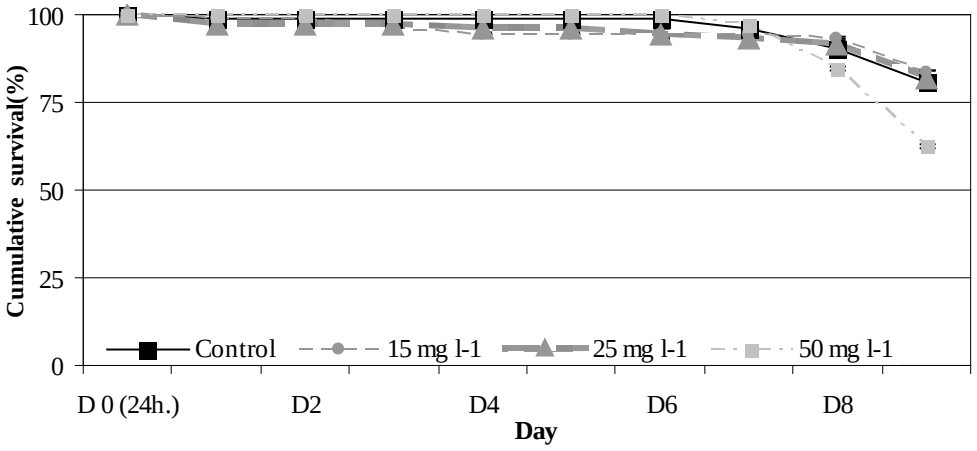


Figure 5.4. Evolution of the cumulative survival rate after incubation of eggs with continuous water renewal of the different doses that showed no significant differences in the hatching rate (control, 15, 25, 50 mg L^{-1} active iodine for 10 minutes). There were no differences among treatments. D0 (24 h) indicates 24 hours after hatching.

5.3.3 Experiment 3

The specific identification of the bacterial flora for the three spawns resulted in the isolation of the following species: coagulase-negative *Staphylococcus spp*., *Sphingomonas paucimobilis*, *Corynebacterium spp*., *Pseudomonas stutzeri*, *Aeromonas hydro/caviae* and *Vibrio alginolyticus*. In this trial, there was no bacterial growth in the eggs treated with 50 mg L^{-1} of iodine. Table 5.3. shows the bacterial species that were isolated for each spawn: coagulase-negative *Staphylococcus spp.* and *S. paucimobilis* appeared in the first two spawns, whereas *Corynebacterium spp*. appeared in one and *P. Stutzeri* appeared in the other. *A. Hydro/caviae* and *V. Alginolyticus* only appeared in the third spawn without the presence of any other bacterial species. In the case of fungus, there was no growth in any of the three spawns studied.

Table 5.3. Bacterial species isolated in the three different spawns (1, 2 and 3) under disinfection with 50 mg L^{-1} of iodine and control conditions. "-" indicates no growth.

	Control			50mg L^{-1}_10 min		
Species	**1**	**2**	**3**	**1**	**2**	**3**
Coagulase-negative Staphylococcus spp.	+	+		-	-	-
Sphingomonas paucimobilis	+	+		-	-	-
Corynebacterium spp.	+			-	-	-
Pseudomonas stutzeri		+		-	-	-
Aeromonas hydro/caviae			+	-	-	-
Vibrio alginolyticus			+	-	-	-

5.4 Discussion

The aim of this study was to determine an egg disinfection protocol for *S. senegalensis* using iodine, identify the toxic doses, and assess the disinfectant effectiveness of the innocuous doses for normal egg development. The disinfection protocol and test concentrations were established from previous studies of other species (see Table 5.4.).

Table 5.4. Response summary of other fish eggs treated with iodine. In the species with an "a" superscript, the hatching rate decreased to 0; in the species with a "b" superscript, the reduction was significant.

Specie	Dose	Contac time	Autor (year)
Micopterus salmonides [a]	200 ppm (Wescodyne)	15 min	Wright & Snow (1975)
Oreochromis mossambicus [a]	200ppm (Buffodine)	15 min	Subasinghe & Sommerville (1985)
Pleuronectes Platessa [a]	250 ppm (Buffodine)	10 min	Salvesen & Vadstein (1995)
Pargus major [a]	500 ppm (Povidon-iodide)	5 min	Hirazawa et al. (1999)
Diplodus sargus sargus [b]	25 ppm (PVP-iodine)	5 min	Katharios et al. (2007)
Osmerus mordax [b]	50 ppm (PVP-iodine)	15 min	Walker et al. (2010)
Pargus pargus [b]	100 ppm (PVP-iodine)	5 min	Katharios et al. (2007)

As in other species, there is a wide range of concentrations that do not affect egg development, hatching and survival rates, but it is necessary to establish which of these has the highest capacity for disinfection (Salvesen and Vadstein, 1995; Tendencia, 2001; Overton *et al.*, 2010). In the case of *S. senegalensis* eggs, for the developmental stage used in this study, we were able to establish a safe range from 0 to 50 mg L^{-1} with a maximum exposure time of 10 minutes.

The egg developmental stage appears to be a key factor when conducting disinfection treatments (Douillet and Holt, 1994; Hirazawa *et al.*, 1999; Tendencia, 2001; Peck *et al.*, 2004). In this study, the developmental stage was chosen by considering the two forms of reproduction of this species in captivity. On one hand, in natural reproduction, the spawns are produced at the beginning or end of the evening and eggs are collected in the morning at the proposed stage at 16-18 °C (Carazo personal communication). On the other hand, in artificial fertilization, the eggs, obtained by stripping and then fertilised, could be incubated without being disinfected and then treated later at the proposed developmental stage. This stage also prevents possible adverse effects resulting from disinfecting eggs shortly after fertilization because at the proposed stage, late I-early II (Laurence & Rogers 1976), hardening of the chorion has already occurred (Bergh and Jelmert 1996).

Unlike other studies with different species, (Rach *et al.*, 1998; Treasurer *et al.*, 2005; Khodabandeh and Abtahi, 2006; Overton *et al.*, 2010), the disinfection with iodine did not improve hatching rates in *S. senegalensis*. This is likely due to the short time the eggs take to hatch (24-48 h at 17-20 ºC), which reduces the contact time between pathogen and egg. For this reason, the advantages of this protocol in this species are focused on other aspects of disease control. First, this protocol reduces or eliminates pathologies caused by vertical transmission. Second, it improves larval survival by reducing the environmental pathogens that may develop into diseases during the larvae culture. Third, it attempts to avoid the spread of diseases among different geographical areas or facilities when transporting eggs or larvae. In this regard, the results obtained in this study met the objectives described without adverse effects on the normal development of eggs and larvae until day 9.

Regarding the survival of larvae hatched from disinfected eggs, initial results showed a decrease compared to the control. This reduction may be due to the limited volume of water used for the incubation of each egg (200 µL) and to traces of iodine that may have remained on the surface of the egg, affecting the hatched larvae, despite the three rinses with seawater. This hypothesis was confirmed with larvae hatched from disinfected eggs (15, 25 and 50 mg L^{-1}) and incubated in 1 L incubators with continuous water renewal. With these larvae, the survival study was repeated and no differences were found among treatments. These results indicate that the microtiter plate incubation method, suitable for eggs and larvae without subjection to any treatment (Panini *et al.*, 2001; Unuma *et al.*, 2004), must be performed with caution in studies with elements that may be toxic in such a low volume.

The total bacterial load was reduced by disinfection with 25 and 50 mg L^{-1}. The reduction was significant in both cases, but the level of CFU reduction achieved with the 50 mg L^{-1} was approximately 90% in all cases (24, 48, 72 h and total bacteria count after 72 h). The level of CFU reduction achieved with the 25 mg L^{-1} was approximately

70%. There were no differences among treatments, but because none of the treatments reduced the normal development of eggs, disinfection seems to be more appropriate due to the greater reduction of the bacterial load with 50 mg L^{-1}. However, it would be advisable to assess the safety margin between the doses of 50 and 75 mg L^{-1} because at 75 mg L^{-1} the hatching rate decreased significantly.

The bacteria species isolated and identified in this study are primarily not pathogens that cause serious illness in fish or humans, with the exception of *Vibrio Alginolyticus* and *Aeromonas hydrophila*. The former is related to diseases in marine fish, such as gilthead sea bream, *Sparus aurata* (Balebona *et al.*, 1998), turbot, *Scophthalmus maximus*, and grouper, *Epinephelus malabaricus* (Lee, 1995). This bacterial species is also responsible for human infections caused by the contact with seawater and food poisoning (Blake *et al.*, 1980). In Senegalese sole, *V. alginolyticus* has been identified as a secondary pathogen associated with flexibacteriosis. *A. hydrophila* causes a microbial disease called motile aeromonad septicaemia (MAS), and despite being more related to freshwater fish outbreaks, cases have also been reported in brackish water and marine fish (Das and Sahoo, 2012). The remainder of the isolated species are not directly related to fish pathogens; however, as Barker reported (1989), bacterial loads can alter the normal development of eggs in cases of overgrowth by consumption of the oxygen necessary for embryonic development. Nevertheless, no bacterial growth was observed with 50 mg L^{-1} in the identification trial (Experiment 3), thus improving the results of Experiment 2 (total bacteria count).

5.5 Conclusions

In conclusion, the authors recommend disinfection with 50 mg L^{-1} of iodine for 10 minutes followed by three 5 minutes rinses with seawater to significantly reduce the bacterial load in *Solea senegalensis* eggs without affecting normal egg development

and the hatching rate. This methodology is simple and easy to implement as a routine in industrial production systems. However, as this is the first published study about the disinfection of *Solea senegalensis* eggs, it would be desirable to test the efficacy of this compound for viruses and fungi, as well as the possible adverse effects of disinfection at different embryonic stages.

Chapter 6. Waterless transport of Senegalese sole, Solea senegalensis, Kaup 1858.

Chapter 6.

Waterless transport of Senegalese sole, *Solea senegalensis*, Kaup 1858.

6.1 Introduction

Transport and distribution of live aquatic animals is becoming an important part of the aquaculture industry, and all the aspects focused on cost reduction, improving product quality, or reducing health problems contribute significantly to a better development of this sector (Berka, 1986). The most common is the transport of larvae or fingerlings between different production units, but occasionally it is necessary to move larger specimens or breeders. Transport of large live individuals is important in the management of broodstocks in the companies or research centers, and in the maintenance of the product quality and hence the price (Southgate, 2008). In order to maintain the organoleptic qualities, product degradation must be avoided. Degradation can be produced by microorganisms but also by autolysis. In the last case, product spoilage occurs due to endogenous enzymes, and a decrease in the final product quality is provoked (FAO, 1998). By transporting live animals, autolysis is prevented and quality is preserved. This improvement in quality could be correlated with higher price in the market; Japan is one of the most important examples where the price of live fish is higher than that of fresh fish (Takashima, 1996).

Among all the transport systems for fish, the most common way is in water, either through an open or closed system (Berka, 1986), but the transport of specimens without water has been successfully used in some species such as turbot

(*Scophthalmus maximus*) (Caillens, 1996; Liu *et al.*, 2009). Even so, the water transport presents some drawbacks, such as a high technical requirements, higher cost, or problems with water and equipment used for transport related to pathologies dissemination (Southgate, 2008). By contrast, transport without water is easy to perform, inexpensive, do not generate waste problems and allow bigger load amount (Yin *et al.*, 1995), although it may not be used for all species. Most of the shellfish are transported without water, and sea products such as lobster, shrimp, oyster or scallop are maintained at low temperatures during transportation without need for water. In fish, some trials have been performed successfully mainly in turbot (Caillens, 1996; Liu *et al.*, 2009), and there have been also positive results in non-flat species as the Giant grouper, *Epinephelus tauvina* (Comandante, 2005). However, the different ways of addressing waterless transport, make it necessary to develop the technique for each species.

Another important aspect in relation to live animals transport is that related to animal welfare. European Union regulates the terms in which the transport of live animals should be performed (Directive (EC) 1/2005, of December 22, 2004 on the protection of animals during transport and related operations), this text aims to prevent injury or undue suffering to animals and to ensure that they have appropriate conditions that meet their needs. One way to determine the welfare status of specimens during transport is to assess stress level to which animals are subjected. The most accepted method to determine stress levels is the study of plasma cortisol as well as other plasmatic parameters (glucose and lactate) (Wendelaar Bonga, 1997; Barton 2002).

The aim of this study was to define the maximum emersion time in specimens of *S. senegalensis* of commercial size or breeders, for its application in transport, determine stress levels produced by this activity, as well as the short-term effects of prolonged emersion (delayed mortality, first food intake, and feeding performance after stress).

6.2 Material and Methods

6.2.1 Experimental procedures

The experiment was carried out in the Marine Aquaculture Plant "El Bocal" of the Spanish Institute of Oceanography (IEO), in Santander (Spain). A total of 56 individuals of *S. senegalensis* (922.33 ± 29.52 g body mass, and 39.80 ± 0.47 cm body length) obtained from a same age and origin stock, were used in the experiment . The tanks used (volume 1.125 m^3) were located inside an industrial warehouse, in open flow circuit, with a water renovation rate of 1.2 m^3/hour and constant moderate aeration. An artificial photoperiod of 16 hours light, and 8 hours dark was used throughout the entire year. The light intensity was reduced using mesh shading over the tanks that allowed a maximum light intensity at the water surface of 50 lux. Fish were fasted 72 hours before the emersion tests. The specimens were randomly divided into 8 groups of 7 individuals. On day 0, the specimens were placed in 2 cm thick polystyrene boxes (59x39x13 cm) on moistened cloths (1 fish per box), and all the boxes were placed in a room with constant temperature (15 ºC). At the beginning of the experiment, 7 individuals were blood sampled to establish the baseline levels of different plasmatic parameters assessed in this study. Then, on 2, 6, 12, 20, 28, 36 and 44 hours after emersion, groups of 7 individuals were also blood sampled. After sampling, each experimental group was introduced into an aerated recovery tank under normal culture conditions to evaluate their performance after the emersion period and the possible delay in mortality. For this purpose, survival rate and food intake were recorded during four weeks after the emersion trial. For the valuation of food intake after the emersion test, fish were daily feed late in the afternoon with one pellet per fish (Vitalis Repro 9 Skretting S.L.), and the detection of uneaten pellets remaining in the tank (Rubio *et al.*, 2003) was conducted the next day early in the morning to avoid the pellets got rid in the water. Tanks were fed from Monday to Thursday according to

the facilities feeding routines. The percentage of food intake was calculated as the number of pellets eaten by the total of pellets offered.

6.2.2 Blood sampling procedures

For blood sampling, each individual was removed from the box, and the blood was withdrawn from the caudal vein using 1 mL heparinized syringes. Sampling began at the times indicated above, but no fish was removed from the box until having finished the previous sample. Moreover, once opened the box each blood sample was taken in less than three minutes to avoid plasma cortisol and glucose enhancement due to handling (Arjona *et al.*, 2007). The extracted blood was transferred to 1.5 mL eppendorfs tubes containing 10 µL of heparin and centrifuged (3000 x g for 15 min. at 4 ºC) to obtain plasma, that was stored at -80 ºC until further analysis.

The experiment was performed following the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

6.2.3 Blood analytical procedures

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske-VT, USA) and expressed as $mOsm \bullet kg^{-1}$. Glucose, lactate and triglycerides concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides ref. 1001311) adapted to 96-well microplates. Plasma protein concentrations were measured on a 50-fold plasma dilution using the bicinchoninic acid method with the BCA protein kit (Pierce P.O., Rockford, USA), with bovine serum albumin serving as

standard. Free amino acid levels were assessed colorimetrically on a 75-fold plasma dilution using the nynhidrin method (Rosen. 1957). All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by KCjunior™ software. Standards and samples were measured in duplicate.

Plasma cortisol levels were measured by Enzyme Immune-Assay (EIA) using microtiter plates (MaxiSorp™, Nunc, Roskilde, Denmark) as previously described by Martos-Sitcha *et al.* (2014) for other teleost species. Steroids were extracted from 5 µL of plasma in 100 µL RB (10 % v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01 % w/v NaN3, 2.34 % w/v NaCl, 0.037 % w/v EDTA, 0.1 % w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac), and evaporated during 48-72 hours at 37 ºC. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate.

6.2.4 Statistical analysis

Results are presented as means ± SEM. After normality and homogeneity of variance were checked (Kolmogorov–Smirnov and Levene tests, respectively), comparison between groups was analysed as appropriate using one-way analysis of variance (ANOVA) taking the time of emersion as main factor, followed by post-hoc comparison with Tukey's test. Significance was taken at $P<0.05$.

6.3 Results

No mortality was observed during the different times of emersion excepting at 44 hours, where 66.7 % of the specimens died (Table 6.1.). Delayed mortality, registered during the 24 hours after introduction of specimens in recovery tanks, was 42.8 % and 16.7 % in the group of 36 and 44 hours of emersion respectively (Table 6.1.). In addition, the group emerged during 20 hours registered 1 individual death (14.3 % mortality) fifteen days after the emersion trial (Table 6.1.).

Table 6.1. Mortality rate during and after the emersion trial. The delayed mortality was evaluated during four weeks.

	Hours of emersion							
	0	**2**	**6**	**12**	**20**	**28**	**36**	**44**
Mortality rate (%)	0	0	0	0	0	0	0	66,6
Delayed mortality rate (%)	0	0	0	0	14,3	0	42,8	16,7
Total mortality rate (%)	**0**	**0**	**0**	**0**	**14,3**	**0**	**42,8**	**83,3**

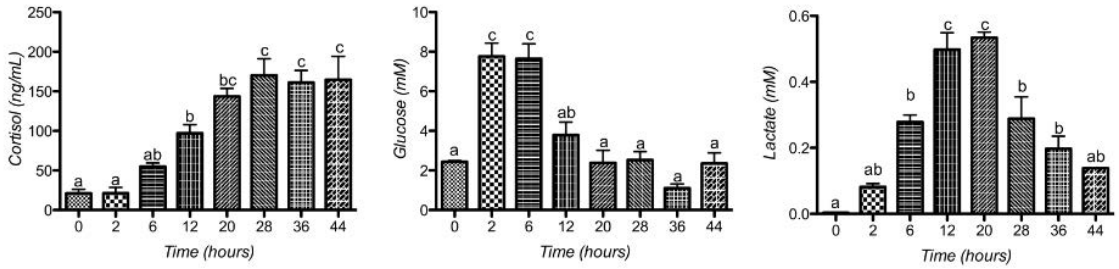
The groups with significantly higher levels of ingestion ($p<0.05$) were those maintained for 2, 6, 12, 20 and 28 hours of emersion (Table 6.2.), ranging between 26.3 and 53.3 % of intake. In addition, the group with a significantly lower level of intake was 44 hours ($p <0.05$) in which food ingestion was not observed during 4 weeks (Table 6.2.). Groups of 0 and 36 hours showed intermediate levels of intake (Table 6.2.). No food intake was observed in any group until seven days after the emersion trials (Table 6.2.).

Table 6.2. Feeding performance of the 8 different groups during four weeks. Values represent the percentage of food eaten over the supplied. Different superscript letters represent significant differences between the means of each experimental group.

	Days after emersion trial																
	1	**2**	**3**	**4**	**8**	**9**	**10**	**11**	**15**	**16**	**17**	**18**	**22**	**23**	**24**	**25**	**Mean± SE**
G.1 (0h.)	_	_	_	_	_	_	_	14,3	_	_	_	28,6	85,7	_	_	7,1	**8,5 ± 5,5^{b,c}**
G.2 (2h.)	_	_	_	_	28,6	28,6	42,9	21,4	42,9	_	28,6	14,3	92,9	100	78,6	85,7	**35,3 ± 8,9^{a}**
G.3 (6h.)	_	_	_	_	_	35,7	42,9	57,1	85,7	42,9	57,1	85,7	100	100	100	100	**50,4 ± 10,3^{a}**
G.4 (12h.)	_	_	_	_	_	35,7	_	42,9	85,7	7,1	_	71,4	71,4	100	100	100	**38,4 ± 10,6^{a}**
G.5 (20h.)		_	_	_	_	_	_	71,4	100	28,6	100	100	100	100	100	100	**53,3 ± 12,2^{a}**
G.6 (28h.)		_	_	_	_	_	_	_	28,6	_	28,6	28,6	66,7	100	41,7	100	**26,3 ± 9,0^{b,a}**
G.7 (36h.)		_	_	_	_	_	_	_	_	_	25	_	_	_	_	50	**5,0 ± 3,5^{c,d}**
G.8 (44h.)		_	_	_	_	_	_	_	_	_	_	_	_	_	_	_	**0,0 ± 0^{d}**

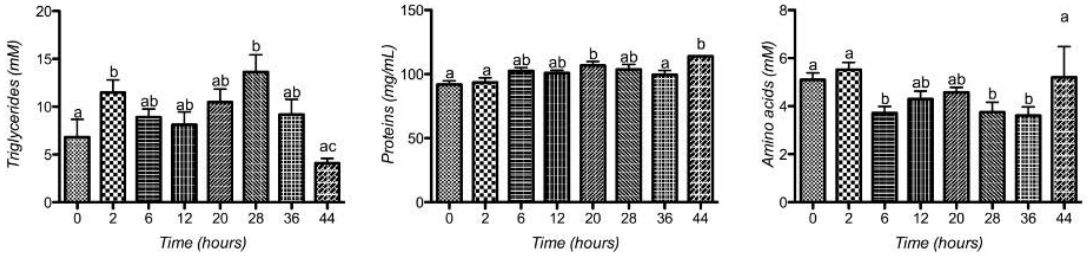
Plasma cortisol levels showed a progressive increase throughout the emersion time, although no significant difference respect to control was observed prior to 12 hours (Figure 6.1.). In addition, the highest levels of this hormone were found in those specimens maintained during 28, 36 and 44 hours out of water, with more than 7-fold increase when compared with control group (0 hours). Moreover, glucose levels enhanced significantly in the groups of 2, 6 and 12 hours at emersion, although no differences in the remaining groups were observed (Figure 6.1.). Furthermore, lactate showed its highest values in those groups maintained during 12 and 20 hours at emersion ($p < 0.05$), decreasing its values after this time (Figure 6.1.).

Figure 6.1. Time course changes in plasma cortisol, glucose and lactate levels after different hours of emersion. Values are represented as mean ± S.E.M. (n = 7 fish per group). Significant differences between sampling points are identified with different letters.



In addition, Figure 6.2. shows plasma values of triglycerides, total proteins and free amino acids after different hours of emersion. Thus, triglycerides levels enhanced significantly in those groups maintained during 2 and 28 hours at emersion (Figure 6.2.), whereas proteins significantly increased its levels in the groups of 20 and 44 hours (Figure 6.2.). Finally free amino acids levels decreased its values after 6 hours of emersion and recovered basal levels at 44 hours (Figure 6.2.).

Figure 6.2. Time course changes in plasma triglycerides, proteins and amino acids levels after different hours of emersion. Values are represented as mean ± S.E.M. (n = 7 fish per group). Significant differences between sampling points are identified with different letters.



6.4 Discussion

This study was aimed to determine the optimum emersion time for *S. senegalensis* specimens (about 1000 g body mass) kept in polystyrene boxes on a wet cloth at 15 °C. For this purpose, mortality rates during and after emersion trial as well as feeding performance during a period of four weeks after trial were assessed. In addition, evaluation of stress system activation was carried out by analysis of different plasma stress (cortisol, glucose, and lactate) and metabolic (triglycerides, proteins and free amino acids) parameters in order to establish the effect of transport in terms of animal welfare. The results will be discussed in relation to its application in transport processes of *S. senegalensis* specimens of large size or/and breeders.

In the case of transport in water, commonly oxygen-enriched, a high percentage of mortality could occur if monitoring systems or oxygen supply fail and the dissolved oxygen is consumed completely (Berka, 1986). The principal advantage of waterless

transport, is that oxygen level available for fish does not change along the time, and only depends on gill epithelium integrity, moreover the oxygen content in air is much higher than in saturated water (Carter, 1975). Oxygen exchange and uptake out of water is possible, while secondary folds on the filaments of the gills do not collapse against each other (Carter, 1957), mortality in the case of waterless transport seems to be related mainly to metabolites excretion inhibition, resulting in excessively high plasmatic content of ammonia nitrogen (Liu *et al.*, 2009).

Survival results obtained in this study, allowed establishing a safe period of time of 28 hours for waterless transport in *S. senegalensis* specimens without direct or delayed effect on mortality under those conditions used in our trial. Even so, the mortality observed in the group of 20 hours (1 specimen) could not be directly associated to our experimental design, because it was a single death recorded 15 days after the emersion trial. Similar survival results have been obtained in other studies performed in *S. maximus* (Calliens, 1996; Liu *et al.*, 2009) or *Dormitator latifrons* (Chang, 1984). Results obtained by Chang (1984) also show the importance of using methodologies that ensure a high level of humidity during air exposure, and how this positively affects the emersion times allowed without mortality. In addition, studies with *S. maximus* indicated that longer times of emersion were achieved without mortality when low temperatures were used, suggesting that environmental temperature could be important factor to be considered in waterless transport. The metabolism decrease induced by low environmental temperature, and the consequent attenuation of oxygen consumption and/or waste metabolite production could explain the longer time of emersion without mortality (Clarke and Johnston, 1999). However, other species such as Pacific halibut, *Hippoglosus stenolepis*, do not exhibit the same tolerance to air exposure, and significant mortality occurred after 40 min and 60 min of air exposure in specimens of 1 and 2 years respectively (Davis and Schreck, 2005).

In terms of behavior, specimens showed no continuous fluttering or stirring during the experiment, and only some low-intensity isolated flutter happened. The relationship between stress and behavior has been widely studied in many species with different types of stress. In our study, *S. senegalensis* specimens showed no behavior clearly associable to emersion. However, chronic or unavoidable stressors may deny this function and so behavioral changes may become maladaptive (Ashley, 2007).

Feeding patterns described under our experimental conditions reflected substantial variability, since the minimum level of management (group 0 hours) inhibited food intake during one week at least. In general, stress response results in an immediate feed intake decrease (Wenderlaar Bonga, 1997). However, Bernier *et al.* (2004) observed how food intake gradually increased in goldfish (*Carassius auratus*) using a low cortisol diet and how this increase was significantly elevated between days 9 and 21, comparing with a non-supplemented with cortisol diet. In addition, Salas-Leiton *et al.*, 2008 and 2010 showed in *S. senegalensis* juveniles, on the one hand an increase in food intake and on the other hand higher cortisol levels associated with high stocking densities (30 Kg/m^2). Thus, the results obtained in the present work confirm both patterns i) an initial inhibition in food intake, and ii) a subsequent increase of the global intake significantly higher in those groups maintained out of water (stressed), which did not register mortalities (G.2, 3, 4, 5 and 6).

Regarding with stress parameters, although these values obtained are consistent with the trends of the parameters along the time, the levels obtained in the 44 hours group must be considered with caution when compared with the other experimental groups, since only 2 individuals were alive at the time of blood sampling, and one of them died within 24 hours after sampling. It has been demonstrated that plasmatic biochemical parameters are altered in fish under comatose condition (Lian-Tien *et al.*, 1994).

Cortisol is the most widely stress indicator used in fish (Wenderlaar Bonga, 1997). Cortisol enhancement was observed after 12 hours emersion, while group submitted to 2 hours of emersion showed similar values that control group. Levels observed in the group 6 hours are comparable to those obtained by Oliveira *et al.* (2013) using *S. senegalensis* specimens (413 ± 36 g body mass) in basal conditions (unstressed), which ranged from 5 to 35 ng/mL over a 24 hour cycle, indicating the existence of a circadian rhythm for plasma cortisol values in this specie. In addition, and due to the existence of a daily rhythms for the different endocrine factors related to hypothalamus-pituitary-interrenal axis as well as in the acute stress responses in *Solea senegalensis*. (López-Olmeda *et al.*, 2013), the influence of time for start emersion trial will be necessary to be considered in future studies. According to these results, we can assume that transport without water could be performed without inducing stress up to 12 hours, first time when cortisol level enhanced significantly respect to control.

In juveniles of *S. senegalensis*, acute stress induced a first stress peak between 5 minutes and 2 hours after the stress challenge (Wunderink *et al.*, 2011; Costas *et al.*, 2011; Herrera *et al.*, 2012). However, our experimental design did not consider these sampling points associated with acute stress situation, because it is focused in a long-term response. In this way, the highest cortisol values observed in this study was those obtained in the group of 28 hours of emersion (170.1 ± 21.1 ng/mL), being these values much lower than those obtained by other authors in stressed specimens of this species (Weber *et al.*, 2009; Costas, 2011; Silva *et al.*, 2010).

Plasma glucose values presented a similar trend than the reported previously in *S. maximus* specimens submitted to emersion (Calliens, 1996). In our case, glucose peak was ahead of cortisol enhancement, so that this energy substrate mobilization seems to be modulated by action of catecholamines, or even by a delay in the response to the cortisol hormone (Wendellar Bonga, 1997). However, its values were reduced from 12 hours of emersion, indicating an increase in its consumption and/or decrease of

hepatic free glucose. Plasma lactate, another energy substrate used in stress situations (Mommsen *et al.*, 1999), presents a similar pattern of change that cortisol and appears to be modulated by this hormone. Indeed, both parameters showed an increase over time, reflecting a stressful situation. Regarding with plasma triglycerides, proteins and amino acids, no marked variations were observed in relation to stress intensity. These results only highlighted the decline in the amino acids levels from 6 hours of emersion. This effect is probably associated with the conversion of these by the gluconeogenesis pathway to glucose (Mommsen *et al.*, 1999), to maintain homeostasis.

6.4 Conclusions

In conclusion, we can establish a safe range of 28 hours for waterless transport in *S. senegalensis* without direct or delayed effect on mortality. Furthermore, in the absence of an in-depth analysis on the evolution of stress parameters between 0 and 2 hours emersion, it seems that this type of activity does not generate significant levels of stress previous to 6 hours of emersion. Nevertheless, it would be also desirable to study aspects such as i) the influence of anesthetics and other compounds on stress level attenuation, ii) the minimum size allowed for this transport system, iii) the appropriate temperature to improve these results. In addition it will be necessary to have a good description of the baseline levels of stress markers in this species at different ages/weights, in order to determine when stress induced by waterless transport is really significant, as well as to determine the recovery time needed to return to the baseline levels.

Chapter 7. General Conclusions and future research.

Chapter 7.

General conclusions and future research.

The objective of this thesis was twofold, first, to contribute to the knowledge of the reproductive biology of the *Solea senegalensis* in captivity to try to explain the reason behind the lack of fertilized spawns in the cultivated broodstocks. On the other hand, to make progress in the zootechnics of the species by adapting egg disinfection and transport protocols from other species to Senegalese sole. According to these general objectives, and to the specific objectives established in the thesis, several experiments were carried out. The results obtained allow the extraction of the following conclusions.

7.1. General conclusions

In this section, the general conclusions are displayed for each of the four study chapters.

Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons.

- The low number of breeders that contribute to the total production of the stock (between 8.7% and 51.7%) reveal the individual importance in the reproduction of this species.

- The couples that contribute more to the total production exhibit fidelity patterns that continue over the successive breeding seasons.

- The females of this species are able to ovulate for at least 6 consecutive days in captivity under the conditions described. In addition, an average ovulation frequency of 7.3 ± 0.6 days has been established.

- The eggs of Senegalese sole females show a decrease of diameter throughout the spawning season. Furthermore, during this decrease, the size is regained during partial breading pauses, so, the evolution of egg diameter fits a cubic regression model.

- The thermoperiod manipulation is effective to induce spawning in *S. Senegalensis*, and allows increasing the spawning periods described in the literature from 4-6 months (depending on the authors) to 8.5 months (Tank B3, 2009).

Reproductive responses of captive Senegalese sole, *Solea senegalensis*, according to the type of feeding and the origin of each gender.

- Natural feeding improves the reproductive response of cultured *Solea senegalensis,* compared with a commercial diet in terms of daily relative fecundity, number of spawns, and therefore total volumes obtained.

- The feeding regime with which fertilized spawns are achieved in wild individuals, fails to enhance natural reproduction of the cultivated individuals of the species in terms of fertilization.

- The housing of mixed broodstock, made up of wild males and cultivated

females allows obtaining fertilized spawns with fertilization and hatching rates similar to those obtained with wild individuals adapted to captivity (65.46±7.48 % and 78.86±9.56 % respectively).

- The spawns obtained with the cultivated males and wild females are fewer and always unfertilized.

Surface disinfection of Senegalese sole, *Solea senegalensis* Kaup 1858, eggs using iodine.

- A disinfection with 50 mg l^{-1} of iodine for 10 minutes followed by three 5 minutes rinses with seawater, significantly reduces the bacterial load in *Solea senegalensis* eggs without affecting the egg development or the hatching rate. The reduction level achieved was around 90 % in the number of new CFU that appeared at 24, 48, 72 hours of incubation, and also in the total bacteria count.

- The microtiter plate incubation method, suitable to evaluate the hatching rate and the larval survival, must be carried out with caution in studies with products that may be toxic in such low volumes.

Waterless transport of Senegalese sole, *Solea senegalensis*, Kaup 1858.

- The results obtained allow establishing a safe range of 28 hours for waterless transport in *S. senegalensis* without direct or delayed effects on mortality, using the methodology described with a temperature of 15ºC.

- In addition, this transport system does not significantly increase circulating Cortisol with emersion times under 12 hours.

7.2 Future research

Despite the knowledge gained on the reproductive biology of the species in captivity, and the management tools proposed for disinfection of eggs or transport of specimens without water, this thesis does not provide the key factor/s to obtaining fertilized natural spawns with individuals born in captivity. The results obtained can improve the management of this species (disinfection and transport), and are the first studied specifically for the Senegalese sole, but it is still necessary to continue studying the processes that can improve or optimize the zootechnics of the species.

In this sense, the main lines of work and issues arising from the results obtained in this thesis are listed below, divided into the two major areas of knowledge presented in this thesis (reproductive biology and zootechnics):

Reproductive biology

- All the factors that allow optimizing the number of breeders for an adequate reproductive performance of this species in captivity, such as the most suitable male:female ratio, the minimum number of breeders necessary to ensure the maximum efficiency of a broodstock, or the determination of the role of individuals that do not participate in spawns.

- The possible influence of feeding in early culture stages on reproduction success.

- The individual relationships established in the tanks with wild males and cultured females.

- Possible ways to stimulate or induce social relations (courtship) in cultivated specimens (hormone therapies, pheromones, tutoring systems, etc).

- Deepen the control of natural reproduction by developing physicochemical manipulation protocols that allow greater predictability in the spawns, and assess the possible modulating effect of the light.

- The importance of the type of tank, the minimum size and or volume needed for reproduction success in this species.

Zootechnics

- The effectiveness of iodine for viruses, the efficacy of other compounds, as well as the possible adverse effects of disinfection at the different embryonic stages.

- Evaluate the stress level differences between the waterless transportation and a conventional transportation in water tanks. To this end, it would be necessary to previously establish the standard baseline levels of the stress markers in this species at different ages, in order to determine when the stress is significant, as well as to determine the recovery time needed to return to baseline levels.

- Other aspects related to the waterless transport conditions, such as the influence of anesthetics or other compounds to reduce stress levels, or the most suitable temperature to carry out this type of transportation.

- The minimum fish size/weight allowed for this transport system.

References

References

A

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

Andrade, J.P. 1990. A importância da Ria Formosa no ciclo biológico de *Solea senegalensis* (Kaup, 1858), *Solea vulgaris* (Quensel, 1806), *Solea lascaris* (Risso, 1810) e *Michrochirus azevia* (Capello, 1868). PhD Thesis, Universidade do Algarve, Portugal.

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

Anguís, V., Moure, M., Cañavate, J.P. 2007. Estudio comparativo de aspectos reproductivos en lenguado senegalés (*Solea senegalensis*) en función de su origen salvaje o de primera generación (F1). *Proceedings of XI Congreso Nacional de Acuicultura*. Xunta de Galicia, Vigo, Spain, vol. II, 1443-1446.

APROMAR 2012. La acuicultura marina de peces en España 2012. Asociación Empresarial de Productores de Cultivos Marinos, Chiclana (Cádiz), Spain.

APROMAR 2014. La acuicultura marina de peces en España 2014. Asociación Empresarial de Productores de Cultivos Marinos, Chiclana (Cádiz), Spain.

Arijo, S., Chabrillón, M., Díaz-Rosales, P., Rico, R.M., Martínez-Manzanares, E., Balebona, M.C., Toranzo, A.E., Moriñigo, M.A. 2005. Bacteria isolated from outbreaks

affecting cultured sole, *Solea senegalensis* (Kaup). *Bulletin of the European Association of Fish Pathologists* **25**, 148-154.

Arjona, F.J., Vargas-Chacoff, L., Ruiz-Jarabo, I., Martín del Río, M.P. Mancera, J.M. 2007. Osmoregulatory response of Senegaleses sole (*Solea senegalensis* Kaup, 1858) to changes in environmental salinity. *Comparative Biochemistry Physiology* **148**, 413-421.

Aristizabal, E., Suarez, J., Vega, A., Bargas, R. 2009. Egg and larval quality assessment in the argentinean red porgy (*pagrus pagrus*). *Aquaculture* **287**(3-4), 329-334.

Ashley, P. J. 2007. Fish welfare: current issues in aquaculture. *Applied Animal Behaviour Science* **104**(3), 199-235.

B

Balebona, M.C., Andreu, M., Bordas, M., Zorrilla, I., Morinigo, M., Borrego, J.J. 1998. Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.). *Applied and Environmental Microbiology* **64**, 4269-4275.

Barker, G.A., Smith, S.N., Bromage, N.R. 1989. The bacterial flora of rainbow trout, *Salmo gairdneri* Richardson, and brown trout, *Salmo trutta* L., eggs and its relationship to developmental success. *Journal of Fish Diseases* **12**, 281-293.

Barton, B.A. 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative Comparative Biology* **42**, 517-525.

Ben-Tuvia, A. 1990. A taxonomic reappraisal of the atlanto-mediterranean soles *Solea solea, S. senegalensis* and *S. lascaris*. *Journal of Fish Biology* **36**(6), 947-960.

Bennetau-Pelissero, C., Kaushik, S. J. 2001. Effect of genistein, a phytoestrogen from soybean on endocrine status and reproductive performance in rainbow trout *World Aquaculture Society*, 143 J.M Parker Coliseum Louisiana State University Baton Rouge LA 70803 USA.

Bergh, O., Jelmert, A. 1996. Iodophor disinfection of eggs of Atlantic halibut. *Journal of Aquatic Animal Health* **8**, 135-145.

Berka, R. 1986. The transport of live fish. A review. FAO, ROME (ITALY).1986.

Bernier, N. J., Bedard, N., Peter, R. E. 2004. Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. *General and Comparative Endocrinology* **135**(2), 230-240.

Blake, P., Weaver, R.E., Hollis, D.G**.** 1980. Diseases of humans (other than cholera) caused by vibrios. *Annual Review of Microbiology* **34,** 341–367.

Blonk, R.J.W., Komen, J., Kamstra, A., Crooijmans, R.P.M.A., van Arendonk, J.A.M. 2009. Levels of inbreeding in group mating captive broodstock populations of Common sole, (*Solea solea*), inferred from parental relatedness and contribution. *Aquaculture* **289**, 26-31.

Bromage, N. Broodstock management and seed quality – general considerations. In: BROMAGE, N.R.; ROBERTS, R.J. (Eds.) Broodstock management and egg and larval quality. London: Blackwell Science Ltd, 1995. p.1-24.

C

Caillens, S. 1996. Survival emersion of turbot (*scophthalmus maximus*) IFREMER CENT. BREST, PLOUZANE (FRANCE).

Cameron Brown, R., Woolliams, J. A., McAndrew, B.J. 2005. Factors influencing effective population size in commercial populations of gilthead seabream, *Sparus aurata*. *Aquaculture* **247**, 219-225.

Cañavate, J.P. 2005. Opciones del lenguado senegalés *Solea senegalensis* Kaup, 1858 para diversificar la acuicultura marina. *Boletín Instituto Español de Oceanografía* **21**, 147-154.

Carazo, I. 2013. Reproductive behaviour and physiology of Senegalese sole, (*Solea senegalensis*) broodstock in captivity. Thesis. University of Barcelona, Spain, 209 pp.

Carazo, I., Martin, I., Hubbard, P., Chereguini, O., Mañanós, E., Canário, A., Duncan, N. 2011. Reproductive behaviour, the absence of reproductive behaviour in cultured (G1 generation) and chemical communication in the senegalese sole (*Solea senegalensis*). *Indian Journal of Science Technology* **4**, 96-97.

Carazo, I., Chereguini, O., Huntingford, F., Martin, I., Norambuena, F. y Duncan, N. 2009. Observaciones del cortejo de lenguado (*Solea senegalensis*, Kaup 1858) salvaje mantenido en cautividad. *Proceedings of XII Congreso Nacional de Acuicultura*. Madrid, Spain.

Carter, G. S. 1957. Air breathing. In: Brown M. E. (Ed) The physiology of fishes: Volume I metabolism. Academic press, p. 65-79.

Castro, J., Pino, A., Hermida, M., Bouza, C., Riaza, A., Ferreiro, I., Sánchez, L., Martínez, P. 2006. A microsatellite marker tool for parentage analysis in Senegal sole (*Solea senegalensis*): Genotyping errors, null alleles and conformance to theoretical assumptions. *Aquaculture* **261**, 1194-1203.

Chang, B. D. 1984. Tolerances to salinity and air exposure of *dormitator latifrons* (pisces: *Eleotridae*). *Revista De Biología Tropical. San Jose* ***32***(1), 155-157.

Chavanne, H., Parati, K., Cambuli, C., Capoferri, R., Jiménez, C. A., Galli, A. 2012. Microsatellites markers to depict the reproductive and genetic patterns of farmed gilthead seabream (*Sparus aurata*): illustration by a case study on mass spawning. *Aquaculture Research* 1 – 14.

Chereguini, O. 2006. Natural spawns of wild Senegal sole obtained by thermoperiod manipulation in Cantabria (Northern Spain). *Report of a 3rd workshop on the cultivation of soles* CIFPA El Toruño, Cádiz, Spain.

Chereguini, O., Rasines, I., Anguis, V., Cal, R., Martín, I., Rodríguez, C., Guzman, J.M., Mylonas, C.C. Mañanós, E. 2007. Primeras fecundaciones artificiales en lenguado senegalés cultivado (generación F1). *Proceedings of XI Congreso Nacional de Acuicultura*. Xunta de Galicia, Vigo, Spain, vol. II, 1435-1438.

Chistiakov, D.A., Hellemans, B., Volckaert, F.A.M. 2006. Microsatellites and their genomic distribution, evolution, function and applications: A review with special reference to fish genetics. *Aquaculture* **255**, 1-29.

Clarke, A., Johnston, N. M. 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. *Journal of Animal Ecology* **68**, 893–905.

Comandante Bonifacio F Jr. 2005. Process for transport of live fish without water. Patent WO2005039280 A1. 6 May 2005.

Costas, B., Conceicao, L. E. C., Aragao, C., Martos, J. A., Ruiz-Jarabo, I., Mancera, J. M., Afonso, A. 2011. Physiological responses of Senegalese sole (*Solea senegalensis* kaup, 1858) after stress challenge: Effects on non-specific immune parameters, plasma free amino acids and energy metabolism. *Aquaculture* **316**(1-4), 68-76.

Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97.

D

Das, S., Sahoo, P.K. 2012. Developments in selective breeding for resistance to *Aeromonas hydrophila* in fish. *World Aquaculture* ***43***, 39-45.

Davis, M. W., Schreck, C. B. 2005. Responses by pacific halibut to air exposure: Lack of correspondence among plasma constituents and mortality. *Transactions of the American Fisheries Society* **134**(4), 991-998.

Dinis, M.T. 1986. Quatre Soleidae de l´Estuaire du Tage. Reproduction el Croissance. Essai d´Elevage de *Solea senegalensis* Kaup 1858. Thése d´Etat ès-Sciences Naturelles. Université de Bretagne Occidentale, France, 347 pp.

Dinis, M. T., Metailler, R., Rodeia, J. 1987. Weaning of *Solea senegalensis* kaup, 1858 with rehydratable pellets. *Proceedings of Jornadas sobre Nutriçao em Aquacultura*, Lisbon (Portugal), 29 Jan 1985.

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

Douillet, P., Holt, G. 1994. Surface disinfection of red drum (*Sciaenops ocellatus* Linnaeus) eggs leading to bacteria-free larvae. *Journal of Experimental Marine Biology and Ecology* **179**, 253-266.

E

Escaffre, A., Bazin, D., Bergot, P. 2001. Disinfection of *Sparus aurata* eggs with glutaraldehyde. *Aquaculture International* **9**, 451-458.

F

FAO 1998. Quality and quality changes in fresh fish 1998. In Huss H. (Ed.)

FAO 2014. The State of World Fisheries and Aquaculture 2014. Rome. 223pp.

FAO. www.fao.org

FEAP 2014. Production Report of the Federation of European Aquaculture Producers.

Fishbase. www.fishbase.org

G

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

García-López, A., Pascual, E., Sarasquete, C., Martinez-Rodriguez, G. 2006b. Disruption of gonadal maturation in cultured senegalese sole *Solea senegalensis* kaup by continuous light and/or constant temperature regimes. *Aquaculture* **261**(2), 789-798.

Guarniero, I., Parma, L., Rongai, F., Bonaldo, A., Trentini, M., Gatta, P.P. 2010. Parentage assignment of common sole, *Solea solea* larvae inferred by microsatellites: Preliminary data. *Proceedings of Aquaculture Europe 10*, Porto, Portugal, October 2010. *European Aquaculture Society*, 534-535.

Guzman, J. M., Norberg, B., Ramos, J., Mylonas, C. C., Mañanos, E. L. 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female senegalese sole (*Solea senegalensis*). *General and Comparative Endocrinology* **156**(2), 285-297.

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

H

Henrotte, E., Overton, J. L., Kestemont, P. 2008. In Fostier A., Le Gac F. and Guiguen Y. (Eds.), Effects of dietary n-3 and n-6 fatty acids levels on egg and larval quality of eurasian perch. *Societe Francaise d'Ichtyologie*, MNHN, Paris (France).

Herlin, M., Delghandi, M., Wesmajervi, M., Taggart, J.B., McAndrew, B.J., Penman, D.J. 2008. Analysis of the parental contribution to a group of fry from a single day of spawning from a commercial Atlantic cod (*Gadus morhua*) breeding tank. *Aquaculture* **274**, 218-224.

Herrera, M., Aragao, C., Hachero, I., Ruiz-Jarabo, I., Vargas-Chacoff, L., Mancera, J. M., Conceicao, L. E. C. 2012. Physiological short-term response to sudden salinity change in the senegalese sole (*Solea senegalensis*). *Fish Physiology and Biochemistry* **38**(6), 1741-1751.

Hirazawa, N., Hara, T., Mitsuboshi, T., Okazaki, J., Hata, K. 1999. Iodophor disinfection of eggs of spotted halibut *Verasper variegatus* and red sea bream *Pagrus major*. *Fisheries Science* **65**, 333-338.

Howell, B., ConceiÇao, L., Prickett, R., Cañavate, P., Mañanos, E. 2009. Sole farming: nearly there but not quite?!. *Aquaculture Europe* **34**, 24-27.

Howell, B. R., Yamashita, Y. 2005. In Gibson R. (Ed.), Aquaculture and stock enhancement Blackwell Publishing Ltd., 9600 Garsington Road Oxford OX4 2DQ UK

Hutchings, J.A., Bishop, T.D., McGregor-Shaw, C.R. 1999. Spawning behaviour of Atlantic cod, *Gadus morhua*: evidence of mate competition and mate choice in a broadcast spawner. *Canadian Journal of Fisheries and Aquatic Sciences* **56**, 97-104.

I

Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P. 2004. A review of the culture potential of *Solea solea* and *S. senegalensis. Reviews in Fish Biology and Fisheries* **13,** 379–407.

J

Jia, Y., Meng, Z., Liu, X., Lei, J. 2014. Biochemical composition and quality of turbot (*Scophthalmus maximus*) eggs throughout the reproductive season. *Fish Physiology and Biochemistry* **40**(4), 1093-1104.

K

Katharios, P., Agathaggelou, A., Paraskevopoulos, S., Mylonas, C.C. 2007. Comparison of iodine and glutaraldehyde as surface disinfectants for red porgy (*Pagrus pagrus*) and white sea bream (*Diplodus sargus sargus*) eggs. *Aquaculture Research* **38**, 527-536.

Khodabandeh, S., Abtahi, B. 2006. Effects of sodium chloride, formalin and iodine on the hatching success of common carp, *Cyprinus carpio*, eggs. *Journal of Applied Ichthyology* **22**, 54-56.

Kusuda, R., Yokoyama, J., Kawai, K. 1986. Bacteriological study on cause of mass mortalities in cultured black sea bream fry. *Bulletin of the Japanese Society of Scientific Fisheries* **52**, 1745-1751.

L

Laurence, G.C., Rogers, C.A. 1976. Effects of temperature and salinity on the comparative embryo development and mortality of Atlantic cod (*Gadus morhua* L.) and haddock (*Melanogrammus aeglefinus* L.). *Conseil permanent International pour l´Exploration de la Mer* **36**, 220-228.

Lee, K. 1995. Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus*, Bloch et Schneider. *Microbial Pathogenesis* ***19***, 39-48.

Lian-Tien, S., Guan-Ru, C., Ching-Fong, C. 1994. Characteristics of blood parameters and gill Na+-K+-ATPase in chilled comatose tilapia cultured in various salinities. *Comparative Biochemistry and Physiology* **107** (4), 641-646.

Liu, X., Liu, X., Lian, J., Wang, Y., Zhang, F., Yu, H. 2008. Large scale artificial reproduction and rearing of senegal sole, *Solea senegalensis* (Kaup). *Marine Fisheries research*/Haiyang Shuichan Yanjiu **29**, 10-16.

Liu, W., Xue, C., Yin, B., Liu, Q. 2009. Physiological and biochemical change of *Scophthalmus maximus* kept alive at low temperature with or without water. *Progress in Fishery science/Yuye Kexue Jinzhan* **30**(5), 69-74.

López-Olmeda J.F., Blanco-Vives B., Pujante I.M., Wunderink Y.S., Mancera J.M., Sánchez-Vázquez F.J. 2013. Daily rhythms in the hypothalamus-pituitary-interrenal axis and acute stress responses in a teleost flatfish, *Solea senegalensis*. *Chronobiology International* **30** (4) 530-539.

M

McFadden, T.W. 1969. Effective disinfection of trout eggs to prevent egg transmission of *Aeromonas liquefaciens*. *Journal of the Fisheries Research Board of Canada* ***26***, 2311-2318.

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

Proceedings of Aquaculture Europe 07, Istanbul, Turkey, October 2007. *European Aquaculture Society*, 330-331.

Marshall, T. C., Slate, J., Kruuk, L. E. B. and Pemberton, J. M. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7,** 639–655.

Martín, I., Rodríguez, C., Chereguini, O. 2007. Puestas naturales de lenguado senegalés, salvajes y cultivados (generación F1), obtenidos por manipulación del termoperiodo en Cantabria. *Proceedings of XI Congreso Nacional de Acuicultura*. Xunta de Galicia, Vigo, Spain, vol. II, 1427-1430.

Martín, I., Carazo, I., Gómez, M., Rodriguez, C., Rasines, I., Duncan, N., Chereguini, O. 2011. Puestas naturales y comportamiento reproductivo en individuos salvajes y cultivados (F1/F2) de *Solea senegalensis* estabulados conjuntamente. *Proceedings of XIII Congreso Nacional de Acuicultura*. Barcelona, Spain.

Martínez, P. 2005. Aplicaciones de la genética para la mejora de la acuicultura. *Boletín Instituto Español de Oceanografía* **21**, 225-238.

Martos-Sitcha, J. A., Wunderink, Y. S., Straatjes, J., Skrzynska, A. K., Mancera, J. M. Martínez-Rodríguez, G. 2014. Different stressors induce differential responses of the CRH-stress system in the gilthead sea bream (*Sparus aurata*). *Comparative Biochemistry and Physiology Part A, Molecular and Integrative Physiology* **177**, 49-61.

Mira, S., Madeira, C., Cabrita, E., Soares, F., Cancela, M.L., Dinis, M.T. 2010. Parental contribution of *Solea senegalensis* assessed with microsatellite markers. *Proceedings of Aquaculture Europe 10*, Porto, Portugal, October 2010. E*uropean Aquaculture Society*, 826-827.

Mommsen, T. P., Vijayan, M. M., Moon, T. W. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9**(3), 211-268.

Morais, S., Aragão, C., Cabrita, E., Conceição, L. E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanós, E., Valente, L. M. P., Yúfera, M. and Dinis, M. T. 2014, New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. *Reviews in Aquaculture* **6**, 1-37.

N

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

Norambuena, F., Estévez, A., Sánchez-Vázquez, F.J., Carazo, I., Duncan, N. 2012b. Self-selection of diets with different contents of arachidonic acid by Senegalese sole (*Solea senegalensis*) broodstock. *Aquaculture* **364-365**, 198-205.

O

Oliveira, C. C. V., Aparicio, R., Blanco-Vives, B., Chereguini, O., Martin, I., Javier Sanchez-Vazquez, F. 2013. Endocrine (plasma cortisol and glucose) and behavioral (locomotor and self-feeding activity) circadian rhythms in senegalese sole (*Solea*

senegalensis kaup 1858) exposed to light/dark cycles or constant light. *Fish Physiology and Biochemistry* **39**(3), 479-487.

Oliveira, C., Dinis, M. T., Soares, F., Cabrita, E., Pousao-Ferreira, P., Sanchez-Vazquez, F. 2009. Lunar and daily spawning rhythms of Senegalese sole *Solea senegalensis*. *Journal of Fish Biology* **75**(1), 61-74.

Oliveira, C., Duncan, N. J., Pousao-Ferreira, P., Mañanos, E., Sanchez-Vazquez, F. 2010. Influence of the lunar cycle on plasma melatonin, vitellogenin and sex steroids rhythms in Senegal sole, *Solea senegalensis*. *Aquaculture* **306**(1-4), 343-347.

Overton, J.L., Bruun, M.S., Dalsgaard, I. 2010. Chemical surface disinfection of eggs of Baltic cod, *Gadus morhua* L. *Journal of fish diseases* **33**, 707-716.

P

Padrós, F., Zarza, C., Estévez, A., Crespo, S., Furones, M.D. 2003. Patología como factor limitante para el cultivo del lenguado. P*roceedings of IX Congreso Nacional de Acuicultura*. Junta de Andalucía, Cádiz, Spain, 343-345.

Panini, E., Mylonas, C., Zanuy, S., Carrillo, M., Ramos, J., Bruce M. 2001. Incubation of embryos and larvae of marine fish using microtiter plates. *Aquaculture International* **9**, 189-195.

Peck, M., Buckley, L., O'Bryan, L., Davies, E., Lapolla, A. 2004. Efficacy of egg surface disinfectants in captive spawning Atlantic cod *Gadus morhua* L. and haddock *Melanogrammus aeglefinus* L. *Aquaculture Research* **35**, 992-996.

Porta, J., Porta, J.M., Martínez-Rodriguez, G., Alvarez, M.C. 2006a. Development of a microsatellite multiplex PCR for Senegalese sole (*Solea senegalensis*) and its application to broodstock management. *Aquaculture* **256**, 159-166.

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

R

Rach, J., Gaikowski, M., Howe, G., Schreier, T. 1998. Evaluation of the toxicity and efficacy of hydrogen peroxide treatments on eggs of warm- and coolwater fishes. *Aquaculture* **165**, 11-25.

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

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

Rasines, I. 2014. Reproducción en cautividad del lenguado senegalés (*Solea senegalensis*): inducción hormonal a la evolución y obtención de huevos para la fecundación artificial. Thesis. University of Oviedo, Spain. 183pp.

Rodriguez, J.L. Peleteiro J.B. Cultivo del lenguado Senegalés (*Solea senegalensis*). 2014. Cuadernos de acuicultura, Fundación Observatorio Español de Acuicultura (OESA).

Rodríguez, L., Begtashi, I., Zanuy, S. and Carrillo, M. 2000. Development and validation of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax*, L.) at puberty. *Fish Physiology and Biochemistry* **23**, 141-150.

Rodriguez, R. B., Pascual, E. 1982. Effect of tuna dried pituitary on maturation and spawning in *Solea senegalensis* and *sparus aurata* , and comparison with carp dried pituitary and HGG treatments.

Rodríguez, R.B. 1984. Biología y cultivo de *Solea senegalensis* Kaup, 1858 en el Golfo de Cádiz. Thesis. University of Sevilla, Spain. 207 pp.

Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. *Archives of Biochemistry and Biophysics* **67**, 10-15.

Ross, A.J., Smith, C.A. 1972. Effect of two iodophors on bacterial and fungal fish pathogens. *Journal of the Fisheries Research Board of Canada* **29**, 1359-1361.

Rubio, V. C., Sanchez-Vazquez, F., Madrid, J. A. 2003. Nocturnal feeding reduces sea bass (*Dicentrarchus labrax*, L.) pellet-catching ability. *Aquaculture* **220**(1-4), 697-705.

Rungruangsak-Torrissen, K., Fosseidengen, J. E. 2007. Effect of artificial feeding on digestive efficiency, growth and qualities of muscle and oocyte of maturing atlantic mackerel (scomber scombrus L.). *Journal of Food Biochemistry* **31**(6), 726-747

S

Salas-Leiton, E., Anguis, V., Manchado, M., Canavate, J. P. 2008. Growth, feeding and oxygen consumption of Senegalese sole (*Solea senegalensis*) juveniles stocked at different densities. *Aquaculture* **285**(1-4), 84-89.

Salas-Leiton, E., Anguis, V., Martin-Antonio, B., Crespo, D., Planas, J. V., Infante, C., Manchado, M. 2010. Effects of stocking density and feed ration on growth and gene expression in the Senegalese sole (*Solea senegalensis*): Potential effects on the immune response. *Fish & Shellfish Immunology* **28**(2), 296-302.

Salvesen, I. Vadstein, O. 1995. Surface disinfection of eggs from marine fish: evaluation of four chemicals. *Aquaculture International* **3**,155-171.

Salvesen, I., Oie G., Vadstein, O. 1997. Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. *Aquaculture International* **5**, 249-258.

Sarasquete, M. C., Muñoz-Cueto, J., Gonzalez, d. C., Garcia-Garcia, A., Rodriguez, R. B. 1993. In Cervino A., Landin A., de Coo A., Guerra A. and Torre M. (Eds.), Histological, histochemical and immunohistochemical characteristics during oogenesis of *solea senegalensis* (kaup, 1858) in the gulf of cadiz (spain) CENTRO DE INVESTIGACIONES MARINAS, PONTEVEDRA (SPAIN).

Sarasquete, M. C., Muñoz-Cueto, J., Gonzalez, d. C., Rodriguez, R. B. 1993. In Cervino A., Landin A., de Coo A., Guerra A. and Torre M. (Eds.), Presumptive pasteurellosis in *Solea senegalensis* (kaup, 1858) from southwestern spain CENTRO DE INVESTIGACIONES MARINAS, PONTEVEDRA (SPAIN).

Silva, P. I. M., Martins, C. I. M., Engrola, S., Marino, G., Averli, A., Conceicao, L. E. C. 2010. Individual differences in cortisol levels and behaviour of senegalese sole (*Solea*

senegalensis) juveniles: Evidence for coping styles. *Applied Animal Behaviour Science* **124**(1-2), 75-81.

Southgate, P. J. 2008. In Branson J. (Ed.), Welfare of fish during transport. Blackwell Publishing Ltd, Oxford.

Springate, J., Bromage, N. R., Cumaranatunga, P. 1985. In Cowey C., Mackie A. and Bell J. (Eds.), The effects of different ration on fecundity and egg quality in the rainbow trout (*salmo gairdneri*).

Subasinghe, R.P., Sommerville, C. 1985. Disinfection of *Oreochromis mossambicus* (Peters) eggs against commonly occurring potentially pathogenic bacteria and fungi under artificial hatchery conditions. *Aquaculture and Fisheries Management **16***, 121-127.

T

Taggart, J. B. 2006. FAP: an exclusion-based parental assignment program with enhanced predictive functions. *Molecular Ecology* **7**, 412-415.

Takashima, F. 1996. Transportation and distribution of live aquatic animals. *Institut International du Froid*, Paris (France).

Teixeira, C. M., Cabral, H. N. 2010. Comparative analysis of the diet, growth and reproduction of the soles, *Solea solea* and *Solea senegalensis*, occurring in sympatry along the portuguese coast. *Journal of the Marine Biological Association of the United Kingdom* **90**(5), 995-1003.

Tendencia, E.A. 2001. Effect of iodine disinfection on the bacterial flora and hatching rate of grouper, *Epinephelus coioides* eggs at the cleavage and eyed stages. *Bulletin of the European Association of Fish Pathologists* **21**, 160-163.

Teng, S. K., El-Zahr, C., Al-Abdul-Elah, K., Almatar, S. 1999. Pilot-scale spawning and fry production of blue-fin porgy, *sparidentex hasta* (valenciennes), in kuwait. *Aquaculture* **178**(1-2), 27-41.

Treasurer, J., Cochrane, E., Grant, A. 2005. Surface disinfection of cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* eggs with bronopol. *Aquaculture* 250, 27-35.

Toranzo, A.E., Avendaño, R., López-Vázquez, C., Magariños, B., Dopazo, C.P., Romalde, J.L., Barja, J.L. 2003. Principales patologías bacterianas y víricas en lenguado cultivado: caracterización y agentes etiológicos. *Proceedings of IX Congreso Nacional de Acuicultura*. Junta de Andalucía, Cádiz, Spain, 355-356.

U

UN. Concise Report on the World Population Situation. 2014

Unuma, T., Kondo, S., Tanaka, H., Kagawa, H., Nomura, K., Ohta, H. 2004. Determination of the rates of fertilization, hatching and larval survival in the Japanese eel, *Anguilla japonica*, using tissue culture microplates. *Aquaculture* **241**, 345-356.

V

Vinagre, C., Maia, A., Amara, R., Cabral, H.N. 2013. Spawning period of Senegal sole, *Solea senegalensis*, based on juvenile otolith microstructure. *Journal of Sea Research* **76**, 89-93.

W

Wagner, E.J., Arndt, R.E., Billman, E.J., Forest, A., Cavender, W. 2008. Comparison of the Efficacy of Iodine, Formalin, Salt, and Hydrogen Peroxide for Control of External Bacteria on Rainbow Trout Eggs. *North American Journal of Aquaculture* **70**, 118-127.

Wagner, E.J., Oplinger, R.W., Arndt, R.E., Forest, A.M., Bartley, M. 2010. The Safety and Effectiveness of Various Hydrogen Peroxide and Iodine Treatment Regimens for Rainbow Trout Egg Disinfection. *North American Journal of Aquaculture* **72**, 34-42.

Walker, A. B., Ward, D., Duclos, K., Peters, M. Berlinsky, D. L. 2010. Surface disinfection and removal of adhesiveness from rainbow smelt eggs. *Progressive Fish-Culturist* **72**(2), 158-163.

Walsh, P.S., Metzger, D.A., Higuchi, R. 1991. Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* **10**, 506–513.

Wang, J. 2002. An estimator for pairwise relatedness using molecular markers. *Genetics* **160**, 1203-1215.

Weber, R.A., Peleteiro, J.B., Martín, L.O. G., Aldegunde, M. 2009. The efficacy of 2-phenoxyethanol, metomidate, clove oil and MS-222 as anaesthetic agents in the Senegalese sole (*Solea senegalensis* Kaup 1858). *Aquaculture* **288**(1), 147-150.

Wendelaar Bonga, S. 1997. The stress response in fish. *Physiological Reviews* **77**(3), 591-625.

Wright, L.D., Snow, J.R. 1975. The effect of six chemicals for disinfection of largemouth bass eggs. *Progresive Fish Culturist* **37**, 213-217.

Wunderink, Y. S., Engels, S., Halm, S., Yufera, M., Martinez-Rodriguez, G., Flik, G., Klaren, P. H. M., Mancera, J. M. 2011. Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): The involvement of cortisol, CRH and CRH-BP. *General and Comparative Endocrinology* **171**(2), 203-210.

Y

Yemm, E. W., Cocking, E. C. and Ricketts, R. E. 1955. The determination of amino-acids with ninhydrin. *Analyst* **80,** 209-214.

Yin, B., Liu, Q., Liang, M., Jiang, Y. 1995. On transportation of live sea fish. *Shandong fisheries/Qilu Yuye.Yantai* **12**(2), 25-26.

Z

Zorrilla, I., Balebona, M. C., Morinigo, M. A., Sarasquete, C., Borrego, J. J. 1999. Isolation and characterization of the causative agent of pasteurellosis,

photobacterium damsela ssp. piscicida, from sole, *Solea senegalensis* (kaup). *Journal of Fish Diseases* **22**(3), 167-172.