Aquaculture 530 (2021) 735740

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Natural feed after weaning improves the reproductive status of *Solea senegalensis* breeders

Ignacio Martín[a,*], Marta F. Riesco[a], Elena Chaves-Pozo[b], Cristina Rodríguez[a], Juan Manuel Martinez-Vázquez[a], Vanesa Robles[a], Olvido Chereguini[a], Inmaculada Rasines[a]

[a] Spanish Institute of Oceanography, Santander Oceanographic Centre, Promontorio de San Martín, s/n. Apdo. 240, 39080 Santander, Spain
[b] Spanish Institute of Oceanography, Aquaculture Facilities of the Oceanographic Centre of Murcia, Crta. De La Azohía s/n, 30860, Puerto de Mazarrón, Murcia, Spain

ARTICLE INFO

Keywords:
Senegalese sole
Reproduction
Sperm quality
Production
Feeding

ABSTRACT

The aim of this study was to evaluate the effect of long term natural feeding in the subsequent reproductive status of 4 years old cultured *Solea senegalensis*, and to determine if the potential changes were structural or feeding dependent. To this aim, two different feeding regimes were used from one year after weaning and during the following 3 years; 1) a commercial dry food diet and 2) a natural feeding regime. After this period, the proportion of fluent males and the evolution of maturity stages of females over a breeding season were studied. A complete sperm quality analysis assessment was carried out, including individual volumes, motility, density and curvilinear, rectilinear and mean velocities of spermatozoa. Moreover, viability and apoptosis indexes were analyzed as indicator of molecular sperm membrane integrity. Additionally, a morphological characterization of the testes during the spawning season was conducted. Finally, both groups were fed with the same commercial pellets during one year to evaluate the effect of the diet of previous years on sperm quality. The results of this study showed how feeding can improve not only sperm quality and quantity, but also the proportion of fluent males and females in advanced maturity stages. All the sperm quality parameters resulted significantly higher in the group fed with a natural diet. Moreover, the number of apoptotic cells was significantly higher in the group fed with a commercial diet. According to the morphological features of the testes, the animals fed with a natural diet presented more basal position, less protuberances and irregular edges when compared with the animals fed with commercial diet. Interestingly, the progression of the spermatogenesis determined by the proportion of germ cells and the production of spermatozoa determined by the wider of the ducts system was also significantly larger in the natural diet group.

After the standardization of the diets, mean volume per male and production of total motile cells were significantly higher in the group that was previously fed a natural diet, confirming structural improvements.

1. Introduction

Senegalese sole has been considered for decades a promising species for European marine aquaculture diversification (Dinis et al., 1999; Imsland et al., 2004; Morais et al., 2016). In the last years, industrial production figures show how this species has achieved an important place in the international market. According to the Federation of European Aquaculture Producers (FEAP) there has been produced over 1600 tons mostly in Spain, France, Portugal and Iceland, doubling the 2013 production levels (FEAP, 2017). An intense research activity mainly in Spain and Portugal, particularly in the last decade, has allowed developing an enough knowledge framework for a sustainable industrial production. However, several aspects, such as the cultured individuals reproductive dysfunctions, or the insufficient control on the artificial reproduction shows the need for further investigation on the reproduction of this species.

It has been demonstrated that only a low percentage of individuals participate in the natural reproduction of the wild individuals of the species adapted to captivity (Mira et al., 2010; Martín et al., 2014), and this is the main reproduction system at industrial scale (Rodriguez and Peleteiro, 2014). So, controlling the reproduction of the cultured individuals is crucial for reducing the dependence on wild populations to obtain larvae, and for setting up genetic breeding programs.

Nowadays the artificial fertilization protocols developed in the last years (Chereguini et al., 2007; Liu et al., 2008; Rasines, 2014) are the most promising tool to approach such breeding programs, since allow

* Corresponding author at: IEO – Spanish Institute of Oceanography, Marine Aquaculture Plant "El Bocal", Barrio de Corbanera s/n, CP 39012 Santander, Spain.
E-mail address: nacho.martin@ieo.es (I. Martín).

https://doi.org/10.1016/j.aquaculture.2020.735740
Received 5 March 2020; Received in revised form 25 June 2020; Accepted 18 July 2020
Available online 22 July 2020

working with all the individuals of a broodstock. However during this development, the scarcity of sperm production in the males of the species has been highlighted (Morais et al., 2016). Moreover, latest researches point to the fact that the use of sperm pools, might affect the individual sperms quality in this species (Rasines and Rodríguez, 2017). In this scenario, all the advances focused on the increase of individual sperm production contribute to improve the zootechnics of the species.

The status of females is another relevant aspect when applying artificial reproduction techniques. The available artificial fertilization protocols include the hormonal induction of the females to achieve the release of the oocytes. For these inductions it is necessary to have females in the advanced sexual maturity stages (Rasines et al., 2012), therefore, all the elements that contribute to improve the reproductive status of the females, also improve the zootechnics of the species.

The influence of nutrition on reproductive performance has been reported in fish (Izquierdo and Fernandez-Palacios, 2001; Valdebenito et al., 2015), and also in the species under study (Anguis et al. 2008, Beirão et al., 2015), nevertheless most of the studies carried out in sole have been always made by changing the feeding regimes after reaching sexual maturity, and the mean results obtained showed some differences when they were compared with wild caught individuals (Cabrita et al., 2006; Chereguini personal communication). So, the possible influence of the feeding from the beginning of the ongrowing of the individuals to achieve structural improvements in the reproductive performance has never been analyzed.

In this general scenario the aim of this study was to evaluate the long term effect of a diet wildly use in the reproduction of the species and based on their natural feeding habits (Garcia-Franquesa et al., 1996; Colen et al., 2014) during the ongrowing, and how this feeding regime could affect cultured individuals, especially males, in order to solve the reproductive problems of these individuals by improving their reproductive performance for artificial fertilization.

2. Material and methods

2.1. Management and broodstock establishment

Two different groups were formed from individuals of the same origin (spawn and larval culture) in the facilities of the Spanish Institute of Oceanography in Santander. One year after weaning, 72 individuals of 127.8 ± 13.6 g of body weight were randomly separated into two groups (n = 36) and fed with different diets (natural or commercial) in order to have enough individuals of each sex for the subsequent reproductive analysis. The natural (N) group was fed *ad libitum* with a natural diet composed of mussels, *Mytilus* spp. (2 days a week, Tuesday and Thursdays), small squids, *Loligo* spp.(2 days a week, Monday and Wednesdays), and polychaete worms, *Nereis* spp. (Topsybaits Ltd., Netherlands) (1 day a week, Fridays). Natural food was chopped to achieve similar sizes to dry commercial diet pellets. The commercial (C) group was fed a diet composed of Elite® and Europa® pellets from Skretting S.L, (Norway), following pellet's size recommendations of the producers and also supplied *ad libitum*. The biomass of the tanks was recorded monthly by individual samplings (size and weight) and the amount of food subsequently adjusted. The non eaten food was controlled daily to ensure *ad libitum* availability. The last year of the study, both groups were fed with a commercial diet for breeders (Vitalis Repro® pellets) from Skretting S.L, (Norway), in order to determine if the possible changes produced in the different treatments were structural or temporarily associated with the experimental diets.

All the tanks were located inside an industrial warehouse, in open flow circuit, with a water renovation rate of 1.7 m^3/h and constant moderate aeration. The average salinity ± SE recorded during the study was 34.4 ± 0.87 PSU. An artificial photoperiod of 16 h of light and 8 h 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 lx. The fish were subjected to a natural thermoperiod during the ongrowing, with temperatures varying between 11.8 (winter) and 22.4 °C (summer).

Four years after weaning, both (natural and commercial) groups were individually characterized in order to differentiate males and females, to determine the maturity stages of the females, and to identify the proportion of fluent males of each stock. This evaluation resulted in two broodstocks (n = 28, the broodstock fed with a natural diet, and n = 31 the one fed with pellets), the rest of individuals up to the initial 36 died during the ongrowing. The male:female ratio was 1:1 and an average density in the tanks was always under 4 kg/m^2. All individuals were tagged with a passive integrated transponder (Trovan®) placed in the dorsal area, allowing the specimens to be identified and monitored individually. All tanks were treated with a prophylactic hydrogen peroxide bath after sampling (80 ppm for 1 h without water renewal). The average total body weight of females and males at the beginning of the reproductive performance evaluation was 1083.43 ± 48.14 g and 678.63 ± 49.98 g, respectively, in the group with the natural diet, and 1124.58 ± 60.63 g and 784 ± 57.81 g, respectively, in the group with a commercial diet.

From this point of the experiment, the thermoperiod was manipulated every year from January to June to induce the maturation and spawns of the broodstocks, according to Martín et al., 2014, to simulate the natural temperatures recorded in the Toruño (IFAPA, Cádiz) during the spawning season of this species (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. In the middle or end of June, when the environmental seawater temperature was similar to that of the artificially heated water, the thermoperiod

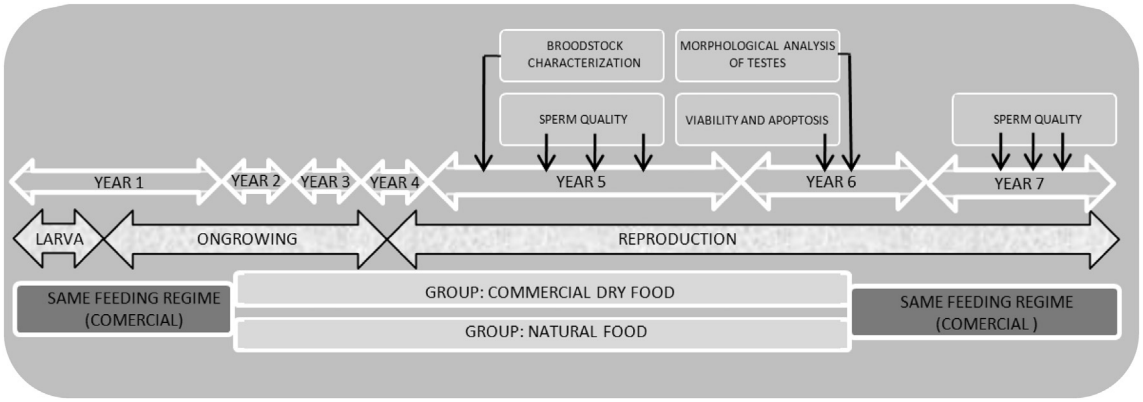

Fig. 1. General outline of the study. The scheme includes a timescale on annual basis. The different culture phases (larva; ongrowing; reproduction), the feeding regime used. Black arrows pointing down show the different samplings that were carried out during the last three years (broodstock characterization, three sperm quality assessments, the morphology analysis of the testes, viability assay of the sperm, and a sperm quality assessments after equalizing the feeding regime).

was no longer manipulated.

Fig. 1 includes a summarized outline of the experimental design and the different phases of the study; feeding regime, time scale in a yearly and culture phase basis, and all the sampling carried out during the last three years of the study.

2.2. Samplings

2.2.1. Characterization of the maturity stages of the females

Monthly from April to September (breeding season), the gonad maturity stage of the females was estimated by external examination of abdominal swelling according to the classification of Anguis and Cañavate (2005): stage E0 corresponds to externally undetectable development; stage E1 corresponds to ovary only detectable by touching the abdominal region; stages E2 and E3 correspond to two levels (initial and intermediate) of externally visible abdominal swelling; and stage E4 corresponds to maximum ovary development, characterized by high abdominal swelling.

2.2.2. Analysis of the sperm quality

Regarding males, the sperm was collected by stripping on May, July and September, (three times over the breeding season, to avoid possible interferences caused by seasonal variations). The 14 individuals of each experimental group were anesthetized with 40 ppm of clove oil (Guinama, Valencia, Spain) for one minute and a half. Samples were taken with a special care to avoid urine contamination and maintained at 4 °C (on an ice bed) until further analysis. After the volume evaluation, 10 μl of sperm were transferred to an ependorff tube with 40 μl of immobilizing solution (Ringer 200 mOsm) for subsequent kinetic analysis, and other 10 μl were transferred to 190 μl of fixative solution (formolic) for density analysis. Both, sperm kinetics and density were analyzed using a computer assisted sperm analysis (CASA) system (ISASv1 integrated system for semen analysis, Proiser, Valencia, Spain) and the following parameters were studied; number of cell per ml, percentage of motile cells, and curvilinear (VCL), rectilinear (VSL) and mean velocity (VAP) (micrometers per second).

For the kinetics evaluation 1 μl of the immobilized sperm solution was activated with 9 μl of cold seawater (on an ice bed) in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), the motility record was made immediately after activation and every male sample was assessed in triplicate (3 videos per sample and 3 samples per male). Density was also evaluated in triplicate by placing 10 μl of the 1:20 diluted sample in the Makler chamber after three seconds vortex agitation. The production of sperm per male Kg was calculated dividing the individual sperm volume by the weight of the male in the month of the sampling. The production of motile spermatozoa was calculated by multiplying the individual volumes by the mean density of each sample, and by the mean proportion of motile spermatozoa.

Another sperm characterization (volume, motility and density) over the breeding season was repeated the last year of study (see Fig. 1) after 8 months feeding both groups with the same commercial diet. There were only 11 males in the group previously fed a natural diet, and 10 males in the group fed always a commercial diet.

2.2.3. Determination of sperm viability and apoptotic rates

Sperm membrane integrity of the two experimental groups was evaluated the following spring of the year of the sperm quality analysis (May), as shown in Fig. 1. For this evaluation PI/DAPI staining was used. Propidium Iodide (PI-Sigma, Spain) was added at 1 μg/ml final concentration to assay cell viability based on the membrane permeability. The total number of sperm cells was counted using 300 nM DAPI (4′,6-diamidino-2-phenylindole), a nuclear staining. For apoptosis analysis, YO-PRO-1 (Invitrogen) was added to the sperm (150 nM) and incubated 10 min in the dark at 4 °C according to the previous protocol described for this species (Valcarce and Robles, 2016).

At least 3 microscope fields were photographed (Nikon Eclipse Ts2R, Japan) recorded and analyzed by NIS-Element software for each sample, in order to have a total of 200 cells. The number of total, non-viable and apoptotic cells was counted and the percentage of viable and apoptotic cells calculated in each experimental group (n = 10–14 samples per group).

2.2.4. Morphological analysis of the testes

Males of each experimental group (n = 3) were sacrificed by cerebral contusion 20 days after the sperm sampling for sperm viability and apoptotic rates evaluation, in accordance with the current regulations in the field of uses of experimental animals. The visceral cavity of each animal was individually photographed, the testes were excised, weighted, and introduced in Bouin's solution (71% saturated picric acid solution, 24% of formalin (40%) and 5% of glacial acetic acid) at 4 °C during 16 h. After fixation in Bouin's solution, proximal, distal and medium portions of each testes were dehydrated in crescents solutions of ethanol in water, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 μm. The sections were dewaxed, rehydrated and stained with hematoxylin-eosin or with Mallory's trichromic to determine the reproductive stage of each male. Transverse section (n = 12) for two different areas of each half (n = 4) of each specimen (n = 3 fish/group) were used to measure the medullar area consisted on the efferent duct system that collects and stores the spermatozoa (García-López et al., 2005). The testicular medullar was drawn manually over the digital image. However, the total area covers all the testicular section was measured using an image analysis thresholding method employed to differentiate borders. The ratio between these two areas was calculated from measurements of gonad tissue images obtained with a Nikon eclipse E600 light microscope and an Olympus SC30 digital camera (Olympus soft imaging solutions GMBH) and the ImageJ software. The areas measured were randomly distributed to cover the whole testes and expressed as the % of the total testicular area. The gonadosomatic index (GSI), was calculated dividing the testes weight by the body mass of the individual and multiplying per 100 the result.

2.3. Statistical analysis

All data 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 or the equivalent nonparametric Kruskal–Wallis, with significance levels at $P < .05$. Monthly and global maturity percentages profiles were compared with the chi-square test ($P < .05$). All data was analyzed using the statistical package SPSS 19.0. (IBM Corp., Armonk, NY).

3. Results

There were no significant differences between groups in the mean weight at the beginning of the reproductive performance evaluation (ANOVA, $p > .05$), and the characterization resulted in two broodstocks composed of 14 males and 14 females in the group fed with a natural diet and 16 males and 16 females in the group fed with a commercial diet.

The identification of fluent males by stripping for the stock establishment (April) resulted in a 100% of fluent males in the broodstock fed with a natural diet, and a 64.3% of fluent males in the group fed with commercial diet. The non fluent males were assigned as fluent males in the subsequent samplings for quality assessment (May, July and September).

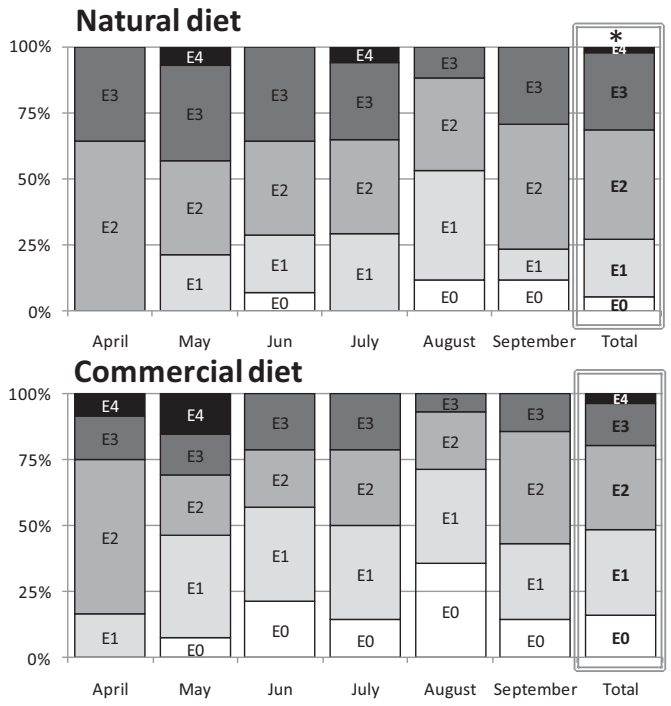

Fig. 2. Evolution of the proportions of the different maturity stages of the females, fed with natural ($n = 14$) or commercial ($n = 17$) diets throughout the study period. The asterisk indicates significant differences ($\chi 2$, $P < .05$) between diets in the total proportions.

3.1. Characterization of the maturity stages of the females

The evolution of the proportions of the maturity stages of each group is represented on Fig. 2. The month to month comparison of the maturity stages proportions did not show significant differences between different diets ($\chi^2, p > .05$). However, the global analysis of the maturity stages proportions showed a significantly different proportions pattern between diets ($\chi^2, p < .05$). The proportion of females in E0, E1 and E4, was higher in the group fed with the commercial diet than in the natural diet group, while the proportion of females in E2, E3, was higher in the group fed with the natural diet.

3.2. Analysis of the sperm quality

Total volumes obtained globally and on each sampling point, mean volumes, motility, density, and production parameters are shown on Fig. 3. All the parameters, globally and on each sampling point were always significantly ($p < .05$) higher in the group fed with the natural diet than in the one fed with commercial diet with the exception of the production (vol) and the mean volume, in which the increase observed in July was not significant ($p > .05$). The proportion of fluent males was 100% in both experimental groups in the three sperm samplings performed for quality evaluation (May, July and September).

There were no differences between treatments in the VSL or the VAP (Table 1). The VCL was significantly ($p < .05$) higher in May and the total analysis in the group fed with a commercial diet (Table 1).

The last year sperm characterization resulted in more homogeneous values, with only significant differences in the mean volume and production of motile cells (analyzed globally). Monthly there were only differences in motility in July, and production of motile cells in May and July (Table 2).

3.3. Determination of sperm viability and apoptotic rates

Viability results obtained by means of PI staining showed no significant differences between the different experimental groups fed with different diets. All viability percentages were higher than 80%, resulting in high viability rates after treatments (Fig. 4). However, when apoptosis was analyzed, significant differences ($p < .05$) were found between experimental groups revealing an increase in sperm apoptosis of the commercial diet samples (26.23%) when compared with the natural diet samples (12.08%) (Fig. 3). The proportion of fluent males during this sampling resulted in a 71.4% of the males fed with commercial diet and a 100% of the males fed with natural diet.

3.4. Morphological analysis of the testes

The GSI of males resulted significantly higher (ANOVA, $p < .05$) in the group fed with a commercial diet than the in the one fed with a natural diet (0.144 ± 0.013 and 0.101 ± 0.003%, respectively). The testes of the animals fed with a commercial diet were located in a more centered position in the visceral cavity than the ones of the animals fed with a natural diet (Fig. 5). Moreover, the animals fed with commercial diet presented testes with more protuberances, irregular edges and a general squared shape than the animals fed with a natural diet that presented testes with any protuberances, continuous edges and a proper kidney shape (Fig. 5).

Based on the cell types observed on the testes section (Fig. 6), all the specimens displayed an active spermatogenetic process. However, when comparing the cortical region of the testes of both experimental groups, we found that in the group fed with the commercial diet the relative percentage of cyst with pre-meiotic germ cells (spermatogonia) was higher than in the group fed with natural diet where the relative amount of cyst of spermatocytes (SC) and spermatids (SD) are increased (Fig. 6A-F). Interestingly, the medullar area where the efferent duct system is being formed as spermatogenesis process is smaller in the group fed with the commercial diet (23.55 ± 4.34% of total testicular area) than in the group fed with natural diet (40.21 ± 4.39% of total testicular area) as is easily observed (Fig. 6G-H) due to the fact that the tubules appeared almost empty as free spermatozoa had been sampled 20 days before the testicular sampling.

4. Discussion

Spontaneous spawning of cultured individuals is still unsolved at a commercial scale in *Solea senegalensis*, and only small and/or infrequent viable spawns have been obtained with cultured individuals (Fatsini Fernandez, 2017) or housing wild males with cultured females (Mañanos et al., 2007; Martín et al., 2019). Moreover, despite the advances registered in the artificial reproduction of the species (Rasines et al., 2012; Rasines et al., 2013), more reliable protocols are needed to achieve full control of the reproduction by artificial fertilization. This is especially important to set up breeding programs since it has been demonstrated the low or no contribution to natural spawning of most of the individuals of the wild broodstocks in captivity (Porta et al., 2006; Martín et al., 2014) that brings about a loss of genetic variability and a strong relatedness among individuals to conform new broodstocks. Having females available in advanced maturity stages and enough sperm to carry out the available artificial fertilizations protocols is essential to implement these techniques at industrial scale or setting up breeding programs which in turn are critical for the long term development of aquaculture (Migaud et al., 2013).

The results of this study show a qualitative and quantitative improvement in several reproductive parameters. Specifically, the number of females ready to apply the available artificial fertilization protocols (the sum of the proportions of females on mature stages E2, E3 and E4) increased significantly in natural feeding females than in females fed with dry pellets. This means a higher number of females to increase the

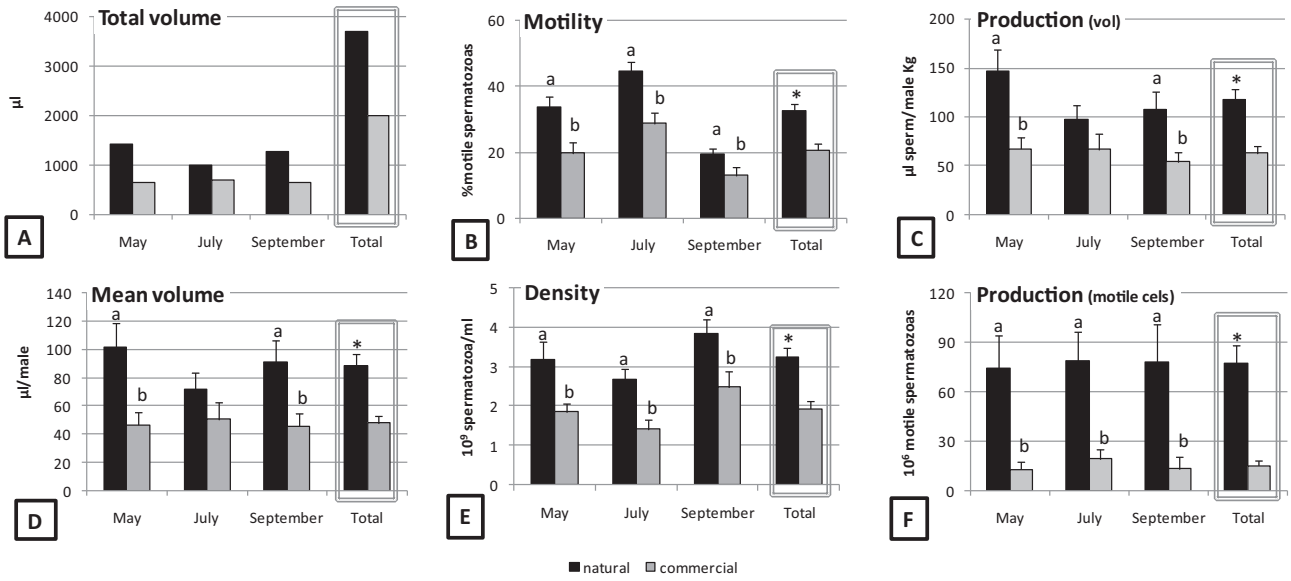

Fig. 3. Sperm quality parameters of the broodstocks fed with natural or commercial diets. (A) the total volume of sperm obtained from n = 14 males sampled at each experimental group. (B) the mean motility at each experimental group. (C) the mean volume obtained per kg of male at each experimental group. (D) the mean volume of sperm obtained per specimen sampled. (E) the mean density at each experimental group. (F) the volumes of each specimen multiplied by their mean density and by the motile cell fraction. Different letters indicate significant differences ($p < .05$) between diets in the same month (sampling). The asterisks indicate significant differences ($p < .05$) between diets in the global analysis of each parameter.

production potential and/or enrich the genetic breeding programs. In the case of males, natural food significantly reduced the sperm apoptotic numbers, increased volume, density, motility and therefore production of sperm, what is a widely recognized limiting factor on this species (Morais et al., 2016).

In terms of production, sperm quality parameter values have been higher than those obtained by other authors in the same species. Thus, the volumes obtained in the specimens fed with a commercial diet are comparable with those obtained by Cabrita et al. (2006) that obtained volumes with wild specimens adapted to the captivity between 10 and 80 µl and between 5 and 20 µl with F1 specimens, or Beirão et al. (2011) with volumes of 20–30 µl, with wild specimens adapted to the captivity, in all cases using natural feeding regime and bigger individuals than the used in the present study (between 1 and 1.8 Kg). On the other hand, the specimens of our study fed with natural feed *ad libitum* presented mean values between 70 and 100 µl, with an average value of 90 µl per sample. These results suggest that a natural diet to satiety used from juvenile stages could maximize the production potential in volume, since the wild specimens used by Cabrita and Beirao were also fed with natural food all their life, but one possible difference, apart from the change of conditions from a wildlife to captivity, would have been the variable availability of food in the wild. Motility, turned out to be less than that obtained by these authors, thus Cabrita et al. (2006) obtained motilities around 70% with wild and F1 indisticntly, and Beirão et al. (2011) with wild individuals adapted to captivity obtained motilities between 40 and 70%. The specimens of the present study fed a natural diet presented significantly greater motilities than with a commercial diet, but varied between 20 and 45%, with an average value of 35%. In terms of density, all the samples obtained in our study were in the range of 1.5–3.5 10^9 cells per ml, although the difference between both feeding regimes was significant, with an average density of 3 using a natural diet. Cabrita et al. (2006) obtained densities between 0.7 and 2 10^9 cells per ml with F1 and wild males, although in 2011 they obtained values around 2–3 10^9 cells per ml in their samples of wild individuals. Beirão et al. (2015), reached densities of 5.2 ± 1.7 10^9 cells per ml with the diet enriched with DHA and supplemented with antioxidants, although it was not significantly greater than their control group that presented densities in the line of the present study. Nevertheless it is important to consider that is difficult to make rigorous comparisons between concrete values of different studies due to the differences that may occur, not only in samples obtaining, but as Gallego et al., 2018 explained, also in the evaluation of samples between different technicians.

Molecular sperm quality parameters, namely apoptosis rate, plays an important role in regulating spermatogenesis and it has been previously analyzed in Senegalese sole during the reproductive season to determine its status. The results obtained in this study suggest that apoptosis may be related to more processes than the elimination of

Table 1
Sperm kinetics obtained with the different feeding regimes at each sampling month and total results.

	VCL				VSL			VAP		
May-Natural	94,95	±	5,18	**b**	68,78	±	4,56	80,31	±	5,04
May-Commercial	107,52	±	5,23	**a**	73,63	±	5,30	84,37	±	5,47
July-Natural	95,26	±	3,72		68,06	±	3,24	81,56	±	3,57
July-Commercial	95,82	±	5,47		66,08	±	5,24	80,94	±	5,70
September-Natural	65,98	±	3,65		43,36	±	2,82	54,04	±	3,25
September-Commercial	78,27	±	6,35		50,91	±	5,41	66,28	±	6,26
Total-Natural	**85,98**	±	**2,79**		**60,59**	±	**2,40**	**72,49**	±	**2,65**
Total-Commercial	**94,68**	±	**3,43**	*	**64,17**	±	**3,18**	**77,70**	±	**3,39**

VCL means curvilinear velocity, VSL means rectilinear velocity and VAP means mean velocity. Velocities are expressed in micrometers per second. Different letters indicate significant differences between treatments each month. The asterisks indicate significant differences ($p < .05$) between diets in the total analysis.

Table 2
Sperm quality parameters obtained during the last year of study, after diet standardization.

	Total volume	Mean volume	Mean motility	Mean density	Production (μl/male kg)	Production (10^6motile cells)
May-Natural-Commercial	1230	111,82 ± 14,26	20,00 ± 3,21	2,33 ± 0,47	116,03 ± 11,57	69,58 ± 30,02[a]
May-Commercial-Commercial	1060	106,00 ± 17,71	10,20 ± 3,79	1,19 ± 0,28	119,76 ± 14,51	21,63 ± 9,26[b]
July-Natural-Commercial	930	84,55 ± 15,28	41,50 ± 5,14[a]	4,38 ± 0,71	84,47 ± 17,35	188,06 ± 74,98[a]
July-Commercial-Commercial	534	53,40 ± 14,98	20,78 ± 5,52[b]	3,66 ± 0,33	52,73 ± 13,61	31,64 ± 14,09[b]
September-Natural-Commercial	860	78,18 ± 14,64	9,12 ± 3,24	5,64 ± 0,78	80,38 ± 14,86	48,43 ± 25,18
September-Commercial-Commercial	399	39,90 ± 10,55	14,52 ± 4,59	4,49 ± 0,83	43,21 ± 9,94	39,94 ± 20,70
Total-Natural-Commercial	**3020**	**91,52 ± 8,63***	**22,98 ± 3,17**	**4,06 ± 0,43**	**93,62 ± 8,73**	**102,02 ± 29,38***
Total-Commercial-Commercial	**1993**	**66,43 ± 9,76**	**14,99 ± 2,62**	**2,84 ± 0,37**	**71,90 ± 9,55**	**31,07 ± 8,69**

The total volume of sperm was obtained from $n = 11$ males sampled in the natural-commercial group and from n = 10 males in the commercial-commercial group. Volumes are expressed in micro liters; motility is expressed in percentage of motile cells; density is expressed in million of cells per ml; production (μl/male Kg) represent the sperm volume obtained divided by each specimen weight (kg); production (10^6 motile cells) expresses the millions of motile cells on each sperm sample obtained by multiplying the individual volume by the density and the proportion of motile cells. Different letters indicate significant differences ($p < .05$) between diets in the same month (sampling). The asterisks indicate significant differences ($p < .05$) between diets in the global analysis of each parameter.

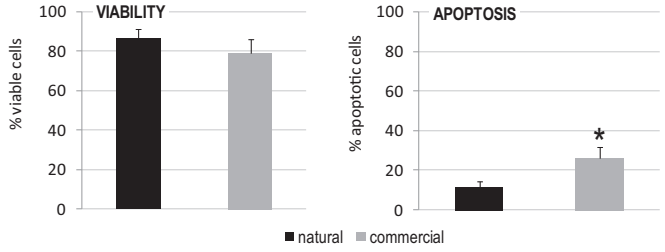

Fig. 4. Sperm viability and apoptosis of Senegalese sole fed with natural and commercial diets. Asterisk represents significant differences ($p < .05$) between experimental groups.

over-mature spermatozoa, as suggested by Beirão et al., 2009, since there were differential levels between treatments depending on the feeding. The viability resulted to be high in both groups (around 80%), and did not showed differences between groups. This levels of viability resulted higher than the obtained by Beirão et al. (2011), using similar techniques, but at the same level to those obtained by Valcarce and Robles (2016) with the same techniques.

The influence of nutrition in the reproductive performance of fish has been widely studied (Izquierdo and Fernandez-Palacios, 2001) and the lipids composition of the diet has been also correlated with sperm quality in several fish species (Asturiano et al., 2001; Nandi et al., 2007; Baeza et al., 2015). Specifically in Senegalese sole, Beirao et al. (2015) tested the effects of two experimental diets on the reproductive performance of the males and showed how the diet supplementation with PUFA (specifically DHA) increased sperm quality particularly when this supplementation was accomplished with antioxidants. However this work is the first time in which the reproductive effect of a natural diet administrated for the long term has been analyzed in this specie.

The lipid class and the fatty acid content composition of the diets used in this study were analyzed in detail by Norambuena (Norambuena Filcun, 2012), and significant differences between natural and commercial diets were highlighted in the proportion of essential fatty acids widely related with reproduction performance, namely DHA, EPA or ARA, although, the compositional differences between the different foods included in the natural diet, make it difficult to establish a correlation between any compound or even with any

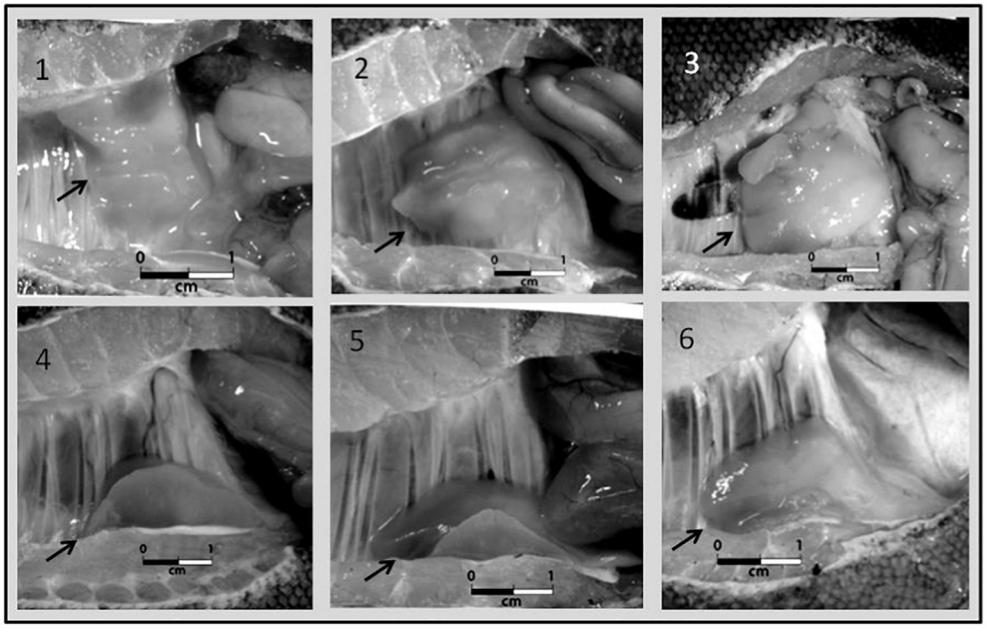

Fig. 5. Photographs of the ocular side testes sampled for performing the morphological study. 1, 2 and 3 correspond to animals fed with a commercial diet. 4, 5 and 6 correspond to animals fed with a natural diet. Arrows point the upper testicular lobes.

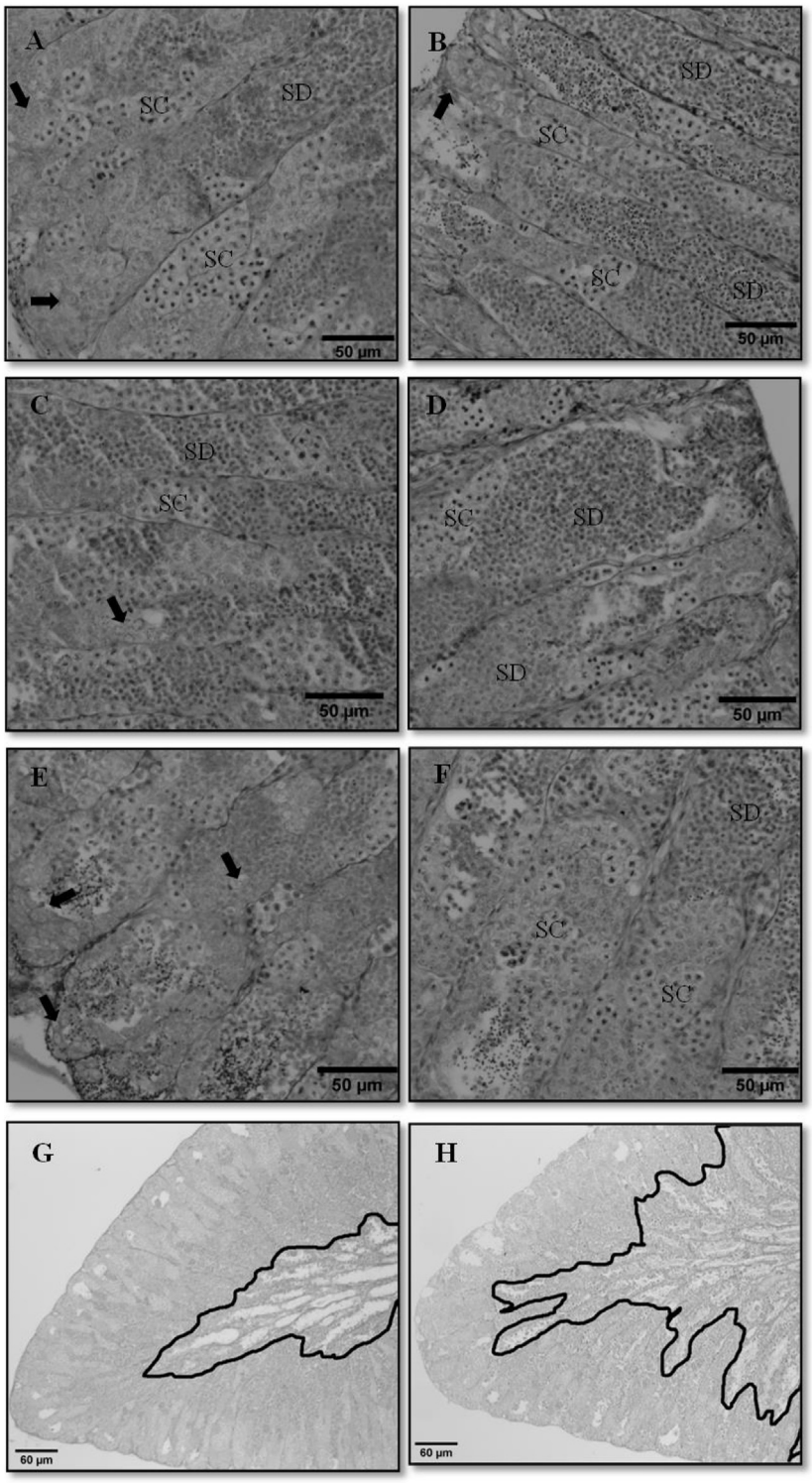

Fig. 6. Testicular sections of *Solea senegalensis* specimens fed with commercial (A,C,E,G) or natural (B,D,F,H) diets. Black arrows denote spermatogonia, SC, spermatocytes, SD, spermatids. The black line encircles the medullar area formed by the efferent duct system that collected and stored the spermatozoa. Scale bars = 50 µm (A-F), 60 µm (G-H).

specific food (mussel, squid or sea worm) and the reproductive improvement.

The amelioration of the reproductive performance obtained with the natural diet in this study could be related with a greater variety of nutrients that could compensate the deficit of any essential compound for any or both sexes in Senegalese sole. And this could happen not only when reaching sexual maturity, but also during the ongrowing and puberty, when sexual tissues and structures are being developed. This fact was suggested by the images obtained in the study, that show a general appearance similar to that shown in wild individuals of the related species sole (*Solea solea*) (ICES, 2010) and confirmed by the better results obtained one year after feeding regime unification in terms of mean total volumes and production of motile cells. Specifically, despite a theoretical reducing of the quality in group N due to a

change from a natural diet to a commercial diet, and a theoretical improvement produced by the change from a fattening diet to a specific diet for breeders in group C, some differences were maintained. Moreover, contrary to what might be expected, a higher GSI (bigger testes) was not related to a higher sperm production, since the group fed with a commercial diet produced significantly lower amounts of sperm and presented a significantly higher testes-body weight ratio.

Structurally, all males analyzed had testicular morphological features to be classified at functional maturation stage (stage IV) as described previously (García-López et al., 2006). However, the sizes of the medullar area where the efferent duct system develops as spermatogenesis process were bigger in the specimens fed with natural diet suggesting this a bigger production of free spermatozoa as demonstrated also by the increase reported in the volume of sperm and density of the group fed with a natural diet. In fish testes, the cysts-generating activity established a zonation in which early stages of germ cells reside in the periphery and the cyst-generating activity that move the advanced germ cells through the center of the testes and the subsequent release of the spermatozoa such that the lumen of the cyst becomes continuous with the lumen of the tubules determine the appearance of the efferent duct system in the medullar area of the testes (Schulz et al., 2010). Moreover, this is the first time in which the morphology of the fish testes and the progression of the spermatogenesis have been related with different dietary intakes. Thus, our data suggest that a natural diet influence gonad capability to produce a bigger amount of spermatozoa probably due to triggering an early development of the asynchronous spermatogenetic process combine or not with the maintenance of this process further in time through promoting spermatogonia stem cell or primary spermatogonia proliferation. Although further studies will be needed to clearly analyze the proliferative process in sole testes to clearly establish this issue, we can stay that specimens fed with natural diet have higher amount of sperm production due to a different timing in the progression of the spermatogenetic process in their testes.

This study present, for the first time, the effect of long-term use of a natural diet after weaning in Senegalese sole, and how this feeding regime can increase the sperm production of the specimens. Furthermore, that the natural diet increases the proportion of females in the higher maturity stages suitable for the application of the hormonal therapies necessary to carry out the current artificial reproduction protocols. However, further studies are needed in order to correlate these reproductive improvements with fertilization or hatching success, larval abnormalities proportions, or the key elements improving each sex reproductive performance to improve artificial diets in this species.

Ethical approval

The fish were always handled (routine management and experimentation) according to the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 53/2013) for the use of laboratory animals. Moreover, all the people involved in the experiments had the required FELASA accreditations for each procedure (ECC556/2015). The project was evaluated by official ethics committee with favorable report number PI-10-16.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledges

Authors would like to acknowledge Planta de Cultivos el Bocal (IEO) staff, AGL2015 68330-C2-1-R project (MINECO/FEDER), PTA2016-11987-I contract (MINECO/FEDER) and AQUA-CIBUS international net 318RT0549 funded by CYTED (Programa Iberoamericano de Ciencia y Tecnologia para el Desarrollo).

References

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., Salas-Leiton E., Martín I., Chereguini O. Influence of the feeding on sperm quality of first generation (F1) Solea senegalensis during an annual cycle in two facilities of Spain. Proceedings of Aquaculture Europe, Krakow (Poland), September 2008. European Aquaculture Society.

Asturiano, J.F., Sorbera, L.A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J.C., Bromage, N., 2001. Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture 194 (1–2), 173–190. https://doi.org/10.1016/S0044-8486(00)00515-9.

Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L., Asturiano, J.F., 2015. Relationship between sperm quality parameters and the fatty acid composition of the muscle, liver and testis of European eel. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 181, 79–86. https://doi.org/10.1016/J.CBPA.2014.11.022.

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

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

Beirão, J., Soares, F., Pousão-Ferreira, P., Diogo, P., Dias, J., Dinis, M.T., Herráez, M.P., Cabrita, E., 2015. The effect of enriched diets on *Solea senegalensis* sperm quality. Aquaculture 435, 187–194. https://doi.org/10.1016/j.aquaculture.2014.09.025.

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

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). In: Proceedings of XI Congreso Nacional de Acuicultura. Xunta de Galicia, Vigo, Spain. II. pp. 1435–1438.

Colen, R., Ramalho, A., Rocha, F., Dinis, M.T., 2014. Cultured Aquatic Species Information Programme. *Solea solea.* Cultured Aquatic Species Information Programme. FAO Fisheries and Aquaculture Department [online], Rome.

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.

Fatsini Fernandez, E., 2017. Reproduction, Olfaction and Dominance Behaviour in Senegalese Sole (*Solea senegalensis*). Thesis. Universitat Politècnica de València, Valencia (Spain). https://doi.org/10.4995/Thesis/10251/81550. (204 pp).

FEAP, 2017. Production Report of the Federation of European Aquaculture Producers Covering European Fish Farming from 2007–2015.

Gallego, V., Herranz-Jusdado, J., Rozenfeld, C., Pérez, L., Asturiano, J., 2018. Subjective and objective assessment of fish sperm motility: when the technique and technicians matter. Fish Physiol. Biochem. 44, 1457. https://doi.org/10.1007/s10695-018-0505-1.

Garcia-Franquesa, E., Molinero, A., Valero, J., Flos, R., 1996. Influence of sex, age and season on the feeding habits of the flatfish *Solea senegalensis*. Environ. Biol. Fish 47, 289–298. https://doi.org/10.1007/BF00000501.

García-López, A., Martínez-Rodríguez, G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole *Solea senegalensis* (Kaup): anatomy, histology and histochemistry. Histol. Histopathol. 20 (4), 1179–1189. https://doi.org/10.14670/HH-20.1179.

García-López, Á., Fernández-Pasquier, V., Couto, E., Canario, A.V.M., Sarasquete, C., Martínez-Rodríguez, G., 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen. Comp. Endocrinol. 147 (3), 343–351. https://doi.org/10.1016/J.YGCEN.2006.02.003.

ICES, 2010. Report of the Workshop on Sexual Maturity Staging of sole, plaice, dab and flounder (WKMSSPDF), 22-26 February 2010, Ijmuiden, The Netherlands. ICES CM 2010/ACOM:50. (96 pp).

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*. Rev. Fish Biol. Fish. 13, 379–407.

Izquierdo, M.S., Fernandez-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197, 25–42.

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). Mar. Fish. Res. Haiyang Shuichan Yanjiu 29, 10–16.

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. In: Proceedings of Aquaculture Europe 07, Istanbul, Turkey, October 2007. European Aquaculture Society, pp. 330–331.

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

Martín, I., Carazo, I., Rasines, I., Rodríguez, C., Fernández, R., Martínez, P., Norambuena, F., Chereguini, O., Duncan, N., 2019. Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender. Span. J. Agric. Res. 17 (4). https://doi.org/10.5424/sjar/2019174-14953. e0608.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish.

Rev. Aquac. 5, S194–S223. https://doi.org/10.1111/raq.12025.
Mira, S., Madeira, C., Cabrita, E., Soares, F., Cancela, M.L., Dinis, M.T., 2010. Parental contribution of *Solea senegalensis* assessed with microsatellite markers. In: Proceedings of Aquaculture Europe 10, Porto, Portugal, October 2010. European Aquaculture Society, pp. 826–827.
Morais, S., Aragao, C., Cabrita, E., ConceiÇao, L.E.C., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., Gisbert, E., Mañanos, E., Valente, L.M.P., Yúfera, M., Dinis, M.T., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Rev. Aquac. 8, 227–263. https://doi.org/10.1111/raq.12091.
Nandi, S., Routray, P., Gupta, S.D., Rath, S.C., Dasgupta, S., Meher, P.K., Mukhopadhyay, P.K., 2007. Reproductive performance of carp, *Catla catla* (ham.), reared on a formulated diet with PUFA supplementation. J. Appl. Ichthyol. 23 (6), 684–691. https://doi.org/10.1111/j.1439-0426.2007.00874.x.
Norambuena Filcun, F., 2012. Senegalese sole (*solea senegalensis*) Broodstock Nutrition: Arachidonic Acid (20:4n-6, ARA) and Reproductive Physiology. TDX (Tesis Dr. en Xarxa). Thesis. Universitat Autònoma de Barcelona, Barclona. Spain (187 pp).
Porta, J., Porta, J.M., Martínez-Rodriguez, G., Alvarez, M.C., 2006. Development of a microsatellite multiplex PCR for Senegalese sole (*Solea senegalensis*) and its application to broodstock management. Aquaculture 256, 159–166.
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).
Rasines, I., Martín, I., Rodriguez, C., 2017. Se pueden formar pooles de esperma en lenguado senegalés? In: Proceedings of XVI Congreso Nacional de Acuicultura. Zaragoza, Spain, pp. 403–404.
Rasines, I., Gomez, M., Martin, I., Rodriguez, C., Mananos, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture 326–329, 129–135. https://doi.org/10.1016/j.aquaculture.2011.11.021.
Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2013. Artificial fertilisation of cultured Senegalese sole (*Solea senegalensis*): effects of the time of day of hormonal treatment on inducing ovulation. Aquaculture 392–395, 94–97. https://doi.org/10.1016/j.aquaculture.2013.02.011.
Rodriguez, J.L., Peleteiro, J.B., 2014. Cultivo del lenguado Senegalés (*Solea senegalensis*). Cuadernos de acuicultura, Fundación Observatorio Español de Acuicultura (OESA).
Schulz, R.W., de França, L.R., Lareyre, J.J., LeGac, F., Chiarini-García, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165 (3), 390–411 (https://doi.org/S0016-6480(09)00074-4 [pii] 10.1016/j.ygcen.2009.02.013).
Valcarce, D.G., Robles, V., 2016. Effect of captivity and cryopreservation on ROS production in *Solea senegalensis* spermatozoa. Reproduction 152, 439–446. https://doi.org/10.1530/REP-16-0270.
Valdebenito, I.I., Gallegos, P.C., Effer, B.R., 2015. Gamete quality in fish: evaluation parameters and determining factors. Zygote 23, 177–197. https://doi.org/10.1017/S0967199413000506.