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RESEARCH ARTICLE

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Reproductive performance of captive Senegalese sole, *Solea senegalensis*, according to the origin (wild or cultured) and gender

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Abstract

Aim of study: The reproductive performance over a five year period in three different sole broodstocks: wild males with cultured females (WM), cultured males with wild females (CM), and a control of cultured males and females (C).

Area of study: Cantabria, Northern Spain.

Material and methods: The parental contribution was evaluated through the allocation of hatched larvae and a behavioural study was performed using underwater video recordings.

Main results: Fertilized natural spawns were only obtained from group WM showing a significantly higher mean annual volume of floating eggs compared with groups CM and C. Over the entire 5-yr a higher number of spawns, total and floating volumes of eggs were obtained compared with groups CM and C. The analysis of four polymorphic microsatellites enabled the identification of the individuals involved in the fertile spawns by parental assignment. The percentage of individuals contributing to spawns ranged between 56.3% and 75% showing fidelity patterns. However between 46.2% and 68.6% of the contribution to production was a result of the crossing of three single couples. According to the behaviour analysis, group WM presented the highest peak of activity at 20:00 h and a higher activity profile during the night, moreover, the courtship behaviours "swim follow" and "swim followed", were only recorded in group WM.

Research highlights: The study has importance for aquaculture as it demonstrated how broodstocks with wild males and cultured females had adequate egg production for a commercial operation to supply eggs and implement single sex breeding programs.

Additional keywords: reproduction; natural spawn; behaviour; parental contribution.

Abbreviations used: C (control); CM (cultured males); SD (standard deviation); SE (standard error); SQ (stay quiet); *r* (relatedness coefficient); WM (wild males).

Authors' contributions: IM: experimental design, experimental work, data analysis, discussion, writing. IC: experimental design, experimental work, data analysis, discussion. IR: experimental design, discussion and general overview. CR: experimental design, experimental work, data analysis. RF: experimental work, data analysis. PM: experimental design, discussion and general overview. FN: experimental work, data analysis. OC: experimental design, experimental work. NC: experimental design, discussion, experimental work, data analysis and general overview.

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Introduction

Reproductive dysfunctions of cultured Senegalese sole (*Solea senegalensis*) that were hatched and reared entirely in captivity (that will be called "cultured" individuals) remain the principal bottleneck for the species culture. Currently, all natural spawns of cultured specimens exhibit low or no fertilization, hindering the possibility of closing the life cycle in terms of reproduction in captivity. Therefore, the entire industrial production of 830 tons in Spain and over 1656 tons in Europe during 2017 (APROMAR, 2018) was produced through natural spontaneous spawning of wild individuals or artificial fertilization carried out with wild and cultured specimens.

There is information about cultured individuals (males and females) that spawned naturally (Guzmán *et al.*, 2008) and that spawned when subjected to different hormonal therapies (Agulleiro *et al.*, 2006; Guzmán *et al.*, 2009). Natural spawning was described over a three-year period during which fecundities increased with the highest fecundities in the final year of the study (Guzmán *et al.*, 2008). However, no fertilized eggs were obtained during the entire three years. The application of hormone therapies increased the fecundities obtained from natural spawns, but again all eggs were not fertilized (Agulleiro *et al.*, 2006; Guzmán *et al.*, 2009). Spawning from wild Senegalese sole acclimated to captivity provided the quantities of eggs required for hatchery production (Dinis *et al.*, 1999; Anguís & Cañavate, 2005; Martín *et al.*, 2014). However, viable egg production was variable with large fluctuations in fecundities obtained within the spawning season and between different spawning seasons.

Molecular markers, such as microsatellites, have been used in aquaculture for parental analysis aimed to identify the effective breeding population or kinship relations among breeders, to study the spawning dynamic along the reproductive period, or to assess individual spawning performances (Brown *et al.*, 2005; Herlin *et al.*, 2008). In this regard, specific microsatellite tools have been validated for Senegalese sole (Castro *et al.*, 2006; Porta *et al.*, 2006a,b; Martín *et al.*, 2014) allowing the description of parental relationships among breeders, production performances or fidelity patterns in wild sole adapted to captivity (Martín *et al.*, 2014). Martín *et al.* (2014) observed that a large percentage of breeders in a broodstock did not contribute to the production of progeny, which was dominated by a few pairs of breeders. These spawning pairs exhibited fidelity both within a spawning season and between spawning seasons, and the variation in individual spawning kinetics contributed to the overall variation in production of viable eggs.

One promising approach to clarify the origin of the reproductive dysfunctions of this species in captivity was the study of Senegalese sole reproductive behaviour (Carazo, 2013; Carazo *et al.*, 2016). Following this approach, a complete description of the stages of the courtship performed by wild individuals adapted to captivity has been detailed. This courtship was briefly described as: 1) a period of intense activity between the female ready to spawn, males and amongst males; 2) the female swam from the bottom of the tank accompanied by a male; 3) the female and male swam in synchrony in the water's surface with the genital ducts held closely together and liberated and fertilized the gametes (Carazo *et al.*, 2016). The description demonstrated that Senegalese sole, through the courtship, select a mate to spawn as a monogamous pair. This courtship and in particular the coupled swim to the surface was a requirement to ensure the fertilisation of the eggs. These observations suggested that behavioural problems could explain the low levels of fertilisation from a broodstock of cultured Senegalese sole.

Therefore, the present study aimed to generate new information related to the reproductive dysfunction observed in cultured broodstock by examining behaviour, spawning and paternity of prodigy from broodstocks of mixed origin and gender. To achieve this, three different broodstocks (cultured males and females; cultured males and wild females; cultured females and wild males) were monitored to describe the reproductive dynamics, the reproductive behaviour during spawning and the parental allocation of offspring (using microsatellites) during five consecutive spawning seasons.

Material and methods

Broodstock and management

At the beginning of 2010, three Senegalese sole broodstocks (n= 16 each) were housed in three 7-m^3 tanks. One tank was composed of wild males, previously adapted to captivity, and cultured females and will be referred to as group WM (wild males); a second tank was composed of cultured males and wild females, previously adapted to captivity to form group CM (cultured males); and the third tank was composed of cultured males and females to form group C (control). The three groups had a male:female ratio of 1:1 and an average density in the tanks of 3 kg/m^2. All individuals were tagged with a passive integrated transponder (Trovan®, Madrid) placed in the dorsal area, allowing the specimens to be identified and monitored. The following individuals died during

the 5-yr period of the experiment: 3 females and 4 males died in the tank WM, 4 females and 1 male in the tank CM, and 4 females and 5 males in the tank C. They were all replaced by another animals of the same characteristics (similar age and weight). The wild fish used in the present study had the same origin and background as wild fish used in the study of Martín *et al.* (2014), which was made with similar holding conditions and during the same period and could be considered as a positive, all wild broodstock, control to the present study. The reproductive behaviour of these wild broodstock (Martín *et al.*, 2014) was described by Carazo *et al.* (2016).

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 ± SD recorded during the study was 34.5 ± 0.84 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 lux. All individuals were weighed and measured (total length) once a month. All tanks were treated with a prophylactic hydrogen peroxide bath after sampling (80 ppm for 1 h without water renewal). The duration of the experiment was 5 years. The average total body weight ± SE of females and males at the beginning of the trial was respectively: 1161 ± 98.1 g and 1241.9 ± 100.8 gin group WM; 1748 ± 294.4 g and 939 ± 110 g in the group CM; and 1499.4 ± 138g and 1494.9 ± 81.5 g in group C. The groups were fed with natural food composed of mussels (*Mytilus* spp.), small squid (*Loligo* spp.) and cultured polychaete worms (*Nereis* spp.) (Seabait Ltd., UK), 6 days per week. In the pre-spawning and spawning period, fish were fed with small squid 3 days per week and with mussels the other 3 days. In addition, frozen worms were added to the diet once a week. The rest of the year, fish were fed with mussels twice a week and with small squid 4 days per week. The daily amount of food was adjusted to 1% of the total biomass of the fish in the tank, which was determined monthly. Cultured individuals were adapted to natural food for 3 months prior to the experiment.

The modification of the tank temperature began each year at the end of January; this thermo-period was manipulated to induce the reproductive response of the broodstock, simulating the natural fluctuations recorded in the Toruño (IFAPA, Cádiz) (Anguís & Cañavate, 2005) and to induce natural spawning (Martín *et al.*, 2014). 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 thermo-period was no longer manipulated. The natural spawns obtained during this time were due to the natural local water temperature fluctuations.

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 placed in 1-L graduated cylinders to estimate the volume of floating and non-floating eggs. All spawns were recorded, but only those with a total volume greater than or equal to 20 mL were used in this study for production analysis. 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 (Anguís & 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 as 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 of each spawn was incubated in individual 70-L incubators with filtered seawater (1 µm) at 18-20 ºC, with a water renewal rate of 1.5 L/min, 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 stocked in the incubator. The number of hatched larvae was determined by counting the number of larvae in a minimum of three 100 mL subsamples.

Genetic analysis and parental assignment

The first stage of the study was to genetically characterize the broodstock with a microsatellite panel. For this purpose, caudal fin clips were obtained for each individual and preserved in absolute ethanol for further processing. The DNA extraction was performed using the synthetic resin Chelex®100 according to Walsh *et al.* (1991), and the genetic characterization of the broodstock was performed by amplifying four

microsatellite loci: F13-7, Smax-02, SseGATA38 and CA13, included in a single multiplex PCR. Once the parents were characterized, the second step was to perform parental allocation of the larvae obtained during the five years of the study. For this, all spawns between 2010 and 2014 with fertilized eggs were incubated. Parental assignment was completed for all fertilized spawns with floating volumes greater than 20 mL. The eggs from spawns (< 20 mL) were incubated in jars with 5 L of filtered seawater (1 µm), controlled temperature (18-20ºC) and moderate aeration. From the hatched larvae, sets of eight larvae were washed with 96% ethanol and placed in an Eppendorf tube with absolute ethanol for subsequent parentage analysis. During the experiment, a total of 792 larvae from 99 spawns were genetically characterized.

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 using CERVUS 3.0 (Kalinowski *et al.*, 2007). The potential of the microsatellite set to allocate offspring from the IEO Santander broodstock was estimated with the same program by calculating the exclusion probability of detecting a false parent when no parent is known (Excl1) and when one parent is known (Excl2). All these estimations were conducted using the software CERVUS3.0 (Kalinowski *et al.*, 2007). 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) implemented in SPAGeDi (Hardy & Vekemans, 2002).

Behaviour analysis

Digital cameras (Square black and white CCD camera, model F60B/N80-50G, KT&C Co. Ltd., Korea Technology and Communications Korea, supplied in waterproof housing by Praesentis S.L. Barcelona, Spain) were used to film the fish behaviour. The cameras were connected to a digital video recorder (model DVR-Camtronics-UCDI-DV4150-1500, supplied by Praesentis S.L.) and 24 h recordings were made during the period of study. Four cameras were installed in each tank. The cameras had an angle of view of 150º (in air) and with the cameras positioned just below the water surface angled downwards, at intervals of 1.5 m along the length of the tank a total of 96º of the entire water column, from the surface to the bottom was captured and recorded. During the recording period night illumination was low intensity red light from fluorescent lights covered with a red filter that only permitted the passage of light >720 nm wavelength (supergel Rosco, Rosco Iberica S.A. Madrid, Spain www.rosco.com). The red light was adjusted to approximately 5 lux at the water's surface, which enable recording and observation of fish behaviour. This illumination system was previously tested and shown to not significantly affect behaviour, locomotor activity or plasma melatonin (Carazo *et al.*, 2013). All recordings to study the behaviour were made during April, which is the peak month of spawning for Senegalese sole.

The entire 24 hour period was observed on all 24 hour periods in which a spawn was collected. The locomotor activity was quantified by scoring each time a sole swam over a line drawn across the middle of the field of vision of each camera as described and validated by Carazo *et al.* (2013; 2016). The different behaviours (Carazo *et al.*, 2016) associated with spawning (stay quiet, SQ with actions, swim solo, swim follow, swim followed, tremor and rest head), were counted during the peak half hour (19:45 to 20:15) of locomotor activity. The period was divided into 3 min sections and the behaviour of each animal recorded at each 3 min time point. The same 30 min period of peak activity was analysed for all 24 h periods with a spawn.

Statistical analysis

All data were expressed as the mean ± 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 $p<0.05$. Profiles of frequency counts of different behaviours during different periods were compared with the chi-square test ($p<0.05$). The analysis of the activity was conducted by a description of daily activity profiles (Bayarri *et al.*, 2004). All data was analyzed using the statistical package SPSS 19.0. (IBM Corp., Armonk, NY, USA) and Sigma Stat (Systat Software Inc., Germany).

Results

Spawning performance

Spawns were obtained from all groups WM, CM and C over the five-year period of the study in relation to the thermo-period (Fig. 1). The spawning period lasted between 1 and 258 days, depending on the group and the

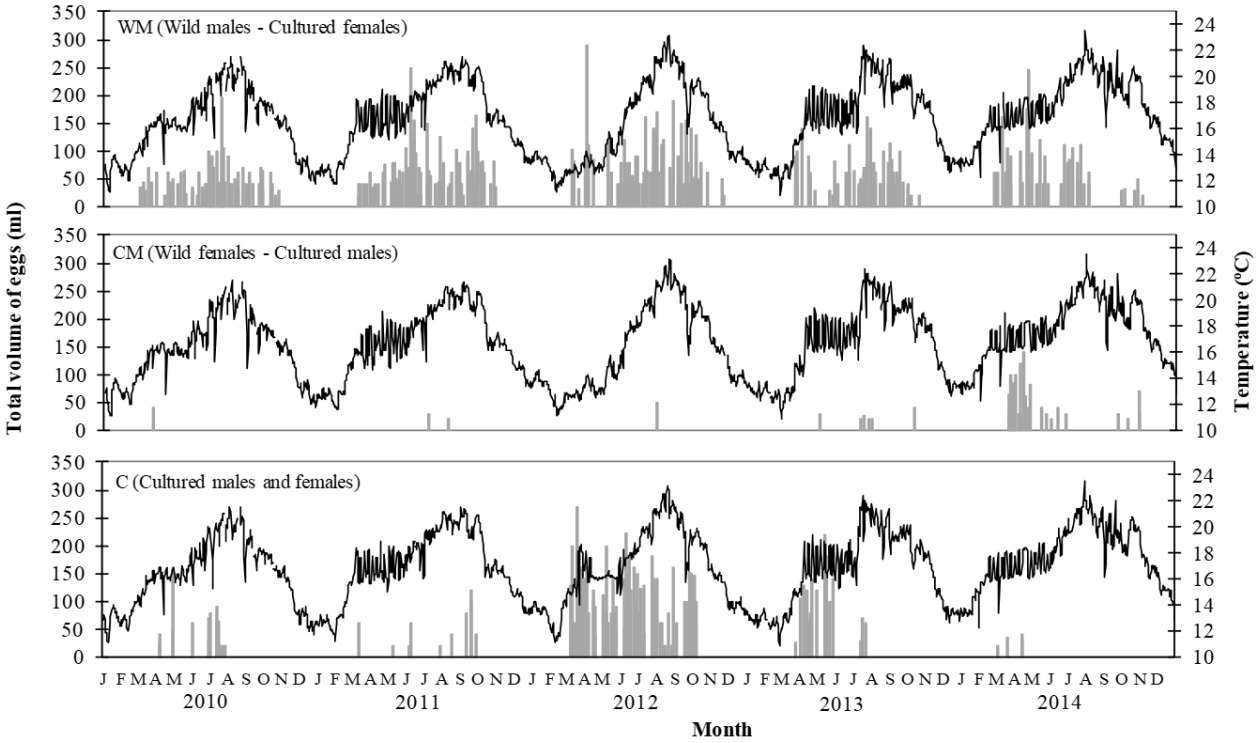

Figure 1. Daily total egg production (grey bars) and temperature regime (black continuous line) of the cultured group C (control) and mixed broodstocks groups WM (wild males with cultured females) and CM (cultured males with wild females) over the 5-yr study period.

year. Group WM produced the highest volume of total eggs, floating eggs and number of spawns followed by group C and group CM, which produced the lowest egg volume (Table 1, Fig. 1). The mean annual total volume of eggs and the mean number of spawns from group WM was significantly higher (p<0.05) than group CM while group C was intermediate with no differences with groups WM and CM (Table 1). Mean annual total floating eggs from group WM was significantly higher (p<0.05) than groups C and CM, which were similar. Only group WM produced fertilized spawns, hatching and viable larvae with mean fertilization and hatching rates ranging between 45.65-82.81% and 42.65-100% respectively (Table 1). There was a large variation of daily relative fecundity in the different spawns ranging between 1021 and 25082 eggs per kg of female. The mean relative fecundity per spawn was similar between groups and there were no significant differences amongst groups in any year or in the total results except in 2013 when group C was significantly higher (p<0.05) than group WM, which was in turn significantly higher (p<0.05) than group CM.

Genetic diversity and parental contribution

Genetic diversity of the broodstock estimated as the mean number of alleles resulted (13 ± 2.450), and expected and observed heterozygosities resulted 0.824 ± 0.082 and 0.844 ± 0.064 respectively. In this scenario, the theoretical potential for paternity inference was very high, with Excl1 and Excl2 probabilities of 0.947 and 0.990, respectively. The real potential was slightly lower than expected due to the kinship observed in females. The average relationship coefficient r among the females was 0.099, while only 0.006 among the males, and -0.017 among females and males.

Using the exclusion method implemented in the FAP package, and solving the trios using the maximum likelihood method (80% and 95% thresholds) implemented in CERVUS 3.0, 96.3% of the larvae analysed along the five breeding seasons were allocated to a single couple. In the remaining 3.7%, the trios could not be solved because none of the couples proposed by FAP reach the minimum threshold with CERVUS using maximum likelihood.

The proportion of females involved on fertile spawns ranged between 67% and 100%, and the proportion of males between 22% and 44%. These individuals maintained the reproductive success over the spawning seasons, although, in general, more than 50% of the production in volume of eggs was the result of the activity of 3-4 couples depending on the breeding season (I-S5; I-S8; VI-S8; VI-S5; IX-S5; IX-S8). These couples maintained fidelity patterns (two or

Table 1. Broodstock production parameters for each year and group, and total and mean annual production for each group.

Year	Group	Total egg volume (mL)	Floating egg volume (mL)	Floating egg rate (%)	MRFec (10^3 eggs /female kg ± SE)	Number of spawns	Fertilisation rate ± SE	Hatching rate ± SE	Larvae
2010	WM	3211	381	10.07±2.06	4.68±0.33	65	45.65±9.11^{a}	42.65±14.62	101724
	CM	40	20	50	8.08	1	0^{b}	-	-
	C	1007	87	5.56±2.65	5.24±0.81	19	0^{b}	-	-
2011	WM	4806	451	8.25±1.45^{a}	6.11±0.44	72	56.72±9.72^{a}	100±0	272930
	CM	50	0	0	7.98±1.58	2	0^{b}	-	-
	C	561	1	0.24±0.24^{b}	5.65±1.02	10	0^{b}	-	-
2012	WM	5686	547	7.94±1.82^{a}	7.33±0.52	71	68.69±9.82^{a}	81.79±10.05	338794
	CM	50	0	0	11.74	1	0^{b}	-	-
	C	7529	169	2.70±1.11^{b}	7.80±0.53	80	0^{b}	-	-
2013	WM	3151	216	6.84±1.67	5.69±0.40^{b}	50	61.79±11.72^{a}	65.67±34.33	42938
	CM	157	27	18.81±8.03	2.86±0.39^{c}	6	0^{b}	-	-
	C	1631	191	9.34±4.34	7.98±1.08^{a}	16	0^{b}	-	-
2014	WM	2931	461	13.12±2.42	6.23±0.62	43	82.81±7.76^{a}	89.34±5.08	340690
	CM	1536	141	8.67±2.96	5.80±0.66	27	0^{b}	-	-
	C	146	26	14.93±14.14	2.82±0.44	5	0^{b}	-	-
Mean anual production	WM	3957±546^{A}	411±55^{A}	9.24±1.10	6.01±0.43	60±6^{A}	63.13±6.19^{A}	75.89±10.02	219415.2± 61975.05
	CM	366±293^{B}	38±26^{B}	15.50±9.30	7.29±1.46	7±5^{B}	0^{B}	-	-
	C	2174±1361^{AB}	95±38^{B}	6.55±2.58	5.90±0.95	26±14^{A,B}	0^{B}	-	-
Total production	WM	19785	2056	9.03±0.84^{A}	6.04±0.21	301	61.67±4.47^{A}	77.95±5.76	1147076
	CM	1833	188	9.64±2.66^{A}	5.59±0.57	37	0^{B}	-	-
	C	10874	474	4.21±1.09^{B}	7.09±0.40	130	0^{B}	-	-

C: cultured males and females. WM: wild males and cultured females. CM: cultured males and wild females. MRFec: mean relative fecundity. Different superscript lower case letter indicate significant differences (ANOVA, $p<0.05$) between groups within each year. Upper case indicate significant differences between groups in the mean annual production and total production.

more seasonal reproductive interactions) throughout the successive spawning seasons (see Table 2.).

The number of breeders involved in each spawn showed that most of the spawns were from crosses of several females with one male (76.8%), followed by a 18.2% of the spawns produced by several males and females. Only 4% of the spawns were the result of the cross of a single male with a single female, and 1% resulted from the cross of several males and one female (Table 3).

Behavioural observations

No nights with spawning were video recorded for group CM and, therefore, no behavioural analysis was made for group CM. During nights with a spawn in groups WM (n=3 fertilised spawns) and C (n=5 liberations of unfertilised eggs), most activity occurred between 15:00 and 01:00 h. Moreover, a peak of activity, probably related to feeding routines, was also described at 11:00 hours in both groups (Fig. 2). Group WM presented the highest peak of activity at 20:00 h within the period of activity (from 15:00 to 01:00 h). During the rest of the day the activity profiles were similar. The entire 24-h period before a spawn was observed to identify the distinctive behaviour of a coupled swim to the surface to release and fertilise the eggs. In group WM, two coupled swimming behaviours to fertilise eggs were clearly observed and these preceded the collection of fertilised eggs from the tank. However, in group C during the five 24-h periods before unfertilised eggs were collected no coupled swimming was observed and it was not possible to identify when the eggs were released by females.

Regarding the description of specific behaviours (stay quiet (SQ), SQ with actions, swim solo, swim follow, swim followed, tremor and rest head) during the peak of activity, all types of behaviour were described in the two tanks except for the "swim follow" and "swim followed" behaviours that were only recorded in

Table 2. Summary of the mating pairs recorded WM (wild males and cultured females) group per year through the parental allocation of larvae born during the study. In each pair, the number of spawns (N) and the floating volume of eggs (mL) derived from these fertilized spawns are shown. During the period of study, the following individuals died: (75 in 2012; Vin 2013 and S5, VI, VIII and XI in 2014).

2010																			
Female	I	V	V	V	VI	VI	VII	IX	X	X									
Male	S5	75	S5	S8	S5	S8	S5	S5	S5	S8									
N	3	6	1	1	5	1	3	7	2	1									
Volume	7.5	30	28.5	3	72	4	9.5	34.5	8	2									
2011																			
Female	I	I	V	V	VI	VI	VI	VIII	IX	IX	X								
Male	S5	S8	S8	S5	S8	S5	S2	S8	S8	S5	S8								
N	5	7	3	3	7	3	2	7	4	4	3								
Volume	25.2	17.7	14.7	5.7	52.6	27.7	7.5	6.9	14.8	10.2	6								
2012																			
Female	I	I	V	V	VI	VI	VI	VIII	IX	IX	IX	X	X						
Male	S5	S8	S5	S8	S8	S5	S2	S8	S5	S8	S2	S8	S5						
N	8	7	4	2	9	10	2	7	9	6	1	1	1						
Volume	42.9	33.6	12.4	5	134.1	68.2	5.7	36.4	44.4	21.7	0.1	22.9	3.6						
2013																			
Female	I	I	V	V	V	VI	VI	VI	VIII	IX	IX	IX	IX	X	X	X	XI	XII	XII
Male	S8	139	S8	139	S5	S8	S5	139	S8	S8	S5	139	S2	S8	139	S5	S8	S8	S5
N	15	2	6	3	2	8	5	2	2	12	4	5	1	4	2	1	2	5	2
Volume	78	11	11	4	2	25	17	6	3	27	9	8	1	5	3	1	3	9	4
2014																			
Female	I	I	VI	VI	VIII	VIII	IX	IX	X	X	XI	XI	XII	XII					
Male	139	S8	139	S8	139	S8	139	S8	139	S8	S8	139	139	S8					
N	10	3	11	5	8	4	10	7	1	1	6	5	6	3					
Volume	28	11	28	17	17	7	13	11	1	1	6	5	7	6					

group WM (Fig. 3). The profiles of the frequency of the different behaviours between groups WM and C were significantly different (χ^2, $p<0.05$).

Discussion

The present study has demonstrated that cultured females have the potential to spawn large qualities of fertilised eggs when kept with wild males (group WM). To date, no study or commercial company has spawned large qualities of fertilised eggs from cultured breeders and this has made the sole culture industry unsustainable, relying on the capture of wild fish without the possibility to make genetic improvement of cultured stocks. The spawning of commercially relevant fecundities from cultured females, as obtained in the present study, shows that genetic breeding programs could be based on cultured female breeders and indicates that cultured males may present a reproductive dysfunction. The quality of the natural spawns obtained from cultured females with wild males (group WM) was comparable to those obtained from broodstocks of only wild Senegalese sole individuals (Martín *et al.*, 2014). The study of Martín *et al.* (2014) was completed in the same installation of this study (IEO Santander, Spain), with wild fish from the same group and during the same period. In the present study, the number of

Table 3. 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.

Individuals involved in the spawns	(%)
One male and one female	4
Multiple males and females	18.2
Multiple males and one female	1
Multiple females and one male	76.8

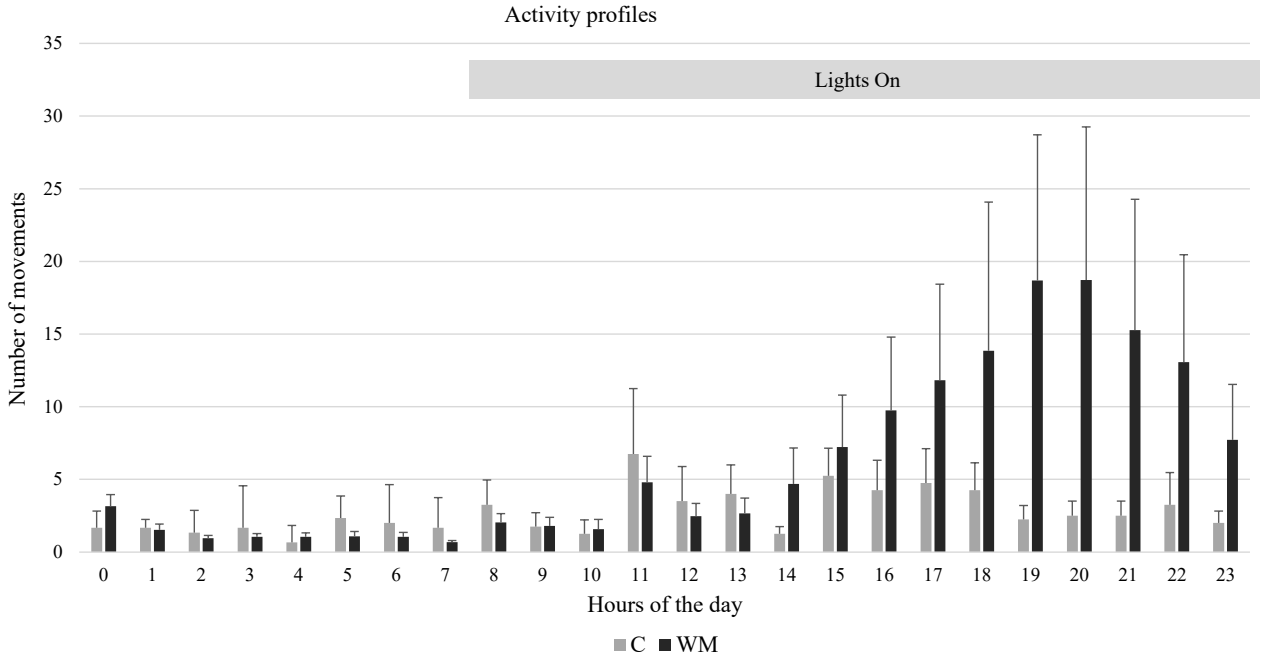

Figure 2. Activity profiles description of the groups studied: group WM (wild males with cultured females) and group C (cultured males and females) on spawning days. The period with the lights on in the facilities is indicated.

annual spawns from group WM was in the same order to those obtained in the wild broodstocks (Martín *et al.*, 2014), and fertilization and hatching rates were similar, achieving larval productions of the order of 200,000 larvae per year, under the conditions described. Differences on spawn quality could be related to the previous feeding regime (commercial dry feed) of the cultured females, since it has been demonstrated the influence of feeding on egg quality (Brooks *et al.*,1997).

In addition, the cultured females with wild males exhibited similar spawning behaviour as the wild stock described by Carazo *et al.* (2016) who studied the same groups reported in Martín *et al.* (2014). In the present study, the mixed broodstock group WM showed an increase in activity during the night (active period of this species) and had an activity profile similar to spawning wild individuals (Carazo, 2013; Carazo *et al.*, 2016). The specific behaviours described highlighted the presence of the "follow" behaviours (swim follow and swim followed). These behaviours have been described as predictors of spawning events (Carazo *et al.*, 2016) and the "follow" behaviours increase during nights prior to spawning in wild individuals contributing to the observed increase in activity. The analysis of behaviours

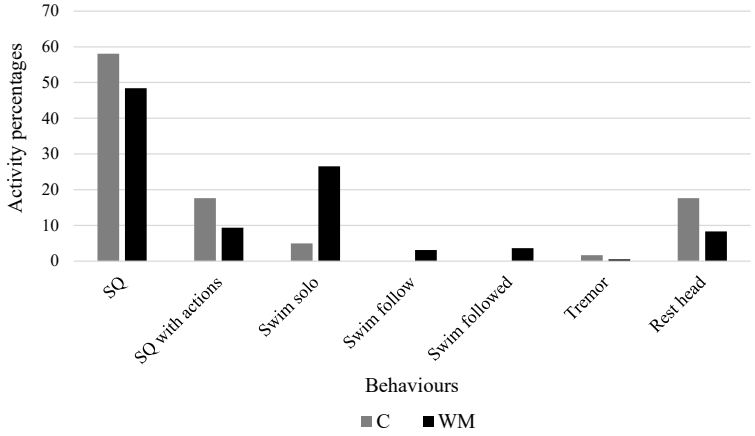

Figure 3. Behavior profiles of the groups studied: group WM (wild males with cultured females) and group C (cultured males and females) on spawning days. SQ means stay quiet. The global profile of each group was significantly different (χ^2, $p<0.05$).

in group WM during the 24h period before fertilised eggs were collected described coupled swimming to the surface to fertilise eggs, which was always observed before the collection of fertilised eggs in wild broodstocks (Carazo *et al.*, 2016). Overall, the increase in activity during the spawning period, the "follow" behaviours, and the "coupled swimming" behaviour before the collection of fertilised eggs indicated that wild males with cultured females (group WM) had similar behavioural courtship as wild broodstocks and did not present any reproductive dysfunction.

The molecular tools used for paternity inference were very useful even in non-ideal conditions, such as in the present study when kinship was observed amongst the cultured females in the mixed stock studied (group WM) and this highlights the possibility of using this technique as support for broodstock management and breeding programs. The paternity allocation revealed similar fidelity patterns to those obtained in wild individuals (Martín *et al.*, 2014), over several spawning seasons. However, the classification of the spawns according to the number of individuals participating from each sex in group WM exhibited a different pattern to that obtained in wild males and females (Martín *et al.*, 2014). In the case of the WM group, most of the spawns were obtained by the participation of multiple females and one male and 67-100% of the females were involved in spawning during a season (or year), while participation of females was less frequent in the wild broodstocks (8-57 %) and most spawns were obtained from couples that exhibited fidelity. However, multi-maternal and multi-paternal spawning (an individual spawning with many other individuals), had similar frequency in both stocks wild and WM group.

Therefore, overall the cultured females in group WM did not show any reproductive dysfunction that can explain the complete failure of cultured stocks to spawn fertilised eggs that has been reported in the literature (Agulleiro *et al.*, 2006; Guzmán *et al.*, 2008; 2009) and from the sole culture industry (Howell *et al.*, 2009). The cultured females with wild males (group WM) in the present study exhibited during five years of holding, fecundities similar to wild broodstocks (Martín *et al.*, 2014), similar reproductive behaviour (Carazo *et al.*, 2016) and participation in spawning was higher than reported for wild females (Martín *et al.*, 2014). The high participation (67-100%) in spawning of randomly selected cultured females would indicate that it is not unreasonable to conclude that cultured females have the capacity to reproduce naturally and spontaneously.

However, the groups of cultured males with cultured females (group C) and cultured males with wild females (group CM) did not spawn any fertilised eggs and mean annual volumes of floating eggs were significantly lower than group WM. The spawning of unfertilised eggs from group C was consistent with all other attempts to reproduce cultured breeders, as to date no cultured broodstock (four research centres and three companies) has spawned large qualities of fertilised eggs (personal observation). This situation has been described for untreated cultured breeders in research centres (Guzman *et al.*, 2008) and industrial companies (Howell *et al.*, 2009) and for hormonally induced cultured breeders (Agulleiro *et al.*, 2006; Guzmán *et al.*, 2009). The present study for the first time studied the reproductive behaviour of a cultured broodstock. The cultured breeders in group C had no nocturnal increase in activity, lower activity than group WM in the night before eggs were collected and did no exhibit the behaviours, "follow(ed)" and "coupled swim" related to courtship, spawning and fertilisation. An increase in nocturnal activity and courtship behaviours (follow and coupled swim) was observed prior to the collection of fertilised eggs in wild broodstocks (Carazo *et al.*, 2016) and a broodstock of cultured females with wild males (group WM). The absence of courtship behaviour, in the present study, indicated that the cultured broodstock (group C) produced unfertilised eggs because there was no reproductive behaviour to fertilise the eggs. Reproductive behaviour appears to be a key factor in solving the absence of fertilized spawns with cultured specimens in this species, and research is being carried out on this line to determine and evaluate the relations between different behaviour approaches and reproduction in this species (Ibarra-Zatarain *et al.*, 2016; Castanheira *et al.*, 2017; Fatsini *et al.*, 2017a,b).

Similarly, cultured males with wild females in group CM produced low volumes of unfertilised eggs. In the final year of the experiment group CM fecundities increased, but as in group C large amounts of unfertilised eggs were collected. This highlighted the importance of long-term studies for this species as fecundities often increase over the years and are variable. Therefore, the two groups containing cultured males did not produce fertilised eggs. Both groups had randomly selected females and females from the same stock did spawn when housed with wild males. Fertilised eggs were spawned by wild females with wild males (Martín *et al.*, 2014) and cultured females with wild males (group WM, present study). Therefore, the behavioural observations, successful spawning of cultured females (group WM) and failure to spawn fertilised eggs by both groups with cultured males would suggest that cultured males exhibit a reproductive behavioural dysfunction. However, perhaps caution is required as although replication of individual breeders was included in the present study an exact tank replica was not included and perhaps it cannot be discounted that both these

groups CM and C did not spawn fertilised eggs due to external factors not related to the origin and sex of the breeders. This would indicate more work is required to confirm the reproductive behavioural dysfunction in cultured males.

Considering the need for further work it should be mentioned that similar results, particularly in terms of obtaining fertilised eggs, were obtained by Mañanos *et al.* (2007) that preceded the present study. Mañanos *et al.* (2007) had the same mixed groups and, as in the present study, the cultured males with wild females group did not produce fertilised spawns compared to the wild males with cultured females that did produce fertilised spawns. However, Mañanos *et al.* (2007) hormonally induced the cultured fish to spawn, only collected the eggs for a short period after hormone induction (the exact period was not reported) and the percentage of fertilised eggs from the cultured females with wild males appeared to be low. Therefore, the present study found a clear and consistent relationship between genders, origin and the lack of fertilized spawns in accordance with Mañanos *et al.* (2007) and has deepened and detailed aspects of production potential and inter-individual relations of the species in captivity.

The percentage of floating eggs has been commonly used as an indicator of spawn quality 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 the present study, egg viability was around 4-5% in group C and significantly increased in the mixed stocks (groups WM and CM) close to the 10%, but this was lower than in wild individuals held in similar conditions (30-40%) (Martín *et al.*, 2014). However, the present study does not support that the floating fraction is a good indication of egg viability as two groups (C and CM) that produced floating "viable" eggs actually had no fertilisation and no viable eggs were obtained. In this study, viable eggs were only obtained from group WM.

In conclusion, firstly the production of high numbers of fertilised eggs over a five year period highlights the possibility of industrial production and the design of genetic breeding programs based on the natural spontaneous spawning of cultured females. Secondly, the production of viable eggs from culture females with wild males, but not with cultured males either with wild or cultured females, suggests that the reproductive dysfunction may be specific to cultured males. However, more research is required on natural reproduction of cultured males to both confirm the existence of a sex specific reproductive behavioural dysfunction and examine underlying factors that may contribute to the reproductive dysfunction in cultured broodstocks. Factors that should be examined include, the influence of feeding in early stages, epigenetics, tank and social environment, early growth and development, the individual relationships that appear to be established in the tanks between wild males and cultured females and ways to stimulate and induce social relations (courtship) in cultured specimens.

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