The presence of wild Senegalese sole breeders improves courtship and reproductive success in cultured conspecifics

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Abstract

The life cycle of Senegalese sole (*Solea senegalensis*) is not closed in captivity due to a reproductive dysfunction related to the lack of participation of cultured male breeders in the courtship. To discern a possible solution to this social reproductive dysfunction, the main objective of this study was to determine the effect of the presence of spawning wild Senegalese sole breeders on the courtship and reproduction of cultured breeders. Three experimental groups were formed: Control group (n = 10) formed with only cultured sole; groups M1 and M2 constituted of mixed-origin sole (10 cultured and 8 or 9 wild breeders). All cultured breeders were from the same stock, which had never successfully reproduced, whilst the wild broodstock had spawned viable eggs in captivity. All groups were held in the same captive conditions and spawning, and behaviour were recorded for each group over four spawning seasons. All spawns were collected, and the parameters registered were floating and non-floating eggs, fertilization and hatching rates. In addition, parental analysis was made of larvae from viable spawns. Behaviour was studied with video recordings to compare locomotor activity and courtship behaviours including the "Follow" behaviours where sole swim after each other in a procession. Fertilised spawns were obtained from the mixed-origin groups (M1 and M2) including spawns involving a cultured male. The cultured males also participated actively in the "Follow" behaviours with the courting wild sole and this participation of cultured males increased significantly over the four years of the experiment. Male percentage participation in the "Follow" behaviours was positively correlated (R = 0.81) to participation in spawning to indicate the importance of increasing cultured male participation in the "Follow" behaviours. A total of seven spawns were obtained from a cultured male that fertilized eggs from one cultured female and two wild females. The same cultured female also spawned with a wild male. No viable spawns were obtained from the Control group and

locomotor activity and courtship behaviour counts were significantly lower than in the experimental mixed-origin groups. This is the first report of cultured male breeders participating in reproductive behaviour and spawning, which could be associated with social learning processes, mate selection and dominance where cultured males copied the behaviour and spawning of wild Senegalese sole breeders.

Key Words: *Solea senegalensis*
Reproductive Behaviour
Social learning
Spawns
Origins

1. Introduction

The Spanish aquaculture producer organisation, APROMAR (2015), stated that Senegalese sole (*Solea senegalensis*) had good characteristics for aquaculture and had in recent years the highest economic return of all aquaculture marine fish species that were commercialised in Spain. The positive characteristics of this species for aquaculture are good growth rates, high larval survival and high capacity to adjust to intensive production. These characteristics and high economic return have aided a recent rapid increase in European aquaculture production of Senegalese sole from 55 t in 2008 to 1616 t in 2018 (APROMAR, 2019).

Nevertheless, some issues exist that must be improved to optimise industrial Senegalese sole production (Morais et al., 2016). One of the main problems that has limited Senegalese sole production is a reproductive dysfunction in cultured breeders that were bred and reared in captivity (Morais et al., 2016). This reproductive dysfunction resulting in the failed spontaneous spawning of viable eggs from cultured breeders, means Senegalese sole egg production has been based on the spawning of wild-origin breeders (Dinis et al., 1999; Anguis and Cañavate, 2005; Martín et al., 2014), which is unsustainable in a long-term. The frequency and volume of eggs obtained from captive wild broodstock is enough for commercial production. However, a low participation of breeders in spawning has been registered leading to a rapid loss of genetic variability between generations (Porta et al., 2006). Parental analysis with microsatellites indicated that spawning was dominated by a few wild breeders that represented only 8.5 - 51.7 % of the broodstock (Porta et al., 2006; Carazo, 2013; Martín et al., 2014; Carazo et al., 2016). In comparison to wild broodstocks, spontaneous spawning from cultured

broodstocks was less frequent and eggs did not hatch (Agulleiro et al., 2007; Guzman et al., 2008; Howell et al., 2009; Norambuena et al., 2012; Rasines et al., 2012). However, cultured breeders do produce viable gametes, which have been extracted with abdominal pressure and successfully fertilised *in vitro* (Chereguini et al., 2007; Rasines et al., 2012; 2013). The cultured females were induced to ovulate with GnRHa before the viable eggs were extracted and fertilized with cryopreserved sperm from cultured males (Rasines et al., 2012; 2013). However, the artificial fertilization method is complicated due to the low sperm volume found in Senegalese sole (Cabrita et al., 2006; Beirao et al., 2009; Cabrita et al., 2011) and requires further research to implement on a commercial scale.

Carazo (2013) observed that the eggs from cultured breeders were not fertilized due to a dysfunction in the reproductive behaviour or courtship. Spawning, courtship and mate selection have been described in wild stocks held in captivity (Carazo, 2013; Carazo et al., 2016). The courtship was characterised as a complex series of distinctive behaviours associated to mate selection and gamete release, which were also registered as an increase in locomotor activity (Carazo, 2013; Carazo et al., 2016). In comparison, these courtship behaviours were less frequent or absent in cultured breeders and consequentially the ova released by females were not fertilised (Carazo, 2013; Martín et al., 2019). Hormone treatments, with either Gonadotropin-Releasing Hormone analogue (GnRHa) (Agulleiro et al., 2006; Guzman et al., 2008; Guzmán et al., 2009; Carazo, 2013), combined GnRHa and human Chorionic Gonadotropin (hCG) (Guzmán et al., 2011; Carazo, 2013) or combined GnRHa and prostaglandin F2α (PGF2α) (Carazo, 2013) increased both the number and frequency of eggs released by cultured breeders, but none of the treatments increased the fertilisation success (Carazo, 2013). Fertilisation success has been increased by setting up broodstocks of mixed origin (wild and cultured) both with (Mañanós et al.,

2007) and without hormonal treatment (Carazo, 2013; Martín, 2016; Martín et al., 2019). These studies could be summed up as follows: cultured females treated with GnRHa slow-release implants (Mañanós et al., 2007) or with no hormone treatment (Carazo, 2013; Martín, 2016; Martín et al., 2019) cohabiting with untreated wild males produced fertilised spawns and the full sequence of courtship behaviours similar to that observed in captive wild stocks (Carazo, 2013¸ Martín et al., 2019). However, cultured males (with or without GnRHa treatment) cohabitating with untreated wild females did not produce fertilised spawns and did not display courtship behaviour to fertilise eggs (Mañanós et al., 2007; Carazo, 2013; Martín, 2016; Martín et al., 2019). In consequence, the spawning failure was shown to be due to a behavioural reproductive dysfunction associated with cultured males suggesting that environmental parameters (Anguis and Cañavate, 2005), sperm quality (Cabrita et al., 2006) and hormonal treatment (Agulleiro et al., 2006; Rasines et al., 2013) did not offer a possible solution to determine the triggers to promote natural spontaneous spawning.

Therefore, the aim of the present study was to observe the effect of the presence of spawning wild breeders on the reproductive success and behaviour of cultured breeders using a new approach of setting up broodstocks from different origin and gender, mixing males (wild and cultured) and females (wild and cultured) from both origins altogether. To our knowledge, this is the first time this approach of mixing wild and cultured broodstocks with different reproductive capacities has been used to study a behavioural reproductive dysfunction. For this purpose, the spawning success and behaviour was analysed in two different mixed (wild and cultured) groups of Senegalese sole and compared with a control group (pure cultured breeders) during four consecutive spawning seasons. Furthermore, mate selection was determined by microsatellite paternity analysis

based on the results of parental assignation of larvae collected during the study period. Understanding the evolution of these broodstocks during these years may help to improve the management of this species under aquaculture conditions and give a better understanding of the reproductive dysfunction of cultured Senegalese sole.

2. Material and Methods

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

2.1 Broodstocks and management

The experiment had a duration of four years from October 2012 until October 2016, which included four spawning periods from March to June during the years 2013 to 2016. Senegalese sole breeders, 30 cultured individuals (1192.8 ± 158.2 g) and 17 wild individuals (907.5 ± 192.4 g) were placed in three different tanks forming three different experimental groups. All individuals were Pit-tagged (ID-100 Unique, Trovan-Zeus, Madrid, Spain) and photographed and videoed for future identification. The age of the cultured animals was on average 8 years with a mean of 5 years holding in IRTA before the experiment was initiated. The age of the wild animals was not known, but were also held in IRTA a mean of 5 years before the experiment and similar weight and length indicated a similar age to cultured animals. The cultured animals came from parents caught in the Atlantic zone and wild animals were caught from around the Ebro Delta

(Mediterranean zone). All cultured animals were F1, which means that the parents were wild origin. The weaning, ongrowing and rearing conditions of the cultured animals were usual intensive rearing conditions applied in the aquaculture industry.

The different experimental groups were set up in October 2012. The experiment was entirely performed in IRTA Sant Carles de la Ràpita and all experimental groups were located in fibre-glass tanks of 10 m^3 (2 m x 6 m x 0.85 m) included in a recirculation system (IRTAmar®). The temperature regimen was controlled to simulate a natural cycle that ranged from 9 – 20 ºC and during the spawning season after a natural rise to 18 ºC a weekly temperature cycle was used to stimulate further spawning (Monday to Thursday at 16 ± 1 °C and Thursday to Monday at 18 ± 1 °C as described in Martín et al. 2014). The fish received a simulated natural photoperiod ranging from Light: Dark (LD) 9:15 to LD 15:9 with approximately LD 12:12 to 14:10 during the spawning season. Daytime lighting was delivered with fluorescent lighting and natural light from windows (50 *lux* at surface) during the entire year. During the spawning season, red night lighting was used that allowed recording and observation of the sole behaviour. Red light was from fluorescent illuminations covered with a red filter that were adjusted to approximately 5 *lux* at the water surface. Carazo et al. (2013) demonstrated that this illumination system did not affect sole behaviour, locomotor activity or plasma melatonin levels. Half of the bottom of each tank was covered by sand. Breeders were fed *ad libitum* with approximately 1 % of the total biomass five days a week at 09:30 h. The diet consisted on fresh feed (cooked mussels, Sariego Intermares, Spain), marine polychaetes (Topsy-Baits, Holland) and balanced feed (Repro-Vitalis, LE-7 mm ELITE, Skretting Co.).

2.1.1 Experimental groups and experimental design

Breeders were distributed in three experimental groups, Control group (C) constituted of only cultured breeders and two experimental groups (M1 and M2) were mixed including breeders from different origins, wild and cultured (Table 1). All cultured breeders used for this study were from the same stock that had never successfully spawned. On the other hand, wild breeders used for this experiment had spawned in captivity, however, the individual identities of spawning fish were unknown. All groups were monitored following Carazo et al. (2016) video recording analysis to evaluate the behaviour. Spawns were collected, assessed and incubated and parental analyses were made of larvae (*see details below*). Moreover, all groups were formed since October 2012 and approximately 30 cultured Senegalese sole juveniles (~ 100 g) provided by Stolt Sea Farm in May 2012 were cohabiting with breeders in each group at the moment the tanks were established. The juveniles were removed from the experimental tanks in October 2015. In addition, in 2014 wild males were removed on the 1 May from the experimental tanks M1 and M2 in order to enhance the participation of cultured males in the spawning and returned when spawning period finished in June. There was a low incidence of mortality during the whole experiment and no mortalities were registered in the control group.

2.2 Spawns analyses

The egg collectors were checked for eggs each morning at 08:00 hours. Spawned eggs were collected and the following parameters were determined: total volume of eggs (ml), volume of floating and non-floating (inviable) eggs (ml), total number of eggs (determined volumetrically counting eggs in 3 x 100 mL subsamples taken from a homogenous known volume containing all the eggs), stage of development and percentage fertilisation of floating eggs (determined by examining 50-100 eggs). The

daily fecundity was calculated as the total number of eggs related to the total weight of the females in the tank in kg. Once the spawns were evaluated, the floating part was incubated in 30 l incubators with open flow water and natural conditions (temperature and photoperiod). The larvae hatched after 36 - 48 h of incubation, depending on the water temperature (13 – 23 °C in open flow) and the embryonic phase at which the eggs were collected. Hatching rate was determined as the total number of larvae hatched divided by the total number of floating eggs incubated, after counting the number of larvae (and previously eggs) in three 100 ml subsamples. Larvae obtained were held in the incubators until 5 - days post hatch (DPH), when larvae were collected to proceed with the paternity analysis.

2.3 Paternity analyses

Ten larvae obtained from every spawn were placed individually in 1.5 ml Eppendorf filled with 96 % ethanol and were sent to GENEAQUA (Facultad de Veterinaria de la Universidad de Lugo, Lugo, Spain) to determine the paternity of the larvae. For this analysis, all breeders from all tanks were genotyped using the specific microsatellites for sole. For individual identity the total spawns per individual was noted to observe the families per year during the spawning season. An initial analysis was made using four microsatellites. This was followed by a second analysis with two additional microsatellites (six in total) for samples that presented three or more possible parents.

2.4 Behavioural analyses

213 Digital cameras (Square black and white CCD camera, model F60B/N80-50G, KT&C,
214 Korea, supplied in waterproof housing by Praesentis S.L. Barcelona, Spain) were used to
215 film the fish behaviour during the spawning season. Two cameras were placed in the
216 Control tank, and three cameras were located in M1 and M2 tanks, eight cameras in total;
217 four cameras were connected to a digital video recorder (model DVR-Camtronics-UCDI-
218 DV4150-1500, supplied by Praesentis S.L.) and the other four were connected to another
219 video recorder (model XMOTION-304H supplied by Praesentis, S.L). The cameras were
220 situated just below the water surface angled downwards. In all tanks, one of the cameras
221 field of view was almost the complete length of the tank and with the other camera the
222 half sand part of the tank (middle of the tank to the water inlet) was observed. In the case
223 of the tanks M1 and M2, another angle was added with another camera, from the middle
224 of the tank to the water outlet. The cameras positions enabled 96 % of the entire water
225 column of the tank to be filmed and recorded. All the tanks (Control, M1 and M2) were
226 studied from 25th of March to 3rd of June of each year coinciding with the Senegalese sole
227 spawning period in IRTA. The behavioural analyses were divided in locomotor activity
228 and behaviours associated to the courtship during the peak hour activity, explained in
229 detail below.
230
231 *2.4.1 Activity*
232
233 The locomotor activity during the spawning season was measured counting the
234 movements of the sole during five randomly selected days that had spawning events and
235 five without spawning events. Locomotor activity was assessed by putting a line across
236 the middle of the screen dividing the field of vision of the camera (the tank) in two, and
237 the number of times a breeder crossed the line was counted for every hour recorded.

Activity was recorded each year from 17:00 to 00:00 with some additional hours in different years. In 2013 and 2014 the recording period was 14:00 to 7:00. In 2015 the period was 14:00 to 00:00 and in 2016 from 17:00 to 01:00. Hours recorded were reduced to focus on the hours of importance and reduce the storage capacity required. To compare the locomotor activity among experimental groups the mean of every hour for the five days and each tank was divided by the number of breeders in the experimental groups.

2.4.2 Behaviours registered during the peak of activity (courtship)

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

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

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

2.4.3 Identification of individuals in "Follow" behaviours

To determine the origin (wild or cultured) of the breeders involved in the behaviours termed as "Follow", the fish participating in "Follow" behaviours in groups M1 and M2 were identified. For this purpose, "Follow" behaviours (n = 30) were randomly selected and analysed by three different observers that watched the videos and used a photo-video identification catalogue to identify which fish were involved in the behaviours. The photo-video identification catalogue consisted of photos (each fish were individually photographed to observe the shape and the caudal fin pattern) and short video recordings to observe the movements (swimming display) of each fish in each tank, using both options, the fish could be distinguished with a 80 % of reliability. To examine the frequency of the participation of cultured breeders over years in both experimental groups, M1 and M2, the number of cultured sole involved in "Follow" behaviours analysed were counted in each "Follow" behaviour (n = 30) for each year (2013 - 2016).

2.5 Statistical analyses

All the results were presented with means ± standard error (mean ± S.E.M). Data were analysed with the Kolmogorov-Smirnov test and found to have a normal distribution. The

286 analysis of the locomotor activity was made according to the description of daily activity
287 profiles (Bayarri et al., 2004; Carazo et al., 2016).
288 The difference between days with spawning events and without spawning events in the
289 frequency of the "locomotor activity" was evaluated using One-way ANOVA ($P < 0.05$).
290 The frequency of the "Follow" behaviour of each individual was presented as a
291 percentage of the total number of "Follow" behaviours analysed (per year per group) to
292 aid comparison among groups and years with no statistical comparison being applied. In
293 addition, the number of cultured breeders participating in "Follow" behaviours amongst
294 the years were compared with One-way ANOVA ($P < 0.05$) for both experimental
295 groups.
296 The number of behaviours (number of times a particular behaviour was observed in each
297 group) for different tanks and different years were compared with mixed-effect Model
298 Repeated-Measures ANOVA ($P < 0.05$) test. Each behaviour was represented in
299 frequency (number of times the behaviour was displayed during the hour of observation)
300 calculated for the 5 days, the same used for locomotor activity for spawning days.
301 The statistical analysis was performed with SPSS Statistics 19.0 software (IBM Co.,
302 Hong Kong). Raw data from both spawns and reproductive behaviour are available in
303 *figshare* (DOI: 10.6084/m9.figshare.6428486).
304

305 # 3. Results

306

307 ## 3.1 Spawns and Paternity

308 The spawning parameters showed a large variation in relation to experimental group and
309 year (Table 2). Despite of this high variation it appeared that egg production was similar
310 between experimental groups considering differences in biomass, although annual

311 production did vary with the highest production in 2015 and the lowest in 2013, while
312 more spawns were obtained from group M2 compared to the control group. However,
313 fertilized spawns were only obtained from mixed groups, M1 and M2. Again, there was
314 high variation in the number of spawns that hatched from groups M1 and M2 and
315 consequentially no differences were found.
316 Paternity analysis was completed for 1,090 larvae from 109 spawns obtained from mixed
317 groups M1 and M2 during the four spawning seasons. A total of 93.5 % of the larvae
318 were assigned to two parents and 6.5% were not assigned as either the DNA extracted
319 was of poor quality or the analysis was inconclusive as three or more possible parents
320 were identified. In group M1, spawns that hatched were registered in 2014, 2015 and
321 2016 (Table 2). Only 4 wild breeders of 18 animals (10 cultured and 8 wild individuals),
322 1 male and 3 females participated in fertilized spawns (Fig. 1A), which represented a
323 participation in the tank ranging from 11 % in 2016, when just one pair spawned, to 21
324 % in 2014 when the same male spawned with three different females. During 2014 and
325 2015 one female (FW2) dominated the spawning with 15 and 10 spawns or events
326 respectively and other females contributed in less events. The dominant female changed
327 in 2016 when one female (FW1) was the only female to spawn with the dominant male
328 with 19 spawns during that year, but this female had also reproduced with the dominant
329 male during 2014 (6 spawns) and 2015 (1 spawn). After the wild males were removed in
330 May 2014 no fertilised spawns were obtained.
331 In group M2, fertilized spawns were obtained in all years (Table 2). The participation in
332 M2 was more variable than M1 involving both wild and cultured breeders (males and
333 females) ranging from 24 % in 2016 to 38.9 % in 2015 (Fig. 1B). During 2013, the larvae
334 were assigned to five breeders, one wild male (MW2) and four wild females with varying
335 participation. In 2014, the same wild male (MW2) was assigned as the father of most of

the larvae analysed (180 larvae) from 18 of the 20 spawns registered. The same three wild females were also assigned as mating with this wild male. The remaining larvae were assigned to a cultured couple, which reproduced for the first time, the cultured male (Mcult2) mated with the cultured female (FCult1). The third fertilised spawn was obtained after 1 May 2014 when the wild males were removed. Paternal analysis did not clearly identify two parents; however, it can be concluded that the father was a cultured male. In 2015, three wild males contributed to hatching spawns, which included the same male from previous years and two males that contributed for the first time. Wild females assigned as the mothers of the larvae were the same females that reproduced in 2013 and 2014, however, there were two cultured females that participated for the first time, each with one spawn. During 2015, no fertilized spawns were obtained from the cultured couple that reproduced in 2014. In 2016, the wild male that dominated the spawning each year died when spawning activity began and the first eggs were collected from the tank, therefore, the contribution of the previously dominant male MW2 was 1 of the 5 spawns obtained this year. The breeders that contributed were the same cultured male that participated in 2014 (MCult2) with two wild females and one cultured female. Just four fertilized spawns were collected, all spawns were fertilized by the cultured male that spawned twice with a wild female (FW4) and once with the second wild female (FW2) and once with a cultured female (FCult1), which was the same female which reproduced in 2014.

3.3 Behavioural analysis (courtship)

3.3.1 Activity

The locomotor activity of the Senegalese sole breeders showed a circadian rhythm associated with spawning in each group presenting repetition during each year (Fig. 2). In general, activity peaked during early evening on all days, however, the mean number of movements during the peak was significantly ($P < 0.01$) higher on days with spawning compared to days without spawning. Activity during periods with spawning was generally two-fold more compared to days without spawning. Activity with spawning began to rise during the afternoon (from 14:00 in 2015, *data not shown*) achieving the maximum at 19:00 and the minimum from 2:00 to 7:00. Thus, the peak hour of activity was registered from 19:00 to 20:00 in all the tanks and each year activity decreased after 20:00.

3.3.2 Behaviours registered during the peak of activity (courtship)

The "Rest the head" behaviour represented the most common behaviour performed during the peak hour of activity in all experimental groups (Fig. 3). The frequency of this behaviour was significantly higher during all years in the two mixed origin groups, M1 (18.8 ± 3.6; $P = 0.001$) and M2 (16.2 ± 3.4; $P = 0.001$) compared to the Control group (6.6 ± 2.0) (Fig. 3). However, no significant differences were observed between M1 and M2 ($P = 0.99$). The "Follow" behaviour was the second most common behaviour performed during the peak hour of activity (Fig. 3). This behaviour exhibited a similar trend and was in general higher during all years in the two groups of mixed origin, M1 (3.3 ± 1.1; $P = 0.001$) and M2 (2.5 ± 0.7; $P = 0.001$) compared to the Control group (1.5 ± 0.3). The behaviours "Guardian" and "Coupled" swimming were observed to a lesser extent and were not observed at all in the Control group (Fig. 3). The "Guardian" behaviour was differentially observed in all years in the mixed origin groups, M1 (2.0 ±

386 0.2; $P = 0.001$) and M2 (1.4 ± 0.4; $P = 0.001$) compared to Control group (0.0 ± 0.0). The
387 "Coupled" swimming behaviour did not present differences in frequency amongst groups,
388 however, the behaviour was only observed three times, twice in group M2 during 2013
389 and once in M1 group during 2016.
390
391 *3.3.3 "Follow" individual identification*
392
393 The Control group was not analysed for this behavioural part because (a) all fish were of
394 cultured origin and obviously all fish involved in any behaviour were of cultured origin
395 (b) previously the swimming behaviours "Leader and Follower" had been infrequently
396 observed in cultured breeders (c) there was no reproductive success in the Control group.
397 In addition, the "Follow" behaviours were significantly lower in the Control group
398 compared to M1 and M2 groups (Fig. 3).
399 Frequent "Follow" behaviours were observed in both mixed groups, M1 and M2.
400 Principally males were involved, although females were also involved. A total of eight
401 females in the two groups (M1 and M2) were involved in "Follow" behaviours with a
402 mean participation of 12.6 ± 2.6 %. Origin and gender appeared to influence the position
403 "Leader or Follower" in the "Follow" behaviour. Wild males were more commonly
404 involved in the "Follower" position and generally two thirds (65 %) of the "Follow"
405 behaviours of a wild male were as a "Follower". Cultured males had an even involvement
406 in the two positions and generally half (50 %) of the "Follow" behaviours of a cultured
407 male were as a "Follower". The females presented the opposite situation and over 90 %
408 of female involvement in the "Follow" behaviours were as a "Leader".
409 Cultured individuals were involved in the "Follow" behaviours ($n = 30$ randomly selected
410 from periods with spawning) in every year in both mixed groups (Figs. 4 and 5). The

411 involvement of each cultured individual in the “Follow” behaviours generally increased
412 with advancing years and consequentially the involvement of each wild individual
413 generally decreased. In group M1, the mean percentage of participation of cultured
414 individuals in "Follow” behaviours increased from 12 ± 7 % in 2013 to 35 ± 14 % in 2016
415 (Fig. 4) and in group M2, the mean percentage of “Follow” behaviours increased from 24
416 ± 9 % in 2013 to 33 ± 18 % in 2016 (Fig. 5). In addition to the increase of individual
417 involvement, the number of cultured fish involved in “Follow” behaviours each year
418 increased significantly ($P < 0.05$). In group M1, the number of cultured males involved
419 increased significantly from 2013 to 2015 ($F_{3, 116} = 6.567$; $P = 0.001$; Fig. 4, *insert*) and
420 2016 ($F_{3, 116} = 4.756$; $P = 0.01$; Fig. 4, *insert*), while in group M2 cultured male
421 involvement increased significantly from 2013 and 2014 to 2015 and 2016 ($P < 0.05$; Fig.
422 5, *insert*).
423 Lastly, male involvement in “Follow” behaviours appeared to be related to spawning
424 success. Percentage participation in “Follow” behaviours (total “Follow” behaviours,
425 “Follower” + “Leader”) of spawning males (wild and cultured) was strongly correlated
426 ($R = 0.81$, $P = 0.008$) to percentage participation as parents of larvae. The separated
427 “Follower” ($R = 0.70$) and “Leader” ($R = 0.67$) behaviours of spawning males were also
428 correlated to percentage participation as parents of larvae. However, here was no
429 correlation ($R = - 0.09$) between percentage participation in “Follow” behaviours of
430 spawning females (wild and cultured) and percentage participation as parents of larvae.
431

432 4. Discussion

433

434 This is the first study that reports the active participation of the cultured males in
435 reproductive behaviour and spawning in Senegalese sole. In terms of reproductive

436 behaviour, from the first year of the experiment, cultured males cohabiting with wild
437 breeders were observed to participate in the “Follow” behaviours and this participation
438 increased significantly over time. In relation to spawning, a cultured male contributed to
439 spawns with a cultured female and two wild females in two different years. In the entire
440 period of the study, the Control group that never had contact with wild breeders did not
441 present fertilized spawns and the behaviours associated with courtship were significantly
442 lower than those observed in the experimental groups (M1 and M2) that housed cultured
443 breeders with wild breeders that successfully spawned. This demonstrates the positive
444 effect that cohabitation with spawning wild Senegalese sole had on the reproductive
445 success and behaviour of cultured breeders.
446 This is the highest reported contribution of a cultured male to spawning, however, it is
447 not the first report as Guzmán et al. (2011) observed that 1 of 60 spawns was fertilised by
448 a cultured male after GnRHa implants were applied to cultured females and hCG
449 treatment in cultured males. In contrast the seven spawns obtained in this study were
450 naturally achieved and appear to be clearly linked with the cohabitation with wild
451 spawning breeders. However, seven spawns does not represent a sufficient advance in
452 egg production and predictability in egg production for the aquaculture sector and the
453 underlying mechanisms must be examined to determine how this small, but significant
454 advance has been achieved.
455 The courtship displaying is directly related to the spawning success in this species
456 (Carazo et al., 2016; Martín et al., 2019). Four behaviours (Rest the head, Follow,
457 Guardian and Coupled) related to the courtship previously described by Carazo et al.
458 (2016) were analysed in the present study. Generally, the frequency of courtship
459 behaviours of cultured males and the total number of cultured males involved in courtship
460 increased significantly over the years of the study. One of the principal courtship

behaviours, the “Follow” behaviour was correlated with spawning success of males. The “Follow” behaviours have only been observed as part of the spawning season and represent one of the main behaviours involved in the first step of the courtship that preceded spawning (Carazo 3013, Carazo et al., 2016; Martín et al., 2019). In addition the follow behaviours were defined as a kind of display or competition, but without aggressive connotations, which agrees with other studies that identified Senegalese sole as a non-aggressive species (Salas-Leiton et al., 2008; Carazo et al., 2016; Fatsini et al., 2017b). The participation of cultured breeders in the “Follow” behaviours in both experimental groups increased significantly over years from 2013 through to 2016. It would appear that during the experimental period the cohabitation of cultured breeders with wild breeders that completed courtship and spawning facilitated the participation of cultured breeders and particularly males in the “Follow” behaviours and in the courtship in general.

This increasing participation could be associated with social learning like in other animal species. There are many processes through which social learning may occur, however in this case, the process could be associated with social transmission of learning (Thorpe, 1963; Kieffer and Colgan, 1992; Brown and Laland, 2003), where the knowledge is acquired by observing other animals. In the present study, from the moment cultured breeders were in the presence of spawning wild sole, the cultured breeders and especially cultured males started to perform the courtship. This process is called observational learning or contextual imitation (Lefebvre and Palameta, 1988; Brown and Laland, 2003). For example, Mazeroll and Montgomery (1995) reported in brown surgeonfish (*Acanthurus nigrofuscus*) that the fish that were following the leaders in local migrations imitated perfectly the route of leaders and even more the same postural changes. In this example, the social learning is associated with migration, however, swimming behaviours

are also implied in this process. Moreover, Brown (2001) demonstrated that chemical cues are important in learning and demonstrated the association between the chemical cues and experience acquired in relation to predation and danger. Therefore, cultured Senegalese sole males might have obtained new behavioural patterns through the observation of spawning wild males.

These new behavioural patterns or "Follow" behaviours were correlated to spawning success. However, the behavioural improvement may not have been fully expressed as spawning success, as mentioned the degree of cultured males spawning success was low. There would appear to be a negative mechanism, such as dominance or mate selection by reproductively successful wild breeders, which reduced the impact of this learning process to recruit cultured males to successfully participation in spawning. Generally, spawning was considered similar in the two mixed broodstocks over the years in terms of spawn numbers, however, the group M2 obtained a slightly higher numbers of spawns than M1 and a more varied contribution involving more individual breeders. In group M1, the contribution was dominated by a single wild male. In group M2, the participation in spawning was more varied amongst different breeders, perhaps indicating that the dominance effect was lower. This dominance and fidelity is common in Senegalese sole broodstocks, however, the reason why females choose particular males remains unknown and has become one of the main research lines to develop a breeding program for sole cultivation. Martín et al. (2014) found reproductive dominance by few couples and a fidelity of mating couples over years, a situation that has been also observed in this study showing the importance of mate choice with a crucial role of females in this species, preferably dominant by females. However, despite of the dominance by wild fish in group M2, there was a couple formed of cultured breeders in 2014 and 2016 and the same cultured male also spawned with two wild females, which had reproduced with another

wild male. In 2016, the removal of the previously dominant wild male (due to mortality) appeared to provide the opportunity for the cultured male to spawn with both a cultured female and wild females. These results showed the possibility of cultured males gaining dominance and contributing in reproduction over time, however, these results were not consistent over years, reinforcing the importance of mate choice of dominant females. No participation by cultured males was observed in group M1, where spawning was completely dominated by wild fish.

Mate choice copying is another social process that might explain our results, and which could be also involved in dominance in Senegalese sole reproductive behaviour. This process has been considered because of the low parental contribution, which has been observed in this species in the present study and previous studies conducted in Senegalese sole (Porta et al., 2006; Martín et al., 2014; Carazo et al., 2016). Mate choice copying can be defined as "an individual selecting a partner because others of the same sex were observed to have previously selected that individual as a partner" (Gibson and Hoglund, 1992). For example, Dugatkin (1992) showed using guppies (*Poecilia reticulata*) that one female considered as observer, chose the same male (there were two males in the same aquarium which did not have physical contact) that a model female considered as a demonstrator had been observed to choose. This behaviour has been observed in several fish species such as mollies (*Poecilia latipina*) (Schlupp et al., 1994) and gobies (*Pomatoschistus microps*) (Reynolds and Jones, 1999). Therefore, in the present study, the females that were prepared for spawning could have chosen the dominant male (either dominant in spawning or participation in the "Follow" behaviours) copying the choice of other females that successfully spawned.

The present study has also for the first time identified the sex of breeders participating in the "Follow" behaviours. This behaviour involves several individuals, usually males

following males, but females were also involved, usually occupying the "Leader" position (*see* Fig. 4 and 5) being followed or chased by males. This means, a female started swimming and one or several males started to follow, in this case chemical communication and specifically olfaction (Fatsini, et al., 2017) might be involved indicating that the female might be excreting or releasing some products through the body fluids to stimulate the courtship in those males also ready to reproduce. Fatsini et al. (2017) demonstrated that sole differentiated between origin, sex and maturity status through olfactory sensitivity to donor urine and intestinal fluids. The position of sole in the "Follow" behaviour was also significant. Interestingly, the males that dominated the "Following" positions also dominated spawning success (correlated) suggesting that this information may be used by females to select mates and could be used in aquaculture operations to identify success or conversely unsuccessful breeders. As found in other studies the "Follow" behaviour was the second most observed behaviour in the peak hour of activity in the days with spawning events, demonstrating that the increase of activity in the tank was due to the presence of this behaviour. All groups exhibited a circadian pattern in each of the four years studied. The peak hour of activity was registered from 19:00 to 20:00, coinciding with dusk, in the four - year period, showing the importance of photoperiod during the spawning season in this species. These results coincided with several studies previously performed with Senegalese sole species (Carazo et al. 2016, Oliveira et al., 2009¸ Martín et al., 2019) from different broodstocks. Other courtship behaviours ("Rest the head", "Guardian" and "Coupled") that were examined had similar significance as in other studies (Gibson, 2005; Carazo et al. 2016). The "Rest the head" and "Guardian" behaviours appeared to have aims towards mate selection and protection where the male gained acceptance to initiate the couple swimming (Carazo et al. 2016) and was similar to studies on largescale flounder courtship (Manabe et al., 2000) and

bothid species studied in the natural habitat (Gibson, 2005). The "Coupled" swim behaviour represented the act to fertilise gametes (Carazo et al., 2016) and as would be expected was only observed in the mixed groups giving further confirmation that courtship was only completed in these groups.

5. Conclusion

In conclusion, this is the first report of cultured breeders participating in the courtship and successful spawning. This participation was stimulated by the presence of spawning wild Senegalese sole breeders. Cultured Senegalese sole male breeders participated in the "Follow" behaviour in mixed-origin groups and this participation increased significantly over the years of the study. The "Follow" behaviour of males was correlated to participation in spawning and one cultured male fertilised a total of seven spawns. These observations could be controlled by underlying mechanisms of social learning, mate selection and dominance. However, these mechanisms may be conflicting. Different processes of social learning, such as observational conditioning and imitating, could be involved in the increased participation in courtship and spawning of the cultured male breeders, while dominance and mate selection may favour reproductively successful wild breeders to suppress the participation of cultured breeders. The present study appears to present a complex interaction suggesting that the behavioural reproductive dysfunction in male cultured sole could be solved by rearing cultured sole in the presence of successfully spawning Senegalese sole, but also by controlling or lowering dominance by reproductively successful breeders.

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Figure 1. Schematic representation of the spawning contribution from Senegalese sole (*Solea senegalensis*) breeders during each year (2013 – 2106). Figure **A** Depicts the spawning contribution of breeders in group M1 and **B** Depicts the spawning contribution from group M2 breeders. Legends: **blue circle** = wild males; **pink circles** = wild females; **blue square** = culture male; **pink square** = culture female. The number in parenthesis represents the spawns or events of each individual during that year out of all spawns registered. The couples are represented in series, i.e. the different females (wild or cultured) are indicated next to a male (wild or cultured) forming a couple for that specific year. The major contribution is denoted by larger size of the form.

Figure 2. Number of times an individual crossed a line in middle of the field of view of the camera that covered the entire length of the tank of Senegalese sole (*Solea senegalensis*) breeders during the different periods that included spawning (n = 5) and periods without spawning (n = 5) for each year and each experimental group studied (Control, M1 and M2). Data was shown in mean ± SEM. Asterisk denoted significant differences (One - Way ANOVA; $P < 0.05$) between days with and without spawning events or egg release.

Figure 3. Behaviour observed during the peak hour of activity (19:00 to 20:00) in periods with spawning (n = 5) for each experimental group (Control, M1 and M2). The mean frequency (counts) of the behaviour “Rest the head”; “Follow”; “Guardian” and “Coupled” swim were represented for each experimental group (Control, M1 and M2). An asterisk indicates significant differences among experimental groups when the number of behaviours (number of times a particular behaviour was observed in each

group) for different tanks and different years were compared running a mixed-effect Model Repeated - Measures ANOVA ($P < 0.05$) test.

Figure 4. Individual identification of the breeders implied in the "Follow" behaviour from M1 group for the four-year spawning period. **M** = male, **F** = female, **W** = wild breeders, **Cult** = cultured breeders. The grey section of the bar corresponds to the percentage by which the individual sole was occupying the "Leader" position and was followed by the other individuals and the green (for wild) and orange (for cultured) section of the bar represents the percentage by which individual sole were followers that followed the lead sole. The number above the bars represents the percentage of contribution of that individual in spawning according to paternity analysis. ***Insert on the right upper part of the figure:*** "Follow" behaviour in cultured Senegalese sole, presents the mean number of cultured individuals involved in each "Follow" behaviour ($n = 30$) for each year. Data are shown in mean ± SEM. Different letter denoted significant differences (One - Way ANOVA; $P < 0.05$).

Figure 5. Individual identification of the breeders implied in the "Follow" behaviour from M2 group for the four-year spawning period. **M** = male, **F** = female, **W** = wild breeders, **Cult** = cultured breeders. The grey section of the bar corresponds to the percentage by which the individual sole was occupying the "Leader" position and was followed by the other individuals and the green (for wild) and orange (for cultured) section of the bar resembles to the percentage by which individual sole were followers that followed the lead sole. The number above the bars represents the percentage of contribution of that individual in spawning according to paternity analysis. ***Insert on the right upper part of the figure:*** "Follow" behaviour in cultured Senegalese sole, presents

the mean number of cultured individuals involved in each “Follow” behaviour (n = 30) for each year. Data are shown in mean ± SEM. Different letter denoted significant differences (One - Way ANOVA; $P < 0.05$).

Figure 1

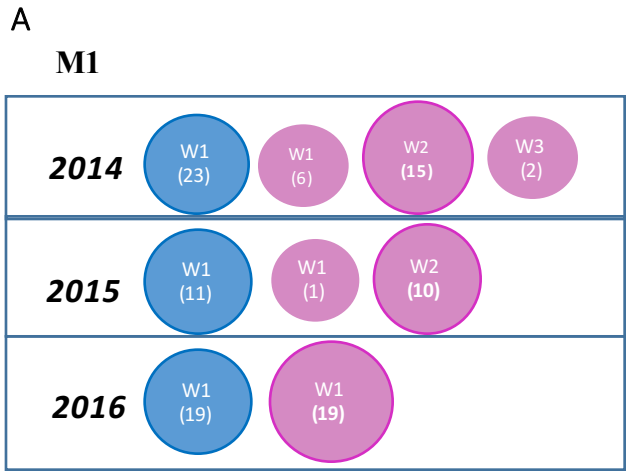

Figure 1

B

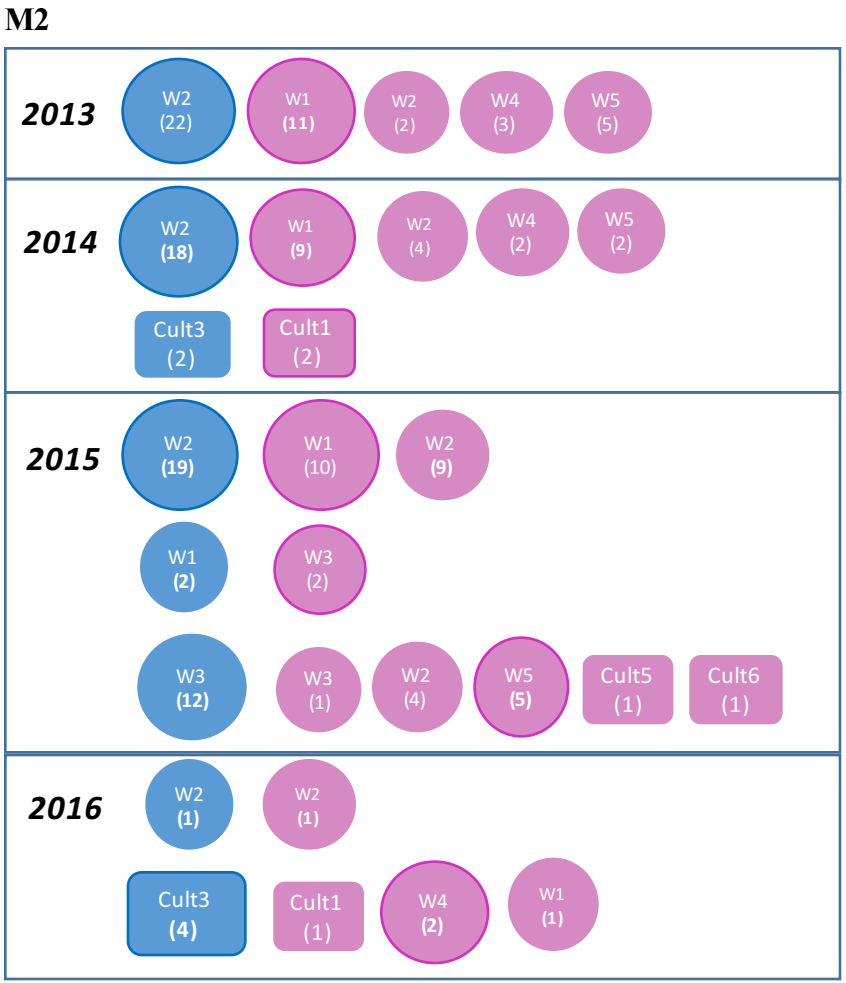
M2
2013
W2 (22)
W1 (11)
W2 (2)
W4 (3)
W5 (5)
2014
W2 (18)
W1 (9)
W2 (4)
W4 (2)
W5 (2)
Cult3 (2)
Cult1 (2)
2015
W2 (19)
W1 (10)
W2 (9)
W1 (2)
W3 (2)
W3 (12)
W3 (1)
W2 (4)
W5 (5)
Cult5 (1)
Cult6 (1)
2016
W2 (1)
W2 (1)
Cult3 (4)
Cult1 (1)
W4 (2)
W1 (1)

Figure 2

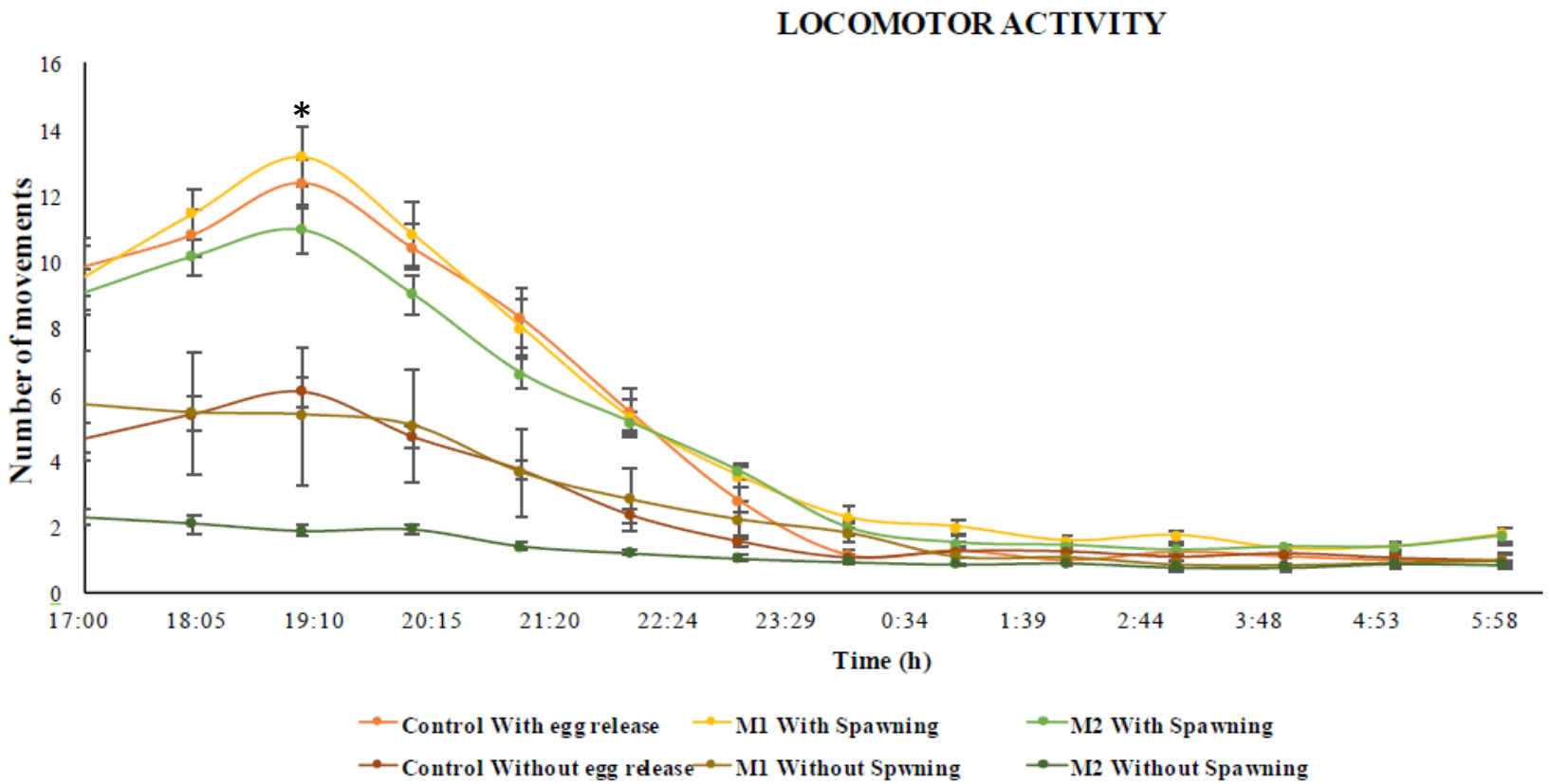
LOCOMOTOR ACTIVITY
Number of movements
16
14
12
10
8
6
4
2
0
*
17:00
18:05
19:10
20:15
21:20
22:24
23:29
0:34
1:39
2:44
3:48
4:53
5:58
Time (h)
Control With egg release
M1 With Spawning
M2 With Spawning
Control Without egg release
M1 Without Spwning
M2 Without Spawning

Figure 3

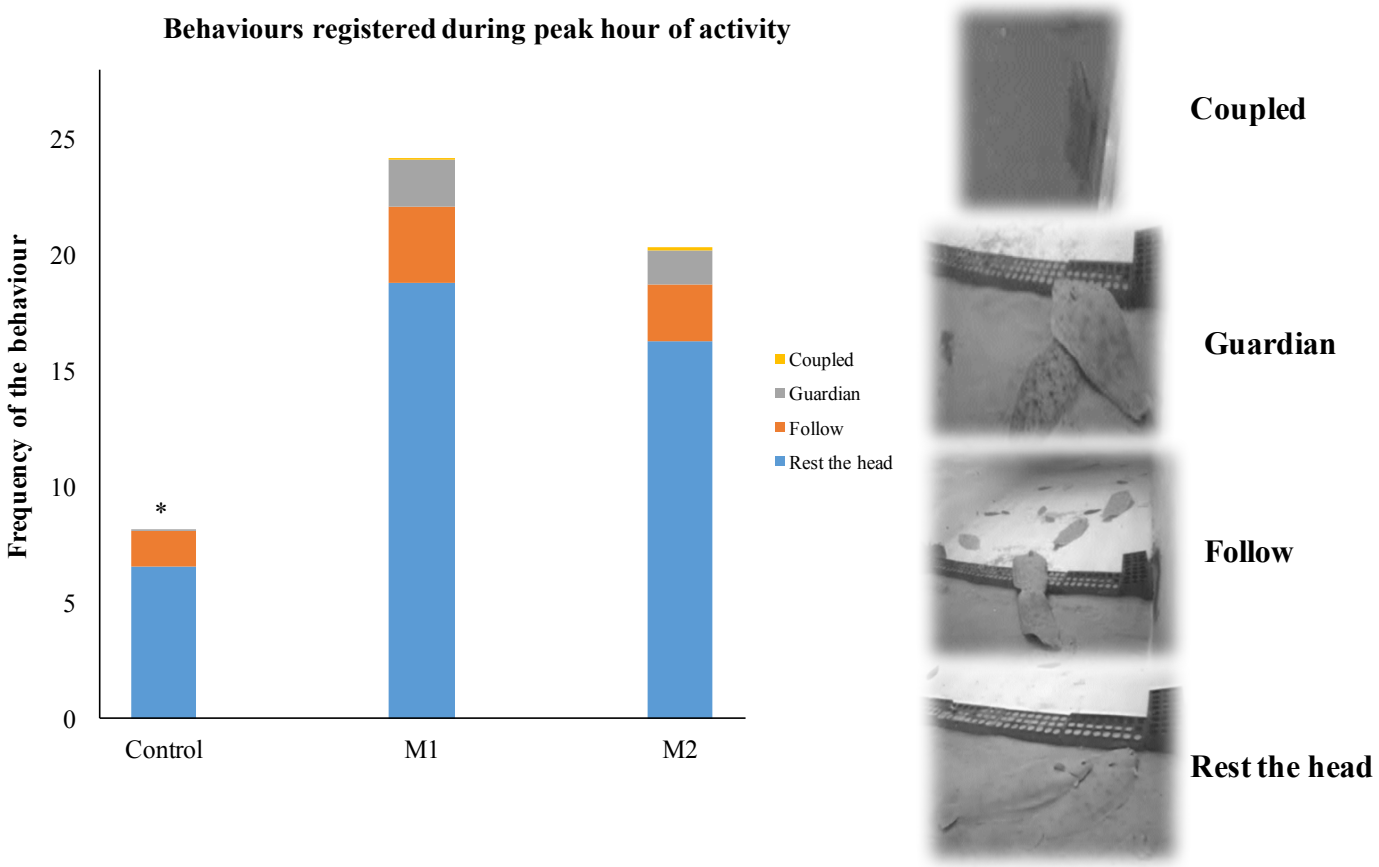
Behaviours registered during peak hour of activity
Frequency of the behaviour
25
20
15
10
5
0
*
Control
M1
M2
Coupled
Guardian
Follow
Rest the head
Coupled
Guardian
Follow
Rest the head

Figure 4

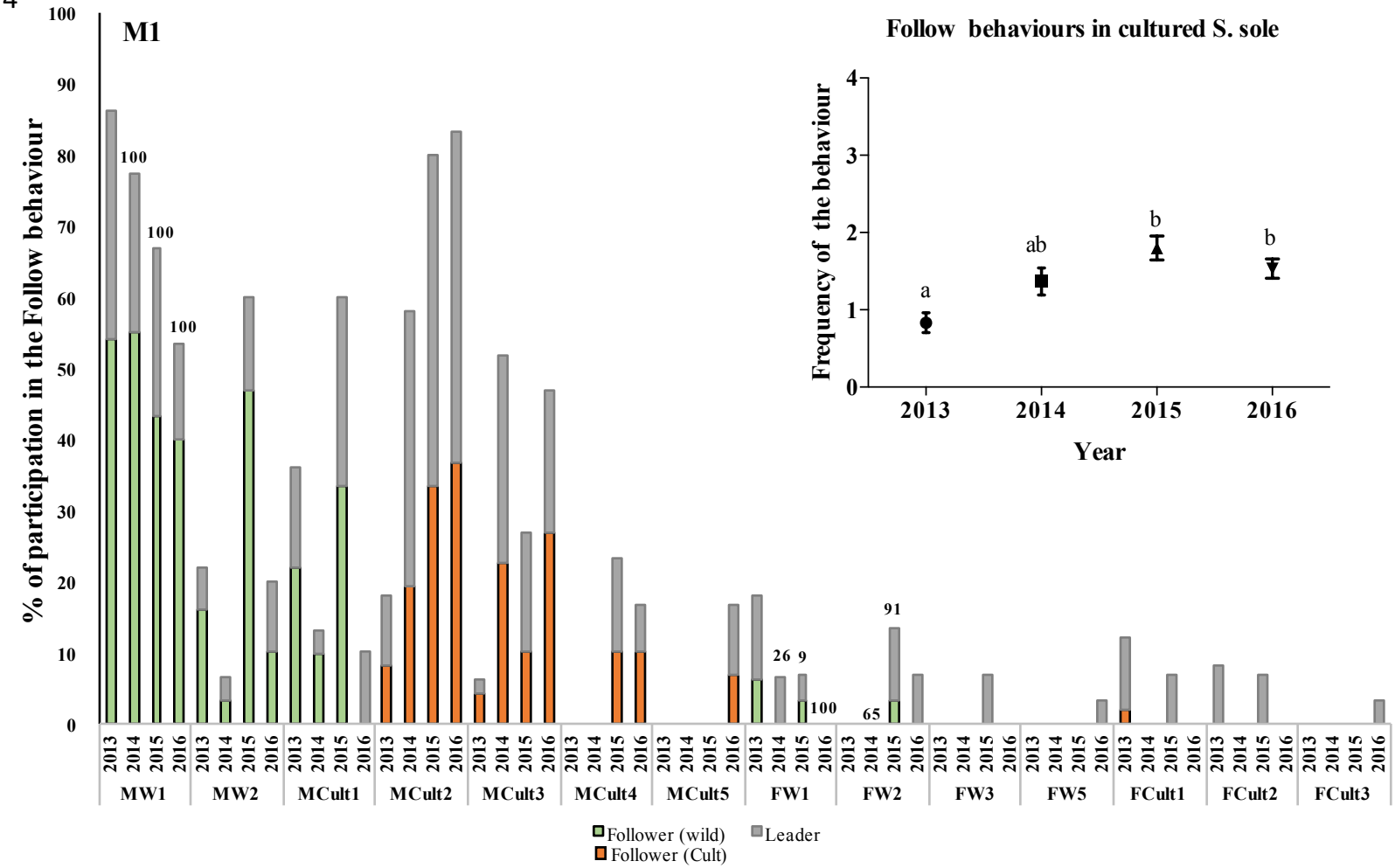
M1
% of participation in the Follow behaviour
100
90
80
70
60
50
40
30
20
10
0
100
100
100
26
9
100
65
91
2013
2014
2015
2016
MW1
MW2
MCult1
MCult2
MCult3
MCult4
MCult5
FW1
FW2
FW3
FW5
FCult1
FCult2
FCult3
Follower (wild)
Leader
Follower (Cult)
Follow behaviours in cultured S. sole
Frequency of the behaviour
Year
a
ab
b
b

Figure 5

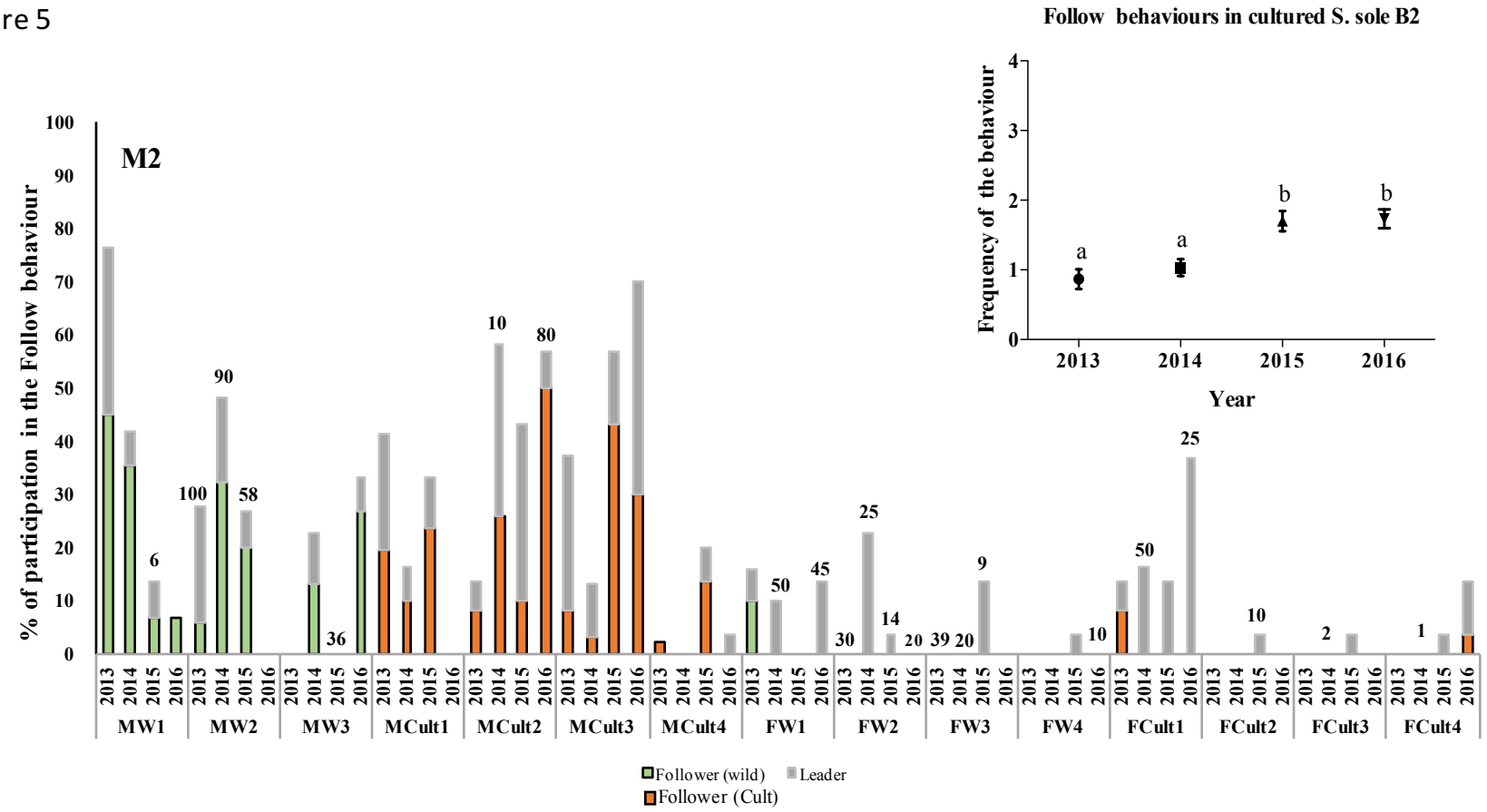
Follow behaviours in cultured S. sole B2
Frequency of the behaviour
Year
a
a
b
b
M2
% of participation in the Follow behaviour
MW1
MW2
MW3
MCult1
MCult2
MCult3
MCult4
FW1
FW2
FW3
FW4
FCult1
FCult2
FCult3
FCult4
Follower (wild)
Leader
Follower (Cult)

Table 1. Broodstock distribution and characteristics of the different tanks stablished for the monitoring during four consecutive spawning seasons, 2013 - 2016. Tank, N (number of breeders inside the tank), weight (mean ± SD), stock density, origin and sex.

Tank	N	Weight (g)	Stock density (kg/m^3)	Origin	Sex
Control	10	1165.3 ± 195.5	1.04	Cultured	5 Males 5 Females
M1	19	1146.9 ± 490.4	2.1	Cultured	5 Males 5 Females
				Wild	3 Males 6 Females
M2	18	1037.2 ± 456.3	1.5	Cultured	5 Males 5 Females
				Wild	3 Males 5 Females

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

Tank	Year	Total egg volume (ml)	Floating egg volume (ml)	Inviable egg volume (ml)	Egg Production (eggs/kg female)	Spawns (N - H)	Fertilization rate (%)	Hatching rate (%)	Number of hatched larvae
Control	2013	1,255	580	675	277,121	18 - 00	0.0	0.0	0
	2014	555	120	435	122,551	25 - 00	0.0	0.0	0
	2015	2,339	944	1,395	516,483	37 - 00	0.0	0.0	0
	2016	663	108	555	122,400	08 - 00	0.0	0.0	0
M1	2013	1,410	895	515	112,209	19 - 00	0.0	0.0	0
	2014	2,050	750	1,300	163,141	38 - 23	50.5 ± 7.6	29.4 ± 5.7	17,404 ± 3,913
	2015	3,851	1,301	2,550	306,468	46 - 11	33.8 ± 5.2	10.1 ± 3.6	6,977 ± 1,962
	2016	2,748	1,453	1,295	189,638	31 - 19	35.7 ± 5.9	23.1 ± 9.4	5,528 ± 2,082
M2	2013	4,595	3,000	1,595	443,287	37 - 23	73.0 ± 4.8	30.0 ± 0.1	29,631 ± 3,853
	2014	2,230	805	1,425	215,131	42 - 20	63.6 ± 4.9	36.3 ± 6.5	17,786 ± 4,052
	2015	5,489	1,799	3,690	529,532	47 - 33	32.8 ± 4.7	22.7 ± 9.2	9,475 ± 3,065
	2016	2,963	1,278	1,685	228,118	28 - 05	21.5 ± 5.1	5.2 ± 3.6	1,606 ± 1,100