

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

Elvira Fatsini^{1,2*}, Wendy González¹, Zohar Ibarra-Zatarain^{1,3}, Juvenal Napuchi¹ and Neil J. Duncan^{1*}

¹IRTA - SCR, Ctra. Poble Nou, km. 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain.

²Centre of Marine Sciences (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

³Centro Nayarita de Innovación y Transferencia Tecnológica (CENIT²), 63173, Tepic, Mexico.

*Corresponding authors: Elvira Fatsini, effernandez@ualg.pt and Neil Duncan, neil.duncan@irta.cat and

Neil John Duncan

IRTA - SCR,
Ctra. Poble Nou, km. 5.5,
43540 Sant Carles de la Ràpita,
Tarragona,
Spain
+34 977745427

neil.duncan@irta.cat

Elvira Fatsini Fernández

Centre of Marine Sciences (CCMAR)
Universidade do Algarve, Campus de Gambelas,
8005-139 Gambelas,
Faro,
Portugal
+351 289 800 900 ext. 7167

effernandez@ualg.pt

1 **Abstract**

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

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

31

32 Key Words: *Solea senegalensis*

33 Reproductive Behaviour

34 Social learning

35 Spawns

36 Origins

37

38

1. Introduction

40

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

49

50 Nevertheless, some issues exist that must be improved to optimise industrial Senegalese
51 sole production (Morais et al., 2016). One of the main problems that has limited
52 Senegalese sole production is a reproductive dysfunction in cultured breeders that were
53 bred and reared in captivity (Morais et al., 2016). This reproductive dysfunction resulting
54 in the failed spontaneous spawning of viable eggs from cultured breeders, means
55 Senegalese sole egg production has been based on the spawning of wild-origin breeders
56 (Dinis et al., 1999; Anguis and Cañavate, 2005; Martín et al., 2014), which is
57 unsustainable in a long-term. The frequency and volume of eggs obtained from captive
58 wild broodstock is enough for commercial production. However, a low participation of
59 breeders in spawning has been registered leading to a rapid loss of genetic variability
60 between generations (Porta et al., 2006). Parental analysis with microsatellites indicated
61 that spawning was dominated by a few wild breeders that represented only 8.5 - 51.7 %
62 of the broodstock (Porta et al., 2006; Carazo, 2013; Martín et al., 2014; Carazo et al.,
63 2016). In comparison to wild broodstocks, spontaneous spawning from cultured

64 broodstocks was less frequent and eggs did not hatch (Agulleiro et al., 2007; Guzman et
65 al., 2008; Howell et al., 2009; Norambuena et al., 2012; Rasines et al., 2012). However,
66 cultured breeders do produce viable gametes, which have been extracted with abdominal
67 pressure and successfully fertilised *in vitro* (Chereguini et al., 2007; Rasines et al., 2012;
68 2013). The cultured females were induced to ovulate with GnRH α before the viable eggs
69 were extracted and fertilized with cryopreserved sperm from cultured males (Rasines et
70 al., 2012; 2013). However, the artificial fertilization method is complicated due to the low
71 sperm volume found in Senegalese sole (Cabrita et al., 2006; Beirao et al., 2009; Cabrita
72 et al., 2011) and requires further research to implement on a commercial scale.

73

74 Carazo (2013) observed that the eggs from cultured breeders were not fertilized due to a
75 dysfunction in the reproductive behaviour or courtship. Spawning, courtship and mate
76 selection have been described in wild stocks held in captivity (Carazo, 2013; Carazo et
77 al., 2016). The courtship was characterised as a complex series of distinctive behaviours
78 associated to mate selection and gamete release, which were also registered as an increase
79 in locomotor activity (Carazo, 2013; Carazo et al., 2016). In comparison, these courtship
80 behaviours were less frequent or absent in cultured breeders and consequentially the ova
81 released by females were not fertilised (Carazo, 2013; Martín et al., 2019). Hormone
82 treatments, with either Gonadotropin-Releasing Hormone analogue (GnRH α) (Agulleiro
83 et al., 2006; Guzman et al., 2008; Guzmán et al., 2009; Carazo, 2013), combined GnRH α
84 and human Chorionic Gonadotropin (hCG) (Guzmán et al., 2011; Carazo, 2013) or
85 combined GnRH α and prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$) (Carazo, 2013) increased both the
86 number and frequency of eggs released by cultured breeders, but none of the treatments
87 increased the fertilisation success (Carazo, 2013). Fertilisation success has been increased
88 by setting up broodstocks of mixed origin (wild and cultured) both with (Mañanós et al.,

89 2007) and without hormonal treatment (Carazo, 2013; Martín, 2016; Martín et al., 2019).
90 These studies could be summed up as follows: cultured females treated with GnRHa
91 slow-release implants (Mañanós et al., 2007) or with no hormone treatment (Carazo,
92 2013; Martín, 2016; Martín et al., 2019) cohabiting with untreated wild males produced
93 fertilised spawns and the full sequence of courtship behaviours similar to that observed
94 in captive wild stocks (Carazo, 2013, Martín et al., 2019). However, cultured males (with
95 or without GnRHa treatment) cohabiting with untreated wild females did not produce
96 fertilised spawns and did not display courtship behaviour to fertilise eggs (Mañanós et
97 al., 2007; Carazo, 2013; Martín, 2016; Martín et al., 2019). In consequence, the spawning
98 failure was shown to be due to a behavioural reproductive dysfunction associated with
99 cultured males suggesting that environmental parameters (Anguis and Cañavate, 2005),
100 sperm quality (Cabrita et al., 2006) and hormonal treatment (Agulleiro et al., 2006;
101 Rasines et al., 2013) did not offer a possible solution to determine the triggers to promote
102 natural spontaneous spawning.

103

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

114 based on the results of parental assignation of larvae collected during the study period.
115 Understanding the evolution of these broodstocks during these years may help to improve
116 the management of this species under aquaculture conditions and give a better
117 understanding of the reproductive dysfunction of cultured Senegalese sole.
118

119 **2. Material and Methods**

120

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

125

126 **2.1 Broodstocks and management**

127

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

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

142 The different experimental groups were set up in October 2012. The experiment was
143 entirely performed in IRTA Sant Carles de la Ràpita and all experimental groups were
144 located in fibre-glass tanks of 10 m³ (2 m x 6 m x 0.85 m) included in a recirculation
145 system (IRTAmor®). The temperature regimen was controlled to simulate a natural cycle
146 that ranged from 9 – 20 °C and during the spawning season after a natural rise to 18 °C a
147 weekly temperature cycle was used to stimulate further spawning (Monday to Thursday
148 at 16 ± 1 °C and Thursday to Monday at 18 ± 1 °C as described in Martín et al. 2014).

149 The fish received a simulated natural photoperiod ranging from Light: Dark (LD) 9:15 to
150 LD 15:9 with approximately LD 12:12 to 14:10 during the spawning season. Daytime
151 lighting was delivered with fluorescent lighting and natural light from windows (50 lux
152 at surface) during the entire year. During the spawning season, red night lighting was
153 used that allowed recording and observation of the sole behaviour. Red light was from
154 fluorescent illuminations covered with a red filter that were adjusted to approximately 5
155 lux at the water surface. Carazo et al. (2013) demonstrated that this illumination system
156 did not affect sole behaviour, locomotor activity or plasma melatonin levels. Half of the
157 bottom of each tank was covered by sand. Breeders were fed *ad libitum* with
158 approximately 1 % of the total biomass five days a week at 09:30 h. The diet consisted
159 on fresh feed (cooked mussels, Sariego Intermares, Spain), marine polychaetes (Topsy-
160 Baits, Holland) and balanced feed (Repro-Vitalis, LE-7 mm ELITE, Skretting Co.).

161

162 *2.1.1 Experimental groups and experimental design*

163

164 Breeders were distributed in three experimental groups, Control group (C) constituted of
165 only cultured breeders and two experimental groups (M1 and M2) were mixed including
166 breeders from different origins, wild and cultured (Table 1). All cultured breeders used
167 for this study were from the same stock that had never successfully spawned. On the other
168 hand, wild breeders used for this experiment had spawned in captivity, however, the
169 individual identities of spawning fish were unknown. All groups were monitored
170 following Carazo et al. (2016) video recording analysis to evaluate the behaviour. Spawns
171 were collected, assessed and incubated and parental analyses were made of larvae (*see*
172 *details below*). Moreover, all groups were formed since October 2012 and approximately
173 30 cultured Senegalese sole juveniles (~ 100 g) provided by Stolt Sea Farm in May 2012
174 were cohabiting with breeders in each group at the moment the tanks were established.
175 The juveniles were removed from the experimental tanks in October 2015. In addition, in
176 2014 wild males were removed on the 1 May from the experimental tanks M1 and M2 in
177 order to enhance the participation of cultured males in the spawning and returned when
178 spawning period finished in June. There was a low incidence of mortality during the
179 whole experiment and no mortalities were registered in the control group.

180

181 **2.2 Spawns analyses**

182

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

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

199

200 **2.3 Paternity analyses**

201

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

210

211 **2.4 Behavioural analyses**

212

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.

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

244

245 *2.4.2 Behaviours registered during the peak of activity (courtship)*

246

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

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

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

266

267 *2.4.3 Identification of individuals in “Follow” behaviours*

268

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

281

282 **2.5 Statistical analyses**

283

284 All the results were presented with means \pm standard error (mean \pm S.E.M). Data were
285 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

336 the larvae analysed (180 larvae) from 18 of the 20 spawns registered. The same three wild
337 females were also assigned as mating with this wild male. The remaining larvae were
338 assigned to a cultured couple, which reproduced for the first time, the cultured male
339 (Mcult2) mated with the cultured female (FCult1). The third fertilised spawn was
340 obtained after 1 May 2014 when the wild males were removed. Paternal analysis did not
341 clearly identify two parents; however, it can be concluded that the father was a cultured
342 male. In 2015, three wild males contributed to hatching spawns, which included the same
343 male from previous years and two males that contributed for the first time. Wild females
344 assigned as the mothers of the larvae were the same females that reproduced in 2013 and
345 2014, however, there were two cultured females that participated for the first time, each
346 with one spawn. During 2015, no fertilized spawns were obtained from the cultured
347 couple that reproduced in 2014. In 2016, the wild male that dominated the spawning each
348 year died when spawning activity began and the first eggs were collected from the tank,
349 therefore, the contribution of the previously dominant male MW2 was 1 of the 5 spawns
350 obtained this year. The breeders that contributed were the same cultured male that
351 participated in 2014 (MCult2) with two wild females and one cultured female. Just four
352 fertilized spawns were collected, all spawns were fertilized by the cultured male that
353 spawned twice with a wild female (FW4) and once with the second wild female (FW2)
354 and once with a cultured female (FCult1), which was the same female which reproduced
355 in 2014.

356

357 **3.3 Behavioural analysis (courtship)**

358

359 *3.3.1 Activity*

360

361 The locomotor activity of the Senegalese sole breeders showed a circadian rhythm
362 associated with spawning in each group presenting repetition during each year (Fig. 2).
363 In general, activity peaked during early evening on all days, however, the mean number
364 of movements during the peak was significantly ($P < 0.01$) higher on days with spawning
365 compared to days without spawning. Activity during periods with spawning was
366 generally two-fold more compared to days without spawning. Activity with spawning
367 began to rise during the afternoon (from 14:00 in 2015, *data not shown*) achieving the
368 maximum at 19:00 and the minimum from 2:00 to 7:00. Thus, the peak hour of activity
369 was registered from 19:00 to 20:00 in all the tanks and each year activity decreased after
370 20:00.

371

372 *3.3.2 Behaviours registered during the peak of activity (courtship)*

373

374 The “Rest the head” behaviour represented the most common behaviour performed
375 during the peak hour of activity in all experimental groups (Fig. 3). The frequency of this
376 behaviour was significantly higher during all years in the two mixed origin groups, M1
377 (18.8 ± 3.6 ; $P = 0.001$) and M2 (16.2 ± 3.4 ; $P = 0.001$) compared to the Control group
378 (6.6 ± 2.0) (Fig. 3). However, no significant differences were observed between M1 and
379 M2 ($P = 0.99$). The “Follow” behaviour was the second most common behaviour
380 performed during the peak hour of activity (Fig. 3). This behaviour exhibited a similar
381 trend and was in general higher during all years in the two groups of mixed origin, M1
382 (3.3 ± 1.1 ; $P = 0.001$) and M2 (2.5 ± 0.7 ; $P = 0.001$) compared to the Control group (1.5
383 ± 0.3). The behaviours “Guardian” and “Coupled” swimming were observed to a lesser
384 extent and were not observed at all in the Control group (Fig. 3). The “Guardian”
385 behaviour was differentially observed in all years in the mixed origin groups, M1 ($2.0 \pm$

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 $12 \pm 7\%$ in 2013 to $35 \pm 14\%$ in 2016
415 (Fig. 4) and in group M2, the mean percentage of “Follow” behaviours increased from 24
416 $\pm 9\%$ in 2013 to $33 \pm 18\%$ 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

461 behaviours, the “Follow” behaviour was correlated with spawning success of males. The
462 “Follow” behaviours have only been observed as part of the spawning season and
463 represent one of the main behaviours involved in the first step of the courtship that
464 preceded spawning (Carazo 3013, Carazo et al., 2016; Martín et al., 2019). In addition
465 the follow behaviours were defined as a kind of display or competition, but without
466 aggressive connotations, which agrees with other studies that identified Senegalese sole
467 as a non-aggressive species (Salas-Leiton et al., 2008; Carazo et al., 2016; Fatsini et al.,
468 2017b). The participation of cultured breeders in the “Follow” behaviours in both
469 experimental groups increased significantly over years from 2013 through to 2016. It
470 would appear that during the experimental period the cohabitation of cultured breeders
471 with wild breeders that completed courtship and spawning facilitated the participation of
472 cultured breeders and particularly males in the “Follow” behaviours and in the courtship
473 in general.

474 This increasing participation could be associated with social learning like in other animal
475 species. There are many processes through which social learning may occur, however in
476 this case, the process could be associated with social transmission of learning (Thorpe,
477 1963; Kieffer and Colgan, 1992; Brown and Laland, 2003), where the knowledge is
478 acquired by observing other animals. In the present study, from the moment cultured
479 breeders were in the presence of spawning wild sole, the cultured breeders and especially
480 cultured males started to perform the courtship. This process is called observational
481 learning or contextual imitation (Lefebvre and Palameta, 1988; Brown and Laland, 2003).
482 For example, Mazeroll and Montgomery (1995) reported in brown surgeonfish
483 (*Acanthurus nigrofuscus*) that the fish that were following the leaders in local migrations
484 imitated perfectly the route of leaders and even more the same postural changes. In this
485 example, the social learning is associated with migration, however, swimming behaviours

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

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

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

518 Mate choice copying is another social process that might explain our results, and which
519 could be also involved in dominance in Senegalese sole reproductive behaviour. This
520 process has been considered because of the low parental contribution, which has been
521 observed in this species in the present study and previous studies conducted in Senegalese
522 sole (Porta et al., 2006; Martín et al., 2014; Carazo et al., 2016). Mate choice copying can
523 be defined as “an individual selecting a partner because others of the same sex were
524 observed to have previously selected that individual as a partner” (Gibson and Hoglund,
525 1992). For example, Dugatkin (1992) showed using guppies (*Poecilia reticulata*) that one
526 female considered as observer, chose the same male (there were two males in the same
527 aquarium which did not have physical contact) that a model female considered as a
528 demonstrator had been observed to choose. This behaviour has been observed in several
529 fish species such as mollies (*Poecilia latipina*) (Schlupp et al., 1994) and gobies
530 (*Pomatoschistus microps*) (Reynolds and Jones, 1999). Therefore, in the present study,
531 the females that were prepared for spawning could have chosen the dominant male (either
532 dominant in spawning or participation in the “Follow” behaviours) copying the choice of
533 other females that successfully spawned.

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

536 following males, but females were also involved, usually occupying the “Leader” position
537 (see Fig. 4 and 5) being followed or chased by males. This means, a female started
538 swimming and one or several males started to follow, in this case chemical
539 communication and specifically olfaction (Fatsini, et al., 2017) might be involved
540 indicating that the female might be excreting or releasing some products through the body
541 fluids to stimulate the courtship in those males also ready to reproduce. Fatsini et al.
542 (2017) demonstrated that sole differentiated between origin, sex and maturity status
543 through olfactory sensitivity to donor urine and intestinal fluids. The position of sole in
544 the “Follow” behaviour was also significant. Interestingly, the males that dominated the
545 “Following” positions also dominated spawning success (correlated) suggesting that this
546 information may be used by females to select mates and could be used in aquaculture
547 operations to identify success or conversely unsuccessful breeders. As found in other
548 studies the “Follow” behaviour was the second most observed behaviour in the peak hour
549 of activity in the days with spawning events, demonstrating that the increase of activity
550 in the tank was due to the presence of this behaviour. All groups exhibited a circadian
551 pattern in each of the four years studied. The peak hour of activity was registered from
552 19:00 to 20:00, coinciding with dusk, in the four - year period, showing the importance
553 of photoperiod during the spawning season in this species. These results coincided with
554 several studies previously performed with Senegalese sole species (Carazo et al. 2016,
555 Oliveira et al., 2009, Martín et al., 2019) from different broodstocks. Other courtship
556 behaviours (“Rest the head”, “Guardian” and “Coupled”) that were examined had similar
557 significance as in other studies (Gibson, 2005; Carazo et al. 2016). The “Rest the head”
558 and “Guardian” behaviours appeared to have aims towards mate selection and protection
559 where the male gained acceptance to initiate the couple swimming (Carazo et al. 2016)
560 and was similar to studies on largescale flounder courtship (Manabe et al., 2000) and

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

565

566 **5. Conclusion**

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

583 **6. Acknowledgements**

584

585 We thank J.L. Celades and other technical staff from IRTA for their technical support.
586 This study was funded by the Spanish National Institute for Agronomic Research (INIA)-
587 FEDER projects RTA2011-00050 and RTA2014-00048 (to N.D). The authors would like
588 to thank Dr Farinha for her advices. This study received Portuguese national funds from
589 FCT - Foundation for Science and Technology through project UID/Multi/04326/2019.

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