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1 **Senegalese sole (*Solea senegalensis*) coping styles are consistent over time:**
2 **behavioural and physiological responses during ontogenesis**

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Highlights

- Individual sole juveniles and breeders had consistent stress coping styles (SCS).
- Individual SCS was consistent across ontogenesis including changing maturity status.
- Proactive juveniles initiated puberty and matured before reactive juveniles.

35 **Abstract**

36 Individuals differ in how they cope with stressful situations along a behavioural
37 continuum, being proactive and reactive at the extremes of this continuum. Proactive
38 individuals are usually bold, highly active and take risks, while reactive organisms are
39 generally shy, exhibit low activity and avoid risky situations. Definitions of stress
40 coping styles state that proactive and reactive traits are consistent over time and across
41 contexts. The present study evaluated the individual differences in stress coping style,
42 physiological changes and reproductive status in Senegalese sole juveniles and
43 breeders over three and two-years, respectively. To determine stress coping style, the
44 fish were subjected to three individual (restraining, new environment, confinement)
45 and one group screening test (risk taking). Both groups were tested on three occasions,
46 juveniles were tested each year and adults were tested in the first year and twice (spring
47 and autumn) in the second year. On the third year, a proportion of the juveniles initiated
48 puberty and the reproductive status of all individuals was assessed and compared with
49 their behavioural responses. Results demonstrated individual differences that were
50 consistent with proactive and reactive traits in juveniles and breeders. Significant intra-
51 individual repeatability and consistency of juveniles and breeder's behavioural
52 responses were observed over time and across situations. In addition, glucocorticoid
53 levels (cortisol) were consistent for individuals. Another result to highlight was that
54 juveniles that past puberty and initiated gametogenesis had significant higher activity,
55 risk predisposition and lower plasma cortisol levels compared to fish that remained
56 immature (did not initiate puberty).

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59 **Keywords:** *Solea senegalensis*; coping styles; individual differences; consistency;
60 gametogenesis; breeders.

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69 **Abbreviation list**

- 70 NetActA = Total activity time inside the net in the air (juvenile sole)
- 71 NetActW = Total activity time inside the net in the water (breeders sole)
- 72 NetEscA = Total number of escape attempts from the net in air (juveniles sole)
- 73 NetEscW = Total number of escape attempts in water (breeders sole)
- 74 NewLat = Latency time to move in the new environment
- 75 NewAct = Total activity time of fish in the new environment
- 76 ConLat = Latency time to move in the confinement
- 77 ConAct = Total activity time of fish in the confinement
- 78 *restraining-PCSj* = Principal component scores in restraining for juveniles
- 79 *environment-PCSj* = Principal component scores in new environment for juveniles
- 80 *environment-PCSj* = Principal component scores in confinement for juveniles
- 81 *restraining-PCSb* = Principal component scores in restraining for breeders
- 82 *environment-PCSb* = Principal component scores in new environment for breeders
- 83 *environment-PCSb* = Principal component scores in confinement for breeders
- 84

85 **Introduction**

86 Individuals from the same population present different behavioural responses to a
87 stressful stimulus or novel context and the responses vary along a behavioural continuum
88 over which the extremes have been defined as proactive and reactive (Wilson et al., 1993;
89 Koolhaas et al., 1999). These different behavioural phenotypes have been commonly
90 referred as stress coping styles (SCS) (Koolhaas et al., 1999). The most significant
91 differences between proactive and reactive individuals are how the organism uses the
92 internal and external information to shape their behavioural response to the environmental
93 stimulus. Hence, proactive individuals tend to be bold, active, dominant, aggressive and
94 prone to take risks, while reactive organisms tend to be shy, exhibit lower levels of
95 activity, are less aggressive and avoid risky situations (Koolhaas et al., 1999; Sih et al.,
96 2004a; Brown et al., 2007). In addition, models have proposed that animals with proactive
97 behaviours tend to create fixed routines, while reactive individuals seem to easily adapt
98 to unpredictable environments (Benus et al., 1991; Koolhaas et al., 1999). In fish
99 physiology, the proactive strategy has been associated with low hypothalamus–pituitary–
100 interrenal (HPI) axis responsiveness, and hence producing lower levels of
101 glucocorticoids, while reactive fish present high HPI axis reactivity and produce higher
102 levels of glucocorticoids (Øverli et al., 2007; Koolhaas et al., 2010) both under basal and
103 stressful situations.

104 To date, the existence of SCS have been confirmed in a number of taxa, such as
105 birds (Dingemanse et al., 2002), mammals (Fernández et al., 2009) and fish (see reviews
106 of Toms et al., 2010; Conrad et al., 2011; Castanheira et al., 2015). Individual coping
107 style has been suggested to influence social relationships, reproduction, social dynamics,
108 and many other physiological and behavioural aspects of an individual's life fitness that
109 can have profound costs or benefits depending upon environmental contexts (Dingemanse
110 and Réale, 2005; Smith and Blumstein, 2008; Mittelbach et al., 2014; Castanheira et al.,
111 2015). Indeed, SCS may be repeatable (*e.g.* refers to a stable individual behaviour through
112 time), consistent (*e.g.* refers to the predictability of repeated measures within individuals)
113 and correlated (*e.g.* refers to individual consistency across different situations or contexts)
114 over periods of time and across contexts (for further detail of definitions see Dall et al.,
115 2004; Sih et al., 2004b; Réale et al., 2007; Bell et al., 2009). Measuring the repeatability
116 and consistency of coping styles is of importance when evaluating the behaviour of
117 animals in novel environments, open field or risky situations since environmental factors
118 have been observed to potentially mask individual behavioural differences (Martin and

119 Réale, 2008). Hence, one way to reduce this slant is to repeat tests several times
120 individually to reliably estimate the intra-individual behavioural variation and once this
121 intra-individual variation has been established the behavioural variation can be reliably
122 assessed (Dingemanse et al., 2002). Being able to forecast whether individuals in a group
123 behave predictably over a certain period of time would be valuable for diverse areas, such
124 as behavioural ecology, conservation biology or aquaculture, since it could increase the
125 possibility to characterize individual status (*e.g.* dominance, growth, reproduction) and
126 could provide information to create suitable habitats for individuals. To date, several
127 studies have investigated the repeatability and consistency of coping style behaviours
128 over time and across different tests or situations in several fish species (Cummings and
129 Mollaghan, 2006; Millot et al., 2009; Chervet et al., 2011; Rey et al. 2013, Boulton et al.,
130 2014; Ferrari et al., 2015). However, the majority of previous studies have investigated
131 fish behavioural traits over a relatively short (days - weeks) and intermediate (week -
132 months) time periods, and only a few studies have been carried out over long time periods
133 (close to a year or more) and have evaluated repeatability (Rey et al., 2013; Biro and
134 Adriaenssens, 2013; Ferrari et al., 2015).

135 Senegalese sole (*Solea senegalensis*), is a flatfish species of high commercial
136 value that has been demonstrated to exhibit proactive and reactive coping styles, with
137 significant differences in activity, risk taking and HPA axis responsiveness (Mota-Silva
138 et al., 2010; Martins et al., 2011; Ibarra-Zatarain et al., 2016). To date, no information is
139 available on the temporal behavioural repeatability or consistency in this fish species for
140 juveniles or adults. Therefore, this work evaluated the repeatability and consistency of
141 Senegalese sole juveniles and breeders across different contexts (three individual and one
142 group tests) and over a long-time period (juveniles tested three times in three consecutive
143 years and breeders tested three times in two years). The aims of the present study were to
144 **a)** investigate the intra-individual behavioural repeatability and consistency of juveniles
145 and breeders over time and across contexts, and **b)** compare the behavioural phenotypes
146 over time between juveniles of the same year class that started gametogenesis early
147 (entered puberty) and those that not initiated gametogenesis (pre-pubescent).

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153 **Materials and methods**

154 **Ethic statement**

155 All experimental work in this study complied with the Spanish and European regulations
156 on animal welfare (Federation of Laboratory Animal Science Associations, FELASA)
157 and was approved by the Animal Ethics Committee of IRTA, Spain.

158

159 **Experimental animals, housing and feeding**

160 Sixty-one Senegalese sole juveniles and fifty-nine breeders were used as experimental
161 animals. Sole juveniles presented an initial average weight of 45.6 ± 1.8 g and length of
162 15.2 ± 0.2 cm, while breeders initial average weight was 1238 ± 55.2 g and length $45.8 \pm$
163 0.6 cm. Juveniles were housed in three 0.5 m^3 square tanks (1 m length x 1 m wide x 0.5
164 m depth), while four 13 m^3 tanks (6 m length \times 3 m wide \times 0.9 m depth) were used for
165 breeders and both systems were in a greenhouse structure. A recirculation system
166 (IRTAMAR[®]) with a daily total water exchange rate of 50 % day⁻¹ was used to maintain
167 optimal water parameters for both groups of fish (T = 18 - 21 °C; O₂ = 5 - 6 mg/L). The
168 IRTAMAR[®] recirculation system included sensors that continually measured and
169 registered temperature (Genebre, Barcelona, Spain) and oxygen (OxyGuard, Farum,
170 Denmark) and in addition daily oxygen levels were checked and registered each morning
171 with an oximeter (Oxi3205, Wissenschaftlich-Technische Werkstätten, Germany).
172 Juveniles were fed *ad libitum* twice a day (10:00 and 15:00 h) on a commercial balanced
173 diet (Elite LE-2mm, Skretting, Co.), while the breeders feeding regime incorporated also
174 non-processed fresh food and was as follows: Monday: dry pellet balanced fish feed
175 (Vitalis Repro-7 mm and LE-7 ELITE, Skretting Co.), Wednesday: cooked mussels
176 *Mytilus edulis* (Sariego Intermares, Spain) and Friday: frozen marine polychaetes
177 *Nereisvirens* (Topsy-Baits, Wilhelminadorp, Holland). One hour after feeding, uneaten
178 food was removed from tanks to maintain optimal physicochemical conditions.

179 All juveniles and adult fish were PIT-tagged (11.5 mm x 2.5 mm diameter; ID-
180 100 Unique, Trovan-Zeus, Madrid, Spain) for individual identification.

181

182 **Experimental procedures**

183 Three runs of coping styles tests were performed (supplementary figure 1). Each run
184 started and finished at the same hour and the same material was used (*i.e.* tanks, nets,
185 etc.). The stress assays consisted in three individual (restraining, new environment and
186 confinement) and one grouping test (risk taking) for both groups (juveniles and breeders).

187 Individual tests were performed in the same day, one after another, while the risk-taking
188 test was realized one month later to allow fish to recover (detailed below, supplementary
189 figure 1). After each set of individual behavioural tests, the blood was extracted from all
190 fish, both in year 1 and 3 for juveniles and in year 1 and 2 for breeders, to quantify plasma
191 levels of cortisol, glucose and lactate (see below) from both juveniles and breeders. At
192 the end of the third run, the sex and the gonadal maturity of juveniles were assessed
193 following the methodology of Anguis and Cañavate (2005).

194 a) In juveniles, the restraining and confinement tests were performed in year 1 (run
195 1), 2 (run 2) and 3 (run 3), the new environment test in year 1 (run 1) and 3 (run
196 3) and the risk-taking tests in year 1 (run 1) and 3 (run 3) (supplementary figure
197 1).

198 b) In breeders, the restraining and confinement tests were performed in year 1 –
199 autumn - (run 1), year 2 – spring - (run 2) and year 2 – autumn - (run 3), the new
200 environment test in year 1 –autumn - (run 1) and year 2 – autumn - (run 3) and the
201 risk-taking tests in year 1 –autumn - (run 1) and year 2 – autumn - (run 3).

202 c) The blood collection was performed in year 1 (run 1) and 3 (run 3) in juveniles
203 and in year 1 –autumn - (run 1) and 2 –autumn - (run 3) for breeders
204 (supplementary figure 1).

205 d) Female stage of oogenesis was estimated by the degree of ovarian swelling as
206 follow: stage I the ovary was detected by touching the ventral area of the female;
207 stages II and III was reached when different degrees of gonad swelling were
208 visible externally (initial and intermediate, respectively), and fish were in stage
209 IV when maximum ovarian swelling was observed as a result of oocyte hydration
210 (from Anguis and Cañavate 2005). Males with gametogenesis were identified by
211 applying gentle pressure on the abdomen to obtain a small amount of milt and the
212 percentage of motile spermatozoa was evaluated with a microscope following the
213 methodology described by Fauvel et al. (2010).

214

215 **Test 1. Restraining test**

216 The behavioural responses of juveniles were evaluated by holding each organism in a net
217 out of the water for 90 s, while the behaviour of breeders was determined in a net inside
218 of the water for the same period. Tests were adapted from Martins et al. (2011),
219 Castanheira et al. (2013) and validated by Ibarra-Zatarain et al. (2016) for Senegalese
220 sole. Two variables were measured in both groups: **i)** the total activity time within the net

221 and in the air for juveniles (**NetActA**), and within the net in the water for breeders
222 (**NetActW**), and **ii**) the total number of escape attempts from the net, in the air for
223 juveniles (**NetEscA**) and in the water for breeders (**NetEscW**).

224

225 **Test 2. New environment test**

226 Each fish was placed for five minutes in a plastic tank (56.5 x 36.5 x 30 cm – 50 L - for
227 juveniles and 114 x 95 x 57 cm – 650 L - for breeders) that simulated a new environment.
228 Tests were adapted from Wilson and Godin (2009), Martins et al. (2012), Carter et al.
229 (2013) and Ibarra-Zatarain et al. (2016). Two parameters were measured for juveniles and
230 breeders: **i**) the latency to move, **NewLat**, considered as the first moment that fish started
231 to explore the new environment and **ii**) the total activity time, **NewAct**, being the total
232 time that each fish spent swimming forward in the tank. If fish did not move at all during
233 the 5-minutes period (freezing), then 300 s (maximum time of the test) was recorded as
234 **NewLat** for further statistical analysis (Farwell and McLaughlin, 2009; Ibarra-Zatarain
235 et al., 2016). To cause minimal disturbance to fish, observers stood stationary 1 m away
236 from the container to avoid disturbing the fish.

237

238 **Test 3. Confinement test**

239 Fish were individually placed for five minutes in a plastic tank with reduced dimensions
240 (25 x 14 x 8 cm – 5 L - for juveniles and 56 x 36 x 30 cm – 25 L - for breeders) that
241 simulated a confinement situation. Tests were adapted from Brelin et al. (2005), Ruiz-
242 Gomez et al. (2008), Kittilsen et al. (2009) and validated by Ibarra-Zatarain et al. (2016)
243 for Senegalese sole. Two parameters were registered for juveniles and breeders: **i**) the
244 latency time to move, **ConLat**, considered as the first moment that fish started to move
245 and **ii**) the total activity time, **ConAct**, restricted to active locomotion in the confinement
246 container. If fish did not move during the test, then 300 s was recorded as **ConLat** for
247 further statistical analysis (Farwell and McLaughlin, 2009; Ibarra-Zatarain et al., 2016).
248 Observers stood stationary 1 m away from the container to not disturb fish.

249

250 **Test 4. Risk taking test in groups**

251 This test was performed on juveniles and breeders under the same behavioural criteria,
252 one month after finalizing individual tests. This test aimed to determine fish willingness
253 to cross from a known area, or safe zone, to an unknown area, or risky zone (adapted from
254 Huntingford et al., 2010, Carter et al., 2013; Herrera et al., 2014; Ferrari et al., 2015 and

255 validated by Ibarra-Zatarain et al., 2016 for Senegalese sole). The safe zone was isolated
256 from light (2 and 3 lux at the surface for juveniles and breeders, respectively) and the
257 bottom covered with sand, to simulate a safe space for fish (similar to their natural
258 environment). On the contrary, the risky zone was more illuminated (15 and 11 lux at the
259 surface for juveniles and breeders, respectively) and devoid of sand. For juveniles, a 500
260 L tank (1 m length x 1 m wide x 0.5 m depth) was divided into two equal zones by a rigid
261 plastic screen and a window (5 cm high x 20 cm width) was located at the bottom of the
262 screen, with a door allowing fish to cross between both areas. For breeders, the test was
263 performed in a 16 m³ tank (6 m length x 3 m wide x 0.9 m depth), divided into two equal
264 areas by a solid wooden screen. A window (30 cm width x 15 cm depth) was opened at
265 the base of the screen covered by a sliding door that could be removed to allow fish to
266 pass from one area to another. The windows in the divisions were placed at the centre of
267 a reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that was employed to
268 read the tag numbers of fish that passed through the window. To corroborate information
269 from the reading antenna, a submersible camera was installed in both the safe and risky
270 zone and videos checked to ensure registered fish crossed (square black and white CCD
271 camera, model F60B/NIR580-50G Korea Technology and Communications Co. Ltd.,
272 Korea supplied in waterproof housing by Praentesis S.L., Barcelona).

273 Before starting the test, both stages, juveniles and breeders were acclimated 24-
274 hours in the safe zone, by keeping windows closed until the beginning of the test, which
275 started at 10:00 hours and lasted 24 hours. Juveniles were tested in groups of 15
276 individuals and breeders in groups of 10 individuals, to avoid stress induced by high
277 stocking densities. Fish that successfully crossed from the safe to the risky zone were
278 defined as proactive, while fish that did not cross were labelled as reactive, considering
279 criteria given by Huntingford et al. (2010), Rey et al. (2013), Tudorache et al. (2013) and
280 Ibarra-Zatarain et al. (2016). The total latency time of each individual to cross from one
281 area to another was also recorded.

282

283 **Blood plasma analysis**

284 In order to compare and determine a possible correlation between blood parameters and
285 SCS, blood was sampled from each juvenile and breeder, to quantify cortisol, glucose and
286 lactate levels. To avoid blood coagulation, needles and syringes were coated with heparin.
287 In addition, the blood samples were mixed with 10 µl of heparin (5%, 25.000 UI;
288 HOSPIRA) and 15 µl of aprotinin (from bovine lung; 0.9% NaCl, 0.9% benzyl alcohol

289 and 1.7 mg of protein; SIGMA) in a 1.5 ml Eppendorfs. Blood samples were centrifuged
290 (M23i, ThermoScientific) at 3000 G and 4 °C during 15 min and plasma supernatant was
291 removed and stored by triplicates at -80 °C prior to analysis. Cortisol levels were
292 measured by means of a competitive conjugated binding ligand with a commercial ELISA
293 kit (Range of detection: 0 - 800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany), whereas
294 glucose and lactate concentrations were measured by means of commercial enzymatic
295 colorimetric kits (SPINREACT, Gerona, Spain) and both analysis were performed
296 following manufacturer's instructions. Cortisol, glucose and lactate absorptions were read
297 using a spectrophotometer (Infinite M-200; TECAN, Switzerland) at 23 °C and 505 nm
298 and plotted on a standard curve to determine their concentration levels.

299

300 **Statistical analysis**

301 All statistical analyses were performed using SPSS 20.0 software for Windows (IBM).
302 Values were presented as means \pm standard error of the mean (SEM). For all analysis, the
303 significance level for statistical difference was $P < 0.05$. Data were checked for normality
304 through Kolmogorov Smirnov test with Lilliefors correction and for homogeneity of
305 variances through a Levene's test. All data was normally distributed with homogeneity
306 of variances. First, three principal components analysis (PCA) were successively
307 performed on: i) NetAct and NetEsc from the restraining test; ii) NewLat and NewAct
308 from the new environment test and iii) ConLat and ConAct from the confinement test.
309 For each PCA, the variable that explained the highest variance and showed eigenvalue
310 over 1 (based on Kaiser-Guttman criterion) was the most representative variable of each
311 test performed and was retained to represent the composite behaviour of each organism,
312 also called individual Principal Component Score (PCS) for each test. Thus, the variables
313 selected for juveniles were: NetEscA (eigenvalue = 4.43, variance = 73.9 %, defined as:
314 *restraining-PCSj*), NewLat (eigenvalue = 2.85, variance = 71.2 %, defined as:
315 *confinement-PCSj*) and ConLat (eigenvalue = 4.36, variance = 72.8 %, defined as:
316 *environment-PCSj*). For breeders, the selected factors were: NetEscW (eigenvalue = 3.04,
317 variance = 50.8 %, defined as: *restraining-PCSb*), NewLat (eigenvalue = 2.53, variance
318 = 63.4 %, defined as: *environment-PCSb*) and ConLat (eigenvalue = 2.86, variance = 48.0
319 %, defined as: *confinement-PCSb*). The correlation coefficient between blood parameters,
320 fish morphometric parameters and each PCS for juveniles and for breeders were analysed
321 with a Pearson's correlation analysis.

322 Second, differences in behavioural responses of juveniles and breeders for new
323 environment, confinement and cortisol, glucose and lactate levels from runs 1 to 3 were
324 assessed by performing a General Linear Model with a Multivariate Repeated Measures
325 analyses of variance (GLM-RM MANOVA), with a Wilk's lambda criterion and Fisher's
326 exact test, including general. GLM-RM ANOVA analyses were performed separately for
327 the restraining test for juveniles and breeders, since total activity and escape attempts
328 variables were measured differently in both groups (in the air and inside the water).
329 Significant differences in the behavioural response of individuals among the different
330 runs supported the interpretation for a high intra-individual variability. When no
331 significant differences were found the relationship between data sets was further
332 examined to determine the existence of low intra-individual variability or repeatability of
333 a behavioural trait within individuals. Low intra-individual variability was indicated by
334 the reliability-consistency test, with an Alpha Cronbach's (α_C), Fisher tests and Intra-
335 class correlation coefficient (ICC), which was performed on responses of juveniles and
336 breeders over time and for each individual tests and blood parameters. An α_C value over
337 0.7 and *P*-values below 0.05 for the behavioural responses of juveniles and breeders
338 among the three runs indicated high inter- and intra-behavioural correlation and
339 consistency. In addition, the parameters from different runs were compared with a
340 Pearson's correlation analysis and a correlation coefficient, *R*, over 0.7 and *P*-values
341 below 0.05 indicated repeatability.

342 Third, two general linear model (GLM-MANOVA) analyses were performed: i)
343 to compare the three PCS of juveniles with and without gametogenesis, and ii) to compare
344 the three PCS of fish that crossed and that did not cross in the risk-taking test.
345 Additionally, a Chi-square test, with a Phi and Cramer's nominal analysis, was performed
346 in the risk-taking test to evaluate whether the proportion of fish that crossed in run 1 was
347 similar to the proportion of fish that crossed in run 2, for juveniles and breeders. Then,
348 the ability to take risk of juveniles, in the risk-taking test, was compared between
349 proportions of fish with and without gametogenesis, by means of a Chi-square test.

350

351 **Results**

352 Senegalese sole juveniles and breeders exhibited behavioural tactics that resembled
353 proactive and reactive coping styles as was previously demonstrated for this species
354 (Ibarra-Zatarain et al., 2016). The SCS ranged from proactive individuals, with high
355 activity and low plasma cortisol levels that crossed to the risky zone, to reactive

356 individuals with low activity and high plasma cortisol levels that remained in the safe
357 zone. Therefore, the consistency and repeatability over time and context was examined
358 for both the classified SCS and the behavioural and physiological parameters tested.

359

360 **Behavioural responses of juveniles**

361 *Repeatability (over time)*: Altogether, comparisons of the behavioural responses between
362 runs converged to the conclusion that SCS behavioural responses of Senegalese sole
363 juveniles showed repeatability over time. The behavioural parameters for restraining, new
364 environment and confinement tests were not significantly different for Senegalese sole
365 juveniles among runs 1 to 3 (Table 1 and 2). The Alpha-Cronbach's reliability test and
366 the Pearson's analysis confirmed a high correlation between performed tests over time
367 (Table 3 and supplementary table 1). Performed statistical tests supported the suggestion
368 that Senegalese sole juveniles show behavioural repeatability. However, juveniles varied
369 in the plasma levels of cortisol, glucose and lactate (Table 2, supplementary table 1). The
370 number and proportion of fish that crossed from the safe to risky area was similar ($P =$
371 0.501) in both runs (Table 1). The percentage of individual fish that repeated the same
372 response to the risk test was 77% (14 crossed and 33 did not cross in both tests) suggesting
373 a high intra individual repeatability

374

375 *Consistency (between context or situations)*: Juveniles that successfully crossed presented
376 significantly higher scores for *restraining-PCSj* ($F_{1,54} = 5.14$ and $P = 0.027$ in run 1 and
377 $F_{1,54} = 3.08$, $P = 0.033$ in run 3, Figure 1) and lower scores for *confinement-PCSj* ($F_{1,54}$
378 $= 10.87$ and $P = 0.002$ for run 1 and $F_{1,54} = 3.66$ and $P = 0.029$ for run 3, Figure 1) than
379 juveniles that did not cross, in both runs. For *environment-PCSj*, no significant
380 differences were observed between juveniles that crossed and those that did not cross in
381 run 1, while juveniles that crossed in run 3 showed significantly lower scores than
382 juveniles that did not cross ($F_{1,54} = 4.57$, $P = 0.025$) (Figure 1). Overall, juveniles that
383 took higher risk exhibited greater activity and lower cortisol levels, when compared to
384 fish that did not cross, and this pattern were according to SCS definition.

385

386 **Behavioural responses of breeders**

387 *Repeatability (over time)*: By analysing parameters with the different statistical models,
388 Senegalese sole breeders were evidenced to show similar behavioural responses among
389 runs, as documented in juveniles. Overall, breeders in the different runs presented no

390 significant differences (Table 1 and 2), high intra-class correlation (ICC) and a high
391 degree of correlation (Table 3 and supplementary table 1). Further, cortisol, glucose and
392 lactate levels were stable over time (Table 2). Performed statistical tests supported the
393 conclusion that Senegalese sole breeders show behavioural repeatability. The number and
394 proportion of fish that crossed from the safe to risky area was similar ($P = 0.059$) in the
395 two tests, run 1 and run 3 (Table 1). The percentage of individual fish that repeated the
396 same response to the risk test was 83% (13 crossed and 36 did not cross in both tests)
397 suggesting a high intra individual repeatability.

398

399 *Consistency (between context or situations)*: Breeders that successfully crossed presented
400 significantly higher scores for *restraining-PCSb* ($F_{1,55} = 3.56$ and $P = 0.036$ in run 1 and
401 $F_{2,55} = 3.25$ and $P = 0.042$ in run 3) and lower scores for *environment-PCSb* ($F_{1,55} = 3.18$
402 and $P = 0.047$ in run 1 and $F_{2,55} = 3.90$, $P = 0.026$ in run 3), however, no significant
403 differences were detected for *confinement-PCSb* neither in run 1 nor in run 3 (Figure 2,
404 first and second row). Fish that successfully crossed showed significant lower basal levels
405 of cortisol concentrations in plasma than fish that did not cross (supplementary table 3).
406 Similar to juveniles, breeders that took risk were comparable to proactive behaviours and
407 breeders that did not cross with reactive behaviours, since their differences in activity,
408 risk and cortisol levels.

409

410 **Relationship between SCS and gametogenesis**

411 Twenty-two of sixty-one juveniles showed gametogenesis (11 females and 11 males).
412 Furthermore, four of the eleven females were found in stage 1 and seven in stage 2, while
413 nine of the eleven males presented 20% of motile sperm cells and two showed 10% of
414 motile sperm cells. In addition, juveniles with gametogenesis were significantly heavier
415 and larger ($F_{1,54} = 4.25$, $P = 0.008$ and $F_{1,54} = 3.58$, $P = 0.022$, respectively) than juveniles
416 without gametogenesis (supplementary table 2). The PCS of juveniles with
417 gametogenesis were significantly higher than fish without gametogenesis for *restraining-*
418 *PCSj* ($F_{1,54} = 3.93$, $P = 0.038$) and lower in *confinement-PCSj* ($F_{1,54} = 4.27$, $P = 0.026$),
419 but they did not differ for *environment-PCSj* ($F_{1,54} = 0.38$, $P = 0.538$) (Figure 1, first
420 row). Moreover, fish that had gametogenesis (in run 3) showed significantly lower
421 cortisol levels (half less) in run 1 ($F_{1,54} = 2.67$, $P = 0.042$) and in run 3 than fish without
422 gametogenesis (supplementary table 2). Interestingly, eighteen fish of twenty-two with
423 gametogenesis (81.2 %) crossed in the risk-taking test (in both runs 1 and 3) and none of

424 the fish without gametogenesis crossed. The Chi-square test detected significant
425 differences in fish disposition to take risk between the proportion of individuals with and
426 without gametogenesis ($X^2 = 13.21$, $df = 1$, $P = 0.021$). These results suggested that
427 behavioural patterns of fish with gonadal development were consistent with proactive
428 strategies: higher escape attempts, lower latency to move and higher risk-taking
429 predisposition. No significant correlations ($P > 0.05$) were detected between the three
430 PCS, morphometric parameters and blood parameters, neither for fish with
431 gametogenesis, not for fish without gametogenesis.

432

433 **Discussion**

434 **Senegalese sole juveniles and breeder's behavioural characterization: individual** 435 **and group tests**

436 Fish that successfully crossed in the risk-taking test presented significantly higher scores
437 in the restraining (juveniles and breeders), in the new environment (breeders) and in the
438 confinement (juveniles) tests and had lower plasma cortisol levels (juveniles and
439 breeders) than fish that did not cross; these behavioural patterns were consistent with the
440 definition of proactive SCS (Koolhaas et al., 1999), while behavioural patterns of fish that
441 did not cross, also presenting significantly lower scores in the individual tests and higher
442 plasma cortisol, resembled reactive traits, for both juveniles and breeders, being in
443 agreement with the study of Ibarra-Zatarain et al. (2016).

444

445 **Evaluation of repeatability and consistency in Senegalese sole juveniles and breeders**

446 The combination of the various performed statistical tests allowed the interpretation of
447 behavioural repeatability over time and consistency across contexts in Senegalese sole
448 juveniles and breeders in the restraining (NetActA and NetEscA for juveniles; NetActW
449 and NetEscW for breeders), new environment (NewLat and NewAct) and confinement
450 (ConLat and ConAct) tests, in runs 1 to 3. However, cortisol levels were not as stable
451 over time and across contexts as the behavioural responses were. The Alpha-Cronbach's
452 reliability test and the Pearson's analysis supported the conclusion that individuals
453 showed a high degree of repeatability and correlation individual behavioural responses of
454 juveniles and breeders to restraining, new environment and confinement tests, in runs 1
455 to 3. Regarding cortisol, juveniles showed a high variation in their levels between runs 1
456 and 3, which may be related to the changing maturation status as in run 1 all fish were
457 immature compared to run 3 when a proportion of fish entered puberty (similar

458 observations have been expanded on below). However, breeders that were all in a similar
459 stage of maturity presented a high repeatability in their cortisol levels (Table 2). Besides,
460 juveniles and breeders were confirmed to exhibit two behavioural reactions, which
461 resembled proactive/reactive traits, in response to the different stress tests performed and,
462 furthermore, these behavioural responses were maintained over time. In other words,
463 juveniles or breeders presenting a high number of escape attempts (proactive) in run 1
464 also showed a high number of escapes in the successive runs (2 and 3) and *vice-versa* for
465 reactive fish. Only a few studies have evaluated fish behaviour over long time periods, as
466 in the present study. The behavioural repeatability and consistency displayed by
467 Senegalese sole juveniles and breeders over three and two years, respectively, were
468 consistent with the results of those studies that evaluated activity in response to similar
469 tests over short time periods, such as in swordtail bluegill sunfish *Lepomis macrochirus*
470 (Wilson and Godin, 2009), gilthead sea bream *Sparus aurata* (Castanheira et al., 2013)
471 and sheephead swordtail *X. birchmanni* (Boulton et al., 2015), and over long time
472 periods, such as cichlid *Neolamprologus pulcher* (Chervet et al., 2011), mosquito fish
473 *Gambusia holbrooki* (Biro and Adriaenssens, 2013), and zebrafish *Danio rerio* (Rey et
474 al., 2013). However, some authors manifested that the intra-individual consistency and
475 correlations decreased over time, while in Senegalese sole, behaviours were consistently
476 maintained over time and in some parameters correlation became stronger (*e.g.* activity
477 in restraining, new environment).

478 As well, the Pearson correlations showed high relationships in restraining, new
479 environment and confinement tests for Senegalese sole juveniles, in year 1, 2 and 3.
480 However, it was observed that correlations in breeders were lower when comparing
481 data/results between year 1-autumn, year 2-spring and year 2-autumn and this might be
482 attributed to the season in which tests were performed in year 2 (June). At this period of
483 the year, Senegalese sole adults were recovering from their breeding season. Thus, it is
484 possible that energy and metabolism were used to recover optimal physiology and then
485 induced lower activity in the broodstock (Careau and Garland, 2012). Another argument
486 would be that maturity status and hormones (*e.g.* testosterone, 17- β -estradiol, etc.)
487 influenced the Senegalese sole breeder's behaviour maybe by interfering with cortisol, as
488 had been observed in other fish species, such as stickleback *Gasterosteus aculeatus*,
489 African cichlid fish *Astatotilapia burtoni* and Siamese fighting fish *Betta splendens* (Bell,
490 2004; Huffman et al., 2013; Hebert et al., 2014), whom observed changes in risk taking
491 ability and aggression. However, this hypothesis should be further analysed. Regarding

492 the plasma analysis, significant correlations over time were observed for cortisol and
493 lactate concentrations in juveniles and only for glucose concentrations in breeders
494 (supplementary table 1). The present results are in line to other studies that analysed
495 overall correlations over time (Castanheira et al., 2013; Ferrari et al., 2015).

496

497 **Behavioural patterns of fish with and without gametogenesis**

498 A key and novel result of the present investigation was to observe that juveniles that
499 started gametogenesis presented higher scores in restraining test and lower scores in the
500 confinement test, showed lower cortisol blood levels in both runs (1 and 3) and exhibited
501 higher disposition to take risk. Indeed, the group in gametogenesis showed significantly
502 higher weight and length than sole with no gametogenesis. These first observations
503 suggest that fish with higher activity and risk predisposition and low glucocorticoids
504 levels (resembling proactive SCS) enter puberty and gametogenesis earlier than fish with
505 low activity and risk predisposition and high glucocorticoids levels (resembling reactive
506 SCS) that were not observed to start gametogenesis. These novel results are in line to
507 those reported by Bell and Stamps (2004) and Edenbrow and Croft (2011), whom
508 documented the significant influence of behavioural traits on first sexual maturity in
509 sticklebacks and mangrove killifish *Kryptolebias marmoratus*, respectively. Indeed,
510 results were similar with studies that evaluated relationships between coping styles,
511 growth, activity and physiological changes over time (Brodin, 2008; Wilson and Godin,
512 2009; Edenbrow and Croft, 2011).

513 A probable explanation about these individual behavioural differences between
514 juveniles with and without gonadal development might rely on their metabolic rates and
515 requirements, which were possibly higher in fish with gametogenesis than in fish without
516 gametogenesis. High demanding metabolisms have been generally hypothesized to be
517 translated into higher activity, aggression and proactiveness in contexts related to
518 dominance or risk taking. Further, individuals with higher metabolic rate have higher
519 possibilities for food acquisition and thereby for energy gain that involved greater growth
520 rates, improved physiological development and earlier maturation (Biro and Stamps,
521 2008, 2010; Huntingford et al., 2010; Réale et al., 2010b; Careau and Garland, 2012). In
522 addition, Réale et al. (2010a, b) proposed, in their pace-of-life theory (POLS), that
523 individuals with a fast lifestyle (those with high metabolism and energy) are associated
524 with boldness, aggressiveness, risk predisposition and early maturation, whilst
525 individuals with slow lifestyle (those with low metabolism and energy) exhibit cautious

526 behaviours and delayed reproduction. Another possible explanation for these behavioural
527 differences between fish with and without gametogenesis is the influence of hormones on
528 Senegalese sole behaviour. Sex hormones (*e.g.* testosterone), produced at the beginning
529 of gametogenesis, have been documented to influence the aggressiveness and dominance,
530 a trait that tends to be linked with coping styles (Koolhaas et al., 2010; Conrad et al.,
531 2011; Sih et al., 2015) as observed in other fish species, such as mangrove rivulus
532 *Kryptolebias marmoratus* (Chang et al. 2012) and the African cichlid fish (Huffman et al.
533 2013). It is important to emphasize that Senegalese sole exhibits defined proactive and
534 reactive SCS at early life stages (40 days post-hatch) (Ibarra-Zatarain et al., 2015) and in
535 the present study it was observed that SCS in juveniles were preserved through time. In
536 accordance with the afore-mentioned and considering that sexual maturation has been
537 shown to be related to a threshold gathering data on energy reserves, size and age (Duncan
538 et al., 2013), SCS was demonstrated to be closely associated to gametogenesis, with
539 proactive fish reaching this physiological threshold and then, maturing, before reactive
540 fish. Nonetheless, it would be highly recommendable to perform more studies focusing
541 on these two aspects to corroborate the link between gonadal development, hormones and
542 behavioural traits during fish ontogeny in Senegalese sole, since it may provide valuable
543 information for the general knowledge on the biology of the species and be used for their
544 conservation in natural environments as well as for aquaculture research and in
545 production sectors.

546

547 **Conclusion**

548 The present study provided novel outcomes on Senegalese sole stress coping styles. This
549 study is one of the first demonstrating the significant high degree of intra-individual
550 repeatability over a long-time period (three and two years, respectively) and consistency
551 across different individual-based and group-based coping style tests in Senegalese sole
552 juveniles and breeders. These physiological and behavioural responses were similar to
553 stress coping styles definition (Koolhaas et al., 2010) and some individuals' behavioural
554 responses were consistent with proactive and reactive SCS. For the first time, it was
555 demonstrated some significant behavioural differences between juveniles with and
556 without gametogenesis related to SCS. Nonetheless, more studies are needed to confirm
557 these first results in Senegalese sole juveniles. The significant and strong degree of
558 repeatability, consistency and correlation of behavioural traits in Senegalese sole
559 juveniles and breeders observed in the present study confirmed that the set of individual-

560 based (restraining, new environment and confinement) and group-based (risk taking) tests
561 were suitable and robust to measure SCS in this fish species, as described previously by
562 the same authors (Ibarra-Zatarain et al., 2016).

563

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570

571 **Author's contribution**

572 ZIZ, SR, ND conceived and designed the experiments. ZIZ, EF, AB, ND performed the
573 experiments. ZIZ, SR, ND analyzed the data. ND contributed reagent/materials/analysis.
574 ZIZ, ND wrote the paper. ZIZ, SR, EF, AB, ND critically reviewed the paper. All authors
575 gave final approval for publication

576

577 **Competing interests**

578 We have no competing interests

579

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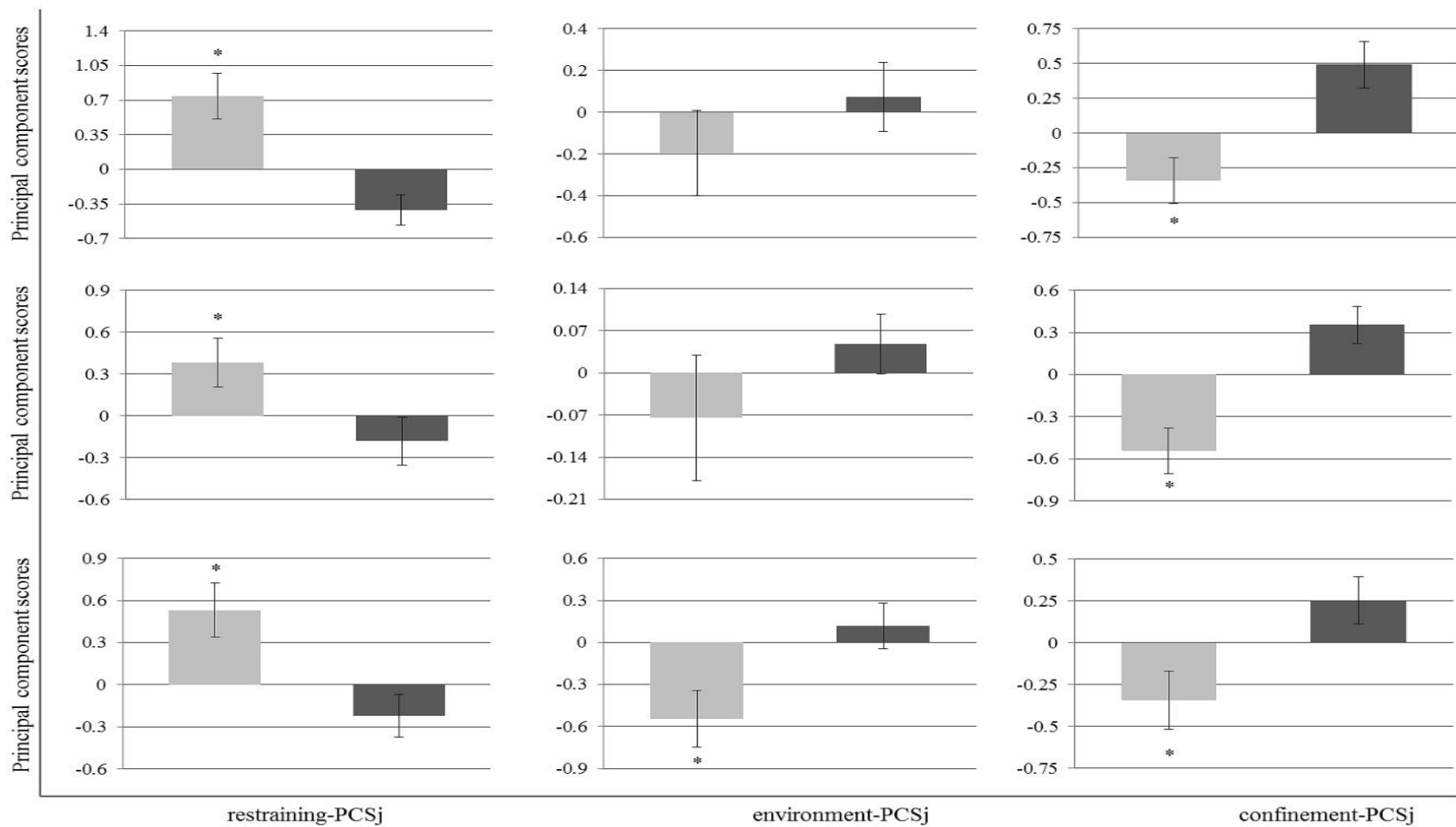


Figure 1. Principal Component Scores of juveniles grouped by gametogenesis (first row, light grey = gametogenesis, dark grey = no gametogenesis), by risk taking run 1 (second row, light grey = crossed, dark grey = not crossed) and by risk taking run 3 (third row, light grey = crossed, dark grey = not crossed). * Indicates significant differences ($P < 0.05$).

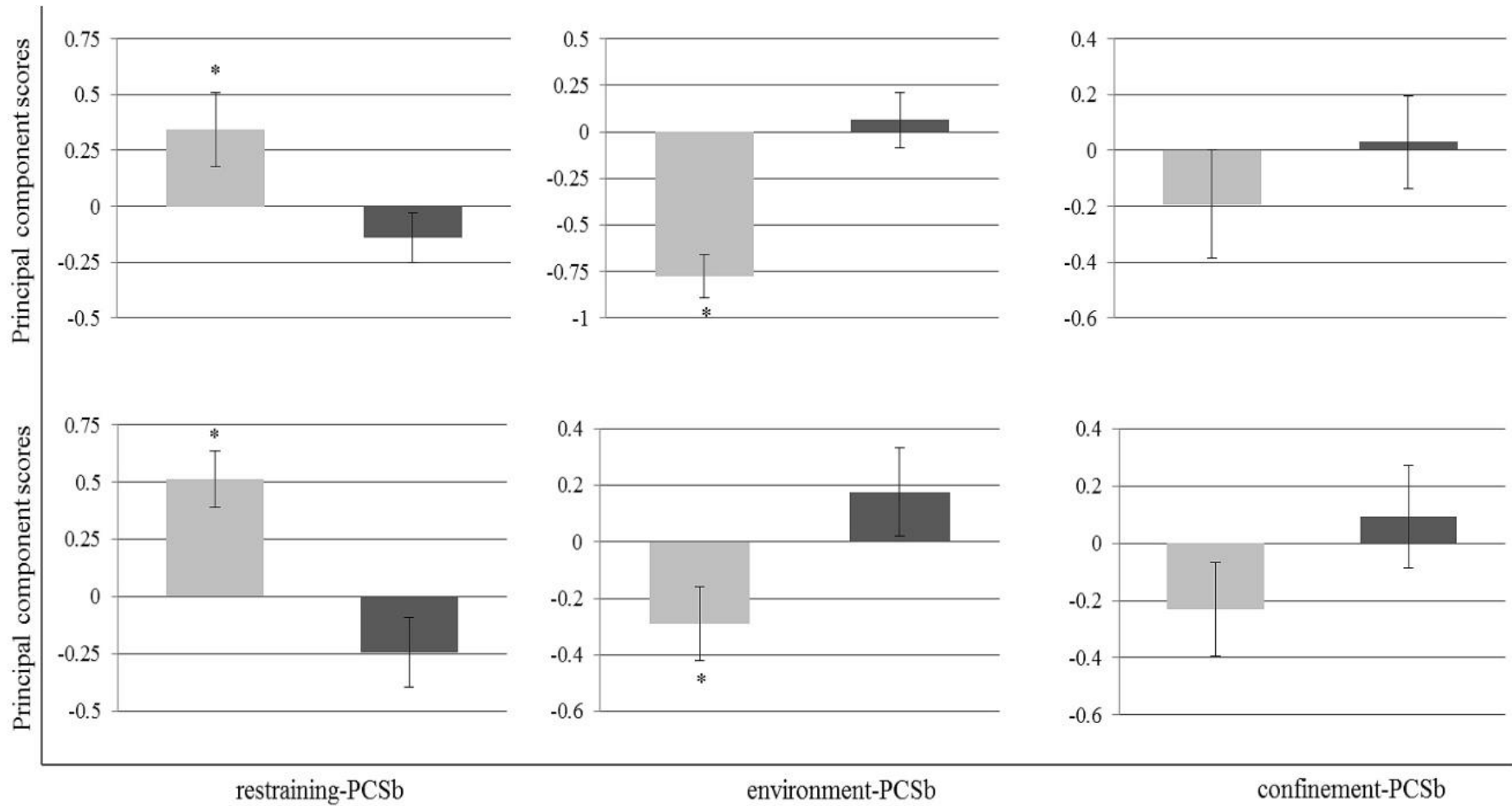


Figure 2. Principal Component Scores of breeders that crossed (light grey) and those that did not cross (dark grey) in the risk-taking run 1 (first row) and run 2 (second row). * Indicates significant differences ($P < 0.05$).

Tables

Table 1. Mean behavioural responses of Senegalese sole juveniles and breeders over time. In juveniles, runs 1, 2 and 3 of individual tests were respectively July 2012, 2013 and 2014, while risk tests were in year 1 (run 1) and 3 (run 3). In breeders, runs 1, 2 and 3 were in year 1 -autumn-, year 2 -spring-, and year 3 -autumn- respectively, while risk-taking tests were performed in autumn of years 1 (run 1) and 2 (run 3).

Tests	Variables	Juveniles			Breeders		
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Restraining	NetActA	10.2 ± 1.0	11.0 ± 0.8	12.0 ± 1.0	na	na	na
	NetEscA	25.0 ± 2.2	23.8 ± 2.1	26.2 ± 1.9	na	na	na
	NetActW	na	na	na	18.1 ± 2.2	15.4 ± 1.7	17.4 ± 1.7
	NetEscW	na	na	na	5.4 ± 0.9	6.6 ± 1.2	7.2 ± 1.0
New environment	NewLat	140.0 ± 16.2	na	134.3 ± 15.5	109.5 ± 16.8	na	93.6 ± 14.2
	NewAct	12.7 ± 2.3	na	17.0 ± 2.4	26.1 ± 4.6	na	28.6 ± 4.6
Confinement	ConLat	126.2 ± 17.0	112.4 ± 16.7	107.8 ± 15.2	112.4 ± 16.3	72.2 ± 14.8	86.5 ± 13.5
	ConAct	36.6 ± 6.1	37.0 ± 6.0	41.5 ± 6.3	24.2 ± 4.7	25.2 ± 3.8	28.0 ± 3.7
Risk taking	Cross	24	na	18	17	na	19
	Not cross	37	na	43	42	na	40
Blood parameters	Cortisol (ng/ml)	58.0 ± 8.1	na	79.6 ± 8.3	20.6 ± 7.2	na	16.8 ± 5.2
	Glucose (mmol/l)	4.3 ± 0.4	na	6.2 ± 0.4	4.7 ± 0.3	na	8.5 ± 0.9
	Lactate (mmol/l)	19.7 ± 0.7	na	26.8 ± 0.7	6.6 ± 0.8	na	10.6 ± 1.1

na = not applied

Table 2. Parameters of the GLM repeated measures MANOVA examining intra and inter-individual consistency of behavioural and physiological responses of Senegalese sole juveniles and breeders for the different tests over time and between breeders and juveniles. λ = Wilk's lambda value, **d.f.** = degrees of freedom, **F** = Fisher value, **P** = significance level. P-values > 0.05 in bold indicated high intra- and inter-individual repeatability

Tests	Variables	Juveniles				Breeders				Juvenile - Breeders			
		λ	d.f.	F	P	λ	d.f.	F	P	λ	d.f.	F	P
Restraining	NetActA	0.748	2, 59	1.69	0.194	na	na	na	na	na	na	na	na
	NetEscA	0.944	2, 59	1.71	0.184	na	na	na	na	na	na	na	na
	NetActW	na	na	na	na	0.973	2, 57	0.77	0.464	na	na	na	na
	NetEscW	na	na	na	na	0.946	2, 57	1.16	0.208	na	na	na	na
New environment	NewLat	0.959	2, 59	2.55	0.115	0.962	2, 57	2.31	0.134	0.969	2, 117	3.81	0.048
	NewAct	0.789	2, 59	6.02	0.175	0.993	2, 57	0.436	0.512	0.976	2, 117	2.96	0.088
Confinement	ConLat	0.959	2, 59	1.25	0.292	0.907	2, 57	2.92	0.062	0.934	2, 117	4.11	0.019
	ConAct	0.901	2, 59	2.90	0.69	0.962	2, 57	2.11	0.335	0.938	2, 117	3.85	0.024
Blood parameters	Cortisol	0.640	2, 59	33.75	0.001	0.997	2, 57	0.19	0.664	0.971	2, 117	64.11	0.000
	Glucose	0.538	2, 59	51.58	0.000	0.966	2, 57	2.06	0.161	0.648	2, 117	3.48	0.065
	Lactate	0.483	2, 59	64.16	0.000	0.966	2, 57	2.03	0.159	0.730	2, 117	43.62	0.004

Table 3. Parameters of the test-retest reliability analysis examining intra and inter-individual variability of behavioural responses of Senegalese sole juveniles and breeders across the different tests and over time. α = Alpha Cronbach's value, ICC = within intraclass correlation, d.f. = degrees of freedom, F = Fisher value, P = significance level. P-values < 0.05 in bold indicated high intra-and inter-individual consistency

Tests	Variables	Juveniles					Breeders					Juvenile-Breeders				
		α	ICC	d.f.	F	P	α	ICC	d.f.	F	P	α	ICC	d.f.	F	P
Restraining	NetActA	0.959	0.872	60, 120	64.16	0.000	na	na	na	na	na	na	na	na	na	na
	NetEscA	0.942	0.844	60, 120	17.37	0.000	na	na	na	na	na	na	na	na	na	na
	NetActW	na	na	na	na	na	0.785	0.548	58, 116	4.64	0.000	na	na	na	na	na
	NetEscW	na	na	na	na	na	0.704	0.285	58, 116	2.19	0.047	na	na	na	na	na
New environment	NewLat	0.989	0.978	60, 120	93.52	0.000	0.871	0.768	58, 58	7.76	0.000	0.938	0.880	119, 119	2.25	0.059
	NewAct	0.948	0.879	60, 120	19.13	0.000	0.794	0.661	58, 58	4.85	0.009	0.840	0.721	119, 119	16.06	0.000
Confinement	ConLat	0.878	0.706	60, 120	8.21	0.001	0.705	0.313	58, 116	2.40	0.046	0.678	0.224	119, 238	4.82	0.054
	ConAct	0.985	0.954	60, 120	67.10	0.000	0.792	0.561	58, 116	4.79	0.000	0.942	0.822	119, 238	17.15	0.000
Blood parameters	Cortisol	0.946	0.851	60, 120	18.51	0.000	0.017	0.009	58, 58	1.01	0.474	0.616	0.129	119, 238	8.46	0.063
	Glucose	0.881	0.669	60, 120	8.34	0.001	0.992	0.885	58, 58	92.28	0.000	0.498	0.216	119, 119	4.52	0.051
	Lactate	0.311	0.100	60, 120	1.45	0.076	0.987	0.687	58, 58	77.08	0.000	0.837	0.620	119, 119	3.13	0.059

Supplementary figure


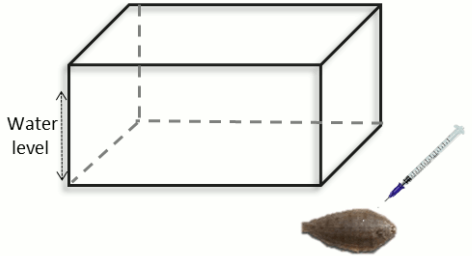
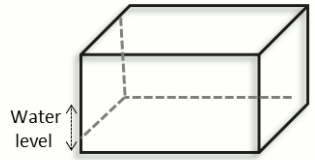
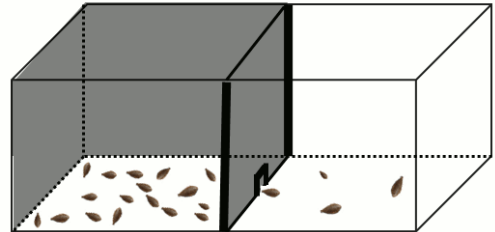


	Series of individual tests			Group test
	1 Restraining test	2 New environment test	3 Confinement test	4 Risk taking test
	 <p>Fish caught in net from tank to evaluate activity and escapes</p>	 <p>New environment test to evaluate first and total activity, afterwards fish was sedated for blood sampling</p>	 <p>Fish passed from net to confinement to evaluate first and total activity</p>	 <p>A risk taking test was performed, one month after individual tests, to evaluate fish disposition to take risk</p>
 <p>Juveniles</p>	Year 1, Year 2 and year 3	Year 1 and year 3	Year 1, Year 2 and year 3	Year 1 and year 3
 <p>Breeders</p>	Year 1 (autumn), year 2 (spring) and year 2 (autumn)	Year 1 and year 2 (autumn)	Year 1 (autumn) year 2 (spring) and year 3 (autumn)	Year 1 and year 2 (autumn)

Figure 1. Chronogram figure explaining the different behavioural tests (restraining, confinement and new environment) and group test (risk taking) applied to Senegalese sole juveniles and breeders during different years

Supplementary tables

Table 1. Pearson's correlations among runs 1 to 3 for Senegalese sole juveniles and breeders. Bold letter indicates significant differences ($P < 0.05$).

Tests	Variables	Values	Juveniles			Breeders		
			run 1 vs run 2	run 1 vs run 3	run 2 vs run 3	run 1 vs run 2 (autumn-spring)	run 1 vs run 3 (autumn-autumn)	run 2 vs run 3 (spring-autumn)
Restraining	NetActA	R	0.788	0.757	0.817	na	na	na
		P	0.001	0.001	0.001	na	na	na
	NetEscA	R	0.739	0.662	0.754	na	na	na
		P	0.001	0.004	0.001	na	na	na
	NetActW	R	na	na	Na	0.422	0.653	0.437
		P	na	na	Na	0.025	0.001	0.019
	NetEscW	R	na	na	Na	0.285	0.458	0.161
		P	na	na	Na	0.035	0.021	0.223
New environment	NewLat	R	na	0.931	Na	na	0.738	na
		P	na	0.001	Na	na	0.001	na
	NewAct	R	na	0.812	Na	na	0.658	na
		P	na	0.001	Na	na	0.001	na
Confinement	ConLat	R	0.551	0.542	0.466	0.042	0.702	0.201
		P	0.009	0.011	0.019	0.762	0.001	0.127
	ConAct	R	0.939	0.897	0.910	0.403	0.805	0.431
		P	0.001	0.001	0.001	0.018	0.001	0.001
Blood parameters	Cortisol (ng/ml)	R	na	0.806	Na	na	0.009	na
		P	na	0.001	Na	na	0.946	na
	Glucose (mmol/l)	R	na	0.034	Na	na	0.457	na
		P	na	0.785	Na	na	0.002	na
	Lactate (mmol/l)	R	na	0.619	Na	na	0.234	na
		P	na	0.008	Na	na	0.071	na

na= not applied

Table 2. Morphometric parameters, behavioural responses and glucocorticoids blood concentrations of Senegalese sole juveniles grouped according to gonadal development and risk taking (year 1 and 3). Capital letters indicates statistical differences ($P < 0.05$).

Variables	Gonadal development		Risk taking run 1		Risk taking run 3	
	Gametogenesis	No gametogenesis	Crossed	Not crossed	Crossed	Not crossed
Weight (g)	290.0 ± 25.4 ^A	189.4 ± 20.4 ^B	46.2 ± 2.8	45.5 ± 2.4	239.7 ± 27.2 ^A	216.2 ± 21.6 ^B
Length (cm)	27.3 ± 0.8 ^A	23.5 ± 0.7 ^B	15.0 ± 0.3	15.4 ± 0.2	25.1 ± 0.8 ^A	24.2 ± 0.8 ^B
<i>restraining-PCSj</i>	0.74 ± 0.23 ^A	-0.41 ± 0.15 ^B	0.38 ± 0.17 ^A	-0.18 ± 0.11 ^B	0.53 ± 0.19 ^A	-0.22 ± 0.15 ^B
<i>environment-PCSj</i>	-0.19 ± 0.20	0.07 ± 0.16	-0.07 ± 0.10	0.35 ± 0.13	-0.54 ± 0.20 ^A	0.25 ± 0.14 ^B
<i>confinement-PCSj</i>	-0.34 ± 0.16 ^A	0.49 ± 0.16 ^B	-0.54 ± 0.16 ^A	0.04 ± 0.04 ^B	-0.34 ± 0.17 ^A	0.11 ± 0.16 ^B
Cortisol (ng/ml)	35.70 ± 10.5 ^A	70.60 ± 10.70 ^B	26.84 ± 4.90 ^A	78.29 ± 11.90 ^B	32.60 ± 7.25 ^A	68.70 ± 10.72 ^B
Glucose (mmol/l)	4.41 ± 1.0	4.11 ± 0.31	4.63 ± 0.90	4.04 ± 0.33	5.0 ± 1.21	3.98 ± 0.29
Lactate (mmol/l)	19.70 ± 1.2	19.74 ± 0.81	20.80 ± 1.16	19.00 ± 0.80	20.92 ± 1.32	19.20 ± 0.76

Table 3. Morphometric parameters, behavioural responses and glucocorticoids blood concentrations of Senegalese sole breeders grouped by risk taking (runs 1 and 3). Capital letters indicates statistical differences ($P < 0.05$).

Variables	Risk taking run 1		Risk taking run 3	
	Crossed	Not crossed	Crossed	Not crossed
Weight (g)	1303 ± 111.4	1211 ± 63.5	1232 ± 91.7	1171 ± 59.2
<i>restraining-PCSb</i>	0.38 ± 0.17 ^A	-0.18 ± 0.11 ^B	0.53 ± 0.19 ^A	-0.22 ± 0.15 ^B
<i>environment-PCSb</i>	-0.07 ± 0.10 ^A	0.35 ± 0.13 ^B	-0.54 ± 0.20 ^A	0.25 ± 0.14 ^B
<i>confinement-PCSb</i>	-0.54 ± 0.16	0.04 ± 0.04	-0.34 ± 0.17	0.11 ± 0.16
Cortisol (ng/ml)	26.84 ± 4.90 ^B	78.29 ± 11.90 ^A	32.60 ± 7.25 ^B	68.70 ± 10.72 ^A
Glucose (mmol/l)	4.63 ± 0.90	4.04 ± 0.33	5.0 ± 1.21	3.98 ± 0.29
Lactate (mmol/l)	20.80 ± 1.16	19.00 ± 0.80	20.92 ± 1.32	19.20 ± 0.76