Accepted refereed manuscript of: Ibarra-Zatarain Z, Rey S, Boglino A, Fatsini E & Duncan N (2020) Senegalese sole (Solea senegalensis) coping styles are consistent over time: behavioural and physiological responses during ontogenesis. Physiology & Behavior, 217, Art. No.: 112803. https://doi.org/10.1016/j.physbeh.2020.112803

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

Ibarra-Zatarain, Z[1, 2], Rey, S[3], Boglino, A[1,2], Fatsini, E[1, 4] and Duncan, N[1*]

[1] IRTA, Sant Carles de la Ràpita, Carretera de Poble Nou, km 5.5, E- 43540 Sant Carles de la Ràpita, Tarragona, Spain

[2] CONACYT-UAN-Centro Nayarita de Innovación y Transferencia Tecnológica A.C., Av. E González s/n. Col. Industrial. CP 63173. Tepic, México

[3] Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK

[4] Centre of Marine Sciences (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

*corresponding author: zohar.ibarra@uan.edu.mx; tel+52 3114566741

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.

Abstract

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

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

Abbreviation list

NetActA = Total activity time inside the net in the air (juvenile sole)

NetActW = Total activity time inside the net in the water (breeders sole)

NetEscA = Total number of escape attempts from the net in air (juveniles sole)

NetEscW = Total number of escape attempts in water (breeders sole)

NewLat = Latency time to move in the new environment

NewAct = Total activity time of fish in the new environment

ConLat = Latency time to move in the confinement

ConAct = Total activity time of fish in the confinement

restraining-PCSj = Principal component scores in restraining for juveniles

environment-PCSj = Principal component scores in new environment for juveniles

environment-PCSj = Principal component scores in confinement for juveniles

restraining-PCSb = Principal component scores in restraining for breeders

environment-PCSb = Principal component scores in new environment for breeders

environment-PCSb = Principal component scores in confinement for breeders

Introduction

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

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

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

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

Materials and methods

Ethic statement

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

Experimental animals, housing and feeding

Sixty-one Senegalese sole juveniles and fifty-nine breeders were used as experimental animals. Sole juveniles presented an initial average weight of 45.6 ± 1.8 g and length of 15.2 ± 0.2 cm, while breeders initial average weight was 1238 ± 55.2 g and length 45.8 ± 0.6 cm. Juveniles were housed in three 0.5 m^3 square tanks (1 m length x 1 m wide x 0.5 m depth), while four 13 m^3 tanks (6 m length × 3 m wide × 0.9 m depth) were used for breeders and both systems were in a greenhouse structure. A recirculation system (IRTAMAR®) with a daily total water exchange rate of 50 % day^{-1} was used to maintain optimal water parameters for both groups of fish (T = 18 - 21 °C; O_2 = 5 - 6 mg/L). The IRTAMAR® recirculation system included sensors that continually measured and registered temperature (Genebre, Barcelona, Spain) and oxygen (OxyGuard, Farum, Denmark) and in addition daily oxygen levels were checked and registered each morning with an oximeter (Oxi3205, Wissenschaftlich-Technische Werkstätten, Germany). Juveniles were fed *ad libitum* twice a day (10:00 and 15:00 h) on a commercial balanced diet (Elite LE-2mm, Skretting, Co.), while the breeders feeding regime incorporated also non-processed fresh food and was as follows: Monday: dry pellet balanced fish feed (Vitalis Repro-7 mm and LE-7 ELITE, Skretting Co.), Wednesday: cooked mussels *Mytilus edulis* (Sariego Intermares, Spain) and Friday: frozen marine polychaetes *Nereisvirens* (Topsy-Baits, Wilhelminadorp, Holland). One hour after feeding, uneaten food was removed from tanks to maintain optimal physicochemical conditions.

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

Experimental procedures

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

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

a) In juveniles, the restraining and confinement tests were performed in year 1 (run 1), 2 (run 2) and 3 (run 3), the new environment test in year 1 (run 1) and 3 (run 3) and the risk-taking tests in year 1 (run 1) and 3 (run 3) (supplementary figure 1).
b) In breeders, the restraining and confinement tests were performed in year 1 – autumn - (run 1), year 2 – spring - (run 2) and year 2 – autumn - (run 3), the new environment test in year 1 –autumn - (run 1) and year 2 – autumn - (run 3) and the risk-taking tests in year 1 –autumn - (run 1) and year 2 – autumn - (run 3).
c) The blood collection was performed in year 1 (run 1) and 3 (run 3) in juveniles and in year 1 –autumn - (run 1) and 2 –autumn - (run 3) for breeders (supplementary figure 1).
d) Female stage of oogenesis was estimated by the degree of ovarian swelling as follow: stage I the ovary was detected by touching the ventral area of the female; stages II and III was reached when different degrees of gonad swelling were visible externally (initial and intermediate, respectively), and fish were in stage IV when maximum ovarian swelling was observed as a result of oocyte hydration (from Anguis and Cañavate 2005). Males with gametogenesis were identified by applying gentle pressure on the abdomen to obtain a small amount of milt and the percentage of motile spermatozoa was evaluated with a microscope following the methodology described by Fauvel et al. (2010).

Test 1. Restraining test

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

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

Test 2. New environment test

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

Test 3. Confinement test

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

Test 4. Risk taking test in groups

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

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

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

Blood plasma analysis

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

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

Statistical analysis

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

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

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

Results

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

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

Behavioural responses of juveniles

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

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

Behavioural responses of breeders

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

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

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

Relationship between SCS and gametogenesis

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

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

Discussion

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

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

Evaluation of repeatability and consistency in Senegalese sole juveniles and breeders

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

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

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

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

Behavioural patterns of fish with and without gametogenesis

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

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

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

Conclusion

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

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

Acknowledgments

We thank Josep Luis Celades and Magda Monllaó for their help and assistance during the sampling and general husbandry of juveniles and breeders. This study was funded by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain (INIA-FEDER RTD2011-00050) coordinated by ND. ZIZ was sponsored by a PhD grant provided by the Consejo Nacional de Ciencia y Tecnologia (México).

Author´s contribution

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

Competing interests

We have no competing interests

References

Adriaenssens, B., Johnsson, J.I., 2011. Learning and context-specific exploration behaviour in hatchery and wild brown trout. App. Anim. Behav. Sci. 132, 90-99

Anguis, V., Cañavate, J.P., 2005. Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. Aquaculture 243, 133-145

Benus, R.F., Bohus, B., Koolhaas, J.M., Van Oortmerssen, G.A., 1991. Heritable variation for aggression as a reflection of individual coping strategies. Experientia 47, 1008–1019

Bell, A.M., 2004. An endocrine disrupter increases growth and risky behaviour in three spined stickleback (*Gasterosteus aculeatus*). Horm. Behav. 45,108-114

Bell, A.M., Hankison, S.J., Laskowski, K.L., 2009. The repeatability of behaviour: a meta-analysis. Anim. Behav. 77, 771-783

592 Bell, A.M., Stamps, J.A., 2004. Development of behavioural differences between
593 individuals and populations of sticklebacks, *Gasterosteus aculeatus*. Anim. Behav.
594 68, 1339-1348
595 Biro, P.A., Adriaenssens, B., 2013. Predictability as a personality trait: consistent
596 differences in intraindividual behavioral variation. Amer. Nat. 182, 621-629
597 Biro, P.A., Stamps, J.A., 2008. Are animal personality traits linked to life-history
598 productivity? Trends Ecol. Evol. 23, 361-368
599 Biro, P.A., Stamps, J.A., 2010. Do consistent individual differences in metabolic rate
600 promote consistent individual differences in behaviour? Trends Ecol. Evol. 25, 653-
601 659
602 Boulton, K., Couto, E., Grimmer, A.J., Earley, R.L., Canario, A.V.M., Wilson, A.J.,
603 Walling, C.A., 2015. How integrated are behavioral and endocrine stress response
604 traits? A repeated measures approach to testing the stress-coping style model.
605 Ecol.Evol. 5, 618-633
606 Boulton, K., Grimmer, A.J., Rosenthal, G.G., Walling, C.A., Wilson, A.J., 2014. How
607 stable are personalities? A multivariate view of behavioural variation over long and
608 short timescales in the sheepshead swordtail, *Xiphophorus birchmanni*. Behav.
609 Ecol. Sociobiol. 68, 791-803
610 Brelin, D., Petersson, E., Winberg, S., 2005. Divergent stress coping styles in juvenile
611 brown trout (*Salmo trutta*). Ann. N.Y. Acad. Sci. 1040, 239-245
612 Brodin, T., 2008. Behavioral syndrome over the boundaries of life-carryovers from larvae
613 to adult damselfly. Behav. Ecol. 20, 30-37
614 Brown, C., Jones, F., Braithwaite, V.A., 2007. Correlation between boldness and body
615 mass in natural populations of the poeciliid *Brachyrhaphis episcopi*. J. Fish Biol.
616 71, 1590-1601
617 Careau, V., Garland, T., 2012. Performance, personality, and energetics: Correlations,
618 causation, and mechanism. Physiol. Biochem. Zool. 85, 543-571
619 Carter, A.J., Feeney, W.E., Marshall, H.H., Cowlishaw, G., Heinsohn, R., 2013. Animal
620 personality: what are behavioural ecologist measuring? Biol. Rev. 88, 465-475
621 Castanheira, M.F., Conceição, L.E.C., Millot, S., Rey, S., Bégout, M.L., Damsgard, B.,
622 Kristianses, T., Höglund, E., Øverli, Ø., Martins, C.I.M., 2015. Coping styles in
623 farmed fish: consequences for aquaculture. Rev. Aquacult. 7, 1-19

624 Castanheira, M.F., Herrera, M., Costas, B., Conceição, L.E.C., Martins, C.I.M., 2013.
625 Can we predict personality in fish? Searching for consistency over time and across
626 contexts. PLoS ONE 8(4), e62037
627 Chang, C., Li, C.Y., Earley, R.L., Hsu, Y., 2012. Aggression and related behavioural
628 traits: The impact of winning and losing and the role of hormones. Integr. Comp.
629 Biol. 56, 801-813
630 Chervet, N., Zöttl, M., Schürch, R., Taborsky, M., Heg, D., 2011. Repeatability and
631 heritability of behavioural types in social cichlid. Int. J. Evol. Biol. Article ID
632 321729
633 Conrad, J.L., Weinersmith, K.L., Brodin, T., Saltz, J.B., Sih, A., 2011. Behavioural
634 syndromes in fishes: a review with implications for ecology and fisheries
635 management. J. Fish Biol. 78, 395-435
636 Cummings, M., Mollaghan, D., 2006. Repeatability and consistency of female preference
637 behaviours in a northern swordtail, *Xiphophorus nigrensis*. Anim. Behav. 72, 217-
638 224
639 Cook, K.V., O´Connor, C.M., McConnachie, S.H., Gilmour, K.M., Cooke, S.J., 2012.
640 Condition dependent intra-individual repeatability of stress-induced cortisol in a
641 freshwater fish. Comp. Biochem. Physiol. Part A. 161, 337-343
642 Dall, S.R., Houston, A.I., McNamara, J.M., 2004. The behavioural ecology of
643 personality: consistent individual differences from an adaptive perspective. Ecol.
644 Let. 7, 734-739
645 Dingemanse, N.J., Both, C., Drent, P.J., VanOers, K., Noordwijk, A.J., 2002.
646 Repeatability and heritability of exploratory behaviour in great tits from the wild.
647 Anim. Behav. 64, 929-938
648 Dingemanse, N.J., Réale, D., 2005. Natural selection and animal personality. Behaviour
649 142, 1165-1190
650 Duncan, N.J., Sonesson, A.K., Chavanne, H., 2013. Principles of finfish broodstock
651 management in aquaculture: control of reproduction and genetic improvement. In
652 Allan, G., Burnell, G. (Eds), Advances in Aquaculture Hatchery Technology
653 Cambridge, UK, Woodhead Publishing Limited. pp. 23-75.
654 Edenbrow, M., Croft, D.P., 2011. Behavioural types and life history strategies during
655 ontogeny in the mangrove killfish, *Kryptolebias marmoratus*. Anim. Behav. 82,
656 731-741.

Farwell, M., McLaughlin, R.L., 2009. Alternative foraging tactics and risk taking in brook charr (*Salvenilus fontinalis*). Behav. Ecol. 20, 913-921

Fauvel, C., Suquet, M., Cosson J., 2010. Evaluation of fish sperm quality. J. Fish Ichthyol. 26, 636-643

Ferrari, S., Millot, S., Leguay, D., Chatain, B., Bégout, M.L., 2015. Consistency in European seabass coping styles: A life-history approach. App. Anim. Behav. Sci. 167, 74-88

Hebert, O.L., Lavin, L.E., Marks, J.M., Dzieweczynski, T.L., 2014. The effects of 17α-ethinyloestradiol on boldness and its relationship to decision making in male Siamese fighting fish. Anim. Behav. 87, 203-212

Herrera, M., Castanheira, M.F., Conceição, L.E.C., Martins, C.I.M., 2014. Linking risk taking and the behavioral and metabolic responses to confinement stress in gilthead seabream *Sparus aurata*. App. Anim. Behav. Sci. 155, 101-108

Hori, T.S., Gamperl, A.K., Hastings, C.E., Vander Voort, G.E., Robinson, J.A.B., Hohnson, S.C., Afonso, L.O.B., 2012. Inter-individual and family differences in the cortisol responsiveness of Atlantic cod (*Gadus morhua*). Aquaculture 3245-325, 165-173

Huffman, L.S., O´Connell, L.A., Hofmann, H.A., 2013. Aromatase regulates aggression in the African cichlid fish *Astatotilapia burtoni*. Physiol. Behav. 112-113, 77-83

Huntingford, F.A., Andrew, G., Mackenzie, S., Morera, D., Coyle, S.M., Pilarczyk, M., Kadri, S., 2010. Coping strategies in a strongly schooling fish, the common carp, *Cyprinus carpio*. J. Fish Biol. 76, 1576-1591

Ibarra-Zatarain, Z., Fatsini, E., Rey, S., Chereguini, O., Martin, I., Rasines, I., Alcaraz, C., Duncan, N., 2016. Characterization of stress coping style in Senegalese sole (*Solea senegalensis*) juveniles and breeders for aquaculture. R. Soc. open. sci. 3: 160495. doi:10.1098/rsos.160495

Kittilsen, S., Ellis, T., Schjolden, J., Braastad, B.O., Øverli, Ø., 2009. Determining stress-responsiveness in family groups of Atlantic salmon (*Salmo salar*) using non-invasive measures. Aquaculture 298, 146-152

Koolhaas, J.M., De Boer, S.F., Coppens, C.M., Buwalda, B., 2010. Neuroendocrinology of coping styles: Towards understanding the biology of individual variation. Front. Neuroendocrin. 31, 307-321

Koolhaas, J.M., Korte, S.M., De Boer, S.F., Van Der Vegt, B.J., Van Reenen, C.G., Hopster, H., De Jong, I.C., Ruis, M.A.W., Blokhuis, H.J., 1999. Coping styles in

691 animals: current status in behavior and stress-physiology. Neurosci. Biobehav. Rev.
692 23, 925-935
693 Martin, J.G., Réale, D., 2008. Temperament, risk assessment and habituation to novelty
694 in eastern chipmunks, *Tamias striatus*. Anim. Behav. 75, 309-318
695 Martins, C.I.M., Castanheira, M.F., Engrola, S., Costas, B., Conceição, L.E.C., 2011.
696 Individual differences in metabolism predict coping styles in fish. App. Anim.
697 Behav. Sci.130, 135-143
698 Martins, C.I.M., Schaedelin, F.C., Mann, M., Blum, C., Mandl, I., Urban, D., Grill, J.,
699 SchöBwender, J., Wagner, R.H., 2012. Exploring novelty: a component trait of
700 behavioural syndromes in a colonial fish. Behaviour 149, 215-231
701 Millot, S., Bégout, M.L., Chatain, B., 2009. Risk-taking behaviour variation over time in
702 sea bass *Dicentrarchus labrax*: effects of day-night alternation, fish phenotype
703 characteristics and selection for growth. J. Fish Biol. 75, 1733-1749
704 Mittelbach, G.G., Ballew, N.G., Kjelvik, M.K., 2014. Fish behavioural types and their
705 ecological consequences. Can. J. Fish. Aquat. Sci. 74, 1-18
706 Mota-Silva, P.I., Martins, C.I.M., Engrola, S., Marino, G., Øverli, Ø., Conceição, L.E.C.,
707 2010. Individual differences in cortisol levels and behaviour of Senegalese sole
708 (*Solea senegalensis*) juveniles: Evidence for coping styles. App. Anim. Behav. Sci.
709 124, 75-81
710 Øverli, Ø., Sørensen, C., Pulman, K.G.T., Pottinger, T.G., Korzan, W., Summers, C.H.,
711 Nilsson, G.E., 2007. Evolutionary background for stress-coping styles:
712 Relationships between physiological, behavioral, and cognitive traits in non-
713 mammalian vertebrates. Neurosci. Biobehav. Rev. 31, 396-412
714 Réale, D., Reader, S.M., Sol, D., McDougall, P.T., Dingemanse, N.J., 2007. Integrating
715 animal temperament within ecology and evolution. Biol. Rev. 82, 291-318.
716 Réale, D., Dingemanse, N.J., Kazem, A.J.N., Wright, J., 2010a. Evolutionary and
717 ecological approaches to the study of personality. Phil. Trans. R. Soc. B. 365, 3937-
718 3946
719 Réale, D., Garant, D., Humphries, M.M., Bergeron, P., Careau, V., Montiglio, P.O.,
720 2010b. Personality and the emergence of the pace-of-life syndrome concept at the
721 population level. Phil. Trans. R. Soc. B 365, 4051-4063
722 Rey, S., Boltana, S., Vargas, R., Roher, N., Mackenzie, S., 2013. Combining animal
723 personalities with transcriptomics resolves individual variation within a wild-type

724 zebrafish population and identifies underpinning molecular differences in brain
725 function. Mol. Ecol. 22, 6100-6115. doi: 10.1111/mec.12556
726 Ruiz-Gomez ML, Huntingford FA, Øyvind Ø, Thörnqvist PO and Höglund E, 2011.
727 Response to environmental change in rainbow trout selected for divergent stress
728 coping styles. Physiol. Behav. 102, 317-322
729 Ruiz-Gomez, M.D., Kittilsen, S., Hoglund, E., Huntingford, F.A., Sorensen, C., Pottinger,
730 T.G., Bakken, M., Winberg, S., Korzan, W.J., Øyvind, Ø., 2008. Behavioral
731 plasticity in rainbow trout (*Oncorhynchus mykiss*) with divergent coping styles:
732 when doves become hawks. Horm.Behav. 54, 534–8
733 Sih, A., Bell, A., Johnson, J.C., 2004a. Behavioral syndromes: an ecological and
734 evolutionary overview. Trends Ecol.Evol. 19, 372-378
735 Sih, A., Bell A, Johnson, J.C., Ziembra, R.E., 2004b. Behavioural syndromes and
736 integrated overview. Q. Rev. Biol. 79, 241-277
737 Sih, A., Mathot, K.J., Moirón, M., Montiglio, P.O., Wolf, M., Dingemanse, N.J., 2015.
738 Animal personality and state-behaviour feedbacks: a review and guide for
739 empiristics. Trends Ecol.Evol. 30, 50-60
740 Smith, B.R., Blumstein, D.T., 2008. Fitness consequences of personality: a meta-analysis.
741 Behav. Ecol. 19, 448-455
742 Sneddon, L.U., 2003. The bold and the shy: individual differences in rainbow trout. J.
743 Fish Biol. 62, 971-975
744 Tudorache, C., Schaaf, M.J.M., Slabbekoorn, H., 2013. Covariation between behaviour
745 and physiology indicators of coping style in zebrafish (*Danio rerio*). J. Endocrinol.
746 219, 251-258
747 Wilson, A.D.M., Godin, J.G.J., 2009. Boldness and behavioral syndromes in the bluegill
748 sunfish, *Lepomis macrochirus*. Behav. Ecol. 20, 231-237
749 Wilson, D.S., Coleman, K., Clark, A.B., Biederman, L., 1993. Shy-bold continuum in
750 pumpkinseed sunfish (*Lepomis gibbosus*): An ecological study of a psychological
751 trait. J. Comp. Psychol. 107, 250-260

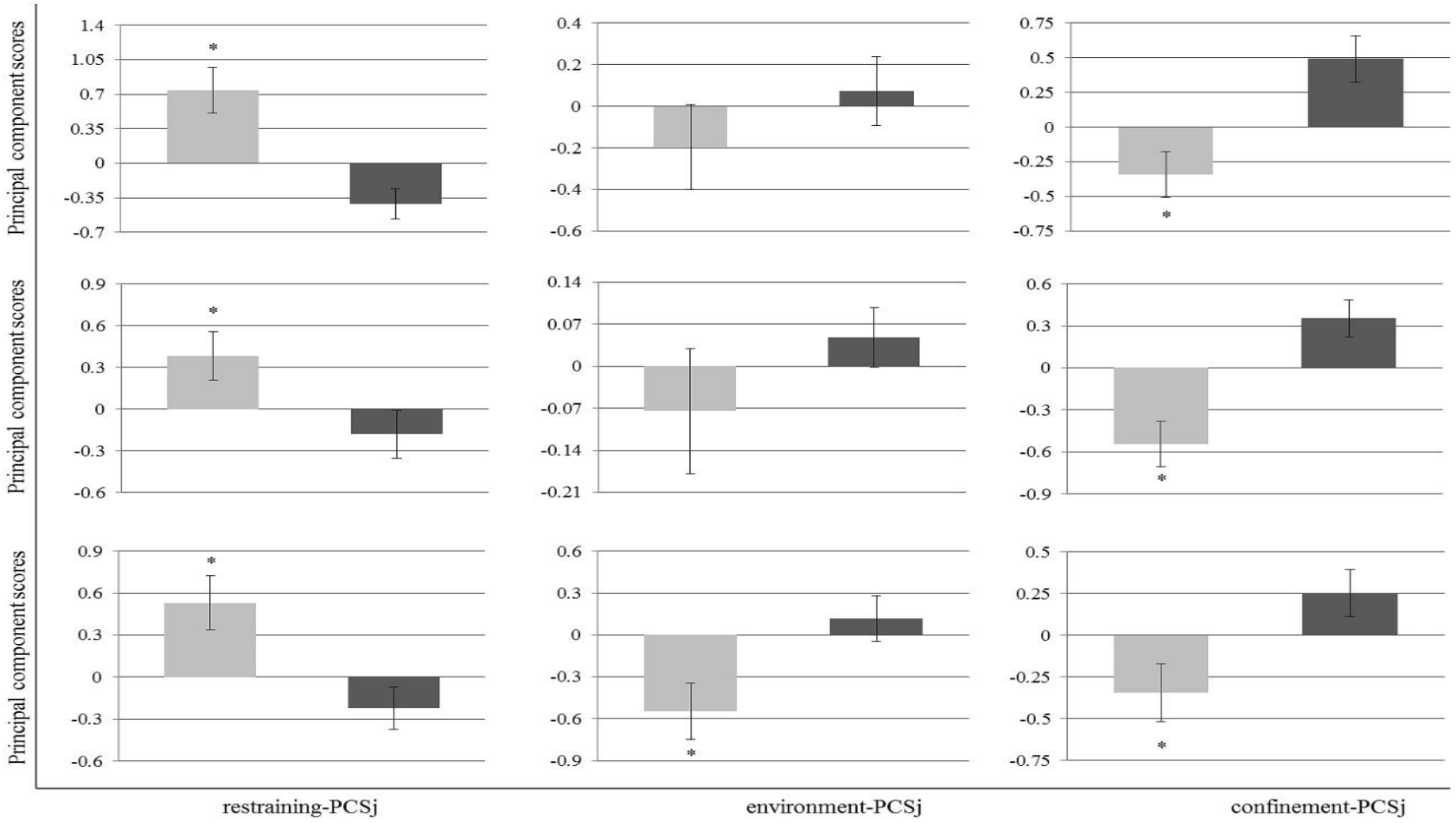

Figure 1. Principal Component Scores of juveniles grouped by gametogenesis (first row, light grey = gametogenesis, dark grey = no gametogenesis), by risk taking run 1 (second row, light grey = crossed, dark grey = not crossed) and by risk taking run 3 (third row, light grey = crossed, dark grey = not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

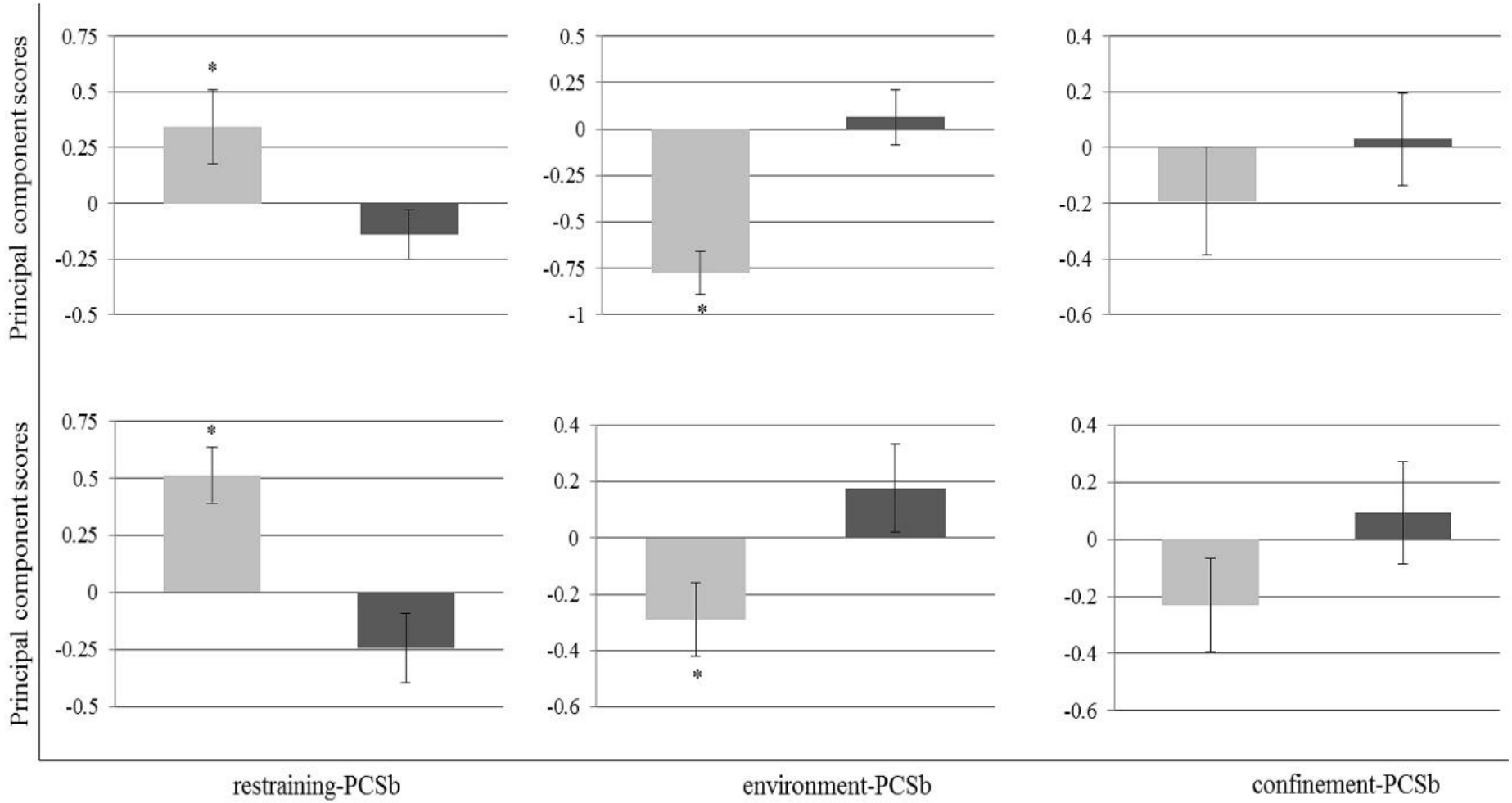

Figure 2. Principal Component Scores of breeders that crossed (light grey) and those that did not cross (dark grey) in the risk-taking run 1 (first row) and run 2 (second row). * Indicates significant differences ($P < 0.05$).

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

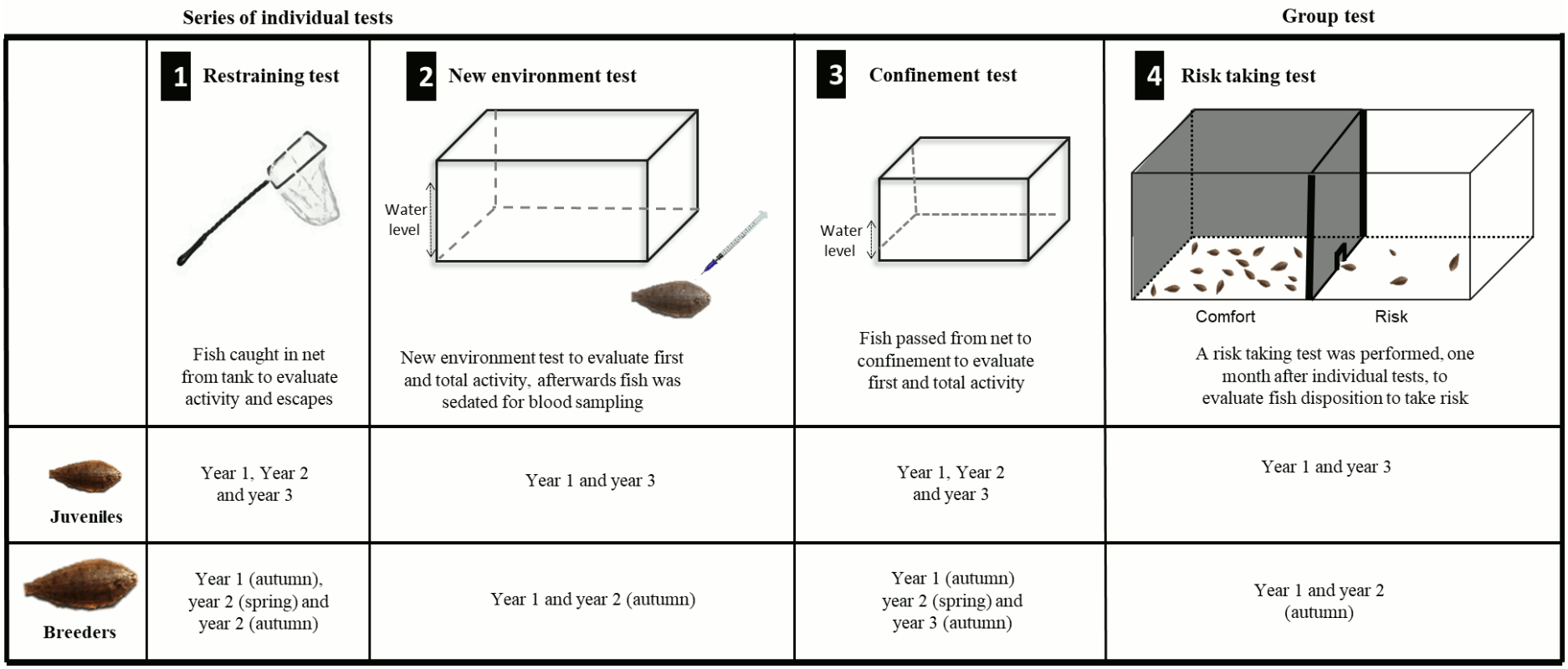

Figure 1. Chronogram figure explaining the different behavioural tests (restraining, confinement and new environment) and group test (risk taking) applied to Senegalese sole juveniles and breeders during different years

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}
restraining-PCSj	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}
environment-PCSj	-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}
confinement-PCSj	-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
restraining-PCSb	0.38 ± 0.17^{A}	-0.18 ± 0.11^{B}	0.53 ± 0.19^{A}	-0.22 ± 0.15^{B}
environment-PCSb	-0.07 ± 0.10^{A}	0.35 ± 0.13^{B}	-0.54 ± 0.20^{A}	0.25 ± 0.14^{B}
confinement-PCSb	-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