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Highlights

- Senegalese sole reproductive success was not linked to stress coping styles.
- The sex of Senegalese sole was not linked to proactive or reactive coping styles.
- The origin, wild or 1st generation hatchery, of sole was not linked to coping styles.
- This nonaggressive social sole had equal opportunities in relation to coping styles.

Exploring the relationship between stress coping styles and sex, origin and reproductive success, in Senegalese sole (*Solea senegalensis*) breeders in captivity

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Abstract

Individual animals commonly adopt different stress coping styles that have been shown to impact reproductive success and differ between sexes (female/male) and origin (wild/hatchery). Hatchery reared Senegalese sole (*Solea senegalensis*) exhibit a behavioural reproductive dysfunction and a complete failure to spawn viable eggs. Hence, the present study examined whether 1) reproductive success of Senegalese sole was linked to coping styles and 2) behavioural differences exist in relation to sex or origin. A total of 198 breeders held in two research institutions were submitted to three individual tests (restraining, new environment and confinement) and one grouping test (risk taking). In addition, a blood sample to quantify cortisol, glucose and lactate levels was obtained from each individual after completing the individual tests. Senegalese sole

breeders showed individual differences in behaviour across the different behavioural tests that were consistent with proactive and reactive coping styles traits. However, the most striking result was that reproductive success, sex and origin of Senegalese sole was not biased to any specific coping style. Indeed, the behavioural responses were similar and consistent between fish grouped by reproductive success, sex and origin. This study presented information that contrasts with different studies on dominant aggressive species and indicated that social non-aggressive species such as Senegalese sole follow a cooperative strategy that favours equal opportunities between stress coping styles and sexes. Therefore, results suggest that maintaining both coping styles strategies are fundamental for a sustainable breeder population approach.

Keywords: Solea senegalensis, coping styles, fitness, reproduction, sex differences

Introduction

Animals including fish when confronted with threatening or stressful situations have been recognized to exhibit different behavioural responses (Koolhaas et al., 1999). These patterns of behavioural responses have been defined as animal personalities (Dall et al., 2004), behavioural syndromes (Sih et al., 2004) or when under challenging situations, stress coping styles (Koolhaas et al., 1999). Different stress coping styles (hereafter SCS), which is the selected term for the present study, have been documented in different taxa of animals, such as birds, (Van Oers et al., 2005), mammals (Réale et al., 2009) and fishes (Castanheira et al., 2015).

Stress coping styles represent a continuous axis of behavioural variation in animals that ranges from two extremes: proactive to reactive (Koolhaas et al., 1999; Øverli et al., 2007). When these two extremes are compared, proactive organisms have been characterised to consistently present bold personalities, are highly active, are motivated to take risk and to explore unfamiliar environments, are more aggressive and have lower basal and post-stress glucocorticoids levels due to their higher hypothalamic-pituitary-interrenal (HPI) axis activity (Koolhaas et al., 1999; Coopens et al., 2010; Sørensen et al., 2013; Mittelbach et al., 2014). However, proactive fish were found to be less flexible to environmental fluctuations and tended to follow routines

(Sih et al., 2004; Réale et al., 2009). On the other hand, reactive fish are less active, avoid taking risk and exploring novel situations, avoid confrontation and usually pay more attention to external stimuli and possess a higher capacity to adjust behaviour to novel situations (Koolhaas, et al., 1999; Sih et al., 2004; Ruiz-Gomez et al., 2011). Such differences in behaviour have been recognized to influence the overall fitness of fishes (Mittelbach et al., 2014; Castanheira et al., 2015, Vargas et al., 2018).

Studies evaluating stress coping styles have documented that sex (female/male), origin (wild/culture) and reproductive success were in some species biased to a specific SCS. For instance, King et al. (2013) demonstrated that stickleback males (*Gasterosteus aculeatus*) were more proactive, being significantly more active and prone to take risk than females. Likewise, Ibarra-Zatarain et al. (2019) submitted the gilthead seabream males and females to different coping styles tests and observed that males were more active, produced lower glucocorticoids levels and took higher risk than females. Regarding fish origin, Lepage et al. (2000) found that wild sea trout (*Salmo trutta*) produced significantly lower plasma glucose and cortisol and showed elevated brain levels of dopamine than domesticated trout after submitting fish to different stress tests. Moreover, Huntingford and Adams (2005) observed that Atlantic salmon (*Salmo salar*) hatched and kept in captivity were more prone to exhibit proactive behaviours and to take higher risk, when submitted to novel situations, than wild individuals transferred to captivity. Concerning reproduction, there is still an ongoing debate about if proactive or reactive stress coping styles are factors that intrinsically influences mating and reproductive success. In this context, proactive males of swordfish (*Xiphophorus helleri*) (Royle et al., 2005), zebrafish (*Danio rerio*) (Ariyomo and Watt 2012; Vargas et al., 2018) and gilthead seabream (*Sparus aurata*) (Ibarra-Zatarain et al., 2019) had higher reproductive success and fertilized higher number of eggs than reactive males. However, no relationship was reported between proactiveness and reproductive success in guppies (*Poecilia reticulate*) (Piyapong et al., 2009) or mosquito fish (*Gambusia holbrooki*) (Wilson et al., 2010). Thus, further investigations are needed to elucidate the mechanisms and routes of action of stress coping styles towards reproduction, particularly in non-aggressive species with social tendency, since most of studies evaluating SCS and reproductive success have been performed in species that uses aggression as strategic behaviour to achieve reproductive success and spawning (Godin

and Dugatkin, 1996; Cook et al., 2011; Ariyomo and Watt, 2012; Ibarra-Zatarain et al., 2019).

Senegalese sole (*Solea senegalensis*) is an important marine aquaculture species that is commonly reared in intensive production systems in Southern European regions (Morais et al., 2016). Senegalese sole is a benthonic, predominantly littoral fish species found in sandy and muddy bottoms down to 100m and occasionally inhabit estuaries. The species diet in the wild consists mainly of benthonic invertebrate, such as polychaetes, molluscs and small crustaceans. However, the control of reproduction is a bottleneck that is compromising the successful production in captivity of this species. Senegalese sole captured from the wild and adapted to captive conditions spawn naturally in captivity (Dinis et al., 1999; Anguis and Cañavate, 2005; Martín et al., 2014). Nonetheless, broodstocks that were hatched and reared entirely in captivity do not produce viable eggs (Guzman et al., 2009) and this has been attributed to a behavioural reproductive dysfunction in the males that do not complete the courtship to fertilise the eggs (Mañanos et al., 2007; Martín, et al., 2019). In this context, several studies have evaluated the behavioural patterns of sole. For instance, Ibarra-Zatarain et al. (2016) showed that sole exhibits well-defined proactive and reactive stress coping styles. Carazo et al. (2016) found that sole have a complex courtship for mate selection and only spawn as pairs. Martin et al. (2014) observed that these pairs showed fidelity within a spawning season and between years and, furthermore, a large number of breeders did not participate in spawning and may have been excluded by the established couples. Lastly, Fatsini et al. (2017 and 2020) suggested that sole is not an aggressive species, but displays a dominant/subordinate behaviour related to site preference or feeding areas. Considering previous background, the present study investigated if reproductive success of Senegalese sole was linked or not to proactive or reactive behaviours and established possible individual behavioural differences according to sex (female / male) and origin (wild / hatchery) of breeders. Results of the present investigation will provide a first insight on the reproductive strategy of this species in relation to different stress coping styles.

Ethic statement

All experimental procedures on fish that formed part of this study was carried out in strict accordance with the Spanish law (RD53/2013) and European regulations on animal welfare (2010/63/UE and Federation of Laboratory Animal Science Associations, FELASA), approved by the Animal Ethics Committee of the Institut de Recerca en Tecnologies Agroalimentaries (IRTA) and in accordance to the Guidelines for the treatment of animals in behavioural research and teaching (2012).

Material and Methods

Fish maintenance

A total of 198 Senegalese sole breeders, 59 held in IRTA (Sant Carles de la Rápita, Spain) and 139 in the Spanish Oceanographic Institute, IEO (Santander, Spain), were used in the present study. All sole breeders were tagged with a passive integrated transducer (PIT-ID-100 Unique, Trovan-Zeus, Madrid, Spain) for individual identification.

Breeders from IRTA presented a mean weight of 1189 ± 50 g and were housed in four 13 m^3 rectangular tanks located in a greenhouse. To provide conditions similar to those for the development of this fish species in nature, water was supplied to the tanks with a recirculation system (IRTAmar® RAS system) that provided parameters similar to those experienced in the species natural habitat with a temperature and oxygen levels adjusted to 9 – 19°C (winter to summer) and 5 – 6 mg/L, respectively. Photoperiod was natural ranging from light dark (L:D) 14:10 during summer to LD 10:14 in the winter. Water temperature was 19°C and oxygen concentration was 6.0 mg/L during the experimental period. Fish were hand-fed *ad libitum* in the morning (10:00 h) according to the following regime: on Monday and Sunday balanced feed (Vitalis REPRO and LE-7 ELITE line, Skretting Co.), on Wednesday cooked mussels (Sariego Intermares, Spain), and on Tuesday and Friday, marine polychaetes (Topsy-Baits, Holland). One hour after feeding, uneaten food was removed from tanks to maintain optimal physicochemical water conditions.

Breeders from IEO presented a mean weight of 1357 ± 28g and were housed in four 14 m^3 rectangular tanks located in a building. The tanks were flow through with simulated natural temperature (11 - 20ºC) and constant photoperiod (L:D) 14:10. Water

temperature was 19ºC and oxygen level was 6 mg/L during the study period. The fish were fed *ad libitum* in the morning according to the following regime: on Monday and Friday cooked mussels (*Mytilus sp.*), and on Tuesday, Wednesday, Thursday and Saturday fresh squid (*Loligo sp.*).

Spawning and paternity analysis

A passive egg collector was placed at the surface outflow of each spawning tank. Spawned eggs were collected daily in the morning between 08:00 - 09:00 h and the following parameters were determined and registered to determine the spawning quality: a) volume of viable (floating) and unviable (sinking) eggs was determined using a 1 L measuring cylinder, b) total fecundity, estimated by determining the number of eggs in a 5-ml sample and multiplying by the total volume of eggs and c) total fertilization rate by counting the eggs with viable embryos in a sample of 50 eggs (by triplicate). Once the quality of spawn was assessed, the fertilized eggs were transferred to a 30L vertical incubators, with continuous water flow and aeration. After 36 - 48 h (at natural conditions for the season, 19 - 23°C) hatching rate was calculated by counting the total estimated hatched larvae / total number of eggs incubated. Three-day old larvae were collected for the paternity analysis.

For paternity analysis, breeders from both centres were genotyped by analysing DNA from caudal fin clips (see methodology by Martin et al., 2014). To assign paternity, a sample of 10 larvae (3-day old) were collected from spawns and placed individually in 1.5 ml Eppendorf tubes filled with absolute ethanol after three washes in 96° alcohol. The paternity assignment was carried out by GENEAQUA (Facultad de Veterinaria de la Universidad de Lugo, Lugo, Spain). The samples were genotyped using 6 microsatellites loci isolated from the species (initially 4 microsatellites were used to determine paternity and 2 extra microsatellites were only used for those samples that presented 3 or more possible parents) in a single multiplex PCR (Martin et al., 2014; Fatsini et al., 2017). All fish that were identified as parents from any spawn in the period 2013-2014 were considered to have had reproductive success.

Stress coping style tests

The selected tests (restraining, new environment, confinement and risk taking) were previously evaluated and confirmed as operational tests to characterize stress coping styles in Senegalese sole (Ibarra-Zatarain et al., 2016). Overall, tests were carried on the first and second week of October, in IRTA and IEO, respectively, and performed between 10:00 – 16:00 h in both locations. Tanks (dimension and colour), nets and other instruments were of the same characteristics in both facilities to avoid possible confounding of the results. Lastly, all tests were performed out of the breeding season in order to reduce the influence of maturity status on fish behavioural responses.

Individual coping style tests

The first test, *restraining test*, consisted in capturing and maintaining a fish in the net out of the water for 90 seconds and two variables were evaluated: Total Activity Time **NetAct** (duration of fish movement in the net in seconds) and the Number of Escape Attempts **NetEsc** (number of contortions or strong movements made by fish to escape in counts) (Figure 1A). The definition of activity for this test was restricted to full body movements made by fish to attempt escape from the net. The second test, *new environment test*, aimed to evaluate the fish reaction to a novel environment. For this instance, fish were placed in a 110 x 110 x 90 cm (width x length x depth) plastic tank (Figure 1B) and during a 5-minutes period, two behavioural parameters were evaluated: First Activity time **NewLat** and the Total Activity time **NewAct** (recorded in seconds). The third test, *confinement test*, consisted of submitting the fish into a small plastic container 56 x 36 x 30 cm (width x length x depth) (Figure 1C), that simulated a confined space, and for 5 minutes, two behavioural parameters were evaluated: First Activity Time **ConLat** and the Total Activity Time **ConAct** (recorded in seconds). The definition of activity, for both new environment and confinement tests, was restricted to active locomotion or swimming. If fish did not move during the 5-minute period, then 300 s was recorded and used for statistical analysis (Farwell and McLaughlin, 2009; Ibarra-Zatarain et al., 2016).

Risk taking in groups test

A single group coping style test, *risk-taking test*, was performed one month after finalizing the three individual tests, to allow fish to recover. This test aimed to determine fish capacity to cross from a known area (safe zone) to an unknown area (risky zone) (Figure 1D). The test was performed in a 16 m^3 tank 6.0 m x 3.0 m x 0.9 m (length x width x depth), divided into two equal water volumes by a wood barrier. The safe zone or shelter was isolated from light (2 lux; OSRAM DULUX 48 and 150W) and covered with sand, to simulate natural conditions in the wild. On the contrary, the risky area was more illuminated (11 lux on the surface of water; OSRAM DULUX 48 and 150W) and the bottom of the tank was devoid of sand. Light intensity was adjusted in each area by two external manual light dimmers. A window (30 cm width x 15 cm tall), which could be opened from outside the tank, was at the base of the wooden barrier and when open the sole could freely pass from the safe zone to explore the risky zone. This window was at the centre of a PIT (passive integrated transducer) tag reading antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that read the tag number of fish that passed through the window, following criteria from Carter et al., 2013; Vargas et al., 2018 and validated by Ibarra-Zatarain et al., 2016 for Senegalese sole).

Before the beginning of the test, breeders were submitted to a 24-hour acclimation in the safe zone. After acclimation, the window was opened to begin the test and any fish that crossed into the risky area during the following 24 hours were recorded by the PIT tag antenna. Breeders were tested in groups of 10 individuals to avoid inducing stress due to high stocking densities. The latency time of each organism to cross from one area to another was recorded. A maximum time of 1440 min was assigned to fish that did not cross during the 24-hour period of the test.

Quantification of blood plasma cortisol, glucose and lactate

Blood samples (0.5 ml) were extracted from the caudal vein of anesthetized fish (MS-222; 100 ppm; Argent, USA,) to measure cortisol, lactate and glucose concentrations. Blood extraction was performed approximately 40 minutes after completing individual tests. To avoid blood coagulation, a solution of 10 µl sodium heparin (5%, 25.000 UI; HOSPIRA) and 15 µl aprotinin (from bovine lung; 0.9% NaCl, 0.9% benzyl alcohol and

1.7 mg of protein; SIGMA) was placed inside the 1.5 ml plastic tubes (Eppendorfs), while syringes and needles were coated with heparin. Blood samples were centrifuged at 3000 G and 4ºC during 15 min (ThermoScientific centrifuge, M23i; Thermo rotor AM 2.18; 24 x 1.5 ml) and plasma supernatant was removed and stored in triplicates at −80ºC prior to analysis (Ibarra-Zatarain et al., 2016). Cortisol level was measured with a commercial ELISA kit (Range of detection: 0 - 800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany), by means of a competitive reaction with a conjugated binding ligand, whereas glucose and lactate concentrations were measured by means of a commercial enzymatic colorimetric kit (SPINREACT, Gerona, Spain) and read by a spectrophotometer (Infinite M-200; TECAN, Switzerland) at 23ºC and 505 nm, following the methodology validated by Ibarra-Zatarain et al. (2016) for this fish species.

Statistical analysis

All statistical analyses were performed using PASW 20 software for Windows. Normality of data was checked through a Kolmogorov-Smirnov test with Lilliefors correction. Two approaches were used to characterise coping styles of Senegalese sole by having a behavioural composite and comparing it with the physiological state, reproductive success, sex and origin of individuals. The first approach considered the performance of fish in each individual test and examined how this was related to reproductive success, sex, origin and holding institution. The second approach considered if components collectively were related to the performance of individual fish in all of the SCS tests were related to reproductive success, sex, origin and holding institution.

First approach consisted in evaluating the variables measured in each individual coping style test (NetAct and NetEsc for the restraining test, NewAct and NewLat for the new environment test and ConAct and ConLat for the confinement test) with three successive PCA (one per test). Then, the three Principal Component Scores resulting from these PCA´s (hereafter defined as *restraining-PCS1*, *new environment-PCS2* and *confinement-PCS3*) were used as single composite score that represented the individual behaviour index for each individual test (Budaev, 1997; Wilson and Godin, 2009) and validated for this fish species by Ibarra-Zatarain et al. (2016). The second approach

consisted in assessing the six variables (two per test) and glucocorticoids hormones (cortisol, glucose and lactate) into a single PCA and two components were generated (*PC1-global* and *PC2-global*). A Bartlett's test of sphericity and a Kaiser-Meyer-Olkin test was performed to test for variable and sample adequacy to the PCA analysis. Correlations among coping styles variables of fish grouped according to their reproductive success, sex, origin and research institute were analysed by Point-biserial correlations, an extension of Pearson analysis for dichotomous variables.

Once the principal component scores of the two approaches were generated, two General Multivariate Linear Models (GLMM) were performed: **i)** on the *restraining-PCS1*, *new environment-PCS2, confinement-PCS3* and cortisol, glucose and lactate concentrations and **ii)** on *PC1-global* and *PC2-global*. The GLMM was performed to identify possible significant differences between fish with different reproductive success (spawned / not spawned), between sex (female / male), origin (wild / hatchery), research institutes (IRTA / IEO) and results obtained in the risk-taking test (crossed / not crossed). A Kolmogorov-Smirnov test (KS-test), with Fisher´s Z-test, was performed to analyse frequency distribution of behaviours in breeders grouped according to their reproductive success, sex, origin and groups. A logistic regression analysis, with a Fisher´s exact test, was performed to establish if the latency time to move in the new environment (NewLat) and in confinement (ConLat) tests were correlated with the fish that crossed and did not cross (yes / no variables) in the risky area. Lastly, a Chi-square test (X^2-test) was executed to establish significant differences in the proportion of fish that crossed in the risk-taking test versus those that did not cross, between the fish separated by reproduction success, sex and origin. Values are presented as means ± standard deviation. Statistical differences were established when $P < 0.05$ for all analysis.

Results

Statistical approaches to analyse stress coping styles

The first statistical approach showed that **NetEsc**, **NewLat** and **ConLat** were the variables that explained the highest variance in the 3 individual tests, 72.58% of the *restraining-PCS1*, 69.27% of the *new environment-PCS2*, 62.26% of the *confinement-*

PCS3, respectively, and presented eigenvalues greater than 1. Moreover, the Pearson´s correlation analysis showed that *restraining-PCS1* was significantly and negatively correlated with *new environment-PCS2* ($R = -0.301$, $P < 0.001$) and *confinement-PCS3* ($R = -0.341$, $P < 0.001$), suggesting that those fish with more escape attempts (higher scores) started to explore the new environment and resumed activity in confinement earlier (lower scores). In addition, *new environment-PCS2* was positively correlated with *confinement-PCS3* ($R = 0.412$, $P < 0.001$). Whilst the second statistical approach (considering all variables together), showed that **NetAct** and **NewAct** explained the higher variance (42.8%). However, these two factors were not significantly correlated (Pearson, $P > 0.05$).

Individual and group coping style characterization

In the individual tests, Senegalese sole ($n = 198$) showed a high behavioural variability in restraining (total activity: min = 0 to max = 80 sec, CV = 93.4%; escape attempts: min = 0 to max = 49, CV = 134.7%), new environment (latency: min = 1 to max = 300 sec, CV = 143.4%; total activity: min = 0 to max = 227 sec, CV = 130.50%) and confinement (latency: min = 1 to max = 300 sec, CV = 203.9%; total activity: min = 0 to max = 132 sec, CV = 132.8%) tests. Minimum and maximum values of these variables showed two extremes of coping styles, proactiveness and reactiveness. In the grouping test (Figure 3), 29 individuals (IRTA = 17, IEO = 12) crossed from the safe to the risk zone and 169 did not cross (IRTA = 42, IEO = 127), and the chi-square test showed that risk taking (fish that crossed or did not cross) and institution (IRTA or IEO) were dependent variables (chi-square $X^2 = 13.496$, F1 = 12.366, $P < 0.001$), suggesting that sole held in IRTA took higher risk than those held in IEO. Furthermore, the first statistical approach demonstrated that sole that crossed had significant higher escape attempts in the *restraining-PCS1* (GLMM, $F_{173} = 3.71$, $P < 0.05$) than fish that did not cross. However, no statistical differences between fish that crossed and did not cross were found in the *new environment-PCS2* (GLMM, $F_{173} = 0.143$, $P = 0.521$), *confinement-PCS3* (GLMM, $F_{173} = 1.15$, $P = 0.285$), cortisol (GLMM, $F_{173} = 0.416$, $P = 0.520$), glucose (GLMM, $F_{173} = 1.91$, $P = 0.169$) and lactate (GLMM, $F_{173} = 0.934$, $P = 0.335$) levels (Figure 2). Fish that crossed and did not crossed did not show statistical differences when considering the second statistical approach, neither for *PC1-global*

(GLMM, $F_{173} = 0.2.878$, $P = 0.092$) nor for *PC2-global* (GLMM, $F_{173} = 0.063$, $P = 0.802$). Latency time to cross was statistically linearly correlated with *confinement-PCS3* ($R = 0.535$, $F_{196} = 8.432$, $P < 0.001$), but not with either *restraining-PCS1* ($R = 0.254$, $F_{196} = 3.947$, $P < 0.05$), *new environment-PCS2* ($R = 0.321$, $F_{196} = 1.158$, $P < 0.05$). No significant correlations (Pearson, $P > 0.05$) were detected between fish that crossed and did not cross regarding the latency time to move in the new environment and confinement tests.

Reproductive success and coping styles

Behavioural responses of fish that successfully spawned (n = 54) were similar to those that did not spawn (n = 144) in the three individual tests (Table 1). Further, the GLMM showed that those fish that successfully spawned behaved similarly to those that did not spawn and no differences were detected when analyse their components with the first approach (restraining-PCS1 GLMM, F173 = 1.45, P = 0.230, new environment-PCS2 GLMM, F173 = 0.593, P = 0.442 and confinement-PCS3 GLMM, F173 = 0.483, P = 0.490) and second approach (GLMM, PC1-global F184 = 0.282, P = 0.596 and PC2-global F184 = 0.193, P = 0.661). Moreover, successful and unsuccessful breeders showed similar frequency distributions (first approach KS-test restraining-PCS1 P = 0.425, new environment-PCS2 P = 0.598 and confinement-PCS3 P = 0.822; second approach KS-test PC1-global P = 0.493 and PC2-global P = 0.982). In addition, blood parameters were similar in fish of both groups (GLMM, cortisol F173 = 0.001, P = 0.999, glucose F173 = 0.021, P = 0.884 and lactate F173 = 0.011, P = 0.916). Lastly, the chi-square test showed that risk taking (fish that crossed or did not cross) and reproductive success (fish that reproduce or did not reproduce) were independent variables (X2 = 0.742, F1 = 0.779, P = 0.268), suggesting that reproduction of sole is not related to coping styles (approach one Figure 4A; approach two Figure 5A).

Sex and coping styles

Males (n = 88) and females (n = 110) behaved similarly in the individual stress coping styles tests (Table 1). The first statistical approach showed that males and females behaved similar in the *new environment-PCS2* (GLMM, $F_{173} = 0.013$, $P = 0.909$) and

confinement-PCS3 (GLMM, $F_{173} = 0.267$, $P = 0.267$) (Figure 4B). Indeed, the KS-test showed that these two components had similar frequency distributions in both groups ($P = 0.790$ and $P = 0.837$, respectively). Likewise, the second approach (Figure 5B) showed no statistical differences between behaviours of males and females (GLMM, PC1-*global* $F_{184} = 0.029$, $P = 0.864$ and GLMM, PC2-*global* $F_{184} = 0.070$, $P = 0.792$) and between their distributions (KS-test, PC1-*global* $P = 0.646$ and PC2-*global* $P = 0.287$). Blood parameters were not significantly different between males and females (GLMM, cortisol $F_{173} = 2.09$, $P = 0.150$, glucose $F_{173} = 0.606$, $P = 0.437$ and lactate $F_{173} = 2.35$, $P = 0.127$). Besides, the X^2 test showed that males and females did not differ in their risk taking ($X^2 = 1.584$, $F_1 = 1.573$, $P = 0.146$).

Origin and coping styles

Behaviours of hatchery breeders ($n = 100$) were similar to wild breeders ($n = 98$) in *restraining-PCS1* (GLMM, $F_{173} = 3.61$, $P = 0.060$), *new environment-PCS2* (GLMM, $F_{173} = 1.37$, $P = 0.243$) and *confinement-PCS3* (GLMM, $F_{173} = 0.220$, $P = 0.883$) analysed with the first approach (Figure 4C; Table 1). Moreover, both groups presented highly similar distributions for the three PCs (KS-test, PCS1 $P = 0.501$, PCS2 $P = 0.268$ and PCS3 $P = 0.311$). The second approach showed no statistical differences and similar frequency distributions between hatchery and wild fish (GLMM, PC1-*global* $F_{184} = 0.003$, $P = 0.959$ and PC2-*global* $F_{184} = 0.863$, $P = 0.354$; KS-test $P = 0.870$ and $P = 0.483$, respectively) (Figure 5C). Likewise, no statistical differences were detected in the risk-taking test between hatchery and wild breeders ($X^2 = 3.063$, $F_1 = 3.110$, $P = 0.065$).

Sole coping styles by research institutions

IEO breeders ($n = 139$) exhibited significantly higher scores for *restraining-PCS1* (GLMM, $F_{173} = 5.21$, $P = 0.024$) (Figure 4D) and produced less glucose and lactate levels (GLMM, $F_{173} = 53.91$, $P < 0.001$; $F_{173} = 49.74$, $P < 0.001$, respectively) than breeders from IRTA ($n = 59$). Nevertheless, the *new environment-PCS2*, *confinement-PCS3* and cortisol were not significantly different (GLMM, $F_{173} = 0.712$, $P = 0.400$, $F_{173} = 0.257$, $P = 0.613$ and $F_{173} = 0.812$, $P = 0.369$, respectively). The KS-test also

showed different behavioural distributions between both groups for *restraining-PCS1* *(P = 0.041)* and *confinement-PCS3* *(P = 0.049)*. The second approach (Figure 5D) showed significant differences between fish from IEO and IRTA and between their distributions in *PC2-global* (GLMM, $F_{173} = 6.178$, $P = 0.010$; KS-test $P = 0.001$), but not in *PC1-global* (GLMM, $F_{173} = 1.969$, $P = 0.162$; KS-test $P = 0.002$).

Discussion

Behavioural characterization of Senegalese sole breeders

Overall, we have described individual differences in behaviour between Senegalese sole breeders and classified individuals as proactive and reactive. Senegalese sole with high activity, low latency to explore novel situations and low glucocorticoids, glucose and lactate blood plasma levels were defined as proactive, whilst sole that exhibited lower activity, high latency to start exploration in a new environment and high glucocorticoids and glucose and lactate blood plasma levels were defined as reactive, in accordance with Ibarra-Zatarain et al. (2016) for this species. Moreover, it was observed that individuals with higher number of escape attempts (high score in *restraining-PCS1)* started to explore the new environment and resumed activity in confinement earlier (low score in *new environment-PCS2* and *confinement-PCS3*) confirming hence the existence of behavioural syndromes in adult individuals of this species. These behavioural criteria, to differentiate the proactive from reactive Senegalese sole, agrees with previous studies performed with this (Silva et al., 2010; Ibarra-Zatarain et al., 2016; Fatsini et al., 2017; Fatsini et al., 2019; Ibarra-Zatarain et al., 2020) and other fish species (Koolhaas et al., 1999; Brelin et al., 2005; Farwell and McLaughlin, 2009; Castanheira et al., 2015).

Sex and stress coping styles

Several fish models have suggested that males are prone to present proactive styles, while females are usually associated to reactiveness (Godin and Dugatkin, 1996; Candolin, 1999; Harris et al., 2010; Ariyomo and Watt, 2012, King et al., 2013; Mamuneas et al., 2014). These interpretations are based on the observation that males

had higher overall activity, foraged more in risky situations, resumed activity earlier than females after a stressful situation and made faster decisions towards food reward in unknown contexts (Harris et al., 2010; Schuett et al., 2010; King et al., 2013). In the present study, Senegalese sole males and females exhibited similar coping abilities to stress, with an exception in the number of escape attempts (*restraining*-PCS1), in which males attempted to escape more than females. Nevertheless, it is important to notice that females were significantly heavier than males and this factor possibly reduced the ability of females to attempt escaping from the net, although no significant correlations were observed between weight and coping style responses. This result is contrary to several hypotheses that have suggested that males and females differs in their personality and in their strategy to counteract stressful situations (Harris et al., 2010; Ariyomo and Watt, 2012, King et al., 2013). Schuett et al. (2010) proposed that behaviour consistently differs between sexes because "*the competition and requirements for accessing to reproduction leads to greater variance in males than of females*". Thus, the hypotheses suggest that males are expected to maximize their fitness by taking higher risks, dominating other males and foraging more distance to increase their opportunities to reproduce and to provide their genetic charge to fry, whereas females give advantage to a longer life-span to maximize their reproductive opportunities, hence, they reduced foraging and risk taking (Andersson, 1994; Piyapong et al., 2009; Harris et al., 2010; Schuett et al. 2010; King et al., 2013). Even in situations where males cannot dominate, males use strategies of "sneaking" to reproduce with as many females as possible. However, in Senegalese sole this appears to not be the case and seems to be more related to "a cooperation system" where coping abilities to stressful situations offer no advantage to either sex and this similitude in behavioural patterns might help both sole, males and females, to reduce competition, defend territories, avoid injuries of individuals or increase breeding success (see Taborsky, 1994; Stiver et al., 2005; Le Vin et al., 2011). Furthermore, Senegalese sole is a social species and their mating systems is characterized by the formation of single monogamous pairs that exhibit fidelity (Martin et al., 2014; Carazo et al., 2016; Martin et al., 2019). Therefore, in a species with these characteristics and where the opportunities for reproductive success are similar for the two sexes the hypothesis of competition and requirements for accessing to reproduction leads to greater variance in males than of females does not apply. Thus, the absence of correlations between sex and SCS in sole is in accordance to the observation that males and females have similar

variation in reproduction and do not compete to attract many mates as is witnessed in other fish species, which uses proactiveness/aggression as behavioural strategy to increase opportunities to find mates and successfully reproduce, as observed in zebrafish (Vargas et al., 2018) and seabream (Ibarra-Zatarain et al., 2019).

Origin and stress coping styles

It has been hypothesized that fish domestication may have profound effects on behaviour and adaptation (Huntingford, 2004; Robinson and Rowland, 2005). In the present study, hatchery and wild breeders showed similar behavioural responses and no significant differences were detected between their behavioural scores and their glucocorticoids levels, but close to significance as they present a clear trend. In addition, morphometric parameters were not significantly correlated with stress coping responses. The lack of significant behavioural differences between wild and hatchery-reared Senegalese sole may be attributed to life experience of individuals, to the fish capacity of adaptation to captivity or that the Senegalese sole were the first-generation breed in captivity with little advance in the domestication process (Huntingford, 2004; Adriaenssens and Johnson, 2011). Nonetheless, hatchery breeders presented a higher, but not statistically different, activity in the individual tests (restraining "NetAct", new environment "NewAct" and confinement "ContAct") and in their risk-taking capacity in comparison of wild individuals. Therefore, this low, but detectable, variability in behaviours between wild and hatchery-reared fish might be considered as the first consequence of domestication and genetic changes, which played a fundamental role on fish personality modelling (Dingemanse et al., 2012). Similar observations and tendencies, in overall activity and risk taking to those observed in the present study have been reported in other fish species, such as zebrafish *Danio rerio* (Robinson and Rowland, 2005), rainbow trout *Oncorhynchus mykiss* (Biro et al., 2006), brown trout *Salmo trutta* (Adriaenssens and Johnson, 2011), seabass *Dicentrarchus labrax* (Benhaïm et al., 2013) and Atlantic salmon *Salmo salar* (Metcalfe et al., 2003). In addition, Huntingford and Adams (2005) reviewed that hatchery-reared salmonids regularly tended to be proactive, more aggressive and took higher risk when foraging than wild specimens. In captivity, fish are involved into a constant selection for improving growth, promoting disease resistance and increasing overall performance and

cognition (Huntingford, 2004; Huntingford and Adams, 2005; Benhaïm et al., 2013). Nevertheless, it is worth to consider that these slight behavioural differences in activity and in risk taking between wild and hatchery-reared fish can be the reflection of a pre-existing genetic variation between both strains, which are innate and independent of domestication, but related to different coping style strategies. However, the fish in the present study are the first generation in captivity and more studies are necessary on future generations of this fish species to confirm previous speculations.

Reproduction success and stress coping styles

The aim of the investigation was to evaluate if SCS and reproduction were correlated in Senegalese sole, as have been observed in different studies performed in different taxa, such in mammals (red squirrels *Tamiasciurus hudsonicus* (Boon et al., 2007), bighorn sheep rams *Ovis candensis* (Réale et al., 2009)), birds (ural owl *Strix uralensis* (Kontiainen et al., 2009), zebra finches *Taeniopygia guttate* (Schuett et al., 2011)), insects (fishing spider *Dolomedes fimbriatus* (Arnqvist and Henriksson, 1997)), lizards (Indian rock agama *Psammophilus dorsalis* (Batabyal and Thaker, 2018)) and fish (swordfish *Xiphophorus helleri* (Royle et al., 2005), zebrafish *Danio rerio* (Vargas et al., 2018), gilthead seabream *Sparus aurata* (Ibarra-Zatarain et al., 2019)). Nevertheless, the coping strategies exhibited by successful and unsuccessful Senegalese sole breeders were similar and no behavioural differences were detected. As initially commented, most of studies that evaluated the correlation between coping styles and reproduction have mainly highlighted aggression as a behavioural tactic linked to proactiveness or boldness, which is used by individuals to increase reproductive success. Even more, Smith and Blumstein (2008) reported that behavioural traits are positively linked to mating success and commented that aggressive and proactive individuals are favoured during reproduction. According to Fatsini et al. (2017), Senegalese sole is a social and non-aggressive specie, but exhibits a dominance / subordination behaviour without aggression or fights amongst conspecifics. In this context, a possible hypothesis could be that social animals tend to create some forms of cooperation to make their subsistence successful by promoting synchronised behaviours to counteract harmful situations, create coalitions and share reproduction. This theory is reinforced by different reviews that have demonstrated that animals living in social dynamic systems

provide benefits to individuals in terms of evolution, adaptation, reduced predation risk, acquire resources, genetic and fitness (Silk, 2007; Pike et al., 2008; Maruska and Fernald, 2013, Fernald, 2015). In terms of reproduction, several studies have suggested that dominant and aggressive individuals monopolize spawning. For instance, Vargas et al. (2018) and Ibarra-Zatarain et al. (2019) described that males and females of zebrafish and gilthead seabream, respectively, utilized aggression to dominate reactive individuals during spawn. Therefore, authors found that SCS were significantly linked with reproductive success. However, the opposite is detected in social animals living in groups, where frequently a change of leadership can be observed (meaning that subordinate individuals can become dominant and vice-versa). Thus, opportunities of reproduction are similar amongst individuals, as it has been reported in studies performed in macaques *Macaca sylvanus* (Kuester et al., 1995), rabbits *Oryctolagus cuniculus* (Von Holst et al., 2002), grey wolves *Canis lupus* (Peterson et al., 2002), zebra *Equus burchelli* (Fischhoff et al., 2009), degus *Octodon degus* (Wey et al., 2013) and cichlid fish *Neolamprologus pulcher* (Dey et al., 2015). Hence, it is possible to conclude that reproductive tactics of Senegalese sole were in line with tactics performed by social species (Fatsini et al., 2017) and are less influenced by proactive-reactive traits, contrary to aggressive species. Although further studies are needed to confirm the hypothesis that all Senegalese sole fish have the same opportunity for reproduction and spawning, independently from their SCS response.

Conclusions

Overall results demonstrated that Senegalese sole exhibit defined stress coping styles. However, the key results were to demonstrate that proactive or reactive patterns were not significantly related to reproductive success, sex and origin of Senegalese sole. The present study is particularly important, since Senegalese sole with proactive or reactive traits have similar opportunities of reproduction, therefore, it is possible to suggest that conserving both coping strategies likely allows this species to improve the ability of individuals to maximize their opportunities for adaptation and subsistence of their future progeny to different environmental situations. Moreover, the present study demonstrated that reproductive dysfunctions of Senegalese sole appeared not to be

biased to proactive or reactive styles, as was initially thought, but is more related to a strategy of social animals living in groups.

Acknowledgments

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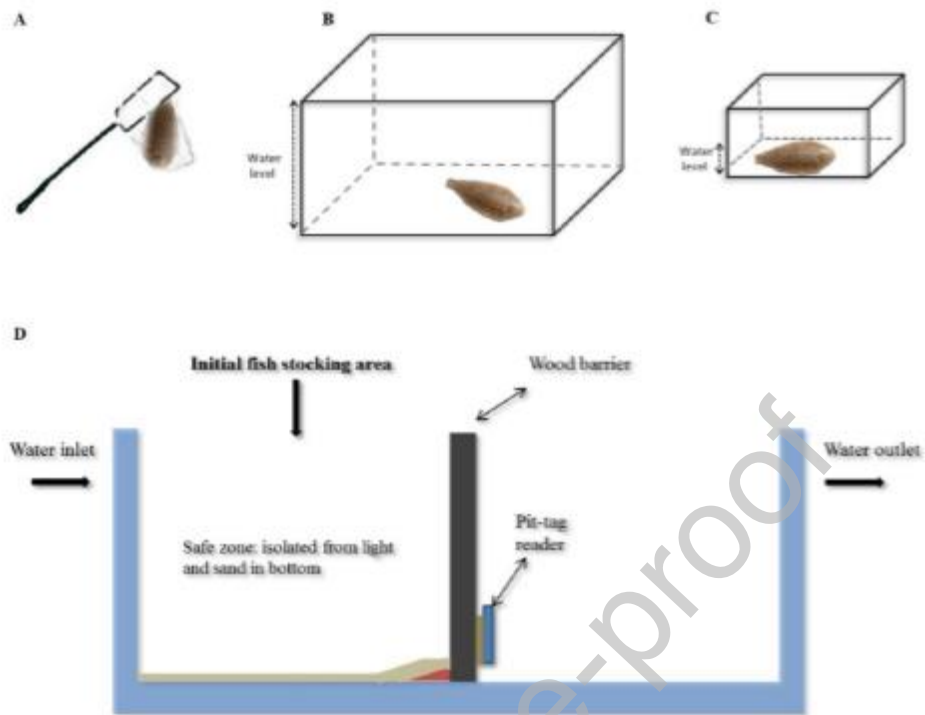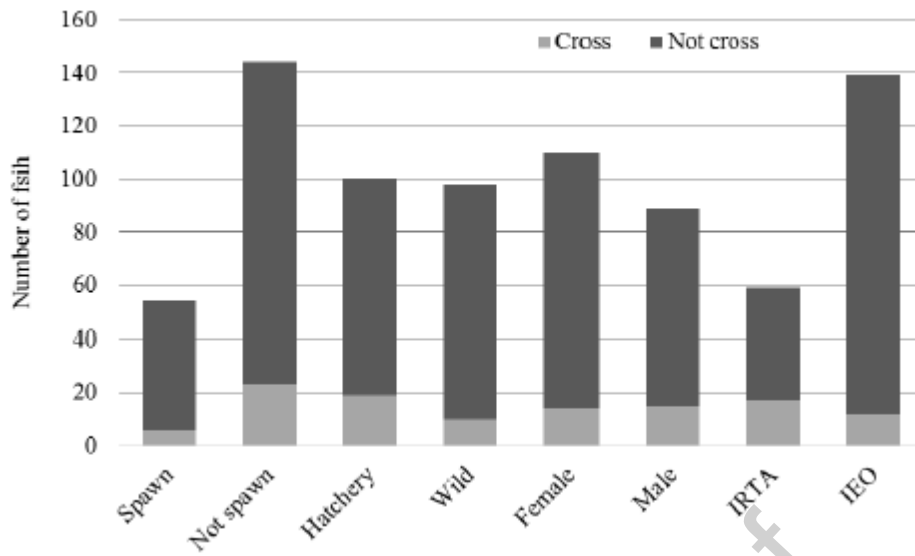
Figures

Figure 1. Description of equipment used to perform the coping styles tests on Senegalese sole breeders. A= Restraining test; **B=** Novel environment test; **C=** Confinement test; **D=** Risk taking test
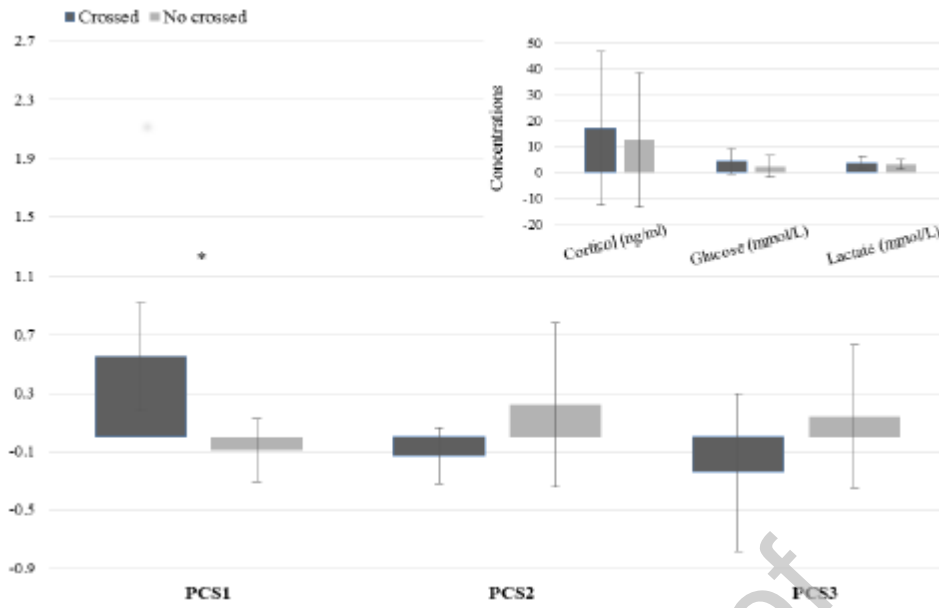
Figure 2. PCS and glucocorticoids concentrations differences between the fish that successfully crossed and those that did not cross. * Indicates significant differences

Figure 3. Number of fish that successfully crossed (light grey) and did not cross (dark grey) in the risk-taking test, grouped by reproductive success, origin, sex, and institute. * Indicates significant differences
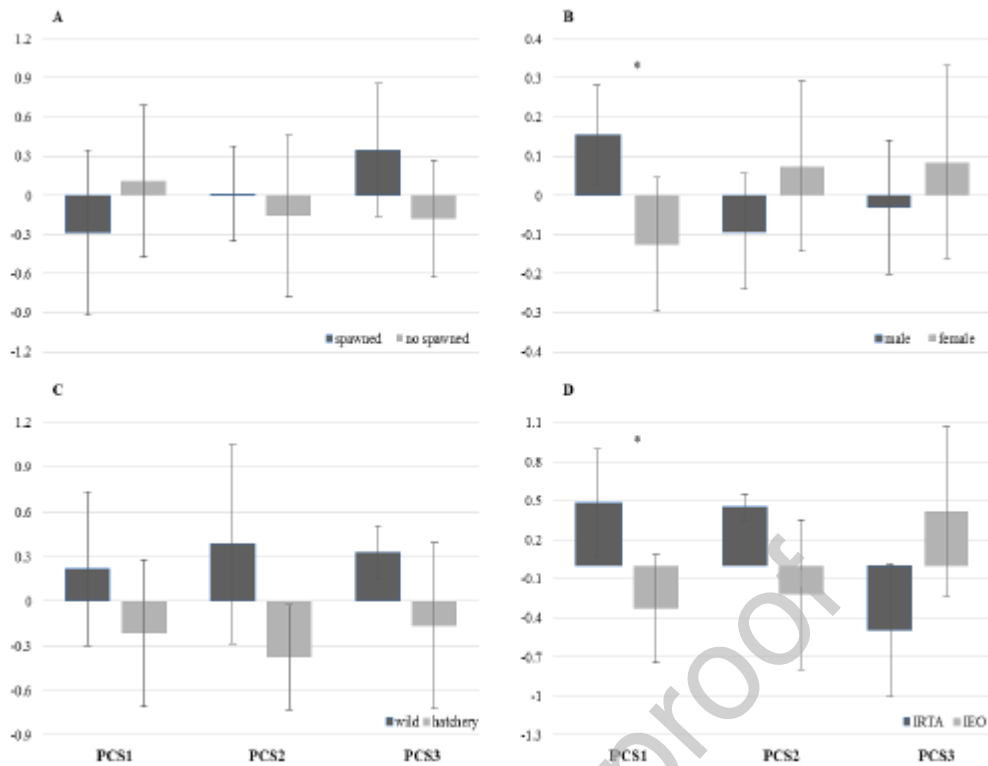
Figure 4. Comparison of principal component scores of sole breeders calculated from the restraining (PCS1), new environment (PCS2) and confinement (PCS3). Graphics split by sex (**A**), origin (**B**), spawning success (**C**) and research institution (**D**). ***** indicates significant differences between groups of fish within a PCS (*P* < 0.05).
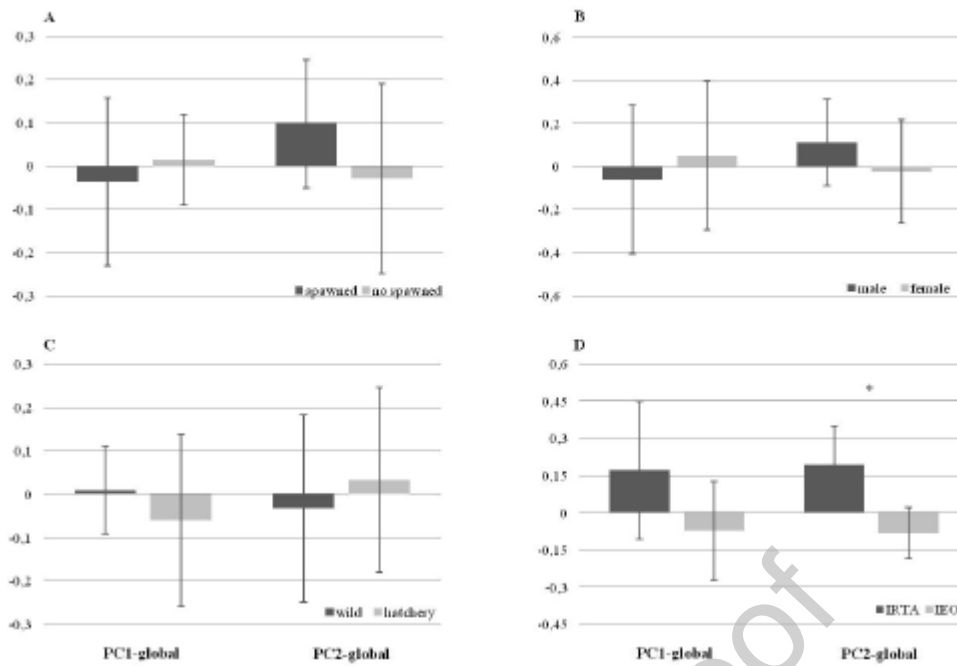
Figure 5. Comparison of principal component scores of sole breeders calculated from the second approach considering all six variables and glucocorticoids levels. Graphics split by sex (**A**), origin (**B**), spawning success (**C**) and group (**D**). ∗ Indicates significant differences between groups of fish within a PCS (*P* < 0.05).

Table 1. Comparison between sexes, origin and spawning success for fish morphology, variables for test 1-3 and biochemical quantifications (means ± sem). Numbers in parenthesis correspond to the number of animals in each group. Capital superscript letters designated significant differences between fish weight and length (resulted from GLMM). Cortisol is expressed in ng/mL, glucose and lactate in mmol/L

Tests	Variable	Female (110)	Male (88)	Hatchery (100)	Wild (98)	Spawn (54)	No spawn (144)	IRTA (59)	IEO (139)
Morphology	**Weight**	1391 ± 37^A	1200 ± 29^B	1240.2 ± 28.6^A	1374.8 ± 41.3^B	1350.0 ± 55	1290.7 ± 28.1	1190 ± 50	1357 ± 29
	Length	47.0 ± 0.6	47.2 ± 0.5	44.0 ± 0.4^A	50.4 ± 0.5^B	50.1 ± 0.7^A	46.0 ± 0.4^B	45.8 ± 0.6	47.7 ± 0.5
Netting the fish	**NetEsc**	17 ± 1.5	21.6 ± 2.1	17.3 ± 1.8	20.7 ± 1.2	20.7 ± 2.1	18.3 ± 1.5	16.4 ± 1.7	20.1 ± 1.6
	NetAct	4.6 ± 0.6	6.2 ± 0.9	5.7 ± 0.8	5.0 ± 0.6	4.6 ± 0.8	5.6 ± 0.6	7.4 ± 1.1	4.5 ± 0.5
New environment	**NewLat**	73.3 ± 11.0	66.4 ± 11.6	73.3 ± 11.1	67.0 ± 11.3	69.2 ± 15.0	70.5 ± 9.3	98.3 ± 14.5	58.3 ± 9.3
	NewAct	19.2 ± 2.2	25.1 ± 3.2	24.3 ± 2.9	19.4 ± 2.5	21.1 ± 3.7	22.2 ± 2.2	26.4 ± 3.9	20.2 ± 2.1
Confinement	**ConLat**	58.9 ± 10.0	49.1 ± 10.3	53.0 ± 10.0	56.1 ± 10.4	50.4 ± 13.2	56.1 ± 8.5	66.1 ± 12.2	49.6 ± 8.8

	ConAct	21.4 ± 3.0	18.7 ± 2.8	22.8 ± 2.8	17.5 ± 2.9	17.0 ± 3.1	21.5 ± 2.5	27.9 ± 3.7	16.9 ± 2.4
Risk taking	**Cross**	14	15	19	10	6	23		
	Not cross	96	74	81	88	48	121		
Blood analysis	**Cortisol**	11.8 ± 2.7	15.1 ± 3.0	15.2 ± 2.6	11.3 ± 3.0	12.2 ± 4.4	13.6 ± 2.2	16.7 ± 5.2	11.7 ± 1.8
	Glucose	3.1 ± 0.2	3.2 ± 0.2	3.3 ± 0.2	3.0 ± 0.2	3.1 ± 0.2	3.1 ±0.1	4.7 ± 0.3[A]	2.5 ± 0.1[B]
	Lactate	2.7 ± 0.4	2.6 ± 0.5	3.0 ± 0.5	2.3 ± 0.4	3.2 ± 0.7	2.5 ±0.3	7.0 ± 0.8[A]	0.8 ± 0.1[B]