LINKING STRESS COPING STYLES WITH BRAIN mRNA ABUNDANCE OF SELECTED TRANSCRIPTS FOR SENEGALESE SOLE (*Solea senegalensis*) JUVENILES.

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Highlights

- Stress coping styles behavioural classification is linked with mRNA abundance in Senegalese sole juveniles.
- Senegalese sole juvenile population in captive conditions could be classified in three different stress coping styles categories; proactive, intermediate and reactive.
- Several transcripts associated with important biological aspects in Senegalese sole are differently expressed depending on the stress coping styles categories.
- This study addressed an emerging field for the fish biology related to physiology, behaviour and individual variation.

Abstract

In fish, proactive and reactive individual stress copying styles (SCS) have been used to resolve variation in molecular expression data. Stress coping styles have been previously described in several stages of *Solea senegalensis* by validating for the species the use of standard behavioural screening tests. The present study aimed to link behavioural SCS tests with brain transcript abundance in early Senegalese sole juveniles in order to observe the natural variation in a molecular pathway in this species. A total of 50 juveniles were subjected to three individual behavioural (Restraining, New environment and Confinement) and one group (Risk-taking) screening tests. The fish were classified in SCS categories by applying a hierarchical cluster to the variable "Total activity" (the total activity time that the fish was moving in each individual test). Three categories were defined, proactive, intermediate and reactive sole. Six transcripts were chosen and tested, one related to basic metabolism (*gapdh-2*), three to feeding behaviour (*per1*, *igf-Ia*, *pparß*) and two to the stress response (*crh-BP* and *hsp90aa*) in 30 juveniles (10 individuals per SCS category) using *rt*-qPCR to observe differences in the abundance of those transcripts among SCS. Four transcripts were differentially expressed (DETs) among them. The transcript *gapdh-2* showed up-regulation for proactive and intermediate SCS sole while reactive individuals showed down-regulation. Target mRNAs *per1*, *igf-Ia* and *pparß*, showed different levels of up-regulation for proactive and reactive fish while intermediates were highly down-regulated. Surprisingly no differences in stress related transcripts were observed. Correlations were found between variation in coping styles and variation in the abundance of mRNAs involved in important biological functions in Senegalese sole. These results are the first evidence of the relationship between the behavioural individual variation and the fluctuation in brain transcripts abundance in Senegalese sole.

Key words: Flatfish; Transcripts; Behavioural traits; Individual variation

Introduction

The study of individual differences in animal behaviour is recognised as an important field in sociobiological studies related to ecology and evolution in animals (Morgan and Dall, 2015). Such behavioural studies have been considered an essential tool that can be used to explain individual variation inside of the same population (Reale et al., 2007; Wolf and Weissing, 2012).

Some research has already shown that wild individuals or non-selected line from the same population behave differently among them (Koolhaas et al., 1999). This difference in behaviour is more evident when stressful factors are present in the environment. Individuals exhibit different responses or stress coping styles (SCS) when subjected to stressful or risky situations and these may range from proactive to reactive responses (Koolhaas et al., 1999). Proactive animals are considered more active, aggressive, tend to grow faster and may have better mating opportunities by higher dominance but show lower plasticity to changes in the natural environment than reactive animals (Koolhaas et al., 1999; Sih et al., 2004; Coppens et al., 2010; Wilson and Godin, 2009). Contrarily, reactive animals are characterized by low levels of conspecific aggression, avoid taking risk in unknown environments with lower rates of activity, and show passive behaviours such as immobility in response to stressful stimuli (Koolhaas et al., 1999; Koolhaas et al., 2007; Castanheira et al., 2017).

Moreover, the proactive versus reactive as stress coping styles extremes has been reinforced by the fact that phenotypical dissimilarity might have a genetic (heritability) and genomic (gene expression) influence with differences in the physiological stress axis (Koolhaas et al., 1999, 2010; Øverli et al., 2007; Driscoll et al., 1998). Physiologically, proactive fish have a lower activity at hypothalamus-pituitary-adrenal/interrenal (HPI)

level than reactive fish, which affects the stress response to different stressors, presenting lower post-stress levels of glucocorticoids, which may be broadly classified to affect two major categories, immunological and metabolic response (Koolhaas et al., 2010; Braithwaite et al., 2011; Castanheira et al., 2017). These coping style profiles may remain consistent across time and between different contexts (predation, confinement, environmental variations, amongst others) for each of the individuals of the population studied (Coppens et al., 2010; Braithwaite et al., 2011; Ibarra-Zatarain et al., 2016).

Therefore, gene expression in relation to SCS in terms of individual variation has other influences and the genetic component would be delimiting the coping strategies of the individuals for several features, such as behavioural responses, genomic and the ecological niche Moreover, genomic methods using fish have already offered discernments into the mechanisms that trigger short and long-term environmental adaptations. Individual variation has been associated with genomic variation in several fish species (Huntingford et al., 2010; MacKenzie et al., 2009; Øverli, 2007; Rey et al., 2013; Rey et al., 2016) and the information of mRNAs differentially expressed between diverse SCS groups could be used for the interpretation of biological responses to resolve variation, knowing that those variations might be adaptive or genetically fixed within the population (MacKenzie et al., 2009). For example, some studies found that proactive fish showed up-regulation of the immune and metabolic related genes (such as *gapdh*) after a simulated infection challenge with LPS (lipopolysaccharide) as a similar bacterial infection while reactive fish showed down-regulation in the same challenge (MacKenzie et al., 2009; Rey et al., 2013).

Senegalese sole (*Solea senegalensis*) is an important marine flatfish species for the European aquaculture industry due to its high market price and demand (Howell et al., 2011). Furthermore, conservation measures are unknown and there exist few data on their

wild population (Monroe et al., 2015). Conversely, besides their aquaculture interest, Senegalese sole could be used as model species to study the difference in gene expression associated with coping styles categories due to the variability of stress responses recently found in this species. Moreover, Senegalese sole possesses different ecological features which make even more interesting the study of this behavioural-molecular association. This marine flatfish species is euryhaline with high range of tolerance to environmental changes (temperature and salinity) (Morais et al., 2016), however, Senegalese sole species does not possess specific phenotypic characteristics to get information about the individual coping styles categories. In other species these coping styles categorization has had influence in the gene expression. Several behavioural tests designed specifically for Senegalese sole have been published characterizing stress coping styles (proactive and reactive) in juveniles and breeders (Ibarra-Zatarain et al., 2016). The same study demonstrated that proactive sole reached the puberty earlier than reactive fish, had better growth rate and lower levels of cortisol (Ibarra-Zatarain, 2015).

Considering the background information related to Senegalese sole, the aim of this study was to test whether stress coping styles traits are involved in gene expression changes using six candidate genes involved in several functions (basic metabolism, feeding behaviour and stress response) analysed in cultured Senegalese sole (*Solea senegalensis*). These mRNAs were chosen because some of them such as *gapdh* has been observed to express differently depending on behavioural traits in other fish species (Mackenzie et al., 2009) and others such as *per1* because is a gene involved in circadian rhythmicity which is very important in species like Senegalese sole due to the change of locomotor activity from day to night. It is critical to uncover the mechanisms that underlie behavioural traits to understand how they have progressed, are sustained and could evolve in the future.

Material and Methods

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

1. Animal rearing conditions

Fish used for this experiment were provided by Stolt Sea Farm (Santiago de Compostela, Spain) and were transported from La Coruña to IRTA's facilities in March of 2012. Fish were kept at the Research Centre facilities of IRTA, in Sant Carles de la Ràpita, North East Spain and were held in 10 m^3 fiberglass tanks with natural photoperiod (40°62'82.42", 0°66'09.37, using artificial lighting). All tanks were located in a greenhouse structure and were connected to a recirculation system (IRTAmar®) to maintain a simulated natural water temperature (9 – 19 °C: winter to summer), oxygen (5 – 6mg l^{-1}) levels and salinity (35 – 38 ‰) levels. Sole were fed *ad libitum* five days per week with balanced feed (LE - 3mm ELITE, Skretting, Co.). Fifty early juvenile Senegalese sole (121.4 ± 8.1 g) were randomly selected to conduct the behavioural tests in November (the temperature registered was 12 – 14 °C). Animals were moved and acclimated to a 400 L fiberglass tank two weeks before tests started. The acclimation tank was also connected to a recirculation system (IRTAmar®) to maintain a constant temperature of 13 ± 1 ºC to avoid the environmental influences on the different behaviours among individuals and oxygen (5 – 6mg l^{-1}) levels. Water quality parameters were registered by computer system using temperature and oxygen probes. The pooled control animals used for RNAs transcripts analysis were from the same batch of the experimental sole used for this study and were acclimated to the same tanks as the experimental fish. Control fish were fed normally and were not used for any experimental procedure to obtain objective data similar to standard husbandry conditions. All fish were

PIT tagged (Passive Integrated Transponder: ID100A, Unique Trovan-Zeuss; Madrid, Spain) intramuscular for individual identification.

2. Behavioural assays

The tests applied were selected as appropriate SCS tests following Ibarra-Zatarain et al., (2016) who demonstrated that one "Risk-taking" in group and three individual tests ("Restraining", "New environment" and "Confinement") screened Senegalese sole juveniles into a range of different coping styles (proactive through to reactive), and those tests were the most representative to explain the individual variation.

2.1. In group testing

The first test performed was *Risk taking in groups*. The objective of this test was to determine the fish willingness to cross from a well-known "safe" area to an unfamiliar area (risky zone). This has been established as a standardised test to screen for SCS in fish and other animals (Smith et al., 1992; Huntingford et al., 2010; van Oers et al., 2004). A 400 L fiberglass tank was divided into two equal zones by a polyvinyl chloride (PVC) wall. The wall had a small window at the bottom to allow fish to cross between both areas. The 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 the fish which crossed through the window to the unfamiliar zone. (*see* Fig. S1A). The known sheltered area simulated natural conditions for the species, the area was isolated from light (2 *lux* on the surface) and covered by sand. On the other hand, the risky or unknown area was provided with more light (15 *lux* (OSRAM DULUX 48W on the surface) and the bottom was lacking substrate. Before beginning the test, the fish were acclimated 24 hours in the well-known sheltered zone keeping the window closed until the beginning of the test. The duration of the test was 24 hours and the Risk-taking test was video

169 recorded to validate the results registered by the antenna. The test was performed for two
170 groups of 25 fish. The number of fish was the variable observed in this group behavioural
171 test.

172 *2.2. Individual testing*

173 The other stress coping style tests were performed to all 50 fish individually in a serial
174 way - when in relation to the risk test (*see* Fig. 1 for experimental design and time line of
175 the behavioural tests). All tests were performed in a serial way to ensure less fish handling
176 and stress.

177 Fish were divided and held in two tanks of 25 fish per tank. The first test performed was
178 the "Restraining" test (REST), which was evaluated by holding individual fish in a small
179 handling net inside the water for 90 seconds (*see* Fig. S1B). The net was 54 x 60 cm
180 rectangular shape, white colour with 6 mm mesh. The variables registered in this test were
181 a) the latency time or time of first activity when the fish started to move inside the net
182 and b) the total activity time that fish was moving inside the net.

183 The next test performed, was the "New environment" test (NE); fish was individually
184 placed in a plastic tank that was novel for them and so considered as a new environment.
185 The novel tank dimensions for this test were 56.5 x 36.5 x 30 cm, rectangular shape and
186 grey colour (*see* Fig. S1C). The duration of the test was of a maximum time of 5 min (300
187 seconds), during which two variables were measured: a) the latency time or time of first
188 activity when the fish started to explore the new environment and b) the total activity
189 time, which was the total time the fish spent exploring, swimming forward in the tank.

190 The last test performed was the "Confinement" test (CON); each fish was individually
191 placed in a plastic tank that simulated a confinement situation. The tank dimensions were
192 25 x 14 x 8 cm, rectangular shape and white colour (*see* Fig. S1D). The duration of the
193 test was again 5 min (300 seconds), during which two variables were measured: a) the

194 latency time or time of first activity when the fish started to move in the tank and b) the
195 total activity time referring to the total time the fish was moving.
196 For the last two tests (New environment and Confinement test), if fish did not move at all
197 during the period of the test, the maximum duration of the test (300s) was noted for
198 statistical analysis. At the end of the "Confinement" test, all animals were euthanized
199 with an overdose of MS-222 (tricaine methanesulfonate; Acros-Organic, New Jersey,
200 USA), brains were dissected, frozen in dry ice and stored at -80 ºC for posterior molecular
201 analysis.
202 *Quantitative real time PCR*
203 The differential expression of brain target transcripts (*gapdh2*, *per1*, *igf-Ia*, *pparß*,
204 *hsp90aa* and *crh-BP*) for stress coping behaviour (Table 1) was measured in brains from
205 thirty sole, ten fish from each phenotypical category (proactive/intermediate/reactive)
206 (see *statistical analyses (behaviour)* section for classification of behavioural traits).
207 Target transcripts were chosen according to their proven relation to stress coping styles
208 in zebrafish (*Danio rerio*) (Rey et al., 2013) and also for their biological significance such
209 as, basic metabolism, lipid metabolism, growth, circadian rhythms and stress response.
210 Primers used were specific for Senegalese sole and already published (Table 2). The
211 mRNAs were analysed by real-time quantitative PCR (*q*PCR). Data were normalised
212 using 18*S* as a housekeeping transcript. Relative mRNA expression for each transcript
213 was determined using the method $(1 + E_T)^{(\Delta Ct)} / (1 + E_R)^{(\Delta Ct)}$ (Pfaffl, 2001). For this
214 purpose, RNA was extracted using TRI Reagent RNA Isolation Reagent following
215 manufacturer's instructions (SigmaAldrich). The complementary DNA was synthesised
216 using 1 µg of total RNA and oligo dT(20) in 20 µl reactions and the SuperScript® III
217 First-Strand Synthesis SuperMix 50 rxn kit following the manufacturer's protocol
218 (Invitrogen, Life technologies, USA). Before performing the *q*PCR, primers were

validated by conventional PCR using a cDNA pool from several samples randomly chosen. The HSX My taq Mix (Bioline) was used to perform the conventional PCR with the following conditions: initial activation step at 98 °C for 1 min, followed by 35 cycles: denaturation at 95 °C for 10 s, annealing at Tm (58 - 60 °C) for 15 s and extension at 72 °C for 15 s. Primers efficiency was evaluated by serial dilutions from 10 to 10,000. The *Q-rt*PCR was run using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific), 1 µl of the primer corresponding to the analysed transcript (10 pmol), 3 µl of RNA / DNA water free and 5 µl of cDNA at the validated dilution. Furthermore, amplifications were carried out with a systematic negative control (NTC; no template control) containing no cDNA. Standard amplification conditions contained a uracil DNA glycosylase (UDG) pre-treatment at 50 °C for 2 min, an initial activation step at 95 °C for 10 min, followed by 35 cycles: 15s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C.

Statistical analyses

Behaviour

Statistical analyses were performed using SPSS Statistics 20.0 (IBM®). A hierarchical clustering algorithm using the Euclidean distance matrix and complete linkage method was run to classify the fifty sole into different SCS categories (proactive, intermediate and reactive) according to the total activity time (in seconds) of all the individual behavioural tests conducted (Ibarra-Zatarain et al., 2016). A coefficient of variation (CV % = SD/mean*100) was calculated for each category representing the inter-individual sole variability in the population studied. Data were not distributed normally (Shapiro-Wilks) in all tests and a Kruskal-Wallis non-parametric test was performed to analyse the significant differences among SCS categories for the behavioural tests with non-normally

distributed data. However, when data was normal, the statistical test performed was One-way ANOVA, followed by Tukey's *post-hoc* test.

Pearson rank correlation test was run to observe the possible relationship between behaviours and between behaviours and genes with the possibility to strengthen the differential analysis of behavioural traits. Significance was set at P - value < 0.05 for all cases.

Q-rtPCR

Results were expressed as mean ± S.E.M (Standard error of the mean) and statistics analyses were performed using SPSS software and plotted with GraphPad Prism 6 software. Outliers of the corrected ratio for every mRNA on the different groups (proactive, intermediate and reactive) were extracted using the Tukey's test formula (k = 1.5). All data sets analysed were normally distributed (Shapiro-Wilks), although logarithmic transformation was performed when needed.

Raw data from both stress coping styles behaviour and mRNA abundance are available in *figshare* (DOI: 10.6084/m9.figshare.6300992). Comparisons of the mRNA transcripts among proactive, intermediate and reactive groups were made using One-way ANOVA, followed by Tukey's *post-hoc* test. A P - value < 0.05 indicated a statistically significant difference in all tests performed.

Results

Behavioural assays

The hierarchical cluster divided the population in three different clusters grouping similar stress responses in terms on total activity (*see* Fig. S2) from the individual tests "Restraining", "New environment" and "Confinement". Therefore, the final classification

of the hierarchical cluster was proactive, intermediate reactive animals according to the total activity displayed in every individual behavioural test.

Senegalese sole individuals presented a wide range of responses to the different tests performed indicative of inter-individual behavioural differences. The variability of the individual tests for the variable total activity was similar for the tests "Restraining" (REST; CV = 123.9 %) and "New Environment" (NE; CV = 132.7 %). However, the "Confinement" test presented the highest variability (CON; CV= 213.9 %). According to the other variables measured as first activity, NE and CON showed similar variability of the data for the first activity (CV = 90.7 % and 120.7 % respectively).

The total activity (Fig. 2) in the "New environment" (NE; K-W = 26.13; $P < 0.001$; Fig. 2B) and "Confinement" (Con; K-W = 25.46; $P < 0.001$; Fig. 2C) were significantly different ($P < 0.05$) among SCS categories. In the case of **NE**, intermediate (Total activity = 34.5 s; CV = 19.5 %; $P < 0.001$) and proactive juveniles (Total activity = 16.2 s; CV= 122.0 %; $P < 0.05$) showed significantly higher total activity than reactive (Total activity = 3.1 s; CV = 178.0 %), but there was no difference between proactive and intermediate individuals. In the case of **CON**, differences were found between proactive (Total activity = 55.5 s; CV = 75.6 %), being significantly higher than intermediate (Total activity = 3.8 s; CV = 108.0 %; $P = 0.001$) and reactive (Total activity = 2.1 s; CV = 147.1 %; $P < 0.001$), but not between intermediate and reactive. In the case of the restraining test, **REST**, marginal differences were found among groups (K-W = 5.491; $P = 0.0642$; Fig. 2A) and there were no significant differences among proactive (Total activity = 14.1 s; CV = 122.7 %), intermediate (Total activity = 13.8 s; CV = 96.7 %) and reactive (Total activity = 4.9 s; CV = 55.3 %).

Regarding first activity (Fig. 3) the situation was similar to the total activity, so the "New Environment" (NE; $F_{2, 47} = 7.822$; $P = 0.0012$; Fig. 3B) and "Confinement" (CON; $F_{2, 47}$

= 3.387; P = 0.0423; Fig. 3C) tests presented differences among SCS categories. In case of the NE, intermediate (first activity = 38.6 s; CV = 167.0 %; P < 0.001) presented significantly lower latencies than reactive sole juveniles (first activity = 203.6 s; CV = 65.4 %), however, proactive animals (first activity = 105.9 s; CV = 117 %; P > 0.05) presented no significant differences in comparison to intermediate and reactive sole. "Confinement" test, CON, showed clearly differences between proactive (first activity = 27.4 s; CV = 225.5 %; P < 0.001) and reactive latencies (first activity = 150.5 s; CV = 96.2 %), however, intermediate sole (first activity = 95 s; CV = 149.0 %; P > 0.05) did not present differences in latencies with the extremes. In the case of REST, no differences were found among coping styles (K-W = 2.366; P = 0.3064; Fig. 3A), where proactive animals (first activity = 10.8 s; CV = 258.1 %), intermediate (first activity = 1.9 s; CV = 149.8 %) and reactive (first activity = 8.2 s; CV = 278.2 %; P > 0.05) showed similar latencies profile.

Analysing the group-test, the risk-taking test, eleven of fifty juveniles (22 %) crossed from the well-known to the unfamiliar area, 6 of them coincided with proactive classification, 4 with intermediate and 1 was classified as reactive by the cluster. According to the results, the classification of the stress coping style groups was considered appropriate to continue with the brain transcripts abundance statistical analysis.

Brain transcripts abundance

Brain mRNAs abundance was analysed in ten individuals from each SCS category (proactive, intermediate and reactive). In the case of the reactive group, the ten fish considered as the most reactive (the last ten fish in the list of the hierarchical cluster) were used to balance the number among categories. According to the brain transcripts abundance in sole juveniles, the abundance or expression of four of the six mRNAs tested

were significantly different among coping styles' categories. In the case of glyceraldehyde-3-phosphate dehydrogenases 2 (*gapdh-2*) proactive and intermediate individuals (up-regulated) exhibited significantly higher expression than reactive individuals (down-regulated) ($F_{2, 27} = 8.173$; P = 0.0017; Fig. 4A). The other transcripts that were differentially expressed, presented similar expression profile for the extremes categories (proactive and reactive), which were up-regulated and were significantly differently expressed than intermediate (down-regulated): Period 1 (*per1*) (K-W = 14.43; P = 0.0007; Fig. 4B), Insuline-like Growth factor (*igf-Ia*) ($F_{2, 27} = 4.606$; P = 0.0190; Fig. 4C) and Peroxisome proliferator-activated receptor (*pparß*) ($F_{2, 25} = 7.554$; P = 0.0027; Fig. 4D). The other two transcripts did not present significant differences in expression, Specific hypothalamic corticotropin-releasing hormone (CRH) binding protein (*crh-BP*) ($F_{2, 24} = 0.4842$; P = 0.6221) and Heat shock protein 90, alpha (cytosolic) class (*hsp90aa*) ($F_{2, 27} = 2.346$; P = 0.1150).

Behavioural and brain mRNA abundance relationship

First of all, correlation among variables from the different behavioural tests was observed to try to discern the association among them. To observe the complete map of the relationship, the data was not split in categories, it was treated in continuous. In this context, the Restraining variables (first and total activity) do not present significantly correlation between them (r = -0.158; P = 0.403), however, negatively correlation was observed between the New environment variables (first and total activity) (r = -0.655; P = 0.001) and also Confinement variables (first and total activity) (r = -0.382; P = 0.037). It is worth mentioning that there was no correlation among the variables from the different behavioural tests observed in this study.

In case of the association among the candidate genes used for this study, *gapdh-2* is slightly correlated with *per1* (r = 0.395; P = 0.031), good correlated with *hsp90aa* (r =

343 0.713; P < 0.001), *igf-a* (*r* = 0.548; P = 0.002), and *pparß* (*r* = 0.619; P = 0.001). The *per1*
344 transcript was strongly correlated with *igf-a* (*r* = 0.774; P < 0.001) and *pparß* (*r* = 0.641;
345 P = 0.001). The *hsp90aa* gene was positively correlated with *igf-a* (*r* = 0.414; P = 0.023)
346 and *pparß* (*r* = 0.596; P = 0.001). The *igf-a* gene was strongly correlated with *pparß* (*r* =
347 0.758; P < 0.001) and slightly correlated with *crh-bp* (*r* = 0.375; P = 0.041). The *pparß*
348 transcript was correlated with *crh-bp* (*r* = 0.549; P = 0.002).
349 After the observation whether genes involved in several biological functions varied in
350 expression with coping styles, the individual correlation was carried out to observe the
351 relationship between coping styles varables from the different behavioural tests applied
352 and gene expression (Table 3). In this case, there were just two variables from the same
353 behavioural test ("New environment") which obtained significant correlation with the
354 expression of 4 mRNAs of the 6 tested (*Per1, hsp90aa, pparß and crh-bp*). However,
355 there exist some association between behavioural variables and gene expression which
356 were not significantly correlated but showed a clear trend. For example, first activity from
357 Confinement test was slightly non-correlated with *gapdh-2* (*r* = 0.315; P = 0.09) and
358 *hsp90aa* (*r* = 0.323; P = 0.082).

359 ## Discussion

360 In the present study natural variation in mRNA brain abundance of selected transcripts
361 was described in cultured Senegalese sole early stage juveniles and whether coping traits
362 were associated with these transcriptional differences. Based on previous studies
363 differences in mRNA brain abundance were expected in relation to the behavioural traits
364 (Mackenzie et al., 2009; Aubin-Horth et al., 2012; Rey et al., 2013).

365 ### Behavioural assays

366 In terms of the behavioural study, previous studies have demonstrated that the same
367 behavioural tests conducted in this study classify animals according to their behavioural

traits (proactive through to reactive) in diverse fish species, such as stickleback (*Gasterosteus aculeatus*) (Bell, 2005), gilt-head seabream (*Sparus aurata*) (Castanheira et al., 2013) and zebrafish (Tudorache et al., 2015). In the present study we classified early stage Senegalese sole juveniles in three SCS categories (proactive, intermediate and reactive) using a hierarchical cluster analysis. The present study considered the intermediate as another category having in mind the association of the presence of this category with captive environment. Oortmerssen and Busser (1989), observed in a natural feral mice population a proactive and reactive bimodal distribution of SCS variables. However, this distribution changed when the experiment was performed under laboratory conditions (controlled), where another coping style category was found, the intermediate, probably due to the low natural selection pressure in captive conditions. In case of the Senegalese sole, domestication could be the reason of the presence of this third coping category, as under captive conditions animals have no biological limited resources such as food, proper habitat conditions (pH, temperature, salinity…) and no predators, so there are no or different selective pressures acting upon them. This model, with proactive, reactive and intermediate coping styles has been observed in a widespread variety of animal species, including fish such as African catfish (*Clarias gariepinus*) (van de Nieuwegiessen et al. 2010), several salmonids species (Huntingford and Adams, 2005), among others. The presence of correlation between the variables of the different behavioural tests denoted the importance of phenotypic pleiotropy to perceive the variability of the population. However, no correlation was observed among variables from the different behavioural tests applied, showing the possibility that the activity in this species fluctuates depending on the test conducted. Hence, in the present study, proactive sole presented lower latencies and higher activity than reactive, indicating higher explorative behaviour and different response to stressful circumstances. However,

intermediate sole is less consistent obtaining a different profile according to the behavioural test conducted.

Brain transcripts abundance

Gene expression data is usually difficult to analyse in terms of variability, which could be influenced by several factors including environmental elements. The interpretation of such interactions with the different variations between individuals inter and intra-populations have remarkable potential for evolution, unravelling the patterns of gene expression and phenotypic variation (Whitehead and Crawford, 2006). In our study, those interactions were considered according to the different coping styles profiles (proactive, intermediate and reactive) where Senegalese sole provided different levels of mRNAs transcript abundances under the same environmental conditions (temperature, photoperiod, salinity, oxygen saturation, feeding regime…) exposing the fish to some kind of challenge which has been considered the stress coping styles behavioural tests. Hence, differences in behavioural traits might reveal a specific outline presenting altogether a specific profile, phenotype and genotype.

The few studies that have been completed have found a clear relationship between stress coping styles classification and gene expression. In this context, the results of the present study were in concordance to previous studies, for example, MacKenzie et al. (2009) found differences in transcript abundance between proactive and reactive common carp (*Cyprinus carpio*) when those animals were under the same environmental circumstances (temperature and photoperiod) and applying an immune challenge afterwards. In that report, coping styles were included in the analysis reducing the unexplained variation and increasing the interpretation of the experimental data.

The transcripts abundance profile was carried out by *q*-rtPCR in 6 specific mRNAs (*gapdh2*, *Per1*, *igf-Ia*, *pparß*, *hsp90aa* and *crh-BP*) where 4 of the 6 candidate mRNAs

418 (*gapdh2*, *pparß*, *igf-Ia* and *Per1*) were considered differential expressed transcripts
419 (DETs) suggesting that there exist variations in the transcriptome among Senegalese sole
420 individuals classified by coping styles. The primers of all these mRNAs have been
421 published before exhibiting the importance of the study of these ones associated with
422 Senegalese sole species. Specifically, the different mRNAs chosen for this study were
423 related to basic metabolism, stress responses and biologic conditions specifics for
424 Senegalese sole, which could provide important information in terms of development (*see*
425 Table 2). Differences in metabolism have been linked with changes in coping styles in
426 some species (Biro and Stamps, 2008; Martins et al., 2011), including fish such as
427 zebrafish (Rey et al., 2013), common carp (MacKenzie et al., 2009; Rey et al., 2016),
428 Nile tilapia (*Oreochromis niloticus*) (Vera Cruz and Brown, 2007) and rainbow trout
429 (*Oncorhynchus mykiss*) (Thomson et al., 2011) where these studies associated
430 physiological and gene expression variation with behavioural phenotypic traits. One of
431 the most recent studies performed on sea bass (*Dicentrarchus labrax*) (Alfonso et al.,
432 2019) found some transcripts linked with stress axis and neurogenesis were differently
433 expressed depending on the behavioural traits, however, this species has not shown
434 consistency in boldness over time using different behavioural tests (group and
435 individual).
436 In the present study, one of the transcripts differentially expressed was Glyceraldehyde-
437 3-phosphate dehydrogenase (*gapdh*), which is habitually used as a housekeeping
438 transcript for its ubiquitous presence in all tissues in quantitative *rt*-PCR. However, there
439 are facts that evidence that *gapdh* levels of expression may vary among tissues,
440 development, or during different physiological processes including behavioural traits
441 (MacKenzie et al., 2009; Rey et al., 2013). Moreover, *gapdh* was discarded as a suitable
442 housekeeping transcript for Senegalese sole (Infante et al., 2008). The metabolic function

might be compromised by acute and chronic stress, explaining why *gapdh-2*, which has been demonstrated to be the *gapdh* isoform more expressed in brain in Senegalese sole (Manchado et al., 2007), was up-regulated in proactive sole relative to reactive fish (down-regulated). MacKenzie et al. (2009) made similar observations with common carp, where *gapdh* presented up-regulation in proactive fish and down-regulation in reactive animals demonstrating differences between coping styles and basic metabolism. These outcomes would be consider similar to the association found by Ibarra-Zatarain et al., 2016 between physiological response and behavioural traits in Senegalese sole, who perceived differences in cortisol concentration between proactive (low concentration) and reactive sole (high concentration). As observed before, *gapdh-2* expression was correlated with the expression of *per1*, *pparβ*, *hsp90aa* and *igf-I* genes, exhibiting that all these transcripts are also involved with metabolism, however, the distinct expression profiles in the different behavioural traits show that there is large inter-individual variation in post-stress responses in early Senegalese sole juveniles affecting gene expression.

The other three mRNAs (*pparβ, igf-Ia* and *per1*) differentially expressed among coping style categories in this study, presented similar expression profiles in proactive and reactive animals which were up-regulated and intermediate animals presented high down-regulation, and these transcripts are associated with feeding behaviour and nutrition. There are no data to compare with in other fish species in relation with these specific transcripts and individual variation in mRNA abundance. Moreover, the expression of these three genes presented a strong correlation, highlighting the relationship among them in functionality and expression profile. In general, intermediate animals present more behavioural plasticity than the extremes coping styles categories, proactive and reactive (Dingemanse et al., 2010). According to these results in mRNAs abundance, intermediate

sole presented also different profiles depending on the behavioural test performed (*for more detail see* Fig. 2).

The first transcript differentially expressed associated with nutrition was peroxisome proliferator-activated receptor (*pparβ*). This transcript is implicated in the skeletal, brain and skin functions in mammals (Lee et al., 2003; Giaginis et al., 2007) and in addition, this nuclear receptor has been associated with the early step towards adipogenesis. Moreover, *pparβ* is a target transcript for fatty acids and vitamin A. The expression of *pparβ* is influenced by nutrition in fish such as gilthead seabream (Fernandez et al., 2011) and sea bass (Vagner et al., 2009) acting as regulators of lipid and lipoprotein metabolism and associated with feeding behaviour. The second transcript associated with nutrition and feeding behaviour was Insuline-like growth factor I (*igf-I*) which shows a central role in postnatal growth in mammals (Baxter, 1994). *Insuline-like growth factor I* mRNA profile in hepatic and non-hepatic tissues are dependent to the growth hormone (GH), which is synthesized in the pituitary gland and secreted into the blood circulation under the regulation of different factors such as neuronal, hormonal and nutritional. Nevertheless, GH does not appear to control the relative expression of *igf-I* in non-hepatic tissues in fish. Duan (1998) demonstrated that *igf-I* is highly conserved between fish and mammals and is found in all development stages in fish. Besides, nutritional status has a deep effect on *igf-I* expression in fish. The third transcript associated with feeding behaviour was period 1 (*per1*), which is one of the clock genes that control the circadian rhythm. The *period* genes (*per1, per2 and per3*) are negative regulators, which inhibit the CLOCK and BMAL1 activators (Reppert and Weaver, 2002). This mechanism is cyclic, where the expression of clock genes is approximately daily. The transcripts, *per* are expressed during daylight (diurnal), however, CLOCK and BMAL1 are expressed at night (nocturnal). Fish have a feeding schedule when they are under captive conditions

493 and feeding can work as a strong synchronizer of circadian rhythms in several animals,
494 increasing the locomotor activity some hours before the food is provided, which is called
495 food anticipatory activity (Mistlberger, 2009). In case of the Senegalese sole, even
496 though, is considered a nocturnal species, it has been observed that feeding schedule can
497 modify the locomotor activity to diurnal when they are in captive conditions, due to
498 operational activities (Carazo et al., 2016). This activity can affect the expression of the
499 clock genes, for example in zebrafish it was observed that the animals exposed to different
500 lights and different feeding schedules, including random feeding presented different *per1*
501 expression profiles (Lopez-Olmeda et al., 2010). In the random feeding regime, the
502 animals did not present food anticipatory activity and *per1* expression rhythm
503 disappeared demonstrating the importance of feeding behaviour in the circadian
504 rhythmicity. In the present study, sole were fasted 24 hours prior to the behavioural tests
505 and according to their feeding regime all sole used for the experiment should present
506 similar expression profile, however, only proactive and reactive presented up-regulation
507 in every transcript of these three and intermediate sole showed high down-regulation, so
508 the different expression among coping styles categories of those genes might be explained
509 just by the behavioural screening prior to molecular analysis.
510 Intriguingly, both stress-related transcripts (*hsp90aa* and *crh-BP*) tested in this study were
511 not differentially expressed among coping styles categories. Curiously, *hsp90aa*
512 expression was also correlated with *pparβ* and *igf-I,* associated with feeding behaviour
513 and nutrition, but the expression of this transcript was not correlated with *crh-bp* that
514 presents another expression profile. The *hsp90* transcript has been associated with
515 nutritional stress in early stages in fish (Cara et al., 2005) and as a protection against
516 different stressors such as infections, heat shock, etc. (Basu et al., 2002). In previous
517 studies performed with Senegalese sole revealed that *hsp90aa* was activated in the

518 moment that sole was under a heat shock treatment, however, no significant differences
519 were found after a cold shock treatment. Nevertheless, in our study, all animals used for
520 the experiment were under the same prior and experimental conditions without any
521 treatment, so the change in the regulation of *hsp90aa* transcript could be caused by the
522 variability between individuals due to the behavioural tests conducted. The crh-binding
523 protein is considered different from the crh receptors and it is very conservative among
524 phylum, suggesting that the functions are also evolutionary conserved. Corticotropin
525 releasing hormone binding protein (*crh-BP*) presented down-regulation in the three
526 groups, but the variability intra- and inter-group resulted higher than the other transcripts.
527 This could be explained whether the animals did not accuse a high influence according to
528 the stressful period performing the different tests. Wunderink et al. (2011) found that *crh-*
529 *BP* levels were not affected at different stocking densities (chronic stress response) in
530 Senegalese sole and in addition, the *crh-BP* expression was improved in both densities
531 when animals were moved to hypersaline seawater (acute stress response) proposing that
532 *crh-BP* worked as a modulator of the acute stress reaction. Another study showed that the
533 exposure to air during 30 seconds in Senegalese sole did not alter the expression of *crh-*
534 *BP* transcript (Lopez-Olmeda et al., 2013). The stress-induced regulation of this transcript
535 in fish, seems to be related to the sort of stress and its duration. Therefore, in the present
536 study, the down-regulation in all groups could be explained that in the moment the fish
537 finished the tests did not present an acute stress, however, the variability in the three
538 coping style categories proposed that the expression of this transcript could be analysed
539 individually. The association of *hsp90aa* transcript to SCS has not been evaluated in other
540 fish species before the present study. However, other transcripts related to stress axis (*mr,*
541 *crf, crf-r2, pomc1, gr1 and gr2*) were tested to associate gene expression and SCS in other
542 fish species, such as, stickleback (Aubin-Horth et al., 2012) and sea bass (Alfonso et al.,

543 2019). Some of those transcripts were differentially expressed depending on behavioural
544 traits, for example in case of sea bass, *mr*, *crf*, and *gr2* were higher expressed in shy fish
545 (considered as reactive). In the present study the expression of *crh-BP* transcript was
546 down-regulated in all behavioural traits, showing a pattern of expression completely
547 different from sea-bass *crf* transcript expression. These differences with our study could
548 be related to the differences in activity and swimming behaviour, which is completely
549 dissimilar between sea bass (constantly swimming and active) and sole (sedentary during
550 long periods). However, it is worth to mention here that the expression of the *crh* and *crh-*
551 *BP* are not always comparable, due to the high variability in mRNA expression inside the
552 CRH system and among species. For example, social status variation using visual cues in
553 African cichlid (*Astatotilapia burtoni*) showed higher expression in whole brain *crf* and
554 *crf-BP* in dominant males than subordinates (Chen and Fernald, 2011). Therefore, social
555 status would be one of the reasons to obtain differences in stress responses. Recent studies
556 have been observed differences in physiological responses in sea bass depending on social
557 hierarchy where dominant fish presented different muscle activity, immune response and
558 stress response (Carbonara et al., 2015, 2019).

559 Nevertheless, the results from this study suggest that the life strategy, the absence of
560 constant swimming, activity, sedentary and non-aggressive behaviour (Salas-Leiton et
561 al., 2010; Fatsini et al., 2017) of Senegalese sole could be behind these differences
562 compared with active species, showing the variability of the data depending on the
563 different behavioural tests conducted. Moreover, there was no relationship between SCS
564 classification and social status in this species (*data not shown*), that means that proactive
565 sole did not always display dominance behaviour, being also variable depending on the
566 dominance test applied. However, Ibarra-Zatarain et al., 2016 demonstrated the presence
567 of two clear stress coping behavioural axes ("fearfulness-reactivity" and "activity-

exploration") in this species, which are also reflected in this study noticing the results from different behavioural test and brain gene expression.

Conclusions

In conclusion, Senegalese sole were classified into three different stress coping style groups, proactive, intermediate and reactive. One transcript, *gapdh-2* was differentially expressed between proactive and reactive behavioural trait and three DETs were differentially expressed between the intermediate group and the other SCS categories. The three DETs may have importance to screen for intermediate individuals. Coping style and molecular expression appear to be linked in this species with clear differential expression between behavioural traits, however, the transcriptional expression pattern of Senegalese sole in relation to SCS was different to the patterns observed in other fish species, these differences may be due to species specific behavioural differences. Altogether indicates the complexity and the potential to explain mechanisms controlling behavioural pleiotropy and increase our understanding of the molecular context of adaptive variation among individuals within and between populations. Besides, this knowledge of coping styles could improve management and welfare under captive conditions, to envisage population dynamics widening information for its status conservation. However, more physiological and functional studies are needed to understand the effects of the stress coping style phenotypes to the development of this species in captivity.

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Competing interests

The authors have no competing interests.

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Figure Legends:

Figure 1. Chronogram illustrating the experimental design of the different stress coping style (SCS) tests performed by early Senegalese sole juveniles (*n* = 50). First activity (1st act), escape attempts and total activity.

Figure 2. Stress coping style tests regarding Total activity variable in seconds in early Senegalese sole juveniles (*n* = 50). A) Restraining, B) New environment and C)

857 Confinement compared among the different stress coping style categories (proactive,
858 intermediate and reactive) classified according to total activity measurement. Data was
859 shown in Mean ± SEM. Different letters means to be significantly different (Kruskal-
860 Wallis $P < 0.05$ level of significance).
861
862 **Figure 3. Stress coping style tests regarding First activity variable in seconds in early**
863 **Senegalese sole juveniles (*n* = 50)**. A) Restraining B) New environment and C)
864 Confinement compared among the different stress coping style categories (proactive,
865 intermediate and reactive) classified according to total activity measurement. Data was
866 shown in Mean ± SEM. Different letters means to be significantly different (Kruskal-
867 Wallis or One-Way ANOVA $P < 0.05$ level of significance).
868
869 **Figure 4. Brain transcripts abundance of different genes which were differentially**
870 **expressed among groups (proactive, intermediate and reactive) in early Senegalese**
871 **sole juveniles (*n* = 30).** A) *gapdh-2*, B) *per1*, C) *igh-Ia* and D) *pparß*. Data was
872 transformed to Log_{10} and was shown in Mean ± SEM. Different letters means to be
873 significantly different expressed (One-Way ANOVA $P < 0.05$ level of significance).

Figure 1

S. senegalensis

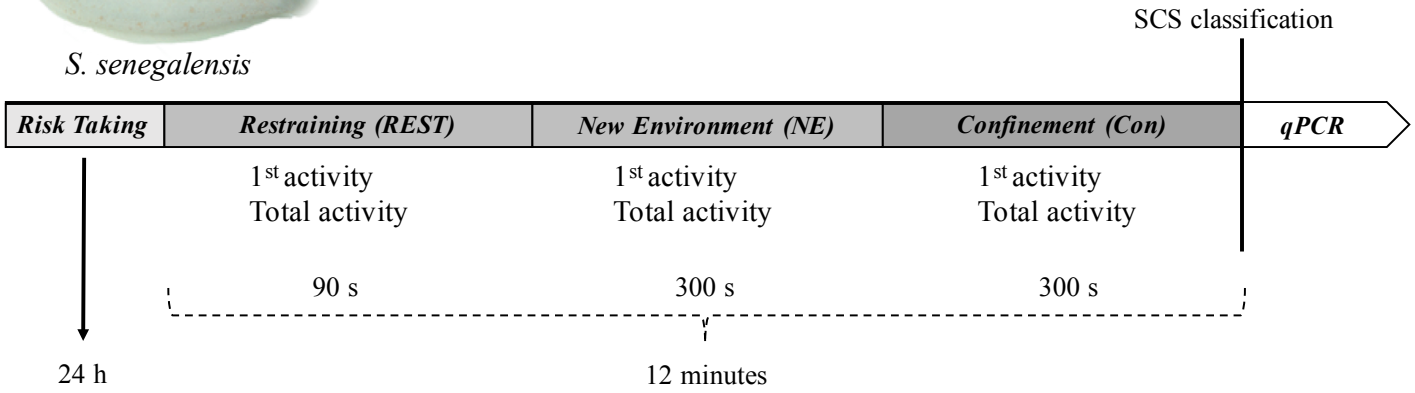


Figure 2

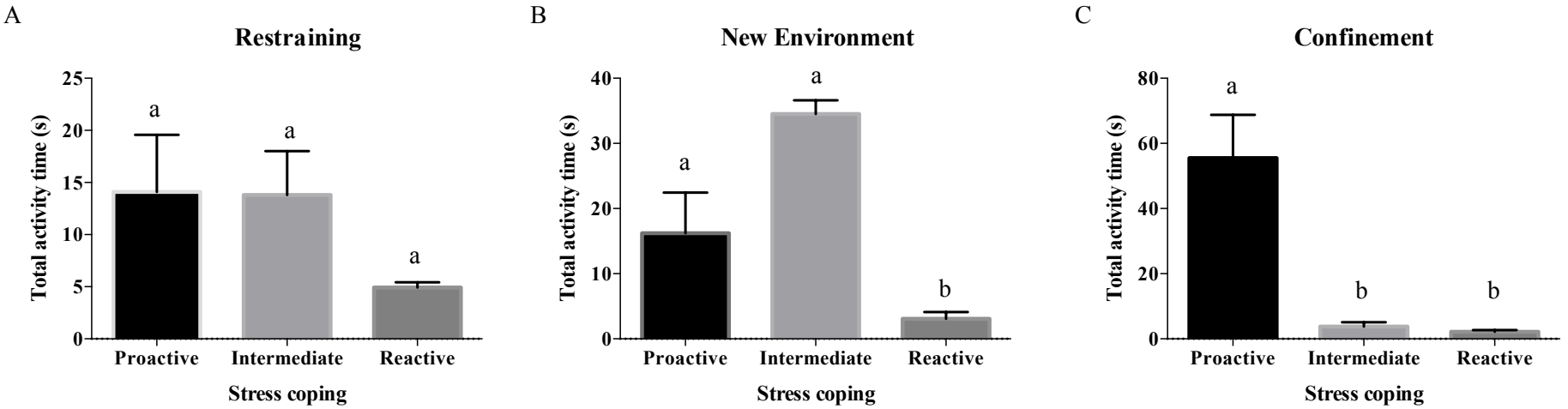

A
Restraining
Total activity time (s)
25
20
15
10
5
0
a
a
a
Proactive
Intermediate
Reactive
Stress coping
B
New Environment
Total activity time (s)
40
30
20
10
0
a
a
b
Proactive
Intermediate
Reactive
Stress coping
C
Confinement
Total activity time (s)
80
60
40
20
0
a
b
b
Proactive
Intermediate
Reactive
Stress coping

Figure 3

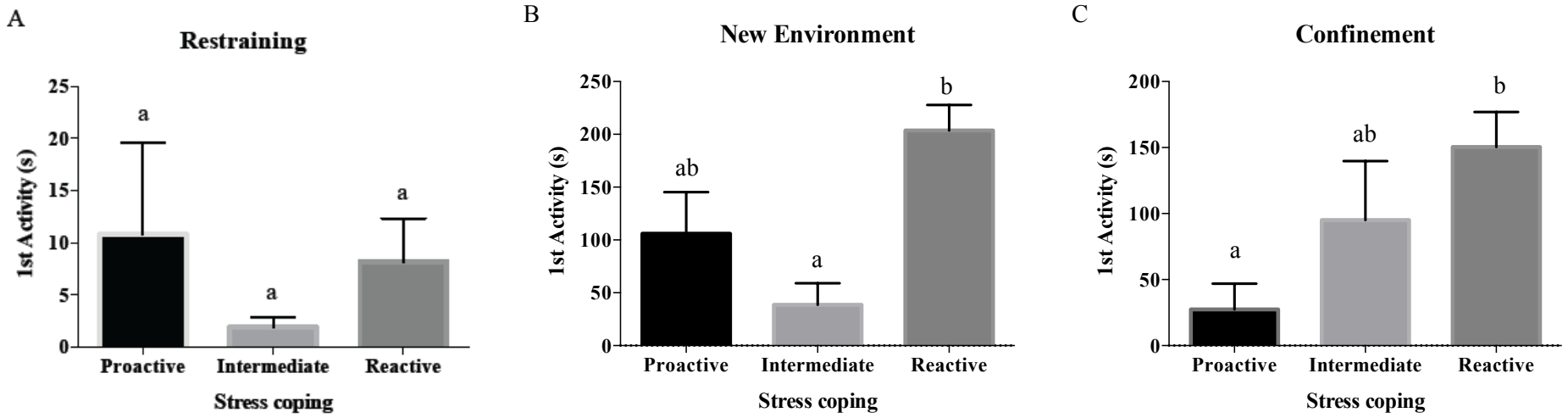
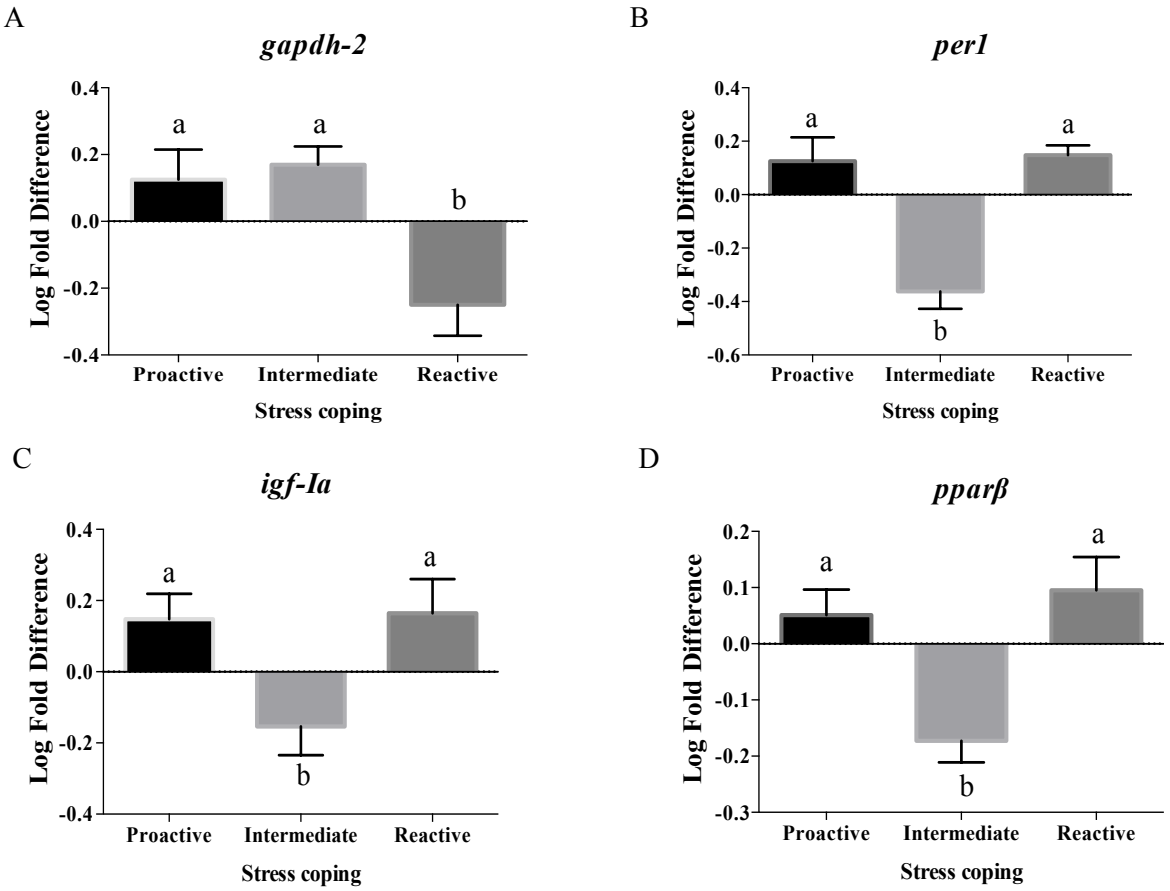

A
Restraining
1st Activity (s)
25
20
15
10
5
0
a
a
a
Proactive
Intermediate
Reactive
Stress coping
B
New Environment
1st Activity (s)
250
200
150
100
50
0
ab
a
b
Proactive
Intermediate
Reactive
Stress coping
C
Confinement
1st Activity (s)
200
150
100
50
0
a
ab
b
Proactive
Intermediate
Reactive
Stress coping

Figure 4

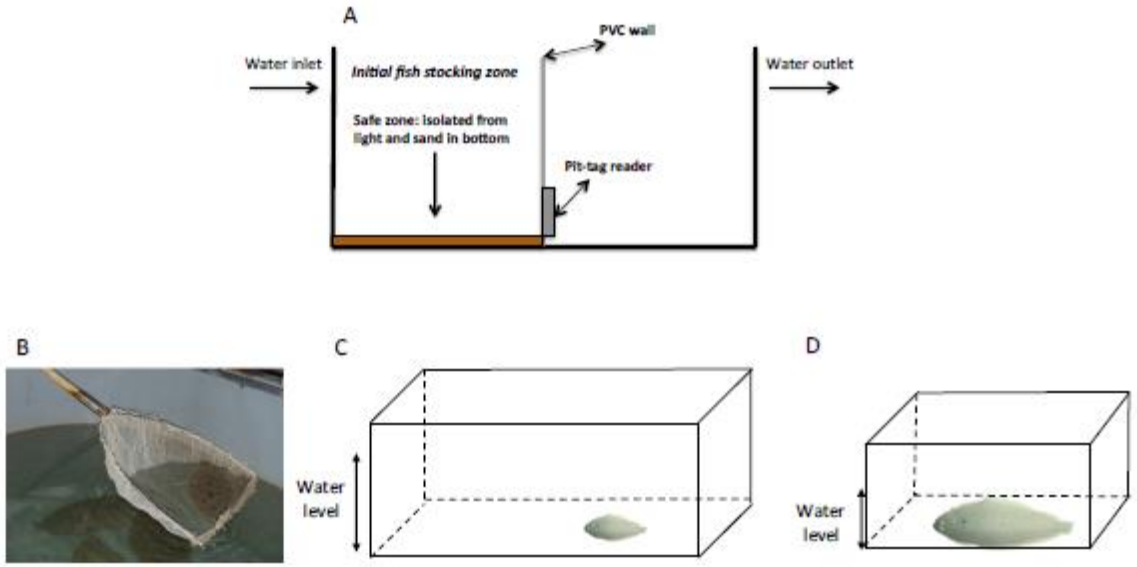

A
gapdh-2
Log Fold Difference
0.4
0.2
0.0
-0.2
-0.4
a
a
b
Proactive
Intermediate
Reactive
Stress coping
B
per1
Log Fold Difference
0.4
0.2
0.0
-0.2
-0.4
-0.6
a
b
a
Proactive
Intermediate
Reactive
Stress coping
C
igf-Ia
Log Fold Difference
0.4
0.2
0.0
-0.2
-0.4
a
b
a
Proactive
Intermediate
Reactive
Stress coping
D
pparβ
Log Fold Difference
0.2
0.1
0.0
-0.1
-0.2
-0.3
a
b
a
Proactive
Intermediate
Reactive
Stress coping

Legends:

Supplementary Figure 1. Description of material and indications to perform the coping styles tests on Senegalese sole juveniles. A Risk-taking test; B Restraining test; C New environment test; D Confinement test.

Supplementary Figure 2. Distribution of the Senegalese sole juveniles (n = 50) applying hierarchical cluster according to the "Total activity" variable of the three behavioural tests (Restraining, New environment and Confinement) conducted.

Supplementary Figure 1



Supplementary Figure 2