

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

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18

## 19 **Abstract**

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

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

## 45 **Introduction**

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

51 Some research has already shown that wild individuals or non-selected line from the same  
52 population behave differently among them (Koolhaas et al., 1999). This difference in  
53 behaviour is more evident when stressful factors are present in the environment.  
54 Individuals exhibit different responses or stress coping styles (SCS) when subjected to  
55 stressful or risky situations and these may range from proactive to reactive responses  
56 (Koolhaas et al., 1999). Proactive animals are considered more active, aggressive, tend to  
57 grow faster and may have better mating opportunities by higher dominance but show  
58 lower plasticity to changes in the natural environment than reactive animals (Koolhaas et  
59 al., 1999; Sih et al., 2004; Coppens et al., 2010; Wilson and Godin, 2009). Contrarily,  
60 reactive animals are characterized by low levels of conspecific aggression, avoid taking  
61 risk in unknown environments with lower rates of activity, and show passive behaviours  
62 such as immobility in response to stressful stimuli (Koolhaas et al., 1999; Koolhaas et al.,  
63 2007; Castanheira et al., 2017).

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

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

76 Therefore, gene expression in relation to SCS in terms of individual variation has other  
77 influences and the genetic component would be delimiting the coping strategies of the  
78 individuals for several features, such as behavioural responses, genomic and the  
79 ecological niche. Moreover, genomic methods using fish have already offered  
80 discernments into the mechanisms that trigger short and long-term environmental  
81 adaptations. Individual variation has been associated with genomic variation in several  
82 fish species (Huntingford et al., 2010; MacKenzie et al., 2009; Øverli, 2007; Rey et al.,  
83 2013; Rey et al., 2016) and the information of mRNAs differentially expressed between  
84 diverse SCS groups could be used for the interpretation of biological responses to resolve  
85 variation, knowing that those variations might be adaptive or genetically fixed within the  
86 population (MacKenzie et al., 2009). For example, some studies found that proactive fish  
87 showed up-regulation of the immune and metabolic related genes (such as *gapdh*) after a  
88 simulated infection challenge with LPS (lipopolysaccharide) as a similar bacterial  
89 infection while reactive fish showed down-regulation in the same challenge (MacKenzie  
90 et al., 2009; Rey et al., 2013).

91 Senegalese sole (*Solea senegalensis*) is an important marine flatfish species for the  
92 European aquaculture industry due to its high market price and demand (Howell et al.,  
93 2011). Furthermore, conservation measures are unknown and there exist few data on their

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

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

119

## 120 **Material and Methods**

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

### 124 *1. Animal rearing conditions*

125 Fish used for this experiment were provided by Stolt Sea Farm (Santiago de Compostela,  
126 Spain) and were transported from La Coruña to IRTA's facilities in March of 2012. Fish  
127 were kept at the Research Centre facilities of IRTA, in Sant Carles de la Ràpita, North  
128 East Spain and were held in 10 m<sup>3</sup> fiberglass tanks with natural photoperiod  
129 (40°62'82.42", 0°66'09.37, using artificial lighting). All tanks were located in a  
130 greenhouse structure and were connected to a recirculation system (IRTAMar®) to  
131 maintain a simulated natural water temperature (9 – 19 °C: winter to summer), oxygen (5  
132 – 6mg l<sup>-1</sup>) levels and salinity (35 – 38 ‰) levels. Sole were fed *ad libitum* five days per  
133 week with balanced feed (LE - 3mm ELITE, Skretting, Co.). Fifty early juvenile  
134 Senegalese sole (121.4 ± 8.1 g) were randomly selected to conduct the behavioural tests  
135 in November (the temperature registered was 12 – 14 °C). Animals were moved and  
136 acclimated to a 400 L fiberglass tank two weeks before tests started. The acclimation tank  
137 was also connected to a recirculation system (IRTAMar®) to maintain a constant  
138 temperature of 13 ± 1 °C to avoid the environmental influences on the different  
139 behaviours among individuals and oxygen (5 – 6mg l<sup>-1</sup>) levels. Water quality parameters  
140 were registered by computer system using temperature and oxygen probes. The pooled  
141 control animals used for RNAs transcripts analysis were from the same batch of the  
142 experimental sole used for this study and were acclimated to the same tanks as the  
143 experimental fish. Control fish were fed normally and were not used for any experimental

144 procedure to obtain objective data similar to standard husbandry conditions. All fish were  
145 PIT tagged (Passive Integrated Transponder: ID100A, Unique Trovan-Zeuss; Madrid,  
146 Spain) intramuscular for individual identification.

## 147 2. *Behavioural assays*

148 The tests applied were selected as appropriate SCS tests following Ibarra-Zatarain et al.,  
149 (2016) who demonstrated that one “Risk-taking” in group and three individual tests  
150 (“Restraining”, “New environment” and “Confinement”) screened Senegalese sole  
151 juveniles into a range of different coping styles (proactive through to reactive), and those  
152 tests were the most representative to explain the individual variation.

153

### 154 2.1. *In group testing*

155 The first test performed was *Risk taking in groups*. The objective of this test was to  
156 determine the fish willingness to cross from a well-known “safe” area to an unfamiliar  
157 area (risky zone). This has been established as a standardised test to screen for SCS in  
158 fish and other animals (Smith et al., 1992; Huntingford et al., 2010; van Oers et al., 2004).  
159 A 400 L fiberglass tank was divided into two equal zones by a polyvinyl chloride (PVC)  
160 wall. The wall had a small window at the bottom to allow fish to cross between both  
161 areas. The window was at the centre of a PIT (passive integrated transducer) tag reading  
162 antenna (SQR series; TROVAN-ZEUS, Madrid, Spain) that read the tag number of the  
163 fish which crossed through the window to the unfamiliar zone. (*see* Fig. S1A). The known  
164 sheltered area simulated natural conditions for the species, the area was isolated from  
165 light (2 *lux* on the surface) and covered by sand. On the other hand, the risky or unknown  
166 area was provided with more light (15 *lux* (OSRAM DULUX 48W on the surface) and  
167 the bottom was lacking substrate. Before beginning the test, the fish were acclimated 24  
168 hours in the well-known sheltered zone keeping the window closed until the beginning

169 of the test. The duration of the test was 24 hours and the Risk-taking test was video  
170 recorded to validate the results registered by the antenna. The test was performed for two  
171 groups of 25 fish. The number of fish was the variable observed in this group behavioural  
172 test.

173

## 174 2.2. Individual testing

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

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

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

192 The last test performed was the “Confinement” test (CON); each fish was individually  
193 placed in a plastic tank that simulated a confinement situation. The tank dimensions were



194 25 x 14 x 8 cm, rectangular shape and white colour (*see* Fig. S1D). The duration of the  
195 test was again 5 min (300 seconds), during which two variables were measured: a) the  
196 latency time or time of first activity when the fish started to move in the tank and b) the  
197 total activity time referring to the total time the fish was moving.

198 For the last two tests (New environment and Confinement test), if fish did not move at all  
199 during the period of the test, the maximum duration of the test (300s) was noted for  
200 statistical analysis. At the end of the “Confinement” test, all animals were euthanized  
201 with an overdose of MS-222 (tricaine methanesulfonate; Acros-Organic, New Jersey,  
202 USA), brains were dissected, frozen in dry ice and stored at -80 °C for posterior molecular  
203 analysis.

#### 204 *Quantitative real time PCR*

205 The differential expression of brain target transcripts (*gapdh2*, *per1*, *igf-Ia*, *pparβ*,  
206 *hsp90aa* and *crh-BP*) for stress coping behaviour (Table 1) was measured in brains from  
207 thirty sole, ten fish from each phenotypical category (proactive/intermediate/reactive)  
208 (*see statistical analyses (behaviour)* section for classification of behavioural traits).

209 Target transcripts were chosen according to their proven relation to stress coping styles  
210 in zebrafish (*Danio rerio*) (Rey et al., 2013) and also for their biological significance such  
211 as, basic metabolism, lipid metabolism, growth, circadian rhythms and stress response.

212 Primers used were specific for Senegalese sole and already published (Table 2). The  
213 mRNAs were analysed by real-time quantitative PCR (*qPCR*). Data were normalised

214 using 18S as a housekeeping transcript. Relative mRNA expression for each transcript  
215 was determined using the method  $(1 + E_T)^{(\Delta Ct)} / (1 + E_R)^{(\Delta Ct)}$  (Pfaffl, 2001). For this

216 purpose, RNA was extracted using TRI Reagent RNA Isolation Reagent following  
217 manufacturer’s instructions (SigmaAldrich). The complementary DNA was synthesised

218 using 1 µg of total RNA and oligo dT(20) in 20 µl reactions and the SuperScript® III

219 First-Strand Synthesis SuperMix 50 rxn kit following the manufacturer's protocol  
220 (Invitrogen, Life technologies, USA). Before performing the *q*PCR, primers were  
221 validated by conventional PCR using a cDNA pool from several samples randomly  
222 chosen. The HSX My taq Mix (Bioline) was used to perform the conventional PCR with  
223 the following conditions: initial activation step at 98 °C for 1 min, followed by 35 cycles:  
224 denaturation at 95 °C for 10 s, annealing at  $T_m$  (58 - 60 °C) for 15 s and extension at 72  
225 °C for 15 s. Primers efficiency was evaluated by serial dilutions from 10 to 10,000. The  
226 *Q-rt*PCR was run using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen,  
227 Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of  
228 Luminaris Color HiGreen *q*PCR Master Mix (Thermo Scientific), 1 µl of the primer  
229 corresponding to the analysed transcript (10 pmol), 3 µl of RNA / DNA water free and 5  
230 µl of cDNA at the validated dilution. Furthermore, amplifications were carried out with  
231 a systematic negative control (NTC; no template control) containing no cDNA. Standard  
232 amplification conditions contained a uracil DNA glycosylase (UDG) pre-treatment at 50  
233 °C for 2 min, an initial activation step at 95 °C for 10 min, followed by 35 cycles: 15s at  
234 95 °C, 30 s at the annealing  $T_m$  and 30 s at 72 °C.

### 235 *Statistical analyses*

#### 236 *Behaviour*

237 Statistical analyses were performed using SPSS Statistics 20.0 (IBM®). A hierarchical  
238 clustering algorithm using the Euclidean distance matrix and complete linkage method  
239 was run to classify the fifty sole into different SCS categories (proactive, intermediate  
240 and reactive) according to the total activity time (in seconds) of all the individual  
241 behavioural tests conducted (Ibarra-Zatarain et al., 2016). A coefficient of variation (CV  
242 % =  $SD/mean*100$ ) was calculated for each category representing the inter-individual  
243 sole variability in the population studied. Data were not distributed normally (Shapiro-

244 Wilks) in all tests and a Kruskal-Wallis non-parametric test was performed to analyse the  
245 significant differences among SCS categories for the behavioural tests with non-normally  
246 distributed data. However, when data was normal, the statistical test performed was One-  
247 way ANOVA, followed by Tukey's *post-hoc* test.

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

## 252 *Q-rtPCR*

253 Results were expressed as mean  $\pm$  S.E.M (Standard error of the mean) and statistics  
254 analyses were performed using SPSS software and plotted with GraphPad Prism 6  
255 software. Outliers of the corrected ratio for every mRNA on the different groups  
256 (proactive, intermediate and reactive) were extracted using the Tukey's test formula ( $k =$   
257  $1.5$ ). All data sets analysed were normally distributed (Shapiro-Wilks), although  
258 logarithmic transformation was performed when needed.

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

## 264 **Results**

### 265 **Behavioural assays**

266 The hierarchical cluster divided the population in three different clusters grouping similar  
267 stress responses in terms on total activity (*see* Fig. S2) from the individual tests

268 “Restraining”, “New environment” and “Confinement”. Therefore, the final classification  
269 of the hierarchical cluster was proactive, intermediate reactive animals according to the  
270 total activity displayed in every individual behavioural test.

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

278 The total activity (Fig. 2) in the “New environment” (NE; K-W = 26.13; P < 0.001; Fig.  
279 2B) and “Confinement” (Con; K-W = 25.46; P < 0.001; Fig. 2C) were significantly  
280 different (P < 0.05) among SCS categories. In the case of **NE**, intermediate (Total activity  
281 = 34.5 s; CV = 19.5 %; P < 0.001) and proactive juveniles (Total activity = 16.2 s; CV=  
282 122.0 %; P < 0.05) showed significantly higher total activity than reactive (Total activity  
283 = 3.1 s; CV = 178.0 %), but there was no difference between proactive and intermediate  
284 individuals. In the case of **CON**, differences were found between proactive (Total activity  
285 = 55.5 s; CV = 75.6 %), being significantly higher than intermediate (Total activity = 3.8  
286 s; CV = 108.0 %; P = 0.001) and reactive (Total activity = 2.1 s; CV = 147.1 %; P <  
287 0.001), but not between intermediate and reactive. In the case of the restraining test,  
288 **REST**, marginal differences were found among groups (K-W = 5.491; P = 0.0642; Fig.  
289 2A) and there were no significant differences among proactive (Total activity = 14.1 s;  
290 CV = 122.7 %), intermediate (Total activity = 13.8 s; CV = 96.7 %) and reactive (Total  
291 activity = 4.9 s; CV = 55.3 %).

292 Regarding first activity (Fig. 3) the situation was similar to the total activity, so the “New

293 Environment” (NE;  $F_{2,47} = 7.822$ ;  $P = 0.0012$ ; Fig. 3B) and “Confinement” (CON;  $F_{2,47}$   
294  $= 3.387$ ;  $P = 0.0423$ ; Fig. 3C) tests presented differences among SCS categories. In case  
295 of the NE, intermediate (first activity = 38.6 s; CV = 167.0 %;  $P < 0.001$ ) presented  
296 significantly lower latencies than reactive sole juveniles (first activity = 203.6 s; CV =  
297 65.4 %), however, proactive animals (first activity = 105.9 s; CV = 117 %;  $P > 0.05$ )  
298 presented no significant differences in comparison to intermediate and reactive sole.  
299 “Confinement” test, CON, showed clearly differences between proactive (first activity =  
300 27.4 s; CV = 225.5 %;  $P < 0.001$ ) and reactive latencies (first activity = 150.5 s; CV =  
301 96.2 %), however, intermediate sole (first activity = 95 s; CV = 149.0 %;  $P > 0.05$ ) did  
302 not present differences in latencies with the extremes. In the case of REST, no differences  
303 were found among coping styles (K-W = 2.366;  $P = 0.3064$ ; Fig. 3A), where proactive  
304 animals (first activity = 10.8 s; CV = 258.1 %), intermediate (first activity = 1.9 s; CV =  
305 149.8 %) and reactive (first activity = 8.2 s; CV = 278.2 %;  $P > 0.05$ ) showed similar  
306 latencies profile.

307  
308 Analysing the group-test, the risk-taking test, eleven of fifty juveniles (22 %) crossed  
309 from the well-known to the unfamiliar area, 6 of them coincided with proactive  
310 classification, 4 with intermediate and 1 was classified as reactive by the cluster.  
311 According to the results, the classification of the stress coping style groups was  
312 considered appropriate to continue with the brain transcripts abundance statistical  
313 analysis.

#### 314 **Brain transcripts abundance**

315 Brain mRNAs abundance was analysed in ten individuals from each SCS category  
316 (proactive, intermediate and reactive). In the case of the reactive group, the ten fish  
317 considered as the most reactive (the last ten fish in the list of the hierarchical cluster) were  
318 used to balance the number among categories. According to the brain transcripts

319 abundance in sole juveniles, the abundance or expression of four of the six mRNAs tested  
320 were significantly different among coping styles' categories. In the case of  
321 glyceraldehyde-3-phosphate dehydrogenases 2 (*gapdh-2*) proactive and intermediate  
322 individuals (up-regulated) exhibited significantly higher expression than reactive  
323 individuals (down-regulated) ( $F_{2,27} = 8.173$ ;  $P = 0.0017$ ; Fig. 4A). The other transcripts  
324 that were differentially expressed, presented similar expression profile for the extremes  
325 categories (proactive and reactive), which were up-regulated and were significantly  
326 differently expressed than intermediate (down-regulated): Period 1 (*per1*) ( $K-W = 14.43$ ;  
327  $P = 0.0007$ ; Fig. 4B), Insuline-like Growth factor (*igf-Ia*) ( $F_{2,27} = 4.606$ ;  $P = 0.0190$ ; Fig.  
328 4C) and Peroxisome proliferator-activated receptor (*ppar $\beta$* ) ( $F_{2,25} = 7.554$ ;  $P = 0.0027$ ;  
329 Fig. 4D). The other two transcripts did not present significant differences in expression,  
330 Specific hypothalamic corticotropin-releasing hormone (CRH) binding protein (*crh-BP*)  
331 ( $F_{2,24} = 0.4842$ ;  $P = 0.6221$ ) and Heat shock protein 90, alpha (cytosolic) class (*hsp90aa*)  
332 ( $F_{2,27} = 2.346$ ;  $P = 0.1150$ ).

### 333 **Behavioural and brain mRNA abundance relationship**

334 First of all, correlation among variables from the different behavioural tests was observed  
335 to try to discern the association among them. To observe the complete map of the  
336 relationship, the data was not split in categories, it was treated in continuous. In this  
337 context, the Restraining variables (first and total activity) do not present significantly  
338 correlation between them ( $r = -0.158$ ;  $P = 0.403$ ), however, negatively correlation was  
339 observed between the New environment variables (first and total activity) ( $r = -0.655$ ;  $P$   
340  $= 0.001$ ) and also Confinement variables (first and total activity) ( $r = -0.382$ ;  $P = 0.037$ ).  
341 It is worth mentioning that there was no correlation among the variables from the different  
342 behavioural tests observed in this study.

343 In case of the association among the candidate genes used for this study, *gapdh-2* is  
344 slightly correlated with *per1* ( $r = 0.395$ ;  $P = 0.031$ ), good correlated with *hsp90aa* ( $r =$   
345  $0.713$ ;  $P < 0.001$ ), *igf-a* ( $r = 0.548$ ;  $P = 0.002$ ), and *ppar $\beta$*  ( $r = 0.619$ ;  $P = 0.001$ ). The *per1*  
346 transcript was strongly correlated with *igf-a* ( $r = 0.774$ ;  $P < 0.001$ ) and *ppar $\beta$*  ( $r = 0.641$ ;  
347  $P = 0.001$ ). The *hsp90aa* gene was positively correlated with *igf-a* ( $r = 0.414$ ;  $P = 0.023$ )  
348 and *ppar $\beta$*  ( $r = 0.596$ ;  $P = 0.001$ ). The *igf-a* gene was strongly correlated with *ppar $\beta$*  ( $r =$   
349  $0.758$ ;  $P < 0.001$ ) and slightly correlated with *crh-bp* ( $r = 0.375$ ;  $P = 0.041$ ). The *ppar $\beta$*   
350 transcript was correlated with *crh-bp* ( $r = 0.549$ ;  $P = 0.002$ ).

351 After the observation whether genes involved in several biological functions varied in  
352 expression with coping styles, the individual correlation was carried out to observe the  
353 relationship between coping styles variables from the different behavioural tests applied  
354 and gene expression (Table 3). In this case, there were just two variables from the same  
355 behavioural test (“New environment”) which obtained significant correlation with the  
356 expression of 4 mRNAs of the 6 tested (*Per1*, *hsp90aa*, *ppar $\beta$*  and *crh-bp*). However,  
357 there exist some association between behavioural variables and gene expression which  
358 were not significantly correlated but showed a clear trend. For example, first activity from  
359 Confinement test was slightly non-correlated with *gapdh-2* ( $r = 0.315$ ;  $P = 0.09$ ) and  
360 *hsp90aa* ( $r = 0.323$ ;  $P = 0.082$ ).

## 361 **Discussion**

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

## 367 **Behavioural assays**

368 In terms of the behavioural study, previous studies have demonstrated that the same  
369 behavioural tests conducted in this study classify animals according to their behavioural  
370 traits (proactive through to reactive) in diverse fish species, such as stickleback  
371 (*Gasterosteus aculeatus*) (Bell, 2005), gilt-head seabream (*Sparus aurata*) (Castanheira  
372 et al., 2013) and zebrafish (Tudorache et al., 2015). In the present study we classified  
373 early stage Senegalese sole juveniles in three SCS categories (proactive, intermediate and  
374 reactive) using a hierarchical cluster analysis. The present study considered the  
375 intermediate as another category having in mind the association of the presence of this  
376 category with captive environment. Oortmerssen and Busser (1989), observed in a natural  
377 feral mice population a proactive and reactive bimodal distribution of SCS variables.  
378 However, this distribution changed when the experiment was performed under laboratory  
379 conditions (controlled), where another coping style category was found, the intermediate,  
380 probably due to the low natural selection pressure in captive conditions. In case of the  
381 Senegalese sole, domestication could be the reason of the presence of this third coping  
382 category, as under captive conditions animals have no biological limited resources such  
383 as food, proper habitat conditions (pH, temperature, salinity...) and no predators, so there  
384 are no or different selective pressures acting upon them. This model, with proactive,  
385 reactive and intermediate coping styles has been observed in a widespread variety of  
386 animal species, including fish such as African catfish (*Clarias gariepinus*) (van de  
387 Nieuwegiessen et al. 2010), several salmonids species (Huntingford and Adams, 2005),  
388 among others. The presence of correlation between the variables of the different  
389 behavioural tests denoted the importance of phenotypic pleiotropy to perceive the  
390 variability of the population. However, no correlation was observed among variables  
391 from the different behavioural tests applied, showing the possibility that the activity in  
392 this species fluctuates depending on the test conducted. Hence, in the present study,



393 proactive sole presented lower latencies and higher activity than reactive, indicating  
394 higher explorative behaviour and different response to stressful circumstances. However,  
395 intermediate sole is less consistent obtaining a different profile according to the  
396 behavioural test conducted.

### 397 **Brain transcripts abundance**

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

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

418 The transcripts abundance profile was carried out by *q*-rtPCR in 6 specific mRNAs  
419 (*gapdh2*, *Per1*, *igf-Ia*, *ppar $\beta$* , *hsp90aa* and *crh-BP*) where 4 of the 6 candidate mRNAs  
420 (*gapdh2*, *ppar $\beta$* , *igf-Ia* and *Per1*) were considered differential expressed transcripts  
421 (DETs) suggesting that there exist variations in the transcriptome among Senegalese sole  
422 individuals classified by coping styles. The primers of all these mRNAs have been  
423 published before exhibiting the importance of the study of these ones associated with  
424 Senegalese sole species. Specifically, the different mRNAs chosen for this study were  
425 related to basic metabolism, stress responses and biologic conditions specifics for  
426 Senegalese sole, which could provide important information in terms of development (*see*  
427 Table 2). Differences in metabolism have been linked with changes in coping styles in  
428 some species (Biro and Stamps, 2008; Martins et al., 2011), including fish such as  
429 zebrafish (Rey et al., 2013), common carp (MacKenzie et al., 2009; Rey et al., 2016),  
430 Nile tilapia (*Oreochromis niloticus*) (Vera Cruz and Brown, 2007) and rainbow trout  
431 (*Oncorhynchus mykiss*) (Thomson et al., 2011) where these studies associated  
432 physiological and gene expression variation with behavioural phenotypic traits. One of  
433 the most recent studies performed on sea bass (*Dicentrarchus labrax*) (Alfonso et al.,  
434 2019) found some transcripts linked with stress axis and neurogenesis were differently  
435 expressed depending on the behavioural traits, however, this species has not shown  
436 consistency in boldness over time using different behavioural tests (group and  
437 individual).

438 In the present study, one of the transcripts differentially expressed was Glyceraldehyde-  
439 3-phosphate dehydrogenase (*gapdh*), which is habitually used as a housekeeping  
440 transcript for its ubiquitous presence in all tissues in quantitative *rt*-PCR. However, there  
441 are facts that evidence that *gapdh* levels of expression may vary among tissues,  
442 development, or during different physiological processes including behavioural traits

443 (MacKenzie et al., 2009; Rey et al., 2013). Moreover, *gapdh* was discarded as a suitable  
444 housekeeping transcript for Senegalese sole (Infante et al., 2008). The metabolic function  
445 might be compromised by acute and chronic stress, explaining why *gapdh-2*, which has  
446 been demonstrated to be the *gapdh* isoform more expressed in brain in Senegalese sole  
447 (Manchado et al., 2007), was up-regulated in proactive sole relative to reactive fish  
448 (down-regulated). MacKenzie et al. (2009) made similar observations with common carp,  
449 where *gapdh* presented up-regulation in proactive fish and down-regulation in reactive  
450 animals demonstrating differences between coping styles and basic metabolism. These  
451 outcomes would be consider similar to the association found by Ibarra-Zatarain et al.,  
452 2016 between physiological response and behavioural traits in Senegalese sole, who  
453 perceived differences in cortisol concentration between proactive (low concentration) and  
454 reactive sole (high concentration). As observed before, *gapdh-2* expression was  
455 correlated with the expression of *per1*, *ppar $\beta$* , *hsp90aa* and *igf-I* genes, exhibiting that all  
456 these transcripts are also involved with metabolism, however, the distinct expression  
457 profiles in the different behavioural traits show that there is large inter-individual  
458 variation in post-stress responses in early Senegalese sole juveniles affecting gene  
459 expression.

460 The other three mRNAs (*ppar $\beta$* , *igf-Ia* and *per1*) differentially expressed among coping  
461 style categories in this study, presented similar expression profiles in proactive and  
462 reactive animals which were up-regulated and intermediate animals presented high down-  
463 regulation, and these transcripts are associated with feeding behaviour and nutrition.  
464 There are no data to compare with in other fish species in relation with these specific  
465 transcripts and individual variation in mRNA abundance. Moreover, the expression of  
466 these three genes presented a strong correlation, highlighting the relationship among them  
467 in functionality and expression profile. In general, intermediate animals present more

468 behavioural plasticity than the extremes coping styles categories, proactive and reactive  
469 (Dingemanse et al., 2010). According to these results in mRNAs abundance, intermediate  
470 sole presented also different profiles depending on the behavioural test performed (*for*  
471 *more detail see Fig. 2*).

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

493 expressed during daylight (diurnal), however, CLOCK and BMAL1 are expressed at  
494 night (nocturnal). Fish have a feeding schedule when they are under captive conditions  
495 and feeding can work as a strong synchronizer of circadian rhythms in several animals,  
496 increasing the locomotor activity some hours before the food is provided, which is called  
497 food anticipatory activity (Mistlberger, 2009). In case of the Senegalese sole, even  
498 though, is considered a nocturnal species, it has been observed that feeding schedule can  
499 modify the locomotor activity to diurnal when they are in captive conditions, due to  
500 operational activities (Carazo et al., 2016). This activity can affect the expression of the  
501 clock genes, for example in zebrafish it was observed that the animals exposed to different  
502 lights and different feeding schedules, including random feeding presented different *perl*  
503 expression profiles (Lopez-Olmeda et al., 2010). In the random feeding regime, the  
504 animals did not present food anticipatory activity and *perl* expression rhythm  
505 disappeared demonstrating the importance of feeding behaviour in the circadian  
506 rhythmicity. In the present study, sole were fasted 24 hours prior to the behavioural tests  
507 and according to their feeding regime all sole used for the experiment should present  
508 similar expression profile, however, only proactive and reactive presented up-regulation  
509 in every transcript of these three and intermediate sole showed high down-regulation, so  
510 the different expression among coping styles categories of those genes might be explained  
511 just by the behavioural screening prior to molecular analysis.

512 Intriguingly, both stress-related transcripts (*hsp90aa* and *crh-BP*) tested in this study were  
513 not differentially expressed among coping styles categories. Curiously, *hsp90aa*  
514 expression was also correlated with *ppar $\beta$*  and *igf-I*, associated with feeding behaviour  
515 and nutrition, but the expression of this transcript was not correlated with *crh-bp* that  
516 presents another expression profile. The *hsp90* transcript has been associated with  
517 nutritional stress in early stages in fish (Cara et al., 2005) and as a protection against

518 different stressors such as infections, heat shock, etc. (Basu et al., 2002). In previous  
519 studies performed with Senegalese sole revealed that *hsp90aa* was activated in the  
520 moment that sole was under a heat shock treatment, however, no significant differences  
521 were found after a cold shock treatment. Nevertheless, in our study, all animals used for  
522 the experiment were under the same prior and experimental conditions without any  
523 treatment, so the change in the regulation of *hsp90aa* transcript could be caused by the  
524 variability between individuals due to the behavioural tests conducted. The crh-binding  
525 protein is considered different from the crh receptors and it is very conservative among  
526 phylum, suggesting that the functions are also evolutionary conserved. Corticotropin  
527 releasing hormone binding protein (*crh-BP*) presented down-regulation in the three  
528 groups, but the variability intra- and inter-group resulted higher than the other transcripts.  
529 This could be explained whether the animals did not accuse a high influence according  
530 to the stressful period performing the different tests. Wunderink et al. (2011) found that  
531 *crh-BP* levels were not affected at different stocking densities (chronic stress response)  
532 in Senegalese sole and in addition, the *crh-BP* expression was improved in both densities  
533 when animals were moved to hypersaline seawater (acute stress response) proposing that  
534 *crh-BP* worked as a modulator of the acute stress reaction. Another study showed that the  
535 exposure to air during 30 seconds in Senegalese sole did not alter the expression of *crh-*  
536 *BP* transcript (Lopez-Olmeda et al., 2013). The stress-induced regulation of this transcript  
537 in fish, seems to be related to the sort of stress and its duration. Therefore, in the present  
538 study, the down-regulation in all groups could be explained that in the moment the fish  
539 finished the tests did not present an acute stress, however, the variability in the three  
540 coping style categories proposed that the expression of this transcript could be analysed  
541 individually. The association of *hsp90aa* transcript to SCS has not been evaluated in other  
542 fish species before the present study. However, other transcripts related to stress axis (*mr,*

543 *crf*, *crf-r2*, *pomc1*, *gr1* and *gr2*) were tested to associate gene expression and SCS in other  
544 fish species, such as, stickleback (Aubin-Horth et al., 2012) and sea bass (Alfonso et al.,  
545 2019). Some of those transcripts were differentially expressed depending on behavioural  
546 traits, for example in case of sea bass, *mr*, *crf*, and *gr2* were higher expressed in shy fish  
547 (considered as reactive). In the present study the expression of *crh-BP* transcript was  
548 down-regulated in all behavioural traits, showing a pattern of expression completely  
549 different from sea-bass *crf* transcript expression. These differences with our study could  
550 be related to the differences in activity and swimming behaviour, which is completely  
551 dissimilar between sea bass (constantly swimming and active) and sole (sedentary during  
552 long periods). However, it is worth to mention here that the expression of the *crh* and *crh-*  
553 *BP* are not always comparable, due to the high variability in mRNA expression inside the  
554 CRH system and among species. For example, social status variation using visual cues in  
555 African cichlid (*Astatotilapia burtoni*) showed higher expression in whole brain *crf* and  
556 *crf-BP* in dominant males than subordinates (Chen and Fernald, 2011). Therefore, social  
557 status would be one of the reasons to obtain differences in stress responses. Recent studies  
558 have been observed differences in physiological responses in sea bass depending on  
559 social hierarchy where dominant fish presented different muscle activity, immune  
560 response and stress response (Carbonara et al., 2015, 2019).

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

568 dominance test applied. However, Ibarra-Zatarain et al., 2016 demonstrated the presence  
569 of two clear stress coping behavioural axes (“fearfulness-reactivity” and “activity-  
570 exploration”) in this species, which are also reflected in this study noticing the results  
571 from different behavioural test and brain gene expression.

## 572 **Conclusions**

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

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591



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597

### 598 **Competing interests**

599 The authors have no competing interests.

600

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850

851 **Figure Legends:**

852

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

856

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

859 Confinement compared among the different stress coping style categories (proactive,  
860 intermediate and reactive) classified according to total activity measurement. Data was  
861 shown in Mean  $\pm$  SEM. Different letters means to be significantly different (Kruskal-  
862 Wallis  $P < 0.05$  level of significance).

863

864 **Figure 3. Stress coping style tests regarding First activity variable in seconds in early**  
865 **Senegalese sole juveniles ( $n = 50$ ).** A) Restraining B) New environment and C)  
866 Confinement compared among the different stress coping style categories (proactive,  
867 intermediate and reactive) classified according to total activity measurement. Data was  
868 shown in Mean  $\pm$  SEM. Different letters means to be significantly different (Kruskal-  
869 Wallis or One-Way ANOVA  $P < 0.05$  level of significance).

870

871 **Figure 4. Brain transcripts abundance of different genes which were differentially**  
872 **expressed among groups (proactive, intermediate and reactive) in early Senegalese**  
873 **sole juveniles ( $n = 30$ ).** A) *gapdh-2*, B) *per1*, C) *igh-Ia* and D) *ppar $\beta$* . Data was  
874 transformed to  $\text{Log}_{10}$  and was shown in Mean  $\pm$  SEM. Different letters means to be  
875 significantly different expressed (One-Way ANOVA  $P < 0.05$  level of significance).