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

Elvira Fatsini[1,2*], Sonia Rey[3], Zohar Ibarra-Zatarain[4], Sebastián Boltaña[5], Simon Mackenzie[3] and Neil J. Duncan[1]

[1]IRTA, Ctra. Poble Nou, km. 5.5, 43540 Sant Carles de la Ràpita, Tarragona (Spain)

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

[3]Institute of Aquaculture, University of Stirling, FK9 4LU Stirling (Scotland)

[4]Centro Nayarita de Innovación y Transferencia Tecnológica (CENIT[2]), 63173 Tepic (Mexico)

[5]University of Concepción, Bío Bío Region, Concepción (Chile)

*Corresponding author:
Elvira Fatsini Fernández, Tel.: +351 289800900, ext. 7167, Email: effernandez@ualg.pt

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

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

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

2.2. Individual testing

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

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

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

The last test performed was the "Confinement" test (CON); each fish was individually 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 ºC for posterior molecular
203 analysis.
204 *Quantitative real time PCR*
205 The differential expression of brain target transcripts (*gapdh2*, *per1*, *igf-Ia*, *pparß*,
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 (*q*PCR). Data were normalised
214 using 18*S* 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

First-Strand Synthesis SuperMix 50 rxn kit following the manufacturer's protocol (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

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

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 = 0.713; P < 0.001), *igf-a* (r = 0.548; P = 0.002), and *pparß* (r = 0.619; P = 0.001). The *per1* transcript was strongly correlated with *igf-a* (r = 0.774; P < 0.001) and *pparß* (r = 0.641; P = 0.001). The *hsp90aa* gene was positively correlated with *igf-a* (r = 0.414; P = 0.023) and *pparß* (r = 0.596; P = 0.001). The *igf-a* gene was strongly correlated with *pparß* (r = 0.758; P < 0.001) and slightly correlated with *crh-bp* (r = 0.375; P = 0.041). The *pparß* transcript was correlated with *crh-bp* (r = 0.549; P = 0.002).

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

Discussion

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

Behavioural assays

In terms of the behavioural study, previous studies have demonstrated that the same 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.

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

(MacKenzie et al., 2009; Rey et al., 2013). Moreover, *gapdh* was discarded as a suitable 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

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 *per1*
503 expression profiles (Lopez-Olmeda et al., 2010). In the random feeding regime, the
504 animals did not present food anticipatory activity and *per1* 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 *pparβ* 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

dominance test applied. However, Ibarra-Zatarain et al., 2016 demonstrated the presence 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.

References

Alfonso, S., Sadoul, B., Gesto, M., Joassard, L., Chatain, B., Geffroy, B. and Bégout, M. L. (2019). Coping styles in European sea bass: The link between boldness, stress response and neurognesis. *Physiol. Behav.* (*In press*)

Aubin-Horth, N., Deschênes, M. and Cloutier, S. (2012). Natural variation in the molecular stress network correlates with a behavioural syndrome. *Horm. Behav.* **61**, 140-146.

Basu, N., Todgham, A. E., Ackerman, P. A., Bibeau, M. R., Nakano, K., Schulte, P. M. and Iwama, G. K. (2002). Heat shock protein genes and their functional significance in fish. *Gene.* **295,** 173-183.

Baxter, R. C. (1994). Insulin-like growth factor binding proteins in the human circulation: a review. *Horm. Res.* **42,** 140-144.

Bell, A. M. (2005). Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). *J. Evol. Biol.* **18,** 464-473.

Biro, P. A. and Stamps, J. A. (2008). Are animal personality traits linked to life-history productivity? *Trends Ecol. Evol.* **23,** 361-368.

Braithwaite, V. A., Huntingford, F. and Van Der Bos, R. (2011). Variation in emotion and cognition among fishes. *J. Agric. Environ. Ethics.* **26,** 7-23.

Campos, C., Valente, L. M., Conceiçao, L. E., Engrola, S., Sousa, V., Rocha, E. and Fernandes, J. M. (2013). Incubation temperature induces changes in muscle cellularity and gene expression in Senegalese sole (*Solea senegalensis*). *Gene*. **516** (2), 209-217.

Cara, J. B., Aluru, N., Moyano, F. J. and Vijayan, M. M. (2005). Food-deprivation induces HSP70 and HSP90 protein expression in larval gilthead sea bream and rainbow trout. *Comp. Biochem. Phys. B.* **142,** 426-431.

Carazo, I., Chereguini, O., Martín, I., Huntingford, F. and Duncan, N. (2016). Reproductive ethogram and mate selection in captive wild Sengalese sole (*Solea senegalensis*). *SJAR* **14(4)**, e0401.

Carbonara, P., **Scolamacchia, M., Spedicato, M. T., Zupa, W., McKinley, R. S. and Lembo, G.** (2015). Muscle activity as a key indicator of welfare in farmed European sea bass (*Dicentrarchus labrax* L. 1758). *Aquaculture Res.* **46,** 2133 – 2146.

Carbonara, P., Dioguardi, M., Cammarata, M., Zupa, W., Vazzana, M., Spedicato, M. T. and Lembo, G. (2019). Basic knowledge of social hierarchies and physiological profile of reared sea bass Dicentrarchus labrax (L.). *PLoS ONE* **14(1),** e0208688.

Carter, C. G. and Bransden, M. P. (2001). Relationships between protein-nitrogen flux and feeding regime in greenback flounder, *Rhombosolea tapirina* (Gunther). *Comp Biochem Physiol A Mol Integr Physiol.* **130,** 799-807.

Castanheira, M. F., Conceiçao, L. E. C., Millot, S., Rey, S., Bégout, M. L., Damsgard, B., Kristiansen, T., Höglund, E., Overli, O. and Martins, C. I. M. (2017).

641 Coping styles in farmed fish: consequences for aquaculture. *Rev. Aquacult.* **9(1),**
642 23-41.
643 **Castanheira, M. F., Herrera, M., Costas, B., Conceicao, L. E. and Martins, C. I.**
644 (2013). Can we predict personality in fish? Searching for consistency over time
645 and across contexts. *PLoS One.* **8,** e62037.
646 **Chen, C. C. and Fernald, R. D.** (2011). Visual information alone changes behavior and
647 physiology during social interactions in a cichlid fish (*Astatotilapia burtoni*).
648 PLoS ONE **6**:e20313.
649 **Coppens, C. M., De Boer, S. F. and Koolhaas, J. M.** (2010). Coping styles and
650 behavioural flexibility: towards underlying mechanisms. *Philos. Trans. R. Soc.*
651 *Lond. B Biol. Sci.* **365,** 4021-4028.
652 **Darias, M. J., Boglino, A., Manchado, M., Ortiz-Delgado, J. B., Estevez, A., Andree,**
653 **K. B. and Gisbert, E.** (2012). Molecular regulation of both dietary vitamin A and
654 fatty acid absoption and metabolisms associated with larval morphogenesis of
655 Senegalese sole (*Solea senegalensis*). *Com. Biochem. Phys. A.* **161** (2), 130-139.
656 **Dingemanse, N. J., Kazem, A. J., Reale, D. and Wright, J.** (2010). Behavioural
657 reaction norms: animal personality meets individual plasticity. *Trends Ecol. Evol.*
658 **25,** 81-89.
659 **Driscoll, P., Escorihuela, R. M., Fernandez-Teruel, A., Giorgi, O., Schwegler, H.,**
660 **Steimer, T., Wiersma, A., Corda, M. G., Flint, J., Koolhaas, J. M., et al.**
661 (1998). Genetic selection and differential stress responses. The Roman
662 lines/strains of rats. *Ann. N. Y. Acad. Sci.* **851,** 501-510.
663 **Duan, C.** (1998). Nutritional and developmental regulation of insulin-like growth factors
664 in fish. *J. Nutr.* **128,** 306S-314S.

Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Mackenzie, S. and Duncan, N. J. (2017). Dominance behaviour in a non-aggrssive flatfish, Senegalese sole (*Solea sengalensis*) and brain mRNA abundance of selected transcripts. *PLoS ONE* **12(9)**, e0184283.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Ann. Rev. Physiol*. **61**, 243-282.

Fernandez, I., Darias, M., Andree, K. B., Mazurais, D., Zambonino-Infante, J. L. and Gisbert, E. (2011). Coordinated gene expression during gilthead sea bream skeletogenesis and its disruption by nutritional hypervitaminosis A. *BMC Dev. Biol.* **11,** 7.

Forthergill-Gilmore, L. A. And Mitchels, P. A. (1993). Evolution of glycolosis. *Prog. Biophys. Mol. Bio.* **59** (2), 105-235.

Giaginis, C., Tsantili-Kakoulidou, A. and Theocharis, S. (2007). Peroxisome proliferator-activated receptors (PPARs) in the control of bone metabolism. *Fundam. Clin. Pharmacol.* **21,** 231-244.

Howell, B., Pricket, R., Cañavate, P., Mañanos, E., Teresa, M. and Valente, C. C. The cultivation of soles. V workshop of the Cultivation of Sole, 5-7 April 2011 CCMAR, University of the Algarve Faro, Portugal.

Huising, M. O., Metz, J. R., van Schooten, C., Taverne-Thiele, A. J., Hermsen, T., Verburg-van Kemenade, B. M. and Flik, G. (2004). Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. *J. Mol. Endocrinol*. **32** (3), 627-648.

Huntingford, F. A. and Adams, C. (2005) Behavioural syndromes in farmed fish: implications for production and welfare. *Behaviour* **142**, 1213–1227.

690 **Huntingford, F. A., Andrew, G., Mackenzie, S., Morera, D., Coyle, S. M., Pilarczyk,**
691 **M. and Kadri, S.** (2010). Coping strategies in a strongly schooling fish, the
692 common carp *Cyprinus carpio*. *J. Fish Biol.* **76,** 1576-1591.
693 **Ibarra-Zatarain, Z.** (2015). The role of stress coping styles in reproduction and other
694 biological aspects in the aquaculture species, Senegalese sole (*Solea senegalensis*)
695 and gilthead seabream (*Sparus aurata*). *PhD in Aquaculture Articles*,
696 Autonomous University of Barcelona.
697 **Ibarra-Zatarain, Z., Fatsini, E., Rey, S., Chereguini, O., Martin, I., Rasines, I.,**
698 **Alcaraz, C. and Duncan, N.** (2016). Characterization of stress coping style in
699 Senegalese sole (*Solea senegalensis*) juveniles and breeders for aquaculture. *R.*
700 *Soc. Open Sci.* **3,** 160495.
701 **Infante, C., Matsuoka, M. P., Asensio, E., Canavate, J. P., Reith, M. and Manchado,**
702 **M.** (2008). Selection of housekeeping genes for gene expression studies in larvae
703 from flatfish using real-time PCR. *BMC Mol. Biol.* **9,** 28.
704 **Iwama, G. K., Thomas, P. T., Forsyth, R. B. and Vijayan, M. M.** (1998). Heat shock
705 protein expression in fish. *Rev. Fish Biol. Fisher*. **8**, 35-56.
706 **Koolhaas, J. M., De Boer, S. F., Buwalda, B. and Van Reenen, K.** (2007). Individual
707 variation in coping with stress: a multidimensional approach of ultimate and
708 proximate mechanisms. *Brain Behav. Evol.* **70,** 218-226.
709 **Koolhaas, J. M., De Boer, S. F., Coppens, C. M. and Buwalda, B.** (2010).
710 Neuroendocrinology of coping styles: towards understanding the biology of
711 individual variation. *Front Neuroendocrinol.* **31,** 307-321.
712 **Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C.**
713 **G., Hopster, H., De Jong, I. C., Ruis, M. A. and Blokhuis, H. J.** (1999). Coping

styles in animals: current status in behavior and stress-physiology. *Neurosci. Biobehav. R.* **23,** 925-935.

Leaver, M. J., Boukouvala, E., Antonopoulou, E., Diez, A., Favre-Krey, L., Ezaz, M. T., Bautista, J. M., Tocher, D. R. and Krey, G. (2005). Three peroxisome proliferator-activated receptor isotypes from each of two species of marine fish. *Endocrinol*. **146** (7), 3150-3162.

Lee, C. H., Olson, P. and Evans, R. M. (2003). Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. *Endocrinol.* **144,** 2201-2207.

Lopez-Olmeda, J. F., Blanco-Vives, B., Pujante, I. M., Wunderink, Y.S., Mancera, J. M. and Sanchez-Vives, F. J. (2013). Daily rhythms in the hypothalamus-pituitary-interrenal axis and acute stress responses in a teleost flatfish, *Solea senegalensis*. *Chronobiol. Int.* **30(4)**, 530-539.

Lopez-Olmeda, J. F., Tartaglione, E. V., De La Iglesia, H. O. and Sanchez-Vazquez, F. J. (2010). Feeding entrainment of food-anticipatory activity and per1 expression in the brain and liver of zebrafish under different lighting and feeding conditions. *Chronobiol. Int.* **27,** 1380-1400.

Mackenzie, S., Ribas, L., Pilarczyk, M., Capdevila, D. M., Kadri, S. and Huntingford, F. A. (2009). Screening for coping style increases the power of gene expression studies. *PLoS One.* **4,** e5314.

Manchado, M., Infante, C., Asensio, E. and Canavate, J. P. (2007). Differential gene expression and dependence on thyroid hormones of two glyceraldehyde-3-phosphate dehydrogenases in the flatfish Senegalese sole (*Solea senegalensis* Kaup). *Gene.* **400,** 1-8.

738 **Manchado, M., Salas-Leiton, E., Infante, C., Ponce, M., Asensio, E., Crespo, A.,**
739 **Zuasti, E. and Cañavate, J. P.** (2008). Molecular characterization, gene
740 expression and transcriptional regulation of cytosolic HSP90 genes in the flatfish
741 Senegalese sole (*Solea senegalensis* Kaup). *Gene*. **416** (1-2), 77-84.
742 **Martin-Robles, A. J., Whitmore, D., Sanchez-Vazquez, F. J., Pendon, C. and**
743 **Munoz-Cueto, J. A.** (2012). Cloning, tissue expression pattern and daily rhythms
744 of Period1, Period2, and Clock transcripts in the flatfish Senegalese sole, *Solea*
745 *senegalensis*. *J. Comp. Physiol. B.* **182,** 673-685.
746 **Martins, C. I. M., Castanheira, M. F., Engrola, S., Costas, B. and Conceiçao, L. E.**
747 **C.** (2011). Individual differences in metabolism predict coping styles in fish. *Appl.*
748 *Anim. Behav. Sci.* **130,** 135-143.
749 **McClennen, S. J., Cortright, D. N. and Seasholtz, A. F.** (1998). Regulation of pituitary
750 corticotropin releasing hormone-binding protein messenger ribonucleic acid
751 levels by restraint stress and adrenalectomy. *Endocrinol*. **139** (11), 4435-4441.
752 **Michalik, L., Desvergne, B., Dreyer, C., Gavillet, M., Laurini, R. N. and Wahli, W.**
753 (2002). PPAR expression and function during vertebrate development. *Int. J. Dev.*
754 *Biol.* **46** (1), 105-114.
755 **Migaud, H., Davie, A., Martinez Chavez, C. C. and Al-Khamees, S.** (2007). Evidence
756 for differential photic of pineal melatonin synthesis in teleosts. *J. Pineal Res.* **43**
757 (4), 327-335.
758 **Mistlberger, R. E.** (2009). Food-anticipatory circadian rhythms: concepts and methods.
759 *Eur. J. Neurosci.* **30,** 1718-1729.
760 **Monroe, T., Adeofe, T. A., Camara, K., Camara, Y. H., Cissoko, K., de Morais, L.,**
761 **Djiman, R., Mbye, E., Sagna, A., Sylla, M. and Tous, P.** (2015) Solea
762 senegalensis. The IUCN Red List of Threatened Species 2015:

763 e.T15622678A15623382. http://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.
764 T15622678A15623382.en
765 **Morais, S., Aragao, C., Cabrita, E., Conceiçao, L., Constenla, M., Costas, B., Dias,**
766 **J., Duncan, N., Engrola, S., Estevez, A., et al.** (2016). New developments and
767 biological insights into the farming of *Solea senegalensis* reinforcing its
768 aquaculture potential. *Rev. Aquacult.* **6,** 1-37.
769 **Morgan, D. and Dall, S. R.** (2015). Unravelling the philosophies underlying "Animal
770 Personality" studies: a brief Re-appraisal of the field. *Ethology.* **122,** 1-9.
771 **Oortmerssen, G. A. and Busser, J.** (1989). House mouse aggression: a model for
772 understanding the evolution of social behavior. *In:* Brain, P. F., Mainardi, D. and
773 Parmigiani, S. (eds.) *Studies in wild house mice III: disruptive selection on*
774 *aggression as a possible force in evolution.*: Harwood Academic Publishers.
775 **Øverli, Ø., Sorensen, C. and Nilsson, G. E.** (2006). Behavioral indicators of stress-
776 coping style in rainbow trout: Do males and females react differently to novelty?
777 *Physiol. Behav.* **87,** 506-512.
778 **Øverli, Ø.** (2007). Preface: plasticity and diversity in behavior and brain function-
779 important raw material for natural selection? *Brain Behav. Evol.* **70,** 215-217.
780 **Øverli, Ø., Sorensen, C., Pulman, K. G., Pottinger, T. G., Korzan, W., Summers, C.**
781 **H. and Nilsson, G. E.** (2007). Evolutionary background for stress-coping styles:
782 relationships between physiological, behavioral, and cognitive traits in non-
783 mammalian vertebrates. *Neurosci. Biobehav. Rev.* **31,** 396-412.
784 **Pfaffl, M. W.** (2001). A new mathematical model for relative quantification in real-time
785 RT-PCR. *Nucleic Acids Res.* **29,** e45.

Reale, D., Reader, S. M., Sol, D., Mcdougall, P. T. and Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biol. Rev. Camb. Philos. Soc.* **82,** 291-318.

Reinecke, M., Bjornsson, B. T., Dickhoff, W. W., McCormick, S. D., Navarro, I., Power, D. M. and Gutierrez, J. (2005). Growth hormone and insulin-like growth factors in fish: where we are and where we go. Gen. Com. Endocrinol. **142** (1-2), 20-24.

Reppert, S. M. and Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature.* **418,** 935-941.

Rey, S., Boltana, S., Vargas, R., Roher, N. and Mackenzie, S. (2013). Combining animal personalities with transcriptomics resolves individual variation within a wild-type zebrafish population and identifies underpinning molecular differences in brain function. *Mol. Ecol.* **22,** 6100-6115.

Rey, S., Ribas, L., Morera Capdevila, D., Callol, A., Huntingford, F. A., Pilarczyk, M., Kadri, S. and Mackenzie, S. (2016). Differential responses to environmental challenge by common carp *Cyprinus carpio* highlight the importance of coping style in integrative physiology. *J. Fish Biol.* **88,** 1056-1069.

Salas-Leiton, E., Anguis, V., Martin-Antonio, B., Crespo, D., Planas, J. V., Infante, C., Cañavate, J. P. and Manchado, M. (2010). Effects of stocking density and feed ration on growth and gene expression in the Senegalese sole (*Solea senegalensis*): potential effects on the immune response. Fish Shellfish Immun. **28** (2), 296-302.

Sih, A., Bell, A. and Johnson, J. C. (2004). Behavioral syndromes: an ecological and evolutionary overview. *Trends Ecol. Evol.* **19,** 372-378.

Sirover, M. A. (1999). New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phophate dehydrogenase. *Biochim. Biophys. Acta* **1432** (2), 159-184.

Smith, R. E., Ptacek, J. T. and Smoll, F. L. (1992). Sensation seeking, stress, and adolescent injuries: a test of stress-buffering, risk-taking, and coping skills hypotheses. *J. Pers. Soc. Psychol.* **62,** 1016-1024.

Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science*. **291** (5503), 490-493.

Thomson, J. S., Watts, P. C., Pottinger, T. G. and Sneddon, L. U. (2011). Physiological and genetic correlates of boldness: characterising the mechanisms of behavioural variation in rainbow trout, *Oncorhynchus mykiss*. *Horm. Behav.* **59,** 67-74.

Tudorache, C., Ter Braake, A., Tromp, M., Slabbekoorn, H. and Schaaf, M. J. (2015). Behavioral and physiological indicators of stress coping styles in larval zebrafish. *Stress.* **18,** 121-128.

Vagner, M., Robin, J. H., Zambonino-Infante, J. L., Tocher, D. R. and Person-Le Ruyet, J. (2009). Ontogenic effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. *Br. J. Nutr.* **101,** 1452-1462.

van de Nieuwegiessen, P. G., Ramli, N. M., Knegtel, B. P. F. J. M., Verreth, J. A. J., Schrama, J. W. (2010). Coping strategies in farmed African catfish *Clarias gariepinus*. Does it affect their welfare? *J. Fish Biol.* **76,** 2486–2501.

Van Oers, K., Drent, P. J., De Goede, P. and Van Noordwijk, A. J. (2004). Realized heritability and repeatability of risk-taking behaviour in relation to avian personalities. *Proc. Biol. Sci.* **271,** 65-73.

Vera Cruz, E. M. and Brown, C. L. (2007). The influence of social status on the rate of growth, eye color pattern and insulin-like growth factor-I gene expression in Nile tilapia, *Oreochromis niloticus*. *Horm. Behav.* **51,** 611-619.

Whitehead, A. and Crawford, D. L. (2006). Variation within and among species in gene expression: raw material for evolution. *Mol. Ecol.* **15,** 1197-1211.

Wilson, A. D. M. and Godin, J. G. J. (2009). Boldness and behavioral syndromes in the bluegill sunfish, *Lepomis macrochirus*. *Behav. Ecol.* **20,** 231-237.

Wolf, M. and Weissing, F. J. (2012). Animal personalities: consequences for ecology and evolution. *Trends Ecol. Evol.* **27,** 452-461.

Wunderink, Y. S., Engels, S., Halm, S., Yufera, M., Martinez-Rodriguez, G., Flik, G., Klaren, P. H. and Mancera, J. M. (2011). Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): the involvement of cortisol, CRH and CRH-BP. *Gen. Comp. Endocrinol.* **171,** 203-210.

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)

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

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

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