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Coping styles in European sea bass: The link between boldness, stress response and neurogenesis

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18 **Abstract**

19 Coping styles consist of a coherent set of individual physiological and behavioral differences
20 in stress responses that are consistent across time and context. Such consistent inter-individual
21 differences in behavior have already been shown in European sea bass (*Dicentrarchus*
22 *labrax*), but the associated mechanisms are still poorly understood. Here, we combine
23 physiological measurements with individual behavioral responses in order to characterize
24 coping styles in fish. Fish were tagged and placed in a tank for group risk-taking tests (GRT)
25 at eight months of age to evaluate boldness using the proxy latency of leaving a sheltered area
26 towards an open area. A subsample of these fish were individually challenged 16 months later
27 using an open field test (OFT), in which the boldness was assessed after being placed in a
28 shelter within an open arena. Latency to exit the shelter, time spent in the shelter, and distance
29 travelled were recorded for this purpose. The blood and brain were then collected to evaluate
30 plasma cortisol concentration and neurotransmitter levels (dopamine, norepinephrine,
31 serotonin, and related metabolites), as well as brain transcription of key genes involved in
32 stress axis regulation (*gr1*, *gr2*, *mr*, *crf*), neurogenesis (*neurod1*, *neurod2*, *pcna*), and neuronal
33 development (*egr1*). Fish acting bolder in the GRT were not necessarily those acting bolder in
34 the OFT, highlighting the relatively low consistency across different types of tests performed
35 with a 16-months interval. There was, however, a significant correlation between stress
36 markers and boldness. Indeed, mRNA levels of *mr*, *crf*, *gr2*, *egr1*, and *neurod2*, as well as
37 norepinephrine levels were higher in shy than bold fish, whereas brain serotonergic activity
38 was lower in shy fish. Overall, our study highlights the fact that boldness was not consistent
39 over time when testing context differed (group vs. alone). This is in agreement with previous
40 literature suggesting that social context play a key role in boldness measurement and that the
41 particular life history of each individual may account in shaping the personality fate of a fish.

42 **Key-word:** Personality; HPI axis; Gene transcription; Neurotransmitters.

43 **1. Introduction**

44 Coping styles have been defined as “a coherent set of individual physiological and behavioral
45 differences in stress responses consistent across time and context” by Koolhaas and
46 colleagues [1]. They have been described in various animal species, including fish, as a
47 *continuum* between two extreme phenotypes, called proactive and reactive [2-5]. In terms of
48 behavior, proactive fish are bolder, more aggressive, explore their environment faster, and
49 display less flexible behavior than reactive fish [4-8]. Moreover, correlations between these
50 behavioral responses across time and context have been established in several fish species [2,
51 5, 9], as well as between behavior and physiology [4, 10-15], generally used for defining
52 coping styles since.

53 Stress can be defined as the cascade of biological events that occur when an organism faces a
54 challenge out of the normal range and attempts to reestablish physiological equilibrium [16].
55 Typical stress responses of fish toward adverse events involve two main neuroendocrine axes,
56 the brain-chromaffin axis and the hypothalamo-pituitary-interrenal (HPI) axis [17-19]. Both
57 axes are important in the primary response to stress [20-22], which is activated in various
58 stressful contexts [21, 23-27] and leads to the release of stress hormones, catecholamines
59 (mostly epinephrine and norepinephrine), and corticosteroids (cortisol in teleost fish) into the
60 systemic circulation. At the central level, several monoaminergic neurotransmitters, such as
61 serotonin (5-HT), dopamine (DA), and norepinephrine (NE), are also known to be involved in
62 the organization of stress responses in vertebrates [27]. Stress induces changes in brain
63 monoaminergic systems, both acutely and chronically, which is especially true for the
64 serotonergic system. For example, serotonergic activity is consistently activated upon both
65 chronic or acute stress exposure in fish [17, 26, 28]. Acute net chasing was for example
66 demonstrated to induce a rapid increase in forebrain serotonergic activity in rainbow trout

67 [17]. Similarly, chronic confinement stress (three times a day during four weeks) also induced
68 elevated concentration of 5HIAA and serotonergic activity in the telencephalon of Artic Charr
69 [28]. The concentration of the brain monoamines DA, NE, and 5HT and the concentration of
70 their associated main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-
71 hydroxyphenylglycol (MHPG), and hydroxyindoleacetic acid (5HIAA), respectively, are
72 generally used to quantify monoaminergic system activity using the turnover ratio between
73 the monoamine and associated metabolite [26, 27, 29]. This activity has been suggested to
74 vary between individuals with different behavioral responses [26, 27, 30-32]. Both the stress
75 axis (HPI) and monoaminergic systems (brain-chromaffin) have been shown to be reliable
76 physiological markers correlated with differences in coping styles [7, 26, 33-35] even if, in
77 some species, physiological markers were not directly linked to behavioral traits related to
78 coping styles [36].

79 The HPI axis, which produces cortisol, its major end-product hormone, acts as an activator of
80 physiological and behavioral responses [18, 37, 38]. Glucocorticoid receptors (Grs) and
81 mineralocorticoid receptor (Mr) bind cortisol [39] and are detected in various fish tissues.
82 They are involved in different physiological functions linked for instance to growth,
83 reproduction, immunity and they participate in the regulation of control cortisol release [40-
84 43]. The production and release of cortisol occurs in interrenal cells, located in the teleost
85 head kidney, and is mainly controlled by adrenocorticotrophic hormone (Acth), which is
86 released by the pituitary gland into the blood [18]. In turn, the release of Acth is mainly
87 controlled by corticotropin releasing-factor (Crf), a neuropeptide produced in the
88 hypothalamus [20, 44]. In addition, recent studies suggest that the regulation of stress axis and
89 neurogenesis are closely related [45-47] and we previously proposed that fish displaying high
90 sensitivity to stress also exhibit high levels of neurogenesis in a non-stressful context [45].
91 Proliferating cell nuclear antigen (Pcna), neurogenic differentiation factors 1 and 2 (Neurod1

92 and Neurod2), and the early growth response protein (Egr1) are important markers of
93 neurogenesis and neuronal development [34, 48]. While the regulation of stress axes might be
94 an important feature to characterize coping styles in fish, there is still a paucity of information
95 regarding the differences in neurogenesis between coping styles. Additional data are therefore
96 needed in order to better understand the mechanisms associated to the behavioral responses.

97 Differences in behavioral and physiological responses to stress may have direct implications
98 on growth, foraging activities, and the resulting fitness of wild fish species [37, 49, 50]. In the
99 context of aquaculture, better knowledge of individual diversity can help to improve the
100 welfare and management of the fish [5], as proactive fish sometimes show better feeding
101 motivation and feed efficiency than reactive fish [2, 5, 51, 52].

102 The European sea bass *Dicentrarchus labrax* is a predatory species, widely distributed around
103 Europe and West Africa [53], that has a key ecological role in the marine food chain [54]. It is
104 also one of the most highly farmed fish species in Europe [55]. Therefore, additional
105 knowledge of coping styles in European sea bass is required to improve both its management
106 and welfare under conditions of aquaculture [4, 5, 56] and to better understand and predict
107 population dynamics [57, 58].

108 Here, we had two main objectives. First, we monitored the boldness of the fish, by assessing
109 the behavior of *European sea bass* over time and context testing for behavioral consistency
110 between shy and bold fish. A group risk taking test (GRT) and open field test (OFT) were
111 performed at 227 and 749 days *post* fertilization (dpf), respectively. Second, we aimed to
112 describe the underlying physiological mechanisms associated with boldness. The blood and
113 brains of the fish were sampled after the OFT to measure cortisol concentration in the plasma,
114 the transcription of genes involved in stress regulation (*mr*, *gr1*, *gr2*, *crf*), neurogenesis (*pcna*,

115 *neurod1*, *neurod2*), and neuronal development (*egr1*) and, finally, the concentration of
116 monoamines (DA, 5HT, NE) and associated metabolites (DOPAC, 5HIAA) in whole brain.

117 **2. Material and methods**

118 Experiments were authorized by ethics committee agreement APAFIS#7098 and all
119 procedures involving animals were in accordance with the ethical standards of the institution
120 and followed the recommendations of Directive 2010/63/EU.

121 **2.1. Fish rearing and pit-tagging**

122 The reproduction and larval rearing of European sea bass, were performed at Ifremer,
123 Palavas-les-flots (France, 34250). Fish were produced from third-generation domesticated
124 West Mediterranean brood stock on February 27, 2016, applying a full factorial mating design
125 combining 10 dams and 50 sires by *in vitro* fertilization. At 223 dpf, ~3000 fish were
126 anesthetized using 200 ppm of benzocaine (benzocaine E1501, Sigma, Saint Louis, MO,
127 USA), measured for body mass (g) and standard length (cm), and pit-tagged in the muscle,
128 just in front of the dorsal fin, with an ISO PIT tag (8 x 1.4 mm) to ensure individual
129 identification.

130 Fish were maintained until 457 dpf following standard procedures [59]. Thereafter, they were
131 reared in six different 5-m³ tanks within a recirculating bio-filtered system at a temperature of
132 $20.9 \pm 1.3^{\circ}\text{C}$, pH = 7.4, and a salinity of 37 PSU under a 10/14 light/dark photoperiod. The
133 fish were fed a standard marine fish aquaculture diet (Neo-grower, Le Gouessant, France) *ad*
134 *libitum* using a self-feeder.

135 **2.2. Group risk-taking test**

136 The group risk-taking test (GRT) is a standardized test to measure boldness in fish and was
137 adapted from Ferrari and colleagues [4]. The GRT was performed at 227 dpf with 1,468 fish

138 and the following day, it was repeated with the other 1,468 naive fish (mass = 18.8 ± 1.2 g;
139 standard length = 10.3 ± 1 cm).

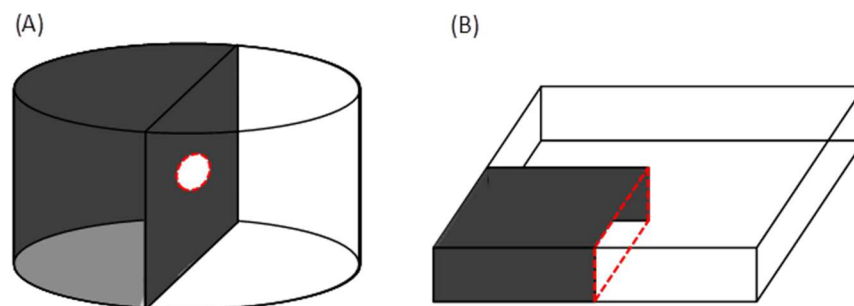
140 The GRT consists of placing all the fish into one half of a 1.5 -m³ tank, separated in two by an
141 opaque PVC divider (**Figure 1.A**). Fish were gently transferred from their home tank to one
142 half of the experimental tank under light anesthesia (100 ppm Benzocaine, same stock
143 solution as before) between 10:00 and 11:00 am. The side containing the fish was covered
144 with a black opaque curtain to create a sheltered area. Both halves of the tank received water
145 of the same quality, oxygenation, and exchange rate. After an habituation period of 2 h, the
146 door (a circular opening of 12 cm Ø in the centre of the divider) was opened and the fish were
147 left with the possibility to exit the sheltered area, therefore exhibiting a risk-prone behavior
148 reflecting boldness level. The door was equipped with a PIT-tag detection antenna (DORSET,
149 The Netherlands) connected to a control device. The PIT-tag detection antenna allowed the
150 monitoring of fish behavior during the GRT by obtaining the exact exit time (*latency to exit*
151 (*s*)) of each fish leaving the sheltered area for the first time. As the test lasted for 24 hours, a
152 latency of 1,440 min was assigned to fish that did not exit the sheltered area before the end of
153 the test. Once the outcome of the GRT was analyzed, the individuals were categorized as bold
154 (fish that exited the shelter) or shy (fish that stayed in the shelter during the entire
155 experiment). They were then transferred back to their home tanks and reared together until the
156 open field test (OFT) was performed, 16 months later.

157 **2.3. Open field test**

158 The open field test (OFT) was adapted from that of Benhaim and colleagues [60] and
159 performed at 749 dpf (341 ± 4 g) using a sub-sample of fish ($n = 57$, half of them bold and the
160 other half shy, based on their GRT categorization). The OFT consists of placing an individual
161 fish in an open and novel arena (75 x 75 cm with a water height of 23 cm, **Figure 1.B**) and
162 tracking its swimming activity. The fish was placed in the closed sheltered area for a

163 habituation period of 5 min. The door was then opened, allowing the fish to explore its novel
164 environment while being video-tracked. Videos were taken with a DMK 31AU03 camera
165 (The Imaging Source, Germany) and the recorded behavior of each fish then analyzed using
166 EthoVision XT 13.1 software (Noldus, The Netherlands) by the same operator (SA). The
167 *latency to exit* the shelter (s) was recorded for each individual. A latency of 1,200 s was
168 assigned to fish that did not exit the shelter at the end of the test. Fish that exited the shelter
169 were categorized as bold, whereas those that did not were categorized as shy. The *total*
170 *distance travelled* (cm), *number of transitions* between shelter and arena, and the *time spent in*
171 *the shelter* (s) were also recorded.

172



173

174 **Figure 1.** Design of the apparatus for the (A) group risk-taking test (GRT) and (B) open field
175 test (OFT). Dark grey boxes represent the shelter; dashed red lines indicate the door position.

176 **2.4. Blood and brain sampling**

177 Following the OFT, fish were gently removed from the arena and euthanized using 1,500 ppm
178 benzocaine. Blood and brain were sampled without knowing the boldness level of the fish.
179 Blood was sampled from the caudal vessels using lithium heparinized syringes [61]. Then,
180 blood was centrifuged (4 min at 13,000 x g) to obtain plasma samples, which were stored at -
181 20°C until analysis. The plasma cortisol concentration was determined by ELISA (RE52061,

182 IBL International, Hamburg, Germany) using a Synergy-HT microplate reader (BioTek
183 Instruments, Winooski, VT, USA) following manufacturer's instructions.

184 The brain was extracted, divided along the sagittal plane, and placed directly in liquid
185 nitrogen. The left half was used for monoamine analyses and the right half for gene
186 transcription analyses.

187 **2.5. Brain monoamine analyses**

188 The left half of the fish brain was used for HPLC analysis of brain monoamines. Tissues were
189 weighed and immediately homogenized in 1 mL perchloric acid (PCA) solution (0.4 M PCA
190 and 0.1 mM EDTA) with a Sonopuls ultrasonic homogenizer (Bandelin, Germany).
191 Homogenates were then centrifuged for 10 min at 14 000 x g (at 4°C). Supernatants were
192 diluted 20 times in the PCA solution and 20 µL of the dilution injected into the high
193 performance liquid chromatography (HPLC) system.

194 The diluted supernatants were analyzed high performance liquid chromatography with
195 electrochemical detection (HPLC-EC). The HPLC mobile phase consisted of 73.9 mM
196 NaH₂PO₄, 0.1 mM Na₂EDTA, and 0.58 mM sodium 1-octanesulfonate in deionized water
197 with 15.3% (v/v) methanol. The pH of the mobile phase was adjusted to 3.0 with
198 orthophosphoric acid. The chromatographic separation was performed at 0.9 mL/min with a
199 Reprosil-Pur 120 C18-AQ column (150 x 4 mm, 5 µm particles) (Dr. Maisch, Germany) and
200 electrochemical detection of the separated compounds was performed using an ESA
201 Coulochem II detector. The detection system included a double analytical cell with oxidation
202 potentials set at 0 mV (first electrode) and +340 mV (second electrode). A conditioning cell at
203 + 400 mV was used before the analytical cell to oxidize potential interfering compounds. The
204 rest of the HPLC system consisted of a Shimadzu LC-10AD pump and a Midas autosampler

205 (Spark Holland, The Netherlands). Acquisition and integration of the chromatograms were
206 performed using Clarity™ HPLC software (DataApex Ltd, Czech Republic).

207 The levels of serotonin (5HT), dopamine (DA), and their main oxidative metabolites 5-
208 hydroxyindoleacetic acid (5HIAA) and 3, 4- dihydroxyphenylacetic acid (DOPAC) were
209 quantified, along with the levels of norepinephrine (NE). The main NE metabolite, 3-
210 methoxy-4-hydroxyphenylglycol (MHPG), was below the detection limit. Quantification was
211 carried out by comparing peak areas with those of the corresponding standards. The ratios
212 between 5HIAA and 5HT and between DOPAC and DA were then calculated as indirect
213 measures of the activity of the serotonergic and dopaminergic neurons, respectively [27].

214 **2.6. Gene transcription analyses**

215 The right half of the fish brain was used for gene transcription analyses.

216 *Extraction and reverse transcription of total RNA from the brain*

217 Each half-brain (\pm 200 mg) was mechanically disrupted using a ball mill in 900 μ L QIAzol
218 lysis Reagent (Quiagen) in 2 ml RNase-free tubes. They were then diluted two-fold before
219 starting RNA extraction, following the manufacturer's instructions (RNeasy Plus Universal
220 Mini Kit protocol, Qiagen, recommended initial sample size: 100 mg). The reverse
221 transcription of total RNA to cDNA was performed as described in Geffroy and colleagues
222 [62]. RNA quantity was assessed by measuring the A260/A280 ratio using the NanoDrop®
223 ND-1000 V3300 Spectrophotometer (Nanodrop Technology Inc., Wilmington, DE, USA).
224 Briefly, an aliquot of each tube was diluted in diethylpyrocarbonate-treated water. Then, 1.5
225 μ g RNA was reverse transcribed using 200 U Moloney murine leukemia virus (MMLV)
226 reverse transcriptase (PROMEGA) and 2 μ g random hexamers (Promega) in a master-mix
227 buffer and supplemented with 25 U RNase inhibitor (RNasin; Promega) in a final volume of

228 15 µl. All cDNA were diluted 50 fold in nuclease-free water prior to quantitative real-time
229 PCR (qPCR).

230 *Quantitative real-time PCR*

231 The transcription of genes involved in stress regulation, namely glucocorticoid receptor 1 and
232 2 (*gr1* and *gr2*), the mineralocorticoid receptor (*mr*), and corticotrophin releasing factor (*crf*)
233 was monitored. We also investigated the expression of genes involved in neurogenesis:
234 neurogenic differentiation factors (*neurod1* and *neurod2*) and proliferating cell nuclear
235 antigen (*pcna*), as well as an early growth response gene (*egr1*), involved in brain
236 development.

237 Primer sequences were retrieved from the literature [45, 63, 64] or specifically-designed using
238 the web version of PRIMER 3 (<http://primer3.ut.ee>; **Table 1**). The qPCR experiments were
239 conducted in a final volume of 1.5 µl, using 1 µl fish brain cDNA and 0.5 µl of an optimized
240 concentration of primers and Fast SYBR Green PCR Master Mix (Applied Biosystems, Life
241 technologies). Distribution of the volume into the plate was performed using a robot (Labcyte
242 Echo525) able to distribute as low as 25 nL by acoustic transfer ensuring reliable repeatability
243 even with small qPCR volumes. The hot start enzyme was activated for 2 min at 95°C and
244 amplification carried out using the following program: 95°C for 3 s and 60°C for 30 s for 40
245 cycles. After amplification, a melting curve was obtained using the following protocol: 10 s
246 holding at 55°C followed by sequential 0.05°C increases repeated 80 times. Technical
247 duplicates were run for each sample. A standard curve was generated by mixing all samples
248 and the efficiency (E) for all genes was calculated (*ef1*: 1.98; *l13*: 1.96; *gr1*: 1.90; *gr2*: 2.14;
249 *mr*: 2; *crf*: 1.83; *egr1*: 2; *neurod1*: 1.88; *neurod2*: 1.88; and *pcna*: 1.80).

250 *Housekeeping genes*

251 Ribosomal protein (*l13*) and eukaryotic translation elongation factor 1 alpha (*eef1-alpha*) have
252 both been recommended as housekeeping genes [65, 66] and were used in this study. The
253 coefficients of variation were below 5% for both *l13* and *eef1-alpha*. The amplification
254 efficiency was 98 and 96 % for *l13* and *eef1-alpha* respectively.

255 Relative levels of gene transcription were obtained using the following equation with the
256 efficiency (E) of the target gene normalized by the efficiency and the geometric mean of the
257 two housekeeping genes as reference (ref; *l13* and *eef1-alpha*), according to Roche diagnostic
258 [67]:

$$259 \quad \text{Relative level of gene transcription} = \frac{(E_{ref})^{Ct_{ref}}}{(E_{target})^{Ct_{sample}}}$$

260

261 **Table 1.** GenBank accession numbers and primer sequences for each gene used for gene
 262 transcription analyses.

Gene	GenBank accession numbers	Forward primer (5' → 3')	Reverse primer (3' → 5')
<i>mr</i>	JF824641.1	GTTCCACAAAGAGCCCCAAG	AGGAGGACTGGTGGTTGATG
<i>gr1</i>	AY549305 ^a	GAGATTTGGCAAGACCTTGACC	ACCACACCAGGCGTACTGA
<i>gr2</i>	AY619996 ^a	GACGCAGACCTCCACTACATTC	GCCGTTCATACTCTCAACCAC
<i>crf</i>	JF274994	GCAACGGGGACTCTAACTCT	GTCAGGTCCAGGGATATCGG
<i>neurod1</i>	b*	TTCTCCTTCAGCGTGCACTA	GGTGCGAGTGTCCATCAAAG
<i>neurod2</i>	d*	TGCGTAAAGTGGTTCCATGC	GTCGTGGGTTGGGAGAGTC
<i>pcna</i>	JQ755266 ^c	CAGAGCGGCTGGTTGCA	CACCAAAGTGGAGCGAACAA
<i>egr1</i>	d*	AACTCCAGCCTCAGTTCTC	AGTCAGGAATCATGGGCACA
<i>eef1-alpha</i>	AJ866727.1	AGATGGGCTTGTTCAAGGGA	TACAGTTCCAATACCGCCGA
<i>l13</i>	DT044910.1	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTGAGAGCAG

263 a*: from Pavlidis and colleagues [63]; b*: from Sadoul and colleagues [45]; c*: from Crespo
 264 and colleagues [64]; and d*: designed from the European sea bass genome from UCSC.

265 2.7. Statistical analyses

266 Statistical analyses were performed using R 3.1.0 software [68]. All statistical analyses were
 267 carried out at the 95% level of significance. Values are expressed in the text as the mean ±
 268 standard error of the mean (SEM) and illustrated in the figures using boxplots. The central
 269 line of the boxplot indicates the median and the boxes the quartiles, with the whiskers
 270 covering 95% of the values. Outliers are represented by points. First, the chi-square test was
 271 performed to compare the proportion of bold versus shy fish obtained between the GRT and
 272 OFT. Then, an exact binomial test was performed to evaluate behavioral consistency between

273 the GRT and OFT by comparing the proportion of consistent behavior with random
274 theoretical probability ($p = 0.5$) for each coping style. The remaining tests (concerning
275 physiology and behavior) were performed on fish screened in the GRT and the OFT
276 respectively.

277 Normality was verified for other comparisons between coping styles on behavioral and
278 physiological variables using the Shapiro test before applying either the Student's t-test or
279 Wilcoxon rank sum test, depending on whether normality was verified or not. Comparisons
280 between individual coping styles in the *distance travelled*, the *number of transitions* between
281 shelter and OFT arena, and the *time spent in the shelter* were performed using the Wilcoxon
282 rank sum test.

283 Concerning physiological variables, two individuals were discarded due to an RNA extraction
284 failure (no gene transcription) and therefore analyses were performed on a total of 55
285 individuals. The Student's t-test was performed to compare plasma cortisol concentrations
286 and monoamine concentrations in the brain (5HT, 5HIAA, 5HIAA/5HT turnover ratio, DA,
287 DOPAC, DOPAC/DA, and NE) between coping styles measured in the OFT. The Wilcoxon
288 rank sum test was performed to compare the transcription of genes (*gr1*, *gr2*, *mr*, *crf*, *neurod1*,
289 *neurod2* and *pcna*) between coping styles (Student t-test for *egr-1*).

290 Finally, a principal component analysis (PCA) was performed among fish that exited the
291 shelter with individual continuous variables recorded in OFT (Latency to exit shelter, distance
292 travelled, time spent in shelter and number of transitions) using ade4 package [69]. Individual
293 PC scores of the two first axes of the PCA were then downloaded. PC scores from axis 2,
294 reflecting coping styles were analyzed using linear regression as in relation to either
295 monoamine concentration or relative level of gene transcription in the brain. Homogeneity of
296 the model residuals was *a posteriori* checked using Shapiro-Wilk.

297 **3. Results**

298 **3.1. Behavioral consistency across context and time between the GRT and OFT**

299 During the GRT, 63% of the fish exited the sheltered area within the 24 hours of the test and
 300 were designated as bold. Consequently, 37% of the fish were designated as shy in the GRT. In
 301 the OFT, 58% of the fish exhibited bold behavior and exited the sheltered compartment vs.
 302 42% which exhibited shy behavior and stayed in the sheltered compartment during the 20 min
 303 of the test. The proportion of fish with bold/shy behavior was similar between the GRT and
 304 OFT ($\chi^2 = 0.51$; $p = 0.48$, **Figure 2A**). However, there was no behavioral consistency across
 305 context between the GRT and OFT for either bold or shy fish designated as such during the
 306 GRT ($p = 0.28$ and 0.85 , respectively). Indeed, 46% of fish designated as shy during the GRT
 307 stayed shy in the OFT, whereas the other 54% became bold one year later in the OFT. For fish
 308 designated as bold during the GRT, 61% stayed bold, whereas the other 39% became shy
 309 during the OFT (**Figure 2B**).

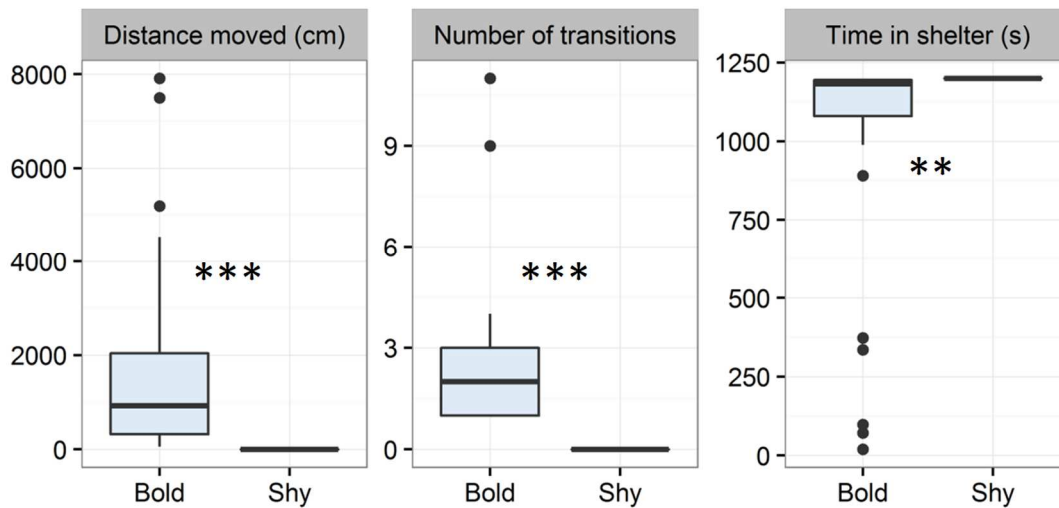
310 **Table 2.** Contingency table showing behavior displayed by the fish during the two tests (GRT
 311 and OFT).

		Open field test		
		New shy	New bold	Total
Group risk- taking test	Original shy	12	14	26
	Original bold	12	19	31
Total		24	33	57

312 **3.2. Open field test**

313 Of the 57 fish screened in the OFT, 58% were categorized as bold. These bold fish actively
 314 swam in the tank during the 20 minutes of the test (912.7 ± 60.2 cm; $W = 792$, $p < 0.001$) and
 315 moved between shelter and open field area (2.4 ± 0.4 times; $W = 0$, $p < 0.001$); whereas the

316 shy fish stayed in the shelter. Although the bold fish exited the shelter and were active in the
 317 open arena, most nonetheless spent most of the test time in the shelter ($1,000 \pm 64$ s), but still
 318 for less than the shy fish ($W = 792$, $p < 0.001$, **Figure 2**).



319

320 **Figure 2.** Distance travelled (cm), number of transitions between shelter and arena, and time
 321 spent in the shelter (s) during the open field test (OFT) between bold and shy fish categories
 322 as assigned in the OFT. Wilcoxon rank sum test: **: $p < 0.01$; ***: $p < 0.001$.

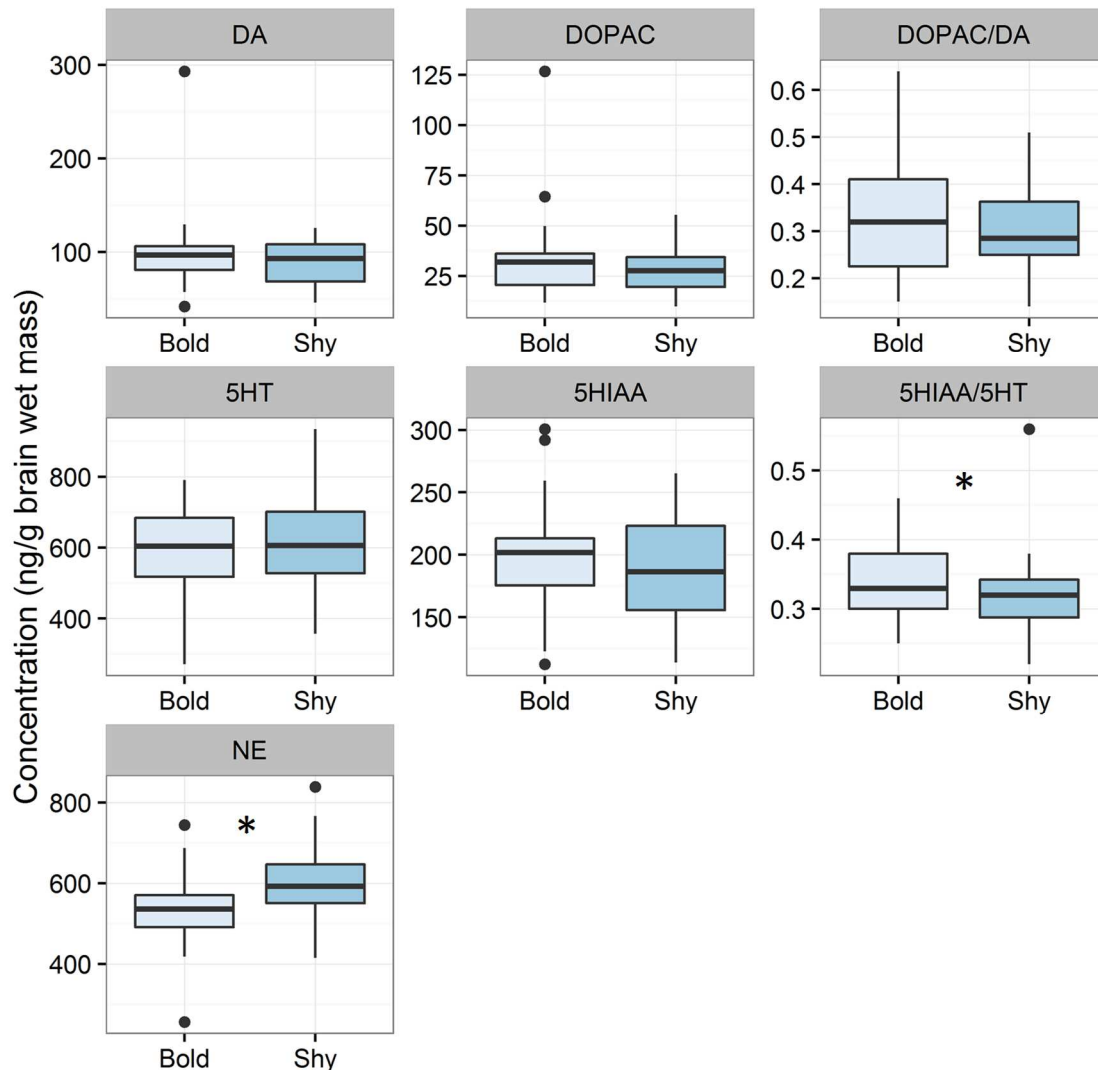
323 3.3. Plasma cortisol concentration

324 Plasma cortisol concentrations were similar between bold and shy fish screened in the OFT (t
 325 = -0.10, $p = 0.92$, data not shown). The mean plasma cortisol concentration in bold fish was
 326 904.3 ± 51.8 ng/mL ($n = 33$) and 912.7 ± 60.2 ng/mL in shy fish ($n = 24$).

327 3.4. Monoamine concentration in the brain

328 There was no difference in the concentration of DA or DOPAC in the brain between bold and
 329 shy fish screened in the OFT, nor the DOPAC/DA ratio ($t = -0.86$, $p = 0.40$; $t = -0.58$, $p =$
 330 0.57 ; and $t = -0.61$, $p = 0.54$, respectively). For the serotonergic system, there was no
 331 difference in the concentration of 5HT or 5HIAA in the brain between bold and shy fish ($t =$
 332 0.45 , $p=0.66$ and $t = -1.15$, $p = 0.25$, respectively). However, shy fish had a significantly

333 lower 5HIAA/5HT ratio in the brain than bold fish ($t = -2.17, p = 0.03$). The concentration of
 334 NE in the brain was also significantly higher in the shy than bold fish ($t = 2.22, p = 0.03$,
 335 **Figure 3**). Note that when considering bold and shy fish categories as assigned after the
 336 GRT, no difference in monoamine concentration or turnover ratio was detected
 337 (**Supplementary material**).

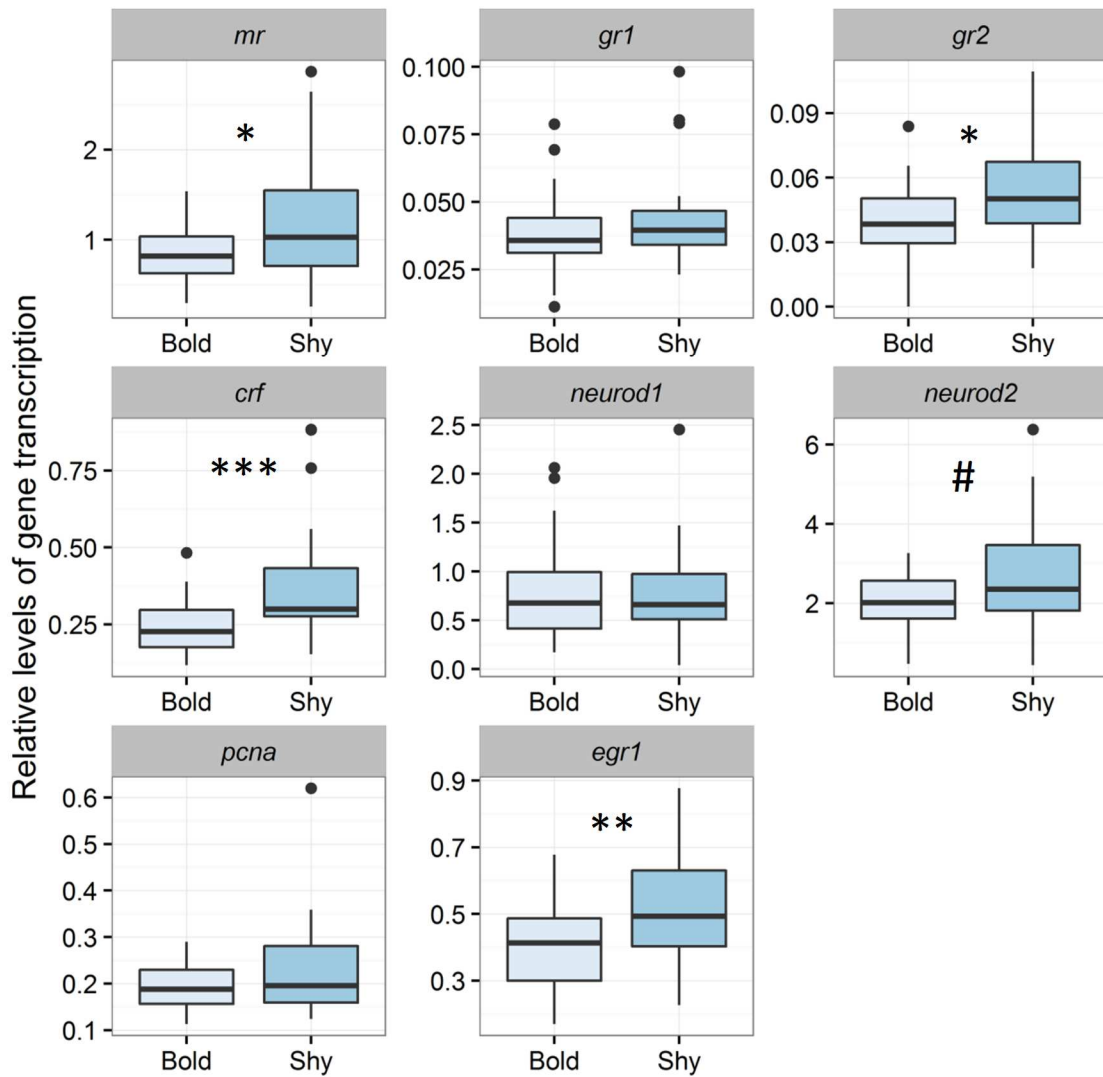


338 **Figure 3.** Concentration (ng/g brain wet mass) of dopamine (DA), 3,4-dihydroxyphenylacetic
 339 acid (DOPAC), the DOPAC/DA turnover ratio, serotonin (5HT), 5-hydroxyindoleacetic acid
 340 acid (DOPAC), the DOPAC/DA turnover ratio, serotonin (5HT), 5-hydroxyindoleacetic acid
 341 (5HIAA), the 5HIAA/5HT turnover ratio, and norepinephrine (NE) in the brains of bold and
 342 shy fish categories as assigned in the open field test (OFT). Student's t-test: *: $p < 0.05$.

343 **3.5. Gene transcription in the brain**

344 The transcription of *gr1* in the brain did not differ between bold and shy fish screened in the
345 OFT ($W = 312$, $p = 0.32$), whereas *gr2*, *mr* and *crf* were significantly upregulated in the
346 brains of shy fish relative to those of bold fish ($W = 171$, $p = 0.01$, $W = 255$, $p = 0.047$ and W
347 $= 145$, $p < 0.001$, respectively).

348 The transcription of *neurod1* and *pcna* in the brain did not differ between bold and shy fish
349 ($W = 358$, $p = 0.82$ and $W = 311$, $p = 0.31$, respectively), whereas there was a trend towards
350 the upregulation of *neurod2* in shy fish relative to bold fish ($W = 269$, $p = 0.08$). The
351 transcription of *egr1* was significantly upregulated in the brains of shy fish relative to those of
352 bold fish ($t = -2.70$, $p < 0.01$, **Figure 4**). Note that when considering bold and shy fish
353 categories as assigned after the GRT, no difference in gene transcription was detected
354 (**Supplementary material**).



355

356 **Figure 4.** Relative levels of gene transcription of the *mineralocorticoid receptor* (*mr*),

357 *glucocorticoid receptor 1* (*gr1*), *glucocorticoid receptor 2* (*gr2*), *corticotropin-releasing*

358 *factor* (*crf*), *neurogenic differentiation factors* (*neurod1* and *neurod2*), *proliferating cell*

359 *nuclear antigen* (*pcna*), and *early growth response* (*egr1*) relative to reference genes (*il13* and

360 *eef1-alpha*) in the brains of bold and shy fish categories assigned after the open field test

361 (OFT). Wilcoxon rank sum test (Student's t-test for *egr1*): #: $p < 0.10$; *: $p < 0.05$; **: $p <$

362 0.01 ; ***: $p < 0.001$. Note that for *gr2*, 6 cDNA samples were lost and are not presented.

363

364

365 **3.6.Principal component analysis (PCA)**

366 On one hand, PCA axis 1, which explained 52 % of the data variability observed, was mainly
367 driven by the time spent in shelter, the distance travelled and the number of transitions
368 between shelter and open field arena during the OFT (**Table 3**). On the other hand, PCA axis
369 2 was mainly driven by the latency to exit the shelter and the number of transitions between
370 shelter and open field arena during the OFT and explained 28 % of the data variability
371 observed (**Table 3**). PCA axis 1 was thus rather representative of activity in the OFT with
372 individuals displaying high values for PCA axis 1 being more active in the OFT than
373 individuals displaying low values. PCA axis 2 was rather representative of coping styles and
374 individuals displaying high values for PCA axis 2 scores were shy and moved more
375 between shelter and open field arena than individuals with low values which were bolder and
376 moved less between the two compartments of the OFT. Because of PCA axis 2 being more
377 representative of coping styles, further analyses were performed on PCA axis 2 scores.

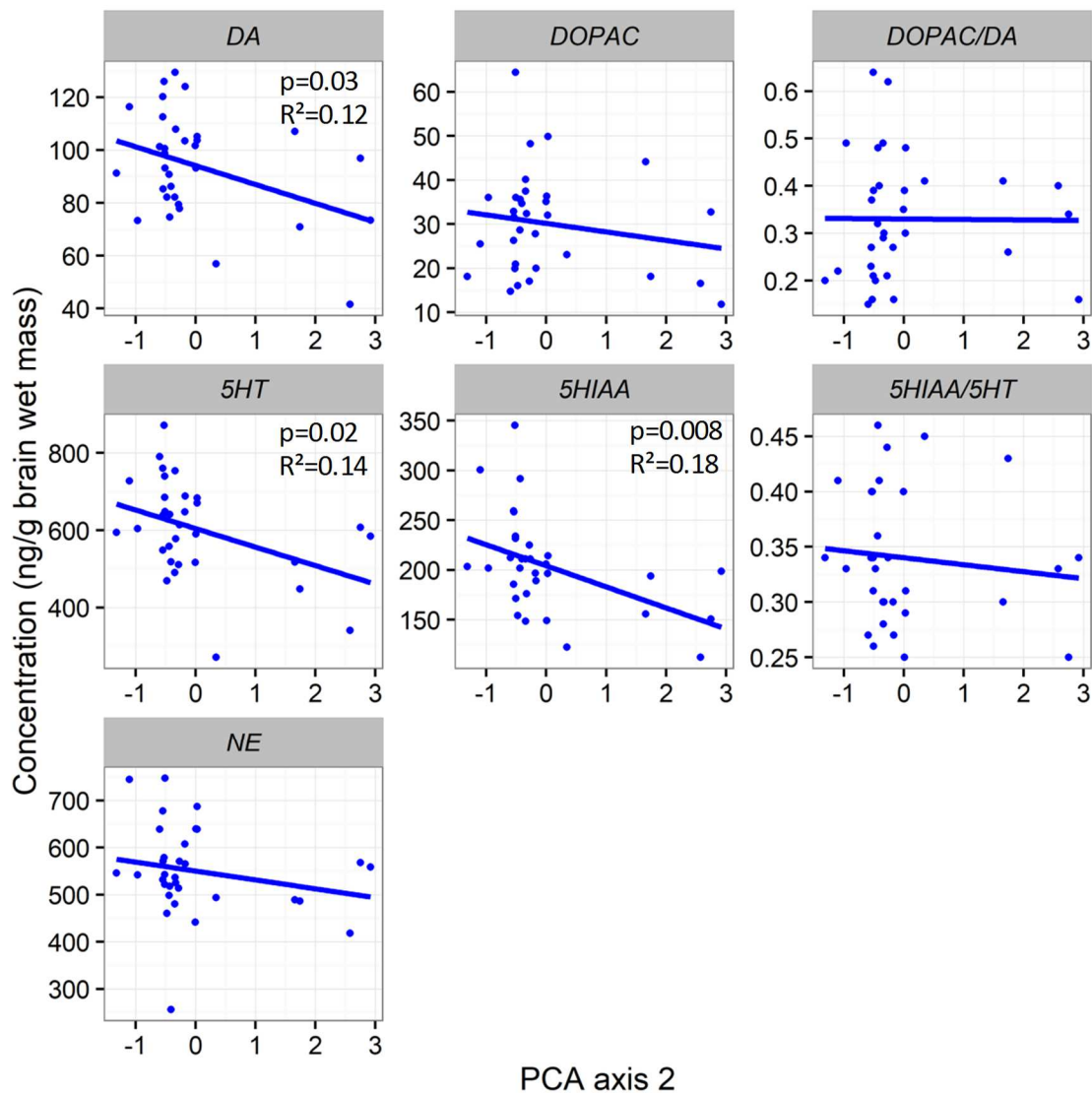
378 **Table 3.** Loading of behavioral variables (Latency to exit shelter, distance travelled, time
379 spent in shelter and number of transitions between shelter and open field arena during the
380 open field test) for the two PCA axes. Values in bold are the main drivers of the related axis.

Variable contribution	PCA axis 1	PCA axis 2
Latency to exit shelter	0.02	0.90
Distance travelled	0.94	-0.18
Time spent in shelter	-0.95	0.13
Number of transitions	0.56	0.50
Variability explained (%)	52	28

381

382 Regarding monoamines concentration in the brain, concentration of DA was negatively
383 correlated with PCA axis 2 ($F = 5.25$, $p = 0.03$, $R^2 = 0.12$), *i.e.*, bolder fish displayed higher
384 level of DA in the brain but there was no correlation for DOPAC or DOPAC/DA ratio with
385 PCA axis 2 ($F = 0.98$, $p = 0.33$ and $F = 0.002$, $p = 0.96$ respectively). Moreover, 5HT and
386 5HIAA also were negatively correlated with PCA axis 2 scores ($F = 6.10$, $p = 0.02$, $R^2 = 0.14$
387 and $F = 7.96$, $p = 0.008$, $R^2 = 0.18$ respectively) but there was no correlations between
388 5HIAA/5HT ratio and PCA axis 2 ($F = 0.39$, $p = 0.54$). Finally there was no correlation
389 between concentration of NE in the brain and PCA axis 2 scores ($F = 1.4$ $p = 0.25$, **Figure 5**).

390



391

392 **Figure 5.** Concentration (ng/g brain wet mass) of dopamine (DA), 3,4-dihydroxyphenylacetic
393 acid (DOPAC), the DOPAC/DA turnover ratio, serotonin (5HT), 5-hydroxyindoleacetic acid
394 (5HIAA), the 5HIAA/5HT turnover ratio, and norepinephrine (NE) in the brain in relation to
395 individual PCA axis 2 scores. P-value (p) and the associated R² are indicated for each
396 significant linear regression.

397 Concerning gene transcription in the brain, there was no correlation between the investigated
398 genes and PCA axis 2 scores except a negative trend between relative level of *pcna* and PCA
399 axis 2 scores ($F = 3.99$, $p = 0.07$, $R^2 = 0.08$). All statistical details concerning correlations
400 between the relative levels of other genes are given in the text of the **Supplementary**
401 **Material** and illustrated in **Figure S1**.

402 **4. Discussion**

403 Here, we evaluated the boldness of European sea bass using two different challenges, which
404 were group risk taking test (GRT) and open field test (OFT) at 227 and 749 dpf, respectively.
405 The proportion of bold/shy fish did not differ between the two tests (approximately 60% of
406 the fish were categorized as bold). The proportion of bold/shy fish however differed from a
407 previous study in European sea bass in which ~80% were identified as shy using a group risk-
408 taking test [56]. The high proportion of bold fish identified in our study may be attributable to
409 small differences with their protocol and/or the level of fish domestication which could affect
410 boldness [6]. We did not find any clear behavioral consistency in boldness across context
411 between the GRT and OFT. Indeed, only 50% of fish with shy behavior in the GRT were also
412 categorized as shy in the OFT (522 days between the two tests). The consistency of boldness
413 over time was previously reported in European sea bass when consecutive GRTs were
414 performed 29 days apart [4]. Boldness, as well as other personality traits, is known to be
415 consistent over a short period of time, but can evolve during the life of the animal [70].

416 Moreover, boldness is influenced by many external factors, both biotic and abiotic [71-74].
417 Abiotic factors, such as temperature, physico-chemical characteristics of the water, or food
418 supply, were kept constant during our experiment. However, some other factors could explain
419 the absence of a clear boldness consistency between the GRT and OFT. The difference in
420 social context between the two tests (group *vs* isolation) could be one and could partly explain
421 the discrepancy between the results, regardless of the time window that separated the two
422 behavioral experiments [75]. Social context is indeed very important in shaping personality
423 traits in fish [76] and may have affected our capacity to evaluate boldness in single fish in a
424 social species, such as sea bass, as already mentioned by Ferrari and colleagues [4].

425 Nevertheless, the fish expressed two divergent behavioral responses during the OFT. The
426 majority of fish (58 %) expressed bold trait, with a high level of activity in the OFT arena,
427 whereas shy fish expressed freezing behavior in the shelter. These two different behavioral
428 responses have been largely described in the literature as typical proactive and reactive coping
429 style characteristics [2, 5, 77].

430 Differential stress regulation of the HPI axis and the brain-chromaffin axis are often
431 associated with the opposite behavioral responses expressed by proactive and reactive
432 individuals [1, 2, 35, 78]. Here, the signal from the dopaminergic system in the whole brain
433 was similar between bold and shy fish. The concentrations of DA and DOPAC and the
434 DA/DOPAC turnover ratio were indeed identical between our two groups, whereas
435 dopaminergic system signaling was found to be different between bold and shy fish in
436 different brain parts such as optic tectum [26, 79]. Possible specific differences between bold
437 and shy fish could also be masked by the fact that the analyses were performed on whole
438 brain [26, 80]. The boldest fish screened in the OFT nevertheless displayed higher DA
439 concentration in the whole brain compared to the less bold ones. This is consistent with
440 previous studies suggesting that dopaminergic system may trigger active behavioral

441 responses, including bolder behavior [81-83]. Nevertheless, it is important to note that our
442 study was performed on the whole brain, therefore we cannot conclude that the differences
443 between bold and shy fish are representative of what would be measured in region-specific
444 areas. Concerning the serotonergic system, the 5HT/5HIAA turnover ratio was lower in shy
445 than bold fish following the OFT but the boldest fish displayed both higher levels of 5HT and
446 5HIAA in the brain than the less bold ones. Similar differences in 5HIAA have been
447 previously observed in the hypothalamus and telencephalon of rainbow trout (*Oncorhynchus*
448 *mykiss*) following an acute stressor [26]; fish with a more reactive coping style showed lower
449 levels of serotonergic activity upon exposure to stress. This supports the view of the shy and
450 bold groups established in the OFT as exhibiting different coping styles, as differences in the
451 serotonergic system could be involved in the determination of the individual coping style
452 [84]. Another interpretation is that the differential behavior observed (exploring vs freezing in
453 the shelter) might have driven the difference in the serotonergic response. In this case, the
454 bold fish were more active and left the shelter and thus exposed themselves out in the open
455 area, which might be more stressful for them, leading to higher 5HT activity. This alternative
456 interpretation has been considered by Koolhaas and colleagues [85] who hypothesized that “it
457 is likely that differential neuroendocrine characteristics are mainly a consequence rather than
458 the cause of behavioral differentiation”. Finally, the noradrenergic system is generally less
459 well-documented than the serotonergic and dopaminergic systems in relation to coping styles.
460 However, the higher NE concentrations we measured in the whole brain of shy fish correlate
461 well with results found in the brainstem, telencephalon and optic tectum of rainbow trout
462 following confinement stress [26, 80].

463 Concerning the HPI axis, plasma cortisol concentrations measured in our study were higher
464 than those previously found in European sea bass [86, 87]. It is likely that the social isolation
465 during the OFT is a very strong stressor for European sea bass, as was shown for rainbow

466 trout [88]. Further, there was no difference in the cortisol response between bold and shy fish
467 categorized using the OFT. This may seem surprising, as high cortisol levels have been shown
468 to be predictive of the reactive coping style in sea bass in another context [4]. Nevertheless, it
469 cannot be excluded that the lack of difference may be due to the fact that, once in the OFT,
470 bold fish take more risk and are further stressed by exploring the open field arena whereas shy
471 fish stay in the shelter resulting in different physiological status when blood sample was
472 taken. Moreover, such an absence of a difference in the peak cortisol response was previously
473 reported following an acute stressor in various fish species [3, 78], but divergences appeared
474 during the recovery phase [3]. The transcription in the whole brain of *gr2*, *mr* and *crf*, which
475 involved in the regulation of the HPI axis, was higher in shy than bold fish, whereas *gr1*
476 transcription did not differ between the two groups. Elevated *gr2*, *mr* or *crf* transcription in
477 shy fish has also been reported in other fish species and contexts [80, 89-91]. For example,
478 HR rainbow trout (considered as shy) displayed higher levels of *crf* than LR (considered as
479 bold) in the forebrain of rainbow trout following confinement stress [80]. These effects were
480 shown to be modulated by binding proteins reducing the activity of Crf but increasing its
481 longevity [92, 93]. In addition to its effects on the regulation of the HPI axis, Crf can interact
482 with neurotransmission and modulate behavior [94-97]. Overall, Crf interacts with
483 serotonergic and noradrenergic pathways, resulting in an increase in locomotor behavior,
484 including non-ambulatory behavior, and has been reported to cause anxiety [95]. In the
485 present study, shy fish (which expressed higher levels of *crf* than bold fish) expressed a lower
486 turnover of serotonergic activity and higher NE concentrations. Shy fish also displayed a
487 freezing behavior in the shelter during the OFT. This echoes a previous study showing that
488 intraventricular Crf injections in rats increase the anxiety state of the animal, as well as the
489 turnover ratio between NE and its metabolite DHPG in the brain-stem locus coeruleus [98].
490 More recently, it was reported in rainbow trout that Crf-injected fish have higher 5HT and 5

491 HIAA levels in various brain regions (*i.e.*, the subpallium, raphe, and preoptic hypothalamus)
492 and/or express head-shaking behavior, which is related to anxiety [99, 100]. Interactions
493 between Crf and both the serotonergic and the noradrenergic system appear promising to
494 further explain the divergent phenotypes observed in fish coping styles [95, 99, 100].

495 Recent studies suggest that stress axis regulation and neurogenesis are tightly linked [45-47].
496 For example, Sadoul and colleagues [45] have shown that high levels of *mr* and *gr* in the
497 brain correlate with high levels of *pcna* and *neurod1* in several fish species, including the
498 European sea bass. Here, we also detected a strong and significant correlation (at the
499 individual level) between genes involved in the stress response and those involved in
500 neurogenesis (**supplementary material**), confirming the results of Sadoul and colleagues
501 [45]. In the present study, we show that the expression of *egr1* and *neurod2*, which are
502 important markers of neurogenesis involved in neural activation and differentiation,
503 respectively [34, 48], were higher in the brains of shy fish than in those of bold fish (only a
504 trend for *neurod2*). This higher propensity to display strong neurogenesis in shy fish is
505 suggested in the literature [34, 89, 90, 101] and may be important for learning [34]. This is in
506 accordance with previous results showing that shy fish are more proficient in learning and
507 triggering self-feeders [60, 102].

508 Here, both monoamine concentrations and gene transcription analyses were performed on the
509 whole brain, including multiple regions with specific functions and patterns of monoamine
510 synthesis and gene transcription [26, 78, 103]. Significant effects were observed using the
511 whole brain, and therefore we could expect even more significant differences between groups
512 in specific areas of the brain. Microdissections or dissection of the different areas of the brain
513 would have probably lead to more robust conclusions. A refined analysis would be necessary
514 to better understand the differential pattern of responses in the different brain areas.

515 Overall, European sea bass characterized by two divergent behavioral phenotypes on the
516 boldness axis, bold and shy, also display two distinct physiological responses which could be
517 reflect the proactive and reactive differentiation, respectively. Altogether, our results suggest
518 that the two coping styles display differences in the HPI axis, the serotonergic and
519 noradrenergic system reactivity, and in neurogenesis. Further, and according to our results,
520 the behavioral responses of a social animal to a challenge in isolation seem to be relevant
521 markers of its physiological state and capacity to regulate and react to a stressor. More data
522 are needed to verify whether such responses are specific to one time point or if they are
523 indicators of a lifelong coping style.

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