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1 Physiological responses during acute stress recovery depend on stress coping style in European sea
2 bass, *Dicentrarchus labrax*.

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16

17 **Abstract**

18 Individual stress coping style (reactive, intermediate and proactive) was determined in 3 groups of
19 120 pit tagged European seabass using the hypoxia avoidance test. The same three groups (no
20 change in social composition) were then reared according to the standards recommended for this
21 species. Then, 127 days later, individuals initially characterized as reactive, intermediate or proactive
22 were submitted to an acute confinement stress for 30 min. Blood samples were taken to measure
23 plasma cortisol levels 30 min (Stress30) or 150 min (Stress150) after the end of the confinement
24 stress. Individuals were then sacrificed to sample the telencephalon in order to measure the main
25 monoamines and their catabolites (at Stress30 only). Individuals from Stress150 were sampled for
26 whole brain for a transcriptomic analysis. The main results showed that reactive individuals had a
27 lower body mass than intermediate individuals which did not differ from proactive individuals. The
28 physiological cortisol response did not differ between coping style at Stress30 but at Stress150 when
29 intermediate and proactive individuals had recovered pre stress levels, reactive individuals showed a
30 significant higher level illustrating a modulation of stress recovery by coping style. Serotonin turnover
31 ratio was higher in proactive and reactive individuals compared to intermediate individuals and a
32 significant positive correlation was observed with cortisol levels whatever the coping style. Further,
33 the confinement stress led to a general increase in the serotonin turnover comparable between
34 coping styles. Stress150 had a significant effect on target mRNA copy number (*Gapdh* mRNA copy
35 number decreased while *ifrd1* mRNA copy number increased) and such changes tended to depend
36 upon coping style.

37 **Key words:** *fish, behaviour, physiology, personality, brain, transcriptomic*

38

39 **Highlights:**

- 40 - Reactive fish showed a slower stress recovery after acute confinement stress
- 41 - Serotonin turnover ratio was affected by acute confinement stress
- 42 - A positive correlation was shown between plasma cortisol and serotonin turnover ratio

43

44 1. Introduction

45 Recently, the scientific community has seen a surging interest in the evolution and
46 development of links between different variable traits and consistent variation between individuals
47 across time and contexts. Adaptive phenotypic variation is principally expressed as correlated
48 behavioural and physiological profiles, in turn conferring a variable vulnerability to competition,
49 stress and disease. Physiological-behavioural trait associations are commonly referred to as stress
50 coping styles (Koolhaas et al, 1999). These individual coping styles occur within a population along an
51 axis (also called a continuum) between pro- and reactive extremes. For simplification, most studies
52 only consider the extreme phenotypes but it should be understood within this continuum and
53 relative to the population composition and situation. Proactive animals tend to engage in active
54 avoidance or cope with stressful stimuli through a “fight or flight” response contrary to reactive ones
55 which display a passive behaviour through a “freeze and hide” response (Koolhaas et al, 1999;
56 Koolhaas, 2008).

57 Physiologically, a proactive fish is characterized with a lower hypothalamus-pituitary-inter-
58 renal (HPI) activity (Øverli et al, 2005, 2007; Silva et al, 2010) and higher sympathetic reactivity
59 compared to a reactive fish (reviewed by Øverli et al, 2007; Castanheira et al, 2013; Sørensen et al,
60 2013). Therefore, proactive fish typically have lower basal concentrations of glucocorticoids (the
61 principal hormones involved in the stress response and the ultimate product of HPI axis activation)
62 and lower stress-induced glucocorticoid concentrations (Øverli et al, 2007) than reactive individuals.
63 In further details, the main endocrine components of stress are an immediate increase in the release
64 of catecholamines (epinephrine and norepinephrine [NE]) into the circulation, accompanied by
65 increased sympathetic tone, and a slightly delayed (within minutes) increase in the release of
66 glucocorticoid hormones (mainly cortisol in teleost fish). The monoamines serotonin (5-
67 hydroxytryptamin, 5-HT), dopamine (DA) and norepinephrine (NE), have been studied as
68 neurotransmitters or neuromodulators potentially involved in the mediation of physiological as well

69 as behavioural stress responses (Winberg et al, 1991; Winberg and Nilsson, 1992, 1993; Lillesaar,
70 2011; Vindas et al, 2014; Thornqvist et al., 2018). The effects of monoaminergic neurotransmitters
71 are terminated by uptake into presynaptic nerve and possibly glial cells. Following uptake,
72 monoamines are deaminated to their main catabolites by monoamine oxydase (MAO). 5-HT is
73 transformed to 5-hydroxyindoleacetic acid (5-HIAA) and DA to 3,4-dihydroxyphenylacetic acid
74 (DOPAC). Both for 5-HT and the catecholamines, the ratio of the tissue concentration of their
75 metabolites to that of the parent monoamine is frequently used as an index of neural activity,
76 increased concentration of the metabolite being taken to indicate increased release and turnover of
77 the neurotransmitter (reviewed in Shannon et al, 1986; Fillenz, 1993).

78 Behavioural inhibition in reactive animal has been suggested to be mediated partly by a
79 stress induced elevation of brain serotonergic activity (Winberg et al, 1993a, b; Øverli et al, 1998)
80 but the opposite, a reduced serotonergic activity, has also been observed (Alfonso et al 2019). 5-HT
81 has also been suggested to stimulate HPI axis in teleosts (reviewed in Höglund et al, 2000). The
82 release of catecholamines is also integral part of the physiological response to stressors in all
83 vertebrates (Hart et al, 1989). The function of catecholamines includes modulation of respiratory and
84 cardiovascular systems, blood oxygen transport capacity, blood glucose and free fatty acid levels.
85 Moreover, in fish, brain dopamine seems to have a generally suppressive effect on HPI axis activity
86 and 5-HT signalling (Höglund et al, 2001).

87 Molecular regulation at the level of the transcriptome underpins the behavioural and
88 physiological characteristics of the two main coping styles described above (MacKenzie et al, 2009).
89 This regulation insures correct adaptive response to changing environmental conditions and include
90 a scaled suite of multi-directional regulatory processes, from transcriptome to behaviour, that
91 interact to optimize individual fitness (Rey et al, 2013). Thus, a transcriptome or gene expression
92 profile is a collection of mRNAs within a cell, tissue or organism that represents the available
93 transcripts at a specific point in time (i.e. phenotype shaped by the organism's genotype, Rey et al,

94 2013). In previous studies, prior screening for coping style before experimentation increased the
95 value of gene expression data and their interpretation (MacKenzie et al, 2009; Rey et al 2016). Such
96 methodology allows for targeted candidate gene expression analysis in follow-up studies (e.g. the
97 current report).

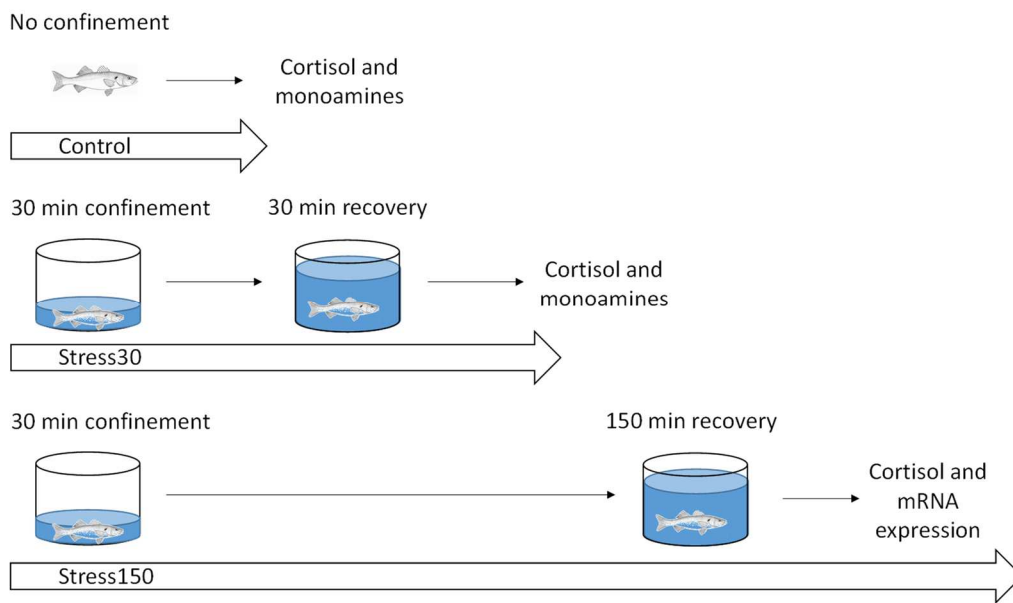
98 Here we present a multidisciplinary study, assessing coping styles at the level of behavioural
99 and physiological responses with the aim to constitute an integrative design to better understand
100 underlying mechanisms related to adaptive phenotypic variation in sea bass (*Dicentrarchus labrax*).
101 Indeed, in this particular species, genetic correlations between weight and risk taking traits showed
102 negative values i.e. reactive individual mass was heavier (Ferrari et al, 2016) contrary to a recent
103 study performed in Atlantic salmon (Damsgård et al, 2019).

104 The objectives of this study were to assess physiological, neurochemical and mRNA
105 expression of selected brain target genes of fish from divergent coping style after an acute
106 confinement stress and during recovery. To achieve this, sea bass were screened by using a hypoxia
107 avoidance test (Laursen et al 2011, translated to sea bass by Ferrari et al, 2015) and were assigned to
108 reactive, intermediate and proactive categories. Although this test does not allow distinguishing
109 between exploration and hypoxia tolerance, it is relevant for coping style assessment in its
110 physiological dimension since hypoxia tolerance is a determinant of individual fitness in sea bass
111 (Joyce et al., 2016) and correlations between risk taking and hypoxia tolerance were shown in sea
112 bass (Ferrari et al. 2016, Alfonso et al. 2019).

113 Following this, fish were reared under standard conditions and were exposed to a
114 confinement stress. Brain and blood were sampled, plasma were analysed for cortisol, telencephalon
115 for monoaminergic neurochemistry and gene expression of target genes were analysed in whole
116 brains. Data obtained for each individual were cross-correlated in order to explore causal links of
117 phenotypic traits.

118 **2. Material and methods**

119 In this study, PIT tagged sea bass were screened using the hypoxia tolerance test (see below).
120 They were then reared for 4 months without changing group composition. At the end of the rearing
121 period, a confinement stress protocol was applied resulting in three treatments (Control, Stress30
122 and Stress150, **Figure 1**). Blood samples were taken to assess cortisol levels, and fish were dissected
123 to sample telencephalon to quantify monoamines whereas target mRNA expression was measured in
124 whole brain.



125
126 **Figure 1:** Scheme of the three treatment conditions (Control, Stress30 and Stress150). “Control”
127 corresponds to cortisol and monoamine sampling just after netting in the home tank. “Stress30”
128 corresponds to cortisol and monoamine sampling after 30 minutes confinement and 30 minutes
129 recovery. “Stress150” corresponds to Cortisol and gene expression sampling after 30 minutes
130 confinement and 150 minutes recovery.

131 2.1. Fish and experimental conditions

132 Fish were hatched and reared at the experimental research station of Ifremer (Palavas-les-
133 Flots, France) according to sea bass rearing standard (Chatain, 1994). Three groups of each 120 fish
134 were used in this experiment (N total=360). Each triplicate was placed in a 1.5 m³ tank under sand
135 filtered open flow system.

136 2.2. Growth follow-up

137 All fish were weighed five times under anaesthesia ((benzocaine, 200 ppm, after tranquilization in
138 the rearing tank with 70 ppm Benzocaine), at 215 days post hatching (dph), 251 dph, 285 dph, 314

139 dph and 342 dph (hereafter termed BW_215dph, BW_285dph etc...). Specific Growth Rate was
140 calculated as follows ($SGR=100*\ln(BW_f)-\ln(BW_i)/t$, in %) with BW_f corresponding to final body mass
141 and BW_i to initial body mass and t corresponded to time in days between two successive mass
142 measurements. Four SGRs were calculated: SGR_1 (215-251 dph), SGR_2 (251-285 dph), SGR_3 (285-
143 314 dph) and SGR_4 (314-342 dph). At the end of the experiment, fish were euthanized with an
144 overdose of anaesthetic for further sampling (see following sections) and phenotypic sex was
145 determined according to the method described by Ferrari et al (2014). Four fish with undetermined
146 sex were removed from statistical analyses.

147 **2.3. Behavioural screening**

148 Three groups of 120 individuals were screened at 215 dph (mass 25.91 ± 0.51 g) for hypoxia
149 tolerance (adapted from Laursen et al, 2011; translated to sea bass by Ferrari et al, 2015) in order to
150 assess their coping styles and then placed back in their home tank without modifying the group
151 composition. Briefly, in the hypoxia test, oxygen concentration was decreased in one out of two
152 adjacent chambers of a test tank, and the escape from the hypoxic to the normoxic compartment
153 was recorded. The test apparatus consisted of two identical circular tanks (70 l, h: 48 cm, diameter:
154 49.5 cm,) attached to each other via a transparent acrylic pipe (diameter: 11 cm, length: 30 cm,
155 height from bottom: 23 cm, see Castanheira et al, (2013) for a detailed diagram of the apparatus).
156 Each tank was considered a separate environment individually equipped with oxygen and air supply
157 that were switched off during the trials in the hypoxia tank (see below). Sixty fish were placed in one
158 chamber of the tank (which subsequently became the hypoxia tank) and were allowed to acclimate
159 to the conditions for 30 minutes before the start of the experiment. The hypoxia tank was supplied
160 with nitrogen, to induce hypoxic conditions during the experiment (nitrogen bubbling decreased
161 oxygen saturation from 90 % to 8 % in 1 hour). The second chamber of the tank, which was supplied
162 with oxygen, is referred to as the normoxia tank. Once an individual escaped from the hypoxic tank
163 into the initially empty normoxia tank, it was immediately netted, then placed in a separate tank
164 before being anesthetized (benzocaine, 200 ppm), tagged with 12 mm ISO PIT tags, measured for

165 mass and replaced into the home rearing tank. Within the coping style continuum, assignment to
166 categories was performed based on Ferrari et al. (2015) previous behavioural and physiological
167 characterisation which demonstrated that hypoxia avoider fish had lower cortisol concentration,
168 higher levels of activity and took more risks, three characteristics of a proactive coping style. Hence it
169 was done as follows: The 20 first fish escaping hypoxic conditions were referred to as proactive (P),
170 the ~20 followers were referred to as intermediate (I) and the last fish that did not escape hypoxic
171 conditions were referred to as reactive (R). The hypoxia test ended when two third of the fish had
172 escaped from the hypoxia tank or when 8% oxygen saturation was reached (water temperature 20°C,
173 salinity 26.9). This operation was repeated until the three triplicates of 120 fish were screened. In
174 total the characterization from each replicate rearing tank yielded: Tank 1: 40 P, 39 I and 40 R (one
175 fish died), Tank 2: 40 P, 61 I and 19 R and finally Tank 3: 40 P, 46 I and 34 R.

176 **2.4. Stress treatment**

177 In total, 276 fish were subsampled at 342 dph (mean mass 89.2 ± 31.8 g) after applying a
178 confinement stress protocol resulting in three treatments: Control, Stress30 and Stress150 (**Figure 1**).
179 Fish were fasted 24 hrs prior to sampling. After decreasing the water level in their home tank, fish
180 were lightly anaesthetised (70 ppm of Benzocaine), netted and transferred in a smaller well aerated
181 holding tank still under light anaesthesia. PIT tags were read, then mass and length were measured
182 and depending on their previously known coping styles and assigned treatment, fish were dispatched
183 in 9 identical tanks (70 l each: Proactive, Intermediate, Reactive x Control, Stress30 and Stress150).
184 Fish thereafter called Control were immediately deeply anaesthetized using 200 ppm of benzocaine
185 and blood samples were obtained from the caudal vein with heparinised syringes within 3 minutes.
186 Thereafter, fish were killed using an overdose of anaesthetic and body kept on ice for further brain
187 dissection. Fish from Stress30 group were placed under confinement (water level at 1/4 in the 70l
188 tanks) during 30 min then allowed to recover (water level back to maximum) during 30 min and were
189 then directly anaesthetized for the same sampling procedure as above. Fish from Stress150 group

190 were submitted to the same stress procedure, but allowed 150 min for recovery and then underwent
191 the same sampling procedure.

192 **2.5. Plasma cortisol**

193 Fish were sampled at 342 dph for circulating cortisol (40 individuals per coping style for the
194 control treatment, 20 individuals per coping styles for Stress30 and Stress150 treatments). The blood
195 was centrifuged (5 min at 1500 g) to obtain plasma samples, which were stored at -22°C for further
196 analyses. Plasma cortisol concentration was determined with an ELISA kit (RE52061, IBL, Germany)
197 following manufacturing instructions.

198 **2.6. Telencephalon monoamine neurochemistry**

199 A subset of 10 individuals per coping styles from Control and Stress30 treatment were
200 selected for analysis of monoamine neurochemistry. Brains were dissected out from each fish in
201 order to extract the telencephalon which was frozen individually in liquid nitrogen and kept at -80°C
202 for later analysis. Before analysis, each frozen telencephalon was individually weighed. After
203 weighing, the brain part was homogenised in a homogenising reagent (4% perchloric acid, 0.2%
204 Ethylene diamine tetraacetic acid, 40 ng ml⁻¹ dihydroxi benzylamine hydroxide solution). The solvent
205 was then centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was assayed by High
206 Performance Liquid Chromatography (HPLC) with electrochemical detection to quantify the
207 concentration of 5-HT and its catabolite 5-HIAA, DA and its catabolite DOPAC and norepinephrine NE.
208 The HPLC system consisted of a mobile phase (buffer solution; 10.35 g l⁻¹ sodium phosphate,
209 0.3252 g l⁻¹ sodium octyl sulphate, 0.0037 g l⁻¹ EDTA, 7% acetonitril in deionised water), a solvent
210 delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6
211 mm 100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with
212 two electrodes at -40 mV and +320 mV. A conditioning electrode with a potential of +40 mV is used
213 to oxidize possible contaminants before analysis. Brain 5-HT, 5-HIAA, DA, DOPAC and NE were
214 quantified by comparing them with standard solutions of known concentrations and corrected for
215 recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic).

216 **2.7 Brain gene expression patterns**

217 A subset of 20 individuals per coping styles from Control and Stress150 treatment were
218 prepared for whole brain gene expression analysis. Brains were extracted and immediately frozen
219 with liquid nitrogen. Total RNA was extracted from the brain using TriReagent (Molecular Research
220 Center) following the manufacturer's instructions and verified for quantity using a NanoDrop ND-
221 1000 (Thermo Scientific) and quality visualized under UV light in a 1% agarose gel containing $1 \mu\text{g}\cdot\text{ml}^{-1}$
222 ethidium bromide. $1 \mu\text{g}$ of total RNA was taken from each individual to synthesize cDNA with
223 SuperScript III RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega). Selected target
224 transcripts were cloned and sequenced. Conventional PCR products were visualized under UV light in
225 a 1.2 % agarose gel containing $1.5 \mu\text{g}/\text{ml}$ ethidium bromide, purified using PCR clean-up Gel
226 extraction MN (Cultek), cloned into pGEM-T Easy Vector (Promega) by T/A cloning and transfected
227 into competent Escherichia coli DH5 α strain TM Competent Cells, Invitrogen (Promega). Plasmid DNA
228 was isolated by Nucleospin Quickpure (Marcherey Nagel). All constructs were verified by DNA
229 sequencing (GATC Biotech).

230 Absolute quantification was performed and the copy number of each transcript, derived from
231 the standard dilution curve obtained from target plasmids was analysed using a Thermocycler
232 Stratagene Mx3005P (Agilent, USA). Each sample was tested in triplicate in a 96-well plate. The
233 reaction mix (20 μl final volume) consisted of 10 μl of SYBR Green mix (Aligent, USA), 0.5 μl of each
234 primer (20 μM), 7 μl of H₂O and 2 μl of a 1/10 dilution of the cDNA sample. The thermocycling
235 program consisted of one hold at 95 °C for 3 min, followed by three-step 35 cycles of 15 s at 95 °C,
236 10 s at 58 °C and 10 s at 72 °C. No template controls (NTCs) were used to assure no false positive
237 signals were calculated. Thresholds were normalized for all genes with the gene project software.

238 *Identification of Target mRNAs*

239 Taking a comparative evolutionary approach, common mRNA transcripts differentially
240 expressed in zebrafish screened for coping styles (Rey et al 2013) were used to identify target genes
241 in *D. labrax*. The zebrafish specific target transcripts that display coping style specific variance were

242 cloned 'in silico'. Cloning was carried out using genomic resources from all available public libraries.
 243 To identify specific European Sea bass targets parallel scale BLAST (Basic Local Alignment Search
 244 Tool) was executed using the filtered target set from the zebrafish resource against the species of
 245 interest. Curated lists of mRNA transcripts were used for primer design and validated using pools of
 246 fish whole brain cDNAs with the objective of individual absolute quantification of gene expression.
 247 From this collection, 4 mRNAs for sea bass were chosen for the gene expression study. The genes
 248 selected for the sea bass study were sodium/potassium-transporting *atpase* subunit alpha-3:
 249 *atpase α 3*, glyceraldehyde-3-phosphate dehydrogenase: *gapdh*, Interferon-related developmental
 250 regulator 1: *ifrd1*, and *nedd8* precursor factor: *nedd8* (see Table 1).

251 **Table1.** mRNA target sequence names, primers, amplicon size and database accession number used
 252 for European sea bass.

	Primer Sequence	Amplicon (bp)	NCBI seq ID
ATPase alpha3_For	AGAACATGGTGCCTCAGCAA	146	AY532637.1
ATPase alpha3_Rev	GCCATGAGCAGAAACAACCC		
Dlabrax_IFRD1_For	GTGACACCACCAGTGTAGCA	237	NM_001076555.1
Dlabrax_IFRD1_Rev	TGCCTTTCTTGAGGCATCGT		
Dlabrax_GAPDH_for	CTGTCCGTCTGGAGAAACCC	210	AY863148.1
Dlabrax_GAPDH_rev	TGTCGTACCATGTGACCAGC		
Dlabrax_NEDD8_for	TTGAGCCCACAGACAAGGTG	148	XM_003457410.2
Dlabrax_NEDD8_rev	ACTGAGCCTCCCTGGATCTT		

253

254 2.8. Data analyses

255 Analyses of growth were carried out using a two factor ANOVA without replication with Sex
 256 and Coping style (Proactive, Reactive and Intermediate) as fixed factors after checking for normality
 257 and homogeneity of variances. Significant ANOVA outcomes were then followed by post hoc
 258 Newman-Keuls test. Plasma cortisol levels and brain monoamine neurochemistry in telencephalon
 259 were analyzed using a two factor ANOVA without replication with Treatments (Control, Stress30 (for

260 monoamines), and Stress30 and Stress150 (for cortisol)) and Coping style (Proactive, Intermediate,
261 Reactive) as fixed factors. A Pearson correlation was used to assess links between plasmatic cortisol
262 concentration and ratio of [5-HIAA]/[5-HT]. In order to fulfil the assumption of normal distribution,
263 data on plasma [cortisol], brain [NE], [DA], [DOPAC], [5-HT], [5-HIAA] and ratios of [5-HIAA]/[5-HT]
264 and [DOPAC]/[DA] were log-transformed. For data analysis of gene expression, brain mass and gene
265 copy number were checked with an ANOVA test and a post-hoc Scheffé test was performed for
266 specific significances. A blind analysis on individual gene expression data for the whole subpopulation
267 with a K-means cluster was performed. These analyses were performed with SPSS v19 (IBM®) and
268 Statistica. For correlations between coping styles selected by behavioural screenings and coping
269 styles identified by clustering of individual gene expression, analyses were carried out using a two
270 factor ANOVA without replication with Sex and Coping style as fixed factors after checking for
271 normality and homogeneity of variances. In case of non-normality or non-homogeneity of variances,
272 data were log transformed. Significant ANOVA were then analyzed using post hoc Newman-Keuls
273 test. Other statistical analyses were performed with Statistica for windows (Statsoft, USA).

274 **3. Results**

275 **3.1. Growth performances**

276 Coping styles were equally distributed between sexes ($\text{Chi}^2=1.25$, $\text{Df}=6$, $p=0.97$) and females
277 sea bass were bigger than males all along the experiment duration (Table 2).

278 Already at the beginning of the experiment (just after the behavioural screening), fish body
279 mass (BW) were different between coping styles (at 215 dph; Table 2, Figure 2). Post hoc NK test
280 showed that reactive fish had lower BW than intermediate and proactive fish ($p<0.05$, Figure 2). At
281 the second measuring point, differences in body mass were still observed between coping styles (at
282 251 dph, Table 2) and post hoc test showed that reactive and proactive fish were similar and reactive
283 fish had lower BW than intermediate fish ($p<0.05$, Figure 2). At the third measuring point, five weeks
284 later, differences in body mass had developed (at 285dph, Table 2, Figure2) and post hoc test
285 showed the same situation ($p<0.01$, Figure 2). At the fourth measuring point, body mass were still

286 different between coping styles (at 314dph, Table 2, Figure 2) and post hoc test showed that reactive
 287 fish had lower BW than intermediate and proactive fish ($p < 0.05$ or 0.01 , Figure 2). At the final
 288 sampling point, the situation was the same (at 342dph, Table 2, Figure 2), post hoc test showed that
 289 reactive fish had lower BW than intermediate and proactive fish ($p < 0.01$ and $p < 0.05$ respectively,
 290 Figure 2).

291 Concerning specific growth rate, no Sex or Coping styles effects were observed on SGR.

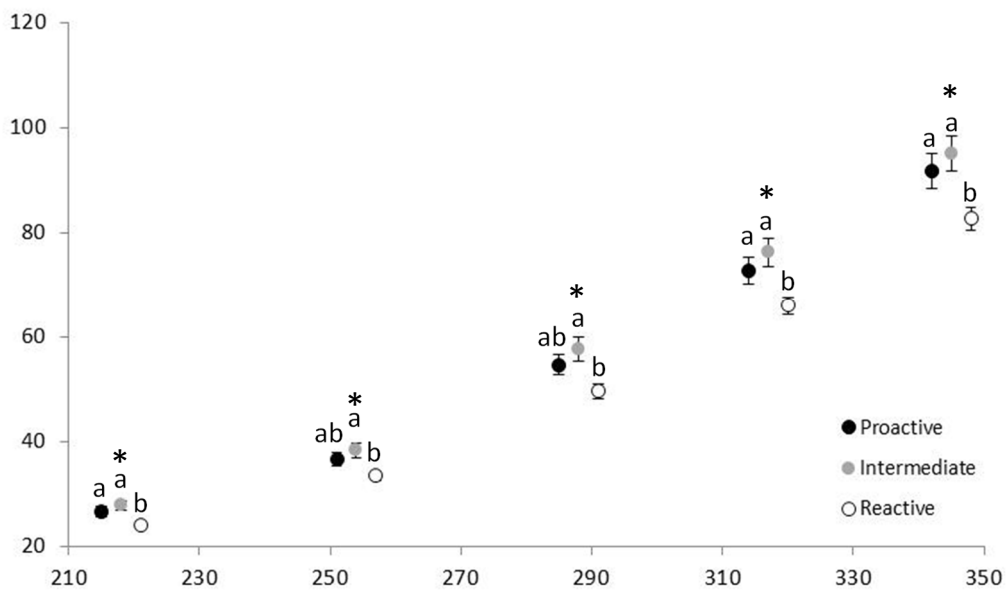
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293 **Table 2.** Results of a two factor ANOVA without replication of Body mass (BW) and Specific Growth
 294 Rate (SGR) in relation to Sex and Coping style. NS: non significant.

Fish age (dph)	Sex		Coping style	
	<i>f</i>	<i>p</i>	<i>f</i>	<i>p</i>
BW_215	48.45	<0.001	3.57	<0.05
BW_251	44.90	<0.001	3.71	<0.05
BW_285	36.30	<0.001	5.42	<0.05
BW_314	39.26	<0.001	5.47	<0.05
BW_342	33.24	<0.001	5.21	<0.05
SGR_215_251		NS		NS
SGR_251_285		NS		NS
SGR_285_314		NS		NS
SGR_314_342		NS		NS

295

296



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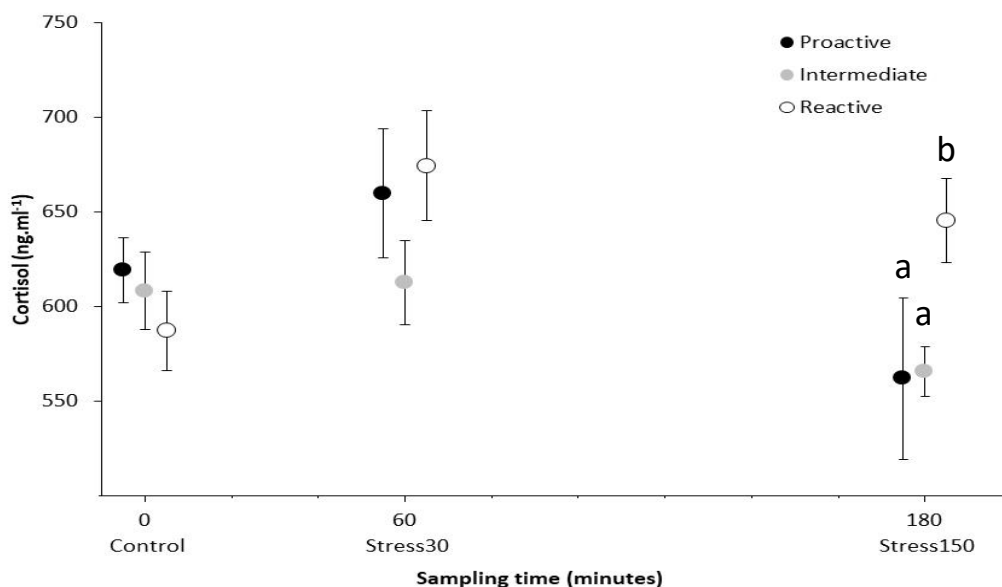
298 **Figure 2:** Fish body mass according to age (dph) and coping style (mean \pm SEM). * indicate significant
299 differences between coping styles and letters indicate Newman-Keuls post hoc test results.

300 3.2. Physiological status

301 3.2.1. Plasma cortisol

302 No significant sex effect was observed on plasma cortisol concentration for Control, Stress30
303 and Stress150 groups tested separately ($F_{(1,113)}=2.59$, $p=0.11$; $F_{(1,55)}=0.16$, $p=0.69$ and $F_{(1,55)}=0.41$,
304 $p=0.52$ respectively). No correlations were observed between cortisol values and fish BW.

305 Cortisol data were then analysed using Treatment (Control, Stress30 and Stress150, Figure 3)
306 and Coping styles (Proactive, Reactive and Intermediate) as fixed factors. No effect of treatment or
307 coping style alone was seen, but a significant interaction between treatment and coping style was
308 present ($F_{(4,229)}=2.55$, $p<0.05$). Post hoc test showed that Stress150-Proactive and Stress150-
309 Intermediate fish had significantly lower plasmatic cortisol concentrations than Stress150-Reactive
310 fish (Figure 3). However, plasma cortisol concentrations were not different for any coping style at
311 baseline and Stress30 sampling points. The lowest values were observed for Stress150-Proactive fish
312 (561.93 ± 42.80 ng ml⁻¹) whereas the highest values were observed for Stress30-Reactive fish
313 (668.16 ± 33.95 ng ml⁻¹).



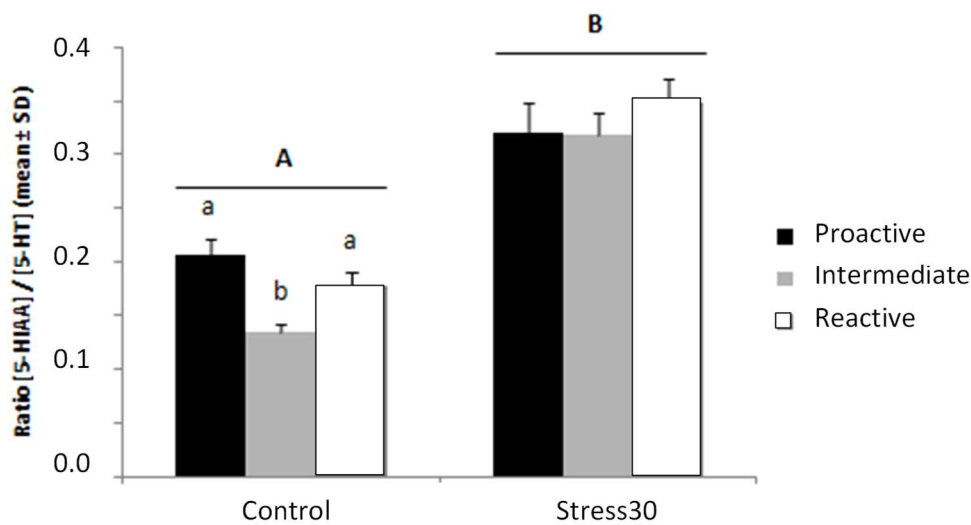
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315 **Figure 3:** Plasma cortisol concentration according to treatment (sampling time post confinement
316 stress) and coping style (mean \pm SEM). Letters indicate significant differences within treatment.

317 **3.2.2. Monoamine neurochemistry**

318 Data on monoamine neurochemistry in the telencephalon are shown in table 3.

319 A significant interaction was observed between coping style and treatment on the ratio [5-HIAA/5HT]
320 ($F_{(2,49)}=4.83$, $p=0.01$, Figure 4), specifically in that intermediate fish showed lower serotonergic
321 turnover than both proactive and reactive categories under control (unstressed) conditions. 5-
322 HIAA/5-HT ratios increased in response to acute stress (sampling time: “Stress30”) in all groups, and
323 at this time point there were no longer any significant effect of coping style on this indicator. The
324 stress-induced increase in 5-HIAA/5-HT ratios could be ascribed to a rise in catabolite (5-HIAA)
325 concentrations in all groups, with a near significant effect of coping style (i.e. reactive fish showed a
326 trend [$p=0.06$] towards generally higher 5-HIAA concentrations, see table 3 for full summary of
327 statistics and effects).



328

329 **Figure 4:** Mean value of the ratio between the concentrations of [5-HIAA/5-HT] in telencephalon
330 according to treatment and coping style (mean ± SEM). Lower case letters indicate significant
331 differences within treatment and upper case letter indicate significant differences between
332 treatments.

333

334 For the DA system, a stress-induced increase in the DA catabolite DOPAC was accompanied by a
335 corresponding increase in concentrations of the parent monoamine, leaving DOPAC/DA ratios
336 unaffected by stress. Norepinephrine [NE] concentrations remained unaffected by stress, and NE

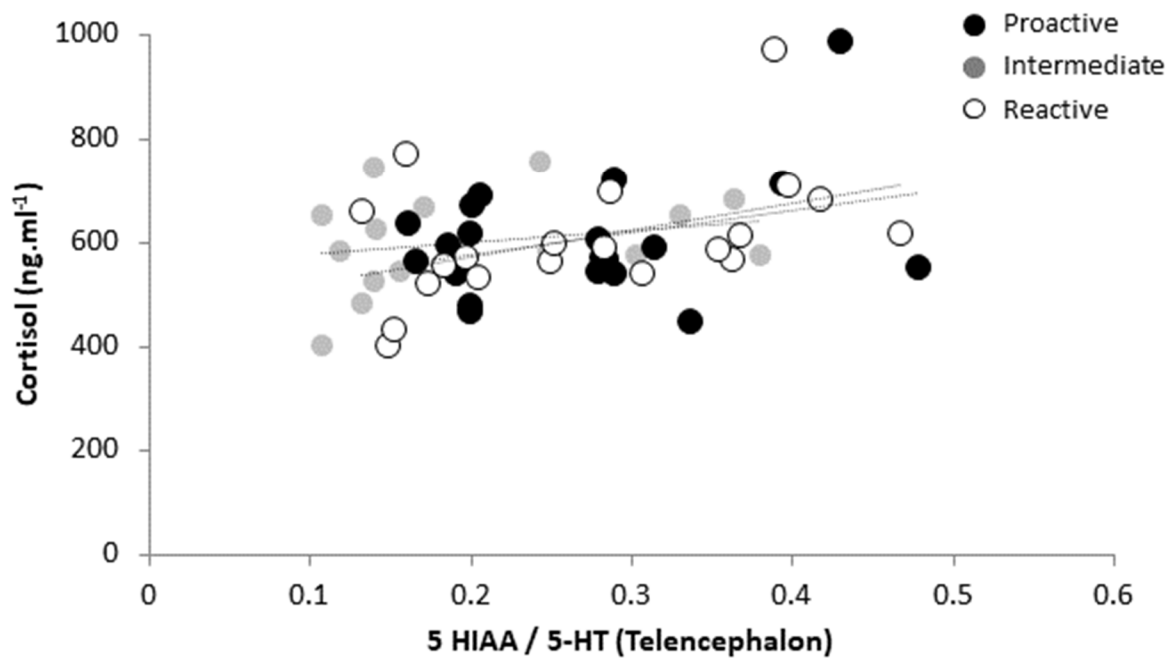
337 metabolites were undetectable in the current analytical set-up. None of the catecholamine indicators
 338 were affected by coping style or an interaction between coping style and stress. (Table 3).

339 **Table 3.** Neurochemistry in the telencephalon according to treatment and coping style (mean \pm SEM).

Treatment	Telencephalon					
	Control			Stress30		
	Intermediate	Proactive	Reactive	Intermediate	Proactive	Reactive
<i>Monoamines</i>						
5-HIAA/5-HT	0.13 \pm 0.01	0.21 \pm 0.02	0.18 \pm 0.01	0.32 \pm 0.02	0.32 \pm 0.03	0.35 \pm 0.02
5-HIAA	41.34 \pm 2.62	56.60 \pm 4.42	58.97 \pm 4.65	94.36 \pm 8.65	91.25 \pm 7.26	107.45 \pm 8.48
5-HT	303.63 \pm 15.77	277.02 \pm 17.03	341.39 \pm 33.60	294.27 \pm 11.73	352.60 \pm 53.85	306.51 \pm 17.29
DOPAC/DA	0.07 \pm 0.01	0.09 \pm 0.01	0.08 \pm 0.01	0.08 \pm 0.01	0.09 \pm 0.01	0.08 \pm 0.01
DOPAC	0.64 \pm 0.09	0.97 \pm 0.10	0.82 \pm 0.09	1.03 \pm 0.12	0.95 \pm 0.05	0.92 \pm 0.06
DA	10.27 \pm 0.40	10.36 \pm 0.50	11.25 \pm 0.75	12.25 \pm 0.87	12.88 \pm 1.44	11.31 \pm 0.50
NE	94.39 \pm 10.28	101.04 \pm 6.21	99.13 \pm 7.81	98.20 \pm 8.93	102.28 \pm 7.71	103.92 \pm 7.67

340

341



342

343 **Figure 5:** Pearson correlation between plasmatic cortisol concentration and ratio of [5-HIAA / 5-HT]
 344 (N=54, $r_p=0.34$, $p<0.01$), different colours indicate different coping style.

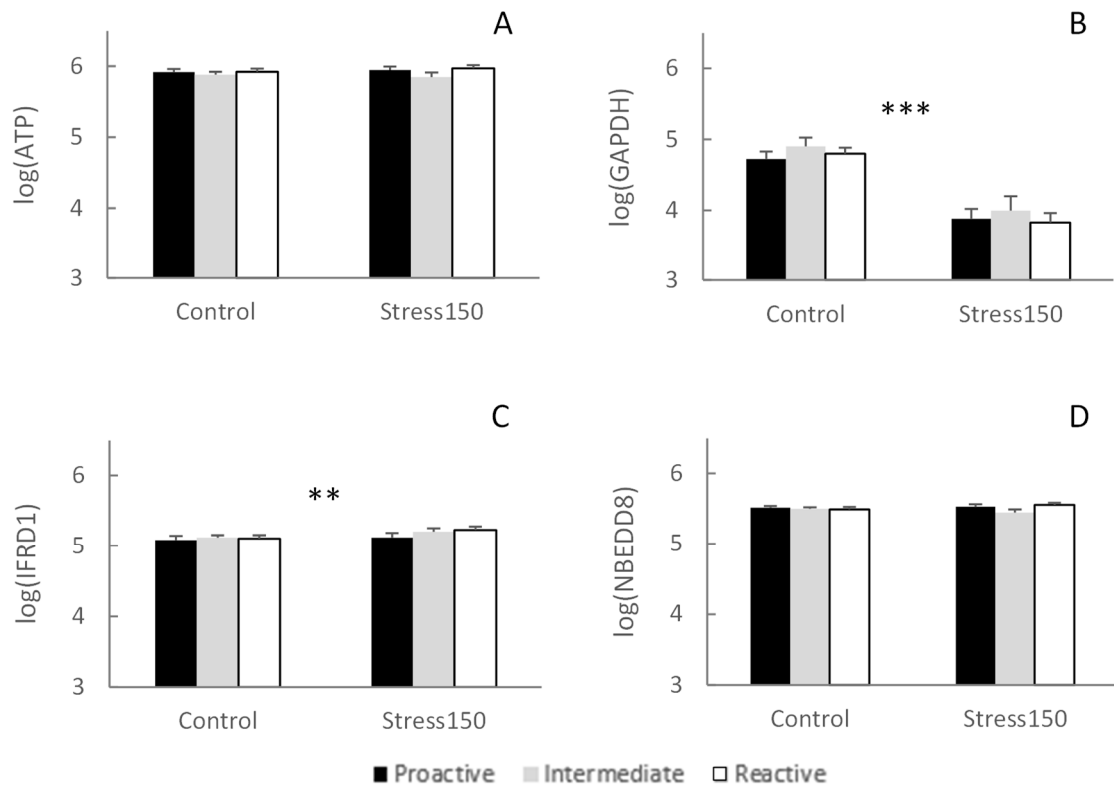
345 A significant positive correlation (N=54, $r_p=0.34$, $p<0.01$) was observed between plasmatic cortisol
 346 concentration and ratio of [5-HIAA / 5-HT], (Figure 5), the correlation did not differ between coping
 347 style.

348

349

350 **3.2.3. Gene expression**

351 Stress150, 150 min post confinement, had a significant effect on target mRNA copy number
352 that was in several cases dependent upon coping style (ANCOVA, $F_{4,96}=30.574$; $p<0.001$). *Gapdh*
353 mRNA copy number decreased after the stress treatment (Post-hoc, $p<0.001$) whereas *ifrd1*
354 transcripts increased (Post-hoc, $p<0.01$). No statistical differences were observed for the other two
355 mRNAs studied however there were observable tendencies for measured mRNA abundances
356 dependent upon coping styles with the intermediate fish being the most different in gene expression
357 (see Figure 6a, b, c and d). No significant interactions between coping style and treatment were
358 observed.



359

360 **Figure 6:** Differential mRNA expression transcripts for fish with different coping style under control
361 conditions and after a confinement stress treatment (Stress150). **A.** Log copy number for *atpasea3*,
362 **B.** Log copy number for *gapdh*, **C.** Log copy number for *ifrd1* and **D.** Log copy number for *nedd8* gene.

363 **4. Discussion**

364 Sea bass are one of the highest commercial value species for European aquaculture, with a
365 current mean European production of about 125,000 metric tons year⁻¹ (Tveteras and Nystoyl, 2011).

366 In addition, sea bass domestication is still in its infancy and studying physiological and behavioural
367 responses of fish from divergent coping style should allow improving domestication process and
368 selecting fish with higher adaptation abilities to rearing conditions. Indeed European sea bass stress
369 coping styles have been characterized and resemble most fish species studied (Ferrari et al 2015,
370 2016; Samaras et al 2016a; Alfonso et al 2019) and when held in groups and fed to satiation, the
371 species does not display aggressive social hierarchy but is rather showing a producers-scroungers
372 social organization instead of a hierarchical one (Di Poï et al 2007, Ferrari et al 2014). Further, the
373 high stress responsiveness of this species (Samaras et al 2016b) makes it a good marine teleost
374 model to study the dynamics of cortisol signalling. Here a medium term experiment (140 days) was
375 performed and specifically targeted neural and transcriptional activity, behaviour and cortisol levels
376 to evaluate stress recovery, a rarely investigated interaction (Wong et al 2019).

377 Differentially expressed traits were observed regarding growth with intermediate fish
378 growing larger and showing a lower baseline serotonergic activity. Confinement stress induced an
379 immediate and higher serotonergic activity whatever the coping style and a slower cortisol recovery
380 rate in reactive fish. Finally mRNA copy numbers for some genes associated to metabolism were also
381 differentially affected.

382 In further details, all along the experiment duration, reactive fish had lower body mass than
383 intermediate fish which were the larger fish but most often not significantly different from proactive
384 ones. This is an interesting result which echoes the findings of Millot (2008) who observed, when
385 comparing selected *versus* wild strain that proactive sea bass from wild population had lower body
386 mass than reactive ones. On the opposite, proactive sea bass issued from selected for growth strain
387 had higher body mass than reactive ones. In addition, Ferrari et al (2016) observed that reactive fish
388 from an unselected sea bass population (close to wild fish) had higher body mass than proactive
389 ones. Overall, this divergent growth potential leads to think that hatchery selection and/or
390 domestication process and/or husbandry practices promote growth of proactive coping style, as

391 already observed in salmonids (Sundström et al, 2004; Huntingford and Adams, 2005, Damsgård et al
392 2019). Further, the high growth observed in intermediate fish has already been observed in emerging
393 rainbow trout fry where early and late emerging individuals grew less than intermediate emerging
394 individuals (Andersson et al 2013). The authors hypothesized that intermediate emerging individuals
395 had a 'stress coping style lying between the proactive and reactive extremes in the pro-reactive
396 continuum', our results corroborate their findings. Further, intermediate fish showed both enhanced
397 growth and reduced resting 5-HT activation under basal conditions. In a previous study, Cubitt et al
398 (2008) showed that slower growing salmon in aquaculture were characterised by enhanced 5-HT
399 neurotransmission, attributing this observation to the presence of a size hierarchy even in relatively
400 large groups of fish. Our contrasting results highlight the fact that interactions between coping style,
401 body size, and social status still little explored outside the salmonids family should be investigated
402 further. The present results, in summary, suggest that intermediate fish with less pronounced coping
403 styles were best adapted to current rearing conditions.

404 No difference in circulating cortisol levels were observed between the different coping styles
405 fish in the Control and Stress30 treatments. This, on one hand, confirms the high susceptibility to
406 stress of this species (Samaras et al 2016b), and demonstrates that a different protocol such as water
407 cortisol (Fanouraki et al 2008) should be favoured to analyse basal cortisol since any manipulation of
408 the fish leads to an immediate increase of plasmatic cortisol concentration. On the other hand, a
409 similar result has recently been observed in rainbow trout where coping style divergent fish had
410 similar cortisol levels both at basal levels and after an acute confinement stress when a confinement
411 stress-challenge test was performed several weeks after the coping style characterization (Gesto
412 2019). As mentioned by Gesto (2019), the known high phenotypical plasticity of fish could also have
413 affected the individual differences during that time. Nonetheless, what pleads for a correct coping
414 style characterization is that after a recovery period (Stress150 treatment, 150 minutes recovery),
415 proactive and intermediate fish had a lower cortisol level than reactive ones. This shows that these
416 individuals were able to recover faster from the stress than reactive ones, highlighting that proactive

417 and reactive sea bass display differential hypothalamus-pituitary-interrenal (HPI) axis reactivity as
418 already observed in rainbow trout (Øverli et al 2005). In other word, proactive and intermediate as
419 opposed to reactive fish have higher capacity to downregulate HPI activity, in line with data from
420 other species such as zebrafish (Tudorache et al, 2013, 2015, Wong et al 2019). Such involved
421 mechanisms, leading to a faster HPI axis down regulation of proactive individuals, may be an
422 evolutionary adaptive process for the proactive individuals to be faster prepared to unpredictable
423 stress since proactive behavioural responses are known to be maladaptive under repeated,
424 uncontrollable or unpredictable stress (Øverli et al 2007). From an operational perspective, the
425 hypoxia tolerance test is thus relevant to assess coping style in sea bass and predict cortisol response
426 after an acute stress (herein a 30 min confinement stress). In our study, fish were screened for
427 coping style 127 days before stress protocol was applied and blood sampling performed, and
428 physiological differences between coping styles were still observed at least for plasmatic cortisol.
429 This underlines that the cortisol response is relatively stable over time (Samaras et al, 2016b),
430 contrary to some personality traits which could be shaped by environmental factors, age or
431 experience (Ferrari et al, 2015, 2016; Alfonso et al 2019).

432 In the present experiment, confinement stress lead to an increase in telencephalic 5-HIAA
433 concentrations and 5-HIAA/5-HT ratios, indicating a general activation of 5-HT neurotransmission in
434 this brain part similar for all behavioural coping styles. Elevated brain serotonergic activity is a
435 general indicator of aversive experiences in all vertebrates and has in fishes been shown to occur
436 after for instance social stress (Øverli et al 1999), predator exposure (Winberg et al 1993b),
437 confinement stress (Øverli et al 2001), salmon louse infestation (Øverli et al 2014) and suboptimal
438 rearing conditions (Laursen et al 2013). Therefore, in all likelihood the telencephalon response
439 reflects a general increase in 5-HT activity throughout the brain at Stress30 following the onset of
440 stress. Here, we however focus on the telencephalon which also in fish contains limbic systems
441 assumed to mediate hippocampal and amygdala like functions (Portavella et al 1998, 2002; Demski
442 2013; Maximino et al 2013; Silva et al 2015). Altered brain 5-HT dynamics in these areas may

443 influence animal welfare through its role in mood control and emotion (Cools et al. 2008; Dayan and
444 Huys 2009; Carhart-Harris and Nutt 2017), neurogenesis, and neural plasticity (Gould 1999; Mahar et
445 al 2014, Castrén et al 2017). In particular, the ability to respond to further acute stressors is an
446 essential indicator of compromised animal welfare (Vindas et al 2016). Therefore, measurements of
447 immediate responsiveness of the 5-HT system are indicative to reveal any effect of contrasting
448 coping ability on animal welfare. These differences did however not translate into an altered ability
449 to respond to further acute stress, i.e. indicative of allostatic overload (Vindas et al 2016). All groups
450 showed significant cortisol, serotonergic and dopaminergic responses 30 min post-stress. No
451 significant effect of coping style was observed after stress, although there was a trend towards
452 reactive fish showing a slightly enhanced response in terms of elevated 5-HIAA concentrations. This
453 differs from the responses observed in shy sea bass after an open field test (Alfonso et al. 2019) and
454 might reveals some context specificities. Relevance of the sampling protocol is illustrated by
455 significant correlation in all groups between telencephalon 5-HIAA/5-HT and cortisol, suggesting co-
456 ordinated activation of these neuroendocrine systems under stress.

457 The dopaminergic system was not differentially activated across coping style as also observed
458 in European sea bass in another context by Alfonso et al (2019). Interestingly, regarding the DA
459 system, it should be noted that a stress induced increase in DOPAC did not result in elevated
460 DOPAC/DA ratios, due to a compensatory simultaneous increase in concentrations of the parent
461 monoamine DA. This again suggests robust coping ability at least under acute stress in our tested
462 fish, and also illustrates the importance of observing both relative and absolute amounts of analytes
463 in neurochemical studies.

464 The genes selected for this study were functionally related to different physiological
465 processes: *atpase α3* is related to osmotic regulation as major mediator of cellular transmembrane
466 ionic gradients. It also plays an important role in signal transduction in the nervous system. It has
467 been found necessary for brain ventricle formation and development in early brain morphogenesis

468 (brain lumen inflation). *Ifrd1* is related to development and alternate splicing results in multiple
469 transcript variants. *Gapdh* is related to general metabolic processes. *Need8* is a neural precursor for
470 Ubiquitin-like protein, which plays an important role in cell cycle control and embryogenesis.

471 The stress treatment had a significant effect on target mRNA copy number with different
472 reaction norms for each mRNA and that were in several cases dependent on coping style. This was
473 mainly due to the differential gene expression of *gapdh* and *ifrd1*; *gapdh* mRNA copy number
474 decreased for all three coping styles in the same way and similar magnitude after the stress
475 treatment showing an effect of stress over the expression of this metabolic gene whereas *ifrd1*
476 transcripts increased. *Ifrd1* is an immediate early gene that encodes a protein related to interferon-
477 gamma. This protein may function as a transcriptional co-activator/repressor that controls the
478 growth and differentiation of specific cell types during embryonic development and tissue
479 regeneration. Mutations in this gene are associated with sensory/motor neuropathy with ataxia. The
480 general increase of *Ifrd1* transcripts for all coping styles could be related to their role in the immune
481 system (Langevin et al 2013) with an immediate response. *Ifrd1* has also been identified in Cyprinids
482 as being able to discern between proactive and reactive stress coping styles (Mackenzie et al 2009)
483 under stress situations. In sea bass there were differences between coping styles but not significantly
484 different in any case. The tendency was for the proactive fish to have higher numbers of mRNA
485 transcripts than the reactive both under control and stress conditions. For the other genes studied,
486 both *atpaseα3* and *nedd8* are quite ubiquitous genes and may play specific roles in cell control but
487 maybe in this case they had not enough time (sampling after 150 min post stress) to be fully
488 differentially expressed or they just are not so affected by this specific confinement stress applied.
489 However, for *atpaseα3* a tendency was observed to increase the number of transcripts after the
490 stress situation for Proactive and Reactive fish, and to decrease for intermediate fish. The same
491 response was observed between intermediate and reactive fish for *nedd8*.

492

493

494 **Conclusions**

495 In the present study as well as in previous ones, we have shown that different coping styles are
496 associated with different growth potential closely linked with the domestication level, husbandry
497 conditions and likely the social context; here intermediate and proactive coping styles appeared
498 favoured. In previous work it was shown that European sea bass extreme coping style (reactive vs.
499 proactive) displayed associated differences in the HPI axis, the serotonergic and noradrenergic
500 system reactivity, and in neurogenesis at one time point immediately post challenge (Alfonso et al
501 2019). The present work highlight the importance of investigating not only the immediate
502 neuroendocrine components responses of coping styles, especially in such a highly stress responsive
503 species, but the post stress recovery phase which is even more discriminant as for the stress axis
504 reactivity and the metabolic transcriptional activity. This implies allowing sufficient time to test the
505 ability to both respond and down regulate and hence tackle the allostatic reaction norm of the
506 species in any particular and well defined context.

507

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517

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