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1 Ocean acidification alters the acute stress response of a marine fish

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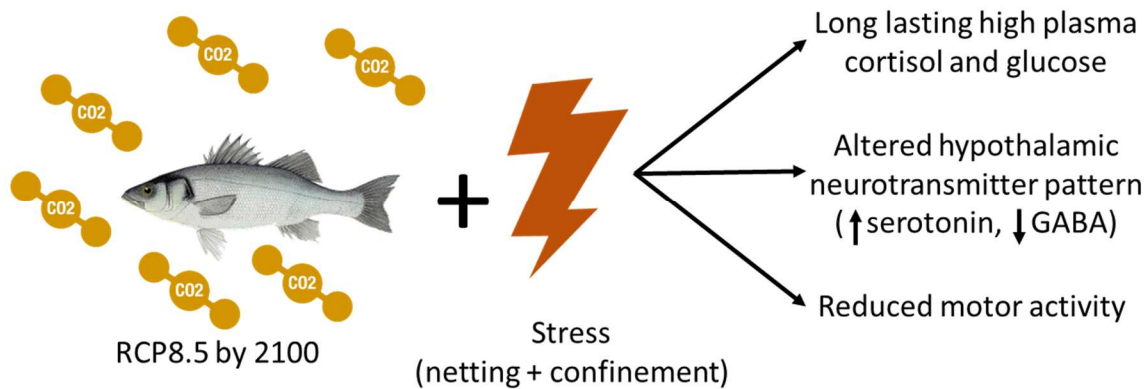
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12

13 Graphical abstract:



14

15

16 Highlights

- 17
- Ocean acidification (OA) impacts the physiological stress response of European sea

18 bass

 - Post-stress return to basal plasma cortisol and glucose levels is delayed under OA
- 19

- 20 • This delay is associated to alteration of hypothalamic neurotransmitters pattern
- 21 • Motor activity is reduced during recovery from stress in fish under OA conditions

22

23 **Keyword:** climate change, allostatic load, phenotypic plasticity, European sea bass,
24 corticotropic axis, neuroendocrine control and behavior

25

26 **Abstract**

27 The absorption of anthropogenic carbon dioxide from the atmosphere by oceans generates
28 rapid changes in seawater carbonate system and pH, a process termed ocean acidification.
29 Exposure to acidified water can impact the allostatic load of marine organism as the
30 acclimation to suboptimal environments requires physiological adaptive responses that are
31 energetically costly. As a consequence, fish facing ocean acidification may experience
32 alterations of their stress response and a compromised ability to cope with additional stress,
33 which may impact individuals' life traits and ultimately their fitness. In this context, we
34 carried out an integrative study investigating the impact of ocean acidification on the
35 physiological and behavioral stress responses to an acute stress in juvenile European sea bass.
36 Fish were long term (11 months) exposed to present day pH/CO₂ condition or acidified water
37 as predicted by IPCC "business as usual" (RCP8.5) scenario for 2100 and subjected to netting
38 stress (fish transfer and confinement test). Fish acclimated to acidified condition showed
39 slower post stress return to plasma basal concentrations of cortisol and glucose. We found no
40 clear indication of regulation in the central and interrenal tissues of the expression levels of
41 gluco- and mineralocorticoid receptors and corticoid releasing factor. At 120 minutes post
42 stress, sea bass acclimated to acidified water had divergent neurotransmitters concentrations
43 pattern in the hypothalamus (higher serotonin levels and lower GABA and dopamine levels)

44 and a reduction in motor activity. Our experimental data indicate that ocean acidification
45 alters the physiological response to acute stress in European sea bass via the neuroendocrine
46 regulation of the corticotropic axis, a response associated to an alteration of the motor
47 behavioral profile. Overall, this study suggests that behavioral and physiological adaptive
48 response to climate changes related constraints may impact fish resilience to further stressful
49 events.

50

51 **1. Introduction**

52 Ocean acidification is a direct consequence of the ongoing anthropogenic climate change and
53 the capacity of the ocean to uptake carbon from the atmosphere. This process leads to an
54 increase in seawater concentration of CO_2 , H^+ and HCO_3^- and a decrease of CO_3^- and pH.
55 Earth system models (CMIP5) corresponding to the high emission scenario (Representative
56 Concentration Pathway, RCP, 8.5 scenario) predicted a 0.38 decrease of pH units of global
57 surface ocean by the end of the century (Bopp, Resplandy et al. 2013). More recently, a new
58 generation of earth system models (CMIP6) reported an even sharper decline in surface ocean
59 pH corresponding to -0,44 pH units by the end of the century (Shared Socioeconomic
60 Pathway 5-8.5)(Kwiatkowski, Torres et al. 2020), raising questions regarding the
61 consequences of such acidification for marine life.

62 These rapid changes in seawater carbonate system and pH can impact directly or indirectly
63 the physiology and behavior of marine organisms. First predictions considered most marine
64 fish as quite resilient to the pH/ $p\text{CO}_2$ values expected for 2100 since they are effective acid-
65 base regulators (Regan, Turko et al. 2016). However, several effects associated to seawater
66 acidification have been observed in some fish species notably on hatching rates and early
67 development (Munday, Donelson et al. 2009, Frommel, Margulies et al. 2016, Pimentel,

68 Faleiro et al. 2016, Stiasny, Sswat et al. 2019, Villalobos, Love et al. 2020, Baumann, Jones et
69 al. 2022), sensory performances (Munday, Dixson et al. 2009, Munday, Dixson et al. 2010,
70 Lai, Jutfelt et al. 2015, Porteus, Hubbard et al. 2018, Williams, Dittman et al. 2019) and
71 reproduction (reviewed by Servili et al., 2020). The effects of ocean acidification on fish
72 behavior remain controversial (Cattano, Fine et al. 2019, Clark, Raby et al. 2020, Munday,
73 Dixson et al. 2020): some studies reported very strong alterations of fish behavior in response
74 to ocean acidification (shelter use, Cattano et al. 2019) while others have stressed the lack of
75 reproducibility of many previous studies on individuals avoidance of predator chemical cues,
76 fish activity, and lateralization (Sundin, Amcoff et al. 2019, Clark, Raby et al. 2020).
77 Irrespective of the heated debate concerning the effect of ocean acidification on fish behavior,
78 a prolonged exposure to acidified water can impact the allostatic load of an organism since
79 maintaining biological parameters within the physiological range in a suboptimal environment
80 is energetically costly (Korte, Koolhaas et al. 2005). As a consequence, brain and other organs
81 involved in the stress-coping response can alter their functioning and compromise individuals'
82 ability to cope with additional stress and hence affect their other life history traits (survival,
83 growth and reproductive success) and fitness (McEwen 2000, McEwen 2007).

84 The physiologic stress response in teleost fish is driven by the activation of two hormonal
85 axes: the brain-sympathetic-chromaffin cells (BSC) axis and the hypothalamic-pituitary-
86 interrenal (HPI) axis. The BSC axis initiates the stress response through the rapid rise in
87 plasma catecholamines (mostly epinephrine and norepinephrine) released by chromaffin cells,
88 leading to increases in ventilation, branchial blood flow, gas exchange and plasma glucose
89 concentration (Wendelaar Bonga 1997). The glucose is oxidized and used as fuel to respond
90 to the increased energy demand associated to the stress factors. Several neurotransmitters,
91 notably monoaminergic neurotransmitters (serotonin, dopamine and norepinephrine) play a
92 central role in the modulation of the stress response in vertebrates (Winberg and Nilsson

93 1993). The HPI axis is responsible for the increase in levels of plasma glucocorticoids, mainly
94 cortisol, and plays an important role in the reallocation and mobilization of energy under
95 stressing condition (Balasch and Tort 2019). When the HPI axis is activated, the
96 corticotropin-releasing hormone (CRH or CRF) is released from the hypothalamus and acts
97 on the pituitary by increasing the adrenocorticotrophic hormone secretion (ACTH), which in
98 turns stimulates the release of cortisol from the interrenal cells (Wendelaar Bonga 1997).
99 Cortisol exerts its action on multiple tissues (e.g. liver, brain, gonads, gills and immune cells)
100 by binding to specific isoforms of glucocorticoid receptors (notably GR1 and GR2) and one
101 mineralocorticoid receptor (MR). After binding, the formed heterocomplex translocates to the
102 nucleus, binds to the gluco- or melano-corticoid response element of the promotor gene and
103 modulates the transcription of the target gene (Prunet, Sturm et al. 2006). In addition, a rapid
104 non-genomic signaling mediated by membrane receptors is likely to play a role in the cortisol
105 driven acute stress adaptation in teleosts (Das, Thraya et al. 2018, Aedo, Ruiz-Jarabo et al.
106 2019, Aedo, Aravena-Canales et al. 2021). Therefore, to better understand the impact of
107 ocean acidification on fish life history traits, it is critical to evaluate its effects on the
108 physiological stress response of fish.

109 A number of past studies have investigated specific traits related to the stress response of fish
110 under acidification conditions. It has been reported an increase of anxiety under elevated CO₂
111 in larvae of barramundi (Rossi, Nagelkerken et al. 2015) and juveniles of yellowtail kingfish
112 (Jarrold, Welch et al. 2020), Californian rockfish (Hamilton, Holcombe et al. 2014) and three-
113 spined sticklebacks (Jutfelt, de Souza et al. 2013) using light/dark test, shelter test or novel
114 object test. By contrast, ocean acidification did not impact the anxiety of juvenile Californian
115 blacksmiths (Kwan, Hamilton et al. 2017). This variability of effects was also observed when
116 the impact of elevated CO₂ on fish activity was tested. For instance, juvenile sea bass showed
117 lower baseline activity and more prolonged freezing behavior under low pH (Porteus,

118 Hubbard et al. 2018), but earlier stages of the same species (during metamorphosis) did not
119 exhibited any effect of acidification on fish activity (Duteil, Pope et al. 2016). Non-significant
120 trend suggesting a decreasing activity score under elevated CO₂ was observed in speckled
121 sanddabs (Andrade, Hurst et al. 2018), whereas increased boldness and swimming activity
122 was recorded in larval clownfish (Munday, Dixson et al. 2010). Such contrasting results on
123 particular stress related traits (anxiety and activity) likely reflect a species-specific response to
124 acidified water and/or methodological differences. It is worthy to note that most of the
125 reported studies focused on short term effects of ocean acidification (ranging from 10 days to
126 1.5 months) on stress response-related traits. Nowadays, the effect of a prolonged exposure to
127 acidified water on fish stress response, which would be more relevant to predict what is like to
128 happen in the wild, is unknown. In this context, we report an integrative approach to
129 investigate whether the prolonged ocean acidification condition impacts the ability of a
130 marine fish to cope with an additional acute stress. We test the hypothesis that neuroendocrine
131 and behavioral responses of fish to netting and to a confinement test would be altered in fish
132 constantly living in acidified seawater by showing i) higher plasma cortisol and glucose
133 concentrations during recovery, ii) variations in expression profiles of key players of the HPI
134 axis, iii) alterations in neurotransmitter hypothalamic levels and iv) modified behavioral traits
135 associated to motor activity. The model species chosen for this study is the European sea bass,
136 *Dicentrarchus labrax*, an economically important marine teleost, whose physiological and
137 behavioral response to acidified condition (Pope, Ellis et al. 2014, Duteil, Pope et al. 2016,
138 Crespel, Zambonino-Infante et al. 2017, Poulton, Porteus et al. 2017, Cominassi, Moyano et
139 al. 2019, Alves, Gregorio et al. 2020, Mazurais, Servili et al. 2020a, Servili, Canario et al.
140 2020) and to an acute stress separately is known (Samaras, Dimitroglou et al. 2016, Alfonso,
141 Sadoul et al. 2019, Ferrari, Rey et al. 2020).

142

143 **2. Materials and methods**

144 All fish experiments fall under the EU Directive 2010/63/EU and French national regulations.
145 Experiments were performed in the accredited animal facilities of Ifremer-Centre de Bretagne
146 (agreement number B29-212-05); the experimental design was subjected to prior
147 authorization by the regional ethic committee to which the facility belongs (CEFEA: Comité
148 d'Éthique Finistérien en Expérimentation Animale, registering code C2EA-74) and by the
149 French Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation
150 (Authorization APAFIS 12718, permit number 2017121516545362 v3); animals were
151 handled by qualified and accredited personnel.

152 **2.1 Animals and experimental design**

153 This experiment is based on sixty-four juvenile European sea bass (11 months post hatching;
154 mean body mass = 144.2 g, SEM = 5.5 g), obtained as larvae from the aquaculture facility
155 'Aquastream' (Ploemeur, Lorient, France) and transferred to Ifremer's experimental facilities
156 at 2 days post-hatching (dph) at the density of 5000 larvae per tank. Larvae were gradually
157 acclimated (- 0.1 pH units per day) to two conditions of water pH/pCO₂ targeting current
158 pH/pCO₂ conditions of Bay of Brest referred to "Current condition" group (Duteil, Pope et al.
159 2016) and pH/pCO₂ conditions as predicted by IPCC's RCP 8.5 for the end of the century
160 referred to 'RCP8.5 condition' group, consisting in a decrease in surface ocean pH by 0.4 unit
161 by 2100 (Meinshausen, Raper et al. 2011, Pörtner, Karl et al. 2014). During their larval stage,
162 fish were fed with artemia ad libitum until 28 dph and progressively acclimated to
163 commercial dry pellets (Neo-Start, Le Gouessant Aquaculture, France) until 45 dph.
164 Afterwards, juvenile from the same treatment were pooled and then randomly transferred to 2
165 juvenile rearing tanks per treatment. All juvenile fish were fed the commercial feed 'Neo
166 Grower Extra Marin' (Le Gouessant Aquaculture, France). Table 1 shows the obtained mean
167 and SEM values of water parameters for each treatment during fish rearing and the

168 confinement test. The respective pH/pCO₂ conditions of each experimental group were
 169 constantly maintained for 11 months. At 11 months, approximately 100 fish per treatment
 170 remained from a series of samplings. The 64 fish used in the present experiment were
 171 randomly picked among them. They were transferred to tanks equipped with cameras and
 172 appropriated set up for the confinement test. Fish were sacrificed for sampling at different
 173 sampling time after the confinement stress. Mortality was constantly checked and no
 174 difference was observed between treatments. The same fish batch has been used in a previous
 175 study (Cominassi, Moyano et al. 2019).

Treatment	pH free scale	Temp (°C)	Salinity (psu)	O2 (% airsat)	TA	pCO ₂ (µatm)
Current condition group						
mean	7.97	15.6	33.8	94.9	2400	632
SEM	0.01	0.0	0.1	0.4	21	14
RCP8.5 condition group						
mean	7.58	15.6	33.8	94.6	2410	1684
SEM	0.01	0.0	0.1	0.5	23	35

176 Table 1: Mean and SEM values of water parameter of fish rearing. Values of pH are expressed in free
 177 proton concentration scale (Waters and Millero 2013). Oxygen saturation (WTW Oxi 340, Xylem
 178 Analytics Germany, Weilheim, Germany) salinity (WTW LF325, Xylem Analytics Germany,
 179 Weilheim, Germany) and total alkalinity were measured once a week in all replicate tanks starting
 180 from juvenile stage (n = 36 values per tank in total).

181 The water of the rearing tanks consisted in natural seawater directly pumped from a depth of
 182 20 m approximately at 500 m from the coastline in the Bay of Brest. Seawater was filtered
 183 (sand filter), heated (tungsten, Plate Heat Exchanger, Vicarb, Sweden), degassed, refiltered
 184 again (2µm membrane) and sterilized (UV lamp, PZ50, 75W, Ocene, France). Replicate

185 treatment tanks consisted of 35 l flow-through tanks (n = 3) with 0.18 l min⁻¹ of flow rate for
186 larvae and of 670 l flow-through tanks (n = 2) with 8.4 l min⁻¹ of flow rate for juvenile rearing
187 per scenario.)During larval rearing, the water supply for the acidified scenario tanks came
188 from a central header tank, where the water pCO₂ was regulated. The water pH was controlled
189 by an IKS Aquastar system (iks Computer Systeme GmbH, Karlsbad Germany), which
190 continuously measured pH in one of the replicate tanks and, when pH in this rearing tank
191 became too high, it opened a magnetic valve to bubble CO₂ into the header tank. During
192 juvenile rearing with higher water exchange rates, additional PVC columns were installed to
193 control the pH in the rearing tanks of acidified scenario. The water arrived at the top of the
194 column and was pumped from the bottom of the column to the rearing tanks. The CO₂
195 bubbling was installed at the bottom of the column and was adjusted by a flow control unit,
196 when needed. The water pH of the Current group was not regulated and corresponded to the
197 pH of the natural seawater that was directly pumped from the Bay of Brest as described
198 above. Water pH and temperature were daily monitored in all replicate tanks before feeding
199 the fish by a WTW 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with
200 electrode: WTW Sentix 41, NBS scale). The pH meters were daily calibrated by using NBS
201 certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim,
202 Germany). For the current study, the rearing temperature was set at 15°C. The photoperiod
203 regime was also set to simulate the natural photoperiod of the Bay of Brest (Halogen lamp at
204 42W, 55-60 lux). The total alkalinity was measured once a week following the protocol
205 described in Cominassi and collaborators (Cominassi, Moyano et al. 2019) adapted from the
206 protocol of Anderson and Robinson (1946) and Strickland and Parsons (1972). Briefly, 50 ml
207 of filtered seawater from tanks was mixed with 15 ml HCl (0.01 M) and pH (NSB scale) was
208 immediately measured. Total alkalinity was calculated with the formula:

$$TA = \frac{V_{HCl} * C_{HCl}}{V_{sample}} - \frac{(V_{HCl} + V_{sample})}{V_{sample}} * \frac{\{H^+\}}{\gamma_{H^+}}, \left[\frac{mol}{l} \right]$$

210 With: total alkalinity (TA), volume of HCl (V_{HCl}, l) and of the sample (V_{sample}, l), HCl
 211 concentration (C, mol l⁻¹), hydrogen activity (H⁺, 10^{-pH}) and hydrogen activity coefficient
 212 (γ_{H⁺} = 0.758). Seawater pH in free scale and pCO₂ was calculated using the Microsoft Excel
 213 macro CO₂sys (Lewis 1998) with the constants after Mehrbach et al. (Mehrbach, Culberson et
 214 al. 1973) refit by Dickson et al. (Dickson and Millero 1987) (as cited in CO₂sys). First pCO₂
 215 and fCO₂ values (μatm) were calculated from measured pH values in NSB scale, total
 216 alkalinity (μmol/kgSW), temperature (°C), atmospheric pressure (dbar) and salinity (‰).
 217 Afterwards, free scale pH values were calculated with the same macro using pCO₂ and fCO₂
 218 values.

219 **2.2 Netting stress**

220 At day 0 (the starting day of each netting stress), 16 fish of one experimental condition
 221 (Current or RCP8.5 condition groups) were transferred from the rearing tanks to four
 222 experimental tanks (1m³ water volume, 4 fish per tank). Netting was realized with large fish
 223 nets enabling to quickly (less than 1 minute) capture simultaneously 4 fish. Fish were
 224 immediately transferred in one of the experimental tanks equipped with a wire-net cage at the
 225 bottom. Water conditions and fish density (7kg/m³) were the same as the corresponding
 226 rearing tanks. The pH conditions of the experimental tanks was stable during the duration of
 227 the experiment. Immediately after the netting and transfer in the experimental tanks, wire-net
 228 cages of three tanks were partially lifted for 4 minutes to confine fish to a reduced volume of
 229 water and increase fish density (confinement test). The acute stress generated from the
 230 transfer of fish to experimental tanks and the confinement test is termed “netting stress”
 231 hereafter. The fish were then euthanized by ethylene glycol monophenyl ether (300 ppm by
 232 balneation; Merck; 807291; USA) at three different times: 30, 120 and 240 minutes after the

233 beginning of the confinement test (one tank per time, n = 4 fish per time), and sampled
234 (sampling points 't30', 't120', and 't240', respectively). The fish of the fourth experimental
235 tank were not submitted to the confinement test. They were let recovered from the transfer
236 and netting for 24 hours (1440 minutes), and then sampled (sampling point termed 't1440'
237 and considered at resting to obtain basal values, as shown by Fanouraki and collaborators in
238 European sea bass after a comparable acute stress; Fanouraki, Mylonas et al., 2011). At each
239 sampling point, blood was immediately sampled from the caudal vessel using heparinized
240 syringes and plasma collected (within 5 minutes) and stored at -20°C until cortisol and
241 glucose analyses. All euthanasia and blood samplings were realized within 5 minutes. Fish
242 were weighted and brain and head kidney removed. Hypothalamus (diencephalon) was
243 dissected from the brains. Experimented personnel, trained to identify and dissect the
244 hypothalamus and head kidney using sterile tweezers and scissors, performed all dissections
245 within 3 minutes. All tissue samples were stored in RNAlater (Qiagen, Hilden, Germany) and
246 placed at 4°C for 24 hours and then to -20°C until gene expression analyses, except for the
247 hypothalami from 't120' which were frozen in liquid nitrogen and stored at -80°C until
248 neurotransmitters analysis. This procedure was repeated twice for each treatment to obtain a
249 total of 32 fish per experimental group (n = 8 per sampling point).

250 **2.3 Plasma cortisol analysis**

251 At all sampling points (t30, t120, t240, t1440), plasma cortisol concentration was determined
252 by ELISA (ELISA kit #500360, Cayman Chemicals, Michigan, USA). This assay was
253 previously used and validated for teleost fish plasma samples (Gamperl, Vijayan et al. 1994),
254 showing intra- and inter-assay variations of 2.9% and 7.6%, respectively.

255 **2.4 Plasma glucose analysis**

256 Commercial kits were used for determining plasma glucose concentration (Glucose GOD-
257 PAC kit #87409, Maizy, France) starting from t120 onward, since glucose is involved in
258 secondary (later) stress response. This assay has intra- and inter-assay variations of 1.3% and
259 1.2% (Stoot, Cairns et al. 2014).

260 **2.5 Gene expression analysis**

261 In order to analyze the expression profile involved in the primary stress response, the
262 hypothalami collected at t30 and t1440 (hypothalami from t120 were processed for
263 neurotransmitters quantification) and head kidneys from t30, t120 and t1440 were processed
264 for total RNA extraction as described elsewhere (Mazurais, Servili et al. 2020b). The RNA
265 integrity number (RIN) of the extracted RNA were higher than 8.5 certifying the high quality
266 of the extraction.

267 **2.6 Reverse transcription and qPCR analysis**

268 The positive and negative (without retro-transcriptase enzyme) reverse transcription for
269 cDNA synthesis was carried out for all samples using iScript™ cDNA Synthesis kit (Bio-Rad
270 Laboratories Inc., Hercules, CA, USA) following the protocol previously described in
271 Mazurais, Servili et al. (2020b). We focused on the following genes: glucocorticoid receptor 1
272 (*gr1*), glucocorticoid receptor 2 (*gr2*), mineralocorticoid receptor (*mr*), corticotropin releasing
273 factor (*crf*, only for hypothalamus). The relative expression of these genes of interest and of
274 one housekeeping gene (the elongation factor 1-alpha, *ef1α*) was determined by qPCR by
275 using the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories Inc.) and a
276 protocol previously described (Mazurais, Servili et al. 2020b). The relative quantities of
277 transcripts were normalized with the $\Delta\Delta C_t$ method using *ef1α* as housekeeping gene since no
278 significant differences in Ct values were observed for *ef1α* between conditions (linear mixed
279 model using duplicate as random factor and Anova function, head kidney: $\text{Chisq}(\text{scenario}) =$

280 3.37, $df(\text{scenario}) = 1$, $p(\text{scenario}) = 0.07$, $\text{Chisq}(\text{time}) = 1.14$, $df(\text{time}) = 2$, $p(\text{time}) = 0.56$;
 281 hypothalamus: $\text{Chisq}(\text{scenario}) = 1.12$, $df(\text{scenario}) = 1$, $p(\text{scenario}) = 0.29$, $\text{Chisq}(\text{time}) = 3.81$,
 282 $df(\text{time}) = 1$, $p(\text{time}) = 0.05$). The primer pairs used, described in Table 2, were designed using
 283 Primer 3 plus tool (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and
 284 checked by 2-fold serial dilution of pools of cDNA.

285

Gene	5'/3' Forward primer	5'/3' Reverse primer	NCBI GenBank
<i>gr1</i>	ATGGATCAGGGTGGACTGAA	CATATCACACGGACCAGCAC	AY549305.1
<i>gr2</i>	AGTCATCTGCAGGCCAGAGT	GGAACACACCAGGCAGATTT	AY619996.1
<i>mr</i>	AGTACCAGCCCTGGGAAGAT	CACGTAGGAGGACTGGTGGT	JF824641.1
<i>crf</i>	ACGAATGTCGGGCTATTGAG	CTTATGAGCGCCCTGATGTT	JF274994.1
<i>ef1α</i>	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT	AJ866727.1

286 Table 2: Primer pairs used for the determination of relative gene expression in hypothalamus and head
 287 kidneys.

288 2.7 Neurotransmitters analysis

289 The hypothalami collected at t120, sampling time showing differential plasma cortisol
 290 concentration between scenarios, were processed for neurotransmitters quantification to
 291 assess potential central disruptions in acidified conditions. The hypothalami were crushed in
 292 2% (v/v) formic acid aqueous solution, centrifuged and injected in an ultra-high-pressure
 293 liquid chromatography coupled with tandem-mass spectrometry (UHPLC-MS/MS) developed
 294 and validated for the quantifications of serotonin (5HT) noradrenaline (NOR), dopamine
 295 (DA), glutamate (GLU) and γ -aminobutyric acid (GABA) concentrations (expressed in pmol
 296 mg^{-1}). The quantification of monoamines was obtained in MRM mode versus a 1/X2
 297 weighted calibration curves using 5-hydroxy-N-methyl tryptamine oxalate (5-HMT) as

298 internal standard. The quantification of neurotransmitters in two hypothalami (one of each
299 scenario) was not successful due to storage problem.

300 **2.8 Behavioral tests**

301 Just before the sampling at t120, ten minutes mp4 videos were recorded in all tanks still
302 containing fish, i.e. tanks used for final sampling at 120 and 240 minutes post-stress (32 fish
303 in total), with mini-dome cameras (HD 960P, 1.3 Mega pixels with backlight function, Sony)
304 connected to a digital recorder (Trybride, AHD 8 channels, H264). The cameras were placed
305 1 meter above the tanks in a central position (one camera per tank), enabling to record the
306 total surface of the tanks. All the cameras were connected to a remote control placed behind
307 black curtains. The remote control was used by the personnel to start and end the recording
308 without been perceived by fish. The videos, recorded at t120, were full-blind analyzed to
309 determine whether there were any difference between experimental groups (pH/pCO₂
310 treatments). A grid pattern, composed of 12 squares of 4 cm², was used to analyze five
311 behavioral traits using the Noldus Observer software [®]. The following behavioral variables
312 were measured for each fish: total time spent without moving around ('stationary position'),
313 total time spent moving slowly ('slow', square crossed in more than 3 sec) or total time spent
314 moving rapidly ('fast', square crossed in less than 3 sec), total distance travelled calculated as
315 number of squares crossed ('squares') and number of times the fish stayed alone ('alone'
316 defined as the number of times the fish remained separated at least one square far from its
317 congeners).

318 **2.9 Statistical analysis**

319 All statistical analyses were performed with R software (R Core Team, 2015). Differences
320 between the two pCO₂/pH treatments and time points were tested using linear mixed models
321 with the 'lme4' package (Bates, Mächler et al. 2015) to which the Anova function was applied

322 to obtain an Analysis of Deviance Table for the fixed factors (Type II Wald chisquare tests).
323 These models used $p\text{CO}_2/\text{pH}$ treatments, time (categorical factor), and their interaction as
324 fixed factors and the identification number of each duplicate as a random factor. The
325 normality of the residuals and homogeneity of the variance were verified graphically. For data
326 concerning the gene expression analysis, the assumption of normal distribution of residuals
327 were not confirmed (strong skewness), and we carried out generalized linear mixed models
328 using the r-package ‘glmm’ (Knudson, Benson et al. 2020). Stepwise backward selection was
329 carried out in all analyses to identify the most parsimonious models. For post-hoc tests we
330 used the function lsmeans (Lenth 2016). Data of neuromodulators levels in hypothalami and
331 behavioural traits at t120 were analysed by two Principal Component Analysis (PCA) with the
332 r-package ‘FactoMineR’ (Lê, Josse et al. 2008) to reveal at the two $\text{pH}/p\text{CO}_2$ conditions the
333 relationships between neuromodulators (first PCA), and between behavioural traits (second
334 PCA). Linear mixed models were used to test whether there were differences in dimensions
335 (PC) 1 and 2 values between experimental groups (duplicate as random factor) followed by an
336 analysis of variance to obtain Analysis of Deviance Table for the fixed factors. Statistical
337 significance were set at $p < 0.05$. Data are presented as boxplots showing the median, the 2nd
338 and 3rd quartiles, and the 95% confidence interval and outside of the 95 percentile range
339 values.

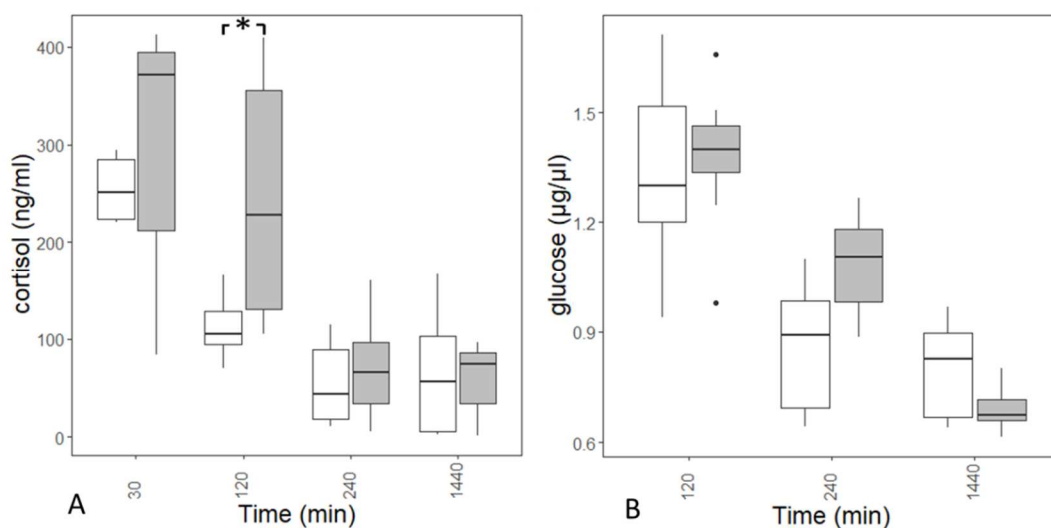
340

341 **3. Results**

342 **3.1 Plasma cortisol concentration**

343 Both scenarios (Chisq = 5.84, df =1, $p = 0.02$) and the sampling time (Chisq = 74.83, df = 3, p
344 < 0.01) showed a clear effect on plasma cortisol. Overall, the netting stress induced a
345 substantial increase in plasma cortisol levels (figure 1A) but this response gradually decreased
346 with the time elapsed since the netting stress (t30, t120 and t240; figure 1A). Both Current

347 and RCP8.5 condition groups had a clear increase in plasma cortisol at t30. The interaction
 348 term between the treatment group and sampling time was not statistically significant, but a
 349 non-significant trend (Chisq = 7.13, df = 3, p = 0.07) could suggest that fish of the RCP8.5
 350 treatment had slightly higher cortisol levels at subsequent time points. The post-hoc test for
 351 this model indicated that plasma cortisol levels were significantly higher in RCP8.5 condition
 352 fish compared to the Current condition fish at 120 minutes post-stress (lsmeans posthoc
 353 testing t120 Current-RCP8.5 conditions: estimate = 132.32, SE = 41.20, df = 62.20, t = 3.21, p
 354 = 0.04). Cortisol concentration of Current condition group had returned to basal level
 355 (lsmeans posthoc testing Current group t1440-t120: estimate = -48.84, SE = 42.50, df = 62.20,
 356 t = -1.15, p = 0.94) while the one at RCP8.5 condition was still higher than basal
 357 concentration (lsmeans posthoc RCP8.5 group t1440-t120: estimate = -185.20, SE = 41.20, df
 358 = 62.20, t = -4.50, p < 0.01).



359 Figure 1: Box plots representing post stress levels at 30, 120 and 240 minutes and 24 hours (1440
 360 minutes) of cortisol (A) and glucose (B) in sea bass plasma. White and grey blocks indicate values for
 361 sea bass acclimated at Current or RCP8.5 conditions, respectively. * indicates p < 0.05 between
 362 scenarios.
 363

364 3.2 Plasma glucose concentration

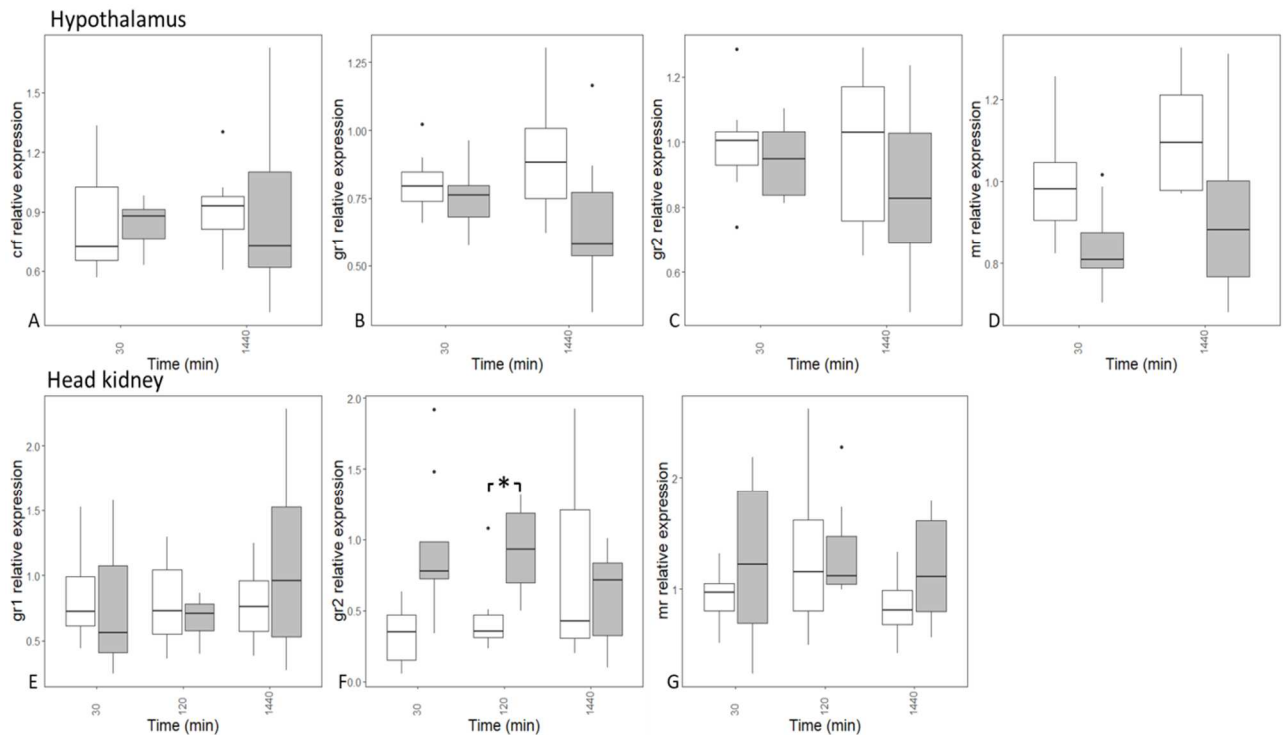
365 After the netting stress we found a significant interaction between treatment groups and
366 sampling time (Chisq = 8.73, df = 2, p = 0.01). The highest glucose concentrations were
367 observed 120 minutes post-stress in both experimental groups but at 240 min post-stress, fish
368 under Current condition presented glucose concentration similar to those measured at t1440,
369 considered the baseline value (lsmeans post hoc test: Current condition t1440 – t240: estimate
370 = -0.06, SE = 0.09, df = 52.70, t = -0.63, p = 0.99), while fish of the RCP8.5 condition
371 scenario had sustained glucose levels compared with t1440 (lsmeans post hoc test: RCP8.5
372 condition t1440 – t240: estimate = -0.39, SE = 0.08, df = 52.6, t = -4.62, p < 0.01) (Figure
373 1B).

374 **3.3 Gene expression analysis**

375 In the hypothalamus, the expression level of *crf* and *gr2* did not vary with sampling time and
376 acidification conditions (*crf*: scenario: Chisq = 0.06, df = 1, p = 0.81; time: Chisq = 0.34, df =
377 1, p = 0.56, figure 2A; *gr2*: scenario: Chisq = 1.46, df = 1, p = 0.23; Chisq = 0.50, df = 1, p =
378 0.48, figure 2C). The relative expression of *gr1* in the hypothalamus was down-regulated in
379 acidified conditions (scenario: Chisq = 5.13, df = 1, p = 0.02; time: Chisq = 0.01, df = 1, p =
380 0.93, figure 2B). Similarly, the hypothalamic *mr* profiles showed a consistent down-regulation
381 in fish under RCP8.5 condition scenario (scenario: Chisq = 11.7, df = 1, p < 0.01, figure 2D)
382 and a down-regulation 30 minutes post-stress compared to t1440 (time: Chisq = 3.88, df = 1,
383 p = 0.048, figure 2D).

384 In the head kidney, the *gr1* and *mr* expression levels were not influenced by the treatment or
385 time (*gr1*: scenario: Chisq = 0.02, df = 1, p = 0.91; time: Chisq = 2.03, df = 2, p = 0.36, figure
386 2E; *mr*: scenario: Chisq = 2.66, df = 1, p = 0.10; time: Chisq = 3.14, df = 2, p = 0.21, figure
387 2G). The relative expression of *gr2* was regulated by scenario and interaction between
388 scenario and time (scenario: Chisq = 4.31, df = 1, p = 0.04; time: Chisq = 0.17, df = 2, p =
389 0.92; scenario:time: Chisq = 9.75, df = 2, p < 0.01, figure 2F). The profile of *gr2* in the head

390 kidney presented an upregulation in RCP8.5 condition group, at 30 minutes post-stress
 391 (lsmeans: estimate = -1.9996, SE = 0.680, df = Inf, z = -2.942, p = 0.0384) compared to fish
 392 exposed to Current condition.



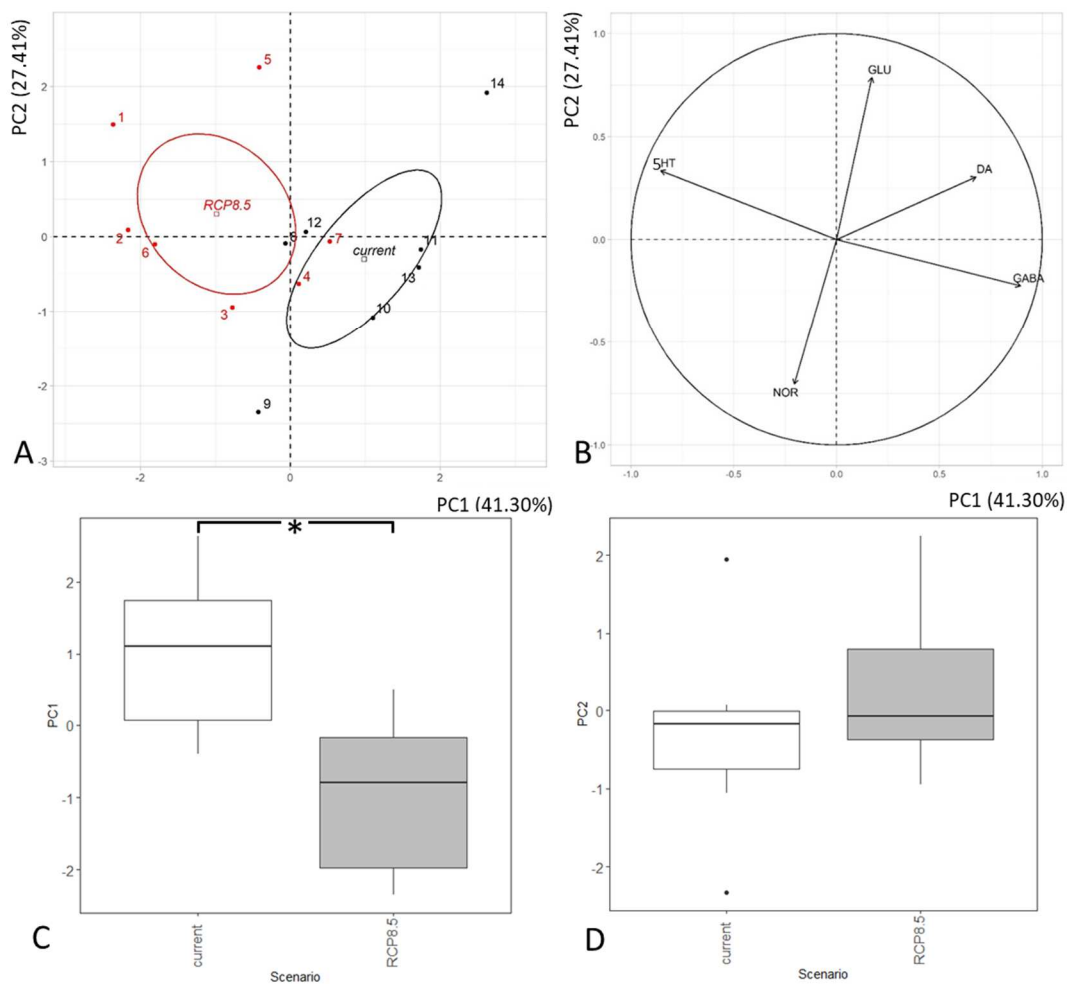
393
 394 Figure 2: Gene expression levels for *crf* (A), *gr1* (B), *gr2* (C) and *mr* (D) in hypothalamus at 30
 395 minutes and 24 hours (1440 minutes) post-stress. E, F and G show gene expression profiles for *gr1*
 396 (E), *gr2* (F) and *mr* (G) in head kidney in fish at 30 and 120 minutes and 24 hours (1440 minutes) after
 397 the netting stress. White and grey blocks indicate values for sea bass acclimated at Current or RCP8.5
 398 condition, respectively. * indicates p < 0.05 between scenarios.

399

400 3.4 Neurotransmitter quantification

401 The first 2 PCs (having an eigenvalue > 1) of the PCA run with the hypothalamic
 402 neurotransmitters levels explained 68.7 % of the total variance (appendix 1A). The first
 403 principal component (PC1) explained 41.30 % of the total variance and was positively related
 404 to GABA levels (corr = 0.89, p-value < 0.001), dopamine levels (DA; corr = 0.68, p-value <
 405 0.001) and negatively related to serotonin levels (5HT; corr = -0.85, p-value < 0.001). The

406 second principal component (PC2) explained 27.41% of the total variance and was primarily
 407 positively related to glutamate (GLU; corr = 0.79, p-value < 0.001) and negatively to
 408 norepinephrine (NOR; corr = -0.70, p-value < 0.001; figures 3A-B). The linear mixed models
 409 run with PC1 and PC2 loadings using $p\text{CO}_2/\text{pH}$ treatment as fixed factor only revealed a
 410 significant difference between scenarios in PC1 loadings (PC1: SE = 0.55, df = 11.86, t = -
 411 3.59, p = 0.003; PC2: SE = 0.58, df = 11.95, t = 1.02, p = 0.33), showing that fish exposed to
 412 RCP8.5 condition scenario presented higher content of serotonin and lower concentration of
 413 GABA in the hypothalamus compared to the Current condition group (figures 3C,D).

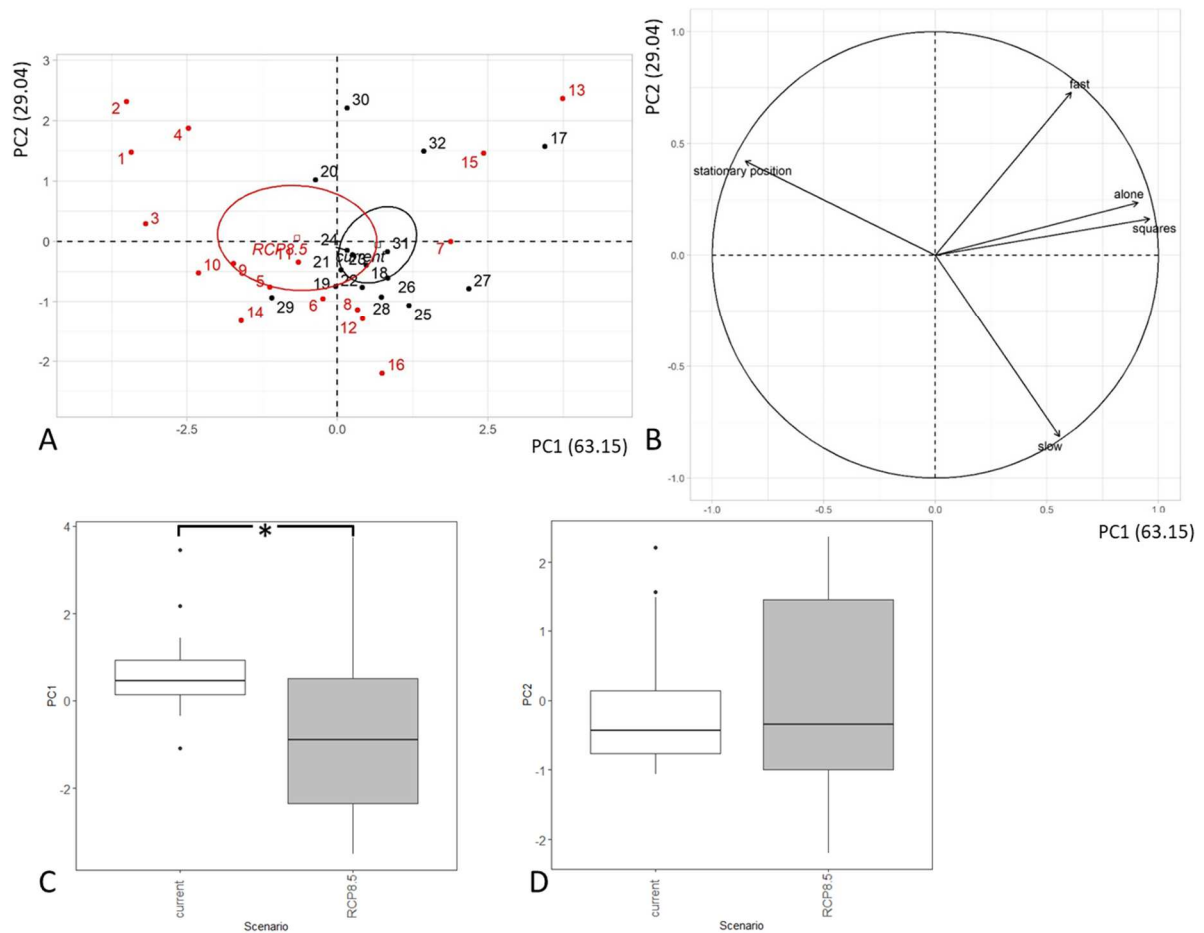


414
 415 Figure 3. Principal component analysis of variability in neurotransmitters levels on the hypothalamus
 416 of sea bass acclimated to acidified (RCP8.5 in red) and present-day condition (Current, in black) at
 417 120 minutes post netting stress. PCA loadings of individuals (dots in A) and variables (arrowheads in

418 B) are represented in graphs displaying plots for principal components 1 and 2 (PC1 and PC2).
419 Ellipses around the mean of RCP8.5 and Current condition groups (red and black rectangles
420 respectively, in A) represents the confidence ellipse of each experimental group. 5HT: serotonin, DA:
421 dopamine, GABA: γ -aminobutyric acid, GLU: glutamate, NOR: norepinephrine. C and D represents
422 boxplots of PC1 and PC2 loadings respectively of fish of each experimental group. * indicates $p <$
423 0.05 between scenarios.

424 **3.5 Behavioral analysis**

425 The first two PCs of the PCA loaded with behavioral variables explained together the 92.19 %
426 of the total variance (eigenvalue value >1 ; appendix 1B). The first component explained
427 63.15% of the total variation and the second component 29.04%. The most important loadings
428 for the PC1 were the total distance travelled (corr = 0.96, p -value < 0.01) and number of times
429 the fish was alone (corr = 0.91, p -value < 0.01), inversely correlated to the total time spent
430 without moving (corr = -0.85, p -value < 0.01 ; figures 4A-B). Therefore, PC1 reflected fish
431 motor activities. The most important loading on the second component were the total time
432 spent moving around slowly (corr = -0.81, p -value < 0.01), inversely related to the total time
433 spent moving rapidly (corr = 0.73, p -value < 0.01) and, to a lesser extent, to the total time
434 spent immobile (corr = 0.42, p -value < 0.01). On this basis, PC2 were named swimming
435 speed axis. During recovery (t120), a significant effect of the $p\text{CO}_2$ /pH treatment was
436 observed for the first component loading, showing a reduced motor activity for the fish
437 exposed to RCP8.5 condition scenario (SE = 0.58, df = 30.00, $t = -2.314$, $p = 0.03$; figures
438 4C,D). No significant treatment difference was observed for the second component (SE =
439 0.42, df = 32.00, $t = 0.27$, $p = 0.79$) (figures 4D).



440

441 Figure 4. Principal component analysis of behavioral traits observed in sea bass acclimated to acidified (RCP8.5
 442 in red) and present-day condition (Current, in black) at 120 minutes post netting stress. PCA loadings of
 443 individuals (dots in A) and variables (arrowheads in B) are represented in graphs displaying plots for principal
 444 components 1 and 2 (PC1 and PC2). Ellipses around the mean of RCP8.5 and Current condition groups (red and
 445 black rectangles respectively, in A) represent the confidence ellipse of each experimental group. C and D
 446 represents boxplots of PC1 and PC2 loadings respectively of fish of each experimental group. * indicates $p <$
 447 0.05 between scenarios.

448

PC	Eigenvalue	% of variance	cumulative % of variance	PC	Eigenvalue	% of variance	cumulative % of variance
1	2.06	41.30	41.30	1	3.16	63.15	63.15
2	1.37	27.41	68.70	2	1.45	29.04	92.19
3	0.75	15.09	83.79	3	0.28	5.55	97.75
4	0.60	11.91	95.70	4	0.06	1.21	98.96
5	0.22	4.30	100.00	5	0.05	1.04	100.00

449

450 Appendix 1. Tables with the Eigenvalue scores and the percentages of total variance explained by each
451 principal component (PC) and the cumulative percentage of the variance calculated by the sum of the
452 percentage of variance explained by each PC and the previous ones for the PCA run with hypothalamic
453 neurotransmitters concentrations (A) and behavioral traits (B).

454

455 **4. Discussion**

456 Juvenile European sea bass long term acclimated to acidified water as predicted by RCP8.5
457 scenario for 2100 exhibited modified physiological and behavioral responses to a netting
458 stress (fish transfer and confinement test). European sea bass naturally shows an intense stress
459 response to acute stress with a substantial post-stress rise in plasma cortisol. The exposure to
460 sustained stress can cause reproductive disruptions and disease outbreaks (Fanouraki,
461 Mylonas et al. 2011). In our study, resting plasma cortisol concentrations were slightly higher
462 than those previously reported (Cerdá-Reverter, Zanuy et al. 1998, Vazzana, Cammarata et al.
463 2002, Rotllant, Ruane et al. 2003, Marino, Di Marco et al. 2008) ($10\text{-}50\text{ ng ml}^{-1}$), but lower
464 than the ones observed by Planas et al., 1990 ($>200\text{ ng ml}^{-1}$) likely reflecting differences in
465 rearing conditions and handling. The maximal plasma cortisol levels were reached in our
466 experiment at 30 minutes after the netting test with the mean value of 283.88 ng ml^{-1} . This is
467 in line with the magnitude of cortisol rise observed in the same species at the same sampling
468 point following a comparable stress (Samaras, Dimitroglou et al. 2016). However, it is
469 possible that we missed the real peak of cortisol plasma rise if it occurred before 30 minutes
470 or between 30 and 120 min post-stress, due to the chosen experimental design.

471 We found that acidification of the water did not affect basal or maximum values of plasma
472 cortisol levels. However, our findings indicate a slower recovery from stress in fish
473 acclimated to the RCP8.5 condition scenario with a return to the resting cortisol concentration
474 at the t240 sampling point whereas Current condition group showed a return to the basal

475 concentration already at t120. A slower recovery from stress in RCP8.5 condition scenario
476 was confirmed by the plasma glucose post-stress kinetic. The delay of the glucose response to
477 stress compared to the plasma cortisol kinetic is not surprising since glucose plasma
478 concentration is considered a marker of the secondary, and thus slower, physiological
479 response to stress in vertebrates (Barton 2002). The main effect of cortisol and glucose plasma
480 rise is the energy reallocation to allow organisms to cope with the potential increase in energy
481 demand in stressful events or environments. It's likely that RCP8.5 condition fish presented
482 sustained cortisol and glucose concentration when facing the additional stress before restoring
483 the homeostasis. Overall, our findings suggest that an additional acute stress reveals the
484 limited coping ability of fish when facing higher $p\text{CO}_2$ levels. More precisely, the reactivity
485 and sensitivity of the corticotropic axis is not disrupted in acidified condition, but fish
486 recovery might be slightly compromised. Similar delay in recovery is also observed in
487 animals facing a chronic stress (Veissier, Boissy et al. 2001).

488 The expression patterns of main genes involved in the central control of the corticotropic axis
489 only indicate a weak overall down-expression of *gr1* and *mr* in RCP8.5 condition without
490 obvious variations with time. The lack of reactivity of the expression pattern of cortisol
491 receptor in the hypothalamus in response to an acute stress is globally in line to what has been
492 reported in freshwater species like rainbow trout and carp in which short term confinement
493 exposure did not influence the expression profiles of *gr1*, *gr2*, and *mr* genes in the
494 hypothalamus (Stolte, de Mazon et al. 2008, Kiilerich, Servili et al. 2018). Possibly, the role
495 of cortisol in acute stress adaptation could be exerted through the rapid nongenomic pathway
496 as suggested in literature and does not require the higher expression of glucocorticoid
497 receptors transcripts (Das, Thraya et al. 2018).

498 Gluco- and mineralocorticoid receptors expression levels were not regulated during stress
499 recovery in sea bass in the interrenal tissue located in the head kidney. This is consistent with

500 the interrenal response of the anadromous species Atlantic salmon to unpredictable chronic
501 and acute stress, but this result is not in agreement with the upregulation of *gr1* reported in
502 freshwater carp after a confinement test (Stolte, Nabuurs et al. 2008, Madaro, Olsen et al.
503 2015). Here, we found a slight trend of an upregulation of *gr* and *mr* expression levels in sea
504 bass head kidney under the RCP8.5 condition scenario with statistical significance reached for
505 *gr2* gene expression at 30 minutes post-stress. Overall, the potential increase in *gr* expression
506 levels in the head kidney of fish under acidified conditions could be linked to the longer
507 lasting high cortisol levels observed in the plasma and be interpreted as a further sign for a
508 slower recovery from stress in RCP8.5 condition. However, the experimental design of the
509 present study did not allow to exclude that the slower return to basal level in RCP8.5
510 condition scenario was not due to a potential higher peak of cortisol and glucose at low pH
511 which was not detectable with the tested sampling time.

512 A prolonged increase in cortisol plasma concentration in response to stress can have a number
513 of metabolic consequences involving the activation of energy demanding pathways to restore
514 homeostasis, such as the modulation of the carbohydrate metabolisms (gluconeogenesis), the
515 increase in protein turnover, the regulation of amino acid metabolism and increase in lipolysis
516 (Mommsen, Vijayan et al. 1999). Cortisol may also influence the regulation of the acid-base
517 balance in fish and high cortisol levels could be beneficial in acidified waters, where an
518 effective acid-base regulation is crucial for maintaining the homeostasis (Cruz, Chao et al.
519 2013, Kwong and Perry 2013, Tucker, Suski et al. 2018). However, the present study showed
520 that fish of both scenarios presented, at the tested sampling times, comparable basal and post-
521 stress cortisol plasma concentrations. Only the recovery from the acute stress seems to be
522 slower in fish acclimated to high $p\text{CO}_2$ levels consistent with the hypothesis that acidification
523 may alter the response of the corticotropic axis in fish.

524 We determined the profiles of main hypothalamic neurotransmitters during recovery (at 120
525 minutes post-stress) to better understand the effects of acidified water on the stress response
526 of fish at central level. Interestingly, the two scenarios showed a divergent pattern with
527 notably higher serotonin and lower GABA concentrations in the RCP8.5 condition fish.
528 GABA is a strong inhibitory neurotransmitter in vertebrate. A decade ago, Nilsson and
529 collaborators (Nilsson, Dixson et al. 2012) proposed the “GABA model” hypothesis to
530 explain the mechanisms underlying the reported effect of ocean acidification on behavior and
531 sensory performances in some fish species (Munday, Dixson et al. 2009, Dixson, Jennings et
532 al. 2015, Lai, Jutfelt et al. 2015, Lai, Li et al. 2016, Munday, Watson et al. 2016). When
533 facing increasing $p\text{CO}_2$ in the ranges predicted by IPCC scenarios, most fish react by retaining
534 additional HCO_3 in their blood (Esbaugh 2018). This is associated to changes in intracellular
535 and extracellular ion concentrations that could interfere with the flux of ions passing through
536 GABA_A receptors after GABA binding. Consequently, GABA would exert a stimulatory role
537 on the activity of GABAergic neurons under acidification condition. Interestingly, a similar
538 stimulatory action of GABA is suggested also during post-stress recovery in rodents (Sarkar,
539 Wakefield et al. 2011). If the described regulation is conserved in fish, one could expect that
540 acute stress and water acidification would have a synergic effect on the GABA regulation of
541 stress axis. The end result would be the robust excitatory action of GABA on CRF neuron
542 activity and a potential sharp increase in plasma cortisol levels. However, in our study we
543 observed higher plasma cortisol levels in acidification condition at 120 minutes post-stress,
544 associated to lower hypothalamic GABA concentrations which is likely in contrast to the
545 GABA model hypothesis. It is possible that this decrease in GABA concentration reflects the
546 action of a negative feedback that is needed at the hypothalamic levels to inhibit CRF neurons
547 activity and, consecutively, inhibit the release of cortisol from the interrenal tissue that is
548 essential to restore the homeostasis.

549 Interestingly, a recent study used molecular approaches to test the direct effect of long term
550 acidification on brain function in wild coral fish (Kang, Nagelkerken et al. 2022). The authors
551 observed that cardiac β 1-adrenergic receptor (ADRB1), which is involved in the stress
552 response, was significantly expressed in fish exposed to elevated CO₂. This condition was
553 also associated with a decreased gene expression of the GABAergic pathway (Kang,
554 Nagelkerken et al. 2022). Similarly, both GABA and serotonin were shown to be
555 differentially expressed in the brain (olfactory bulb) of coho salmon acclimated to acidified
556 water at resting (Williams, Dittman et al. 2019). Altogether, this suggests an activation of the
557 stress response, associated to a repression of GABAergic signaling, even in fish under ocean
558 acidification that are not recovering from a further acute stress. Monoamines are one major
559 class of neuromodulators and their sensitivity to environmental factors, including
560 acidification, has been documented (Libersat 2009, Paula, Repolho et al. 2019). It is therefore
561 not surprising that there is a high variation in serotonin content in the brain of sea bass
562 acclimated to different p CO₂ conditions. In teleost, serotonin acts stimulating CRF secretion
563 in the hypothalamus resulting in the rise of the secretion of cortisol from the head kidney
564 (Lim, Porteus et al. 2013). Similarly, the higher serotonin hypothalamic concentration in
565 RCP8.5 condition fish, observed at 120 minutes post-stress in the present study, explains the
566 sustained cortisol plasma level in this group.

567 Studies in salmonids suggests that serotonin is linked to an increase in motor activity,
568 (Clements, Schreck et al. 2002, Clements, Moore et al. 2003, Carpenter, Watt et al. 2007). In
569 the case of the European sea bass, during recovery from acute stress (t120), hypothalamic
570 serotonin levels are increased in animals acclimated to acidification which exhibit a more
571 static attitude (longer duration of stationary position, shorter duration of time moving around
572 and less total distance travelled) compared to Current condition group. Thus, the relationship
573 between serotonin and motor activity is apparently inverted in the case of sea bass compared

574 with what has been reported in salmonids. However, it is worth noting that it is plausible that
575 the interaction between serotonin, corticotropic axis and motor behavior may vary during
576 post-stress recovery or long-term exposure to challenging environments (i. e. acidification).

577 The way an animal perceives, processes, and copes with stressful stimuli determines the
578 magnitude of the physiological response to stress and is modulated by their behavioral coping
579 style (Koolhaas, Korte et al. 1999). In general, individuals presenting proactive behavior type
580 show active coping style with bold and aggressive score, high swimming activity and low
581 behavioral flexibility (Øverli, Sørensen et al. 2007). Conversely, reactive animals are passive
582 copers, with non-aggressive and cautious score, prefer the immobility response and show
583 flexible behavior immobility (Øverli, Sørensen et al. 2007). The physiological response to
584 stress of proactive animals is associated to low reactivity of HPI axis, while reactive
585 individuals show the opposite pattern. Individual-specific cortisol stress responses is shown to
586 exist and to be a repeatable trait in European sea bass (Samaras, Dimitroglou et al. 2016).
587 Even though more specific behavioral tests should be performed to validate this hypothesis,
588 our data suggest that sea bass under RCP8.5 condition scenario recovering from an additional
589 acute stress, adopt a more reactive coping style (higher cortisol and serotonin levels, freezing
590 strategy and stationary attitude), whereas fish exposed to current condition would display a
591 more proactive behavior coping style (lower plasma cortisol and hypothalamic serotonin
592 content and higher motor activity). A number of previous studies have already examined
593 potential changes in the activity and also anxiety, two traits related to stress response and
594 individual coping style, in fish under elevated CO₂ conditions. Globally they reported
595 contrasting effects in different fish species and stages, ranging from increased anxiety (Jutfelt,
596 de Souza et al. 2013, Hamilton, Holcombe et al. 2014, Rossi, Nagelkerken et al. 2015, Jarrold,
597 Welch et al. 2020) and lower motor activity scores (associated sometime with prolonged
598 stationary behavior) (Porteus, Hubbard et al. 2018), to no effects (Duteil, Pope et al. 2016,

599 Kwan, Hamilton et al. 2017, Andrade, Hurst et al. 2018), or even to increased boldness and
600 swimming behavior in larval clownfish (Munday, Dixson et al. 2010). This discrepancy could
601 be likely explained by a species specific sensitivity to acidification and/or by the different
602 tests used to assess anxiety and activity scores. We should also keep in mind that these
603 experiments reported short term effects of exposure to elevated CO₂ in fish that were not
604 recovering from a further and acute stress. This makes difficult the comparison to the current
605 study since neurotransmitters and activity were assessed during recovering from an acute
606 stress in fish acclimated to long term acidification. Anyways, what it appears evident, from
607 the present and past studies, is that both physiological and behavioral responsiveness to stress
608 would be plastic and that changing environments can modify the individual coping style of
609 the animals.

610 **5. Conclusions**

611 Long term exposure to pH/pCO₂ conditions as predicted by IPCC RCP8.5 scenario for the end
612 of the century impacts the physiological and motor behavioral responses to an acute stress in
613 juvenile sea bass. Fish acclimated to RCP8.5 condition scenario showed slower post-stress
614 return to basal concentrations of plasma cortisol and glucose. This is not associated to a clear
615 central (hypothalamus) and interrenal (head kidney) regulation of gluco- and
616 mineralocorticoid receptors and corticoid releasing factor expression levels. Acclimated sea
617 bass to acidified water showed altered neurotransmitters' concentration pattern in the
618 hypothalamus, at 120 minutes post-stress, with higher concentration of serotonin and lower
619 levels of GABA and dopamine compared to the current condition scenario. At the same time
620 post-stress, behavioral traits analysis revealed a reduction in motor activity in fish exposed to
621 RCP8.5 conditions. Overall, these findings suggest that behavioral and physiological adaptive
622 response to climate changes related constraints may impact fish resilience to further stressful
623 events.

624

625 **Competing interests**

626 The authors have no competing interests to declare.

627

628 **CRedit author statement**

629 Arianna Servili: conceptualization, methodology, formal analysis, project administration,
630 funding acquisition, writing (original draft). Etienne Lévêque: investigation, data curation.
631 Olivier Mouchel: methodology, data curation, writing (review and edits). Jimmy Devergne:
632 formal analysis, writing (review and edits). Christophe Lebigre: methodology, validation,
633 writing (review and edits). Sabine Roussel: resources, methodology, validation, writing
634 (review and edits). David Mazurais: validation, writing (review and edits). José-Luis
635 Zambonino Infante: writing (review and edits).

636

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641 development and quantification of hypothalamic neurotransmitters concentrations.

642

643 **Data availability**

644 The raw data of this study been uploaded as supplemental data (S1).

645

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