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

2   Arianna Servili[1*], Etienne Lévêque[1], Olivier Mouchel[1], Jimmy Devergne[1], Christophe

3   Lebigre[2], Sabine Roussel[1], David Mazurais[1], José-Luis Zambonino-Infante[1].

4   [1]Ifremer, Université de Brest, CNRS, IRD, LEMAR, Plouzané, France

5   [2]UMR DECOD (Ecosystem Dynamics and Sustainability), Institut Agro, IFREMER, INRAE,

6   F-29280 Plouzané, France

7

8   [*]Corresponding author

9   Email: arianna.servili@ifremer.fr

10   Full postal address: Ifremer, Unit of Functional Physiology of Marine Organisms,

11   (PFOM/ARN), Technopole Iroise, ZI de la Pointe du Diable, 29280 Plouzané, France

12

13   **Graphical abstract:**
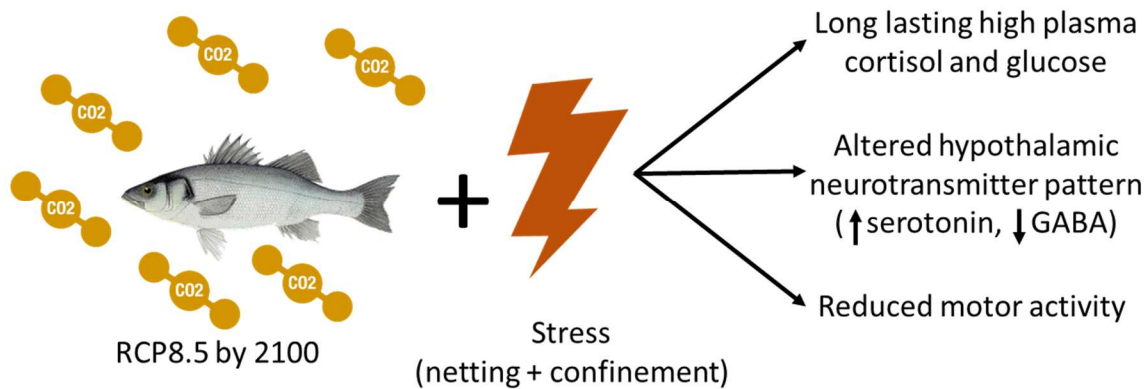
14

15

16   **Highlights**

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

18   bass

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

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

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/CO_2 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/pCO$_2$ 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 adrenocorticotropic 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 glucorticoid 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 CO_2

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 CO_2 on fish activity was tested. For instance, juvenile sea bass showed

117 lower baseline activity and more prolonged freezing behavior under low pH (Porteus,

Hubbard et al. 2018), but earlier stages of the same species (during metamorphosis) did not

exhibited any effect of acidification on fish activity (Duteil, Pope et al. 2016). Non-significant

trend suggesting a decreasing activity score under elevated CO_2 was observed in speackled

sanddabs (Andrade, Hurst et al. 2018), whereas increased boldness and swimming activity

was recorded in larval clownfish (Munday, Dixson et al. 2010). Such contrasting results on

particular stress related traits (anxiety and activity) likely reflect a species-specific response to

acidified water and/or methodological differences. It is worthy to note that most of the

reported studies focused on short term effects of ocean acidification (ranging from 10 days to

1.5 months) on stress response-related traits. Nowadays, the effect of a prolonged exposure to

acidified water on fish stress response, which would be more relevant to predict what is like to

happen in the wild, is unknown. In this context, we report an integrative approach to

investigate whether the prolonged ocean acidification condition impacts the ability of a

marine fish to cope with an additional acute stress. We test the hypothesis that neuroendocrine

and behavioral responses of fish to netting and to a confinement test would be altered in fish

constantly living in acidified seawater by showing i) higher plasma cortisol and glucose

concentrations during recovery, ii) variations in expression profiles of key players of the HPI

axis, iii) alterations in neurotransmitter hypothalamic levels and iv) modified behavioral traits

associated to motor activity. The model species chosen for this study is the European sea bass,

Dicentrarchus labrax, an economically important marine teleost, whose physiological and

behavioral response to acidified condition (Pope, Ellis et al. 2014, Duteil, Pope et al. 2016,

Crespel, Zambonino-Infante et al. 2017, Poulton, Porteus et al. 2017, Cominassi, Moyano et

al. 2019, Alves, Gregorio et al. 2020, Mazurais, Servili et al. 2020a, Servili, Canario et al.

2020) and to an acute stress separately is known (Samaras, Dimitroglou et al. 2016, Alfonso,

Sadoul et al. 2019, Ferrari, Rey et al. 2020).

6

2. Materials and methods

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

2.1 Animals and experimental design

This experiment is based on sixty-four juvenile European sea bass (11 months post hatching; mean body mass = 144.2 g, SEM = 5.5 g), obtained as larvae from the aquaculture facility 'Aquastream' (Ploemeur, Lorient, France) and transferred to Ifremer's experimental facilities at 2 days post-hatching (dph) at the density of 5000 larvae per tank. Larvae were gradually acclimated (- 0.1 pH units per day) to two conditions of water pH/pCO$_2$ targeting current pH/pCO$_2$ conditions of Bay of Brest referred to "Current condition" group (Duteil, Pope et al. 2016) and pH/pCO$_2$ conditions as predicted by IPCC's RCP 8.5 for the end of the century referred to 'RCP8.5 condition' group, consisting in a decrease in surface ocean pH by 0.4 unit by 2100 (Meinshausen, Raper et al. 2011, Pörtner, Karl et al. 2014). During their larval stage, fish were fed with artemia ad libitum until 28 dph and progressively acclimated to commercial dry pellets (Neo-Start, Le Gouessant Aquaculture, France) until 45 dph. Afterwards, juvenilefrom the same treatment were pooled and then randomly transferred to 2 juvenile rearing tanks per treatment. All juvenile fish were fed the commercial feed 'Neo Grower Extra Marin' (Le Gouessant Aquaculture, France). Table 1 shows the obtained mean and SEM values of water parameters for each treatment during fish rearing and the

168   confinement test. The respective pH/pCO$_2$ conditions of each experimental group were

169   constantly maintained for 11 months. At 11 months, approximatively 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 pCO$_2$ 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 CO$_2$ 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 CO$_2$

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:

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

209

210　With: total alkalinity (TA), volume of HCl (VHCl, l) and of the sample (Vsample, l), HCl

211　concentration (C, mol l-1), hydrogen activity (H+, 10-pH) and hydrogen activity coefficient

212　(yH+ = 0.758). Seawater pH in free scale and pCO_2 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 (1m^3 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/m^3) 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 forth 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 ΔΔCt 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: Chisq(scenario) =

12

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

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

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

2.7 Neurotransmitters analysis

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

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/pCO$_2$

310   treatments). A grid pattern, composed of 12 squares of 4 cm^2, 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).

2.9 Statistical analysis

319   All statistical analyses were performed with R software (R Core Team, 2015). Differences

320   between the two pCO$_2$/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

to obtain an Analysis of Deviance Table for the fixed factors (Type II Wald chisquare tests).

These models used pCO_2/pH treatments, time (categorical factor), and their interaction as

fixed factors and the identification number of each duplicate as a random factor. The

normality of the residuals and homogeneity of the variance were verified graphically. For data

concerning the gene expression analysis, the assumption of normal distribution of residuals

were not confirmed (strong skewness), and we carried out generalized linear mixed models

using the r-package 'glmm' (Knudson, Benson et al. 2020). Stepwise backward selection was

carried out in all analyses to identify the most parsimonious models. For post-hoc tests we

used the function lsmeans (Lenth 2016). Data of neuromodulators levels in hypothalami and

behavioural traits at t120 were analysed by two Principal Component Analysis (PCA) with the

r-package 'FactoMineR' (Lê, Josse et al. 2008) to reveal at the two pH/pCO_2 conditions the

relationships between neuromodulators (first PCA), and between behavioural traits (second

PCA). Linear mixed models were used to test whether there were differences in dimensions

(PC) 1 and 2 values between experimental groups (duplicate as random factor) followed by an

analysis of variance to obtain Analysis of Deviance Table for the fixed factors. Statistical

significance were set at $p < 0.05$. Data are presented as boxplots showing the median, the 2nd

and 3rd quartiles, and the 95% confidence interval and outside of the 95 percentile range
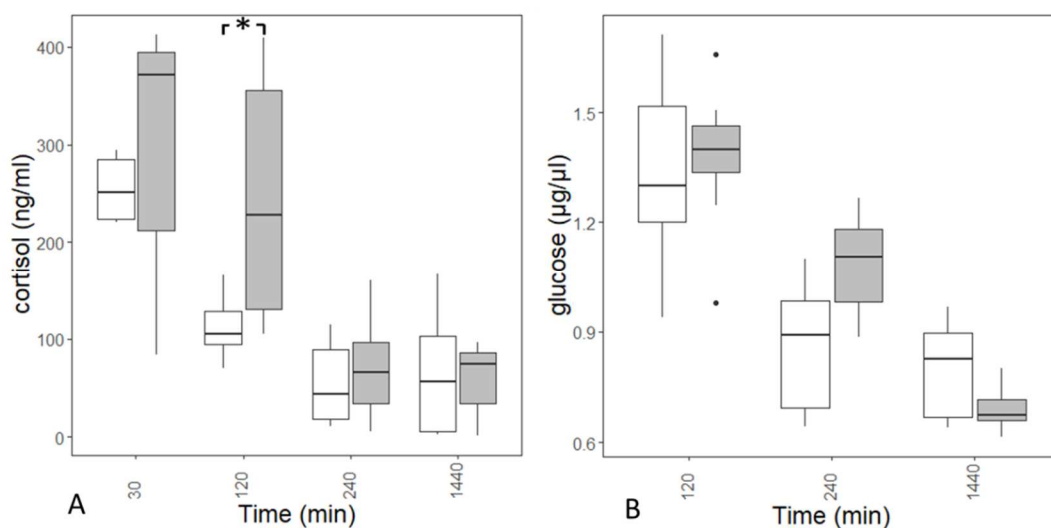
values.

3. Results

3.1 Plasma cortisol concentration

Both scenarios (Chisq = 5.84, df =1, p= 0.02) and the sampling time (Chisq = 74.83, df = 3, p

< 0.01) showed a clear effect on plasma cortisol. Overall, the netting stress induced a

substantial increase in plasma cortisol levels (figure 1A) but this response gradually decreased

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

360 Figure 1: Box plots representing post stress levels at 30, 120 and 240 minutes and 24 hours (1440

361 minutes) of cortisol (A) and glucose (B) in sea bass plasma. White and grey blocks indicate values for

362 sea bass acclimated at Current or RCP8.5 conditions, respectively. * indicates p < 0.05 between

363 scenarios.

3.2 Plasma glucose concentration

16

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

3.3 Gene expression analysis

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

In the head kidney, the *gr1* and *mr* expression levels were not influenced by the treatment or time (*gr1*: scenario: Chisq = 0.02, df = 1, p = 0.91; time: Chisq = 2.03, df = 2, p = 0.36, figure 2E; *mr*: scenario: Chisq = 2.66, df = 1, p = 0.10; time: Chisq = 3.14, df = 2, p = 0.21, figure 2G). The relative expression of *gr2* was regulated by scenario and interaction between scenario and time (scenario: Chisq = 4.31, df = 1, p = 0.04; time: Chisq = 0.17, df = 2, p = 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.
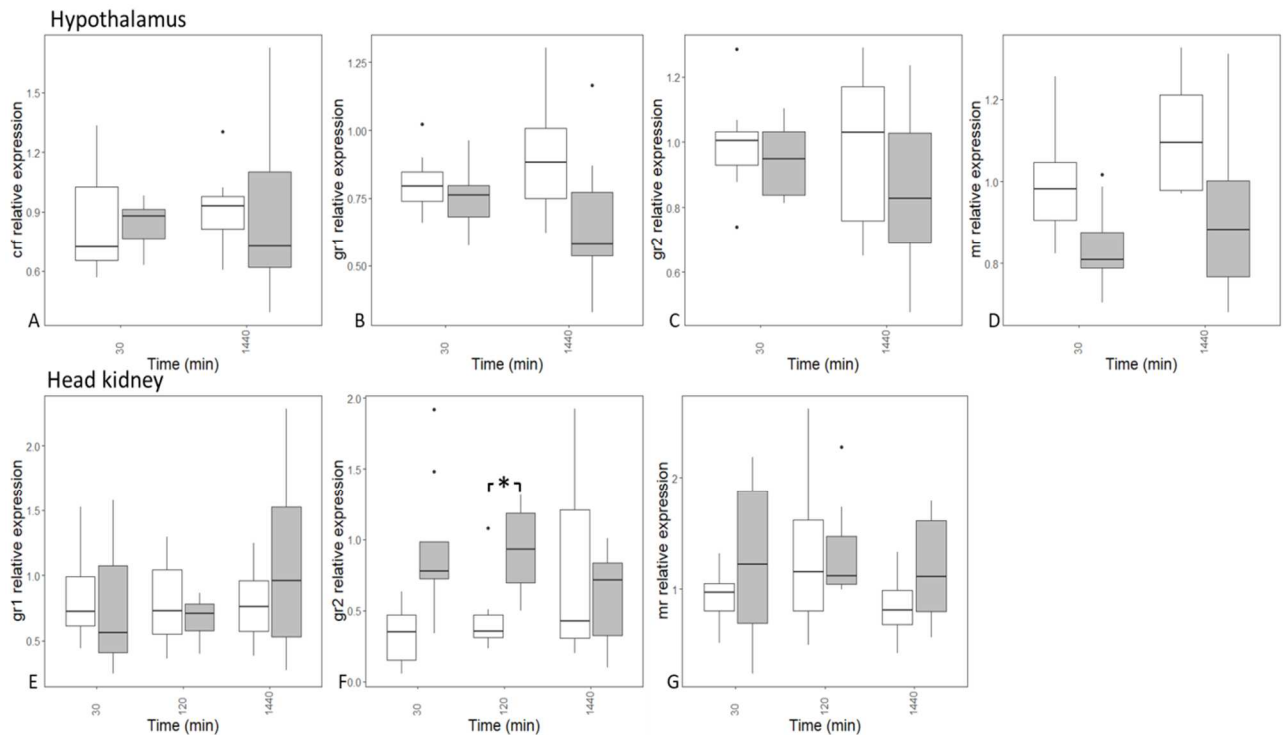
393

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

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

18

second principal component (PC2) explained 27.41% of the total variance and was primarily positively related to glutamate (GLU; corr = 0.79, p-value < 0.001) and negatively to norepinephrine (NOR; corr = -0.70, p-value < 0.001; figures 3A-B). The linear mixed models run with PC1 and PC2 loadings using pCO_2/pH treatment as fixed factor only revealed a significant difference between scenarios in PC1 loadings (PC1: SE = 0.55, df = 11.86, t = -3.59, p = 0.003; PC2: SE = 0.58, df = 11.95, t = 1.02, p = 0.33), showing that fish exposed to RCP8.5 condition scenario presented higher content of serotonin and lower concentration of GABA in the hypothalamus compared to the Current condition group (figures 3C,D).
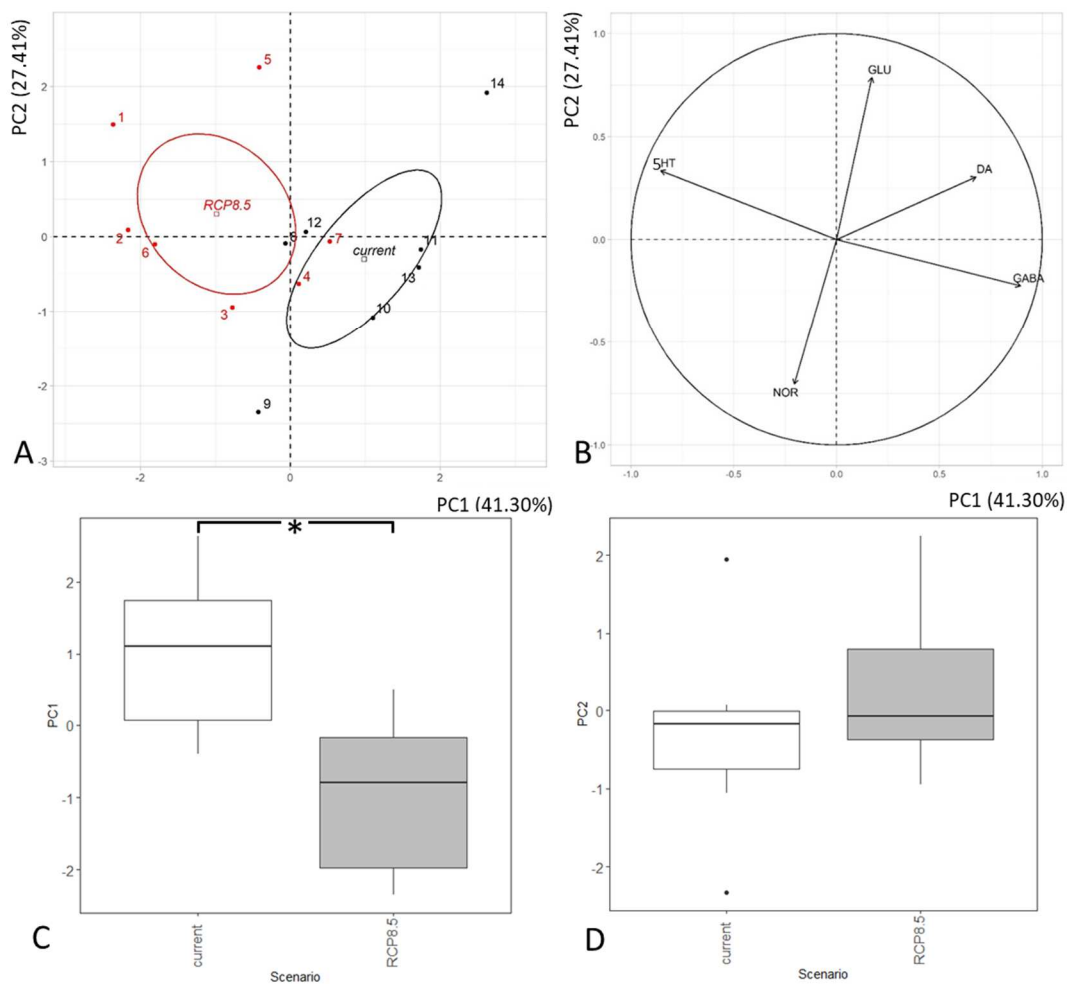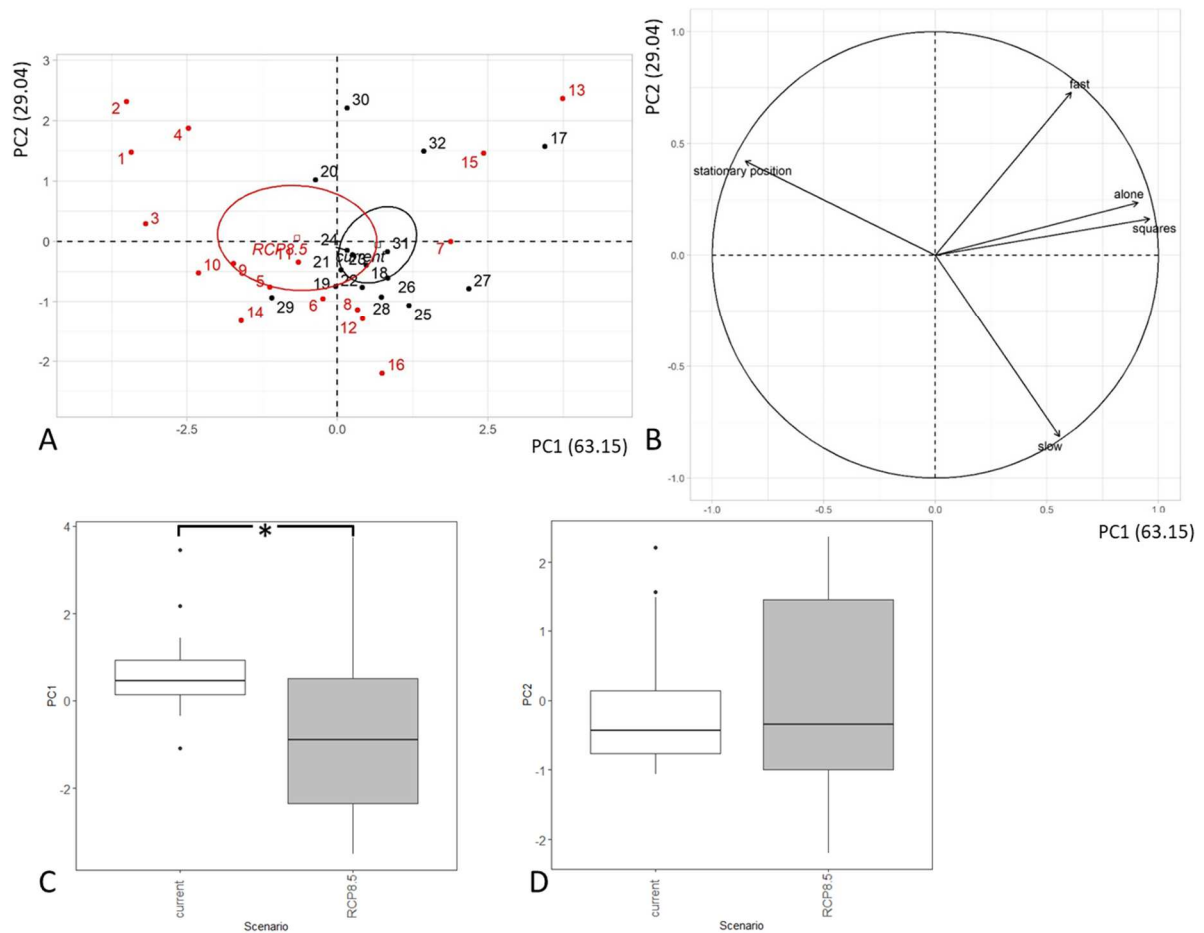

Figure 3. Principal component analysis of variability in neurotransmitters levels on the hypothalamus of sea bass acclimated to acidified (RCP8.5 in red) and present-day condition (Current, in black) at 120 minutes post netting stress. PCA loadings of individuals (dots in A) and variables (arrowheads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

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

3.5 Behavioral analysis

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

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

PC	Eigenvalue	% of variance	cumulative % of variance
1	2.06	41.30	41.30
2	1.37	27.41	68.70
3	0.75	15.09	83.79
4	0.60	11.91	95.70
5	0.22	4.30	100.00

A

PC	Eigenvalue	% of variance	cumulative % of variance
1	3.16	63.15	63.15
2	1.45	29.04	92.19
3	0.28	5.55	97.75
4	0.06	1.21	98.96
5	0.05	1.04	100.00

B

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

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) (10-50 ng ml^{-1}), but lower

464 than the ones observed by Planas et al., 1990 (>200 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 pCO_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 pCO$_2$ 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 pCO$_2$ 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, consequently, 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 β1-adrenergic receptor (ADRB1), which is involved in the stress

552   response, was significantly expressed in fish exposed to elevated CO_2. 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 pCO_2 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 CO_2 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 clowfish (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 CO_2 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/pCO$_2$ 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.

643   **Data availability**

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

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