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Ocean acidification alters the acute stress response of a marine fish

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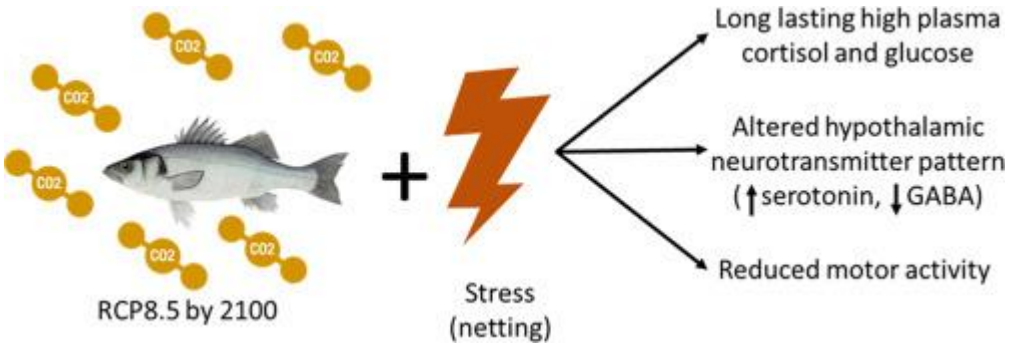
Abstract :

The absorption of anthropogenic carbon dioxide from the atmosphere by oceans generates rapid changes in seawater carbonate system and pH, a process termed ocean acidification. Exposure to acidified water can impact the allostatic load of marine organism as the acclimation to suboptimal environments requires physiological adaptive responses that are energetically costly. As a consequence, fish facing ocean acidification may experience alterations of their stress response and a compromised ability to cope with additional stress, which may impact individuals' life traits and ultimately their fitness. In this context, we carried out an integrative study investigating the impact of ocean acidification on the physiological and behavioral stress responses to an acute stress in juvenile European sea bass. Fish were long term (11 months) exposed to present day pH/CO2 condition or acidified water as predicted by IPCC “business as usual” (RCP8.5) scenario for 2100 and subjected to netting stress (fish transfer and confinement test). Fish acclimated to acidified condition showed slower post stress return to plasma basal concentrations of cortisol and glucose. We found no clear indication of regulation in the central and interrenal tissues of the expression levels of gluco- and mineralocorticoid receptors and corticoid releasing factor. At 120 min post stress, sea bass acclimated to acidified water had divergent neurotransmitters concentrations pattern in the hypothalamus (higher serotonin levels and lower GABA and dopamine levels) and a reduction in motor activity. Our experimental data indicate that ocean acidification alters the physiological response to acute stress in European sea bass via the neuroendocrine regulation of the corticotropic axis, a response associated to an alteration of the motor behavioral profile. Overall, this study suggests that behavioral and physiological adaptive response to climate changes related constraints may impact fish resilience to further stressful events.

Graphical abstract



Highlights

► Ocean acidification (OA) impacts the physiological stress response of European sea bass. ► Post-stress return to basal plasma cortisol and glucose levels is delayed under OA. ► This delay is associated to alteration of hypothalamic neurotransmitters pattern. ► Motor activity is reduced during recovery from stress in fish under OA conditions.

Keywords : climate change, allostatic load, phenotypic plasticity, European sea bass, corticotropic axis, neuroendocrine control and behavior

1. Introduction

Ocean acidification is a direct consequence of the ongoing anthropogenic climate change and the capacity of the ocean to uptake carbon from the atmosphere. This process leads to an increase in seawater concentration of CO_2, H^+ and HCO_3^- and a decrease of CO_3^- and pH. Earth system models (CMIP5) corresponding to the high emission scenario (Representative Concentration Pathway, RCP, 8.5 scenario) predicted a 0.38 decrease of pH units of global surface ocean by the end of the century (Bopp, Resplandy et al. 2013). More recently, a new generation of earth system models (CMIP6) reported an even sharper decline in surface ocean

pH corresponding to -0,44 pH units by the end of the century (Shared Socioeconomic Pathway 5-8.5)(Kwiatkowski, Torres et al. 2020), raising questions regarding the consequences of such acidification for marine life.

These rapid changes in seawater carbonate system and pH can impact directly or indirectly the physiology and behavior of marine organisms. First predictions considered most marine fish as quite resilient to the pH/pCO_2 values expected for 2100 since they are effective acid-base regulators (Regan, Turko et al. 2016). However, several effects associated to seawater acidification have been observed in some fish species notably on hatching rates and early development (Munday, Donelson et al. 2009, Frommel, Margulies et al. 2016, Pimentel, Faleiro et al. 2016, Stiasny, Sswat et al. 2019, Villalobos, Love et al. 2020, Baumann, Jones et al. 2022), sensory performances (Munday, Dixson et al. 2009, Munday, Dixson et al. 2010, Lai, Jutfelt et al. 2015, Porteus, Hubbard et al. 2018, Williams, Dittman et al. 2019) and reproduction (reviewed by Servili et al., 2020). The effects of ocean acidification on fish behavior remain controversial (Cattano, Fine et al. 2019, Clark, Raby et al. 2020, Munday, Dixson et al. 2020): some studies reported very strong alterations of fish behavior in response to ocean acidification (shelter use, Cattano et al. 2019) while others have stressed the lack of reproducibility of many previous studies on individuals avoidance of predator chemical cues, fish activity, and lateralization (Sundin, Amcoff et al. 2019, Clark, Raby et al. 2020). Irrespective of the heated debate concerning the effect of ocean acidification on fish behavior, a prolonged exposure to acidified water can impact the allostatic load of an organism since maintaining biological parameters within the physiological range in a suboptimal environment is energetically costly (Korte, Koolhaas et al. 2005). As a consequence, brain and other organs involved in the stress-coping response can alter their functioning and compromise individuals' ability to cope with additional stress and hence affect their other life history traits (survival, growth and reproductive success) and fitness (McEwen 2000, McEwen 2007).

The physiologic stress response in teleost fish is driven by the activation of two hormonal axes: the brain-sympathetic-chromaffin cells (BSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis. The BSC axis initiates the stress response through the rapid rise in plasma catecholamines (mostly epinephrine and norepinephrine) released by chromaffin cells, leading to increases in ventilation, branchial blood flow, gas exchange and plasma glucose concentration (Wendelaar Bonga 1997). The glucose is oxidized and used as fuel to respond to the increased energy demand associated to the stress factors. Several neurotransmitters, notably monoaminergic neurotransmitters (serotonin, dopamine and norepinephrine) play a central role in the modulation of the stress response in vertebrates (Winberg and Nilsson 1993). The HPI axis is responsible for the increase in levels of plasma glucocorticoids, mainly cortisol, and plays an important role in the reallocation and mobilization of energy under stressing condition (Balasch and Tort 2019). When the HPI axis is activated, the corticotropin-releasing hormone (CRH or CRF) is released from the hypothalamus and acts on the pituitary by increasing the adrenocorticotropic hormone secretion (ACTH), which in turns stimulates the release of cortisol from the interrenal cells (Wendelaar Bonga 1997). Cortisol exerts its action on multiple tissues (e.g. liver, brain, gonads, gills and immune cells) by binding to specific isoforms of glucorticoid receptors (notably GR1 and GR2) and one mineralocorticoid receptor (MR). After binding, the formed heterocomplex translocates to the nucleus, binds to the gluco- or melano-corticoid response element of the promotor gene and modulates the transcription of the target gene (Prunet, Sturm et al. 2006). In addition, a rapid non-genomic signaling mediated by membrane receptors is likely to play a role in the cortisol driven acute stress adaptation in teleosts (Das, Thraya et al. 2018, Aedo, Ruiz-Jarabo et al. 2019, Aedo, Aravena-Canales et al. 2021). Therefore, to better understand the impact of ocean acidification on fish life history traits, it is critical to evaluate its effects on the physiological stress response of fish.

A number of past studies have investigated specific traits related to the stress response of fish under acidification conditions. It has been reported an increase of anxiety under elevated CO_2 in larvae of barramundi (Rossi, Nagelkerken et al. 2015) and juveniles of yellowtail kingfish (Jarrold, Welch et al. 2020), Californian rockfish (Hamilton, Holcombe et al. 2014) and three-spined sticklebacks (Jutfelt, de Souza et al. 2013) using light/dark test, shelter test or novel object test. By contrast, ocean acidification did not impact the anxiety of juvenile Californian blacksmiths (Kwan, Hamilton et al. 2017). This variability of effects was also observed when the impact of elevated CO_2 on fish activity was tested. For instance, juvenile sea bass showed 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 (Dutil, 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).

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 confinement test. The respective pH/pCO_2 conditions of each experimental group were constantly maintained for 11 months. At 11 months, approximatively 100 fish per treatment remained from a series of samplings. The 64 fish used in the present experiment were randomly picked among them. They were transferred to tanks equipped with cameras and appropriated set up for the confinement test. Fish were sacrificed for sampling at different sampling time after the confinement stress. Mortality was constantly checked and no difference was observed between treatments. The same fish batch has been used in a previous study (Cominassi, Moyano et al. 2019).

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

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

The water of the rearing tanks consisted in natural seawater directly pumped from a depth of 20 m approximately at 500 m from the coastline in the Bay of Brest. Seawater was filtered (sand filter), heated (tungsten, Plate Heat Exchanger, Vicarb, Sweden), degassed, refiltered again (2µm membrane) and sterilized (UV lamp, PZ50, 75W, Ocene, France). Replicate treatment tanks consisted of 35 l flow-through tanks (n = 3) with 0.18 l min^{-1} of flow rate for larvae and of 670 l flow-through tanks (n = 2) with 8.4 l min^{-1} of flow rate for juvenile rearing per scenario.)During larval rearing, the water supply for the acidified scenario tanks came from a central header tank, where the water pCO_2 was regulated. The water pH was controlled by an IKS Aquastar system (iks Computer Systeme GmbH, Karlsbad Germany), which continuously measured pH in one of the replicate tanks and, when pH in this rearing tank became too high, it opened a magnetic valve to bubble CO_2 into the header tank. During juvenile rearing with higher water exchange rates, additional PVC columns were installed to control the pH in the rearing tanks of acidified scenario. The water arrived at the top of the column and was pumped from the bottom of the column to the rearing tanks. The CO_2 bubbling was installed at the bottom of the column and was adjusted by a flow control unit, when needed. The water pH of the Current group was not regulated and corresponded to the

pH of the natural seawater that was directly pumped from the Bay of Brest as described above. Water pH and temperature were daily monitored in all replicate tanks before feeding the fish by a WTW 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NBS scale). The pH meters were daily calibrated by using NBS certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany). For the current study, the rearing temperature was set at 15˚C. The photoperiod regime was also set to simulate the natural photoperiod of the Bay of Brest (Halogen lamp at 42W, 55-60 lux). The total alkalinity was measured once a week following the protocol described in Cominassi and collaborators (Cominassi, Moyano et al. 2019) adapted from the protocol of Anderson and Robinson (1946) and Strickland and Parsons (1972). Briefly, 50 ml of filtered seawater from tanks was mixed with 15 ml HCl (0.01 M) and pH (NSB scale) was immediately measured. Total alkalinity was calculated with the formula:

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

With: total alkalinity (TA), volume of HCl (VHCl, l) and of the sample (Vsample, l), HCl concentration (C, mol l-1), hydrogen activity (H+, 10-pH) and hydrogen activity coefficient (yH+ = 0.758). Seawater pH in free scale and pCO_2 was calculated using the Microsoft Excel macro CO2sys (Lewis 1998) with the constants after Mehrbach et al. (Mehrbach, Culberson et al. 1973) refit by Dickson et al. (Dickson and Millero 1987) (as cited in CO2sys). First pCO2 and fCO2 values (µatm) were calculated from measured pH values in NSB scale, total alkalinity (µmol/kgSW), temperature (°C), atmospheric pressure (dbar) and salinity (‰). Afterwards, free scale pH values were calculated with the same macro using pCO2 and fCO2 values.

2.2 Netting stress

At day 0 (the starting day of each netting stress), 16 fish of one experimental condition (Current or RCP8.5 condition groups) were transferred from the rearing tanks to four experimental tanks ($1m^3$ water volume, 4 fish per tank). Netting was realized with large fish nets enabling to quickly (less than 1 minute) capture simultaneously 4 fish. Fish were immediately transferred in one of the experimental tanks equipped with a wire-net cage at the bottom. Water conditions and fish density ($7kg/m^3$) were the same as the corresponding rearing tanks. The pH conditions of the experimental tanks was stable during the duration of the experiment. Immediately after the netting and transfer in the experimental tanks, wire-net cages of three tanks were partially lifted for 4 minutes to confine fish to a reduced volume of water and increase fish density (confinement test). The acute stress generated from the transfer of fish to experimental tanks and the confinement test is termed "netting stress" hereafter. The fish were then euthanized by ethylene glycol monophenyl ether (300 ppm by balneation; Merck; 807291; USA) at three different times: 30, 120 and 240 minutes after the beginning of the confinement test (one tank per time, n = 4 fish per time), and sampled (sampling points 't30', 't120', and 't240', respectively). The fish of the forth experimental tank were not submitted to the confinement test. They were let recovered from the transfer and netting for 24 hours (1440 minutes), and then sampled (sampling point termed 't1440' and considered at resting to obtain basal values, as shown by Fanouraki and collaborators in European sea bass after a comparable acute stress; Fanouraki, Mylonas et al., 2011). At each sampling point, blood was immediately sampled from the caudal vessel using heparinized syringes and plasma collected (within 5 minutes) and stored at -20°C until cortisol and glucose analyses. All euthanasia and blood samplings were realized within 5 minutes. Fish were weighted and brain and head kidney removed. Hypothalamus (diencephalon) was dissected from the brains. Experimented personnel, trained to identify and dissect the hypothalamus and head kidney using sterile tweezers and scissors, performed all dissections

within 3 minutes. All tissue samples were stored in RNAlater (Qiagen, Hilden, Germany) and placed at 4°C for 24 hours and then to -20°C until gene expression analyses, except for the hypothalami from 't120' which were frozen in liquid nitrogen and stored at -80°C until neurotransmitters analysis. This procedure was repeated twice for each treatment to obtain a total of 32 fish per experimental group (n = 8 per sampling point).

2.3 Plasma cortisol analysis

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

2.4 Plasma glucose analysis

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

2.5 Gene expression analysis

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

2.6 Reverse transcription and qPCR analysis

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

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

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

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

2.8 Behavioral tests

Just before the sampling at t120, ten minutes mp4 videos were recorded in all tanks still containing fish, i.e. tanks used for final sampling at 120 and 240 minutes post-stress (32 fish in total), with mini-dome cameras (HD 960P, 1.3 Mega pixels with backlight function, Sony) connected to a digital recorder (Trybride, AHD 8 channels, H264). The cameras were placed 1 meter above the tanks in a central position (one camera per tank), enabling to record the total surface of the tanks. All the cameras were connected to a remote control placed behind black curtains. The remote control was used by the personnel to start and end the recording without been perceived by fish. The videos, recorded at t120, were full-blind analyzed to determine whether there were any difference between experimental groups (pH/pCO_2 treatments). A grid pattern, composed of 12 squares of 4 cm^2, was used to analyze five

behavioral traits using the Noldus Observer software ®. The following behavioral variables were measured for each fish: total time spent without moving around ('stationary position'), total time spent moving slowly ('slow', square crossed in more than 3 sec) or total time spent moving rapidly ('fast', square crossed in less than 3 sec), total distance travelled calculated as number of squares crossed ('squares') and number of times the fish stayed alone ('alone' defined as the number of times the fish remained separated at least one square far from its congeners).

2.9 Statistical analysis

All statistical analyses were performed with R software (R Core Team, 2015). Differences between the two pCO_2/pH treatments and time points were tested using linear mixed models 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 and RCP8.5 condition groups had a clear increase in plasma cortisol at t30. The interaction term between the treatment group and sampling time was not statistically significant, but a non-significant trend (Chisq = 7.13, df = 3, p = 0.07) could suggest that fish of the RCP8.5 treatment had slightly higher cortisol levels at subsequent time points. The post-hoc test for this model indicated that plasma cortisol levels were significantly higher in RCP8.5 condition fish compared to the Current condition fish at 120 minutes post-stress (lsmeans posthoc testing t120 Current-RCP8.5 conditions: estimate = 132.32, SE = 41.20, df = 62.20, t = 3.21, p = 0.04). Cortisol concentration of Current condition group had returned to basal level (lsmeans posthoc testing Current group t1440-t120: estimate = -48.84, SE = 42.50, df = 62.20, t = -1.15, p = 0.94) while the one at RCP8.5 condition was still higher than basal concentration (lsmeans posthoc RCP8.5 group t1440-t120: estimate = -185.20, SE = 41.20, df = 62.20, t = -4.50, p < 0.01).

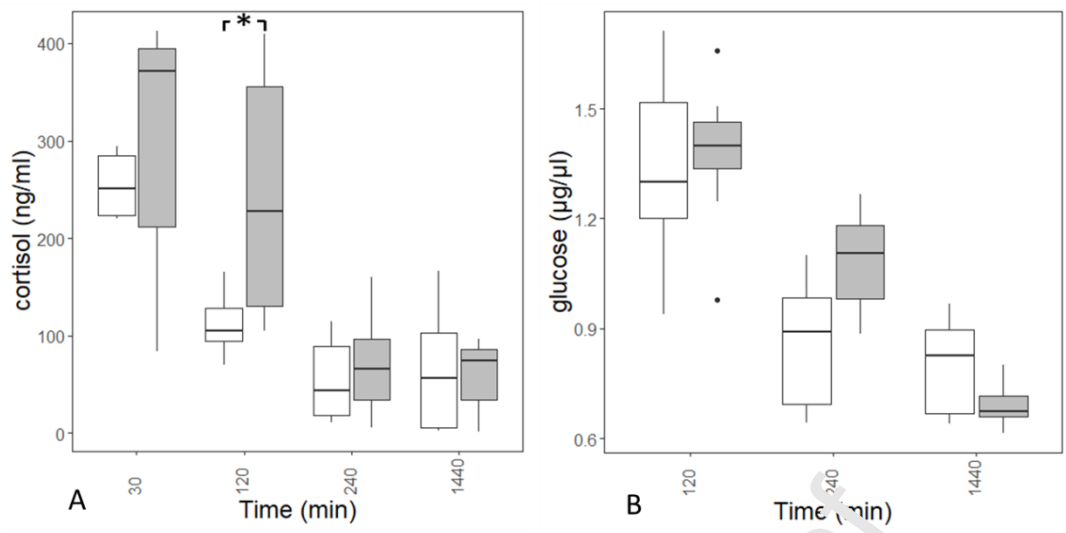


Figure 1: Box plots representing post stress levels at 30, 120 and 240 minutes and 24 hours (1440 minutes) of cortisol (A) and glucose (B) in sea bass plasma. White and grey blocks indicate values for sea bass acclimated at Current or RCP8.5 conditions, respectively. * indicates $p < 0.05$ between scenarios.

3.2 Plasma glucose concentration

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

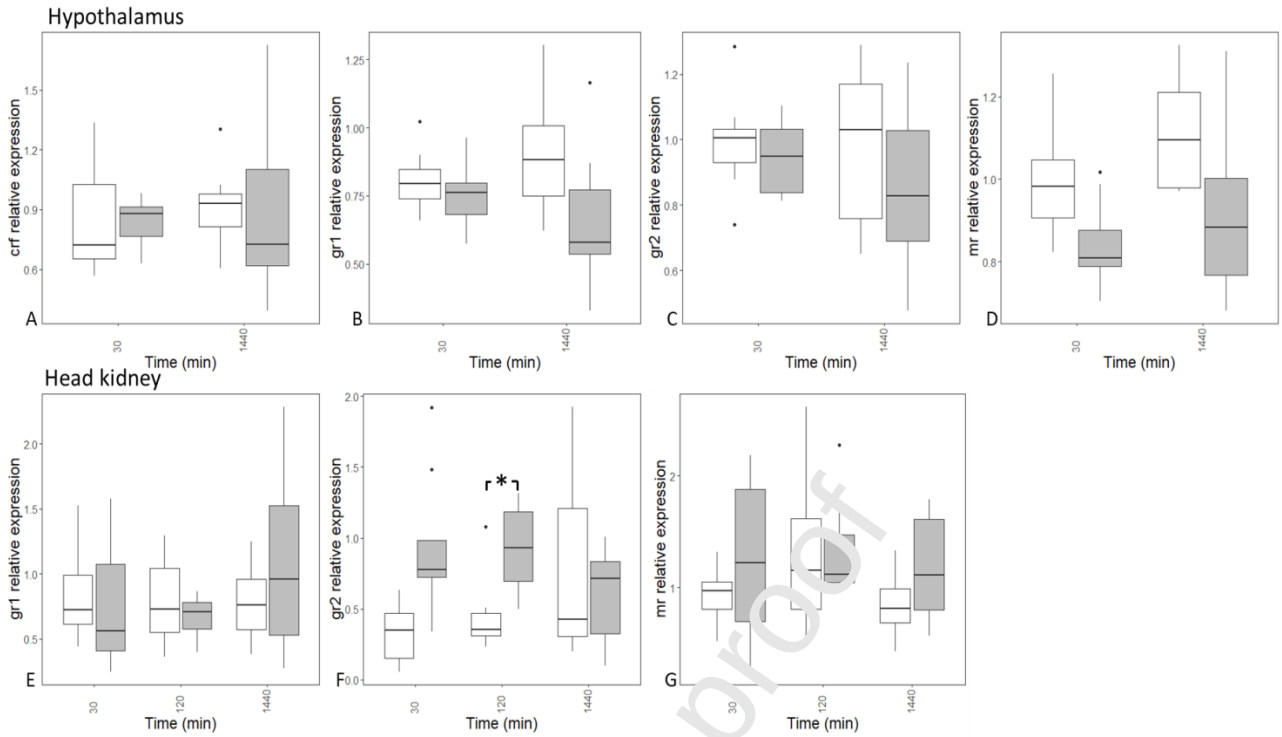


Figure 2: Gene expression levels for *crf* (A), *gr1* (B), *gr2* (C) and *mr* (D) in hypothalamus at 30 minutes and 24 hours (1440 minutes) post-stress. E, F and G show gene expression profiles for *gr1* (E), *gr2* (F) and *mr* (G) in head kidney in fish at 30 and 120 minutes and 24 hours (1440 minutes)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

3.4 Neurotransmitter quantification

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

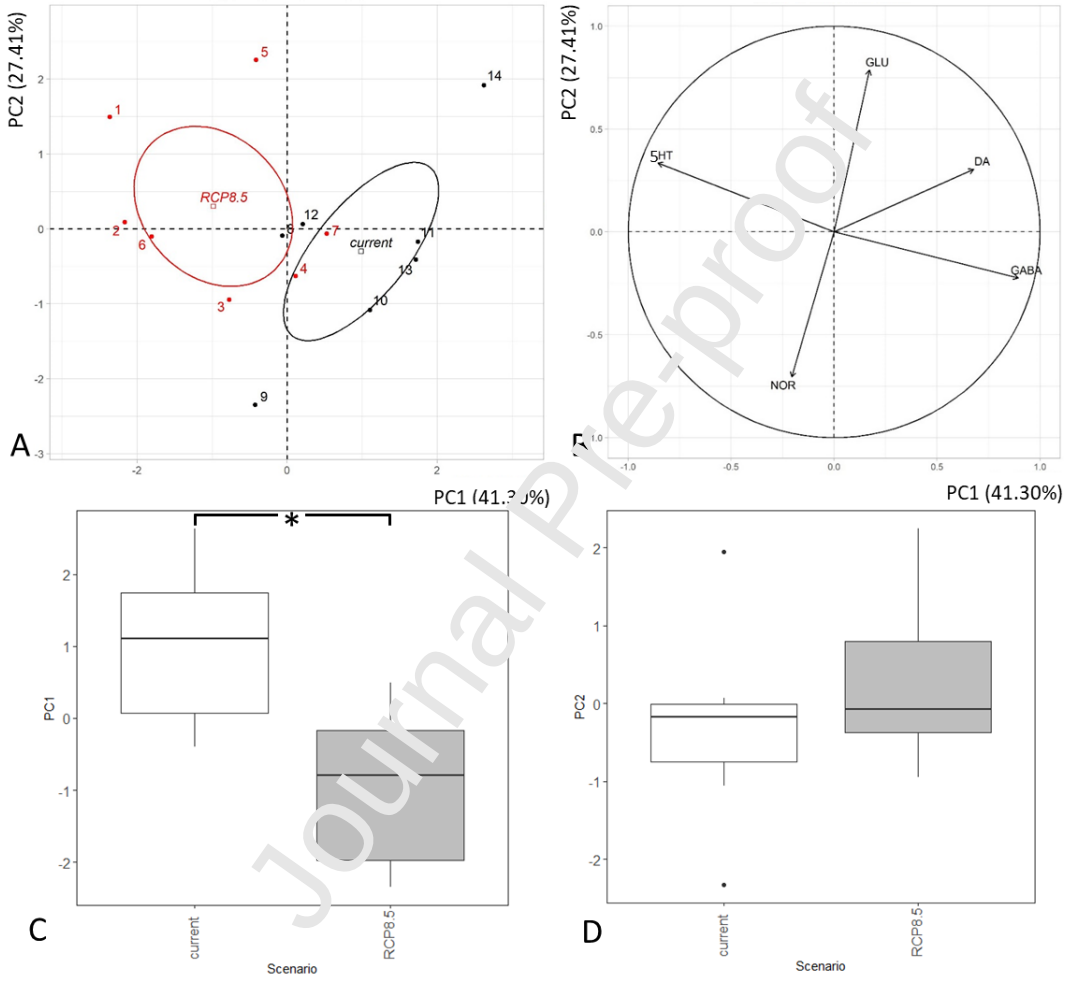


Figure 3. Principal component analysis of variability in neurotransmitters levels on the hypothalamus of sea bass acclimated to acidified (RCP8.5 in red) and present-day condition (Current, in black) at 120 minutes post netting stress. PCA loadings of individuals (dots in A) and variables (arrowheads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

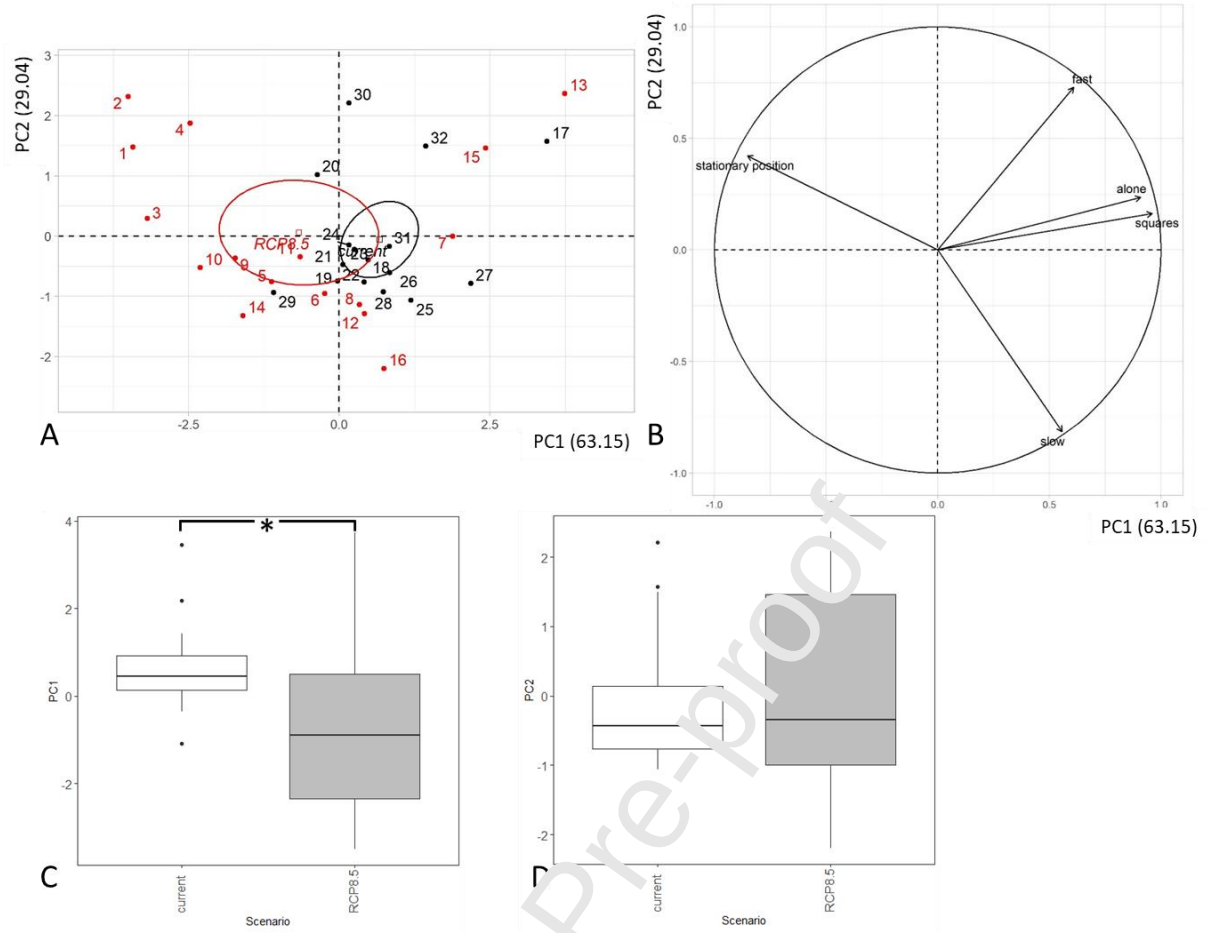


Figure 4. Principal component analysis of behavioral traits observed in sea bass acclimated to acidified (RCP8.5 in red) and present-day condition (Current, in black) at 120 minutes post netting stress. PCA loadings of individuals (dots in A) and variables (arrowheads in B) are represented in graphs 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.

4. Discussion

Juvenile European sea bass long term acclimated to acidified water as predicted by RCP8.5 scenario for 2100 exhibited modified physiological and behavioral responses to a netting stress (fish transfer and confinement test). European sea bass naturally shows an intense stress

response to acute stress with a substantial post-stress rise in plasma cortisol. The exposure to sustained stress can cause reproductive disruptions and disease outbreaks (Fanouraki, Mylonas et al. 2011). In our study, resting plasma cortisol concentrations were slightly higher than those previously reported (Cerdá-Reverter, Zanuy et al. 1998, Vazzana, Cammarata et al. 2002, Rotllant, Ruane et al. 2003, Marino, Di Marco et al. 2008) (10-50 ng ml^{-1}), but lower than the ones observed by Planas et al., 1990 (>200 ng ml^{-1}) likely reflecting differences in rearing conditions and handling. The maximal plasma cortisol levels were reached in our experiment at 30 minutes after the netting test with the mean value of 283.88 ng ml^{-1}. This is in line with the magnitude of cortisol rise observed in the same species at the same sampling point following a comparable stress (Samaras, Dimitroglou et al. 2016). However, it is possible that we missed the real peak of cortisol plasma rise if it occurred before 30 minutes or between 30 and 120 min post-stress, due to the chosen experimental design.

We found that acidification of the water did not affect basal or maximum values of plasma cortisol levels. However, our findings indicate a slower recovery from stress in fish acclimated to the RCP8.5 condition scenario with a return to the resting cortisol concentration at the t240 sampling point whereas Current condition group showed a return to the basal concentration already at t120. A slower recovery from stress in RCP8.5 condition scenario was confirmed by the plasma glucose post-stress kinetic. The delay of the glucose response to stress compared to the plasma cortisol kinetic is not surprising since glucose plasma concentration is considered a marker of the secondary, and thus slower, physiological response to stress in vertebrates (Barton 2002). The main effect of cortisol and glucose plasma rise is the energy reallocation to allow organisms to cope with the potential increase in energy demand in stressful events or environments. It's likely that RCP8.5 condition fish presented sustained cortisol and glucose concentration when facing the additional stress before restoring the homeostasis. Overall, our findings suggest that an additional acute stress reveals the

limited coping ability of fish when facing higher pCO_2 levels. More precisely, the reactivity and sensitivity of the corticotropic axis is not disrupted in acidified condition, but fish recovery might be slightly compromised. Similar delay in recovery is also observed in animals facing a chronic stress (Veissier, Boissy et al. 2001).

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

Gluco- and mineralocorticoid receptors expression levels were not regulated during stress recovery in sea bass in the interrenal tissue located in the head kidney. This is consistent with the interrenal response of the anadromous species Atlantic salmon to unpredictable chronic and acute stress, but this result is not in agreement with the upregulation of *gr1* reported in freshwater carp after a confinement test (Stolte, Nabuurs et al. 2008, Madaro, Olsen et al. 2015). Here, we found a slight trend of an upregulation of *gr* and *mr* expression levels in sea bass head kidney under the RCP8.5 condition scenario with statistical significance reached for *gr2* gene expression at 30 minutes post-stress. Overall, the potential increase in *gr* expression levels in the head kidney of fish under acidified conditions could be linked to the longer lasting high cortisol levels observed in the plasma and be interpreted as a further sign for a slower recovery from stress in RCP8.5 condition. However, the experimental design of the

present study did not allow to exclude that the slower return to basal level in RCP8.5 condition scenario was not due to a potential higher peak of cortisol and glucose at low pH which was not detectable with the tested sampling time.

A prolonged increase in cortisol plasma concentration in response to stress can have a number of metabolic consequences involving the activation of energy demanding pathways to restore homeostasis, such as the modulation of the carbohydrate metabolisms (gluconeogenesis), the increase in protein turnover, the regulation of amino acid metabolism and increase in lipolysis (Mommsen, Vijayan et al. 1999). Cortisol may also influence the regulation of the acid-base balance in fish and high cortisol levels could be beneficial in acidified waters, where an effective acid-base regulation is crucial for maintaining the homeostasis (Cruz, Chao et al. 2013, Kwong and Perry 2013, Tucker, Suski et al. 2018). However, the present study showed that fish of both scenarios presented, at the tested sampling times, comparable basal and post-stress cortisol plasma concentrations. Only the recovery from the acute stress seems to be slower in fish acclimated to high pCO_2 levels consistent with the hypothesis that acidification may alter the response of the corticotropic axis in fish.

We determined the profiles of main hypothalamic neurotransmitters during recovery (at 120 minutes post-stress) to better understand the effects of acidified water on the stress response of fish at central level. Interestingly, the two scenarios showed a divergent pattern with notably higher serotonin and lower GABA concentrations in the RCP8.5 condition fish. GABA is a strong inhibitory neurotransmitter in vertebrate. A decade ago, Nilsson and collaborators (Nilsson, Dixson et al. 2012) proposed the "GABA model" hypothesis to explain the mechanisms underlying the reported effect of ocean acidification on behavior and sensory performances in some fish species (Munday, Dixson et al. 2009, Dixson, Jennings et al. 2015, Lai, Jutfelt et al. 2015, Lai, Li et al. 2016, Munday, Watson et al. 2016). When facing increasing pCO_2 in the ranges predicted by IPCC scenarios, most fish react by retaining

additional HCO_3 in their blood (Esbaugh 2018). This is associated to changes in intracellular and extracellular ion concentrations that could interfere with the flux of ions passing through $GABA_A$ receptors after GABA binding. Consequently, GABA would exert a stimulatory role on the activity of GABAergic neurons under acidification condition. Interestingly, a similar stimulatory action of GABA is suggested also during post-stress recovery in rodents (Sarkar, Wakefield et al. 2011). If the described regulation is conserved in fish, one could expect that acute stress and water acidification would have a synergic effect on the GABA regulation of stress axis. The end result would be the robust excitatory action of GABA on CRF neuron activity and a potential sharp increase in plasma cortisol levels. However, in our study we observed higher plasma cortisol levels in acidification condition at 120 minutes post-stress, associated to lower hypothalamic GABA concentrations which is likely in contrast to the GABA model hypothesis. It is possible that this decrease in GABA concentration reflects the action of a negative feedback that is needed at the hypothalamic levels to inhibit CRF neurons activity and, consecutively, inhibit the release of cortisol from the interrenal tissue that is essential to restore the homeostasis.

Interestingly, a recent study used molecular approaches to test the direct effect of long term acidification on brain function in wild coral fish (Kang, Nagelkerken et al. 2022). The authors observed that cardiac β1-adrenergic receptor (ADRB1), which is involved in the stress response, was significantly expressed in fish exposed to elevated CO_2. This condition was also associated with a decreased gene expression of the GABAergic pathway (Kang, Nagelkerken et al. 2022). Similarly, both GABA and serotonin were shown to be differentially expressed in the brain (olfactory bulb) of coho salmon acclimated to acidified water at resting (Williams, Dittman et al. 2019). Altogether, this suggests an activation of the stress response, associated to a repression of GABAergic signaling, even in fish under ocean acidification that are not recovering from a further acute stress. Monoamines are one major

class of neuromodulators and their sensitivity to environmental factors, including acidification, has been documented (Libersat 2009, Paula, Repolho et al. 2019). It is therefore not surprising that there is a high variation in serotonin content in the brain of sea bass acclimated to different pCO_2 conditions. In teleost, serotonin acts stimulating CRF secretion in the hypothalamus resulting in the rise of the secretion of cortisol from the head kidney (Lim, Porteus et al. 2013). Similarly, the higher serotonin hypothalamic concentration in RCP8.5 condition fish, observed at 120 minutes post-stress in the present study, explains the sustained cortisol plasma level in this group.

Studies in salmonids suggests that serotonin is linked to an increase in motor activity, (Clements, Schreck et al. 2002, Clements, Moore et al. 2003, Carpenter, Watt et al. 2007). In the case of the European sea bass, during recovery from acute stress (t120), hypothalamic serotonin levels are increased in animals acclimated to acidification which exhibit a more static attitude (longer duration of stationary position, shorter duration of time moving around and less total distance travelled) compared to Current condition group. Thus, the relationship between serotonin and motor activity is apparently inverted in the case of sea bass compared with what has been reported in salmonids. However, it is worth noting that it is plausible that the interaction between serotonin, corticotropic axis and motor behavior may vary during post-stress recovery or long-term exposure to challenging environments (i. e. acidification).

The way an animal perceives, processes, and copes with stressful stimuli determines the magnitude of the physiological response to stress and is modulated by their behavioral coping style (Koolhaas, Korte et al. 1999). In general, individuals presenting proactive behavior type show active coping style with bold and aggressive score, high swimming activity and low behavioral flexibility (Øverli, Sørensen et al. 2007). Conversely, reactive animals are passive copers, with non-aggressive and cautious score, prefer the immobility response and show

flexible behavior immobility (Øverli, Sørensen et al. 2007). The physiological response to stress of proactive animals is associated to low reactivity of HPI axis, while reactive individuals show the opposite pattern. Individual-specific cortisol stress responses is shown to exist and to be a repeatable trait in European sea bass (Samaras, Dimitroglou et al. 2016). Even though more specific behavioral tests should be performed to validate this hypothesis, our data suggest that sea bass under RCP8.5 condition scenario recovering from an additional acute stress, adopt a more reactive coping style (higher cortisol and serotonin levels, freezing strategy and stationary attitude), whereas fish exposed to current condition would display a more proactive behavior coping style (lower plasma cortisol and hypothalamic serotonin content and higher motor activity). A number of previous studies have already examined potential changes in the activity and also anxiety, two traits related to stress response and individual coping style, in fish under elevated CO_2 conditions. Globally they reported contrasting effects in different fish species and stages, ranging from increased anxiety (Jutfelt, de Souza et al. 2013, Hamilton, Holcombe et al. 2014, Rossi, Nagelkerken et al. 2015, Jarrold, Welch et al. 2020) and lower motor activity scores (associated sometime with prolonged stationary behavior) (Porteus, Hubbard et al. 2018), to no effects (Duteil, Pope et al. 2016, Kwan, Hamilton et al. 2017, Andrade, Hurst et al. 2018), or even to increased boldness and swimming behavior in larval clowfish (Munday, Dixson et al. 2010). This discrepancy could be likely explained by a species specific sensitivity to acidification and/or by the different tests used to assess anxiety and activity scores. We should also keep in mind that these experiments reported short term effects of exposure to elevated CO_2 in fish that were not recovering from a further and acute stress. This makes difficult the comparison to the current study since neurotransmitters and activity were assessed during recovering from an acute stress in fish acclimated to long term acidification. Anyways, what it appears evident, from the present and past studies, is that both physiological and behavioral responsiveness to stress

would be plastic and that changing environments can modify the individual coping style of the animals.

5. Conclusions

Long term exposure to pH/pCO_2 conditions as predicted by IPCC RCP8.5 scenario for the end of the century impacts the physiological and motor behavioral responses to an acute stress in juvenile sea bass. Fish acclimated to RCP8.5 condition scenario showed slower post-stress return to basal concentrations of plasma cortisol and glucose. This is not associated to a clear central (hypothalamus) and interrenal (head kidney) regulation of gluco- and mineralocorticoid receptors and corticoid releasing factor expression levels. Acclimated sea bass to acidified water showed altered neurotransmitters' concentration pattern in the hypothalamus, at 120 minutes post-stress, with higher concentration of serotonin and lower levels of GABA and dopamine compared to the current condition scenario. At the same time post-stress, behavioral traits analysis revealed a reduction in motor activity in fish exposed to RCP8.5 conditions. Overall, these findings suggest that behavioral and physiological adaptive response to climate changes related constraints may impact fish resilience to further stressful events.

Competing interests

The authors have no competing interests to declare.

CRediT author statement

Arianna Servili: conceptualization, methodology, formal analysis, project administration, funding acquisition, writing (original draft). Etienne Lévêque: investigation, data curation.

Olivier Mouchel: methodology, data curation, writing (review and edits). Jimmy Devergne: formal analysis, writing (review and edits). Christophe Lebigre: methodology, validation, writing (review and edits). Sabine Roussel: resources, methodology, validation, writing (review and edits). David Mazurais: validation, writing (review and edits). José-Luis Zambonino Infante: writing (review and edits).

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Data availability

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

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

A

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

B

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

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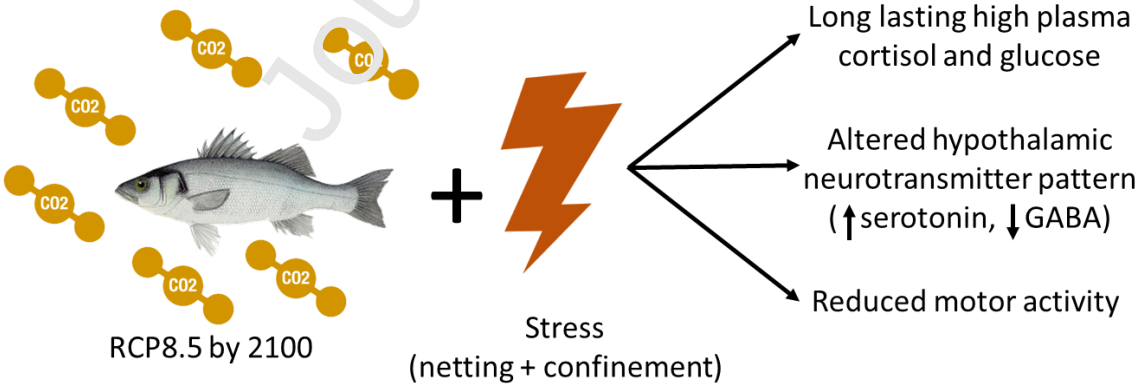
Graphical abstract:



Highlights

- Ocean acidification (OA) impacts the physiological stress response of European sea bass
- Post-stress return to basal plasma cortisol and glucose levels is delayed under OA
- This delay is associated to alteration of hypothalamic neurotransmitters pattern
- Motor activity is reduced during recovery from stress in fish under OA conditions