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► **To cite this version:**

Bastien Sadoul, Sébastien Alfonso, Xavier Cousin, Patrick Prunet, Marie-Laure Bégout, et al.. Global assessment of the response to chronic stress in European sea bass. *Aquaculture*, 2021, 544, pp.737072. 10.1016/j.aquaculture.2021.737072 . hal-03275735

**HAL Id: hal-03275735**

**<https://hal.inrae.fr/hal-03275735v1>**

Submitted on 2 Aug 2023

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# 1 **Global assessment of the response to chronic stress in** 2 **European sea bass**

3

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17

## 18 **Highlights**

- 19 • Stress load following a chronic stress protocol was assessed in European sea  
20 bass
- 21 • Our broad approach shows that osmoregulatory functions are the most  
22 sensitive
- 23 • Measures taken individually can be misleading when evaluating welfare in  
24 aquaculture
- 25 • Multiple endpoints are needed to properly assess health and welfare in  
26 aquaculture

27

## 28 **Abstract**

29 Stress modifies energy allocation in fishes by redirecting energy from growth and  
30 reproduction to coping mechanisms. However, these adjustments become  
31 inappropriate when the challenge consists of sustained or repeated stressors, with the

32 animal entering a maladaptive state. Capacities to cope with additional threats are  
33 then altered and compromise survival. The characterization of the responses to  
34 chronic stress in fishes helps better understanding the physiological limits in an  
35 aquaculture or ecological context. Here, we investigate the coping capacities of  
36 European sea bass to multiple and diverse stressors applied over a 3-weeks period.  
37 Multiple behavioural (group dispersion and swimming activity) and physiological  
38 responses (blood cortisol, osmoregulatory mechanisms, stress-related gene  
39 expression, etc.) were evaluated in resting fish or in fish exposed to additional  
40 challenges. Resilience to the chronic stress protocol was evaluated 4 months after the  
41 end of the chronic stress. Chronically stressed individuals showed reduced growth,  
42 lower cortisol response, increased chloride and sodium concentration in the plasma  
43 and modified gill gene expression translating osmoregulatory dysfunctions. Chronic  
44 stress had no significant effect on plasmatic calcium, lysozyme concentration and  
45 osmotic pressure. Increased thigmotaxic behaviour was observed in a new  
46 environment behavioural test. Four months after the chronic stress, no significant  
47 difference was observed in growth performances and in plasma parameters.  
48 Altogether, gills and more generally osmoregulatory functions were found to be the  
49 most sensitive to the chronic stress, while only limited changes in growth, activity of  
50 the HPI axis, immunity and swimming behaviour were observed when assessed  
51 individually. This work demonstrates the necessity of using multiple and diverse  
52 endpoints related to different functions to properly assess health and welfare in fishes.

53

#### 54 **Keywords**

55 Welfare, Plasticity, Fish, Osmoregulation, Robustness, Resilience

56

#### 57 **1. Introduction**

58 Welfare is gaining increasing attention in fish research with the rise of societal  
59 concerns regarding fishing methods, aquaculture practices and slaughtering  
60 techniques (Browman et al., 2019; Sneddon et al., 2016). In aquaculture, fish can be  
61 exposed to physical stressors (e.g. confinement, handling), or low water qualities all  
62 potentially altering fish welfare (Sanahuja et al., 2020; Sneddon et al., 2016; Toni et  
63 al., 2019). Most of welfare assessment are related to stress responses, experience of  
64 pain, growth problems, incidence of disease or abnormal behaviour (Sneddon et al.,  
65 2016; Stevens et al., 2017; Toni et al., 2019; van de Vis et al., 2020). Detection and

66 assessment of poor welfare conditions have benefited from outcomes of multiple  
67 research studies over the past years on physiological and behavioural responses to  
68 acute or chronic stressors, and has led to the development of numerous animal-based  
69 physiological and behavioural indicators mostly linked to stress responses  
70 (Huntingford et al., 2006; Martins et al., 2012; Noble et al., 2018; Sadoul et al., 2014;  
71 Stien et al., 2020).

72 Stress in fish is classically defined as “the physiological cascade of events that occurs  
73 when the organism is attempting to resist death or re-establish homeostatic norms in  
74 face of insults”(Schreck, 2000). Stress responses have been classified in fish as  
75 primary, secondary and tertiary for which numerous studies have been dedicated  
76 (Barton, 2002; Schreck and Tort, 2016; Wendelaar Bonga, 1997). Activation of  
77 endocrine pathways, i.e. the hypothalamo-sympathetic and the hypothalamus-  
78 pituitary-interrenal (HPI) axes, constitutes the primary responses to stressors (Barton,  
79 2002; Gorissen and Flik, 2016), and help reallocating energy for downstream systems  
80 involved in the secondary stress responses (Sadoul and Vijayan, 2016). These  
81 secondary responses involve cardiovascular and respiratory responses as  
82 consequences of hormone rises (Barton, 2002). Osmoregulatory modifications are  
83 also observed as body fluid homeostasis is regulated by catecholamine and cortisol  
84 which act on the gill ion transports and blood circulation at the level of gill lamellae  
85 (Takei and Hwang, 2016). Tertiary responses refer to aspects of whole-animal  
86 performance and are generally maladaptative; they include not only changes in  
87 growth but also in cognition, learning and behaviour such as swimming capacity and  
88 modified behavioural patterns (feeding, aggression) (Noakes and Jones, 2016;  
89 Wendelaar Bonga, 1997).

90 All mechanisms involved from the primary to the tertiary stress response can be  
91 integrated in a generic framework describing consequences on energetic trade-offs.  
92 Primary and secondary stress responses tend to increase the energetic cost for  
93 maintenance, while the tertiary response reduces the capacities to assimilate energy.  
94 Consequently, due to limited available energy, long term stress inevitably reduces  
95 energy allocated towards growth, maturity and reproduction (Sadoul and Vijayan,  
96 2016). The Dynamic Energy Budget (DEB) theory has been demonstrated to properly  
97 describe energy allocations towards growth, maturation and maintenance throughout  
98 the life cycle of many species including fish species (Marques et al., 2018; Sadoul and  
99 Vijayan, 2016). Based on longitudinal body mass and length data, the DEB model can

100 provide estimations of the energetic trade-offs related to environmental perturbations  
101 (Kooijman, 2010). Such an approach can help bridging data obtained at the cellular  
102 level and life history traits at the individual level and providing biological pertinence  
103 across all levels of organization.

104 During the last decade, effects of environmental or physical chronic stressors on fish  
105 biology have been evaluated in various fish species through studies focusing on the  
106 HPI axis and the neuroendocrine regulation of the stress response including the  
107 serotonergic system (Höglund et al., 2020; Madaro et al., 2016, 2015; Moltesen et  
108 al., 2016; Pavlidis et al., 2015; Samaras et al., 2018; Vindas et al., 2016). In addition,  
109 several authors were also interested in effects of chronic stressors on tertiary stress  
110 responses, including growth and metabolism, immune response or behaviour  
111 (Carbonara et al., 2019; Martos-Sitcha et al., 2019; Mateus et al., 2017; Millot et al.,  
112 2010; Person-Le Ruyet et al., 2008; Piato et al., 2011; Rambo et al., 2017; Santos et  
113 al., 2010; Uren Webster et al., 2018). Overall, these studies highlight the difficulty we  
114 have to evaluate resistance to chronic stress as it involves multiple physiological and  
115 behavioural regulatory mechanisms which vary depending on the fish species or the  
116 nature of the chronic stressor (Balasch and Tort, 2019). In this context, assessment of  
117 fish health and welfare in farmed animals exposed to chronic stress condition is still a  
118 challenging issue which deserves more studies.

119

120 In the present study, we used one-year old juvenile European sea bass, *Dicentrarchus*  
121 *labrax*, a marine species of ecological and commercial importance in Europe  
122 (Vandeputte et al., 2019) to develop a global assessment of the effects of chronic  
123 stress on fish welfare. This study aims to test whether a chronic stress protocol has  
124 clear consequences on some physiological and behavioural responses allowing  
125 establishment of reliable biomarkers of welfare in aquaculture. We made the  
126 hypothesis that gill-related functions are more sensitive to chronic stress in a  
127 euryhaline fish such as the European sea bass. In nature, the species lives in coastal  
128 waters mostly of the eastern Atlantic Ocean and the Mediterranean Sea and can be  
129 exposed to a wide range of salinities during its life cycle (Pickett and Pawson, 1994).  
130 The species is also one of the most cultured finfish species in the Mediterranean Sea,  
131 but is known to show intense and high physiological and behavioural responses to  
132 stress (Fanouraki et al., 2011; Millot et al., 2014). Nevertheless, our capacity to  
133 provide robust biomarkers of chronic stress in this species is still limited. This is

134 particularly relevant in the context of increasing societal expectations regarding  
135 rearing conditions (Toni et al., 2019).

136

## 137 **2. Material and Methods**

138

### 139 *2.1. Rearing conditions*

140 All experiments were performed at the experimental research station of Ifremer  
141 Palavas-Les-Flots. Experiments were authorized by ethics committee agreement  
142 APAFIS#10745 and all procedures involving animals were in accordance with the  
143 ethical standards of the institution and followed the recommendations of Directive  
144 2010/63/EU.

145 European sea bass eggs (*Dicentrarchus labrax* from West Mediterranean population)  
146 were obtained from 10 females and *in vitro* fertilized with the frozen sperm of 13  
147 males using a full factorial crossing method. Eggs and larvae were then reared  
148 following previously optimized standards for European sea bass (Chatain, 1994).  
149 Briefly, eggs were reared at 13 $\pm$ 3°C degrees in 9 different tanks until hatching.  
150 Temperature was then set at 15 degrees. Larvae were reared following previously  
151 optimized standards for European sea bass in 9 different tanks with the exact same  
152 rearing conditions. At 121 days post fertilization (dpf), random subpopulations of 600  
153 fish per tank were transferred in 9 larger rearing tanks and reared at 21°C. At 175 $\pm$ 3  
154 dpf, a subpopulation of 2025 fish was individually tagged using PIT tags and  
155 randomly distributed in three 1.5 m<sup>3</sup> tanks under anesthesia, as described in (Alfonso  
156 et al., 2019b). The fish were then monitored over time for growth and for their  
157 response to chronic stress through multiple physiological and behavioural tests as  
158 described below and illustrated in Fig. 1.

159 Over the entire experiment, rearing densities were below 40 kg/m<sup>3</sup> considered as an  
160 intermediate density in recirculating system and shown to have no effect on fish stress  
161 level nor growth (Sammouth et al., 2009).

162

### 163 *2.2. Chronic stress protocol*

164 At 309 dpf, fish from all 3 tanks were evenly distributed in 6 experimental tanks of 1  
165 m<sup>3</sup> (n=291 fish per tank). Mean body mass was 41.1 g ( $\pm$ 13.5SD). All experimental  
166 tanks were isolated with opaque plastic curtains ensuring independency between tanks  
167 and avoiding external rearing routine disturbances. Fish were fed using an automatic

168 feeder delivering 20 portions over 6 h. The delivered food was readjusted every 3  
169 days in order to make sure that fish were fed ad libitum: until uneaten pellets were  
170 observable at the bottom of the tank. These uneaten pellets were removed daily.

171 At 336 dpf, and for a period of 3 weeks, the following stressors reflecting common  
172 aquaculture practices were applied on 3 tanks :

- 173 - Every day, 6 randomly programmed one minute flash of light (including 3  
174 overnight),
- 175 - Every week, 3 randomly planned one minute chasing with a net,
- 176 - Every week, 3 randomly planned thirty minutes confinement stress obtained  
177 by reducing initial tank volume to  $\frac{1}{4}$  .

178 All stressors were chosen because they potentially regularly occur in aquaculture  
179 practices. The chasing and confinement stressors were selected based on previous  
180 studies, showing that they induce acute stress responses (Karakatsouli et al., 2012;  
181 Athanasios Samaras et al., 2016). The one minute flash light was identified as a  
182 potential strong stressor based on the intense behavioural response we observed. They  
183 were all randomly programmed in order to avoid predictability shown to reduce the  
184 stress load (Cerqueira et al., 2020). Similar random repeated stressors have already  
185 been shown to induce chronic stress in seabass (Samaras et al. 2018).

186 No stress regime was applied to the three other tanks over the 3 weeks. Although all  
187 in the same room and supplied with the same water, the three tanks used for the  
188 chronic stress protocol were placed as far as possible from the control tanks in order  
189 to avoid disturbing the control tanks when performing the planned stressors.

190

### 191 *2.3. Biometrics*

192 A total of 6 biometrics were performed from the tagging to the start of the chronic  
193 stressor protocol (175, 207, 233, 256, 289 and  $336 \pm 4$  dpf) (see figure 1). This  
194 consisted in anesthetizing the fish in the rearing tank using  $15 \text{ mg.L}^{-1}$  Benzocaine  
195 (benzocaine E1501, Sigma, Saint Louis, MO, USA) to reach loss of equilibrium  
196 (Stage I of anesthesia), transferring them with a net in a 80L oxygen-aerated tank filled  
197 with rearing water and  $37.5 \text{ mg.L}^{-1}$  Benzocaine to provoke stage II of anesthesia  
198 (Iwama et al., 1989), reading the tag and measuring the fork length and body mass of  
199 each fish using computer connected ruler and scale. Each fish spent less than 30  
200 seconds out of the water for each biometry.

201 At the end of the three weeks of chronic stress, only sampled fish (see below) were  
202 measured for body mass and fork length. Two more biometries were performed on all  
203 fish 66 and 105 days after the end of the chronic stress protocol. Fish were fasted for  
204 24 hours prior each biometry.

205

#### 206 *2.4.Sampling protocols*

207 Prior each sampling, an algorithm was used to randomly assign each fish of the tank  
208 to a specific treatment based on the number of fish required for each protocol (see  
209 below). All samplings were performed in a random order by experimenters blind to  
210 treatments.

211 Right after the end of the three weeks of chronic stress, one tank of the control  
212 treatment and one tank of the chronic stress treatment were fasted for 24 h. Fish were  
213 then slightly anesthetized (Stage I of anesthesia) with Benzocaine ( $15 \text{ mg.L}^{-1}$ ) in their  
214 home tank, simultaneously fished in both tanks and fully anesthetized (Stage II)  
215 with  $37.5 \text{ mg Benzocaine.L}^{-1}$ . They were then identified based on their RFID tag and  
216 accordingly dispatched in order to perform one of the following 6 protocols:

217 - Protocol 1 (P1): A total of 18 individuals per treatment (36 fish in total) were  
218 euthanized in high dose of benzocaine ( $225 \text{ mg.L}^{-1}$ ) immediately after  
219 dispatching. Blood was collected from the caudal vein using a heparinized  
220 syringe. The brain, the pituitary and the head kidney were extracted and  
221 immediately frozen in liquid nitrogen. The gills of 12 individuals per  
222 treatment were also collected and frozen in liquid nitrogen.

223 - Protocol 2 (P2): A total of 18 fish per treatment were first allowed to rest in  
224 80L tank filled with clear water for 20 minutes. They were then exposed to an  
225 acute stress test (AST) consisting in confining the fish for 4 minutes in a 10 L  
226 aerated bucket. The fish were then allowed to recover from the stressor for one  
227 hour in a 100 L tank supplied with water renewed twice per hour. Fish were  
228 then euthanized in  $225 \text{ mg benzocaine.L}^{-1}$  and blood was collected.

229 - Protocol 3 (P3): A total of 18 fish per treatment were sampled following the  
230 same procedure as described for P2 but allowing the fish to recover during 3  
231 hours in another identical 100 L tank.

232 - Protocol 4 (P4): The same procedure as P3 was also performed on 18 fish per  
233 treatment but with a 6 hours recovery period.



- 234 - Protocol 5 (P5): A total of 12-13 fish per treatment were exposed to a 24 hours  
235 osmotic challenge (OC) consisting in transferring the fish directly in  
236 freshwater (Bossus et al., 2011; Masroor et al., 2019) individually in a 10 L  
237 aquarium supplied with freshwater at 21°C. Full water volume was renewed  
238 over an hour. Fish were then euthanized in high dose of benzocaine (225mg.L<sup>-1</sup>),  
239 blood collected and gills dissected and frozen in liquid nitrogen.  
240 - The other fish were put back in the tank for future analyses.

241 Protocols P1 to P4 aim at describing the acute confinement stress response of each  
242 condition, while P5 allows to investigate stress response and homeostatic capacities to  
243 extreme conditions. The dispatching process among protocols took 15 minutes after  
244 fish were anesthetized (Stage I of anesthesia). In the first sampled fish (P1), blood  
245 was collected within 15 minutes following end of dispatch, hereafter called ‘fish  
246 sampled after sorting and dispatching’ (see results section, Fig. 3). The same  
247 protocols were performed over the next two days on the remaining tanks, by testing  
248 each day one tank of each treatment. All euthanized fish were measured for their  
249 length and body mass, and sexed. The number of 18 fish sampled per tank in  
250 protocols P1 to P4 was chosen to be sufficient to account for sex differences. Since  
251 proportion of females in our population was unknown and suspected to range from 25  
252 to 50% (Vandeputte et al., 2020), sampling numbers were increased to get sufficient  
253 females. In P5, gills were sampled on reduced number of individuals (12 fish per  
254 tank) due to the cumbersome and cost of procedures.

255 The remaining fish were then mixed and transferred in three new tanks making sure  
256 each treatment was equally represented in each tank. Four months after the chronic  
257 stress (478+/-1 dpf), protocols P1, P2 and P3 were applied on 12 fish per protocol and  
258 tank-replicate. The reduced number of fish sampled is explained by the unbiased sex-  
259 ratio in the population (54.1%) observed after the first experiment and increasing the  
260 probability of getting sufficient numbers of females and males.

261

## 262 *2.5.Plasmatic measurements*

263 After collection, blood was immediately centrifuged at 13000 rcf for 4 minutes. An  
264 aliquot was frozen at -80°C for analysis of lysozyme, and ion concentrations whereas  
265 another aliquot was frozen at -20°C for further cortisol analysis.

266

### 267 *2.5.1. Cortisol measurement using ELISA*

268 Cortisol is the major stress hormone in fishes (Sadoul and Geffroy, 2019). It was  
269 therefore quantified from plasma samples following a slightly modified competitive  
270 ELISA assay protocol previously described (Faught et al., 2016). The assay was  
271 performed by an experimenter blind to the treatment and the plasma were placed in a  
272 random order on the plates. Briefly, 96-well plates were coated for 16 hours at 4°C  
273 with a cortisol monoclonal antibody (1.6 µg.mL<sup>-1</sup>; East Coast Bio, ME, USA) diluted  
274 in PBS. Standards ranging from 0 to 25 ng.mL<sup>-1</sup> were obtained by diluting  
275 hydrocortisone (Sigma). Cortisol conjugated to horseradish peroxidase (East Coast  
276 Bio, ME, USA) diluted in PBS (1:1600 dilution) was added to aliquots of standards  
277 and samples (1 to 1 ratio). All resulting mixes were then distributed (100 µL) in  
278 duplicates in the 96-well coated plate, and the plate was incubated at room  
279 temperature for 2 hours. After washing the plate three times with PBS-tween, each  
280 well was filled with a detection solution (50 µL) for 30 minutes and the reaction was  
281 stopped using 1M sulfuric acid (50 µL). Absorbance was read at 450 nm (EL800  
282 Universal Microplate Reader, BIO-TEK INSTRUMENTS, INC.).

283 Eighteen samples were used to check for ELISA assay validity. Results obtained with  
284 the ELISA assay were compared with results obtained using liquid chromatography  
285 tandem mass spectrometry (LC-MS/MS) (Dufour-Rainfray et al., 2015). The  
286 correlation between the measures obtained using both techniques rendered a R<sup>2</sup> of 0.9  
287 with intercept 0 but a slope of 2, indicating that concentrations measured in ELISA  
288 were always overestimated by a factor 2. LC-MS/MS is an expensive method  
289 requiring heavy equipment and extensive maintenance, but is considered to be the  
290 reference method for absolute quantification of steroids. We used that technic to  
291 validate the ELISA measures. Consequently, all ELISA measures were divided by 2.

292

### 293 2.5.2. Lysozyme concentration

294 Lysozyme is released by the non-humoral defence system to protect from bacteria and  
295 was shown to be affected by stressful conditions (Demers and Bayne, 1997). Plasma  
296 lysozyme activity was determined using a turbimetric assay following a previously  
297 published protocol (Doux fils et al., 2012). Briefly, 20 µl of plasma was mixed with  
298 160 µl of *Micrococcus lysodeikticus* (Sigma) solution (1.25 mg.mL<sup>-1</sup> 0.05M sodium  
299 phosphate buffer, pH 6.2). Absorbance was measured at 450 nm every 3min during  
300 30 min at 25°C (Synergy2, BioTek Instruments, France). Using a standard lysozyme

301 chloride from chicken egg white (Sigma) in sodium phosphate buffer, the lysozyme  
302 concentration in the plasma was expressed in U.mL<sup>-1</sup>.

303

304 2.5.3. Plasmatic measures of sodium, calcium and chloride concentrations and  
305 osmotic pressure

306 Maintenance of hydromineral balance is generally assessed using the main plasmatic  
307 ion concentrations and osmotic pressure (McCormick, 2001). Plasma sodium was  
308 analysed using flame atomic absorption spectrophotometry (Varian AA240FS,  
309 Agilent Technologies, Massy, France). Plasma concentrations of chloride and calcium  
310 were measured using colorimetric kits (chloride with a mercuric-thiocyanate method  
311 and calcium with Arsenazo III (Biolabo, France)), following manufacturers  
312 recommendations. Absorbance was measured with the micro plate reader Synergy2  
313 (BioTek instruments, France). Osmotic pressure was measured with a Wescor Vapor  
314 Pressure Osmometer (Model 5500; Wescor Inc., Logan, UT, USA).

315

316 2.6. *Behavioural test*: Novel environment and hypoxia test in group

317

318

319 The novel environment in group was adapted from (Alfonso et al., 2020) and shown  
320 to be sensitive to environmental perturbations (Alfonso et al., 2020). Briefly, at 422,  
321 424 and 425 dpf (2 months after the end of the chronic stress protocol), two groups of  
322  $8 \pm 1$  fish from each treatment were transferred in 4 different tanks. After one night, the  
323 group of fish (either group of control or group of stressed fish) was placed in a 1 m<sup>2</sup>  
324 observation tank (75cm x 75cm x 21.5 cm of water height, 120 L) for measuring  
325 behavioural response to a new environment. A corner (15x15 cm, 225 cm<sup>2</sup>) of the  
326 tank was separated from the open field arena by a grid and contained a pump, oxygen  
327 and nitrogen aerators to maintain targeted oxygen concentration and an oxygen probe  
328 (Odéon, NEOTEK-PONSEL, Caudan, France) to record oxygen saturation every  
329 minute all along the experiment. After 1 hour in the new environment, oxygen  
330 saturation was reduced over 20 minutes using nitrogen bubbling, in order to reach a  
331 saturation around 20%. This hypoxic period lasted 40 minutes. Fish behaviour was  
332 recored over the whole duration of the test for a total of 2 hours using a DMK  
333 31AU03 camera and IC Capture software (The Imaging Source, Germany) at 25  
334 frames.s<sup>-1</sup>. Data extraction and analyses were performed using EthoVision XT 13.1

335 software (Noldus, The Netherlands). Swaps between individuals were manually  
336 corrected using the track editor module. For behavioural analyses, the arena was  
337 virtually separated into two areas: the centre area composed of one half of the surface  
338 and the periphery area including the other half; time spent in periphery (s), indicative  
339 of thigmotaxis behaviour, was recorded. The velocity of each fish ( $\text{cm}\cdot\text{s}^{-1}$ ), indicative  
340 of individual fish activity, and the interindividual distances (cm), indicative of group  
341 cohesion (Alfonso et al., 2020), were also assessed. Variables were averaged over 1  
342 minute every 10 minutes in order to record the kinetics of behavioural responses.

343

## 344 2.7. Gene expression analyses

### 345 2.7.1. RNA extraction

346 All samples used for gene expression analyses were stored at  $-80^{\circ}\text{C}$  until RNA  
347 extraction. Brain, pituitary and head kidneys samples were grinded using 2 ball mills  
348 (45 seconds at 30 rpm) in 500  $\mu\text{L}$  MR1 and 1  $\mu\text{L}$  of TCEP from NucleoMag® RNA  
349 extraction kit (Macherey-Nagel). Samples were then centrifugated at 13000 rpm for  
350 two minutes and a sub-sample of 200  $\mu\text{L}$  was collected, and diluted in 150  $\mu\text{L}$  of MR1  
351 in a 96 deep-wells plate. The plate was then placed in the KingFisher automatic  
352 extraction robot to perform the extraction protocol following manufacturer's  
353 instructions for the NucleoMag® RNA kits.

354 The gills were extracted using Trizol according to manufacturer's recommendations  
355 (Invitrogen, Carlsbad, CA, USA).

356

### 357 2.7.2. RNA integrity

358 Twelve samples per extraction plate were analysed for RNA integrity on the 2100  
359 bioanalyzer® (Agilent) following manufacturer's instructions. All samples had a RIN  
360 above 8.

361

### 362 2.7.3. Reverse transcription

363 RNA quantities were estimated by measuring the A260/A280 ratio with the  
364 NanoDrop® ND-1000 V3300 Spectrophotometer (Nanodrop Technology Inc.,  
365 Wilmington, DE, USA).

366 Reverse transcriptions were performed on RNA extracted from the brains, gills, and  
367 head kidneys following a previously published protocol (Kiilerich et al., 2018).  
368 Briefly, 1  $\mu\text{g}$  RNA was treated with 0.5 units RQ1 DNase (Promega, Madison, WI,

369 USA) for 30 min at 37°C in a total volume of 21 µl. DNase was inactivated by 5 min  
370 at 75°C. Reverse transcription was carried out with the following Promega chemicals:  
371 1 µg random primers, 200 units MMLV reverse transcriptase, 0.5 mM dNTPs and 25  
372 units RNasin RNase inhibitor. The mix was incubated for 1 h at 37°C in a total  
373 volume of 25 µl. cDNA samples were diluted 43 times and stored at -20°C until real-  
374 time PCR (qPCR).

375 Total RNA extracted from pituitaries were below 1 µg, and we therefore used the  
376 Qscript cDNA supermix kit® (Quantabio) able to extract on lower RNA amounts.  
377 cDNA from pituitaries were diluted 10 times and stored at -20°C until real-time PCR  
378 (qPCR).

379

#### 380 2.7.4. Primers selection

381 Primers were either obtained from the literature (Table 1) or specifically designed for  
382 this study (Table 2). Primer designs were performed using the PerlPrimer software  
383 (Marshall, 2004) which enables to target exon-exon junctions when the mRNA and  
384 the genomic sequences are available which is the case for European Sea bass (Tine et  
385 al., 2014). They were designed to target expression of:

386 - the main genes of the HPI axis (*crf*, *crfbp*, *pomcb*, *star*, *mc2r*, *hsd11b2*, *cyp11a2*,  
387 *cyp11b1 mr*, *gr1*, *gr2*)

388 - genes coding for cell proliferation and neural differentiation and known to covary  
389 with the stress axis (Sadoul et al., 2018) (*egr1*, *pcna*, *neurod1*, *neurod2*, *bdnf*),

390 - one key genes related to the appetite and known to be affected by stress (Sadoul and  
391 Vijayan, 2016) (*npv*)

392 - genes involved in ions and water movements previously published for European sea  
393 bass (Blondeau-Bidet et al., 2019a; Bodinier et al., 2009; Bossus et al., 2011, 2013;  
394 Giffard-Mena et al., 2007) (*atp1a1a*, *atp1a1b*, *slc12a2*, *slc12a3-like*, *clcn3*, *slc4a4*,  
395 *atp6v1a*, *atp6v1b2*, *slc9a2b*, *slc9a2c*, *slc9a3*, *trpv4*, *cftr*, *aqp3*) or for other fish  
396 species (de Polo et al., 2014; Shahsavarani and Perry, 2006; Su et al., 2020) (*ecac*,  
397 *cazh*, *ca15b*)

398 - genes involved in ammonia transport in European sea bass (Blondeau-Bidet et al.,  
399 2019a) (*rhcgl*, *rhcg2*, *rhbg*) or in other fish species (Wood and Nawata, 2011) (*rhag*).

400 - genes coding for proteins controlling gill permeability (Chasiotis et al., 2012)  
401 (*cldn5a*, *cldn5b*, *cldn7a*, *cldn8like*, *cldn12*, *cldn23a*, *oclna*, *oclnb*, *tjp1*).

402 These primers were tested for their efficiency over a minimum of 6 dilution points  
403 and kept when their efficiency was comprised between 80%-105% (Table 2).

404

#### 405 2.7.5. Real-time PCR protocol

406 An Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA) was used to  
407 dispense 0.5 µL of diluted cDNA and 1 µL of a mix containing 0.75 µL of  
408 SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK), and 0.25 µL of primers  
409 at a concentration leading to a final well-concentration ranging from 0.2 to 0.8 µM  
410 depending on the primer (Table 1). Each sample was run in duplicate. The qPCR  
411 steps were as follows: denaturation at 95°C for 2 min, followed by 45 cycles of  
412 amplification (95°C, 15 s), hybridization (60°C, 5 s) and elongation (72°C, 10 s), and  
413 a final step at 40°C for 30 s. A melting curve program was performed to control the  
414 amplification specificity using the following protocol: 10 s holding at 55°C followed  
415 by sequential 0.05°C increases, repeated 80 times. Ultra-pure water was used as a no-  
416 template control in the qRT-PCR. Relative levels of gene transcription were obtained  
417 using the following equation  $(2^{-(Ct_{ref})}) / (2^{-(Ct_{target})})$  with the target gene  
418 normalized by the geometric mean of two housekeeping genes as reference. Seven  
419 different reference genes (*eef1-alpha*, *l13*, *gapdh*, *18S*, *actb*, *fau*, *rpl17*) were tested  
420 for all organs and the R function “selectHKS” from the “NormqPCR” package  
421 (Perkins et al., 2012) was used to select the best two reference genes, based on their  
422 stability across all conditions. For the 4 organs, the function selected the same two  
423 reference genes: *l13* and *fau*.

424

#### 425 2.8. A bioenergetic model to compare growth data overtime between treatments

426 The DEB model has previously been parametrized for European sea bass (Lika et al.,  
427 2018) and used to explain metabolic trends both in nature (Sadoul et al., 2020) and in  
428 controlled conditions (Stavrakidis-Zachou et al., 2019). Based on longitudinal data of  
429 body mass and length, we used the DEB model (equations provided in Table S1) to  
430 estimate the amount of assimilated energy and test whether chronic stress has  
431 significant effects on this variable. In addition, we used this approach to test possible  
432 impact of chronic stress on other metabolic trait. For this purpose, we tested whether  
433 allowing individual DEB parameters to vary individually improved the predictions of  
434 body mass and length values. We tested all primary metabolic parameters of Table S2  
435 leading to a significant change in body mass and/or length over time prior puberty:

- 436 - The “surface-area-specific maximum assimilation rate” ( $\{\dot{p}_{Am}\}$ , in  $J.d^{-1}.cm^{-2}$ ),  
 437 driving the maximum amount of energy the animal can assimilate per unit of  
 438 structural surface when food is *ad libitum*.
- 439 - The “allocation fraction to soma” ( $\kappa$ ), specifying the fraction of energy  
 440 mobilized from the reserve compartment allocated to the production and  
 441 maintenance of structure.
- 442 - The “specific cost for structure” ( $[E_G]$ ,  $J.cm^{-3}$ ), which represents the cost  
 443 (biomass and overhead) to the animal of transforming the energy allocated  
 444 towards growth ( $\dot{p}_G$ ) in structure.
- 445 - The “somatic maintenance cost” ( $[\dot{p}_M]$ ,  $J.cm^{-3}.d^{-1}$ ), corresponding to energy  
 446 requirement to maintain a unit of structure.
- 447 - The “energy conductance” ( $v$ ,  $d^{-1}$ ), corresponding to the rate of energy  
 448 mobilization from the reserve compartment.

449 To estimate the difference in assimilated energy between chronically stressed and  
 450 control fish, calculations were run during different periods, i.e. until the first biometry  
 451 (6 month old), until transfer to experimental tanks, during chronic stress treatment and  
 452 after this treatment.

453

#### 454 2.9. Statistical analyses

455 Statistical analyses and illustrations were performed using R version 3.6.1. All  
 456 statistical analyses were carried out at the 95% level of significance. A linear mixed  
 457 model was fitted to analyse body mass and length at 11, 12 and 14 months using  
 458 “chronic stress”, “sex” and their interaction as fixed effects, and the “rearing tank” as  
 459 random effect.

460 Plasmatic values of cortisol, lysozyme, chloride, and calcium concentrations and  
 461 osmotic pressure, before and after the osmotic challenge, were also analyzed using a  
 462 similar mixed model but “length” and “osmotic challenge” were added as fixed  
 463 effects and “sampling day” as a random crossed effect with “chronic stress” (model  
 464 1). Genes from the gills were analysed using the same model. Expression of these  
 465 genes were also analysed only on individuals before the salinity challenge using the  
 466 same model without “salinity challenge” as fixed effect (model 2). Gene expressions  
 467 of the HPI axis were measured only before the salinity challenge and were therefore  
 468 analysed using model 2.

469 The lmer function from the lme4 package was used for all these mixed models (Bates  
470 et al., 2014).

471 A PCA was performed on the expression of all genes studied in the brain, pituitary  
472 and head kidney using the FactomineR package (Lê et al., 2008). The coordinates for  
473 each individual on the first 3 dimensions were extracted and evaluated using model 1.  
474 The same procedure was performed for the expression of the genes evaluated in the  
475 gills using model 2.

476 From the novel environment and hypoxia test, the behaviour data (inter-individual  
477 distances, velocity and time spent in the center) were analysed before (<60 min) and  
478 after ( $\geq 60$  min) the hypoxia challenge using a linear mixed model using chronic  
479 stress, time (categorical) and their interaction as fixed effects and the day of  
480 experiment as random effect.

481 The step function was used to remove non-significant fixed effects from the mixed  
482 models. The lmerTest package was used to provide p-values based on Satterthwaite's  
483 degrees of freedom approximation (Kuznetsova et al., 2017). Degrees of freedom  
484 reported in the result section were rounded to the nearest integer. The approximation,  
485 required to obtain significances from linear mixed models, might provide small  
486 differences between variables measured on the same individuals. When one fixed  
487 effect (or the interaction) was found significant, a Tukey post hoc test was completed  
488 with the glht function from the multcomp package to test significant differences  
489 between the levels of the fixed effect.

490 Finally, correlations between all variables of interest across all individuals were  
491 calculated and illustrated using the rcorr and the corrplot functions from the Hmisc  
492 and corrplot packages respectively.

493

### 494 **3. Results**

495

#### 496 **Growth performances**

497 The chronic stress protocol had no significant effect on survival. However, after 3  
498 weeks of chronic stress, we observed a significant lower weight ( $F_{1,486} = 9.01$ , p-value  
499 = 0.003) and length ( $F_{1,485} = 6.42$ , p-value = 0.01) in stressed individuals highlighting  
500 reduced growth performances in the stressed group compared to control (Fig. 2).  
501 When compared to controls, chronically stressed individuals showed slower growth ,  
502 but both treatments had positive growth over the three weeks (Fig. S1). However,



503 growth reduction started before the stress protocol, during the acclimation period  
504 presumably because of a unfavourable localisation of the stress tanks in the  
505 experimental hall. Two months after the end of the exposure, no more difference was  
506 observed between stressed and control fish.

507 The DEB model applied to growth data fits well body mass and lengths of control and  
508 chronically stressed fish by optimizing only the amount of energy assimilated (Fig. 2).  
509 Both biometric values have a mean relative error (MRE) of less than 5%. The  
510 goodness of fits for chronically stressed individuals is slightly inferior with a MRE  
511 higher than controls (4.17 vs 4.59). The DEB model estimates that chronically  
512 stressed fish assimilated 24.8% less energy than controls. According to the model,  
513 after the chronic stress, the chronically stressed fish were catching up the body mass  
514 and length of controls by assimilating more energy (6.9% more).

515 Allowing any DEB parameter to vary individually did not help to increase the  
516 goodness of fits of the model for both treatments (Fig. S2).

517

### 518 **Physiology of the HPI axis**

519 High levels of plasma cortisol were measured in fish sampled right after sorting and  
520 dispatching them (P1) (Fig. 3). A significant difference between chronically stressed  
521 and control fish was observed with chronically stressed individuals showing lower  
522 cortisol values ( $F_{1,6} = 9.45$ ,  $p$ -value = 0.02). During the recovery periods (P2 to P4,  
523 i.e. 1h, 3h and 6h respectively) after the acute stress test (AST), no significant  
524 difference in plasma cortisol levels was observed between the two treatments. No  
525 significant difference was also observed after 24 hours of freshwater challenge (P5)  
526 (Fig. 3).

527 Despite the effect of chronic stress on plasma cortisol levels after sorting and  
528 dispatching, no effect of the chronic stress was observed on the expression of  
529 candidate genes in the HPI axis, except for *pomcb* significantly upregulated in the  
530 pituitary of chronically stressed fish ( $F_{1,14} = 4.78$ ,  $p$ -value = 0.047) (Data not shown).  
531 Moreover, PCA analysis of gene expressions in the brain, pituitary and head-kidney  
532 did not reveal significant effect of chronic stress. In agreement with these results, we  
533 observed in both, stressed and control groups, the same correlation at the individual  
534 level between all measures performed in the tissues (gene expressions and plasmatic  
535 measures, Fig. 4). Similarly, some interesting intra- and inter- organ correlations  
536 could be observed in both experimental groups (Fig. 4B). The expression of genes

537 related to neurogenesis (*neurod*, *pcna*, *egr1*) and genes related to cortisol receptors  
538 and pathway synthesis (*mr*, *gr1*, *gr2*, *crf*) in the brain showed significant positive  
539 correlations (Fig. 4). Within the interrenal, the expressions of most genes related to  
540 cortisol synthesis (*mc2r*, *star*, *hsd11b2a*, *hsd11b2b*, *cyp11b1*, *p450scc*) and genes  
541 related to the expression of cortisol receptors (*mr*, *gr1*, *gr2*) are positively correlated.  
542 Finally, positive correlations were observed between the expressions of *pomcb*, *gr1*,  
543 *gr2* and *mr* in the pituitary.

544

## 545 **Homeostasis regulation of ions and water before and after an osmotic test**

### 546 *Effects of chronic stress before the osmotic test*

547 Chronic stress induced a significant increase in plasmatic chloride ( $F_{1,19} = 5.26$ , p-  
548 value = 0.033) and sodium ( $F_{1,25} = 13$ , p-value = 0.001) levels (Fig. 5C and D) but had  
549 no significant effect on osmotic pressure or on calcium concentration. Moreover,  
550 when analysing changes in gill transcript levels, a significant increase was observed  
551 for two genes: *slc12a3like* ( $F_{1,61} = 19.5$ , p-value < 0.001) and *aqp3* ( $F_{1,62} = 13.6$ , p-  
552 value < 0.001).

553

### 554 *Effects of the osmotic challenge*

555 The 24 hours freshwater challenge (OC) strongly reduced the overall osmotic pressure  
556 ( $F_{1,137} = 228.3$ , p-value < 0.001) in both control and chronically stressed individuals  
557 (Fig. 5A). This was concomitant with a significant reduction in chloride ( $F_{1,130} =$   
558 220.7, p-value < 0.001) and sodium ( $F_{1,133} = 447.8$ , p-value < 0.001) concentrations  
559 but not in calcium levels (Fig. 5). However, differences in chloride and sodium  
560 concentrations between control and stressed fish were not maintained after 24 hours  
561 of freshwater challenge.

562 This OC also induced significant modification of the expression of several gill genes  
563 related to ion and water homeostasis, ammonia transport, cortisol receptors and most  
564 genes involved in gill permeability (Table 3). Finally, expression of these genes did  
565 not significantly differ between control and chronically stressed fish, but a significant  
566 interaction effect (p<0.05) between the OC and the chronic stress treatment was  
567 observed for several of these genes, including *atp1a1b* ( $F_{1,122} = 4.1$ , p-value = 0.045),  
568 *slc12a3like* ( $F_{1,128} = 4.1$ , p-value = 0.045), *cftr* ( $F_{1,130} = 4.3$ , p-value = 0.04), *slc9a2c*  
569 ( $F_{1,125} = 10.4$ , p-value = 0.002), *cldn5a* ( $F_{1,127} = 5.03$ , p-value = 0.027), *cldn8like*

570 ( $F_{1,128} = 5.2$ , p-value = 0.024), *ocldnb* ( $F_{1,129} = 7.6$ , p-value = 0.007), *gr2* ( $F_{1,127} = 4.6$ ,  
571 p-value = 0.034).

572 The PCA analysis using the expression of all genes measured in the gills show a clear  
573 separation on the first axis of individuals based on the OC (Fig. 6). The genes  
574 contributing the most to this separation are given in Fig. 6C. Overall, *trpv4*, *oclnb*,  
575 *rhchl1*, *aqp3* and *atplala* are the genes the most upregulated in the 24 hours  
576 freshwater fish, while *slc12a2*, *cfr*, *rhcg2*, *ca15b*, *slc9a2c*, and *slc4a4* are the genes  
577 showing the highest expression in fish that were not challenged. All expressions of  
578 these genes showed significant differences between seawater and freshwater fish  
579 when analyzed individually (Table 3).

580 The second axis of the PCA displayed a significant difference between control and  
581 chronically stressed individuals ( $F_{1,132} = 7.2$ , p-value = 0.008). The genes contributing  
582 the most to this difference are listed in Fig. 6C. Genes *cldn8like*, *mr*, *cldn12*, *ocln* and  
583 *gr2* were the one contributing the most although their expression did not significantly  
584 differ between treatments when evaluated individually (Table 3).

585

#### 586 **Innate immune system**

587 Investigation of the effects of chronic stress or osmotic test on innate immune system  
588 was also carried out. While OC significantly reduced plasma lysosyme levels ( $F_{1,123} =$   
589 11.1, p-value = 0.0012), no significant effect of chronic stress on this parameter was  
590 observed (Fig. S3).

591

#### 592 **Behaviour**

593 In the novel environment challenge in group, the three variables of interest measured  
594 (inter-individual distances, velocity and time spent in the center) changed  
595 significantly through time both before and after the start of the hypoxia challenge  
596 (Table S3). Prior to the hypoxia challenge, a significant interaction between the time  
597 and the treatment (chronic stress) was observed for the mean inter-individual  
598 distances ( $F_{5,457} = 7.4$ , p-value <0.001) and the time spent in periphery ( $F_{5,456} = 7.4$ , p-  
599 value <0.001). However, the post-hoc tests ran on each independent time point  
600 highlighted only a significant effect of the chronic stress on the time spent in the  
601 periphery during the first two time points of the measure (z-values = 2.33 and 2.32,  
602 and p-values = 0.02 and 0.02 respectively, Fig. 7C). The chronically stressed fish  
603 spent more time in the periphery of the tank than controls at the beginning of the trial,

604 indicative of higher thigmotaxis behaviour. After the start of the hypoxia challenge, a  
605 significant interaction between time and treatment was observed only for the mean  
606 distance between individuals ( $F_{5,468} = 3.36$ ,  $p\text{-value} = 0.005$ ). Nevertheless, the post-  
607 hoc test did not show any significant differences between treatments for any of the  
608 time points during the hypoxia challenge. In addition, the velocity and the associated  
609 changes over time were not different between treatments neither before nor after the  
610 start of hypoxia challenge (no interaction, Table S3).

611

#### 612 **Parameters measured 4 months later**

613 Four months after the chronic stress, no significant difference was observed in growth  
614 performances and in plasma parameters (cortisol, lysozyme, sodium, calcium,  
615 chloride) measured between treatments.

616

#### 617 **4. Discussion**

618

619 The present study aimed to assess responses of fish repeatedly exposed during  
620 3 weeks to a variety of acute stressors commonly observed in aquaculture. To assess  
621 their welfare status, we investigated a large spectrum of physiological and  
622 behavioural markers analysed from molecular to whole-body levels and involved in  
623 various biological functions including growth, activity of the HPI axis, gill functions,  
624 immunity and swimming behaviour.

625

#### 626 **Consequences on growth**

627 In the present study, the most apparent effects of the chronic stress protocol were  
628 detected on biometric results, with a clear reduction in growth of body mass and  
629 length. It is well known that stress is energy consuming, decreases appetite through  
630 well-described molecular mechanisms and thus reduces growth performances in fish  
631 (Sadoul and Vijayan, 2016). These growth data are therefore not surprising and in  
632 agreement with previous studies (Samaras et al., 2018; Santos et al., 2010). Samaras  
633 et al. (2018) also observed growth reduction for European sea bass exposed to a “high  
634 stress regime” over three weeks, and this reduction led to a 6.5% difference in final  
635 body mass. Based on body mass results (9% difference), the stress protocol (also over  
636 three weeks) could be considered to lead to a more severe stress load. Nevertheless,  
637 while Samaras et al. (2018) observed a complete interruption of growth over the 3

638 weeks with both, medium and high stress regimes, chronically stressed fish continued  
639 growing in our experiment and gained 13%. This illustrates the complexity behind  
640 chronic stress, and highlights the need for multiple and diverse markers of stress;  
641 growth taken individually is not a reliable indicator of stress intensity.

642 The DEB model applied to biometric data suggests that growth reduction induced by  
643 the chronic stress protocol can be entirely explained by reduced energy assimilation.  
644 DEB is often used to highlight a mode of action of a contaminant based on changes in  
645 biometric values (Ashauer and Jager, 2018). To our knowledge, this is the first time  
646 DEB theory was applied to physical stressors. Here, based on available data, the  
647 results from the DEB model suggest that none of the metabolic mechanisms involved  
648 in energy allocation was impacted by the treatment, apart from total energy  
649 assimilated. This translates into reduced foraging and/or reduced nutrient assimilation  
650 by the digestive tract. Reduced food consumption was previously reported in sea bass  
651 exposed to chronic stress (Samaras et al., 2018; Santos et al., 2010). However, Millot  
652 et al. (2010) reported, on the contrary, that repeated acute stress increases feed  
653 demand and intake in sea bass, while growth was still reduced, suggesting that  
654 nutrient assimilation and/or energy allocated to maintenance were affected. A very  
655 recent study also demonstrates that elevated cortisol levels affect growth mainly as a  
656 consequence of reduced digestibility rather than feed intake (Pfalzgraff et al., 2021).  
657 The outputs from the DEB model analysis suggest that our chronic stress protocol had  
658 no dramatic consequence on other metabolic functions than assimilation. This argues  
659 for only subtle physiological alterations due to the chronic stress and translates in a  
660 very quick recovery in terms of body mass and length.

661

#### 662 **Biomarkers related to corticotropic axis**

663 Assessment of chronic stress by studying the HPI axis activity can be misleading  
664 when solely based on basal plasma cortisol levels. Indeed, a negative feedback of  
665 cortisol production causes down regulation of the HPI axis in chronically stressed fish  
666 (Barton et al., 2002, 1987; Pickering and Stewart, 1984; Vijayan and Leatherland,  
667 1990; Wendelaar Bonga, 1997). In this study, it was not possible for practical reasons  
668 to measure basal levels of cortisol, the major stress hormone in fishes (Sadoul and  
669 Geffroy, 2019). However, assessment of the reactivity of the HPI axis to acute stress  
670 has already been used to study chronic stress effects (Madaro et al., 2015; Pavlidis et  
671 al., 2015; Samaras et al., 2018; Santos et al., 2010). In our protocol, sorting and

672 dispatching of the fish represents a first acute stressor as indicated by the high cortisol  
673 levels (Fig. 3). This response was expected as sea bass is a very stress-sensitive  
674 species as shown by rapid and high cortisol response after exposure to acute stressors  
675 (Fanouraki et al., 2011; Ordóñez-Grande et al., 2020; A. Samaras et al., 2016;  
676 Samaras et al., 2018). Interestingly, chronic stress treatment significantly decreases  
677 the acute stress response observed after sorting and dispatching fish when compared  
678 to control. Nevertheless, both treatments reacted similarly to the additional acute  
679 stress (confinement during 4 minutes, AST) suggesting that the HPI axis was not  
680 deeply affected by the chronic stress protocol. Cortisol values after 6 hours were still  
681 much higher than basal levels previously observed for European sea bass (Acerete et  
682 al., 2009; Samaras et al., 2018), indicating that the total stress recovery were not met  
683 after 6 hours.

684 Our post-AST cortisol values differ from previous studies. Samaras et al. (2018)  
685 observed a decrease in the maximum cortisol response 1 h after acute stress in sea  
686 bass chronically exposed to acute stressors of different load intensities. Santos et al.  
687 (2010) and Di Marco et al. (Di Marco et al., 2008) observed an increase in plasma  
688 cortisol levels after an acute challenge in sea bass chronically exposed to high  
689 stocking density but not when using low or medium densities. Similar inconsistent  
690 results in HPI reactivity in response to chronic stress were also observed in other  
691 species, such as in salmon, zebrafish or seabream (Madaro et al., 2015; Moltesen et  
692 al., 2016; Pavlidis et al., 2015; Samaras et al., 2018; Vindas et al., 2016). Overall,  
693 these results highlight that chronic stress effects vary according to age, experimental  
694 protocols and/or species.

695 In the present study, the limited effect of chronic stress on HPI-axis reactivity also  
696 translates in terms of gene expression related to the HPI axis. Despite a wide analysis  
697 of key genes involved in stress response in the brain, pituitary or interrenal, only  
698 *pomcb* was affected by the chronic stress, with a significant upregulation. In sea bass,  
699 two orthologous genes have been identified and annotated as *pomca* and *pomcb*  
700 (Rousseau et al., 2021). Measures of *pomcb* transcripts in the pituitaries of seabream  
701 (a fish species phylogenetically close to sea bass) suggest that expression values  
702 reflect ACTH activity (Cardoso et al., 2011). Our study indicates a stimulatory effect  
703 of chronic stress on *pomcb* gene expression measured before the AST. Although we  
704 did not measure basal cortisol levels, this increased expression of *pomcb* is in  
705 agreement with increased basal cortisol levels measured by Samaras et al. (2018) in

706 sea bass. On the contrary, significant changes of *cfr*, *gr1* and *mr* gene expressions  
707 were previously also described by Samaras et al. (2018) in the pre-optic area (POA).  
708 Our non-significant results might be explained by the fact that they were obtained in  
709 the entire brain, diluting potential signal variations. Overall, our repeated acute stress  
710 protocol has only minor effects on the HPI axis which suggests a moderate stress load  
711 of the experimental protocol. Additional plasmatic parameters, including lactate and  
712 glucose levels, would have been interesting to investigate in light of previous results  
713 on chronic stress in fish (Santos et al., 2010).

714

### 715 **Biomarkers related to ions and water homeostasis**

716 To our knowledge, the present study is the first to investigate the effect of chronic  
717 stress on ions and water homeostasis in sea bass. The interplay between the stress axis  
718 and osmoregulatory functions is well described (Takei and Hwang, 2016), but data on  
719 the effects of chronic stress on osmoregulatory functions are scarce. In seawater  
720 (SW), chronic stress induced a significant increase in plasma sodium and chloride  
721 levels and an upregulation of the expression of two gill genes: *aqp3* (a water channel)  
722 and *ncc2* (a co-transporter of Na<sup>+</sup> and Cl<sup>-</sup>). Relationships between such effects on  
723 plasma ions and expression of these genes remain complex. *Aqp3* and *ncc2* genes are  
724 well known to play an important role in osmoregulation under freshwater (FW)  
725 conditions for several euryhaline fishes (Madsen et al., 2015; Takei et al., 2014)  
726 including sea bass (Blondeau-Bidet et al., 2019a; Giffard-Mena et al., 2007). *Aqp3* is  
727 involved in the water flow through the basolateral side of gill's epithelium to prevent  
728 swelling, and may take part in nitrogen excretion (Madsen et al., 2015). *Ncc2* allows  
729 gill absorption of NaCl to maintain ion homeostasis (Takei et al., 2014). However,  
730 several studies suggest that *aqp3* and *ncc2a* are also implicated in gill functions since  
731 SW adapted fish show significant levels of the transcripts (Blondeau-Bidet et al.,  
732 2019a; Breves et al., 2020; Giffard-Mena et al., 2007; Jung et al., 2012; Moorman et  
733 al., 2015; Tipsmark et al., 2010). *Aqp3* immunoreactivity appears quantitatively  
734 similar whatever the salinity in medaka (Ellis et al., 2019) and protein abundance does  
735 not change significantly in killifish (Jung et al., 2012). *Ncc2* protein was also  
736 quantified in SW-acclimated mummichogs using western blot approach (Breves et al.,  
737 2020). We propose that *aqp3* and *ncc2* genes are important targets of the chronic  
738 stress in SW-adapted sea bass but their exact functions in relation to gill ion transport  
739 and epithelial permeability still need to be clarified.

740 Fish from both treatments (control and chronic stress) responded to the OC with a  
741 significant decrease in blood osmotic pressure and NaCl levels in agreement with  
742 previous results (Bossus et al., 2011; L'Honoré et al., 2019). In parallel significant  
743 changes in the expression of genes implicated in ions and water movements were  
744 observed in agreement with previous studies in sea bass measuring gene expressions  
745 24 h or several weeks after a transfer in FW: (i) decrease in *nkaa1b*, *nkcc1a*, *cftr*,  
746 *nhe2c*, *vha-a* and *vha-b*, *ca15b* transcript levels and (ii) increase in gene expression of  
747 *nkaa1a*, *ncc2*, *clcn3*, *nhe3*, *trpv4*, *rhcgl*, *aqp3* (Blondeau-Bidet et al., 2019a; Bodinier  
748 et al., 2009; Bossus et al., 2011, 2013; Giffard-Mena et al., 2007; L'Honoré et al.,  
749 2020; Lorin-Nebel et al., 2006; Masroor et al., 2019). In both treatments, we also  
750 measured significant changes in the expression of genes involved in paracellular  
751 movement of solutes with *cldn5a*, *cldn7*, *oclna* and *oclnb* up-regulated and *cldn5b*,  
752 *cldn23a* and *zo-1* down-regulated in FW. In Atlantic killifish, *ocln* gene expression  
753 was also up-regulated following hypo-osmotic challenge (Whitehead et al., 2011) and  
754 acclimation of goldfish to ion-poor water induced an increase in *ocln* and *cldn7*  
755 mRNA and a decrease of *zo-1* transcript (Chasiotis et al., 2012). More work would be  
756 needed to better understand the functional roles in osmotic challenge for genes such  
757 as *cldn5a* and *cldn5b*.

758 An important majority of the gene expressions evaluated were not affected by the  
759 chronic stress (2 in seawater and 8 significant interaction out of 33 tested after  
760 freshwater challenge), suggesting that many cellular mechanisms, such as ammonia  
761 transport, were not altered by our stress protocol. Nevertheless, the PCA analysis  
762 performed on all 33 genes measured in the gills shows that the chronic stress explains  
763 part of the variability in the expression of SW-adapted and FW-challenged sea bass,  
764 with a significant effect on the second dimension. In addition, expression of several  
765 gill genes presented a significant interaction between chronic stress and salinity.  
766 Altogether, these analyzes suggest that our chronic stress protocol significantly alters  
767 abilities of sea bass to withstand a FW challenge. Further experiments will be  
768 necessary to understand the exact role of the most affected genes and get a clearer  
769 view on the consequences of chronic stress on this coping ability. Although such a  
770 challenge has no biological reality in nature or in captivity, it provides an interesting  
771 test for assessing health and adaptive capacities of fishes.

772

773 **Biomarkers related to behaviour.**



774 In terms of behaviour, our results show that chronically stressed fish spent more time  
775 on the periphery of the experimental tank, indicating a higher thigmotaxis during the  
776 first 20 minutes in the new environment. Thigmotaxis is generally considered as an  
777 indicator of stress, related to anxiety and is evolutionarily conserved across multiple  
778 fish species including European sea bass (Alfonso et al., 2020; Prut and Belzung,  
779 2003; Schnörr et al., 2012). This behaviour was previously demonstrated to be a good  
780 marker of contaminant-related disruptions in ecotoxicology (Alfonso et al., 2019a) or  
781 altered welfare in aquaculture (Colson et al., 2015; Sadoul et al., 2016; Tonkins et al.,  
782 2015). Moreover, exposure of sea bass to environmental stressors such as high  
783 ammonia levels, hyperoxia or hypoxia also induced a decrease in thigmotaxis  
784 associated with a decrease in activity and changes in group cohesion (Alfonso et al.,  
785 2020). Our results over the first 20 minutes in the new environment suggest that  
786 chronic stress might have subtle effects on some emotional reactivity traits in sea bass  
787 increasing thigmotactic response without altering behavioural coping responses to  
788 threatening stress, such as hypoxia.

789

## 790 **Resilience**

791 Body mass and length, plasma ions and cortisol levels were among the most  
792 significant physiological parameters for which differences were observable in  
793 response to repeated acute stress during three weeks. In order to evaluate the lasting  
794 effect of the chronic stress, they were therefore measured again 4 months after the  
795 chronic stress protocol. No more differences were observable in any of the measures  
796 performed. Therefore, in parallel to the recovery of the biometric measures, fish  
797 reinstated also their physiological parameters after the chronic stress, indicating a  
798 good resilience of European sea bass to our chronic stress conditions. Compensatory  
799 growth effects after a period of chronic stress has already been observed in sea bass  
800 and was attributed to an increase in feed intake (Millot et al., 2010), in accordance to  
801 our modeling approach.

## 802 **Conclusions**

803 The present study illustrates the benefit of using several endpoints related to different  
804 functions to assess health and welfare in European sea bass exposed to a chronic  
805 stress protocol. This was particularly important with a protocol leading overall to a  
806 low stress load. The modelling approach suggested that only subtle physiological  
807 consequences were affected by the stress protocol, and this was confirmed by limited

808 significant differences in measures taken individually, despite a large spectrum of  
809 performed analyses. Altogether, the present study suggests that 1) growth or HPI  
810 reactivity are not always relevant taken individually for assessing chronic stress in  
811 European sea bass, 2) gill functions are more sensitive to chronic stress and should be  
812 included when assessing a chronic stress protocol and 3) behavioural tests are useful  
813 measures when included in a multi-parameters approach.

814 Reasons for the small difference in stress load, despite a protocol supposed to be  
815 stressful are unclear. One could suggest that the multiple biometrics performed prior  
816 the chronic stress protocol, increased the stress load of both conditions prior the  
817 experiment (Moraes et al., 2017) and attenuated the differences between conditions.  
818 Nevertheless, regardless of the reason, this work demonstrates the importance of  
819 integrating within a multivariate analysis a large spectrum of measures to be able to  
820 show subtle differences of stress load in European sea bass. In aquaculture conditions,  
821 multiple random acute stressors can have consequences on welfare which are difficult  
822 to highlight because of tenuous changes in physiology and behaviour. Thus, we  
823 propose that our multivariate approach is necessary to get a relevant assessment of  
824 welfare in fish exposed to chronic stress protocols.

825

### 826 **Acknowledgments**

827 We are grateful to Marie-Odile Vidal, François Ruelle, and Alain Vergnet for  
828 hatching and rearing the fish. The authors would also like to thank Dr. D. Dufour-  
829 Rainfray, Dr. P. Emond and Mr. A. Lefevre for LC-MS-MS analysis of seabass  
830 plasma samples. The authors are also grateful to Emilie Levavasseur for the European  
831 sea bass drawing in Fig. 1.

832

### 833 **Competing interests**

834 'No competing interests declared'

835

### 836 **Data availability**

837 The data that support the findings of this study are available upon request.

838

### 839 **Funding**

840 This work was funded by the ERANET COFASP project SUSHIFISH (ANR-15-  
841 COFA-0002-01).

842

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1322 Fig. 1. Timeline of the experimental protocol performed.

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1324 Fig. 2. Effects of chronic stress on the evolution of biometric values in European sea  
1325 bass. At 309 dpf, fish were dispatched in 6 different tanks. At 336 dpf, fish from three  
1326 tanks were chronically stressed with repeated acute stressors during 3 weeks (red  
1327 zone). Differences to the controls are presented for the body mass (A) and the length  
1328 (B). Data are represented as mean  $\pm$  SEM. The continuous lines represent the  
1329 simulated outputs from the DEB model.

1330 (n=873 and 879 until 309 dpf, n=865 and 877 at 336 dpf, n=247 and 252 at 358 dpf,  
1331 n=586 and 593 at 424 dpf, n=573 and 581 at 463 dpf for control and chronic stress  
1332 respectively). The pink period illustrates the 4 weeks acclimation period to the  
1333 experimental tanks prior the chronic stress. Difference to the control are highlighted  
1334 by an asterisk.

1335  
1336 Fig. 3. Stress response of European sea bass chronically stressed (blue) or control  
1337 (yellow). Cortisol values are represented as mean  $\pm$  SEM. Fish were first anesthetized,  
1338 sorted and dispatched for experimental purposes, and cortisol was measured on a  
1339 subsample (n=54 and 55, left panel). Fish were then stressed by a 3 minutes  
1340 confinement stress and sampled 1, 3 and 6 hours later (n= 54 and 50 for 1h, n=51 and  
1341 57 for 3h, n=52 and 60 for 6h, middle panel). Finally, a group of fish was osmotically  
1342 challenged during 24 hours in fresh water.

1343  
1344 Fig. 4. Correlogram of the correlation matrix between gene expressions in the Brain,  
1345 the Interrenal and the pituitary (Pit.) and measures performed in the plasma for  
1346 individuals in the control group (A) or for individuals chronically stressed for 3 weeks  
1347 (B). Pearson linear correlations coefficient are illustrated using colors ranging from  
1348 dark red (-1) to dark blue (+1). Non-significant (p-value>0.05) correlations are  
1349 crossed.

1350  
1351 Fig. 5. Osmotic markers in plasma of European sea bass chronically stressed (blue) or  
1352 control (yellow) before and after a 24 hours challenge in freshwater. Data are  
1353 represented as mean  $\pm$  SEM. Significant effects of the salinity challenge are  
1354 highlighted by asterisks on the x-label. Significant difference between control and  
1355 chronic stress are highlighted by asterisks on the graph (\*: p<0.05; \*\*: p<0.01; \*\*\*:  
1356 p<0.001).

1357

1358 Fig. 6. Principal component analysis (PCA) on the gene expression in the gills of  
1359 individuals chronically stressed or controls and before or after a 24 h freshwater  
1360 challenge. (A) Individual plot. (B) Box plots of the coordinates on dimension 2 of the  
1361 PCA for all individuals and compared between the control and the chronic stress  
1362 groups. Significant difference is highlighted by asterisks (\*\*:  $p < 0.01$ ). (C) List of the  
1363 genes with the most significant contributions to the variability on the first and the  
1364 second dimensions of the PCA.

1365

1366 Fig. 7. Group behaviour in a novel environment before and during a hypoxia  
1367 challenge of fish previously exposed to chronic stress treatment or control conditions.  
1368 Mean inter-individual distance (A), velocity (B) and time spent in the center (C) are  
1369 illustrated during a 60 min acclimation period to the novel environment and during a  
1370 hypoxia challenge obtained by reducing the oxygen saturation down to 20% (D). Data  
1371 are represented as mean  $\pm$  SEM. The difference between chronically stressed  
1372 individuals and control individuals at a single time point are illustrated with an  
1373 asterisk (p<0.05).

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Table 1. Published primers used for real-time PCR on European sea bass cDNA.

Gene	Protein name	GenBank accession numbers	Forward primer (5' → 3')	Reverse primer (5' → 3')	Concentration in qPCR well	Tm	Ref
<i>eef1-alpha</i>	elongation factor 1-alpha 1	AJ866727.1	AGATGGGCTTGTCAAGGGA	TACAGTTCCAATACCGCCGA	0,4	60	(Sadoul et al., 2018)
<i>l13</i>	ribosomal Protein L13	DT044910.1	TCTGGAGGACTGTCAAGGGCATGC	AGACGCACAATCTTGAGAGCAG	0,4	60	(Sadoul et al., 2018)
<i>18S</i>	18S ribosomal RNA	AM419038.1	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCACA	0,4	60	(Pavlidis et al., 2011)
<i>fau</i>	ubiquitin like and ribosomal protein S30 fusion	FM004681	GACACCCAAGGTTGACAAGCAG	GGCATTGAAGCACTTAGGAGTTG	0,4	60	(Mitter et al., 2009)
<i>rpl17</i>	ribosomal Protein L17	AF139590	TTGAAGACAACGCAGGAGTCA	CAGCGCATTCTTTTGCCACT	0,2	60	(Samaras et al., 2018)
<i>mr</i>	mineralocorticoid receptor	JF824641.1	GTTCCACAAGAGCCCAAG	AGGAGGACTGGTGGTTGATG	0,4	60	(Sadoul et al., 2018)
<i>gr1</i>	glucocorticoid receptor 1	AY549305	GAGATTTGGCAAGACCTTGACC	ACCACACCAGGCGTACTGA	0,4	60	(Pavlidis et al., 2011)
<i>gr2</i>	glucocorticoid receptor 2	AY619996	GACGCAGACCTCCACTACATTC	GCCGTTCACTCTCAACCAC	0,2	60	(Pavlidis et al., 2011)
<i>mc2r</i>	melanocortin 2 Receptor	FR870225.1	CATCTACGCCTCCGCATTG	ATGAGCACCGCTCCATT	0,4	60	(Tsalafouta et al., 2017)
<i>cyp11b1 (11B-hydroxylase)</i>	cytochrome P450 family 11 subfamily B member 1	AF449173.2	GGAGGAGGATTGCTGAGAACG	AGAGGACGACACGCTGAGA	0,4	60	(Samaras et al., 2018)
<i>atp1a1a</i>	Na/K ATPase alpha subunit isoform 1a	KP400258	CCTCAGATGGCAAGGAGAAG	CCCTGCTGAGATCGGTTCC	0,4	60	(Blondeau-Bidet et al., 2016)
<i>atp1a1b</i>	Na/K ATPase alpha subunit isoform 1b	KP400259	AGCAGGGCATGAAGAACAAG	CCTGGGCTGCGTCTGAGG	0,4	60	(Blondeau-Bidet et al., 2016)
<i>slc12a2 (nkcc1)</i>	sodium-potassium-chloride cotransporter 1	AY954108	TCAGCTCACAGTTCAAGGCC	TTGTGGAGTCCATAGCGGC	0,4	60	(Blondeau-Bidet et al., 2019b)
<i>slc12a3-like (ncc2)</i>	sodium-chloride cotransporter 2		ATGATGAGCCTCTTCGAGCC	GCTGCTCATCACCTTCTGT	0,6	60	(Blondeau-Bidet et al., 2019a)
<i>clcn3</i>	chloride channel 3	JN998891	CAAGTACAGCAAGAACGAGGC	ACAGCGTCTTGAGAGGGAAG	0,4	60	(Bossus et al., 2013)
<i>slc4a4 (nbc1)</i>	sodium bicarbonate cotransporter	FM001880	ACAGAGCACGGAACACACGG	CGTCCACAGCCAGCAGTTCCG	0,4	60	(Blondeau-Bidet et al., 2019a)
<i>atp6v1a (vha-a)</i>	V-type proton ATPase catalytic subunit a		GGCAGTCACATCACAGGAGG	CCAGCTCCATCACCATCG	0,4	60	(Blondeau-Bidet et al., 2019a)
<i>atp6v1b2 (vha-b)</i>	V-type proton ATPase catalytic subunit b2		TTGCCATAGTCTTCGAGCC	CTTCTCGACTGGTAGGCC	0,4	60	(Blondeau-Bidet et al., 2019a)
<i>slc9a3 (nhe3)</i>	sodium/hydrogen exchanger isoform 3	CX660524	GGATACCTCGCTACCTGAC	AAGAGGAGGGTGAGGAGGAT	0,4	60	(Blondeau-Bidet et al., 2019a)
<i>slc9a2b (nhe2b)</i>	sodium/hydrogen exchanger isoform 2b		CTGTCAGATCGAGGCGTTTG	TCAAACACACTCAGCACAGC	0,4	60	(Blondeau-Bidet et al., 2019a)
<i>slc9a2c (nhe2c)</i>	sodium/hydrogen exchanger isoform 2c		CGTTTCACCCACAATGTCCG	GCACCAGAATGCCAATTTCC	0,4	60	(Blondeau-Bidet et al., 2019b)
<i>trpv4</i>	transient receptor potential cation channel subfamily V member 4	GQ396264	CGGGAGAGATTGTACCTTG	CCATCACGGACACATAAGCC	0,4	60	(Bossus et al., 2011)
<i>rhbg</i>	rhesus blood group, b glycoprotein		CCTCATGGTGACCCGAATCC	TATGTGGACAGAGTGACGGC	0,4	60	(Blondeau-Bidet et al., 2019b)
<i>rhcg1</i>	rhesus blood group, c glycoprotein 1		TCAGGGAATTGTGTGACCGC	CCCAGCGTGGACTTGATTCT	0,4	60	(Blondeau-Bidet et al., 2019b)
<i>rhcg2</i>	rhesus blood group, c glycoprotein 2		TGGTACCTGTTTGTACGC	TATAAAGCCGCCGAGCATCC	0,4	60	(Blondeau-Bidet et al., 2019b)
<i>pcna</i>	proliferating cell nuclear antigen		CAGAGCGGCTGGTTGCA	CACCAAAGTGGAGCGAAACAA	0,4	60	(Crespo et al., 2013)
<i>neurod1</i>	neuronal differentiation 1		TTCTCTTCAGCGTGCACTA	GGTGCGAGTGTCCATCAAAG	0,4	60	(Sadoul et al., 2018)

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Table 2. Designed primers used for real-time PCR on European sea bass cDNA.

Gene	Protein name	GenBank accession numbers	Forward primer (5' → 3')	Reverse primer (5' → 3')	amplicon size	Concentration in qPCR well	<a href="http://sea.bass.mpi-pz.de/cgi-bin/hgGateway">http://sea.bass.mpi-pz.de/cgi-bin/hgGateway</a>	Efficacy
<i>gapdh</i>	glyceraldehyde-3-Phosphate Dehydrogenase	AY863148	GAGAAACCCGCCAAATATGAC	TACCATGTGACCAGCTTGAC	193	0,4	DLAgn_00059000	86,3
<i>actb</i>	actin beta	AY148350.1	TGACCTCACAGACTACCT	GCTCGTAACTCTTCTCCA	140	0,4	DLAgn_00187660	92,3
<i>crf</i>	corticotropin-releasing hormone		GCAACGGGGACTCTAACTCT	GTCAGGTCCAGGGATATCGG	217	0,6	DLAgn_00076040	86,0
<i>crfbp</i>	corticotropin-releasing hormone binding protein		CCAGAGGGCAGTTTACCAT	ACATAGTCACCTGACCCCGA	173	0,4	DLAgn_00120190	92,0
<i>star</i>	steroidogenic acute regulatory protein		TGAGCTGAACAGACTGGCAG	TCTCATTTCGACGCCACAAT	216	0,4	DLAgn_00115570	92,0
<i>hsd11b2</i>	hydroxysteroid (11-beta) dehydrogenase 2		CCGAGCTGTCCCTAATGTCG	TGAGGAGGGTAGGATGGTGG	263	0,4	DLAgn_00234370	87,3
<i>pomcb</i>	pro-opiomelanocortin-b		GGATACTGGACTGTATTACCT	GAAATGCCCTCAGAAGATCC	291	0,4	DLAgn_00069720	94,2
<i>cyp11a2 (p450scc)</i>	p450 side chain cleaving		CCCCCGTCAGTGTTTAGGAC	TTGCGCTGTTTCTCCACTCT	96	0,4	DLAgn_00168500	91,3
<i>cfr</i>	cystic fibrosis transmembrane conductance regulator	DQ501276.2	GAACCAACAGGACAACCA	GCAAGTCGATGAACTTAAACTC	149	0,4	DLAgn_00172160	89,6
<i>cazh</i>	carbonic anhydrase	FK944087	GACTAACGGACCTGATACATGG	TCTGTGTGCTCCACAAGTC	223	0,4	DLAgn_00000090	92,7
<i>ca15b</i>	carbonic anhydrase 15b	CX660749	GGCAAGACAGTCAAAGTCAG	CCTCAATAAAGAAACCAAGAGCAG	255	0,6	DLAgn_00101240	86,0
<i>trpv6 (ecac)</i>	epithelial calcium channel		TTCCATGTTATCCTTATCGGCT	CATAAACTTTGTCAGGTCTCCA	209	0,4	DLAgn_00059190	90,9
<i>aqp3</i>	aquaporin 3	DQ647191	CATGTACTACGATGCCCTGTG	CATAGCCAGAGTTAAAGCCCA	271	0,4	DLAgn_00117370	96,8
<i>rhag</i>	rhesus blood group, a glycoprotein		CAAGTTCCTTTCTCCATCCT	GTAACAACACCTCCAACAG	206	0,4	DLAgn_00071020	92,0
<i>egr1</i>	early growth response 1		AACTCCAGCCTCAGTTCCTC	AGTCAGGAATCATGGGCACA	202	0,4	DLAgn_00110040	91,6
<i>neurod2</i>	neuronal differentiation 2		TGCGTAAAGTGGTTCCATGC	GTCGTGGGTTGGGAGAGTC	172	0,4	DLAgn_00193890	95,7
<i>bdnf</i>	brain-derived neurotrophic factor	DQ915807.1	TGAGACCAAATGCAACCCCA	CACGTAGGACTGGGTTGTCC	100	0,2	DLAgn_00159270	94,5
<i>cldn5a</i>	claudin 5a		GTAATTGGCTCGCTCCTGGT	GTCTGAGCCACCACGATGTT	92	0,4	DLAgn_00120450	104,9
<i>cldn5b</i>	claudin 5b		CAAGGTCCAGACTCGGTTT	CGCCTCATCCTTGATGCAGT	145	0,4	DLAgn_00250470	87,6
<i>cldn7a</i>	claudin 7a		CCCGTCAATACCAAGTATGAG	ACTTCGGTGTAGATTTCCCT	123	0,4	DLAgn_00040400	87,3
<i>cldn8-like</i>	claudin 8like		AGCCAACATCAGGATGCAGT	CTGCAACGATGAGGGCAAAG	125	0,4	DLAgn_00035220	104,0
<i>cldn12</i>	claudin 12		CCTTCATCATTGCCATTGTGTC	GGTCAACTTTAGAGTACCACTCTG	200	0,4	DLAgn_00060570	81
<i>cldn23a</i>	claudin 23		GACCATCATACCCATGCCT	TAAACATGACGAAACCGCCC	145	0,4	DLAgn_00086250	81,6
<i>oclna</i>	occludin a		ACTTTCATCGTGAATTCCTCC	GAGTCCGTTTCATGTTCTTTATCC	227	0,4	DLAgn_00081130	100,9
<i>oclnb</i>	occludin b		CCCAAGAGGTTATAGCTATTGTCC	AGGTAAGAGGCTTGCTGTG	229	0,4	DLAgn_00258740	94,2
<i>tjp1b</i>	tight junction protein 1b		GACAACAGGCCCAAATACCA	CAGCGTTTCTCCTTTCTCCT	290	0,8	DLAgn_00169520	90,9
<i>npy</i>	neuropeptide Y		GAGACACTACATCAACCTCATCAC	TGGGTCATATCTCGACTGTGG	132	0,4	DLAgn_00199940	93,4

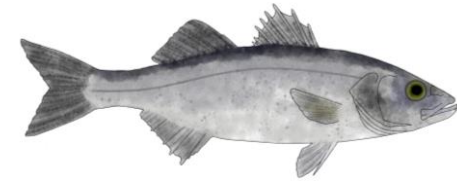
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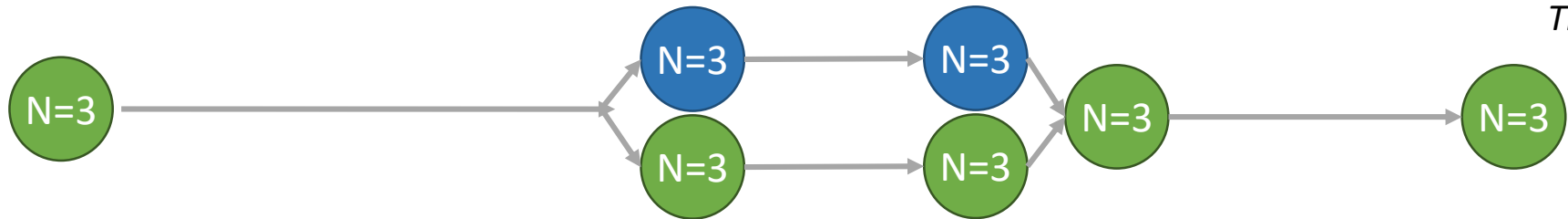
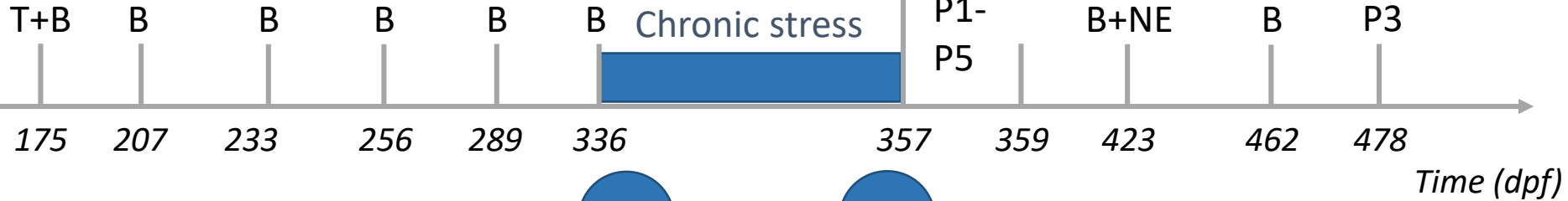
1383 Table 3. Gill gene expression of European sea bass chronically stressed or control before and after a  
 1384 24 hours challenge in freshwater. Values are presented as mean  $\pm$  SEM of 32-37 fish. Significances  
 1385 are represented with asterisks (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).  
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		Control		Chronic stress		statistic		
		SW	FW	SW	FW	chronic stress	salinity	interaction
<i>ion and water homeostasis</i>	<i>atp1a1a</i>	0,8 $\pm$ 0,02	1,28 $\pm$ 0,04	0,82 $\pm$ 0,03	1,31 $\pm$ 0,04		***	
	<i>atp1a1b</i>	1,1 $\pm$ 0,03	0,94 $\pm$ 0,03	1,14 $\pm$ 0,03	0,89 $\pm$ 0,02		***	*
	<i>slc12a2</i>	1,81 $\pm$ 0,05	0,53 $\pm$ 0,02	1,92 $\pm$ 0,06	0,5 $\pm$ 0,02		***	
	<i>slc12a3 like</i>	0,77 $\pm$ 0,03	1,22 $\pm$ 0,06	0,96 $\pm$ 0,04	1,24 $\pm$ 0,06		***	*
	<i>clcn3</i>	0,89 $\pm$ 0,02	1,16 $\pm$ 0,03	0,89 $\pm$ 0,02	1,17 $\pm$ 0,03		***	
	<i>cftr</i>	1,72 $\pm$ 0,05	0,55 $\pm$ 0,03	1,88 $\pm$ 0,08	0,52 $\pm$ 0,02		***	*
	<i>slc4a4</i>	1,73 $\pm$ 0,1	0,69 $\pm$ 0,03	1,44 $\pm$ 0,08	0,61 $\pm$ 0,03		***	
	<i>atp6v1a</i>	1,12 $\pm$ 0,03	0,96 $\pm$ 0,03	1,08 $\pm$ 0,03	0,9 $\pm$ 0,03		***	
	<i>atp6v1b2</i>	1,09 $\pm$ 0,04	0,97 $\pm$ 0,03	1,07 $\pm$ 0,04	0,89 $\pm$ 0,02		***	
	<i>slc9a2b</i>	1,15 $\pm$ 0,05	0,94 $\pm$ 0,04	1,14 $\pm$ 0,04	0,9 $\pm$ 0,05		***	
	<i>slc9a2c</i>	1,59 $\pm$ 0,11	0,68 $\pm$ 0,04	1,9 $\pm$ 0,09	0,53 $\pm$ 0,04		***	**
	<i>slc9a3</i>	0,85 $\pm$ 0,03	1,31 $\pm$ 0,05	0,78 $\pm$ 0,03	1,3 $\pm$ 0,05		***	
	<i>ecac</i>	0,74 $\pm$ 0,04	1,36 $\pm$ 0,08	0,87 $\pm$ 0,04	1,37 $\pm$ 0,06		***	
	<i>trpv4</i>	0,35 $\pm$ 0,03	3,17 $\pm$ 0,11	0,37 $\pm$ 0,03	3,3 $\pm$ 0,13		***	
	<i>cazh</i>	1,16 $\pm$ 0,05	0,89 $\pm$ 0,06	1,17 $\pm$ 0,06	0,96 $\pm$ 0,07		***	
	<i>ca15b</i>	2,89 $\pm$ 0,25	0,51 $\pm$ 0,09	2,95 $\pm$ 0,32	0,42 $\pm$ 0,06		***	
<i>aqp3</i>	0,44 $\pm$ 0,03	2,2 $\pm$ 0,17	0,7 $\pm$ 0,06	2,49 $\pm$ 0,2		***		
<i>ammonia transport</i>	<i>rhcg1</i>	0,75 $\pm$ 0,03	1,39 $\pm$ 0,04	0,77 $\pm$ 0,03	1,39 $\pm$ 0,04		***	
	<i>rhcg2</i>	1,59 $\pm$ 0,07	0,72 $\pm$ 0,05	1,48 $\pm$ 0,05	0,61 $\pm$ 0,04		***	
	<i>rhbq</i>	1,26 $\pm$ 0,07	0,84 $\pm$ 0,04	1,31 $\pm$ 0,06	0,79 $\pm$ 0,04		***	
	<i>rhag</i>	1,03 $\pm$ 0,07	0,98 $\pm$ 0,06	1,18 $\pm$ 0,06	0,94 $\pm$ 0,06		**	
<i>gill permeability</i>	<i>cldn5a</i>	0,78 $\pm$ 0,04	1,37 $\pm$ 0,06	0,89 $\pm$ 0,05	1,27 $\pm$ 0,06		***	*
	<i>cldn5b</i>	1,13 $\pm$ 0,04	0,96 $\pm$ 0,03	1,12 $\pm$ 0,05	0,92 $\pm$ 0,04		***	
	<i>cldn7a</i>	0,8 $\pm$ 0,03	1,32 $\pm$ 0,04	0,81 $\pm$ 0,03	1,3 $\pm$ 0,04		***	
	<i>cldn8like</i>	1,07 $\pm$ 0,04	1,14 $\pm$ 0,04	0,97 $\pm$ 0,03	0,91 $\pm$ 0,04			*
	<i>cldn12</i>	1,02 $\pm$ 0,03	1,01 $\pm$ 0,03	1,02 $\pm$ 0,03	0,97 $\pm$ 0,03			
	<i>cldn23a</i>	1,14 $\pm$ 0,05	1,03 $\pm$ 0,04	0,99 $\pm$ 0,03	0,93 $\pm$ 0,03		**	
	<i>oclna</i>	1,02 $\pm$ 0,05	1,13 $\pm$ 0,04	0,94 $\pm$ 0,04	1,01 $\pm$ 0,05		*	
	<i>oclnb</i>	0,6 $\pm$ 0,03	1,98 $\pm$ 0,07	0,58 $\pm$ 0,03	1,71 $\pm$ 0,07		***	**
	<i>tjp1</i>	1,18 $\pm$ 0,06	0,89 $\pm$ 0,05	1,18 $\pm$ 0,06	0,9 $\pm$ 0,06		***	
<i>hormonal regulation</i>	<i>mr</i>	0,99 $\pm$ 0,03	1,09 $\pm$ 0,03	0,97 $\pm$ 0,03	1,01 $\pm$ 0,05		**	
	<i>gr1</i>	1,11 $\pm$ 0,04	0,92 $\pm$ 0,02	1,08 $\pm$ 0,04	0,9 $\pm$ 0,03		***	
	<i>gr2</i>	1,04 $\pm$ 0,03	1,01 $\pm$ 0,03	1,05 $\pm$ 0,04	0,91 $\pm$ 0,03		**	*

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Sorting and dispatching



T: Tagging using RFID-tag

B: Biometry

P1: Blood/brain sampling

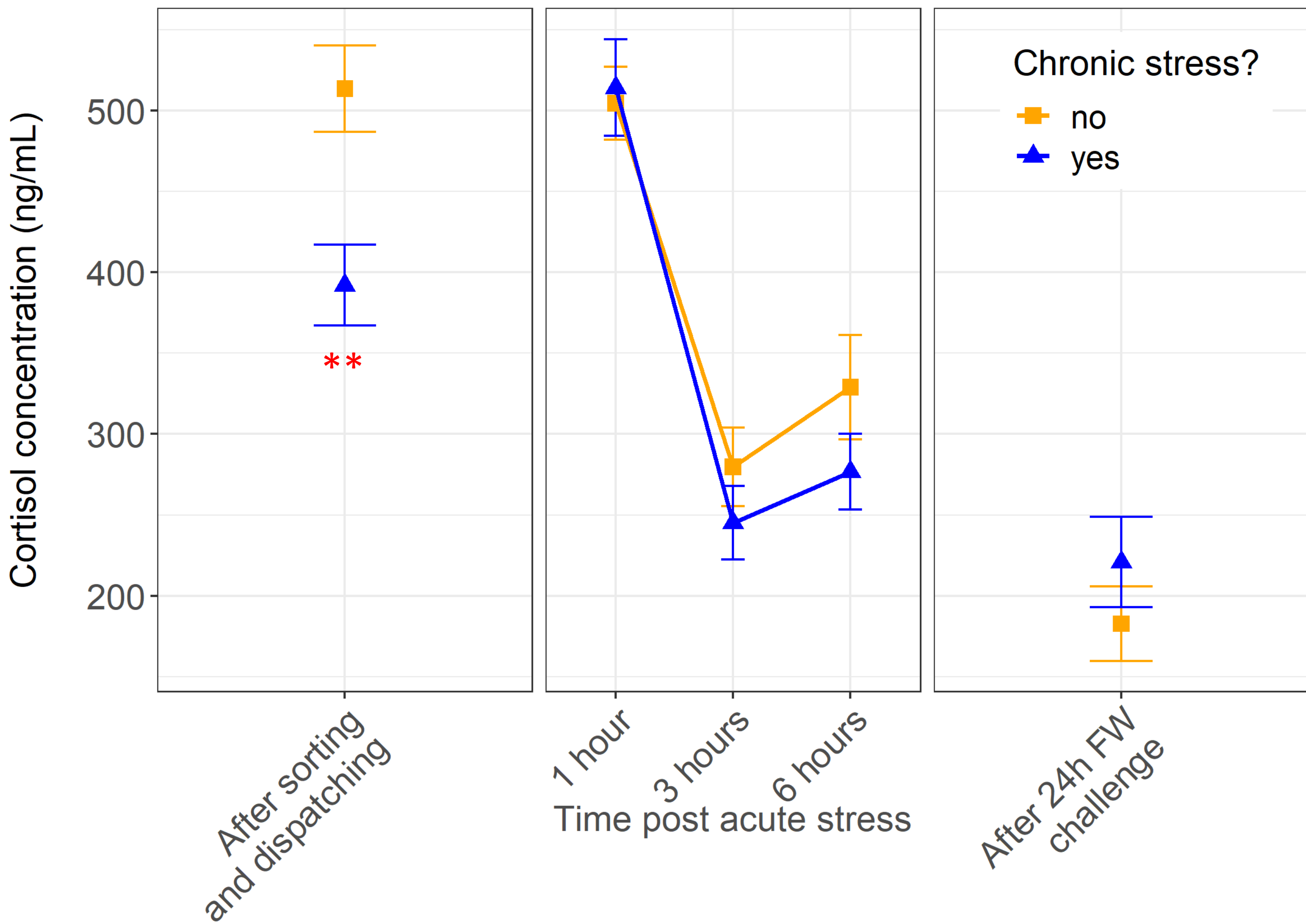
P2: Acute stress test + blood sampling 1h later

P3: Acute stress test + blood sampling 3h later

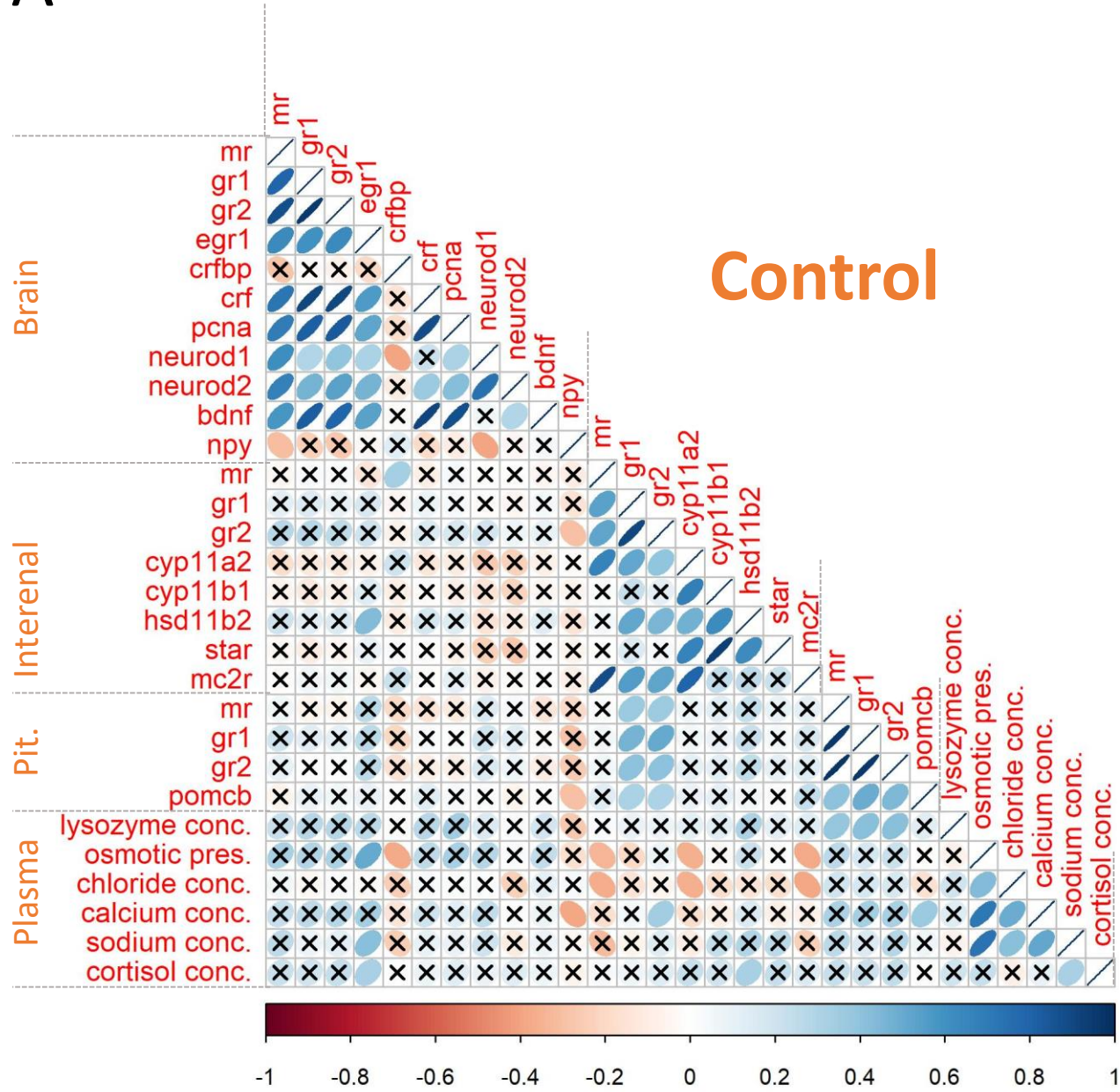
P4: Acute stress test + blood sampling 6h later

P5: Osmotic test + blood/gills sampling

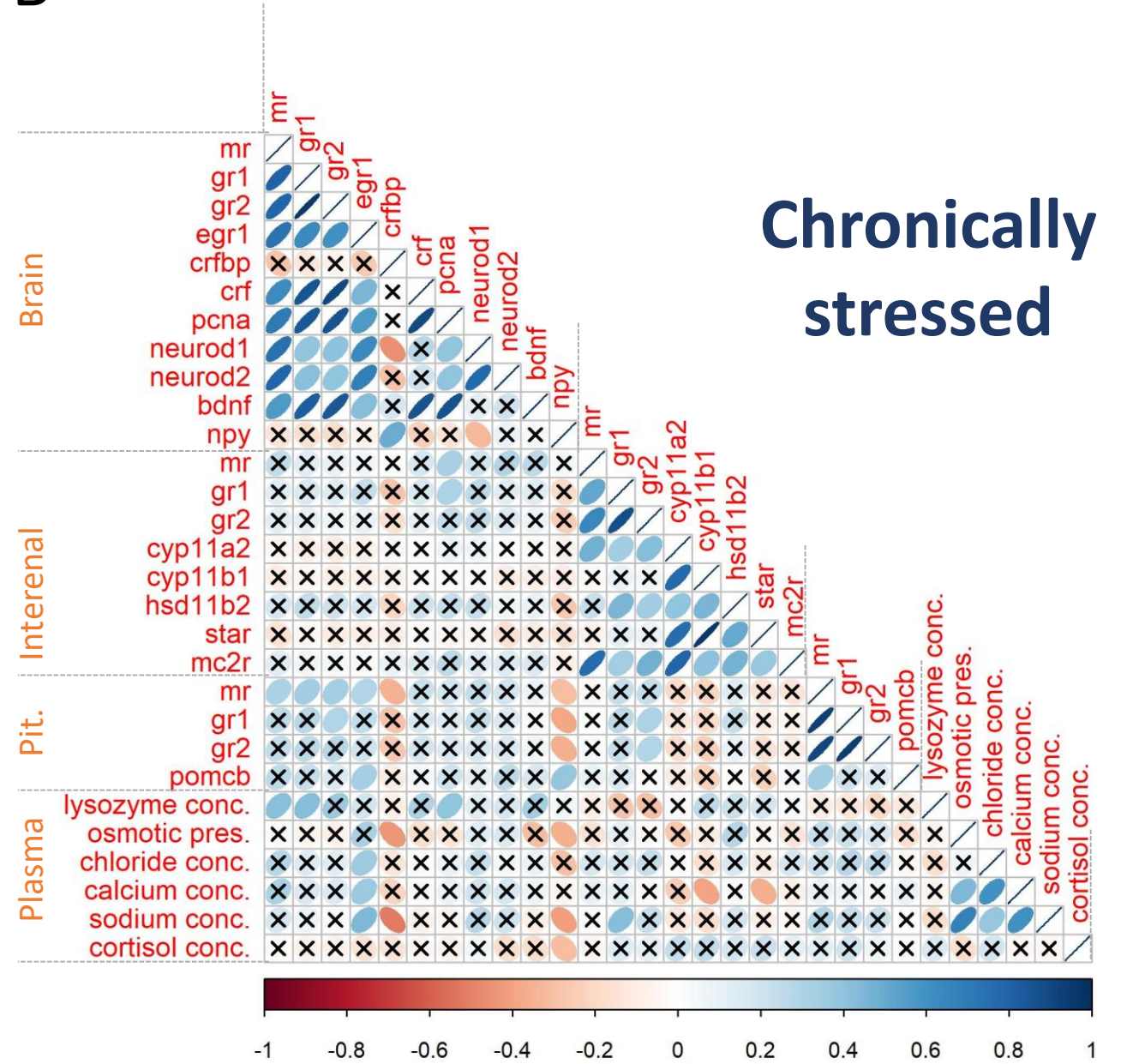
NE: Novel environment + hypoxia test

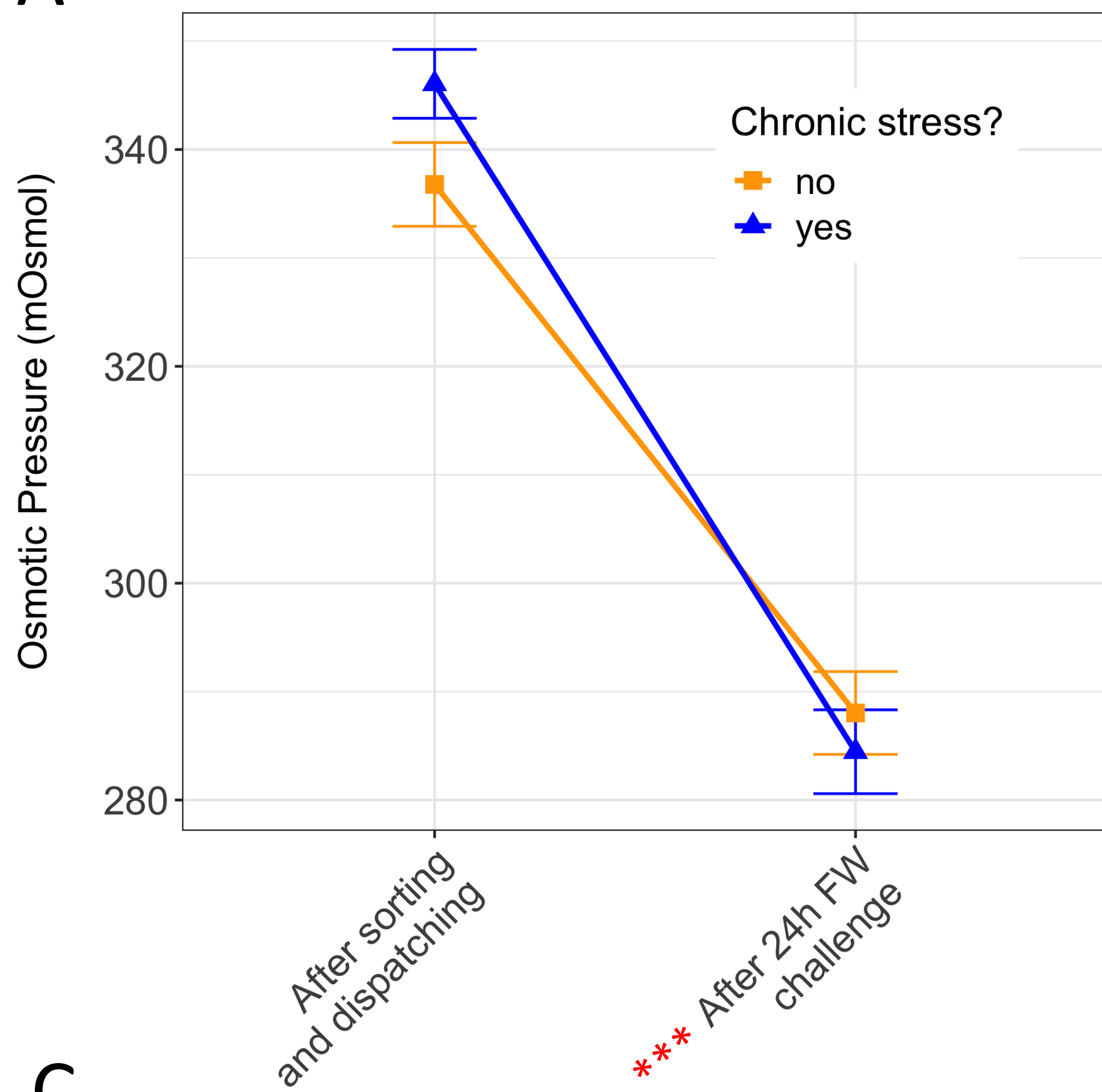
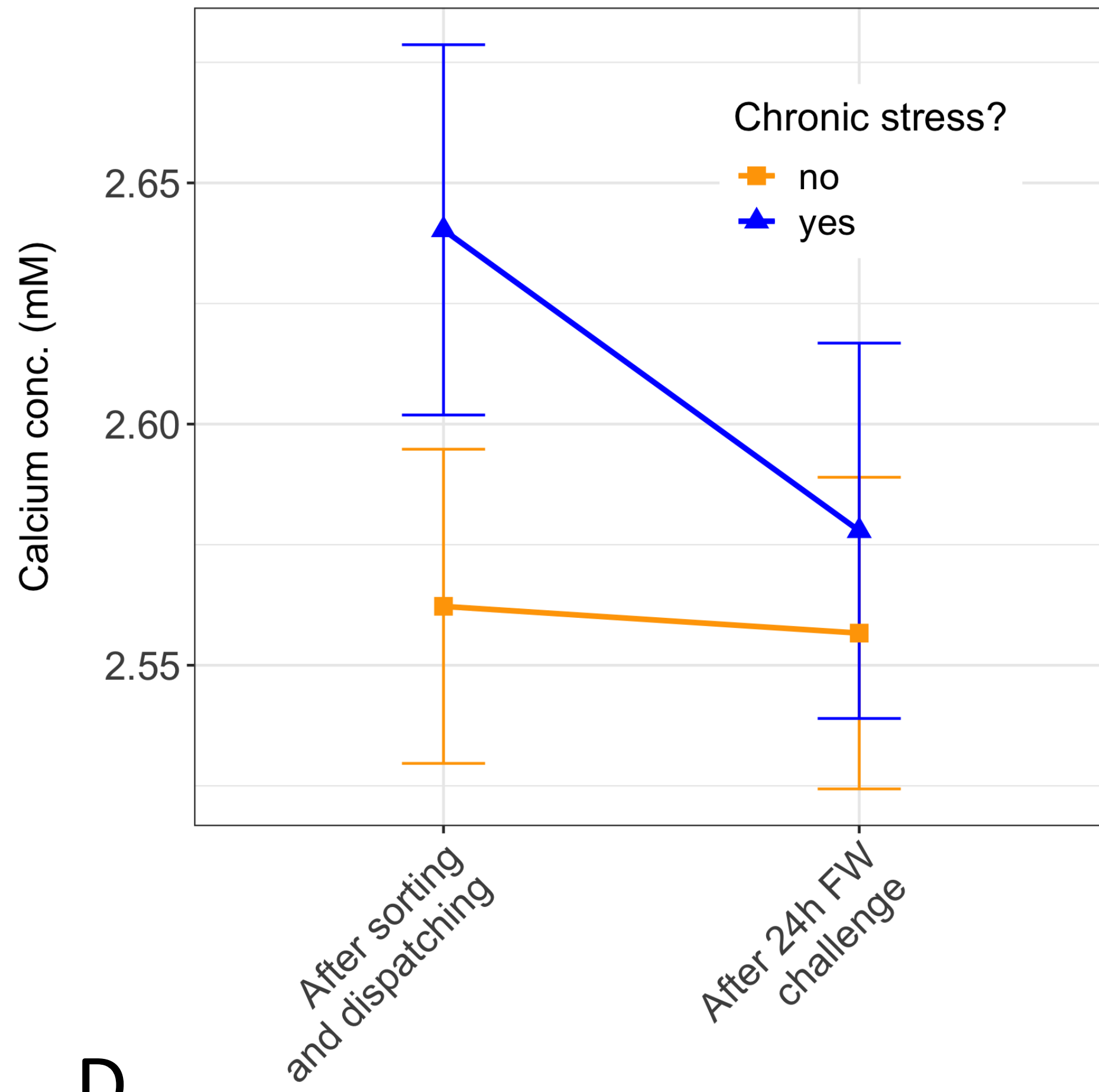
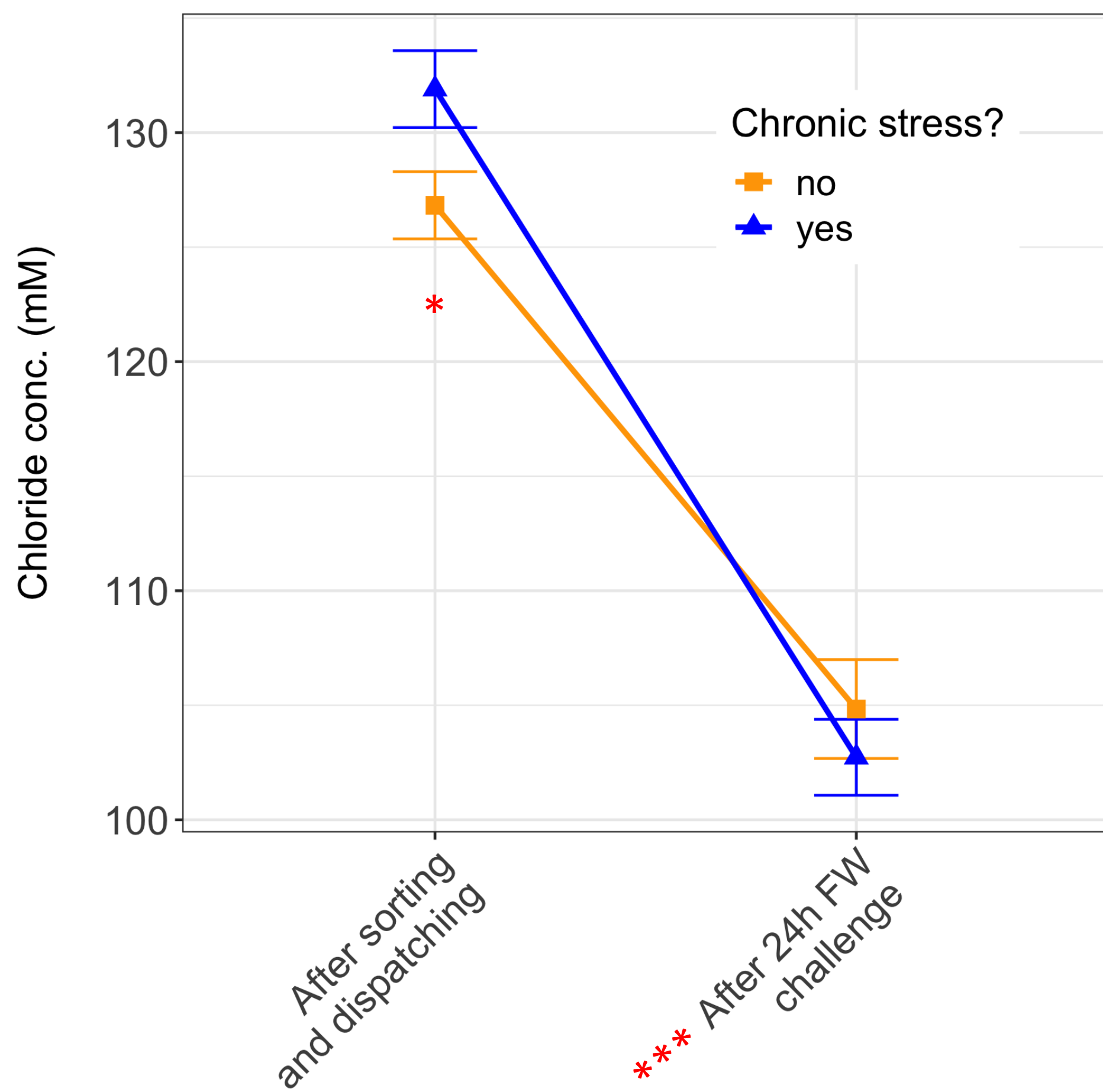
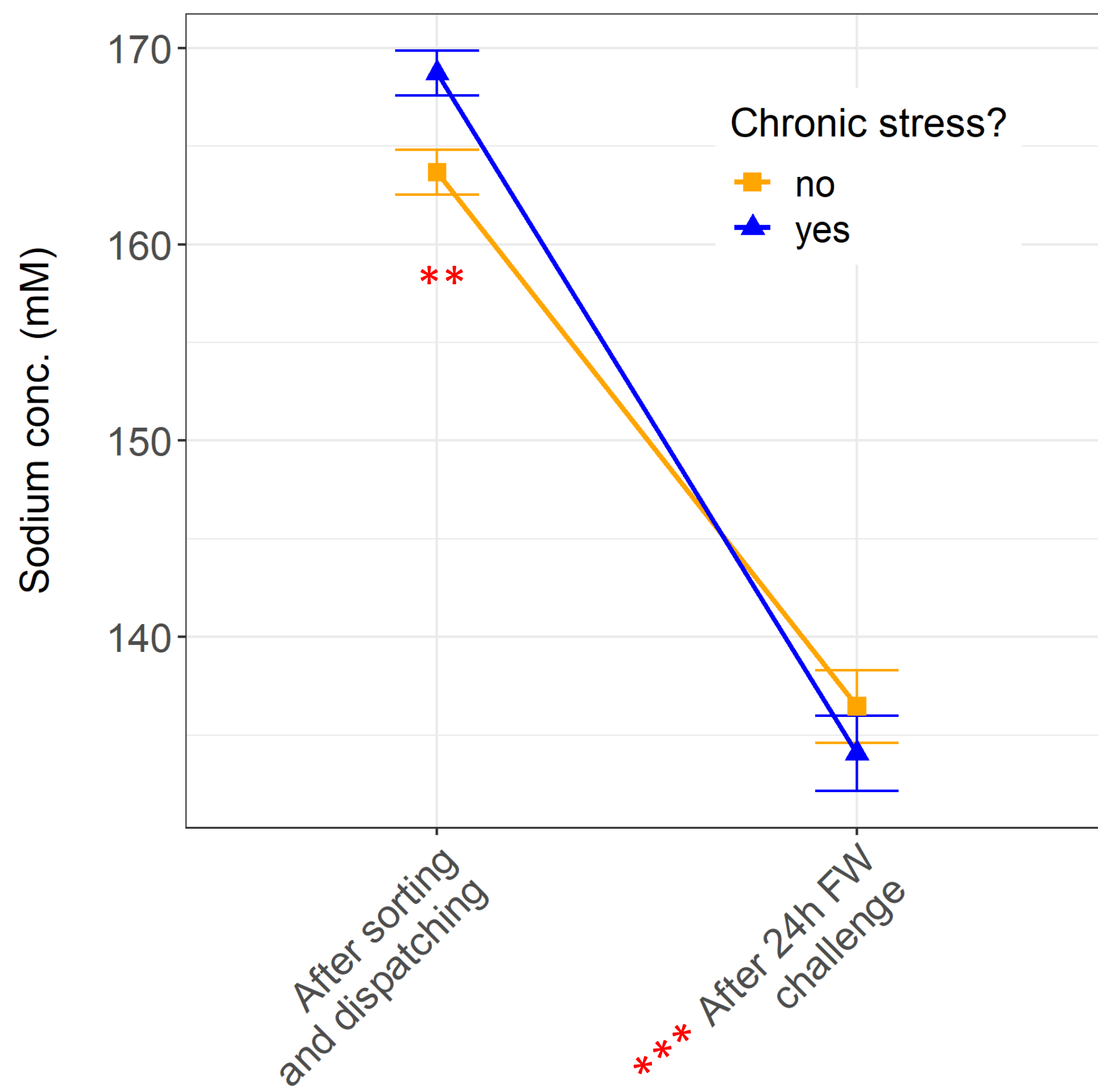


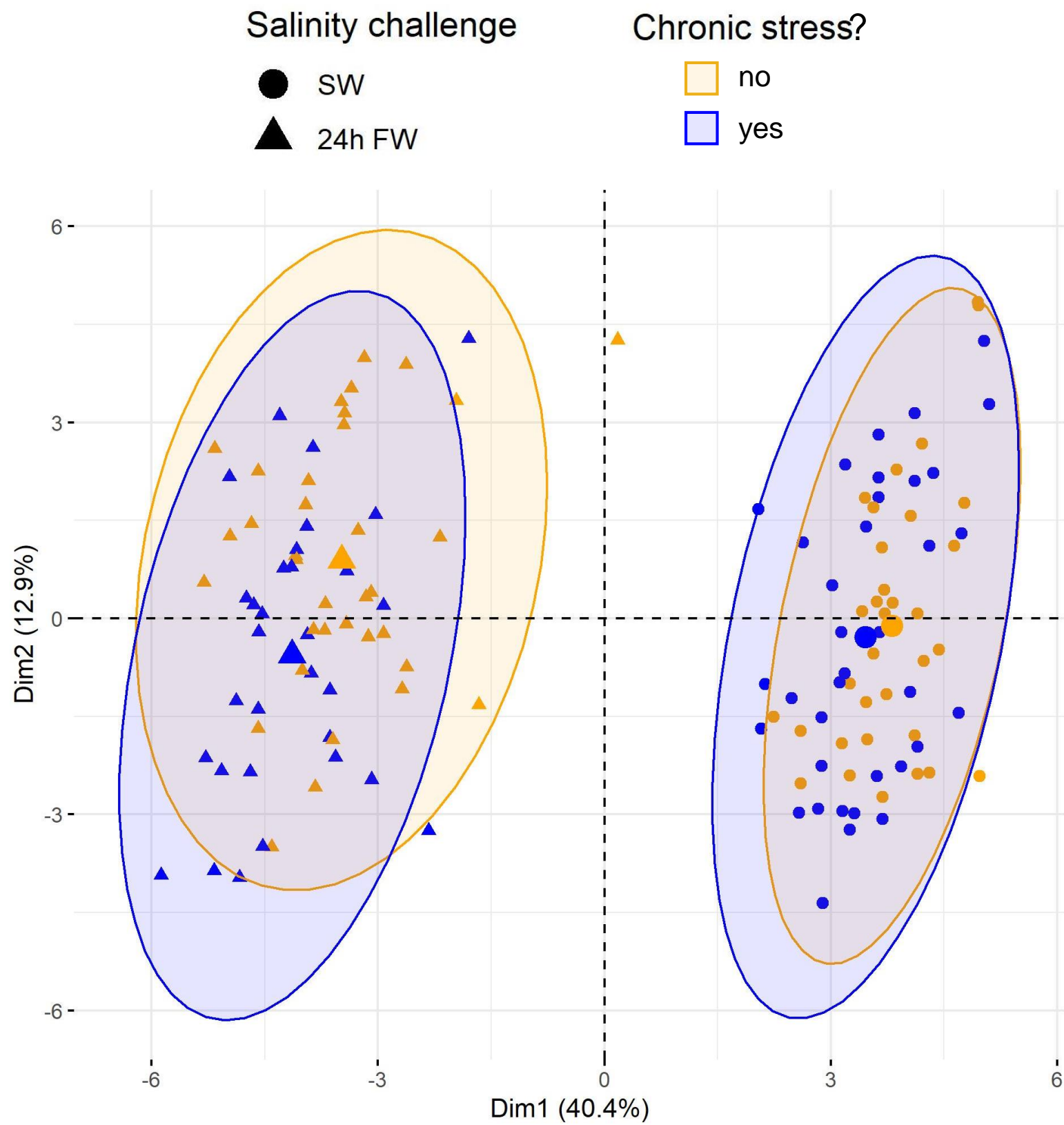
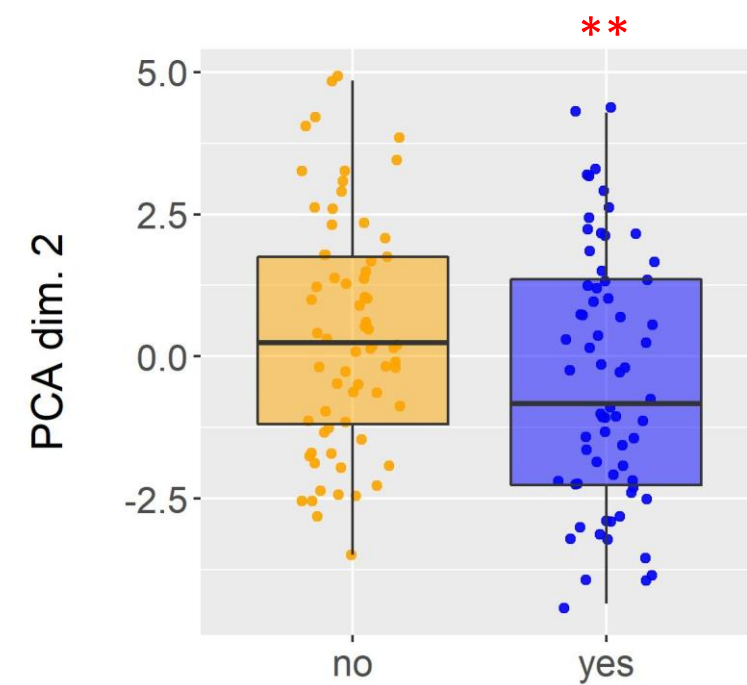
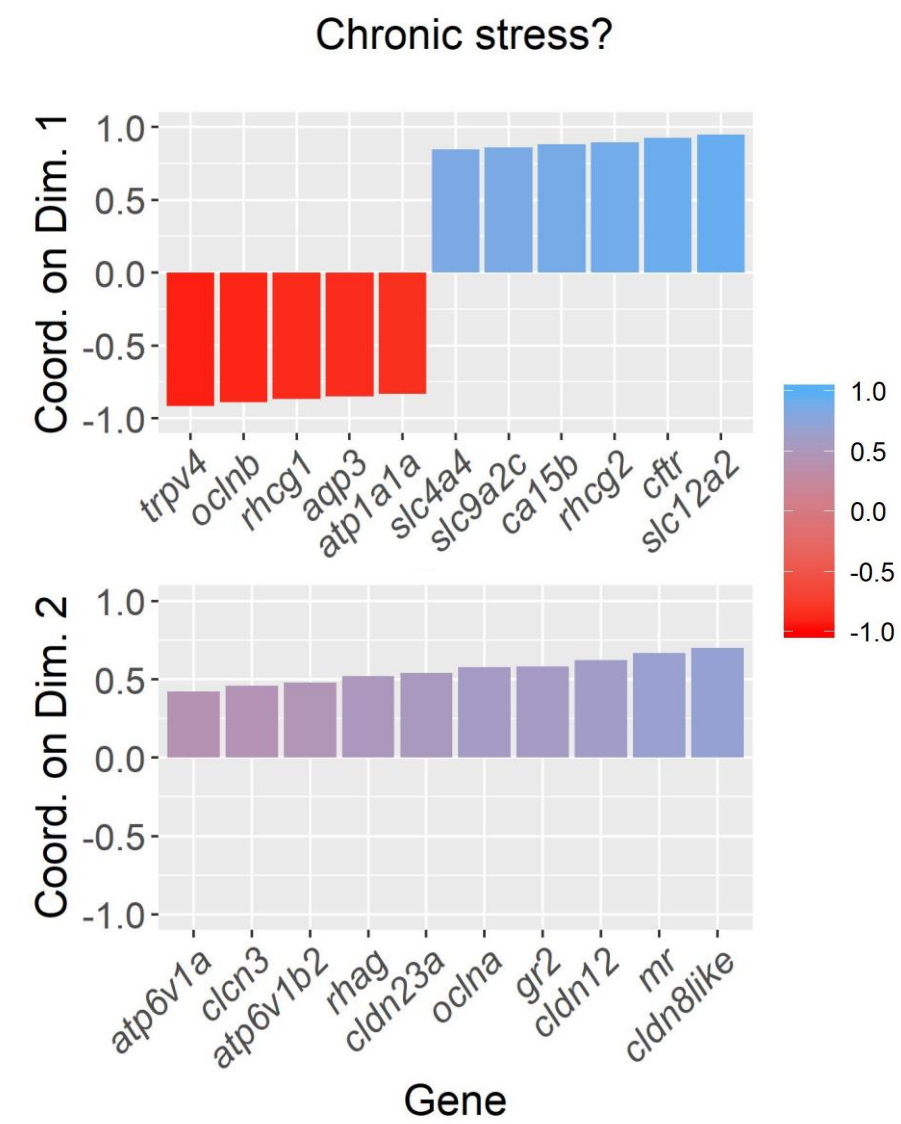
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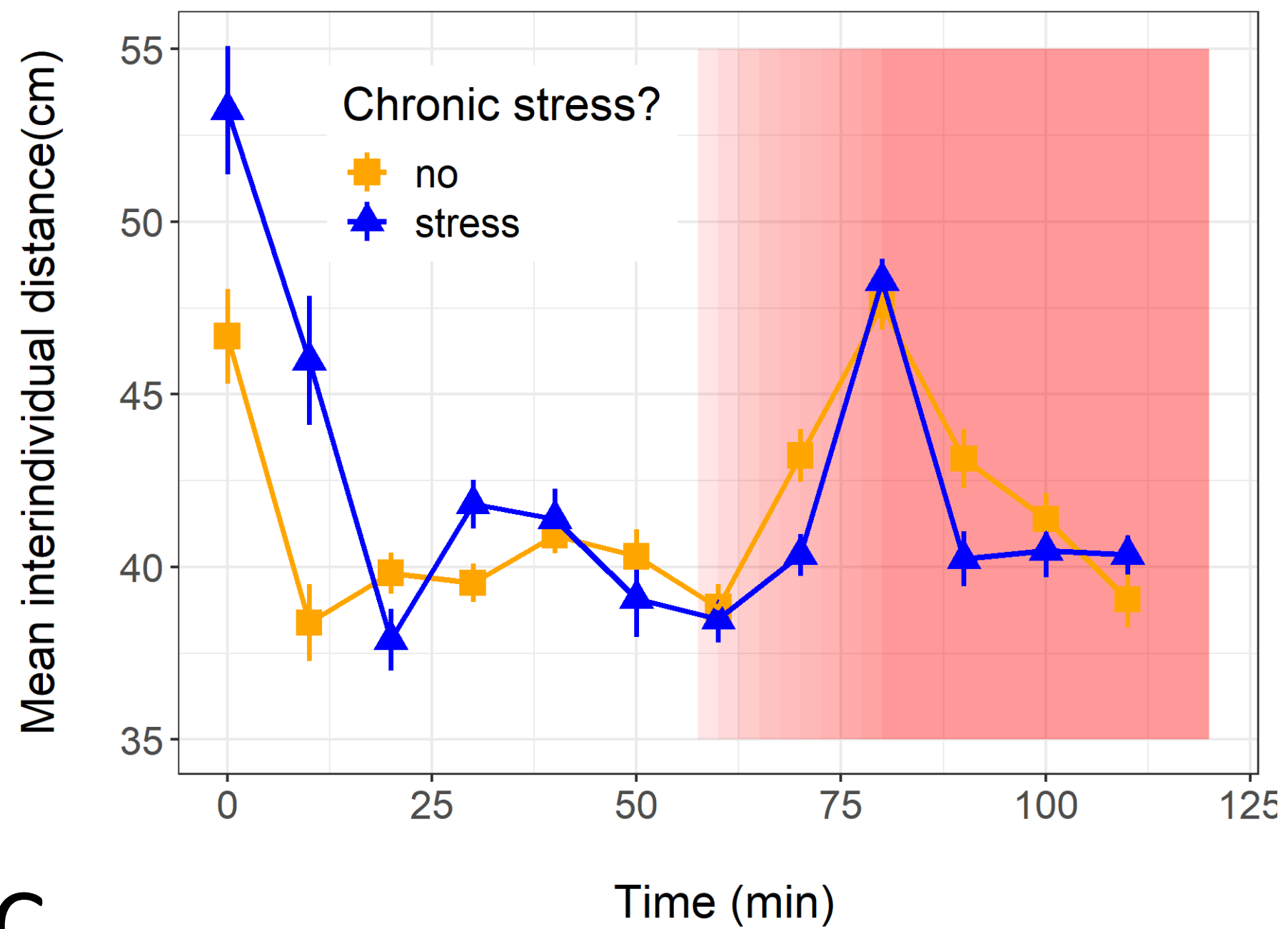
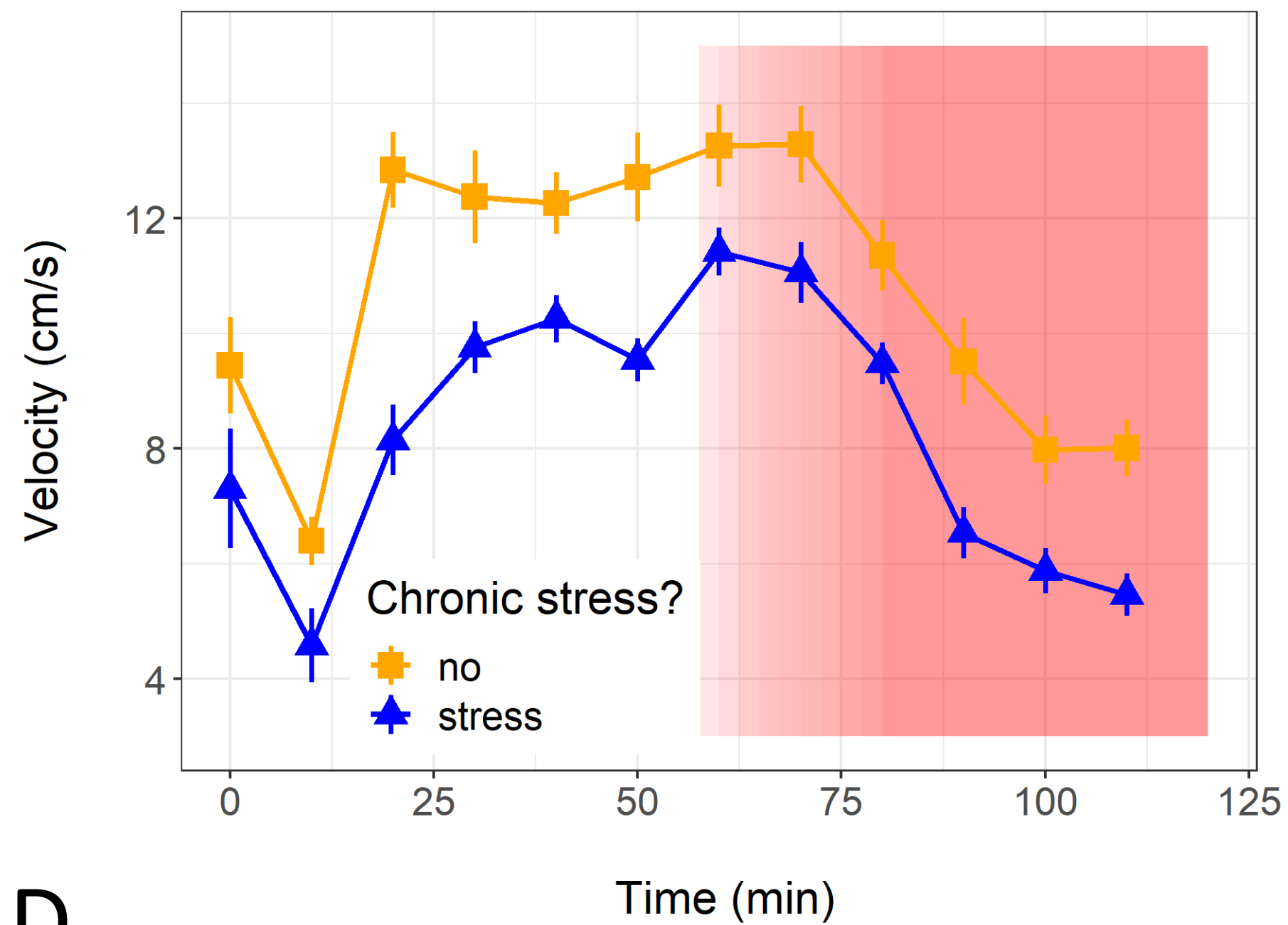
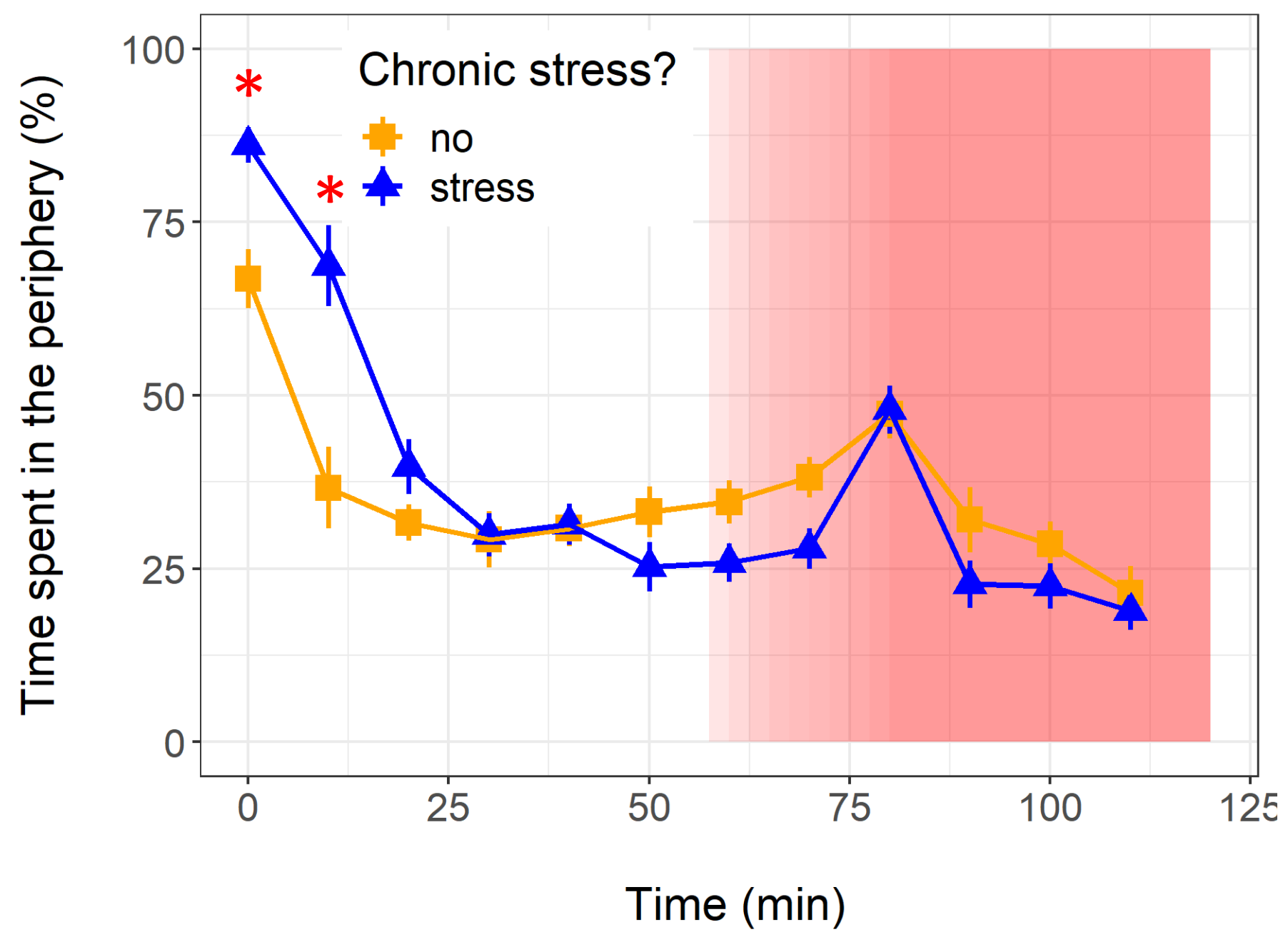


B



**A****B****C****D**

**A****B****C**

**A****B****C****D**