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Highlights

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

Abstract

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

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

Keywords

Welfare, Plasticity, Fish, Osmoregulation, Robustness, Resilience

1. Introduction

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

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

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

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

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

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

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

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

2. Material and Methods

2.1.Rearing conditions

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

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

Over the entire experiment, rearing densities were below 40 kg/m3 considered as an intermediate density in recirculating system and shown to have no effect on fish stress level nor growth (Sammouth et al., 2009).

2.2.Chronic stress protocol

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

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

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

- Every day, 6 randomly programmed one minute flash of light (including 3 overnight),
- Every week, 3 randomly planned one minute chasing with a net,
- Every week, 3 randomly planned thirty minutes confinement stress obtained by reducing initial tank volume to ¼ .

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

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

2.3.Biometries

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

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

2.4.Sampling protocols

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

Right after the end of the three weeks of chronic stress, one tank of the control treatment and one tank of the chronic stress treatment were fasted for 24 h. Fish were then slightly anesthetized (Stage I of anesthesia) with Benzocaine (15 $mg.L^{-1}$) in their home tank, simultaneously fished in both tanks and fully anesthetized (Stage II) with37.5 mg $Benzocaine.L^{-1}$. They were then identified based on their RFID tag and accordingly dispatched in order to perform one of the following 6 protocols:

- Protocol 1 (P1): A total of 18 individuals per treatment (36 fish in total) were euthanized in high dose of benzocaine (225 $mg.L^{-1}$) immediately after dispatching. Blood was collected from the caudal vein using a heparinized syringe. The brain, the pituitary and the head kidney were extracted and immediately frozen in liquid nitrogen. The gills of 12 individuals per treatment were also collected and frozen in liquid nitrogen.
- Protocol 2 (P2): A total of 18 fish per treatment were first allowed to rest in 80L tank filled with clear water for 20 minutes. They were then exposed to an acute stress test (AST) consisting in confining the fish for 4 minutes in a 10 L aerated bucket. The fish were then allowed to recover from the stressor for one hour in a 100 L tank supplied with water renewed twice per hour. Fish were then euthanized in 225 mg $benzocaine.L^{-1}$ and blood was collected.
- Protocol 3 (P3): A total of 18 fish per treatment were sampled following the same procedure as described for P2 but allowing the fish to recover during 3 hours in another identical 100 L tank.
- Protocol 4 (P4): The same procedure as P3 was also performed on 18 fish per treatment but with a 6 hours recovery period.

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

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

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

2.5.Plasmatic measurements

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

2.5.1. Cortisol measurement using ELISA

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

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

2.5.2. Lysozyme concentration

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

chloride from chicken egg white (Sigma) in sodium phosphate buffer, the lysozyme concentration in the plasma was expressed in $U.mL^{-1}$.

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

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

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

The novel environment in group was adapted from (Alfonso et al., 2020) and shown to be sensitive to environmental perturbations (Alfonso et al., 2020). Briefly, at 422, 424 and 425 dpf (2 months after the end of the chronic stress protocol), two groups of 8±1 fish from each treatment were transferred in 4 different tanks. After one night, the group of fish (either group of control or group of stressed fish) was placed in a 1 m^2 observation tank (75cm x 75cm x 21.5 cm of water height, 120 L) for measuring behavioural response to a new environment. A corner (15x15 cm, 225 cm^2) of the tank was separated from the open field arena by a grid and contained a pump, oxygen and nitrogen aerators to maintain targeted oxygen concentration and an oxygen probe (Odéon, NEOTEK-PONSEL, Caudan, France) to record oxygen saturation every minute all along the experiment. After 1 hour in the new environment, oxygen saturation was reduced over 20 minutes using nitrogen bubbling, in order to reach a saturation around 20%. This hypoxic period lasted 40 minutes. Fish behaviour was recored over the whole duration of the test for a total of 2 hours using a DMK 31AU03 camera and IC Capture software (The Imaging Source, Germany) at 25 $frames.s^{-1}$. 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 ($cm.s^{-1}$), 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°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 µL MR1 and 1 µl 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 µl was collected, and diluted in 150 µl 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 µg RNA was treated with 0.5 units RQ1 DNase (Promega, Madison, WI,

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

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

2.7.4. Primers selection

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

- the main genes of the HPI axis (*crf, crfbp, pomcb, star, mc2r, hsd11b2, cyp11a2, cyp11b1 mr, gr1, gr2*)
- genes coding for cell proliferation and neural differentiation and known to covary with the stress axis (Sadoul et al., 2018) (*egr1, pcna, neurod1, neurod2, bdnf*),
- one key genes related to the appetite and known to be affected by stress (Sadoul and Vijayan, 2016) (*npy*)
- genes involved in ions and water movements previously published for European sea bass (Blondeau-Bidet et al., 2019a; Bodinier et al., 2009; Bossus et al., 2011, 2013; Giffard-Mena et al., 2007) *(atp1a1a, atp1a1b, slc12a2, slc12a3-like, clcn3, slc4a4, atp6v1a, atp6v1b2, slc9a2b, slc9a2c, slc9a3, trpv4, cftr, aqp3)* or for other fish species (de Polo et al., 2014; Shahsavarani and Perry, 2006; Su et al., 2020) (*ecac, cazh, ca15b*)
- genes involved in ammonia transport in European sea bass (Blondeau-Bidet et al., 2019a) (*rhcg1, rhcg2, rhbg*) or in other fish species (Wood and Nawata, 2011) (*rhag*).
- genes coding for proteins controlling gill permeability (Chasiotis et al., 2012) (*cldn5a, cldn5b, cldn7a, cldn8like, cldn12, cldn23a, oclna, oclnb, tjp1*).

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

2.7.5. Real-time PCR protocol

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

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

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

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

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

2.9. Statistical analyses

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

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

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

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

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

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

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

3. Results

Growth performances

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

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

Homeostasis regulation of ions and water before and after an osmotic test

Effects of chronic stress before the osmotic test

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

Effects of the osmotic challenge

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

This OC also induced significant modification of the expression of several gill genes related to ion and water homeostasis, ammonia transport, cortisol receptors and most genes involved in gill permeability (Table 3). Finally, expression of these genes did not significantly differ between control and chronically stressed fish, but a significant interaction effect (p<0.05) between the OC and the chronic stress treatment was observed for several of these genes, including *atp1a1b* ($F_{1,122}$ = 4.1, p-value = 0.045), *slc12a3like* ($F_{1,128}$ = 4.1, p-value = 0.045), *cftr* ($F_{1,130}$ = 4.3, p-value = 0.04), *slc9a2c* ($F_{1,125}$ = 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 *rhch1, aqp3 and atp1a1a* are the genes the most upregulated in the 24 hours
576 freshwater fish, while *slc12a2, cftr, 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,

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

Parameters measured 4 months later

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

4. Discussion

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

Consequences on growth

In the present study, the most apparent effects of the chronic stress protocol were detected on biometric results, with a clear reduction in growth of body mass and length. It is well known that stress is energy consuming, decreases appetite through well-described molecular mechanisms and thus reduces growth performances in fish (Sadoul and Vijayan, 2016). These growth data are therefore not surprising and in agreement with previous studies (Samaras et al., 2018; Santos et al., 2010). Samaras et al. (2018) also observed growth reduction for European sea bass exposed to a “high stress regime” over three weeks, and this reduction led to a 6.5% difference in final body mass. Based on body mass results (9% difference), the stress protocol (also over three weeks) could be considered to lead to a more severe stress load. Nevertheless, 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^+ and Cl^-). 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 *nnc2* 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.

Fish from both treatments (control and chronic stress) responded to the OC with a significant decrease in blood osmotic pressure and NaCl levels in agreement with previous results (Bossus et al., 2011; L'Honoré et al., 2019). In parallel significant changes in the expression of genes implicated in ions and water movements were observed in agreement with previous studies in sea bass measuring gene expressions 24 h or several weeks after a transfer in FW: (i) decrease in *nkaα1b*, *nkcc1a*, *cftr*, *nhe2c*, *vha-a* and *vha-b*, *ca15b* transcript levels and (ii) increase in gene expression of *nkaα*1a, *ncc2*, *clcn3*, *nhe3*, *trpv4*, *rhcg1*, *aqp3* (Blondeau-Bidet et al., 2019a; Bodinier et al., 2009; Bossus et al., 2011, 2013; Giffard-Mena et al., 2007; L'Honoré et al., 2020; Lorin-Nebel et al., 2006; Masroor et al., 2019). In both treatments, we also measured significant changes in the expression of genes involved in paracellular movement of solutes with *cldn5a*, *cldn7*, *oclna* and *oclnb* up-regulated and *cldn5b*, *cldn23a* and *zo-1* down-regulated in FW. In Atlantic killifish, *ocln* gene expression was also up-regulated following hypo-osmotic challenge (Whitehead et al., 2011) and acclimation of goldfish to ion-poor water induced an increase in *ocln* and *cldn7* mRNA and a decrease of *zo-1* transcript (Chasiotis et al., 2012). More work would be needed to better understand the functional roles in osmotic challenge for genes such as *cldn5a* and *cldn5b*.

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

Biomarkers related to behaviour.

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

Resilience

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

Conclusions

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

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

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

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Competing interests

'No competing interests declared'

Data availability

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

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Fig. 1. Timeline of the experimental protocol performed.

Fig. 2. Effects of chronic stress on the evolution of biometric values in European sea bass. At 309 dpf, fish were dispatched in 6 different tanks. At 336 dpf, fish from three tanks were chronically stressed with repeated acute stressors during 3 weeks (red zone). Differences to the controls are presented for the body mass (A) and the length (B). Data are represented as mean ± SEM. The continuous lines represent the simulated outputs from the DEB model.
(n=873 and 879 until 309 dpf, n=865 and 877 at 336 dpf, n=247 and 252 at 358 dpf, n=586 and 593 at 424 dpf, n=573 and 581 at 463 dpf for control and chronic stress respectively). The pink period illustrates the 4 weeks acclimation period to the experimental tanks prior the chronic stress. Difference to the control are highlighted by an asterisk.

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

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

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

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 ± SEM. The difference between chronically stressed
1372 individuals and control individuals at a single time point are illustrated with an
1373 asterisk ($p<0.05$).

1374 Table 1. Published primers used for real-time PCR on European sea bass cDNA.
1375
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Gene	Protein name	GenBank accession numbers	Forward primer (5' → 3')	Reverse primer (5' → 3')	Concentration in qPCR well	Tm	Ref
eef1-alpha	elongation factor 1-alpha 1	AJ866727.1	AGATGGGCTTGTTCAAGGGA	TACAGTTCCAATACCGCCGA	0,4	60	(Sadoul et al., 2018)
l13	ribosomal Protein L13	DT044910.1	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTGAGAGCAG	0,4	60	(Sadoul et al., 2018)
18S	18S ribosomal RNA	AM419038.1	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCACA	0,4	60	(Pavlidis et al., 2011)
fau	ubiquitin like and ribosomal protein S30 fusion	FM004681	GACACCCAAGGTTGACAAGCAG	GGCATTGAAGCACTTAGGAGTTG	0,4	60	(Mitter et al., 2009)
rpl17	ribosomal Protein L17	AF139590	TTGAAGACAACGCAGGAGTCA	CAGCGCATTCTTTTGCCACT	0,2	60	(Samaras et al., 2018)
mr	mineralocorticoid receptor	JF824641.1	GTTCCACAAAGAGCCCCAAG	AGGAGGACTGGTGGTTGATG	0,4	60	(Sadoul et al., 2018)
gr1	glucocorticoid receptor 1	AY549305	GAGATTTGGCAAGACCTTGACC	ACCACACCAGGCGTACTGA	0,4	60	(Pavlidis et al., 2011)
gr2	glucocorticoid receptor 2	AY619996	GACGCAGACCTCCACTACATTC	GCCGTTCATACTCTCAACCAC	0,2	60	(Pavlidis et al., 2011)
mc2r	melanocortin 2 Receptor	FR870225.1	CATCTACGCCTTCCGCATTG	ATGAGCACCGCCTCCATT	0,4	60	(Tsalafouta et al., 2017)
cyp11b1 (11B-hydroxylase)	cytochrome P450 family 11 subfamily B member 1	AF449173.2	GGAGGAGGATTGCTGAGAACG	AGAGGACGACACGCTGAGA	0,4	60	(Samaras et al., 2018)
atp1a1a	Na/K ATPase alpha subunit isoform 1a	KP400258	CCTCAGATGGCAAGGAGAAG	CCCTGCTGAGATCGGTTCC	0,4	60	(Blondeau-Bidet et al., 2016)
atp1a1b	Na/K ATPase alpha subunit isoform 1b	KP400259	AGCAGGGCATGAAGAACAAG	CCTGGGCTGCGTCTGAGG	0,4	60	(Blondeau-Bidet et al., 2016)
slc12a2 (nkcc1)	sodium-potassium-chloride cotransporter 1	AY954108	TCAGCTCACAGTTCAAGGCC	TTGTGGAGTCCATAGCGGC	0,4	60	(Blondeau-Bidet et al., 2019b)
slc12a3-like (ncc2)	sodium-chloride cotransporter 2		ATGATGAGCCTCTTCGAGCC	GCTGCTCTCATCACCTTCTGT	0,6	60	(Blondeau-Bidet et al., 2019a)
clcn3	chloride channel 3	JN998891	CAAGTACAGCAAGAACGAGGC	ACAGCGTCTTGAGAGGGAAG	0,4	60	(Bossus et al., 2013)
slc4a4 (nbc1)	sodium bicarbonate cotransporter	FM001880	ACAGAGCACGGAACACACGG	CGTCCACAGCCAGCAGTTCG	0,4	60	(Blondeau-Bidet et al., 2019a)
atp6v1a (vha-a)	V-type proton ATPase catalytic subunit a		GGCAGTCACATCACAGGAGG	CCAGCTCCATCACCACATCG	0,4	60	(Blondeau-Bidet et al., 2019a)
atp6v1b2 (vha-b)	V-type proton ATPase catalytic subunit b2		TTGCCATAGTCTTCGCAGCC	CTTCTCGCACTGGTAGGCC	0,4	60	(Blondeau-Bidet et al., 2019a)
slc9a3 (nhe3)	sodium/hydrogen exchanger isoform 3	CX660524	GGATACCTCGCCTACCTGAC	AAGAGGAGGGTGAGGAGGAT	0,4	60	(Blondeau-Bidet et al., 2019a)
slc9a2b (nhe2b)	sodium/hydrogen exchanger isoform 2b		CTGTCAGATCGAGGCGTTTG	TCAAACACACTCAGCACAGC	0,4	60	(Blondeau-Bidet et al., 2019a)
slc9a2c (nhe2c)	sodium/hydrogen exchanger isoform 2c		CGTTTCACCCACAATGTCCG	GCACCAGAATGCCAATTCCC	0,4	60	(Blondeau-Bidet et al., 2019b)
trpv4	transient receptor potential cation channel subfamily V member 4	GQ396264	CGGGAGAGATTGTCACCTTG	CCATCACGGACACATAAGCC	0,4	60	(Bossus et al., 2011)
rhbg	rhesus blood group, b glycoprotein		CCTCATGGTGACCCGAATCC	TATGTGGACAGAGTGCAGGC	0,4	60	(Blondeau-Bidet et al., 2019b)
rhcg1	rhesus blood group, c glycoprotein 1		TCAGGGAATTGTGTGACCGC	CCCAGCGTGGACTTGATTCT	0,4	60	(Blondeau-Bidet et al., 2019b)
rhcg2	rhesus blood group, c glycoprotein 2		TGGCTACCTGTTTGTCACGC	TATAAAGCCGCCGAGCATCC	0,4	60	(Blondeau-Bidet et al., 2019b)
pcna	proliferating cell nuclear antigen		CAGAGCGGCTGGTTGCA	CACCAAAGTGGAGCGAACAA	0,4	60	(Crespo et al., 2013)
neurod1	neuronal differentiation 1		TTCTCCTTCAGCGTGCACTA	GGTGCGAGTGTCCATCAAAG	0,4	60	(Sadoul et al., 2018)

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1378 Table 2. Designed primers used for real-time PCR on European sea bass cDNA.
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Gene	Protein name	GenBank accession numbers	Forward primer (5' → 3')	Reverse primer (5' → 3')	amplicon size	Concentration in qPCR well	http://seabass.mpipz.de/cgi-bin/hgGateway	Efficiency
gapdh	glyceraldehyde-3-Phosphate Dehydrogenase	AY863148	GAGAAACCCGCCAAATATGAC	TACCATGTGACCAGCTTGAC	193	0,4	DLAgn_00059000	86,3
actb	actin beta	AY148350.1	TGACCTCACAGACTACCT	GCTCGTAACTCTTCTCCA	140	0,4	DLAgn_00187660	92,3
crf	corticotropin-releasing hormone		GCAACGGGGACTCTAACTCT	GTCAGGTCCAGGGATATCGG	217	0,6	DLAgn_00076040	86,0
crfbp	corticotropin-releasing hormone binding protein		CCAGAGGGCAGTTTCACCAT	ACATAGTCACCTGACCCCGA	173	0,4	DLAgn_00120190	92,0
star	steroidogenic acute regulatory protein		TGAGCTGAACAGACTGGCAG	TCTCCATTCGCAGCCACAAT	216	0,4	DLAgn_00115570	92,0
hsd11b2	hydroxysteroid (11-beta) dehydrogenase 2		CCGAGCTGTCCCTAATGTCG	TGAGGAGGGTAGGATGGTGG	263	0,4	DLAgn_00234370	87,3
pomcb	pro-opiomelanocortin-b		GGATACTGGACTGTATTCACCT	GAAATGCCCTCAGAAGATCC	291	0,4	DLAgn_00069720	94,2
cyp11a2 (p450scc)	p450 side chain cleaving		CCCCCGTCAGTGTTTAGGAC	TTGCGCTGTTTCTCCACTCT	96	0,4	DLAgn_00168500	91,3
cftr	cystic fibrosis transmembrane conductance regulator	DQ501276.2	GAACCAACCAGGACAAACCA	GCAAGTCGATGAACTTAAACACTC	149	0,4	DLAgn_00172160	89,6
cazh	carbonic anhydrase	FK944087	GACTAACGGACCTGATACATGG	TCTGTGTCGTCCACAAAGTC	223	0,4	DLAgn_00000090	92.7
ca15b	carbonic anhydrase 15b	CX660749	GGCAAGACAGTCAAAGTCAG	CCTCAATAAAGAAACCAAGAGCAG	255	0,6	DLAgn_00101240	86,0
trpv6 (ecac)	epithelial calcium channel		TTCCATGTTATCCTTATCGGCT	CATAAACTTTGTCAGGTCTCCA	209	0,4	DLAgn_00059190	90,9
aqp3	aquaporin 3	DQ647191	CATGTACTACGATGCCCTGTG	CATAGCCAGAGTTAAAGCCCA	271	0,4	DLAgn_00117370	96,8
rhag	rhesus blood group, a glycoprotein		CAAGTTCCTTTCTCCCATCCT	GTAACAACACCTCCAACCAG	206	0,4	DLAgn_00071020	92,0
egr1	early growth response 1		AACTCCAGCCTCAGTTCCTC	AGTCAGGAATCATGGGCACA	202	0,4	DLAgn_00110040	91,6
neurod2	neuronal differentiation 2		TGCGTAAAGTGGTTCCATGC	GTCGTGGGTTGGGAGAGTC	172	0,4	DLAgn_00193890	95,7
bdnf	brain-derived neurotrophic factor	DQ915807.1	TGAGACCAAATGCAACCCCA	CACGTAGGACTGGGTTGTCC	100	0,2	DLAgn_00159270	94,5
cldn5a	claudin 5a		GTAATTGGCTCGCTCCTGGT	GTCTGAGCCACCACGATGTT	92	0,4	DLAgn_00120450	104,9
cldn5b	claudin 5b		CAAGGTCCACGACTCGGTTC	CGCCTCATCCTTGATGCAGT	145	0,4	DLAgn_00250470	87,6
cldn7a	claudin 7a		CCCGTCAATACCAAGTATGAG	ACTTCGGTGTAGATTTCCCT	123	0,4	DLAgn_00040400	87,3
cldn8-like	claudin 8like		AGCCAACATCAGGATGCAGT	CTGCAACGATGAGGGCAAAG	125	0,4	DLAgn_00035220	104,0
cldn12	claudin 12		CCTTCATCATTGCCATTGTGTC	GGTCAACTTTAGAGTACCACTCTG	200	0,4	DLAgn_00060570	81
cldn23a	claudin 23		GACCATCATACCCATCGCCT	TAAACATGACGAAACCGCCC	145	0,4	DLAgn_00086250	81,6
oclna	occludin a		ACTTTCATCGTGAATTTCCTCC	GAGTCCGTTTCATGTTCTTTATCC	227	0,4	DLAgn_00081130	100,9
oclnb	occludin b		CCCAAGAGGTTATAGCTATTGTCC	AGGTAAAGAGGCTTGCTGTG	229	0,4	DLAgn_00258740	94,2
tjp1b	tight junction protein 1b		GACAACAGGCCCAAATACCA	CAGCGTTTCTCCTTTCTCCT	290	0,8	DLAgn_00169520	90,9
npy	neuropeptide Y		GAGACACTACATCAACCTCATCAC	TGGGTCATATCTCGACTGTGG	132	0,4	DLAgn_00199940	93,4

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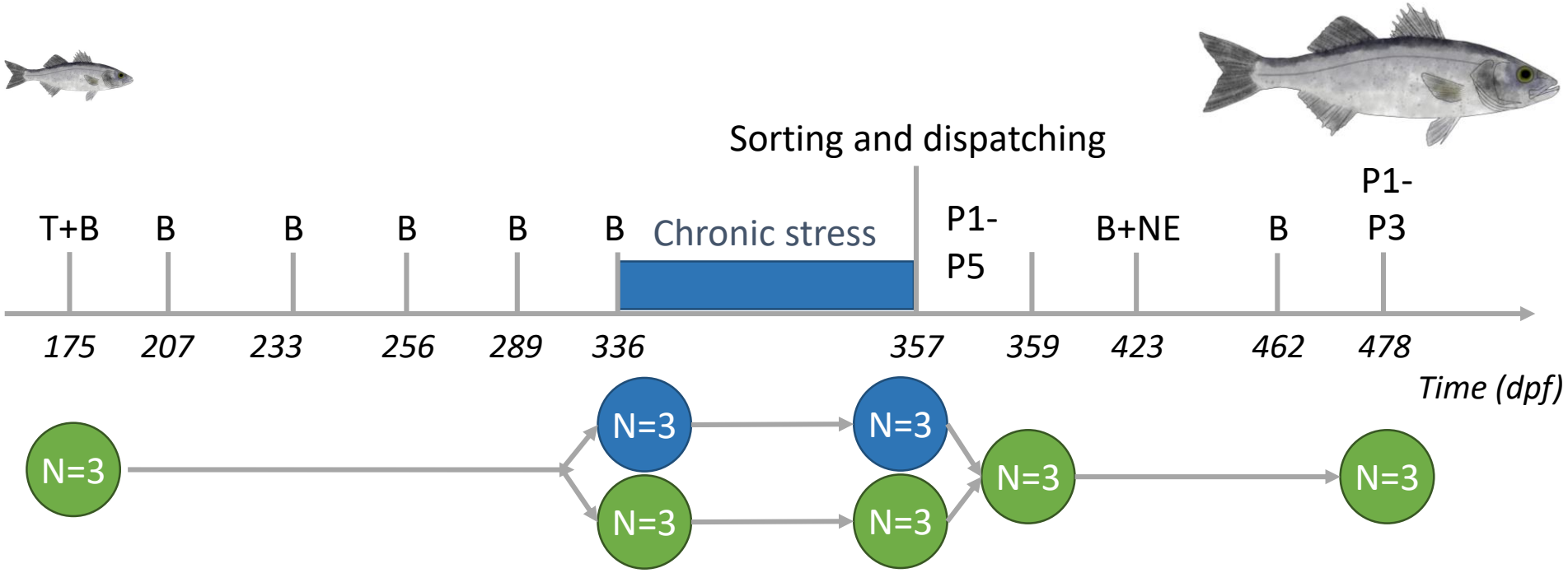
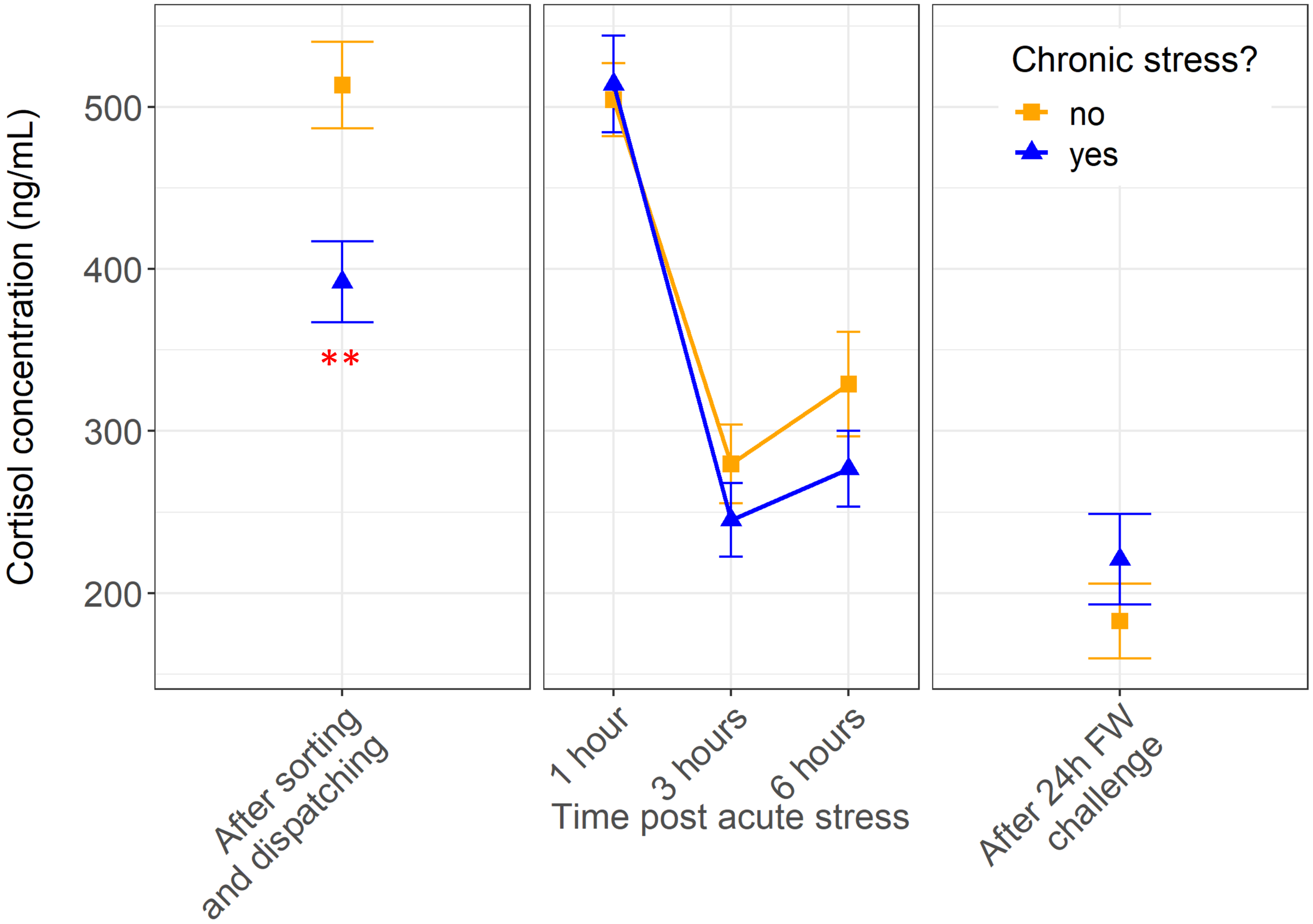
Table 3. Gill gene expression of European sea bass chronically stressed or control before and after a 24 hours challenge in freshwater. Values are presented as mean ± SEM of 32-37 fish. Significances are represented with asterisks (*: p<0.05; **: p<0.01; ***: p<0.001).

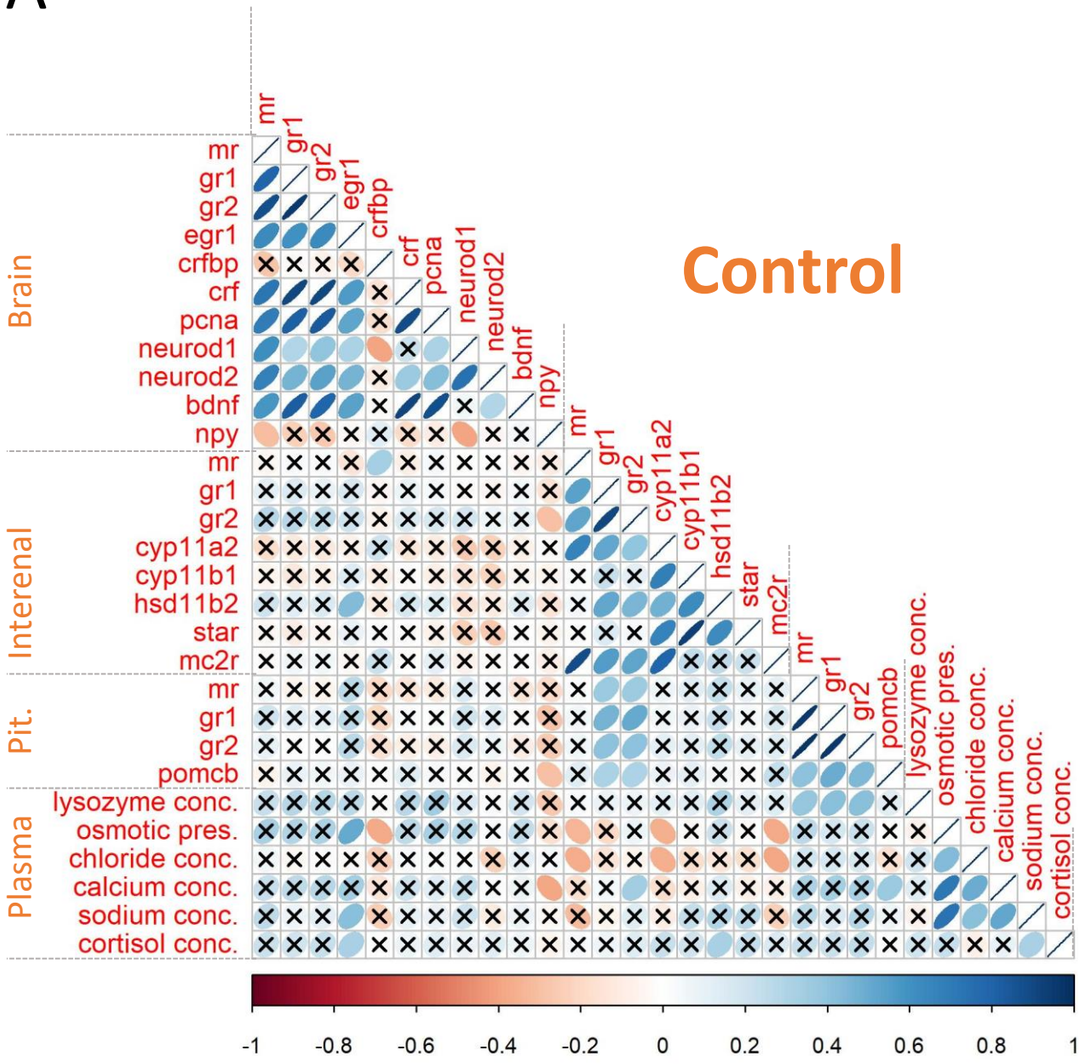
		Control		Chronic stress		statistic		
		SW	FW	SW	FW	chronic stress	salinity	interaction
ion and water homeostasis	*atp1a1a*	0,8 ± 0,02	1,28 ± 0,04	0,82 ± 0,03	1,31 ± 0,04		***	
	atp1a1b	1,1 ± 0,03	0,94 ± 0,03	1,14 ± 0,03	0,89 ± 0,02		***	*
	slc12a2	1,81 ± 0,05	0,53 ± 0,02	1,92 ± 0,06	0,5 ± 0,02		***	
	slc12a3 like	0,77 ± 0,03	1,22 ± 0,06	0,96 ± 0,04	1,24 ± 0,06		***	*
	clcn3	0,89 ± 0,02	1,16 ± 0,03	0,89 ± 0,02	1,17 ± 0,03		***	
	cftr	1,72 ± 0,05	0,55 ± 0,03	1,88 ± 0,08	0,52 ± 0,02		***	*
	slc4a4	1,73 ± 0,1	0,69 ± 0,03	1,44 ± 0,08	0,61 ± 0,03		***	
	atp6v1a	1,12 ± 0,03	0,96 ± 0,03	1,08 ± 0,03	0,9 ± 0,03		***	
	atp6v1b2	1,09 ± 0,04	0,97 ± 0,03	1,07 ± 0,04	0,89 ± 0,02		***	
	slc9a2b	1,15 ± 0,05	0,94 ± 0,04	1,14 ± 0,04	0,9 ± 0,05		***	
	slc9a2c	1,59 ± 0,11	0,68 ± 0,04	1,9 ± 0,09	0,53 ± 0,04		***	**
	slc9a3	0,85 ± 0,03	1,31 ± 0,05	0,78 ± 0,03	1,3 ± 0,05		***	
	ecac	0,74 ± 0,04	1,36 ± 0,08	0,87 ± 0,04	1,37 ± 0,06		***	
	trpv4	0,35 ± 0,03	3,17 ± 0,11	0,37 ± 0,03	3,3 ± 0,13		***	
	cazh	1,16 ± 0,05	0,89 ± 0,06	1,17 ± 0,06	0,96 ± 0,07		***	
	ca15b	2,89 ± 0,25	0,51 ± 0,09	2,95 ± 0,32	0,42 ± 0,06		***	
	aqp3	0,44 ± 0,03	2,2 ± 0,17	0,7 ± 0,06	2,49 ± 0,2		***	
ammonia transport	*rhcg1*	0,75 ± 0,03	1,39 ± 0,04	0,77 ± 0,03	1,39 ± 0,04		***	
	rhcg2	1,59 ± 0,07	0,72 ± 0,05	1,48 ± 0,05	0,61 ± 0,04		***	
	rhbg	1,26 ± 0,07	0,84 ± 0,04	1,31 ± 0,06	0,79 ± 0,04		***	
	rhag	1,03 ± 0,07	0,98 ± 0,06	1,18 ± 0,06	0,94 ± 0,06		**	
gill permeability	*cldn5a*	0,78 ± 0,04	1,37 ± 0,06	0,89 ± 0,05	1,27 ± 0,06		***	*
	cldn5b	1,13 ± 0,04	0,96 ± 0,03	1,12 ± 0,05	0,92 ± 0,04		***	
	cldn7a	0,8 ± 0,03	1,32 ± 0,04	0,81 ± 0,03	1,3 ± 0,04		***	
	cldn8like	1,07 ± 0,04	1,14 ± 0,04	0,97 ± 0,03	0,91 ± 0,04			*
	cldn12	1,02 ± 0,03	1,01 ± 0,03	1,02 ± 0,03	0,97 ± 0,03			
	cldn23a	1,14 ± 0,05	1,03 ± 0,04	0,99 ± 0,03	0,93 ± 0,03		**	
	oclna	1,02 ± 0,05	1,13 ± 0,04	0,94 ± 0,04	1,01 ± 0,05		*	
	oclnb	0,6 ± 0,03	1,98 ± 0,07	0,58 ± 0,03	1,71 ± 0,07		***	**
	tjp1	1,18 ± 0,06	0,89 ± 0,05	1,18 ± 0,06	0,9 ± 0,06		***	
hormonal regulation	*mr*	0,99 ± 0,03	1,09 ± 0,03	0,97 ± 0,03	1,01 ± 0,05		**	
	gr1	1,11 ± 0,04	0,92 ± 0,02	1,08 ± 0,04	0,9 ± 0,03		***	
	gr2	1,04 ± 0,03	1,01 ± 0,03	1,05 ± 0,04	0,91 ± 0,03		**	*



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

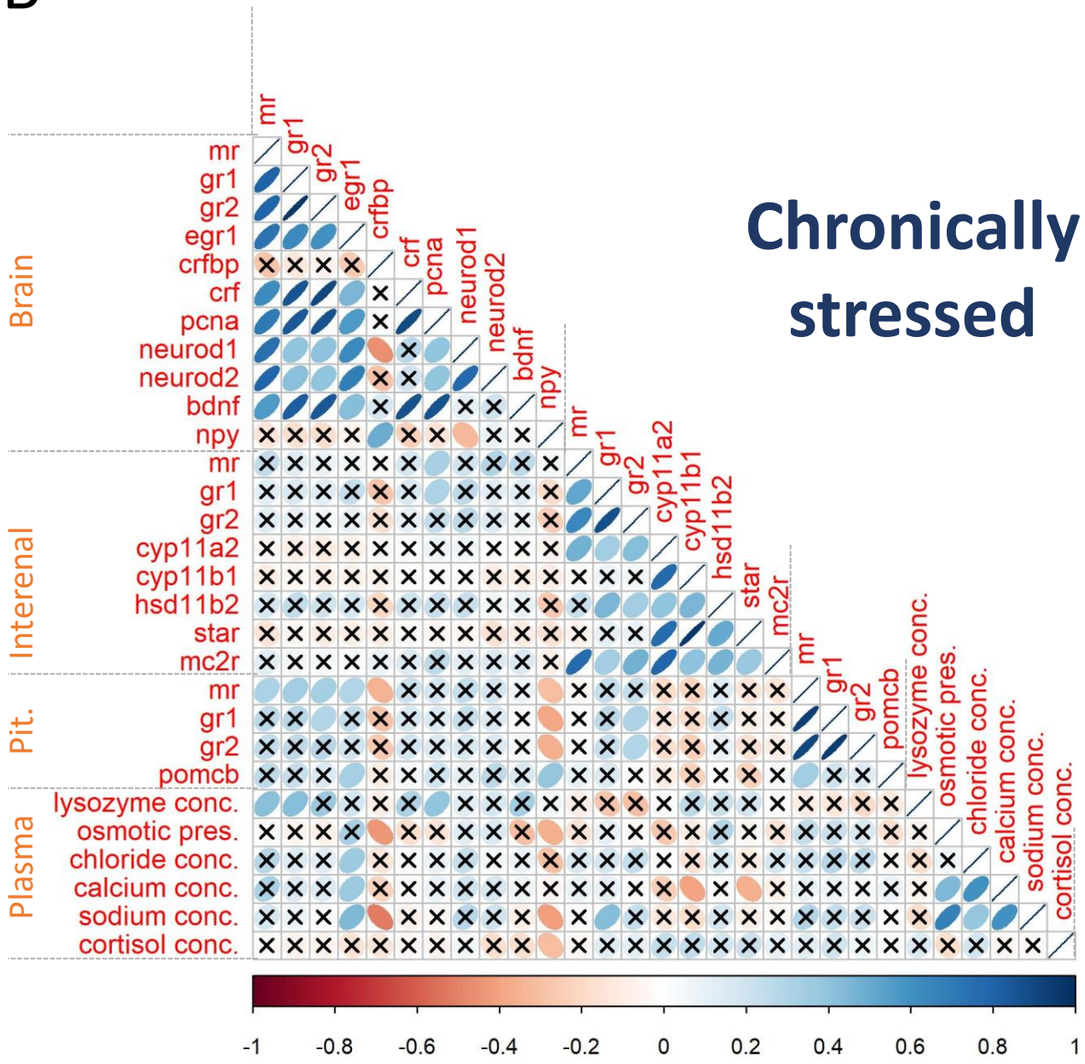

Cortisol concentration (ng/mL)
500
400
300
200
**
Chronic stress?
no
yes
After sorting and dispatching
1 hour
3 hours
6 hours
Time post acute stress
After 24h FW challenge

A



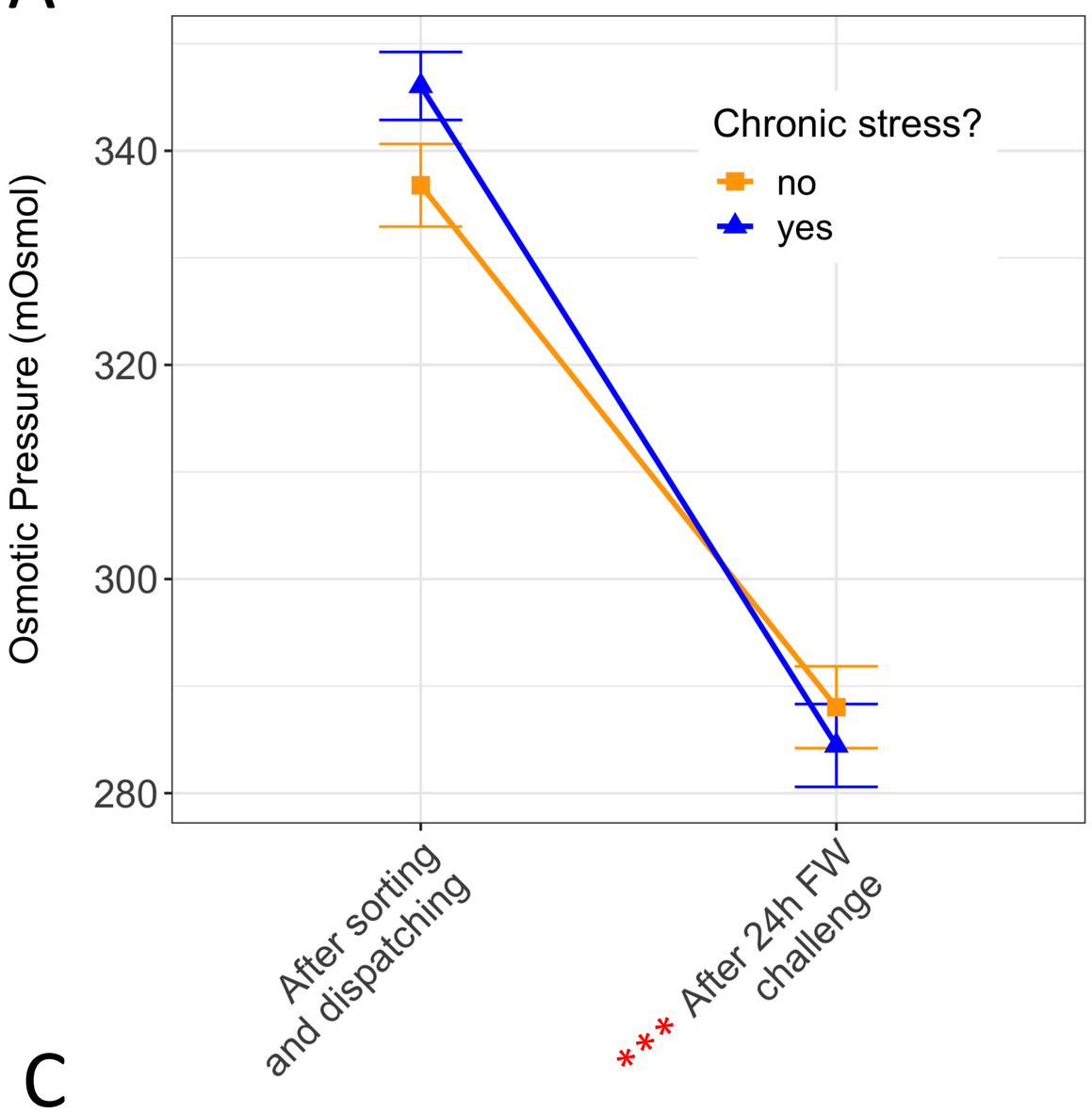
Control

B

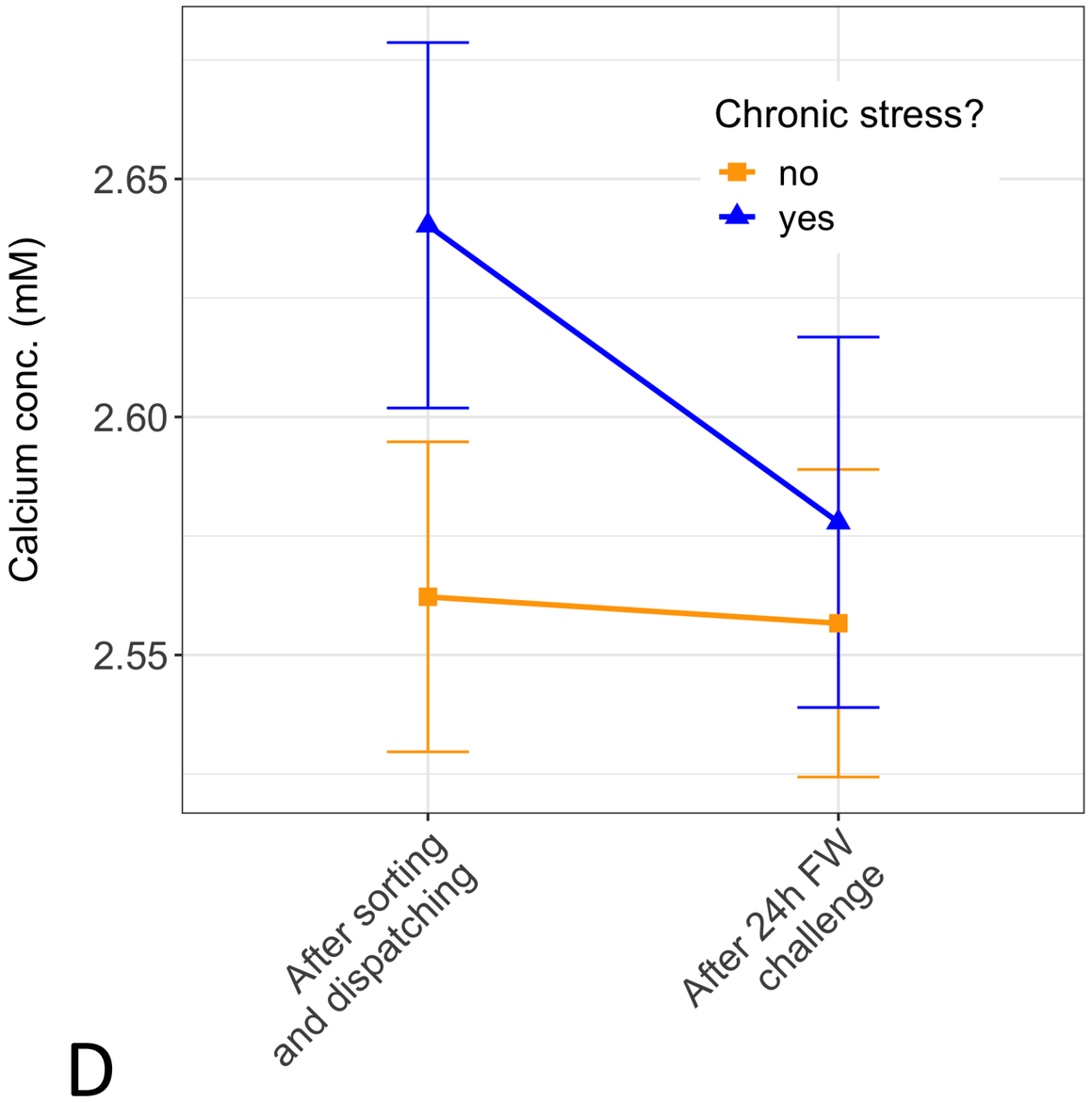


Chronically stressed

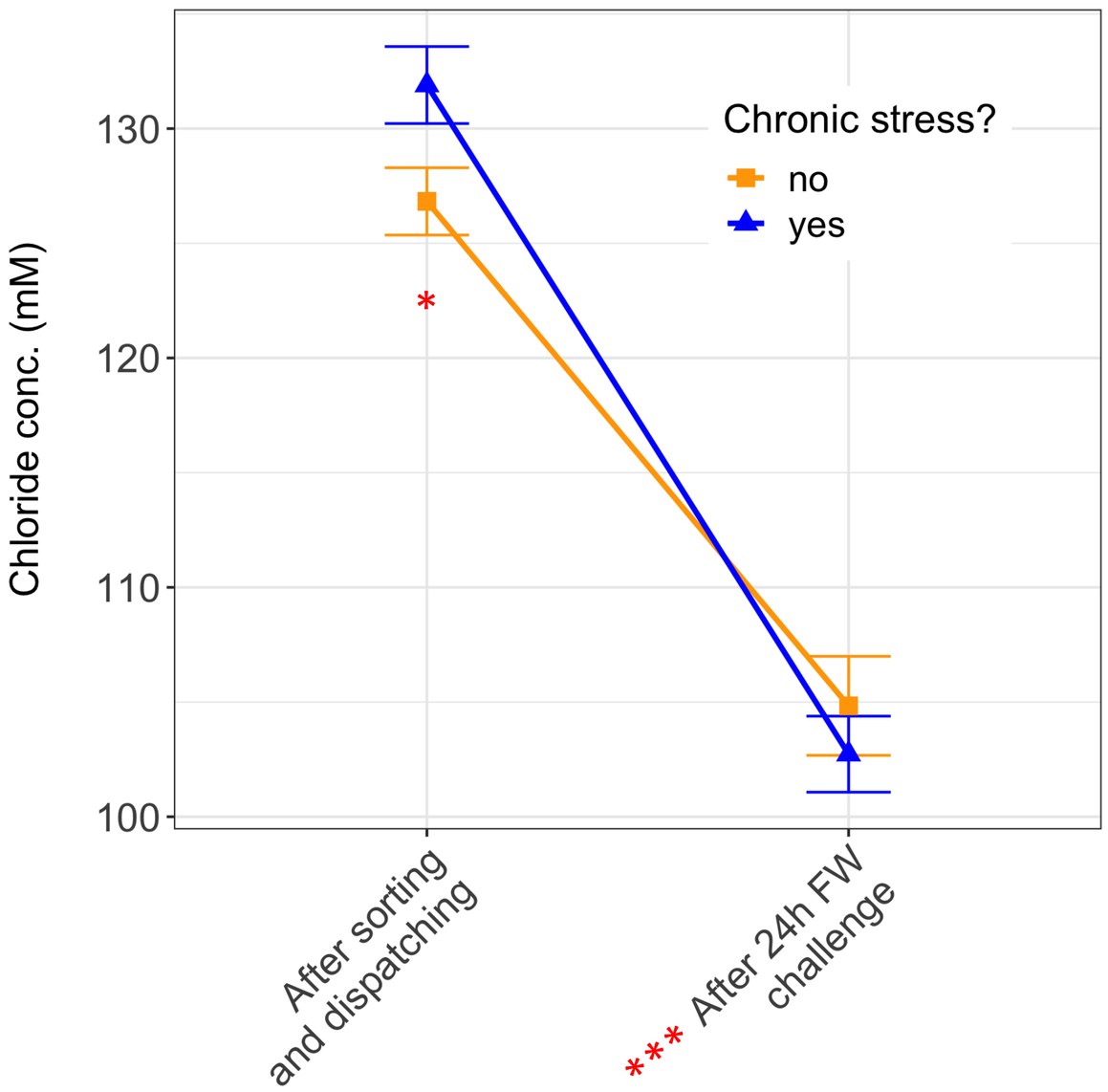
A


Osmotic Pressure (mOsmol)
340
320
300
280
Chronic stress?
no
yes
After sorting and dispatching
*** After 24h FW challenge

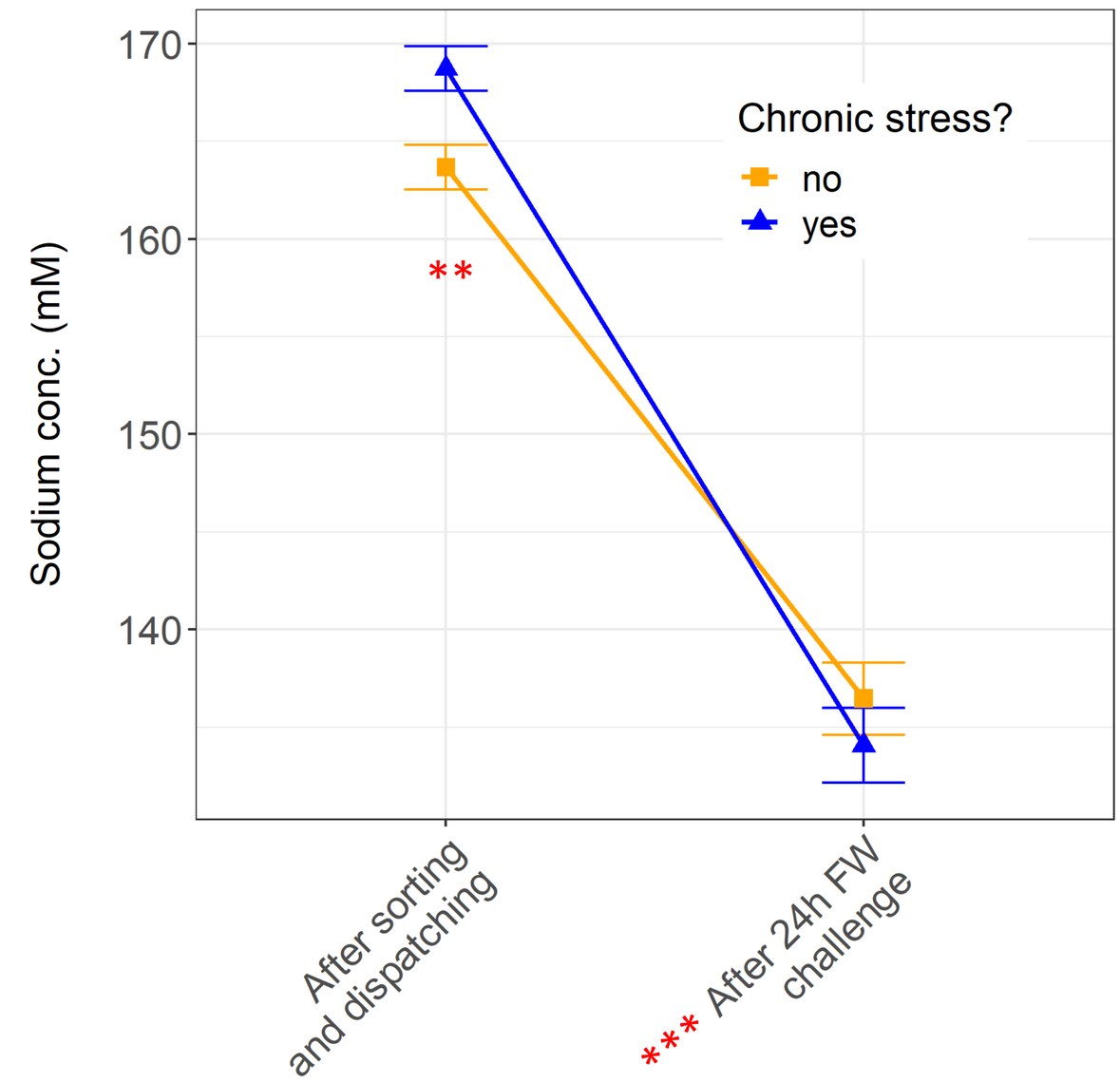
B


Calcium conc. (mM)
2.65
2.60
2.55
Chronic stress?
no
yes
After sorting and dispatching
After 24h FW challenge

C


Chloride conc. (mM)
130
120
110
100
*
Chronic stress?
no
yes
After sorting and dispatching
*** After 24h FW challenge

D


Sodium conc. (mM)
170
160
150
140
**
Chronic stress?
no
yes
After sorting and dispatching
*** After 24h FW challenge

A

Salinity challenge

SW

24h FW

Chronic stress?

no

yes

Dim2 (12.9%)

Dim1 (40.4%)

B

PCA dim. 2

**

no

yes

Chronic stress?

C

Coord. on Dim. 1

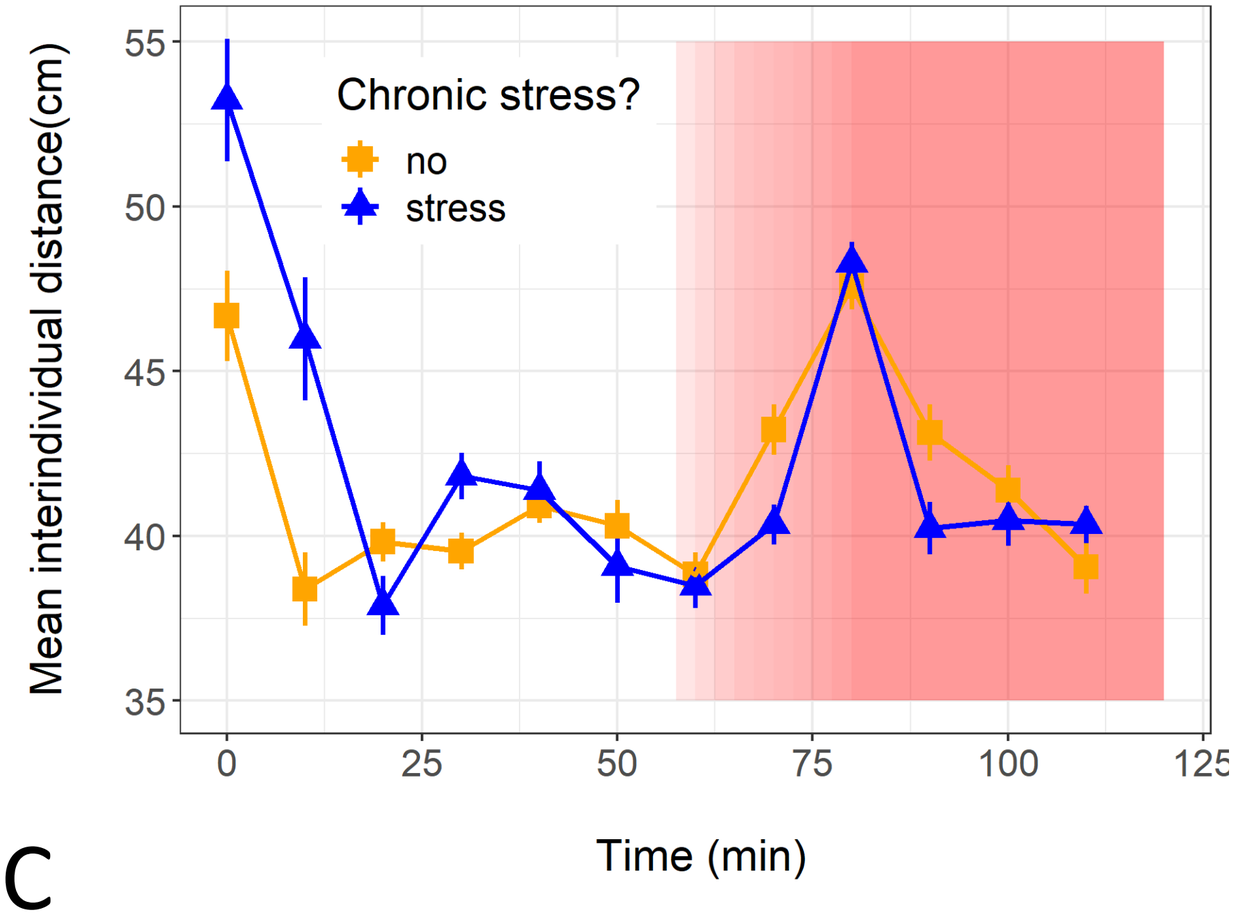
trpv4 oclnb rhcg1 aqp3 atp1a1a slc4a4 slc9a2c ca15b rhcg2 cftr slc12a2

Coord. on Dim. 2

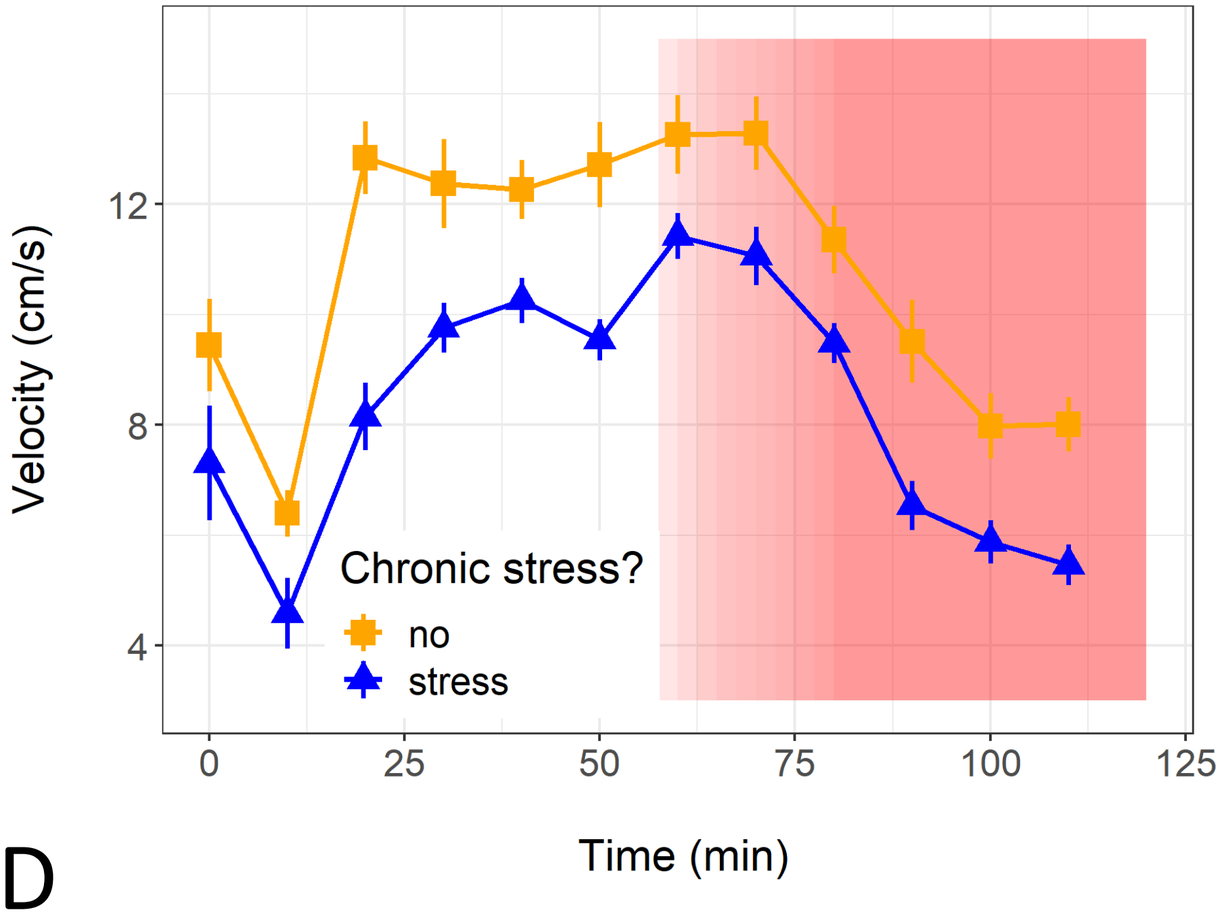
atp6v1a clcn3 atp6v1b2 rhag cldn23a oclna gr2 cldn12 mr cldn8like

Gene

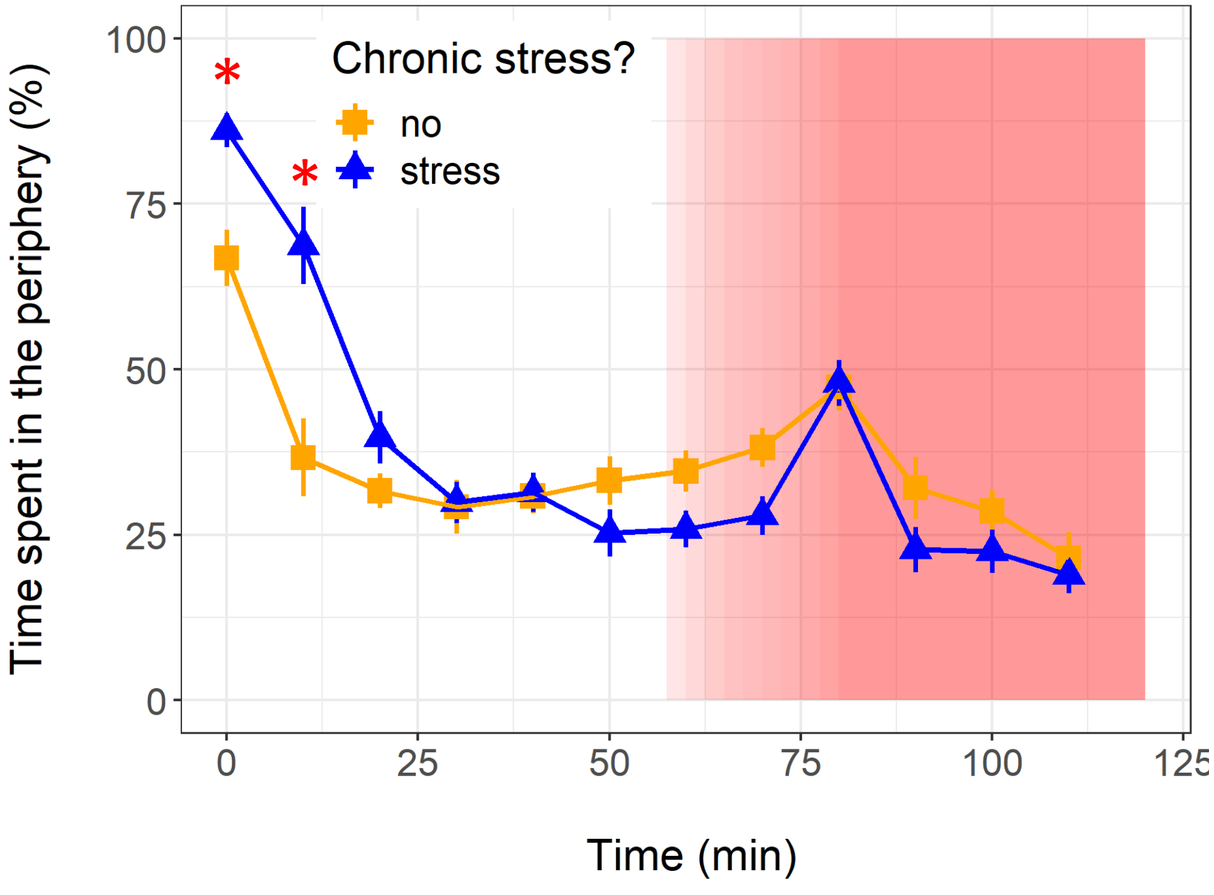
A


Mean interindividual distance(cm)
55
50
45
40
35
Chronic stress?
no
stress
0
25
50
75
100
125
Time (min)

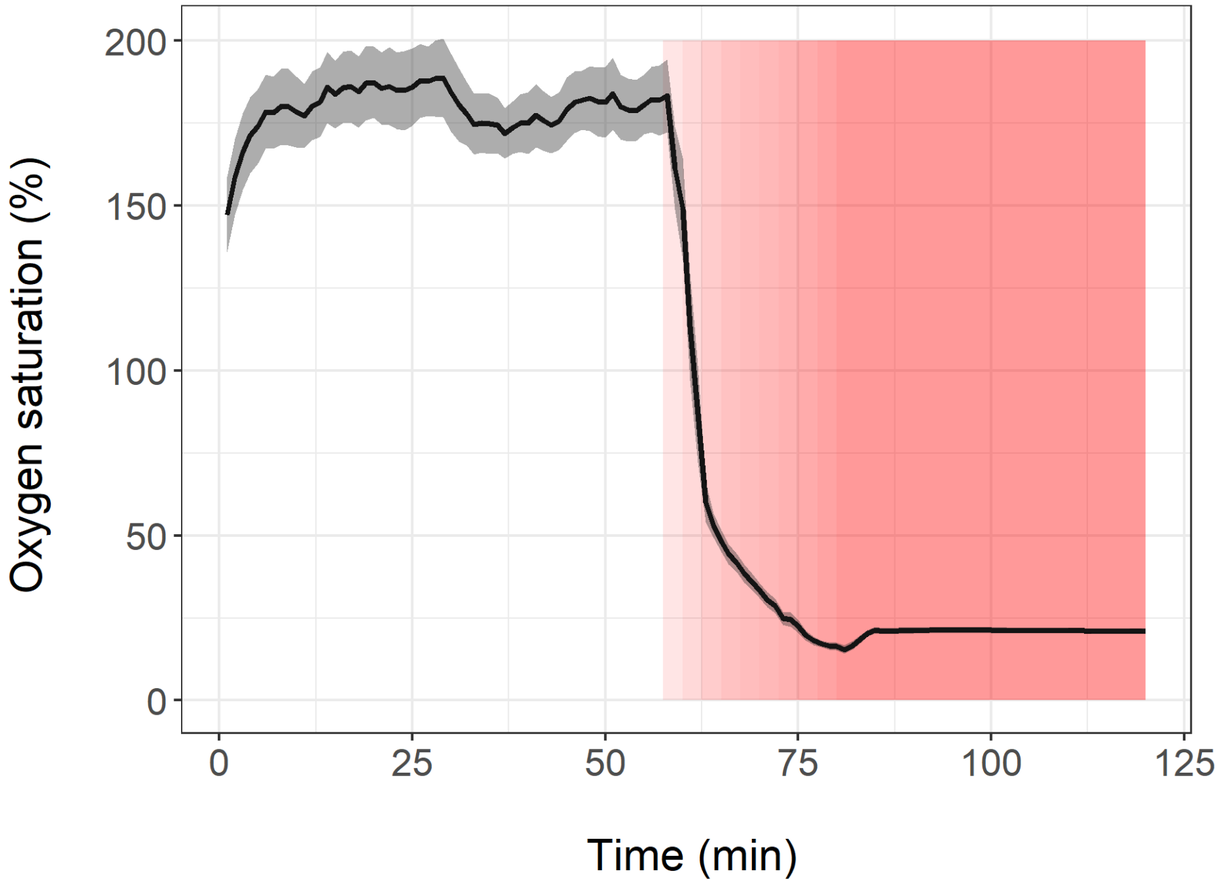
B


Velocity (cm/s)
12
8
4
Chronic stress?
no
stress
0
25
50
75
100
125
Time (min)

C


Time spent in the periphery (%)
100
75
50
25
0
*
*
Chronic stress?
no
stress
0
25
50
75
100
125
Time (min)

D


Oxygen saturation (%)
200
150
100
50
0
0
25
50
75
100
125
Time (min)