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

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**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.

**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

**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 (Canahuja 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, reactivity 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 330 ± 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 turbidimetric 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 software (Noldus, The Netherlands). Swaps between individuals were manually corrected using the track editor module. For behavioural analyses, the arena was virtually separated into two areas: the centre area composed of one half of the surface and the periphery area including the other half; time spent in periphery (s), indicative of thigmotaxis behaviour was recorded. The velocity of each fish (cm.$s^{-1}$), indicative of individual fish activity, and the interindividual distances (cm), indicative of group cohesion (Alfonso et al., 2020), were also assessed. Variables were averaged over 1 minute every 10 minutes in order to record the kinetics of behavioural responses.

*2.7.Gene expression analyses*

2.7.1. RNA extraction

All samples used for gene expression analyses were stored at -80°C until RNA extraction. Brain, pituitary and head kidneys samples were grinded using 2 ball mills (45 seconds at 30 rpm) in 500 µL MR1 and 1 µl of TCEP from NucleoMag® RNA extraction kit (Macherey-Nagel). Samples were then centrifugated at 13000 rpm for two minutes and a sub-sample of 200 µl was collected, and diluted in 150 µl of MR1

in a 96 deep-wells plate. The plate was then placed in the KingFisher automatic extraction robot to perform the extraction protocol following manufacturer's instructions for the NucleoMag® RNA kits.

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

### 2.7.2. RNA integrity

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

### 2.7.3. Reverse transcription

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

Reverse transcriptions were performed on RNA extracted from the brains, gills, and head kidneys following a previously published protocol (Kiilerich et al., 2018). 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; Boussus 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" ($\kappa$), 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, growth reduction started before the stress protocol, during the acclimation period presumably because of a unfavourable localisation of the stress tanks in the experimental hall. Two months after the end of the exposure, no more difference was observed between stressed and control fish.

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

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

### Physiology of the HPI axis

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

Despite the effect of chronic stress on plasma cortisol levels after sorting and dispatching, no effect of the chronic stress was observed on the expression of candidate genes in the HPI axis, except for *pomcb* significantly upregulated in the pituitary of chronically stressed fish ($F_{1,14}$ = 4.78, p-value = 0.047) (Data not shown). Moreover, PCA analysis of gene expressions in the brain, pituitary and head-kidney did not reveal significant effect of chronic stress. In agreement with these results, we observed in both, stressed and control groups, the same correlation at the individual level between all measures performed in the tissues (gene expressions and plasmatic measures, Fig. 4). Similarly, some interesting intra- and inter- organ correlations could be observed in both experimental groups (Fig. 4B). The expression of genes related to neurogenesis (*neurod, nr4a, 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* ($F_{1,128}$ = 5.2, p-value = 0.024), *ocldnb* ($F_{1,129}$ = 7.6, p-value = 0.007), *gr2* ($F_{1,127}$ = 4.6, p-value = 0.034).

The PCA analysis using the expression of all genes measured in the gills show a clear separation on the first axis of individuals based on the OC (Fig. 6). The genes contributing the most to this separation are given in Fig. 6C. Overall, *trpv4, oclnb, rhch1, aqp3 and atp1a1a* are the genes the most upregulated in the 24 hours freshwater fish, while *slc12a2, cftr, rhcg2, ca15b, slc9a2c, and slc4a4* are the genes showing the highest expression in fish that were not challenged. All expressions of these genes showed significant differences between seawater and freshwater fish when analyzed individually (Table 3).

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

**Innate immune system**

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

**Behaviour**

In the novel environment challenge in group, the three variables of interest measured (inter-individual distances, velocity and time spent in the center) changed significantly through time both before and after the start of the hypoxia challenge (Table S3). Prior to the hypoxia challenge, a significant interaction between the time and the treatment (chronic stress) was observed for the mean inter-individual distances ($F_{5,457}$ = 7.4, p-value <0.001) and the time spent in periphery ($F_{5,456}$ = 7.4, p-value <0.001). However, the post-hoc tests ran on each independent time point highlighted only a significant effect of the chronic stress on the time spent in the periphery during the first two time points of the measure (z-values = 2.33 and 2.32, and p-values = 0.02 and 0.02 respectively, Fig. 7C). The chronically stressed fish 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 weeks with both, medium and high stress regimes, chronically stressed fish continued growing in our experiment and gained 13%. This illustrates the complexity behind chronic stress, and highlights the need for multiple and diverse markers of stress; growth taken individually is not a reliable indicator of stress intensity.

The DEB model applied to biometric data suggests that growth reduction induced by the chronic stress protocol can be entirely explained by reduced energy assimilation. DEB is often used to highlight a mode of action of a contaminant based on changes in biometric values (Ashauer and Jager, 2018). To our knowledge, this is the first time DEB theory was applied to physical stressors. Here, based on available data, the results from the DEB model suggest that none of the metabolic mechanisms involved in energy allocation was impacted by the treatment, apart from total energy assimilated. This translates into reduced foraging and/or reduced nutrient assimilation by the digestive tract. Reduced food consumption was previously reported in sea bass exposed to chronic stress (Samaras et al., 2018; Santos et al., 2010). However, Millot et al. (2010) reported, on the contrary, that repeated acute stress increases feed demand and intake in sea bass, while growth was still reduced, suggesting that nutrient assimilation and/or energy allocated to maintenance were affected. A very

recent study also demonstrates that elevated cortisol levels affect growth mainly as a consequence of reduced digestibility rather than feed intake (Pfalzgraff et al., 2021). The outputs from the DEB model analysis suggest that our chronic stress protocol had no dramatic consequence on other metabolic functions than assimilation. This argues for only subtle physiological alterations due to the chronic stress and translates in a very quick recovery in terms of body mass and length.

**Biomarkers related to corticotropic axis**

Assessment of chronic stress by studying the HPI axis activity can be misleading when solely based on basal plasma cortisol levels. Indeed, a negative feedback of cortisol production causes down regulation of the HPI axis in chronically stressed fish (Barton et al., 2002, 1987; Pickering and Stewart, 1984; Vijayan and Leatherland, 1990; Wendelaar Bonga, 1997). In this study, it was not possible for practical reasons to measure basal levels of cortisol, the major stress hormone in fishes (Sadoul and Geffroy, 2019). However, assessment of the reactivity of the HPI axis to acute stress has already been used to study chronic stress effects (Madaro et al., 2015; Pavlidis et al., 2015; Samaras et al., 2018; Santos et al., 2010). In our protocol, sorting and dispatching of the fish represents a first acute stressor as indicated by the high cortisol levels (Fig. 3). This response was expected as sea bass is a very stress-sensitive species as shown by rapid and high cortisol response after exposure to acute stressors (Fanouraki et al., 2011; Ordóñez-Grande et al., 2020; A. Samaras et al., 2016; Samaras et al., 2018). Interestingly, chronic stress treatment significantly decreases the acute stress response observed after sorting and dispatching fish when compared to control. Nevertheless, both treatments reacted similarly to the additional acute stress (confinement during 4 minutes, AST) suggesting that the HPI axis was not deeply affected by the chronic stress protocol. Cortisol values after 6 hours were still much higher than basal levels previously observed for European sea bass (Acerete et al., 2009; Samaras et al., 2018), indicating that the total stress recovery were not met after 6 hours.

Our post-AST cortisol values differ from previous studies. Samaras et al. (2018) observed a decrease in the maximum cortisol response 1 h after acute stress in sea bass chronically exposed to acute stressors of different load intensities. Santos et al. (2010) and Di Marco et al. (Di Marco et al., 2008) observed an increase in plasma cortisol levels after an acute challenge in sea bass chronically exposed to high

stocking density but not when using low or medium densities. Similar inconsistent results in HPI reactivity in response to chronic stress were also observed in other species, such as in salmon, zebrafish or seabream (Madaro et al., 2015; Moltesen et al., 2016; Pavlidis et al., 2015; Samaras et al., 2018; Vindas et al., 2016). Overall, these results highlight that chronic stress effects vary according to age, experimental protocols and/or species.

In the present study, the limited effect of chronic stress on HPI-axis reactivity also translates in terms of gene expression related to the HPI axis. Despite a wide analysis of key genes involved in stress response in the brain, pituitary or interrenal, only *pomcb* was affected by the chronic stress, with a significant upregulation. In sea bass, two orthologous genes have been identified and annotated as *pomca* and *pomcb* (Rousseau et al., 2021). Measures of *pomcb* transcripts in the pituitaries of seabream (a fish species phylogenetically close to sea bass) suggest that expression values reflect ACTH activity (Cardoso et al., 2011). Our study indicates a stimulatory effect of chronic stress on *pomcb* gene expression measured before the AST. Although we did not measure basal cortisol levels, this increased expression of *pomcb* is in agreement with increased basal cortisol levels measured by Samaras et al. (2018) in sea bass. On the contrary, significant changes of *cfr*, *gr1* and *mr* gene expressions were previously also described by Samaras et al. (2018) in the pre-optic area (POA). Our non-significant results might be explained by the fact that they were obtained in the entire brain, diluting potential signal variations. Overall, our repeated acute stress protocol has only minor effects on the HPI axis which suggests a moderate stress load of the experimental protocol. Additional plasmatic parameters, including lactate and glucose levels, would have been interesting to investigate in light of previous results on chronic stress in fish (Santos et al., 2010).

**Biomarkers related to ions and water homeostasis**

To our knowledge, the present study is the first to investigate the effect of chronic stress on ions and water homeostasis in sea bass. The interplay between the stress axis and osmoregulatory functions is well described (Takei and Hwang, 2016), but data on the effects of chronic stress on osmoregulatory functions are scarce. In seawater (SW), chronic stress induced a significant increase in plasma sodium and chloride levels and an upregulation of the expression of two gill genes: *aqp3* (a water channel) and *ncc2* (a co-transporter of $Na^+$ and $Cl^-$). Relationships between such effects on

plasma ions and expression of these genes remain complex. *Aqp3* and *ncc2* genes are well known to play an important role in osmoregulation under freshwater (FW) conditions for several euryhaline fishes (Madsen et al., 2015; Takei et al., 2014) including sea bass (Blondeau-Bidet et al., 2019a; Giffard-Mena et al., 2007). *Aqp3* is involved in the water flow through the basolateral side of gill's epithelium to prevent swelling, and may take part in nitrogen excretion (Madsen et al., 2015). *Ncc2* allows gill absorption of NaCl to maintain ion homeostasis (Takei et al., 2014). However, several studies suggest that *aqp3* and *ncc2a* are also implicated in gill functions since SW adapted fish show significant levels of the transcripts (Blondeau-Bidet et al., 2019a; Breves et al., 2020; Giffard-Mena et al., 2007; Jung et al., 2012; Moorman et al., 2015; Tipsmark et al., 2010). *Aqp3* immunoreactivity appears quantitatively similar whatever the salinity in medaka (Ellis et al., 2019) and protein abundance does not change significantly in killifish (Jung et al., 2012). Ncc2 protein was also quantified in SW-acclimated mummichogs using western blot approach (Breves et al., 2020). We propose that *aqp3* and *nnc2* genes are important targets of the chronic stress in SW-adapted sea bass but their exact functions in relation to gill ion transport 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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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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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 (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

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

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

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 |
|---|---|---|---|---|---|---|---|
| *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 | GGCATTGAAGCACTT[illegible]GGAGTTG | 0,4 | 60 | (Mitter et al., 2009) |
| *rpl17* | ribosomal Protein L17 | AF139590 | TTGAAGACAACGCAGGAGTCA | CAGCGCATTCTTTTGCC[illegible]CT | 0,2 | 60 | (Samaras et al., 2018) |
| *mr* | mineralocorticoid receptor | JF824641.1 | GTTCCACAAAGAGCCCCAAG | AGGAGGACTGC[illegible]GG[illegible]TG[illegible]TG | 0,4 | 60 | (Sadoul et al., 2018) |
| *gr1* | glucocorticoid receptor 1 | AY549305 | GAGATTTGGCAAGACCTTGACC | ACCACAC[illegible]GC[illegible]TA[illegible]TGA | 0,4 | 60 | (Pavlidis et al., 2011) |
| *gr2* | glucocorticoid receptor 2 | AY619996 | GACGCAGACCTCCACTACATTC | GCCG[illegible]TC[illegible]ATAC[illegible]CTCAACCAC | 0,2 | 60 | (Pavlidis et al., 2011) |
| *mc2r* | melanocortin 2 Receptor | FR870225.1 | CATCTACGCCTTCCGCATTG | ATG[illegible]GCAC[illegible]CCTCCATT | 0,4 | 60 | (Tsalafouta et al., 2017) |
| *cyp11b1 (11B-hydroxylase)* | cytochrome P450 family 11 subfamily B member 1 | AF449173.2 | GGAGGAGGATTGCTGAGAACG | AGA[illegible]GACGACACGCTGAGA | 0,4 | 60 | (Samaras et al., 2018) |
| *atp1a1a* | Na/K ATPase alpha subunit isoform 1a | KP400258 | CCTCAGATGGCAAGGAG[illegible]AC | C[illegible]CTGCTGAGATCGGTTCC | 0,4 | 60 | (Blondeau-Bidet et al., 2016) |
| *atp1a1b* | Na/K ATPase alpha subunit isoform 1b | KP400259 | AGCAGGGCATGAAG[illegible]A[illegible]AG | CCTGGGCTGCGTCTGAGG | 0,4 | 60 | (Blondeau-Bidet et al., 2016) |
| *slc12a2 (nkcc1)* | sodium-potassium-chloride cotransporter 1 | AY954108 | TCAGCTC[illegible]CAGT[illegible]CA[illegible]GGCC | TTGTGGAGTCCATAGCGGC | 0,4 | 60 | (Blondeau-Bidet et al., 2019b) |
| *slc12a3-like (ncc2)* | sodium-chloride cotransporter 2 | | AT[illegible]ATGAGC[illegible]TCTTCGAGCC | GCTGCTCTCATCACCTTCTGT | 0,6 | 60 | (Blondeau-Bidet et al., 2019a) |
| *clcn3* | chloride channel 3 | JN998891 | CAAC[illegible]ACAGC[illegible]AGAACGAGGC | ACAGCGTCTTGAGAGGGAAG | 0,4 | 60 | (Bossus et al., 2013) |
| *slc4a4 (nbc1)* | sodium bicarbonate cotransporter | FM001880 | AC[illegible]GA[illegible]CACGGAACACACGG | CGTCCACAGCCAGCAGTTCG | 0,4 | 60 | (Blondeau-Bidet et al., 2019a) |
| *atp6v1a (vha-a)* | V-type proton ATPase catalytic subunit a | | [illegible]GC[illegible]AGTCACATCACAGGAGG | 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 | CN660524 | 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 glycoprotei... | | 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) |

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

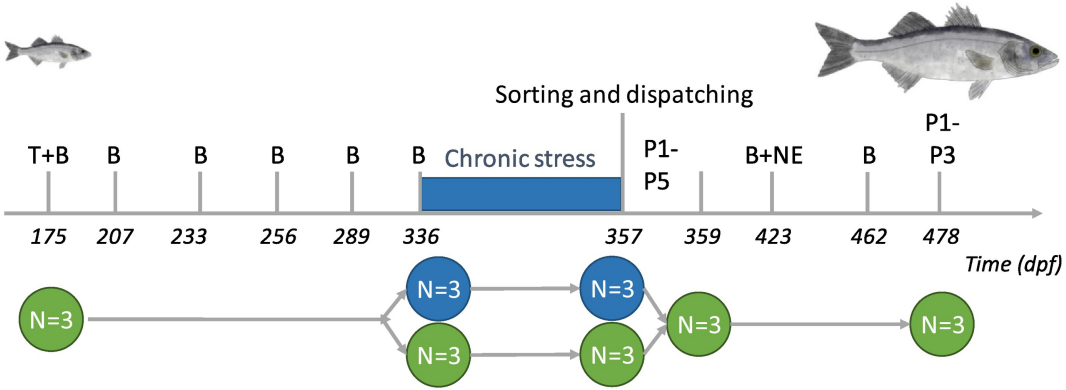
| Gene | Protein name | GenBank accession numbers | Forward primer (5' → 3') | Reverse primer (5' → 3') | amplicon size | Concentration in qPCR well | http://seabass.mpipz.de/cgi-bin/hgGateway | Efficiency |
|---|---|---|---|---|---|---|---|---|
| *gapdh* | glyceraldehyde-3-Phosphate Dehydrogenase | 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 | GAAATGCCCTCAGAAGATA | 291 | 0,4 | DLAgn_00069720 | 94,2 |
| *cyp11a2 (p450scc)* | p450 side chain cleaving | | CCCCCGTCAGTGTTTAGGAC | TTGCGCTGTTTCTCC CTC | 96 | 0,4 | DLAgn_00168500 | 91,3 |
| *cftr* | cystic fibrosis transmembrane conductance regulator | DQ501276.2 | GAACCAACCAGGACAAACCA | GCAAGTCGA GAA TTA ACACTC | 149 | 0,4 | DLAgn_00172160 | 89,6 |
| *cazh* | carbonic anhydrase | FK944087 | GACTAACGGACCTGATACATGG | TCTGTG GTC ACA AAGTC | 223 | 0,4 | DLAgn_00000090 | 92.7 |
| *ca15b* | carbonic anhydrase 15b | CX660749 | GGCAAGACAGTCAAAGTCAG | CCT A TA AGAAACCAAGAGCAG | 255 | 0,6 | DLAgn_00101240 | 86,0 |
| *trpv6 (ecac)* | epithelial calcium channel | | TTCCATGTTATCCTTATCGGCT | CA AAA TTGTCAGGTCTCCA | 209 | 0,4 | DLAgn_00059190 | 90,9 |
| *aqp3* | aquaporin 3 | DQ647191 | CATGTACTACGATGCCCTGTG | ATA CCAGAGTTAAAGCCCA | 271 | 0,4 | DLAgn_00117370 | 96,8 |
| *rhag* | rhesus blood group, a glycoprotein | | CAAGTTCCTTTCTCCCATCC | GTAAC ACACCTCCAACCAG | 206 | 0,4 | DLAgn_00071020 | 92,0 |
| *egr1* | early growth response 1 | | AACTCCAGCCTC TTC | AGTCAGGAATCATGGGCACA | 202 | 0,4 | DLAgn_00110040 | 91,6 |
| *neurod2* | neuronal differentiation 2 | | TGCGTAAAG GGTT CAT C | GTCGTGGGTTGGGAGAGTC | 172 | 0,4 | DLAgn_00193890 | 95,7 |
| *bdnf* | brain-derived neurotrophic factor | DQ915807.1 | TGAG CCAAA CAACCCCA | CACGTAGGACTGGGTTGTCC | 100 | 0,2 | DLAgn_00159270 | 94,5 |
| *cldn5a* | claudin 5a | | GTAAT GCTCG CCTGGT | GTCTGAGCCACCACGATGTT | 92 | 0,4 | DLAgn_00120450 | 104,9 |
| *cldn5b* | claudin 5b | | CA AG TC ACGACTCGGTTC | CGCCTCATCCTTGATGCAGT | 145 | 0,4 | DLAgn_00250470 | 87,6 |
| *cldn7a* | claudin 7a | | CC GTC TACCAAGTATGAG | ACTTCGGTGTAGATTTCCCT | 123 | 0,4 | DLAgn_00040400 | 87,3 |
| *cldn8-like* | claudin 8like | | A GC ACATCAGGATGCAGT | CTGCAACGATGAGGGCAAAG | 125 | 0,4 | DLAgn_00035220 | 104,0 |
| *cldn12* | claudin 12 | | CC TTCATCATTGCCATTGTGTC | 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 |

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 ± 0,0[illegible] | | *** | |
| | *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 ± 0,03 | 1,13 ± 0,04 | 0,94 ± 0,04 | 1,01 ± 0,05 | | * | |
| | *oclnb* | 0,6 ± 0,0[illegible] | 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 | | ** | * |

**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




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

Figure 1

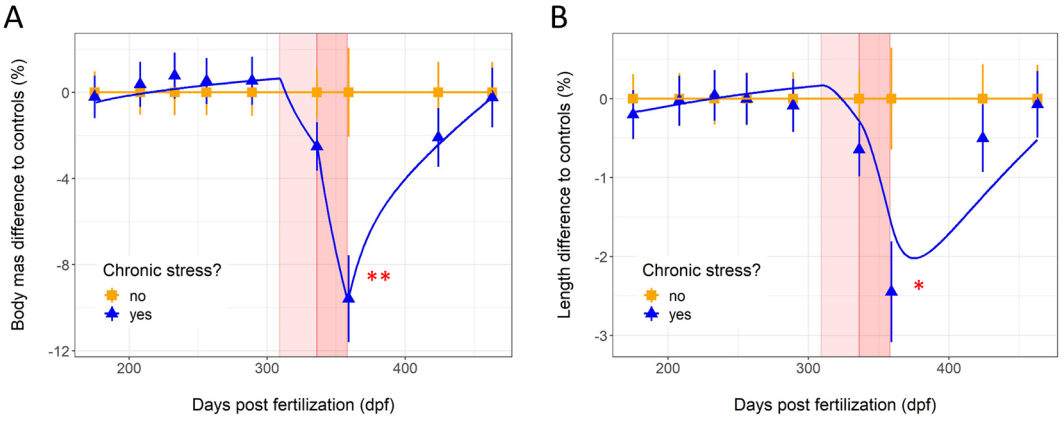

A
Body mas difference to controls (%)
0
-4
-8
-12
Chronic stress?
no
yes
**
200
300
400
Days post fertilization (dpf)
B
Length difference to controls (%)
0
-1
-2
-3
Chronic stress?
no
yes
*
200
300
400
Days post fertilization (dpf)

Figure 2

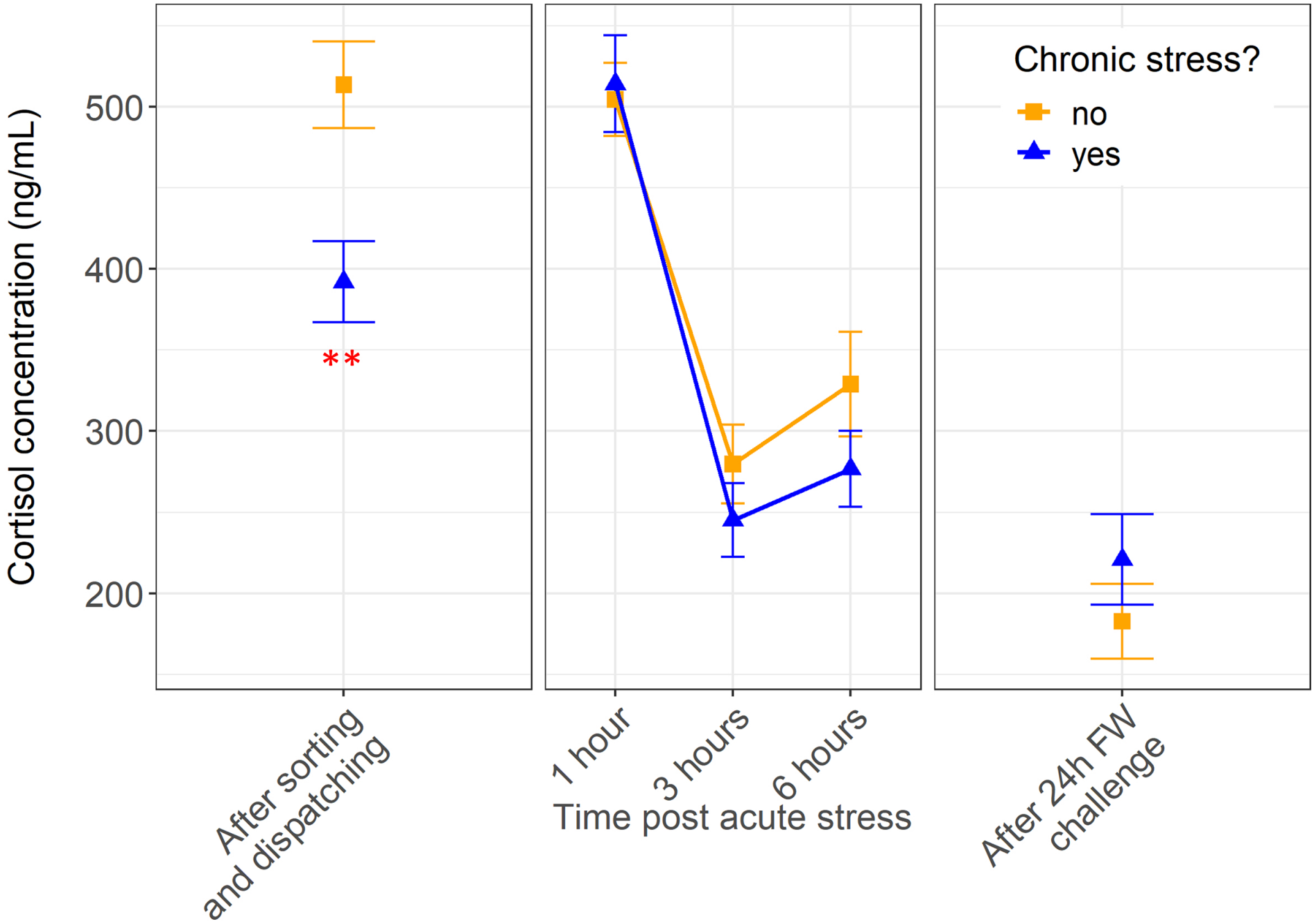

Cortisol concentration (ng/mL)
500
400
300
200
**
Chronic stress?
no
yes
After sorting and dispatching
1 hour
3 hours
6 hours
Time post acute stress
After 24h FW challenge

Figure 3

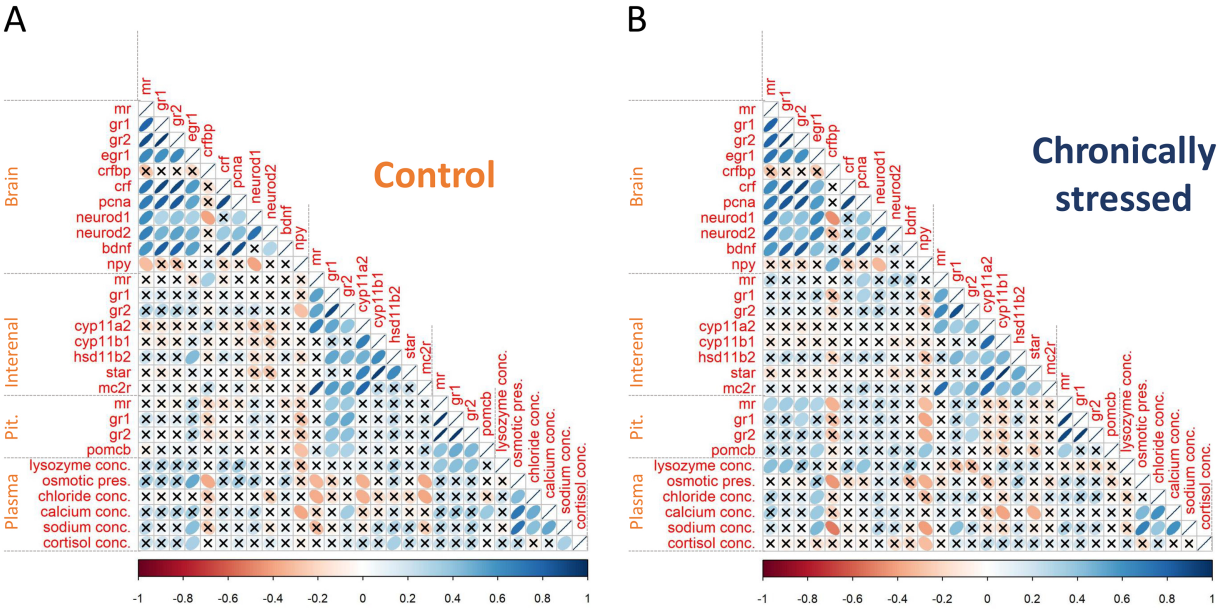

A
B
Control
Chronically stressed
Brain
Interenal
Pit.
Plasma
mr
gr1
gr2
egr1
crfbp
crf
pcna
neurod1
neurod2
bdnf
npy
cyp11a2
cyp11b1
hsd11b2
star
mc2r
pomcb
lysozyme conc.
osmotic pres.
chloride conc.
calcium conc.
sodium conc.
cortisol conc.
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 4

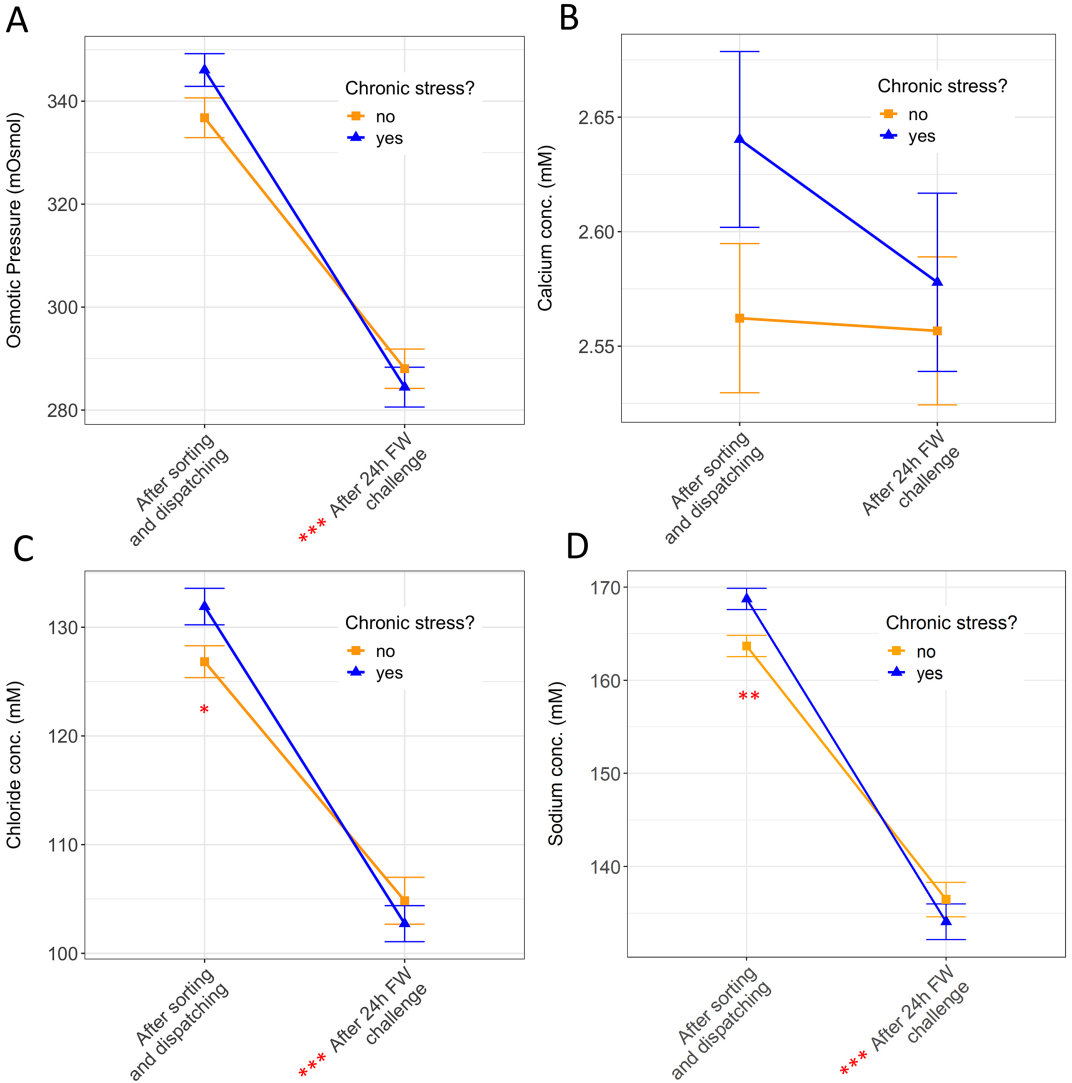

A
Osmotic Pressure (mOsmol)
340
320
300
280
Chronic stress?
no
yes
After sorting and dispatching
*** After 24h FW challenge
B
Calcium conc. (mM)
2.65
2.60
2.55
Chronic stress?
no
yes
After sorting and dispatching
After 24h FW challenge
C
Chloride conc. (mM)
130
120
110
100
*
Chronic stress?
no
yes
After 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

Figure 5

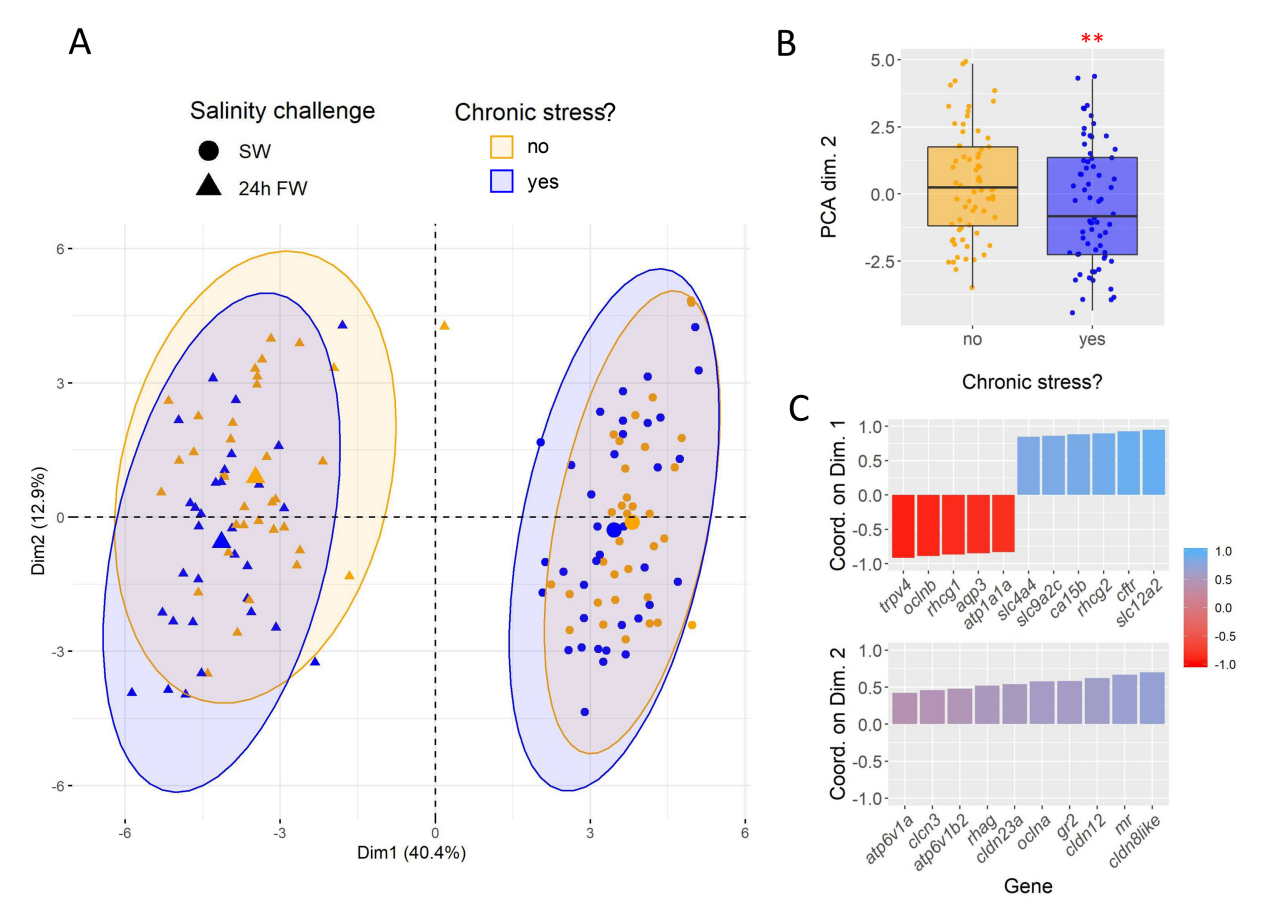

A
Salinity challenge
SW
24h FW
Chronic stress?
no
yes
Dim2 (12.9%)
Dim1 (40.4%)
B
**
PCA dim. 2
Chronic stress?
C
Coord. on Dim. 1
trpv4
ocln b
rhcg1
aqp3
atp1a1a
slc4a4
slc9a2c
ca15b
rhcg2
cftr
slc12a2
Coord. on Dim. 2
atp6v1a
clcn3
atp6v1b2
rhag
cldn23a
ocln a
gr2
cldn12
mr
cldn8like
Gene

Figure 6

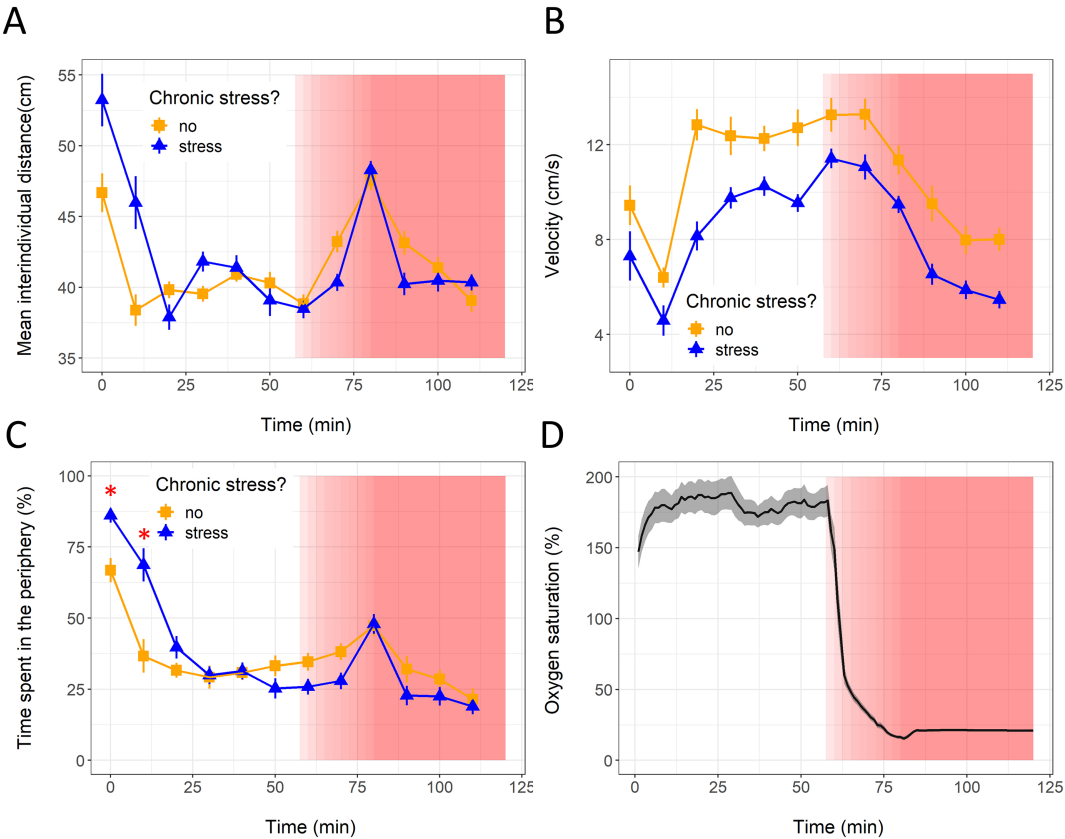

A
Mean interindividual distance(cm)
Chronic stress?
no
stress
Time (min)
B
Velocity (cm/s)
Chronic stress?
no
stress
Time (min)
C
Time spent in the periphery (%)
Chronic stress?
no
stress
Time (min)
D
Oxygen saturation (%)
Time (min)

Figure 7