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Genetic pathways underpinning hormonal stress responses in fish exposed to short- and long-term warm ocean temperatures

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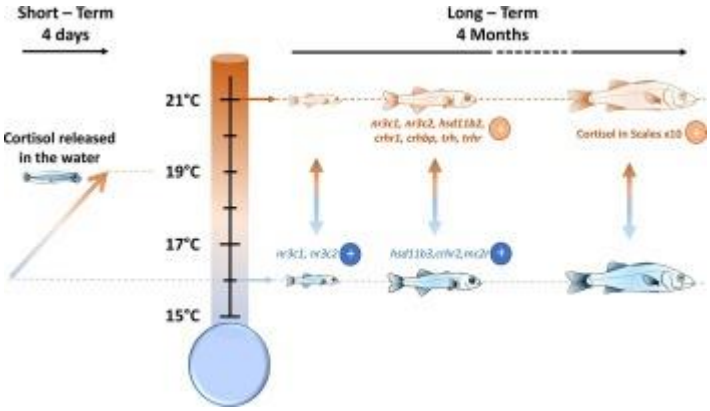
Abstract :

Changes in ocean water temperature associated with global climate change are bound to enormously affect fish populations, with potential major economic consequences in the aquaculture and fisheries industries. A link between temperature fluctuations and changes in fish stress response is well established. In this study, we aimed to assess the effects of a short- (4 days) or a long-term (4 months) exposure to warm temperature in the stress physiology of European sea bass (Dicentrarchus labrax) larvae and juveniles. First, cortisol (i.e. the main stress hormone in fishes) analysis was used to confirm that a steady and short-term elevation of temperature acts as a physiological stressful event in these fish, and cortisol release is indeed above a metabolic increase linked to temperature. Moreover, our results verified that measurement of cortisol released into the water can be reliably employed as a non-invasive indicator of acute thermal stress in experimental conditions. Secondly, the different effects on the genetic cascade underlying the stress response between long-term low or high thermal treatments were evaluated at two larval development stages via candidate-gene and whole-transcriptome approaches. Interestingly, opposite expression for some key stress genes (nr3c1, nr3c2 and hsd11b2) were observed between developmental stages, highlighting the distinct adaptive mechanisms controlling the primary and secondary responses to a stressor. Surprising expression patterns for some understudied genes involved in the stress axis were also revealed, including crhr1, mc2r, mc5r, trh or trhr, which should be further explored. Finally, evaluation of cortisol content in scales was successfully used as a biomarker of chronic thermal stress, with 10x more cortisol in fish kept at 21 °C vs 16 °C after 4 months, supporting the gene expression results observed. The use of such a method as a proxy of long-term stress, unprecedented in the literature, holds a vast array of applications in further research, in particular, in the investigation of the impact of global warming on wild fish populations.

Graphical abstract



Highlights

► Small within-day increases in temperature are physiologically stressful. ► Whole-fish transcriptomic analysis revealed a signature of warming. ► Fish kept at high temperature accumulate more cortisol in scales than those kept at low temperature. ► We propose fish scales as a relevant indicator of global warming in wild populations.

Keywords : Cortisol, Glucocorticoid receptors, Transcriptomics, Scales, Commercial fish

1 Introduction

In the current context of climate change, rising global temperatures and rapid changes in ocean acidification may be expected to greatly affect fish populations (Free et al., 2019). However, the effects of global warming on stress physiology are not fully understood yet. To shed light on the potential impact exposure to warmer temperatures will have on fish stress is of the utmost importance to ensure the adaptation and survival of fish species. In particular, understanding how fishes respond to a thermal stressor at the physiological level and its underlying mechanisms will be very valuable in aquaculture and fisheries settings (Faught et al., 2019), where species of great economic importance may be subject to the harmful effects of temperature elevation. Although global warming constitutes a gradual process and does not entail a heat shock *per se*, ensuing marine heatwaves are becoming more likely to arise and could last for several months in specific locations, with long-term effects on fish communities' structures (Wernberg et al., 2013). Even naturally occurring temperature increases (*i.e.* over the course of a day) observed in lagoons have drastic consequences on fish survival (Hobbs and McDonald, 2010). A complete understanding of how these short-term and chronic events differently affect stress physiology and endocrine pathways in fish is thus paramount. Furthermore, existing thermal life-cycle bottlenecks in fishes need also be considered, as thermal tolerance has been demonstrated to shift along ontogeny in multiple fish species (Dahlke et al., 2020). Elucidating which life stages are more temperature-sensitive and how this affects their adaptation to thermal stressors of diverse magnitude and duration would help characterise the vulnerability of these species against climate change.

Cortisol is the main glucocorticoid in fishes and the hormone most directly linked to stress (Goikoetxea et al., 2017; Sadoul and Geffroy, 2019). When an environmental factor acts as a

stressor (e.g. a temperature increase), two major neuroendocrine axes, the hypothalamic-pituitary-interrenal (HPI) axis (or so-called stress axis) and the brain-sympathetic-chromaffin-cell (BSC) axis, become activated leading to the release of cortisol and catecholamines, respectively (Kalamarz-Kubiak, 2018; Wendelaar Bonga, 1997). Multiple genes involved in the regulation of the HPI axis have been investigated for their potential roles in the stress response. One such gene is 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2*), responsible for breaking down cortisol to its inactive derivative, cortisone (Arterbery et al., 2010; Sadoul and Geffroy, 2019). Modulation of *hsd11b2* is involved in the regulation of the primary stress response (Alderman and Vijayan, 2012; Faught et al., 2019), whereby increased levels of cortisol would lead to cortisol-to-cortisone conversion driven by Hsd11b2 for protection against the potentially detrimental effects of high circulating corticosteroids in peripheral tissues (Kusakabe et al., 2003; Patiño et al., 1987). Another steroidogenic gene critical for glucocorticoid metabolism is 11β-hydroxylase (*cyp11b*), which converts 11-deoxycortisol to cortisol (Frisch, 2004). High basal levels of enzymes involved in cortisol biosynthesis, such as Cyp11b, have been shown to be essential for a prompt cortisol production following a sudden exposure to an acute stressor (Jiang et al., 1998).

Further genes of growing interest are *nr3c1* and *nr3c2*, encoding glucocorticoid receptor and mineralocorticoid receptor, respectively (Faught and Vijayan, 2018). Although the detailed involvement of *nr3c1* and *nr3c2* in the control of the HPI axis in teleost fishes remains unclear (Alderman and Vijayan, 2012), their contribution to the stress response has been nevertheless described in several species (Alderman et al., 2012; Faught et al., 2019; Faught and Vijayan, 2018; Kiilerich et al., 2018; Sathiyaa and Vijayan, 2003). Moreover, during the commonly named secondary stress response, activation of Nr3c1 and Nr3c2 via cortisol-mediated genomic

signaling has been demonstrated, facilitating energy mobilization to promote adaption to the new environmental conditions (Faught et al., 2016; Sadoul and Vijayan, 2016).

The aim of this work was to evaluate the impact of a short and a long-term increase of temperature on the stress physiology of European sea bass (*Dicentrarchus labrax*) larvae and juveniles using two experimental set-ups. Of great historical value, European sea bass is one of the most widely farmed fish species in the Mediterranean sea (Vandeputte et al. 2019). Sea bass are group-spawners that reproduce between December and March in the Mediterranean Sea. After egg hatching in the open sea, larvae reach the post-larval stage in 2-3 months while migrating to inshore nursery areas and lagoons (Vandeputte et al. 2019). Hence growing juveniles settle in lagoons mainly between April and June and are exposed to relatively high temperatures from that point. In the context of global warming, fish (and specifically juveniles) are now experiencing abnormally high water temperatures throughout the year, as exemplified by recent observations (2019, 2020) in the Thau lagoon (Western Mediterranean) (Supplementary Figure 1; https://wwz.ifremer.fr/velyger/Acces-aux-Donnees/Lagune-de-Thau/Temperature). For instance, in this lagoon, the mean water temperature was 21.9°C the 8th of May 2020, while the mean temperature over the past 15 years, on the same date, was 16°C (Supplementary Figure 1B). Relatively quick increase of temperature that last for a few days are also noticeable (Supplementary Figure 1A, B). We sought to investigate the effect of a short-term gradual increase of temperature on the concentration of cortisol detected across different stages of larval development, as well as on cortisol release into the tank water (Experiment 1), and compare basal stress physiology between European sea bass individuals at the earliest stages of development maintained at low (16 °C) vs high temperatures (21 °C) for 4 months (Experiment 2). Specifically, during Experiment 1, our main goal was to assess if a short-term temperature surge could act as a physiologically stressful event or if it would merely

reflect an increase in metabolism. During Experiment 2, we were interested on ascertaining whether such long-term thermal treatments had a different effect on expression of key genes involved in stress response (*hsd11b2*, *cyp11b*, *nr3c1* and *nr3c2*) as well as at the whole-transcriptome level (using 68 fishes) at two different larval stages. Finally, we took advantage of a new method to measure cortisol concentration in ontogenetic scales and used it as a biomarker of chronic stress (Aerts et al., 2015) to detect differences between the two temperature treatments after four months of growth. A recent study allowed to provide an overview of the effect of 4 different temperatures (8, 16, 24, and 32°C) on the stress of European sea bass juveniles grown for 1 month, focusing mostly on biochemical plasma metabolites, histology and growth parameters (Islam et al., 2020). The present experiment brings thus a complementary light on the effect of temperature on stress, by deepening our understanding of these processes and investigating two opposite time-frames (*i.e.* 4 days and 4 months) on larvae and juveniles.

2 Materials and Methods

2.1 Experiment 1: Assessing the effect of a short-term increase of temperature on fish stress

2.1.1 Experimental fish

The fish population used was the result of a complete factorial mating design including 4 males and 4 females from a wild west Mediterranean sea strain of European sea bass, performed by artificial fertilization as described in (Grima et al., 2010). Fertilized eggs were incubated at 14 °C until 48 hours post fertilization (hpf), then stocked at a density of 170 larvae per liter in eight 110-L tanks (Figure 1A). This system was designed with 2

recirculating aquaculture systems (RAS), each with 4 tanks connected to a common biofilter tank, where water was sampled (Figure 1A). A hatching rate of 82% was obtained, and temperature was gradually increased from 14 °C to 16 °C. From 10 days post hatching (dph) onwards, fish were fed *Artemia* nauplii for 40 days, then weaned on a commercial sea bass diet (Pro Start and Pro Wean, BioMar, Nersac, France). All fish were reared at the experimental aquaculture station of Ifremer (Palavas-les-Flots, France).

For the 2 RAS (8 tanks in total), the temperature-increase protocol started at 17 dph and 16 °C, with a progressive increase of 0.5 °C per day until reaching 19 °C at 22 dph (Figure 1A). Temperature was controlled following automatised instructions, and monitored in the biofilter tank twice a day throughout the experiment (see Figure 2, red line represents real temperature). We minimized all possible sources of disturbance (e.g. no swim-bladder sorting was performed, daily husbandry tasks were performed by only one person, who ensured minimum noise) and larvae were fed *Artemia* using an automated peristaltic pump delivering food continuously.

2.1.2 Water and larval sampling for cortisol measurement

Gloves were used during every step to avoid cross-contamination. Water was sampled at 7 different time-points: 13, 17, 18, 19, 20, 21 and 28 dph, encompassing the increase of temperature starting at 17 dph. Briefly, at 1pm, the open water system (delivering fresh seawater) was closed for 1 hour, so that each RAS was fully closed (as in Geffroy et al., 2018). During every sampling, at 2pm, three 500-mL samples of water were collected in new plastic beakers from the 2 tanks containing the biofilter (n=6 x 500 mL at each time). The 500-mL beakers were then stored at -20 °C before being processed all at once.

Cortisol extraction was performed using C18 solid phase extraction cartridge (Sep-pak Plus C18, Waters Ltd.; www.waters.com), as described in (Sadoul and Geffroy, 2019).

2.1.3 Hormone quantification

Cortisol in the water was analyzed by competitive ELISA, following manufacturer's recommendations (Neogen Lexington, HI, USA). Cross-reactivity of the antibody pre-coated on the plate is 15.7% for cortisone, 15% for 11-deoxycortisol and 4.8% for corticosterone according to the supplier. First, standards (50µL) or sample (50µL) were added to each well in duplicate. Each well then received cortisol conjugated to horseradish peroxidase (HRP), and was filled with a detection solution which turns blue in contact with HRP. Absorption was read at 650 nm using a Synergy-HT microplate reader (BioTek Instruments, Winooski, VT, USA) following manufacturer's instructions. To estimate the amount of cortisol lost during the extraction process, we added 10 µl of a hydrocortisone (Sigma) solution (10 ng/µl) to 3 samples of fresh seawater (500 mL) and processed them as previously described. Quantification via ELISA reported mean cortisol values of 101 ± 11 SD ng (11.16 ± 12 SD ng/ml), confirming that cortisol is entirely recovered from the extraction.

2.1.4 Predicting cortisol release using metabolic performances over time and temperature

We assumed that cortisol release was comparable to any other metabolic process; and, therefore, that this release rate follows the thermal performance curve supposed to drive the rate of biological processes (Schulte et al., 2011). The thermal performance curve for

European sea bass has been previously estimated using the Dynamic Energy Budget (DEB) theory (Stavrakidis-Zachou et al., 2019). The DEB theory integrates temperature to estimate metabolic processes driving assimilation, growth, maintenance and reproduction in animals (Kooijman, 2010). Briefly, the theory, based on first principals, assumes that energy is first assimilated from the environment into a reserve compartment at a daily rate proportional to its squared structural length (L^2). Energy is then mobilized and a proportion (κ) is allocated towards the development of structure after having paid for the associated maintenance. The other fraction of the mobilized energy ($1-\kappa$) is allocated towards maturity of the animal and its associated maintenance. The temperature impacts all rates of the model through a thermal coefficient following a thermal performance curve, described by a temperature tolerance range and a thermal optimum. A detailed description of the theory can be found in the book by Kooijman (2010).This theory has been demonstrated to properly describe the life cycle of many species, including European sea bass (Stavrakidis-Zachou et al., 2019). We used the parameters available online (Lika et al., 2018) and the temperature profile of our experiment to model the growth of our experimental individuals. We took into consideration that fish were fed *ad libitum* starting at 10 dph. In addition, we used the associated thermal performance curve for European sea bass to simulate the effect of temperature on the amount of cortisol released from individuals in the tank. Cortisol released at the beginning of the experiment (13 dph) was used as reference. We assumed that cortisol released in the water is proportional to the weight of the fish, and we therefore corrected the amount of cortisol released in the tank by the simulated weight of the fish over time.

2.2 Experiment 2: Assessing the effect of chronic exposure to warm temperature on fish stress

The aim of this experiment was to compare basal stress physiology of fish kept at 16 °C (low temperature, LT) vs fish kept at 21 °C (high temperature, HT) over 4 months. To enable data comparison between experimental groups, samples were taken at the same sum of degree-day (base 10 °C, $DD_{10\ °C}$), a procedure previously used to allow standardized measurement of growth in fishes (Chezik et al., 2014), and specifically in juvenile European sea bass (Le Boucher et al., 2013; Vandeputte et al., 2014). Here we collected larvae at 242 $DD_{10\ °C}$ (25 dph for the HT and 40 dph for the LT) and 550 $DD_{10\ °C}$ (53 dph for the HT and 78 dph for the LT), so that LT individuals were always older than HT individuals. One might argue that comparing individuals of different age might render the results difficult to interpret. However, weak to no differences were observed in previous experiments comparing those different developmental stages (*i.e.* flexion, all fins and juveniles) at a given temperature for various stress-related genes (*i.e. gr2, mr, crf*); although *gr1* did differs (Pavlidis et al., 2011; Tsalafouta et al., 2017). The effect of age for these specific stages might thus be negligible compared to the effect of temperature *per se* and detecting differences within developmental stages would thus underline a real thermal stress. The last sampling, aimed at obtaining samples for cortisol-content analysis in scales and was based on size instead of thermal age, since growth difference between treatments declined with the latter. For this reason, juveniles were taken at 117 dph (HT) and at 124 dph (LT), where the same development stage was achieved.

2.2.1 Experimental fish

The fish population used was the result of a complete factorial mating design including 15 males and 7 females from a wild west Mediterranean sea strain of European sea bass, performed by artificial fertilization as described in (Grima et al., 2010). Fertilized eggs were incubated at 14 °C until 48 hpf, then stocked at a density of 60 larvae per liter at 15 °C (Figure 1B). A hatching rate of 59% was obtained. Larvae were then exposed to either HT or LT treatment. HT treatment consisted in temperature gradually increasing to reach 21 °C between hatching (0 dph) and 8 dph. For the LT treatment, temperature was gradually increased from hatching until 1 dph, reaching 16 °C (Figure 1B). Fish were fed and maintained as described in Experiment 1 but in triplicates of 500L tanks per condition (Section 2.1.1).

2.2.2 Sampling

Sixty larvae, 35 from the HT group and 25 from the LT group, were collected at the flexion stage (242 $DD_{10\,°C}$, 25 dph for the HT and 40 dph for the LT) and used for gene expression analysis of target genes (*nr3c1*, *nr3c2*, *cyp11b*, *hsd11b2*). A second sampling of 38 HT larvae and 24 LT larvae was performed at the same stage for wet-weight evaluation. Seventy fish were also collected at the 'all fins' stage (550 $DD_{10\,°C}$, 53 dph for the HT and 78 dph for the LT), 40 from the HT group and 30 from the LT group, and used for transcriptomic analysis. Another sampling to evaluate weight (79 HT and 60 LT) and length (40 HT and 60 LT) was performed. For juveniles (124 dph in the case of LT and 117 dph in the case of HT), weight, length and scales from 35 individuals in each treatment were collected.

2.2.3 Gene expression of whole bodies at the flexion stage

2.2.3.1 Extraction and reversed transcription of total RNA from fish samples

Larvae were placed individually in 2 mL RNase-free tubes containing 1 mL of Tri reagent (Molecular Research Centre, Cincinnati, OH, USA) and disrupted using ball mill (two times 30 seconds at 30 rpm). Total RNA was extracted according to manufacturer's instructions. RNA quantity was assessed by measuring the A260/A280 ratio using the NanoDrop® ND-1000 V3300 Spectrophotometer (Nanodrop Technology Inc., Wilmington, DE, USA). In addition, RNA quality was checked on an Agilent bioanalyzer (Agilent) using electrophoretic trace method. The RNA integrity number was between 8.5 and 10 for all samples. An aliquot of each tube was diluted in RNase- and DNase-free water to obtain a total RNA quantity of 1 µg for the reverse transcription. cDNA was synthesized using the qScript cDNA SuperMix (Quantabio, QUIGEN, Beverly, MA, USA) following manufacturer's instructions. cDNA was diluted 40-fold in nuclease-free water prior to quantitative real-time PCR (qPCR).

2.2.3.2 Quantitative real-time PCR

All primer sequences were extracted from the literature (Martins et al., 2015; Pavlidis et al., 2011; Sadoul et al., 2018; Socorro et al., 2007) with the exception of *hsd11b2* primers, which were designed specifically (Table 1).

Gene	GenBank accession numbers	Primers	Primer sequence 5' to 3'	Amplicon size (bp)	Efficiency	Reference
gr1	AY549305.1	gr1-F gr1-R	GAGATTTGGCAAGACCTTGACC ACCACACCAGGCGTACTGA	401	2.07	Pavlidis et al. 2011
gr2	AY619996	gr2-F gr2-R	GACGCAGACCTCCACTACATTC GCCGTTCATACTCTCAACCAC	403	2.21	Pavlidis et al. 2011
mr	JF824641.1	mr-F mr-R	GTTCCACAAAGAGCCCCAAG AGGAGGACTGGTGGTTGATG	197	2.27	Sadoul et al. 2018
cyp11b	AF449173.2	cyp11b-F cyp11b-R	GGAGGAGGATTGCTGAGAACG AGAGGACGACACGCTGAGA	80	1.86	Socorro et al. 2007
hsd11b2	DLAgn_00250230	hsd11b-F hsd11b-R	CAGGCACGTTACTTCGCTGG TGACTGCTTCCTTAGAGCGC	141	1.94	This study
ef1-α	AJ866727.1	ef1-F ef1-R	AGATGGGCTTGTTCAAGGGA ACAGTTCCAATACCGCCGA	166	2.05	Sadoul et al. 2018
actin	AY148350.1	act1-F act1-R	TGACCTCACAGACTACCT GCTCGTAACTCTTCTCCA	176	1.89	Martins et al. 2015
L13	DLAgn_00023060	L13-F L13-R	TCTGGAGGACTGTCAGGGGCATGC AGACGCACAATCTTGAGAGCAG	148	1.78	Sadoul et al. 2018

Table 1: List of Primers used for whole body gene expression at the flexion stage.

PCR products were sequenced by Sanger sequencing in order to validate the identity of the amplified sequences in comparison with the European sea bass genome. We attempted to run *gr2*, but the mean cycle threshold was very high (31.7), so results were discarded. The *nr3c1* we refer to throughout the manuscript is considered to be *gr1* (after alignment of the sequence on NCBI). Ribosomal protein L13 (*L13*), eukaryotic translation elongation factor 1 alpha (*eef1a*) and beta-actin (*β-actin*) are all recommended reference genes for qPCR and have been successfully used previously in European sea bass (Alfonso et al., 2019; Sadoul et al., 2018). An Echo® 525 liquid handling system (Labcyte Inc., San Jose, CA, USA) was used to dispense 0.75 µL of SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK), 0.037 to 0.111 µL of each primer (forward and reverse primers between 0.2 to 0.6 µM final concentration), sufficient amount of ultra-pure water and 0.5 µL of diluted cDNA into a 384-well reaction plate. Each sample was run in duplicate. qPCR conditions were as follows: denaturation at 95 °C for 2 min, 45 cycles of amplification (95 °C, 15 s), hybridization (60 °C or 64 °C depending on the gene, 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. Ultra-pure water was used as a no-template control.

2.2.4 RNA-Sequencing of whole bodies at the 'all fins' stage

2.2.4.1 cDNA Libraries:

Total RNA extraction and quantification of a total of 70 samples was performed for the larvae at the flexion stage. RNA extraction of two larvae (1 LT and 1 HT) failed, as confirmed by the low RNA Integrity Number (RIN) observed. Those samples were thus discarded, and 68 libraries were then constructed using the Truseq stranded mRNA sample prep kit (Illumina, San Diego, CA, USA) according to manufacturer instructions. Briefly, poly-A RNAs were purified using oligo-d(T) magnetic beads from 1 µg of total RNA. The poly-A RNAs were fragmented into small pieces using divalent cations under elevated temperature and reverse transcribed using random hexamers, Super Script II (Thermo Fisher Scientific, Carlsbad, CA) and Actinomycin D. During the second strand generation step, dUTP substituted dTTP. This prevents the second strand to be used as a matrix during the final PCR amplification. Double stranded cDNAs were adenylated at their 3' ends before ligation was performed using Illumina's (dual) indexed adapters. Ligated cDNAs were amplified following 15 cycles PCR and PCR products were purified using AMPure XP Beads (Beckman Coulter Genomics, Brea, CA, USA). Libraries were validated on a Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified using the KAPA Library quantification kit (Roche, Bâle, CHE).

2.2.4.2 Libraries sequencing

A total of 68 libraries were pooled in equimolar amounts, denatured with NaOH, and diluted to 20-22 pM before clustering on a cBot system (Illumina, San Diego, CA, USA). Sequencing was performed on an HiSeq 2500 (Illumina, San Diego, CA, USA) using the single-end 1*50 nt protocol on 9 lanes of a flow cell V4 or V2. Sequencing produced between 5 and 34 million passed-filter clusters per library.

2.2.4.3 Sequencing quality control

Image analyses and base calling were performed using the Illumina HiSeq Control Software and the Real-Time Analysis component. Demultiplexing was performed using Illumina's conversion software (bcl2fastq 2.20). The quality of the raw data was assessed using FastQC from the Babraham Institute and the Illumina software SAV (Sequencing Analysis Viewer). Potential contaminants were monitored with the FastQ Screen software from the Babraham Institute.

2.2.5 Scales

Ontogenetic scales were placed in 1.5-mL Eppendorf tubes and stored at 4 °C. After removing the mucus, scales were cut into fine pieces. The amount of sample used for analysis was standardized to 100 mg and homogenized using PowerBead tubes (ceramic 2.8 mm, Qiagen) and a bead ruptor (PowerLyzer 24, Qiagen). Each sample was quantitatively transferred using 8000 µL of methanol (HPLC grade) into a 12-mL tube to which 10 µL of a cortisol-d4 solution of 0.5 µg/L was added as internal standard.

Whenever other sample volumes were used, results were corrected accordingly. Each sample was vortex-mixed for 30 s until homogenization and placed on an overhead shaker for 60 min at 90 rpm. Subsequently, each sample was centrifuged for 10 min at 3452 g (= 4000 rpm on a swing-out) at 7 °C and the supernatant was transferred to a new 12-mL tube. Each sample was evaporated to dryness under nitrogen at 60 °C using a Turbovap and resuspended in 5000 µL H_2O/MeOH (80:20; v/v). After conditioning a C18 SPE column (C18-Max, 500 mg, 6 mL) with 3 mL of MeOH (HiPerSolv) followed by 3 mL of H_2O (Type I), each sample was loaded. The column was washed with 4.5 mL H_2O/MeOH (65:35; v/v) and retained compounds eluted with 2.5 mL H_2O/MeOH (20:80; v/v) into a 12-mL test tube and evaporated to dryness under a stream of nitrogen at 60 °C using a nitrogen evaporator.

Samples were then reconstituted in 50 µL H_2O/MeOH (80:20; v/v) in a vial with insert and analyzed on an Acquity UPLC BEH C18 (1.7 µm; 2.1 mm x 100 mm) column by means of UPLC-MS/MS (Xevo TQS, Waters, Milford, USA). Calibration curves were made in H_2O/MeOH (80:20, v/v). Subsequently, the stock factor was 1000 and results were corrected for this (Aerts et al., 2015).

2.2.6 Statistical Analysis

Cortisol concentrations in the water were compared using the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) followed by post-hoc comparisons using pairwise Wilcoxon analysis (Wilcoxon, 1945). Results were considered significantly different when p-value < 0.05. The differences between values were also compared to the DEB estimates at 17, 18, 19, 20, 21, and 28 days using t-test. For gene expression at 242 dph, we first compared expression of housekeeping genes, to evaluate whether they differed between

treatments (HT vs LT), using a t-test. We did not detect significant differences for any of the housekeeping genes (Supplementary Figure 2). For target genes (*nr3c1*, *nr3c2, cyp11b* and *hsd11b2*) and cortisol content in scales, we tested for differences between treatments using t-tests. For the RNA-Seq data analysis, a splice junction mapper, TopHat 2.1.1 (Kim et al., 2013) (using Bowtie 2.2.9; (Langmead and Salzberg, 2012)) was used to align the RNA-Seq reads to the *Dicentrarchus labrax* genome (NCBI, reference GCA_000689215.1) with a set of gene model annotations (Tine et al., 2014). Final read alignments having more than 3 mismatches were discarded. Samtools (v.1.5) was used to sort alignment files and counting was performed with Featurecounts (v.1.6.2) (Liao et al., 2014). When data come from a strand-specific assay (libraries with high starting material), the read has to be mapped to the opposite strand of the gene (-s 2 option).

Before statistical analysis, genes with less than 195 reads (cumulating all the analyzed samples) were filtered out. Differentially expressed genes between HT and LT treatments were identified using the Bioconductor (Gentleman et al., 2004) package DESeq2 v.1.26.0 (Love et al., 2014). Data were normalized using the default method of DESeq2. Genes with adjusted p-value of less than 5% (according to the FDR method from Benjamini-Hochberg (Benjamini and Hochberg, 1995)) were considered differentially expressed. During filtering, and for this purpose, we only kept genes involved in 4 different gene ontology pathways in mouse (*Mus musculus*): 'GO 085121 glucocorticoid metabolic process'; 'GO 101540 cellular response to heat'; 'GO 101649 cellular response to cold' and 'GO 0006950 response to stress', available at http://www.informatics.jax.org/vocab/gene_ontology/ and downloaded on the 26/03/20. We used mouse GOs instead of zebrafish GOs, since much more genes are identified in the former compared to the latter. For the glucocorticoid metabolic process pathway, we also added all genes recently highlighted

to play a role in cortisol synthesis (Gorissen and Flik, 2016). All statistical analyses were performed in R (v. 3.6.2) (Core Team, 2013).

3 Results

3.1 Cortisol measurement and DEB analysis

Cortisol concentration in the water readily increased as water temperature (red line, Figure 2) rose over time, with a significantly higher cortisol concentration at 19, 20 and 21 dph compared to 17 dph (Wilcoxon test, p-value < 0.05, Figure 2). Hence, an increase of just 0.5 °C/day for 4 days triggers a significant increase in cortisol production. Cortisol released in the water was greater than estimated by the DEB (Blue line, Figure 2) at 19, 20 and 21 days (t-test, p-values<0.01). At 28 days, the difference was no longer significant. For the experiment on chronic exposure to high and low temperature, at 242 $DD_{10\,°C}$, wet weight significantly differed between treatments, with larvae from the LT group being heavier than those from the HT group (7.9 ± 0.8 mg vs 3.7 ± 0.3 mg; t-test, p-value < 0.001). At 550 $DD_{10\,°C}$, wet weight was also significantly different between treatments, with LT larvae being heavier than HT larvae (134 ± 8.3 mg vs 77 ± 2.7 mg; t-test, p-value < 0.001). LT larvae were also slightly longer than HT larvae (24 ± 0.4 mm vs 22 ± 0.3 mm; t-test, p-value < 0.001). At 117 (HT) and 124 dph (LT), wet weight and length did not significantly differ between treatments (weight: 4.54 ± 0.2 g vs 4.7 ± 0.2 g, t-test, p-value = 0.6; length: 7.2 cm ± 0.1 vs 7.4 ± 0.1 cm, t-test, p-value = 0.14).

3.2 Gene expression and whole-transcriptome analyses

In terms of gene expression, at 242 $DD_{10\ °C}$, LT fish presented a significantly higher expression of *nr3c1* (t-test, p-value < 0.01) and *nr3c2* (t-test, p-value < 0.01) compared to HT fish, but there were no differences for *hsd11b2* and *cyp11b* (Figure 3).

RNA-seq analysis performed on 550 $DD_{10\ °C}$ old larvae revealed that a total of 15966 genes were differentially expressed between HT and LT treatments. Among those genes, 21 were from the gene ontology of glucocorticoids metabolism (Figure 4A), which included *nr3c1*, *nr3c2* and *hsd11b2* significantly more expressed at HT (Figure 4B).

The gene ontology related to 'Cellular response to heat' highlighted 27 genes differentially expressed, 12 more expressed in the LT and 15 more expressed in the HT treatment (Figure 5A). Gene ontology 'Cellular response to cold' highlighted 6 genes differentially expressed, 3 more expressed in the LT and 3 more expressed in the HT treatment (Figure 5B). Gene ontology 'Response to stress', which included 3718 genes, enabled detection of 2285 genes differentially expressed between the HT and LT treatments, 1236 with higher expression in the LT and 1049 with greater expression in the HT (Supplementary Figure 3).

3.3 Scale cortisol content

At the end of the experiment (*i.e.* 124 dph), cortisol content in the scales of fishes reared at 21 °C was 10 times higher compared to the scales of those individuals reared at 16 °C (21 ± 6.3 µg/g and 2.1 ± 0.3 µg/g; t-test, p-value < 0.01, Figure 6).

4 Discussion

Very little ecologically relevant information is available on the effects of temperature increases on the teleost HPI axis, despite its central role in coping abilities of fish species (Faught et al., 2019). Elucidating the link between temperature fluctuations, physiological stress and the associated variation in key gene expression is becoming even more ecologically relevant in the current ever-changing landscape of global warming. Thermal stressors in connection with climate change are not only expected to affect interspecific interactions between fishes, but may also negatively impact the health and fitness of these animals (Kordas et al., 2011). Therefore, understanding the mechanisms underlying the physiological and genetic cascades triggered by short-term or chronic thermal stressors is essential for the accomplishment of a sustainable growth of the aquaculture and fisheries industries. The work hereby discussed contributes to shed light on how fish physiologically respond to such thermal changes at the hormonal and genetic levels. To fully comprehend how exposure to warmer temperatures affects fish stress physiology, a crucial question is how the duration and magnitude of a thermal stressor relate to the amount of cortisol released and to changes in key gene expression, especially of those genes involved in glucocorticoid metabolism or thermal tolerance at a cellular level. In this study, we evaluated European sea bass response to stress after the application of a short- or a long-term thermal stressor in two independent experimental set-ups using multiple approaches (hormonal, candidate gene, RNA-Seq). Our multivariate analysis enables the consideration of new biological markers as potential candidates for future ecological indicators of altered coping abilities in response to climate change in fishes. Using these novel and effective biomarkers, such as water cortisol measurement and scale cortisol content, may also help assess the potential

negative repercussions of global warming on fish using less invasive tools than those traditionally employed.

Cortisol release increases with small within-day rises of temperature

In teleost fishes, an increase in temperature is hypothesized to promote HPI axis activity, leading to higher circulating levels of cortisol (Conde-Sieira et al., 2018; Faught et al., 2019; Vargas-Chacoff et al., 2018). However, it is still unknown whether this reflects a physiological stress (*i.e.* similar to the one following any acute stressful event) or if this is merely the result of an increase in metabolism related to thermal performance, whereby all enzymes are more active at higher temperatures, particularly in poikilothermic species (Schulte et al., 2011). Our results from Experiment 1 advocate for the former hypothesis, since cortisol release rate was higher than what expected based on the DEB theory (Figure 2). In addition, the possibility that the temperature end-point chosen for the experimental set-up (i.e. 19 °C) induced eustress (whereby a stressful environment can lead to beneficial effects), impacting the observed results by preventing the measurement of a true physiological response, cannot be conclusively rejected.

Long-term thermal stress decreases glucocorticoid and mineralocorticoid receptors gene expression at the flexion stage

Steroid hormones and hormone receptors are known mediators of the stress response. Among the genes implicated in steroidogenesis evaluated via qPCR in Experiment 2, which involved long-term exposure to warm temperature, *nr3c1* and *nr3c2* expression appeared to be significantly affected by temperature, but not that of *cyp11b* and *hsd11b2*

(Figure 3). In our study, lower *nr3c1* and *nr3c2* mRNA levels were detected in the flexion stage larvae from the HT group, which underwent a greater thermal stress, resulting in higher levels of cortisol, compared to those from the LT group (Figure 3). This downregulation of *nr3c1* mRNA by stress is contrary to results observed in studies with rainbow trout (*Oncorhynchus mykiss*) involving long-term cortisol exposure (Rosewicz et al., 1988; Vijayan et al., 2003; Yudt and Cidlowski, 2002), and suggests that older and bigger larvae (LT) produce more glucocorticoids receptors than younger ones (HT), independently of their thermal age (Figure 3). This appears to be consistent with the production of mineralocorticoid receptors observed in the case of LT European sea bass larvae, as shown in Figure 3. Indeed, despite cortisol being the physiological ligand for Nr3c1, it has been suggested that Nr3c2 may display a higher affinity to cortisol than Nr3c1 (Prunet et al., 2006), likely influenced by the absence of aldosterone (the main mineralocorticoid hormone) in fishes. The lack of gene expression differences between LT and HT treatments detected for *cyp11b* and *hsd11b2* (Figure 3), involved in cortisol biosynthesis, suggests that although larvae are still responding to a thermal stressor at 34 dph, plasma cortisol may have gone back to basal levels. In fact, during chronic stress, circulating levels of cortisol are expected to fall back to resting levels even if the stressor is still being applied (Mommsen et al., 1999; Vijayan and Leatherland, 1990).

RNA-Seq highlighted a high number of key stress genes with differential expression between thermal treatments

Among such genes, whole-transcriptome analysis revealed opposite expression patterns between HT and LT individuals at the 'all fins' stage for stress-related genes *nr3c1*, *nr3c2* and *hsd11b2* when compared to qPCR results for the same genes in the flexion stage larvae

(Figure 4). We hypothesized that circulating levels of cortisol in the European sea bass larvae increased for a second time as a long-term response to adapt to the thermal transition. Therefore, the higher expression of genes *nr3c1*, *nr3c2* and *hsd11b2* in the HT larvae compared to LT individuals at the 'all fins' stage likely reflects adaptive mechanisms of negative feedback on the HPI axis (Figure 4). A mid-term response to exposure to warm temperature translated into increased cortisol levels accompanied by glucocorticoid receptors upregulation has also been reported in other species, such as the rainbow trout and the Senegalese sole (*Solea senegalensis*) (Benítez-Dorta et al., 2017; Rosewicz et al., 1988; Vijayan et al., 2003; Yudt and Cidlowski, 2002) supporting the data here presented. Nevertheless, exceptions have been reported in some species such as the emerald rockcod (*Trematomus bernacchii*), where a correlation between a temperature increase and changes in basal cortisol levels was not observed (Hudson et al., 2008), suggesting that this relationship may be, to some extent, species-specific. The different expression patterns observed for the same key genes (*nr3c1*, *nr3c2* and *hsd11b2*) between the flexion (qPCR) and 'all fins' (RNA-Seq) stages were also expectedly affected by ontogeny throughout sampling stages.

Corticotropin-releasing hormone receptors (Crhrs) play a key role during the stress response in fishes. Binding of corticotropin releasing hormone (Crh) to its receptors activates the HPI axis by promoting the release of ACTH (corticotropin) into the circulatory system (Wendelaar Bonga, 1997). Genes *crhr1* and *crhr2* had opposite responses to the chronic thermal treatment, with a higher expression of *crhr1* and lower expression of *crhr2* observed in the HT larvae, and vice versa for the larvae from the LT treatment at 550 $DD_{10\,°C}$ (Figure 4). Upregulation of *crhr1* mRNA levels in the HT larvae was unexpected. Following a physiological stress, ACTH-mediated release of cortisol is hypothesized to inhibit further production of Chr and ACTH,

negatively regulating additional cortisol release (Polson, 2007). Therefore, under a higher thermal stress (*i.e.* HT treatment), we might have predicted lower expression of both *crhr1* and *crhr2*. Indeed, *crhr1* was shown to be downregulated in the pituitary, gills and skin of the common carp (*Cyprinus carpio*) in response to an acute stressor (Huising et al., 2004; Mazon, 2006). However, current knowledge of Crhr1 and Crhr2 is based, to a certain extent, on their mammalian counterparts, and further investigation is needed to evaluate potential species- and/or tissue-specific roles in fishes. Statistically significantly higher expression of *crhbp* (corticotropin-releasing hormone-binding protein) in the HT larvae compared to the LT group (Figure 4) suggests a stronger regulation of cortisol synthesis during the high-temperature treatment. Crhbp is hypothesized to mediate the HPI axis response to stress by sequestering Crh and preventing its binding to its receptors, so as to contribute to a faster regaining of the homeostatic status following exposure to stress (Doyon et al., 2005). Our results imply higher Crhbp activity at elevated temperatures, likely associated with the mid-term response to the long-term thermal stressor applied.

Once ACTH reaches the interrenal cells harboured in the head kidney, it binds and activates Mc2r (melanocortin 2 receptor), resulting in the synthesis and release of cortisol by interrenal tissue (Mommsen et al., 1999; Wendelaar Bonga, 1997). The exact functional regulation of Mc2r and other melanocortin receptors identified to date in fishes, which vary from species to species, remains to be elucidated (Agulleiro et al., 2013). In our study, contrary to expected, *mc2r* expression was downregulated in larvae exposed to a higher thermal stress (HT). Expression of *mc2r* has been shown to parallel whole-body cortisol concentrations following application of a stressor (Tsalafouta et al., 2017). Therefore, we might have expected a higher *mc2r* expression in the HT larvae, as higher levels of circulating cortisol were hypothesized to be released as response to the long-term higher thermal stressor applied. Upregulation of *mc2r*

after exposure to an acute stress was shown in both flexion- and 'all fins'-stage European sea bass larvae in a study by Tsalafouta et al (2017), in agreement with work in other species, such as the rainbow trout (Aluru and Vijayan, 2008) and zebrafish (*Danio rerio*) (Tokarz et al., 2013). It may be that *mc2r* role during the stress response is dependent on the magnitude and/or duration (*i.e.* short-term vs long-term) of the stressor, which deserves further investigation. Contrarily to *mc2r*, higher levels of *mc5r* mRNA were detected in the HT larvae compared to the LT larvae. Little is known about the role of this melanocortin receptor subtype during the stress response in teleost fishes. Interestingly, it has been suggested that *mc5r* may have arisen after a gene duplication event, whereby an ancestral *mc5r*/*mc2r* gene would have acquired a new function through gene duplication mechanisms (Baron et al., 2009). Indeed, *mc5r*, and not *mc2r*, is hypothesised to be the ACTH receptor par excellence in some species, such as the horn shark (*Heterodontus francisci*) (Baron et al., 2009). It has also been suggested that *mc5r* could mediate cortisol release induced by α-Melanocyte-stimulating hormone (α-MSH) but this hypothesis remains ambiguous (Metz et al., 2006). Our data suggest that *mc2r* and *mc5r* may play opposite roles during sea bass response to a thermal stress.

Thyrotropin-releasing hormone (Trh) and its receptor (Trhr) are some of the main components of the hypothalamic-pituitary-thyroid (HPT) axis, which is known to cross-talk with the HPI axis (Castañeda Cortés et al., 2014). Data from Experiment 2 showed an upregulated expression of both *trh* and *trhr* in the larvae from the HT treatment compared to those from the LT group (Figure 4). Although a causal relationship cannot be established from this data, these results suggest that *trh* and *trhr* could stimulate cortisol release to adapt to the higher temperature, in line with cortisol content in scales at the end of the experiment. A Trh-regulated cortisol production via α-MSH has been previously suggested in the Mozambique tilapia (*Oreochromis mossambicus*) (Lamers et al., 1997; Van den Burg et al., 2003), supporting our observations.

Multiple genes associated with the physiological response to stress at the level of the cell also showed differential expression between HT and LT treatment groups (Figure 5). Interestingly, among those genes involved in the cellular response to heat, *hsp90aa1* was observed to have a statistically significantly biased expression towards the LT treatment. Although accumulation of proteins belonging to the HSP (heat-shock proteins) family after exposure to high thermal stressors is widely established (Feder and Hofmann, 1999; Hofmann, 2005; Madeira et al., 2012), cold temperatures have also been shown to affect HSP expression (Matz et al., 1995). Higher expression of *hsp90aa1* in the LT group suggests that synthesis of the homonymous protein may be involved in protection against low temperatures at the cellular level in the case of European sea bass larvae. A downregulation of *hsp70* might have also been expected owing to previous work on the rainbow trout showing that high cortisol had a suppressive effect on stress-induced tissue levels of Hsp70 (Basu et al., 2001). However, we did not detect a significant difference in *hsp70* expression in the whole body, which is concordant with findings in the liver and the kidney of European sea bass larvae (Islam et al., 2020), suggesting a potential species-specific response regarding heat-shock proteins. A hypothetical species-specific role of these heat-shock proteins is further supported by a recent study on the sensitivity and molecular changes of sea bream (*Sparus aurata*) larvae towards ocean warming by Madeira et al. (2016). Madeira and co-authors predictably detected an increase in Hsp90 in larvae exposed to warming, offering protection to heat stress (Madeira et al., 2016). However, Hsc70 (or Hspa8, a member of the Hsp70 family) was observed to be downregulated in this species, suggesting that the downregulation of this constitutive chaperone may influence other cell functions (e.g. cell cycle regulation) as well as being involved in protein folding regulation (Madeira et al., 2016).

Scale cortisol content confirms correlation between temperature and cortisol production

Cortisol concentrations detected in scales of European sea bass juveniles at the end of the long-term exposure to warmer temperature experiment (Figure 6) demonstrated a clear pattern of higher scale cortisol content (x10) under elevated temperatures, supporting gene expression differences between treatments observed during the whole-transcriptome analysis, whereby greater cortisol production would be expected over time after recovery of the primary stress response and promotion of the secondary stress response. Resting circulating cortisol concentrations have been reported to be higher upon a prolonged temperature increase in other fish species (Kim et al., 2019; Madaro et al., 2018; Samaras et al., 2018), while no significant changes were detected in European sea bass juveniles when comparing those raised at 16 °C vs 24 °C after 10, 20 and 30 days (Islam et al., 2020). Resting circulating cortisol was only higher at 32 °C (Islam et al., 2020). The novel work presented here thus allowed a finer approach to detect chronic exposure to relatively high (but physiologically sound) temperatures. Such a discovery could provide new avenues for future research aiming to assess the effects of climate change on wild fish populations suffering heat waves or chronic warming. Not only in terms of biomarker application, but it will also be of great interest to use the gene expression data provided in this study to draw comparisons between wild larvae and adults of European sea bass and other species in regard to their physiological stress response in the face of global warming. As we know that the vulnerability of species to climate change varies according to the different life stages (Dahlke et al., 2020) elucidating how thermal events affect life stages differently at the metabolic and genetic pathway levels and how this impacts development (e.g. size reached at a certain stage, time between life stages) will advance our understanding of the response to stressful events in teleost fishes.

5 Conclusions

In this study, the effect of a short- and a long-term increase of temperature on the hormonal and genetic cascades underlying the stress response in European sea bass larvae was successfully investigated via manipulative experiments. Experiment 1 not only demonstrated that a short-term steady temperature elevation is perceived as a physiologically stressful event, but also highlighted the effectivity of the measurement of cortisol released into the water as a proxy for acute thermal stress. Gene expression differences evaluated between long-term low or high thermal treatments at two larval development stages (Experiment 2) showed opposing patterns for some key stress genes (*nr3c1*, *nr3c2* and *hsd11b2*), likely reflecting adaptive mechanisms of negative feedback on the HPI axis during the secondary stress response. Interestingly, whole-transcriptome analysis also revealed some surprising expression patterns (*crhr1*, *mc2r*, *mc5r, trh* and *trhr*) of the genetic mechanisms that control the physiological response to thermic stress, which deserve further investigation. Importantly, measurement of cortisol concentrations in ontogenetic scales supported the gene expression results observed. The use of such a novel biomarker for evaluation of chronic stress proved a very valuable tool, with enormous possibilities in future studies, in which the potential accumulation of cortisol in scales following a stressor other than a chronic thermal stressor should also be explored. These promising results open doors to the application of this biomarker in the assessment of the effect of climate change on wild fish populations, allowing for the minimizing of animal usage during sampling, and thus contributing to a more ethical use of animals in research that respects the 3Rs principles (Russell and Burch, 1959). Going forward, the combination of mRNA data from this study with genetic data from other species of interest in aquaculture, boosted by the application of emerging multi-omics technologies, will help unravel the evolution of the hormonal response to heat stress

across species. Moreover, the genetic pathways involved in the response to rising ocean temperatures identified in the present study may help predict physiological phenotypes of fish breeds undergoing stress exposure, with great ecological and economic implications in aquaculture practices.

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8 Figures

Figure 1. Experimental design for (A) Experiment 1 aiming at assessing cortisol production over time by European sea bass larvae exposed to a gradual increase of temperature (16 -> 19°C) over 4 days; and (B) Experiment 2 aiming at assessing the impact of long-term (4 months) exposure to warming (21°C) on gene expression, whole-body transcriptomic and cortisol accumulation in scales during the development process of European sea bass. Dashed blue lines represent sampling time of individuals kept at 16°C and dashed red lines represent sampling time of individuals kept at 21°C. Complementary information are available in Material and Method section.

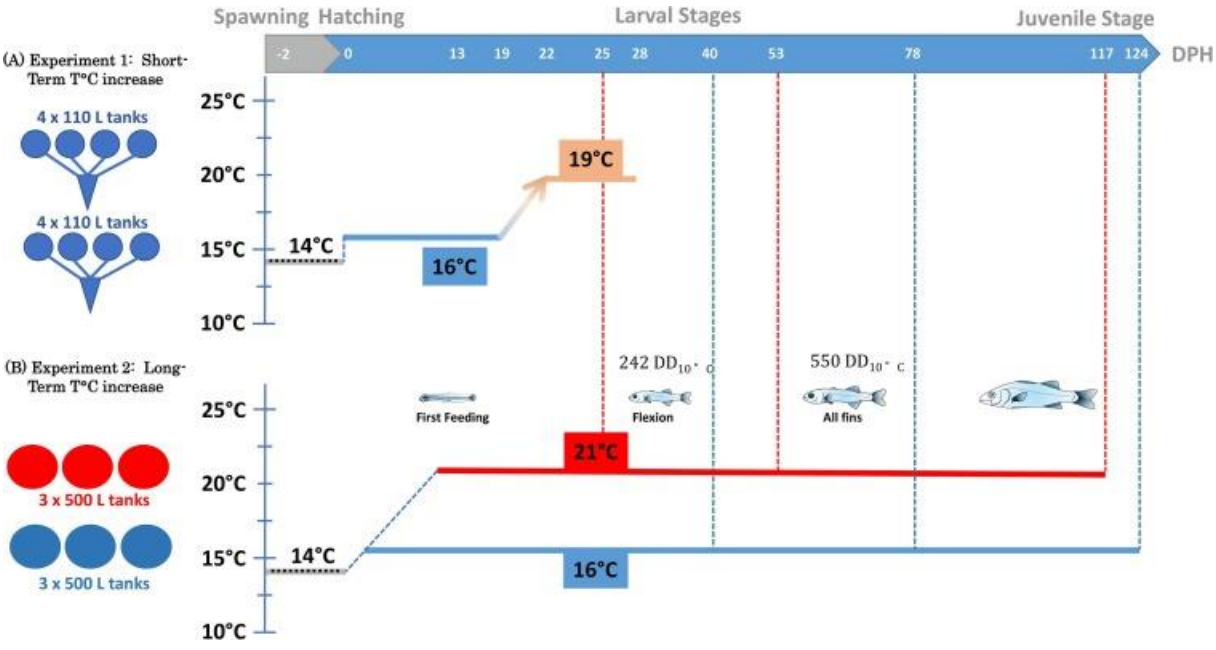

Figure 2. Cortisol production over time by European sea bass larvae exposed to a gradual increase of temperature over 4 days (Experiment 1). Mean cortisol (ng/L) represented by black points (mean + SE), released into the water by developing larvae (from 13 to 28 dph) exposed to a gradual increase of temperature (red line, second y axis). The blue line represents the estimated cortisol produced under the Dynamic Energetic Budget (DEB) theory by growing larvae exposed to the same temperature protocol (see Materials and Methods for complementary information).

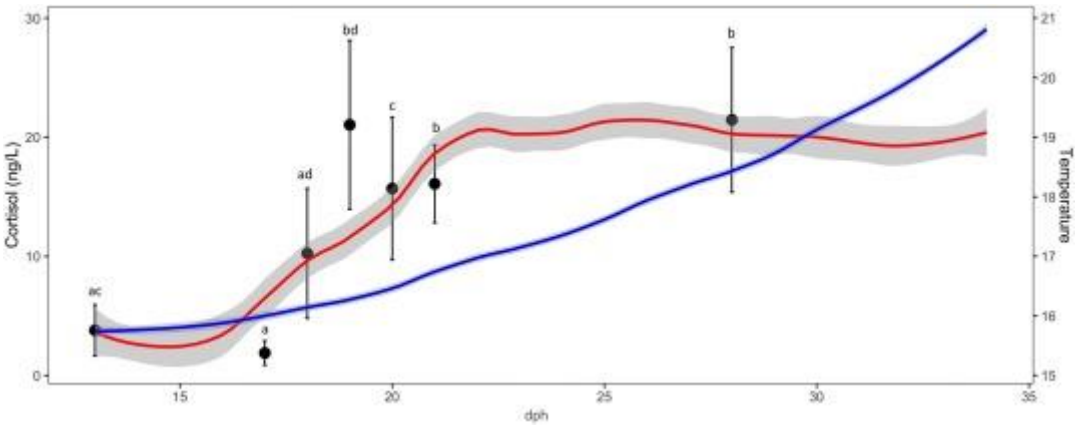

Figure 3. Whole-body gene expression of *nr3c1*, *nr3c2*, *cyp11b* and *hsd11b2* from larvae kept at 21 °C (HT, n=35) and 16 °C (LT, n=25) sampled at 242 $DD_{10\,°C}$. Note that these are values relative to the geometric mean of the following housekeeping genes: ribosomal protein L13 (*L13*), eukaryotic translation elongation factor 1 alpha (*eef1a*) and beta-actin (*β-actin*). Box plots show the mean represented by yellow diamonds, the median is represented by lines, and the first and third quartiles as well as outliers are represented by full circles outside the box. "**" indicates a p-value < 0.01 and "***" indicates a p-value < 0.001.

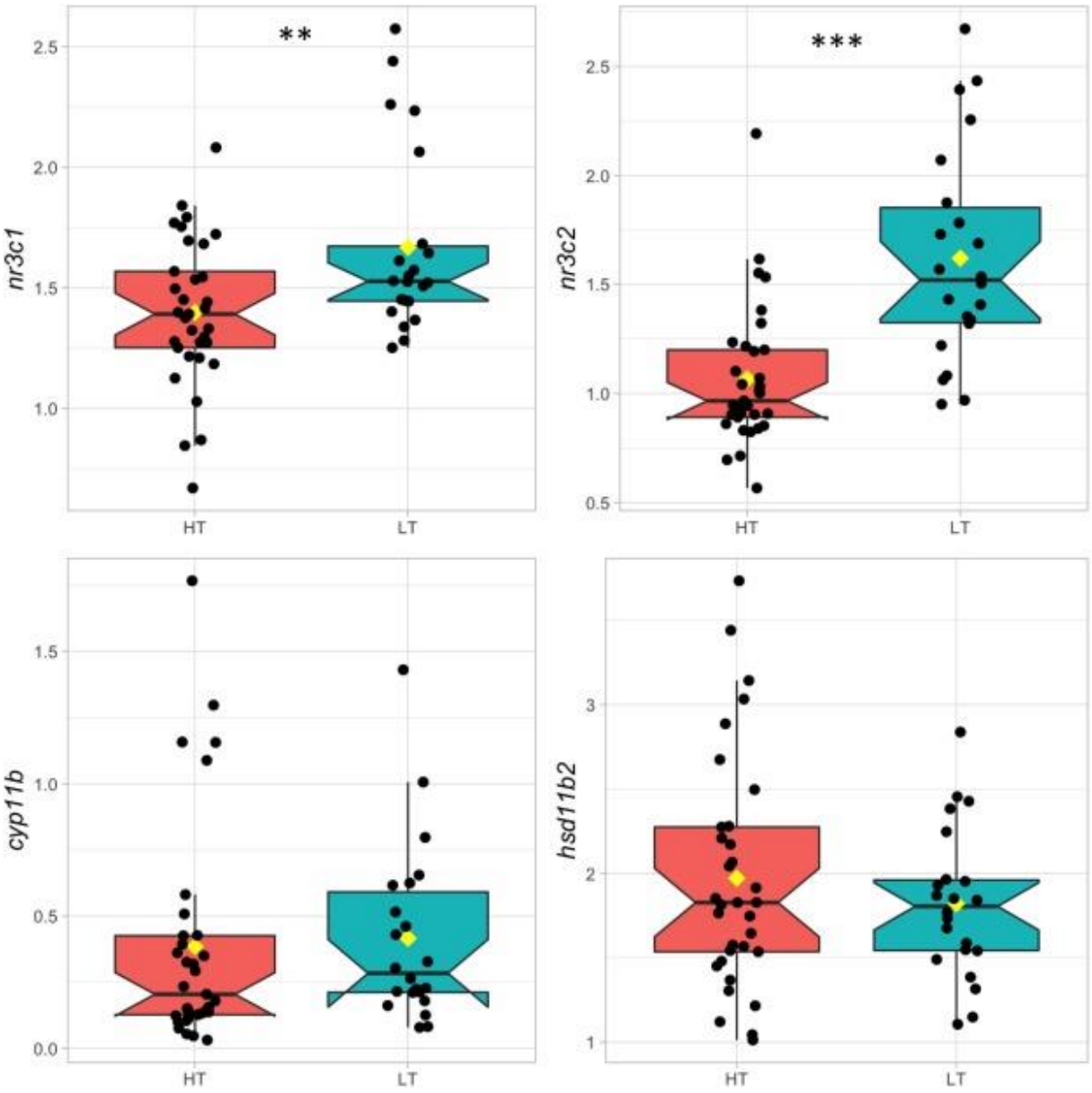

Figure 4. A) Heatmap representing normalized number of reads from the RNA sequencing of whole larvae at 550 $DD_{10\,°C}$ in which represented genes are only those from the gene ontology pathway in mouse (*Mus musculus*): 'GO 085121 glucocorticoid metabolic process', from which we selected those recently highlighted to play a role in cortisol synthesis (Gorissen and Flik, 2016). Only those genes with a significant difference between treatments (HT individuals in red vs LT individuals in green) are represented. Each column represents an individual (total number = 68). The horizontal cluster dendrogram allowed to split those genes highly expressed at HT compared to those highly expressed at LT. Specific colors represent different expression levels. Red, high expression; blue, low expression. B) Relative number of reads from some key genes: *nr3c1*, *nr3c2* and *hsd11b2*. Box plots show the mean represented by yellow diamonds, the median is represented by lines, and the first and third quartiles as well as outliers are represented by full circles outside the box. "***" indicates a p-value < 0.001.

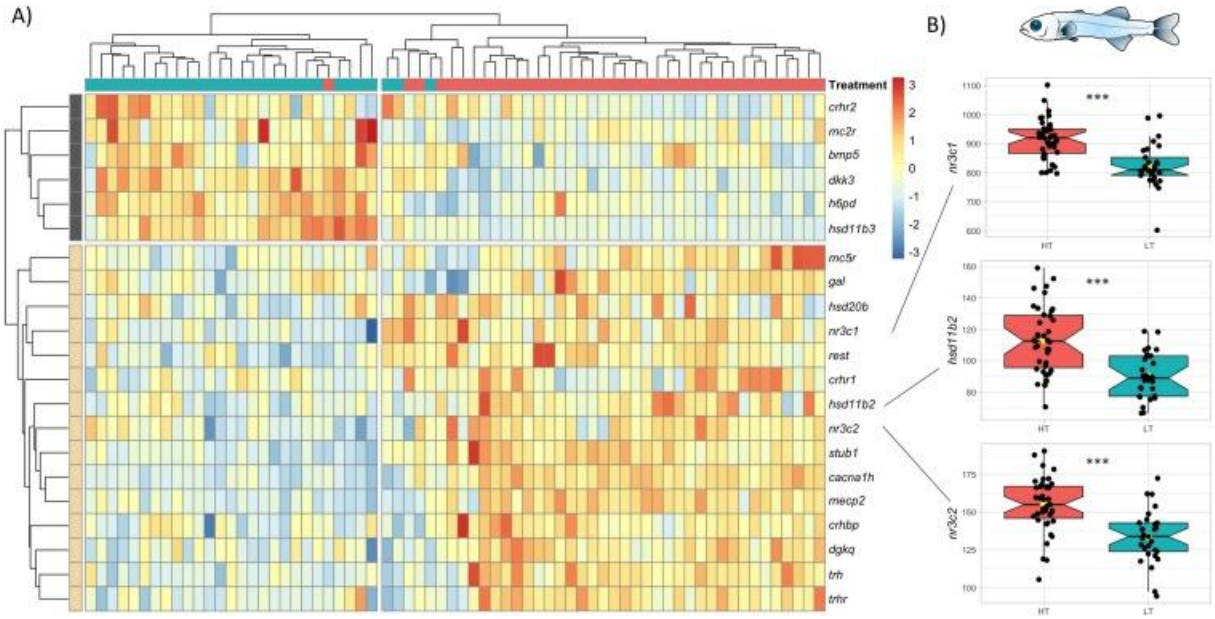

Figure 5. A) Heatmap representing normalized number of reads from the RNA sequencing of whole larvae at 550 $DD_{10\,°C}$ in which represented genes are only those from the gene ontology pathways in mouse (*Mus musculus*): ''GO 101540 cellular response to heat'' and B) those from the 'GO 101649 cellular response to cold'. Only those genes with a significant difference between treatments (HT individuals in red vs LT individuals in green) are represented. Each column represents an individual (total number = 68). The horizontal cluster dendrogram allowed to split those genes highly expressed at HT compared to those highly expressed at LT. Specific colors represent different expression levels. Red, high expression; blue, low expression.

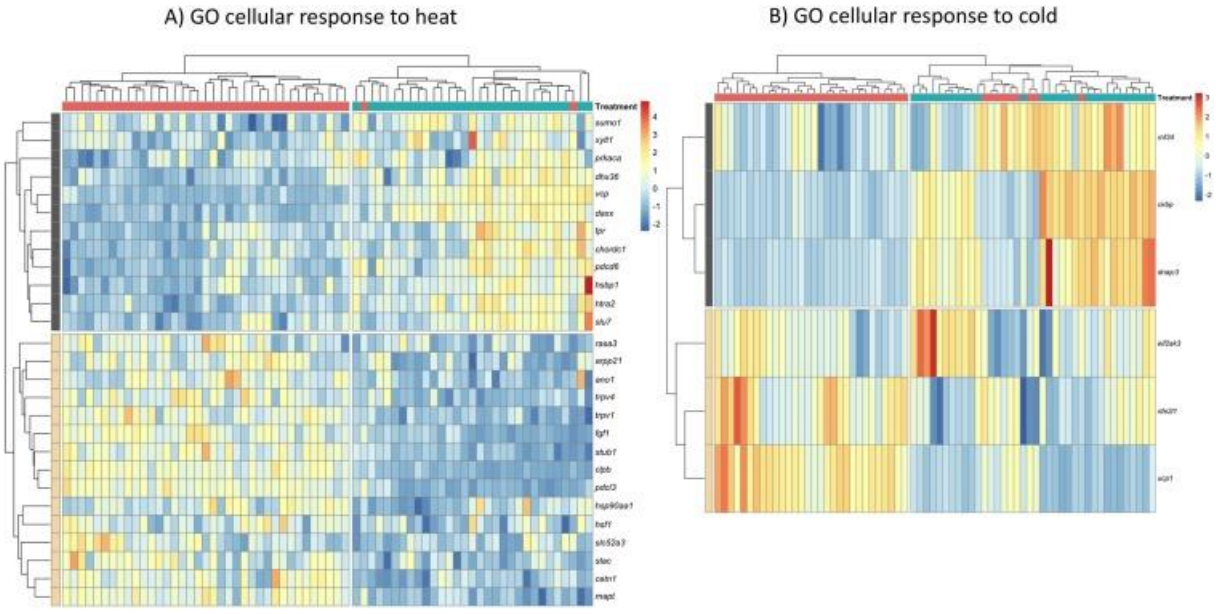

Figure 6. Cortisol content (µg/g) in ontogenetic scales of fishes of the same size and weight sampled at 117 (HT, n = 35) and 124 dph (LT, n = 35). Note that a log scale has been applied for graphical purposes. Box plots show the mean represented by yellow diamonds, the median is represented by lines, and the first and third quartiles as well as outliers are represented by full circles outside the box. "***" indicates a p-value < 0.001.

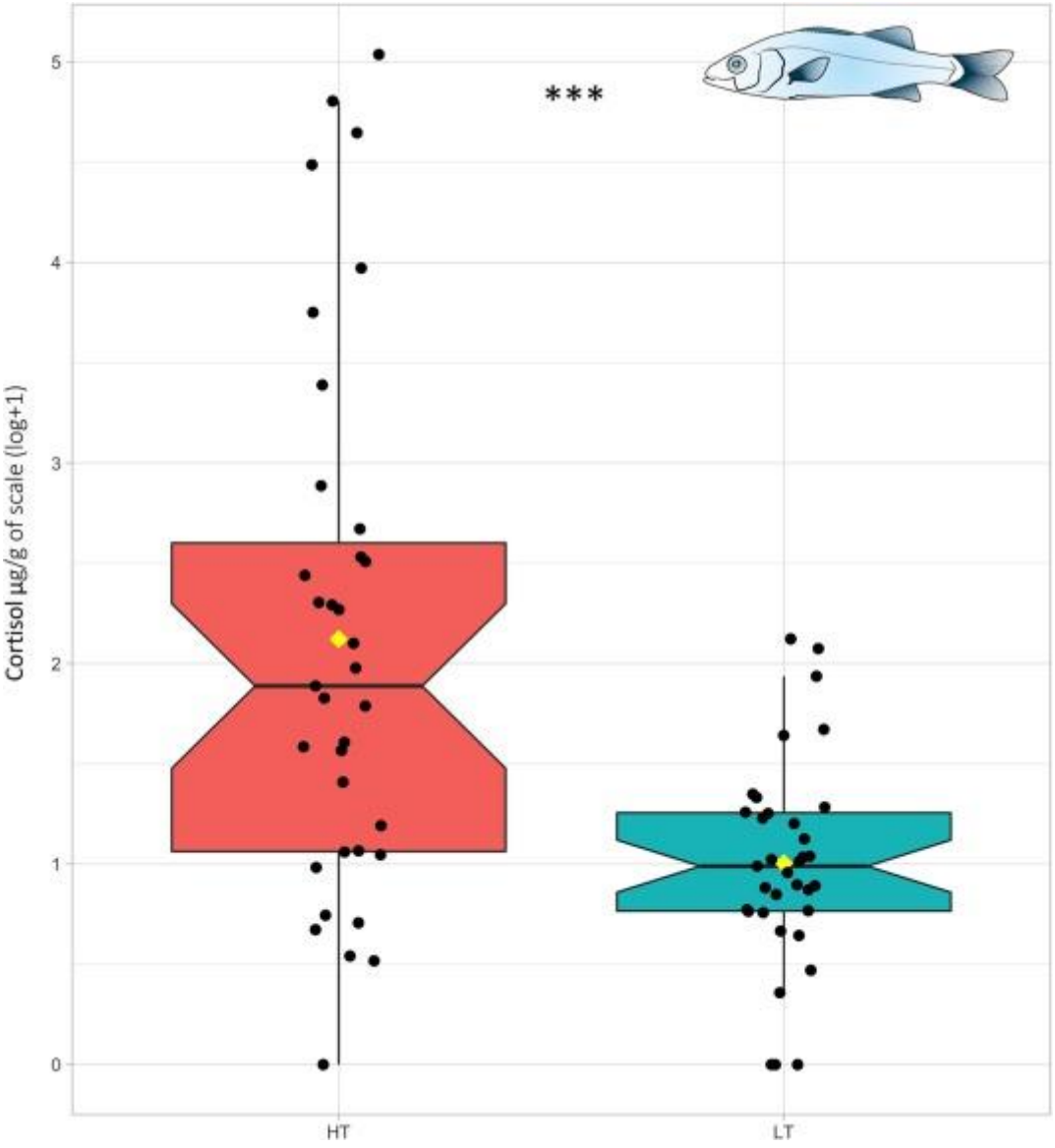

Figure S1: Temperatures in the Thau Lagoon (western Mediterranean) over the year (A) 2019 and (B) 2020. Overall, positive temperature anomalies (red lines) are detected in the two years when compared to the mean water temperature of the pas 15 years (gray line). Note that the 8th of May 2020, the mean water temperature was 21.9°C compared to 16°C for the mean water temperature over the last 15 years at the same date. Data are available in open access through the Ifremer Velyger network https://wwz.ifremer.fr/velyger/Acces-aux-Donnees/Lagune-de-Thau/Temperature.

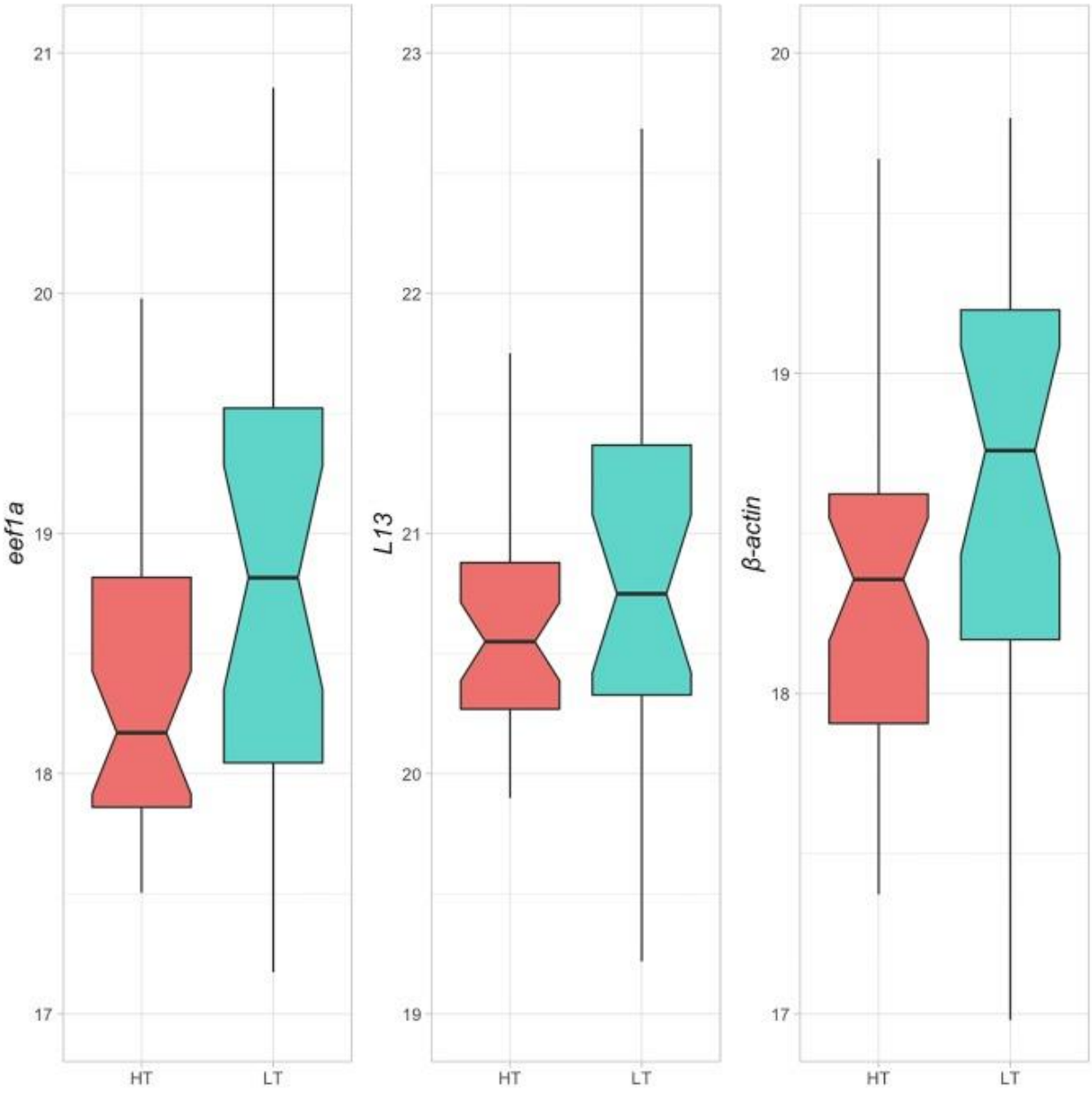

Figure S2. Housekeeping genes: eukaryotic translation elongation factor 1 alpha (*eef1a*), ribosomal protein L13 (*L13*), and beta-actin (*β-actin*) for the groups sampled at the two temperatures. Box plots show the median that is represented by lines, and the first and third quartiles.

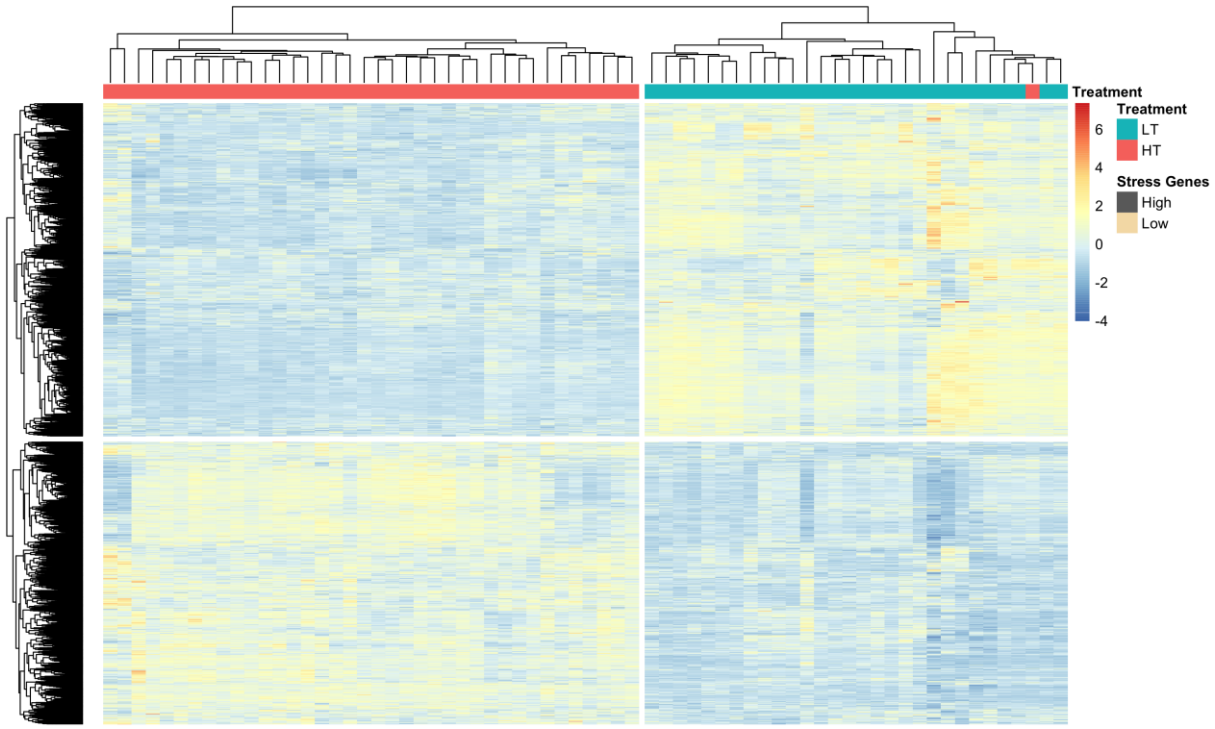

Figure S3. Heatmap representing normalized number of reads from the RNA sequencing of whole larvae at 550 $DD_{10\ °C}$ and in which represented genes are only those from the gene ontology pathway in mouse (*Mus musculus*): 'GO 0006950 response to stress'. Only those genes with a significant difference between treatments (HT individuals in red vs LT individuals in green) are represented, adding up to a total of 2285 genes. Each column represents an individual (total number = 68). The horizontal cluster dendrogram allowed to split those genes highly expressed at HT compared to those highly expressed at LT. Specific colors represent different expression levels. Red, high expression; blue, low expression.