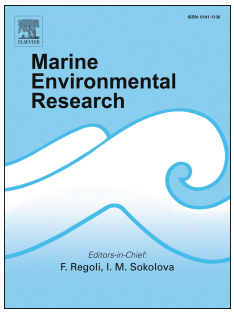

Different sensitivity to heatwaves across the life cycle of fish reflects phenotypic adaptation to environmental niche

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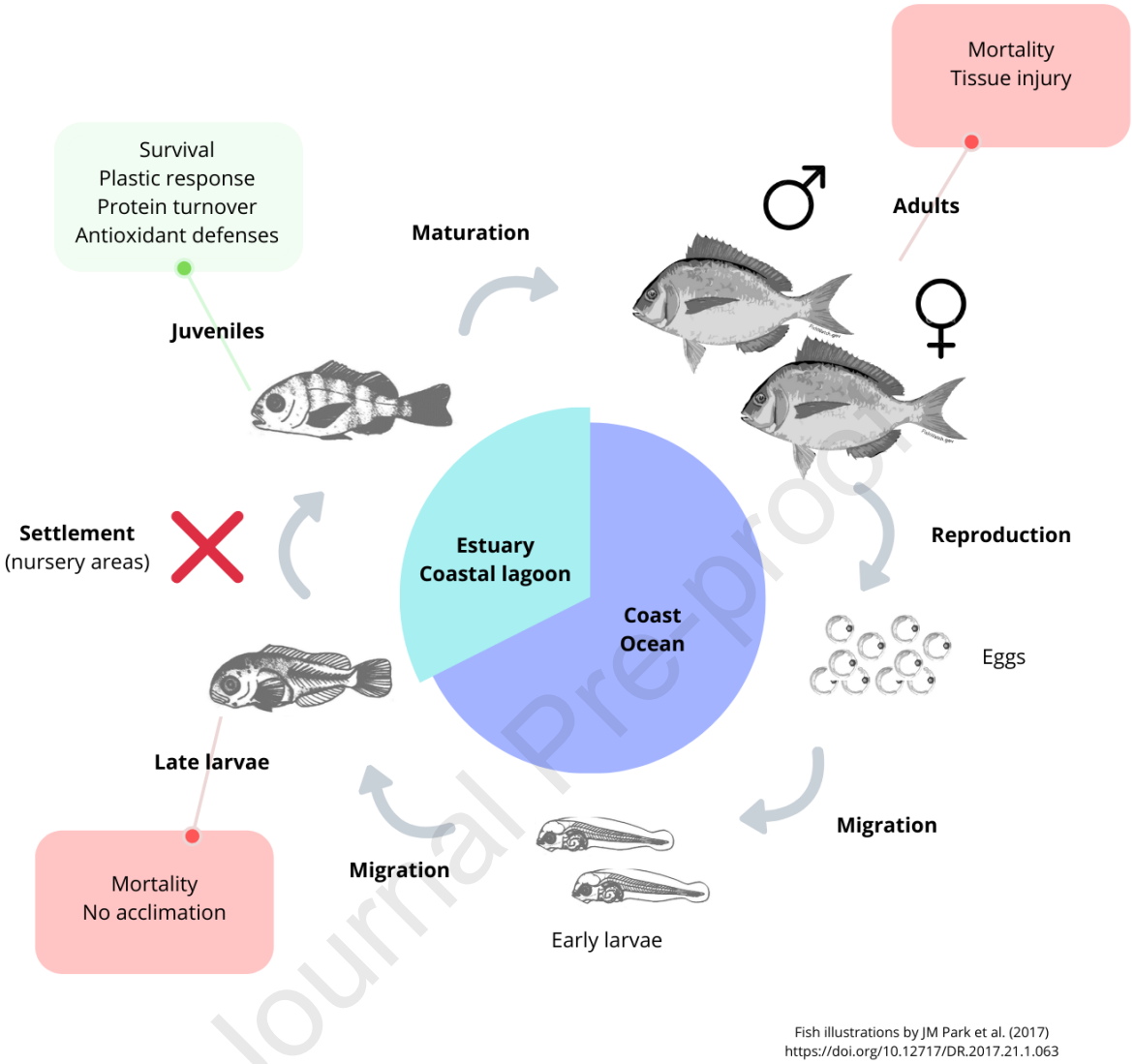
Author statement

D. Madeira: investigation, formal analysis, data curation, writing – original draft, visualization; **C. Madeira**: investigation; **P.M. Costa**: methodology, investigation, formal analysis, visualization, supervision, resources, writing – review and editing; **C. Vinagre**: conceptualization, methodology, writing – review and editing, supervision; **H.O. Pörtner**: writing – review and editing; **M. S. Diniz**: conceptualization, methodology, validation, resources, writing – review and editing, supervision, project administration, funding acquisition.



Running head: Vulnerability of fish to heatwaves

Different sensitivity to heatwaves across the life cycle of fish reflects phenotypic adaptation to environmental niche

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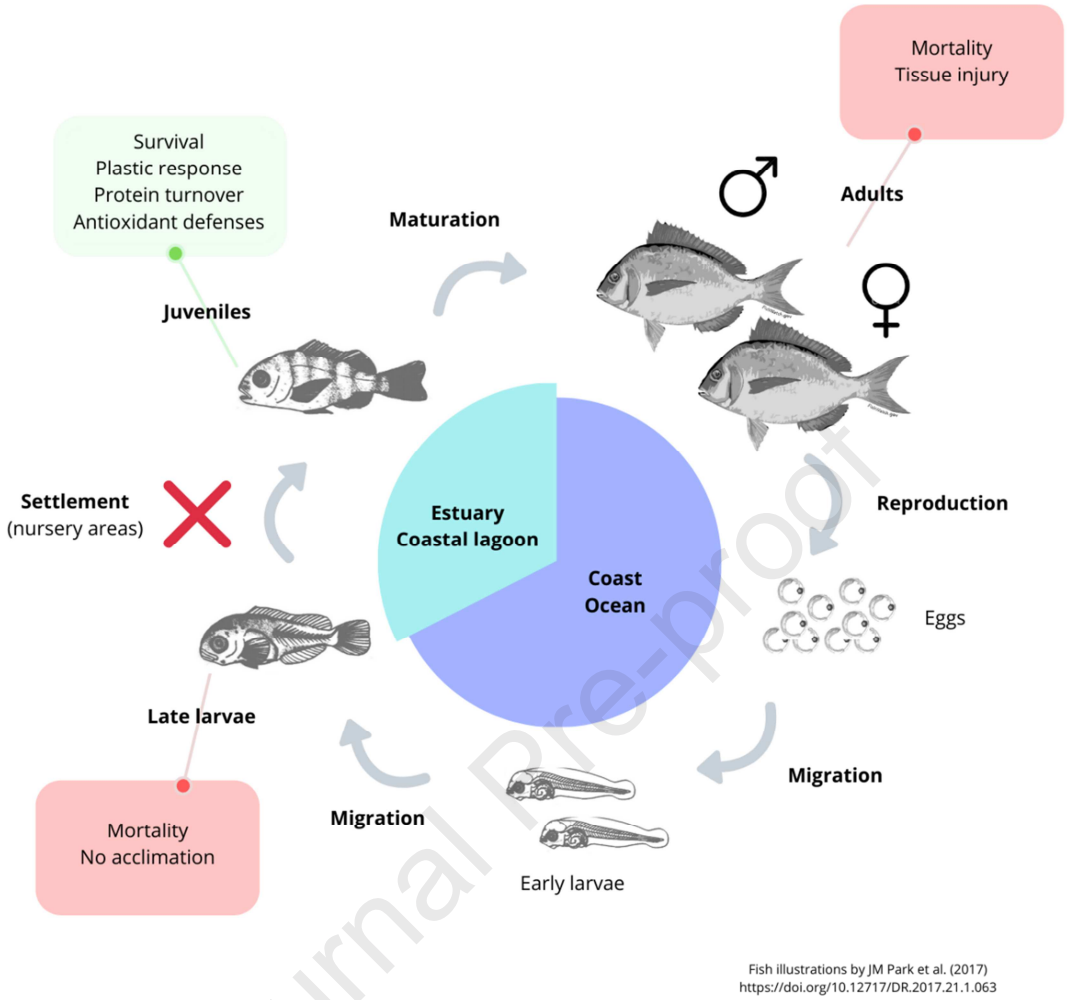
6861 words (main text)

Graphical abstract



Abstract (193 words)

Predicting responses of marine organisms to global change requires eco-physiological assessments across the complex life cycles of species. Here, we experimentally tested the vulnerability of a demersal temperate fish (*Sparus aurata*) to long-lasting heatwaves, on larval, juvenile and adult life-stages. Fish were exposed to simulated coastal (18 ºC), estuarine (24 ºC) summer temperatures, and heatwave conditions (30 ºC) and their physiological responses were assessed based on cellular stress response biomarkers and phenotypic measures (histopathology, condition and mortality). Life-stage vulnerability can be ranked as larvae > adults > juveniles, based on mortality, tissue pathology and the capacity to employ cellular stress responses, reflecting the different environmental niches of each life stage. While larvae lacked acclimation capacity, which resulted in damage to tissues and elevated mortality, juveniles coped well with elevated temperature. The rapid induction of cytoprotective proteins maintained the integrity of vital organs in juveniles, suggesting adaptive phenotypic plasticity in coastal and estuarine waters. Adults displayed lower plasticity to heatwaves as they transition to deeper habitats for maturation, showing tissue damage in brain, liver and muscle. Life cycle closure of sea breams in coastal habitats will therefore be determined by larval and adult stages.

Key words: temperature, phenotypic plasticity, life cycle stages, biomarkers, global change

Introduction 951 words

Average global temperature is predicted to increase by 2-6ºC in the next century, owing to anthropogenic climate change (Hansen et al., 2010; IPCC, 2013, 2007). In addition to changes in mean temperatures, extreme events like marine heat waves are predicted to expand in frequency and amplitude as well as spatially, acting as strong selection pressures with severe and possibly irreversible ecological impacts (Grant et al., 2017; Smale et al., 2019; Stillman, 2019). In literature reviews, fish have been pointed out as one of the main taxonomic groups with a high risk of impact under business-as-usual CO_2 emission scenarios (Gattuso et al., 2015; Nagelkerken and Connell, 2015). Distributional shifts, abundance changes, altered migration patterns, lower recruitment success, and changes in trophic cascades have already been reported (Nagelkerken and Connell, 2015; Rijnsdorp et al., 2009; Sims et al., 2004; Ullah et al., 2018; Vinagre et al., 2019; Walther et al., 2002). Ultimately, climate warming may lead to a convergence of traits that enable adaptation of fish communities to novel environments. Smaller and fast growing species with a preference for higher temperatures and pelagic water column position should be favored in this new scenario (McLean et al., 2019).

Temperature drives physiological processes in ectotherms (Brett, 1971), affecting metabolic rates, immune responses, growth, reproduction, foraging and performance (Ettinger-Epstein et al., 2007; Motani and Wainwright, 2015; Pittman et al., 2013; Pörtner and Farrell, 2008). In order to cope with environmental change and maintain homeostasis, organisms can modify their gene expression patterns and physiological functions (Hofmann and Todgham, 2010; Logan and Somero, 2011) by up-regulating the minimal stress proteome (Kültz, 2005; Madeira et al., 2017). The main proteins involved in the cellular stress response (CSR) to mitigate cellular damage include i) heat shock proteins, which are chaperones with an adaptive value, repairing denatured

proteins upon thermal stress and maintaining the integrity of the protein pool (Feder and Hofmann, 1999; Hofmann and Todgham, 2010; D. Madeira et al., 2012; Narum and Campbell, 2015; Sørensen et al., 2003), ii) ubiquitin, which targets irreversibly damaged proteins for proteasome degradation preventing cytotoxic aggregations (Hofmann and Somero, 1995; Logan and Somero, 2011; Madeira et al., 2014; Tang et al., 2014), iii) antioxidant enzymes which neutralize ROS (reactive oxygen species) and oxidation products (e.g. lipid peroxides) that arise due to higher metabolic rates at higher temperatures (Bagnyukova et al., 2007; Heise et al., 2006; Lushchak and Bagnyukova, 2006; Vinagre et al., 2012).

Despite the vast literature on thermal eco-physiology of fish, most studies have not considered their complex life cycles, hampering accurate predictions of climate change impacts on fish populations. Successive life stages have different requirements (habitat, food, physiology, size, form, behavior, thermal niche) and therefore climate change is expected to differently affect eco-physiological traits of organisms throughout their life cycles, impacting mostly survival and dispersal processes in larvae and fitness in adults (Kingsolver et al., 2011; Petitgas et al., 2013; Rijnsdorp et al., 2009; Webster et al., 2013). Constraints in oxygen supply capacity related to body size and the development of tissue functional capacity during ontogeny have been hypothesized to cause differences in thermal ranges across the life cycle of fish and thus thermal stress phenomena at systemic and cellular levels (Dahlke et al., 2020; Pörtner et al., 2017; Pörtner and Farrell, 2008). Accordingly, thermal strategies are known to vary across life cycle stages (Truebano et al., 2018) and an ontogenetic shift in temperature tolerance is expected (Pörtner and Farrell, 2008; Rijnsdorp et al., 2009). In general, thermal window widths are narrower for eggs and larvae while increasing in juveniles and becoming constrained again at a large body size (adult stage) (Pörtner and Farrell, 2008; Truebano

et al., 2018). Consequently, determining the life history stage(s) most critical for life cycle closure under ocean warming and heat wave scenarios is essential for understanding the consequences and selection pressures imposed by global change upon organisms. Such studies are especially relevant in commercial species considering that fishing reduces genetic variability and alters the structure and thus reproductive capacity of the population (Anderson et al., 2008; Caddy and Agnew, 2003; Ottersen et al., 2006), leading to increased sensitivity of fish stocks to adverse climate conditions (Ottersen et al., 2006; Planque and Fredou, 1999). The scientific community is thus faced with the challenge of projecting the effect of climate forcing on exploited fish species and subsequently design adequate management guidelines, following a climate-smart conservation strategy (Bozinovic and Pörtner, 2015; Kingsolver et al., 2011; Petitgas et al., 2013; Radchuk et al., 2013; Stein et al., 2014).

We hypothesize that plasticity in traits related to thermal physiology is modulated non-linearly in fish species with complex life cycles, in which life stages occupy different environmental niches. Specifically, we aim at disclosing which life stages are most vulnerable to extreme heatwaves by being less metabolically competent to swiftly deploy mechanisms of cellular defense, resulting in deleterious consequences at the whole-organism level and leading to pathophysiological anomalies and increased mortality.

To test our hypothesis, we integrate sub-cellular to whole-organism endpoints to compare the vulnerability and acclimation capacity of fish life cycle stages toward long lasting heat waves using a proxy for common demersal predatory fish, the commercial sea bream *Sparus aurata*. We chose life-stages that transition from open ocean to coastal and estuarine environments (at larval stage) and that exclusively inhabit shallow coastal waters, lagoons and estuarine environments (juveniles), subsequently moving to

coastal and open ocean waters again (growing adults). These life stages have a high probability of exposure to extreme temperatures, not only due to the increase in intensity, duration and frequency of heat waves but also due to the small thermal inertia of shallow habitats. Hence, embryos and spawners were excluded as they only occur in colder open sea waters.

2. Material and Methods (1838 words)

2.1 Ethical statement

This study was approved by *Direcção Geral de Alimentação e Veterinária* and followed EU legislation for animal experimentation (Directive 2010/63/EU).

2.2 Assessment of Sparus aurata*'s thermal environments*

Temperature data were obtained for both coastal and estuarine waters using several tools: 1) studies in Portuguese coastal waters and estuaries (Minho, Douro, Ria de Aveiro, Mondego, Tejo, Sado, Mira, Ria Formosa, Guadiana; data between 1978-2005, not continuous - Azevedo et al., 2006; Cabral et al., 2007; Costa, 1990; Coutinho, 2003; Madeira et al., 2012); 2) sea temperature database (satellite data available from http://seatemperature.info/portugal-water-temperature.html) which has monthly sea surface temperatures for the main coastal cities of Portugal (2011 to 2015) and 3) the Marine and Environmental Sciences Centre database (data from several estuaries including Tagus' temperatures measured by YSI loggers from 1978 to 2006.

2.3 Housing and husbandry of fish

Sparus aurata (life cycle stages determined by body size and age: 35d post-hatch larvae, n=180, 1.0 to 1.5 cm total length (TL); juveniles, n=75, mean±sd TL of

8.93±1.16 cm and 12.76±4.60 g weight, 5-6 months old; and adults, n=60, mean±sd TL of 13.15±1.40 cm and 45.78±12.25 g weight, 11-12 months old) were obtained from a fish farm (MARESA, Mariscos de Estero .S.A., Ayamonte, Huelva, Spain). All animals were produced from a brood stock of 50 males and 25 females. The first parental fish of the hatchery (collected in the late '90s) were wild fish caught in the nearby coastal lagoon mixed with adults obtained from an aquaculture in Almería region (Spain). The current breeding stock has about 400-600 animals. The breeding scheme consists of replacing the males that turn into females by new males (annual replacement; usually the 200-300 largest of one generation are chosen). Accordingly, the largest 200-300 females are removed. Larvae are reared under tightly controlled conditions in indoor tanks (20ºC, high water quality) until they reach 0.1 g (approximately at 60 days post-hatch). Afterwards, they are placed in other less controlled indoor tanks but keep being reared at 20ºC. When they reach 1g (approximately 90 days post-hatch), they are moved into land-based outdoor ponds (with water from the nearby coastal lagoon) and subjected to a natural temperature regime (temperate climate with seasonal variation: colder during winter and warmer during summer). According to data obtained from the Spanish Agencia Estatal de Meteorologia (from 1984 to 2010), mean air temperatures in the area range from 11 ºC in January to 26 ºC in July/August. Maximum air temperatures can reach 33 ºC and minimum air temperatures can reach approximately 6ºC.

Fish were transported to the laboratory and randomly placed in a re-circulating system (total of 2,000 L, Fig. S1a) as follows:

i) Larvae were placed in six transparent polyvinyl containers ($17.5 \times 17.5 \times 15$ cm, approximately 4.5 L; n=30 larvae.tank^{-1}), each positioned within a 70 L-tank with water gently flowing through small punctures;

ii) Juveniles and adults were placed in 70 L white plastic tanks (35 × 35 × 55 cm) (juveniles, n=13 individuals.tank^{-1}, 6 tanks; and adults, n=10 individuals.tank^{-1}, 6 tanks).

All tanks were filled with clean aerated sea water (95-100 % air saturation), with a stable temperature of 18±0.5°C, salinity 35‰ and pH 8±0.1 and summer photoperiod 15h light: 09h dark. Inflow of clean sea water in each individual tank was 300 mL.min^{-1}. All tanks were provided with a filter (ELITE Underwater Mini-Filter Hagen, 220L.h^{-1}). Fish were conditioned (juveniles and adults: one week; larvae: one day) and their health status was assessed (i.e. wounds or disease symptoms). During the conditioning and experimental trial larvae were exposed to periodic feeding (every 6 h) with *Artemia salina* metanauplii and two different grain-sized feeds (0.3-0.6 mm and 0.6-1.0 mm). Juveniles and adults were fed with commercial food pellets once a day (BRM3, Aquasoja, Portugal) mixed with cyanobacterium *Spirulina* sp. (Tropical®, Poland).

2.4 Experimental setup

After the conditioning period, temperature was gradually increased (0.25 ºC.h^{-1}) until the experimental temperatures were reached (control 18±0.5ºC; experimental temperatures 24±0.5ºC and 30±0.5ºC; n=2 tanks for each temperature). Temperatures were maintained for 28 experimental days using thermostats (TetraTec® HT 100, 100-150L, Tetra Werke, GmbH, Melle, Germany). Water quality parameters (temperature, salinity, pH, ammonia, nitrites, nitrates) were monitored every 48 h and kept within optimum range. Fish were euthanized through cervical transection at day 0 (only 18ºC), 7, 14, 21 and 28 (all temperatures), following OECD guidelines for ecotoxicology studies (supplemental material, Fig. S1b). At each time point, 4 to 5 individuals were randomly sampled (2 to 3 from each tank) for biochemical and histopathological

analyses. The entire body of larvae was collected while several organs were collected separately in juveniles and adults i.e. brain (b), gills (g), intestine (i), liver (l) and muscle (m). All analyses were carried out separately for each individual. The total length of individuals (and weight in juveniles and adults) was measured at each sampling point.

2.5 Temperature effects on sea breams

2.5.1 Cellular stress response (CSR)

Sample treatment

Whole-larvae (n=4-5 per treatment) or approximately 150-200 mg of tissue (brain, gills, intestine, liver and muscle) of juveniles (n=5 per treatment) and adults (n=4 per treatment) were homogenized individually in 0.5 and 1 mL (respectively) of cold phosphate buffered saline (pH 7.4) using a Tissue Master 125 homogenizer (Omni International, Kennesaw, USA). Afterwards, homogenates were centrifuged (10 min at 16,000 ×g) and the supernatant fractions were stored at -80ºC until further analysis.

Biochemical analyses

Total protein content was determined through the Bradford method (Bradford, 1976) for data normalization and followed the protocol described by (Madeira et al., 2014). Heat Shock Protein 70 (Hsc70/Hsp70) was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) (Njemini et al., 2005) in 96-well microplates using a primary antibody against Hsp70/Hsc70 (AM03140PU-N, Acris, USA), a secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) and the substrate SIGMA FAST™ p-Nitrophenyl Phosphate Tablets (Sigma-

Aldrich, USA). Total ubiquitin was quantified through a direct ELISA in 96-well microplates using the primary antibody Ub P4D1 (sc-8017, HRP conjugate, Santa Cruz, USA) and the substrate TMB/E (Temecula California, Merck Millipore). Both quantifications followed the protocols described by (Madeira et al., 2014). The enzymatic assay of Catalase (CAT) (EC 1.11.1.6) was adapted from (Johansson and Borg, 1988) and performed as described in (Vinagre et al., 2014) in 96-well microplates. Catalase activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The enzymatic assay of glutathione S-transferase (GST) activity (EC 2.5.1.18), using the substrate CDNB (1-Chloro-2,4-dinitrobenzene), was adapted from (Habig et al., 1974) and performed as described by (Vinagre et al., 2014) in 96-well microplates and using a molar extinction coefficient for CDNB of 0.0053εmM (adapted for microplates). The enzymatic assay of superoxide dismutase (SOD) activity was adapted from (Sun et al., 1988) and performed using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), as described in (Vinagre et al., 2014). The lipid peroxides assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978) to quantify malondialdehyde bis(dimethylacetal) (MDA) following the procedure described by (Vinagre et al., 2014). For more details see supplementary Table S1.

2.5.2 Phenotypic endpoints

Mortality and condition index

Mortality rates were calculated at the end of the experiment in each tank (n=2 tanks per treatment). Additionally, Fulton's K condition index was calculated using the formula:

$$K = 100\ M_t/L_t^3 \qquad (1)$$

Where M_t is the total wet mass (mg) and L_t is the total length (mm) (Ricker, 1975). This index was calculated for fish in each temperature treatment after 21 days of exposure (n=5 juveniles and n=4 adults per temperature treatment). No calculations were made at 28 days nor for larvae due to mortality rates.

Histopathological assessment

Histological sections were obtained from whole-body sections of larvae and tissue sections of juveniles and adults fixed in Bouin's solution for 24 h or 48 h at room temperature and embedded in paraplast. Sections (5 µm thick, cut in a Jung RM2035 rotary microtome) were stained with Haematoxylin and Eosin (H&E) for general histopathological screening, Periodic Acid-Shiff's (PAS) plus Haematoxylin (for glycogen detection and general structural analysis) and a trichrome stain using Weigert's Iron Haematoxylin and van Giesons' dye (Acid Picrofuchsin) in larvae to assist differentiation of multiple structures. More details may be found in (Martins et al., 2015). Histological sections were analyzed with a DMLB model microscope equipped with a DFC480 camera, all from Leica Microsystems (Germany). The histopathological analyses were qualitative and based on the presence/absence of lesions, as well as type and extent of the identified lesions. Such assessment was used as phenotypic anchoring to help assist the interpretation of biomarker results (Paules, 2003).

2.5.3 Statistical analysis

Cellular Stress Response (CSR) biomarkers

Generalized linear models (GLM) and linear discriminant analysis (LDA)

Data were analysed through generalized linear models (GLM) and linear discriminant analysis (LDA) using packages 'glm2' and 'MASS' for R (Ihaka and Gentleman, 1996), respectively. Data were fitted to GLMs using a Gamma distribution with log link. Deviance analysis (based on regressive *F*-tests) was employed to determine the significance of three explanatory variables (life stage, time, and temperature) in the models. A second set of models were run on a subset of the data (only for juveniles and adults) to determine the significance of four explanatory variables (life stage, time, temperature, and organ). All the models were ranked according to Akaike's Information Criterion (AIC). Quality of fit was determined by dispersion of residuals, Cook's *h* statistic and qq-plots of ordered deviance residuals. Analysis follows McCullagh & Nelder (1989). Linear discriminant analysis was conducted for multi-class classification and separability, after Xanthopoulos, Pardalos, & Trafalis (2013). Quality assessment and visualisation was done through the proportion of trace attributed to each Fisher linear discriminant, percentage of correct classifications and two-dimensional plots of discriminants. Histograms were plotted to compare data distribution per group.

Integrated Biomarker Response Index (IBR)

To detect which life stage and tissue was more susceptible or responsive to warming, the Integrated Biomarker Response was calculated according to (Beliaeff and Burgeot, 2002) (see supplementary Table S1 for details). The IBR provides a synthesis of biomarker responses, providing a numeric value that integrates all responses, previously standardized. It is represented as the "sum of the area defined by k biomarkers arranged in a radar diagram" (Devin et al., 2014). A heatmap was constructed in Cluster 3.0 & Java TreeView to visualize IBR data following the parameters (i) adjust data: log

transform, center rows, (ii) hierarchical cluster: cluster rows (correlation uncentered) and cluster columns (Euclidean distance), (iii) clustering method: complete linkage.

Mortality and condition index

Data was tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test) prior to statistical analysis. Due to invalidation of assumptions, mortality data were analysed through non-parametric Kruskal-Wallis analyses to test the effect of temperature and life stage on mortality levels. Fulton's K condition index was analysed via one-way ANOVA in juveniles (18ºC *vs* 24ºC *vs* 30ºC) and the Mann-Whitney U test in adults (18ºC *vs* 24ºC; mortality at 30ºC). All analyses were carried out in Statistica v10 (StatSoft Inc., USA), considering a significance level of 0.05.

3. Results (1208 + 1081 of tables and legends)

3.1 Assessment of Sparus aurata*'s thermal environments*

Monthly mean sea surface temperature (SST) along the Portuguese coast registered values from 12 (at the Northern coast, Viana do Castelo) to 16ºC (at the Southern coast of Algarve) during the winter months (December to March). Monthly mean±sd (averaging all locations) was 15.6±0.2 ºC in December, 14.8±0.3 ºC in January, 13.9±0.6 ºC in February, and 14.2±0.4 ºC in March (satellite dataset from 2011 to 2015). During summer months (June to September) monthly mean SST ranged from 15 to 23ºC, according to the location as well. The monthly mean±sd averaging all locations was 17.9±0.8 ºC in June, 18.7±0.8 ºC in July, 18.9±0.5 ºC in August and 19.3±0.7 ºC in September. Seasonal variation considering monthly mean coastal SST is around 4 ºC,

335 with the lowest and highest temperatures occurring during February and September,
336 respectively. According to regional projections based on the HadRM model (emission
337 scenario IS92a), Portuguese water temperatures will rise by 3-4ºC until 2100 (Miranda
338 et al., 2002). Considering a +3ºC increase, monthly mean SST will be in the range of 15
339 to 19ºC and 18 to 26ºC during the winter and summer, respectively (Fig. 1a). Present
340 estuarine temperatures range approximately from 10-11 to 14ºC during the winter and
341 20 to 24ºC during summer (monthly SST). During heat waves, estuaries can reach
342 maximum temperatures between 25 and 28ºC, persisting for over 2 weeks. Following
343 the scenario of a +3ºC increase in Portuguese waters by 2100, estuaries' mean
344 temperature during summer would be in the range of 23 to 27ºC, reaching over 30ºC
345 during heat waves (Fig. 1b).
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347

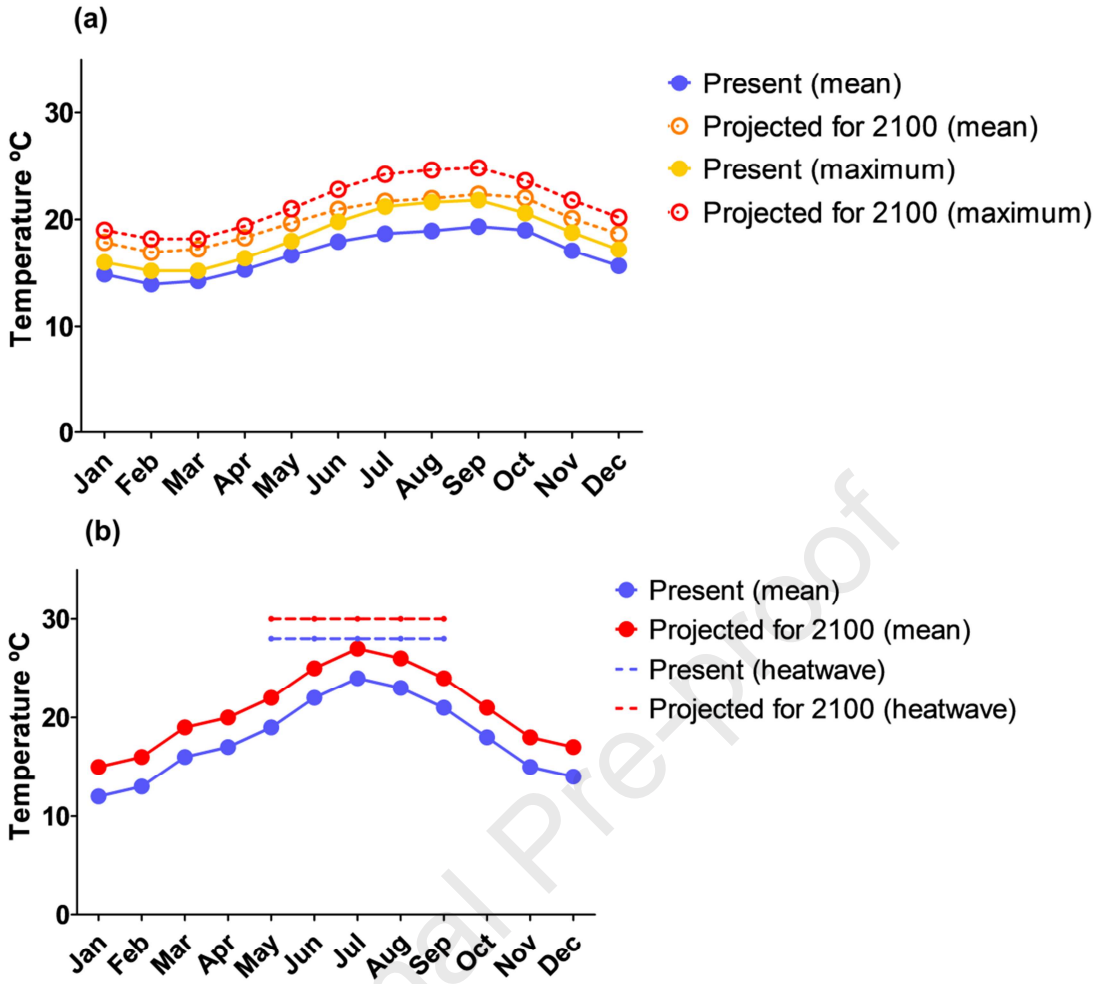


Fig. 1 Present and projected temperatures for 2100 (+3ºC) in **(a)** Portuguese coastal waters (monthly average sea surface temperature for the main coastal cities from 2011-2015) and **(b)** estuaries (based on data from the Tagus estuary considering monthly average temperatures collected from 1978 to 2006).

3.2 Temperature effects on S. aurata *throughout its life cycle*

3.2.1 Cellular stress response (CSR)

Life stage consistently affected CSR biomarkers ($p<0.05$ in all GLM models, except for GST) (Table 1). The explanatory variable temperature was also consistently significant in the models (GST, SOD, LPO, Hsp70, TUB, $p<0.0001$), except for CAT model

(p=0.2). The effect of time was less evident as this explanatory variable was only significant for LPO (p=0.001) and TUB (p<0.0001). Based on AIC, the top three models were those for TUB (AIC=-1907.9), LPO (AIC= -1295.9) and Hsp70 (1018.1) (Table 1). In both TUB and LPO, the three explanatory variables were significant, while in Hsp70, only life stage and temperature were significant (Table 1). A second set of models was run on juvenile and adult data to include organ as a fourth explanatory variable, highlighting that organ has a significant effect on all CSR biomarkers (Table 2). Based on AIC, the top three models in this case were still TUB (AIC=-1016.6), LPO (AIC=-719.91) and Hsp70 (AIC=435.93) (Table 2). Model diagnostic plots can be consulted in Figs. S2 and S3.

Linear discriminant analysis (LDA) showed a clear separation between life stages, especially larvae from juveniles and adults, with an overall percentage of correct classifications of 87% (correct classifications per group were 97% for adults, 79 % for juveniles and 63 % for larvae) (Fig. 2a). Overall, larvae showed greater inter-individual variation but higher levels of CSR biomarkers, followed by juveniles and the lowest levels were recorded in adults (Fig. 2a, b). Separation of groups by temperature was observed, especially differentiating 30 ºC from 18 and 24 ºC. Group separation was more evident when only juveniles and adults were analyzed, with an overall percentage of correct classifications of 70 % (correct classifications per group were 76% for 18 ºC, 68 % for 24 ºC and 60% for 30 ºC) (Fig. 2c, d). A similar pattern was observed for factor time, in which group separation was only evident after excluding larval data, for which several time-points were missing due to mortality (Fig. 2e, f). The model showed an overall percentage of correct classifications of 78% (correct classifications per group were 40 % for T0, 64 % for T7, 85 % for T14, 85% for T21 and 100% for T28).

Separation between organs was also observed, especially liver, muscle, gills and brain

(Fig. 2g, h). Intestine highly overlaps with other organs. In summary, fish generally showed an induction of CSR biomarkers, especially at 30ºC, and more noticeable at 7 and 14 days of exposure. A heatmap of individual biomarker data can be consulted in Fig S4. Raw biomarker data have been deposited to the Knowledge Network for Biocomplexity Repository with the dataset identifier urn:uuid:575bfeee-8df2-4058-9712-8d95c729722b (D. Madeira et al., 2020).

Table 1. Generalized linear models produced to address the effect of three explanatory variables (life stage: larvae, juveniles, adults; time: 0, 7, 14, 21 and 28 days; and temperature: 18, 24 and 30 ºC) on cellular stress response biomarkers in the gilt-head seabream *Sparus aurata* (CAT – catalase, GST – glutathione-S-transferase, SOD – superoxide dismutase, LPO – lipid peroxidation, Hsp70 – heat shock protein 70kDa, TUB – total ubiquitin). Model fit was evaluated by Akaike's Information Criterion (AIC). Significant results are highlighted by asterisks (*** p<0.0001, ** p<0.001, * p<0.01). Models are designated by dependent variable (biomarker endpoint).

Model	***Explanatory variable***	***F***	***p-value***	***Model AIC***
CAT	*Life stage*	*53.50*	*<2e-16****	*2695.5*
	Time	*0.39*	*0.532*	
	Temperature	*1.57*	*0.210*	
GST	*Life stage*	*1.58*	*0.207*	*4679.4*
	Time	*1.22*	*0.270*	
	Temperature	*12.75*	*0.0004*	
SOD	*Life stage*	*46.31*	*<2.2e-16****	*3285.2*
	Time	*0.58*	*0.464*	
	Temperature	*43.74*	*1.063e-10****	
LPO	*Life stage*	*10.06*	*5.315e-05****	*-1295.9*
	Time	*10.88*	*0.001***	
	Temperature	*19.60*	*1.195e-05****	

Hsp70	*Life stage*	*153.68*	*<2.2e-16****	*1018.1*
	Time	*0.03*	*0.873*	
	Temperature	*39.95*	*6.176e-10****	
TUB	*Life stage*	*179.91*	*<2.2e-16****	*-1907.9*
	Time	*25.87*	*5.347e-07****	
	Temperature	*11.17*	*0.0009****	

Table 2. Generalized linear models produced for a subset of the data (juveniles and adults) to address the effect of four explanatory variables (life stage: juveniles, adults; time: 0, 7, 14, 21 and 28 days; temperature: 18, 24 and 30 ºC; and organ: muscle, brain, gills, liver, intestine) on cellular stress response biomarkers in the gilt-head seabream *Sparus aurata* (CAT – catalase, GST – glutathione-S-transferase, SOD – superoxide dismutase, LPO – lipid peroxidation, Hsp70 – heat shock protein 70kDa, TUB – total ubiquitin). Model fit was evaluated by Akaike's Information Criterion (AIC). Significant results are highlighted by asterisks (*** p<0.0001, ** p<0.001, * p<0.01). Models are designated by dependent variable (cellular stress response biomarker).

Model	***Explanatory variable***	***F***	***p-value***	***Model AIC***
CAT	*Life stage*	*32.83*	*3.384e-08****	*1142.7*
	Time	*0.49*	*0.482*	
	Temperature	*1.76*	*0.186*	
	Organ	*45.62*	*2.2e-16****	
GST	*Life stage*	*0.39*	*0.532*	*2106.6*
	Time	*3.19*	*0.075*	
	Temperature	*13.55*	*0.0003****	
	Organ	*46.03*	*2.2e-16****	
SOD	*Life stage*	*19.51*	*1.586e-054****	*1523.3*
	Time	*0.35*	*0.552*	

	Temperature	*29.92*	*1.250e-07****	
	Organ	*15.47*	*3.938e-11****	
LPO	*Life stage*	*0.67*	*0.413*	*-719.91*
	Time	*1.45*	*0.230*	
	Temperature	*8.28*	*0.004***	
	Organ	*9.65*	*3.313e-07****	
Hsp70	*Life stage*	*187.69*	*2.2e-16****	*435.93*
	Time	*0.01*	*0.916*	
	Temperature	*29.07*	*1.806e-07****	
	Organ	*7.55*	*1.023e-05****	
TUB	*Life stage*	*227.30*	*2.2e-16****	*-1016.6*
	Time	*15.43*	*0.0001****	
	Temperature	*17.10*	*5.069e-05****	
	Organ	*7.19*	*1.873e-05****	

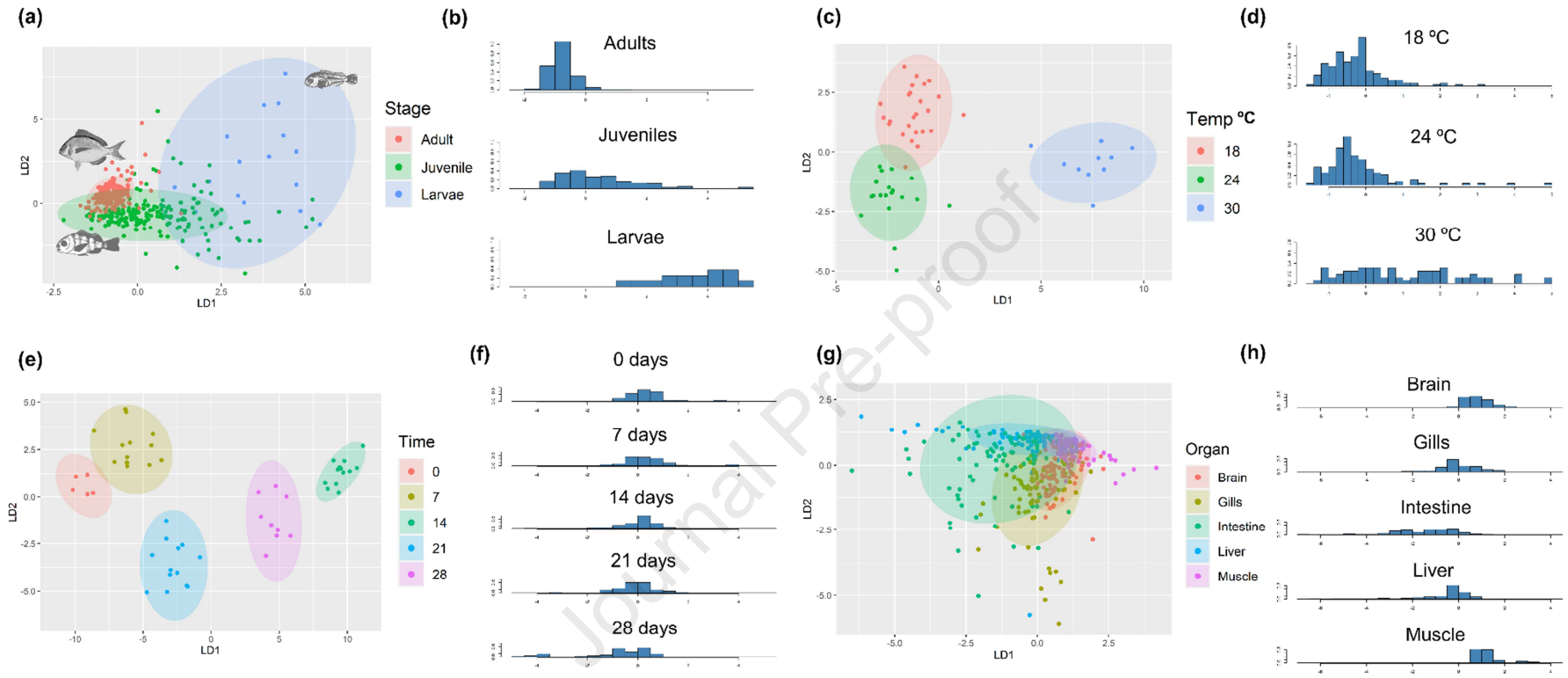


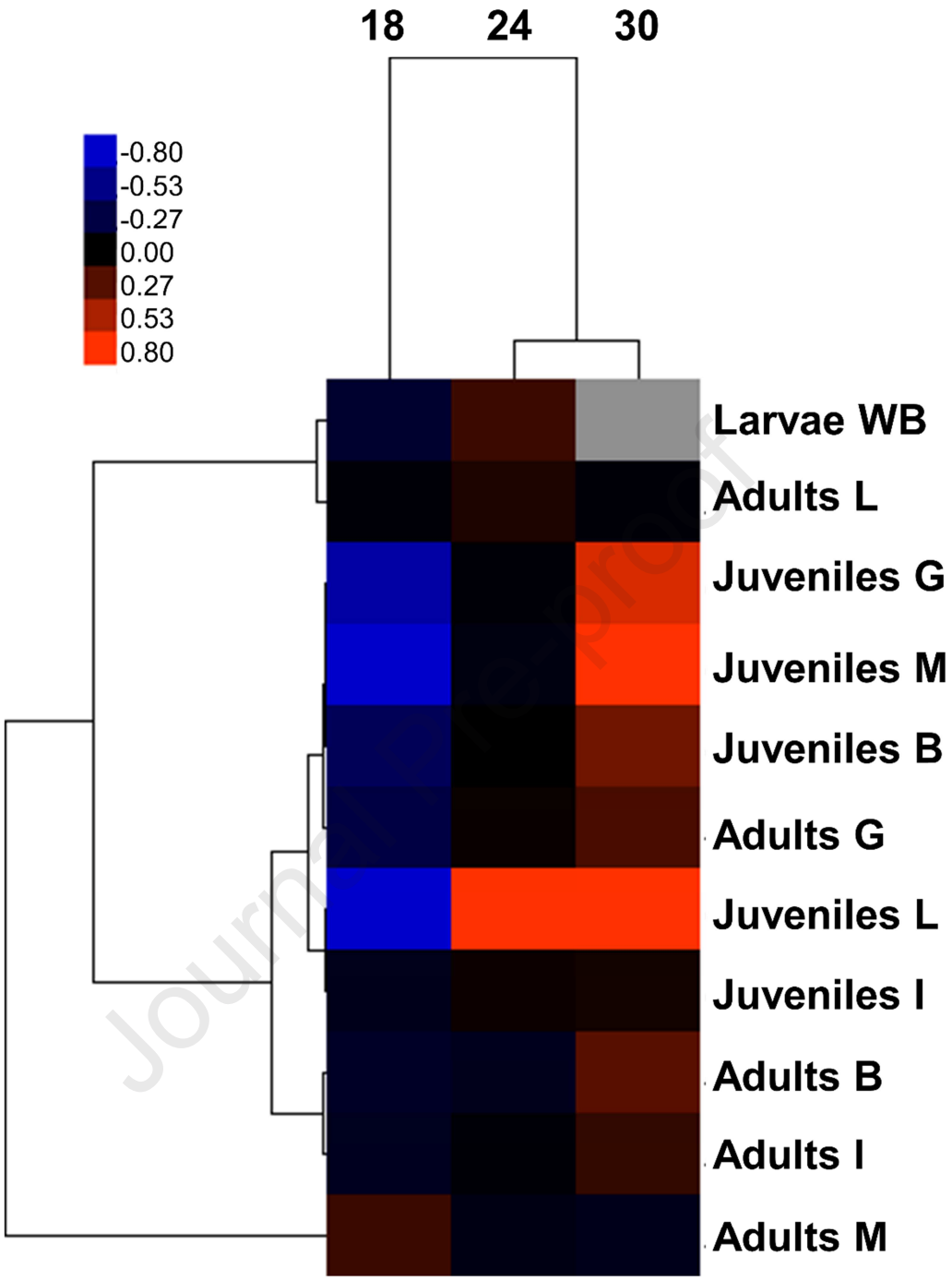
Fig. 2 Plots of linear discriminants per class and corresponding histograms showing the overall profile of cellular stress response (CSR) biomarkers across life stages of the seabream fish *Sparus aurata* exposed to different temperatures over time. Ellipses represent the 95 % confidence intervals to centres assuming a multivariate *t* distribution. Dots represent real observations. Plots for life stages **(a, b)**, temperatures **(c, d)**, exposure times **(e, f)** and organs **(g, h).** Only juvenile and adult data were used for temperature, time and organ plots, as group separation was evident after the removal of larval data. Fish were sampled at day 0 (only 18ºC), 7, 14, 21 and 28 days (all temperatures). At each time point, 4 to 5 individuals were randomly sampled (2-3 from each tank, n=2 tanks per temperature). CSR biomarkers were quantified

424 in the whole body of larvae and different organs in juveniles and adults. CSR biomarkers include heat shock protein 70 kDa, total ubiquitin,
425 catalase, glutathione-S-transferase, superoxide dismutase and lipid peroxidation.

426 *Integrated Biomarker Response (IBR)*

427 An overview of the response capacity upon exposure to increased temperature was
428 provided by IBR values, which were similar between larvae exposed to 18ºC and 24ºC
429 (Fig. 3). In juveniles, IBR was always higher at 24ºC and 30ºC when compared to 18ºC,
430 the largest increases occurring in liver, gills and muscle. In adults, IBR was higher at
431 24ºC and 30ºC in all organs except muscle and liver. In muscle, the IBR at both 24ºC
432 and 30ºC was lower than that at 18ºC. In liver, the IBRs at 18ºC and 30ºC were very
433 similar and lower than 24ºC (Fig. 3).

434



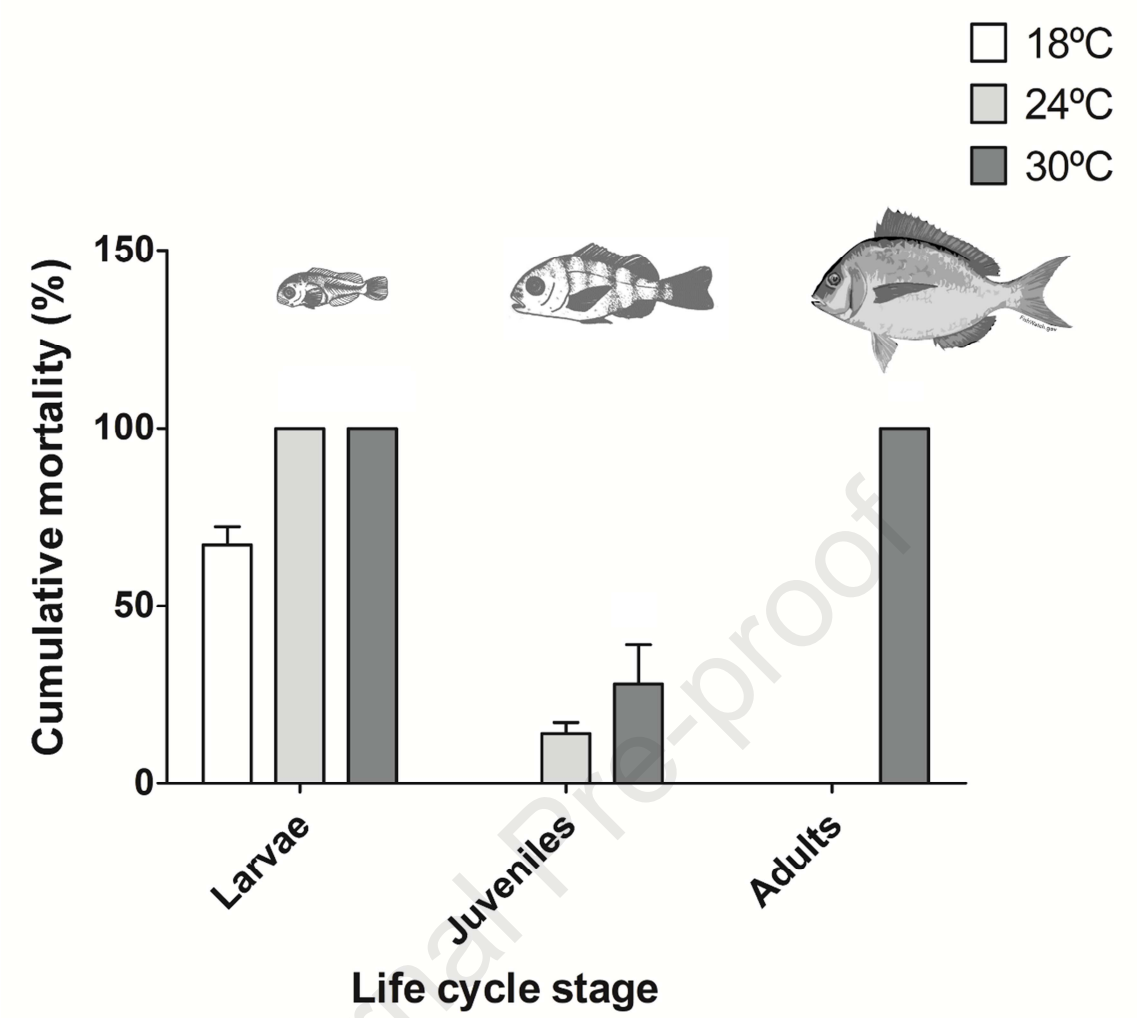
435

436 **Fig. 3** Integrated Biomarker Response index (IBR) plotted as a heatmap with clustered
437 data to detect which life stage and tissue was more responsive to warming. Columns
438 represent different temperatures (18, 24 and 30ºC) and rows represent different life
439 stages and organs (WB – whole body; L – liver; G – gills; M – muscle; I – intestine; B-
440 brain). Orange represents higher than mean IBR values and blue represents lower than
441 mean IBR values. Gray cells represent missing values due to mortality. IBR was
442 calculated based on data from all time-points (0, 7, 14, 21 and 28 days). At each time
443 point, 4 to 5 individuals were randomly sampled (2-3 individuals from each tank, n=2
444 tanks per temperature).

3.2.1 Phenotypic endpoints

Mortality and condition index

Significant differences were detected between cumulative mortalities among temperature treatments (Kruskal-Wallis, H=6.25 ; p=0.0439), namely 18 and 30 ºC (multiple comparisons, p<0.05) and life stages (Kruskal-Wallis, H=7.46; p=0.0240), namely larvae and juveniles (multiple comparisons, p<0.05). Cumulative mortalities (after 28 days of exposure) of sea bream larvae were generally higher than for other life stages, namely 67±5% at 18ºC, 100±0% at 24ºC and 100±0% at 30ºC. Cumulative mortalities of juveniles were 0±0% at 18ºC, 14±3% at 24ºC and 28±11% at 30ºC; and cumulative mortalities of adults were 0±0% at 18ºC, 0±0% at 24ºC and 100±0% at 30ºC (Fig. 4). Fulton's K condition index did not vary with temperature in any of the developmental stages tested (juveniles, 18 *vs* 24 *vs* 30ºC: Kruskal-Wallis, H=1.28, p>0.05; adults, 18 *vs* 24ºC: Mann-Whitney, U=7, p>0.05).



458

459 **Fig. 4** Cumulative mortalities (mean±SD) of *Sparus aurata* larvae, juveniles and adults
460 exposed to 18ºC, 24ºC and 30ºC for a period of 28 days. The experimental design for
461 each life stage consisted of 2 tanks per temperature (density was based on body size,
462 n=30 larvae tank^{-1}, n=13 juveniles tank^{-1}, and n=10 adults tank^{-1}). Significant
463 differences were detected between temperature treatments (Kruskal-Wallis, H=6.25 ;
464 p=0.0439), namely 18 and 30 ºC ($p<0.05$) and life stages (Kruskal-Wallis, H=7.46;
465 p=0.0240), namely larvae and juveniles ($p<0.05$). Illustrations by (Park et al., 2017)
466 and FishWatch.gov.

467

468 ***Histopathology***

469 Skeletal muscle yielded changes in all three age groups, from larvae (Fig. 5A) to

470 juveniles and adults (Fig. 5B), with a clear trend to increase with time and temperature.

471 However, muscular dystrophies were more severe and diffuse in adults and juveniles,

472 where infiltration of inflammatory cells accompanied focal (towards diffuse in animals

subjected to 24ºC and higher) autolytic processes of muscle bundles and atrophy of connective tissue. The brain was seemingly affected only in adults (Fig. 5C) exposed to 24ºC or higher for longer periods of time. However histopathological changes were limited to vacuolation of glial cells in the medulla. The liver (hepatopancreas) was the most affected organ in adults and overall the organ that yielded the most severe histopathological changes, affecting both hepatic and pancreatic tissue, the latter of which presented diffuse dystrophy of acini in animals exposed to 30ºC, with a clear time-dependent trend. The main alterations hitherto observed were fat vacuolation, inflammation (revealed by foci of infiltrating inflammatory cells and hyperemia, see Fig. 5D, inset), loss of glycogen storage disclosed by PAS reaction and loss of zymogen granules in acinar cells, which appeared degenerated in shape and size, with loss of acinar structure. Control animals presented the normal structure of the organ throughout the experiment (Fig. 5E). Gills did not reveal any significant changes that could be pinpointed to thermal stress in any of the age groups. Changes observed solely relate to benign infections, mostly by *Chlamidia*-like bacteria (Fig. 5F). Similarly, no histopathological changes were observed in digestive tracts. Larvae subjected to higher temperatures presented minor alterations to kidneys, namely cuboidal cell vacuolation and loss of tubular shape (not shown). Overall, muscle was the organ that was most consistently affected in all life stages.

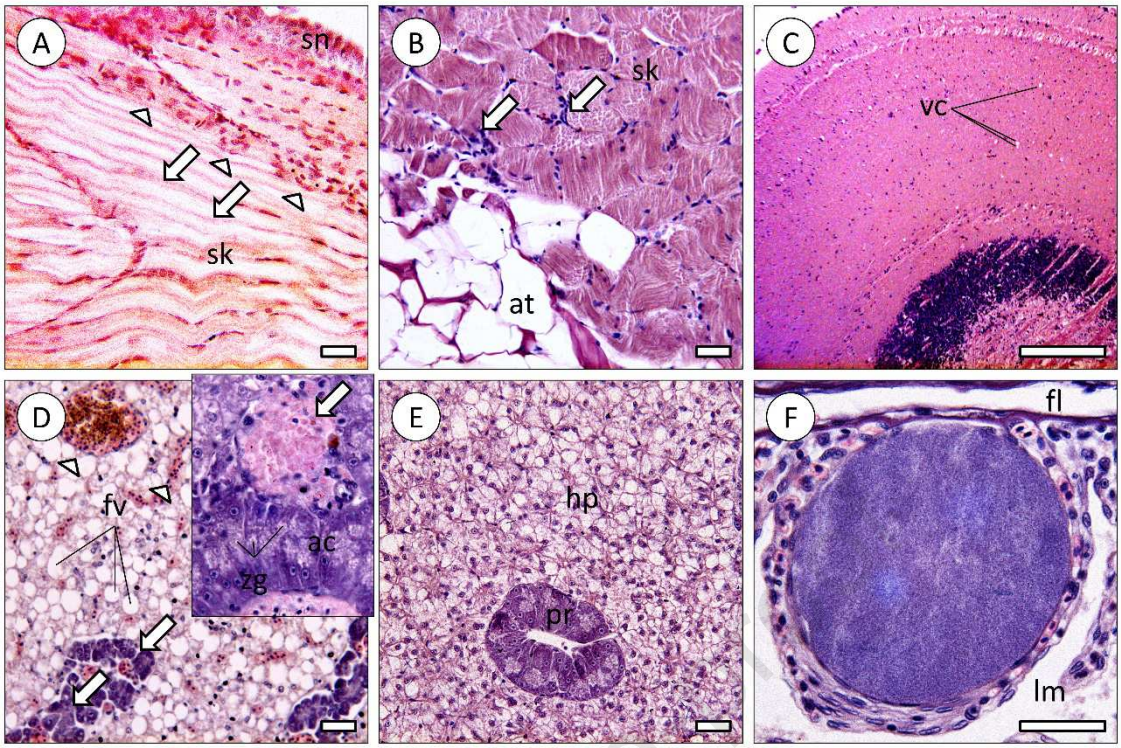


Fig. 5 Histopathological sections of multiple organs from larva, juvenile and adult *Sparus aurata* subjected to different temperatures (18, 24 and 30ºC). **(A)** Muscle of a larva subjected to 24 ºC for seven days. Note the infiltration of inflammatory cells along the junction between skeletal muscle segments (arrowheads). Bundles of disorganized (atrophied) muscle bundles are also visible (arrows). sn) Skin. Staining: Weigert's Iron Haematoxylin + van Gieson. **(B)** Skeletal muscle of an adult subjected to 24 ºC for 21 days, revealing diffuse atrophy of skeletal muscle (sk) bundles and inflammatory foci (H&E). The subcutaneous adipose tissue (at) was frequently observed to infiltrate affected muscle (H&E). **(C)** Section through the optic lobe of an adult fish exposed to 24 ºC for 21 days, revealing low-moderate diffusion of vacuolation (vc) in the medullar area, likely affecting glial tissue (H&E). **(D)** Liver (hepatopancreas) of an adult fish subjected to 30 ºC for seven days, with diffuse fat vacuolation (fv) plus inflammation, indicated by hyperaemia and infiltration of inflammatory cells into hepatic tissue (arrow heads). The pancreatic acini are severely affected (arrows), revealing loss of zymogen granules (H&E). Inset: Focus of inflammatory cells (likely melanomacrophages) in the vicinity of pancreatic acini in an adult exposed to 24 ºC for 14 days. Here the acini (ac) still presented a normal structure. Note zymogen granules (zg). PAS-Haematoxylin. **(E)** Hepatopancreas of a control (18 ºC) adult fish at 21 days, for comparative purposes. These animals presented the normal architecture of hepatic (hp) and pancreatic tissue (pr), similar to juveniles (H&E). **(F)** Benign bacterial infection by *Chlamydia*-like bacteria in the interlamellar space in gills of a control fish collected at 14 days of exposure. fl) filament; lm) lamella. Note the absence of inflammation. These benign infections were present in virtually all animals, regardless of test or age class (H&E). Scale bars: 25 µm except C (250 µm).

4. Discussion (1721 words)

Accurate predictions of the impacts of climate change on populations and ecosystems ideally require the assessment of thermal tolerance of all life-history stages (Freitas et al., 2010; Levy et al., 2015; Zeigler, 2013). Here, we show that while demersal fish larvae lack the ability to tolerate or acclimate to elevated summer temperatures and heat waves, juveniles were able to cope with all warming scenarios. Larger adult fish survived elevated summer temperatures but were also severely affected under future heatwave scenarios predicted for estuaries, despite no differences in condition index. This outcome is supported by the high mortality rates endured by larvae at both 24ºC and 30ºC and endured by adults at 30ºC, confirming that high temperatures lead to a bottleneck effect not only in early life stages of fish, in accordance with other studies in marine organisms (Bartolini et al., 2013; Faria et al., 2011; Houde, 1989) but also at later mature stages, possibly linked to larger body size. Mortality levels were still elevated for larvae under control conditions, but this was expected as planktonic larval fish are extremely fragile and have critical periods in development that explain significant portions of mortality (Garrido et al., 2015; Sifa and Mathias, 1987).

Our findings suggest that molecular mechanisms underpinning thermal tolerance of fish varied with organ as well as with life stage. Tissue-specific expression of the CSR has been widely reported and has been associated with the level of oxygenation and metabolic functions of each tissue (Colin et al., 2016; Dietz and Somero, 1993; C. Madeira et al., 2016; Madeira et al., 2014). The most responsive organs in the juvenile phase were liver, gills and muscle, as shown by IBR values, suggesting relevant phenotypic plastic responses. However, in adults, most organs seemed less able to employ efficient cellular protection. Histopathological observations corroborated these results, as juveniles only show mild alterations in muscle whereas relevant

545 modifications and potential loss of function were detected in adults' muscle, liver and
546 brain, with potential effects on energy metabolism and neural function. Thus, it seems
547 that juveniles are able to prevent cytotoxic effects of damaged proteins and oxidative
548 stress during heatwave events as opposed to adults, which show depletion of energy
549 reserves (namely loss of glycogen storage) and experience function loss in vital organs
550 due to inflammation, dystrophy, and damage to lipids and proteins (mainly in muscle
551 and liver). Like adults, larvae showed reduced molecular plasticity, with a lower ability
552 to employ a strong and efficient CSR (as shown by the similar IBRs between 24ºC and
553 30ºC), potentially resulting in oxidative damage to lipids and damage to muscle and
554 kidneys. Alterations in muscle contraction and osmoregulatory imbalance could thus be
555 potentially accountable for the elevated mortality rates of larvae. Differences in thermal
556 tolerance and molecular mechanisms between life cycle stages may be associated not
557 only with development and functional capacity but also with an ontogenetic shift in
558 thermal scope for activity (see Pörtner et al., 2017), which should be concomitant with
559 the habitat transition that usually accompanies metamorphosis and sexual maturation in
560 many fish species.

561 Divergence in life stage thermal tolerance may be related to oxygen supply constraints
562 related to body size (Dahlke et al., 2020; Pörtner et al., 2017; Pörtner and Farrell, 2008).
563 Moreover, it may also relate to different levels of plasticity through epigenetic
564 regulation of the genome in response to the environment that each life stage inhabits
565 (Webster et al., 2013). This could lead to different expression of phenotypic plasticity
566 and thus life-stage specific phenotypic landscapes. Such phenotypic plasticity could be
567 adaptive, given that it has an effect on fitness (Arnold et al., 2019) (for instance
568 allowing juveniles to mature and return to spawning areas to reproduce). Still,
569 phenotypic plasticity has costs, limits and trade-offs (Arnold et al., 2019; Fox et al.,

570 2019), possibly related to energy budgets of organisms and physiological constraints.
571 Ultimately the capacity to be plastic at the individual level and the capacity to adapt at
572 the population level will determine winners and losers in the Anthropocene (Fox et al.,
573 2019).

574 While embryonic and mature adult stages of demersal fish species may depend on stable
575 environmental conditions in their spawning habitat (cf. Dahlke et al., 2018), planktonic
576 larval stages switch from oceanic into coastal or estuarine habitats in spring, during or
577 following metamorphosis. The eurythermal and euryhaline traits known for seabream
578 and similar fishes (e.g. seabass) enable this type of migratory behavior (Moyano et al.,
579 2017). For example, the Critical Thermal Maximum (CT_{max}) calculated for seabream
580 larvae is ~30ºC (Madeira et al., 2016), increasing to 35.5 ºC in juvenile stages (Madeira
581 et al., 2014). In seabass, CT_{max} values range from 28 to 33 ºC during early ontogeny,
582 increasing up to 35 ºC during juvenile stages (Madeira et al., 2012; Moyano et al.,
583 2017). Growth as juveniles during summer months can, however, be stressful especially
584 considering a difference of 4 to 6 ºC between oceanic and estuarine waters, not to
585 mention the selective pressure that will be imposed by heat waves in the next century
586 (Stillman, 2019). However, migration into estuaries is not obligatory for the species to
587 complete its life cycle. Hence, populations that remain in coastal waters may have more
588 thermal refugia to escape extreme conditions.

589 One could argue that the higher surface-to-volume ratio of early life stages could lead to
590 a more efficient uptake of oxygen and respiration (see Leiva et al., 2019), especially
591 given that larvae can respire through skin (Yúfera et al., 2011). Skin respiration
592 balances oxygen demand while circulatory and ventilatory capacities of larval fish
593 develop. However, at high temperature extremes, organisms may not be able to sustain
594 metabolic needs, and thus performance and fitness, due to a mismatch between supply

595 and demand. Energy homeostasis depends on physiological traits (oxygen intake
596 capacity, surface-area-specific assimilation rate and efficiency of food-ATP
597 conversion), volume-specific somatic maintenance costs and energy allocation trade-
598 offs, ultimately determining tolerance limits and metabolic strategies (compensation vs
599 conservation, *sensu* Petitjean et al., 2019) upon exposure to stress (Freitas et al., 2010;
600 Sokolova, 2013; Sokolova et al., 2012). Moreover, parallel increases in protein
601 denaturation, disturbance to neural function and to mitochondrial membrane integrity
602 (Clark et al., 2017; Pörtner, 2012) might be observed during stress, contributing to
603 performance decrements. Thus, respiration dynamics, energy balance and cellular
604 defenses are factors paramount in stress tolerance (Pörtner, 2012; Sokolova, 2013).

605 Previous studies on seabream larvae showed that elevated temperature induces cellular
606 changes such as cytoskeleton reorganization, protein damage and lowered oxygen
607 transport (Madeira et al., 2016), following the framework of the oxygen and capacity
608 limited thermal tolerance hypothesis OCLTT (Pörtner et al., 2017). Other studies also fit
609 well within the concepts described above; for instance, Truebano *et al.* (2018)
610 summarized that early life stages may be more susceptible to heat stress (especially
611 acute) due to several complementary reasons including (i) energy is mostly allocated to
612 growth and cellular rearrangements, (ii) thermal tolerance mechanisms such as the heat
613 shock response may be underdeveloped in early life stages possibly because
614 overexpression impairs developmental processes, (iii) cellular defenses prioritize
615 developmental stability and acute stress may disrupt these defenses leading to altered
616 development and mortality. Therefore, all of these interlinked processes combined with
617 high mass-specific oxygen consumption rates typical of fish larvae (see Hess et al.,
618 2015; Motani and Wainwright, 2015) contribute to shape the performance of early life
619 stages in warming seas.

Overall, we conclude that the susceptibility of demersal sea breams to ocean warming can be ranked as larvae > adults > juveniles. Despite some study imitations such as the somewhat short laboratory acclimation period and the difficulty in analyzing target organs in larval stages, we highlight that larval migration to coastal and estuarine waters might be the most crucial phase of demersal fish life cycle. This migration enables fish to exploit new resources but may coincide with enhanced exposure to warm periods, despite the widened thermal ranges of seabreams. Here we showed that larvae are quite sensitive to future estuarine temperatures due to a lack of biochemical acclimation, damage to muscle and kidneys and associated mortality rates. Moreover, larvae could not cope with warming for more than a few days, as opposed to juveniles and adults which were able to endure stressful events for much longer timeframes. Sub-lethal effects of temperature in seabream larvae can arise at ~22ºC (D Madeira et al., 2016; Polo et al., 1991) supporting the idea that future coastal (~16-23ºC during spring and summer) and estuarine thermal regimes (~20-26ºC during spring and summer; heat waves 30ºC) will impose temperatures beyond the thermal envelope of larvae, negatively affecting sea bream populations through high mortality and lowered recruitment success. Thus, life cycle closure may depend on the success of the first spawning events (autumn/winter) and subsequent migration to nursery grounds before heatwaves strike shallow water environments. Adult performance was also strongly affected by extreme warming, with vital organs showing the greatest tissue injury suggesting damage rather than a plastic response. However, adult fish may revert to behavioral thermoregulation (Gräns et al., 2010; Pulgar et al., 1999; Thums et al., 2012; Ward et al., 2010) and have thermal refugia available to them in open water to counterbalance the loss of plasticity with age. This could be involved in eliciting return to deep water environments and spawning habitat. Juvenile breams, which are confined

to estuaries, proved to be metabolically more competent to deal with warming, showing the greatest survival and acclimation potential. A plastic response to temperature enables juveniles to cope with their habitat's fluctuating conditions and lack of thermal refugia. Recent reports highlight the importance of using physiological data to inform and direct future research and conservation plans of fishes, directing management actions towards the most vulnerable life stages (Rodgers et al., 2019). Based on GLMs, the best biomarkers to assess thermal stress effects on seabreams were Hsp70, TUB and LPO. Thus, their potential use in monitoring programs should be addressed in future studies. Finally, it must be pointed out that sea breams and similar demersal species are main targets of traditional fisheries and aquaculture in southern Europe and part of the basic Mediterranean diet. Thus, the successful recruitment and population viability of wild and cultured seabreams will have impacts on both ecosystems and human society. Climate-smart strategies in resource management and aquaculture production are thus also paramount to the blue bioeconomy, ensuring the sustainability of fish supply and food security.

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Author contributions

D. Madeira: investigation, formal analysis, data curation, writing – original draft, visualization; **C. Madeira**: investigation; **P.M. Costa**: methodology, investigation, formal analysis, visualization, supervision, resources, writing – review and editing; **C. Vinagre**: conceptualization, methodology, writing – review and editing, supervision; **H.O. Pörtner**: writing – review and editing; **M. S. Diniz**: conceptualization, methodology, validation, resources, writing – review and editing, supervision, project administration, funding acquisition.

Competing Financial Interests Statement

The authors have no conflicts of interest to declare.

Data accessibility

Data have been deposited to the Knowledge Network for Biocomplexity Repository with the dataset identifier urn:uuid:575bfeee-8df2-4058-9712-8d95c729722b (a final DOI will be provided upon publication).

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Highlights

1. Life-cycle assessments are crucial to forecast global change impacts on biota
2. Heatwave effects were tested in seabreams across their life cycle
3. Sub-cellular, cellular and whole organism indicators were assessed
4. The vulnerability of life cycle stages can be ranked as larvae>adults >juveniles
5. Life-cycle closure may be in jeopardy due to the sensitivity of larvae to heat

Conflict of Interest

The authors have no conflicts of interest to declare.