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Histopathological alterations, physiological limits, and molecular changes of juvenile *Sparus aurata* in response to thermal stress

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ABSTRACT: Current concerns about climate change have led to a great body of literature on thermal tolerance. However, it is still difficult to understand and relate biological changes at different organizational levels. This is especially important in commercial species. We aimed to test the effects of temperature stress in juvenile gilthead seabream *Sparus aurata* in order to contribute to the understanding of the vulnerability of this species to temperature changes, heat waves, and potentially climate warming. Here we applied an integrative approach, considering indicators at the biochemical, cellular, and physiological levels. Firstly, the upper thermal limit was estimated via the critical thermal maximum (CTMax); secondly, levels of Hsp70 (reversible protein damage) and total ubiquitin (irreversible protein damage) were quantified in several tissues via ELISAs; and thirdly, histological analyses were performed to identify cellular structural damage due to temperature and how it correlates to biochemical alterations. Results showed that mean (±SD) CTMax was 35.5 ± 0.5°C. Absolute amounts of both Hsp70 and total ubiquitin varied significantly among organs, with gills having the highest amounts of both. Biomarker and histopathological results indicated that *S. aurata* might be particularly sensitive to water temperatures ≥28°C. At 30°C, *S. aurata* juveniles showed severe signs of stress, with increased biomarker levels in almost every organ tested and significant cellular damage (atrophy, inflammation, micro-hemorrhage, hyperemia, hyperplasia). Therefore, *S. aurata* may be vulnerable to heat wave events that currently make water temperature attain 28°C for several weeks (30°C by 2100 under a climate change scenario). Thus, this species might be vulnerable to a rise in sea temperature, and our research may be important from a management perspective, as *S. aurata* is a major commercial species.

KEY WORDS: Hsp70 · Ubiquitin · Cellular alterations · CTMax · Sea bream · Temperature

INTRODUCTION

Stressful conditions can act as evolutionary forces, leading to adaptive changes in populations (Sørensen et al. 2003). A variety of stressors (physical, chemical, biological) operate in aquatic ecosystems, and among them, temperature is one of the most important. All organisms have a thermal performance curve, meaning that when temperature reaches values close to the organism's upper thermal limits, performance decreases (see Huey & Stevenson 1979). Moreover, several studies defined the concept of oxygen-limited thermal tolerance, implying that the oxygen supply to organs is optimal between limits (lower and upper pejus temperatures; Pörtner 2001, 2010). Furthermore, organisms cannot survive (except for a limited

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period of time) at temperatures above or below the pejus temperatures (see Pörtner & Peck 2010 for a review).

Metabolic pathways and biochemical reactions are affected by temperature, determining an organism's physiological state and behavior. Therefore, activities such as foraging, reproduction, and growth will be altered and may change the fitness of the individuals (Mora & Ospina 2001, Hochachka & Somero 2002, Pörtner & Peck 2010) and community and ecosystem structure (e.g. Pörtner & Farrell 2008).

Within marine aquatic environments, estuaries and other confined coastal areas are amongst the most variable. These areas are economically important, providing food resources and serving as nursery areas for juveniles of many species (Haedrich 1983, Vinagre et al. 2010). Nevertheless, as estuaries are shallow water bodies, anthropogenic impacts (e.g. pollution, overfishing) and environmental change are greater than in deep sea or open-ocean sites. Additionally, significant variations in abiotic variables that follow seasonal or even tidal rhythms may be permanent sources of stress to residing biota. Hence, juvenile fish inhabiting these ecosystems may endure stressful conditions, including significant temperature variations (see Madeira et al. 2012a). Considering climate change scenarios for Portuguese waters (a 2°C increase in mean seawater temperature by 2100; Miranda et al. 2002), juveniles may undergo severe thermal stress which may compromise their growth (Vinagre et al. 2012a, 2013a,b). Madeira et al. (2012a) showed that sea bream juveniles inhabiting Portuguese estuaries may already be subjected to thermal stress under the present conditions. It is therefore important to study the molecular and biochemical mechanisms behind the thermal stress response in commercial species to determine whether they may be undergoing cellular and tissue damage. Biomarker quantification may be very useful to achieve this aim. A biomarker is defined as 'any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction' (World Health Organization 1993). Knowing and measuring these biomarkers may help us understand ecological patterns of response to increasing seawater temperature and, therefore, improve the management of commercial fish populations.

From this perspective, the model chosen for this study was the gilthead seabream *Sparus aurata*, which is a very important commercial species, both in fishing activities and aquaculture. It is a euryhaline, warm-temperate species that occurs in coastal seagrass beds, on sandy bottoms, and in the surf zone, as well as in estuaries and coastal lagoons (Altimiras et al. 1994). It usually inhabits shallow waters, from 1 to 150 m deep (commonly 1 to 30 m). Juveniles tend to school in coastal lagoons and estuarine brackish waters (Craig et al. 2008), which are warmer than coastal waters. Its native distribution is subtropical and extends from the Mediterranean to the east Atlantic coast (including Cape Verde and the Canaries to the English Channel).

Amongst stress biomarkers, heat shock proteins (Hsps) have been the most widely studied, especially Hsp70 (Tomanek 2011). Temperature leads to proteotoxic stress and induces a heat shock response in several species (e.g. Somero 1995, Madeira et al. 2012b). Hsps have chaperoning activity and can be seen as molecular protection mechanisms with a selective value, contributing to the organism's and species' success across environmental gradients (Hofmann 2005). They confer stress tolerance (Li et al. 1995, Feder 1996, Basu et al. 2002, Sorte & Hofmann 2005) and are associated with an increase in the ability to temporarily survive temperature extremes (Feder & Hofmann 1999, Hofmann et al. 2002, Sørensen et al. 2003). Hsps are considered an indirect measure of protein damage and biochemical indicators of reversible protein unfolding in the cell (Hofmann 2005, Tomanek 2010, 2011). Another important biomarker, which complements the information provided by Hsps, is ubiquitin. Ubiquitination is critical to cell cycle regulation, transcription, apoptosis, protein sorting, and protein quality control, among other cell processes (Hanna et al. 2007). The ubiquitin metabolic pathway involves the selective tagging of proteins for proteasomal degradation (Hershko & Ciechanover 1992) and falls into 4 categories: (1) continuous turnover to rapidly modulate protein levels; (2) elimination of proteins in response to specific signals; (3) elimination of defective proteins that arise through physical or chemical factors and transcriptional/translational errors or gene mutations; and (4) the turnover of excess free forms of proteins (from multi subunit complexes; Finley 1991). Regarding these aspects, ubiquitin levels are considered a direct measure of protein loss (Ciechanover 1998), indicating irreversible protein damage in the cells (Hofmann & Somero 1995). Moreover, a relationship may exist between the heat shock and ubiquitin systems (Finley et al. 1984, Alberti et al. 2002). However, it is still unclear which features of damaged proteins

mediate proteasome activity, whether they are eliminated through ubiquitination or recovered by Hsps (Finley 1991, Patterson & Höhfeld 2006). Nonetheless, substrate recognition and processing may require cooperation between these 2 systems (Patterson & Höhfeld 2006). During stress, accumulations of misfolded proteins (aggresomes) tend to form in the cells (Johnston et al. 1998, Kopito 2000). Interestingly, components of the ubiquitin system and chaperone system are present and seem to be actively recruited to these aggresomes (García-Mata et al. 1999, Wyttenbach et al. 2000). Moreover, increased Hsp70 can reduce aggresome formation by increasing protein degradation (Dul et al. 2001). Thus, there seems to be a protein triage system within the cells, in which Hsps seem to play an important part. However, little information is available on these matters concerning marine species. All of these hypotheses were tested in mammals or human cell lines, so further studies are needed using aquatic organisms as models.

Histopathological changes are also important indicators of health changes in species of aquatic and other ecosystems (e.g. Bignell et al. 2011, Costa et al. 2013a,b). Even though several studies have investigated histopathological changes induced by temperature, they mainly focused on freshwater fish (e.g. Day & Butler 2005, Sollid & Nilsson 2006). These changes included morphometric changes in the attributes of muscle tissue, changes in axon and fiber diameter and thickness, changes in the respiratory surface area, and changes in several tissues (gills, liver, kidney, and heart), such as hyperplasia, prominent vacuolization, increased melanin production, canaliculi formation, hypertrophy, and necrosis of cells.

The aims of this study were to test the effects of temperature stress in juvenile gilthead seabream *S. aurata* in order to contribute to the understanding of the vulnerability of this species to temperature changes, heat waves, and potentially climate warming. In detail, our aims were (1) to test the upper thermal limit, (2) to assess the biochemical thermal stress response, quantifying the levels of Hsp70 (reversible protein damage) and total ubiquitin (irreversible protein damage) in several tissues of this species (gills, intestine, muscle, brain, hepatopancreas–liver, and infiltrated pancreatic acini), (3) to identify any correlations between Hsp70 levels and total ubiquitin levels, and (4) to perform histological analysis of the samped tissues in order to identify cellular structural damage due to temperature and how it correlates to biochemical alterations.

MATERIALS AND METHODS

Thermal tolerance method

Juvenile *Sparus aurata* (mean ± SD total length of 92.1 ± 8.1 mm and weight of 12.2 ± 2.9 g) were obtained from a fish farm (MARESA, Spain) and acclimated in a re-circulating system with 150 l aquaria containing clean, permanently aerated sea water, at a constant temperature of 18°C and salinity of 35‰ (maintenance conditions in the fish farm). The dissolved O_2 level of the water varied between 95 and 100%. The fish were acclimated for 1 wk prior to bioassays and were fed commercial pellets *ad libitum* twice a day. They were starved for 24 h before the experiments. The use of farmed organisms reduces potential confounding factors (i.e. thermal history). According to several authors, thermal history and parental effects are determinant and induce irreversible changes in the thermal tolerance of the species (Cossins & Bowler 1987, Schaefer & Ryan 2006).

Thermal tolerance was determined using the dynamic method described by Mora & Ospina (2001). The parameter measured was the critical thermal maximum (CTMax, given in °C), which is defined as the 'arithmetic mean of the collective thermal points at which the end-point is reached' (Mora & Ospina 2001, p. 766). This end-point is defined as the loss of equilibrium. The CTMax method was chosen for this study because it has been the most used to determine upper thermal limits (Lutterschmidt & Hutchison 1997) and because dynamic trials are accurate predictors of the responses of organisms to natural conditions (Bennett & Judd 1992, Bennett & Beitinger 1997).

To determine the CTMax, the fish were subjected to a thermostatized bath, using a heated/refrigerator circulator (MultiTemp III, Pharmacia Biotech). They were placed in 40.5 l white plastic tanks (n = 5, 30 × 30 × 45 cm) with lids to prevent evaporation (10–11 fish $tank^{-1}$). Each tank was equipped with an aeration system. During the experiment, animals were exposed to a constant rate of water temperature increase of 1°C h^{-1}, and observed continuously, until they reached the end-point. Fish were collected every 2°C, killed by cervical decapitation with the aid of a scalpel, and organs were removed. Samples (brain, gills, intestine, hepatopancreas, muscle) from 6 individuals were taken and immediately frozen in liquid nitrogen or processed fresh for histological analyses (in this case, samples of 3 animals per sampling point, out of the total of 6). The total number of individuals was 54.

The temperature at which each animal reached its end-point was measured with a digital thermometer and registered, and then CTMax (and its standard deviation) were calculated as:

$$\mathrm{CTMax} = \frac{\sum_1^n (T_{\text{end-point } n})}{n} \quad (1)$$

Where $T_{\text{end-point}}$ is the temperature at which the end-point (loss of equilibrium) was reached for individual 1, individual 2, individual n, divided by the n individuals that were in the sample. The experiments were carried out in shaded daylight (15 h light:9 h dark). Total length and weight were measured at the end of each trial using an ichthyometer and a scale, respectively.

Protein extraction

Samples (approximately 200–250 mg of gills, muscle, intestine, brain, and hepatopancreas) were placed in 1.5 ml microtubes and homogenized in 1 ml of phosphate-buffered saline (PBS) solution (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.4) to extract most soluble cytosolic proteins, using a glass and teflon Potter Elvehjem tissue grinder, in ice-cold conditions. The crude homogenates were then centrifuged for 15 min at 10 000 × g. Afterwards, the supernatant was collected, transferred to new microtubes (1.5 ml), and frozen immediately (–80°C) until further biochemical analyses.

Hsp70 quantification

Hsp70 was quantified using an indirect enzyme-linked immunosorbent assay (ELISA) (Njemini et al. 2005) with 96-well microplates (Greiner). Either ELISA or Western blot can be successfully employed in Hsp quantification (Brun et al. 2008). Samples were diluted to 1:100 in 0.05 M carbonate-bicarbonate buffer (Sigma-Aldrich). This dilution was chosen to give an optimum signal and was achieved after previous optimization.

Three replicates of 50 µl were taken from each diluted sample, transferred to the microplate wells, and incubated overnight at 4°C. The microplate was washed 3 times in PBS 0.05% Tween-20 and then blocked by adding 200 µl of 1% BSA (%w/v; Sigma-Aldrich) in PBS. The microplate was incubated at 37°C for 90 min. After microplate washing, the primary antibody (anti-HSP70/HSC70; Acris), diluted to 0.5 µg ml^{-1} in 1% BSA in PBS, was added to the microplate wells (50 µl each). The microplates were then incubated for 90 min at 37°C. After another washing stage, the secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate; Sigma-Aldrich) was diluted (1 µg ml^{-1} in 1% BSA in PBS) and added (50 µl) to each well followed by incubation at 37°C for 90 min. After the washing stage, 100 µl of substrate (SIGMA *FAST*™ p-nitrophenyl phosphate tablets, Sigma-Aldrich) was added to each well and incubated for 30 min at room temperature. Stop solution (3N NaOH, 50 µl) was added to each well, and the absorbance was read in a 96-well microplate reader at 405 nm (Benchmark, BIO-RAD). For quantification purposes, a calibration curve was constructed using serial dilutions of purified Hsp70 active protein (Acris) to give a range from 0 to 2 µg ml^{-1}.

Ubiquitin and ubiquitinated/poly-ubiquitinated protein quantification

Quantification was achieved through a direct ELISA with 96-well microplates (Greiner). After previous dilution optimization, samples were diluted to 1:100 in 0.05 M carbonate-bicarbonate buffer (Sigma-Aldrich) to give an optimum signal.

Three replicates of 50 µl were taken from each diluted sample, transferred to the microplate wells, and incubated overnight at 4°C. The microplate was washed 3 times in PBS 0.05% Tween-20 and then blocked by adding 200 µl of 1% BSA in PBS (Sigma-Aldrich). The microplate was incubated at 37°C for 90 min. After microplate washing, the primary antibody (Ub P4D1, sc-8017, HRP conjugate, Santa Cruz Biotechnology), diluted to 0.5 µg ml^{-1} in 1% BSA in PBS, was added to the microplate wells (50 µl each). The microplates were then incubated for 90 min at 37°C. After another washing stage, 100 µl of substrate (TMB/E, Merck Millipore) were added to each well and incubated for 30 min at room temperature. Stop solution (1 N HCl, 100 µl) was added to each well, and the absorbance was read in a 96-well microplate reader at 415 nm (Benchmark, BIO-RAD). For quantification purposes, a calibration curve was constructed using serial dilutions of purified ubiquitin (UbpBio, E-1100) to give a range from 0 to 2 µg ml^{-1}.

For normalization purposes, the Bradford Assay was used to quantify the total amount of protein in each sample (Bradford 1976). The analysis was carried out in 96-well microplates (Nunc) by adding 200 µl of Bradford reagent in each well and 10 µl of each sample or standards. After 10 min of reaction, the absorbance was read at 595 nm in a microplate

reader (Benchmark, BIO-RAD). A calibration curve was constructed using BSA standards (0–10 µg ml^{-1}). The results for Hsp70 and total ubiquitin were divided by the total amount of protein in order to give the final data values.

Histological analysis

The procedure followed essentially Martoja & Martoja-Pierson (1967) for light microscopy. In brief, freshly-dissected samples from 3 individuals per temperature group (of gills, muscle, intestine, hepatopancreas), were fixed in Bouin's solution (10% v/v formalin and 7% v/v acetic acid with picric acid added to saturation) for 48 h at room temperature. The samples were dehydrated in a progressive series of ethanol and embedded in liquid paraffin (Panreac) overnight at 60°C. Sectioning was done with a Jung RM2035 model rotary microtome. Sections (5 µm thick) were stained with Harris' alum hematoxylin (Riedel-de Haën) and counterstained with alcoholic eosin Y (Panreac) for structural analysis. The slides were mounted with DPX resinous media (VWR International). Microscopy analysis was performed with a DMLB model optical microscope equipped with a DFC 480 digital camera (all from Leica Microsystems).

Statistical analysis

Failure to comply with the assumptions of parametric ANOVA (normality of the data and homoscedasticity) led to the application of non-parametric statistics. Kruskal-Wallis ANOVA by ranks and multiple comparisons tests were used to compare Hsp70 and ubiquitin levels between different temperature groups and different organs. Correlation analyses (Spearman R, since the data did not follow bivariate normality) were performed for Hsp70 and ubiquitin levels in each tissue. A significance level of $\alpha = 0.05$ was set for all statistics. Statistics were carried out using the software Statistica (Version 10, StatSoft).

RESULTS

CTMax and Hsp70

Following Eq. (1), *Sparus aurata* juveniles had a mean ± SD CTMax of 35.5 ± 0.5°C.

The Kruskal-Wallis results showed significant differences in Hsp70 levels among temperature groups in every tissue tested. For easier interpretation, Fig. 1a only shows significant differences to the control group (18°C). The results showed significant increases from control at temperatures ≥30°C, namely in the brain (1.4-fold, peak at 30°C), gills (2.1-fold, peak at 34°C), muscle (2.2-fold, peak at 34°C), and intestine (3.1-fold, peak at 36°C). These results show that during increasing temperatures, changes in the brain are observed sooner than in the rest of the organs. Gills expressed the highest absolute amount of Hsp70 when compared to other tissues ($p < 0.001$). Values of Hsp70 in the gills were followed by those measured in the brain and muscle (these 2 showed equal amounts of Hsp70 but higher amounts in relation to other

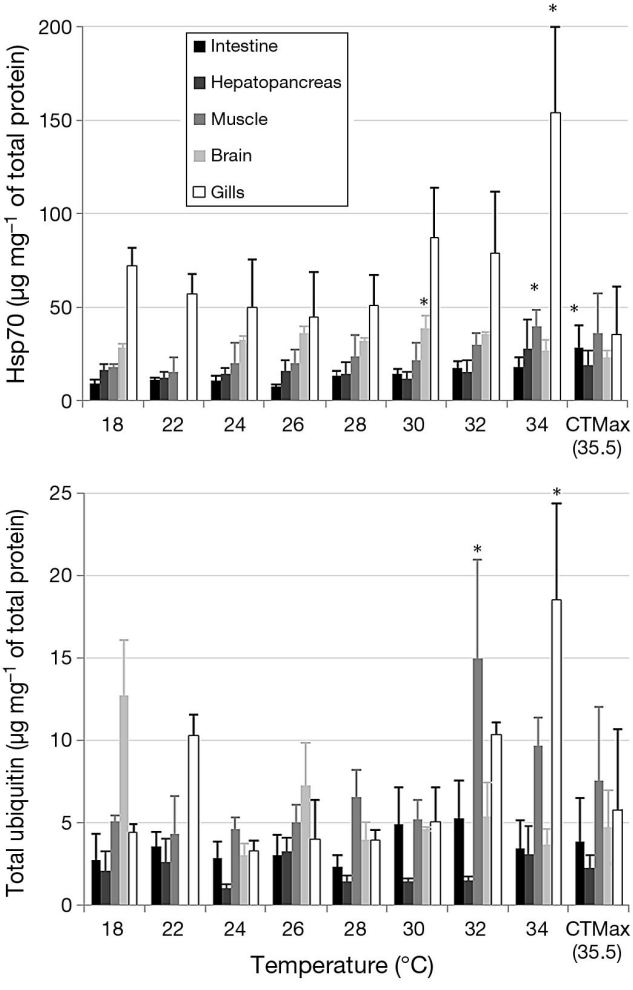

Fig. 1. (a) Mean + SD levels of (a) Hsp70 (hsc+hsp70) and (b) ubiquitin, ubiquitinated and polyubiquitinated proteins in juvenile *Sparus aurata* along a temperature gradient ranging from the control (18°C) to the critical thermal maximum (CTMax). Six individuals were sampled at each temperature point (except 22°C for brain tissue). Groups with Hsp70 or total ubiquitin levels significantly different from controls are marked with an asterisk ($p < 0.05$)

tissues; $p < 0.001$). Hepatopancreas and intestine showed equivalent amounts of Hsp70, but these levels were lower compared to other tissues ($p < 0.001$). Intraspecific variability in Hsp70 was elevated in all tissues.

Total ubiquitin

The Kruskal-Wallis results showed significant differences in total ubiquitin levels between temperature groups in all tissues tested except intestine and brain. For easier interpretation, Fig. 1b only shows significant differences to the control group (18°C). The results showed significant increases from controls in muscle (+2.9-fold, peak at 32°C) and gills (+4.1-fold, peak at 34°C), indicating that muscle responds faster than gills. When comparing total ubiquitin amounts in different tissues, gills, brain, and muscle had equivalent levels to each other, but had significantly higher levels than hepatopancreas and intestine ($p < 0.001$). Hepatopancreas and intestine showed significant differences, with intestine having higher amounts of total ubiquitin ($p < 0.01$). Intraspecific variability in total ubiquitin was also elevated in all tissues.

Correlation between Hsp70 and total ubiquitin

Spearman R correlations showed that Hsp70 and total ubiquitin were significantly correlated in gills (Spearman $R = 0.43$, $p < 0.005$), hepatopancreas (Spearman $R = 0.44$, $p < 0.002$), and muscle (Spearman $R = 0.67$, $p < 0.000$). Brain ($p > 0.61$) and intestine ($p > 0.18$) did not show a significant correlation between these 2 biomarkers.

Histological observations

Gills. Lesions in this organ started to occur at 28–30°C. The normal microanatomy of gills (i.e. in control individuals) consisted of lamellae attached to gill filament. Lamellae were covered by a single layer of squamous epithelial cells (1 cell thick) jointed by pillar cells between which were located the lamellar capillaries, with few erythrocytes (Fig. 2a). Goblet (mucus-secreting) and chloride cells (presenting a dense cytoplasm and a visible crypt) were mostly (but not exclusively) found in the interlamellar space (usually 1 or 2 cells per interlamellar space). At low temperatures (20–22°C), some occasional and localized alterations were visible, such as epithelial hyperplasia (especially in the interlamellar space), occasional hyperemia in the lamellar capillaries, and localized epithelial lifting from lamellae. From 28°C to CTMax, alterations to lamellar structure and epithelia became more frequent and diffuse. Between 28 and 30°C, the epithelial cells showed some degree of hypertrophy, and a few chloride cells became vacuolated (hypertrophied) in aspect due to fluid retention (see e.g. Costa et al. 2010; Fig. 2b). Compared to preceding conditions, epithelial lifting also became more disseminated (Fig. 2d). At 32–34°C, the aforementioned alterations were also conspicuous; however, at CTMax, deformation of lamellae (convoluted lamellae; Fig. 2e) was common, as was focal or partial lamellar fusion due to hyperplastic events (Fig. 2c) and some epithelial desquamation (shedding of squamous epithelial cells). Despite significant interlamellar epithelial hyperplasia, rod-shaped filaments did not occur.

Muscle. The normal microanatomy of muscular fibers (as observed in control animals) consisted of polynucleated striated myocytes (rhabdomyocytes) grouped into fascicles surrounded by a sheath of connective tissue, between which blood vessels were common (Fig. 3a). Overall, gross lesions were either reduced or absent in animals exposed to elevated temperatures; however, at 34°C and CTMax, a few muscle bundles showed atrophy of the fibers, probably caused by focal necrosis or autophagy (Fig. 3b).

Liver. The normal structure of liver tissue, observed in control animals, consisted of parenchymal tissue composed of polygonal hepatocytes with 1 or 2 round nuclei holding conspicuous nucleoli (Fig. 4a). Sinusoids were visible in the parenchyma, as well as intrusion of pancreatic tissue (thus forming the hepatopancreas). Small fat vacuoles or microvesicles were common features in control organisms. At 24°C, a higher number of defense cells were visible inside blood vessels, albeit without significant intrusion into the tissue. At 26°C, extensive areas of vacuolization (Fig. 4b) were prominent, and at 28°C, both hyperemia and melanomacrophage aggregates were visible (Fig. 4c) inside sinusoids and infiltrating the adjacent tissue. When the temperature reached 30°C and 32°C, vacuolization became more diffuse, and small necrotic foci appeared in the parenchyma, typically involving hyperemia, micro-hemorrhage, and macrophage infiltration (Fig. 4d), thus indicating an inflammatory response to the lesions. At 34°C, the alterations were similar to those that occurred at 30–32°C, but hepatocyte degeneration became apparent (involving altered shape and differential cytoplasm

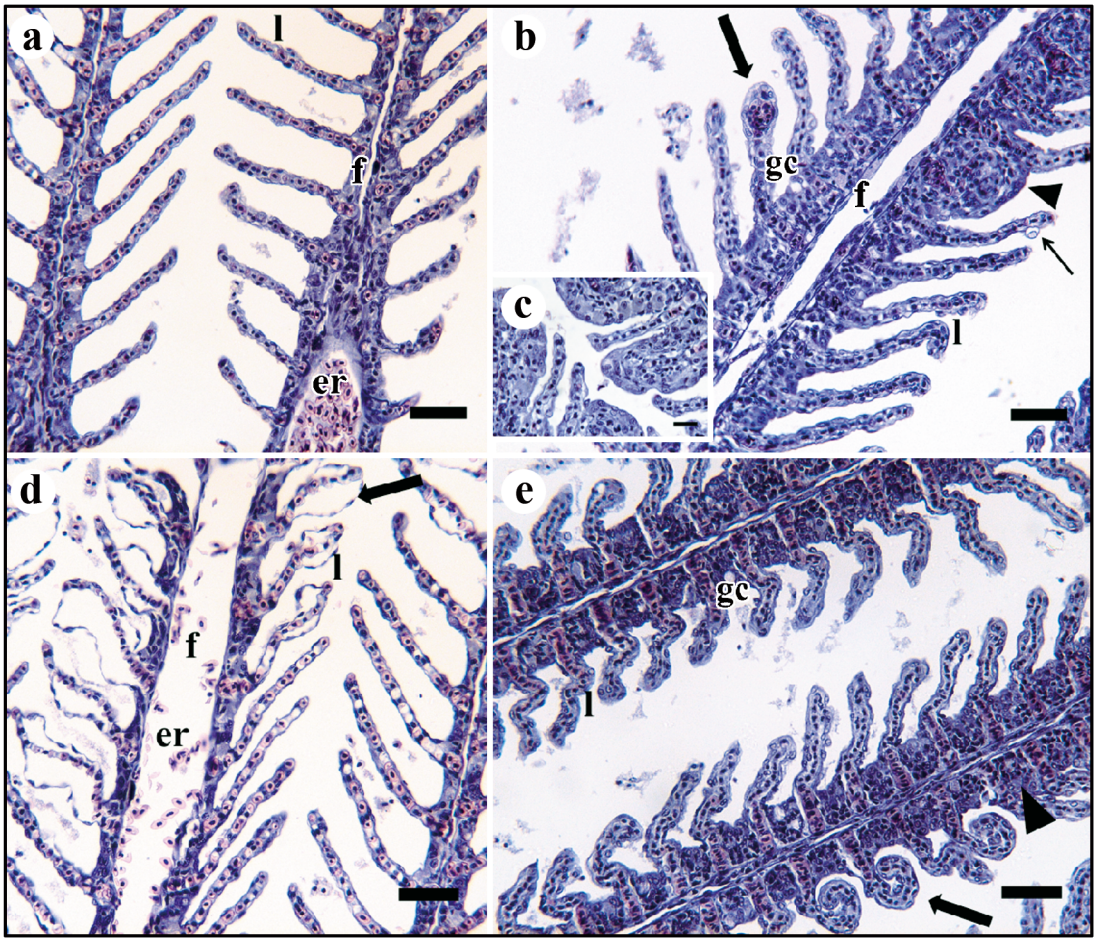

Fig. 2. Representative micrographs of the gills of juvenile *Sparus aurata* exposed to control or high temperatures. (a) Normal structure of the gills at control temperature (18°C). (b) Gill filament showing a lamellar edema (thick arrow), hyperplasia (arrow head), and hypertrophied chloride cell (thin arrow) (32°C). (c) Detail of several hyperplasic foci (34°C). (d) Epithelial lifting of lamellae (34°C). (e) Convoluted lamellae (arrow) at the critical thermal maximum temperature (35.5°C) and moderate hyperplasia in the interlamellar space (arrow head); f: filament, l: lamellae, er: erythrocyte, gc: goblet cell. Scale bars: (a, b, d, e) 25 µm; (c) 15 µm

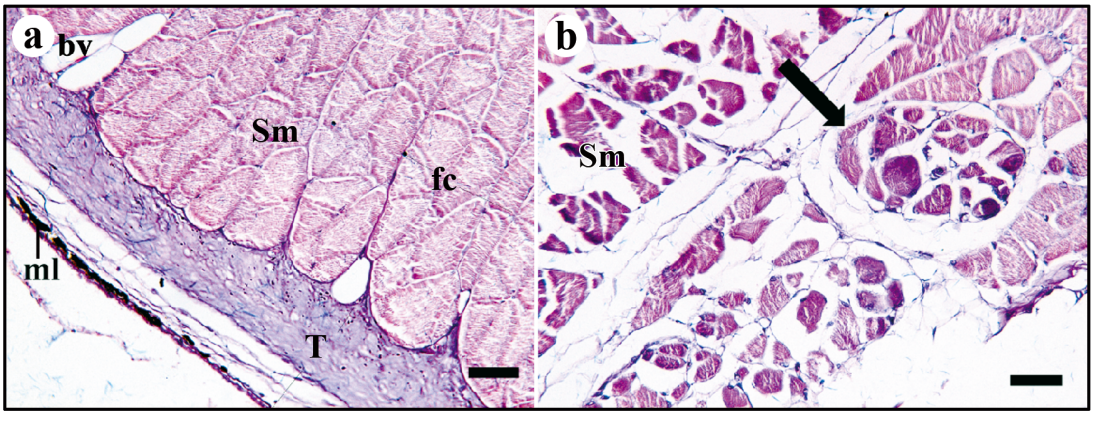

Fig. 3. Representative micrographs of the muscle of juvenile *Sparus aurata* exposed to control or high temperatures. (a) Normal structure of skeletal muscle at control temperature (18°C) with striated myocytes (Sm) grouped into fascicles (fc) and surrounded by a sheath of connective tissue. Blood vessels (bv) are evident between fascicles and adjacent to the tegument (T), in which melanin is visible (ml). (b) Muscle at the critical thermal maximum temperature (35.5°C) showing localized atrophy of muscle fibers, probably caused by focal necrosis or autophagy. Scale bars: 25 µm

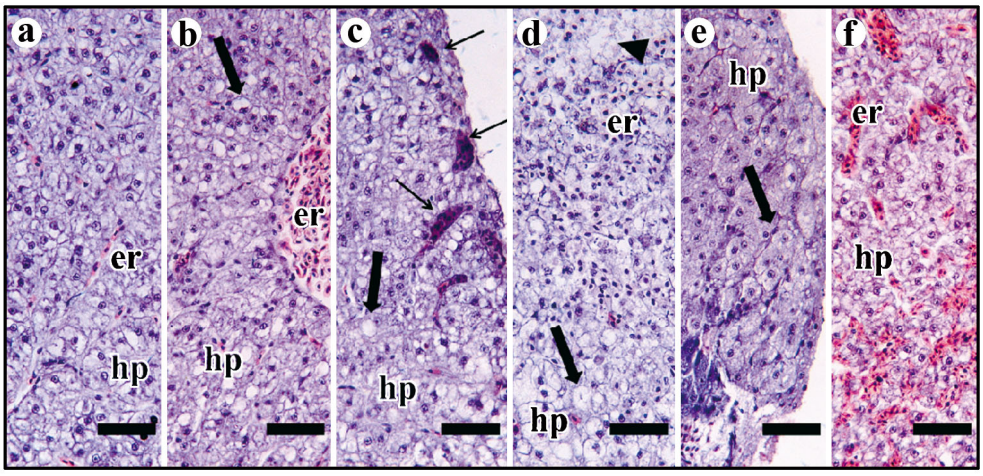

Fig. 4. Representative micrographs of the liver of juvenile *Sparus aurata* exposed to control or high temperatures. (a) Normal structure of the liver at control temperature (18°C) consisting of parenchymal tissue composed of polygonal hepatocytes (hp) with round nuclei (1 or 2) and conspicuous nucleoli. Sinusoids with few erythrocytes (er) are visible in the parenchyma. (b) Fat vacuoles (arrow) and slight hyperemia in hepatic vessels (26°C). (c) Fat vacuoles (thick arrow), hyperemia, and melanomacrophage aggregates (thin arrows) (28°C). (d) Fat vacuoles (thick arrow), small necrotic foci in the parenchyma (arrow head) involving hyperemia, micro-hemorrhage, and melanomacrophage infiltration (30°C). (e) Moderate hepatocellular alteration (arrow) and melanomacrophage infiltration (34°C). Note the alteration to hepatocyte size and shape. (f) Increased hyperemia and hemorrhage, with hepatocytes clearly more eosinophilic (at the critical thermal maximum temperature of 35.5°C). Scale bars: 25 µm

staining, probably indicative of a change in function; Fig. 4e). When organisms reached their CTMax, there was a boost in hyperemia, and degenerate hepatocytes were clearly more eosinophilic, likely indicating an early stage of hepatocellular alteration (Fig. 4f).

Pancreatic acini. Control animals (18°C) revealed normal microanatomy of pancreatic acini, consisting of highly basophilic (with zymogen granules) cells arranged concentrically in clusters (Fig. 5a). Pancreatic tissue infiltrated the hepatic parenchyma, forming the hepatopancreas typical of this species and other teleosts. Overall, this tissue yielded lesions that developed in a gradual mode as temperature increased, similar to the liver. Occasional alterations started to occur at 24°C, namely moderate hyperemia and macrophage infiltration, which may indicate the beginning of an inflammation process (Fig. 5b). At 28°C, vacuolization of acinar cells became evident, accompanied by hyperemia (Fig. 5c). At temperatures around 30–32°C, agglomerates of lipofuscin-like pigments were visible inside pancreatocytes and nearby melanomacrophages (Fig. 5d). At 34°C and CTMax, the acini were atrophied, showing diffuse inflammation and necrosis (Fig. 5e,f).

Intestine. Control animals (18°C) exhibited the normal intestinal architecture, consisting of mucosal folds lined internally by microvilli-bearing absorptive cells and goblet cells that secrete mucin (which, in contact with water, forms mucus). Underneath the epithelial layer, a thin layer of connective tissue in the middle (the lamina propria) was visible, with scattered lymphocytes inside adjacent lymph vessels (Fig. 6a,b). The muscularis of the mucosa was also visible as well as the tunica submucosa, tunica muscularis externa, and tunica serosa at the edge. At low temperatures (20 and 22°C), no alterations were visible except for occasional epithelial vacuolization. Lesions started to become visible in fish subjected to 28 and 30°C, namely with atrophy of epithelial cells and defense cell infiltration within the lamina propria, accompanied by a slight hyperemia, which became more prominent at higher temperatures (Fig. 6c,d). At 34°C, hyperplasia of absorptive cells seemed to occur, as well as moderate focal necrosis in the muscle layer, which was more obvious in animals subjected to CTMax.

DISCUSSION

Sparus aurata juveniles had a CTMax value of 35.5 ± 0.5°C. As gilthead seabream is a warm temperate marine species and juveniles inhabit warmer waters of estuaries and coastal lagoons, a relatively high CTMax value was expected. Living in these confined habitats, with a lower thermal inertia (in comparison

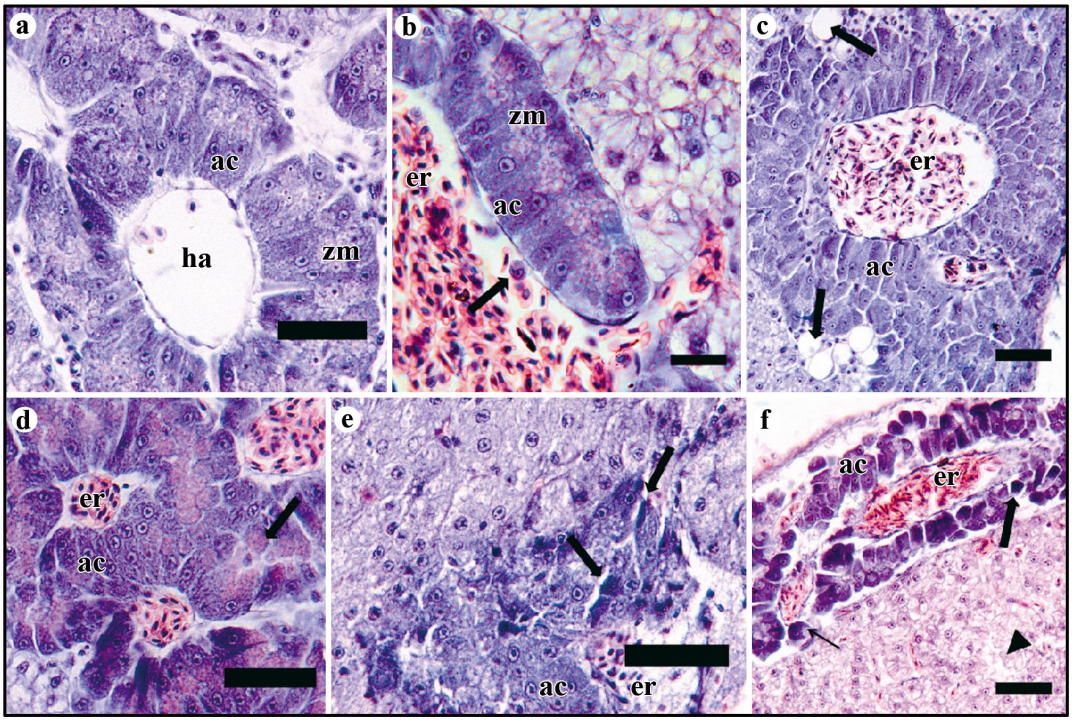

Fig. 5. Representative micrographs of the hepatopancreas of juvenile *Sparus aurata* exposed to control or high temperatures. (a) Normal structure of the pancreas at control temperature (18°C) consisting of acini composed of highly basophilic acinar cells (ac) (with zymogen granules, zm) arranged concentrically in clusters. Hepatic arterioles (ha) are visible holding few erythrocytes. (b) Detail of the pancreas at 24–26°C showing some hyperemia (erythrocytes, er) and leucocytes (arrow). (c) Vacuolization of acinar cells (arrows) accompanied by hyperemia (28°C). (d) Detail of lipofuscin-like pigments (arrow) accumulated in acinar cells (30–32°C). (e) Pancreatic tissue at 34°C with atrophied acini, showing diffuse inflammation and necrosis (arrows). (f) Macrophage infiltration (thick arrow) and hyperemia at the critical thermal maximum temperature (35.5°C). Lipofuscin-like pigments (thin arrow) and necrotic liver tissue (arrow head) are visible. Scale bars: (a, c, d, e, f): 25 µm; (b) 15 µm

to the sea), increases the probability of exposure to high temperature conditions. Thus, this species must be adapted to the thermal regime of estuaries and coastal lagoons. The intraspecific variability in CTMax was somewhat low, which is in accordance with other studies (Mora & Ospina 2001, Madeira et al. 2012a, Vinagre et al. 2013c).

Hsp70 levels significantly increased with rising temperature in several tissues (i.e. gills, muscle, brain, intestine), starting at warmer temperatures of ≥30°C. Hsp70 levels generally undergo a gradual increase until they reach a maximum amount and start to decrease as the thermal stress becomes more extreme and exhaustion of the biological system starts to take place. This is a common pattern that has been identified in other marine species (Madeira et al. 2012b). There are some exceptions, considering that neither hepatopancreas nor intestine followed this trend. Total ubiquitin levels showed significant increases from control (18°C) in gills and muscle at temperatures ≥32°C. In other marine organisms, biochemical stress is indicated by an increase in Hsps and ubiquitin levels, and these proteins can be overexpressed in response to thermal stress (Hofmann & Somero 1995, Iwama et al. 1998, Feder & Hofmann 1999). During thermal stress, an increase in denaturation and misfolding of proteins occurs, creating a demand for chaperoning activity and/or protein degradation mechanisms. However, ubiquitin levels are not always increased during thermal stress, probably due to adaptation to stressful habitats (i.e. the concentration of Hsps is adequate to remediate protein denaturation, reducing irreversible damage to proteins; Berger & Emlet 2007).

In the present study, Hsp70 and ubiquitin levels varied according to the tissue examined. These results are in accordance with several other studies in marine species (i.e. Feidantsis et al. 2009, Yamashita et al. 2010). In the current study, gills showed the highest amount of Hsp70 and also high amounts of total ubiquitin which may be due to direct contact with water. As warm water passes directly through the gills, they are more exposed to heat. High levels of these proteins may help this tissue maintain its

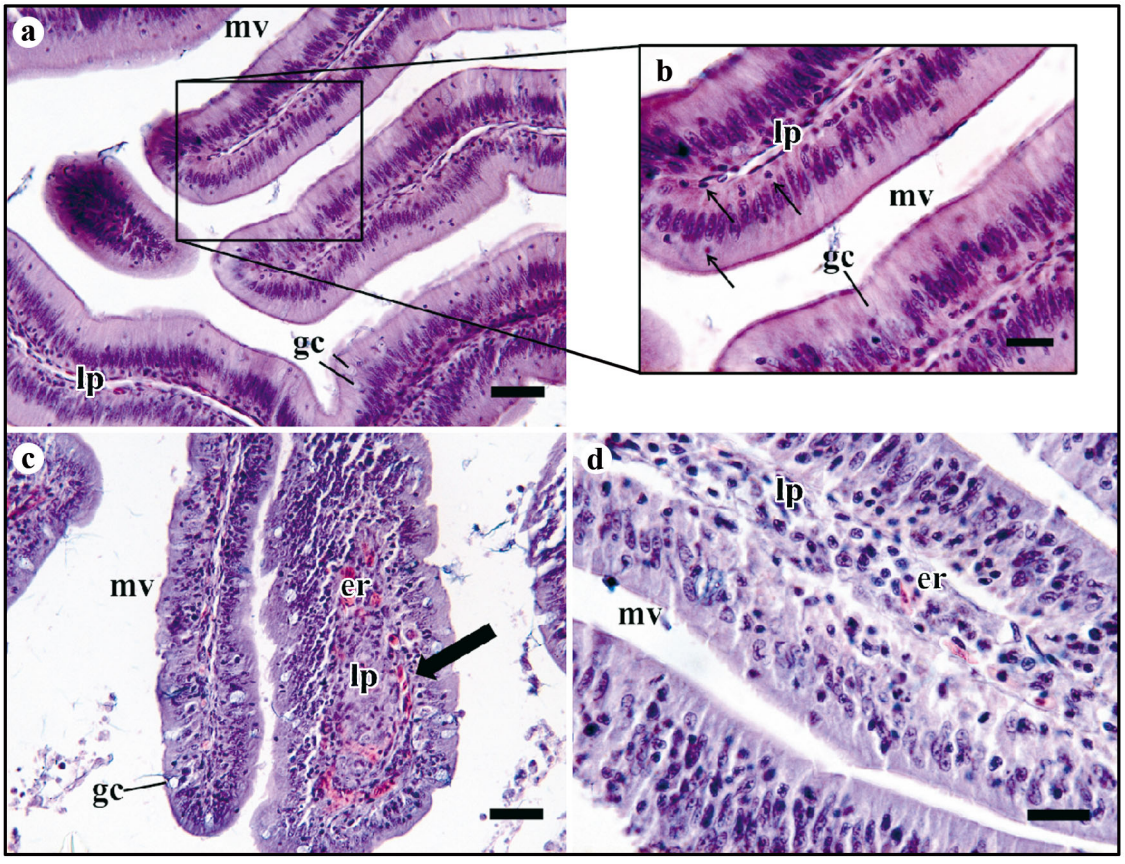

Fig. 6. Representative micrographs of observed histopathological alterations in the intestine of juvenile *Sparus aurata* exposed to control or high temperatures. (a, b) Normal intestinal architecture (18°C) consisting of mucosal folds lined internally by microvilli (mv) bearing absorptive cells and goblet cells (gc). Underneath the epithelial layer lies the lamina propria (lp) with scattered lymphocytes (thin arrows in b) inside adjacent lymph vessels. (c, d) Atrophy of epithelial cells, lymphocyte infiltration, dilated lamina propria (caused by fluid retention), and hyperemia (arrow) at 34°C. Scale bars: (a, c) 25 µm; (b, d): 15 µm

integrity and vital functions (i.e. gas exchange, osmoregulation, and acid-base regulation). This may be achieved by chaperoning activity of Hsp70 and by triage events, preventing irreversibly abnormal proteins to accumulate. Increased Hsp70 can also reduce abnormal protein accumulation by increasing protein degradation (Dul et al. 2001). In addition, maximum Hsp70 and ubiquitin level peaks in gills occurred only at 34°C. This may indicate that gills are able to deal with high temperatures. In fact, according to histopathological results, structural alterations only start taking place between 28 and 30°C, peaking at 34°C and CTMax, supporting the biochemical biomarker results. Other studies in freshwater species (e.g. Sollid et al. 2005, Sollid & Nilsson 2006) have shown that gill structure is altered under high temperatures; however, those were major restructuring events, different from the reported alterations in this study. In addition, our results showed that Hsp70 and total ubiquitin were significantly correlated in gills, indicating that there may be an important interaction of these 2 biomarkers in the protein quality control system.

Brain and muscle had the second highest Hsp70 levels but showed equivalent ubiquitin levels when compared to gills. Brain tissue showed signs of biochemical stress earlier than muscle, when water temperature reached 30°C (Hsp70 peak). However, in relation to total ubiquitin levels, brain and muscle showed different responses: brain did not show any increases with higher temperature, unlike muscle (peak at 32°C). Thus, muscle apparently processes higher amounts of irreversibly damaged proteins than brain. In addition, the tissue-specific expression of stress proteins might be due to different rates of tissue protein synthesis (Kawabe & Yokoyama 2009). Interestingly, Sayegh & Lajtha (1989) showed that protein synthesis in brain is approximately 3 times higher than in muscle in all species examined (goldfish, bullfrog, white Leghorn chicken, mouse, and

Tokay lizard). That study and another study by Lajtha & Sershen (1975) also showed that protein metabolic rates increase with temperature in the brain of fishes and lizards. The increase in Hsp70 in the brain during thermal stress may be necessary due to the high amount of proteins there and their higher metabolic rate. In muscle, which is an organ of low protein turnover rate (Loughna & Goldspink 1985), the cytoprotective efforts may be directed towards the maintenance of existing proteins.

The fact that Hsp70 and ubiquitin were correlated in muscle but not in brain tissue may support this hypothesis of tissue-specific protein expression and metabolism. Histopathological results did not show any gross lesions in muscle, only atrophy of some muscle bundles at higher temperatures (34°C and CTMax). In fact, some degree of atrophy of muscle fibers at higher temperatures has also been shown in other organisms including mammals (Wang & Chen 1999). In addition, Garcia de la serrana et al. (2012) found persistent effects of development temperature on muscle growth patterns during early life stages of *S. aurata* (20% more fibers of lower average diameter at low temperature, i.e. 17.5–18.5°C, than at high temperature, i.e. 21–22°C; myogenesis and gene expression patterns are not fixed during growth). Furthermore, the responses in brain and muscle may be linked to the loss of equilibrium. This loss of righting reflex may indicate that nerve impulse transmission is disturbed (Aslanidi et al. 2008). As described above, brain started to show signs of stress at 30°C and muscle at 32–34°C. The combination of brain damage with an abnormal amount of aberrant proteins in the muscle plus fiber atrophy led to the loss of equilibrium at 35–36°C. This explains why the CTMax value was 35.5°C.

Hepatopancreas and intestine showed equivalent amounts of Hsp70 but showed different response patterns, as intestine had a significant increase at CTMax while hepatopancreas did not show any increase. In addition, hepatopancreas had lower total ubiquitin levels. These results somewhat match the results of Feidantsis et al. (2013), who failed to find significant changes in Hsp70 in the liver of *S. aurata* exposed to seasonal temperature changes. However, these same authors reported changes in Hsp90, so perhaps other chaperones are more important in the liver. Moreover, Hsp70 and Hsp90 expression is tissue- and species-specific, which may explain the different responses (see Deane & Woo 2003). Nevertheless, liver and pancreatic acini started to show histopathological lesions at 26–28°C, with more significant alterations (inflammation and necrosis) at 32–34°C. Additionally, intact hepatocytes showed some degree of hypertrophy and eosinophilia, which may be regarded as a change in function, reflecting an alteration of regular metabolic pathways.

Hsps confer thermal tolerance (Moseley 1997). Still, this adaption is of short duration (hours to days) and undergoes a fast decline when Hsp contents start to decrease (Moseley 1997, Kregel 2002). Therefore, our results may indicate that *S. aurata* is particularly sensitive to prolonged exposures to elevated water temperatures (i.e. ≥28–30°C). This result is supported by other studies (e.g. Feidantsis et al. 2009), in which *S. aurata* started to show signs of stress at temperatures above 20°C and were unable to acclimate to temperatures beyond 26°C. These results were based on the analysis of several chaperones, kinases, dehydrogenases, and citrate synthase. The authors also concluded that 30°C is the critical threshold for this species (yielding a high increase in mortality, lactate accumulation, high Hsp70 levels, and behavioral alterations). Even though the study of Feidantsis et al. (2009) was a 10 d experiment, their results somewhat match our study. Nevertheless, Requena et al. (1997) stated that after a temperature rise from 20 to 28°C (1°C d^{-1}), the basal metabolism (oxygen consumption) of *S. aurata* was compensated within 2 wk. However, other studies (Madeira et al. 2012a, Vinagre et al. 2012a,b,c, 2013a,b) concerning different species (i.e. *Dicentrarchus labrax, Solea senegalensis*, and *Diplodus* sp.) also showed no acclimation to temperatures of 27–28°C, potentially rendering environmental heat waves detrimental to juvenile fish. Thus, there still might be some controversy about the ability of warm-temperate fish to acclimate to high temperatures. It is also reasonable to consider that fish are very mobile organisms and thus may escape stressful conditions. Nonetheless, considering that the growth of juveniles occurs mainly in estuaries or lagoons, they might be more restrained to these shallow warm habitats during this phase, limiting their escape possibilities (as shown in other species of comparable size, life cycle, and ecological requirements, see Vinagre et al. 2008, 2011). In the Tagus estuary (the largest in Western Europe), mean water temperature during the winter is 12°C, increasing to 24°C during the summer (Madeira et al. 2012a). The seasonal variation in temperature is thus 12°C, and the fish may be exposed to extreme temperatures during heat wave events. In a climate warming scenario, water temperature is expected to increase 2°C by 2100. During current heat waves, water temperature attains 28°C in several locations (e.g. Portuguese waters: Vinagre et al. 2012a; Mediterranean:

Damianidis & Chintiroglou 2000), which means that by 2100, the seawater temperature may remain at 30°C for several weeks. As mentioned above, at this temperature, *S. aurata* juveniles show significant signs of stress, with adverse consequences to their health, growth, and therefore individual and populational fitness. In addition, as *S. aurata* is a very important commercial species, fishing pressure may also contribute to the negative impact upon *S. aurata* populations. Moreover, this species is one of the most important in saline and hypersaline aquaculture in the Mediterranean area (Feidantsis et al. 2009, 2013). It can be farmed in coastal ponds and lagoons, with extensive and semi-intensive methods, or in land-based installations and in sea cages, with intensive farming systems. In many cases, the fish are kept in low volumes of water and shallow depths, which makes the animals rather susceptible to warming. This potentially affects the health status of the fish and the quality of the end-product, with obvious impacts on this economic activity.

It is also relevant to mention that thermal windows may differ between life stages (Pörtner & Farrell 2008). According to these authors, juveniles have the greatest aerobic thermal window. Thus, eggs, larvae, and adults may experience physiological and molecular changes earlier than juveniles. Moreover, Polo et al. (1991) showed that eggs of *S. aurata* did not hatch at high temperatures such as 30°C, and outside the range of 16–22°C, mortality and abnormalities increased significantly. Georgakopoulou et al. (2010) also detected a significant effect of water temperature on the development of several deformities in *S. aurata*, such as inside folded gill-cover, hemal lordosis, and malformations of the caudal and dorsal fin-supporting elements. Therefore, it is important to evaluate other life stages in relation to biochemical and physiological alterations resulting from exposure to increased temperature.

CONCLUSIONS

The current work demonstrates that it is extremely important to know the level of impact of rising seawater temperature, either due to natural environmental change or anthropogenic disturbances, on *Sparus aurata*, since it has great commercial value. From this perspective, studies considering other life stages of *S. aurata* are currently being performed by our team, and these may be crucial to understand the population dynamics, recruitment success, survival rates, fitness, and ultimately the vulnerability and resilience of the species to climate and anthropogenic change. These results may be important from an environmental management perspective considering a bottom-up approach. In detail, subcellular (e.g. proteins) and intermediate-level biomarkers (e.g. histopathological alterations) may serve as early warning signals of stress and may help to understand the health status of the individual. Responses occurring at higher levels of biological organization (population, ecosystem) can ultimately be linked to biomarkers. Integrating biomarker information for several life stages can thus be important in predicting impacts at an ecological level. All together, the information can influence decision makers to improve marine resource sustainability by constraining catch limits (in all or specific life stages) and applying financial penalties to those with unsustainable practices, or giving incentives to those with sustainable ones (see Roberts & Brink 2010 for more detailed information on marine resources management).

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