



Energetic, antioxidant, inflammatory and cell death responses in the red muscle of thermally stressed *Sparus aurata*

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Abstract

For a comprehensive understanding of fish responses to increasing thermal stress in marine environments, we investigated tissue energetics, antioxidant levels, inflammatory and cell death responses in *Sparus aurata* (gilthead seabream) red muscle during exposure to elevated temperatures (24 °C, 26 °C, 30 °C) compared to the control temperature of 18 °C. Energetic aspects were assessed by determining lactate, glucose and lipids levels in blood plasma, ATP, ADP and AMP levels, and AMPK phosphorylation as an indicator of regulatory changes in energy metabolism, in tissue extracts. Oxidative defence was assessed by determining superoxide dismutase, catalase and glutathione reductase maximum activities. Moreover, xanthine levels were determined as an indicator of purine conversion to xanthine and associated ROS production. In the context of inflammatory response and cell death due to oxidative stress, pro-inflammatory cytokines (IkB α phosphorylation, IL-6 and TNF α) levels, and LC3 II/I ratio and SQSTM1/p62 as indicators of autophagic-lysosomal pathway were also determined. A recovery in the efficacy of ATP production after a marked decrease during the 1st day of exposure to 24 °C is observed. This biphasic pattern is paralleled by antioxidant enzymes' activities and inflammatory and autophagy responses, indicating a close correlation between ATP turnover and stress responses, which may benefit tissue function and survival. However, exposure beyond 24 °C caused tissue's antioxidant capacity loss, triggering the inflammatory and cell death response, leading to increased fish mortality. The results of the present study set the thermal limits of the gilthead seabream at 22–24 °C and establish the used cellular and metabolic indicators as tools for the definition of the extreme thermal limits in marine organisms.

Keywords Fish · Temperature · Metabolic responses · Antioxidant responses · Inflammation · Cell death

Introduction

In the last decades, while temperature has been rising in the Mediterranean Sea, several marine areas have been identified as climate change “hotspots” with respective effects on marine biodiversity and productivity (Bethoux et al. 1990;

Calvo and Marsh 2011; Gambaiani et al. 2009; Nicholls and Hoozemans 1996). The gilthead seabream (*Sparus aurata*), one of the commercially most important species in saline and hypersaline aquaculture in the Mediterranean Sea, is a potent candidate for investigations of its physiological and molecular stress responses to warming. *S. aurata* is found in the northeast and central-eastern Atlantic Ocean, the Mediterranean and the Black Sea, in brackish and seawater in a depth of 1–150 m. The gilthead seabream, belonging to a trophic level of ≥ 2.8 , is a predator that feeds mainly on macrofauna (zoobenthos) (Bauchot and Hureau 1990). It is a protandric hermaphrodite species, maturing first as male (during the first or second year of age) and after the second or third year of age, as female. Spawning happens generally from October to December, with sequenced spawning during the whole period (Sadovy de Mitcheson and Liu 2008). Accordingly, investigation on the impacts of warming in the

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context of climate change on the physiological output of *S. aurata* is of great interest.

Previous laboratory investigations on metabolic, signaling and stress patterns including HSR (Heat Shock Response) and MAPKs (Mitogen Activated Protein Kinases) showed that *S. aurata* reaches its upper thermal limits at 22–24 °C which corresponds to the upper “*pejus temperature*” (OCLTT concept), at which aerobic performance becomes constrained (Feidantsis et al. 2009, 2013, 2015; Pörtner 2010). Heat-induced biochemical responses (e.g. HSR) are closely related to secondary responses of the fish including changes in the metabolism of carbohydrates, proteins and lipids. These secondary stress responses are believed to be adaptive mechanisms enabling the fish to cope with the stress and defend homeostasis (Burton 2002). As an increase in temperature can lead to metabolic activation and subsequently to the initiation of oxidative stress, combined with an increase in oxygen consumption (Abele and Puntarulo 2004), the investigation of the possible interactions between metabolic responses and oxidative stress, is of great interest. Moreover, oxidative damage and the associated mitochondrial dysfunction beyond thermal limits (critical or denaturation temperatures) may result in impaired cellular function (Li et al. 2014; Madeira et al. 2013), leading to cell signaling disruption, extensive DNA damage and therefore cell death (Chandra et al. 2000). As a result, autophagy contributes in cleaning misfolded proteins and destroying organelles, resulting to the recycling of substrates and nutrients, thus

increasing evidence that autophagy is involved in metabolic regulation (Dodson et al. 2013; Hardie et al. 2016). Although autophagy can serve as an anti-apoptotic process, by removing mitochondria damaged by oxidative stress and by the generation of nutrients (Dodson et al. 2013; Portt et al. 2011), when prolonged or in certain developmental procedures autophagy can play a pro-death role and cells will eventually succumb to cell death. Recent field investigation revealed that oxidative and probably inflammatory responses in the oxidative tissues of *S. aurata* are elicited by high summer sea temperatures in the marine area where this species is farmed (Feidantsis et al. 2018). Moreover, transcriptomic analyses in other fish species also indicate inflammatory responses under exposure to thermal stress (Jeffries et al. 2016; Lewis et al. 2010; Windisch et al. 2014). Such responses might be correlated with energy turnover or oxidative stress responses (Bowden et al. 2007; Hakim 1993).

However, little is known about the link between energy turnover, inflammation and antioxidant capacity in fish thermal tolerance. Therefore, the present study aimed to complement the existing integrated picture of cellular stress responses in a highly oxidative tissue, such as red muscle of the gilthead seabream to increased temperature (Feidantsis et al. 2009, 2013, 2015) (Fig. 1). For an assessment of energetics, levels of blood plasma metabolites such as glucose, triglycerides and L-lactate were analysed. In addition, the levels of adenylates (ATP, ADP and AMP) and the AMP/

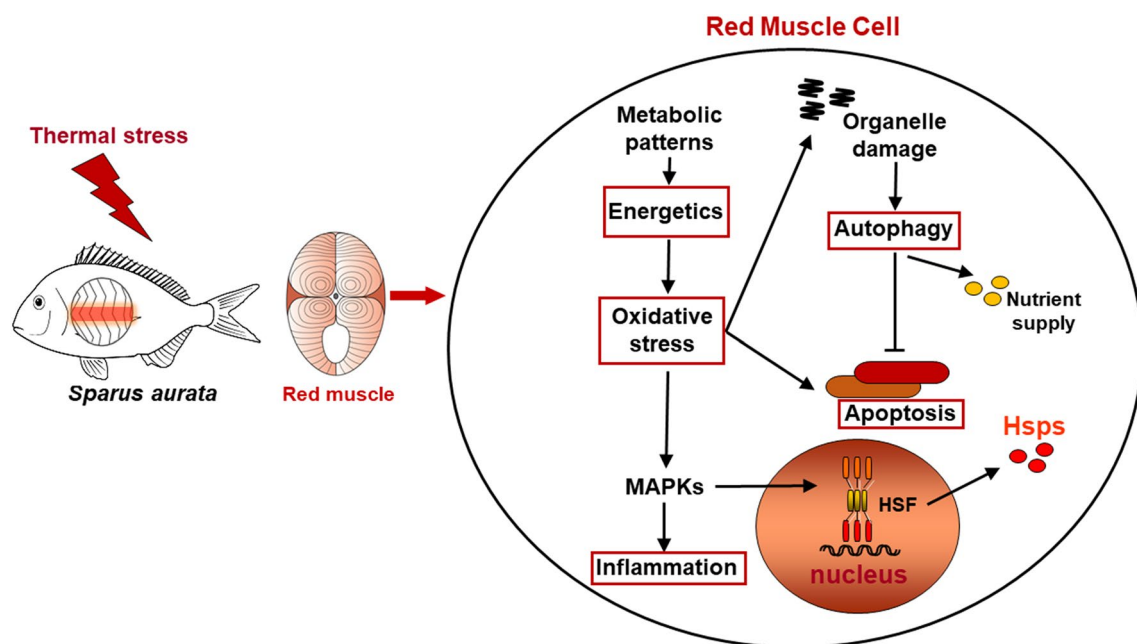


Fig. 1 A model of the cellular responses elicited by thermal stress in *S. aurata*. The present study aimed to complement the existing integrated picture (Feidantsis et al. 2009, 2013, 2015) of stress responses

in a highly oxidative tissue, such as red muscle of the gilthead seabream to increased temperature, by further or newly investigating cellular and biochemical parameters (red squares)

ATP ratio were determined in red muscle. AMP-activated protein kinase (AMPK) is a highly conserved master regulator of metabolism, which restores energy balance during metabolic stress both at cellular and whole-organism levels (Hardie 2004; Hardie and Sakamoto 2006; Hardie et al. 2016). Therefore, AMPK levels and its activation by phosphorylation in relation to an elevated AMP/ATP ratio were determined. Moreover, recent field study revealed elevated xanthine oxidase (XO) activity during summer (Feidantsis et al. 2018). Under conditions of tissue hypoxia, ATP is broken down to ADP, AMP, adenosine, inosine, and hypoxanthine. The enzyme XO generates oxygen free radicals as hypoxanthine is further metabolized to uric acid in the final steps of purine degradation (Barsotti and Ipata 2004; Farthing et al. 2015). Thus, the levels of xanthine in the red muscle were determined as an indicator of ATP degradation to adenosine triggering the production of Reactive Oxygen Species (ROS) and resulting in oxidative stress. These relationships also stimulated the determination of the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR) and catalase. To examine whether such stress responses are correlated with inflammatory phenomena we determined well-established indicators of inflammation: the phosphorylation of I κ B α , which releases the NF- κ B/Rel transcription factors, which in turn trigger the release of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF α). Since oxidative stress is closely related to apoptotic and autophagic phenomena, we determined Bax/Bcl-2 ratio as an indicator of apoptosis, and LC3 II/I ratio and SQSTM1/p62 as indicators of autophagic-lysosomal pathway.

Materials and methods

Animals

Gilthead seabream *S. aurata*, with a mean (\pm SD) weight of 275.24 ± 17 g, total length 25.23 ± 1.32 cm, were purchased from a local hatchery (DIAS Fish Farming, Lamia, Greece), where the final part of the growing procedure entirely occurs in open-sea aquaculture units. Fish were fed regularly (daily) and until satiety. Mechanical (blower) feeders were used for

this purpose. Fish were purchased from the hatchery when the ambient seawater temperature was around 18 °C. In laboratory fish were kept in fiberglass tanks (capacity 1000 l) with recirculating aerated natural seawater. Water temperature was controlled at $18 \text{ °C} \pm 0.5 \text{ °C}$. Salinity and pH were kept at $32 \pm 3.2\text{‰}$ and 8.05 ± 0.02 , respectively.

Experimental procedures

Treatment of animals

After 2 weeks, the fish were placed in four tanks (55–60 individuals in each tank) containing 500 l of recirculating aerated natural seawater at 18 °C and remained under these conditions for 2 days. Thereafter, the water temperature of three tanks was adjusted to 24, 26 and 30 °C at a 1 °C/h rate of temperature increase in all water tanks (Bennett and Judd 1992; Bennett et al. 1997; Madeira et al. 2014). The time that the temperature reached its desired levels, was set as time 0. Fish maintained in a fourth tank at 18 °C were used as controls. The animals were kept at each temperature for 20 days. Fish were fed daily prior to the 20-day experimentation period. However, all four groups (18, 24, 26 and 30 °C) were starved throughout the experimentation period (Madeira et al. 2016). The mean (\pm SD) weight and mean (\pm SD) total length of fish before and at the end of experimentation are shown in Table 1.

Blood and tissue sampling

For blood and tissue sampling, fish ($n = 8$ at each time point) were collected from each tank at day 1, 5, 10 and 20 days and they were then gently placed into five small aquaria containing seawater at 18, 24, 26 and 30 °C, respectively. Thereafter, a concentrated solution of buffered MS-222 was slowly added to the water to a final concentration of 0.15 g/l. Within 2–3 min, the fish lost balance and could be removed from the water without struggling. Whole blood was collected according to Smith and Bell (1964). Fish were then dissected, and samples from red skeletal muscle were removed and immediately frozen in liquid nitrogen and stored at -80 °C for later analysis. All fish were designated as male.

Table 1 Mean (\pm SD) weight and mean (\pm SD) total length of *S. aurata*, before and after the 20-day exposure

Before			After	
Weight (g)	Total length (cm)	Temperature (°C)	Weight (g)	Total length (cm)
287.23 ± 19.63	24.47 ± 1.47	18	284.40 ± 16.71	26.26 ± 1.25
287.51 ± 23.05	25.70 ± 1.27	24	277.51 ± 19.15	23.71 ± 1.95
293.50 ± 22.06	26.06 ± 0.94	26	282.63 ± 21.30	24.56 ± 1.42
289.70 ± 28.47	25.35 ± 1.68	30	286.53 ± 26.74	24.67 ± 0.92

Physicochemical water parameters were measured daily as follows: salinity (g/l), O₂ (mg/l) and pH using Consort C535, Multiparameter Analysis Systems (Consort, bvba, Belgium) while NH₃ (µg/l), NO₂⁻ (µg/l) and NO₃⁻ (µg/l) were analysed using commercial kits by Tetra (Tetra Werke, Germany) (Table 2).

Analytical procedures

SDS/PAGE and immunoblot analysis

The preparation of tissue samples for SDS-PAGE and the immunoblot analysis are based on well-established protocols. Specifically, in the present study, equivalent amounts of proteins (50 µg) were separated either on 10% and 0.275% or 15% and 0.33% (w/v) acrylamide and bisacrylamide (depending on the molecular weight of the detected proteins) slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 µm, Schleicher and Schuell, Keene N. H. 03431, USA). All nitrocellulose membranes were dyed with Ponceau stain to assure a good transfer quality and equal protein loading. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies. Antibodies used were as follows: anti-phospho AMPK (2523, Cell Signaling), anti-phospho IκB α (sc-8404, Santa Cruz Biotechnology), anti-IL-6 (CSB-PA06757A0Rb, Cusabio), anti-TNFα (CSB-PA07427A0Rb, Cusabio), monoclonal rabbit anti-LC3B (3868, Cell Signaling), polyclonal rabbit anti-p62/SQSTM1 (5114, Cell Signaling), anti-Bcl2 (7973, Abcam), anti-Bax (B-9) (2772, Cell Signaling) and anti-β-actin (3700, Cell Signaling).

Determination of ATP, ADP and AMP

For metabolite extraction, 200–300 mg of frozen tissue were ground under liquid nitrogen and extracted using ice-cold 0.6 M perchloric acid (PCA) containing 150 mM EDTA as described elsewhere (Sokolova et al. 2012). Neutralized, deproteinized PCA extracts were stored at -80 °C and used to determine concentrations of tissue metabolites using standard spectrophotometric NADH- or NADPH linked enzymatic assays at 340 nm absorbance wavelength (Adam 1963; Lamprecht and Trautschold 1963).

Determination of plasma metabolites

The plasma metabolites glucose, lactate and triglycerides were measured using commercial kits based on enzymatic–colorimetric methods from Spinreact, Spain (glucose kit, cod. 1001191; triglycerides kit, cod. 1001312; lactate kit, cod. 1001330).

Determination of xanthine

Xanthine levels in the red muscle were measured using a commercial kit (MAK 186) from Sigma-Aldrich (St. Louis, USA).

Determination of activities of antioxidant-enzymes in the tissue homogenates

Supernatant of homogenised tissues where the activities of mitochondrial enzymes were measured was prepared according to Salach (1978). SOD (total activity of mitochondrial Mn- and cytosolic Cu/Zn-superoxide dismutase, SOD EC 1.15.1.1) was assayed according to the protocol described in Paoletti and Mocali (1990). Catalase V_{max} (CAT, EC 1.11.1.6) was determined according to the method of Cohen et al. (1970) and Glutathione reductase V_{max} (GR, EC 1.8.1.7) was determined according to the method of Carlberg and Mannervik (1985).

Statistics

Changes in the phosphorylation of AMPK, ATP, ADP and AMP levels, xanthine levels, antioxidant enzyme activities, plasma metabolites levels, levels of inflammation, apoptosis and autophagy indicators were tested for significance at the 5% level by using one way (GraphPad InStat 3.0) or two-way (GraphPad Prism 5.0) analysis of variance (ANOVA) depending on the number of factors tested each time, with sampling days and treatment as fixed factors. Post-hoc comparisons were performed using the Bonferroni test. Values are presented as means ± SD. Statistically significant differences were observed between all applied temperatures. Main effects of treatment and exposure time, as well as factor interactions, were significant ($P < 0.0001$).

Table 2 Mean values of sea water parameters

Temperature (°C)	Salinity (g/l)	pH	O ₂ (mg/l)	NH ₃ (mg/l)	NO ₂ ⁻ (mg/l)	NO ₃ ⁻ (µg/l)
18	33.5	8.05	7.9	0.2	0.2	< 12.5
24	33.7	8.04	7.8	0.2	0.3	13
26	33.1	8.02	7.8	0.3	0.3	14
30	33.3	8.00	7.5	0.3	0.4	14

Multivariate principal components analysis

Principal components analysis (PCA) in the FactoMineR package in R was employed to assess patterns of possibly correlated variables, and more specifically to detect how cellular stress responses varied between temperatures and exposure time.

Results

Effect of water temperature on fish morphometric characteristics

As shown in Table 1, no differences between weight and total length of the purchased fish, and the initial and the final weight and total length before and after experimentation at all temperatures were observed.

Effect of water temperature on fish mortality

Figure 2 shows the development of mortality overtime during exposure of *S. aurata* at different temperatures. As shown, exposure to 26 °C raised mortality to 5% on the 10th and at 13% on the 20th day. Fish exposed to 30 °C exhibited higher mortality, as 20–40% of fish died between 5 and 15 days of exposure. Thereafter, the mortality rose rapidly to 100%.

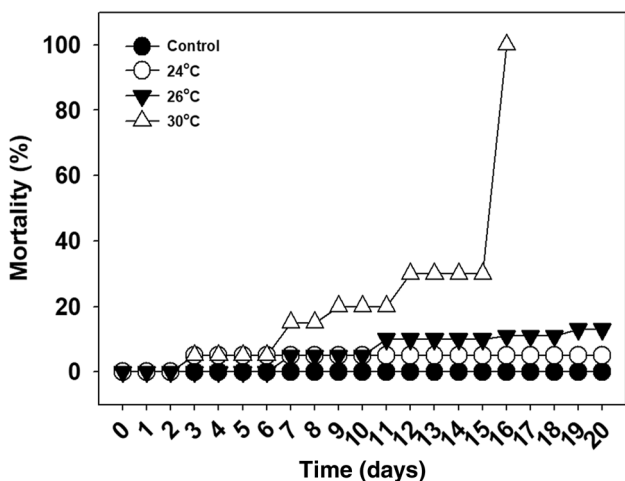


Fig. 2 Mortality (%) of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open triangle)

Effect of increased temperature on blood plasma metabolites

As shown in Fig. 3a, warming to 24 °C caused a significant increase in glucose levels after the 5th day and they remained at high levels by the 20th day. An initial drop in glucose levels and subsequent recovery to control was

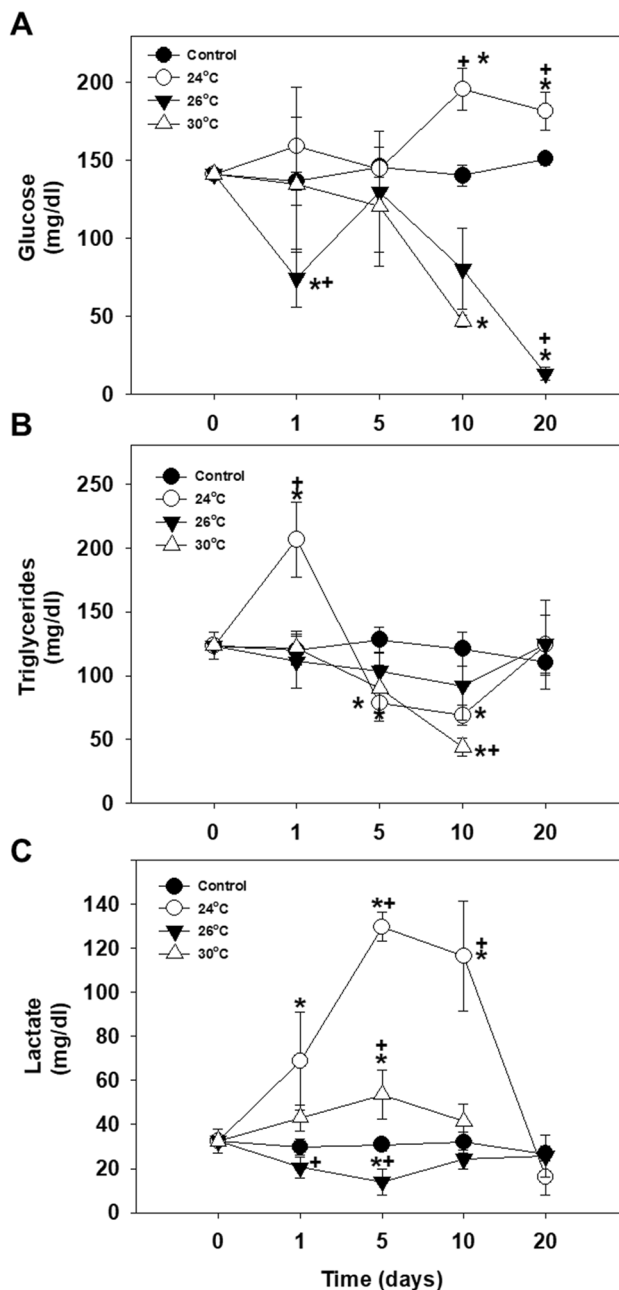


Fig. 3 Glucose (a), triglycerides (b) and lactate (c) levels in the blood plasma of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open triangle). Values are means ± SD; n = 8 preparations from different animals. *P < 0.05 compared to control, +P < 0.05 compared to other temperatures

observed within the first 5 days of exposure to 26 °C. Thereafter, glucose levels dropped markedly until the 20th day. During exposure to 30 °C, the levels of glucose showed a marked decrease after the 5th day (Fig. 3a).

A significant increase in triglyceride levels was observed within the 1st day of exposure to 24 °C. Thereafter, levels dropped below control values but recovered after the 10th day. No significant changes in triglyceride levels were observed when fish were exposed to 26 °C. In contrast, they dropped after the 5th day of exposure to 30 °C (Fig. 3b).

The patterns of lactate levels show a marked increase within the first 5 days of exposure to 24 °C. Thereafter they gradually decreased to control levels. No significant changes were observed when fish were exposed to 26 °C and 30 °C (Fig. 3c).

The main effects of treatment and exposure time, as well as factor interactions, were significant ($P < 0.0001$).

Effect of increased temperature on the levels of ATP, ADP, AMP and on AMPK phosphorylation levels

Exposure of fish to 24 °C caused a sharp decrease of ATP levels within the 1st day. Thereafter ATP levels recovered almost to control by the 20th day (Fig. 4a). In contrast to 24 °C, however, the drop of ATP levels either when fish exposed to 26 °C or to 30 °C, did not exhibit a concomitant recovery (Fig. 4a).

ADP levels started decreasing by the 10th day and thereafter recovered nearly to control levels when fish were exposed to 24 °C. On the contrary, they showed a marked decrease within the 1st day of exposure to 26 °C and thereafter started increasing gradually reaching control levels. During exposure to 30 °C, ADP levels were gradually decreasing and no recovering was observed by the 10th day (Fig. 4b).

AMP levels increased significantly on the 1st day during fish exposure at 24 °C and 26 °C. Thereafter it dropped below control levels and remained at low levels until the 20th day. However, a significant decrease in AMP levels was observed from the 1st day when fish were exposed to 30 °C (Fig. 4c).

Figure 4d depicts a significant increase in the ratio AMP/ATP mainly when fish were exposed to 24 °C, and especially on the 1st and 5th day. Thereafter the value of the above ratio dropped at control levels. Comparatively, exposure of fish to 26 °C and 30 °C resulted in milder increases.

Increased AMPK phosphorylation was observed when fish were exposed to all experimental treatments. Specifically, it gradually increased until the 10th day and thereafter dropped to control levels by the 20th day (Fig. 4e).

Main effects of treatment and exposure time, as well as factor interactions, were significant ($P < 0.0001$).

Effect of increased temperature on the levels of xanthine

Exposure of fish to 24 °C resulted in a sharp increase in the levels of xanthine in the red muscle of *S. aurata* within the 1st day, while they were gradually decreasing by the 20th day. Similarly, levels of xanthine were increased and remained at high levels by the 5th day of fish exposure to 26 °C. Thereafter they recovered to control levels. During exposure to 30 °C the xanthine levels fluctuated but remained higher than those of control by the 10th day (Fig. 5). Main effects of treatment and exposure time, as well as factor interactions, were significant ($P < 0.0001$).

Effect of increased temperature on the activity of antioxidant enzymes

SOD and catalase activities were gradually increasing and peaked at 10th day of fish exposure to 24 °C. Thereafter, they decreased at control level. On the contrary, except for a sudden increase within the 1st day, no significant changes were observed in the enzymatic activities when fish were exposed to 26 °C and 30 °C (Fig. 6a, c). Regarding GR activity, Fig. 6b depicts a marked increase within the 1st day of fish exposure to 24 °C and 26 °C and a subsequent decrease to control levels until the 5th day. Thereafter, the activity of GR exhibited a gradual increase until the 20th day. When fish were exposed to 30 °C the activity of GR remained at higher levels compared to control in a less extent compared to 24 °C and 26 °C. Main effects of treatment and exposure time, as well as factor interactions, were significant ($P < 0.0001$).

Effect of increased temperature on the inflammatory indicators

Exposure of fish to all experimental temperatures resulted in increased phosphorylation of I κ B α with the most potent changes observed at 24 °C and 26 °C, peaking on the 5th and 1st day respectively. Phosphorylation levels thereafter dropped to control levels on the 20th day at both temperatures. Exposure to 30 °C resulted in a mild increase of I κ B α phosphorylated levels only on the 10th day (Fig. 7a). Concerning interleukin 6 and TNF α levels, mostly exposure to 26 °C has resulted in elevated levels of the above-mentioned indicators, while 24 °C and 30 °C had little effect, with levels of these indicators fluctuating to control

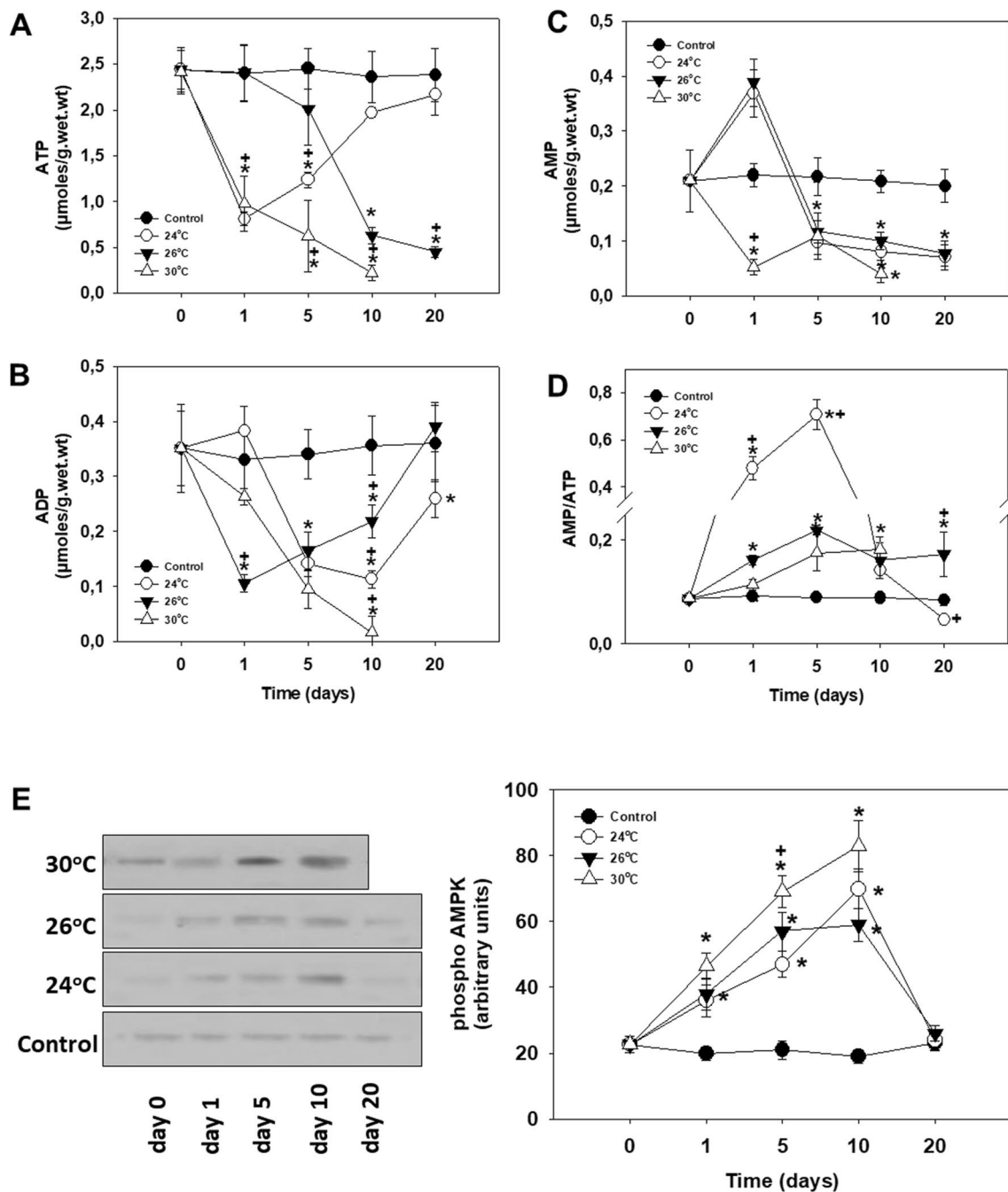


Fig. 4 Levels of ATP (a), ADP (b) and AMP (c), AMP/ATP ratio (d), and phosphorylation levels of AMPK (e), in the red muscle of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open trian-

gle). Values are means \pm SD; $n=8$ preparations from different animals. * $P<0.05$ compared to control, + $P<0.05$ compared to other temperatures

levels (Fig. 7b, c). Exposure to 26 °C resulted in the most significant changes in all three examined indicators of inflammation compared to the other two applied temperatures. Main effects of treatment and exposure time, as well as factor interactions, were significant ($P<0.0001$).

Effect of increased temperature on the indicators of apoptosis and autophagy

Exposure of fish to elevated temperatures resulted in a sharp increase of Bax/Bcl-2 ratio, indicating apoptotic phenomena

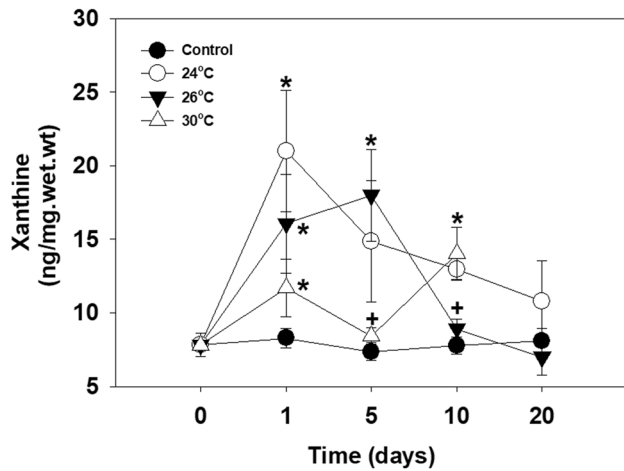


Fig. 5 Xanthine levels in the red muscle of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open triangle). Values are means \pm SD; $n=8$ preparations from different animals. * $P<0.05$ compared to control, + $P<0.05$ compared to other temperatures

(Fig. 8). Specifically, exposure to 24 °C resulted in increased values mainly on the 1st and 5th day, while exposure to 26 °C resulted in increased levels on the 1st day, dropping thereafter and increasing again on the 20th day. Similarly, Bax/Bcl-2 levels increased during exposure to 30 °C and peaked on the 5th day.

Likewise, as seen in Fig. 9, exposure of fish to increased temperatures resulted in a sharp decrease of SQSTM1/p62 levels and a sharp increase of LC3 II/I ratio, indicating the triggering of autophagy. Specifically, SQSTM1/p62 levels decreased until the 10th day during exposure to 24 °C and thereafter increased towards the 20th day. Exposure to 26 °C resulted in decreased levels up to the 20th day, while exposure to 30 °C resulted in a markedly drop on the 5th day of exposure. Then the levels started increasing towards control. LC3 II/I ratio increased at all exposure temperatures, peaking on the 1st and 5th day for 26 °C and 30 °C respectively, and on the 5th day for 24 °C. Thereafter, this ratio dropped close to control levels. Main effects of treatment and exposure time, as well as factor interactions, were significant ($P<0.0001$).

Multivariate analysis reveals strong correlations of temperature and exposure time with cellular stress responses

The PCA analysis (principal components as extraction method) was applied to statistically define differences in cellular stress responses. PC1 explained 34.85% of the variance. Specifically, the physiological variables that were positively correlated with scores on PC1 were mortality, AMPK phosphorylation, GR and the apoptotic Bax/Bcl-2 ratio. In contrast to the above, energy substrates such as ATP, ADP, AMP, triglycerides and glucose, and autophagy indicator

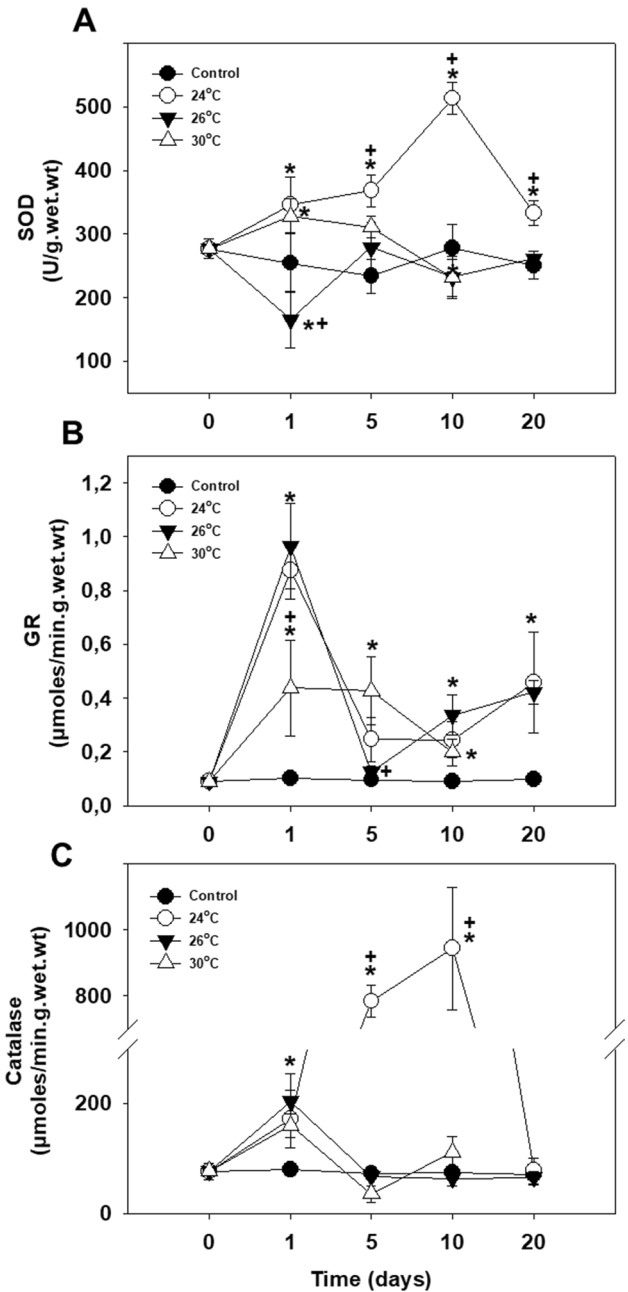


Fig. 6 Activity levels (V_{max}) of antioxidant enzymes SOD (a), GR (b) and catalase (c) in the red muscle of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open triangle). Values are means \pm SD; $n=8$ preparations from different animals. * $P<0.05$ compared to control, + $P<0.05$ compared to other temperatures

p62/SQSTM1 were negatively correlated with scores on PC1. However, mortality was not correlated with individual scores on PC2, which explained 18.81% of the variance. The cumulative value of PC1 and PC2 was 53.66% (Fig. 10b). Contribution of biochemical parameters studied according to factor loadings are presented in the embedded table in Fig. 10a.

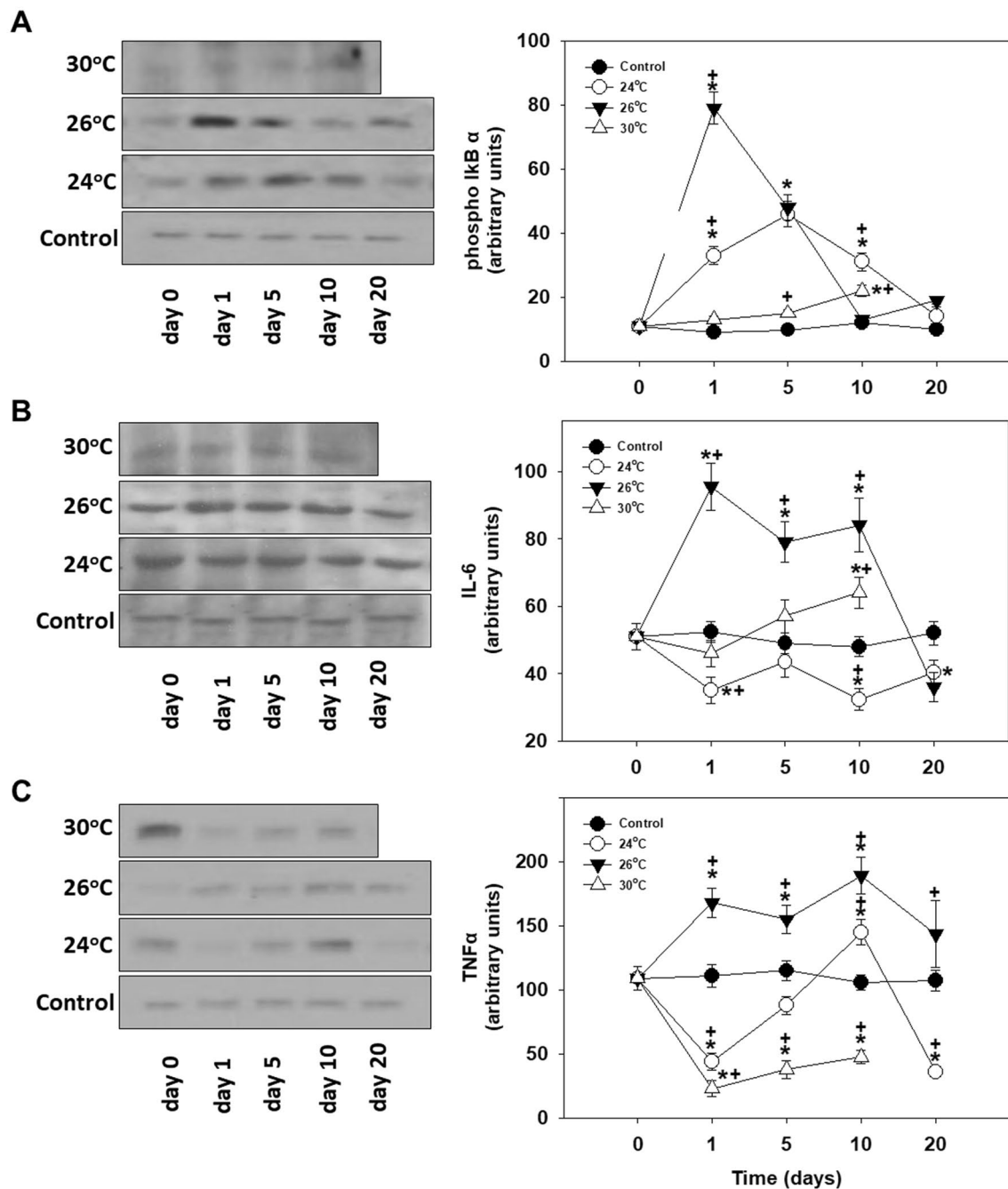


Fig. 7 Phosphorylation levels of IκBα (a), levels of interleukin 6 (b) and levels of TNFα (c) in the red muscle of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled

inverted triangle) and 30 °C (open triangle). Values are means ± SD; $n=8$ preparations from different animals. * $P<0.05$ compared to control, + $P<0.05$ compared to other temperatures

Discussion

The present data show that *S. aurata* is able to compensate for the reduction of ATP turnover after 5 days of exposure to 24 °C. However, despite of the increased phosphorylated levels of AMPK, the ability of ATP recovery is lost when fish are exposed to 26 °C and 30 °C, suggesting that temperatures

beyond 24 °C impact on the efficacy of ATP production. Moreover, the efficiency for oxidative defense seems to be diminished after exposure of *S. aurata* to temperatures beyond 24 °C, indicating a close correlation between ATP turnover and oxidative responses, which may benefit tissue function and survival. This antioxidant response, probably triggers the inflammatory and cell death response, leading

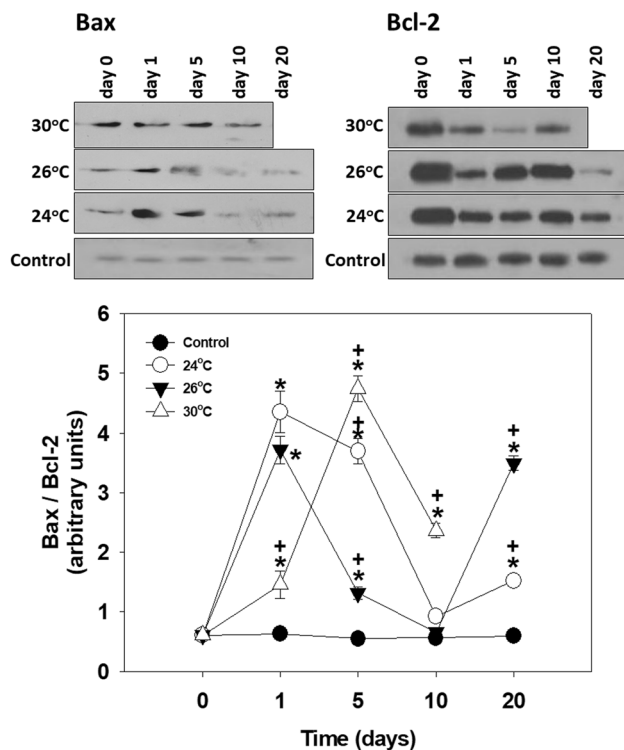


Fig. 8 Levels of Bax/Bcl-2 ratio in the red muscle of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open triangle). Values are means \pm SD; $n=8$ preparations from different animals. * $P<0.05$ compared to control, + $P<0.05$ compared to other temperatures

to increased fish mortality at temperatures beyond 24 °C. The present results complement previous findings (Feidantsis et al. 2009, 2013, 2015) on *S. aurata*, which suggest that temperature increase provokes cellular stress phenomena in the gilthead seabream, thus providing an integrated picture about cellular responses under thermal stress.

The above are consistent with previous studies indicating that temperature threshold beyond stress phenomena are developed in *S. aurata* is set at 22 °C, since cellular stress indicators are mostly profound after exposure to temperatures beyond this limit (Feidantsis et al. 2009, 2015). According to OCLTT hypothesis, increased fish mortality beyond thermal optima may be due to cardiovascular system failure and therefore insufficient oxygen delivery to tissues, resulting in insufficient ATP turnover and insufficient whole animal performance (Pörtner 2002a, b; Pörtner and Knust 2007; Pörtner and Farrell 2008; Somero 2010). This seems to be the case for the present data as acclimation at 24 °C beyond the 5th day compensates for ATP production, while higher temperatures (especially at 30 °C) diminish ATP efficacy and the ability of fish to acclimate through biochemical and cellular responses, and thus result to death. Mitochondria functionality and efficacy for ATP synthesis in fish and other aquatic ectotherms under thermal stress acclimation

is significantly depressed in mitochondria (Chung and Schulte 2015; Iftikar and Hickey 2013; Iftikar et al. 2015; Khan et al. 2014; Kraffe et al. 2007). However, when isolated mitochondrial suspensions were warmed beyond whole organism thermal limits, respiration levels remained intact (Hardewig et al. 1999; Weinstein and Somero 1998). This difference between organismal and mitochondrial level may be attributed to the fact of thermal window narrowing when molecular and mitochondrial functions are integrated into larger units up to the whole organism (Pörtner 2002a, b). This might be beneficial as temperature acute increase may result in unsustainable biochemical reaction rates and generation of ROS (Abele et al. 2002). Moreover, transcriptomic data and metabolic enzyme activity data from warm acclimated fish indicated a decline in metabolic capacity particularly in the atrium. This could be attributed to a decrease in proteins associated with glycolysis, the citric acid cycle and, especially, oxidative phosphorylation, suggesting an overall decrease in levels of proteins supporting ATP production (Jayasundara et al. 2013, 2015).

Previous studies on the enzymatic activities of intermediary metabolism indicated a reorganization of the metabolic patterns in tissues of *S. aurata* exposed to 24 °C. Specifically, increased activities of the glycolytic enzymes PK and L-LDH reflect enhanced glycolytic potential, probably due to increased energy demand for thermal stress defense (Feidantsis et al. 2009, 2015). This energy demand observed in the red muscle of *S. aurata* at 24 °C is parallel to the increase of AMPK levels within the first 10 days of exposure. However, hypoxia-induced factor (Hif-1 α) in the red muscle of warm exposed *S. aurata* and especially at temperatures higher than 24 °C (Feidantsis et al. 2013) indicates a mismatch between oxygen delivery and energy demand, thus switching aerobic to anaerobic metabolism. The latter is in line with the marked drop in the CS activity and accumulation of lactate in the red muscle of *S. aurata* (Feidantsis et al. 2009, 2015). The present data show a marked accumulation of lactate in *S. aurata* plasma when exposed to 24 °C supporting the increased glycolytic potential especially within the first 10 days of fish exposure. As reported elsewhere the expression of L-LDH in the warmth likely is the result of a stress response involving enhanced oxidative stress and a signaling function for hypoxia-inducible factor Hif-1a (Heise et al. 2006). On the other hand, the increased activity of HOAD within the first 5 days of fish exposure to 24 °C indicates a higher contribution of fatty acid oxidation to ATP turnover. However, after the 5th day of exposure the activity of HOAD decreased and CS recovered indicating a switch to a higher contribution of carbohydrate oxidation to ATP turnover (Feidantsis et al. 2015). It has been reported that limited cardiocirculatory performance is crucial for the onset of thermal limitation and the associated decrease in performance (Pörtner and Knust 2007). Increase in lipid

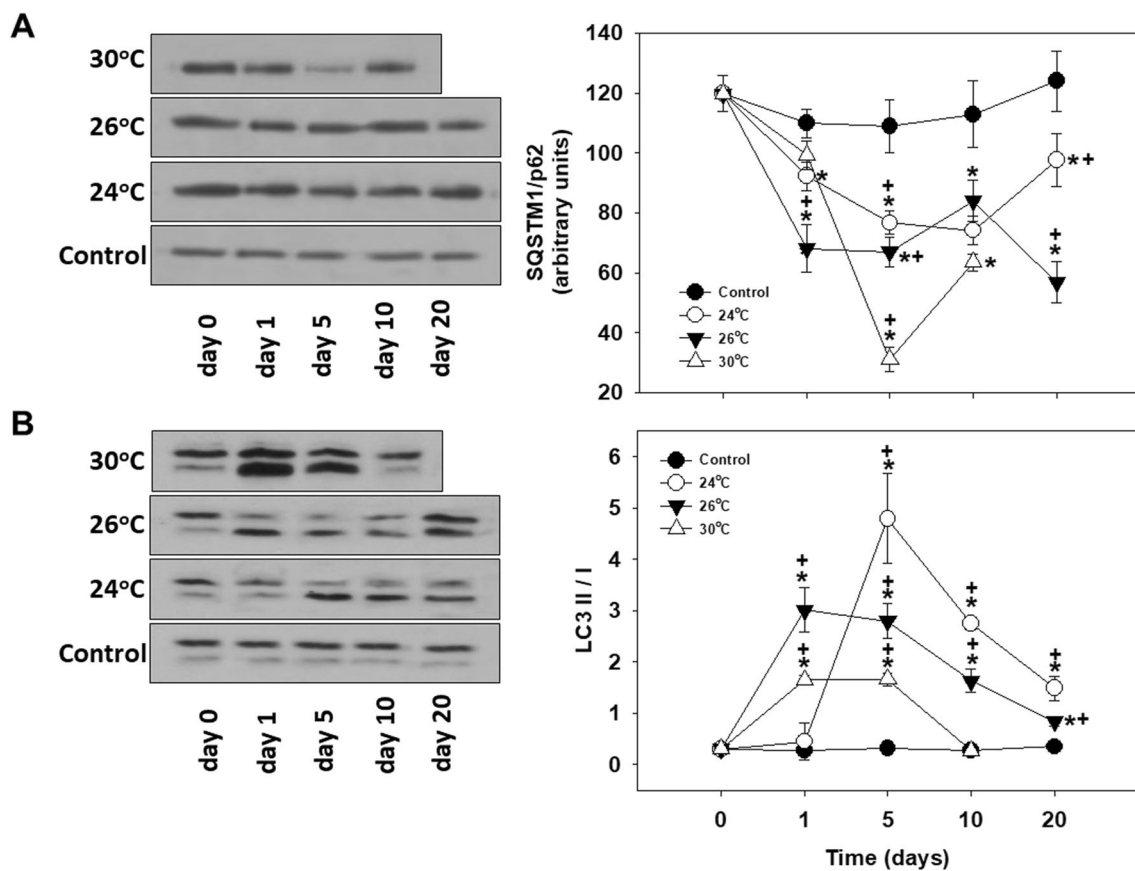


Fig. 9 Levels of SQSTM1/p62 (**a**) and LC3 II / I ratio in the red muscle of *S. aurata* during exposure for 20 days to 18 °C (filled circle), 24 °C (open circle), 26 °C (filled inverted triangle) and 30 °C (open

triangle). Values are means \pm SD; $n=8$ preparations from different animals. * $P<0.05$ compared to control, + $P<0.05$ compared to other temperatures

oxidation would cover enhanced ATP requirements associated with the cellular stress response and the restoration of cellular homeostasis during and after thermal stress (Kültz 2005). Accordingly, increase in the activity of HOAD may counteract the loss in performance and enhance ATP synthesis in the red muscle of *S. aurata*, thereby supporting muscle functionality to deliver oxygen to tissues within the first 5 days of fish exposure to 24 °C. The increase in plasma triglycerides within the 1st day and glucose levels after the 5th day of exposure to 24 °C, support the above patterns of metabolism. During stress, the production of glucose via either glycogenolysis or gluconeogenesis appears beneficial as it provides substrates to tissues such as the brain, gills and muscles (Iwama 1999). Both, the mobilization of liver glycogen and FBPase (Fructose 1,6-bisphosphatase) activity increased in *S. aurata* after 35 days of acclimation to 26 °C (Vargas-Chacoff et al. 2009).

Recent investigations revealed that metabolic impairment in mitochondria from arctic char *Salvelinus alpinus* are accompanied by increased ROS production at elevated temperatures (Christen et al. 2018). The activities of the

antioxidant enzymes examined in the present study confirm an increase in the production of ROS mainly when *S. aurata* was exposed to 24 °C until the 5th day, followed by a compensating mechanism, similar to the metabolic profile of this species, exhibiting *S. aurata*'s capacity to compensate for the provoked oxidative stress. Madeira et al. (2016) have also observed that when *S. aurata* was acclimated until its Critical Thermal Maximum (≈ 35 °C), showed signs of oxidative stress in every tissue tested (gills, muscle, liver, brain and intestine), the most affected being muscle and liver, which showed greater increases in lipid peroxidation. These results seem to be in line with a parallel increase in the levels of oxidative stress indicator TBARS during exposure to the above temperature (Feidantsis et al. 2015). However, neither SOD nor catalase exhibited elevated activities when individuals were exposed to 26 °C and 30 °C. This loss of enzymatic antioxidant activities beyond critical temperatures might be related to heat-induced protein denaturation or disturbances of protein synthesis which inhibit physiological oxidative defense mechanisms (Kregel 2002; Pörtner 2002a, b; Shin et al. 2018). Moreover, transcriptomic data indicated

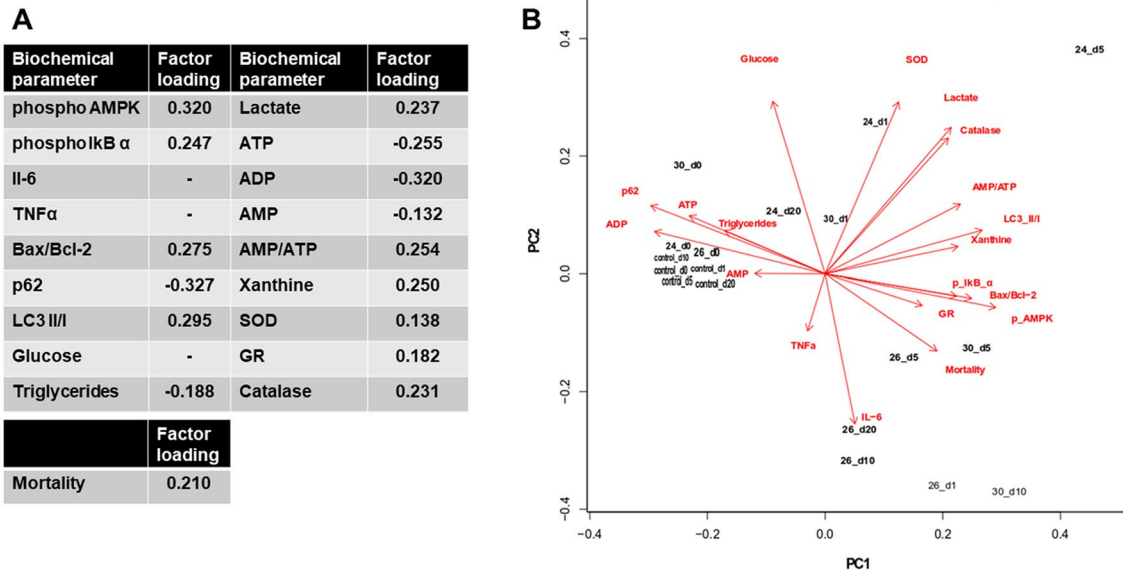


Fig. 10 **a** Analytical table of the contribution of biochemical parameters studied according to factor loadings and **b** variable correlations with each of the first two principal components (PCs) in the multivariate analysis. The PCA was generated from the complete biochemical and physiological dataset. Parameters with red vector arrows were included as predictors in constructing the PCA

decreased expression of genes involved in molecular chaperones and oxidative stress responses, and a parallel increase in expression of apoptotic genes in the atrium of warm acclimated fish (Jayasundara et al. 2013).

Although it has been suggested that under internal hypoxia, due to limited oxygen availability, the carriers of electron-transport chains are reduced and therefore more electrons escape from the chains, joining oxygen molecules, another possible mechanism of ROS production may be related to purine metabolism due to ATP depletion via xanthine reductase/xanthine oxidase system function (Boueiz et al. 2008; Lushchak 2011). XO seems to be the major endogenous source of ROS in the rat during hypoxia promoting oxidative stress (Baudry et al. 2008; Becker et al. 1999; Terry et al. 1997; Zajaczkowski et al. 2018). The increased xanthine levels in the red muscle of *S. aurata* coincide well with the time course of ATP decrease in the first 5 days when fish were exposed to 24 °C and 26 °C, indicating possible ATP degradation towards xanthine production, during this period. Thereafter, both the decreases of ATP and xanthine levels indicate that fish may compensate for the stress response. To our knowledge, it is the first time that such a metabolic response may take place under thermal stress in fish.

It is well known that the elevation of ROS levels may induce inflammatory responses (Forrester et al. 2018). The present results underline the triggering of pro-inflammatory cytokines' production, such as TNF α and interleukins and, moreover, I κ B α phosphorylation in *S.*

aurata, highlighting the nuclear translocation of active NF- κ B and regulation of inflammatory gene expression. Specifically, the expression of NF- κ B-regulated inflammatory genes results in the production of TNF α , IL-1b and IL-6 inflammatory cytokines, amplifying inflammation through activation of IKK/NF- κ B and JNK MAPK signaling (Tornatore et al. 2012). Although the results of the present study show I κ B α phosphorylation at 24 °C and 26 °C, TNF α and IL-6 levels increased mainly when fish were exposed to 26 °C. While there are many studies showing that TNF- α is constitutively expressed in several fish tissues (Garcia-Castillo et al. 2002; Laing et al. 2001), there are no in vivo studies of temperature-dependent TNF- α and interleukin regulation in fish, since most studies focus on interleukin expression only after antigen administration. JNKs activation (Feidantsis et al. 2009), as well as I κ B α phosphorylation in the red muscle, are observed in elevated temperatures of 24 °C and 26 °C, suggesting a linkage between the MAPK signalling pathway and inflammation in fish. However, both MAPKs and inflammatory indicators seem to decrease toward the end of the exposure period, suggesting the acclimation of fish to these conditions. Moreover, thermal stress-induced inflammatory responses in the Antarctic Plunderfish, *Harpagifer antarcticus* (Thorne et al. 2010), while Tomalty et al. (2015) indicated an inflammatory response as well as an increase in innate immune activity, particularly in warm acclimated *Oncorhynchus tshawytscha*. In addition, Jeffries et al. (2014) found inflammatory regulators

involved with NF- κ B activity and other 24 inflammatory/immune regulatory genes in the gill tissue of chronically heat-stressed adult Pink and Sockeye Salmon. Similarly, transcriptomic analysis showed inflammatory responses in the Antarctic fish *Pachycara brachycephalum* (Windisch et al. 2014).

It is well known that oxidative stress adversely affects cellular proteins (Davies 1987), DNA (Imlay and Linn 1988) and membrane lipids (Fridovich 1986), leading to cell signaling disruption, extensive DNA damage and therefore cell death (Chandra et al. 2000). As a result, the autophagy-lysosomal cell death pathway is activated, removing misfolded proteins, mitochondria and other organelles, resulting in the recycling of substrates and nutrients, contributing to metabolism regulation (Dodson et al. 2013; Hardie et al. 2016). The obtained data in the present study show a close relation between the patterns of changes in apoptotic and autophagic indicators' levels and those of activity levels of antioxidant enzymes mainly when fish were exposed to 24 °C for the first 5 days. Thereafter, the levels of above-mentioned indicators are decreased to control, indicating compensatory cellular mechanisms and enhanced capacity for acclimation. This is also supported by the observed changes in the phosphorylation levels of AMPK, which indicate the link between autophagy and the regulation of energy metabolism under the above thermal conditions and exposure time.

Conclusively, the present results are in line with previous findings, which suggest that temperature increase provokes cellular stress phenomena in the gilthead seabream (Feidantsis et al. 2009, 2015, 2018) (Fig. 11). The biphasic

response of these phenomena at least during exposure at 24 °C revealed an “*hormetic response*” and activation of adaptive cellular stress response pathways (ACSRPs) in the first 5 days of exposure that benefits tissue function and survival. Specifically, the PCA analysis showed that this “*hormetic response*” and survival of *S. aurata* is correlated with the restoration of energy turnover and ATP levels. However, a further increase in temperature seems to cause a cumulative cost (“*allostatic load*”) to the body of the maintenance of stability through change (allostasis) (McEwen and Wingfield 2003; Schulte 2014). The continuous AMPK phosphorylation during exposure to temperatures higher than 24 °C indicates a higher energy demand to compensate for cellular thermal stress. According to PCA analysis, *S. aurata* mortality is correlated not only to AMPK phosphorylation, but also to several other factors including apoptosis, oxidative stress and inflammation. The above stress responses seem to depend on both the magnitude and duration of heat stress, as several investigators have reported (Buckley and Hofmann 2004; Logan and Somero 2010). Although the latter biochemical processes are recruited due to thermal stress, their efficacy is not adequate since the mortality of fish increases beyond 24 °C and temperature of 30 °C is not life compatible when they are long term exposed. Probably, this temperature extreme is the denaturation temperature where molecular structure disruption (as shown by increased apoptosis) and limited survival due to deficiency of ATP production are observed (Pörtner 2001, 2002a, b; 2010, 2012). The data obtained in the present work are in line with previous investigations reporting that the capacity to generate

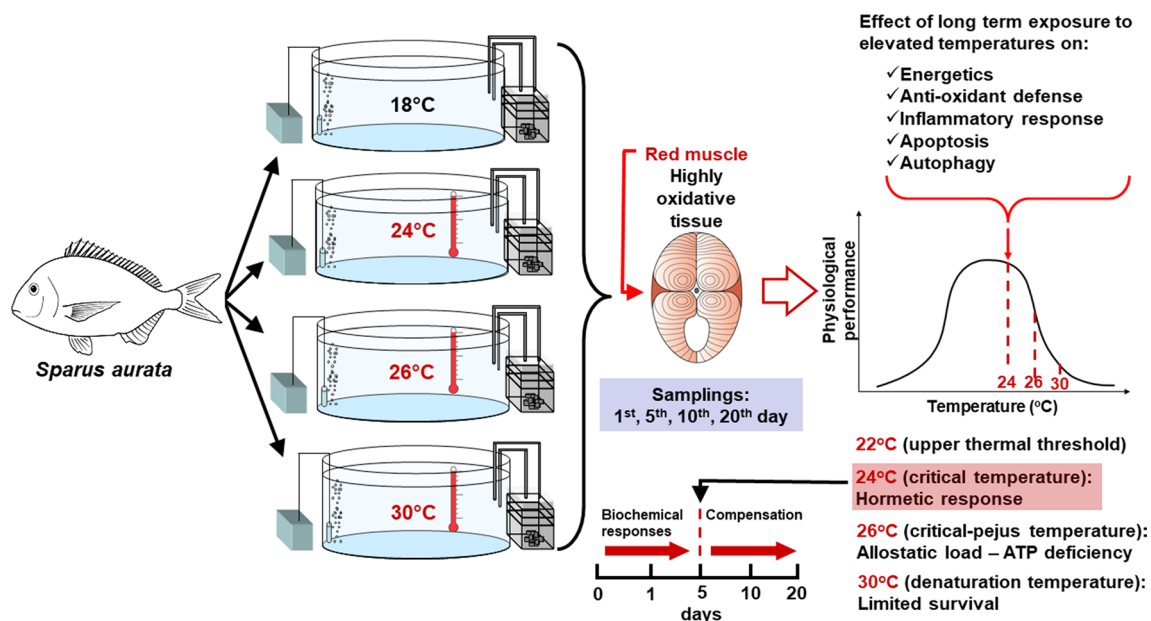


Fig. 11 Summarized model of cellular stress and energy demand patterns in the red muscle of *S. aurata* during exposure to elevated temperatures

adequate levels of ATP and to compensate for temperature effects on energy metabolic processes might be crucial in determining thermal plasticity of tissues function in aquatic organisms (Sokolova et al. 2012; Jayasundara and Somero 2013; Jayasundara et al. 2013). Moreover, the present data highlight the significance of molecular and biochemical responses to the shaping of threshold temperatures beyond thermal stress where fish mortality is initiated.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval Animals received proper care in compliance with the “Guidelines for the Care and Use of Laboratory Animals” published by US National Institutes of Health (NIH publication No 85–23, revised 1996) and the “Principles of laboratory animal care” published by the Greek Government (160/1991) based on EU regulations (86/609). The protocol as well as surgery and sacrifice were approved by the Committee on the Ethics of Animal Experiments of the Directorate of Veterinary Services of Prefecture of Thessaloniki under the license number EL 54 BIO 05.

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