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Integrated behavioural and physiological responses of sand smelt larvae to the effects of warming and hypoxia as combined stressors

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ABSTRACT

Forecasts indicate that rising temperatures towards the future and the expansion of dead zones will change environmental suitability for fish early stages. Therefore, we assessed the chronic effects of warming (26 °C), hypoxia (<2–2.5 mg L^{-1}) or their combination on mortality rate, growth, behaviour, energy metabolism and oxidative stress using *Atherina presbyter* larvae as a model species. There were no differences between the treatments in terms of mortality rate. The combination of warming and hypoxia induced faster loss of body mass (+22.7%). Warming, hypoxia or their combination enhanced boldness (+14.7–25.4%), but decreased exploration (−95%–121%), increased the time in frozen state (+60.6–80.5%) and depleted swimming speed (−45.6–50.5%). Moreover, routine metabolic rate was depleted under hypoxia or under the combination of warming and hypoxia (−56.6 and 57.2%, respectively). Under hypoxia, increased catalase activity (+56.3%) indicates some level of antioxidant defence capacity, although increased DNA damage (+25.2%) has also been observed. Larvae also exhibited a great capacity to maintain the anaerobic metabolism stable in all situations, but the aerobic metabolism is enhanced (+19.3%) when exposed to the combination of both stressors. The integrative approach showed that changes in most target responses can be explained physiologically by oxidative stress responses. Increased oxidative damages (lipid peroxidation and DNA damage) and increased interaction between antioxidant enzymes (superoxide dismutase and catalase) are associated to increased time in frozen state and decreased swimming activity, growth rates and boldness. Under all stressful situations, larvae reduced energy-consuming behaviours (e.g. depleted exploration and swimming activity) likely to stabilize or compensate for the aerobic and anaerobic metabolisms. Despite being an active small pelagic fish, we concluded that the sensitive larval phase exhibited complex coping strategies to physiologically acclimate under thermal and hypoxic stress via behavioural responses.

1. Introduction

Fishes have evolved complex physiological capacities to optimize fitness-related performance, such as growth, behaviour and locomotion, over a wide range of temperatures, often depending on the O_2 demand to fuel aerobic processes (Clark et al., 2013). However, warmer zones with low availability of dissolved oxygen are spreading in coastal seas and these situations will become more frequent by the end of the century (Altieri and Gedan, 2015). The average global sea surface temperature has been consistently higher during the past three decades compared to any other time series since 1880 (NOAA, 2016). In the subtropical North Atlantic Ocean, for example, surface temperatures increased by ~0.85 °C since the 1980s (Bates and Johnson, 2020). The oxygen content of the ocean has declined by ~2% due to global warming and nutrient enrichment since the middle of the 20th century (Claireaux and Chabot, 2019). According to the Coupled Model Intercomparison Project (CMIP5), the global average ocean temperature is expected to rise between 0.9 °C and 2.9 °C by 2100 for the ensemble averages under

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SSP1-2.6 and SSP5-8.5, which will drive a decline of 3–4% in average oceanic oxygen levels (Schmidtko et al., 2017). Nevertheless, the extent at which such global changes will affect the different life stages of fishes is challenging to assess.

The effects of rising temperatures and O_2 depletion are known to induce putative changes in energy metabolism, growth, swimming ability, oxidative stress and may even lead to high mortality rates in fish. Warming increases the rate of oxygen consumption or decreases the anaerobic metabolism of fishes, as observed in *Sparus aurata* (Remen et al., 2015; Madeira et al., 2016) and *Oncorhynchus kisutch* (Little et al., 2020), respectively, which would probably reduce their potential to deal with hypoxic situations. (Little et al., 2020). Other species are able to supplement swimming activity using the anaerobic metabolism when exposed to hypoxia, such as *Mugil cephalus* (Vagner et al., 2008). These stressors can also induce fishes to alter risk-taking related behaviours. *Pomacentrus moluccensis* larvae, for example, became bolder and swam faster when exposed to warming (Biro et al., 2010). Faster larval growth was observed when *Salmo salar* (Braun et al., 2013) and *Amphiprion melanopus* (Green and Fisher, 2004) were exposed to warming. On the other hand, hypoxia decreased the growth rate of *Morone saxatilis* (Brandt et al., 2009). Finally, severe hypoxic conditions led to increased mortality rates in *Gadus morhua* and *Anchoa mitchilli* (Vaquer-Sunyer and Duarte, 2008).

Nevertheless, the negative impacts that hypoxia has on biological performance often outweigh those of warming and the various reports of the co-occurrence of these stressors prompt the study of their combined effects (Sampaio et al., 2021). Fish exposed to warmer dead zones will need to improve performance to support high metabolic rates in areas with limited O_2 supply (McBryan et al., 2013). Despite this, studies evaluating how the combined effect of these stressors alter target responses in fish are still scarce (Schurmann and Steffensen, 1997; McBryan et al., 2016; Zhou et al., 2019; Pettinau et al., 2022). The combined effects of warming and hypoxia induced reduced growth in *Paralichthys lethostigma* (Del Toro-Silva et al., 2008), weight loss in *M. saxatilis* (Brandt et al., 2009) and increased gill diffusion area in *S. salar* (Anttila et al., 2013). Even rarer are works on fish early stages. Larvae have not developed specific tissues/organs needed for homeostasis and can have relatively narrow windows of environmental tolerance. As a result, the combined effects of warming and hypoxia can act synergistically and increase mortality (Verberk et al., 2022).

To fulfil some of the knowledge gaps on the impacts of climate change on fish early stages, this study aimed to integrate behavioural and physiological outcomes to understand which coping strategies are used by larvae of the sand smelt (*Atherina presbyter*) to withstand the single and the combined effects of warming and hypoxia. This is a small pelagic fish of ecological relevance as it is an important prey for commercial coastal predators (Faria et al., 2014). The species inhabit the northeastern Atlantic and very sporadically the Mediterranean, with a cohesive population from Portugal to Germany (Francisco et al., 2008). Therefore, our hypotheses aim to answer: (I) whether the effects of chronic exposure (15 days) to warming and hypoxia can be amplified by their combination on mortality rate, growth patterns, boldness, swimming activity, metabolic rate, oxidative stress and energy metabolism; and (II) whether chronic alterations on physiological performance, measured as oxidative stress and energy metabolism, can explain the variability in mortality rate, growth, behaviour and metabolic rate.

2. Material and methods

2.1. Experimental design

Fish larvae were collected in the very nearshore waters of Portinho da Arrabida beach (38°28′35.09″N; 8°59′01.05″W) on June 2022, in the Arrábida Marine Park (Portugal). Larvae were immediately transported to the laboratory and maintained in quarantine for 5 days at a mean temperature of 18 °C and a salinity of 35 g/L (the variables at the sampling site) to recover from transport and handling stress. During this period, larvae were daily fed with recently hatched *Artemia* nauplii.

Geo-referenced occurrence data representing spawning habitats for the species were used to extract present-day and future sea surface temperature (SST) (quarterly-averages from daily-means) from remotely sensed climate models [2100 – Representative Concentration Pathway (RCP) 8.5]. The highest value of SST extracted from the National Oceanic and Atmospheric Administration's World Ocean Atlas (NOAA's WOA) (Garcia et al., 2019; Locarnini et al., 2019) was 22 °C and from Couple Model Intercomparison Project 5 (CMIP5) (Scott et al., 2016) was 26 °C. These SST were set as probable conditions for both present-day and future, respectively. Hypoxic condition was set as 2–2.5 mg L^{-1} dissolved oxygen (DO) to mimic the coastal dead zones found across the distribution range of the species (Breitburg et al., 2018).

After quarantine, larvae were randomly distributed over 8 experimental tanks of 25 L (18 °C and 100% DO), at a density of ~60 larvae per tank to reduce biased results from the proposed tests. There were 4 different treatments, each with 2 replicate tanks:

A: 22 °C & 80–100% DO (5.8–7.3 mg L^{-1} DO) (control treatment)
B: 26 °C & 80–100% DO (5.3–6.7 mg L^{-1} DO) (warming treatment)
C: 22 °C & 27.2–34.1% DO (2.0–2.5 mg L^{-1} DO) (hypoxia treatment)
D: 26 °C & 30–37.6% DO (2.0–2.5 mg L^{-1} DO) (warming x hypoxia treatment)

We gradually change the water parameters to acclimatize the larvae to the new conditions, avoiding stress and heat shock. Temperature was increased using heaters at a rate of 1 °C day^{-1} until reaching 22 °C in all tanks. After that, temperature was increased in treatments B and D at a rate of 1 °C day^{-1} until the desired temperature of 26 °C. The oxygen level was steadily decreased in treatment C and D over a 4-day period until matching the desired O_2 level between 2 and 2.5 mg L^{-1}. Solenoid valves controlled by an oxygen regulator computer (Loligo® Systems OXY-REG) maintained O_2 at the desired level by pumping pure nitrogen (N_2) into the tanks. A cycle of 14L:10D using fluorescent light was followed. Larvae were daily fed ad libitum with *Artemia* nauplii. Exposure to extreme conditions was run for 15 days and the number of dead larvae was counted after the exposure period to assess mortality rate.

This study was carried out under the approval of Direção Geral de Alimentação e Veterinária (DGAV, Portuguese Authority for Animal Health, permit 0421/000/000/2020) and according to the University's animal ethics guidelines.

2.2. Growth patterns

The chronic effects of warming and hypoxia on growth in both length and weight were evaluated in twenty-six larvae randomly collected from both replicate tanks per treatment after the exposure period. The standard length (S_L) and weight (Wg) were used as dependent variables (y) and the total length (T_L) as the independent variable to run the allometric growth model $y = \beta_0 T_L^{\beta 1} + \varepsilon$, where β_0 is the intercept and β_1 is the growth coefficient (van Snik et al., 1997). When β_1 is 1 for length and 3 for weight, the growth is said to be isometric (Lima et al., 2013). When β_1 is smaller than the isometric value, the growth is negatively allometric (slower); when higher, the growth is positively allometric (faster) (Lima et al., 2013).

2.3. Behavioural analysis

Fourteen larvae were randomly sourced after the exposure period from both replicate tanks per treatment in a temperature-controlled room. Fish were individually tested once. All tests were performed over a period of 2 days (28 larvae/day), changing the order of the tanks analysed to reduce differences in individual peaks of activity. The experimental arena used to assess fish boldness and activity consisted of a rectangular aquarium (56x38 × 40 cm) covered with an opaque lining to block the larva from viewing the external movements. The aquarium was divided into a small starting compartment (18 × 38 cm) with gravel on the bottom to simulate a shelter, separated by an opaque barrier from a larger testing arena without structural elements. The bottom of the larger compartment had a gridded white surface underneath, dividing the area in three square zones: zone 1 was closer to the tank wall, zone 2 an intermediate zone, and zone 3 was the inner central zone of the arena (Magnhagen et al., 2014). The last was assumed to be perceived as an unsafe environment because it was unknown to the larva at the start of the trial (Magnhagen et al., 2014). The water level was set to 12.5 cm. Water temperature and oxygen in the arena matched the conditions of the treatment of origin and ~20% of the water was replaced in between evaluation of each fish of the tested conditions. Fish were only fed at the end of the trial day to avoid postprandial effects which could affect behaviour (Höjesjö et al., 1999). All tests were recorded from above using a camera (Canon PowerShot G7X Mark II, Sony RX100 IV).

2.3.1. *Boldness assay*

An emergence assay was used to test boldness (Brown and Braithwaite, 2004; Miller et al., 2006). Each fish was placed in the starting compartment to acclimate for a period of 5 min. After that, the door was lifted for 1 min, allowing the fish to swim into the larger compartment. A small aquarium dip net was used to move those fish that do not leave the starting area (Chen et al., 2018). A bolder fish is expected to leave shelter faster and move further away from the protected area than a shyer fish (Toms et al., 2010). Therefore, we recorded: (1) average emergency to exit shelter (ms) and (2) average time spent in zones 1, 2 and 3 (ms). Video analysis was carried out using BORIS 7.3 (Friard and Gamba, 2016).

2.3.2. *Activity assay*

Activity was measured as routine swimming traits. We used BORIS to evaluate (1) the average time in frozen state (ms) and (2) the average time swimming (ms). Image-J (v1.48; U. S. National Institutes of Health, Bethesda, Maryland) was used to evaluate (3) the distance swum (mm) and (4) the average swimming speed (mm/s). For 3 and 4, each video was divided into frames associated to a period in seconds and the pixels of each frame were converted into millimetres. The distance swum by the larva between consecutive frames over a given period was measured using a manual tracking plugin.

2.4. *Routine metabolic rate (RMR)*

Twelve larvae were randomly sourced after the exposure period from both replicate tanks per treatment in a temperature-controlled room. Fish were individually tested once. All tests were performed over a period of 3 days (4 larvae/day), changing the order of the tanks analysed to reduce differences in individual peaks of activity. To estimate RMR, oxygen consumption was measured in a closed respirometry system composed by 4 cylindrical o-ring sealed chambers with a water volume of 100 ml, according to Almeida et al. (2022). The larvae were allowed to acclimate to the respirometry chamber for 1 h with constant water circulation with temperature and oxygen levels matching the conditions of each treatment. After that, the circulation of water was interrupted, and contactless oxygen sensor spots (OXSP5, Pyroscience) measured oxygen levels inside the chamber every 15 s for 25 min. The software Pyro Oxygen Logger (Pyroscience, Denmark) was used to monitor oxygen consumption. The background bacterial oxygen consumption was recorded after each test to account for the effects of bacterial respiration. The RMR (mg O_2/kg/h) was calculated as (Almeida et al., 2022):

$$MO_2 = \frac{\text{Chamber volume (L)} * \text{Slope of oxygen consumption (mg/L)}}{\text{fish weight (kg)} * \text{Time (h)}} \text{ - Background}$$

Temperature coefficients (Q_{10}) for treatments B (warming) and D (the combination of warming and hypoxia) were calculated to evaluate whether RMR is thermally sensitive(Almeida et al., 2022):

$$Q_{10} = \left(\frac{\text{RMR } 26^\circ\text{C}}{\text{RMR } 22^\circ\text{C}}\right)^{22^\circ\text{C}/(26^\circ\text{C-}22^\circ\text{C})}$$

The resulting Q_{10} is 1 when RMR is completely independent of temperature. Q_{10} is $>$ 1 when RMR has a positive thermal dependence and $<$1 when RMR has a negative thermal dependence.

2.5. *Biomarkers analysis*

Seven larvae per treatment were randomly sourced after the exposure period from both replicate tanks per treatment. These fish were not tested for behavioural or metabolic responses. Oxidative stress was evaluated by assessing whether the formation of reactive oxygen species (ROS) (Socci et al., 1999) can be controlled by superoxide dismutase (SOD) (McCord and Fridovich, 1969) and catalase (CAT) (Claiborne, 1985) activities in order to prevent oxidative damages to DNA (Olive, 1988) and lipid peroxidation (LPO) (Ohkawa et al., 1979; Bird and Draper, 1984) (see Supplementary Material for more information).

Energy metabolism was evaluated as the rate of cellular energy consumption represented by the electron transport system (ETS) (De Coen and Janssen, 1997). Isocitrate dehydrogenase activity (IDH) was evaluated as a proxy for the aerobic metabolism (Ellis and Goldberg, 1971; Lima et al., 2007), while lactate dehydrogenase activity (LDH) for the anaerobic metabolism (Vassault, 1983; Diamantino et al., 2001) (see Supplementary Material for more information).

3. Statistical analysis

To check whether growth in length and weight differ among treatments, we used T-tests to compare growth coefficients. Generalized Linear Mixed Models (Breslow and Clayton, 1993) were performed to investigate whether the different treatments affect the measured traits [mortality, growth, behaviour (boldness and activity), RMR and biomarkers]. Each tank was used as nested random repeated factor within the main effects to reduce the chances of a type I error. A Poisson distribution, with the Poisson variance $V(\mu) = \mu$ and a logarithmic-link log (μ) function, was used to analyse mortality rate, boldness, time in frozen state and time swimming (McCullagh and Nelder, 1989). A log-normal distribution, with the lognormal variance $V(\mu) = \exp^{(2\mu + \sigma^2)} [\exp^{(\sigma^2)} - 1]$ and the logarithmic-link $\log(\mu)$ function, was used to analyse swimming speed, distance swan, RMR and biomarkers (Dick, 2004). A Tukey HSD *post hoc* test was performed whenever significant differences were detected. The statistical significance level was $\alpha = 0.05$ (p-value <0.05). The values are expressed as averages (±S.E).

A Redundancy Analysis (RDA) (Rao, 1964) was performed to evaluate whether the chronic effects of warming and hypoxia on the measured traits [mortality, growth, behaviour (boldness and activity), RMR] could be explained by alterations on oxidative stress and energy metabolism biomarkers. A matrix of the groups (i.e. different treatments and tanks) containing the measured traits was used as response variable, while a matrix of values related to alterations on oxidative stress and energy metabolism was used as explanatory variable. Response variables were Hellinger-transformed before running the model (Legendre and Gallagher, 2001). The overall statistical significance of each RDA models, of constrained axis and of each variable were assessed using an ANOVA permutation function in order to exclude explanatory variables not significant to the model. The explanatory variables selected by stepwise procedures appear as vectors radiating from the origin of the ordination. The length of the vector is related to the importance of the explanatory variables to the dataset (response variables and the groups). The direction and proximity among variables, groups and vectors inform the type of relationship (positive or negative correlations). Statistical analyses were performed with R (R Core Team, 2022).

4. Results

4.1. Mortality rate after chronic exposure to heat and hypoxic stress

Warming and hypoxia or their combination did not exhibit direct effects in mortality rate when compared to control conditions (Fig. 1). Mortality rate averaged ~66.6% in all treatments.

4.2. Changes in growth patterns after chronic exposure to heat and hypoxic stress

By comparing growth coefficients (or slopes) among treatments, our results showed that growth in standard length did not differ among treatments ($p > 0.05$) (Fig. 2a). However, gains in weight were significantly slower (or allometric negative) when fish were exposed to the combination of warming and hypoxia when compared to all other treatments ($p < 0.001$) (Fig. 2b).

4.3. Behavioural responses (boldness and activity) after chronic exposure to heat and hypoxic stress

Warming and hypoxia significantly affected boldness and activity. The emergency of sand smelt to exit the shelter for the first time differed significantly between stressed conditions and the control (Fig. 3a). Fish exposed to warming, hypoxia or their combination left the shelter faster (were bolder) ($p < 0.001$). Overall, these larvae also spent significantly more time in zone 1 (protected or closer to the arena wall) ($p < 0.001$) and spent significantly less time in zone 3 (less protected or far from the arena wall), on average ($p < 0.001$) (Fig. 3b–d). In addition, sand smelt exposed to warming, hypoxia or their combination spent more time in the frozen state ($p < 0.01$), swam slower ($p < 0.01$) and covered shorter distances ($p < 0.01$) when compared to the control (Fig. 3e–g and h). There were no differences between the treatments in terms of time spent in swimming activity ($p > 0.05$) (Fig. 3f).

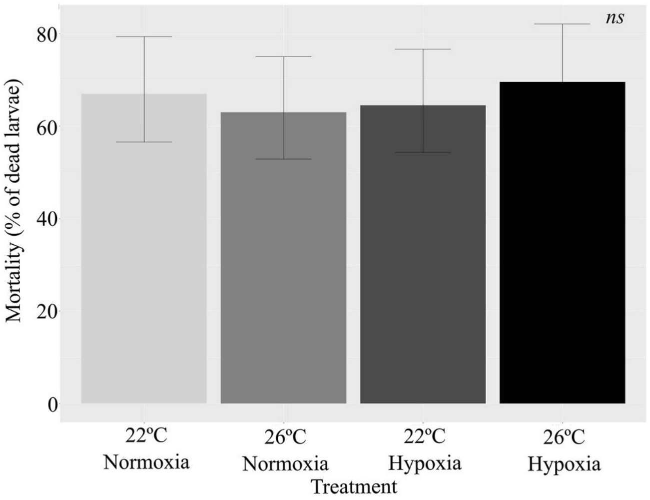


Fig. 1. Average mortality rate (±S.E.) measured as the number of dead sand smelt larvae chronically exposed to different treatments. ns, non-significant.

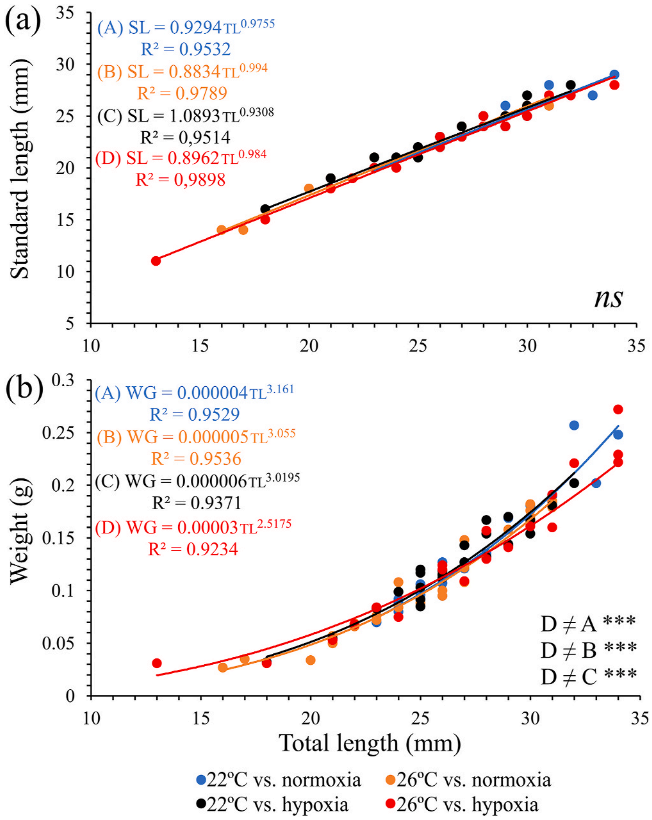


Fig. 2. Relationships between morphometric variables [standard length vs. total length (a) and weight vs. total length (b)] of sand smelt larvae ($N = 26$) chronically exposed to different treatments simulating (A) control (or natural) condition, (B) warming, (C) hypoxia and (D) the combination of both stressors. Growth coefficients (β1) and R2 values were calculated according to the model $y = \beta0\ TL^{\beta1} + \varepsilon$. ns, non-significant; ***$p < 0.001$.

4.4. Effects on physiology after chronic exposure to heat and hypoxic stress

4.4.1. Effects on RMR

Hypoxia and the combination of warming and hypoxia significantly decreased routine metabolic rate (RMR) when compared to all other treatments ($p < 0.01$) (Fig. 4). RMR exhibited a positive thermal dependence under warming ($Q_{10} = 1.82$) and a negative thermal dependence under the combination of warming and hypoxia ($Q_{10} = 0.23$).

4.4.2. Oxidative stress responses

ROS formation, SOD activity and LPO did not differ among treatments (Fig. 5a, b and d), while CAT activity was induced under hypoxia when compared to all other treatments ($p < 0.001$) (Fig. 5c). As a consequence of higher oxidative stress, damage to DNA was significantly higher under hypoxia, when compared to the control ($p < 0.05$) (Fig. 5e).

4.4.3. Energy metabolism responses

ETS activity did not differ among treatments (Fig. 6a). The anaerobic

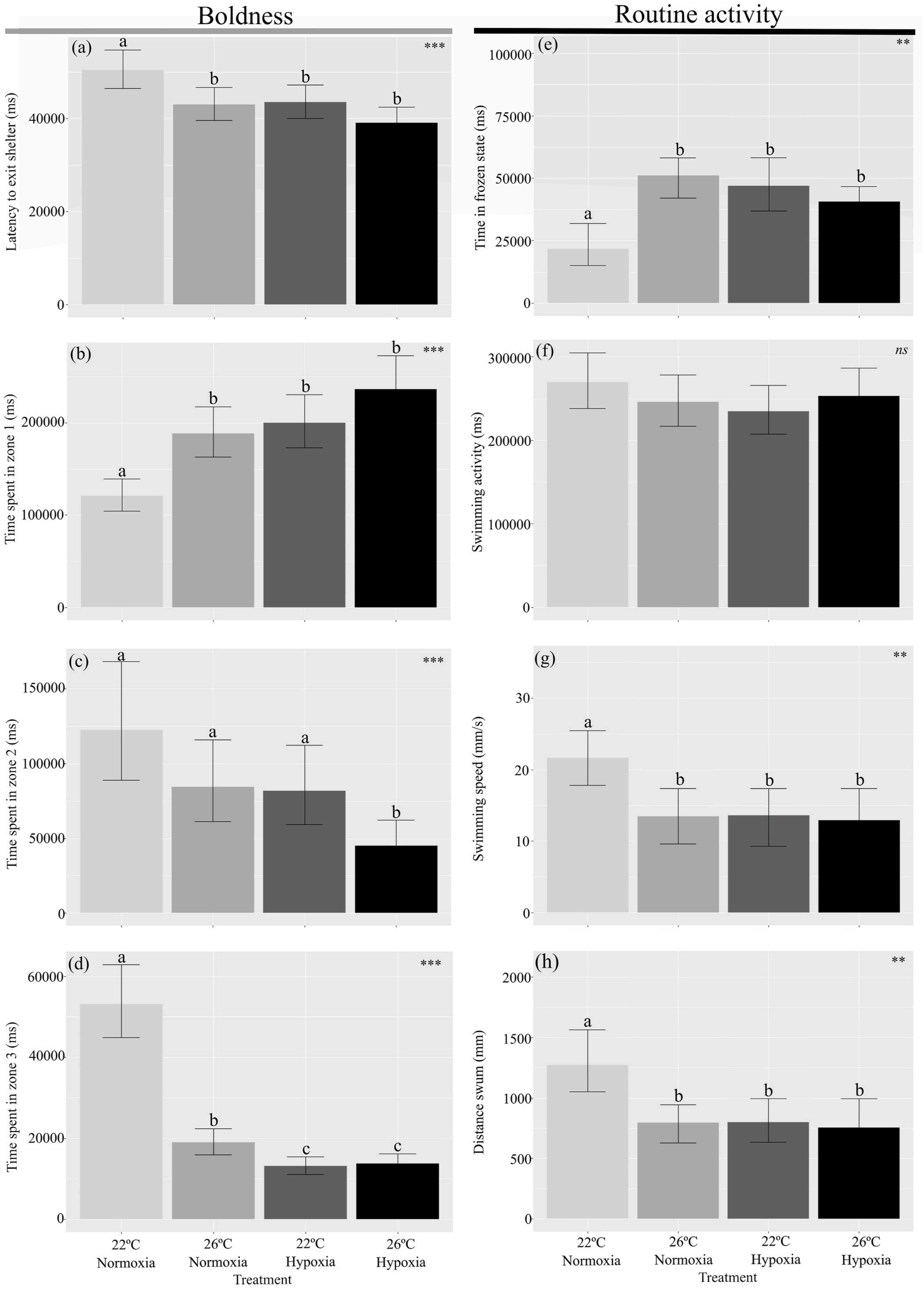


Fig. 3. Average (±S.E.) of behavioural responses [boldness (a–d) and activity (e–h)] of sand smelt larvae (N = 14) chronically exposed to different treatments. Letters represent homogeneous groups. ns, non-significant; $^{***}p < 0.001$; $^{**}p < 0.01$.

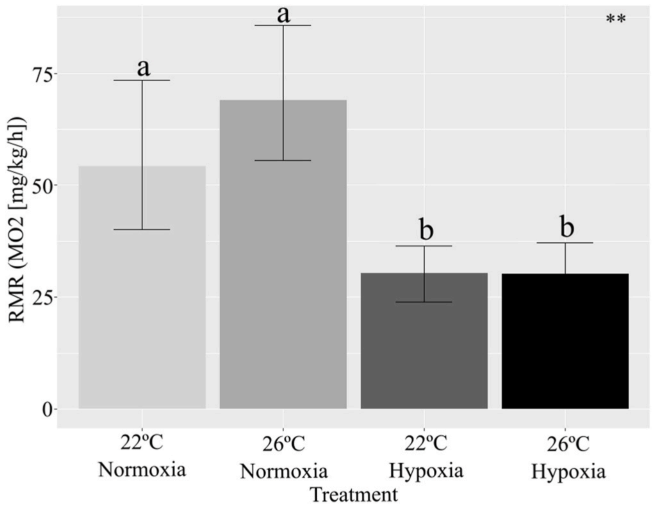


Fig. 4. Average routine metabolic rate (RMR ±S.E.) of sand smelt larvae (N = 12) chronically exposed to different treatments. Letters represent homogeneous groups. **p < 0.01.

metabolism (LDH activity) was significantly higher under the combination of warming and hypoxia when compared to the warming treatment ($p < 0.01$) but did not differ from the control and the hypoxia treatment (Fig. 6b). The aerobic metabolism (IDH activity) was significantly increased under the combination of warming and hypoxia when compared to the warming treatment and the control ($p < 0.05$) but did not differ from the hypoxia treatment (Fig. 6c).

4.5. *Relationship among target responses and physiology after chronic exposure to heat and hypoxic stress*

The RDA showed that oxidative stress, but not energy metabolism (i.e. biomarkers), significantly influenced variability in behaviour, growth and RMR of *A. presbyter* early stages exposed to the different treatments ($p < 0.01$) (Fig. 7). All biomarkers selected by the model had significant influence on the RDA ($p < 0.01$). The axis RDA1 explained 95.88% of the variance of the relationship among variables and is represented by the variability on the oxidative stress responses, except for LPO ($p < 0.01$) (Fig. 7). The axis RDA2 did not significantly influence the dataset and is represented by the oxygen availability (the larvae exposed to hypoxia were positively loaded along RDA2, whilst larvae exposed to normoxia were negatively loaded along RDA2) (Fig. 7). The first axis showed both a strong positive ($r = 0.7956$) and negative correlations ($r = -0.9238$) to damaged DNA and lipid peroxidation (LPO), respectively. This means that these two variables strongly drove the variation in the data. Growth, boldness, activity and RMR decreases when fish are exposed to hypoxia and increases under normoxia, regardless the temperature (Fig. 7). These target responses had also a negative correlation with the first axis, meaning that when these responses decrease, it is also possible to observe increases in DNA damage, LPO and in the interaction between SOD and CAT activities. On the other hand, time in frozen state had a strong positive correlation with the first axis and increases under the same conditions in which damage to DNA, LPO and the interaction between SOD and CAT activities also increase (Fig. 7). This last response also increases ($p = 0.005$) when fish are exposed to hypoxia, regardless the temperature.

5. Discussion

5.1. *Warming, hypoxia or their combination had no effect on mortality rate*

Our results show that sand smelt larvae have a relative high

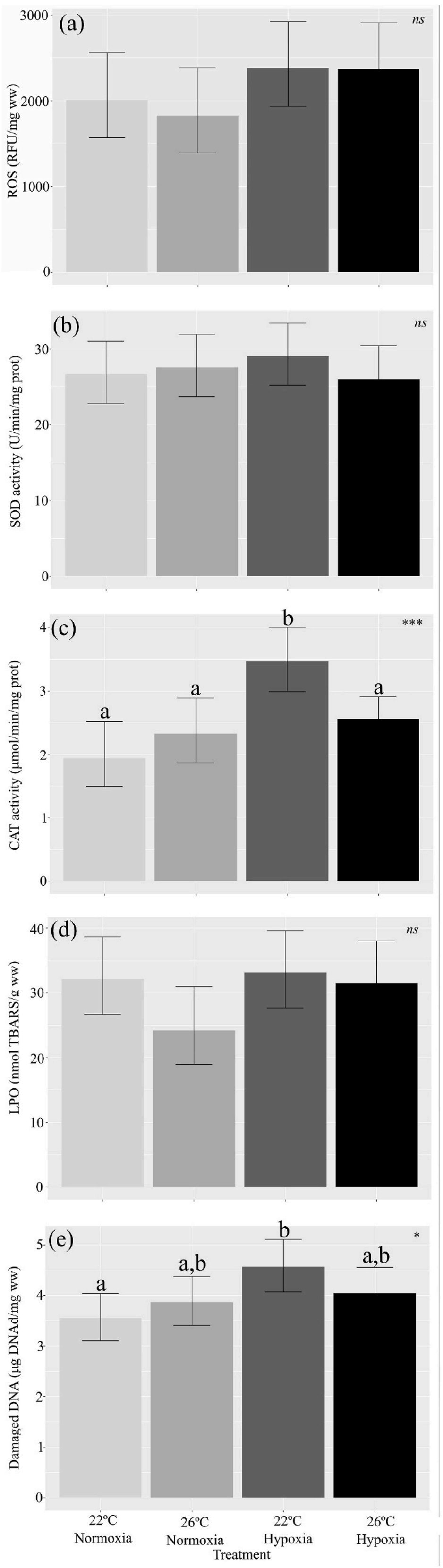


Fig. 5. Variability of biomarkers (average ± S.E.) of oxidative stress responses (a–e) in larval tissues of sand smelt larvae (N = 7) chronically exposed to different treatments. ROS = reactive oxygen species, SOD = superoxide dismutase, CAT = catalase, LPO = lipid peroxidation, DNA = DNA damage. Letters represent homogeneous groups. ns, non-significant; ***p < 0.001; *p < 0.05.

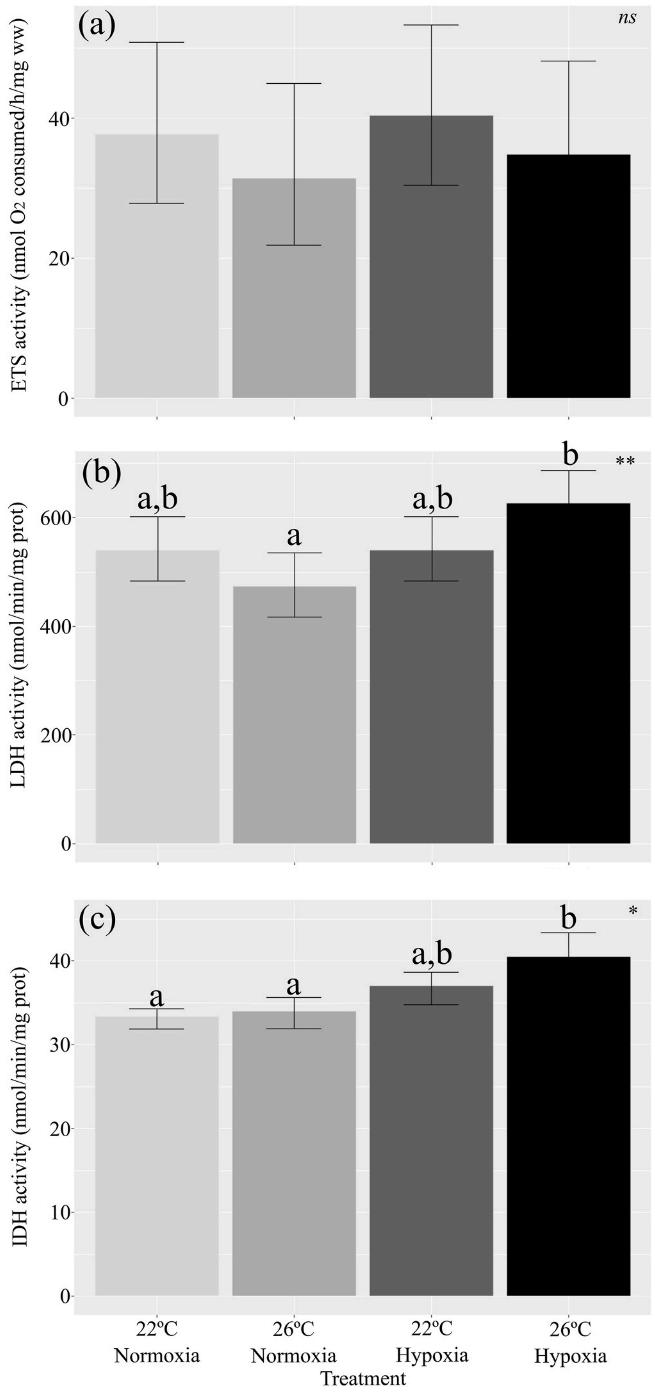


Fig. 6. Variability of biomarkers (average ± S.E.) of energy metabolism responses (a–c) in larval tissues of sand smelt larvae (N = 7) chronically exposed to different treatments. ETS = Electron transport system, LDH = lactate dehydrogenase, IDH = isocitrate dehydrogenase. Letters represent homogeneous groups. ns, non-significant; $**p < 0.01$; $*p < 0.05$.

mortality rate >60% even in optimal rearing conditions. Despite this, warming, hypoxia or their combination had no effect on mortality as there was no difference between stressed conditions and the control. The combination of these stressors had also no effect in larvae of *Dicentrarchus labrax* (Vanderplancke et al., 2015), whilst acted synergistically increasing the average mortality of juvenile of *Acipenser oxyrinchus* by 92% (Secor and Gunderson, 1998). Thus, many fish species are likely to experience reduced survival in the future because long-lasting warmer dead zones are expanding across their distribution range (Diaz and Rosenberg, 2008; NASA, 2010). Some other species, such as the sand smelt, are unlikely to experience additional population-level effects in the future, as mortality under hypoxic or thermal stress is no higher than that observed under current conditions.

5.2. The combination of warming and hypoxia induced slower gains in weight

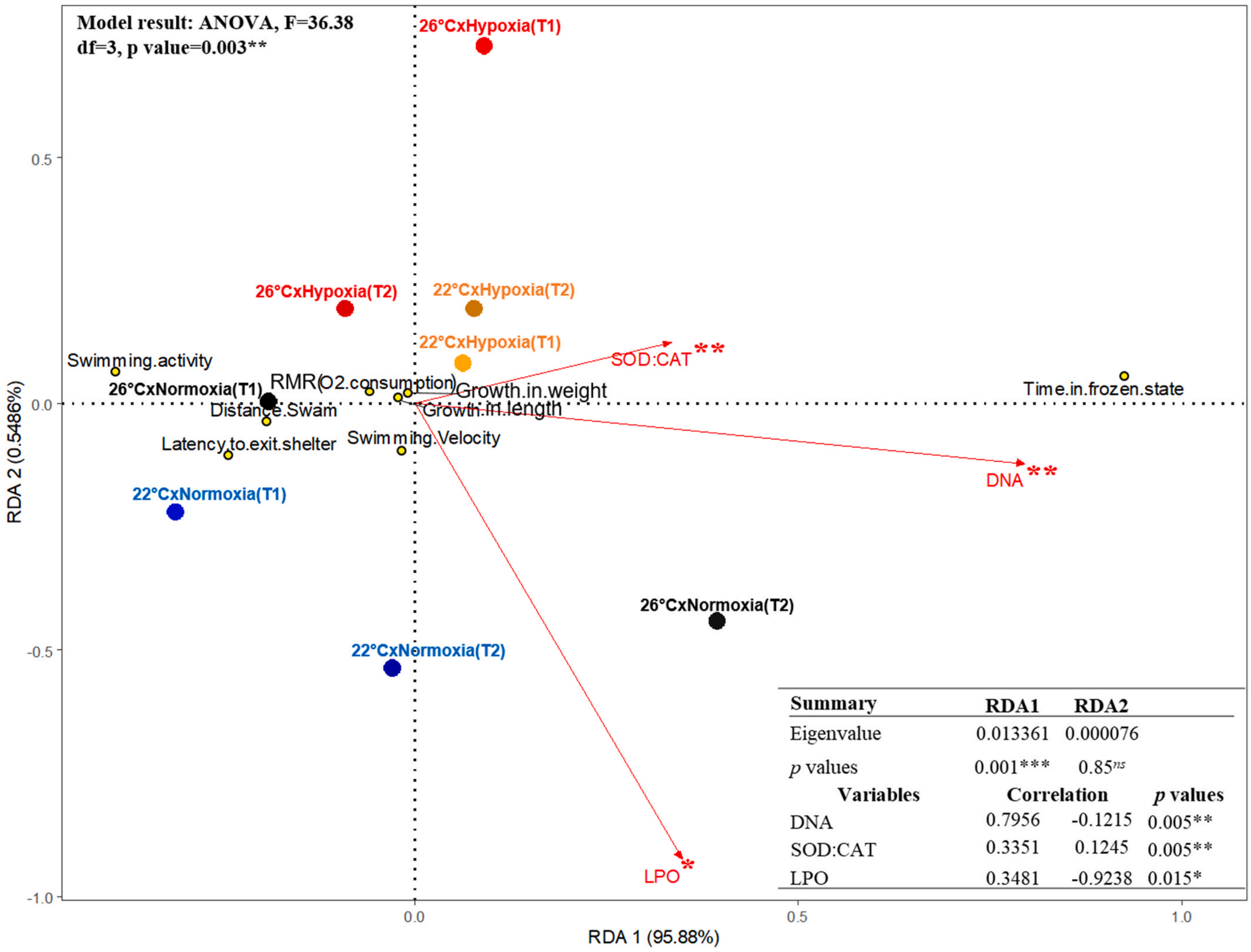
The combination of warming and hypoxia affected growth patterns by inducing faster loss of body mass, resulting in thinner sand smelt larvae. Controversially, the larval stage is generally the period of rapid growth over a short time interval (Pepin, 1995; Lima et al., 2013). Larvae commonly gain weight ~100 times more rapid than length under optimal natural conditions due to the faster development of organ and tissue (Pepin, 1995; Yang et al., 2020). According to our results, growth in weight was faster in larvae exposed to warming or hypoxia, similarly to the control condition. Under these situations larvae prioritized the development of body mass such as observed during the larval period (Yang et al., 2020). In addition, warming, hypoxia or their combination had no effect on growth in length. According to our biochemical results, it is likely that sand smelt have the ability to offset metabolic and developmental costs associated to these stressors as metabolism was maintained stable in all treatments, except when larvae were exposed to the combination of warming and hypoxia. Under such situation, the increased aerobic and anaerobic activities coincided with faster loss of body mass in sand smelt, suggesting that the larvae may be using energetic reserves to improve metabolism when exposed to combined heat and hypoxic stress (Del Rio et al., 2019).

Although there is little work on the effects of warming and hypoxia on allometric growth patterns, the combination of warming and hypoxia also caused body mass loss in early stages of *Oncorhynchus tshawytscha* (Del Rio et al., 2019) and *M. saxatilis* (Brandt et al., 2009), while hypoxia alone decreased body mass in *S. salar* (Remen et al., 2013), *D. labrax* (Vanderplancke et al., 2015) and the *Seriola lalandi* (Bowyer et al., 2014). Our results indicate that weight loss will be faster if sand smelt larvae stay longer in habitats where the coexistence of warming and hypoxia persists. This means that such habitat conditions are not suitable for larval growth. It is likely that larvae exposed to the combination of these stressors prioritizes the use of energetic reserves to improve metabolism rather than to invest in development to maintain body shape. This is enough to change growth strategies that are important to life history success (Baltazar-Soares et al., 2023). The exposure to these stressors during early stages may also have adverse carry-over effects in later stages (Cadiz et al., 2018). Thus, it is likely that later stages of sand smelt growing under the combination of warming and hypoxia will have delayed development when compared to those growing in optimal conditions. Larvae with poor body condition will hardly ever succeed along their development, which could compromise fitness and population recruitment success (Lima et al., 2013).

5.3. Warming, hypoxia and their combination enhance boldness and protection and deplete larval activity

Our study indicates that warming, hypoxia or their combination affect the reaction of sand smelt larvae to a situation perceived as dangerous and the willingness to take risks in novel environments (Fraser et al., 2001; Brown et al., 2005). These larvae left the shelter more quickly but had less willingness to take risks by avoiding more exposed areas of a novel environment. Moreover, warming, hypoxia or their combination decreased activity in sand smelt larvae as they swam significantly slower and for shorter distances than when they were under control conditions. These stressors also increased the time larvae spent in frozen state.

Actually, the effects of warming and hypoxia on boldness, exploration and activity are highly variable among species and they have often been linked to metabolic requirements (Lienart et al., 2014). Warming



Summary	RDA1	RDA2	
Eigenvalue	0.013361	0.000076	
p values	0.001***	0.85^{ns}	
Variables	**Correlation**		***p* values**
DNA	0.7956	-0.1215	0.005**
SOD:CAT	0.3351	0.1245	0.005**
LPO	0.3481	-0.9238	0.015*

Fig. 7. Redundancy analysis (RDA) for the relationships among physiological responses (oxidative stress and energy metabolism) and target responses (boldness, activity, RMR, growth and mortality) after chronic exposure. T1 = tank 1, T2 = tank 2, LDH = lactate dehydrogenase, IDH = isocitrate dehydrogenase, ROS = reactive oxygen species, LPO = lipid peroxidation, DNA = DNA damage, SOD = superoxide dismutase. ns, non-significant; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

decreased boldness in juveniles of *Pomacentrus chrysurus* when fish were in low physiological condition (low food availability) thus prioritizing metabolic requirements rather than escaping a predator cue (Lienart et al., 2014). Rising temperatures also affected behaviour in *P. moluccensis* as those that were active at a given temperature also tended to be bolder, suggesting that individual differences in metabolism may contribute to personality (Biro et al., 2010). Increased activity in *S. lalandi* larvae was positively related to resting oxygen uptake rates when exposed to high temperature (Laubenstein et al., 2018). Activity and risk-taking increased in *D. labrax* exposed to hypoxia, and both were positively correlated with metabolic rate (Killen et al., 2012).

Despite being an active small pelagic fish with larval critical swimming speed of ~670 m h^{-1} (Faria et al., 2014), when sand smelt larvae were exposed to all stressful situations the swimming speed was reduced by ~63% and the time spent in frozen state increased by >50% in relation to the control. It is thus likely that, when faced with those conditions, sand smelt larvae will choose to respond behaviourally by not taking risks and reducing activity to maintain metabolic requirements (Lienart et al., 2014; Domenici et al., 2019; Mattiasen et al., 2020; Kua et al., 2020). Therefore, our results suggest that such behavioural changes will affect both access to resources and vulnerability to predators that are tolerant to these stressors.

5.4. Larvae use behavioural responses as coping strategies to overcome the effects of warming and hypoxia or their combination on physiology

ROS are byproducts of the normal metabolism of oxygen and their formation may be disturbed by warming and hypoxia leading to oxidative damages. To prevent oxidative damages to cell membrane (LPO) and to DNA, SOD and CAT act to convert and decrease superoxide anion and hydrogen peroxide levels within the cell, respectively (Silva et al., 2018). In sand smelt, responses regarding oxidative stress did not differ between stressed conditions and the control, except when fish were exposed to hypoxia. In this case, although the antioxidant system was stimulated by the induction of CAT activity, that was not enough to prevent an increase in DNA damage. Regarding energy metabolism, rising temperatures often result in higher energy demand supplied via aerobic metabolism, which in turn becomes limited under low availability of oxygen (Schulte, 2015; Earhart et al., 2022). For sand smelt larvae, stressful conditions did not affect cellular respiration (here studied as ETS activity) and the anaerobic metabolism (assessed through LDH activity) was maintained similar as that of the control when fish were exposed to any of the situations. However, the aerobic metabolism (assessed through IDH activity) increased when fish were exposed to the combination of warming and hypoxia.

The effects of warming and hypoxia on fish physiology is highly variable among and within species as tolerance to these stressors are modulated by body mass, salinity, cell size and metabolic rate (Verberk et al., 2022; Duskey, 2023). A study evaluating 195 fish species concluded that hypoxia tolerance in fishes with larger bodies and larger cell sizes is lower in warmer water (Verberk et al., 2022). On the other hand, larger *Maccullochella peelii* were more tolerant to the combined effects of warming and hypoxia (McPhee et al., 2023). Tolerance to these stressors has often been assessed as to whether a given species has the ability to improve physiology. For example, *S. aurata* exposed to warming (Madeira et al., 2016) and *Leiostomus xanthurus* exposed to severe hypoxia (Cooper et al., 2002) had increased antioxidant defence. Reduced anaerobic metabolism was observed in *O. kisutch* exposed to warming (Little et al., 2020), while increased anaerobic metabolism was observed in *L. xanthurus* exposed to severe hypoxia (Cooper et al., 2002). Moreover, species such as *Diplodus puntazzo*, *S. aurata* and *Pagrus auratus* are able to sustain metabolic demands under extreme conditions through anaerobic reliance (Cerezo and García García, 2004; Domenici et al., 2007; Cook and Herbert, 2012; Cook et al., 2013; Remen et al., 2015).

To date it is still unclear what are the mechanisms used by fishes to cope with warming and hypoxia, especially considering ecological interactions among species in a changing environment (Duskey, 2023). To fulfil these knowledge gaps, some studies point to the need of integrative approaches to investigate how environmental change will affect populations and communities by predicting how species respond physiologically and behaviourally (Duskey, 2023). In *M. cephalus*, for example, the anaerobic metabolism was used to supplement swimming activity under hypoxic conditions (Vagner et al., 2008). Therefore, fishes can optimize the relationships between physiology and behaviour, through plasticity or evolution, to overcome the effects of extreme events (Domenici et al., 2007; Dwyer et al., 2014).

Our study also ascertained that the variability in responses related to oxidative stress could significantly explain ~96% of the variability of the relationship among behaviour, growth and RMR after chronic exposure, while responses associated with energy metabolism had no influence. There are clear negative relationships between most of the target responses and oxidative stress. When oxidative stress was high on average, fish spent more time in frozen state and depleted activity, growth rates and oxygen consumption (RMR). In addition, RMR of sand smelt increased with increased temperature ($Q_{10} > 1$). Under this condition, oxygen consumption was higher than those exposed to normoxia, on average (i.e. higher RMR). RMR was significantly lower when sand smelt were exposed to hypoxia or to the combination of warming and hypoxia. This indicates that under hypoxia, oxygen consumption decreases when temperature increases ($Q_{10} < 1$). Coincidently, routine swimming activity was depleted in all stressful situation, possibly as a strategy to overcome their effects on metabolism (Mandic and Regan, 2018). However, although hypoxia and thermal tolerance may be beneficial, the depleted activity will act in favour of the predator, especially those tolerant to the same stressors (Shoji et al., 2005). Therefore, we hypothesize that sand smelt larvae have a great capacity of using behavioural responses to buffer shifts in aerobic and anaerobic metabolisms, whilst reducing oxidative stress. These results suggest that sand smelt exhibit physiological tolerance to cope with the tested conditions of warming, hypoxia or their combination.

6. Conclusion

Collectively, the outcomes of this study show evidence that sand smelt larvae are able to use behavioural responses as a coping strategy to sustain the aerobic and anaerobic metabolisms and to regulate oxidative stress when exposed to warming, hypoxia or their combination. This strategy could be explained by variability in responses to oxidative stress among the different situations. Larvae exposed to these stressful situations increased the time spent in frozen state, enhanced boldness, decreased swimming activity and growth in weight. Under these conditions, the larvae exhibited some level of antioxidant defence capacity, although increased levels DNA damage could be observed under hypoxia. Larvae were also able to keep the anaerobic metabolism (LDH activity) similar to control situations (i.e. when temperature and O_2 levels are optimal). Moreover, this study has shown that under the combination of warming and hypoxia, larvae uptake less oxygen (low RMR) while boost aerobic demands (IDH activity). Coincidently, larvae exhibited faster loss of body mass under such high metabolic demands, possibly using energetic reserves to enhance aerobic metabolism even when oxygen consumption is reduced. Therefore, despite being an active small pelagic fish, larvae exhibited thermal and hypoxia tolerance through physiological and behavioural acclimations. We highlight that more replicate tanks, endpoints (e.g. protein carbonylation) and types of exposure (e.g. ability to recover from stress) could have been evaluated to increase the reliability of fish larvae responses to climate change. Future work should focus on the ecological costs of these strategies, integrating these biological responses into predictions of future habitats for fish larvae.

CRediT authorship contribution statement

André R.A. Lima: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Resources. **Ana Rita Lopes:** Formal analysis, Methodology, Validation, Writing – review & editing. **Sara Martins-Cardoso:** Formal analysis, Methodology, Validation, Writing – review & editing. **Ariana B. Moutinho:** Formal analysis, Methodology, Validation. **Marco F.L. Lemos:** Funding acquisition, Resources. **Sara C. Novais:** Formal analysis, Methodology, Resources, Validation, Writing – review & editing. **Ana M. Faria:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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References

Almeida, J., Lopes, A.R., Ribeiro, L., Castanho, S., Candeias-Mendes, A., Pousão-Ferreira, P., Faria, A.M., 2022. Effects of exposure to elevated temperature and different food levels on the escape response and metabolism of early life stages of white seabream, *Diplodus sargus*. Conserv. Physiol. 10 (1), 1–13. https://doi.org/10.1093/conphys/coac023.

Altieri, A.H., Gedan, K.B., 2015. Climate change and dead zones. Global Change Biol. 21, 1395–1406. https://doi.org/10.1111/gcb.12754.

Anttila, K., Dhillon, R.S., Boulding, E.G., Farrell, A.P., Glebe, B.D., Elliott, J.A.K., Wolters, W.R., Schulte, P.M., 2013. Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level. J. Exp. Biol. 216, 1183–1190. https://doi.org/10.1242/jeb.080556.

Baltazar-Soares, M., Lima, A.R.A., Silva, G., Gaget, E., 2023. Towards a unified eco-evolutionary framework for fisheries management: coupling advances in next-generation sequencing with species distribution modelling. Front. Mar. Sci. 9, 1–9. https://doi.org/10.3389/fmars.2022.1014361.

Bates, N.R., Johnson, R.J., 2020. Acceleration of ocean warming, salinification, deoxygenation and acidification in the surface subtropical North Atlantic Ocean. Commun. Earth Environ. 1, 33. https://doi.org/10.1038/s43247-020-00030-5.

Bird, R.P., Draper, A.H., 1984. Comparative studies on different methods of melondyhaldehyde determination. Methods Enzymol. 90, 105–110. https://doi.org/10.1016/S0076-6879(84)05038-2.

Biro, P.A., Beckmann, C., Stamps, J.A., 2010. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. Proc. Royal Soc. B Biol. Sci. 277 (1678), 71–77. https://doi.org/10.1098/rspb.2009.1346.

Bowyer, J.N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomson, M.J.S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquacult. Res. 45, 2010–2020. https://doi.org/10.1111/are.12146.

Brandt, S.B., Gerken, M., Hartman, K.J., Demers, E., 2009. Effects of hypoxia on food consumption and growth of juvenile striped bass (*Morone saxatilis*). J. Exp. Mar. Biol. Ecol. 381 (Suppl. L.), S143–S149. https://doi.org/10.1016/j.jembe.2009.07.028.

Braun, D.C., Patterson, D.A., Reynolds, J.D., 2013. Maternal and environmental influences on egg size and juvenile life-history traits in Pacific salmon. Ecol. Evol. 3, 1727–1740. https://doi.org/10.1002/ece3.555.

Breitburg, D., Levin, L.A., Oschlies, A., Grégoire, M., Chavez, F.P., Conley, D.J., et al., 2018. Declining oxygen in the global ocean and coastal waters. Science 359 (6371). https://doi.org/10.1126/science.aam7240.

Breslow, N.E., Clayton, D.G., 1993. Approximate inference in generalized linear mixed models. J. Am. Stat. Assoc. 88, 9–25. https://doi.org/10.1080/01621459.1993.10594284.

Brown, C., Braithwaite, V.A., 2004. Size matters: a test of boldness in eight populations of the poeciliid *Brachyraphis episcopi*. Anim. Behav. 68 (6), 1325–1329. https://doi.org/10.1016/j.anbehav.2004.04.004.

Brown, C., Jones, F., Braithwaite, V., 2005. In situ examination of boldness-shyness traits in the tropical poeciliid, *Brachyraphis episcopi*. Anim. Behav. 70 (5), 1003–1009. https://doi.org/10.1016/j.anbehav.2004.12.022.

Cadiz, L., Ernande, B., Quazuguel, P., Servili, A., Zambonino-Infante, J.L., Mazurais, D., 2018. Moderate hypoxia but not warming conditions at larval stage induces adverse carry-over effects on hypoxia tolerance of European sea bass (*Dicentrarchus labrax*) juveniles. Mar. Environ. Res. 138, 28–35. https://doi.org/10.1016/j.marenvres.2018.03.011.

Cerezo, J., García García, B., 2004. The effects of oxygen levels on oxygen consumption, survival and ventilatory frequency of sharpsnout sea bream (*Diplodus puntazzo* Gmelin, 1789) at different conditions of temperature and fish weight. J. Appl. Ichthyol. 20 (6), 488–492. https://doi.org/10.1111/j.1439-0426.2004.00601.x.

Chen, B. jian, Liu, K., Zhou, L. jun, Gomes-Silva, G., Sommer-Trembo, C., Plath, M., 2018. Personality differentially affects individual mate choice decisions in female and male Western mosquitofish (*Gambusia affinis*). PLoS One 13 (5), e0197197. https://doi.org/10.1371/JOURNAL.PONE.0197197.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, FL, pp. 283–284.

Claireaux, G., Chabot, D., 2019. The significance of ocean deoxygenation for the physiology of marine organisms. In: Laffoley, D., Baxter, J. (Eds.), Ocean Deoxygenation: Everyone's Problem. Causes, Impacts, Consequences and Solutions. IUCN, International Union for Conservation of Nature, Gland, Switzerland, pp. 461–484.

Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J. Exp. Biol. 216, 2771–2782. https://doi.org/10.1242/jeb.084251.

Cook, D.G., Herbert, N.A., 2012. Low O_2 avoidance is associated with physiological perturbation but not exhaustion in the snapper (*Pagrus auratus*: Sparidae. Comp. Biochem. Physiol. Mol. Integr. Physiol. 162 (4), 310–316. https://doi.org/10.1016/j.cbpa.2012.03.024.

Cook, D.G., Iftikar, F.I., Baker, D.W., Hickey, A.J.R., Herbert, N.A., 2013. Low-O2 acclimation shifts the hypoxia avoidance behaviour of snapper (*Pagrus auratus*) with only subtle changes in aerobic and anaerobic function. J. Exp. Biol. 216 (3), 369–378. https://doi.org/10.1242/jeb.073023.

Cooper, R.U., Clough, L.M., Farwell, M.A., West, T.L., 2002. Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J. Exp. Mar. Biol. Ecol. 279, 1–20. https://doi.org/10.1016/S0022-0981(02)00329-5.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. J. Aquatic Ecosyst. Stress Recovery 6, 43–55. https://doi.org/10.1023/A:1008228517955.

Del Rio, A.M., Davis, B.E., Fangue, N.A., Todgham, A.E., 2019. Combined effects of warming and hypoxia on early life stage Chinook salmon physiology and development. Conserv. Physiol. 7 (1), 1–14. https://doi.org/10.1093/conphys/coy078.

Del Toro-Silva, F.M., Miller, J.M., Taylor, J.C., Ellis, T.A., 2008. Influence of oxygen and temperature on growth and metabolic performance of *Paralichthys lethostigma* (Pleuronectiformes: paralichthyidae). J. Exp. Mar. Biol. Ecol. 358, 113–123. https://doi.org/10.1016/j.jembe.2008.01.019.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M., Guillermino, L., 2001. Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* strauss. Chemosphere 45, 553–560. https://doi.org/10.1016/S0045-6535(01)00029-7.

Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science 321 (5891), 926–929. https://doi.org/10.1126/science.1156401.

Dick, E.J., 2004. Beyond "lognormal versus gamma": discrimination among error distributions for generalized linear models. Fish. Res. 70, 351–366. https://doi.org/10.1016/j.fishres.2004.08.013.

Domenici, P., Allan, B.J.M., Lefrançois, C., McCormick, M.I., 2019. The effect of climate change on the escape kinematics and performance of fishes: implications for future predator-prey interactions. Conserv. Physiol. 7 (1), coz078 https://doi.org/10.1093/conphys/coz078.

Domenici, P., Lefrançois, C., Shigles, A., 2007. Hypoxia and the antipredator behaviours of fishes. Phil. Trans. R. Soc. B. 362, 2105–2121. https://doi.org/10.1098/rstb.2007.2103.

Duskey, E., 2023. Metabolic prioritization of fish in hypoxic waters: an integrative modeling approach. Front. Mar. Sci. 10, 1206506 https://doi.org/10.3389/fmars.2023.1206506.

Dwyer, G.K., Stoffels, R.J., Pridmore, P.A., 2014. Morphology, metabolism and behaviour: responses of three fishes with different lifestyles to acute hypoxia. Freshw. Biol. 59 (4), 819–831. https://doi.org/10.1111/fwb.12306.

Earhart, M.L., Blanchard, T.S., Harman, A.A., Schulte, P.M., 2022. Hypoxia and high temperature as interacting stressors: will plasticity promote resilience of fishes in a changing World? Biol. Bull. 243 (2), 149–170. https://doi.org/10.1086/722115.

Ellis, G., Goldberg, D.M., 1971. An improved manual and semi-automatic assay for NADP dependent isocitrate dehydrogenase activity, with a description of some kinetic properties of human liver and serum enzyme. Clin. Biochem. 2, 175–185. https://doi.org/10.1016/S0009-9120(71)91363-4.

Faria, A.M., Borges, R., Gonçalves, E.J., 2014. Critical swimming speeds of wild-caught sand-smelt *Atherina presbyter* larvae. J. Fish. Biol. 85, 953–959. https://doi.org/10.1111/jfb.12456.

Francisco, S.M., Congiu, L., Stefanni, S., Castilho, R., Brito, A., Ivanova, P.P., Levy, A., Cabral, H., Kilias, G., Doadrio, I., Almada, V.C., 2008. Phylogenetic relationships of the north-eastern atlantic and mediterranean forms of *Atherina* (pisces, Atherinidae). Mol. Phylogenet. Evol. 48, 782–788. https://doi.org/10.1016/j.ympev.2007.12.009.

Fraser, D.F., Gilliam, J.F., Daley, M.J., Le, A.N., Skalsk, G.T., 2001. Explaining leptokurtic movement distributions: intrapopulation variation in boldness and exploration. Am. Nat. 158, 124–135. https://doi.org/10.1086/321307.

Friard, O., Gamba, M., 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol. Evol. 7 (11), 1325–1330. https://doi.org/10.1111/2041-210X.12584.

Garcia, H.E., Weathers, K., Paver, C.R., Smolyar, I., Boyer, T.P., Locarnini, R.A., Zweng, M.M., Mishonov, A.V., Baranova, O.K., Seidov, D., Reagan, J.R., 2019. World Ocean Atlas 2018, volume 3: dissolved oxygen, apparent oxygen utilization, and oxygen saturation. In: Mishonov Technical, A. (Ed.), NOAA Atlas NESDIS 83, p. 38pp. https://www.nodc.noaa.gov/OC5/woa18/pubwoa18.html.

Green, B.S., Fisher, R., 2004. Temperature influences swimming speed, growth and larval duration in coral reef fish larvae. J. Exp. Mar. Biol. Ecol. 299 (1), 115–132. https://doi.org/10.1016/j.jembe.2003.09.001.

Höjesjö, J., Johnsson, J.I., Axelsson, M., 1999. Behavioural and heart rate responses to food limitation and predation risk: an experimental study on rainbow trout. J. Fish. Biol. 55 (5), 1009–1019. https://doi.org/10.1111/J.1095-8649.1999.TB00736.X.

Killen, S.S., Marras, S., Ryan, M.R., Domenici, P., Mckenzie, D.J., 2012. A relationship between metabolic rate and risk-taking behaviour is revealed during hypoxia in juvenile European sea bass. Funct. Ecol. 26 (1), 134–143. https://doi.org/10.1111/j.1365-2435.2011.01920.x.

Kua, Z.X., Hamilton, I.M., McLaughlin, A.L., Brodnik, R.M., Keitzer, S.C., Gilliland, J., et al., 2020. Water warming increases aggression in a tropical fish. Sci. Rep. 10 (1), 1–13. https://doi.org/10.1038/s41598-020-76780-1.

Laubenstein, T.D., Rummer, J.L., Nicol, S., Parsons, D.M., Pether, S.M.J., Pope, S., et al., 2018. Correlated effects of ocean acidification and warming on behavioral and metabolic traits of a large pelagic fish. Diversity 10, 2. https://doi.org/10.3390/D10020035.

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129 (2), 271–280. https://doi.org/10.1007/s004420100716.

Lienart, G.D.H., Mitchell, M.D., Ferrari, M.C.O., McCormick, M.I., 2014. Temperature and food availability affect risk assessment in an ectotherm. Anim. Behav. 9, 199–204. https://doi.org/10.1016/j.anbehav.2013.12.031.

Lima, A.R.A., Barletta, M., Dantas, D.V., Ramos, J.A.A., Costa, M.F., 2013. Early development of marine catfishes (Ariidae): from mouth brooding to the release of juveniles in nursery habitats. J. Fish. Biol. 82 (6), 1990–2014. https://doi.org/10.1111/jfb.12128.

Lima, I., Moreira, S.M., Osten, J.R., Soares, A.M.V.M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the northwestern coast of Portugal. Chemosphere 66, 1230–1242. https://doi.org/10.1016/j.chemosphere.2006.07.057.

Little, A.G., Hardison, E., Kraskura, K., Dressler, T., Prystay, T.S., Hendriks, B., Pruitt, J. N., Farrell, A.P., Cooke, S.J., Patterson, D.A., Hinch, S.G., Eliason, E.J., 2020. Reduced lactate dehydrogenase activity in the heart and suppressed sex hormone levels are associated with female-biased mortality during thermal stress in Pacific salmon. J. Exp. Biol. 223 https://doi.org/10.1242/jeb.214841.

Locarnini, R.A., Mishonov, A.V., Baranova, O.K., Boyer, T.P., Zweng, M.M., Garcia, H.E., Reagan, J.R., Seidov, D., Weathers, K., Paver, C.R., Smolyar, I.V., 2019. World Ocean Atlas 2018, volume 1: temperature. In: Mishonov Technical, A. (Ed.), NOAA Atlas NESDIS 81, p. 52. http://www.nodc.noaa.gov/OC5/indprod.html.

Madeira, D., Vinagre, C., Diniz, M.S., 2016. Are fish in hot water? Effects of warming on oxidative stress metabolism in the commercial species *Sparus aurata*. Ecol. Indicat. 63, 324–331. https://doi.org/10.1016/j.ecolind.2015.12.008.

Magnhagen, C., Wacker, S., Forsgren, E., Myhre, L.C., Espy, E., Amundsen, T., 2014. Context consistency and seasonal variation in boldness of male two-spotted gobies. PLoS One 9 (3), e93354. https://doi.org/10.1371/journal.pone.0093354.

Mandic, M., Regan, M.D., 2018. Can variation among hypoxic environments explain why different fish species use different hypoxic survival strategies? J. Exp. Biol. 221 (21), jeb161349 https://doi.org/10.1242/jeb.161349.

Mattiasen, E.G., Kashef, N.S., Stafford, D.M., Logan, C.A., Sogard, S.M., Bjorkstedt, E.P., Hamilton, S.L., 2020. Effects of hypoxia on the behavior and physiology of kelp forest fishes. Global Change Biol. 26, 3498–3511. https://doi.org/10.1111/gcb.15076.

McBryan, T.L., Anttila, K., Healy, T.M., Schulte, P.M., 2013. Responses to temperature and hypoxia as interacting stressors in fish: implications for adaptation to environmental change. Integr. Comp. Biol. 53 (4), 648–659. https://doi.org/10.1093/icb/ict066.

McBryan, T.L., Healy, T.M., Haakons, K.L., Schulte, P.M., 2016. Warm acclimation improves hypoxia tolerance in *Fundulus heteroclitus*. J. Exp. Biol. 219 (4), 474–484. https://doi.org/10.1242/jeb.133413.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5.

McCullagh, P., Nelder, J.A., 1989. Generalized Linear Models, second ed. Chapman & Hall, London, p. 509.

McPhee, D., Watson, J.R., Harding, D.J., Prior, A., Fawcett, J.H., Franklin, C.E., Cramp, R.L., 2023. Body size dictates physiological and behavioural responses to hypoxia and elevated water temperatures in Murray cod (*Maccullochella peelii*). Conserv. Physiol. 11, coac087. https://doi.org/10.1093/conphys/coac087.

Miller, K.A., Garner, J.P., Mench, J.A., 2006. Is fearfulness a trait that can be measured with behavioural tests? A validation of four fear tests for Japanese quail. Anim. Behav. 71 (6), 1323–1334. https://doi.org/10.1016/j.anbehav.2005.08.018.

NASA, 2010. NASA earth observatory. Revised 17 July 2010. https://earthobservatory.nasa.gov/images/44677/aquatic-dead-zones. (Accessed 17 January 2010).

NOAA, 2016. National oceanic and atmospheric administration. Monthly Global Climate Report for Annual 2016. National Centers for Environmental Information. Published online January 2017. Retrieved 4 September 2023. https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/201613.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358. https://doi.org/10.1016/0003-2697(79)90738-3.

Olive, P., 1988. DNA precipitation assay - a rapid and simple method for detecting DNA damage in mammalian cells. Environ. Mol. Mutagen. 11, 487–495. https://doi.org/10.1002/em.2850110409.

Pepin, P., 1995. An analysis of the length-weight relationship of larval fish: limitations of the general allometric model. Fish. Bull. 93 (2), 419–426.

Pettinau, L., Lancien, F., Zhang, Y., Mauduit, F., Ollivier, H., Farrell, A.P., et al., 2022. Warm, but not hypoxic acclimation, prolongs ventricular diastole and decreases the protein level of Na^+/Ca^{2+} exchanger to enhance cardiac thermal tolerance in European sea bass. Comp. Biochem. Physiol. Mol. Integr. Physiol. 272, 111266 https://doi.org/10.1016/j.cbpa.2022.111266.

R Core Team, 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.r-project.org/index.html.

Rao, R.C., 1964. The use and interpretation of principal component analysis in applied research. Indian J. Statistics Series A 26 (4), 329–358.

Remen, M., Nederlof, M.A.J., Folkedal, O., Thorsheim, G., Sitjà-Bobadilla, A., Pérez-Sánchez, J., et al., 2015. Effect of temperature on the metabolism, behaviour and oxygen requirements of *Sparus aurata*. Aquacult. Env. Interac. 7 (2), 115–123. https://doi.org/10.3354/aei00141.

Remen, M., Oppedal, F., Imsland, A.K., Olsen, R.E., Torgersen, T., 2013. Hypoxia tolerance thresholds for post-smolt Atlantic salmon: dependency of temperature and hypoxia acclimation. Aquaculture 416–417, 41–47. https://doi.org/10.1016/j.aquaculture.2013.08.024.

Sampaio, E., Santos, C., Rosa, I.C., Ferreira, V., Pörtner, H.O., Duarte, C.M., Levin, L.A., Rosa, R., 2021. Impacts of hypoxic events surpass those of future ocean warming and acidification. Nat. Ecol. Evol. 5, 311–321. https://doi.org/10.1038/s41559-020-01370-3.

Schmidtko, S., Stramma, L., Visbeck, M., 2017. Decline in global oceanic oxygen content during the past five decades. Nature 542 (7641), 335–339. https://doi.org/10.1038/nature21399.

Schulte, P.M., 2015. The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. J. Exp. Biol. 218, 1856–1866. https://doi.org/10.1242/jeb.118851.

Schurmann, H., Steffensen, J.F., 1997. Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod. J. Exp. Mar. Biol. Ecol. 50, 1166–1180. https://doi.org/10.1111/j.1095-8649.1997.tb01645.x.

Scott, J.D., Alexander, M.A., Murray, D.R., Swales, D., Eischeid, J., 2016. The climate change web portal: a system to access and display climate and earth system model output from the CMIP5 Archive. Bull. Am. Meteorol. Soc. 97 (4), 523–530. https://doi.org/10.1175/BAMS-D-15-00035.1.

Secor, D.H., Gunderson, T.E., 1998. Effects of hypoxia and temperature on survival, growth, and respiration of juvenile Atlantic Sturgeon *Acipenser oxyrinchus*. Fish. Bull. 96, 603–613.

Shoji, J., Masuda, R., Yamashita, Y., Tanaka, M., 2005. Effect of low dissolved oxygen concentrations on behavior and predation rates on red sea bream *Pagrus major* larvae by the jellyfish *Aurelia aurita* and by juvenile Spanish mackerel *Scomberomorus niphonius*. Mar. Biol. 147, 863–868. https://doi.org/10.1007/s00227-005-1579-8.

Silva, C.S.E., Lemos, M.F.L., Faria, A.M., Lopes, A.F., Mendes, S., Gonçalves, E.J., Novais, S.C., 2018. Sand smelt ability to cope and recover from ocean's elevated CO_2 levels. Ecotoxicol. Environ. Saf. 154, 302–310. https://doi.org/10.1016/j.ecoenv.2018.02.011.

Socci, D.J., Bjugstad, K.B., Jones, H.C., Pattisapu, J.V., Arendash, G.W., 1999. Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the H-Tx rat model. Exp. Neurol. 155, 109–117. https://doi.org/10.1006/exnr.1998.6969.

Toms, C.N., Echevarria, D.J., Jouandot, D.J., 2010. A methodological review of personality-related studies in fish: focus on the shy-bold Axis of behavior. Int. J. Comp. Psychol. 23, 1–25. https://doi.org/10.46867/ijcp.2010.23.01.08.

Vagner, M., Lefrançois, C., Ferrari, R.S., Satta, A., Domenici, P., 2008. The effect of acute hypoxia on swimming stamina at optimal swimming speed in flathead grey mullet *Mugil cephalus*. Mar. Biol. 155 (2), 183–190. https://doi.org/10.1007/s00227-008-1016-x.

van Snik, G.M.J., van den Boogaart, J.G.M., Osse, J.W.M., 1997. Larval growth patterns in *Cyprinus carpio* and *Clarias gariepinus* with attention to finfold. J. Fish. Biol. 50, 1339–1352. https://doi.org/10.1111/j.1095-8649.1997.tb01657.x.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Huelvan, C., Corporeau, C., Mazurais, D., Zambonino-Infante, J.L., 2015. Exposure to chronic moderate hypoxia impacts physiological and developmental traits of European sea bass (*Dicentrarchus labrax*) larvae. Fish Physiol. Biochem. 41 (1), 233–242. https://doi.org/10.1007/s10695-014-0019-4.

Vaquer-Sunyer, R., Duarte, C.M., 2008. Thresholds of hypoxia for marine biodiversity. Proc. Natl. Acad. Sci. U.S.A. 105 (40), 15452–15457. https://doi.org/10.1073/pnas.0803833105.

Vassault, A., 1983. Methods of Enzymatic Analysis. Academic Press, NY, pp. 118–126.

Verberk, W.C.E.P., Sandker, J.F., van de Pol, I.L.E., Urbina, M.A., Wilson, R.W., McKenzie, D.J., Leiva, F.P., 2022. Body mass and cell size shape the tolerance of fishes to low oxygen in a temperature-dependent manner. Global Change Biol. 28 (19), 5695–5707. https://doi.org/10.1111/gcb.16319.

Yang, G., Wang, Z., He, J., Li, W., Yuan, D., 2020. Allometric growth patterns of fine scale fish larvae and its ecological significance. J. Phys. Conf. Ser. 1575, 012157 https://doi.org/10.1088/1742-6596/1575/1/012157.

Zhou, L.Y., Fu, S.J., Fu, C., Ling, H., Li, X.M., 2019. Effects of acclimation temperature on the thermal tolerance, hypoxia tolerance and swimming performance of two endangered fish species in China. J. Comp. Physiol. B. 189 (2), 237–247. https://doi.org/10.1007/s00360-018-01201-9.