Early life stage mechanisms of an active fish species to cope with ocean warming and hypoxia as interacting stressors[☆]

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ABSTRACT

Ocean's characteristics are rapidly changing, modifying environmental suitability for early life stages of fish. We assessed whether the chronic effects of warming (24 °C) and hypoxia ($<$2–2.5 mg L^{-1}) will be amplified by the combination of these stressors on mortality, growth, behaviour, metabolism and oxidative stress of early stages of the white seabream *Diplodus sargus*. Combined warming and hypoxia synergistically increased larval mortality by $>$51%. Warming induced faster growth in length and slower gains in weight when compared to other treatments. Boldness and exploration were not directly affected, but swimming activity increased under all test treatments. Under the combination of warming and hypoxia, routine metabolic rate (RMR) significantly decreases when compared to other treatments and shows a negative thermal dependence. Superoxide dismutase and catalase activities increased under warming and were maintained similar to control levels under hypoxia or under combined stressors. Under hypoxia, the enzymatic activities were not enough to prevent oxidative damages as lipid peroxidation and DNA damage increased above control levels. Hypoxia reduced electron transport system activity (cellular respiration) and isocitrate dehydrogenase activity (aerobic metabolism) below control levels. However, lactate dehydrogenase activity (anaerobic metabolism) did not differ among treatments. A Redundancy Analysis showed that ~99% of the variability in mortality, growth, behaviour and RMR among treatments can be explained by molecular responses. Mortality and growth are highly influenced by oxidative stress and energy metabolism, exhibiting a positive relationship with reactive oxygen species and a negative relationship with aerobic metabolism, regardless of treatment. Under hypoxic condition, RMR, boldness and swimming activity have a positive relationship with anaerobic metabolism regardless of temperature. Thus, seabreams may use anaerobic reliance to counterbalance the effects of the stressors on RMR, activity and growth. The outcomes suggests that early life stages of white seabream overcame the single and combined effects of hypoxia and warming.

1. Introduction

In the past 50 years, rising sea water temperatures have driven a ~2% decline in global dissolved oxygen levels (Schmidtko et al., 2017). The Intergovernmental Panel on Climate Change (IPCC) predicts a future temperature rise of ~1–4 °C by 2100 (Pörtner et al., 2014; IPCC,

2022), which will likely drive an average decline of ~7% in oceanic oxygen levels (Keeling et al., 2010). In addition, eutrophication-induced hypoxia increases at higher temperatures as the metabolism of micro-organisms that drive eutrophication also increases, enhancing the co-occurrence of local episodes of hypoxia and high temperatures (McBryan et al., 2013). Recent research has found that oxygen-minimum zones have expanded by 4.5 million km^2, and more than 700 coastal sites can have oxygen concentrations below 2 mg L^{-1}, which can impair habitat suitability for marine species (Breitburg et al., 2018; Limburg et al., 2020; Baltazar-Soares et al., 2023).

The short-term response of ectotherms, such as fish, to warming involves thermal acclimation to the novel thermal environment by adjusting their physiology to counter the thermal effect (Jutfelt, 2020). On longer timescales, fish often adapt their physiology and performance to a large range of temperatures (Jutfelt, 2020). The effects of higher temperatures have been extensively reported for fishes. Warming increased routine metabolic rate (Remen et al., 2015), oxidative damages and antioxidant defence (Madeira et al., 2016) of *Sparus aurata*. A reduction of anaerobic metabolism (i.e. lactate dehydrogenase) in the heart of *Oncorhynchus kisutch* was observed after warming exposure (Little et al., 2020). High temperatures can also alter risk-taking related behaviours by increasing boldness and swimming activity in *Pomacentrus moluccensis* larvae (Biro et al., 2010). Warmer conditions can induce faster egg hatching and faster larval growth, shortening the pelagic larval phase and enhancing swimming capacities in *Salmo salar* (Braun et al., 2013) and in *Amphiprion melanopus* larvae (Green and Fisher, 2004).

Hypoxia, on the other hand, is often a new stressor for many coastal fishes (Roman et al., 2019). Although fishes inhabiting oceanic minimum zones (OMZ) have co-evolved with low dissolved oxygen concentrations, most coastal species do not appear to have developed specific physiological adaptations to hypoxia (Roman et al., 2019). Species such as *Gadus morhua* and *Anchoa mitchilli* experienced mortality and sublethal responses at oxygen levels even above 2 mg L^{-1} (Vaquer-Sunyer and Duarte, 2008). The hypoxia tolerant species *Mugil cephalus* can use anaerobic metabolism to supplement swimming activity in hypoxic conditions (Vagner et al., 2008). *Sebastes caurinus* and *Sebastes mystinus* exhibited decreased aerobic scope under hypoxia (Mattiasen et al., 2020), while severe hypoxia increased anaerobic metabolism and the antioxidant defence in Leiostomus xanthurus (Cooper et al., 2002). Decreased swimming activity was observed as O$_2$ declined below the limiting oxygen saturation (i.e. P$_{crit}$) in *Embiotoca lateralis* (Cook et al., 2014) and *Silurus meridionalis* (Zhang et al., 2010). In *Oncorhynchus mykiss*, exposure to hypoxia during the embryo stage led to reduced metabolic and locomotor activities of resulting larvae (Johnston et al., 2013). Hypoxia also decreased the growth of *Morone saxatilis* (Brandt et al., 2009) and induced body mass loss in *S. salar* (Remen et al., 2013) and in *Seriola lalandi* (Bowyer et al., 2014).

Nevertheless, studies on the effects of combined stressors related to climate change in fish are rare (Schurmann and Steffensen, 1997; McBryan et al., 2016; Zhou et al., 2019; Pettinau et al., 2022). Even rarer are works on larval stages, which are highly vulnerable, with mortality rates often exceeding 80% under optimal conditions (Lima et al., 2019). The combined effects of acidification and hypoxia had a synergistic negative effect on post-hatch survival of *Menidia* (DePasquale et al., 2015). Combined hypoxia and warming increased the adverse short-term effects of polycyclic aromatic hydrocarbons (PAHs) on development and mortality *Fundulus grandis* larvae (Serafin et al., 2019). Positive effects on growth and survival were observed when *D. labrax* larvae were exposed to the combination of warming and acidification, demonstrating a certain coping ability to such conditions (Pope et al., 2014). However, another study that evaluated several marine organisms, including fish, reported a general trend of lower average survival, growth and development after exposure to combined acidification and warming (Kroeker et al., 2013).

There have been few studies evaluating the combined effects of

warming and hypoxia in fish. It is likely that these stressors interact synergistically in fishes. Ectotherm's metabolic rate and oxygen demand tend to increase with temperature, while hypoxia simultaneously limits oxygen supply (McBryan et al., 2013). The combined effects of warming and hypoxia reduced growth rates by 50% in *Paralichthys lethostigma* (Del Toro-Silva et al., 2008), caused weight loss in *M. saxatilis* (Brandt et al., 2009) and increased the mortality of *Acipen oxyrinchus* juveniles by 92% (Secor and Gunderson, 1998). The combination of these stressors resulted in increased gill diffusion area and increased thickness of the myocardium in *S. salar*, enhancing the heart's ability to contract under hypoxia (Anttila et al., 2013). Regarding early life stages, the combined effects of warming and hypoxia caused reduced hatching success in *Oncorhynchus tshawytscha* (Del Rio et al., 2019) and had no effect on weight loss and loss of equilibrium in *Dicentrarchus labrax* (Cadiz et al., 2018). Thus, there still is no simple cause-and-effect relationship of their interaction, as each species has its own behavioural and physiological mechanisms to withstand the combined effects of these stressors (Zambonino-Infante et al., 2013; Del Rio et al., 2019).

However, most studies to date have evaluated few endpoints or responses that often leads to knowledge gaps in a species' potential to combine behavioural and physiological strategies to withstand the effects of warming and hypoxia. Here, we used early life stages of the white seabream (*Diplodus sargus*) as a model species. This is a demersal fish of ecological and economic importance inhabiting the eastern Atlantic (from South Africa to the English Channel) and the Mediterranean with no population structure differentiation (Bargelloni et al., 2005). Actually, hypoxic coastal areas and oxygen-minimum zones as well as a gradual increase of sea water temperature have been detected along the entire distribution range of this species (Breitburg et al., 2018; Lima et al., 2022a,b). Here we provide an integrative approach to assess: (I) whether the chronic effects of warming and hypoxia can be amplified by the combination of these two stressors on the mortality, growth, behaviour (boldness, exploration and activity levels), metabolic rate, oxidative stress and energy metabolism systems; and (II) whether the variability in mortality, growth, behaviour and metabolic rate could be related to molecular alterations in oxidative stress and energy metabolism systems as coping strategies.

2. Material and methods

2.1. Experimental design

Approximately 600 fish larvae were transferred from the aquaculture research station of the Portuguese Institute for the Sea and Atmosphere (IPMA-EPPO), in Olhão, to the University Institute of Psychological, Social and Life sciences (ISPA) fish facilities. We selected larvae of approximately 55 days post hatch (dph) due to their higher swim capacity and likelihood of inhabiting areas closer to the coast, where hypoxic zones may occur (Azeiteiro et al., 2006; Félix-Hackradt et al., 2013; Baptista et al., 2019, 2020; Torrado et al., 2021). Larvae were maintained in quarantine (20 °C and 100% DO) for 1 week to recover from transport and handling stress. Present-day and future sea surface temperature (SST), represented as quarterly-averages from daily-means, were extracted from NOAA's WOA (Garcia et al., 2019; Locarnini et al., 2019) and CMIP5 (Scott et al., 2016) remotely sensed climate models (2100 - RCP 8.5), respectively, from geo-referenced occurrence data representing habitats where larvae can be collected from the spawning season to the recruitment period. The highest values of SST were set as probable conditions for both present-day and future treatments. Hypoxic conditions simulate the coastal dead zones found along the distribution range of the species (Breitburg et al., 2018).

After quarantine, larvae were randomly distributed over 8 experimental tanks of 25 L at a starting temperature of 20 °C and 100% DO and a density of ~60 larvae per tank. There were 4 different treatments, each with 2 replicate tanks:

A: 20 °C & 80–100% DO (5.9–7.3 mg L^{-1} DO) (control treatment).

B: 24 °C & 80–100% DO (5.5–6.8 mg L^{-1} DO).
C: 20 °C & 27–33.9% DO (2.0–2.5 mg L^{-1} DO).
D: 24 °C & 29–36.3% DO (2.0–2.5 mg L^{-1} DO).

Exposure to treatment conditions was run for 15 days. Temperature was increased in treatments B and D using heaters at a rate of 1 °C/day until the desired temperature of 24 °C. The oxygen level was steadily decreased in treatment C and D over a 4-day period by pumping pure nitrogen (N$_2$) into the tanks using an oxygen regulator computer (Loligo® Systems OXY-REG) until matching the desired O$_2$ level between 2 and 2.5 mg L^{-1}. Solenoid valves controlled the amount of nitrogen that was bubbled into the tanks to maintain O$_2$ at the defined threshold. The set-up was sealed with a clear glass lid and plastic to limit O$_2$ exchange with the atmosphere and reduce evaporation of water to keep the salinity level steady. A cycle of 14 L:10D using fluorescent light was followed. Larvae were fed in all treatments 4 times per day, *ad libitum*, with the commercial ration WINfast (SPAROS Lda®, Portugal). Mortality was checked every day and measured as the number of dead fish during the exposure period. Dead fish were removed from tanks.

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

Thirty-six fish per treatment were randomly collected from both replicate tanks to evaluate the chronic effects of warming and hypoxia on growth in both length and weight. We used the allometric growth model $y = \beta_0 \, T_L^{\beta 1} + \varepsilon$, where y is the dependent variable, T_L is the independent variable, β_0 is the intercept and β_1 is the slope or growth coefficient (van Snik et al., 1997). For isometric growth, the slope β_1 is 1 for length and 3 for weight (Lima et al., 2013). When the slope β_1 is smaller than the isometric slope, the growth is said to be negatively allometric (slower); when higher, the growth is said to be positively allometric (faster) (Lima et al., 2013).

2.3. Behavioural analysis

Experimental trials were conducted over a period of 4 days in a temperature-controlled room. The experimental arena consisted of a rectangular aquarium (56 × 38 × 40 cm), divided into a small starting compartment (18 × 38 cm) and a larger testing arena (Magnhagen et al., 2014) (see supplementary material and Fig. S1). The starting compartment had gravel on the bottom to simulate a shelter. The larger compartment had no structural elements, and the bottom had a gridded white surface underneath, dividing the area in three square zones: zone 1 was closer to the tank wall (protected), zone 2 an intermediate zone (partially protected), and zone 3 was the inner zone of the arena (less protected). Twenty fish were randomly sourced from both replicate tanks per treatment to reduce differences in individual peaks of activity and individually tested once. All tests were recorded from above with the aid of a camera (Canon PowerShot G7X Mark II, Sony RX100 IV). Video analysis was carried out using BORIS 7.3 (Friard and Gamba, 2016).

2.3.1. Boldness assay

Boldness was tested using an emergence assay (Brown and Braithwaite, 2004; Miller et al., 2006). The focal fish was placed in the starting compartment to acclimate for 5 min. After that, the door was lifted which allowed the fish to swim into the test arena. If the fish did not exit the starting area for 5 min (N = 2), we gently moved them outside the container with the help of a small aquarium dip net (Chen et al., 2018). The following response variables were recorded: (1) average latency to exit shelter for the first time; (2) average time spent in the shelter after first exit and (3) average time spent in zones 1, 2 and 3. A bolder fish is expected to enter the large compartment faster, stay

longer, and move further away from the sheltered area than a shyer fish (Toms et al., 2010).

2.3.2. Exploration assay

Exploration was tested with the use of a novel object assay conducted consecutively to the boldness assay. A red Lego® cube was gently sunk in the middle of the large compartment (zone 3) until the middle of the water line using a fish line to avoid human intervention. The following response variables were recorded over 10 min: (1) average latency of fish to approach the novel object for the first time; and (2) average time spent by fish in the novel area (adapted from Villegas-Ríos et al., 2018).

2.3.3. Activity assay

Activity was tested as routine swimming responses by measuring the distance swum and the average swimming velocity (speed). Each video was converted into frames with an associated period in seconds, excluding the first and last 3 min of the exploration trials to exclude the influence of the researcher's proximity to the arena. Pixels were converted into millimetres in each frame. A manual tracking plugin measured the distance swum by the larva between consecutive frames over the chosen period of time. All video analysis we carried out using Image-J (v1.48; U. S. National Institutes of Health, Bethesda, Maryland).

2.4. Oxygen consumption measured by routine metabolic rate (RMR)

Oxygen consumption was measured to estimate individual RMR, resorting to a closed respirometry system, according to Almeida et al. (2022) (see supplementary material). Sixteen fish were randomly selected from both replicate tanks per treatment to reduce differences in individual peaks of activity and only tested once over a period of 4 days. While in the respirometry chamber, the individuals were allowed to acclimate to the new environment for 1 h with constant water circulation with temperature and oxygen levels matching the conditions of each treatment. By the end of acclimation period, the circulation of water was interrupted, and the oxygen levels inside the chamber measured every 15 s using contactless oxygen sensor spots (OXSP5, Pyroscience) during a 25-min period, and monitored using the software Pyro Oxygen Logger (Pyroscience, Denmark). To minimize the effects of bacterial respiration, the background bacterial oxygen consumption was recorded after each test. The RMR was calculated using the following formula:

$$MO_2 = \frac{\text{Chamber volume} * \text{Slope of oxygen consumption}}{\text{Weight (Kg)} * \text{Time (hours)}} - \text{Background}$$

To check whether RMR is thermally sensitivity, temperature coefficients (Q$_{10}$) for the warming (B) and warming *vs.* hypoxia (D) treatments were analysed using the following formula:

$$Q_{10} = \left(\frac{RMR24°C}{RMR20°C} \right)^{20°C/(24°C-20°C)}$$

If RMR is completely temperature independent, the resulting Q$_{10}$ will be 1. If RMR increases with increasing temperature, Q$_{10}$ will be > 1. If RMR decreases with increasing temperature, Q$_{10}$ will be < 1.

2.5. Biomarkers analysis

A total of eight fish per treatment were randomly sourced from both replicate tanks per treatment. These fish were not tested for behavioural responses or RMR. Oxidative stress biomarkers were used to evaluate the antioxidant defence capacities by assessing how the formation of reactive oxygen species (ROS) (Socci et al., 1999) could be controlled by the enzymatic activities of superoxide dismutase (SOD) (McCord and Fridovich, 1969) and catalase (CAT) (Claiborne, 1985). Oxidative damages were checked by observing the levels of lipid peroxidation (LPO) (Ohkawa et al., 1979; Bird and Draper, 1984) and DNA damage (Olive,

1988).

Energy metabolism biomarkers were used to check the rate of cellular energy consumption based on the electron transport system (ETS) (De Coen and Janssen, 1997). Aerobic metabolism was measured by the isocitrate dehydrogenase activity (IDH) (Ellis and Goldberg, 1971; Lima et al., 2007). Anaerobic metabolism was measured by the lactate dehydrogenase activity (LDH) (Vassault, 1983; Diamantino et al., 2001) (see Supplementary Material).

3. Statistical analysis

T-tests were used to compare growth coefficients and check whether growth in length and weight differ among treatments. Generalized Linear Mixed Models (Breslow and Clayton, 1993) were used to investigate whether the variability of mortality, growth, behavioural responses (boldness, exploration and activity), routine metabolic rate and biomarkers [response variables (y_i)] are affected by the different treatments [or categorical explanatory variables (x_i)]. To reduce the chances of a type I error, we used each tank as a nested random repeated factor within the main effects to run all models. Mortality, boldness and exploration were analysed through a Poisson distribution, using the Poisson variance $V(\mu) = \mu$, and a logarithmic-link $\log(\mu)$ functions (McCullagh and Nelder, 1989). Growth, activity, routine metabolic rate and biomarkers were analysed through a log-normal distribution, using the lognormal variance $V(\mu) = \exp^{(2\mu + \sigma2)} [\exp^{(\sigma2)} - 1]$ and the logarithmic-link $\log(\mu)$ functions (Dick, 2004). Whenever significant differences were detected, a Tukey HSD *post hoc* test was performed to examine the source of variance among treatments. P-values below 0.05 were considered significant.

A Redundancy Analysis (RDA) (Rao, 1964) was performed to investigate whether the chronic effects of warming and hypoxia on larval responses could be explained by chronic alterations on physiology. A matrix of the groups (i.e. different treatments and tanks) containing behaviour, activity, growth and metabolic rate was used as response variable in the RDA, while a matrix of values related to alterations on oxidative stress and energy metabolism was utilized as explanatory variables. Response variables were Hellinger-transformed before run the model (Legendre and Gallagher, 2001). The overall statistical significance of the RDA model, of constrained axis and the effects and significance of each variable were assessed using an ANOVA permutation function. Explanatory variables not significant to the model were excluded from the full model. The selected explanatory variables appear as vectors radiating from the origin of the ordination. The length of the vector is related to the power that explanatory variables exert over the response variables and the groups. The direction and proximity among variables and groups dictates a positive or negative relationship. All statistical analyses were performed with R statistical software (R Core Team, 2022).

4. Results

4.1. Effects of warming and hypoxia on mortality

Mortality was significantly higher when seabreams were exposed to the combination of warming and hypoxia when compared to treatments A (control) and B (warming) (p < 0.001) (Fig. 1). In addition, warming and hypoxia acted synergistically, increasing average mortality by >51% relative to the control. Although not significantly different, the second highest mortality was observed in the hypoxia treatment.

4.2. Effects of warming and hypoxia on morphological strategies

By comparing growth coefficients (or slopes) among treatments, our results showed that warming induced putative changes in growth patterns. Growth in weight was significantly slower (allometric negative) under warming, when compared to the isometric growths observed in all
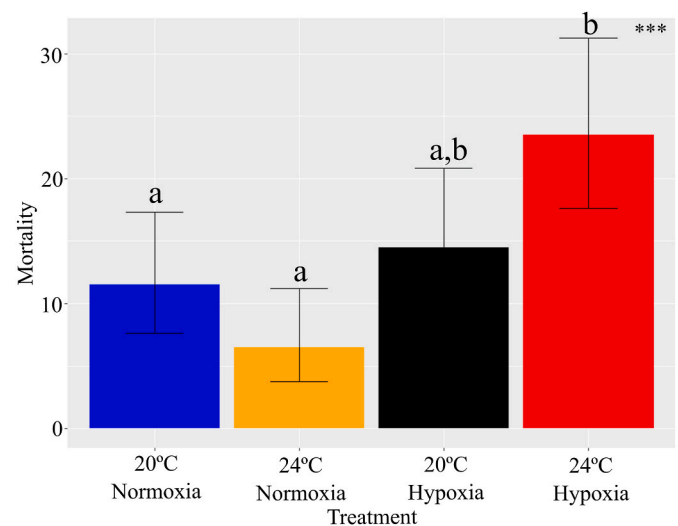

Fig. 1. Average mortality (±S.E.) measured as the number of dead seabream early stages chronically exposed to different treatments. Letters represent homogeneous groups. ns, non-significant; ***p < 0.001; **p < 0.01; *p < 0.05.

other treatments (p < 0.05) (Fig. 2a). Standard length grew significantly faster (allometric positive) under warming, when compared to the allometric negative growths observed in all other treatments (p < 0.05) (Fig. 2b).

4.3. Effects of warming and hypoxia on larval behaviour: boldness, exploration and activity

Warming and hypoxia did not exhibit direct effects in overall boldness and exploration but significantly affected activity. The latency of the fish to exit the shelter for the first time differed significantly among test treatments, but not between test treatments and the control (Fig. 3a). Seabreams exposed to the combination of warming and hypoxia left the shelter faster, followed by seabreams from the hypoxia treatment; and both differed from the warming treatment (p < 0.01). The different treatments did not prevent fish from seeking protection during the entire trial, as there were no significant differences in the time spent in shelter after first exit (p > 0.05) (Fig. 3b).

Overall, fish spent less time in zone 3 (less protected or far from the wall), on average, indicating that fish avoid more exposed areas, regardless of treatment (Fig. 3e). When exposed to the combination of warming and hypoxia, fish spent significantly less time in zone 1 (protected or closer to the wall) when compared to zone 2 (partially protected or between the centre and the wall) (p < 0.001) (Fig. 3c and d). Fish exposed to warming or hypoxia spent significantly less time in zone 2 when compared to the control and the warming & hypoxia treatment (p < 0.001) in the same zone (Fig. 3c and d).

When a novel object was introduced in the middle of the arena, the latency to approach the object for the first time was not significant among treatments (p > 0.05), with fish taking a long time (~420 ± 38 to 470 ± 42 s) to approach the novel object (Fig. 3f). The time seabreams spent closer to the novel object did not differ between test treatments and the control. However, this behaviour was significantly higher under hypoxia when compared to the warming & hypoxia treatment (p < 0.05) (Fig. 3g).

The single and combined effects of warming and hypoxia enhanced larval activity as they swim significantly faster and over longer distances when compared to control conditions (p < 0.05) (Fig. 3h and i).
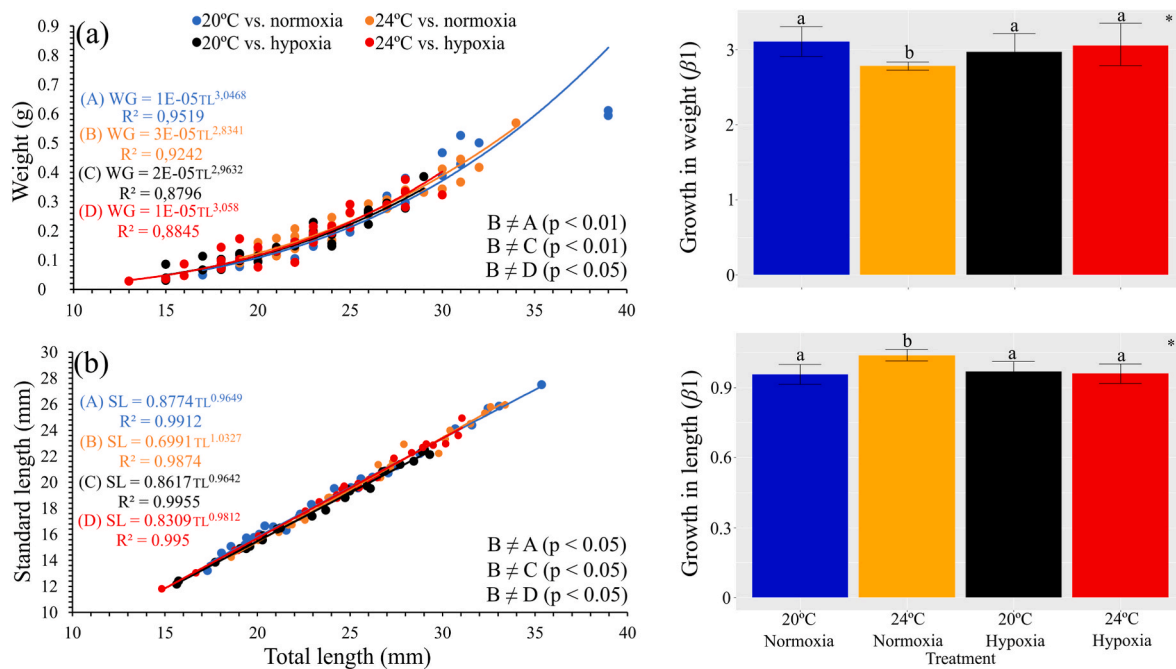
Fig. 2. Relationships between morphometric variables and the average (±S.E.) of growth coefficients of seabream early stages (N = 36) chronically exposed to different treatments simulating (A) control (or natural) condition, (B) warming, (C) hypoxia and (D) the combination of both stressors. Growth coefficients (β1) and R2 values were calculated according to the model y = β0 TL β1 + ε. Letters represent homogeneous groups. ns, non-significant; ***p < 0.001; **p < 0.01; *p < 0.05.

4.4. Effects of warming and hypoxia on physiology

4.4.1. Effects of warming and hypoxia on routine metabolic rates (RMR)

The combined effects of warming and hypoxia significantly decreased routine metabolic rate (RMR), compared to all other treatments ($p < 0.05$) (Fig. 4). RMR was thermally independent ($Q_{10} = 1.03$) under warming and exhibited a negative thermal dependence under warming *vs.* hypoxia ($Q_{10} = 0.29$).

4.4.2. Variability of biomarkers as a response to oxidative stress

ROS formation did not differ among treatments (Fig. 5a). Enzymatic activity was higher under warming. Fish reared in warming exhibited higher SOD activities than those reared in the combination of warming and hypoxia and control treatment ($p < 0.001$) (Fig. 5b). The highest CAT activity is also observed under warming when compared to all other treatments ($p < 0.05$) (Fig. 5c). LPO increased in the hypoxia treatment during the chronic phase, exhibiting higher levels than control treatment ($p < 0.05$) (Fig. 5d). DNA damage was also significantly higher under hypoxia, when compared to all other treatments ($p < 0.001$) (Fig. 5e).

4.4.3. Variability of biomarkers as a response to energy metabolism

ETS activity is significantly reduced in the hypoxia treatment when compared to the warming treatment and the control ($p < 0.05$), but it is similar to the warming *vs.* hypoxia treatment ($p < 0.05$) (Fig. 6a). Anaerobic metabolism (LDH activity) did not differ among treatments (Fig. 6b). On the other hand, the IDH activity is significantly decreased in the hypoxia treatment when compared to the warming treatment ($p < 0.05$), but it is similar to the warming *vs.* hypoxia treatment and the control (Fig. 6c).

4.5. Relationship among behaviour, activity, growth and metabolic rate as a response to alterations on oxidative stress and energy metabolism systems

The RDA showed that oxidative stress and energy metabolism (biomarkers) significantly influenced variability in mortality, behaviour, growth and RMR of *D. sargus* exposed to the different treatments ($p <$

0.01). The first axis explained 99.16% of the variance of the relationship among variables and is represented by the variability in biomarkers ($p < 0.01$) (Fig. 7). All selected biomarkers had significant influence on the RDA model ($p < 0.05$). The first axis showed a strong negative correlation with ROS and a strong positive correlation with IDH. This means that these two variables strongly drove the variation in the data. The second axis explained 0.62% and is represented by the different experimental treatments ($p < 0.05$). The second axis also showed a strong negative correlation with ROS. The upper portion of the image represents mostly fish exposed to 20 °C, whilst the lower portion to 24 °C. The left side of the image represents fish mostly exposed to hypoxia, whilst the right side to normoxia. Growth in both length and weight are located in the centre of the ordination, meaning that variability in oxidative stress and energy metabolism had great influence in growth patterns. Growth decreases with increased oxidative damages (DNA and LPO), increased ROS and with increased activity of SOD, IDH and LDH. The RMR is higher (or positively correlated) when fish are only exposed to hypoxia and increases with increased LPO (Fig. 7). Mortality is high when fish are exposed to warming *vs.* hypoxia and increases with increased ROS, increased DNA damage and with increased IDH and LDH activities. Latency to exit shelter is higher when fish are only exposed to warming or to the control condition and increases with increased DNA damage, SOD activity and aerobic metabolism (IDH). Swimming velocity and distance swam increases when fish are exposed to all test treatments and with increased ROS and anaerobic metabolism (LDH).

5. Discussion

5.1. The combination of warming and hypoxia acted synergistically on mortality

Our results show that warming and hypoxia acted synergistically and significantly increased average mortality of seabreams by >51% relative to the control. Data on the interactive effects of stressors is limited and highly variable among species. They often act synergistically and can even lead to high mortality rates (Verberk et al., 2022). Combined acidification and hypoxia induced negative effects on post-hatch
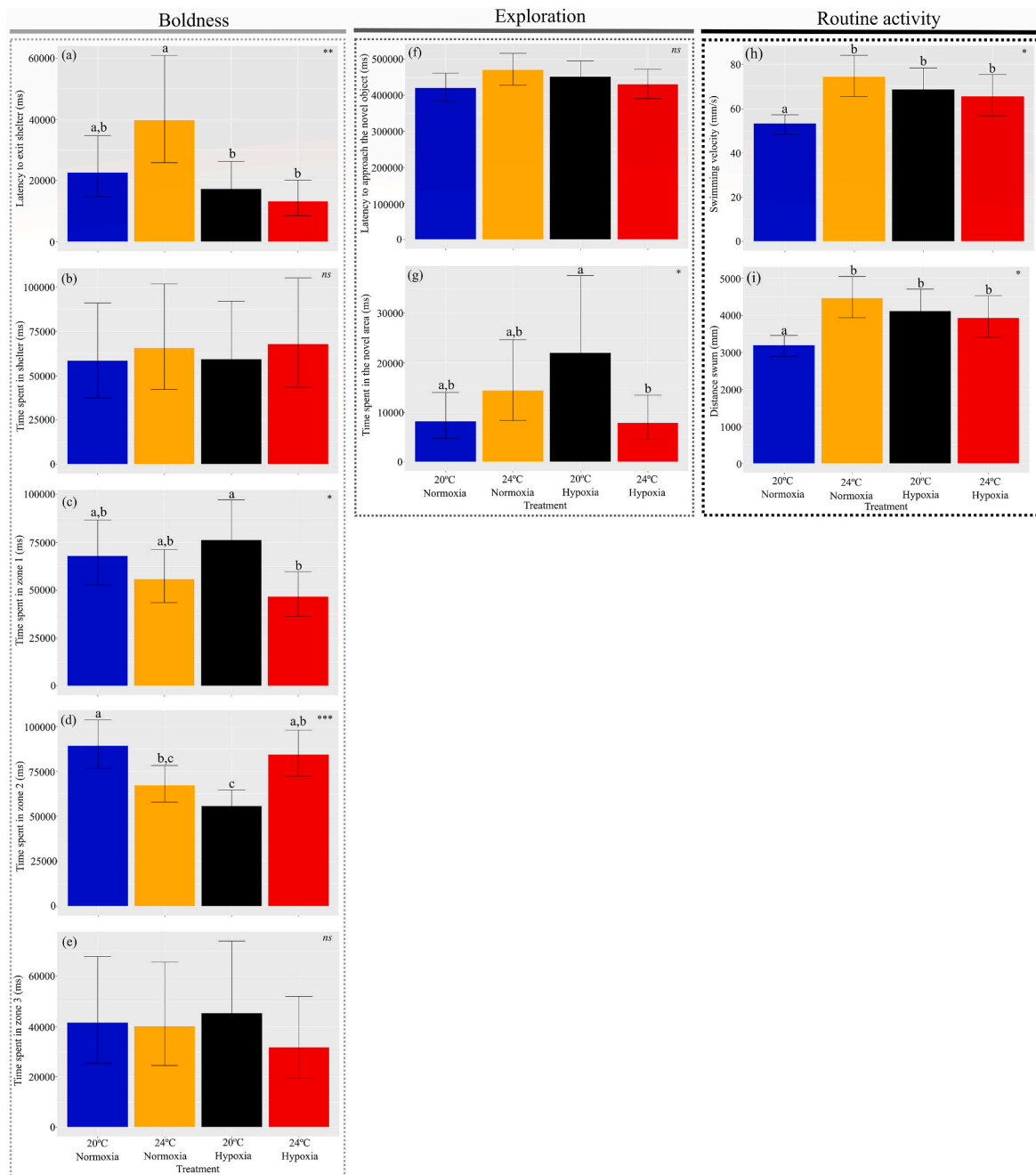
Fig. 3. Average (±S.E.) of behavioural responses [boldness (a–e), exploration (f–g) and activity (h–i)] of seabream early stages (N = 20) chronically exposed to different treatments. Letters represent homogeneous groups. ns, non-significant; ***p < 0.001; **p < 0.01; *p < 0.05.

survival of *Menidia beryllina*, no effect on survival of *Cyprinodon varie-gatus* and synergistically depleted survival of *M. menidia* (DePasquale et al., 2015). Combined warming and acidification synergistically reduced survival of *Gymnodraco acuticeps* embryos (Flynn et al., 2015), while increased survival in larvae of *D. labrax*, suggesting coping ability to such conditions for the later species (Pope et al., 2014). Juveniles of *Gadus morhua* also experienced 50% mortality when gradually exposed to dissolved $O_2 \leq 2.3$ mg L^{-1} regardless of temperature (Schurmann and Steffensen, 1992; Breitburg, 2002). Juveniles of *A. oxyrinchus* experienced 92% of mortality after chronic exposure to ~3 mg L^{-1} and warming (Secor and Gunderson, 1998). Eggs of *A. mitchilli* reached 50% of mortality after acute exposure to ~2.8 mg L^{-1} in ambient temperature (Chesney and Houde, 1989). Meanwhile, no mortality was observed when *D. labrax* larvae were chronically exposed to dissolved O_2 of ~2.8

mg L^{-1} and 19 °C (Vanderplancke et al., 2015).

Such results are insufficient to ascertain that early stages are under severe stress when exposed to the combination of hypoxia and higher temperatures. Mortality will only be high if avoidance to these conditions is ineffective (Breitburg, 2002). It is likely that early stages of seabreams will experience the consequences of warming and hypoxia after approximately 4 weeks of dispersal (~55 dph). This is the period when larvae orient from offshore to settle in shallow sandy or rocky bottoms in coastal lagoons or estuaries (Giacalone et al., 2022). How-ever, early stages of seabreams will likely choose to escape rather than to acclimate to a stressful situation. *Clupea harengus*, for example, increased their speed to avoid hypoxia and find favourable conditions (Domenici et al., 2000). In optimal environmental condition, seabream larvae (45–55dph) can swim at a sustainable critical speed of ~432 m h^{-1} and
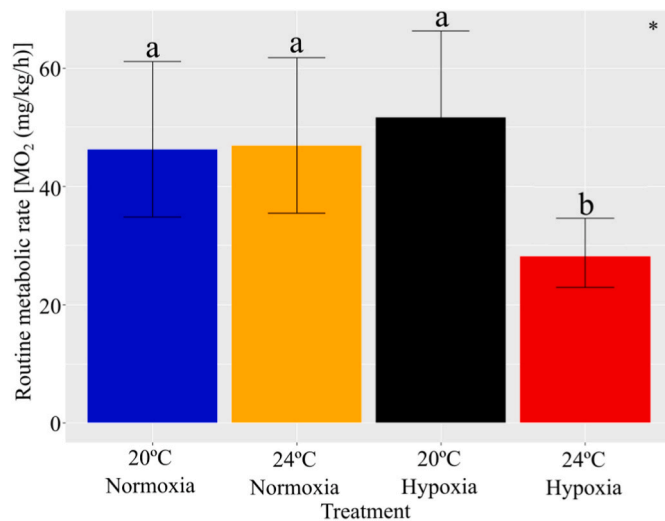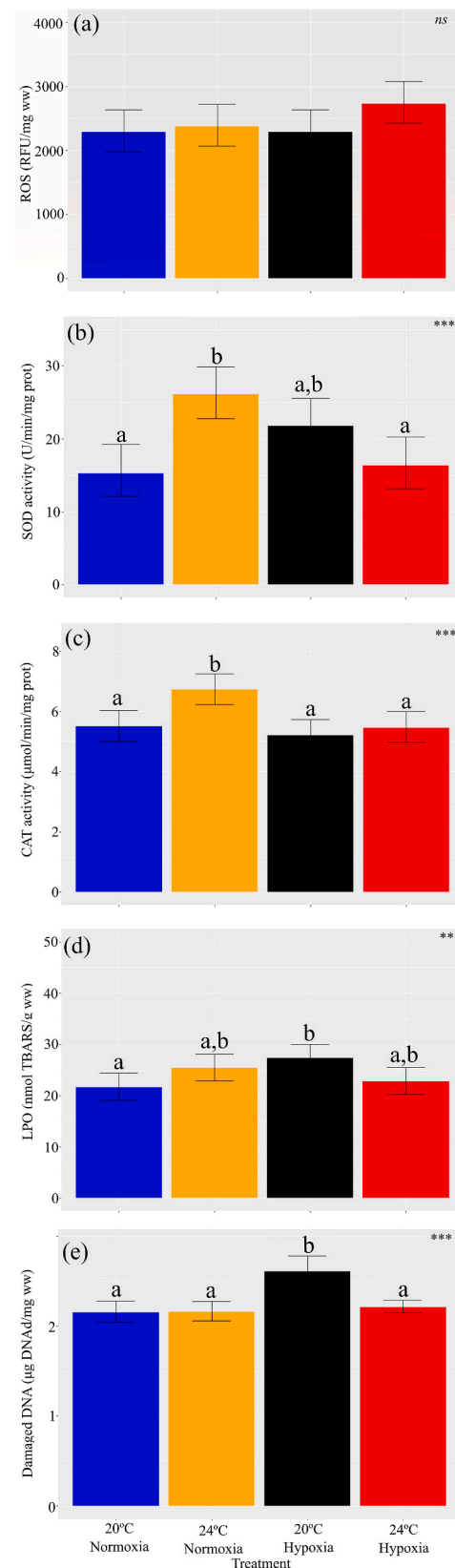
Fig. 4. Average RMR (±S.E.) of seabream early stages (N = 16) chronically exposed to different treatments. Letters represent homogeneous groups. ns, non-significant; ***p < 0.001; **p < 0.01; *p < 0.05.

a total distance of 54 km until reaching exhaustion when maximum water flow is ~792 m h^{-1} (Baptista et al., 2019). Our results also suggested that although combined warming and hypoxia decreases RMR and increases mortality, it enhances swimming activity of seabreams. Thus, it is likely that the high metabolic demand of fish trying to escape such stressful situation overpassed the metabolic demands required when seabreams are under critical oxygen tension (P$_{crit}$), leading to higher mortality. However, forecasts indicate that early stages of seabreams will encounter temperatures above 24 °C in the future. Additionally, dead zones bigger than 15 000 km^2 and a lot of dead zones of unknown size are already spread along the Mediterranean and Iberian Peninsula coasts and in the English Channel (Diaz and Rosenberg, 2008; NASA, 2010). These fish will therefore have reduced survival if these long-lasting warmer dead zones expand across their distribution range to the point that prevent them from escaping quickly, which could have larger population-level effects.

5.2. Warming induced faster growth in length and slower gains in weight

According to our results, the single effect of warming resulted in a significant faster growth in standard length and slower gains in weight. There is minimal work done on how single and combined effects of stressors affect larval growth patterns. Hypoxia alone caused body mass loss in *S. salar* (Remen et al., 2013), *D. labrax* (Vanderplancke et al., 2015) and the *S. lalandi* (Bowyer et al., 2014). Combined acidification and hypoxia induced negative effect on larval growth of *M. beryllina*, while hypoxia alone significantly reduced larval growth of *M. menidia* and *C. variegatus* (DePasquale et al., 2015). Combined warming and acidification had a positive effect on growth of *D. labrax* larvae (Pope et al., 2014), while the development of *G. acuticeps* embryos was synergistically reduced (Flynn et al., 2015). Acidification caused a significant reduction in length, growth and development rate, while warming increased length and development rate of *Rachycentron canadum* larvae, but no combined effects could be observed (Bignami et al., 2017). The combination of warming and hypoxia caused body mass loss in early stages of *O. tshawytscha* (Del Rio et al., 2019) and juveniles of *M. saxatilis* (Brandt et al., 2009). Most of these studies have assessed the effects of the stressors on body mass without focusing on developmental strategies, limiting proper comparisons to our results. Assessing allometric relationships diminishes the likelihood that the differences in growth among treatments are not the result of having different ontogenetic stages in the same tank, but rather the effects of the stressors.

Fig. 5. Variability of biomarkers (average ± S.E.) as responses to oxidative stress in larval tissues of seabream early stages (N = 8) 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 < 0.01; *p < 0.05.
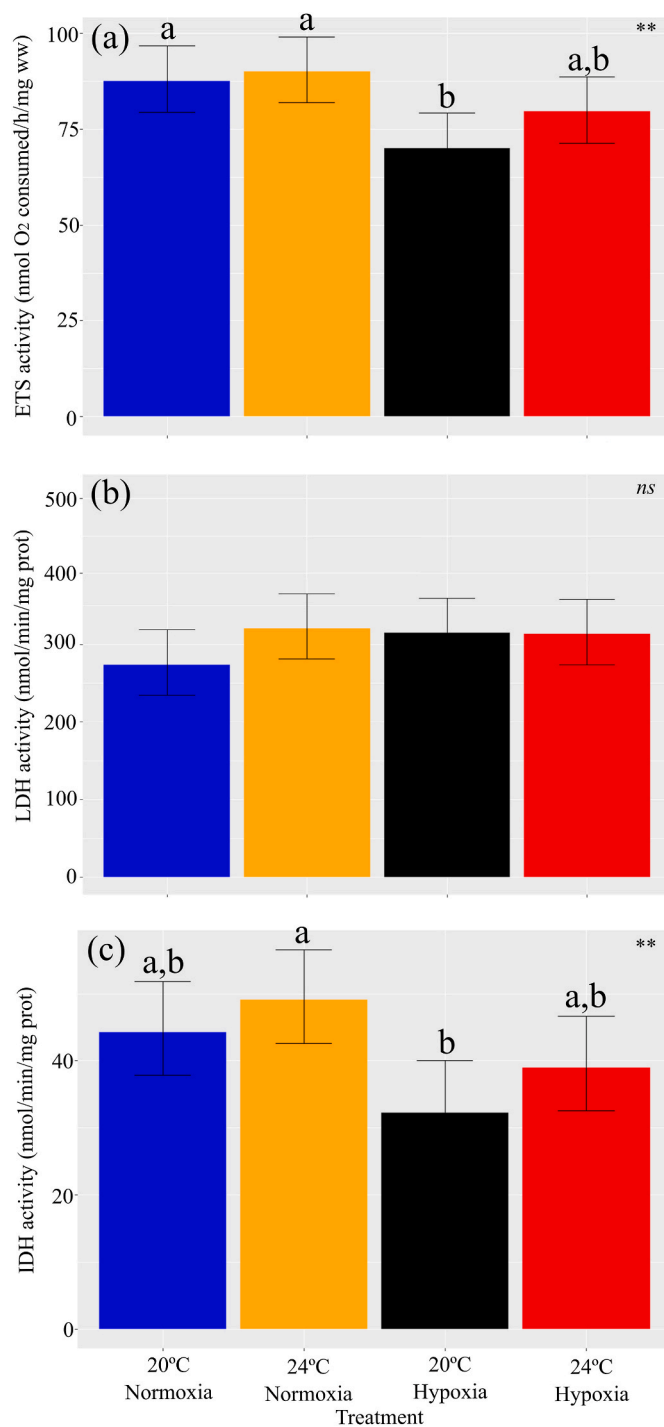
Fig. 6. Variability of biomarkers (average ± S.E.) as responses to energy metabolism in larval tissues of seabream early stages (N = 8) chronically exposed to different treatments. ETS = Electron transport system, LDH = lactate dehydrogenase, IDH = isocitrate dehydrogenase. Letters represent homogeneous groups. ns, non-significant; ***p < 0.001; **p < 0.01; *p < 0.05.

In fish, the larval stage generally consists of a period of rapid growth over a short time interval (Pepin, 1995; Lima et al., 2013). Under optimal conditions, weight may increase by a factor ~100 times more rapid than standard length during the larval period (Pepin, 1995). According to our results, hypoxia had no effect on weight, regardless of temperature. Our molecular results indicated that seabreams are hypoxia tolerant. It may then suggest that they are able to offset metabolic and developmental costs associated with hypoxic conditions through anaerobic reliance, prioritize the development of body mass such as in optimal condition (Yang et al., 2020). On the other hand, exposure to warming is known to increase the rates of biochemical reactions, metabolic demands and consumption of energy supply to support aerobic metabolism (Earhart et al., 2022). Our molecular results also indicated that when exposed to warming, enzymatic activities (SOD and CAT) of seabreams increased. Such high metabolic requirement coincides with the faster growth in length and faster loss of body mass when seabreams were exposed to warming (McMahon et al., 2020; Huss et al., 2021).

Some studies have suggested that the positive relationship between warming and growth can benefit recruitment success (McMahon et al., 2020). However, our results show that growth rate of the seabreams exposed to warming is not maintained and may shorten the duration of the larval stage. As a result, fast-growing larvae are likely to recruit earlier in the future warmer scenario, but survival will depend on whether warming will also affect the timing of favourable condition, such as food availability, flow dynamics, upwelling events and the presence of predators (Shoji et al., 2011).

### 5.3. The single and combined effects of warming and hypoxia enhanced larval activity

According to our results, no direct effects of warming and hypoxia on boldness and exploration were observed. Seabreams behaved differently only among the test treatments, but such differences were similar to the control. They also sought equally for protection, no matter the treatment. Individuals may become bolder and more explorative in order to secure access to resources (Kua et al., 2020), which in turn may increase their vulnerability to predators. Boldness has often been linked with elevated temperature (Lienart et al., 2014), such as observed in *P. moluccensis* (Biro et al., 2010), while hypoxia may lead to disruption of the school unit, altering antipredator behaviour (Domenici et al., 2007). In this study, the high behavioural variability within a specific treatment (i.e. high error bars), suggests that larger differences in larval personality may hide the effects of the stressors on individual sensitivity. This indicates that the temperature and dissolved oxygen levels used in this experiment were not enough to affect the reaction of seabreams to a situation perceived as dangerous, the willingness to take risks in novel environments (Fraser et al., 2001; Brown et al., 2005) or to investigate a novel object (Conrad et al., 2011).

Nevertheless, the single and combined effects of warming and hypoxia enhanced swimming activity in early stages of seabreams as they swam significantly faster and for longer distances when compared to control. Such result coincides with the findings of Almeida et al. (2022), reported that a temperature of 22 °C increased the speed attained during acute escape response of seabream larvae, but long-term exposure did not affect RMR, locomotor parameters or escape response, pointing to the existence of thermal acclimation (Almeida et al., 2022). Some studies have also reported increased swimming activity at higher temperature (Bignami et al., 2017) in *S. lalandi* (Laubenstein et al., 2018), *P. moluccensis* larvae (Biro et al., 2010) and *S. aurata* (Pimentel et al., 2016). Oppositely, hypoxia significantly reduced swimming activity in *E. lateralis* (Cook et al., 2014), *Liza aurata* (Lefrançois et al., 2005), *D. labrax* (Jourdan-Pineau et al., 2010), *S. meridionalis* (Zhang et al., 2010) and *S. aurata* (Remen et al., 2015). The single or combined effect of acidification and warming did not affect swimming activity, but warming alone induced higher swimming ability (Ucrit) in *R. canadum* larvae (Bignami et al., 2017).

5.4. Larvae use physiological coping strategies to overcome the single and combined effects of warming and hypoxia on target responses

Studies have suggested that there have been significant relationships among morphology, lifestyle, behaviour and metabolism as a response to extreme conditions in fishes (Dwyer et al., 2014). Our study also
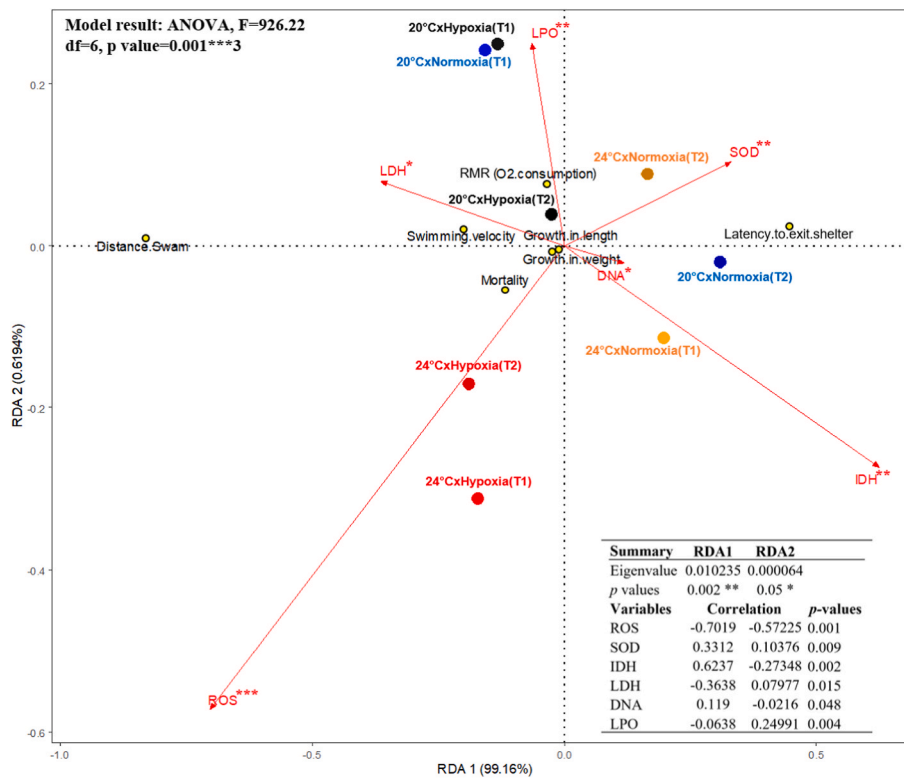
Fig. 7. Redundancy analysis (RDA) for the relationships among physiological responses (oxidative stress and energy metabolism) and target responses (boldness, activity, RMR, growth and mortality) after chronic exposure. T1 = tank 1, T2 = tank 2, LDH = lactate dehydrogenase, IDH = isocitrate dehydrogenase, ROS = reactive oxygen species, LPO = lipid peroxidation, DNA = DNA damage, SOD = superoxide dismutase. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

ascertained that the variability in physiological responses, as represented by the oxidative stress and energy metabolism systems, could significantly explain ~99% of the variability in mortality, behaviour, growth and RMR after chronic exposure. Our integrative analysis indicates that mortality and growth are highly influenced by oxidative stress and energy metabolism, exhibiting a positive relationship with reactive oxygen species and a negative relationship with aerobic metabolism, regardless of treatment. Under hypoxic condition, RMR, boldness and swimming activity have a positive relationship with anaerobic metabolism regardless of temperature. Such positive correlation between behavioural and physiological traits indicates that selection on one trait will enhance the other and accelerate the rate of adaptation. If the traits of interest are heritable, our study then informs that the environmental change discussed herein can influence natural selection processes on early stages of seabreams (Killen et al., 2013; Sunday et al., 2014; Laubenstein et al., 2018; Baltazar-Soares et al., 2023).

In our study, RMR of seabreams is thermally independent ($Q_{10} \geq 1$) under warming. Under this condition seabreams consumed oxygen as efficiently as those exposed to normoxia (i.e. higher RMR), leading to enhanced energetic efficiency of metabolic processes. RMR was only significantly lower when seabreams were exposed to the combination of warming and hypoxia, pointing to a negative thermal dependence under hypoxia ($Q_{10} < 1$). Despite this, no sign of metabolic rate depression could be observed since combined warming and hypoxia increased swimming (Mandic and Regan, 2018). These outcomes suggest that early stages of seabreams may be able to sustain swimming activity under hypoxia via anaerobic reliance (Pollock et al., 2007); or that seabreams still did not reach its critical oxygen tension (P_{crit}) and such mechanism is therefore sufficient to cope with dissolved O_2 of 2–2.5 mg L^{-1} (Mandic and Regan, 2018). The use of anaerobic metabolism to supplement swimming activity in hypoxia has also been reported for the hypoxia tolerant *M. cephalus* (Vagner et al., 2008). In *S. aurata*, RMR increased with temperature (Remen et al., 2015) and decreased at

dissolved $O_2 < 2.4$ mg L^{-1} (Cook et al., 2013), but activity declined with decreasing oxygen level, indicating metabolic stress or a coping strategy to severe hypoxia (Remen et al., 2015).

Molecular responses were then used to better understand thermal tolerance and anaerobic reliance in seabreams. Our results showed that the single and combined effects of warming and hypoxia did not affect ROS formation when compared to the control. Under warming, SOD and CAT activities increased significantly when compared to all other treatments suggesting increased antioxidant defense, while oxidative damages (LPO and DNA damage) increased significantly above control levels under hypoxia. Increased antioxidant defence was also observed in *S. aurata* exposed to warming (Madeira et al., 2016) and in L. xanthurus exposed to severe hypoxia (Cooper et al., 2002). Under the combination of warming and hypoxia, SOD and CAT activities and oxidative damages (LPO and DNA damage) did not change relative to the control. In this last situation, the hypoxia-inducible factor (HIF-1α) gene can be overexpressed (Vitale et al., 2018; Mandic et al., 2020). HIFs are transcription factors that upregulate several genes to promote survival in low-oxygen conditions in the cellular environment and are possibly being overexpressed due to the higher metabolic demands under warming and low availability of O_2 in the cells (Vitale et al., 2018; Mandic et al., 2020).

Regarding energy metabolism systems, the thermodynamic effects that rising temperatures exert over metabolism result in higher energy demand often supplied through aerobic metabolism. If oxygen declines, the ability to supply this demand aerobically becomes limited (Schulte, 2015; Earhart et al., 2022). Warming exposure reduced anaerobic metabolism in *O. kisutch* (Little et al., 2020), while severe hypoxia increased anaerobic metabolism in L. xanthurus (Cooper et al., 2002). Seabreams were able to maintain the anaerobic metabolism (LDH activity) similar to control levels in all test treatments. On the other hand, under hypoxia, ETS activity (cellular respiration) and IDH activity (aerobic metabolism) decreased significantly below control levels. Even

so, when exposed to the combination of warming and hypoxia, fish maintained the aerobic metabolism similar to control levels, even under reduced RMR. This suggests that seabreams are able to sustain metabolic demands by fuelling activities anaerobically (Domenici et al., 2007).

Hypoxia tolerance has also been confirmed in many other Sparidae species. For *Diplodus puntazzo*, the critical oxygen level is around 2.4 mg L^{-1}, becoming lethal and thermally dependent at 0.8 mg L^{-1} (Cerezo and García García, 2004). Critical oxygen levels increased exponentially with temperature in *S. aurata*, being around 1.5, 1.7 and 2.6 mg L^{-1} at 12, 16 and 20 °C, respectively (Remen et al., 2015). *Pagrus auratus* has a P_{crit} around 2.11 mg L^{-1} at 18 °C, however, despite physiological stress, the species does not avoid hypoxia until reaching an oxygen level of around 1.14 mm L^{-1} at this temperature (Cook and Herbert, 2012; Cook et al., 2013). This study corroborates our finding and suggests that snappers and seabreams can prioritize O_2 delivery and utilisation over O_2 uptake during chronic hypoxia, conveying a significant survival benefit to hypoxia tolerant species (Cook et al., 2013).

6. Conclusion

Our study highlights that early stages of seabreams can improve physiological performance to reduce costs associated with the single and combined effects of warming and hypoxia on growth, behaviour and RMR. Although they can physiologically tolerate warming, signs of heat stress were observed in growth strategies. In addition, seabreams were not able to control oxidative damages, exhibited depleted aerobic metabolism and depleted cellular respiration when exposed to the single effect of hypoxia. Finally, the combination of warming and hypoxia induced a large depletion of RMR. Despite this, seabreams were able to consistently use anaerobic metabolism to fuel energetic costs associated with increased swimming activity when exposed to all extreme situations. We were then able to identify that fish exposed to warming or to the combination of warming and hypoxia uses both enzymatic control of oxidative damages and anaerobic reliance as coping strategies, while those exposed to hypoxia can only use anaerobic reliance. The combination of warming and hypoxia acted synergistically leading to high mortality rates. Fish that survived the exposure period overcame hypoxia and thermal stress, suggesting signs of natural selection by these two stressors in early life stages. However, whether sub-lethal effects of exposure to these stressors in early life will influence fitness and survival in later life stages remains to be addressed.

Author contribution statement

André R. A. Lima: Project administration, Funding acquisition, Investigation, Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization. **Emily M. Booms**: Methodology, Formal analysis, Writing – review & editing. **Ana Rita Lopes** Methodology, Formal analysis, Writing – review & editing, Validation. **Sara Martins-Cardoso**: Methodology, Formal analysis, Writing – review & editing, Validation. **Sara C. Novais**: Methodology, Formal analysis, Writing – review & editing, Resources, Validation. **Marco F. L. Lemos**: Funding acquisition, Resources. **Laura Ribeiro**: Resources, Writing – review & editing. **Sara Castanho**: Resources. **Ana Candeias-Mendes**: Resources. **Pedro Pousão-Ferreira** Funding acquisition, Resources. **Ana M. Faria**: Project administration, Funding acquisition, Investigation, Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization, supervision.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122989.

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