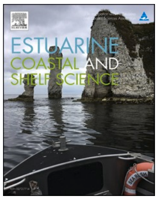
Estuarine, Coastal and Shelf Science 305 (2024) 108849

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

journal homepage: www.elsevier.com/locate/ecss



Shallow water fish display low phenotypic plasticity to ocean warming and extreme weather events regardless of previous thermal history

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ARTICLE INFO

Keywords:
Global warming
Marine heatwaves
Thermal tolerance
Acclimation
Fish

ABSTRACT

Shallow water environments have long been recognized by scientists as sentinels for climate change. By analysing the impacts of ocean warming and marine heatwaves (MHW) in species from these areas, we can estimate their plasticity and hence vulnerability to thermal challenges. *Pomatoschistus microps* is a benthic intertidal fish species inhabiting coastal lagoons where temperature fluctuations are common. Here, we tested the effects of "Present" and "Future summer" scenarios (22 °C and 25 °C) and their respective heatwaves (27 °C and 30 °C) *versus* a "Control" scenario of 19 °C on warm (summer)- and cold (winter)- acclimatized fish. Then, we estimated phenotypic plasticity of critical thermal maximum (CTmax), oxygen consumption and cellular stress responses (CSR). Temperature seasonal variation and body weight (as proxy for body size) effects on fish thermal tolerance were also determined. Fish exposed to higher temperature treatments exhibited higher thermal tolerance, with this pattern being consistent for both warm- and cold-acclimatized fish. However, this difference was subtle (<4.6 %), suggesting a low capacity for acclimation. Nonetheless, warm-acclimatized fish (collected in summer) displayed significantly higher CTmax than cold-acclimatized fish (collected in winter), indicating that CTmax is influenced by seasonal thermal variation. Weight also represents a constraint factor for *P. microps* thermal tolerance, as heavier animals displayed lower CTmax. No alterations in O_2 consumption, neither in CSR biomarkers were detected across temperature treatments, suggesting that fish were otherwise relatively insensitive to thermal fluctuations, independently of thermal history, within the thermal scenarios tested. Overall, the studied population of *P. microps* seems well adapted to temperature variations in their natural environment, exhibiting a large thermal safety margin (average of 11.02 °C).

List of Acronyms

T	Temperature	RMR	Routine Metabolic Rate
S	Salinity	TP	Total Proteins
L	Light	TAC	Total Antioxidant Capacity
D	Dark	LPO	Lipid Peroxides
PHW	Present heatwave	UBI	Ubiquitin
FHW	Future heatwave	HSP-70	Heat Shock Protein 70 kDa
MHW	Marine heatwave	SOD	Superoxide Dismutase
C	Control	Tmax	Maximum temperature
PS	Present summer	Tmin	Minimum temperature
FS	Future Summer	Amax	Maximum amplitude
d	Days of exposure	Amin	Minimum amplitude

(*continued on next page*)

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https://doi.org/10.1016/j.ecss.2024.108849
Received 2 February 2024; Received in revised form 6 June 2024; Accepted 13 June 2024

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ARR	Acclimation Response Ratio	HW	Heatwave
AC	Acclimation Capacity	CV	Coefficient of Variation
TSM	Thermal Safety Margin	MHT	Maximum Habitat Temperature

1. Introduction

Phenotypic plasticity is defined as the ability of one genotype to produce various phenotypes across environmental gradients (Fox et al., 2019). Plastic responses of organisms can thus play a role in population persistence and even species adaptations to changing environments (Fox et al., 2019). However, not all forms of plasticity are adaptive. Various studies report nonadaptive or maladaptive plastic responses resulting from stress or other factors (Merilä and Hendry, 2014). While adaptive plasticity may bring the population into the new favoured optimum phenotype, allowing persistence, nonadaptive or maladaptive plasticity may shift the average phenotype away from the optimum (Ghalambor et al., 2007). Phenotypic plasticity, and more specifically acclimation, has been pointed out as a relevant mechanism for organisms living in variable environments (Romero-Mujalli et al., 2021). Acclimation is a term that refers specifically to the process by which organisms adjust to specific environmental conditions (without undergoing genetic changes) under controlled laboratory settings (Foo and Byrne, 2016). Acclimatization, on other hand, is the process by which organisms adjust to environmental changes in the natural habitat (Foo and Byrne, 2016). If physiological acclimatization results in higher fitness across environments, then organisms with greater acclimatization capacity may be selected for in highly variable settings (Drown et al., 2021). However, if acclimatization processes come with costs (energetic, reproductive, etc.), there could be a variation in how plastic the organisms can be (Drown et al., 2021). Overall, the capacity of organisms to acclimatize to new conditions may strongly influence how populations and species respond to global changes.

Global change and its impacts on biodiversity have been a major focus of the scientific community in the past decades (den Elzen et al., 2005; Dessler, A., & Parson, 2019). Currently, almost all ecosystems are experiencing alterations that are a direct or indirect consequence of global changes, including climate change (Birchenough et al., 2015; Huang et al., 2017; Menezes-Silva et al., 2019; Sturrock et al., 2011). Among marine ecosystems, climate change-driven regime shifts, or habitat loss have already been reported, particularly in shallow or nearshore marine environments (Duarte et al., 2018; Graham et al., 2015; McPherson et al., 2021). Given that warming may be intensified in shallow and intertidal habitats, with warming rates potentially three times higher than those observed in deeper waters (Oczkowski et al., 2015), shallow marine environments have been utilized as natural laboratories for ecological research and as indicators of climate change (Morley et al., 2016; Oczkowski et al., 2015). These key environments harbour high biodiversity and provide a wealth of ecosystem services, and the risks posed by multiple environmental drivers are threatening the sustainability of coastal economic development and livelihoods (James et al., 2023; Magalhães Filho et al., 2022; Mooney et al., 2009). Species inhabiting shallow marine environments are particularly exposed to temperature and salinity shifts, as well as to oxygen depletion. According to IPCC's (Intergovernmental Panel on Climate Change) report AR6 (Assessment Report), sea surface temperature may increase up to 5 °C globally (IPCC, 2022). Extreme weather events such as marine heatwaves (MHW) have also increased 34 % in frequency and 17 % in duration worldwide (Oliver et al., 2018). According to Hobday et al. (2016) a marine heatwave can be described as an anomaly in temperature in which the 90th percentile of the local long-term climatology is exceeded for at least five consecutive days (Hobday et al., 2016).

Shallow marine environments are particularly exposed to sudden temperature shifts associated with MHW. Therefore, the assessment of species thermal limits is crucial to evaluate organism vulnerability to ocean warming and extreme weather events (Fernandes et al., 2023; Gibson, 2003; Madeira et al., 2021b; Missionário et al., 2022; Morley et al., 2016). Several studies have already revealed that some species do display acclimation capacity to increasing temperatures, with phenotypic plasticity and interspecific variability having key roles in individuals' and species' ability to endure environmental change (Faulkner et al., 2014; Nati et al., 2021; Vinagre et al., 2016). However, individuals that are already subjected to high temperatures in their natural habitat, may exhibit high thermal limits but low acclimation capacity upon unusual heat exposure (Madeira et al., 2018; Vinagre et al., 2016). Moreover, species thermal limits might be dependent of their body size (Leiva et al., 2019). This is often observed in the intertidal-subtidal axis, with several intertidal species, such as *Tegula funebralis* and *Petrolisthes cinctipes,* displaying reduced acclimation capacity when compared to subtidal species (Somero, 2005; Stillman, 2002). Goby fish, *Bathygobius soporator* and *Pomatoschistus microps*, have been reported to display very low acclimation capacity in heat tolerance (Vinagre et al., 2016), but the underlying physiological and molecular mechanisms have not been explored.

Metabolic requirements of ectothermic organisms are known to shift under thermal stress (Sokolova, 2021). The temperature dependence of metabolism in ectotherms has been widely established, driven by the deterministic effects of temperature on the kinetic energy of molecules (Gillooly et al., 2001). However, other authors also consider that adaptation to different temperature regimes and associated energetic trade-offs overall dictate the slope of the relationship between temperature and metabolic rate (Clarke and Fraser, 2004). By measuring oxygen consumption through respirometry assays, researchers can measure metabolic rates, and assess the energy demand pertaining to functions such as basal metabolism and maintenance of body homeostasis, and how they shift under global change scenarios (Leeuwis et al., 2021; Nelson, 2016). In this context, routine metabolic rate (RMR) represents the "average rate of metabolism when the animal is undergoing normal behaviour" and maximum metabolic rate (MMR) denotes the organism's capacity for work, such as when it requires more physical effort (e.g., escaping a predator or capturing prey) (Metcalfe et al., 2016; Norin and Metcalfe, 2019).

Under elevated temperature, metabolic rates of ectotherms often increase, subsequently declining again as the organism acclimates to the new conditions, controlling for maintenance costs (Norin and Metcalfe, 2019). Plasticity in whole-animal metabolic rates can be related to the underlying cellular physiology, associated with mitochondrial density, the function of key mitochondrial enzymes and mitochondrial proton leakage (Norin and Metcalfe, 2019). The ability of organisms to maintain mitochondrial and overall cellular functioning under thermal challenge has been associated with the capacity to activate cellular protection/repair processes, termed the cellular stress response (CSR) (Kültz and Somero, 2020; Somero, 2020). Heat stress is known to induce macromolecular damage in cells, including protein denaturation and oxidative injury (Madeira et al., 2014, 2016; Somero, 2020). Thus, organisms gradually activate the CSR depending on stress intensity and degree of damage in cells (Somero, 2020). Understanding these cellular level responses may help us understand how global warming and extreme weather events impact marine fauna (Madeira et al., 2016).

Oxidative stress biomarkers and heat shock proteins (Hsp) have been commonly used to evaluate species health and metabolic status in response to abnormal heat exposure (Birnie-Gauvin et al., 2017; Tomanek, 2010). High temperatures increase reactive oxygen species (ROS) formation, leading to oxidative stress (Abele et al., 2002; Dowling and Simmons, 2009). ROS oxidize lipids from cells' membranes and induce covalent modifications in proteins and nucleic acids, promoting cell death (Gaschler and Stockwell, 2017). Hsp are a highly conserved type of proteins, that refold damaged proteins to their proper structure. If refolding is not possible, ubiquitin, a protein responsible for the degradation of short-lived regulatory cellular proteins, signals them for degradation in the proteasome. The proteasome is comprised of protein complexes that degrade unneeded or damaged proteins through proteolysis (Ciechanover and Schwartz, 1998; Hofmann and Somero, 1995; Lindquist and Craig, 1988; Yusof et al., 2022). By quantifying lipid peroxidation, antioxidants, Hsp and ubiquitin, it is possible to: i) assess the level of oxidative stress and protein denaturation occurring in cells as a result of heat exposure and ii) assess species capacity to counteract the action of ROS in cells (Baag and Mandal, 2023; Carney Almroth et al., 2019; Madeira et al., 2014).

Pomatoschistus microps, the model animal used in the present study, is a benthonic goby fish that inhabits shallow coastal lagoons and estuaries in which abrupt variations of both temperature and salinity are common (Berrebi et al., 2005). This species is both widely distributed and abundant in European waters and is also easy to collect and to maintain in captivity.

Here, we aim to unravel how *P. microps* physiological performance will be affected by global warming and marine heatwaves, and whether its cellular stress response is sufficient to counteract the deleterious effects of heat exposure. Different environmental scenarios were simulated in an experimental biology setting, with “Present” and “Future summer” and respective projected heatwave conditions being replicated and compared to “Control” scenarios. Species upper thermal limit and routine metabolic rate, as well as cellular stress response biomarkers were then quantified to assess the species vulnerability to climate change and extreme weather events. We hypothesise that this goby fish species i) will display some level of seasonal acclimatization in upper thermal limits but ii) we also expect that previous (seasonal) thermal history will affect the way fish respond to thermal challenges; iii) fish will display an overall low acclimation capacity in upper thermal limits under future summer and heatwave conditions since it already lives in an extreme environment. Still, if its thermal safety margin is positive, we predict that populations will persist now and in the future. We also hypothesise that iv) body size will shape thermal tolerance, with larger individuals potentially being less tolerant to heat exposure. Due to the temperature-dependence of metabolic rates, we expect v) an increased oxygen consumption rate, indicative of a higher metabolic activity needed to uphold body function under elevated temperatures. However, acclimatization or adaptation to a shallow water environment (susceptible to higher temperatures and thermal variation) could shape this response. Lastly, we hypothesise that vi) goby fish will activate the cellular stress response under “Future summer” and heatwave conditions as means to maintain cellular functioning.

2. Materials and methods

Ethical statement

All procedures complied with EU legislation for animal experimentation (Directive, 2010/63/EU) and the three Rs principles of animal welfare (Replacement, Reduction and Refinement). Collection of fish was authorized by Directorate-General for Natural Resources, Safety and Maritime Services (DGRM) (annual collection license numbers 573/2021/DRI and 1746/2022/DRI). All experimental procedures were authorized by the Portuguese National Authority for Food and Animal Health (DGAV) (project license number 013161, dated September 29, 2021), after approval by the Commission for Animal Wellbeing and Experimentation of the University of Aveiro (process number CREBEA-02-2021). Authors C.M. and D.M. hold a B level certification (under the Functions’ System Accreditation) for animal experimentation in compliance with FELASA (Federation of European Laboratory Animal Science Association) (license type 0421/000/000, personal license numbers 024940 dated 29-12-2016 and 025250 dated 26-10-2015, respectively, registered by DGAV).

2.1. *Pomatoschistus microps* as a model species

Pomatoschistus microps, more commonly known as the common goby, is a highly abundant fish species with a broad distribution extending from the Baltic Sea to the western Mediterranean (Bouchereau and Guelorget, 1998; Jones and Miller, 1966). This species thrives in diverse habitats including tide pools, estuaries, salt marshes, and brackish lagoons, displaying remarkable tolerance to a wide range of temperatures and salinities (Berrebi et al., 2005). With an average lifespan of one to two years and a maximum total length of 64 mm, the common goby primarily feeds on amphipods, isopods, polychaetes, and chironomid larvae (Jones and Miller, 1966; Magnhagen and Wiederholm, 1982). Additionally, *P. microps* serves as prey for larger fish species (Pockberger et al., 2014). Pockberger et al. (2014) have assessed *P. microps*’ role in benthic community food webs and their respective trophic interactions, revealing both direct and indirect relationships within the food web (Pockberger et al., 2014). Given these extensive interactions, the common goby serves as an excellent model species for assessing the impact of disturbances, such as global warming, on the normal functioning of their habitats (Vinagre et al., 2016).

2.2. *Environmental data collection in P. microps sampling site*

Temperature data was collected every half an hour (with a resolution of 0.1 °C) with a EnvLogger data logger (27 mm EnvLogger with zip-tie mounting holes v2.4, ElectricBlue©, Portugal), between May 28th, 2021 and February 2nd, 2022 in Praia de Biarritz, Costa Nova, Ria de Aveiro, Portugal (40°62′87.47″N, −8°74′58.88″W). The logger was attached to the bottom part of a buoy (in contact with the seafloor) positioned at a maximum depth of 2 m during high tide. The logger was placed precisely at the spot where the fish were subsequently collected.

2.3. *Animal collection and husbandry*

Goby specimens were collected in Praia de Biarritz, Costa Nova, Ria de Aveiro (40°62′87.47″N, -8°74′58.88″W), Portugal (Fig. 1) in two different seasons with a small trawling net. The first animal collection occurred at the end of summer (September 30th, 2021) (n = 400, henceforth called warm-acclimatized fish) and the second collection during the end of winter (March 8th, 2022) (n = 375, henceforth called cold-acclimatized fish). Average fish weight and length were 0.03 ± 0.015 g and 3.40 ± 0.36 cm, respectively, during winter, and 0.04 ± 0.06 g and 3.21 ± 0.39 cm during summer.

In both seasons, after collection animals were placed inside 50 L containers for transport, with water from the sampling site (salinities (S) were S31.3 ± 0.05/S30.5 ± 0.2 and temperatures (T) were T17.2 ± 0.3 °C/T13.8 ± 0.05 °C in summer and winter days of collection, respectively) (VWR® pHenomenal® MU 6100 H Multi-Parameter Meter (pH/Conductivity/DO), Handheld), with constant aeration provided by transportable aeration pumps and air stones.

After arriving at lab facilities (6 min’ drive) (see Supplementary Fig. S5), individuals were randomly placed inside fifteen 50 L plastic tanks (n = 23 fish *per* tank), containing artificial seawater (S33 and ~T19 ± 1 °C (room temperature), a sand layer (30 ± 10 mm high) and constant aeration, with a photoperiod of 12L:12D. Fish remained under these conditions for fifteen days prior to the beginning of the experiments, to acclimate to the indoor system and captivity, as well as to reduce stress levels caused by capture. The adjustment rate from the field to the lab temperature was of 1 °C *per* hour, which is a common rate utilized in similar studies (Cereja et al., 2018) and commonly observed in the sampling location (see Supplementary Fig. S3). Although the time required for a species to adjust to housing conditions is species-specific, similar studies often employ time windows ranging from 1 to 4 weeks (Barker et al., 2018; Vieira et al., 2021). Considering this information, the two-week acclimation period should be sufficient to stabilize any stress triggered by transport or temperature adjustments. Water

Fig. 1. Sampling location: Coastal lagoon at Praia de Biarritz, Costa Nova, Ria de Aveiro (40°62′87.47″N, −8°74′58.88″W), Portugal.

parameters (pH kept at 8.1 ± 0.1, total ammonia kept below 0.5 mg. L^{-1}, nitrites kept below 0.1 mg.L^{-1}, nitrates kept below 0.1 mg.L^{-1}, and dissolved oxygen kept above 6.0 ± 0.3 mg. L^{-1} saturation) were measured twice weekly to ensure water quality, with partial water exchanges performed every other day at 25 % of the total volume. Fish were fed everyday with mussels at 2 % of their body weight. Recently, Chrétien et al., 2023 suggested that studies with wild animals should control for parasite infections. However, no treatment was administered to control for parasite or pathogen infections in this study, aiming to mimic natural environment conditions more accurately. An advantage of using wild animals (*vs*. lab bred ones) is their ability to portray the existing inter-individual variability in physiological responses that characterizes different populations and species. Nevertheless, previous studies on gobies suggest that parasitisation alone does not seem to affect energetics, swimming performance or body condition in these animals (e.g. see Cereja et al., 2018; du Toit et al., 2024.

2.4. Experimental setup

2.4.1. Warm- vs. cold-acclimatized fish

The same experiment was performed (sequentially) on warm- *vs*. cold-acclimatized individuals from the field. The temperature increase and time of exposure selected and described below were based on the EURO-CORDEX and WRF regional climate projections for Portugal and information released by IPCC for the Iberian Peninsula in AR6 (Cardoso et al., 2019; IPCC, 2022).

For fish captured in each season, following the acclimation period to the indoor conditions described previously, tanks were randomly allocated to three treatments (n = 5 tanks *per* treatment, n = 23 fish *per* tank yielding a total of n = 115 fish *per* treatment) in order to simulate 1) average annual seawater temperature (T19 °C – "Control" scenario), 2) “Present summer” temperature conditions (T22 °C – current warm season scenario) and 3) “Future summer” temperature conditions (T25 °C – climate change-driven ocean warming scenario of +3 °C) (see also Supplementary Table S1).

Temperature adjustment to the intended values was performed gradually to avoid stress, at a rate of 1 °C.h^{-1}, as commonly carried out in thermal stress studies (Lu et al., 2016; Missionário et al., 2023). Water parameters were monitored daily, and feeding was carried out as detailed above. Individuals were exposed to the three temperature scenarios (henceforth called “Control”, “Present summer” and “Future summer”) for 30 days, after which random fish were sampled for specific purposes (described later in this section).

After the 30 day period, the experiment continued with the simulation of marine heatwaves in the two summer scenarios, using a +5 °C increase in water temperature (IPCC, 2022), whereas the "Control" scenario was maintained (no heatwave). Briefly, fish tanks that were previously set at 22 °C and 25 °C (“Present” and “Future summer”), were subjected to further temperature increase of 1 °C.h^{-1} until reaching 27 °C (22 + 5 °C) and 30 °C (25 + 5 °C) to simulate “Present” and “Future marine heatwaves”, respectively. For the “Present (PHW)” and “Future (FHW) marine heatwaves”, the simulation had a duration of 7 and 12 days, respectively. The latter was intended to simulate an increase in MHW duration in the Future (Fig. 2). After the heatwaves’ simulation period, the remaining fish were also sampled (at day 37 for “Control” and “Present Heatwave”; and at day 42 for “Control” and “Future Heatwave”) (Fig. 2).

In all sampling timepoints, both warm- and cold-acclimatized fish were sampled for Critical Thermal Maximum (CTmax, n = 1 fish *per* tank *per* sampling timepoint, $n_{\text{per treatment}} = 5$, $n_{\text{total}} = 15$), to test fish acclimation capacity to the different warming scenarios as well as differences in thermal tolerance between warm- *vs*. cold-acclimatized fish. Additionally, warm-acclimatized fish were also sampled for respirometry assays, with the specific aim of testing if fish with prior history of heat exposure display low metabolic plasticity. The respirometry system only allowed to perform measurements in 4 fish at a time, so one random tank of each treatment had to be excluded *per* sampling timepoint, $n_{\text{total}} = 12$; the assay is described in the next section). Regarding cold-acclimatized

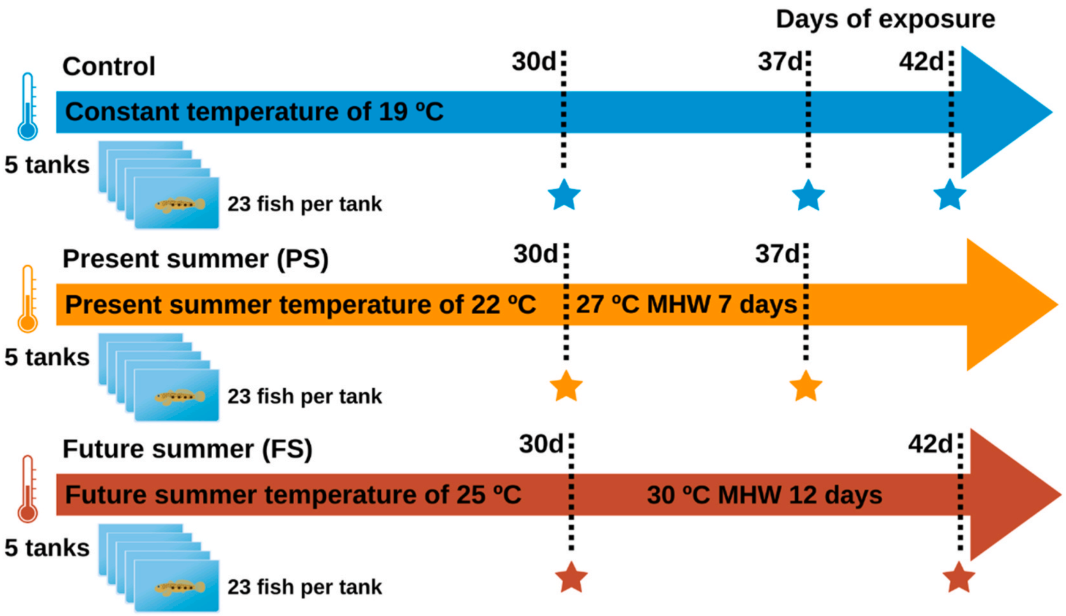

Fig. 2. Experimental design: "Control" - Constant temperature of 19 °C, simulating the average annual seawater temperature at the collection site; "Present summer PS" - 30 days (d) at 22 °C simulating Present warm season seawater temperature followed by another 7 days at 27 °C simulating a Present heatwave (5 °C increase); "Future summer (FS)"- 30 days at 25 °C simulating Future warm season seawater temperature under ocean warming followed by another 12 days at 30 °C simulating a Future heatwave (5 °C increase). Coloured stars represent sampling days in each treatment. Both warm- and cold-acclimatized fish (collected at the end of summer and end of winter, respectively) were exposed to these treatments. Warm-acclimatized fish were sampled for CTmax and respirometry, whereas cold-acclimatized fish were sampled for CTmax and biomarkers at the defined sampling days (30d, 37d, 42d).

fish, these were euthanized with a cervical cut and muscle tissue was sampled and stored at −80 °C for cellular stress response biomarkers quantification (n = 1 fish *per* tank *per* sampling timepoint, $n_{total} = 15$). The aim was to test if intertidal species that normally produce high constitutive (and thus less inducible) concentrations of cytoprotective proteins and antioxidants when frequently exposed to high temperatures (see Cereja et al., 2018) also do so without prior exposure to heat.

At the end of both experiments (warm- *vs*. cold-acclimatized fish), all the remaining fish were euthanized with cervical dissection.

2.4.2. *Critical thermal maximum experiment (CTmax) in warm- and cold-acclimatized fish*

A Critical Thermal Maximum assay (CTmax) was performed to estimate individuals' upper thermal limits (Mora and Ospína, 2001). At each sampling time-point, one individual from each tank was randomly taken and distributed into 1 L glass flasks with a sand layer (30 mm high), previously placed in a thermostatized bath (n = 5 fish tested *per* treatment *per* sampling point, $n_{total} = 15$). This water bath consisted of a 260 L tank filled with freshwater and equipped with a digitally controlled heater (JUMO Quantrol PID LC300 with a temperature probe PT1000 coupled to an aluminium box IP66 and 3 a kW heater, Aqualgae Soc. Lda). Individuals were exposed to a thermal ramp that increased at a rate of 2 $°C.h^{-1}$ (similar rate as observed in the wild in shallow waters) (Fernandes et al., 2023; Missionário et al., 2022) (see also Supplementary Figs. S1A–B). Starting temperature was 19 °C (the same as in "Control" tanks), with individuals from the "Control" group being the first to be placed in the system. As temperature sequentially reached the values of the treatments tested in the experiment, the remaining individuals from the respective tanks were transferred to the flasks in the water-bath. Fish were under continuous observation to detect abnormal behaviour caused by heat exposure (e.g., motionless, excessive swimming or time spent at water surface). CTmax was defined as the temperature at which fish lost their equilibrium and righting response (Madeira et al., 2012). As fish reached their thermal limit, they were removed from the glass flasks, and weight and length measurements were taken. Fish were then euthanized by cervical dissection.

Intraspecific variability in CTmax was calculated via the coefficient of variation (CV), following equation (1):

$$CV = (SD / mean) * 100. \tag{1}$$

Acclimation capacity of CTmax was calculated as the difference between CTmax values in each treatment, following equation (2) and based on (Vinagre et al., 2016):

$$\text{Acclimation capacity } (°C) = \text{CTmax treatment} - \text{CTmax control} \tag{2}$$

On the first sampling point (day 30), average annual seawater temperature (T 19 °C) was used as control to "Present summer" (T 22 °C) and "Future summer" (T 25 °C) conditions. Then, following MHW simulations, "Present summer" (T 22 °C) was used as control for "Present MHW" (T 27 °C, sampling at day 37) and "Future summer" was used as a control for "Future MHW" (T 30 °C, sampling at day 42).

Moreover, acclimation response ratio (ARR) was calculated as a measure of heat tolerance plasticity, following equation (3) (Clausen, 1977):

$$ARR = \frac{\Delta CTmax}{\Delta T_{acc}}, \tag{3}$$

Where ARR represents the change in heat tolerance (ΔCTmax) for a given change in acclimation temperature (ΔT_{acc}). Overall, an ARR = 0 means no plasticity in heat tolerance whereas ARR = 1 means complete physiological compensation for environmental warming. Such calculations were carried out using the same control-treatment comparisons as described above. Lastly, the Thermal Safety Margin (TSM) was also calculated, serving as proxy for how close a species lives to its upper thermal limits. TSM was estimated as the difference between the average CTmax and the Maximum Habitat Temperature (MHT), following equation (4) (Miller et al., 2013; Missionário et al., 2022).

$$TSM = CTmax - MHT \tag{4}$$

2.4.3. *Respirometry in warm-acclimatized fish*

The respirometry measurement complied with the best practices described by Killen et al. (2021) and Clark et al. (2013), in which, an Optical Oxygen Meter - FireSting-O2 (PyroScience, Germany) was used. The system contained 4 channels, each one connected to a closed respirometry chamber (100 mL syringe with an internal oxygen sensor spot) (Clark et al., 2013; Killen et al., 2021). One temperature sensor was used to ensure that the target temperature of the assay was maintained. This system was connected to a laptop and data was obtained and processed using the software Pyro Oxygen Logger (v.3.311, 2018 by www.pyro-science.com, Firmware 3.10) (see Supplementary Fig. S2). Oxygen concentrations in seawater inside the chambers were recorded every second (see Supplementary Table S3) for 90 min or until the O_2 concentration dropped below 70 %, defined as the threshold limit to avoid fish suffering from hypoxia. Prior to the beginning of the measurements, fish were acclimated to the respirometry chambers for 30 min, so that confinement stress would not interfere with the results. As the system had 4 channels, only n = 4 fish *per* treatment *per* sampling point were used to measure oxygen consumption rates. Each individual was placed inside a chamber, filled with seawater from their tank (see Supplementary Table S2). Fish weight, length and volume were taken at the end of the assay. The assay was repeated without fish in the chamber (blanks), so that microorganisms' O_2 consumption was measured as control (background respiration). This value was then subtracted from the total O_2 consumed in the chamber with fish in the final calculations, to obtain the accurate measure exclusive of fish.

Oxygen consumption rate (as proxy for routine metabolic rate - RMR) was calculated resorting to the R software (R version 4.2.3) package *respR* (Harianto et al., 2019; R studio Team, 2020) (for further details see **Appendix 2**).

All respirometry assays were carried out in a climatized room to maintain the target temperature of each treatment.

2.4.4. Biomarkers in cold-acclimatized fish

For cellular stress response biomarkers quantification, fish were dissected (n = 5 fish *per* treatment *per* sampling point), and muscle tissue (~8–67 mg) was used in downstream analyses. Muscle samples were homogenized with 150–300 μL of PBS buffer (Phosphate Buffered Saline (see Appendix 1)) using an OMNI Tissue Homogenizer (OMNI TH, United States). Samples were then centrifuged (10,000 × *g*) for 14 min at 4 °C and the supernatants were stored at −80 °C.

To quantify total proteins (TP) content in muscle samples, Bradford protocol for 96-well plates was used (Bradford, 1976). Samples were diluted 1:10 and 10 μL were placed in each microplate well (in duplicates). Then, 190 μL of Bradford reagent (#B6916, Supelco, Merck) was added and absorbance was read at 595 nm in a microplate reader (SYNERGY H1 BioTek, United States). A seven-point calibration curve was prepared with BSA (Bovine Serum Albumin) at known concentrations (0–1 mg mL^{-1}) and was used to calculate protein quantities in each sample. All biomarkers (see below) were then normalized by TP content. Moreover, TP concentrations were also used as an energy reserve biomarker. For that purpose, TP content was normalized by fish wet weight and expressed as mg of total protein *per* g of wet weight.

To determine the total antioxidant capacity (TAC), 10 μL of each sample was placed in microplate wells (duplicates) and a protocol adapted from Kambayashi et al. (2009) was followed (see Appendix 1) (Kambayashi et al., 2009). Absorbance was then read at 410 nm in a microplate reader (SYNERGY H1 BioTek, United States) (Kambayashi et al., 2009). A Trolox calibration curve (0–0.330 mM) was prepared to estimate TAC concentration through absorbance readings.

The thiobarbituric acid reactive substances method was used to quantify lipid peroxides (LPO) (Uchiyama and Mihara, 1978) (see Appendix 1). Absorbance was read at 530 nm (SYNERGY H1 BioTek, United States). An 8-point calibration curve was prepared with MDA (malondialdehyde bis(dimethylacetal)) (Merk, Germany) (Madeira et al., 2015). Concentrations ranged between 0 and 0.3 μM and this curve was used to estimate LPO concentration based on the absorbance values attained for known MDA concentrations.

An enzyme linked immunosorbent assay (ELISA) was used to quantify ubiquitin (UBI) and Heat Shock Protein 70 kDa (HSP-70) proteins (Madeira et al., 2014; Njemini et al., 2005). Samples were diluted 1:10 for UBI quantification and 1:5 for HSP-70, respectively (see Appendix 1). Absorbance was read at 405 nm in the microplate reader (SYNERGY H1 BioTek, United States). For both biomarkers quantification, calibration curves within a concentration range between 0 and 1 μg mL^{-1} were constructed (standards UbpBio, E−1100, USA for UBI and standards #AR03018PU-N, OriGene, USA for HSP-70).

The percentage of inhibition of superoxide dismutase (SOD) was calculated based on the protocol described by (Madeira et al., 2019) (see Appendix 1). Absorbance was read in a microplate reader (SYNERGY H1 BioTek, United State) at 560 nm every 5 min for a total of 20 min.

SOD % of inhibition was calculated following equations (5) and (6):

$$\text{SODAbs560} \Big/ \text{min} = \frac{Abs560\ final\ read - Abs560\ initial\ read}{Reaction\ time\ (min)} \tag{5}$$

$$\text{SOD \% inhibition} = \frac{\frac{Abs560}{min} negative\ control - \frac{Abs560}{min} sample}{\frac{Abs560}{min}\ negative\ control} \times 100 \tag{6}$$

2.5. Statistical analysis

2.5.1. Physiological traits

CTmax and respirometry data analyses were performed in R Software (R version 4.2.3). When data complied with the assumptions of normality and homoscedasticity for parametric tests, Student's T-tests or analysis of variance (ANOVA) and post-hoc Tukey tests were applied accordingly for comparison between two or more groups, respectively (package *stats)*. If assumptions were not met, non-parametric tests were applied: Kruskal-Wallis and Dunn test (package *stats* and *dunn. test)*. Pearson or Spearman correlation tests were also performed to test the relationship between fish CTmax and fish weight, depending on whether the data followed assumptions, with the *stats* package.

2.5.2. Biomarkers

Since the CSR biomarkers quantified were expressed in different measurement units, the datasets were scaled through a "min-max normalization" method.

A Permutational Multivariate Analysis of Variance (PERMANOVA) using a resemblance matrix based on Euclidean distance and a Principal Components Analysis (PCA) were then performed in PRIMER 6.1 version. To visualize biomarkers' differences in fish from different treatments, heatmaps were prepared using R software (package *ggplots*), after quantiles normalization of the data.

3. Results

3.1. Environmental data collection at *P. microps*' habitat

Maximum temperature recorded in the sampling site was 23.6 °C, on September 3rd, 2021. Average daily temperature and standard deviation for that same day was 21.3 ± 1.4 °C. The minimum temperature was detected during November and December 2021, with the lowest value being 11.1 °C (Fig. 3A). Average daily temperature for those two days were 12.1 ± 0.7 °C and 12.4 ± 0.5 °C, respectively (for further information see Supplementary Table S4).

Thermal amplitude tends to be more pronounced during warmer months, with temperature records oscillating up to 6.3 °C on August 4th, 2021 (Amax), and only 0.4 °C on December 24th, 2021 (Amin) (Fig. 3B). The rate of temperature increases also showed a peak of 2.4 °C *per* hour, as detected on August 17th, 2021, where the temperature rose from 15.6 to 18.0 °C between 13:05 and 14:05 p.m. The average daily maximum temperature increase *per* hour observed during the summer months was 1.3 ± 0.4 °C (see Supplementary Fig. S3).

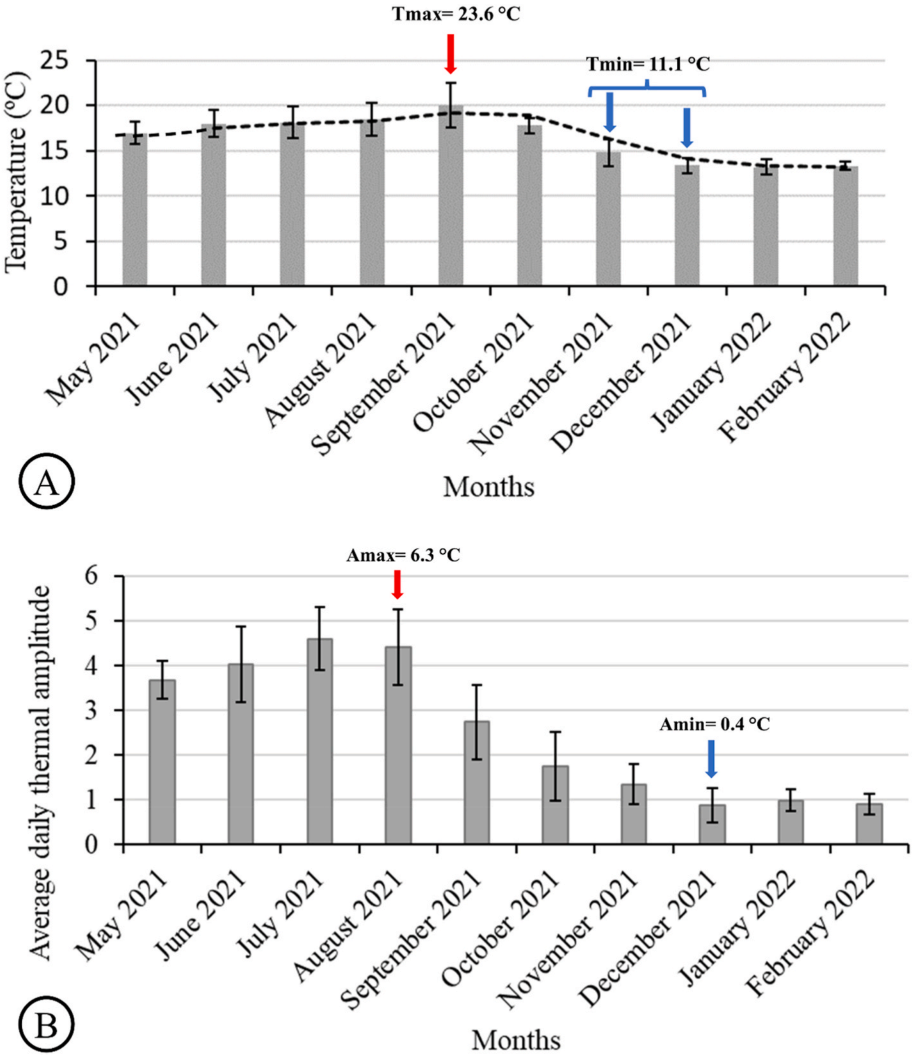

Fig. 3. Water thermal regime at the sampling site (Ria de Aveiro, Portugal). A: Water temperature (mean ± SD) recorded in Ria de Aveiro coastal lagoon, at Praia de Biarritz (Costa Nova, Aveiro, Portugal, 40°62′87.47″N, -8°74′58.88″W) between May 2021 and February 2022. Temperatures were recorded every 30 min with EnvLoggers. Tmax: Maximum temperature recorded (23.6 °C in September); Tmin: Minimum temperature recorded (11.1 °C in both November and December 2021). The dotted line represents the moving average. B: Average daily thermal amplitude found in Ria de Aveiro coastal lagoon, at Praia de Biarritz (Costa Nova, Aveiro, Portugal, 40°62′87.47″N, -8°74′58.88″W) between May 2021 and February 2022. Temperatures were recorded every 30 min with EnvLoggers. Maximum daily thermal amplitude (Amax) was 6.3 °C detected on August 4th, 2021 and the minimum (Amin) was 0.4 °C detected on December 24th, 2021.

3.2. *CTmax: warm-acclimatized fish*

Warm-acclimatized fish exhibited significant differences in their CTmax across all five exposure treatments ($p = 0.0006$; chi-squared = 19.6). Particularly noteworthy differences were observed between the “Control” and the “Future summer” ($p = 0.04$; chi-squared = 19.6), the “Present heatwave” ($p = 0.03$; chi-squared = 19.6), and the “Future heatwave” ($p = 0.0004$; chi-squared = 19.6). Additionally, significant differences were found solely between the “Present summer” and the “Future heatwave” ($p = 0.002$; chi-squared = 19.6) (see Supplementary Fig. S4). Moreover, when analysing CTmax values after only the first 30 days of exposure, significant differences were also detected comparing the "Control", “Present summer”, and “Future summer” conditions ($p = 0.018$, chi-squared = 7.98). Notably, distinctions were mainly observed between the "Control" and the “Future summer” ($p = 0.009$, chi-squared = 7.98), as well as between the “Present and Future summer” ($p = 0.038$, chi-squared = 7.98). Fish exposed to “Future summer” had CTmax average values +1.9 % greater than those from the “Control” group, and +1.6 % greater than fish from the “Present summer” (Fig. 4A) at day 30. The min-max CTmax values measured for the species across the conditions tested were 34.1–35.5 °C.

Following exposure to heatwaves, significant differences were observed in CTmax before and after exposure in both “Present” and “Future” scenarios ($p = 0.014$, $t = -3.22$ and $p = 0.0004$, $t = -6.24$, Fig. 4B and C, respectively**).** Individuals exposed to each of the heatwaves, showed to be, respectively, +1.8 % more tolerant to heat exposure in the “Present HW” and +4.2 % in the “Future HW”, when compared with their respective summer conditions before the heatwaves. Minimum and maximum CTmax values recorded after heatwave exposure were 34.1–35.5 °C in the “Present” scenario and 34.5–36.7 °C in the “Future” one.

A negative correlation between fish CTmax and their weight was also observed in warm-acclimatized individuals exposed to temperature scenarios for 30 days ($p = 0.008$, rho = −0.658) and before and after the heatwave exposure in the “Future” scenario ($p = 0.017$; rho = −0.729). However, no significant differences were, detected in fish weight among the different treatments. Average fish weight and length were 0.04 ± 0.02 g and 3.50 ± 0.35 cm, respectively.

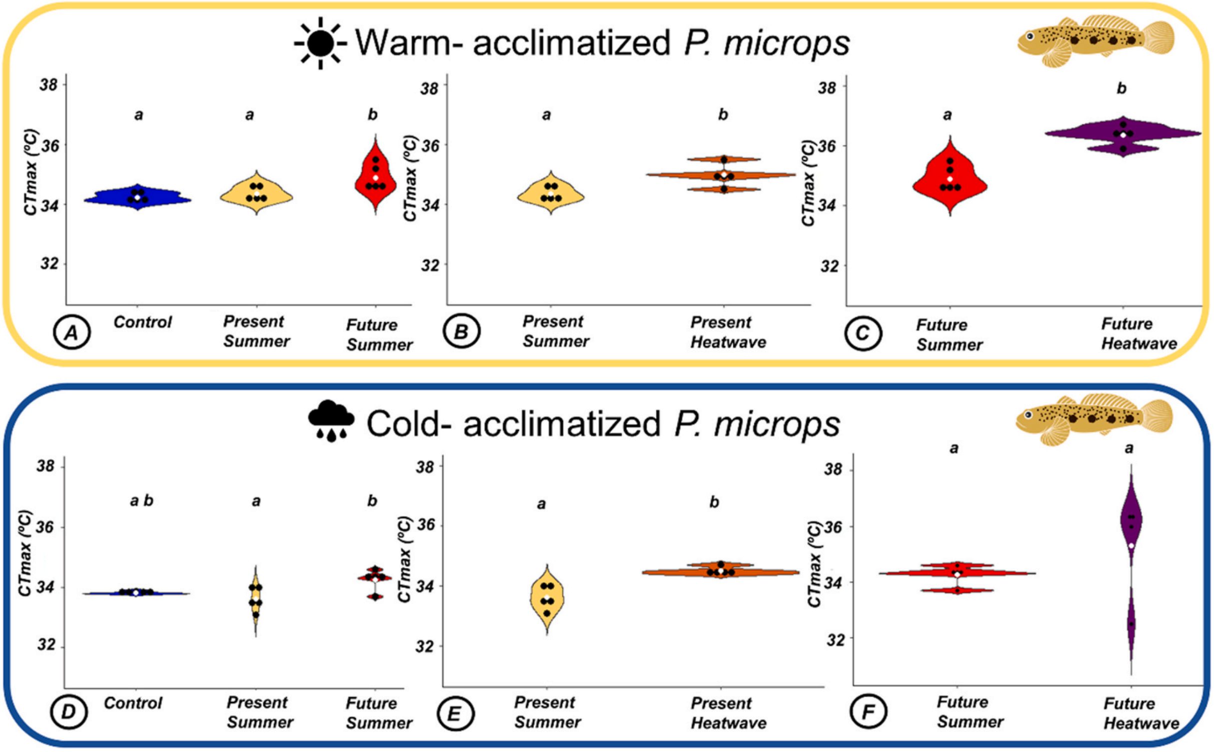

Fig. 4. Violin plots displaying individual and average CTmax values of warm-acclimatized (A–C) and cold-acclimatized (D–F) goby fish (*Pomatoschistus microps*) exposed to different environmental scenarios, namely. A/D: Global warming scenario comparing "Control", "Present summer" and "Future summer" conditions; B/E: "Present heatwave" scenario comparing the "Present summer" and heatwave; C/F: "Future summer" scenario comparing the "Future summer" and respective heatwave.

3.3. *CTmax: cold-acclimatized individuals*

Cold-acclimatized fish also exhibited significant differences in their CTmax across the five different scenarios tested ($p = 0.03$; chi-squared $= 10.98$). These differences were primarily observed between the "Control" and the "Present heatwave" ($p = 0.04$; chi-squared $= 10.98$), as well as between the "Present Summer" and its respective heatwave ($p = 0.04$; chi-squared $= 10.98$), and the "Future heatwave" ($p = 0.04$; chi-squared $= 10.98$) (see Supplementary Fig. S4). When considering only the results obtained during the first 30 days of exposure, significant differences were also detected between the three treatments ($p = 0.015$; $F = 6.137$), as well as before and after the heatwave in the "Present" scenario ($p = 0.005$; $t = -4.889$) (Fig. 4D).

In the first case, greater differences were observed between the "Present summer" and "Future summer" at day 30 ($p = 0.013$, diff $= 0.640$). Individuals in the "Future summer" scenario display a CTmax +1.9 % greater than those from the "Present summer" scenario. In individuals exposed to the "Present scenario", fish are +2.6 % more tolerant after the heatwave simulation (Fig. 4E). No significant differences were observed in CTmax between individuals in the "Future" scenario before and after heatwave exposure (Fig. 4F).

The min-max CTmax recorded in the first sampling timepoint across temperature scenarios (prior to heatwave exposure) was 33.1–34.6 °C, whereas in the second sampling timepoint following heatwave simulations, min-max CTmax were 34.0–34.7 °C in the "Present" and 32.5–36.4 °C in the "Future".

No correlation between fish CTmax and their weight was observed in any of the sampling points for cold-acclimatized fish, neither a significant difference in fish weight according to the exposure treatment. Average fish weight and length were 0.03 ± 0.14 g and 3.54 ± 0.36 cm, respectively.

3.4. *CTmax: warm- vs. cold-acclimatized fish*

Fish exposed to similar treatments but collected in different seasons (summer = warm-acclimatized *vs.* winter = cold-acclimatized), also displayed significant differences in their CTmax levels ($p < 0.001$; $F = 11.380$). Significant differences were observed when comparing warm-*vs.* cold-acclimatized individuals in both "Present" ($p = 0.007$; diff $= -0.74$) and "Future summer" ($p = 0.025$; diff $= 0.64$) conditions. Warm-acclimatized fish exposed to "Present summer" conditions displayed a CTmax + 2.2 % higher than cold-acclimatized fish exposed to the same treatment (Fig. 4B–E). In the case of the "Future summer" the difference was 2.6 %.

Average TSM values considering all scenarios tested were 11.4 ± 0.75 °C and 10.7 ± 0.59 °C for warm- and cold-acclimatized fish, respectively. TSM calculations were performed using the maximum temperature recorded in the sampling site during the course of the present study (23.6 °C).

When comparing Acclimation Capacity (AC), Acclimation Response Ration (ARR) and Thermal Safety Margin between warm- and cold-acclimatized individuals, we observed that warm-acclimatized fish displayed higher AC as well as wider TSM values. However, no changes were detected in ARR between different seasonally acclimatized fish (Tables 1 and 2).

Lastly, greater coefficients of variation were detected comparing conditions before and after heatwave in the "Future" scenario in both warm- and cold-acclimatized fish, where a greater variance occurred after the heatwave strike (Table 1).

3.5. *Respirometry: warm-acclimatized individuals*

No significant differences were detected in oxygen consumption rates between temperature treatments after 30 days of exposure ($p = 0.305$; $F = 1.360$) or before and after exposure to heatwaves, both in the

Table 1
Differences in Acclimation Capacity (AC, °C), Acclimation Response Ratio (ARR, °C) and Coefficient of Variation (% CV) in thermal tolerance (Critical Thermal Maximum) of warm- and cold-acclimatized goby fish *Pomatoschistus microps* tested under different temperature scenarios including “Control”, “Present summer” and “Future summer” (30 d exposure period), followed by the corresponding marine heatwave (MHW) simulations, namely + 5 °C for 7 days to simulate a “Present MHW” and +5 °C for 12 days to simulate a “Future MHW”.

Comparisons						
		Present summer – Control	Future summer – Control	Future summer – Present summer	Present MHW – Present summer	Future MHW – Future summer
Warm-acclimatized fish	**Acclimation capacity (AC)**	0.12	0.66	0.54	0.62	1.46
	Acclimation Response Ratio (ARR)	0.04	0.11	0.18	0.12	0.29
	Coefficient of Variation (% CV)	0.55	1.27	1.19	1.19	2.25
Cold-acclimatized fish	**Acclimation capacity (AC)**	−0.20	0.44	0.64	0.88	1.04
	Acclimation Response Ratio (ARR)	−0.07	0.07	0.21	0.18	0.21
	Coefficient of Variation (% CV)	0.78	0.90	1.34	0.57	3.91

Table 2
Thermal Safety Margin (TSM, °C) of warm- and cold-acclimatized goby fish *Pomatoschistus microps* tested under different temperature scenarios including “Control”, “Present summer” and “Future summer” (30 d exposure period), followed by the corresponding marine heatwave (MHW) simulations, namely + 5 °C for 7 days to simulate a “Present MHW” and +5 °C for 12 days to simulate a “Future MHW”.

Thermal Safety Margin (TSM)					
	Control	Present summer	Future summer	Present MHW	Future MHW
Warm-acclimatized fish	10.64	10.76	11.3	11.38	12.76
Cold-acclimatized fish	10.22	10.02	10.66	10.9	11.7

“Present” as well as “Future summer” scenarios ($p = 0.250$; $t = -1.304$ for “PHW” and $p = 0.973$; $t = 0.036$ for “FWH” scenario) (Fig. 5). Average weight and length of fish were and 0.03 ± 0.02 g and 3.20 ± 0.39, respectively.

3.6. *Biomarkers: cold-acclimatized individuals*

No significant differences were detected in biomarkers' concentrations/activities, when comparing individuals exposed to “Control”, “Present Summer” and “Future Summer” for 30 d ($p = 0.812$; Pseudo-$F = 0.613$) (Fig. 6A) and neither when comparing individuals before and after exposure to heatwaves, both in “Present” ($p = 0.172$; Pseudo-$F = 1.513$) (Fig. 6B) and “Future” scenarios ($p = 0.319$; Pseudo-$F = 0.784$) (Fig. 6C). Although no significant differences were detected in biomarkers quantities across the different environmental scenarios tested, according to the heatmaps: i) “Present summer” and its respective heatwave do appear to have a somewhat distinct type of response, with a trend for greater biomarkers abundance under “Present heatwave” conditions (Fig. 6E) and ii) gradual ocean warming temperature tested in “Future summer” showed a trend toward increasing protein folding-related biomarkers (Hsp70 and UBI), whereas iii) “Future heatwave” showed an increasing trend mostly of oxidative stress related biomarkers (LPO, TAC, SOD). The other treatments of exposure revealed a great heterogeneity of biomarkers' abundances, with no specific patterns being evident (Fig. 6 D/F).

4. Discussion

This study aimed to test whether *P. microps* display heat tolerance, metabolic and CSR plasticity to different temperature scenarios, namely whether cold- *vs.* warm-acclimatized fish show distinct acclimation ability to “Present” and “Future summer” scenarios (gradual warming and heatwaves). In *P. microps'* natural habitat, temperatures start to

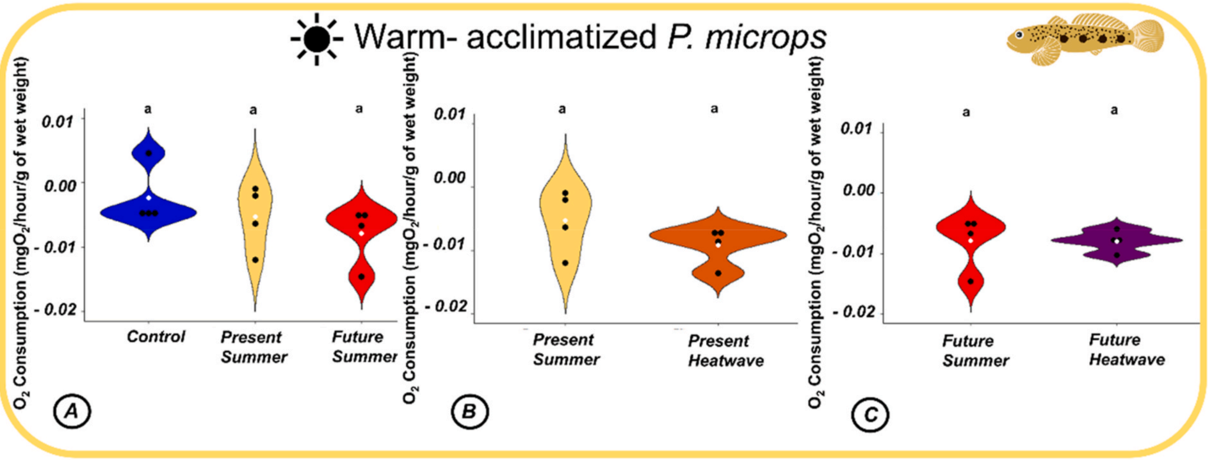

Fig. 5. Violin plots displaying oxygen consumption values (black dots) in warm-acclimatized goby fish (*Pomatoschistus microps*) exposed to different environmental scenarios and their respective means (white dots). A: Global warming scenario comparing “Control”, “Present summer” and “Future summer” conditions; B: “Present” scenario comparing current summer and heatwave; C: “Future” scenario comparing future summer and heatwave.

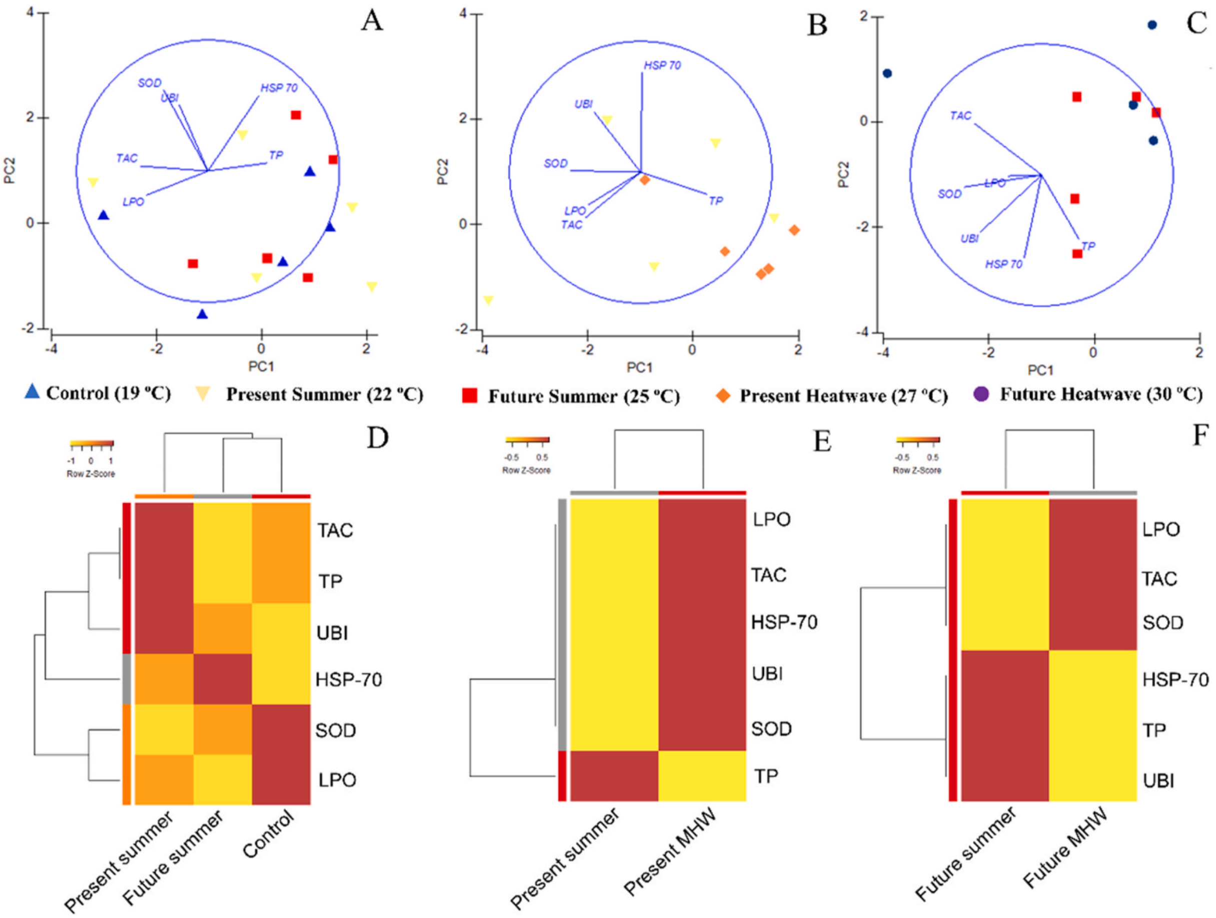

Fig. 6. Cellular stress response (CSR) profiles in the muscle of cold-acclimatized goby fish *Pomatoschistus microps* (collected in the end of winter) and exposed to different environmental scenarios. (A–C) Principal components analysis score plots A: Global warming scenario comparing "Control", "Present summer" and "Future summer" conditions; B: "Present heatwave" scenario comparing the "Present summer" and heatwave; C: "Future summer" scenario comparing the "Future summer" and heatwave. (D–F) Heatmaps, in which each cell denotes the quantiles average biomarker abundance/activity in a specific environmental scenario. D: Global warming scenario comparing "Control", "Present summer" and "Future summer"; E: "Present heatwave" scenario comparing "Present summer" and "Present heatwave"; F: "Future heatwave" scenario comparing "Future summer" and heatwave. Red colours correspond to higher relative abundances/activities and yellow colours correspond to lower relative abundances/activities of biomarkers. Abbreviations: Hsp70 – heat shock protein 70 kDa, UBI – ubiquitin, TP – total protein, SOD – superoxide dismutase, LPO – lipid peroxidation, TAC – total antioxidant capacity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

increase during spring, when fish are still cold-acclimatized and may display less ability to deal with heat when compared to warm-(summer)-acclimatized fish. Given the intensification of heatwaves in all seasons (Cardoso et al., 2023; Thoral et al., 2022), temperature conditions during seasonal transitions may be critical for the persistence of shallow water fish populations.

Overall, we found significant differences between the CTmax of warm- *vs*. cold-acclimatized fish (i.e., between individuals collected at the end of summer *vs*. end of winter). Warm-acclimatized fish were 2.2 % more tolerant to elevated temperatures than cold-acclimatized fish. Previous studies have reported similar differences in CTmax in animals collected in different periods of the year, suggesting that thermal tolerance in a population can display seasonal oscillation (Hopkin et al., 2006; Lattuca et al., 2023). For instance, Hopkin et al. (2006) and Lattuca et al. (2023) found that both crustaceans and fish, exposed to summer conditions endure higher temperatures when compared to those from winter (Hopkin et al., 2006; Lattuca et al., 2023). Since *P. microps* is short-lived (average lifespan of 1–2 years) (Miller, 1975), with most populations reproducing in spring and/or autumn (Leitão et al., 2006), this can differentially impact the tolerance and performance of fish cohorts that co-exist in the same population, depending on the season they hatched and their consequent thermal history. Although most extreme weather events such as heatwaves are expected to occur in the warm season (IPCC, 2022), current climate projections also suggest an increased probability of heatwave occurrence in spring in the Portuguese coast, which could disproportionally affect cold-acclimatized fish (Parente et al., 2018).

In warm-acclimatized fish, CTmax slightly increased with higher rearing temperature treatments, with the maximum value of CTmax recorded being 36.7 °C. Such results corroborate the hypothesis that the temperature at which an organism is subjected prior to an acute heat exposure somewhat impacts their endurance to thermal stress (Morley et al., 2017). However, this species acclimation capacity seems quite low when compared to others from similar habitats (Fernandes et al., 2023; Missionário et al., 2022; Vinagre et al., 2016). For example, fish in the "Future summer" scenario subjected to a heatwave were only able to endure temperatures 4.2 % (~1.46 °C) greater than individuals prior to the heatwave simulation. However, other intertidal fish species can shift their CTmax by 4.0 °C, as is the case of *Coryphoblennius galerita* (Vinagre et al., 2016).

This species low capacity for acclimation was also previously suggested by other authors, who compared the CTmax and acclimation capacity of several marine shallow water species, from both tropical and temperate regions (Christensen et al., 2021; Vinagre et al., 2016). In a study by Vinagre et al. (2016), acclimation temperatures tested for *P. microps* were 20, 23 and 26 °C, where the maximum temperature tested (26 °C) corresponded to a future heatwave simulation with a duration of 10 days. Average CTmax of fish in this condition was 34.7 °C, which did not change much from the other temperatures (20 and 23 °C), in which the CTmax was 32 and 34.8 °C, respectively. Still, the average CTmax obtained by Vinagre et al. (2016) is 1.7 °C lower than the average CTmax we obtained for fish subjected to a more intense and longer Future heatwave (T30 °C for 12 days, warm acclimatized fish). In the present study, *P. microps* was able to slightly increase its CTmax with rising temperature but its heat tolerance plasticity was still limited, as ARR values were always below 0.3 in both warm and cold-acclimatized fish, indicating that there is only a small physiological compensation for environmental warming. Nevertheless, our results suggest that warm-acclimatized individuals, specifically, possess slightly larger acclimation capacity (AC) and thermal safety margins (TSM) than cold-acclimatized individuals, demonstrating that prior thermal history significantly affects species ability to change their physiology to improve performance (Dowd et al., 2015). Moreover, our results show that larger differences occur in *P. microps* CTmax levels when comparing individuals before *vs.* after heatwave exposure in the “Future summer” scenario, independently of the season in which individuals were collected. This indicates that both warm- and cold-acclimatized fish will likely employ a similar physiological strategy to adapt to new temperature conditions, despite the opportunities or limitations imposed to acclimation by their previous thermal history, respectively.

Overall, slight differences in thermal tolerance plasticity between studies likely have to do with the collection of specimens from different *P. microps* populations. Interestingly, the use of different warming ramps to test CTmax in different studies (e.g. 2 °C/h in our study vs. 1 °C/h in Vinagre et al., 2016) is not expected to produce significant differences in the measured CTmax in intertidal gobies, as pointed out by Vinagre et al. (2015). In this study, three different thermal ramps for calculating CTmax were compared (1 °C/min vs. 1 °C/30 min vs. 1 °C/h) in several species, and gobies such as *Bathygobius soporator* showed that their thermal tolerance was independent of the thermal ramp used. This suggests that this methodological difference is not the cause for the difference observed in the measured CTmax between studies, but rather the potential differences in local stressors that the populations have been pre-exposed to at their locations prior to sampling (Tróia in Vinagre et al., 2016 and Ria de Aveiro in the present study). Also, for example, previous authors have reported a significant degree of population differentiation in *P. microps* across its distributional range, including between northern and southern populations in the northeastern Atlantic region (Gysels et al., 2004). Similarly, Berrebi et at. (2009) also report that populations of *P. microps* inhabiting adjacent lagoons, display almost no common haplotypes. Nevertheless, populations from different biogeographic regions have also been reported to display different migratory behaviours, with Mediterranean populations being mainly sedentary, whereas Atlantic ones may migrate, changing the degree of gene flow (Gysels et al., 2004). Consequently, the level of local genetic differentiation between populations, which can be the foundation of local adaptations (Berrebi et al., 2009), can influence the response of this fish species to warming. Notwithstanding, controversy still seems to exist on the genetic differentiation of *P. microps* populations (Tougard et al., 2014), as these studies were generally based on a low number of genes. Thus, future studies should resolve the degree of genetic differences in this goby fish across large and small biogeographic scales. Even if no genetic differentiation exists, processes influenced by regional or local conditions, such as transgenerational plasticity, could result in different responses across populations (Colicchio and Herman, 2020; Munch et al., 2021). A clear negative correlation between CTmax and weight was also observed in warm-acclimatized individuals, whereas no correlation was observed in cold-acclimatized fish. These results suggest that differences in fish surface-to-volume ratio and consequent lag time in response to external temperature affects disproportionally more individuals with prior history of heat exposure. For these individuals, higher body weight results in lower tolerance to elevated temperature, as also observed in other marine species (e.g. Fernandes et al., 2023; Missionário et al., 2022).

A hypothesis proposed by Pörtner et al. (2017) states that thermal range of species, and ultimately thermal tolerance, may in part be determined by tissue oxygen demand and supply, a process inherently dependent on whole-organism metabolic rate (Pörtner et al., 2017). However, this hypothesis also emphasizes the relevance of not neglecting links between biological organizations, which may also modulate thermal tolerance (Pörtner et al., 2017). According to Norin and Metcalfe (2019), metabolic rate connects environmental conditions and animal fitness, and is a proxy for the organism’s underlying cellular respiration. In the present study, no alterations were detected in oxygen consumption rates in the tested fish (warm-acclimatized), suggesting that routine metabolic rate of the goby fish *P. microps* was not affected by temperature, or that their metabolic plasticity is low, and hence cellular respiration is maintained. Based on previous reports that adaptation may influence the relationship between temperature and metabolic rate (Clarke and Fraser, 2004), one could argue that *P. microps* inhabits quite extreme environments and may thus be adapted to temperature shifts and extremes. As such, they could have developed a mechanism to keep steady metabolic rates throughout temperature fluctuations to save energy. Accordingly, a previous study comparing the oxygen consumption rates of 4 different *Pomatoschistus* species (*Pomatoschistus microps*, *P. pictus*, *P. lozanoi* and *P. minutus*), tested under different temperatures, swimming demands and in the presence and absence of food showed similar results (Fonds and Veldhuis, 1973). *Pomatoschistus microps* presented an enhanced swimming performance and respiratory scope for activity when compared with the remaining gobies of the same genus. Temperature increase (from 5 to 25 °C) led to higher respiratory maintenance costs in all species. Yet, in *P. microps* this increase was very subtle, slow, and stable when compared with the others. Authors suggested that this characteristic implied that this species was very well adapted to the substantial temperature fluctuations that are frequent in their shallow water habitats (Fonds and Veldhuis, 1973).

Another example is the round goby, *Neogobius melanostomus,* in which a similar pattern was observed. This species is known to have broad thermal tolerance and is highly adaptable to new environments, being invasive in many regions (e.g., Baltic and Black Sea) (Christensen et al., 2021). Previous experiments have shown that maximum metabolic rate in this species did not differ much after these fish were exposed to temperatures that ranged from 15 to 25 °C (Christensen et al., 2021). This ability to maintain metabolic rates despite temperature change has likely contributed to this species ability to thrive in a wide range of environments. Oxygen consumption only increased considerably after temperature surpassed 25 °C. Collectively, these results suggest that routine and maximum metabolic rates in gobies may be relatively insensitive to temperature shifts within the thermal niche of the species or until a certain threshold is reached. Still, acclimation in metabolic rates is known to occur and thus time of exposure and time of sampling may be crucial aspects of assessing metabolic rate plasticity and aerobic scope. For example, in the fish *Myoxocephalus scorpius*, standard metabolic rate (SMR) (therein defined as minimum metabolism when an individual is non-reproductive, unstressed, inactive and unfed) increased by 82 % in the first week of heat exposure (Metcalfe et al., 2016; Sandblom et al., 2014). However, after 8 weeks of exposure, SMR was restored to control levels, with fish displaying thermal compensation of metabolism (Sandblom et al., 2014).

The susceptibility of fish to temperature may be dependent on their ability to keep cellular homeostasis (Cereja et al., 2018). Cellular stress responses, that could suggest oxidative stress, or protein denaturation as result of heat exposure, also did not show any changes in the muscle of cold-acclimatized *P. microps* subjected to different thermal regimes. This result is somewhat in accordance with a previous study on *P. microps*, in which authors analysed antioxidant markers, lipid peroxidation and

Hsp70 in the muscle of *individuals* subjected to heat stress, infection and the combination of both factors (Cereja et al., 2018). Under the sole influence of heat stress, no changes were detected in superoxide dismutase, catalase or lipid peroxidation. However, Hsp70 did rise in fish after a +4 °C increase in temperature. In our study, there is no indication of a similar response. Nevertheless, biomarker responses are quite susceptible to exposure time (Madeira et al., 2021a) which was much shorter (10 days) in the study of Cereja et al. (2018) when compared to our study (30 days of warming plus 8 or 12 days of an additional heatwave simulation). Interestingly, previous studies on other fish species report that cellular stress responses might peak within 7–14 days of exposure to thermal stress, declining afterwards as the animals acclimate to the new environment (Madeira et al., 2018, 2020). Nevertheless, studies on other intertidal goby species also support the idea that cellular stress responses might not be strongly induced in these fishes upon thermal stress. For example, Paul et al. (2021), which performed a study with *Gobius paganellus*, has also referred that no major changes were observed in catalase and superoxide dismutase activity in the livers of fish in response to chronic thermal stress, reinforcing the hypothesis that intertidal gobies display high tolerance to increasing temperatures. However, we must also consider that responses may depend on the intensity of stress. For example, other authors testing the effect of an acute heat exposure in *G. paganellus* have found increased levels of antioxidant enzymes and lipid peroxidation with increasing temperature (Vinagre et al., 2014). In addition, results may depend on the tissue analysed. In the latter study with *G. paganellus*, fish displayed lower baseline values of cellular stress response biomarkers in the muscle, but an induction was detected in this tissue under acute exposure to thermal stress (20–34 °C). On the contrary higher baseline values were detected in the gills, but these were unresponsive to thermal stress (Vinagre et al., 2014). The fact that some tissues present high baseline levels of several biomarkers may be indicative of a preparative defence strategy, widely reported for intertidal species (Dong et al., 2008; Madeira et al., 2012). This strategy might be more beneficial in terms of energy expenditure and cellular homeostasis, maintaining a constant ability to repair cellular damage in variable and extreme environments (Dong et al., 2008; Madeira et al., 2012). Therefore, it is not fully correct to state that *P. microps* exposure to the different thermal regimes did not trigger any sort of cellular stress response, since only muscle tissue was analysed. Other tissues besides the muscle (e.g., liver or gills), need to be assessed before ascertaining final conclusions. Nevertheless, given the importance of muscle tissue in fish daily activities (e.g., swimming ability, on which many other behaviours depend on, such as foraging/predation, escaping, nest building, etc.), the ability of muscle to maintain function is crucial for fish performance (Domenici et al., 2008).

Overall, if we consider that the maximum temperature recorded in *P. microps* sampling site was 23.6 °C, and the minimum CTmax detected was 33.1 °C in cold-acclimatized individuals, this population still appears to have a quite wide positive thermal safety margin (~9.5 °C). Although the average daily thermal amplitude during cooler months being much lower (0.4 °C) than during warmer ones (6.3 °C), this species is able to cope with the thermal challenges imposed here. Results from the respirometry and cellular stress response biomarkers also corroborate this conclusion, since no signs of stress were detected in any of the traits measured. However, no heatwaves occurred during the year in which we monitored temperature in the field, which means that higher temperatures might be reached in the habitat under MHW conditions. Either way, it is possible for other populations of this same species to reach their thermal limit in the years to come (Vinagre et al., 2018). In other areas of Portugal, where *P. microps* also inhabits, temperatures can reach 30.6 °C, which in this case, considering the lowest CTmax recorded (33.1 °C), it would mean that such population's thermal safety margin is only 2.5 °C (Vinagre et al., 2018). Besides, in Ria Formosa, a site in Portugal where *P. microps* is quite abundant (Ribeiro et al., 2012), shallow water temperatures have already reached 36.5 °C during the warm season (Newton and Mudge, 2003), which in this case, would surpass the minimum CTmax detected in our study by approximately 3.35 °C. If *P. microps* thermal limit is reached, mass mortality can be expected in these areas, unless local adaptation or behavioural thermal regulation can buffer these effects (Vinagre et al., 2018). Although this species seems to display low capacity to alter its body response to heat, independently of acclimation temperature, we must take into account the somewhat small sample size (n = 5) utilized in the present study. Additionally, it is also important to consider that some studies have already described that local adaptation may have a crucial role in this fish potential to survive in a changing world (Berrebi et al., 2009). It is also worth considering that the thermal ramp of 2 °C/h was detected in the field during a year when no heatwaves occurred (2021). This implies that during an actual heatwave event, the rate of water temperature increase may be faster than the one tested in the present study. Further research could also explore the diurnal fluctuation of temperature to more accurately simulate environmental conditions.

Overall, we conclude that for now, *P. microps* populations in the central-northwestern coast of Portugal are not yet threatened by the temperature increase triggered by global warming and MHW. However, if this species low acclimation capacity is also revealed in other Atlantic or Mediterranean populations or even if we consider the multi-stressor context of shallow marine habitats, ocean warming, or extreme weather events may indeed represent a threat for this species persistence in the long run.

CRediT authorship contribution statement

Madalena Missionário: Writing – original draft, Visualization, Investigation, Formal analysis. **Célia Almeida:** Investigation. **Joana Filipa Fernandes:** Investigation. **Catarina Vinagre:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Carolina Madeira:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Diana Madeira:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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

Data are available on ZENODO (https://doi.org/10.5281/zenodo.8375756).

Acknowledgements

This work was supported by project ExtremeOceans (PTDC/BIA-BMA/1494/2020, doi: https://sciproj.ptcris.pt/159421PRJ) and project AQUATROPHYS (2021.09718.CBM), funded by the Portuguese Foundation for Science and Technology (FCT). This work is also financed by national funds i) UIDP/04378/2020 and UIDB/04378/2020 (UCIBIO), ii) LA/P/0140/2020 (i4HB), iii) UIDP/50017/2020+UIDB/50017/2020+LA/P/0094/2020 (CESAM), iv) UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020 (CCMAR), v) UIDB/04292/2020 (MARE). FCT is also acknowledged for the grants 2022.10575.BD awarded to M.M., 2021.04675.BD awarded to J.F.F., CEECIND/01526/2018 (doi: https://sciproj.ptcris.pt/6833EEC) awarded to C.M. and CEECIND/01250/2018 and 2022.00153.CEECIND (https://doi.org/10.54499/2022.00153.CEECIND/CP1720/CT0016) awarded do D.M.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2024.108849.

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