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Impact of marine heatwaves on *Carcinus maenas* crabs: Physiological and biochemical mechanisms of thermal stress resilience

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ABSTRACT

Marine heatwaves (MHWs), characterized by prolonged periods of elevated sea temperatures, pose significant threats to marine ecosystems, particularly affecting the physiology and behavior of marine organisms, including crustaceans. This study investigates the physiological and biochemical responses of males and females of *Carcinus maenas* crabs, after an acute exposure to an MHW, focusing on energy metabolism, oxidative status, and potential neurotoxicity. Specimens were exposed to controlled laboratory conditions simulating a temperature increase from 17 °C to 23 °C, and responses were analyzed in gills and hepatopancreas. Results revealed sex-specific differences in thermal stress resilience, with males showing higher glycogen storage in gills after MHW exposure, while females exhibited a significant reduction in glycogen reserves and an increase in antioxidant enzyme activity. Superoxide dismutase and glutathione reductase activities were notably elevated in females subjected to MHW, suggesting a more robust antioxidant response to counteract oxidative stress. Additionally, acetylcholinesterase activity, an indicator of neurotoxicity, was significantly reduced in females post-MHW, hinting at potential neurotoxic effects. Despite these biochemical changes, lipid peroxidation levels remained stable across both sexes and tissues, indicating that short-term MHW exposure did not cause significant oxidative damage to cell membranes. This study highlights the importance of considering sex differences in assessing the impacts of climate change-induced stressors on marine organisms, as males and females display distinct metabolic and physiological strategies for coping with thermal stress.

1. Introduction

Marine heatwaves (MHWs) events are characterized by sustained periods of elevated water temperatures, lasting at least five consecutive days, surpassing the 90th percentile threshold derived from the region's climatological data over the past three decades (Hobday et al., 2018). These abnormally warm conditions pose significant threats to the structure and resilience of marine ecosystems, leading to various consequences such as the displacement of mobile species and widespread mortality events, particularly among sessile organisms (Sen Gupta et al., 2020; Holbrook et al., 2020).

One of the most immediate and visible impacts of MHWs is the disruption of marine biodiversity. These extreme temperature events can lead to widespread bleaching of coral reefs, not only jeopardising the survival of these vital ecosystems but also threatening the myriad species that depend on them for habitat and food (Donovan et al., 2021;

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Van Woesik and Kratochwill, 2024). Beyond coral reefs, MHWs can trigger shifts in several species' distribution and abundance. Many marine species, including fish, invertebrates, and marine mammals, have specific temperature preferences and are highly sensitive to even slight changes in water temperature. As MHWs persist, these species may migrate to cooler waters searching for suitable habitats, leading to altered community compositions and potential conflicts with existing ecosystems (Cheung and Frölicher, 2020; Monteiro et al., 2023; Smith et al., 2023). MHWs also have cascading effects on marine food webs and ecosystem functioning. Temperature changes can disrupt the timing of biological events such as spawning, migration, and feeding, leading to mismatches between predators and their prey. This disruption can have repercussions throughout the food chain, impacting the abundance of commercially important species and altering ecosystem dynamics (Robinson et al., 2022; Shalders et al., 2023). It was indeed reported by Anacleto et al. (2014) that temperature changes can negatively affect clams, causing a decrease in protein and fatty acids in bivalves.

Concerning crustaceans, and in particular crabs, one of the primary impacts of MHWs is altered physiology and behavior. As water temperatures rise, crabs may experience thermal stress, affecting their metabolic rates, growth, reproduction, and survival (Bartolini et al., 2013). Dudley et al. (2021) and Santora et al. (2020) highlighted that the MHW in 2014–2016 caused nutrient conditions that led to a significant harmful algal bloom (HAB) dominated by the diatom *Pseudo--nitzschia*, which produced high levels of domoic acid (DA). Dungeness crabs, which feed on bivalves, accumulated DA in their tissues, making them unsafe for human consumption. This resulted in a regulatory delay of the crab season, impacting the fishery's timing and reducing fishing effort, catch, and revenue. MHWs can also disrupt the timing of important life cycle events for crabs, such as molting and reproduction. Elevated water temperatures may accelerate molting cycles, leaving crabs vulnerable during the soft-shell stage when they are more susceptible to predation (Sen Gupta et al., 2020). Brylawski and Miller (2006) reported that during periods of elevated water temperatures, blue crabs (*Callinectes sapidus*) may experience premature molting, resulting in increased vulnerability to predation and reduced survival rates. Additionally, temperature changes can affect reproductive behaviors and decrease reproductive success, potentially leading to declines in crab populations over time (Darmaraki et al., 2019; Lotze et al., 2019). When under temperature stress, crabs may seek refuge in cooler, deeper waters or migrate to areas with more favorable temperature conditions. However, this can lead to overcrowding and competition for resources in these refuges, further stressing crab populations (Hobday et al., 2018; Straub et al., 2019). Sanford et al. (2019) showed that during periods of high water temperatures the purple shore crab (*Hemigrapsus nudus*), in the Pacific Northwest, sought refuge in cooler microhabitats or migrated to higher latitudes in search of more suitable environmental conditions. A different distribution of crabs can impact fisheries and ecosystem services. Recent research on crabs (*Chionoecetes opilio*) in the Bering Sea has shown that rising ocean temperatures can lead to changes in crab abundance and distribution, affecting the productivity and profitability of fisheries (Litzow et al., 2019). Other effects have also been noted concerning crabs subjected to MHWs, namely on larval development. Spitzner (2021) showed that in *Carcinus maenas,* high water temperatures can accelerate larval growth rates and increase larval mortality due to physiological stress and predation pressure.

Carcinus maenas has been the subject of many studies, but no studies on the effects of MHWs in males and females of this species have been reported. *C. maenas* (Linnaeus, 1758), known as the "shore crab" or "European green crab," has earned notoriety as one of the world's 100 worst invaders according to the IUCN (Lowe et al., 2000). Originally native to Atlantic Europe, its remarkable adaptability, spanning phenotypic plasticity, broad tolerance to temperature and salinity, and diverse omnivorous diet, contribute to its proficiency as an invader (Howard et al., 2019; Young and Elliott, 2020). Although exhibiting eurythermal and euryhaline traits as adults, this species is constrained to temperate coastlines due to specific temperature requirements crucial for breeding and larval development (Young and Elliott, 2020). *C. maenas* habitat preference leans towards wave-protected sheltered areas such as bays, estuaries, and harbors, as they cannot withstand the harsh conditions of wave-swept open shores.

The present study aimed to understand the effects of MHWs on the crab *C. maenas*, assessing physiological and biochemical changes after acute exposure. To this end, males and females were identified and subjected to laboratory experimental conditions. After exposure, the tissues believed to be target organs of stress, such as gills and hepatopancreas, were used to investigate the impacts. The selected biomarkers for studying the effects of MHWs in crabs were chosen based on their relevance to key physiological processes that are sensitive to environmental stressors. Protein (PROT) and glycogen (GLY) concentrations were included to evaluate the crabs' energy reserves and metabolic adjustments, as these are critical indicators of overall health and energy balance under stress. Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), were assessed to measure the antioxidant capacity of the crabs, reflecting their ability to mitigate oxidative stress induced by MHWs. Lipid peroxidation (LPO) levels were analyzed as markers of cellular damage, providing insights into the degree of oxidative damage sustained by tissues. Finally, acetylcholinesterase (AChE) activity was measured as an indicator of neurological impairments, linking potential effects of heat stress on nervous system function. Together, these biomarkers offer a comprehensive view of the impacts of MHWs on *C. maenas*.

2. Materials and methods

2.1. Organisms sampling and experimental design

Carcinus maenas specimens were collected from the Ria de Aveiro (NW Portugal) in May 2023 and immediately transported to the laboratory in plastic containers filled with water from the sampling site. Upon arrival, they were acclimated to laboratory conditions for two weeks. During this period, organisms were maintained in artificial seawater (salinity 30 ± 1) by mixing commercial Red Sea Salt ® with osmosis water, a temperature of 17 °C, constant aeration and a photoperiod of 14 h of light and 10 h of darkness. Crabs were fed every 2 days with frozen mussels *ad libitum*. After the acclimation period, 7 mature males (maximum carapace width (CW) ranging from 37 to 49 mm) and 7 mature females non-ovigerous (CW varying between 35 and 44 mm) were used per temperature scenario (CTL-17 °C and MHW-17-23-17 °C), with 1 individual per replicate/glass vessel containing 1 L of seawater each (salinity 30 ± 1). A temperature of 17 °C was used as the control (CTL) since the mean temperature recorded at Ria de Aveiro in 2020 was 16.31 ± 1.83 °C (based on data from National Oceanic and Atmospheric Administration 1/4° daily Optimum Interpolation SST version 2.1 (NOAA OISST V2.1)). For the MHW simulation, the temperature was increased up to 23 °C, with a 2 °C increase per day. A temperature of 23 °C was maintained for 7 days, after which the temperature dropped 2 °C per day to control levels. This gradual increase or decrease of 2 °C per day balances the need to avoid any confounding stressor due to temperature shock while realistically simulating the rapid but progressive temperature changes characteristic of marine heatwaves. The duration and intensity (temperature) of the MHW event were chosen based on diverse published literature (Cheng et al., 2023; Hobday et al., 2018) but also considering the short duration and low intensity of MHWs events along the Portuguese coast (Monteiro et al., 2024). In various marine ecosystems, particularly in temperate and subtropical regions, a temperature of 23 °C falls within the threshold typically associated with MHW events. For instance, in temperate regions, sea surface temperatures (SSTs) generally range between 15 and 20 °C, with MHWs often surpassing 22 °C. Similarly, in subtropical zones, 23 °C may represent an upper extreme during such anomalous warming events (Hobday et al., 2018). Considering the wide spatial distribution of the

species, we wanted to test the worst-case scenario. The experimental period lasted 14 days, during which mortality and molting process were daily recorded.

At the end of the experiment (14 days), crabs were weighted and sacrificed with ice and hepatopancreas, gills, and gonads (if present) were dissected from each organism. The hepatopancreas and ovary in females and the entire male reproductive system were weighed (accuracy 0.01 mg) to estimate: hepatosomatic index (HSI, %) = (wet weight of hepatopancreas/total weight) *100 and gonadosomatic index (GSI, %) = (wet weight of ovary or male reproductive system/total wet weight) *100. The gills and hepatopancreas of each organism were dissected and frozen at a temperature of −80 °C until the biochemical analyses were carried out.

2.2. Homogenates preparation and biochemical parameters

A total of seven biochemical parameters were analyzed using the cytosolic fraction: protein (PROT), glycogen (GLY) concentrations, to assess the energy content; superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione reductase (GR) activity to assess crabs antioxidant capacity; lipid peroxidation (LPO) levels as an indicator of cellular damage; and acetylcholinesterase (AChE) activity as a measure of neurotoxicity. Tissue samples from gills and hepatopancreas were first homogenized using TissueLyser II (Qiagen) with a frequency of 20 1/s for 90 s. Secondly, tissue samples were centrifuged for 20 min at 10,000 *g* and 4 °C, after which cytosolic fraction (where most of the cell soluble components, including enzymes, are found), was removed and immediately used or stored at −80 °C. Phosphate buffer (1 mmol/L EDTA, 1 % Triton X-100 and 1 mmol/L DTT at pH of 7.0) was used to perform GLY, PROT, SOD, CAT, GR and AChE. For LPO the extraction buffer consisted of a solution of 20 % (w/v) trichloroacetic acid (TCA).

Overall, the results were expressed on a fresh weight (FW) basis rather than per protein to normalise the data to the total tissue mass, thereby avoiding potential variations in protein concentration across samples. This approach allows for more direct comparisons between treatments or experimental conditions, particularly in cases where factors such as stress, development, or storage may affect total protein levels and, consequently, the calculations. Furthermore, expressing results per FW provides a broader representation of the overall physiological state of the tissue, capturing the metabolic response as a whole, independent of fluctuations in protein synthesis or degradation.

The concentration of GLY was measured using the sulfuric acid method described by DuBois et al. (1956). A calibration curve was created using eight glucose standards with concentrations ranging from 0 to 5 mg/mL. This approach measures the formation of a yellow-orange compound produced by the reaction between sugars (or their derivatives) and phenol in the presence of sulfuric acid. The intensity of the resulting color is proportional to the sugar content. Sulfuric acid facilitates the breakdown of sugars, enabling their quantification. Absorbance was measured at 490 nm after incubating the mixture for 30 min at room temperature. GLY concentrations were expressed in mg/g FW.

The PROT concentration was analyzed using the biuret spectrophotometric method, as outlined by Robinson and Hogden (1940). A standard calibration curve was generated with bovine serum albumin (BSA) at concentrations ranging from 0 to 40 mg/mL. This method relies on the formation of complexes in an alkaline solution, which occurs due to the interaction between copper (II) ions and peptide bonds in amino acids. Before measuring absorbance, the solution was incubated in the dark for 10 min at 30 °C and then analyzed at a wavelength of 540 nm. Based on Beer's Law, the absorbance values are directly correlated with the protein concentration in the sample. Results were reported in mg/g FW.

Superoxide dismutase activity was determined following the method of Magnani et al. (2000). The reaction mixture included the sample and Tris-EDTA buffer, and the reaction was initiated by adding pyrogallol dissolved in 0.01 mmol/L HCl. After a 1-min reaction time, the absorbance was measured at 420 nm. Enzymatic activity was expressed as U/g FW, where one unit (U) corresponds to the enzyme activity required to inhibit 50 % of pyrogallol autoxidation.

The activity of CAT was measured using the method described by Johansson and Borg (1988), with modifications by Carregosa et al. (2014). This method involves the reaction of catalase with methanol in the presence of hydrogen peroxide, leading to the production of formaldehyde, which is then quantified spectrophotometrically using purpald. Absorbance was measured at 540 nm, and the enzymatic activity was expressed in U/g FW, where one unit (U) corresponds to the amount of enzyme that generates 1 nmol of formaldehyde.

Glutathione reductase activity was measured using the method of Carlberg and Mannervik (1985). The assay quantified NADPH oxidation during the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). The reaction was performed in a phosphate buffer (pH 7.6) with NADPH and GSSG, and the decrease in absorbance at 340 nm for 5 min at 15-s intervals was recorded. The extinction coefficient (ε = 6.22 mM^{-1} cm^{-1}) was used to calculate activity, expressed as U/g FW, where one unit (U) represents the quantity of enzyme which catalyzes the conversion of 1 nmol of NADPH per min.

Lipid peroxidation levels were assessed following the method outlined by Ohkawa et al. (1979) with modifications by Carregosa et al. (2014), which involves measuring malondialdehyde (MDA) concentration. The LPO levels were determined by quantifying thiobarbituric acid reactive substances (TBARS) formed during the reaction between MDA, a lipid peroxidation by-product, and 2-thiobarbituric acid (TBA). The samples were incubated at 96 °C for 25 min, after which the reaction was halted by cooling on ice. Absorbance was recorded at 532 nm using an extinction coefficient (ε) of 156 $mM^{-1}cm^{-1}$. The results were reported as nmol MDA /g FW.

The neurotoxicity effects were assessed by determining AChE activity, using acetylthiocholine iodide (ATChI 5 mmol/L) as substrate, following the method developed by Ellman et al. (1961) and modified by Mennillo et al. (2017). The activity was monitored spectrophotometrically at 412 nm over a 5-min period, with readings taken at 15-s intervals. The results were expressed as nmol per min/g FW.

2.3. Statistical analysis

The HSI and GSI were analyzed using One-Way Analysis of Variance (ANOVA) or Student's t-test. All data were checked for normality of distribution and homogeneity of variance using the Shapiro-Wilk normality test and Bartlett's test, respectively. Statistical analyses were performed using R Commander 3.6.1. The level of significance was set at 5 %.

The non-parametric permutational analysis of variance (PERMANOVA + add-on in PRIMER v6) was performed with the biochemical data (Anderson et al., 2008). The null hypotheses tested were: i) for each organ (gills and hepatopancreas) and biochemical responses, no significant differences were observed between CTL and MHWs in males and females (significant differences are represented in figures with an asterisk); ii) for each organ (gills and hepatopancreas) and biochemical responses, no significant differences were observed between male and female in CTL and MHW treatments (significant differences are represented in figures with lowercase letters for CTL and uppercase letters for MHW).

The multivariate analysis was performed with the biochemical data using the cluster analysis of PRIMER v6 software. Hierarchical clustering was carried out using the Unweighted Pair Group Method with Arithmetic Mean, which enabled the comparison of similarities between temperatures (CTL and MHW), organs (gills and hepatopancreas), and sexes (male and female). The biochemical data were also subjected to ordination analysis, performed by non-metric Multidimensional Scaling (MDS), using the same software, to generate a two-dimensional (horizontal and vertical axes) diagram with the respective stress value. This value represents the distortion relative to the two-dimensional multidimensional distance matrix representation. If this value is < 0.10 the

representation is identified as very good. On the contrary, when the value is above 0.30, the diagram obtained is not a reliable representation of the distance matrix (Clarke and Warwick, 2001).

3. RESULTS

3.1. Physiological and biochemical parameters

3.1.1. Physiological alterations

At the end of the assay, mortality and molting process were null for control and MHW males and the mean weight did not present significant differences between control and MHW treatments (Table 1; Student's t-test $p = 0.90$). The hepatopancreas was yellow or brown in 42 % and 58 % of the males, respectively, without differences between temperature scenarios. The HSI was 3.65 ± 1.89 % and 3.80 ± 1.38 % for control and MHW males, respectively, without significant differences (ANOVA, $p = 0.87$; Table 1). All males had well-developed reproductive systems (Fig. 1A). The GSI of the males was 0.30 ± 0.31 % and 0.50 ± 0.11 % for control and MHW males, respectively, without significant differences between treatments (Student's t-test $p = 0.15$).

At the end of the assay, the mortality and molting process were null for control and MHW females and the mean weight did not present significant differences between control and MHW treatments (Table 1; ANOVA, $p = 0.06$). The hepatopancreas was yellow or brown (Fig. 1B) without macroscopic deferences between temperature scenarios. The HSI from females were 5.56 ± 1.71 % and 3.98 ± 1.54 % for control and MHW, respectively without significant differences (ANOVA, $p = 0.09$; Table 1). All the females from the control group did not differentiate the ovary while 42.8 % ($n = 3$) of MHW females presented an orange-thin ovary (Fig. 1B). The seminal receptacles were developed in 42.8 % ($n = 3$) of the females with no spermatophores and without differences between temperature scenarios. GSI of MHW females was 1.94 ± 0.5 %, while at CTL was 0 % which corresponded to no differentiation of the ovary (Table 1).

3.1.2. Biochemical alterations

3.1.2.1. Energy reserves content. In the gills, the protein (PROT) concentration did not change in males and females regardless of the temperature scenario, with no significant differences between males and females at each temperature. A similar response was observed in the hepatopancreas, with no significant differences among treatments (Fig. 2A and B; Tables 2 and 3).

The glycogen (GLY) concentration in the gills of males was significantly higher after acute exposure to the MHW scenario, while females showed an opposite answer, although without statistical significance. In the gills, females showed higher GLY content than males under CTL temperature. In the hepatopancreas, females exposed to the MHW scenario significantly decreased their GLY content in comparison to the CTL temperature. Nevertheless, no significant differences were observed between males and females in each temperature scenario (Fig. 2C and D; Tables 2 and 3).

3.1.2.2. Antioxidant capacity. In the gills and hepatopancreas females significantly increase their superoxide dismutase (SOD) activity after exposure to the MHW scenario. An opposite response was observed in the male's gills. For both organs, no significant differences were observed between males and females at the control temperature, while after the MHW females presented significantly higher SOD activity than males (Fig. 3A and B; Tables 2 and 3).

The activity of catalase (CAT) remained unchanged regardless of the organ and the temperature scenario in males and females; no significant differences were observed between males and females regardless of the temperature scenario (Fig. 3C and D; Tables 2 and 3).

The activity of glutathione reductase (GR) was similar regardless of the organ and the temperature, both in males and females, with no significant differences between males and females regardless of the temperature scenario (Fig. 3E and F; Tables 2 and 3).

3.1.2.3. Cellular damage. Lipid peroxidation (LPO) levels showed no statistical alterations in the gills and hepatopancreas of males and females after exposure to CTL temperature and MHW scenarios. However, in the hepatopancreas, females showed an increase in LPO levels after MHW compared to those found in females at CTL temperature and values found in males after MHW exposure (Fig. 4A and B; Tables 2 and 3).

3.1.2.4. Neurotoxicity. In the gills, compared to the CTL, the acetylcholinesterase (AChE) activity increased significantly after the MHW in males, while a significant decrease was observed in females. Differences between males and females were noticed at CTL and MHW treatments, with higher activity in CTL females and an opposite pattern in MHW females. In the hepatopancreas, differences between CTL and MHW treatments were only noticed in females, with lower values after the MHW stress. Under CTL temperature, males showed lower AChE activity than females (Fig. 5A and B; Tables 2 and 3).

3.2. Multivariate analysis

The cluster analysis (Fig. 6A) showed a first division (groups A and B) that distinguished gills (group A) from the hepatopancreas (group B) treatments. Group A is divided into two subgroups (A1 and A2), separating MHW females (A1) from the other treatments (A2). Group A2 was further subdivided isolating CTL females (A2.1) from the remaining treatments (CTL and MHW males, A2.2). Regarding the hepatopancreas, this group (B) was subdivided into 2 subgroups B1 and B2, separating MHW females (B1) from the remaining treatments (B2). The B2 group was further subdivided, isolating CTL females (group B2.1) from the CTL and MHW males (group B2.2).

The results of the MDS analysis (Fig. 6B) revealed a clear division of treatments into two main groups: Group I, comprising all hepatopancreas treatments, and Group II, containing all gill treatments. These groups align with Groups A and B from the cluster analysis, further emphasizing the distinction between treatments within each organ and highlighting the degree of separation among them.

Table 1
Mean weight (g), carapace width (mm), gonadosomatic index and hepatosomatic index (%) in *Carcinus maenas* males and females exposed to a control temperature (17 ± 1 °C) and a MHW scenario (17-23-17 °C) for 14 days. Data are presented as means ± standard deviation.

	Males		Females	
	Control	MHW	Control	MHW
Mean weight	19.09 ± 6.6	19.41 ± 1.70	14.27 ± 3.21	17.77 ± 3.30
Carapace width	41.43 ± 5.25	43.49 ± 1.03	38.68 ± 2.43	40.76 ± 2.36
Gonadosomatic index	0.30 ± 0.31	0.50 ± 0.11	0[a]	1.94 ± 0.5
Hepatosomatic index	3.65 ± 1.89	3.80 ± 1.38	5.56 ± 1.71	3.98 1.54

[a] Since control females did not differentiate the ovary the GSI is zero.

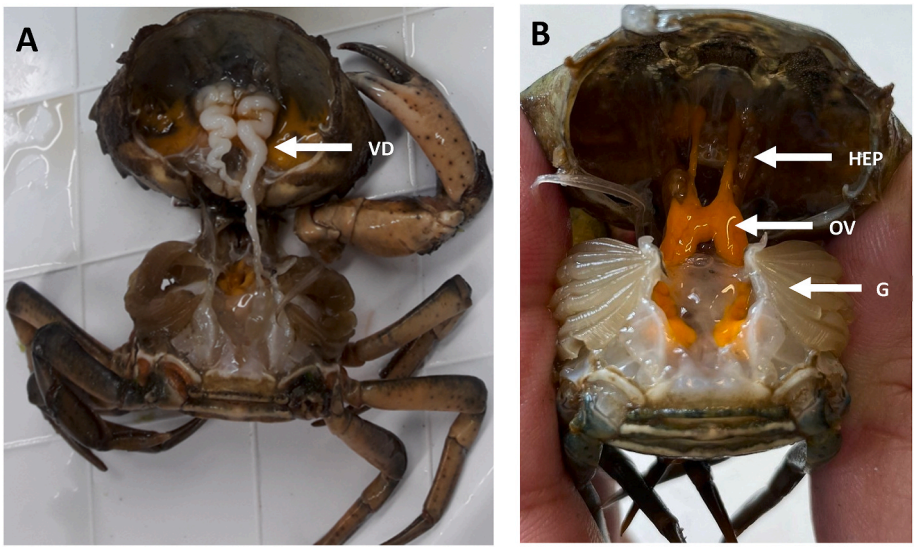

Fig. 1. A: Male of *Carcinus maenas* showing developed vas deferens (VD); B: Female of *Carcinus maenas* (MHW-treatment) showing developed orange ovary (OV), hepatopancreas (HEP) and gills (G).

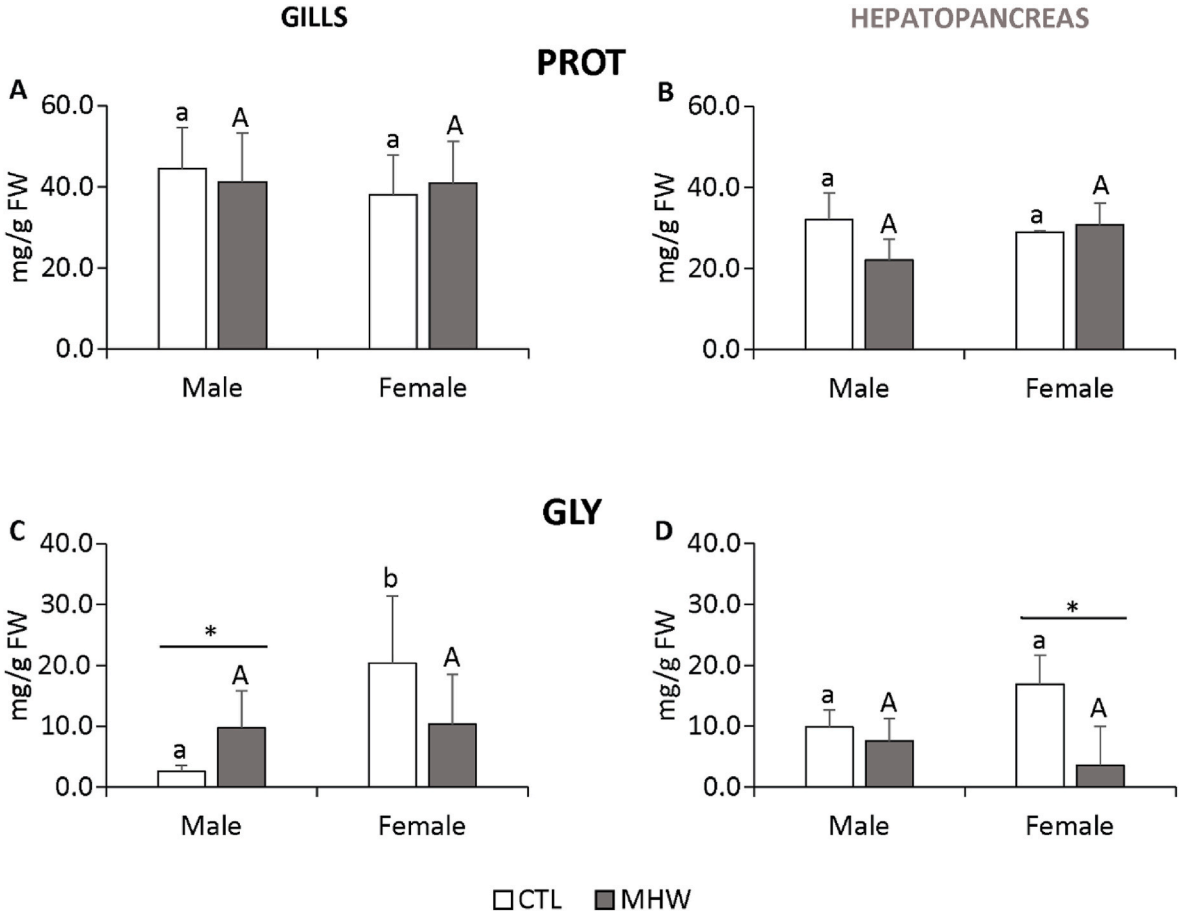

Fig. 2. Protein (PROT) concentration in gills (A) and hepatopancreas (B) and glycogen (GLY) concentration in gills (C) and hepatopancreas (D) of *Carcinus maenas* exposed to different treatments (CTL-17 °C; MHW). Results are reported as mean +SD. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between CTL and MHW in males and females are represented with an asterisk. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between males and females are represented with lowercase letters for CTL and uppercase letters for MHW.

4. Discussion

In the case of *Carcinus maenas,* and also other species like *C. aestuarii,* sea surface temperature is likely a key limiting factor for its global expansion, as it influences larval survival and impacts the physiological performance of both juveniles and adults (Troitiño Vinuesa, 2007; Tepolt and Somero, 2014; Aliko et al., 2015; Behrens Yamada et al., 2021). In this context, the present study aimed to investigate the effects

Table 2
P-values for pairwise comparisons for each biomarker in gills of *Carcinus maenas*. Significant differences ($p < 0.05$) are highlighted in bold. ns: non-significant differences.

	Comparison	PROT	GLY	SOD	CAT	GR	LPO	AChE
Male	CTL 17 vs MHW	ns	**0.0171**	**0.0277**	ns	ns	ns	**0.0014**
Female	CTL 17 vs MHW		0.1057	**0.0022**				**0.0031**
CTL	Male vs Female		**0.0029**	0.1573				**0.0021**
MHW	Male vs Female		0.8877	**0.0007**				**0.007**

Table 3
P-values for pairwise comparisons for each biomarker in hepatopancreas of *Carcinus maenas*. Significant differences ($p < 0.05$) are highlighted in bold. ns: non-significant differences.

	Comparison	PROT	GLY	SOD	CAT	GR	LPO	AChE
Male	CTL 17 vs MHW	ns	0.4449	0.6676	ns	ns	ns	0.1107
Female	CTL 17 vs MHW		**0.0311**	**0.0045**				**0.0072**
CTL	Male vs Female		0.0917	0.533				**0.0002**
MHW	Male vs Female		0.8229	**0.0053**				0.3026

of a marine heatwaves (MHWs) on the physiological and biochemical performance of *C. maenas*, focusing on sex- and organ-dependent responses.

4.1. Physiological alterations

Temperature is a strong extrinsic factor that modulates ectotherm physiology involving the whole energy availability for general metabolism, growth and reproduction (DeLong et al., 2017). In crustaceans, reproduction including ovary maturation and the egg carrying period is strongly influenced by temperature (Ramirez Llodra (2002) and references therein). In the present study control females did not develop the ovary at the assayed temperature (17 °C) while 50 % of the MHWs females did it, even considering that they were exposed only 14 days. It means a quick metabolic response and energy deviation towards growing oocytes. In this sense, further research is needed to assess if such quick energy deviation to the ovary has a significative impact on female metabolic global performance (e.g. compromising future survival), somatic growth (e.g. leading to lower size) and/or in the egg number/progeny quality (Sasaki et al., 1986; García Guerrero et al., 2003) with a strong impact in future recruitment. The observed changes in ovary growth under the assayed MHW scenario need long-term exposition to address the question of a more global impact of heat waves on reproduction, e.g. biochemical composition of eggs, number of hatched larvae, and early survival among other critical variables for this relevant economic species besides the ecological impact because of its role in trophic webs.

Concerning males, there was also a tendency for a greater gonadosomatic index (GSI) in MHW males which could also be demonstrating an accelerated accumulation of secretions in the vasa deferentia (they are mainly proteins, Mutti et al., 2024 for revision). This and the analysis of sperm number and sperm viability require more research to address the impact of water-increasing temperature on *C. maenas* reproduction.

In decapod crustaceans, the main metabolic integrative organ is the hepatopancreas, which involves nutrient resorption, digestive enzyme synthesis, detoxifying function, providing proteins and lipids to the ovary during vitellogenesis, and is the most important antioxidant defense organ (Vogt, 2019). Because of its integrative physiological role, it is a monitor organ for the health status of decapods (Vogt, 2019, 2021; Pedrazzani et al., 2023) and their nutritional condition (Lopeztegui-Castillo, 2021). The analyzed variable, hepatosomatic index (HSI), is one of the proxies of hepatopancreas functionality, and it did not show differences between constant temperature and MHWs for both sexes. Hence, according to this variable, MHWs would not impair hepatopancreas functionality, although a more profound analysis at the cellular level is required to understand the "real" physiological impact of fluctuating temperature.

4.2. Biochemical alterations

In terms of biochemical responses, the present study investigated the effects of an acute MHW on the energy reserves, antioxidant capacity, cellular damage, and neurotoxicity of male and female *C. maenas* crabs. The results revealed notable differences between organs, highlighting the distinct responses given by gills and hepatopancreas. From the multivariate analysis, the results obtained further demonstrated that, within each organ, males and females responded differently to thermal stress. Cluster analysis (Fig. 6A) showed a primary separation between gills and hepatopancreas treatments, with further subdivisions highlighting the distinct responses of males and females to thermal stress. Similarly, the MDS analysis (Fig. 6B) reinforced this pattern, grouping all hepatopancreas treatments separately from gill treatments, further emphasizing the organ-specific effects of MHWs. This differentiation underscores the importance of considering both organ- and sex-specific adaptations when assessing the impact of climate-induced stressors on marine invertebrates.

In the present study, the glycogen (GLY) and protein (PROT) concentrations were assessed in an attempt to understand crabs' energy reserves' variation to thermal stress. The results demonstrated that the PROT levels in male and female crabs were not affected by the temperature scenario, regardless of the organ studied. This suggests that short-term thermal stress does not significantly impact PROT content in gills and hepatopancreas, possibly because PROT catabolism is not the primary source of energy under such conditions. Nevertheless, males showed a significant increase in GLY content in the gills following acute MHW exposure, indicating a potential GLY storage in response to the elevated temperature. In contrast, females subjected to the thermal stress treatment (MHW) exhibited a reduction in GLY content in the gills and hepatopancreas. This suggests that females may rely more on GLY reserves than males when facing thermal stress. However, females presented higher GLY content at CTL temperature than males, regardless of the organ. These sex-specific responses may reflect differing energy allocation strategies, with females possibly preserving GLY for reproductive processes or other critical functions. The study by Capparelli et al. (2019) explored the combined effects of copper exposure and temperature on the physiological responses of the fiddler crab *Minuca rapax*. The primary findings revealed an increase in oxygen consumption with temperature, peaking at 35 °C. Although this study did not differentiate between females and males, higher oxygen consumption may be linked to a greater metabolic capacity, resulting in the use of energy reserves. Similarly, the study by Rodrigues et al. (2015) explored the biochemical and physiological responses of *C. maenas* to temperature

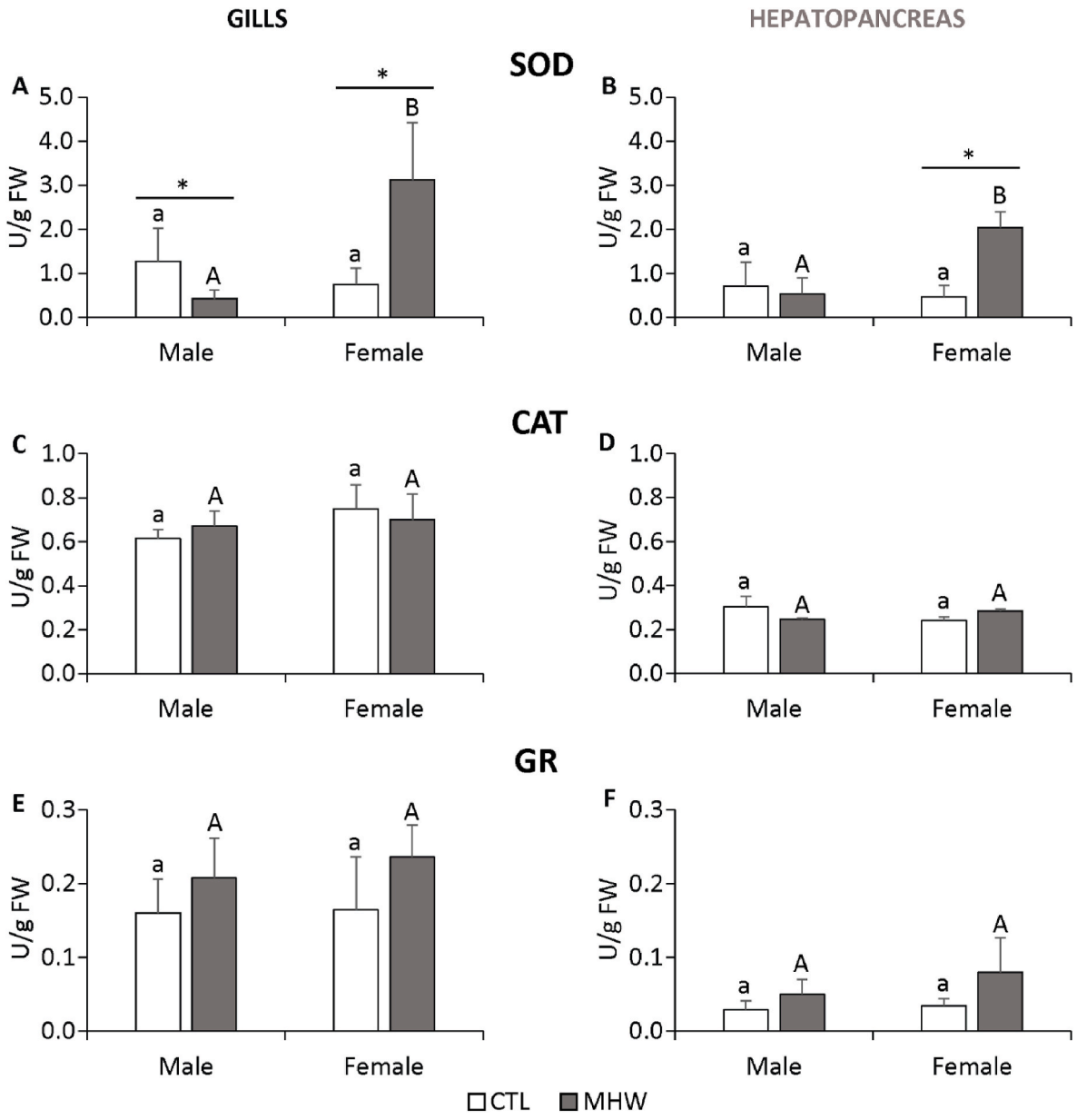

Fig. 3. Superoxide dismutase (SOD) activity in gills (A) and hepatopancreas (B); catalase (CAT) activity in gills (C) and hepatopancreas (D) and glutathione reductase (GR) activity in gills (E) and hepatopancreas (F) of *Carcinus maenas* exposed to different treatments (CTL-17 °C; MHW). Results are reported as mean +SD. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between CTL and MHW in males and females are represented with an asterisk. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between males and females are represented with lowercase letters for CTL and uppercase letters for MHW.

variations and exposure to the fungicide azoxystrobin. While the authors did not distinguish between males and females, their findings revealed that elevated temperatures (27 °C) resulted in higher oxygen consumption, pointing to thermal stress. High oxygen consumption and heat stress were also found at critical temperatures of 25 °C in the same species by Nancollas and McGaw (2021). Therefore, the results reported by other authors might align with the findings observed in the present study, with females using GLY as the energy source under increased stress conditions, while males adopt a different strategy, accumulating GLY and probably using other energy reserves. Hayden et al. (2007) investigated the sex-specific differences in feeding responses of *C. maenas* throughout the year and demonstrated the seasonality of feeding responses. Substantial sex-specific differences exist over the summer months, when males are less active than during winter months, whilst female shore crabs show increased feeding activity during the summer. This behavior may explain why, in the present study, males accumulated GLY when exposed to elevated temperatures, likely as a protective strategy to compensate for reduced feeding activity during thermal stress.

The antioxidant defense mechanisms also demonstrated differences between males and females, particularly under MHWs conditions. In females, superoxide dismutase (SOD) and glutathione reductase (GR) activities increased in both the gills and hepatopancreas following MHW exposure, which may indicate a stronger antioxidant response in females to counteract the elevated levels of reactive oxygen species (ROS) generated by heat stress. Increased antioxidant capacity in females after MHW might be associated with GLY decrease under this treatment, indicating the use of this energy source to fuel up antioxidant defense mechanisms. In contrast, males showed minimal changes in their antioxidant capacity after exposure to the MHW scenario. This suggests that

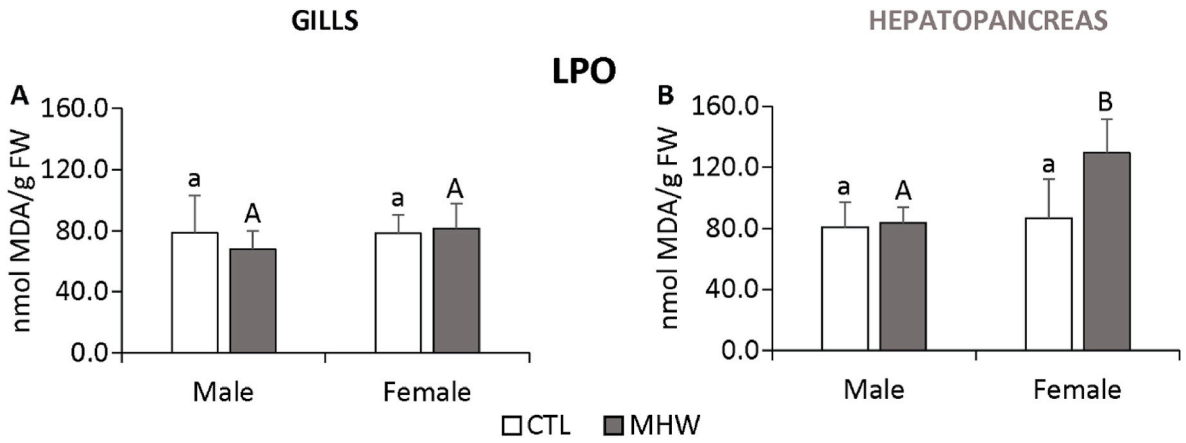

Fig. 4. Lipid peroxidation (LPO) levels in gills (A) and hepatopancreas (B) of *Carcinus maenas* exposed to different treatments (CTL-17 °C; MHW). Results are reported as mean +SD. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between CTL and MHW in males and females are represented with an asterisk. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between males and females are represented with lowercase letters for CTL and uppercase letters for MHW.

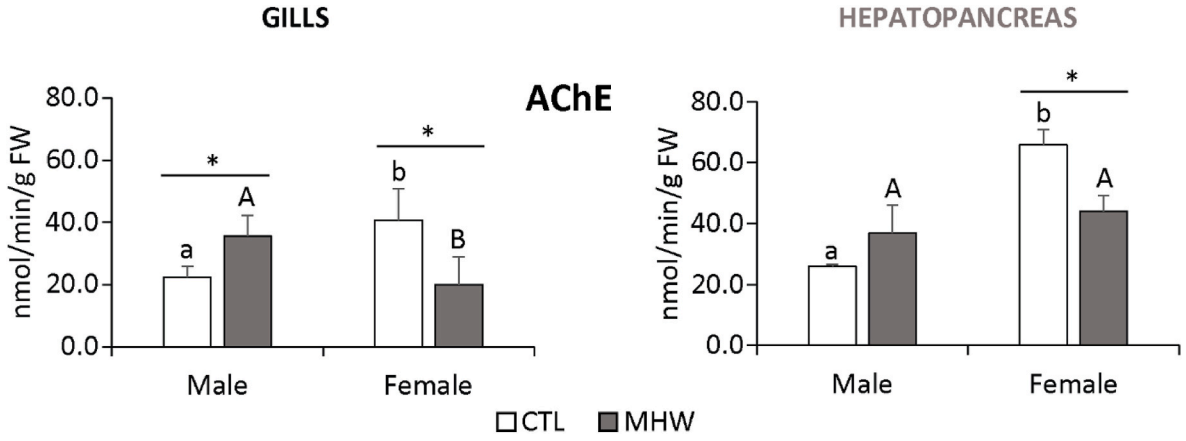

Fig. 5. Acetylcholinesterase (AChE) activity levels in gills (A) and hepatopancreas (B) of *Carcinus maenas* exposed to different treatments (CTL-17 °C; MHW). Results are reported as mean +SD. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between CTL and MHW in males and females are represented with an asterisk. For each organ (gills and hepatopancreas) and biochemical responses, significant differences ($p < 0.05$) between males and females are represented with lowercase letters for CTL and uppercase letters for MHW.

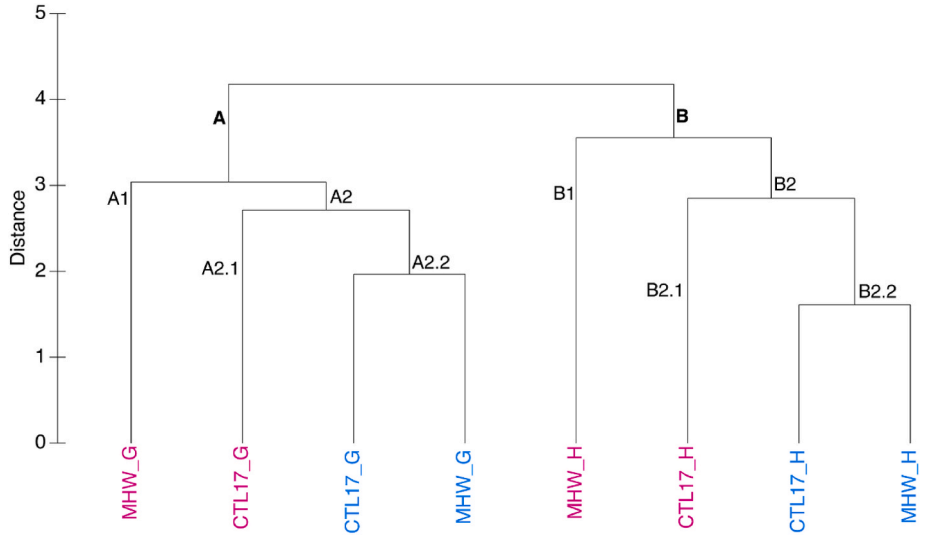

Fig. 6A. Cluster analysis based on biochemical markers in gills (A) and hepatopancreas (B) of *Carcinus maenas* males (light blue) and females (pink) exposed to control temperature (17 °C) and MHW.

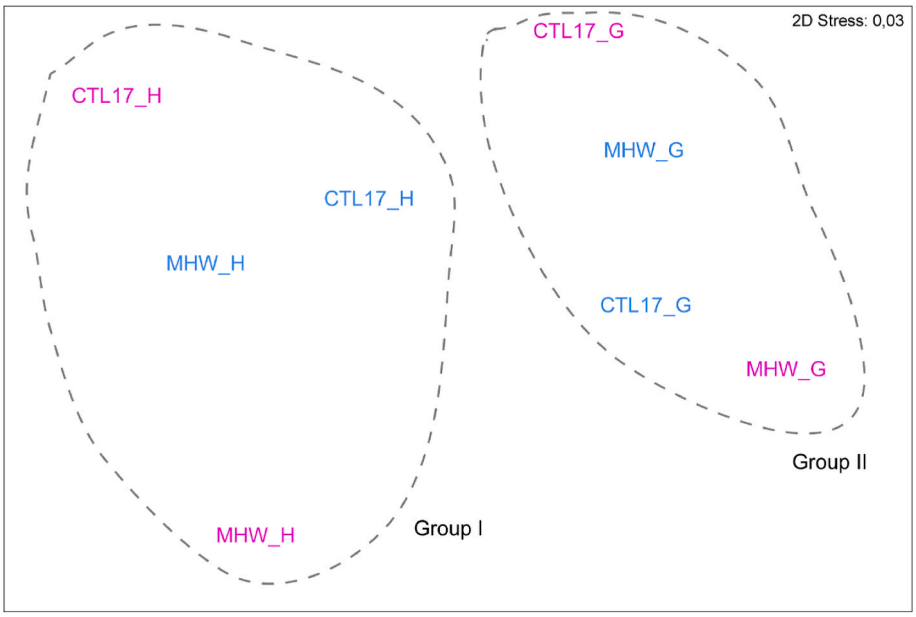

Fig. 6B. Multidimensional scaling (MDS) based on biochemical markers in gills (A) and hepatopancreas (B) of *Carcinus maenas* males (light blue) and females (pink) exposed to control temperature (17 °C) and MHW.

thermal stress has a limited impact on them or that they may be less capable of managing oxidative stress under such conditions. These findings indicate a greater oxidative stress burden in males, which could have potential physiological consequences if their antioxidant defenses are inadequate. Although the studies available did not distinguish males' and females' responses, all reported results identified increased antioxidant capacity in crabs after exposure to increased temperature. Capparelli et al. (2019) reported temperature-dependent antioxidant patterns, with glutathione peroxidase presenting higher activity in the hepatopancreas at 35 °C while decreasing the activity of this enzyme at 15 °C, indicating that oxidative stress defenses were enhanced at higher temperatures but compromised at lower ones. Matozzo et al. (2011) investigated the effects of temperature (4, 17 and 30 °C) on the antioxidant responses of the crab *Carcinus aestuarii*. The authors found that catalase (CAT) activity in the gills significantly decreased at 4 °C, while no significant changes were observed in SOD activity in the gills or digestive gland across temperature conditions. These findings suggest that low temperatures may impair the crabs' ability to manage oxidative stress through reduced CAT activity. The key findings by Rodrigues et al. (2015) study indicated that temperature had a significant impact on *C. maenas* biological responses, with an increase in SOD activity along the increasing temperature gradient (5, 22 and 27 °C). Differences between organs were also reported by Ghedira et al. (2011) in the same species. These authors showed higher CAT activity in the gills of *C. maenas* crabs from a reference site compared to values measured in the hepatopancreas. Dghim et al. (2020) also reported higher CAT activity in the gills of the Portunid crab *Portunus segnis* from polluted areas, compared to the levels observed in the digestive gland.

Alongside the observed sex differences in antioxidant capacity, specifically higher SOD activity in females than in males exposed to the MHW treatment, lipid peroxidation (LPO) levels were detected in the hepatopancreas of females. Nevertheless, no significant differences in LPO levels were observed between treatments (CTL vs MHW) for either sex, regardless of the organ examined. This suggests that the antioxidant mechanisms, and the overall oxidative stress defense mechanisms, in both sexes were sufficient to prevent oxidative damage to lipids during short-term exposure to MHW. The absence of significant LPO implies that crabs can maintain membrane integrity under acute thermal stress, which may be fundamental for preserving cellular function. Although Rodrigues et al. (2013) did not analyze different organs or distinguish between males and females, their study found that LPO levels in *C. maenas* significantly increased only in crabs exposed to 2.6 μg/L of fluoranthene for seven days, while those exposed to higher concentrations exhibited LPO levels close to control values. The authors suggested that this pattern aligns with an upregulation of biotransformation and antioxidant defense mechanisms, which may have helped mitigate oxidative stress at higher exposure levels. Nevertheless, more frequent or longer exposures, resembling actual and predicted scenarios of extreme weather events (namely MHWs) might lead to cellular oxidative damage, in particular in males that demonstrated a limited antioxidant capacity.

The activity of acetylcholinesterase (AChE), an enzyme involved in neurotransmission, revealed significant sex differences. This enzyme activity is a well-established biomarker to monitor environmental pollution caused by neurotoxic compounds, such as organophosphorus and carbamate pesticides. For example, Khedher et al. (2017) reported a reduction of AChE activity over time in *C. maenas* digestive gland exposed to chlorpyrifos-ethyl pesticide, cadmium, copper and the mixture of both metals. In the same species, Ghedira et al. (2009) and Nogueira and Nunes (2021) found a decrease in AChE activity following exposure to chlorpyrifos-ethyl, chlorpyrifos and carbofuran, respectively. In the present study, AChE activity was measured to assess potential neurological effects due to thermal stress. In the gills and hepatopancreas, females had significantly lower AChE activity following MHW exposure. This reduction in females could indicate possible neural impairments induced by heat stress, which may have implications for their overall physiological function under prolonged thermal stress. Furthermore, the present study revealed that, regardless of the organ, at control temperature, females presented higher AChE activity than males. Such findings could indicate a baseline sex-specific difference in cholinergic function.

5. Conclusions

This study provides novel insights into the physiological and biochemical responses of *Carcinus maenas* to marine heatwaves (MHWs), highlighting important sex-specific differences in thermal stress resilience. Males and females exhibited different strategies to cope with thermal stress, with males exhibiting greater thermal resilience by maintaining glycogen reserves, while females exhibited significant

metabolic shifts in response to MHWs. Specifically, females showed a decrease in glycogen concentration, which coincided with an increase in antioxidant enzyme activities (superoxide dismutase and glutathione reductase). The observed increase in antioxidant enzyme activity in females suggests a coordinated metabolic adjustment between the gills and hepatopancreas, where the former plays a frontline role in oxidative stress management while the latter serves as the main site for energy storage and metabolic regulation. The reduction in acetylcholinesterase activity observed in females after MHW exposure suggests potential neurotoxic effects, likely linked to the metabolic demands of coping with thermal stress. This may further impact energy metabolism and disrupt physiological balance across tissues. These findings underscore the importance of considering sex differences when assessing the impacts of climate change-related stressors on marine organisms, as males and females may exhibit distinct vulnerabilities to environmental changes. By integrating multiple biomarkers, including energy metabolism, antioxidant defenses, and neurotoxicity indicators, it offers a comprehensive assessment of the crabs' adaptive mechanisms. However, some limitations should be acknowledged. The short-term exposure does not account for long-term effects, and further research should focus on understanding the long-term consequences of repeated heatwave exposure and its effects on reproductive success and survival in both sexes. A relatively small sample size may impact generalizability, and the study focuses on a single MHW scenario (23 °C), which may not capture the full range of potential responses. Investigating a wider range of temperatures and durations, along with behavioral assessments, would provide a more comprehensive understanding of thermal stress impacts. Integrating additional biomarkers, such as immune responses and heat shock proteins, could further clarify the species' adaptive mechanisms. These efforts will enhance predictions of climate change effects on marine invertebrates, supporting conservation and management strategies.

Despite these limitations, the study provides valuable ecological insights, informing future research on crustacean adaptation to climate change and contributing to conservation and management strategies. The present findings align with the United Nations Sustainable Development Goals (SDGs), particularly SDG 13 (Climate Action) and SDG 14 (Life Below Water). By enhancing our understanding of how climate change affects marine biodiversity, this research supports conservation and sustainable management efforts, helping to protect marine ecosystems from ongoing environmental stressors. The knowledge gained from this study can contribute to the development of strategies aimed at mitigating the effects of MHWs, reinforcing the need for global action to preserve marine life in the face of climate change.

CRediT authorship contribution statement

Federica Arrigo: Writing – original draft, Methodology, Formal analysis, Data curation. **Marta Cunha:** Writing – original draft, Methodology, Data curation. **Hugo C. Vieira:** Methodology. **Amadeu M.V. M. Soares:** Resources, Funding acquisition. **Caterina Faggio:** Writing – review & editing, Supervision. **Ximena González-Pisani:** Writing – original draft, Methodology, Formal analysis. **Laura López Greco:** Writing – review & editing, Methodology, Formal analysis. **Rosa Freitas:** Writing – review & editing, Supervision, Resources, 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.

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Data availability

Data will be made available on request.

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