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Combined effects of temperature and copper on oxygen consumption and antioxidant responses in the mudflat fiddler crab *Minuca rapax* (Brachyura, Ocypodidae)

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ABSTRACT

This study investigates the combined effects of waterborne copper exposure and acute temperature change on oxygen consumption and the oxidative stress biomarkers, glutathione S-transferase (GST) and glutathione peroxidase (GPx), in the gills and hepatopancreas of the fiddler crab *Minuca rapax*. Crabs held at 25 °C were acclimated to 0 (control), 50, 250 or 500 μg Cu L^{-1} for 21 days, and were then subjected to 15, 25 and 35 °C for 24 h. Aerial oxygen consumption rates of crabs in copper free media increased with increasing temperature from 15 to 35 °C, Q_{10} values reaching ≈3. Crabs exposed to increasing copper concentrations exhibited variable responses, Q_{10} values falling to ≈1.5. Copper had no effect on oxygen consumption at 25 °C. However, at 35 °C, rates decreased in a clear concentration-response manner in the copper exposed crabs, revealing impaired aerobic capability. At 15 °C, oxygen consumption rates increased with copper concentration, except for a decrease at 500 μg Cu L^{-1}. Gill GST activity was ≈2-fold that of the hepatopancreas, while hepatopancreas GPx activity was 3-fold that of the gills. Gill GST activities were reduced by copper exposure only at 25 °C while hepatopancreas GST activities were altered by copper at all temperatures. Hepatopancreas GST and GPx activities increased in crabs exposed to copper at 35 °C, revealing oxidative stress induction. Hepatopancreas GST and GPx activities were reduced in copper exposed crabs at 15 °C, suggesting a diminished capability to mitigate the effects of copper exposure at low temperature. These findings reveal that copper exposure increases oxygen consumption at low temperatures but decreases consumption at high temperature. Hepatopancreas GPx activities decreased at low temperature and increased at high temperature. These novel findings demonstrate that the interaction between copper exposure and temperature should be considered when evaluating biomarker activities in semi-terrestrial crabs.

1. Introduction

Copper is an essential micronutrient required by all living organisms for a wide variety of physiological and biochemical processes. This heavy metal is a cofactor in diverse enzymatic processes but is potentially toxic when encountered in high concentrations (Martins et al., 2011). Copper occurs naturally at low levels in many aquatic systems, ranging from < 1–30 μg Cu L^{-1}, depending on the geochemical properties of the particular environment. In areas affected by anthropogenic activities such as industry, agriculture and ports, copper is found at higher levels, reaching as high as 200 mg Cu L^{-1} in heavily impacted areas (Bidone, 2001; USEPA, 2007).

In crustaceans, copper is a natural component of hemocyanin, the respiratory pigment used in oxygen transport (Rainer and Brouwer, 1993), and also plays a role in the molting cycle and in the synthesis of metallothioneins (Engel and Brouwer, 1989). The acute toxic action of waterborne copper primarily affects the gill epithelium, leading to cytological damage, although cellular metabolism can be impaired (Nonnotte et al., 1993). In aquatic species, essential gill functions like gas exchange are severely affected causing a marked decrease in oxygen partial pressure in the hemolymph (Solan and Whiteley, 2016; Vernberg and Vernberg, 1974).

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Metal-induced stress may limit metabolic energy demand at elevated temperatures, which has important implications for crustaceans inhabiting polluted estuaries. In general, metal toxicity and oxygen consumption rates increase at elevated temperatures (Holmstrup et al., 2010; Depledge, 1984). Various mechanisms are involved in oxidative stress induction resulting from fluctuations in ambient temperature, and all metabolic processes are stimulated by increased temperature, leading to oxidative stress. High temperatures tend to increase diffusion rate and alter cell membrane permeability, accelerating biochemical reactions and the rates of subsequent processes that may become uncoordinated, resulting in metabolic imbalance (Reynolds and Casterlin, 1980; Freire et al., 2011; Spicer and Weber, 1992). Elevated temperature also affects ionic regulation and exacerbates copper toxicity (Lemus and Chung, 1999). Changes in membrane permeability and increased ventilation frequency, which may accelerate copper absorption, underlie the increased toxicity of copper at higher temperatures (Gaetke et al., 2014; Sappal et al., 2014). Similarly, reduced temperatures also may induce oxidative stress, weakening the degradation of reactive oxygen species, and/or enhancing their production (Holmstrup et al., 2010).

Oxidative stress is an important component of stress responses in invertebrates exposed to environmental stressors at different temporal and spatial scales (Freire et al., 2011). Temperature variation alone is responsible for oxidative stress induction, and increased temperature stimulates all metabolic processes, increasing oxygen consumption and energy demand, triggering the production of reactive oxygen species *via* secondary metabolic processes (Lushchak, 2011). Sub-optimal water temperatures (below 12 °C or above 35 °C, Vernberg and Vernberg, 1967) and unsuitable salinities (over 60‰ S, Thurman et al., 2013) may interact in an antagonistic, additive or synergistic manner with toxicants, leading to altered tolerance capacities in aquatic crustaceans. The toxicity of copper as regards osmoregulation and metabolism also increases synergistically as a function of the interaction among environmental stressors such as salinity and temperature, both of which are key drivers of ecological function in estuaries. Thus, the combined effects of temperature and copper stress constitute an environmentally relevant challenge for populations of intertidal poikilotherms habiting polluted environments (Freire et al., 2011).

Fiddler crabs are remarkably tolerant of natural variations in salinity and temperature (Baldwin and Kirschner, 1976, Graszynski and Bigalke, 1986, Zanders and Rojas, 1996; Faria et al., 2017) but are sensitive to pollution-induced stress (Zanders and Rojas, 1996; Capparelli et al., 2016; Capparelli et al., 2019). The mudflat fiddler crab *Minuca rapax* (Crustacea, Ocypodidae) is distributed from Florida (USA) to southern Brazil, including the Gulf of Mexico, the Antilles and Venezuela, and from Pará to Santa Catarina state in Brazil (Thurman et al., 2013). This crab is a semi-terrestrial estuarine species associated with mangroves that lives in direct contact with the sediment into which it burrows. It is a scavenger and feeds mainly on microscopic algae and protozoa found on sand grains, and on organic matter brought in on the high tide (Crane, 1975). *Minuca rapax* encounters ample daily and seasonal variations in salinity (Thurman, 2003; Faria et al., 2017) and temperature (Faria et al., 2017) and resists environmental contamination (Capparelli et al., 2016, 2017) for which it is an excellent model species. In the laboratory, *M. rapax* survives high copper concentrations (500 μg Cu L^{-1}, Capparelli et al., 2017) and is present in estuaries highly contaminated by metals and polyaromatic hydrocarbons, such as the Santos Estuarine System (Capparelli et al., 2019), a critically polluted area in southeastern Brazil. Despite the abundance of *M. rapax* in such chronically contaminated regions, and its resistance to high metal concentrations, the crab does show osmoregulatory and metabolic impairment when exposed experimentally to copper (Capparelli et al., 2017) and to metal contamination *in situ* (Capparelli et al., 2016). Fluctuations in precipitation and salinity also influence the crab's antioxidant responses in addition to those caused by contamination *in situ* (Capparelli et al., 2019).

Nevertheless, the combined effects of temperature change and copper exposure remain poorly understood in semi-terrestrial estuarine crustaceans like *M. rapax*. The present study aimed to investigate the interaction between temperature change and waterborne copper exposure on oxygen consumption and the oxidative stress biomarkers, glutathione S-transferase and glutathione peroxidase, in the gills and hepatopancreas of this sentinel species. Comprehending how temperature and copper toxicity influence physiological and biochemical responses is a key factor to consider when using model species to monitor impacted estuarine regions, especially considering scenarios of global temperature change and increasing heavy metal contamination. The present investigation is part of a series of studies designed to better comprehend the effect of environmental contamination, both *in situ* and experimentally, on a key sentinel species from an estuarine ecosystem.

2. Materials and methods

2.1. Crab collection and experimental procedures

Male and female *Minuca rapax* of 10–12 mm carapace width were collected by hand during the austral winter of 2015 from the margins of the Itapanhaú River, Bertioga, São Paulo, (23° 50′ 8.35″ S; 46° 9′ 12.29″ W). The crabs were collected directly from the substrate surface or by digging into their burrows, and were transported to the laboratory in lots of 20 each in plastic boxes (15 × 15 × 6 cm) containing small sponges moistened with water from the collection site (12‰ salinity). Mean yearly air temperature measured at three crab collecting sites in the region was 25.8 °C, ranging from 23 to 29 °C (Capparelli et al., 2019). The mean summer air temperature for the São Paulo coast is 27 °C (SiMCOSTA database, Brazilian Coastal Monitoring System). For this reason, 25 °C was chosen as the reference temperature for these experiments.

Before beginning the experiments, the crabs were acclimated for three days in the laboratory at room temperature (25 °C, air conditioning) in the same boxes in which they were transported, containing water from the collection site. The boxes were inclined slightly to provide access to a dry surface and were cleaned daily when the fiddler crabs were fed fish food pellets, and the water was replaced.

2.2. Copper exposure

The experimental media consisted of isosmotic artificial seawater (25‰S, Faria et al., 2017) prepared from distilled water and Instant Ocean sea salts, containing copper as $CuCl_2$, in concentrations of 0 (control, no copper), 50, 250 or 500 μg Cu L^{-1}. The experimental media were allowed to stand for 8 h before introducing the crabs.

The exposure assays were conducted at 25 °C under a natural 14 h light: 10 h dark photoperiod. Ten, 500-mL plastic vessels with tops were used as test chambers, each containing 50 mL of experimental medium such that the five crabs in each were only partially submerged in a thin layer (3 mm deep) of solution to enable gill wetting. Every 24 h the experimental media were replaced and the crabs were checked for mortality and fed briefly. After 21 days, the crabs were removed from the experimental chambers and used for oxygen consumption measurements (whole crabs) and enzyme assays (gill and hepatopancreas, see below for details). No mortality was registered for any copper concentration during the acclimation period at 25 °C, or during oxygen consumption measurements at the different temperatures.

2.3. Measurement of oxygen consumption rate

Ten crabs from each experimental group were weighed (Gehaka BG 400 balance, 1.0 mg precision), placed individually into acrylic respirometer chambers (153 mL in volume, 4.0 cm internal height and 7.0 cm internal diameter) and held during a 24-h adjustment period at one of three temperatures: 15, 25 (reference temperature) or 35 °C, in a

constant temperature chamber (Fanem BOD incubator, ± 0.5 °C precision) under a 14 h light: 10 h dark photoperiod. The respirometers were coupled by Crystal plastic tubing to a multiplexer manometer and a gas analyzer (Sable Systems, Las Vegas, NV), and were perfused with a constant flow of air from a small aquarium pump. Each respirometer contained a thin layer (3 mm deep) of the respective experimental medium to allow gill wetting.

Oxygen consumption was measured employing an intermittent respirometric technique (Steffensen, 1989). After the 24-h adjustment period, each respirometer was perfused with air at a standard rate of flow (180 mL min^{-1}) *via* the multiplexer manometer leading to the O_2 sensor and pump in the gas analyzer, establishing the baseline percentage of atmospheric O_2 (20.8%). The respirometers containing the individual crabs were then sealed by closing the respective manometer stopcocks and remained without airflow for 50 min. Airflow was then resumed and the output from each respirometer pumped to the O_2 sensor that monitored the respective percentage of atmospheric O_2 present in the air stream. The decrease in this percentage corresponds to the amount of O_2 consumed by each crab in each respirometer during the sealed phase, compared to the baseline reading.

Two consecutive measurements were performed, one immediately after the other, for each crab at each temperature, the lowest value being used to calculate the oxygen consumption rate, as this is most indicative of the standard metabolic rate. The data were collected using the Sable Systems Expedata application that enables calculation of the percentage decrease in oxygen concentration in the air flushed through each respirometer.

Mass specific oxygen consumption rate was calculated from the formula:

$$\text{Oxygen consumption rate (mL } O_2 \cdot g^{-1} \cdot h^{-1}) = \text{IA} \times \text{flow rate (mL min}^{-1}) / [100 \times \text{dry mass (g)} \times \text{duration of sealed phase (50 min)}] \times 60 \text{ min}$$

where IA is the integral of the signal (%O_2) received from the respirometer provided by the Expedata application; flow rate is the flux of air (180 mL min^{-1}) through the respirometer during the open phase; dry mass is the mass (g) of each crab after standard drying for 24 h at 60 °C; duration of the sealed phase is the length of time during which each respirometer remained sealed (50 min). The factor '100' in the denominator converts the percentage O_2 values obtained from the IA values to volume of O_2. The factor '60' transforms the value obtained to an hourly rate.

2.4. Temperature coefficient (Q_{10})

The temperature coefficient is a measure of the rate of variation in chemical or biological systems as a consequence of a 10 °C increase in temperature (Hoar, 1966). Q_{10} values were calculated by applying the equation:

$$Q_{10} = (R_2/R_1)\text{^}(10/T_2 - T_1)$$

where R_1 and R_2 are the rates of mass specific oxygen consumption at temperatures T_1 and T_2 in degrees Celsius where $T_2 > T_1$. Q_{10} was calculated using the mean mass-specific oxygen consumption rate of the 10 crabs at each temperature. The Q_{10} values were calculated for the range 15–25 °C and the range 25–35 °C.

After the final reading, the crabs were removed from the respirometers, anesthetized in crushed ice and killed by freezing at −20 °C for 10 min. They were then transferred to a drying oven (MA 033, Marconi) at 60 °C for 24 h and allowed to cool quickly in a desiccator for subsequent calculation of their dry weights.

2.5. Biochemical biomarkers

Ten different crabs were likewise acclimated to each experimental medium for 21 days at concentrations of 0 (control), 50, 250 or 500 μg Cu L^{-1} as above. They were then were held for a 24-h adjustment period at one of three temperatures: 15, 25 (reference) or 35 °C, in a constant temperature chamber (Fanem BOD incubator, ± 0.5 °C precision) under a 14 h light: 10 h dark photoperiod. To obtain gill and hepatopancreas samples for enzyme and protein assays, individual crabs were cryoanesthetized by cooling on ice for 10 min and then dissected. The anterior and posterior gills and the hepatopancreas were removed, weighed and immediately frozen at −80 °C until the analyses were performed. The homogenates were prepared from tissues pooled from 2 to 3 crabs each.

For the assays, the tissues were gradually thawed on ice, homogenized in ice-cold potassium phosphate buffer (0.1 M, pH 7.2, 1:5 [*w/v*]) and centrifuged at 4 °C (10,000 rpm) for 30 min. The supernatants were divided into three aliquots, according to the analyses conducted (glutathione S-transferase, glutathione peroxidase, total protein). All analyses were performed within three weeks of tissue homogenization and storage in samples that were thawed only once, using a microplate reader (Biotek-Synergy HT).

Glutathione S-transferase (GST) activity was measured using a procedure modified from Keen et al. (1976). The reaction mixture consisted of 1.5 mM glutathione (GSH) and 2.0 mM 1-chloro-2,4-dinitrobenzene in 0.1 M potassium phosphate buffer (pH 6.5). Increases in absorbance were measured at 340 nm at 50-second intervals, and enzyme activity was calculated using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Glutathione peroxidase (GPx) activity was measured using an adaptation of the method developed by Sies and Masumoto (1996). The reaction medium consisted of 0.1 M sodium phosphate buffer (pH 7.0), 3.08 mM sodium azide, 0.308 mM NADPH, 3.08 mM GSH and 1.54 U mL^{-1} glutathione reductase to which a hydrogen peroxide solution was added (5 mM hydrogen peroxide in 0.1 M sodium phosphate buffer at pH 7.0). Decreases in absorbance were measured immediately at 340 nm for 2 min, at 50-second intervals. GPx activity was estimated using the molar extinction coefficient for NADPH (6.22 mM^{-1} cm^{-1}).

Protein concentration was measured spectrophotometrically at 595 nm (Bradford, 1976), using bovine serum albumin as the standard.

2.6. Statistical analyses

All data are expressed as the mean ± SEM (N). After satisfying criteria for normality of distribution and homogeneity of variance, the data sets (oxygen consumption, enzyme activities) were analyzed using 2-way (temperature and copper concentration) or 3-way analyses of variance (temperature, copper concentration and tissue). Differences between means within a given parameter were established using the Student–Newman–Keuls (SNK) *post hoc* multiple comparisons procedure. A minimum significance level of $P = 0.05$ was employed for all procedures.

3. Results

3.1. Oxygen consumption rate

Temperature, copper concentration and their interaction all affected mass specific oxygen consumption rate (2-way ANOVA, $P < 0.05$). Oxygen consumption increased with temperature, while the effects of copper concentration varied. Rates increased markedly at 35 °C and decreased notably at 15 °C compared to crabs at 25 °C (Fig. 1). Copper had no effect on oxygen consumption rate at 25 °C. However, at 35 °C rates decreased significantly by ≈40% in crabs exposed to high [Cu] (500 μg Cu L^{-1}). At 15 °C, consumption rates increased in crabs exposed to 50 and 250 μg Cu L^{-1}, but decreased in 500 μg Cu L^{-1} compared to unexposed control crabs (Fig. 1).

Q_{10} values, reflecting metabolic sensitivity, were lower in the copper exposed crabs (≈1.5) for both temperature intervals compared

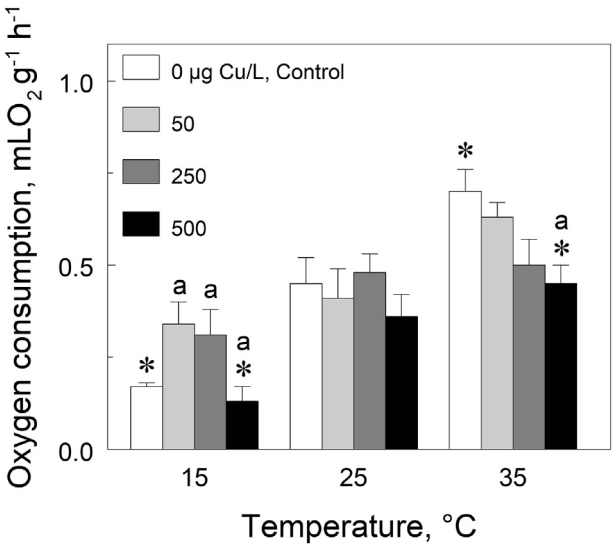

Fig. 1. Oxygen consumption rates (mL O_2·g dry weight^{-1}·h^{-1}) of *Minuca rapax* exposed to different waterborne copper concentrations (0 [control], 150, 250 or 500 µgCu L^{-1}) for 21 days at 25‰S, and to different temperatures (15, 25 [reference] or 35 °C) for 24 h. Data are the mean ± SEM ($N = 10$). *Significantly different from crabs at 25 °C in the same [Cu] ($P \leq 0.05$). [a]Significantly different from control crabs (0 µg Cu L^{-1}) at the same temperature ($P \leq 0.05$).

Table 1
Temperature coefficients (Q_{10}) for *Minuca rapax* exposed for 21 days to increasing concentrations of waterborne copper (0 [control], and 150, 250 or 500 µg Cu L^{-1}), and different temperatures (15, 25 [reference] or 35 °C) for 24 h.

[$CuCl_2$] (µg Cu L^{-1})	0	50	250	500
Temperature range (°C)				
15–25	2.9	1.2	1.6	2.0
25–35	2.8	1.5	1.2	1.2

to unexposed controls (≈3.0) (Table 1).

3.2. Biochemical biomarkers

Gill glutathione S-transferase activity was affected by both temperature and copper concentration with an interaction effect for tissue (3-way ANOVA, $P < 0.05$), activity being significantly higher in the gills than in the hepatopancreas. GST activity decreased at both 15 and 35 °C compared to crabs at 25 °C, and also decreased with increasing [Cu] only in crabs at 25 °C (Fig. 2A). Copper had no effect on gill GST activity at 15 and 35 °C. In contrast, hepatopancreas GST activity increased at both 15 and 35 °C compared to crabs at 25 °C (Fig. 2B). Hepatopancreas GST activity also increased markedly with increasing [Cu] at 25 and 35 °C, but decreased notably at 15 °C (Fig. 2B).

Gill glutathione peroxidase activity was affected by temperature and copper concentration with an interaction effect for tissue (3-way ANOVA, $P < 0.05$), activity being significantly higher in the hepatopancreas than in the gills. GPx activity decreased at both 15 and 35 °C compared to crabs at 25 °C (Fig. 3A). GPx activity decreased in crabs exposed to 500 µg Cu L^{-1} at 15 °C, but increased in crabs at 25 and 35 °C. Hepatopancreas GPx activity also decreased at 15 and 35 °C compared to crabs at 25 °C (Fig. 3B). At 15 and 25 °C, hepatopancreas GPx activity decreased in 500 µg Cu L^{-1}. At 35 °C, activity increased in all [Cu].

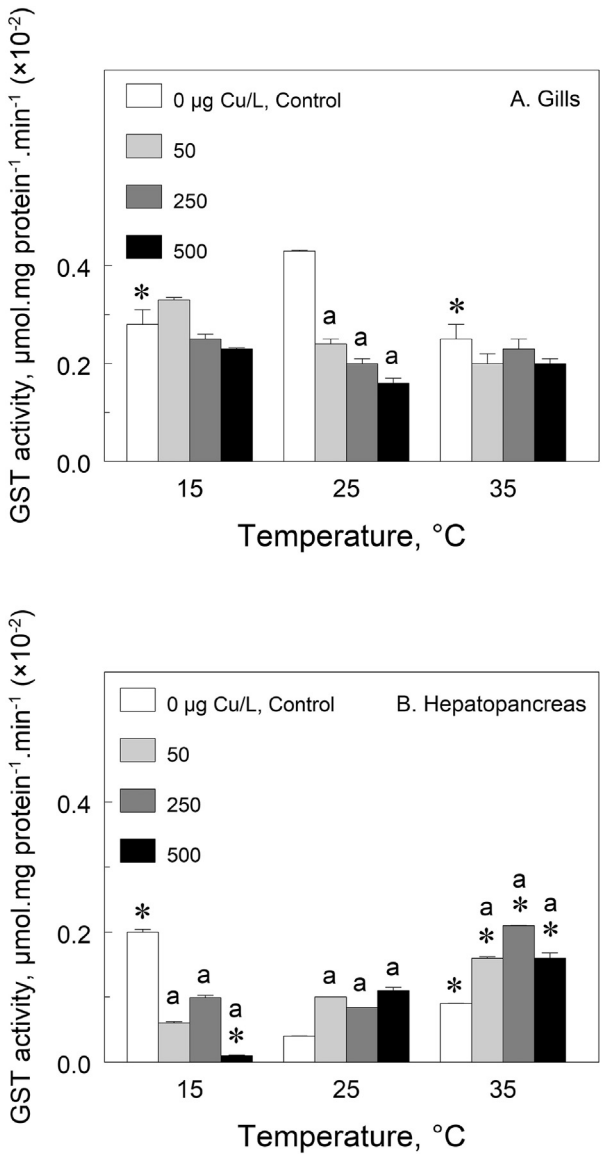

Fig. 2. Glutathione S-transferase (GST) activity in homogenates of gills (A) and hepatopancreas (B) from *Minuca rapax* exposed to increasing copper concentrations (0 [control], 50, 250 or 500 µg Cu L^{-1}) for 21 days at 25‰S, and subsequently for 24 h at different temperatures (15, 25 [reference] or 35 °C). Data are the mean ± SEM ($N = 7–10$). *Significantly different from crabs at 25 °C and same [Cu] ($P \leq 0.05$). [a]Significantly different from control crabs (0 µg Cu L^{-1}) at the same temperature ($P \leq 0.05$).

4. Discussion

Understanding how different species respond physiologically to environmental contamination within and at their thermal limits is an important aspect of comparative ecological toxicology. Investigations of the thermal physiology of the Gelasiminae (formerly *Uca*, Shih et al., 2016) have revealed that these crabs show high tolerance of high temperatures. Their critical thermal limits range from approximately 12 to 35 °C in temperate species (Vernberg and Tashian, 1959; Vernberg and Costlow, 1966) and from approximately 20 to 41 °C in tropical and subtropical species (Darnell et al., 2015; Allen et al., 2012; Munguia et al., 2017; Faria et al., 2017). Thus, the lowest and highest temperatures to which *M. rapax*, a subtropical species, was exposed in the present study (15 and 35 °C) lie close to the critical thermal limits of survival for the species.

Minuca rapax exhibits a metabolic plateau between 20 and 25 °C,

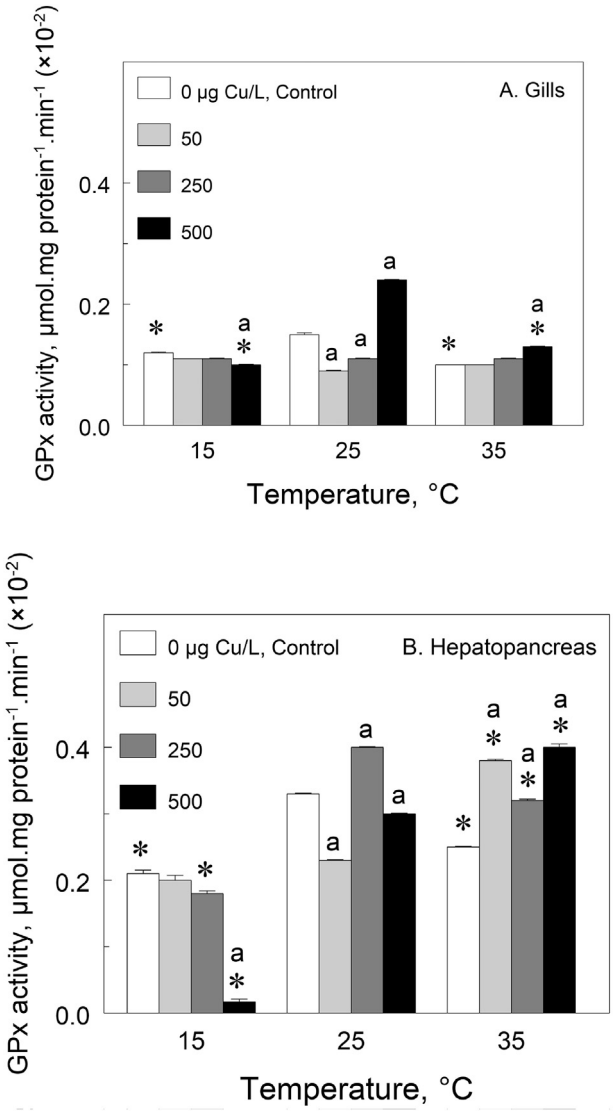

Fig. 3. Glutathione peroxidase (GPx) activity in homogenates of gills (A) and hepatopancreas (B) of *Minuca rapax* exposed to increasing copper concentrations (0 [control] and 50, 250 or 500 µg Cu L^{-1}) for 21 days at 25‰S, and subsequently for 24 h at different temperatures (15, 25 [reference] or 35 °C). Data are the mean ± SEM (N = 7–10). *Significantly different from crabs at 25 °C and same [Cu] ($P \leq 0.05$). [a]Significantly different from control crabs (0 µg Cu L^{-1}) at the same temperature ($P \leq 0.05$).

with oxygen consumption increasing above 30 °C (Vernberg and Tashian, 1959; Vernberg and Vernberg, 1967). Our findings reveal a similar pattern for this crab at 25 and 35 °C. However, our data for *M. rapax* exposed to high temperature and elevated copper (35 °C and 500 µg Cu L^{-1}), show smaller increases in oxygen consumption rates, which exhibit an inhibitory dose response relationship, reflected in their reduced Q_{10} values (≈1.5), corroborating findings on metal toxicity at elevated temperatures (Helmuth et al., 2002; Vernberg and Vernberg, 1972). This metal-induced decrease in oxygen consumption rate suggests that processes other than structural damage to the gill epithelia may be the underlying cause, i.e., decreased ventilation, decreased gas exchange at the lamellar surface, decreased gas transport to the tissues, and direct inhibition by copper of cellular respiration (Spicer and Weber, 1991; Nonnotte et al., 1993). Exposure to a sublethal copper concentration affects the activity of enzymes involved in glycolysis and in the Krebs cycle, especially in the anterior respiratory gills of hyper-osmoregulating crabs like *M. rapax* (Lauer et al., 2012). Nevertheless, as defense mechanisms against metal contamination are costly from an energetic point of view in addition inducing gill damage (Leung et al., 2000), it is unlikely that higher metabolic rates can be maintained. *Minuca rapax* exposed to copper thus loses its aerobic capacity to respond to temperature increase compared to unexposed crabs at 35 °C. Oxygen consumption is likewise impaired in *M. rapax* chronically contaminated *in situ* when subjected to high temperature (Capparelli et al., 2016), corroborating our current findings on crabs exposed experimentally to copper in the laboratory.

At temperatures below 25 °C, oxygen consumption decreases in *M. rapax* (Vernberg and Tashian, 1959) as also seen here. Copper had no effect at 25 °C. However at 15 °C, oxygen consumption increased in crabs exposed to 50 and 250 µg Cu L^{-1}, and decreased at 500 µg Cu L^{-1}. Increased consumption at intermediate copper concentrations may reflect a compensatory mechanism commonly seen at low metal concentrations, i.e., the hormesis phenomenon, where low concentrations stimulate a process while high concentrations inhibit it (Calabrese and Baldwin, 1997). These findings suggest that the effects of copper on oxygen consumption are exacerbated at higher experimental temperatures. The potential impact of metal contamination on energy demand by *M. rapax* both *in situ* (Capparelli et al., 2016, 2019) and when exposed experimentally to copper (present study and Capparelli et al., 2017), depends on the crab's metabolic capability to adjust to temperature challenge in addition to contamination by copper.

Antioxidant defense systems may be significantly induced under challenge from metals or temperature stress, and a relationship between thermal stress responses and oxidative stress responses has been established (Madeira et al., 2013). The increased anti-oxidant capacity of crab tissues at high temperatures may counteract xenobiotics, reactive oxygen species (ROS) induced by chemical and environmental stressors, and oxidative stress levels (Paital and Chainy, 2013, 2010). Critical temperatures evidently influence metabolic rates, which alone frequently produce oxidative stress. Thus, the activation of anti-oxidant defenses is an essential constituent of stress responses to oxidative stress.

It is consensual that changes in GPx activity can result from factors such as metabolic responses to environmental changes (e. g., temperature and salinity) or exposure to contaminants (Freire et al., 2011; Paital and Chainy, 2013; Mieiro et al., 2009). GPx catalyzes the oxidation of reduced glutathione (GSH) to GSSG in the process of hydrogen peroxide reduction. In addition, GPx plays an important role in protecting cellular membranes from damage due to lipoperoxidation, owing to its function in terminating radical chain propagation by rapid reduction to yield further radicals (Adams et al., 1983). GST, in turn, in addition to its detoxifying function in the metabolism of organic xenobiotics (Freire et al., 2011) is important in the ROS scavenging process (Liu et al., 2014), being frequently used as a biochemical biomarker of exposure to metal pollution (Walters et al., 2016; Mieiro et al., 2009; Macfarlane et al., 2006).

The activities of the antioxidant enzymes GST and GPx in the hepatopancreas of *M. rapax* exposed to copper show a direct relationship with temperature. At 15 °C these activities are inhibited and at 35 °C are increased for nearly all copper concentrations. The crustacean hepatopancreas is considered to be the main site of toxicant metabolism and the biotransformation of ROS (Livingstone, 1998). At the same copper concentrations, GPx activities in the hepatopancreas were higher than in the gills at all temperatures, suggesting that the hepatopancreas is likely a more sensitive tissue (Linhua et al., 2009). Although the posterior gills also detoxify metals (Masui et al., 2002), the activity of the enzymes evaluated here is induced mainly in the hepatopancreas.

Hepatopancreas GST and GPx activities were particularly elevated in *M. rapax* exposed to copper at 35 °C. Since higher enzyme activity implies augmented detoxification capacity (Pinho et al., 2005), this finding indicates induction of oxidative stress owing to the combined effects of copper and high temperature, likely due to enhanced ROS production as a consequence of intensified aerobic metabolism at high

temperature (Rajagopal et al., 2005). Copper exposure potentiates this effect to a point at which the crabs are unable to increase oxygen consumption. Further, copper is a cofactor for GST and GPx enzyme activities, and can bind to reduced glutathione (GSH), decreasing the amount of substrate for GST and GPx, further reducing their activities (Elumalai et al., 2002; Martín-Díaz et al., 2007, 2008). This may explain the reduced activities seen at 15 °C in the high copper concentration (500 μg Cu L^{-1}). In contrast, increased activity may result from the synthesis of more enzyme in response to stress caused by exposure to elevated temperature and copper.

At 25 °C, a temperature corresponding to a metabolic plateau (Vernberg and Tashian, 1959), oxygen consumption by *M. rapax* was not affected by copper exposure. However at this temperature, GST and GPx activities in the gills were generally more responsive than at 15 and 35 °C. GST activity was inhibited in the gills and stimulated in the hepatopancreas, while GPx activity was inhibited at lower copper concentrations in the gills and inhibited or stimulated in the hepatopancreas. The effect of copper exposure and temperature on GPx and GST activity in the gills was more evident at 25 °C; in the hepatopancreas the effect was clearly seen at all temperatures. Apparently, in the hepatopancreas, copper exposure causes enzyme inhibition at low temperature (15 °C) but activation at higher temperatures. Since the solubility of oxygen is higher at lower temperatures, the responses of some antioxidant system pathways will correlate inversely with temperature (Vinagre et al., 2016). Our findings suggest that GST and GPx responses are generally greatest at high temperature and copper concentrations, and lowest under highest stress conditions (for example, 50 to 500 μg Cu L^{-1} at 15 °C). High temperatures (25 and 35 °C) seem to increase enzyme activities synergistically with copper exposure, possibly underlying the decreased oxygen consumption seen at 35 °C.

Minuca rapax is abundant in areas chronically affected by metal contamination, and survives high copper concentrations on laboratory exposure. We conclude that exposure to copper decreases the aerobic regulatory capability of *M. rapax* at high temperatures and, in contrast, decreases aerobic regulatory capability at low temperatures, likely related to the inhibition of GST and GPX activities at low temperatures and activation at high temperature particularly in the hepatopancreas. The gills apparently are more stimulated at 25 °C and in the highest copper concentrations. This suggests elevated copper toxicity at both low and high temperatures. These findings are relevant to global climate change scenarios, since under long-term temperature increase, *M. rapax* may be unable to respond to contamination-induced stress.

Compliance with ethical standards

Declaration of Competing Interest

All authors declare that they have no conflict of interest with any governmental agency or commercial entity.

Ethical approval

Specimens of *Minuca rapax* were collected under permit #29594–1/2013 issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis to JCM. All applicable international, national, and institutional guidelines for the care and use of animals in research were followed during the undertaking of this investigation.

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