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Seasonal environmental parameters influence biochemical responses of the fiddler crab *Minuca rapax* to contamination *in situ*

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ABSTRACT

The mudflat fiddler crab *Minuca rapax*, typical of mangroves and intertidal zones in the Western Atlantic Ocean, responds to fluctuations in environmental parameters by biochemical and physiological adjustments. Such biochemical effects are commonly employed in environmental studies as biomarkers of estuarine contamination. This study evaluates biochemical responses in the gills and hepatopancreas of *M. rapax in situ* from localities exhibiting different types and levels of contamination, against a backdrop of fluctuations in environmental parameters like salinity and temperature common to estuarine regions. The biochemical biomarkers metallothionein (MT)-like protein titers and glutathione S-transferase (GST), glutathione peroxidase (GPx) and acetylcholinesterase (AChE) activities were used to evaluate responses to environmental contamination and seasonal changes in environmental parameters. Crabs were collected during two seasons, the austral winter and summer, at three sites along the coast of the state of São Paulo, Brazil that present decreasing degrees of environmental contamination: Ilha Diana, Santos (ID) > Rio Itapanhaú, Bertioga (RI) > Picinguaba, Ubatuba (P), a pristine control site. Our findings show that MT were induced in crabs from the contaminated sites (ID and RI) mainly during winter, revealing the activation of detoxification mechanisms; however MT were also induced in P crabs during the summer rainy season. GPX, GST and AChE activities were altered in P crabs during summer and in ID and RI crabs in winter. While enzyme activities in summer crabs may reflect seasonal changes in precipitation and salinity, in winter these altered activities appear to reflect contamination, although an effect of environmental parameters cannot be excluded. These findings reveal a strong seasonal influence on biochemical biomarker responses in *Minuca rapax*, a relevant factor to consider when interpreting the impact of environmental contamination in estuaries.

1. Introduction

Estuarine organisms are often exposed to a wide variety of chemical compounds, released in ever increasing amounts into the environment, owing to the continuous expansion of human activities (Kennish, 2002). The uptake of contaminants in target tissues takes place from sediments, suspended particulate matter, the water column, or *via* the diet, depending on the trophic level and ecological niche of the exposed organism (van der Oost et al., 2003).

Metals and organic xenobiotics are environmentally relevant classes of pro-oxidant chemicals that exhibit different pathways of oxidative challenge at the biochemical level. Various mechanisms of detoxification may exist for a single compound, determining a very complex network of oxidative interactions and cascade effects (Regoli et al., 2011). The physiological challenges confronted by aquatic species as a result of such contamination are considerable, and even more so for estuarine inhabitants. These species often already exhibit mechanisms of physiological homeostasis as a consequence of the challenges arising from natural physico-chemical variations in ambient parameters like salinity, temperature, oxygen availability, pH, turbidity and, frequently, from contaminant input (Romano and Zeng, 2010).

Regardless of the degree of contamination, exposure to ambient challenge often leads to oxidative stress and other kinds of cellular damage, which require cells to redirect their resources towards maintenance and mitigation (Sokolova et al., 2012). The presence of a single chemical species or of complex mixtures of environmental

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contaminants may impair an organism's antioxidant defense system and other metabolic pathways, producing oxidative stress, protein oxidation, lipid peroxidation and alterations in the activities of anti-oxidant enzymes or in potential non-specific biomarkers that respond similarly to various stressors (Bainy et al., 1996; Geracitano et al., 2004; Gusso-Choueri et al., 2015). These biochemical responses to contaminants are widely used as biomarkers, which provide an indirect measure of the health of a habitat (Giguere et al., 2003). To illustrate, the activation of antioxidant responses can be evaluated employing a set of enzymes such as glutathione peroxidase (GPx), catalase (Cat), glutathione reductase (GR) and peroxiredoxins (PrX), while the conjugation or depuration process is routinely measured using glutathione S-transferase (GST) activity. Acetylcholinesterase (AChE) activity provides evidence of neurotoxicity, while increased total metallothionein or metallothionein-like protein (MT) titers indicates exposure to metals.

In aquatic and semi-terrestrial crustaceans, the gills act mostly as a transient tissue store for contaminants accumulated during waterborne exposure to xenobiotics (Soegianto et al., 1999a, 1999b). The gills are also the main site through which many toxic xenobiotics are taken up by aquatic crustaceans, and thus they play an important role in the toxicology of these species (Henry et al., 2003). Waterborne toxic metals accumulated *via* the gills impair numerous biochemical and physiological functions in aquatic crustaceans. Such toxic xenobiotics interact with the gills by adsorption to the cuticular surface, and after crossing the epithelium, are transported *via* the hemolymph to the hepatopancreas (Brouwer and Lee, 2007); in crustaceans the hepatopancreas accumulates higher xenobiotic titers than do the gills (Martin-Diaz et al., 2008; Capparelli et al., 2016). The hepatopancreas tissues are effectively involved in a variety of physiological processes and play an important role in the detoxification and storage of contaminants (Viarengo, 1989; Rainbow, 1997a, 1997b).

Toxic metals are detoxified through their binding to metallothionein-like proteins, by accumulation in intracellular vacuolar granules, or through the formation of extracellular granules. Increased MT titers are found in response to elevated metal concentrations (Hogstrand and Haux, 1991; Mouneyrac et al., 2002). The positive correlation between tissue MT titers and environmental concentrations of toxic metals suggests that MTs are useful biomarkers of environmental contamination. In contrast, semi-terrestrial species, which spend much of their life in contact with sediment, may exhibit other mechanisms for detoxifying metal contaminants, related to this particular route of exposure.

The mudflat fiddler crab *Minuca rapax* is a semi-terrestrial estuarine species that lives in direct contact with the sediment (Crane, 1967). Fiddler crabs pick up sediment and use their mouthparts to separate edible detritus and algae from the mineral material. Their intense and extensive burrowing and feeding activities constitute part of the bioturbation process (Bertness, 1985). *Minuca rapax* is abundant in both pristine and chronically contaminated areas along the Atlantic coast of Brazil (Masunari, 2006; Capparelli et al., 2016). The crabs encounter ample daily and seasonal variations in environmental parameters such as salinity (Thurman, 2003;Thurman et al., 2017) and temperature (Faria et al., 2017a, 2017b) and are exposed to environmental contamination (Capparelli et al., 2016, 2017). Thus, they constitute an attractive model in which to examine the biochemical mechanisms available to mitigate these different factors *in situ,* particularly because the crabs build burrows and live in direct contact with contaminated sediment from which they feed on organic matter adhering to the sediment particles, absorbing contaminants *via* the diet. Being territorial and lacking extensive mobility, they can be exposed chronically to environmental contamination.

To better comprehend the biochemical mechanisms underpinning chronic exposure to contamination *in situ*, this study evaluates how *Minuca rapax*, a species inhabiting localities showing different levels of environmental contamination along the coast of São Paulo State, responds to exposure to pollution *in situ*. Further, *M. rapax* may incorporate and detoxify such contaminants differently from fully aquatic crustacean model species, since it is a semi-terrestrial crab. Just how fiddler crabs deal with environmental contaminants is very poorly known. To this end, we analyzed seasonal variation in the biochemical biomarkers MT, GST, GPx and AChE in the gills and hepatopancreas of crabs from three differentially contaminated sites. Unraveling how seasonal parameters influence biochemical responses is a key factor to consider when using model species as sentinels for monitoring impacted estuarine regions.

2. Materials and methods

2.1. The test-species, Minuca rapax

The species used as a model in this study is the mudflat fiddler crab *Minuca rapax* (Brachyura, Ocypodidae), a semi-terrestrial estuarine decapod (Crane, 1975). *Minuca rapax* is distributed from Florida, throughout the Gulf of Mexico, the Antilles and Venezuela to Brazil where it ranges from Pará to Santa Catarina states (Thurman et al., 2013). One hundred adult specimens of both male and female *M. rapax* were collected by hand, during low tide, during the austral winter of 2012 (June–July) and summer of 2013 (January–February) from three study sites located on the southern Atlantic coast of the State of São

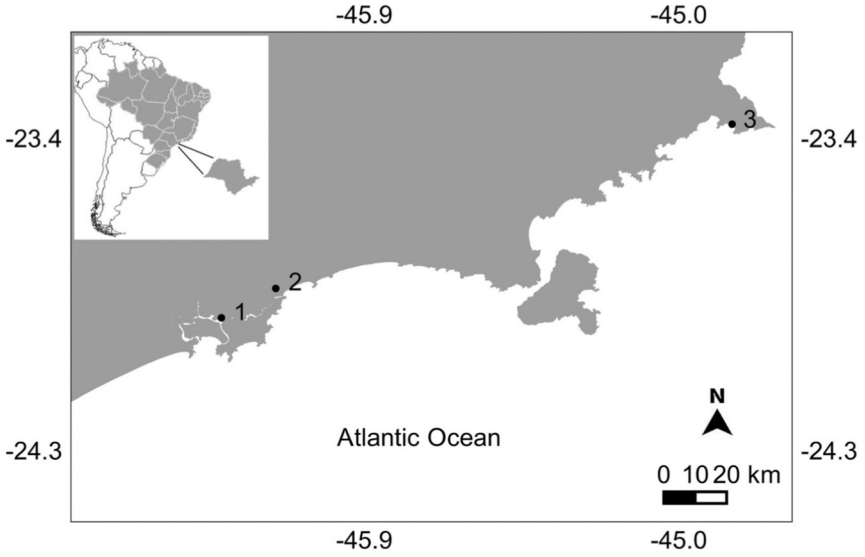


Fig. 1. Location map showing the collection sites for the mudflat fiddler crab *Minuca rapax* along the northern coast of the State of São Paulo, Brazil. 1, Ilha Diana; 2, Rio Itapanhaú, both metal contaminated sites within the Santos Estuarine System; 3, Picinguaba, a pristine control area in the Serra do Mar State Park.
Figure adapted from Capparelli et al. (2016).

Table 1
Compilation of literature data available for overall contamination levels at the three study sites. Ilha Diana and Rio Itapanhaú are both metal contaminated sites located within the Santos Estuarine System. Picinguaba is a pristine control area in the Serra do Mar State Park.

Site	Contaminant	Reference
Ilha Diana	Zn, Ni, Cd > TEL; Pb, Hg and ΣPAHs > PEL	Capparelli et al., 2016 Abessa et al., 2018 Lamparelli et al., 2001 Quináglia, 2006
	Pb > TEL and SQG = high, acenaphthene and acenaphthylene > TEL	Perina et al., 2018
	Cr, Cu, Ni, Zn > EF 4	Kim et al., 2016
	Hg > TEL	Bordon et al., 2011
	Hg > SQG1, SQV1; Cr > SQV1; Cu, Ni > SQV1; Zn > SQG2, SGV2	Buruaem et al., 2012
	Zn > SQG2; PAH > TEL	Buruaem et al., 2013 Quináglia, 2006
Rio Itapanhaú	As, Cr, Ni > EF 4	Kim et al., 2016 Quináglia, 2006
	Pb > TEL	Tramonte et al., 2016 Capparelli et al., 2016
Picingaba	No contamination	Capparelli et al., 2016 CETESB, 2013

TEL, threshold effect level; PEL, probable effect level; SQG1, Brazilian sediment quality guideline level 1; SQG2, Brazilian sediment quality guideline level 2; SQV1, local sediment quality level 1; SQV2, local sediment quality level 2; EF, enrichment factor.

Paulo, Brazil (Fig. 1).

The crabs were collected individually, removing them from their burrows, and were transported to the laboratory in large plastic boxes containing 1–2 cm of water and a thin layer of sediment from the respective collection sites. The boxes were maintained on a slightly inclined plane so that the crabs had free access to a dry surface. Crabs were not fed before or during experiments. Crabs were processed to obtain tissues for posterior analysis of biochemical biomarkers immediately on arrival at the laboratory.

2.2. Site locations and contamination characteristics

Table 1 provides a compilation of overall contamination data available in the literature for the three study areas located along the northern coast of São Paulo state.

1. Ilha Diana, Santos (ID) (23° 55′ 4.5” S; 46° 18′ 31.5” W) is located within the central region of the Santos Estuarine System (SES), and is potentially influenced by multiple sources of contamination from industries, sewage from specific and diffuse sources, urban drainage and storm waters, domestic and industrial landfills, and the Port of Santos. The SES is considered to be a critical area in terms of pollution, and various contaminants such as metals and polyaromatic hydrocarbons have been detected in potentially toxic concentrations.
2. The second sampling locality was chosen in the lower reaches of the Rio Itapanhaú, Bertioga (RI) (23° 50′ 0.2” S; 46° 9′ 10.6” W). This site is located in the northern sector of the SES, and shows some degree of environmental degradation owing to local urban expansion and domestic effluent, and the establishment of nautical infrastructure and sport fishing marinas.
3. The third sampling site was situated within the estuary of the Rio da Fazenda, Picinguaba (P) (23° 22′ 73.0” S; 44° 50′ 50.0” W), located in the Serra do Mar State Park (SMSP) near the Picinguaba Park headquarters, in Ubatuba. The SMSP constitutes an ecological corridor of Atlantic rainforest that is legally protected. This site is considered to be pristine, since it is well removed from relevant sources of anthropogenic pollution.

2.3. Biochemical biomarkers

Immediately upon arrival at the laboratory, the crabs were cryoanesthetized in crushed ice for 10 min after which all gill pairs and the hepatopancreas were dissected from each crab, weighed, placed separately in labeled micro-Eppendorf tubes, frozen at −20 °C and held at −80 °C until the biochemical and enzymatic analyses were performed. Ten crabs were used for each locality, and seven to ten samples of each tissue were processed for the activity of each biochemical biomarker.

For assays, the tissues were gradually thawed on ice, once only, 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 supernatant fractions were divided into five aliquots, according to the analyses to be performed (GST, GPx, AchE, MT and total protein). All samples were stored in an ultrafreezer at −80 °C until the assays were carried out. All biochemical analyses were performed within three weeks of tissue homogenization using a microplate reader (Biotek-Synergy™ HT). Each sample was thawed only once, immediately prior to assay.

Glutathione S-transferase (GST) activity was measured according to Keen et al. (1976) with adaptations. 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 the method developed by Sies et al. (1979) with adaptations. 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). Decreased absorbances 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 $mM^{-1}\,cm^{-1}$).

Acetylcholinesterase (AChE) activity was measured employing the colorimetric method of Ellman et al. (1961) with adaptations. A solution of 5,5′-dithiobis-2-nitro-benzoate (DTNB) followed by an acetylthiocholine iodide solution were added to the samples to begin the reaction. Absorbance at 415 nm was measured every 50 s for 3 min. The molar extinction coefficient for DTNB was 13.6 $mM^{-1}\,cm^{-1}$.

The titers of metallothionein-like proteins were quantified according to the method described by Viarengo et al. (1997). A fraction of partially purified metallothionein was obtained by treating the homogenates with ethanol and chloroform followed by precipitation using ethanol and hydrochloric acid. Subsequently, the low mass molecules containing sulfhydryl radicals were extracted from the precipitate. Quantitation of the metallothionein-like proteins was performed by adding Ellman's reagent containing DTNB and measuring absorbance at

412 nm. About 30% of the total sulfhydryls are estimated to be metallothioneins.

All biomarker data were normalized against total protein content. Total protein concentration in the homogenates was measured spectrophotometrically at 595 nm using Bradford's (1976) method, employing bovine serum albumin as a standard.

2.4. 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 were analyzed using a three-way analysis of variance (ANOVA) (Season, Locality and Tissue) for the enzyme activities measured. Two-way analyses of variance (Locality and Tissue) were also performed, independently of season as a factor for the same parameters. Differences between means within a given parameter were established using the Student–Newman–Keuls *post-hoc* multiple comparisons procedure. A minimum significance level of $P = 0.05$ was employed for all analyses.

3. Results

3.1. Salinity, precipitation and temperature at the study sites

Table 2 provides values for the salinities of pools near the burrows where the crabs were collected, and for rainfall and temperature at the collecting sites. Salinity was constant between seasons except for Picinguaba during the summer where the salt content was much lower (< 0.5‰S) than at the other localities. Precipitation was far greater during the summer months, the southern rainy season. In summer, temperatures ranged from 27 to 28 °C, and in winter from 23 to 25 °C.

3.2. Biomarker enzyme activities

Metallothionein-like protein (MT) concentrations were dependent on collection locality and tissue but there was no effect of season or any interactive effect (3-way ANOVA, $P < 0.05$). Crabs from ID, RI and P showed higher MT titers in the hepatopancreas than in the gills for both seasons ($P < 0.05$) (Fig. 2).

When seasons were evaluated separately, MT concentrations in crabs collected in winter or summer were affected only by tissue (2-way ANOVA, $P < 0.05$). Crabs collected in winter from ID showed greater MT titers in both tissues, while those from RI showed greater titers only in the gills, compared to crabs from P (Fig. 2). In summer, crabs from RI exhibited greater gill MT titers than crabs from P (2-way ANOVA, $P < 0.05$).

Glutathione peroxidase (GPx) activity was affected by season and by the interaction between season and collection site (3-way ANOVA, $P < 0.05$) (Fig. 3).

Considering seasons separately, GPx activities in crabs collected in winter were higher in crabs from RI and ID compared to P (2-way ANOVA, $P < 0.05$). For crabs from RI, GPx activity was greater in the gills than in the hepatopancreas. GPx activity in crabs collected in summer was affected only by tissue (2-way ANOVA, P < 0.05), and showed an inverse response to that found in winter, *i. e.*, crabs from ID and RI had lower GPx activities than those from P in most cases (Fig. 3).

Glutathione S-transferase activity (GST) was affected by season, tissue and the interaction between season and collection site (3-way ANOVA, $P < 0.05$) (Fig. 4).

When evaluating seasons separately, crabs collected in winter from RI and ID showed greater GST activities compared to those from P (2-way ANOVA, $P < 0.05$), and higher GST activities in the hepatopancreas than in the gills. GST activity in crabs collected in summer was affected only by tissue (2-way ANOVA, $P < 0.05$), specimens from RI showing lower activities for the gills, and those from ID exhibiting lower GST activity in the hepatopancreas compared to crabs from P (Fig. 4).

Hepatopancreas tissue did not respond to the AChE assay. In the gills, AChE activity was affected by season and collection site (2-way ANOVA, $P < 0.05$) (Fig. 5). In summer, crabs from RI and ID showed higher gill AChE activities than crabs from P, while in winter, activity levels were reversed: crabs from RI and ID showed lower AChE activities.

4. Discussion

Biochemical approaches have often been employed to examine the effects of xenobiotics on the metabolism of aquatic crustaceans (Wang and Rainbow, 2006; Martin-Diaz et al., 2008). However, very few studies have focused on fiddler crabs, and most of these concern laboratory exposure to a specific contaminant (Zanders and Rojas, 1996; Bartolini et al., 2009; Franco et al., 2018; Capparelli et al., 2017). Even fewer studies have examined the effects of multiple contaminants *in situ* (Bergey and Weis, 2007; Capparelli et al., 2016), particularly alterations in the depuration (*e. g.* GST and MT) and antioxidant systems (*e. g.* GPx), or neurotoxicity (*e. g.* AChE), as warning signs of chemical effects in fiddler crabs.

Our findings, showing increased GST and GPx activities, and inhibition of AChE activity in *M. rapax* chronically exposed *in situ* from RI and ID, particularly in winter, suggest the activation of detoxification and antioxidant response systems, possibly due to environmental contamination at these sites, or to environmental factors acting synergistically and capable of triggering this type of defense. Activated GST and GPx activities can be used as biomarkers indicative of defense against oxidative damage and the peroxidation products of DNA and lipids induced by pollutants (Tlili et al., 2010a, 2010b). Inhibition of AChE activity is widely recognized as both a direct neurotoxic effect and as a biomarker of exposure to neurotoxic compounds in invertebrates, including crustaceans (Lionetto et al., 2003; Fulton and Key, 2001; Van der Oost et al., 2003; Munari et al., 2014).

The increase in tissue MT titers in *M. rapax* exposed to contamination *in situ* corroborates the presence of certain metals, or their complex mixture, in environmental compartments, including sediments, water and organic matter (Bebianno and Serafim, 2003; Hamza-Chaffai et al., 2000). The induction of MT in *M. rapax* from ID and RI indicates exposure to and incorporation of metals in the tissues, which corroborates findings on metal contamination at these sites (Table 1).

Metals absorbed by the gills and/or epidermis of aquatic organisms can only be transferred proportionally to other tissues such as muscle if in excess, and depending on the accumulation pattern for each species

Table 2
Salinity (‰S) measured in pools located close to each collection site, and air temperature (°C) and total mean precipitation (mm) at each site for the austral winter (June–July) of 2012 and summer (January–February) of 2013, for each of the three study sites located along the southern Atlantic coast of the State of São Paulo, Brazil. Precipitation data are from the Instituto Nacional de Meterologia, MAPA, Brazil (http://www.inmet.gov.br/portal)/.

	Ilha Diana			Rio Itapanhaú			Picinguaba		
	Salinity	Rainfall	Temperature	Salinity	Rainfall	Temperature	Salinity	Rainfall	Temperature
Winter	12	100	23	12	85	25	12	84	23
Summer	13	317	27	12	278	28	< 0.5	343	29

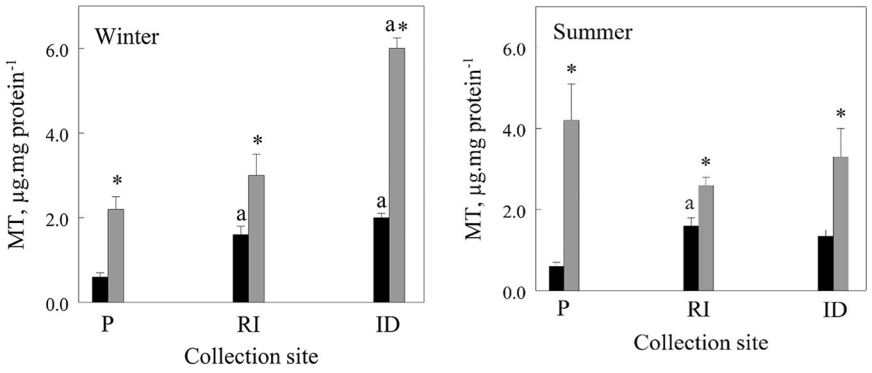


Fig. 2. Metallothionein-like protein concentrations in crude homogenates of gills (black bars) and hepatopancreas (gray bars) of *Minuca rapax* collected from the Picinguaba (P), Rio Itapanhaú (RI) and Ilha Diana (ID) sites during the austral winter (June–July) of 2012 (left panel) and summer (January–February) of 2013 (right panel). Data are the mean ± standard error of the mean (N = 7–10). *Significantly different from gills for the same collection site ($P < 0.05$). [a]Significantly different from crabs from Picinguaba (pristine site) for the same tissue.

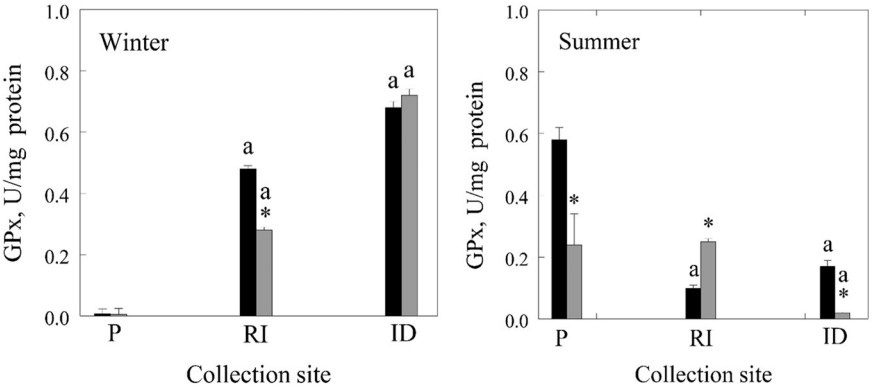
and the chemical properties of the element considered (Rainbow, 1987; Bordon et al., 2018). For bivalves, polychaetes and fish, the bioavailable, stored and detoxified fractions of metals are well described (Wallace et al., 2003; Campana et al., 2013). Crustaceans assimilate non-essential metals mostly in the detoxified form bound to MT with little excretion; however, they can excrete metals in a dynamic equilibrium, since excretion rate is similar to total uptake rate (Rainbow and Black, 2002).

These strategies may be different for semi-terrestrial crabs that maintain constant contact with the sediment through which they acquire contaminants, considering uptake not only from solution but also from the sediment and diet. Food is an important source of metal uptake. In *Callinectes danae* exposed to lead *via* water and diet (Bordon et al., 2018), increased induction of metallothionein-like protein expression is seen in the gills, particularly in crabs exposed to waterborne contamination. However, *C. danae* is a fully aquatic swimming crab and may employ a different depuration strategy from *M. rapax*. In *M. rapax*, the highest MT titers were seen in the hepatopancreas. When this species is exposed to waterborne copper, titers are higher in the hemolymph and hepatopancreas compared to the gills (Capparelli et al., 2017), which also corroborates the higher hepatopancreas MT titers seen in the present study. These findings suggest that in semi-terrestrial crabs, the hepatopancreas is an important organ for both the metal accumulation and depuration processes which appear to differ from the gill-based mechanisms seen in aquatic crabs.

In addition to chronic exposure to contaminants, fiddler crabs also must confront ample variations in environmental parameters. Metal uptake likely takes place across the posterior gill epithelia, the most probable targets of metal toxicity (Péqueux, 1995). Salinity can affect metal uptake indirectly *via* osmoregulatory mechanisms. The accumulation and toxic effects of metals appear to increase at low ambient salinity (Bjerregaard and Depledge, 1994). Gill Na^+/K^+-ATPase and carbonic anhydrase activities are inhibited in copper-exposed *M. rapax*, showing that while overall osmoregulatory ability is unaltered, effects are manifested at the biochemical enzymatic level (Capparelli et al., 2017). Although fiddler crabs are remarkably tolerant of variation in salinity and temperature (Baldwin and Kirschner, 1976a, 1976b; Graszynski and Bigalke, 1986; Zanders and Rojas, 1996), biochemical biomarkers are sensitive to pollution-induced stresses (Zanders and Rojas, 1996; Capparelli et al., 2016).

Both salinity and temperature in the study area fluctuate due to the annual rainfall regime and to the austral summer/winter seasons. These challenges are intensified in the intertidal zone and can alter the bioavailability and consequently, the toxicity of pollutants, which can trigger antioxidant responses (Chapman, 2007). Activation of GPx in the gills, and GST and MT in the gills and hepatopancreas of *M. rapax* from Picinguaba during the summer also may have been induced or potentialized by such environmental factors, including intrinsic physiological alterations, subsequent to intense reproductive activity during the summer. Altered biomarker activities in *M. rapax* during the summer may reflect the low salinity (< 0.5‰S) encountered in the estuary during that particular collection (Table 1) as a consequence of heavy rains. In *C. danae* during the austral summer (low salinity/elevated temperature), physiological and biochemical processes are also considerably altered, hampering detection of effects caused by environmental contaminants, especially metals (Araújo, 2014). However, when salinities are normalized against winter values, clear metal-related responses are discernible. Thus, environmental factors like salinity and temperature appear to affect biochemical and physiological processes in aquatic crabs (Araújo, 2014).

Alterations in salinity and temperature owing to the tidal cycle can induce fluctuations in metabolism and may affect antioxidant responses in estuarine organisms. High GST and GPx activities also are related to biochemical adjustments resulting from seasonal changes in salinity in the copepod *Eurytemora affinis* (Cailleaud et al., 2007) and in the crabs *Callinectes ornatus* (Freire et al., 2011), *Paralomis granulosa* (Sáenz et al., 2010) and *Neohelice granulata* (de Oliveira et al., 2005). Thus, seasonal patterns may overlap with pollution effects on biomarkers indicative of induction of oxidative stress (Niyogi et al., 2001). Some studies suggest increased AChE activity related to temperature and salinity variation



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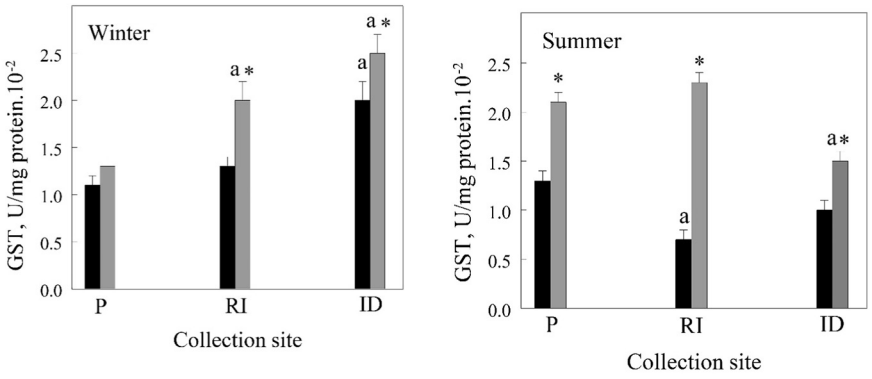

Fig. 4. Glutathione S-transferase (GST) activity in crude homogenates of gills (black bars) and hepatopancreas (gray bars) of *Minuca rapax* collected from the Picinguaba (P), Rio Itapanhaú (RI) and Ilha Diana (ID) sites during the austral winter of 2012 (June–July) (left panel) and summer (January–February) of 2013 (right panel). Data are the mean ± standard error of the mean (N = 7–10). *Significantly different from gills for the same collection site ($P < 0.05$). [a]Significantly different from crabs from Picinguaba (pristine site) for the same tissue.

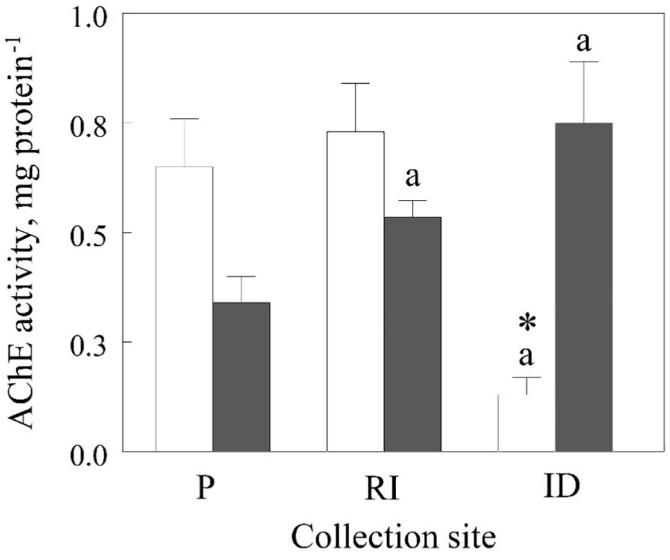

Fig. 5. Acetylcholinesterase (AChE) activity in crude homogenates of the gills of *Minuca rapax* collected from the Picinguaba (P), Rio Itapanhaú (RI) and Ilha Diana (ID) sites during the austral winter (white bars) of 2012 (June–July) and summer (gray bars) of 2013 (January–February). Data are the mean ± standard error of the mean (N = 7–10). *Significantly different from for the same collection site in summer ($P < 0.05$). [a]Significantly different from crabs from Picinguaba (pristine site) for the same season.

and metal contamination (Araújo, 2014; Huynh Thi Tu et al., 2012; Cailleaud et al., 2007). The induction of MT under different salinity regimes rather than as a consequence of elevated metal concentrations is well known (Legras et al., 2000; Monserrat et al., 2008). Thus, abiotic factors should be considered as modulators of antioxidant responses to oxidative stress caused by the presence of xenobiotics.

The set of biochemical biomarker responses evaluated here in *M. rapax* should be interpreted as a warning sign regarding exposure to xenobiotics *in situ*. Metallothionein induction may be related to exposure to a complex mixture of metals, with greater relevance in ID crabs and less in RI crabs, but also may be modulated by environmental factors. However, the altered GST, GPx and AChE activities all suggest a defense response against stress in crabs from all collection sites. Apparently, during the winter, crabs from ID and RI, chronically contaminated sites, exhibited greater disturbances in these biomarkers. Indeed, these areas showed greater metal contamination during winter than in summer (Capparelli et al., 2016). In summer, crabs from all three localities showed changes in these biochemical biomarkers. Because crabs from the pristine site adopted as a control area show high enzyme activities, alterations also may result from elevated temperatures or significant decreases in salinity, typical of summer rains, and/or their combination with exposure to contaminants.

Our findings reveal that there is a strong seasonal influence on biochemical biomarker responses in *Minuca rapax*. Fluctuations in environmental parameters like precipitation and salinity can influence biomarker responses in addition to those caused by pollutants, and should be taken into account when monitoring impacted estuarine regions using sentinel species.

Compliance with Ethical Standards

Conflict of interest

All authors declare that they have no conflict of interest with any government 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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