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Contamination gradient affects differently carbonic anhydrase activity of mollusks depending on their feeding habits

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Abstract

Aquatic organisms that inhabit coastal areas are often exposed to several contaminants. It is known that the bioaccumulation of contaminants can be amplified according to the species feeding habits and contaminant properties. As a consequence, species can experience different effects to contaminant exposure even if they inhabit the same area. The present study aimed to investigate the activities of carbonic anhydrase (CA), Ca^{2+}-ATPase, and Mg^{2+}-ATPase in different tissues (soft tissue, mantle, and gill) of three mollusk species (*Lottia subrugosa*, *Stramonita brasiliensis*, and *Crassostrea brasiliana*) with different feeding habits (herbivore, carnivore, and filter-feeder, respectively) which were sampled within a known contamination gradient at Santos Estuarine System (Southeastern Brazil). From the three enzymes tested, only CA was affected by the presence of contaminants within the contamination gradient evaluated. In general, the CA activity from the three species were lower in contaminated sites when compared to the reference site. The contrasting CA activity response observed in *S. brasiliensis* compared to *L. subrugosa* and *C. brasiliana* could be related to the tissue-specificity of this enzyme activity and species feeding habits (filter-feeders can accumulate more contaminants than herbivores and even carnivores). Results indicated that *C. brasiliana* mantle is the most suitable tissue for the use of CA analysis as a biomarker.

Keywords *Crassostrea brasiliana* · *Lottia subrugosa* · *Stramonita brasiliensis* · Biomarkers · Ca^{2+}-ATPase · Mg^{2+}-ATPase

Introduction

Coastal areas are highly impacted by human activities. Such activities can lead to changes in different biological levels by releasing contaminants from domestic, urban, and industrial wastes (Castro 2019). It is known that these impacts jeopardize the health of aquatic organisms and induce physiological alterations that are often used as biomarkers (Nikinmaa 2014; Harayashiki et al. 2020). Thus, the use of biochemical biomarkers as tools in environmental monitoring assessments is frequent (Galloway et al. 2004; Blaise et al. 2017; Harayashiki et al. 2020).

Carbonic anhydrase (CA) is a zinc metalloenzyme found in all living organisms (Supuran 2008, 2016, 2018; Lionetto et al. 2016). This enzyme catalyzes the reversible hydration of carbon dioxide to bicarbonate (Tripp et al. 2001; Supuran 2016, 2018; Lionetto et al. 2016) and, therefore, is related to several metabolic processes. Among them are respiration and transport of carbon dioxide and bicarbonate between different tissues, homeostasis control of carbon dioxide and pH, electrolyte secretion and, calcification (Supuran 2008, 2016; Lionetto et al. 2016; Zebral et al. 2019).

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According to Lionetto et al. (2012, 2016), studies with both invertebrates and vertebrates showed a general pattern of CA activity inhibition in several tissues after in vivo and in vitro exposure to metals and pesticides, indicating that this enzyme has the potential to be used as a biomarker in environmental monitoring.

Ca^{2+}-ATPase and Mg^{2+}-ATPase are P-type ATPase enzymes that regulate calcium and magnesium concentrations, respectively, across biological membranes (Xuan et al. 2017). Both calcium and magnesium play important roles in organisms' metabolism (Erulkar 1981; Nozadze et al. 2015). Therefore, their regulation is essential to keep the biological functions. Studies regarding the impairment of these enzymes on mollusks exposed to metals have been undertaken under laboratory conditions (Pivovarova et al. 1992; Jorge et al. 2013). However, it is known that, in realistic environmental conditions, aquatic organisms are concomitantly exposed to a combination of stressors. Despite this, field studies assessing activities of these enzymes along contamination gradients have not been carried out so far.

Mollusks are often used in toxicological studies because of several factors, such as economic and ecological importance, broad distribution, possibility to choose different life stages in studies, sessile lifestyle which accurately reflects local environmental conditions, and ability to bioaccumulate contaminants (Rittschof and McClellan-Green 2005; Harayashiki et al. 2020). Considering that different mollusk species can occupy different niches within the same habitat, these species might respond differently even though they are subjected to the same levels of local contamination. For example, it is known that organisms from the base of food web tend to accumulate lower contaminant amounts than organisms from the top (Weis 2014). Thus, it is expected that herbivore mollusks would be less affected to contaminants than carnivore ones. On the other hand, filter-feeder animals, such as oysters and mussels, are usually highly exposed to chemical partitioning in the water column and in particulate material (Castro 2019). As consequence, biological responses induced by contaminant exposure depend on organisms' trophic ecology (Ferraz et al. 2020).

Studies on the effects of contaminants in Santos Estuarine System (SES; Southeastern Brazil) have been undertaken in *Lottia subrugosa* and showed alterations in shell structure and composition, and in DNA damage and lipoperoxidation in soft tissues of this herbivore gastropod (Begliomini et al. 2017; Gouveia et al. 2019; Oliveira et al. 2020; Harayashiki et al. 2021). In fact, a study investigating the morphological alterations in shells of *L. subrugosa* during a transplantation experiment showed a relation between ATPases activities in soft tissues and shell parameters, which could indicate a possible role of these enzymes in the biomineralization process (Harayashiki et al. 2021). Additionally, other mollusk species commonly used as test organisms in toxicological studies, such as the bivalve filter-feeder *Crassostrea brasiliana* and the gastropod carnivore *Stramonita brasiliensis*, cohabit the same rocky shore environments of SES (Torres et al. 2015; Catharino et al. 2015; Abreu et al. 2020). Previous studies have investigated the levels of contaminants in SES and showed higher contaminant levels inside the estuary followed by the dilution of these substances towards the ocean (Abessa et al. 2005; Buruaem et al. 2013; Torres et al. 2015; Begliomini et al. 2017; Kim et al. 2017; Pusceddu et al. 2019). Considering that shells are mainly composed by calcium carbonate and previous studies showed that shells can be altered by the presence of contaminants, the present study aimed to investigate changes in the activity of enzymes (CA, Ca^{2+}-ATPase, and Mg^{2+}-ATPase) that are possibly related to shell formation of three mollusk species (*L. subrugosa*, *S. brasiliensis*, and *C. brasiliana*) sampled in sites under different contaminant levels at SES. The species were selected according to their feeding habits, as this parameter might impact the accumulation of contaminants and, consequently, the activity of these enzymes can be altered.

Materials and methods

Study area and sampling

The samples were obtained in sites at Santos Estuarine System (SES) in São Paulo State (Brazil) in 2019. Contamination degrees in this area have already been investigated by several studies which assessed the occurrence of legacy and emergent chemicals in environmental matrices (Abessa et al. 2005; Buruaem et al. 2013; Torres et al. 2015; Begliomini et al. 2017; Kim et al. 2017; Pusceddu et al. 2019). Based on these studies, three sites were selected according to contamination levels (Fig. 1), with the most contaminated site (Balsa) located close to port terminals and high concentrations of trace metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and dichlorodiphenyltrichloroethane-related compounds (Table 1). The least contaminated site (Palmas) is an island, with restricted access nearby to the mouth of the estuary. Low concentrations of these contaminants were detected at Palmas, but only estrogen was present in higher concentration than at Balsa (Table 1). The site presenting intermediate contamination levels is a beach from Guarujá city (Góes), located between the other two sites. It is important to highlight that these sites are distributed within a range of 5 km at the mouth of the SES presenting similar salinity and pH throughout the year (Gimiliani et al. 2016).

Adult specimens of all species (*L. subrugosa*: >5 mm length; *S. brasiliensis*: >30 mm height; *C. brasiliana*: >60 mm length) were manually captured during low tide on

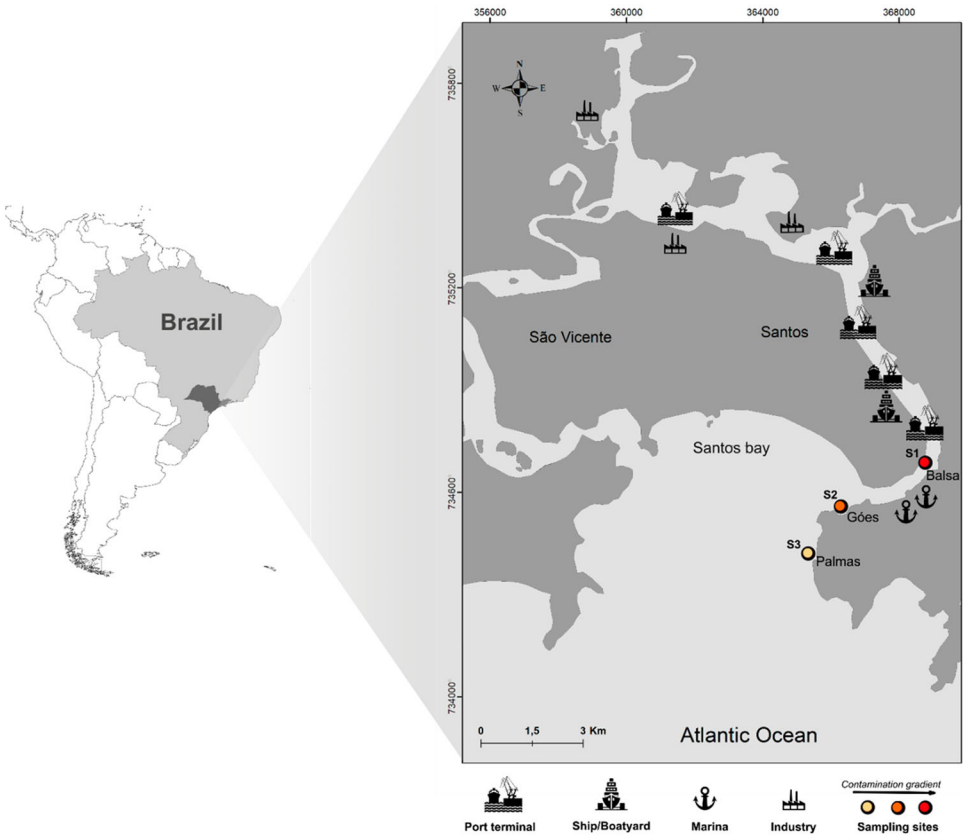

Fig. 1 Sampling sites (Balsa, Góes, and Palmas) in Santos Estuarine System (São Paulo, Brazil). Arrow shows the direction of contamination gradient

the rocky shores, immediately transported to the laboratory at São Paulo Federal University (UNIFESP - Santos, São Paulo, Brazil) and kept at −80 °C to euthanize specimens, which facilitates the removal of soft tissues without damaging the shells. The sampling campaigns were simultaneously performed, in each site, seeking to avoid tide variations, which may lead to higher temperatures, hypoxia and desiccation potentially inducing alteration in enzymatic activities (Sokolova and Pörtner 2001; Oh et al. 2018). For each species, 30 specimens per site were dissected. Due to the small size of *L. subrugosa*, the whole soft tissue was used as sample. Samples of the mantle were removed from *S. brasiliensis*, and samples from both mantle and gill tissues from *C. brasiliana* were collected. The tissues were weighted and, again, stored at −80 °C for subsequent enzymatic assays. Shell biometric parameters (length, width, and height; Online Resource 1 - Fig. S1) were measured with a digital caliper (0.01 ± 0.005 mm), and shells were also weighted in a precision scale (0.0001 g).

Enzymatic assays

Homogenate preparation

Samples were homogenized, with HEPES buffer (1 mM EDTA, 10 mM HEPES, 250 mM Sucrose, 1 mM PMSF; pH 7.4) as described by Santini et al. (2011), in the proportion of 1:10, with an ultrasonic cell disruptor (Eco-Sonics; 30 kHz; 2 × 20 s). Homogenates were centrifuged (Heraeus Fresco 17 Centrifuge, Thermo Scientific™; 12000 G, 20 min and 4 °C) and supernatants were aliquoted for carbonic anhydrase (CA), Ca^{2+}-ATPase, and Mg^{2+}-ATPase assays.

Carbonic anhydrase (CA)

The determination of CA activity was based on the method described by Vitale et al. (1999). A reaction medium (pH 7.4) was prepared with sodium phosphate monobasic monohydrate (10 mM), mannitol (225 mM), sucrose (75 mM) and tris-base (10 mM). The analysis consisted in mixing the HEPES buffer (as blank; 50 µL) or the samples (50 µL) with the reaction medium (7.5 mL) and ultrapure water enriched with CO_2 (1 mL) and writing down the pH drop at 2.5 °C during 20 s measured by a bench pHmeter (SP2000, Sensoglass). Blanks were measured several times, including one at the beginning and one at the end of analysis, and after 10 consecutive reads of samples. The catalyzed reaction rate ($b_{catalyzed}$) and the non-catalyzed reaction rate ($b_{non\text{-}catalyzed}$) were calculated by the slope estimated in the linear regression between pH values (samples and blanks, respectively) and time. The specific carbonic anhydrase activity (SCA) was calculated by the formula: SCA = [($b_{catalyzed}$ average of $b_{non\text{-}catalyzed}^{-1}$) −1] mg protein^{-1}. SCA was expressed in specific activity mg protein^{-1}.

Table 1 Concentrations of contaminants at Santos Estuarine System (SES - São Paulo State, Brazil) measured in biological and sedimentary matrices

Contaminant	Matrix	Balsa	Góes	Palmas	Reference
Cd (μg g^{-1})	Shell	722.3 ± 157.8	607.7 ± 108.8	274.0 ± 56.8	Begliomini et al. 2017
Cr (μg g^{-1})	Shell	30.7 ± 15.3	45.9 ± 16.8	23.0 ± 3.6	Begliomini et al. 2017
Cu (μg g^{-1})	Shell	3.8 ± 0.9	2.3 ± 1.1	2.7 ± 0.7	Begliomini et al. 2017
Fe (μg g^{-1})	Shell	513.3 ± 98.4	378.3 ± 77.3	223.0 ± 42.4	Begliomini et al. 2017
Mn (μg g^{-1})	Shell	24.2 ± 8.4	21.2 ± 6.4	15.6 ± 5.3	Begliomini et al. 2017
Ni (μg g^{-1})	Shell	359.7 ± 34.6	386.0 ± 20.3	264.8 ± 18.4	Begliomini et al. 2017
Pb (μg g^{-1})	Shell	17.9 ± 3.2	15.3 ± 3.6	8.6 ± 1.9	Begliomini et al. 2017
Sr (μg g^{-1})	Shell	2502.7 ± 45.2	2255.7 ± 123.5	2217.8 ± 137.8	Begliomini et al. 2017
Zn (μg g^{-1})	Shell	20.9 ± 2.2	6.4 ± 1.2	2.8 ± 0.7	Begliomini et al. 2017
Polycyclic aromatic hydrocarbons (ng g^{-1})	Sediment	2667	NI	27	Begliomini et al. 2017
Polychlorinated biphenyls (ng g^{-1})	Sediment	11.8	12.4	1.13	Begliomini et al. 2017
Dichlorodiphenyltrichloroethane-related compounds (ng g^{-1})	Sediment	29.2	NI	1.1	Begliomini et al. 2017
Estriol (ng g^{-1})	Sediment	20.9 ± 4.1	NI	169.3 ± 16.4	Pusceddu et al. 2019
17β-estradiol (ng g^{-1})	Sediment	<20.0	NI	< 20.0	Pusceddu et al. 2019
17α-ethynyl estradiol (ng g^{-1})	Sediment	20.0 ± 6.2	NI	42.9 ± 2.3	Pusceddu et al. 2019

NI Not informed

Ca^{2+}- ATPase and Mg^{2+}-ATPase

The activities of both Ca^{2+}-ATPase and Mg^{2+}-ATPase were measured as described by Jorge et al. (2013) based on Vajreswari et al. (1983) method. Reaction medium for Ca^{2+}-ATPase consisted in 5 mM $CaCl_2$, 5 mM $MgCl_2$, 189 mM NaCl, 20 mM Tris-base, 2 mM ouabain, and 3 mM ATP; and for Mg^{2+}-ATPase in 5 mM $MgCl_2$, 14 mM KCl, 189 mM NaCl, 0.2 mM EDTA, 20 mM Tris-base, 2 mM ouabain, and 3 mM ATP. Homogenized samples were mixed with each reaction medium, incubated for 30 min at 30 °C and then chilled on ice for 10 min to stop reaction. The activity of the ATPases were measured spectrophotometrically by the release of inorganic phosphate (Pi) that reacts with the color solution (10% ascorbic acid and 0.42% of ammonium molybdate in 0.5 mM H2SO4; Ames 1966) during an incubation period of 20 min followed by the measurement of absorbance at 620 nm. The phosphorous standard solution 0.65 mM (P3869, Sigma) was used for the standard curve of Pi. The enzymatic activity was expressed as μmol Pi mg $protein^{-1}$ min^{-1}.

Protein determination

Bradford's (1976) method was applied to determinate the total protein content of supernatants, required for the calculations of specific enzymatic activities, and bovine serum albumin was used as standard.

Statistical analysis

Tukey's fences test was applied in all data to remove outliers (Online Resource 1 - Fig. S2–4). The upper limits of shell dimension ranges (length, width, and height) were not strictly defined during the sampling campaigns. However, enzymatic activities can be attributed to mollusk's size and not to contamination degree. Therefore, a linear regression between shell's biggest dimension (*L. subrugosa*: length; *S. brasiliensis*: height; *C. brasiliana*: height) and specific enzymatic activity was executed. Results from this preliminary analysis showed that the activity of both CA in *S. brasiliensis* and Ca^{2+}-ATPase in *C. brasiliana* could be predicted by specimens' height (Online Resource 1 - Fig. S5). In this sense, analysis of covariance (ANCOVA) was used to test differences in Ca^{2+}-ATPase activity among sites, using height as cofactor. Considering that shell height was different among sites only for *S. brasiliensis*, ANCOVA was not applied for CA activity in *C. brasiliana*.

For biometric data and the other enzymatic activities, normality and homoscedasticity were tested by Shapiro-Wilk test and Levene's test, respectively. If data was parametric, analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to test the

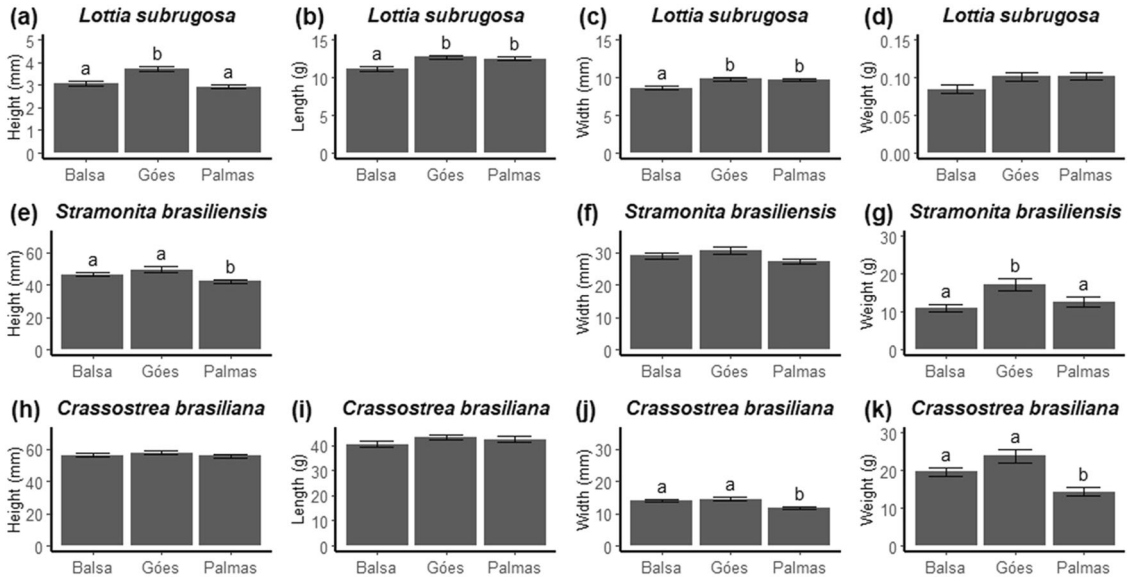

Fig. 2 Shell biometric parameters (mean ± SE) from (**a**–**d**) *Lottia subrugosa* (herbivore), **e**–**g** *Stramonita brasiliensis* (carnivore), **h**–**k** *Crassostrea brasiliana* (filter-feeder) sampled in three sites along a contamination gradient (Balsa > Góes > Palmas) in Santos Estuarine System (São Paulo, Brazil)

differences of variables between sampling sites. For non-parametric data, Kruskal-Wallis followed by Dunn's multiple comparison test was applied. Additionally, the correlation among all variables (biometric parameters and enzymatic activities) was tested by Spearman's rank-order correlation. The software R (version 4.0.0) was used for these analyses. For all analyses, significative differences were considered for $p < 0.05$.

Results

Shell biometric parameters varied according to species (Fig. 2). *L. subrugosa* from Balsa (most contaminated site) and Palmas (least contaminated site) presented similar height ($p > 0.05$) and were shorter ($p < 0.05$) than shells from Góes (intermediate contamination) (Fig. 2a). However, specimens from Palmas and Góes were bigger ($p < 0.05$) in length and width than specimens from Balsa (Fig. 2b, c). *S. brasiliensis* shells from Balsa and Góes were taller than specimens from Palmas ($p < 0.05$; Fig. 2e), but only shells from Góes were heavier than Palmas ($p < 0.05$; Fig. 2g). For *C. brasiliana*, shells from Balsa and Góes were bigger in width ($p < 0.05$; but not in height or length) and heavier ($p < 0.05$) than shells from Palmas (Fig. 2h–k).

Considering all analyzed species, CA presented a higher activity in specimens from Palmas than from the other sites for *L. subrugosa* ($p < 0.05$ just between Palmas and Góes; Fig. 3a) and *C. brasiliana* mantle ($p < 0.05$; Fig. 3d). The *S. brasiliensis* mantle CA activity from Góes was higher than the ones from Balsa and Palmas ($p < 0.05$; Fig. 3b).

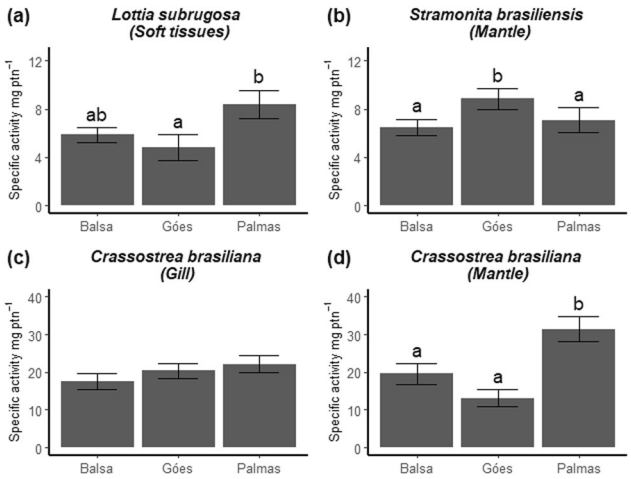

Fig. 3 Carbonic anhydrase activity (mean ± SE) in (**a**) the herbivore *Lottia subrugosa* soft tissues, **b** Carnivore *Stramonita brasiliensis* mantle, **c** Filter-feeder *Crassostrea brasiliana* gill, **d** *C. brasiliana* mantle sampled within a contamination gradient (Balsa > Góes > Palmas) in Santos Estuarine System (SES). Different letters show significant differences between sites ($p < 0.05$)

The activity of both ATPases (Ca^{2+}- and Mg^{2+}-) was not significantly different among sites in any tissue or species analyzed ($p > 0.05$; Table 2).

The Spearman's correlation results for *L. subrugosa* (Online Resource 1 - Fig. S6) showed that all biometric parameters are positively correlated to each other ($p < 0.01$), which is an allometric effect (proportional growth of biometric parameters) already expected. Among the enzymes tested, only the ATPases were correlated to each other ($p < 0.01$, $r_s = 0.83$).

Table 2 Ca^{2+}-ATPase and Mg^{2+}-ATPase activities (mean ± SE) in three mollusk species sampled in Santos Estuarine System (SES - São Paulo, Brazil)

Species (Feeding habit)	Tissue	Ca^{2+}-ATPase (μmol Pi mg protein^{-1} min^{-1})			Mg^{2+}-ATPase (μmol Pi mg protein^{-1} min^{-1})		
		Balsa	Góes	Palmas	Balsa	Góes	Palmas
Lottia subrugosa (Herbivore)	Soft tissues	0.55 ± 0.14 ($n = 29$)	0.63 ± 0.17 ($n = 30$)	0.61 ± 0.21 ($n = 29$)	1.28 ± 0.39 ($n = 30$)	1.12 ± 0.35 ($n = 30$)	1.12 ± 0.44 ($n = 30$)
Stramonita brasiliensis (Carnivore)	Mantle	0.92 ± 0.31 ($n = 30$)	0.81 ± 0.27 ($n = 30$)	0.88 ± 0.49 ($n = 28$)	1.15 ± 0.44 ($n = 30$)	0.98 ± 0.44 ($n = 30$)	0.97 ± 0.63 ($n = 28$)
Crassostrea brasiliana (Filter-feeder)	Gill	2.68 ± 1.05 ($n = 29$)	3.3 ± 1.58 ($n = 29$)	3.32 ± 1.72 ($n = 29$)	2.56 ± 0.66 ($n = 30$)	2.94 ± 1.32 ($n = 29$)	3.20 ± 1.64 ($n = 28$)
	Mantle	1.58 ± 0.38 ($n = 30$)	1.63 ± 0.50 ($n = 30$)	1.6 ± 0.37 ($n = 29$)	1.85 ± 0.46 ($n = 30$)	1.89 ± 0.43 ($n = 30$)	1.86 ± 0.48 ($n = 26$)

As observed in *L. subrugosa*, specimens from *S. brasiliensis* have also presented an allometric effect ($p < 0.01$; Online Resource 1 - Fig. S7). All the biometric parameters were weakly and negatively correlated to CA activity in *S. brasiliensis* mantle ($p < 0.05$). Additionally, the activity of all enzymes was positively related to each other ($p < 0.01$).

The allometric effect on *C. brasiliana* shells was also observed ($p < 0.05$; Online Resource 1 - Fig. S8). A weak negative correlation was observed between biometric parameters and enzymatic activities, but these relations varied according to biometric parameter, enzyme and tissue tested. Length was correlated only with Ca^{2+}-ATPase activity in both tissues ($p < 0.01$; r_s (gill) = −0.33, r_s (mantle) = −0.24). While width was associated with CA in gill ($p < 0.01$, $r_s = -0.32$), and weight was related with CA in mantle ($p < 0.05$, $r_s = -0.25$). Height was correlated to both Ca^{2+}-ATPase in gill ($p < 0.01$, $r_s = -0.31$) and CA activity in mantle ($p < 0.05$, $r_s = -0.25$). In contrast, enzymatic activities were mainly positively correlated to each other. The Mg^{2+}-ATPase activities were strongly correlated only to their respective Ca^{2+}-ATPase activities ($p < 0.01$; r_s (gill) = 0.84, r_s (mantle) = 0.72). CA activities in both gill and mantle were correlated with each other ($p < 0.05$, $r_s = 0.29$) and with Ca^{2+}-ATPase in gill (gill - $p < 0.01$; $r_s = 0.314$; mantle - $p < 0.05$, $r_s = 0.25$).

Discussion

Different mollusk species, even inhabiting the same area, might present different biological responses to contaminants exposure because of their feeding habits (Weis 2014). In the present study, an herbivore patelliform gastropod (*L. subrugosa*), a carnivore muricide gastropod (*S. brasiliensis*) and a filter-feeder bivalve (*C. brasiliana*) were sampled within a contamination gradient area in Brazil (Abessa et al. 2005; Buruaem et al. 2013; Torres et al. 2015; Begliomini et al. 2017; Kim et al. 2017; Pusceddu et al. 2019). Despite the differences detected in biometric parameters, linear regression analysis showed that specimens' size did not affect enzymatic activities (with exception of CA activity in *S. brasiliensis*). The activity of CA varied according to tissue tested and positive correlations among enzymatic activities were detected in the three species.

In the present study, CA was the only enzyme that presented significant differences in its activity between sampling sites. The activity of CA was attributed to different functions, which include osmoregulation, ion transport, respiration, pH regulation and shell biomineralization (Supuran 2016; Zebral et al. 2019). Therefore, the variations in CA activity responses observed in the different tissues from the species evaluated in the present study could be related to the distinct functions that this enzyme performs.

In fact, previous studies have shown that different tissues (e.g., digestive gland, gills, hemolymph, and mantle) from a same mollusk species can either increase or decrease CA activity after a laboratory exposure to a contaminant or field exposure to a mixture of contaminants (Lionetto et al. 2006; Caricato et al. 2010, 2018, 2019; Azevedo-Linhares and Freire 2015; dos Santos et al. 2017). These authors attributed the tissue-specific CA activity variation to the relevance of CA activity in the tissue (e.g., H^+ formed after the hydration of carbon dioxide by CA is required for the lysosome acidification in the digestive gland), exposure period (i.e., acute x chronic exposures), and seasonal variation in enzymatic activity. There is a consensus from these previous studies that this enzyme can be used as a biomarker in monitoring contamination assessments. However, caution must be taken when using CA as biomarker, as its activity vary according to the tissue evaluated.

In addition to the tissue-specific CA activity response to contaminants, the present study showed that the activity of this enzyme varied among the three species studied, mainly regarding CA activity in mantle samples from *S. brasiliensis* and *C. brasiliana*. These variations could be attributed to the differences in bioaccumulation capacity due to the feeding habits of these species. It is well-known that herbivore species, such as *L. subrugosa*, accumulate less contaminants than species with other feeding habits and, therefore, would be less subjected to contamination effects (Weis 2014). On the other hand, Cabrini et al. (2018) showed that metal concentrations in mollusk species that feed from suspended particles can be higher than in carnivorous species. Similar patterns have also been reported for several polychlorinated biphenyls and polycyclic aromatic hydrocarbons in experimental conditions (Hickey et al. 1995; Kaag et al. 1997). Therefore, the clearly lower CA activity observed in the mantle tissue of the filter-feeder *C. brasiliana* from Balsa (highly contaminated) and Góes (intermediate contamination) in comparison to Palmas (least contaminated site) could be related to the higher accumulation of contaminants due to the ingestion of contaminated particles from the water, as stated by Castro (2019). However, further studies relating the different bioaccumulation patterns and CA activity need to be performed to confirm this hypothesis. Considering results from the present study, mantle tissue of *C. brasiliana* is the most suitable tissue to use CA activity as biomarker in monitoring assessments.

In contrast to CA, neither of the ATPase enzymes presented differences in their activities in any of the species tested in the present study, which indicates that local contamination was not affecting the functioning of these enzymes. Previous studies have shown that these enzymes from mollusks can be affected by metals exposure (Pivovarova et al. 1992; Burlando et al. 2004; Pattnaik et al. 2007; Santini et al. 2011; Jorge et al. 2013). The lack of differences found in the present study might be related to the findings reported by Jorge et al. (2013) in freshwater mussel *Lampsilis siliquoidea* exposed to copper. The activity of both Ca^{2+}-ATPase and Mg^{2+}-ATPase in soft tissue of *L. siliquoidea* was higher in specimens exposed to copper for 7 days, but with the increase of the exposure period, the difference disappeared. A similar temporal response was observed in Ca^{2+}-ATPase activity in kidneys and gills of the freshwater mussel *Anodonta anatina* exposed to copper (Santini et al. 2011). Although these previous studies were performed under laboratory conditions, similar responses were observed by Harayashiki et al. (2021) in *L. subrugosa* after both transplantation experiment and field sampling at Palmas and Balsa. At the end of the transplantation experiment (2 months), Ca^{2+}-ATPase activity from specimens caged at Balsa was higher than specimens caged at Palmas. However, specimens investigated applying the field sampling approach did not show differences in the activities of both ATPases. The effect observed during the transplantation experiment was related to different stressors (e.g., contaminants and possible caging stress), while the lack of effect from field sampling approach was attributed to the adaptation to the presence of contaminants in specimens from Balsa. Considering that the three species used in the present study were sampled in sites with different degrees of contamination, the lack of ATPase activity responses are most likely related to the resilience of these species to the local contamination, as already reported for *L. subrugosa*.

Mollusk shells are composed mainly of calcium carbonate. Thus, the activity of CA and Ca^{2+}-ATPase could be related to the mollusk shell biomineralization process since these enzymes are important for the transportation of carbonate and calcium, respectively, to the extrapallial space (between mantle and periostracum shell layer) where the shell is formed (Marin et al. 2012). In fact, Lionetto et al. (2006) attributed the smaller shells of mussel (*M. galloprovincialis*) found during the 'Mussel-Watch' programs to a lowered CA activity observed in their experiment. However, their study used specimens with similar shell size, preventing the evaluation of biometric correlations. In a recent study with transplantation experiment and field sampling, Harayashiki et al. (2021) showed a correlation between ATPases activities and shell size in *L. subrugosa* from the transplantation experiment, but not for specimens from field sampling. Such differences in the correlations were attributed to the fact that specimens from the field were adapted to the contamination levels in their natural environment. The results from the present study showed different correlations between shell size and enzymatic activities. For *L. subrugosa*, there was no correlation between these two parameter types, as previously reported by Harayashiki et al. (2021). Similarly, the activities of both

ATPases from *S. brasiliensis* were not correlated to specimens' size. However, CA activity was negatively related to all biometric parameters. The activities of both CA and Ca^{2+}-ATPase from *C. brasiliana* gills and mantle were negatively correlated to different shell size parameters, contrasting to the other two species. Considering the differences observed among the species, the lack of correlation in *L. subrugosa* could be related to the fact that enzymes were evaluated in the whole soft tissues and not in a specific tissue as it was performed for the other species. Additionally, despite the different relevance of the relation between biometry and enzymatic activities among the species, our findings suggest that these enzymes could be related to shell formation.

Previous studies have suggested the participation of these enzymes in the biomineralization process (Mann et al. 2012; Mann and Edsinger 2014; Zebral et al. 2019; Liao et al. 2019). However, the low r_s found in the correlation between the enzymatic activities and shell sizes also suggests that the correlations found can be related to other processes. In fact, the inverse correlation between CA and shell size in both *S. brasiliensis* and *C. brasiliana* might be related to the mechanism used to avoid desiccation by these organisms. Gastropods such as *S. brasiliensis* close their operculum to avoid desiccation, while bivalves (e.g., *C. brasiliana*) close their shell valves (Diederich et al. 2015; Harayashiki et al. 2020). Regardless of the mechanism adopted, the issue associated with desiccation avoidance is the acidification of extrapallial fluid present in the extrapallial space, which might dissolve recently deposited calcium carbonate crystals on the shell (Wilbur 1964; Wilbur and Saleuddin 1983). The catalyzation of bicarbonate dehydration is one of CA's functions (Supuran 2008; Lionetto et al. 2012). Therefore, the correlation found in the present study could be related to the fact that bigger specimens retain more water inside their shells, and, consequently, lower CA activity is necessary to reduce the acidification caused by the desiccation avoidance. In fact, Chapman (1997) showed that the higher relative shell weights (although not consistently related to size) of littorinid species provided a higher internal volume to accumulate extra-corporeal water and increased the water reserves within these mollusk shells.

Conclusion

It was expected that different feeding habits observed in mollusk species would promote variations in the impact of contaminants in an organism's physiology. However, from the three enzymes evaluated, only CA activity was affected by the presence of local contamination. This enzyme presented a generally lower activity in contaminated sites when compared to the reference site. The contrasting CA activity response observed in *S. brasiliensis* compared to *L. subrugosa* and *C. brasiliana* could be related to the tissue-specificity of this enzyme activity and species feeding habits. In this respect, laboratory based studies should be performed in order to confirm the relationship among feeding habits, bioaccumulation of contaminants and enzymatic activities. Results from the present study indicated that *C. brasiliana* mantle is the most suitable tissue for the use of CA analysis as a biomarker.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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