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Hemolymph and gill carbonic anhydrase are more sensitive to aquatic contamination than mantle carbonic anhydrase in the mangrove oyster *Crassostrea rhizophorae*

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ABSTRACT

Carbonic anhydrase (CA) is a ubiquitous metalloenzyme of great importance in several physiological processes. Due to its physiological importance and sensitivity to various pollutants, CA activity has been used as biomarker of aquatic contamination. Considering that in bivalves the sensitivity of CA to pollutants seems to be tissue-specific, we proposed here to analyze CA activity of hemolymph, gill and mantle of *Crassostrea rhizophorae* collected in two tropical Brazilian estuaries with different levels of anthropogenic impact, in dry and rainy season. We found increased carbonic anhydrase activity in hemolymph, gill and mantle of oysters collected in the Paraíba Estuary (a site of high anthropogenic impact) when compared to oysters from Mamanguape Estuary (inserted in an area of environmental preservation), especially in the rainy season. CA of hemolymph and gill were more sensitive than mantle CA to aquatic contamination. This study enhances the suitability of carbonic anhydrase activity for field biomarker applications with bivalves and brings new and relevant information on hemolymph carbonic anhydrase activity as biomarker of aquatic contamination.

1. Introduction

Carbonic anhydrase (CA) is a ubiquitous metalloenzyme, present in prokaryotes and eukaryotes, that catalyzes the reversible hydration of CO_2 producing HCO_3^- and H^+, using zinc as cofactor. This enzyme is involved in several physiological processes including respiration, ionic transport, acid-base regulation and calcification (Henry, 1988; Henry, 1996; Kupriyanova et al., 2017). In molluscs, CA activity was identified in several species, in different tissues (Freeman and Wilbur, 1948; Nielsen and Frieden, 1972) and its physiological role has been studied ever since. There are evidences that, in marine bivalves, gill and mantle CA are involved in the deposition of calcium carbonate ($CaCO_3$) in the process of shell formation (Wilbur and Jodrey, 1955; Duvail et al., 1998; Cudennec et al., 2006). And although hemolymph CA activity in oysters is quite expressive when compared to other oyster tissues (Nielsen and Frieden, 1972; Wang et al., 2017) or even compared to other bivalves (Henry, 1987), its physiological role has not yet been clarified.

Several *in vitro* and *in vivo* studies have demonstrated inhibition of CA activity by heavy metals in aquatic animals (Lionetto et al., 1998; Vitale et al., 1999; Lionetto et al., 2000; Gilbert and Guzmán, 2001; Skaggs and Henry, 2002; Lionetto et al., 2006; Soyut et al., 2008), as well as inhibition of CA by organic compounds, such as pesticides (Doğan, 2006; Ceyhun et al., 2010; Kolayli et al., 2011; Mela et al., 2013). Thus, due to its physiological importance and sensitivity to various pollutants, recently some authors began to consider the potential of using CA activity as biomarker of aquatic contamination (Gilbert and Guzmán, 2001; Lionetto et al., 2006; Lionetto, Caricato et al., 2012a).

CA activity measurement is a simple and low cost method, and some studies already show that it is indeed suitable for field biomarker applications in fish (de Andrade Brito et al., 2012; Prodocimo et al., 2015; Souza-Bastos and Freire, 2011) and molluscs (Caricato et al., 2010; Azevedo-Linhares and Freire, 2015). CA activity analysis in ecotoxicological studies is more common in gills. Moreover, gill carbonic anhydrase of *Crassostrea rhizophorae* was recently used as biomarker in the assessment of Brazilian estuaries and was quite responsive to aquatic contamination (Azevedo-Linhares and Freire, 2015). However,

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in bivalves the sensitivity of CA to pollutants seems to be tissue-specific (Lionetto et al., 2000; Lionetto et al., 2006; Caricato et al., 2010; Lionetto, Erroi et al., 2012b). So, we proposed here to analyze CA activity of hemolymph, gill and mantle of *C. rhizophorae* to assess two Brazilian tropical estuaries with different levels of anthropogenic impact.

C. rhizophorae is a native mangrove oyster widely distributed along Brazilian coast. It is a euryhaline and osmoconformer bivalve that has some characteristics which makes it a good sentinel organism (sessile, filter-feeding, potential to bioaccumulation of heavy metals and abundance), and therefore is widely used as bioindicator in ecotoxicological studies (Silva et al., 2003; Rebelo et al., 2005; Zanette et al., 2006; Domingos et al., 2007; Ramdine et al., 2012; Azevedo-Linhares and Freire, 2015).

This study aims to contribute to enhance the knowledge of carbonic anhydrase activity as a relevant biomarker of aquatic contamination in oysters. The goal is to identify possible differences in sensitivity of hemolymph, gill and mantle carbonic anhydrase to pollution of impacted aquatic environments.

2. Materials and methods

2.1. Study areas

The study was carried out with oysters collected in 2 tropical estuaries of Paraíba State, Northeast Brazil: The Paraíba Estuary and The Mamanguape Estuary. The Paraíba Estuary (3012 ha) is located in the metropolitan area of João Pessoa (Capital of Paraíba State), a region inhabited by about one million people, and on its right bank is located the Cabedelo Harbor, a seaport of great economic importance for the region. The estuary shows visible signs of anthropogenic impact such as degradation of mangrove areas and waste accumulation. In its surroundings there are sugarcane plantations and activities of shrimp aquaculture. The Paraíba River receives throughout its extension effluents containing domestic sewage. The Mamanguape Estuary (690 ha) is located to the north of João Pessoa and is inserted in an area of environmental preservation (APA Barra de Mamanguape, IUCN category V since 1993). The estuary has extensive well preserved mangrove areas which represents the largest mangrove area in the State. At the mouth, a bay of 6 km width is formed where there is a sandstone reef formation in a straight line that protects the entire bay. The estuary is surrounded by traditional communities of fishermen and indigenous villages. However, shrimp farming and sugarcane plantation also occur in the region, mainly near to the upstream part of the estuary.

Two sites were chosen in each estuary for oyster collection taking into consideration degree of impact and salinity gradient. In the Paraíba Estuary, collection site 1 (P1) is the point upstream and is close to the mouth of an effluent that brings a high load of domestic sewage. Collection site 2 (P2) is the point downstream, at the mouth of the estuary and near to Cabedelo Harbor (Fig. 1B). In the Mamanguape Estuary, collection site 1 (M1) is the point upstream in an area where the mangrove ecosystem is quite preserved. Collection site 2 (M2) is the point downstream, located in the bay of the estuary in a very protected and preserved area (Fig. 1A). For this study, we considered Mamanguape collection sites as our reference, especially M2.

2.2. Animals sampling

The license for oyster collection was granted by ICMBio, the Brazilian environmental agency within the Ministry of Environment (Authorization for scientific activities n° 46357–1/46357–2). The oysters were collected in October 2014 (dry season in Northeastern Brazil) and in May 2016 (rainy season in Northeastern Brazil). 10 specimens (5–9 cm long) were randomly collected at each site of each estuary in both seasons (total of 80 oysters). The animals were placed in plastic boxes with water from the collection site and were transported to the laboratory, with constant aeration. The physical-chemical parameters of the water at the collection site were measured and are shown in Table 1.

2.3. Tissue sampling

In the laboratory, the oysters were kept in plastic boxes with water from the collection site and with constant aeration for 18 to 24 h. The oysters were then measured using a digital caliper and were weighed. The shell was opened and hemolymph was sampled, with a syringe through the adductor muscle, and frozen (− 20 °C) until analysis of carbonic anhydrase activity. The total visceral content was removed and weighed, the shell was weighed and these values were used to calculate the condition index (visceral content wet weight/shell wet weight × 100) according to Lucas and Beninger (1985). Gill and mantle were then dissected and stored (freezer − 20 °C) to analyze tissue carbonic anhydrase activity.

2.4. Carbonic anhydrase activity assay

Carbonic anhydrase activity was determined according to Vitale et al. (1999) based on Henry (1991). Gills and mantle sampled were homogenized at 10% (weight (g)/volume (mL)) with tris-phosphate buffer (225 mM Mannitol, 75 mM Sucrose, 10 mM Tris base and 10 mM NaH_2PO_4, pH 7.4) using a tissue homogenizer (Homomix, Biosystems, Brazil). The homogenate was centrifuged (2000*g*, 5 min, Eppendorf 5810R, Germany) and one aliquot of the supernatant was used for the CA activity assay, and another for total protein quantification. For hemolymph CA activity assay, an aliquot of the hemolymph sample was used without centrifugation. CA activity was quantified based on the rate of pH drop along time after the addition of 1 mL of ice-cold (4 °C) CO_2 saturated distilled water in a mixture containing 0.05 mL of the homogenized or hemolymph, and 7.5 mL of tris-phosphate buffer. The drop in pH was monitored during 20 s with pH readings every 4 s (Labmeter PHS-3E, Biosystems, Brazil). The slope of the linear regression of pH against time corresponds to the rate of the catalyzed reaction (catalyzed rate, CR). Non-catalyzed rate (NCR) was assessed as the rate of pH drop along time without tissue homogenate. Specific activity was calculated as [(CR/NCR) − 1]/mg total protein in the sample or /ml of hemolymph. Total protein concentration in gill and mantle homogenates was measured according to Bradford (1976).

2.5. Statistical analysis

Data were presented as mean ± standard error of the mean. Normality of distribution and equality of variance were verified and, if necessary, data were rank transformed. Two Way ANOVA, followed by Holm-Sidak method, was used to analyze carbonic anhydrase activity and condition index. Factors were: collection site (P1, P2, M1 and M2) and season (dry and rainy).

3. Results

3.1. Abiotical factors

3.2. Condition index

Two Way ANOVA revealed that for condition index the two factors analyzed are relevant (collection site $F = 6.916$, $p < 0.001$; season $F = 13.951$, $p < 0.001$), but there is not a statistically significant interaction between them ($F = 0.647$, $p = 0.587$). High condition index was found at Paraíba Estuary collection site 1 (P1) in the dry season (Fig. 2).