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Capacity of tissue water regulation is impaired in an osmoconformer living in impacted estuaries?

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ABSTRACT

Estuarine osmoconformes rely on their ability to perform tissue and cell water regulation to cope with daily osmotic challenges that occur in the estuary. In addition, these animals currently must deal with pollutants present in the estuarine environment, which can disturb their capacity of water regulation. We collected the mangrove oyster *Crassostrea rhizophorae* in two tropical estuaries in the Northeast region of Brazil with different degrees of human interference: the Paraíba Estuary (impacted) and the Mamanguape Estuary (preserved). Tissue water content was analyzed after exposure to salinities 12, 24 and 36 for 24 h. Gill cell volume regulation was analyzed *in vitro* upon hypo- and hyper-osmotic conditions. We also analyzed gill MXR (multi-xenobiotic resistance) mechanism, as reference of environmental pollution. Gill and muscle of oysters from two sites of Paraíba Estuary, and from one site of Mamanguape Estuary were not able to maintain tissue water content upon hypo- and hyper-osmotic conditions. Gill cells of oyster from the same sites exhibited swelling followed by regulatory volume decrease upon hypo-osmotic condition. Gill MXR activity was increased in oysters from these sites. The best tissue and cell water regulation, and the lowest MXR activity, was found in oyster from downstream of Mamanguape Estuary, our reference site and the one most preserved. Tissue and cell water regulation proved to be a sensitive parameter to environmental pollution and could be considered as biomarker of aquatic contamination.

1. Introduction

Estuaries are cyclically changing environments which imposes natural stressful conditions on animals that inhabit it. Salinity is highly variable in an estuary and is one of the most important abiotic factors affecting species distribution and physiology of estuarine animals (Attrill and Rundle, 2002; Willmer et al., 2009; Telesh and Khlebovich, 2010; Maisano et al., 2016). To cope with salinity variation, these animals must, in some way, regulate their osmotic and ionic balance.

Some animals, called osmoregulators, perform Anisosmotic Extracelular Regulation (AER) through the activation of salt transport mechanisms present at the interface between the extracellular fluid and the external medium (mainly in the gill epithelium), which results in the maintenance of the osmotic homeostasis of the extracellular fluid (Evans, 2008; Larsen et al., 2014). On the other hand, animals called osmoconformers do not have this ability to regulate their body fluids, which vary in function of external osmotic variations and necessarily imposes an osmotic stress on their cells (Deaton, 2008; Foster et al., 2010; Santos et al., 2013). Thus, marine/estuarine osmoconformers that tolerate salinity variations do this by performing Isosmotic Intracellular Regulation (IIR) through the activation of cell volume regulation mechanisms (Santos et al., 2013; Castellano et al., 2016a, 2016b; Freire et al., 2008; Foster et al., 2010; Amado et al., 2015).

Cell volume regulation is of great physiological importance and its mechanisms involve several membrane transporters which direct the flux of organic and inorganic solutes to promote, through osmotic movement of water, the correction of cell volume (Wehner et al., 2003; Strange, 2004; Hoffmann et al., 2009). Upon hypo-osmotic conditions, cells can recover volume performing regulatory volume decrease (RVD) through the efflux of osmolytes, and upon hyper-osmotic conditions, cells perform regulatory volume increase (RVI) through intracellular accumulation of osmolytes (Strange, 2004; Hoffmann et al., 2009).

Oysters are euryhaline osmoconformer animals and, therefore, must have a good ability to perform tissue water regulation, since their cells

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are subject to large osmotic variation in their extracellular environment (Deaton, 2008). The oyster´s euryhaline mechanism adaptations has been studied for years, especially for the genus *Crassostrea*, and involves mobilization of intracellular free amino acids (FAAs) as the most important effectors to cell volume regulation (Pierce and Warren, 2001; Hosoi et al., 2003; Lee et al., 2004; Meng et al., 2013).

In estuaries, in addition to salinity cyclical variations, which itself is an important physiological challenge for the animals, the presence of pollutants can make the challenge even greater. A wide variety of hazardous chemicals can reach the estuaries through domestic, industrial and agricultural effluents. Heavy metals, pesticides, herbicides and fertilizers, among others, can lead to deleterious effects on estuarine animals including changes in the osmotic balance (Monserrat et al., 2007; Lewis et al., 2011), and changes in osmolytes involved in the osmotic regulation (Fasulo et al., 2012; Cappello et al., 2017).

There are several reports in the literature of pollutants (mainly heavy metals) affecting the ability of aquatic invertebrates to maintain tissue water content (Pelgrom et al., 1994; Bianchini et al., 2005; Amado et al., 2006) or to perform cell volume regulation (Amado et al., 2012; Morabito et al., 2013; Torre et al., 2013a). Thus, recently, cell volume regulation capacity has been suggested as a potential biomarker of environmental pollution (Ayrapetyan, 2012; Morabito et al., 2013; Naceur et al., 2016).

It is known that a variety of aquatic animals are constitutively equipped with a mechanism that prevents cells from being harmed by pumping potentially toxic xenobiotics out of the cell before manifesting its deleterious potential (Minier et al., 1999; Kurelec et al., 2000). These animals express the multi-xenobiotic resistance phenotype (MXR), a mechanism that has been described in various phyla of aquatic animals (Bard, 2000). The expression of MXR phenotype results mainly from the activity of a transmembrane ABC family protein, the P-glycoprotein (P-gp). P-gp binds to a variety of substrates and facilitates their efflux, thereby preventing the intracellular accumulation of xenobiotics and acting as the first line of defense against them (Kurelec, 1995; Minier et al., 1999). Due to its lack of specificity, the MXR mechanism is considered a generalized biomarker of aquatic contamination (Smital and Kurelec, 1998; Minier et al., 1999; Kurelec et al., 2000; Bard, 2000), and has been often used as a biomarker in biomonitoring studies using molluscs (Minier et al., 2006; Pain and Parant, 2007; Viarengo et al., 2007; Yawetz et al., 2010).

Therefore, the goal here was to analyze the ability of the mangrove oyster *Crassostrea rhizophorae,* collected in preserved and impacted estuaries, to perform tissue and cell water regulation, using MXR activity, a well-established biomarker of aquatic contamination, as reference for environmental pollution in the estuaries.

2. Material and methods

2.1. Study areas

The study was conducted in two tropical Brazilian estuaries with different degree of anthropogenic impact: The Paraíba Estuary and The Mamanguape Estuary, both located in the Paraíba State, Northeast Brazil (Fig. 1). The Paraíba Estuary (3012 ha) crosses the metropolitan region of the capital João Pessoa, an area in increasing urban expansion and inhabited by about 1 million people. In addition to the urban occupation that promoted mangrove degradation, discharge of domestic sewage and waste accumulation, in areas surrounding the estuary there are also sugarcane agriculture and shrimp aquaculture activities. In the right bank of the estuary, in front of the Restinga Island, is located The Cabedelo Harbor, an important seaport for the Paraíba State. Environmental degradation of the estuary can be visually noticed, and a local study detected high levels of water pollution (Sousa et al., 2015). The Mamanguape Estuary (690 ha), located in the northern part of Paraíba coast, about 80 km from the capital João Pessoa, presents the mangrove ecosystem quite preserved. The estuary is inserted in an area of environmental protection (APA Barra de Mamanguape, IUCN category V since 1993) with little human interference and is considered preserved, although in the upstream region there are also sugar cane plantations and shrimp farming activities.

2.2. Sampling and maintenance of animals

In each estuary two sampling sites of different salinity were selected: point 1 upstream and point 2 downstream (see Fig. 1). The sampling sites were also chosen according to level of impact. In the Paraíba Estuary, P1 is close to an effluent that brings a high load of domestic sewage, and P2 is near to Cabedelo Harbor. In the Mamanguape Estuary, M1 is located in an area with well-preserved mangrove ecosystem, although in its surroundings there are activities of agriculture and aquaculture, and M2 is located in a very preserved and protected area. We considered Mamanguape samplings sites as our reference sites, especially M2.

The oysters were collected with the permission of ICMBio, the Brazilian environmental agency within the Ministry of Environment (Authorization for scientific activities n° 46357–1/46357–2). Specimens (5–9 cm long) collected, manually and randomly at low tide, were placed in plastics boxes containing local water and transferred to a laboratory. Salinity, water temperature (°C) and pH were measured *in situ* using a multi-parameter probe (Horiba/U-50). In the laboratory, oysters were kept in local water, in 15-liter aquaria, at the density of 3 oysters per liter. Temperature and salinity were monitored, and water was partially renewed daily. Oysters were not fed. The aquaria were kept under constant aeration and natural photoperiod until experimentation (1 or 2 days). Independent samplings were carried out between the years 2014 and 2016 for analyses of tissue water content, cell volume regulation and MXR activity.

2.3. Tissue water content

Capacity of tissue water maintenance was investigated subjecting the oysters to salinity variation experiment. Firstly, 10 oysters from each sampling site had their shells opened and the gill and adductor muscle were sampled for tissue water content analysis (described below). The objective was to obtain a reference value of tissue water content of the oysters from each sampling site before being submitted to experimental salinity variation. Then, 15 oysters from each sampling site were subjected to salinities 12, 24 and 36 (obtained with distilled water and commercial sea salt) for 24 h. Gill and adductor muscle were sampled, and tissue water content was determined according to Freire et al. (2008). Briefly, tissue samples were weighed on an analytical balance (0.1 mg precision, Shimadzu AUY220, Brazil) and then dried at 60 °C for 24 h. The dried tissues were weighed again, and total water content was expressed as a percentage of the wet mass.

2.4. Cell volume regulation

Cell volume regulation analysis was performed on freshly dissociated cells submitted to a fluorescence self-quenching technique using Calcein-AM (Sigma–Aldrich) as described by Hamann et al. (2002) and Capó-Aponte et al. (2005) and employed in marine invertebrate cells by Amado et al. (2012) and Castellano et al. (2016b). The principle of the method is based on the property of fluorescence emission intensity increases in diluted solutions and decreases in concentrated solutions, as result of fluorescence self-quenching. This property can be used to measure relative changes in cell water volume in a directly proportional relationship between fluorescence intensity and cell volume.

Oysters collected at two sites in each estuary were acclimated for 3 days to salinity 30 (artificial sea water). Gills were removed, and the cells mechanically dissociated in a calcium-free solution (NaCl 400 mM; Na_2HPO_4 25 mM; K_2HPO_4 3.5 mM; KCl 20 mM; EDTA 5 mM). Gill cells