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Physiological and biochemical responses of three Veneridae clams exposed to salinity changes

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ABSTRACT

Given their global importance, coastal marine environments are a major focus of concern regarding the potential impacts of climate change, namely due to alterations in seawater salinity. It is known that environmental characteristics, such as salinity, affect immune and physiological parameters of bivalves. Nevertheless, scarce information is available concerning the biochemical alterations associated with salinity changes. For this reason, the present work aimed to evaluate the biochemical responses of three venerid clam species (*Venerupis decussata*, *Venerupis corrugata*, *Venerupis philippinarum*) submitted to salinity changes. The effects on the native (*V. decussata* and *V. corrugata*) and invasive (*V. philippinarum*) species collected from the same sampling site and submitted to the same salinity gradient (0 to 42 g/L) were compared. The results obtained demonstrated that *V. corrugata* is the most sensitive species to salinity changes and *V. decussata* is the species that can tolerate a wider range of salinities. Furthermore, our work showed that clams under salinity associated stress can alter their biochemical mechanisms, such as increasing their antioxidant defenses, to cope with the higher oxidative stress resulting from hypo and hypersaline conditions. Among the physiological and biochemical parameters analyzed (glycogen and protein content; lipid peroxidation levels, antioxidant enzymes activity; total, reduced and oxidized glutathione) Catalase (CAT) and especially superoxide dismutase (SOD) showed to be useful biomarkers to assess salinity impacts in clams.

1. Introduction

According to recent reports, increases in temperature, water acidification and changes in seawater salinity are predicted to occur in the next 100 years (IPCC, 2013). Therefore, identifying the effects of predicted climate changes in aquatic ecosystems must be a priority in order to maintain their biodiversity.

Among climate changes there is an increasing concern about future alterations in seawater salinity values, mainly in estuarine and coastal areas (Booij, 2005; Kay et al., 2006), which will affect the performance of native and invasive species. When different stressors act together, namely biological invasions associated with salinity changes, they may have unexpected and irreversible consequences for the native communities (Occhipinti-Ambrogi and Savini, 2003; Whitfield et al., 2007). Salinity is one of the dominant environmental factors controlling species distribution and influencing physiological processes in marine and estuarine organisms (Navarro and Gonzalez, 1998). In bivalves the impact of salinity on host–pathogen interactions (Hauton et al., 2000; Reid et al., 2003; Malagoli and Ottaviani, 2005; Gagnaire et al., 2006; Matozzo et al., 2007; Bussell et al., 2008; Dang et al., 2010; Kuchel et al., 2010; Perrigault et al., 2012), immune responses (Reid et al., 2003; Matozzo et al., 2007), metabolic and physiological alterations (Sarà et al., 2008; Coughlan et al., 2009; Carregosa et al., 2014), and endogenous rhythm (Kim et al., 2001) has been also documented. Nevertheless few studies have looked at the biochemical alterations on bivalves due to salinity changes (Pfeifer et al., 2005; Hamer et al., 2008).

It is well known that estuaries and coastal lagoons are subjected to wide variations in salinity under the impact of tidal and seasonal changes. The ebb and flood of the tide, combined with freshwater inputs from rivers and climate changes can dramatically alter the salinity of these aquatic systems. Since bivalves are mostly estuarine or nearshore in nature, they are highly influenced by these salinity variations. Furthermore, bivalves are good aquatic bioindicators because they have a wide geographical distribution, sedentary behavior, easy to sample, tolerance to a wide range of environmental conditions, and high capacity to bioaccumulate contaminants. The typically euryhaline clams *Venerupis philippinarum*, *Venerupis decussata* and *Venerupis corrugata* have been widely used as biomonitor or ecotoxicological test organisms, since they are found world-wide (Flassch and Leborgne, 1992; Usero et al., 1997; Allam et al., 2000; Kim et al., 2001; Elston et al., 2003; Jensen et al., 2004; Pranovi et al., 2006; Delgado and Pérez-Camacho,

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2007; Bebianno and Barreira, 2009; Coughlan et al., 2009; Dang et al., 2010; Ramos-Gómez et al., 2011; Figueira et al., 2012; Moschino et al., 2012; Anacleto et al., 2013; Figueira and Freitas, 2013; FAO, 2014). Apart from their ecological relevance, these species are economically important worldwide, namely in Portugal where the most recent statistics shows that the national annual production of clams represents 42% of the total shellfish production (INE, 2013), being fundamental to the national socioeconomic framework.

Different works showed that stress related biomarkers are powerful tools to assess the biological effects of contamination in bivalves, both under environmental and laboratory conditions. Biomarkers such as antioxidant enzyme activity (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) or oxidative stress parameters (lipid peroxidation, DNA strand breaks) have shown to be useful to assess organisms impacts due to stress conditions (among others, Dellali et al., 2004; Geret and Bebianno, 2004; Bebianno and Barreira, 2009; Ramos-Gómez et al., 2011; Wang et al., 2011, 2012; Freitas et al., 2012a; Silva et al., 2012).

Thus, the present work aims to study the biochemical alterations resulting from impacts of salinity changes in three Veneridae clams (*V. philippinarum*, *V. decussata* and *V. corrugata*) and assess how oxidative stress markers are correlated with these alterations. Furthermore, the present study aims to compare the performance of native and invasive species, to evaluate the potential spatial distribution of these species under the predicted climate change scenario. Since little information is available about *V. corrugata*, the present work will further enrich the knowledge on this species that inhabits coastal areas along the Atlantic.

2. Materials and methods

2.1. Sampling and experimental conditions

In the present study, the invasive clam *V. philippinarum* (Adams and Reeve, 1850), and the native species *V. decussata* (Linnaeus, 1758) and *V. corrugata* (Gmelin, 1791), formerly known as *Venerupis pullastra* (Montague, 1983) were collected in October from a sampling site at the Ria de Aveiro, a shallow coastal system located in the northwest of Portugal.

In order to minimize the effect of body size on biochemical and physiological responses to salinity changes, organisms with similar size were used in the laboratory experiments. Thus, the *V. decussata* individuals presented an average length of 49 ± 2 mm and 38 ± 2 mm width. *V. philippinarum* clams showed an average length of 50 ± 2.7 mm and 39 ± 3 mm width. *V. corrugata* specimens presented, in average, 38 ± 3 mm length and 25 ± 2 mm width.

In the laboratory, organisms were acclimated for 48 h (Freitas et al., 2012b), by placing individuals in plastic tanks with artificial seawater (salinity of 28 g/L), under continuous aeration. According to measures in the sampling site, the salinity of 28 g/L was selected as representing control conditions. After depuration, the organisms were exposed for 144 h to salinity assays, consisting on the exposure of 9 organisms/salinity (3 replicates, 3 individuals/replicate) to different salinities (0, 7, 14, 21, 28, 35, 42 g/L). Salinity was set up by the addition of artificial sea salt to deionized water. The range of salinity used was selected taking into account the salinity range found in the Ria de Aveiro, where the 3 species were harvested. In summer and winter periods these values easily reach salinities of 10 and 38 g/L, respectively (Santos et al., 2007). A plastic container with 1 L of water was used for each replicate. A temperature of 18 ± 1 °C was maintained during depuration and experimental period and the photoperiod was fixed at 12 h. During the experiment, clams were not fed, the water of each container was continuously aerated and renewed every other day and dead organisms were removed from the containers whenever the water was changed. Organisms were considered dead when their shells gaped and failed to shut again after external stimulus. At the end of the experiment, surviving organisms were frozen at −80 °C for further analysis.

2.2. Laboratory analysis

For all analyses, shells of frozen organisms were removed and the soft tissues were mechanically pulverized, in a mill, with liquid nitrogen. For each organism, the pulverized tissue was distributed in aliquots of 0.5 g. Extraction was performed using 0.5 g of pulverized clam tissues and the specific buffer for each biochemical analysis. Before biomarker quantification, samples were sonicated for 15 s at 4 °C and centrifuged for 10 min at 10,000 *g* at 4 °C. Supernatants were stored at − 80°C or used immediately to determine: glycogen (Gly) content, total protein content, lipid peroxidation (LPO) levels, superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione S-transferase (GST) activity, total glutathione (GSHt) content, and reduced glutathione (GSH) content. All the biochemical parameters were performed twice, using five replicates (n = 5).

2.2.1. Physiological analysis

For protein and glycogen content determination, supernatants were obtained using 0.5 g of pulverized clams and sodium phosphate buffer (1:2 w/v), pH 7.0 (sodium dihydrogen phosphate monohydrate 50 mM, disodium hydrogen phosphate dihydrate 50 mM, 0.1% Triton X-100).

Total protein content was determined according to the spectrophotometric method of Biuret (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standards (0–40 mg/mL). Biuret reagent (600 μL) was added to the samples (50 μL) and the mixture was shaken and let to incubate at 30 °C for 10 min. At the end of this time absorbance was read at 540 nm. Results were expressed in mg per g of fresh tissue.

Following the procedure described by Yoshikawa (1959), *glycogen* was quantified by the sulphuric acid method and glucose standards (0–5 mg/mL) were used as comparison with glycogen concentrations. Samples were previously diluted 25 times with the same sodium phosphate buffer, using 50 μL of supernatant for *V. philippinarum* and 10 μL for *V. decussata* and *V. corrugata*. For every sample, 100 μL of phenol (5%) and 600 μL of H_2SO_4 (96%) were added, shaken and then incubated at room temperature for 30 min. For *V. decussata* and *V. corrugata* the volume was made up to 750 μL with sodium phosphate buffer. Absorbance was measured at 492 nm and results were expressed as mg per g of fresh tissue.

2.2.2. Biochemical analysis

Lipid peroxidation (*LPO*) was measured by the quantification of TBARS (thiobarbituric acid reactive substances), according to the protocol described by Ohkawa et al. (1979). The supernatants were extracted using 0.5 g of pulverized clams and 1 mL trichloroacetic acid (TCA; 20% (1:2 w/v)) and then 300 μL of thiobarbituric acid (TBA) were added (0.5% in 20% (v/v) TCA). 100 μL of each sample was incubated at 96 °C for 25 min with 400 μL of thiobarbituric acid and 300 μL of TCA. The reaction was stopped by transferring samples to ice. Lipid peroxidation levels were expressed in nmol of MDA formed per g of fresh tissue. The amount of malondialdehyde (MDA) was quantified spectrophotometrically at a wavelength of 532 nm. The calculation of MDA concentration was made using its extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

Superoxide dismutase activity (*SOD*, EC 1.15.1.1) was determined based on the method of Beauchamp and Fridovich (1971), with some modifications to the microplate method. Supernatants were extracted using 0.5 g of pulverized clams and 50 mM potassium phosphate buffer (1:2 w/v), pH 7.0 (dipotassium phosphate 50 mM; potassium dihydrogen phosphate 50 mM; EDTA 1 mM; Triton X-100 1% (v/v); PVP 1% (v/v); DTT 1 mM). The standard curve was performed with SOD standards (0.25–60 U/mL). In a microplate, 25 μL of each sample (previously diluted 4 times) was incubated, at room temperature for 10 min, with 25 μL of xanthine oxidase, 56.1 mU/mL and 250 μL of reaction buffer (Tris–HCl 50 mM, pH 8.0); diethylene triamine pentaacetic acid (DTPA) 0.1 mM; hypoxanthine 0.1 mM and nitro blue tetrazolium