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Seasonal modulation of oxidative stress biomarkers in mangrove oyster (*Crassostrea gasar*) from an Amazon estuary

Carla Carolina Miranda dos Santos [a,b,1], Johnata Azevedo Ferreira [b], Cléverson Rannieri Meira dos Santos [c], Lílian Lund Amado [a,b,*]

[a] Programa de Pós-Graduação em Ecologia Aquática e Pesca, Universidade Federal do Pará, Belém, PA, Brazil
[b] Laboratório de Ecotoxicologia (ICB) e Laboratório de Pesquisa em Monitoramento Ambiental Marinho (LAPMAR), Universidade Federal do Pará, Belém, PA, Av. Augusto Corrêa, 01, Guamá, 66075110, Belém, Pará, Brazil
[c] Museu Paraense Emílio Goeldi, Av. Perimetral, 1901, Terra Firme, 66077830 Belém, PA, Brazil

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ABSTRACT

Estuaries are the final destination of many pollutants derived from anthropogenic activity. Therefore, it is difficult to find this kind of ecosystem in a pristine condition. In this context, biomonitoring studies that characterize the organism's conditions against the environment' s natural variation are essential for future impact analysis due to anthropic activity. The present study aims to characterize the natural modulation of biochemical biomarkers in oysters *Crassostrea gasar*. The research was conducted in Japerica Bay, an estuary region located in the Eastern Amazon (Pará, Brazil), which has remained in pristine condition for many years. The samplings were carried out throughout one year during the rainy-dry transition period (June/2013), dry period (September/2013), dry-rainy transition period (November / 2013), and rainy period (February / 2014) in the lower and upper estuary. The activity of glutathione-S-transferase (GST) and total antioxidant capacity (ACAP) were evaluated as biomarkers of exposure and lipid peroxidation (LPO) as an effect biomarker. In gills, GST decreased during the rainy season in both sites and increased during the salinity peak (dry-rainy transition period) for the upper estuary's organisms. In this organ, the lowest levels of LPO occurred during the dry season for both points. There was an induction of ACAP in muscle during the rainy-dry transition period compared to the dry and dry-rainy transition periods for the lower estuary's organisms, and there were no differences for GST suggesting low tissue sensitivity. There was an increase in LPO during the rainy season compared to the rainy-dry transition period for the lower estuaries animals. Biomarkers in gills suggest a metabolic challenge to the rainy season and stability during the dry season. The species shows high viability of use in biomonitoring programs. However, these seasonality-induced alterations in biomarkers responses must be taken into account to interpret the results.

1. Introduction

Coastal ecosystems present striking physical and chemical features. Salinity is on that stands out once it affects not just the bioavailability of toxic elements but also the physiology of the aquatic (Urbina and Glover, 2015). One of the primary adaptations of the estuaries organisms to wide variations of salinity is an osmotic regulation strategy such as osmoconforming that adjusts the organism's internal osmotic levels according to the environment (Schmidt-Nielsen, 1997). Therefore, the salinity changes in the environment directly affect the rate of metabolic functioning of the organisms, including the costs related to osmotic regulation, which is energetically expensive (Urbina and Glover, 2015).

Processes that require an increase in the production of ATP contribute to the formation of reactive oxygen species (ROS) from oxidative metabolism (Burdon, 1999). Thus, the imbalance between pro-oxidant molecules and antioxidant defense molecules of the organism is called oxidative stress (Pizzino et al., 2017). In addition to the high natural energy costs to survive, the increase of anthropogenic activities, and pollution (Shahidul Islam and Tanaka, 2004), can be causing the death and illness incidents of organisms in coastal

* Corresponding author at: Instituto de Ciências Biológicas, Laboratório de Ecotoxicologia e Laboratório de Pesquisas em Monitoramento Ambiental Marinho, Universidade Federal do Pará, Av. Augusto Correa, 01 66075-110 Belém, Pará, Brazil.
E-mail address: lla@ufpa.br (L.L. Amado).
[1] all authors contributed equally to the study

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environments (Kannan et al., 2005). Therefore, increasing the importance of monitoring programs that assess the conditions of the environment and its organisms.

In this context, biomonitoring is an environmental health analyse that uses samples of tissues and fluids from an organism since stress sources, whether natural or synthetic, leave markers that reflect the exposure of the organisms (Zhou et al., 2008). For this proposition, the biomarkers are used as tools in this evaluation, which are defined as molecular, cellular, histopathological, physiological, and behavioral alterations to the exposure or effect of a xenobiotic (Lam and Gray, 2003) or due to the general alterations of the environmental conditions (Livingstone, 1993). The biomarkers results provide information on the biological responses to the natural or synthetic chemical substances absorbed by the organisms and the corresponding induced effects (Zhou et al., 2008). Thereby, they can indicate the exposure and evaluate it (biomarkers of exposure), in addition to elucidating the deleterious effects of exposure (effect biomarkers) (Sogorb et al., 2014).

Many tissues can be used in biochemical analyses, but the gills are one of the most important organs for aquatic species as they are responsible for breathing and ionic regulation, besides being the main access for substances enter the animal's system (Chakraborty et al., 2010). Also, the adductor muscle can be used for biochemical analyses. This organ is important in keeping valves stable and giving the mollusks the ability to open and close their valves in situations of danger or environmental instability, therefore, being fundamental to animal's survival (Castro-Claros et al., 2020).

Among the mollusks in coastal areas, the mangrove oysters of the genus Crassostrea are highly abundant and economically important as sources of food and income for much of the population in this region (Duncan, 2003). Several characteristics make them good biomonitors, such as their wide geographical distribution and accessibility to their natural environment or through aquaculture (Férnandez et al., 2012); they have a benthic habit and are low mobility filters, which allows direct contact with the sediment, thus reflecting the local reality of the environment (Monserrat et al., 2003a, 2003b).

A major controversy in the biomonitoring of estuarine environments is the ambiguity of results because of natural, physical, and chemical variations. Added to the presence of pollutants, there is an increase in the metabolic demand on the animals that live there since physical and chemical fluctuations influence the bioavailability of many toxic compounds (Witters, 1998). Therefore, knowledge of the natural modulation of biological markers prior to anthropic impacts is essential. The present study aims to characterize the seasonal modulation of oxidative stress biomarkers in oysters *Crassostrea gasar* over a year in an Amazon estuary (Japerica Bay).

2. Materials and methods

2.1. Crassostrea gasar sampling

Japerica Bay is an estuary located in the northeast of Pará, within the "Salgado paraense", a micro-region of the Amazonian coastal zone, characterized by estuaries with a low fluvial discharge due to the significant influence of the Atlantic Ocean (Fig. 1). One of the main municipalities on the margin of Japerica Bay is the city of Primavera, which has several different rivers joining with their respective mangroves until it meets the Atlantic Ocean.

Two sites in the mangroves were chosen according to the salinity gradient to achieve the proposed objectives: A) Lower estuary, an area under conditions of higher salinity and longer distance from the city of Primavera; B) Upper estuary, an area closest to Primavera (Fig. 1). The sites selection also followed a gradient of influence of anthropic pressure, where the higher impact zone was the city of Primavera. It should also be noted that at the time of the sampling, close to the upstream of the Japerica Bay estuary, a cement factory was being constructed and began operating in April 2016. Therefore, these results reflect the condition of the animals prior to 2013 and the influence of the factories activity.

The sampling took place quarterly over a year. The first sampling during the transition period I (rainy-dry, June/2013), the second in the dry period (September/2013), the third in the transition period II (dry-rainy, November/2013), and the last during the rainy period (February/2014). The sampling months were selected based on rainfall data taken from the station of the National Water Agency (ANA) located in the city of Primavera.

Manual picking by 20 to 30 individuals during the low tide was the

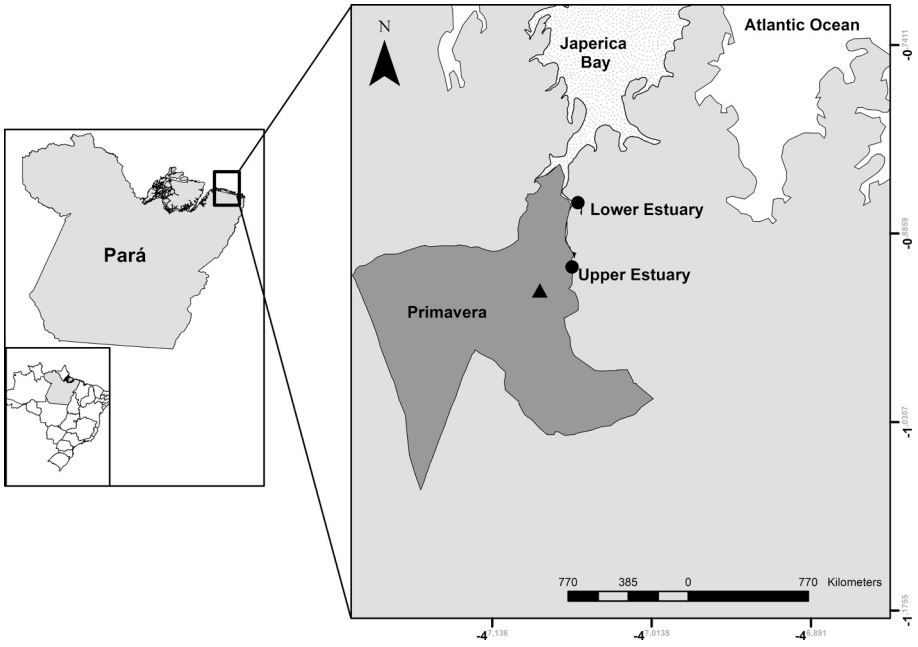

Fig. 1. Geographic location of the Japerica Bay area (north of the municipality of Primavera), indicating the two sampling sites in the Japerica Bay: Lower estuary - 00° 51′43.7"S; 47° 04′ 12.4" W andUpper estuary −00° 54 '41.7"S; 47° 04′28.6"W. The triangle indicates a National Water Agency (ANA) station- 00°55″45.84" S; 47° 05′ 57.84" W.

technique used in sampling the organisms. The larger animals were prioritized in the sampling effort, and straight after the capture, the individuals were cryoanesthetized in ice (0 °C) in order to minimize stress on the animals and preserve their metabolic characteristics. At the time of each sampling (low tide), the water's physical and chemical parameters were recorded at all sites, such as salinity, pH, dissolved oxygen, conductivity, and water temperature. During the same day of sampling, the animals were measured using a caliper (0.01 mm). The dimensions analyzed were: the greatest width of the valves in frontal view and the length and height of the valves. The animals were also weighed using a semi-analytical scale (0.01 g). First, the total weight of the organisms was measured, and later, after removing all the tissues from the animals, only the valves were weighed (to obtain the soft tissue weight). The gills and portions of the adductor muscle were removed and immediately placed in eppendorf tubes for storage in an ultra-freezer (−80 °C) until the moment of biochemical analysis.

2.2. Oxidative stress biomarkers analyses

The gill and muscle samples were homogenized (1: 4, *w/v*) in buffer with the pH adjusted to 7.6 according to the methodology used by Bainy et al. (1996). The homogenates were centrifuged at 20,000 x*g* for 20 min at 4 °C. The supernatant was removed, aliquoted, and stored at −80 °C until the dosing time.

The total proteins analysis was performed with a commercial kit (Doles Ltda, Brazil) based on the Biuret test (0.114 M citrato trisodium, 0.21 M sodium carbonate, and 0.01 M copper sulfate) for proteins. The essay was performed on a multimodal microplate reader (Victor X3, Perkin Elmer) at 550 nm, and the results were expressed in milligrams of protein / mL.

The total antioxidant capacity against peroxyl radicals was analyzed following Amado et al. (2009). The readings were performed in a fluorescence microplate reader (485 and 530 for excitation and emission, respectively) for one hour (Victor 2, Perkin Elmer). The results were then expressed as the inverse of the relative area.

The measurement of GST was based on the work of Habig et al. (1974) and Habig and Jakoby (1981). The readings were performed on a spectrofluorimeter (Victor 2, Perkin Elmer) with a microplate reader. The results were expressed in UGST / mg of protein that represents the necessary amount of the enzyme to conjugate 1 μMol of CDNB (1-Chloro-2,4-dinitrobenzene) / min/ mg of protein, at 25 °C and pH 7.0.

Lipoperoxidation was determined according to Hermes-Lima et al. (1995) and adapted for microplates according to Monserrat et al. (2003a). The samples were homogenized (1: 6 m/v for adductor muscle and 1:10 for gills) in cold 100% methanol (4 °C). The homogenates were centrifuged at 1000 ×*g* for 10 min at 4 °C. The readings were taken in a spectrophotometer at a length of 550 nm, and the lipid peroxides content was expressed as nM CHP (Cumene hydroperoxide) / g of wet weight.

2.3. Statistical analyses

For the parametric analysis, the assumptions of normality (Shapiro Wilks test) and homoscedasticity (Levene test) were tested. The Analysis of Variance (ANOVA) and post hoc tests (Tukey's test) were applied to help verify the significant differences between the sampling sites and climatic periods, and the data was expressed in mean ± standard error. In cases where the parametric analysis was not possible, we used the non-parametric test of Kruskal-Wallis to verify the significant differences between sampling sites and climatic periods. The data was expressed in median ± first quartile. The level of significance adopted was 5% (Zar, 1984).

3. Results

3.1. Pluviometric data and physical-chemical parameters of water

Fig. 2 shows the pattern of precipitation in the Primavera region over the period 2013–2014. Based on these standards, the months of sampling were selected. Among the months in which the samplings occurred, the highest precipitation occurred in February 2014 with 386 mm, while November 2013 presented the lowest value of 31 mm. June 2013 and September 2013 presented values of 97 mm and 68 mm, respectively.

An inversely proportional relation was observed between the values of salinity and rainfall, in which the periods of increased precipitation showed lower salinity values and the periods of low rainfall showed an increase in salinity values on both sites (Fig. 2).

During the transition period I (rainy-dry), the upper and lower estuaries showed salinity values of 2 and 3, respectively. During the dry season, they showed values of 20 and 14, respectively (Fig. 2), following the decrease in rainfall levels. The transition period l seems to be influenced by the history of physical and chemical conditions from previous months. Therefore, it is a transition season between the high freshwater inflow into the estuary by the previous rainy months and the beginning of the dry period's drop in rainfall.

During the transition period II (dry to rainy), the upper and lower and estuaries showed salinity values of 40 and 38, respectively (Fig. 2), evidencing the process of salinization of the environment from the drop in rainfall. In the rainy season, they showed salinity values of 11.5 and 7, respectively, a decrease in relation to transition II and in accordance with higher rainfall levels (Fig. 2).

In transition periods I and II, no significant differences occurred between the points in the salinity values, while in dry and rainy periods, the lower estuary point presented with higher values. Both points had a peak in salinity during transition period II when low precipitation occurred (Fig. 2). The lowest values were recorded during transition period I (Table1).

It was observed that the water temperature did not vary between the points and also between climatic periods, maintaining an average of 30 °C. The pH remained neutral (7–7.5) at all sampled sites throughout all periods (Table 1).

In the lower estuary, dissolved oxygen and conductivity were higher in all periods presenting values of 5–7 mg / L and 24–60 μS / cm, respectively (Table 1).

3.2. Biometry

Among the sampling sites during the dry season, there was a difference in the measures of total length, height, and weight, where the organisms from the lower estuary had the highest values (7.28 ± 6.81 cm, 9.6 ± 8.89 cm, and 37.5 ± 30 g respectively) (Table 2).

During the transition period I, the animals from the lower estuary differed from all of the animals sampled in the dry season measurements where there was an increase in body dimensions (Table 2). Animals sampled in the rainy season (5.84 ± 5.2 cm and 8.64 ± 7.27 cm respectively) also differed from organisms collected in the dry season (7.28 ± 6.81 and 9.6 ± 8.89 cm respectively) in total length and height, presenting smaller values. There were also differences in weight between oysters from transition period I and those from the rainy season, where the latter had the highest value (31 ± 19.75 g).

There were no differences in the animals total length and width from the upper estuary between climatic periods. The animals sampled during the transition I and dry periods (6.73 ± 6.13 cm and 7.09 ± 6.21 cm respectively) differed in height from those sampled during the transition period II and the rainy periods (9.52 ± 8.94 cm and 9.19 ± 7.85 cm respectively). The differences in weight were only recorded during the rainy period (Table 2).

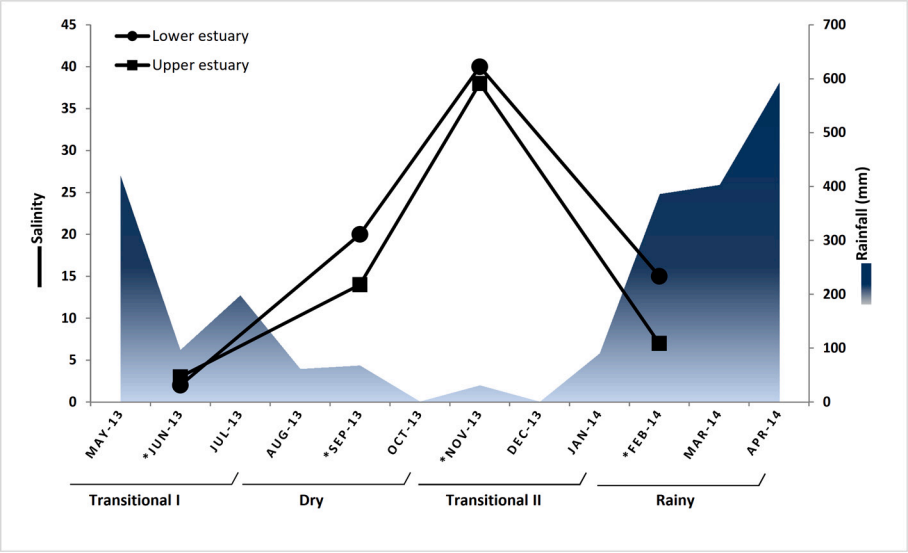

Fig. 2. Precipitation recorded in Primavera, PA from May/2013 to April/ 2014 (data obtained from ANA website <https://www.ana.gov.br/>) represented by the area graph. The lines represent salinity variation of both sites throughout the study period. Dots represent salinity values of Lower estuary and squares represent salinity values of Upper estuary.

Table 1
Physical-chemical parameters of the sites sampled over four seasonal periods in the estuary of Japerica Bay, PA. The data are presented as the values recorded at low tide. (Temp. = Temperature; DO = Dissolved Oxygen; Conduct. = Conductivity; Salt. = Salinity; Lower Est. = Lower Estuary; Upper Est. = Upper Estuary).

Period	Site	Water Temp. (°C)	pH	DO (mg/L)	Conduct. (µS/cm)	SAL.
Transition I	LOWER EST.	29	7.52	7.96	27.6	2
	UPPER EST.	29.8	7.41	5.33	14.64	3
Dry	LOWER EST.	30	7.54	6.58	44.5	20
	UPPER EST.	28.7	7.35	3.07	31.8	14
Transition II	LOWER EST.	28	7.43	6.58	61.2	40
	UPPER EST.	28.4	7.32	3.07	59.8	38
Rainy	LOWER EST.	28.45	7.32	5.69	24.15	11.5
	UPPER EST.	27.6	7.32	3.66	15.33	7

Table 2
Biometrics of *Crassostrea* sp. for all sites sampled over four seasonal periods in the estuary of Japerica Bay, PA (n = 25–35).Data are expressed in median ± quartile. Different capital letters represent differences between periods for the same location and different small letters represent differences between locations in the same period.

Period	Site	Total length (cm)	height (cm)	Width (cm)	Tissue weight (g)
Transition I	Lower EST.	5,3 ± 4,83 Aa	7,2 ± 6,69 Aa	2,9 ± 2,7 Aa	20,5 ± 17,5 Aa
	Upper EST.	5,4 ± 4,87 Aa	6,73 ± 6,13 Aa	2,92 ± 2,48 Aa	16,5 ± 12,25 Aa
Dry	Lower EST.	7,28 ± 6,81 Ba	9,6 ± 8,89 Ba	3,78 ± 3,57 BCa	37,5 ± 30 Ba
	Upper EST.	5,19 ± 4,73 Ab	7,09 ± 6,21 Ab	3,25 ± 2,55 Aa	18 ± 12 Ab
Transition II	Lower EST.	6,49 ± 5,52 BCa	7,9 ± 7,02 ACa	4,69 ± 3,25 Ba	25 ± 18 ABa
	Upper EST.	5,86 ± 5,29 Aa	9,52 ± 8,94 Ba	2,71 ± 2,31 Aa	27,5 ± 19,25 ABa
Rainy	Lower EST.	5,84 ± 5,2 ACa	8,64 ± 7,27 Ca	3,25 ± 2,38 ACa	31 ± 19,75 Ba
	Upper EST.	6,15 ± 5,36 Aa	9,19 ± 7,85 Ba	3,21 ± 2,56 Aa	26 ± 19 Ba

3.3. Oxidative stress biomarkers

3.3.1. Gills

There were no differences in total antioxidant capacity between the organisms at the different sampling sites or between climatic periods (Fig. 3a). There were also no differences in GST activity and lipoperoxidation between the sites for any of the sample periods (Fig. 3b and Fig. 3c).

For animals from the lower estuary, the transition I and dry periods (11.3 ± 10.14 and 13.32 ± 11.17 UGST / mg of protein; $p = 0.016$ and $p = 0.041$ respectively) differed from the rainy season where oysters presented with less enzyme activity (6.49 ± 4.50 UGST / mg protein), as illustrated in Fig. 3b. For animals from the upper estuary, the organisms sampled in the transition II (17.29 ± 15.25 UGST / mg of protein) differed ($p = 0.008$) from those sampled in the rainy season (6.01 ± 4.31 UGST / mg of protein), presenting more significant activity of this enzyme (Fig. 3b).

For organisms from the lower estuary, there was a difference between the dry period - which presented a lower lipoperoxidation content (11.34 ± 2.07 nMol of CHP / g of wet tissue) - transition I and rainy periods (24.45 ± 2.04 and 53.98 ± 1nMol of CHP / g of wet tissue; $p = 0.014$ and $p < 0.0001$ respectively) as illustrated in Fig. 3c. For oysters from the upper estuary, the dry period differed from the others ($p = 0.001$ in transition I, $p = 0.002$ in transition II, and $p < 0.0001$ in the rainy season), presenting the lowest peroxidized lipid content (7.93 ± 1.01 nMol of CHP / g of wet tissue) (Fig. 3c).

3.3.2. Adductor muscle

There was no difference in the total antioxidant capacity and lipoperoxidation (Fig. 4a and c) between the sites in any of the climatic periods. A decrease in the antioxidant capacity of the lower estuary organisms was observed during the dry and transition II periods (0.321 ± 0.24 and 0.367 ± 0.29 $p = 0.023$ and $p = 0.005$ respectively) in comparison to the transition period I (1.062 ± 0.65), while for the

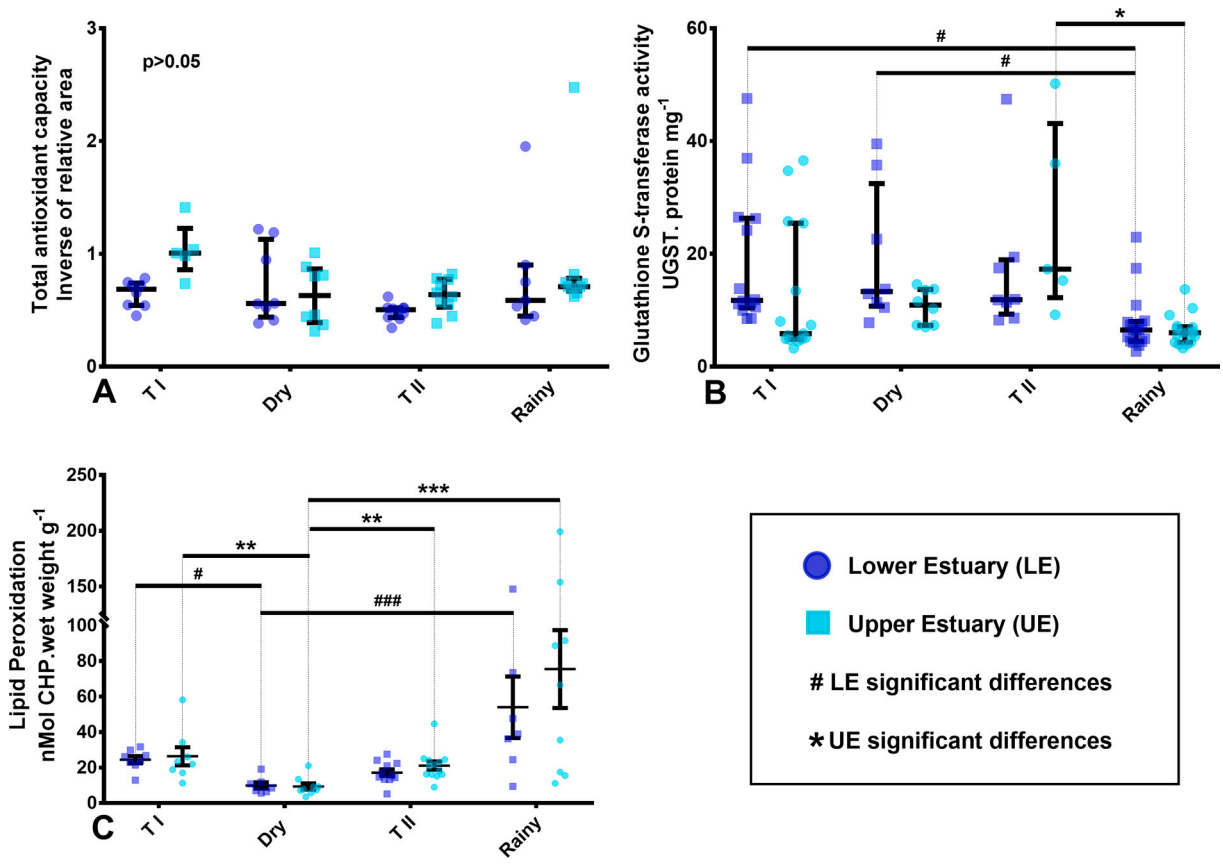

Fig. 3. (a) Total antioxidant capacity (n = 5–10); (b) Activity of Glutathione-S-transferase (n = 5–17); (c) Lipoperoxidation (n = 6–19) in gills of *Crassostrea* gasar from lower and upper estuary along different seasonal periods. Graphs (a) and (b) express data in median ± first quartile. Graph (c) expresses data in mean ± standard error. TI = Transition I (rainy-dry); TII = Transition II (dry-rainy); LE = Lower Estuary; UE = Upper Estuary. Dots represent data from each treatment plotted. The hashtag symbol (#) represents differences between climatic periods for Lower Estuary organisms, the asterisk symbol (*) represents differences between climatic periods for Upper Estuary organisms. One single symbol = $p < 0.05$, double symbol = $p < 0.005$, triple symbol = $p < 0.0005$.

animals from the upper estuary there were no differences between the sampled periods (Fig. 4a).

There were also no differences in GST activity between locations or between climatic periods for animals from both sampled sites (Fig. 4b).

The animals in the lower estuary presented with differences ($p < 0.0001$) between the transition period I and rainy periods, with higher lipoperoxidation content in the latter (10.62 ± 9.26 and 30.23 ± 24.23 nMol of CHP / g of wet tissue, respectively). The animals in the upper estuary did not differ between climatic periods regarding lipoperoxidation (Fig. 4c).

4. Discussion

4.1. Physical-chemical parameters of water

All sampled sites had an average temperature of around 30 °C. This pattern is expected in tropical estuaries where there are low variations in the sunshine and high rainfall during the whole year, expecting an average temperature of 27 °C (Monteiro et al., 2015). Neutral pH is typical of rivers of white water in the Amazon (Holland et al., 2017) due to high concentrations of carbonate salts, bicarbonates, alkali metals, and mineral salts with an average of 6.2–7.2 (Val and Almeida-Val, 1995; Oliveira et al., 2011).

The area with the most significant estuarine influence had the highest values of dissolved oxygen and conductivity. This may be associated with the influence of hydrodynamics, from the entry of ocean waters that promote greater turbulence and increased diffusion of atmospheric oxygen in the water (Moriarty, 1986). In addition, they inject a large amount of salts, changing the water's ionic composition throughout the tidal cycles (Millero, 1984; Sumner and Belaineh, 2005), thus affecting conductivity. According to Boyd (1989), the solubility of dissolved oxygen is inversely proportional to salinity and temperature and is, therefore, more soluble in freshwater and cold environments.

During the transition periods, salinity did not vary between sites since these periods are characterized by high hydrodynamic and the physical and chemical instability of water in the estuary. While in the dry season, when there is a higher concentration of salts, higher values were registered in the lower estuary, which is located where the influence of marine water is more significant (Fairbridge, 1980), contributing even more to the salinization process. Both sites presented salinity and conductivity peaks during transition period II, which can be explained by the cumulative effect of the drop in rainfall initiated during the dry period. This promotes an increase in the concentration of salts dissolved in water through tidal currents (Krumme et al., 2012) that can penetrate up to 60 km within estuaries from Pará (Berrêdo et al., 2008). In transition period I, the lowest values were recorded, possibly due to intense rainfall during the previous four months since these parameters are highly dependent on the volume of rainfall (Sumner and Belaineh, 2005). This inverse relationship between salinity and rainfall verified in the present study confirms the division into four distinct climatic periods.

4.2. Biometry

The higher biometric values recorded in the animals from the lower estuary during the dry season, compared to the other site and other

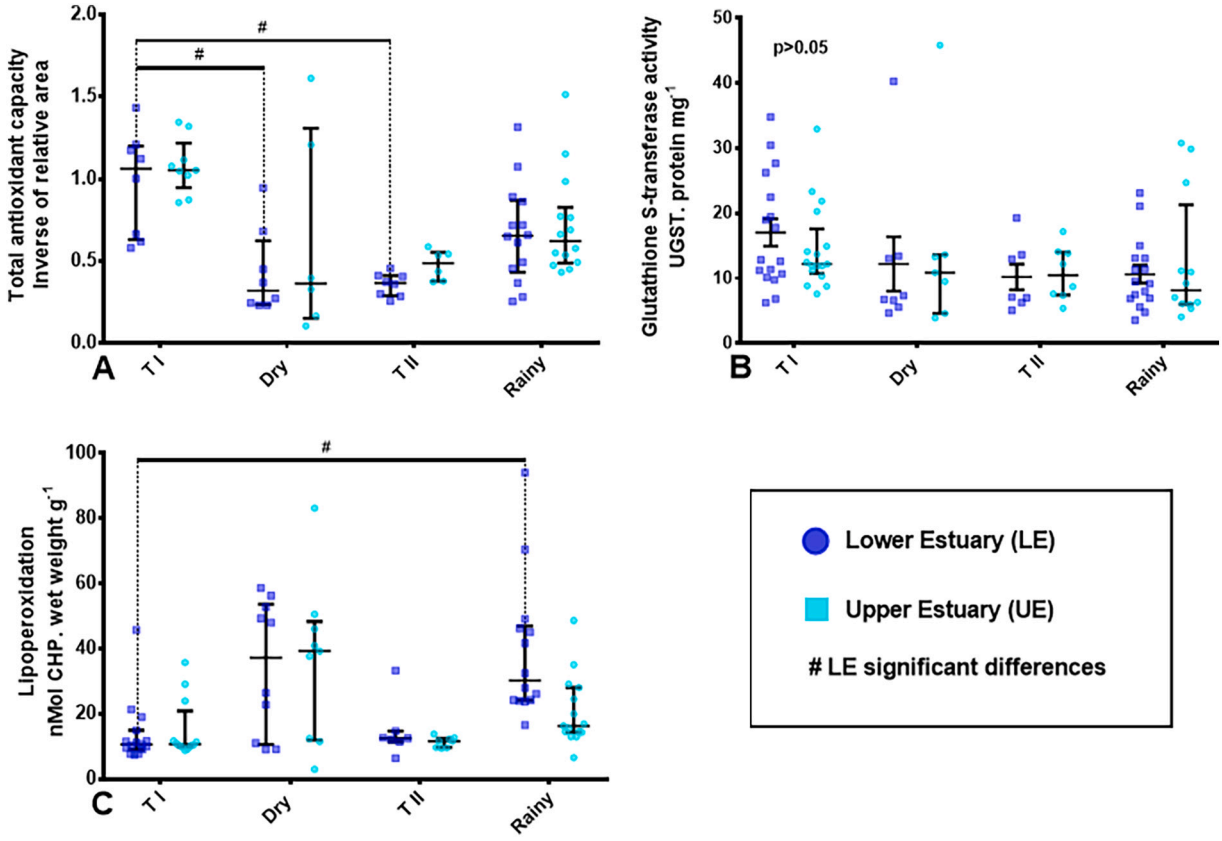

Fig. 4. (a) Total antioxidant capacity (n = 5–10); (b) Activity of Glutathione-S-transferase (n = 7–17); (c) Lipoperoxidation (n = 7–16) in adductor muscle of *Crassostrea gasar* from lower and upper estuary along different seasonal periods. Graphs (a) and (c) express data in median ± first quartile. Graph (b) expresses data in mean ± standard error. TI = Transition I (rainy-dry); TII = Transition II (dry-rainy); LE = Lower Estuary; UE = Upper Estuary. Dots represent data from each treatment plotted. The hashtag symbol (#) represents significant differences between climatic periods for Lower Estuary organisms, the asterisk symbol (*) represents differences between climatic periods for Upper Estuary organisms. A single symbol = $p < 0.05$.

periods (transition period I and rainy periods), reflect greater energy and metabolic stability of the organisms caused by the increased salinity. It is also noteworthy that the oysters from the upper estuary had the highest height values during the transition period II when the peak of salinity was recorded in both locations. Funo et al. (2015), in an experimental study on the influence of salinity on the growth and survival of *C. gasar* oysters, found that the highest biometric values recorded were: in salinity 25 for height, in salinity 20 for length, in salinities 20 and 25 for width and salinity of 20–30 for weight. They found an even higher survival rate for individuals in the range of 20–25, and the value recorded in the dry period for the lower estuary occurred within all the ranges mentioned.

Still, in the work of Funo et al. (2015), the lowest values for all dimensions were recorded under conditions of low salinity (around 5). Oliveira et al. (2018), in a study on the influence of seasonality on the growth performance of *C. gasar*, verified that organisms from areas with low salinity showed a limited growth and weight gain and also concluded that growth performance is highly related to the proximity of the oyster farm to the ocean. This corroborating the data from the present study where the values recorded for the transition periods I and rainy were close to 5 (especially in the lower estuary during transition I) and marked by the drop in biometric values.

It is known that oysters, as well as most aquatic mollusks, are osmoconformers (Berger and Kharazova, 1997a, 1997b; Larsen et al., 2014). Moreover, because they are typical of estuarine environments, they have a high capacity to control their cell volume (Wilmer et al., 2004). However, to survive under situations of osmotic stress, they can reduce the energy spent on growth (Funo et al., 2015) doing this through a series of measures such as closing the valves to stop the passage of salts (Berger and Kharazova, 1997a, 1997b), decreasing the filtration rate that affects the growth (Guimarães et al., 2008). Chang et al. (2016), in a study about the effect of salinity on the filtration rate of juveniles *C. iredalei,* verified lower filtration rates in salinities 15 and 45, considered extreme values that impose osmotic stress.

The increase in the weight of oysters from both sampling sites, during the rainy season, may be related to the accumulation of energetic compounds for the time of spawning. This event has already been reported during periods of high rainfall and low salinity in some species of the genus, such as *C. virginica* (Joshep and Madhyastha, 1984; Martínez et al., 1995; Zamora et al., 2003) and during the dry season for native species such as *C. gasar*. It can also be linked to an increase in the filtration rate and the availability of nutrients that also influence reproductive events and gonadal maturation (Giese and Pearse, 1979; Zamora et al., 2003).

4.3. Biomarkers

4.3.1. Gills

The gills proved to be sensitive to seasonal changes in the environment since they are the main organ of the animal to come into contact with water, coordinating filtration, respiration, and ion exchange and also functioning as the main interface of animals with contaminants (Viarengo, 1989). This knowledge is important in biomonitoring programs, once the choice of responsive organs is fundamental in the evaluation of results since the modulatory capacity of the antioxidant system is not standardized being tissue and species-specific (Crawford et al., 2000).

The results obtained showed that the animals at both sites had

decreased GST activity during the rainy season. This suggests a greater expenditure of energy in maintaining homeostasis (especially relating to the osmotic balance) in periods of greater rainfall and hydrodynamics, with more significant daily fluctuations in the physical and chemical parameters.

Mangrove oysters are osmoconformers, so their internal ionic concentrations follow the variations in the surrounding environment by adjusting their cell volume (Hosoi et al., 2003). Since environmental fluctuations affect the physiology of animals (Oliva et al., 2012) and promote changes in the metabolic rate linked to the generation of pro-oxidants (Pariechar et al., 1997), suppression of the antioxidant defense system may occur generated by the increased osmotic demand of this period promoted by the dilution of the waters. This is reinforced by the high amount of peroxidized lipids recorded, especially with animals in the lower estuary.

It is known that changes in salinity can induce varied physiological responses to bivalve mollusks by altering other basic functions such as the filtration rate (Bernard, 1983). This occurs because metabolic adjustments related to osmotic regulation can result in energy expenditure to maintain levels of salt and water (Cheng et al., 2002). Ivanina et al. (2020). in a study about the effect of salinity and pH on cellular metabolism in biomineralizing tissues of *C. gigas* found the highest metabolic rate at intermediate levels of salinity (18) when compared to high (30) and low (10) salinities. The organisms presented the lowest cellular metabolism at low salinity, and the authors mentioned that it could be a response to osmotic stress.

The suppression of the antioxidant defense system can occur due to the excess of reactive species leading to oxidative stress (Gurer-Orhan et al., 2004). Several experimental studies with Crassostrea oysters point to high mortality and low animal development at low levels of salinities (Heilmayer et al., 2008; Funo et al., 2015; Ivanina et al., 2020) or during the rainy season (Ajana, 1980). Animals of this genus show better development and survival in salinity ranges of 15–25 in experimental conditions and tolerance of 10–40 in estuaries from Pará (Funo et al., 2015).

It is also worth noting that during the rainy season, the nutritional factor contributes to the levels of oxidative stress since the filtration rate is positively correlated with salinity (Ehrich and Harris, 2015), decreasing the intake of antioxidants during the rainy season. González-Fernández et al. (2017), in a study with the mollusk *Mytillus galloprovincialis,* observed a strong influence of the nutritional status on the expression of many genes related to detoxification such as those of cytochrome P450, GST-α, and metallothioneins. He also concluded that low nutrition leads to a significant decrease in this expression, thus increasing the chances of oxidative damage. These results corroborate with the data visualized in the present study, with lipid peroxidation occurring mainly in the animals from the lower estuary during a period of low salinity (rainy period and transition I).

There was a tendency to increase (although not statistically significant) GST activity in transition period II, and it is worth noting that the peak salinity occurred during this period. Therefore, in addition to all environmental factors, the ecological strategies of animals also affect the behavior of the biomarkers. The increase in salinity can serve as a reproductive trigger for some species of mangrove oysters, such as *Crassostrea gasar*, activating the release of gametes (Paixão et al., 2013). Basic vital functions that require increased metabolic activity, such as reproduction, can act as a source of oxidative stress by decreasing antioxidant defenses with or without increasing the generation of ROS, therefore weakening the body's redox system (Alonso-Alvarez et al., 2004).

An increase in body dimensions was observed during the transition period II, especially with animals from the lower estuary, along with high efficiency of the organism's antioxidant defenses from both estuaries' sites, evidenced by a low content of peroxidized lipids. Therefore, it is suggested that *Crasssotrea gasar* are better adapted to the highest salinities in the Amazon and can improve reproduction and growth without deleterious effects on their antioxidant defenses. Therefore, it can be expected that biomarkers will be affected by seasonality, especially in the rainy season, while in other periods, it will be easier to gouge the potential effects of future human activities.

The non-differentiation between points for both biomarkers shows that seasonality is a fundamental factor in modulating the animals' antioxidant defense system. According to Sheehan and Power (1999), seasonality is an essential factor in the physiology of mollusks because it affects important parameters in the development of these animals, such as food availability, reproductive phase, and the growth rate, among others, hence causing fluctuations in oxidative stress throughout the year. Studies already analyzed the seasonal influences on feed dynamics and their results on energy allocation for vital functions such as reproduction in oysters *Crassostrea corteziensis* (Hurtado et al., 2012).

4.3.2. Adductor muscle

The results obtained suggest that the adductor muscle antioxidant response is not very sensitive to physical and chemical (especially salinity) changes in water, evidenced by the non-differentiation between sites or seasons for GST activity in both locations. The primary function of the adductor muscle in bivalves is to keep the valves connected and stabilized (de Simone, 2019) and is also important energy storage of carbohydrates and proteins (Barber and Blake, 2006). According to Grieshaber and Gäde (1977), exposure to air is the main modulator of muscle capacity and muscle exhaustion level. Therefore, its metabolic machinery is mainly aimed at the periods of low tide in which there is a need to close the valves due to the oxygen deficit, performing anaerobic reactions. These reactions require a large endogenous energy store, such as glycogen, to initiate the main anaerobic pathway of mollusks known as the opine pathway (Livingstone, 1983). Due to this, the adductor muscle has a high activity of enzymes such as analopine and strombine dehydrogenase (Zwaan et al., 1983), both from the oxidoreductase family. Strombine is the main opine resulting from pyruvate, and its accumulation occurs only in this tissue under hypoxia conditions Zurburg et al. (1982). Thus, saline stress and the variation of other physical-chemical parameters were not easily detected by this tissue due to its low metabolic apparatus in terms of osmoconforming.

The animals from the lower estuary showed a high content of peroxidized lipids during the rainy season, and also during the dry season. Concomitantly, the antioxidant capacity increased during the transition period I (low salinity) in relation to the periods of higher salinity (dry and transition II). The demand for osmotic regulation caused by the decrease in salinity may generate an increase in adductor muscle activity in oysters by closing valves as a protection strategy against osmotic stress (either by decreasing or increasing salinity outside the tolerance limits of species) (Shumway et al., 1977; McFarland et al., 2013), the so-called facultative pseudo-osmoregulation (Berger and Kharazova, 1997a, 1997b).

The closure of the valves has a significant impact on the physiology of animals and can lead to a significant decrease in metabolic rate and ATP consumption, to support the system only by fermentation reaction (Meng et al., 2018) or even cause death through low oxygen consumption during long-term periods (Lombardi et al., 2013). The recovery period after ischemia plays a key role in the increase of reactive species production. Experiments suggest that only when oxygen returns during reperfusion are there damages to the cellular structure (McCord, 1985). According to Dykens and Shick (1988), the process initiates from the conversion of enzyme xanthine dehydrogenase to xanthine oxidase after an increase of intracellular Ca^{2+} concentration during ischemia, at the same time an accumulation of xanthine oxidase substrates such as xanthine and hypoxanthine (both derivative from ATP degradation) occurs. The oxidase uses molecular oxygen as a cofactor, generating superoxide radicals ($O_2^{\bullet-}$) at the end of the reaction. Added to this fact, the intense anaerobic activity in the adductor muscle can lead to exhaustion culminating with an accumulation of opines as previously mentioned (Zwaan et al., 1983), but these substances are also produced

during the effort and the recovery period (Livingstone et al., 1981) impacting on the functioning of cells mainly by decreasing the hemolymph pH (Ocaño-Higuera et al., 2011).

The low levels of lipoperoxidation visualized during the transition period I still point to efficient mitigation of oxidative damage. Therefore, periods with less metabolic spending from the osmotic regulation (dry) can leave room for deleterious effects arising from the energy metabolism of other physiological needs (such as reproduction) or even from possible future human impacts.

Therefore, variations in physical and chemical parameters, especially salinity, although it does not directly affect the modulation of the antioxidant system of the muscles, have indirect effects through adaptive strategies that modify cellular homeostatic conditions, transmitting secondary metabolic effects of these fluctuations.

Similarly, during the rainy season, there was an increase in the values of soft weight, especially with the animals of the upper estuary –influenced mostly by hyposaline waters - suggesting that despite all the metabolic demand imposed by the environment, there is still no energy impairment of the other vital functions, such as growth.

5. Conclusion

The results allowed us to conclude that the biomarkers in gills present modulation influenced by seasonality, with a more significant metabolic challenge for organisms during the rainy season with the suppression of antioxidant defenses. This period may set precedents for potential deleterious effects from future sources of anthropic activity, given the greater weakness of antioxidant defenses.

The organisms appear to be better adapted to the conditions of high salinity, appearing to be the period of drought that is energetically stable in terms of osmoconformation. However, other vital functions associated with increased salinity naturally impose cell damage in the body. While muscle biomarkers were no less sensitive to natural changes in the environment, the tissue modulation was related more to its activity during the periods of low tide. The applicability of these organisms in biomonitoring programs is high since they are sensitive to changes in the environment and present physiological plasticity conducted by climatic periods. However, these natural modulations in oxidative stress biomarkers must be taken into account in the interpretation of the results of biomonitoring studies.

Declaration of Competing Interest

None

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