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Native and exotic oysters in Brazil: Comparative tolerance to hypercapnia

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ABSTRACT

Environmental hypercapnia in shallow coastal marine ecosystems can be exacerbated by increasing levels of atmospheric CO_2. In these ecosystems organisms are expected to become increasingly subjected to pCO_2 levels several times higher than those inhabiting ocean waters (e.g.: 10,000 µatm), but still our current understanding on different species capacity to respond to such levels of hypercapnia is limited. Oysters are among the most important foundation species inhabiting these coastal ecosystems, although natural oyster banks are increasingly threatened worldwide. In the present study we studied the effects of hypercapnia on two important oyster species, the pacific oyster *C. gigas* and the mangrove oyster *C. brasiliana*, to bring new insights on different species response mechanisms towards three hypercapnic levels (ca. 1,000; 4,000; 10,000 µatm), by study of a set of biomarkers related to metabolic potential (electron transport system - ETS), antioxidant capacity (SOD, CAT, GSH), cellular damage (LPO) and energetic fitness (GLY), in two life stages (juvenile and adult) after 28 days of exposure.

Results showed marked differences between each species tolerance capacity to hypercapnia, with contrasting metabolic readjustment strategies (ETS), different antioxidant response capacities (SOD, CAT, GSH), which generally allowed to prevent increased cellular damage (LPO) and energetic impairment (GLY) in both species. Juveniles were more responsive to hypercapnia stress in both congeners, and are likely to be most sensitive to extreme hypercapnia in the environment. Juvenile *C. gigas* presented more pronounced biochemical alterations at intermediate hypercapnia (4,000 µatm) than *C. brasiliana*. Adult *C. gigas* showed biochemical alterations mostly in response to high hypercapnia (10,000 µatm), while adult *C. brasiliana* were less responsive to this environmental stressor, despite presenting decreased metabolic potential.

Our data bring new insights on the biochemical performance of two important oyster species, and suggest that the duration of extreme hypercapnia events in the ecosystem may pose increased challenges for these organisms as their tolerance capacity may be time limited.

1. Introduction

Shallow coastal marine ecosystems are major contributors in global carbon dioxide (CO_2) cycling, functioning as both sinks and sources of atmospheric CO_2 (Frankignoulle et al., 1998). The CO_2 flux between air and water in these ecosystems has received increasing attention under the eminence of global climate change (Cai, 2011; Feely et al., 2010). In brackish and marine waters pCO_2 levels can be naturally high (up to 10,000 µatm), in comparison to that of open ocean seawater (400 µatm). This fact raises the question of how the increase of atmospheric CO_2 levels expected for the upcoming decades (IPCC, 2013) may further exacerbate high pCO_2 levels in seawater of these ecosystems, and how this may affect resident biota (Tomanek et al., 2011; Melzner et al., 2013).

Organisms inhabiting shallow marine water bodies are known to possess compensation mechanisms to withstand elevated pCO_2 in seawater (hypercapnia), to prevent deleterious effects of acidification of tissues and body fluids that affect physiological fitness (Burnett, 1997). However, such mechanisms are time limited and may lead to negative energetic trade-offs (Sokolova et al., 2012), alterations in acid-base balance (Lindinger et al., 1984), as well as alterations of oxidative status (Tomanek, 2015; Matoo et al., 2013). Among these faunal inhabitants, bivalves are generally less tolerant to elevated levels of hypercapnia than vertebrates (Melzner et al., 2009), despite possessing adaptive mechanisms to thrive in constantly fluctuating environmental parameters (Ringwood and Keppler, 2002). They can partially compensate for hypercapnia-induced acidosis (Burnett, 1997), through mechanisms such as shell dissolution to increase internal bicarbonate

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levels (Shirayama and Thornton, 2005; Lannig et al., 2010) and metabolic adjustment (e.g. metabolic arrest, shifts in metabolic pathways) (Michaelidis et al., 2005; Lannig et al., 2010).

Extensive research on the impacts of seawater acidification on marine bivalves have been published (for reviews see Parker et al., 2013; Gazeau et al., 2014), however most studies focus on the effects of projected CO_2 levels for open ocean waters (up to 1,000 μatm by year 2100) (IPCC, 2013), while the effects of seawater acidification on organisms inhabiting shallow coastal ecosystems has been comparatively overlooked, even though in these systems pCO_2 can reach significantly higher levels (between 400 and 10,000 μatm) (Frankignoulle et al., 1998; Cai, 2011; Noriega and Araujo, 2014; Evans et al., 2013). Considering predictions for increased hypercapnia in shallow coastal systems (Melzner et al., 2013), competitive advantages between species may be altered (Byers, 2002), and zoogeographical shifts in species distribution may occur (Somero, 2010). Therefore, it is important to understand different species ability to cope with such stressors in a changing environment (Parker et al., 2013).

Oysters are important ecosystem engineers in estuarine systems worldwide, providing a variety of ecosystem services and holding a high socio-economic value (Grabowski et al., 2012). However, natural oyster reefs have become severely impacted at a global level due to human pressure and need to be protected (Beck et al., 2011). *Crassostrea brasiliana* is the most important native oyster species occurring in Brazilian estuaries, and is mainly harvested from natural populations, presenting a high socio-economic value, and is especially important for local extractivist communities (Mendonça and Machado, 2010; Neto et al., 2013). *Crassostrea gigas*, a non-native species to Brazil and virtually distributed all over the world, is currently cultured in the southern state of Santa Catarina, and accounts for over 90% of the national oyster aquaculture production (Melo et al., 2010). Since the natural occurrence of *C. gigas* in Brazil has already been registered (Melo et al., 2009), special concerns must be risen in order to understand how the increased frequency of climate change related events (e.g. hypercapnia) may influence different species, and shift competitive advantages towards each other.

Hence, the present study aimed to assess how two important oyster species currently harvested in Brazil, the mangrove oyster *Crassostrea brasiliana*, and the pacific oyster *Crassostrea gigas*, respond to hypercapnic conditions by assessment of a suit of biochemical markers, bringing new insights on how native and non-native oysters species may perform in an acidified estuary.

2. Methods

2.1. Species collection and experimental setup

Crassostrea brasiliana specimens were collected from submerged oyster racks in the Cananéia estuary (25°00′29.50″S 48°01′29.35″W) in the Extractive Reserve of the Mandira (SE Brazil). *Crassostrea gigas* individuals were obtained from the Laboratory of Marine Molluscs of the University of Santa Catarina (SE Brazil). Juvenile and adult specimens of both species were selected for laboratory exposures. Average shell height of *C. brasiliana* and *C. gigas* juveniles was 4.0 ± 0.8 cm and 4.2 ± 0.2 cm respectively. Average shell height of adults was 7.2 ± 0.4 cm for *C. brasiliana*, and 7.8 ± 0.3 cm for *C. gigas*.

Experiments took place during April and May 2015 and were performed in separate for each species. Acclimation to laboratory conditions followed one week prior to the beginning of exposures. During this period, juvenile and adult specimens were maintained in separate tanks, in recirculated artificial seawater (Ocean Fish – Prodac®) (pH 7.8; temperature 24 °C, salinity 25) and daily fed with AlgaMac Protein Plus® (10^9 cells L^{-1} initial cell density). After acclimation, oysters were randomly distributed into testing systems, consisting of 50 L aquaria with individual filters and circulation pumps (total seawater flow of 500 L^{-1}). Each condition was replicated in three separate aquaria, and aquaria were stocked with 4 adults and 8 juveniles each (12 adults and 24 juveniles per condition). Three different hypercapnia levels were tested 1,000 (pH 7.8), 4,000 (pH 7.4) and 10,000 (pH 7.0) μatm pCO_2. Hypercapnia levels were selected based on maximum pH recorded during summer (i.e.: pH 7.85) in submerged oyster beds in the Cananéia estuary (Miraldo and Valenti, unpublished data), high hypercapnia pH 7.0 (10,000 μatm pCO_2) based on reported $pCO2$ in estuarine systems worldwide (Cai, 2011), and an intermediate hypercapnia level pH 7.4 (4,000 pCO_2) to assess transient changes between low and extreme hypercapnia, and values reported for hypoxic estuaries (Melzner et al., 2013).

To achieve targeted hypercapnia levels, food grade CO_2 was diffused into each aquarium (conditions pH 7.4 and pH 7.0) through bubble-counter CO_2 diffusers, at gas releasing rates that were pre-established for each condition, and regulated through six-needle valves (ISTA Products®) allowing for constant and stable gas flow (Duarte et al., 2015). During the entire experimental procedures, pH of each tank was measured and checked three times per day (Hanna Instruments®). After acclimation to laboratory conditions, oysters that were exposed to intermediate and high hypercapnia (pH 7.4 and pH 7.0 respectively) were progressively acclimated to hypercapnia by −0.2 pH units per day until targeted pH values were achieved. This procedure added 4 extra days of acclimation time to each testing group.

After pH equilibration in testing aquaria, exposures carried on for 28 days. During this period water parameters (temperature, dissolved oxygen, salinity) were daily monitored (YSI Pro plus®). Faecal debris were removed prior to feeding (AlgaMac Protein Plus®) 5 days a week, giving partial water renewals of 5%. Oysters were checked for mortality on a daily basis. Water samples were collected every week, prior to total water renewals to determine total alkalinity (TA) for each aquarium by potentiometric titration (Gran, 1952) with an automatic titrator (Mettler Toledo®). Determined TA for each aquarium was plotted against pH, temperature and salinity average values measured during each week on CO2SYS software, to determine carbonate system variables (Robbins et al., 2010), using dissociation constants K1 and K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 from Dickson (1990) (Table 1).

At the end of the experiment (28 days), oysters were frozen at −80 °C until further analysis.

Table 1
Carbonate system physicochemical parameters for pH experiments (mean ± SD; n = 4). Measured pH, and determined total alkalinity (A_t) from weekly water sampling (Temperature 24.5 °C ± 0.3, salinity 25.5, and 77% dissolved oxygen). Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate ion concentrations (CO_3^{2-}), and saturation states of calcite (ΩCal) and aragonite (ΩAg), calculated with CO2SYS software (Robbins et al., 2010).

	Condition	pH	A_t (μmol. Kg^{-1})	pCO_2 (μatm)	HCO_3^-(μmol.kg^{-1})	CO_3^{2-}(μmol.kg^{-1})	ΩCal	ΩAra
C. gigas	pH 7.8	7.78 ± 0.03	2,087 ± 88	1,182 ± 76	1937 ± 99	69.0 ± 6.3	1.8 ± 0.2	1.1 ± 0.1
	pH 7.4	7.38 ± 0.02	2,679 ± 115	3,927 ± 305	2591 ± 113	37.5 ± 2.1	1.0 ± 0.1	0.6 ± 0.04
	pH 7.0	7.01 ± 0.04	2,881 ± 107	10,101 ± 862	2840 ± 105	17.7 ± 1.9	0.5 ± 0.05	0.3 ± 0.03
C. brasiliana	pH 7.8	7.78 ± 0.02	1,919 ± 109	1,068 ± 66	1764 ± 101	63.6 ± 5.2	1.7 ± 0.14	1.1 ± 0.09
	pH 7.4	7.38 ± 0.04	2,508 ± 175	3,751 ± 423	2428 ± 170	34.4 ± 4.1	0.9 ± 0.1	0.6 ± 0.07
	pH 7.0	7.00 ± 0.04	2,789 ± 105	9,992 ± 1010	2751 ± 104	16.7 ± 1.5	0.4 ± 0.04	0.3 ± 0.03

2.2. Biochemical analysis

For biochemical analysis, each juvenile and adult oyster from each species was individually and manually homogenized with a mortar and a pestle under liquid nitrogen. Homogenates from each specimen were further separated in aliquots (0.1 g for juveniles, 0.5 g for adults) to perform individual extractions for each parameter analyzed. For the electron transport system (ETS) activity assay, supernatants were extracted in 0.1 M Tris–HCl buffer (15% (w/v) polyvinylpyrrolidone (PVP); 153 mM magnesium sulfate ($MgSO_4$); 0.2% (v/v) Triton X-100) (pH 8.5). For superoxide dismutase (SOD), catalase (CAT) and glycogen (GLY) assays, supernatants were extracted in phosphate buffer 50 mM sodium dihydrogen phosphate monohydrate; 50 mM disodium hydrogen phosphate dehydrate; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) (PVP); 1 mM dithiothreitol (DTT) (pH 7.0). For reduced (GSH) and oxidized (GSSG) glutathione quantification assays, extraction buffer consisted of 0.6% sulfosalicylic acid in potassium phosphate buffer (0.1 M dipotassium phosphate, 0.1 M potassium dihydrogen phosphate, 5 mM EDTA, 0.1% Triton X-100, pH 7.5). For LPO assay supernatants were extracted in 20% (v/v) trichloroacetic acid (TCA).

Specific buffers were added to aliquots in a 2:1 vol: weight ratio, and homogenates sonicated for 15 s (55 W cm^{-2} at 4 °C), and centrifuged for 15 min at 3,000*g* (for ETS activity) or 10,000*g* (for the remaining biomarkers) at 4 °C.

Supernatants were stored (−80 °C) or directly used to measure: ETS activity; ii) SOD and CAT activities; iv) GSH and GSSG concentrations; v) LPO; vi) GLY content; vii) protein content.

Biomarkers were assessed at room temperature (22 °C) using analytical grade reagents for all analyses. Enzymatic activities (ETS, SOD, CAT) and glutathione (GSH and GSSG) content were standardized by protein concentration of each sample. Protein was quantified by the Biuret method (Robinson et al., 1940), using bovine serum albumin (BSA) as standard. Results on LPO and GLY were standardized by samples fresh weight (FW).

2.2.1. Electron transport system

The ETS activity was measured according to King and Packard (1975) and modifications introduced by Coen and Janssen (1997). Reaction mixture consisted of 0.13 M Tris-HCL buffer (pH 8.5, 0.3% (v/v) Triton X-100), 0.25 mM NADH, 36.5 μM NADPH, and 2.3 mM INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium Chloride). Formazan production rate was determined spectrophotometrically at 490 nm during 10 min (25 s intervals), and determined using ε = 15,900 M^{-1} cm^{-1}. Results were expressed in nmol min^{-1} mg^{-1} protein.

2.2.2. Antioxidant scavengers

SOD activity was quantified following Beauchamp and Fridovich (1971) using SOD standard 0.25–60 U mL^{-1}. Reaction mixture consisted of phosphate buffer 50 mM (pH 8.0), 68.4 μM NBT (nitroblue tetrazolium chloride), 0.1 mM DTPA (diethylenetriamineppent-acetic acid), 0.1 mM hypoxanthine. Enzyme activity was determined at 560 nm in a microplate reader after adding xanthine oxidase (5 mU), diluted in phosphate buffer 50 mM (pH 8.0). Absorbance was measured after 20 min incubation at 22 °C, and the rate of NBT reduction determined. SOD activity was expressed in U mg^{-1} protein (U = μmol min^{-1}).

CAT activity was determined according to Johansson and Håkan Borg (1988), using formaldehyde as standard (0–150 μM). Reaction was made in phosphate buffer (pH 7.0), 5.6 M methanol, and the presence of 35.28 mM H_2O_2. Reaction was stopped by adding 10 M KOH and 34.2 mM purpald. Absorbance was measured at 540 nm in a microplate reader. CAT activity was expressed in U mg^{-1} protein (U = nmol min^{-1}).

GSH and GSSG were determined spectrophotometrically at 412 nm following Rahman et al. (2007), using analytical grade (GSH and GSSG) standards (0–60 μmol L^{-1}). GSH and GSSG concentrations (nmol mg-prot^{-1}) were further expressed as a ratio (GSH/GSSG) and as total glutathione (tGSH), considering the number of thiol equivalents (GSH/GSSG = [GSH] / 2x [GSSG]), and (tGSH = [GSH] + 2x [GSSG]) (Rahman et al., 2007).

2.2.3. Cellular damage

LPO levels were quantified following an adaptation of the thiobarbituric acid (TBA) assay from Buege and Aust (1978). Reaction mixture consisted of TBA at 5% (v/v) in TCA at 20% (v/v). Samples were incubated at 96 °C for 30 min and then cooled on ice. Absorbance was measured at 535 nm (ε = 156 mM^{-1} cm^{-1}). LPO levels were expressed in nmol MDA g^{-1} FW.

2.2.4. Energy reserves

GLY content was determined following Yoshikawa (1959), using glucose as standard (0–5 mg/mL). Samples were incubated at room temperature for 30 min after reacting with phenol (5%) and sulphuric acid (98%). Absorbance was measured at 492 nm, and GLY content expressed in mg g^{-1} FW.

2.3. Biochemical data analysis

Biochemical parameters (ETS, GLY, SOD, CAT, GSH/GSSG; tGSH LPO) were submitted to hypothesis testing using permutational analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008).

Parameters were analyzed following a one-way hierarchical design, with hypercapnia level for juvenile or adult oyster, of each species as the main fixed factor. Concerning each descriptor, null hypothesis tested were: H_0') for each species and each life stage (juvenile or adult), no significant differences exist among hypercapnic levels; H_0'') for each hypercapnic level, and for each species no significant differences exist between life stages; H_0''') at each condition and life stage no significant differences exist between species.

Data for each biomarker are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among groups representing each condition were identified in figures with different letters (minuscule for juvenile, and majuscule for adult specimens). At each hypercapnic level, significant differences between juvenile and adults of each species were represented with an asterisk. Comparative analyses between species are given in Supplementary table I, as Monte-Carlo *p*-values for each biomarker.

3. Results

3.1. Electron transport system activity

Results obtained concerning the ETS activity for both species are depicted in Fig. 1. Juvenile and adult *C. gigas* presented an increase of ETS activity with the increase of hypercapnia, with significant differences towards control (pH 7.8) at the intermediate hypercapnic level (7.4) in juveniles, and at the highest hypercapnic level (pH 7.0) in adults. Comparisons between adult and juvenile metabolic potential showed significantly higher ETS activity in juvenile oysters at the intermediate hypercapnic level (pH 7.4) (Fig. 1A).

The ETS activity in *C. brasiliana* showed a decreasing trend in both juveniles and adults with the increase of hypercapnia (Fig. 1 B), with significant differences at both hypercapnic levels (pH 7.4 and pH 7.0) comparing to control (pH 7.8). No significant differences were observed concerning ETS activity between adult and juvenile *C. brasiliana* at each condition (Fig. 1).

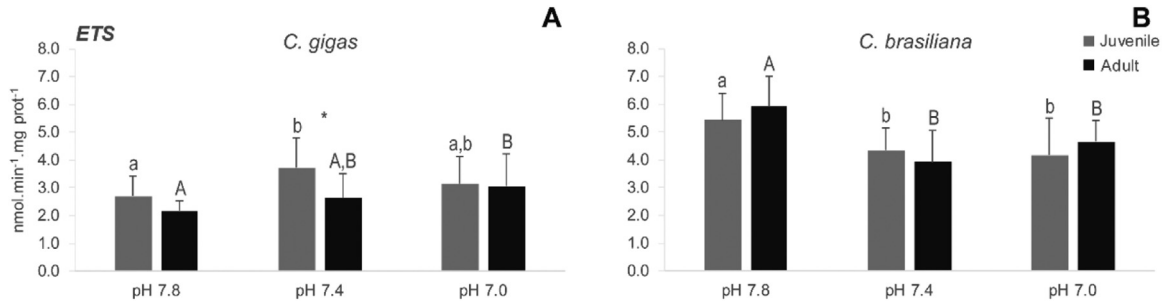

Fig. 1. Electron transport system (ETS) activity in *C. gigas* (A) and *C. brasiliana* (B) exposed to different hypercapnia conditions (pH 7.8, 7.4 and 7.0). Significant differences ($p \leq 0.05$) among hypercapnia conditions are represented with different letters (lowercase for juvenile, and uppercase for adult specimens). For each hypercapnia level, significant differences ($p \leq 0.05$) between juvenile and adult oysters are represented with an asterisk (mean + SD).

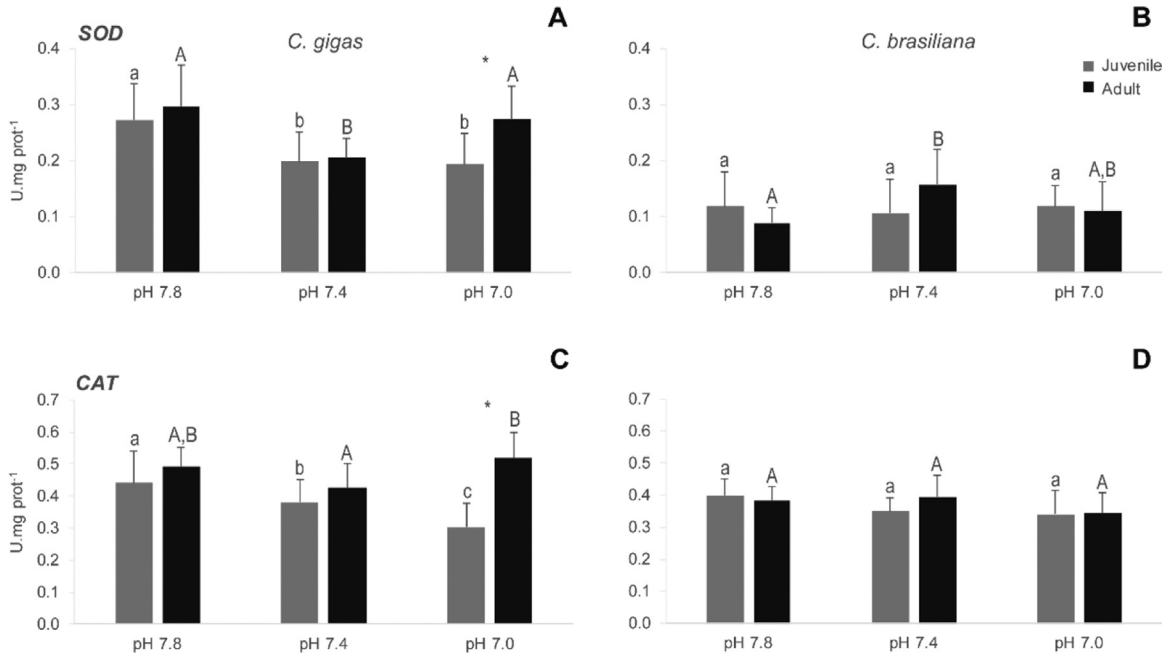

Fig. 2. Antioxidant enzymes of *C. gigas* and *C. brasiliana* exposed to different hypercapnia conditions (pH 7.8, 7.4 and 7.0). SOD activity in *C.* gigas (A) and *C. brasiliana* (B); CAT activity in *C. gigas* (C) and *C. brasiliana* (D). Significant differences ($p \leq 0.05$) among hypercapnia conditions are represented with different letters (lowercase for juvenile, and uppercase for adult specimens). For each hypercapnia level, significant differences ($p \leq 0.05$) between juvenile and adult oysters are represented with an asterisk (mean + SD).

3.2. Antioxidant enzymes

Results obtained on antioxidant enzymes SOD and CAT activity for both species are presented in Fig. 2. In juvenile *C. gigas*, SOD activity was significantly lower at both hypercapnic levels (pH 7.4 and pH 7.0) comparing to control (pH 7.8) (Fig. 2A). Adult *C. gigas* showed a significant decrease of SOD activity at the intermediate hypercapnic condition (pH 7.4) comparing to control. At the highest hypercapnia condition (7.0) SOD activity in adult *C. gigas* was similar to that observed at control (pH 7.8). Comparisons between adult and juvenile SOD activity at each testing condition, showed significantly higher SOD activity in adults at the highest hypercapnic level (pH 7.0) (Fig. 2A).

In juvenile *C. brasiliana* SOD activity was similar among tested conditions (Fig. 2B). In adult *C. brasiliana* a significant increase of SOD activity was observed at the intermediate hypercapnic condition (pH 7.4) comparing to control. At the highest hypercapnic level (pH 7.0), SOD activity in adult *C. brasiliana* was not significantly different from neither of the remaining conditions (pH 7.0). No significant differences were observed between adult and juvenile SOD activity at each condition (Fig. 2B).

Results obtained on CAT for *C. gigas* are presented in Fig. 2C. In juveniles, a decrease of CAT activity was observed with the increase of hypercapnia, with significant differences among all testing conditions (Fig. 2C). Adult oysters presented highest CAT activity at the highest hypercapnic level (pH 7.0), with significant differences towards the intermediate hypercapnia level (pH 7.4), but no significant differences to control (pH 7.8) (Fig. 2C). Significantly higher CAT activity was observed in adults at highest hypercapnia level (pH 7.0) comparing to juveniles (Fig. 2C).

Results obtained concerning CAT activity in *C brasiliana* (Fig. 2D) showed no significant differences among conditions, concerning juveniles and adult oysters. Comparisons between adult and juvenile CAT activity at each hypercapnia condition showed no significant differences throughout (Fig. 2D).

3.3. Glutathione redox balance

Results obtained concerning GSH/GSSG and tGSH for both oyster species are depicted in Fig. 3. Juvenile *C. gigas* presented significantly higher GSH/GSSG at the highest hypercapnic condition (pH 7.0), with significant differences towards both the intermediate hypercapnia level (pH 7.4) and to control (pH 7.8) (Fig. 3A). Adult *C. gigas* presented no significant differences in GSH/GSSG among conditions. Comparisons between adult and juvenile *C. gigas* at each condition, showed significantly higher GSH/GSSG in juvenile oysters at the highest hypercapnia level (pH 7.0) (Fig. 3A).

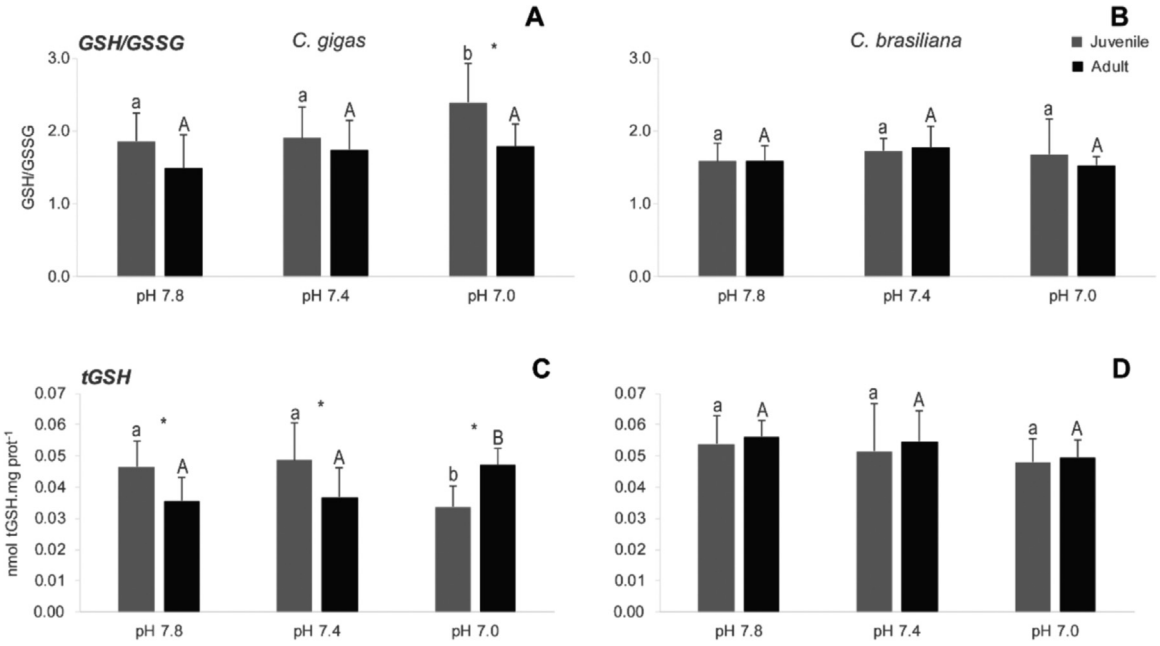

Fig. 3. Glutathione redox balance in *C. gigas* and *C. brasiliana* exposed to different hypercapnia conditions (pH 7.8, 7.4 and 7.0). GSH/GSSG in *C. gigas* (A) and *C. brasiliana* (B); tGSH in *C. gigas* (C) and *C. brasiliana* (D). Significant differences ($p \leq 0.05$) among hypercapnia conditions are represented with different letters (lowercase for juvenile, and uppercase for adult specimens). For each hypercapnia level, significant differences ($p \leq 0.05$) between juvenile and adult oysters are represented with an asterisk (mean + SD).

Regarding *C. brasiliana* no significant changes were observed in GSH/GSSG among different conditions for either juveniles nor adults. Moreover, no significant differences were observed between adults and juveniles GSH/GSSG within each condition (Fig. 3B).

Total glutathione content (tGSH) in juvenile *C. gigas* was significantly lower at the highest hypercapnic level (pH 7.0) comparing to the remaining conditions (Fig. 3C). In contrast, adult oysters presented significantly higher tGSH at the highest hypercapnic level (pH 7.0), compared to the remaining conditions (Fig. 3C). Comparisons between adult and juvenile tGSH at each testing condition, showed significantly higher tGSH in juvenile oysters at low (pH 7.8) and intermediate (pH 7.4) hypercapnia, and lower tGSH at the highest hypercapnic level (pH 7.9) than adults (Fig. 3C).

Concerning *C. brasiliana*, results obtained showed no significant changes in tGSH content among conditions in either juveniles nor adults exposed to different hypercapnic levels (Fig. 3D). No differences were observed between juvenile and adults tGSH at each testing condition (Fig. 3D).

3.4. Cellular damage

Results obtained concerning LPO levels for each species are presented in Fig. 4. In juvenile *C. gigas* LPO was significantly higher at the intermediate hypercapnic level (pH 7.4) compared to that observed in juveniles maintained at low (pH 7.8) and high hypercapnic level (pH 7.0) (Fig. 4A). In adult *C. gigas* no significant differences were observed among testing conditions (Fig. 4A). Comparisons between adult and juvenile LPO levels at each testing condition, showed overall higher LPO in juveniles than in adults, and differences were significant for both hypercapnic conditions (pH 7.4 and pH 7.0) (Fig. 4A).

Regarding *C. brasiliana,* juvenile oysters presented a significant decrease of LPO levels at the highest hypercapnia level (pH 7.0) in comparison to control and intermediate hypercapnia conditions (pH 7.8 and pH 7.4) (Fig. 4B). In adult *C. brasiliana* no significant differences in LPO levels were observed among testing conditions. Comparisons between adult and juvenile oysters at each condition showed significantly higher LPO in juveniles in all hypercapnic levels (Fig. 4B).

3.5. Energetic fitness

Results obtained concerning GLY content for both oyster species are presented in Fig. 5. In juvenile *C. gigas* no significant differences in GLY content were observed among different hypercapnia conditions (Fig. 5A). In adult specimens however, significantly lower GLY content was observed at the highest hypercapnia level (pH 7.0) in comparison to the remaining conditions (Fig. 5A). Comparisons between adult and juvenile GLY at each condition showed no significant differences among testing conditions (Fig. 5A).

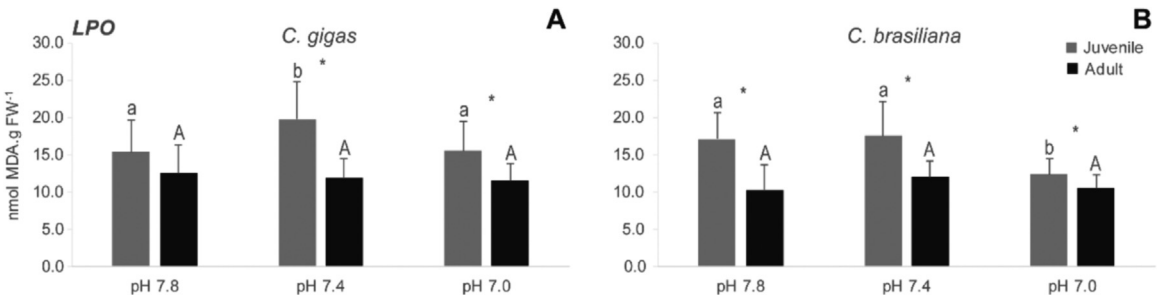

Fig. 4. Lipid peroxidation (LPO) in *C. gigas* (A) and *C. brasiliana* (B) exposed to different hypercapnia conditions (pH 7.8, 7.4 and 7.0). Significant differences ($p \leq 0.05$) among hypercapnia conditions are represented with different letters (lowercase for juvenile, and uppercase for adult specimens). For each hypercapnia level, significant differences ($p \leq 0.05$) between juvenile and adult oysters are represented with an asterisk (mean + SD).

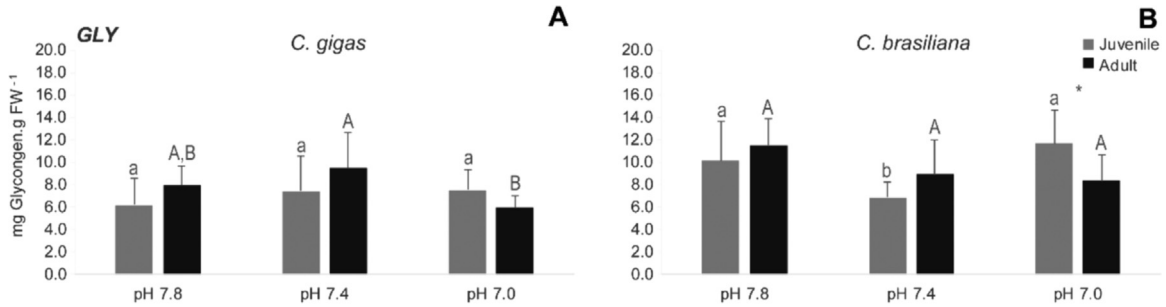

Fig. 5. Glycogen content (GLY) in *C. gigas* (A) and *C. brasiliana* (B) exposed to different hypercapnia conditions (pH 7.8, 7.4 and 7.0). Significant differences ($p \leq 0.05$) tested among hypercapnia conditions are represented with different letters (lowercase for juvenile, and uppercase for adult specimens). For each hypercapnia level, significant differences ($p \leq 0.05$) between juvenile and adult oysters are represented with an asterisk (mean + SD).

Concerning *C. brasiliana,* juveniles presented significantly lower GLY content at the intermediate hypercapnia level (pH 7.4), in comparison to the remaining conditions (Fig. 5B). In adult *C. brasiliana* no significant differences were observed in GLY content among conditions, despite an apparent decreasing trend of GLY with the increase of *p*CO2 (Fig. 5B). Differences in GLY content between adult and juveniles at each condition were significant at the highest hypercapnic level, with higher GLY content in juveniles compared to adults (Fig. 5B).

4. Discussion

The effects of hypercapnia on marine organisms have been focus of research, despite recent advances indicate that the current understanding of the mechanisms involved is still poor (Tomanek, 2015). The present study assessed the effects of hypercapnia on two oyster species, through a suit of biochemical markers related to previously reported effects of hypercapnia (e.g. metabolic shift, oxidative stress response, energetic fitness), to elucidate different species tolerance capacities to this environmental stressor.

4.1. Electron transport system

Ectothermic metazoans exposed to hypercapnia experience tissue and body fluids acidosis, due to diffusive entry of CO_2 into the organisms (see Lindinger et al., 1984; Burnett, 1997; Pörtner et al., 1998; Pörtner et al., 2004). So far, studies show that these organisms increase bicarbonate levels in body fluid compartments as a compensatory mechanism to withstand respiratory acidosis (Pörtner et al., 1998; Strobel et al., 2013). In bivalves this is achieved by dissolution of the internal shell (Michaelidis et al., 2005; Harms et al., 2014). However, the increase of bicarbonate levels can competitively inhibit citrate synthase and, therefore, constrain the tricarboxylic acid cycle (TCA) (Simpson, 1967). This has recently been pointed as one of the drivers for metabolic adjustment in aquatic organisms as a response to hypercapnia, given that the inhibition of the first step of oxidative phosphorylation (citrate synthase) elicits the need for alternative anaplerotic pathways (Strobel et al., 2013; Langenbuch and Pörtner, 2003). Marine bivalves may shift to decarboxylation of amino-acids instead of pyruvate for respiration (Müller et al., 2012), thus altering the electron transport chain functioning, by changing preferential subtracts for energetic turnover (Müller et al., 2012; Tomanek, 2015).

Results obtained in the present study showed alterations of metabolic performance of oysters under hypercapnic conditions, measured by the activity of the electron transport system (ETS). This biomarker gives a proxy of maximum potential metabolic activity (Schmidlin et al., 2015), and has been employed to study the influence of several abiotic factors on oyster metabolism (e.g.: Le Moullac et al., 2007; García-Esquivel et al., 2002; Moreira et al., 2017).

The increase of ETS activity observed in *C. gigas* (adults and juveniles) with the increase of pCO_2, indicate the development of increased metabolic potential in response to hypercapnia in this species. These results are in line with recent studies on other ectothermic marine metazoans exposed to hypercapnia. Strobel et al. (2013) observed increased aerobic capacity (higher activities of citrate synthase and cytochrome oxidase enzymes) in red muscle of *Notothenia rossii* fish exposed to hypercapnia, and suggested that this could either be a mechanism to sustain elevated costs of acid-base balance regulation, or as a compensation mechanism for alterations in mitochondria metabolism. Similarly, Harms et al. (2014) observed upregulation of ETS related genes in *Hyas araneus* crab exposed to > 900 µatm pCO_2, and their results were justified as a mechanism to compensate for increased energetic costs of acid-base maintenance in hypercapnia exposed animals. The ETS activity in *C. gigas* oysters has been shown to increase in conditions of hypoxia (Le Moullac et al., 2007; Samain and McCombie, 2008), and could likely be a common response mechanism triggered by these stressors, since hypoxia and hypercapnia often occur simultaneously in the environment (Willson and Burnett, 2000).

In contrast, *C. brasiliana* presented a decrease of metabolic potential with the increase of hypercapnia, with lower ETS activity in both intermediate and high hypercapnia conditions (pH 7.4 and 7.0) towards low hypercapnia (pH 7.8) in both juvenile and adult specimens, indicating a down regulation of metabolic capacity. Some studies have described metabolic depression in marine invertebrates exposed to high CO_2 concentrations (e.g.: Michaelidis et al., 2005; Pörtner et al., 1998; Reipschläger and Pörtner, 1996), which can be indicative of organisms incurring stress (Guppy and Withers, 1999; Lannig et al., 2010; Parker et al., 2013). Metabolic depression in response to hypercapnia can imply shifts in preferential metabolic pathways (Pörtner et al., 2005), as observed for *C. gigas* through shotgun sequencing (Timmins-Schiffman et al., 2014). In the present study, *C. brasiliana* appears to have achieved a new state in metabolic respiration, with lower potential aerobic capacity after four weeks of exposure to hypercapnic conditions, possibly to reconfigure energetic balance. In a previous study, a decrease in ETS activity was observed in juvenile *C. brasiliana* oysters in response to high temperature and results were explained as trade-off mechanisms to prevent energetic reserves depletion (Moreira et al., 2017). The ETS activity has also been shown to decrease in *Scrobicularia plana* clams (pCO2 > 5000 µatm), and authors suggested these results could relate to metabolic depression to maintain energetic fitness (Freitas et al., 2016).

4.2. Antioxidant scavengers and cellular damage

Alterations in mitochondria respiration capacity and electron transport flow through the electron transport chain modulate reactive oxygen species (ROS) production (Harms et al., 2014; Gibbin et al., 2017). Changes in the electron transport chain functioning might also be employed as a defence mechanism to prevent deleterious effects of ROS (see Abele et al., 2007). Hence, the results concerning the ETS activity can influence the overall antioxidant response (SOD and CAT

activities), glutathione redox status (GSH/GSSG) and ultimately cellular damage (LPO). In *C. gigas* the increase of ETS activity observed in both hypercapnic conditions, could have induced an increase of ROS production, since complexes I and III of the electron transport chain are major sources of superoxide anion, hydrogen peroxide and hydroxyl radicals (Guzy and Schumacker, 2006; Murphy, 2009). ROS can negatively interact with DNA, proteins, carbohydrates and lipids (see Almeida et al., 2007), however the negative effects of ROS at the cellular level can be mitigated by antioxidant enzymes (SOD and CAT) as well as non-enzymatic antioxidant scavengers (GSH). *C. gigas* adults showed increased SOD and CAT activities at the highest hypercapnic level (pH 7.0), which coincided with increased metabolic potential (ETS). SOD and CAT increased activities could have been triggered to mitigate the negative effects of higher ROS production occurring from the mitochondria electron transport chain. Similar relationships between increased ETS and higher antioxidant capacities (SOD and CAT) have been proposed for *Crassostrea angulata* adult oysters (Moreira et al., 2016) exposed to low salinity. However, at the intermediate hypercapnic level both juvenile and adult *C. gigas* did not present increased antioxidant enzymes activities, despite the increase of the ETS activity (significant only for juveniles), rather lower SOD and CAT activities were observed comparing to low hypercapnia condition (pH 7.8). It is possible that other antioxidants could have been in play, namely glutathione (GSH), an important non-enzymatic antioxidant scavenger that is a key participant in processes of ROS neutralization (Rahman et al., 2007). Our data suggest that both juvenile and adult *C. gigas* shifted towards the preferential use of GSH as primary detoxification mechanism, despite presenting differentiated capacities. The significant decrease of both reduced (GSH) and oxidized (GSSG) glutathione content observed in juvenile *C. gigas* with the increase of hypercapnia (data not presented), that resulted in lower tGSH levels and higher GSH/GSSG at the highest hypercapnic level, indicate that glutathione was being involved in detoxification mechanisms in response to hypercapnia, as reported for other bivalve species under hypercapnic or hypoxic conditions (Nardi et al., 2017; Khan and Ringwood, 2016). These findings could explain results showing lower SOD and CAT activities in juvenile *C. gigas* at both hypercapnic levels (pH 7.4; pH 7.0), that together indicate a metabolic shift towards glutathione mediated ROS-quenching pathways, as observed in Mytilid species exposed to heat stress (Tomanek, 2014). The increase of GSH/GSSG observed in juveniles at the highest hypercapnia level (pH 7.0) further indicate oysters were actively transporting glutathione in its oxidized form (GSSG) out of the organism, also reflecting in a lower *t*GSH content. Under oxidative conditions, excessive GSSG can react with thiol groups of proteins, a process known as glutathionylation, leading to alterations of protein functioning (Hawkins et al., 2010; Hurd et al., 2005). The loss of cellular GSH/GSSG redox control makes glutathionylation a deleterious event (Ghezzi and Di Simplicio, 2009), and therefore GSSG is generally exported from the cell to the extracellular matrix (Garcia et al., 2010; Han et al., 2006). Given this, our findings suggest that juvenile *C. gigas* antioxidant capacity at the highest hypercapnia level (pH 7.0) was exceeded, with excess glutathione oxidation, and GSSG excretion resulting in lower total glutathione content, as seen in other bivalve species experiencing oxidative stress (Hannam et al., 2010; Peña-Llopis et al., 2002 Regoli et al., 1998). Although juveniles appeared to present a preferential use of GSH as major antioxidant defence in detriment of antioxidant enzymes (SOD and CAT), possibly because it is energetically less costly (Pannunzio and Storey, 1988), the capacity to replenish tGSH levels showed to be insufficient at the highest hypercapnic level (pH 7.0). In contrast, adult *C. gigas* maintained redox balance (GSH/GSSG) among all hypercapnia conditions, likely due to increased synthesis of glutathione observed (significantly higher GSH at pH 7.0, data not presented). Similarly, Philipp et al. (2008) observed a more pronounced decrease of glutathione in young *Aequipecten opercularis* scallops than adults after swimming bursts, and postulated that younger animals were less effective on homeostatic regulation.

Overall, results obtained concerning the antioxidant capacity of *C. brasiliana* showed a lower degree of oxidative stress response than *C. gigas*. In *C. brasiliana*, significant changes in antioxidant enzymes among different conditions were only observed for SOD activity, in adults at the intermediate hypercapnic level. The relatively low antioxidant response observed, as well as the decrease of metabolic potential (ETS) with the increase of hypercapnia observed in *C. brasiliana* (both adults and juveniles), indicate rearrangement of metabolic pathways towards lower ROS production, as suggested by Tomanek (2015). Under extreme environmental conditions, facultative anaerobes such as oysters, may switch to anaerobic metabolism to extend energetic resources until favourable environmental conditions return (Sokolova et al., 2012). This mechanism also allows for a decrease of ROS production (Abele et al., 2007; Anestis et al., 2007; Pörtner, 2010), and has been shown in *Mytilus edulis* under hypoxia (Rivera-Ingraham et al., 2013). Considering this, our data suggest that *C. brasiliana* developed a depressed metabolic status, preventing excessive ROS production through alterations on the electron transport chain functioning, as well as maintaining energetic balance.

4.3. Cellular damage

The antioxidant capacity of each species likely reflects the oxidative status of the entire organism, and could ultimately impact lipid peroxidation (LPO) levels (Almeida et al., 2007). Increased oxidative stress could be expected to occur with the increase of hypercapnia, considering that CO_2 can directly induce ROS production (Harms et al., 2014) or indirectly through metabolic rearrangement (Timmins-Schiffman et al., 2014; Tomanek, 2015). LPO in adult *C. gigas* was similar among all tested conditions, indicating that cellular or physiological mechanisms could have been employed to prevent membrane oxidative damage. Indeed, we observed increased SOD and CAT activities at the highest hypercapnia tested, as well as an increase of the glutathione pool (tGSH), which all together may have helped prevent LPO increased formation. By the contrary juvenile *C. gigas* were more susceptible to membrane damage, with an observed increase of LPO levels in oysters exposed to the intermediate hypercapnic level (pH 7.4), which could have resulted from significantly lower SOD and CAT activities previously discussed. At the highest hypercapnia level (pH 7.0), LPO in juvenile *C. gigas* was similar to that observed at low hypercapnia (pH 7.8), possibly as a result of glutathione mediated ROS quenching capacity (see above). However, tGSH depletion associated to excessive GSSG may be a precursor of increased LPO (Ringwood et al., 1999). Therefore, this mechanism is likely to become time limited for juvenile *C. gigas*.

Concerning *C. brasiliana*, no change in LPO levels were observed among tested conditions. In juveniles however, lower LPO at the highest hypercapnic level (pH 7.0), corroborates the hypothesis that oysters were depressing metabolism, also in accordance with results obtained for ETS activity and antioxidants (SOD, CAT and GSH) previously described. This hypothesis is further supported by Rivera-Ingraham et al. (2013) studies on *M. edulis*, that showed decreased ROS production in mussels exposed to anoxia, accompanied by no change in oxidative damage parameters (MDA and protein carbonyl).

4.4. Energetic fitness

The energetic status of bivalves can reflect the level of environmental stress (Storey, 1998). Results obtained for *C. gigas* indicate higher energetic expenditure in adult oysters exposed to the highest level of hypercapnia (pH 7.0) (lower GLY content), consistent with results obtained with high metabolic costs of increased ETS and antioxidant enzymes activities observed at the same condition (SOD and CAT). In contrast, Timmins-Shiffman and co-authors (2014) found no change in GLY content of *C. gigas* exposed to hypercapnia, although

testing lower pCO_2 levels (2,800 μatm) than in the present study. Hence, our results suggest that higher levels of hypercapnia (10,000 μatm) may further challenge adult *C. gigas* energetic fitness. In contrast, juvenile oysters presented no change in GLY content among hypercapnic conditions, which could be explained by GSH mediated stress response observed, which can be energetically less costly (Pannunzio and Storey, 1998).

Together, results obtained concerning GLY, ETS and antioxidant capacity in *C. gigas* (adults and juveniles) indicate hypercapnia induced a transition to a moderate stress status, according to the concept of energy-limited stress tolerance (Sokolova et al., 2012), when an increase of metabolic capacity and energetic turnover occurs as a compensation mechanism for homeostatic maintenance and damage repair in response to a given stressor.

C. brasiliana adult oysters showed no change in GLY content among hypercapnic levels. However, at the intermediate hypercapnia level (pH 7.4) juvenile *C. brasiliana* presented significantly lower GLY content, indicative of oysters enduring energetic burden in response to stress (Sokolova and Lannig, 2008). Energetic reserves expenditure has also been demonstrated in juvenile oysters, namely *C. brasiliana* under thermal stress (Moreira et al., 2017), and *Crassostrea virginica* under hypercapnia (ca. 800 μatm pCO_2) (Dickinson et al., 2012). At the highest hypercapnic level (pH 7.0) however, high GLY content in juvenile *C. brasiliana* (similar to values at low hypercapnia (pH 7.8)), indicate these oysters were under an arrested metabolic state at the highest hypercapnic level (pH 7.0), a mechanism employed to conserve energy also reported in other mollusc species (Michaelidis et al., 2005; Gazeau et al., 2014). The rate of carbohydrate catabolism in facultative anaerobes such as oysters is reduced during transition to the *pessimum* range of tolerance to environmental stressors (Sokolova et al., 2012), which could explain similar GLY content observed between low (pH 7.8) and high hypercapnia (pH 7.0). Similarly, *Mytilus galloprovincialis* mussels presented low energetic expenditure (high GLY content) when exposed to hypercapnia (Freitas et al., 2017). Metabolic depression is only a time limited mechanism to endure extreme stress, and therefore the impacts of extended exposure to hypercapnia likely pose greater challenges to this species.

5. Concluding remarks

The present study brings new insights on two important oyster species biochemical responses to hypercapnia. Our data show marked differences in each species response pattern to this environmental stressor, in accordance with other studies assessing comparative performances between other closely related bivalve species enduring abiotic stress, namely Mytilid (Tomanek, 2014) and Venerid (Velez et al., 2016) congeners. An opposite trend was demonstrated regarding metabolic potential between both species, assessed by the electron transport system activity, with *C. gigas* presenting increased metabolic capacity (ETS) with the increase of hypercapnia. Higher antioxidant capacity observed in *C. gigas*, demonstrated by the increase of antioxidant enzymes SOD and CAT, as well as changes in non-enzymatic ROS scavenger GSH oxidation form and concentration, indicate that these conditions induced a prooxidant status. Our data further show that *C. gigas* employed GSH as preferential antioxidant to cope with hypercapnia induced oxidative stress, with observed effects on the glutathione pool (tGSH) and GSH/GSSG, most evident for juvenile specimens at the highest hypercapnia level. The antioxidant capacity of *C. gigas* resulted in no increase of cellular damage (LPO), except for juveniles held at the intermediate hypercapnic level. Nonetheless, data on GSH mediated antioxidant response suggest that this mechanism is time limited.

In contrast, *C. brasiliana* presented a decrease of metabolic potential, noted by lower ETS activity with the increase of hypercapnia. These results suggest metabolic depression to withstand hypercapnia by this species, and were further supported by low antioxidant capacity, no change or even decrease (juveniles at pH 7.0) of LPO, indicating reduced aerobic scope to sustain energetic fitness under hypercapnia.

These results highlight different strategies to cope with increased pCO_2 by different oyster species, bringing new insights on species tolerance capacity and differentiated response mechanisms. The time duration of environmental hypercapnia in estuarine systems may be of upmost importance, since oyster response mechanisms to high environmental pCO_2 suggest to be time limited. According to the energy-limited tolerance concept (Sokolova et al., 2012) our study indicates that the mangrove oyster (*C. brasiliana*) transitioned into the *pessimum* tolerance range under hypercapnia as a conservation mechanism to endure extreme stress, while the pacific oyster (*C. gigas*) response patterns reflected a moderate stress status, which can generally imply a wider range of tolerance towards hypercapnia than *C. brasiliana*, as well as a longer and more sustainable energetic balance. The differentiated response pattern observed can have further implications at the population level, and therefore may influence species competitive advantages towards one another in a hypercapnic environment. In a scenario of coexistence of the two species in the same areas, it appears that *C. gigas* may be more resilient than the native species (*C. brasiliana*) to environmental hypercapnia, with ecological repercussions that are difficult to predict. Therefore, efforts should be made to prevent the spread of the non-native species into pristine environments where *C. brasiliana* still thrives.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2017.10.035.

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