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Are the effects induced by increased temperature enhanced in *Mytilus galloprovincialis* submitted to air exposure?

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ABSTRACT

Intertidal mussel species are frequently exposed to changes of environmental parameters related to tidal regimes that include a multitude of stressors that they must avoid or tolerate by developing adaptive strategies. In particular, besides air exposure during low tides, intertidal mussels are also subjected to warming and, consequently, to higher risk of desiccation. However, scarce information is available regarding the responses of mussels to tidal regimes, particularly in the presence of other stressors such as increased temperature. Investigating the impacts of such combination of conditions will allow to understand the possible impacts that both factors interaction may generate to these intertidal organisms. To this end, the present study evaluated the impacts of different temperatures (18 ºC and 21 ºC) on *Mytilus galloprovincialis* when continuously submersed or exposed to a tidal regime for 14 days. Results showed that in mussels exposed to increased temperature under submersion conditions, the stress induced was enough to activate mussels' antioxidant defenses (namely glutathione peroxidase, GPx), preventing oxidative damage (lipid peroxidation, LPO; protein carbonylation, PC). In mussels exposed to tides at control temperature, metabolic capacity increased (electron transport system activity, ETS), and GPx was induced, despite resulting in increased LPO levels. Moreover, the combination of tides and temperature increase led to a significant decrease of lipid (LIP) content, activation of antioxidant defenses (superoxide dismutase, SOD; GPx) and increase of oxidized glutathione (GSSG), despite these mechanisms were not sufficient to prevent increased cellular damage. Therefore, the combination of increased temperature and air exposure induced higher oxidative stress in mussels. These findings indicate that increasing global warming could be more impacting to intertidal organisms compared to organisms continuously submersed. Furthermore, our results indicate that air exposure can act as a confounding factor when assessing the impacts of different stressors in organisms living in coastal systems.

Keywords: mussels, temperature increase, oxidative stress, metabolism, tidal regime.

1. INTRODUCTION

Organisms present in estuaries and coastal lagoons, must thrive and endure one of the harshest environments. Inhabiting these areas frequently exposed to tides, intertidal organisms are subjected to a large variation of abiotic conditions between aquatic and aerial environments, such as temperature, salinity, oxygen availability and high desiccation risk (Davis, 1985; Freire et al., 2011; Horn et al., 1999; Underwood and Kromkamp, 1999). As a consequence of air exposure, intertidal organisms may face prolonged hypoxic and/or anoxic conditions, with bivalves among the most tolerant to hypoxia (Abele et al., 2009; Gray et al., 2002). Accordingly, alterations on the physiological performance of a diversity of bivalve species experiencing air and tidal exposure have already been demonstrated. While some intertidal bivalves, as the mussel *Mytilus galloprovincialis*, may close their valves when exposed to air and face complete anoxia at ebb tides to avoid desiccation, others may periodically open their valves to maintain a more efficient aerobic metabolism with higher risk of desiccation (Dowd and Somero, 2013; Nicastro et al., 2010; Rivera-Ingraham et al., 2013). *Ruditapes philippinarum* clams showed lower survival and growth when exposed to increased duration of air exposure (Yin et al., 2017). Biochemical alterations can also be induced in bivalves exposed to tidal environment. Previous studies demonstrated an increase of antioxidant defenses in the mussel species *Perna perna* and *M. galloprovincialis* as a defense mechanism against oxidative stress generated during reoxygenation (Almeida and Bainy, 2006; Andrade et al., 2018). Similar biochemical response was observed in *R. philippinarum* clams exposed to daily rhythms of air (Yin et al., 2017). After emergence, *M. edulis* mussels demonstrated over-expression of proteins specially involved in cytoskeleton, chaperoning, energetic metabolism and transcription regulation while presenting decreased activity of antioxidant enzyme superoxide dismutase (Letendre et al., 2011). Rivera-Ingraham et al. (2013) further demonstrated that exposure of *M. edulis* to severe anoxia caused an onset of anaerobiosis (succinate accumulation), while the concentrations of reactive oxygen species (ROS) strongly decreased during anoxic exposure and increased upon reoxygenation.

Intertidal organisms may not only face air exposure during tidal regimes but may, at the same time, be exposed to environmental changes derived from climate change. In particular, as a consequence of global climate change, daily and seasonal environmental variations can be enhanced, such as the increase of temperature. The projected increase of atmospheric CO_2 until the end of the 21st century is considered one of the most important factors contributing to global warming and, consequently, to an increase of global mean air and ocean temperatures (IPCC, 2014). Consequently, intertidal organisms that are exposed to increased temperatures associated with aerial exposure may be subjected to deleterious effects. Different studies have demonstrated that temperatures exceeding the organisms' thermal tolerance range can cause physiological perturbations, namely concerning individuals' growth and reproduction (Pörtner and Knust, 2007; Boukadida et al., 2016), adding to the decrease of aerobic capacity, metabolic rate and respiratory capacity (Jansen et al., 2009; Pörtner, 2005, 2010; Velez et al., 2017). Furthermore, warming can also enhance reactive oxygen species (ROS) production in the cells (Kefaloyianni et al., 2005; Verlecar et al., 2007), leading to oxidative stress. In particular, transcriptomic and biochemical alterations have been observed in different bivalve species in response to temperature rise. *M. galloprovincialis* mussels showed an increase of antioxidant enzymes and metallothionein gene expression levels when exposed to heat stress (Banni et al., 2014). In the same species, significant variations in the immune system were also observed due to increased temperature (Nardi et al., 2017). *M. coruscus* mussels displayed an increase of antioxidant enzymatic activity with increased temperature (Hu et al., 2015).

M. galloprovincialis (Lamarck, 1819) is a common mussel species present in infra littoral areas across the globe (Mitchelmore et al., 1998; FAO, 2016; Vazzana et al., 2016), in rocky areas, cliffs, boulders or other substrates to which it adheres (FAO, 2016; Vazzana et al., 2016). Along coastal areas *M. galloprovincialis* presents a wide spatial distribution and abundance, sedentary and filter feeding behavior and high tolerance to a wide range of environmental conditions, and for these reasons is considered a good sentinel and bioindicator species (Banni

et al., 2014; Coppola et al., 2017; 2018a, 2018b; Faggio et al., 2016; Freitas et al., 2018; Kristan et al., 2014; Sureda et al., 2011; Viarengo et al., 2007). Several studies have also demonstrated the ecological relevance of this species. In particular, *M. galloprovincialis* has shown to be able to improve water quality thought the filtration of particles and excess of nitrogen in aquatic environment (Shumway et al., 2003). The expansion of *M. galloprovincialis* into new habitats has also benefited a near-threatened bird species, the African black oystercatcher *Haemotopus moquini*, which switched its diet to this mussel species thus increasing its food availability (Hockey and Schurink, 1992). Mussel beds can also provide refuge to fish or act as nurseries for juvenile fish and crustaceans (Shumway et al. 2003).

Considering that scarce information is available regarding marine organisms' responses to tidal regime in the presence of other abiotic stressors, the investigation of such scenarios will generate knowledge that will contribute to better understand the impacts resulting from the interaction of factors such as air exposure and temperature rise. Within this context, the present study investigated the possible interactions of air exposure and warming in *M. galloprovincialis* performance, by evaluating the physiological and biochemical alterations induced in organisms exposed to different tidal regimes and temperature conditions, testing the hypothesis: tidal exposure changes the physiological and biochemical performance of *M. galloprovincialis* submitted to warming conditions.

2. METHODOLOGY

2.1. Sampling and experimental conditions

Mytilus galloprovincialis specimens were collected in September 2017 during low tide in an intertidal area at the Mira Channel (Ria de Aveiro, a coastal lagoon, northwest of Portugal). After sampling mussels were transported to the laboratory, where they were placed in aquaria for depuration and acclimation to laboratory conditions for 7 days. Acclimation system operated with synthetic saltwater, prepared by mixing a commercially available salt mixture (Tropic Marin Pro Reef salt; Tropic Marine, Germany) with freshwater purified by reverse osmosis (four stage unit, Aqua-win RO-6080, Thailand). During this acclimation period, organisms were maintained at 18 ± 1.0 ºC (control temperature), pH 8.0 ± 0.1 (control pH) and salinity 35, resembling estuarine conditions while being kept under continuous aeration during a 12 h light: 12 h dark photoperiod.

For the laboratory experiment, mussels were distributed into different 20 L aquaria (with synthetic saltwater, salinity 35), with 6 individuals *per* aquarium and 3 aquaria *per* treatment. The treatments tested were: submersion under control temperature (Sub); submersion under increased temperature (Sub+Temp); exposure to tides simulation under control temperature (Tide); exposure to tides simulation under increased temperature (Tide+Temp). Aquaria were placed in two different climatic rooms to maintain the temperature levels at 18 ± 1.0 ºC (control temperature) and 21 ± 1.0 ºC (increased temperature). For the tidal simulation, an automatic system that mimicked estuarine tidal regime typical of this species habitat (5 hours of low tide and 7 hours of high tide cycles) was developed and used.

The control temperature of 18 ± 1.0 ºC was chosen considering the average temperature of the sampling area during September (IPMA, 2017). To simulate warming conditions, temperature of 21 ºC, was selected taking in account the annual range of average temperatures (13.4-22.9 ºC) for *M. galloprovincialis* habitats in Ria de Aveiro (Coelho et al., 2014; Santos et

al., 2009; Velez et al., 2015) and considering the projected global temperature change to the year 2100 (up to 4.0 ºC, IPCC, 2014). The temperature range tested was also selected considering previously published data on bivalves responses to warming conditions (Banni et al., 2014; Boukadida et al., 2016; Hu et al., 2015; Nardi et al., 2017; Velez et al., 2017).

During the experimental period of 14 days, organisms were fed three times *per* week with Algamac protein plus. Saltwater was renewed after 7 days of the experiment onset and seawater parameters re-established, including salinity, temperature and pH. An experimental period of 14 days was chosen taking in account previous studies performed on mussel species (Andrade et al., 2018; Hu et al., 2015; Huang et al., 2018; Letendre et al., 2011; Verlecar et al., 2007) which allowed to observe physiological or biochemical effects after this period.

At the end of the experimental period (14 days), two organisms *per* aquarium were used for respiration rate and condition index determination, and the remaining ones were immediately frozen at -80 ºC until analysis.

2.2. Biological responses: physiological parameters

Physiological indicators such as respiration rate (RR) may be used to assess alterations induced in bivalves exposed to biotic and abiotic stressors (Gestoso et al., 2016; Freitas et al., 2017; Wang et al., 2015). The condition index (CI) is also frequently used to provide information on the general physiological status of bivalves (Andral et al., 2004) and has been considered as a biomarker of stress in bivalves (Hiebenthal et al., 2012; Pampanin et al., 2005).

2.2.1. Respiration Rate

After 14 days of exposure, respiration rate (RR) was measured in six mussels per condition (two per aquarium/replicate). Measurements were performed by simple static respirometry, using two organisms of the same aquarium per respirometric chamber. Each of

these chambers, which were equipped with an oxygen sensor spot glued to its inner wall using silicon paste, was filled with the same seawater used during the experimental period. Organisms were placed in these chambers under dark and fully-oxygenated concentrations where they were allowed to acclimate for 30 min to avoid the influence of manipulation on RR. After this period, chambers were filled to their maximum capacity (1L) to avoid the formation of air bubbles and were then air-tight sealed. Measurements started in fully oxygenated medium and RR was recorded as a function of declining O2 concentration (mg/L) over time every 15 min during 2h, with a multi-channel fiber optic oxygen meter (Multi channel oxygen meter, PreSens GmbH, Regensburg, Germany) for simultaneous read-outs. Data were recorded using the software PreSens Measurement Studio 2. Twenty-two measurements were carried out at a time (including a blank, i.e. chamber containing no organisms to account for background respiration). Organisms were posteriorly dried and weighed. Respiration rate was expressed in mg O2 consumed per h per g dry weight (DW).

2.2.2. Condition Index

Mussels used from RR measurements were used for Condition Index (CI) determination. Briefly, the soft tissue of six frozen organisms per condition (two *per* aquarium/replicate) were carefully separated from the shells. Both shell and tissue were dried in an oven at 60 ºC for 48 h. After this period, the dry soft tissues and shells were weighed and CI calculated. Following Matozzo et al. (2012), CI values were expressed as the ratio between the DW of soft tissue and the DW of shell x 100. The dry tissue was stored and used for lipid content quantification.

2.3. Biological responses: biochemical parameters

The metabolic capacity and energy reserves of marine organisms can provide information on the organisms' health status. In this respect, the energy production at the

mitochondrial level such as the electron transport activity (ETS) and energy reserves such as glycogen (GLY) and total lipid (LIP) content give an indication of organisms' metabolic capacity (Coen and Janssen, 1997, 2003), and alterations on these biomarkers may indicate organisms' injuries due to different stressful conditions (Gagné et al., 2007; Hummel et al., 1989; Pernet et al., 2010). Furthermore, in organisms exposed to stress, the production of reactive oxygen species (ROS) is increased, which can result in cellular damage, including lipid peroxidation (LPO) (Taylor and Maher, 2010) and protein carbonylation (PC) (Suzuki et al., 2010), if organisms' antioxidant defenses, including enzymatic and non-enzymatic mechanisms, are not effective in removing the excess of ROS produced (among others, Regoli and Giuliani, 2014). Therefore, parameters related to organisms' metabolic capacity (ETS), energy reserves (GLY and LIP content), cellular damage and redox status (LPO and PC levels, oxidized (GSSG) glutathione content), and antioxidant defenses (the activity of antioxidant enzymes superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx) were selected to evaluate the impacts caused in *M. galloprovincialis* by air exposure and increased temperature.

After the 14 days of exposure, the soft tissue of six frozen organisms *per* condition (used previously for the CI and RR) were used to determinate mussels' LIP content. Dry tissue from each individual were homogenized with a mortar and pestle, divided in 0.5 g aliquots and stored for LIP quantification.

For all other parameters, shells of the frozen organisms (four *per* aquarium/replicate, twelve *per* condition) were removed and the frozen whole soft tissue was homogenized using a mortar and pestle with liquid nitrogen. The homogenized tissue of each organism was distributed in 0.5 g aliquots.

For each biochemical parameter, a specific buffer was used in the extraction of the supernatant (Andrade et al., 2018; De Marchi et al., 2017) using a proportion of 1:2 (w/v). Tissue samples from each specimen were individually homogenized using a TissueLyser II (Qiagen) during 1 min, after which they were centrifuged 20 min at 10,000 g or 3,000 g

(depending on the biomarker) and 4 ºC. Supernatants were stored at -80 ºC or immediately used to determine: ETS activity; GLY content; LPO levels; GSSG content; PC levels; and activity of SOD, CAT, and GPx. Two replicates *per* sample were used for the determination of each biochemical parameter.

2.3.1. Metabolic capacity

The ETS activity was measured based on the method of King and Packard (1975) and modifications by Coen and Janssen (1997). Absorbance measurement was performed during 10 min at 490 nm in 25 s intervals and the extinction coefficient of 15.900 $M^{-1}cm^{-1}$ was used to calculate the amount of formazan formed per unit time. Results were expressed in nmol min *per* g fresh weight (FW).

2.3.2. Energy reserves

The GLY content was determined according to the sulfuric acid method (Dubois et al, 1956), using glucose standards. Absorbance was measured at 492 nm after 30 min incubation at room temperature and results were expressed in mg *per* g of FW.

The LIP content was determined following the methods developed by Folch et al. (1957) and Cheng et al. (2011). A standard curve was determined using cholesterol standards (0–100%). After 1 h of color development in the dark at room temperature, absorbance was measured at 520 nm. Results were expressed in percentage *per* mg DW.

2.3.3. Oxidative damage

The quantification of LPO levels followed the method described in Ohkawa et al. (1979) with modifications referred by Carregosa et al. (2014a). Absorbance was measured at 535 nm (ε=156 mM^{-1} cm^{-1}) and results expressed in nmol of MDA equivalents formed *per* g FW.

The quantification of GSSG content was measured according to Rahman et al. (2007), using GSSG as standards (0–90 μmol L^{-1}). Absorbance was measured at 412 nm and results expressed in μmol *per* g FW.

The PC levels were measured according to the DNPH alkaline method described by Mesquita et al., (2014). Absorbance was measured at 450 nm (ε=22 mM^{-1} cm^{-1}), and results expressed in nmol of protein carbonyl groups formed *per* g FW.

2.3.4. Antioxidant enzymes

The activity of SOD was quantified based on the method of Beauchamp and Fridovich (1971). SOD standards (0.25-60 U/mL) were used to generate a standard curve. After 20 min incubation at room temperature, absorbance was measured at 560 nm. Results were expressed in U *per* g FW where one unit (U) of enzyme activity corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity of CAT was quantified following Johansson and Borg (1988). Formaldehyde standards (0-150 μM) were used to perform the standard curve. Absorbance was measured at 540 nm and results expressed in U *per* g FW. One unit (U) is defined as the formation of 1 nmol formaldehyde *per* min.

The activity of GPx was determined following the method of Paglia and Valentine (1967). Absorbance was measured at 340 nm (ε=0.00522 μM^{-1} cm^{-1}) during 5 min in 10 s intervals. Results were expressed in U *per* g FW, one unit (U) corresponds to the quantity of enzyme which catalyzes the conversion of 1 μmol nicotinamide adenine dinucleotide phosphate (NADPH) *per* min.

2.4. Data analysis

Due to lack of homogeneity of variance, CI, RR, ETS, GLY, LPO, PC, GSSG, SOD, CAT and GPx were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7) with a two factor design: submersion condition (submersed and exposed to tide) as factor 1 and temperature condition (control and increased temperature) as factor 2. PERMANOVA main test was performed to test the effect of submersion condition, temperature condition and the interaction between these two factors on each biomarker response. PERMANOVA main tests were considered significant for $p \leq 0.05$ and followed by PERMANOVA pair-wise tests. Pair-wise tests were used to test the effect of temperature condition within each submersion condition and the effect of submersion condition within each temperature level. PERMANOVA pair-wise tests results are represented in figures with lower case letters, with different letter representing significant differences. The interaction between factors is presented in the main text by *p*-values.

3. RESULTS

3.3. Physiological parameters

3.3.1. Mortality

No mortality was observed in all tested treatments after 14 days of exposure.

3.3.2. Respiration Rate

Concerning respiration rate (RR), in mussels submersed during the entire experiment, significantly lower values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Sub vs Sub+Temp). In mussels exposed to tides, no significant differences were observed between organisms exposed to control and to increased temperature (Tide vs Tide+Temp) (Figure 1A). Comparing submersion and tidal exposure conditions, at the control temperature significantly lower RR were observed in mussels exposed to submersion during the entire experiment (Sub vs Tide). For the increased temperature, no significant differences were observed between mussels submersed during the entire experiment and mussels exposed to tides (Sub+Temp vs Tide+Temp) (Figure 1A). The interaction between tidal exposure and the increased temperature showed no significant effects on the RR (p=0.3914).

3.3.3. Condition Index

Concerning condition index (CI) values and comparing both temperatures (control and increased temperature), both for organisms submersed during the entire experiment (Sub vs Sub+Temp) and organisms exposed to tidal regime (Tide vs TideTemp) no significant differences were observed (Figure 1B). Comparing submersion and tidal exposure conditions, no significant differences were observed concerning organisms under control temperature (Sub vs Tide). Regarding increased temperature exposures, although with no statistical differences,

higher CI values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Temp vs Tide+Temp) (Figure 1B). No significant effect of the interaction between tidal exposure and increased temperature on the CI was observed (p=0.9243).

3.2. Biochemical parameters

3.2.1. Metabolic capacity

Concerning ETS activity and comparing both temperatures (control and increased temperature), for organisms submersed during the entire experiment no significant differences were observed (Sub vs Sub+Temp). For mussels exposed to tides, significantly higher values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (Figure 2A). Comparing submersion and tidal exposure conditions, at control temperature significantly lower ETS values were observed in organisms submersed during the entire experiment in comparison to mussels exposed to tides (Sub vs Tide). Concerning increased temperature exposures, no significant differences were observed between mussels submersed during the entire experiment and mussels exposed to tides (Sub+Temp vs Tide+Temp) (Figure 2A). The interaction between tides exposure and the increased temperature showed no significant effects on the ETS activity (p=0.312).

3.2.2. Energy reserves

Concerning GLY content and comparing both temperatures (control and increased temperature), significantly lower GLY values were observed in organisms exposed to increased temperature both for organisms submersed and exposed to tides (Sub vs Sub+Temp, Tide vs Tide+Temp) (Figure 2B). Comparing submersion and tidal exposure conditions, no significant differences were observed both for organisms under control (Sub vs Tide) and increased

(Sub+Temp vs Tide+Temp) temperatures (Figure 2B). The interaction of both stressors (tides and increased temperature) showed no significant effect on the GLY content (p=0.6155).

Regarding LIP content and comparing both temperatures (control and increased temperature), for organisms submersed during the entire experiment no significant differences were observed (Sub vs Sub+Temp). Regarding mussels exposed to tides, significantly higher values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (Figure 2C). Comparing submersion and tidal exposure conditions, no significant differences were observed in organisms under control temperature (Sub vs Tide). Concerning increased temperature, significantly higher LIP values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Temp vs Tide+Temp) (Figure 2C). The interaction of both stressors (tides and increased temperature) showed significant effects on the LIP content (p=0.0065).

3.2.3. Oxidative damage

Regarding LPO levels and comparing both temperatures (control and increased temperature), significantly lower LPO values were observed in organisms exposed to increased temperature both for organisms submersed and exposed to tides (Sub vs Sub+Temp, Tide vs Tide+Temp) (Figure 3A). Comparing submersion and tidal exposure conditions, significantly lower LPO values were observed in organisms submersed during the entire experiment in comparison to mussels exposed to tides for control (Sub vs Tide) and increased (Sub+Temp vs Tide+Temp) temperatures (Figure 3A). The interaction of both stressors (tides and increased temperature) showed no significant effect on LPO levels (p=0.3795).

Concerning GSSG and comparing both temperatures (control and increased temperature), no significant differences were observed concerning organisms submersed during the entire experiment (Sub vs Sub+Temp). For mussels exposed to tides, significantly lower values were observed in organisms exposed to control temperature in comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (Figure 3B). Comparing submersion and tidal exposure conditions, no significant differences were observed considering organisms under control temperature (Sub vs Tide). Regarding increased temperature exposures, significantly lower GSSG values were observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Tide vs Tide+Temp) (Figure 3B). The interaction between tidal exposure and increased temperature showed no significant effects on the GSSG content (p=0.2163).

Concerning PC and comparing both temperatures (control and increased temperature), significantly lower PC values were observed in organisms exposed to increased temperature, considering both organisms submersed during the entire experiment (Sub vs Sub+Temp) and organisms exposed to tides (Tide vs Tide+Temp) (Figure 3C). Comparing submersion and tidal exposure conditions, no significant differences were observed between organisms under control (Sub vs Tide) and increased (Sub+temp vs Tide+Temp) temperatures (Figure 3C). The interaction of both stressors (tides and increased temperature) showed no significant effect on the PC levels (p=0.7076).

3.2.4. Antioxidant enzymes

Concerning SOD activity and comparing both temperatures (control and increased temperature), no significant differences were observed considering organisms submersed during the entire experiment (Sub vs Sub+Temp). Regarding mussels exposed to tides, significantly lower values were observed in organisms exposed to control temperature in

comparison to mussels exposed to increased temperature (Tide vs Tide+Temp) (Figure 4A). Comparing submersion and tidal exposure conditions, at control temperature significantly higher SOD was observed in organisms submersed during the entire experiment in comparison to mussels exposed to tides (Sub vs Tide). Concerning increased temperature exposures, significantly lower SOD was observed in mussels submersed during the entire experiment in comparison to mussels exposed to tides (Sub+Temp vs Tide+Temp) (Figure 4A). The interaction between tidal exposure and increased temperature showed significant effects on the SOD activity (p=0.0011).

Regarding CAT activity and comparing both temperatures (control and increased temperature), no significant differences were observed considering organisms submersed (Sub vs Sub+Temp) and organisms exposed to tides (Tide vs Tide+Temp) (Figure 4B). Comparing submersion and tidal exposure conditions, no significant differences were observed for organisms under control temperature (Sub vs Tide) and for organisms exposed to increased temperatures (Sub+Temp vs Tide+Temp) (Figure 4B). The interaction between tidal exposure and the increased temperature showed no significant effects on CAT activity (p=0.9979).

Concerning GPx activity and comparing both temperatures (control and increased temperature), significantly higher GPx was observed at increased temperature, both in organisms submersed during the entire experiment (Sub vs Sub+Temp) and in organisms exposed to tides (Tide vs Tide+Temp) (Figure 4C). Comparing submersion and tidal exposure conditions, significantly lower GPx was observed in organisms submersed during the entire experiment in comparison to mussels exposed to tides, both for control (Sub vs Tide) and increased temperature (Sub+Temp vs Tide+Temp) (Figure 4C). The interaction of both stressors (tidal and increased temperature) showed no significant effect on GPx activity (p=0.7171).

4. DISCUSSION

Aquatic organisms are facing unprecedented threats on the eminence of climate change, namely due to thermal warming. In fact, different studies demonstrated several consequences to these aquatic organisms due to temperature rise (Boukadida et al., 2016; Kefaloyianni et al., 2005; Jansen et al., 2009; Pörtner et al., 2005, 2010; Pörtner and Knust, 2007; Velez et al., 2017; Verlecar et al., 2007). Furthermore, ecologically relevant intertidal species such as *M. galloprovicianlis* are subjected to air exposure during tidal changes which could enhance the effects resulting from increased temperature and bring future negative impacts to these species populations. However, scarce information is available about the possible physiological and biochemical alterations induced in intertidal organisms resulting from the combined exposure to tidal regimes and increased temperature. Hence, the present study evaluated the physiological and biochemical performance of *M. galloprovincialis* exposed to increased temperature under both continuous submersion and tidal regime, aiming to understand if effects due to warming conditions would be enhanced in organisms exposed to air during ebb tide.

Physiological responses

Respiratory capacity

Results demonstrated that warming conditions increased *M. galloprovincialis* respiratory capacity in both tidal treatments, most evident in organisms exposed to continuous submersion. These findings indicate that the increase of temperature up to 21 ºC represented a moderately stressful condition, affecting mussels' RR. Similarly, previous studies reported an increase of RR with the increase of temperature in *Perna perna* (Resgalla Jr. et al., 2007) and in *M. galloprovincialis* (Gestoso et al., 2016; Jansen et al. 2009).

Furthermore, results indicate that mussels exposed to control temperature tended to present higher RR when exposed to tides in comparison to organisms submersed during the entire experiment. The exposure to tides, with re-oxygenation periods, may require high metabolic capacity (Andrade et al., 2018), which may result in high RR. Similarly, Yin et al. (2017) showed a significant increase in oxygen consumption in *R. philippinarum* clams exposed to different daily air exposure periods (3h, 6h and 9h) followed by immersion, suggesting that after hypoxia, clams compensated for oxygen debt during re-immersion. Under warming conditions, the effect of tidal regime on RR was not observed probably because when exposed to increased temperature organisms may have employed adaptive mechanisms, such as valves closure to avoid stress resulting from dissection effects of warmer temperature during aerial exposure.

Condition Index

In the present study, CI was not significantly affected by temperature or air exposure. Similarly, studies conducted by Gestoso et al. (2016) showed no significant differences on the CI of *M. galloprovincialis* exposed to increased temperature (21 ºC) for 22 days. Thus, the present findings indicate that tested conditions were not stressful enough to result in weight loss and differences in terms of CI. Yet, the lack of differences in organisms CI among tested conditions may also indicate an adaptive behavior of this species to withstand the stress induced both from air exposure and temperature rise, common to intertidal environments. However, the short experimental period probably was not enough to induce significant differences between conditions.

Biochemical responses

Metabolic capacity and energy reserves

In the present study, in comparison to organisms submersed during the entire experiment at control temperature, submersed mussels exposed to increased temperature showed unaltered ETS activity, despite presenting higher RR under warming conditions. Nevertheless, mussels submitted to tidal regime and increased temperature tended to present lower metabolic capacity (ETS) than mussels exposed to tides at control temperature, but similar to ETS in mussels submersed at control temperature. The fact that organisms under warming conditions (submersed or exposed to tidal regime) maintained or even decreased their metabolic capacity in comparison to organisms at control temperature, may indicate that under increased temperature organisms were able to prevent the increase of metabolic capacity by activating behavioral adaptations such as valves closure (Anestis et al., 2007; Gosling, 2003). Accordingly, Anestis et al. (2007) demonstrated that *M. galloprovincialis* exposed to warming at 24 °C lead mussels to keep their valves closed for long periods and caused metabolic depression. Coppola et al. (2017, 2018a) also observed a significant decrease of ETS activity in *M. galloprovincialis* exposed to increased temperature (21 ºC).

Results further revealed that mussels at control temperature showed higher ETS activity when exposed to tides, in comparison to submersed mussels, which may have resulted from re-immersion periods at which the mussels were subjected. These findings may indicate that re-oxygenation after ebb tide, at control temperature, increased mussels' metabolic capacity, probably because after enduring anoxia mussels needed to re-establish their physiological and biochemical performance. Similarly, Andrade et al. (2018) observed that daily air exposure conditions (3h or 6h) followed by immersion, induced increased ETS activity in *M. galloprovincialis*. However, the present findings also revealed that under warming conditions organisms submersed and exposed to tides presented similar metabolic capacity. Such findings may corroborate the hypothesis that organisms deploy adaptive behaviors, such as valve closure, to avoid increased stress from increased temperature, which may have prevented metabolic alterations due to air exposure.

Results showing lower energetic reserves (GLY) in mussels exposed to increased temperature, evidence higher energetic burden in these mussels. These findings are indicative that temperature tested was stressful enough to increase expenditure of energy reserves, probably associated with its use in defense mechanisms (e.g. antioxidant enzymes) or/and repair of damaged cellular structures through the renewal of damaged LIP reserves. Nonetheless, other studies have shown increase of energy reserves in *M. galloprovincialis* with the increase of temperature (Coppola et al., 2017, 2018a). Even so, the expenditure of energy reserves in bivalves under different stressful conditions has also been observed. Velez et al. (2016) observed that clams *R. philippinarum* mobilized GLY as energy source under osmotic stress. Dickinson et al. (2012) demonstrated that *Crassostrea virginica* oysters exposed to osmotic and hypercapnic stress presented partial depletion of GLY and LIP reserves.

The present results further demonstrated that at control temperature exposure to tides did not alter GLY and LIP content in comparison to submersed mussels. Similarly, Ivanina et al. (2011) did not observe differences in the GLY content in the oyster *C. virginica* exposed to hypoxia conditions for 2 weeks. Nevertheless, in mussels submitted to 6h of daily air exposure, Andrade et al. (2018) found that LIP content was decreased. In another study, Yin et al. (2017) demonstrated energy expenditure (through LIP and fatty acid composition analysis) in *R. philippinarum* clams submitted to different daily air exposure regimes, and explained those findings by elevated aerobic metabolism during re-immersion periods. However, it seemed that mussels exposed to tides mobilized LIP reserves at increased temperature. The mobilization of LIP content may be associated with the repair of damaged cellular structures through the renewal of damaged LIP, due to increased LPO under this condition. In the present study, the combination of tidal regime and increased temperature showed to induce LIP mobilization, likely as an energy source to fuel mussels defense mechanisms against the stress induced in this. Therefore, the tidal regime and increased temperature exposure may be the most condition stressful in terms of energy reserves expenditure.

Oxidative damage

Mussels exposed to increased temperature, presented lower LPO levels in comparison to organisms exposed at control temperature, in both tidal treatments. These results were accompanied by an increase of GSSG content, especially noticed in mussels exposed to tides, evidencing that organisms were experiencing oxidative stress as GSSG results from the oxidation of reduced glutathione (GSH), the most abundant cytosolic scavenger participating in the antioxidant defense system, neutralizing directly ROS. Furthermore, GSH also acts as a co-factor of other antioxidant enzymes such as GPx, which in fact significantly increased in organisms under increased temperature and/or exposed to tides. The highest LPO decrease observed in mussels under warming conditions and submersed compared to organisms exposed to tides may indicate that higher ROS production occurred in mussels exposed to tides which were not eliminated at the same rate by antioxidant defenses. For the same temperature (21 ºC), Coppola et al. (2017, 2018a) observed a decrease of GSH/GSSG in *M. galloprovincialis* after 28 days of exposure, which may indicate oxidative stress as GSSG increased relative to GSH. In the same species, Kamel et al. (2012) observed no differences in LPO in mussels exposed for 7 days to 20 ºC, but showed significant increase of LPO levels at 22 ºC.

The present study further demonstrated that mussels exposed to tides, both under control and increased temperature, presented higher LPO levels. These findings further demonstrate that submersion was the least stressful condition to mussels since exposure to air led to cellular damage, likely due to the lack of activation of antioxidant defenses. The highest LPO levels in mussels exposed to tides, especially at control temperature, could also have resulted from higher ROS production due to increased ETS activity in these conditions as a result of re-oxygenation after emersion. Studies have shown that the mitochondrial electron system activity is one of the major cellular generators of ROS (Loschen et al., 1971; Boveris et al., 1972; Chance et al., 1979). Similarly, Yin et al. (2017) showed increased LPO levels in clams exposed to daily air exposure cycles of 3h, 6h and 9h, despite not significantly. Likewise,

in mussels exposed to daily air exposure cycles of 6h Andrade et al. (2018) observed an increase of LPO in mussels. Rivera-Ingraham et al. (2013) demonstrated an increase of ROS with an induction of LPO in the mantle of *M. edulis*, exposed to anoxic conditions and reoxygenation. These authors suggested that during ebb tide emersion intertidal bivalves used a shell closure strategy to avoid oxidative stress during usual anoxia-hyperoxia conditions. In addition, ATP degrades to AMP under anoxic conditions, which is further degraded to hypoxanthine. Upon reoxygenation, hypoxanthine and xanthine are oxidized and generate ROS (Jones 1986) that in turn could explain the increase of LPO levels in mussels exposed to tides.

Concerning results on PC, lower levels were observed in mussels exposed to increased temperature in both submersed and tidal conditions. Consistent with results showing lower LPO in the same conditions, mussels exposed to increased temperature, seemed to be able to prevent protein carbonylation by activating GSSG and/or antioxidant enzymes activity due to high ROS production in this condition. Paital and Chainy (2013) observed an increase of carbonyls in the muscles of mud crabs *Scylla serrata* collected in summer in comparison to organisms captured during winter, although overall the difference was not significant in the gills and hepatopancreas of these organisms.

The present findings further demonstrated similar PC levels between submersed and tides exposure conditions, in mussels from both control and increased temperatures. In accordance to our results, Rivera-Ingraham et al. (2013) demonstrated that mussels *M. edulis* presented unaltered protein carbonyl content after reoxygenation, followed by a burst of LPO in same condition. Furthermore, Ivanina and Sokolova (2016) also observed no differences in carbonyl levels in *Mercenaria mercenaria* clams exposed to anoxia or hypoxia followed by re-oxygenation. Thus, the present findings indicate that air exposure during ebb tide did not produce enough stress to generate protein oxidation in mussels.

Antioxidant enzymes

Our results showed that, in comparison to control temperature, mussels submitted to warming, both submersed and exposed to tides, were able to increase their antioxidant defenses which led to lower LPO and PC levels in mussels exposed to these conditions. Coppola et al. (2017) showed that *M. galloprovincialis* exposed to increased temperature (21 ºC) presented increased SOD activity after 28 days of exposure. Kamel et al. (2012) showed increased CAT activity and LPO in the digestive gland of *M. galloprovincialis* under 22 ºC. Hu et al. (2015) showed an increase of SOD and CAT activities in *M. coruscus* mussels. Thus, our findings clearly suggest that warming conditions greatly increased the antioxidant defenses to prevent oxidative damage.

The present findings generally showed higher antioxidant enzymes activities in organisms exposed to tidal regimes especially in mussels exposed to increased temperature, likely evidencing an adaptation mechanism to high levels of ROS produced during re-oxygenation typical of the tidal environments that mussels inhabit. Yin et al. (2017) demonstrated an increase of SOD activity in *R. philippinarum* after daily exposure to air. Additionally, Andrade et al. (2018) observed increased SOD activity in *M. galloprovincialis* submitted to 3h and 6h of air exposure followed by immersion, evidencing the increase of antioxidant enzymes activity to neutralize ROS originated during re-oxygenation. Likewise, Almeida and Bainy (2006) showed increased SOD activity in mussels *P. perna* under 4h of air exposure. However, non-significant differences of GPx activity in the digestive gland of *P. perna* mussels where demonstrated after 4h of air exposure and re-immersion (Almeida and Bainy, 2006; Almeida et al., 2005). Also a non-significant increase of GPx activity in *M. edulis* was observed by Letendre et al. (2011) after exposure to a simulated tidal cycle during 14 days.

CONCLUSIONS

The present study demonstrated that increased temperature may affect *M. galloprovincialis* physiological and biochemical performance, especially in mussels exposed to tidal emersion cycles. Our findings further revealed that mussels were generally able to cope with high levels of ROS produced during air exposure and warmer temperature by inducing defense mechanisms and using energy reserves to activate them. However, although mussels were able to prevent oxidative damage due to the increase of temperature, air exposure augmented the physiological and biochemical alterations induced by warmer temperature in *M. galloprovincialis*, demonstrating the importance of this species in the evaluation of the effects of different abiotic factors on intertidal organisms in future studies.

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Highlights

- Temperature induced antioxidant defenses avoiding oxidative damage
- Air exposure increased metabolic capacity and cellular damages
- The combination of temperature and air exposure caused energy reserves expenditure
- The combination of stressors activated antioxidant defenses but cellular damage occurred
- Under tidal conditions the oxidative stress generated by warming is enhanced

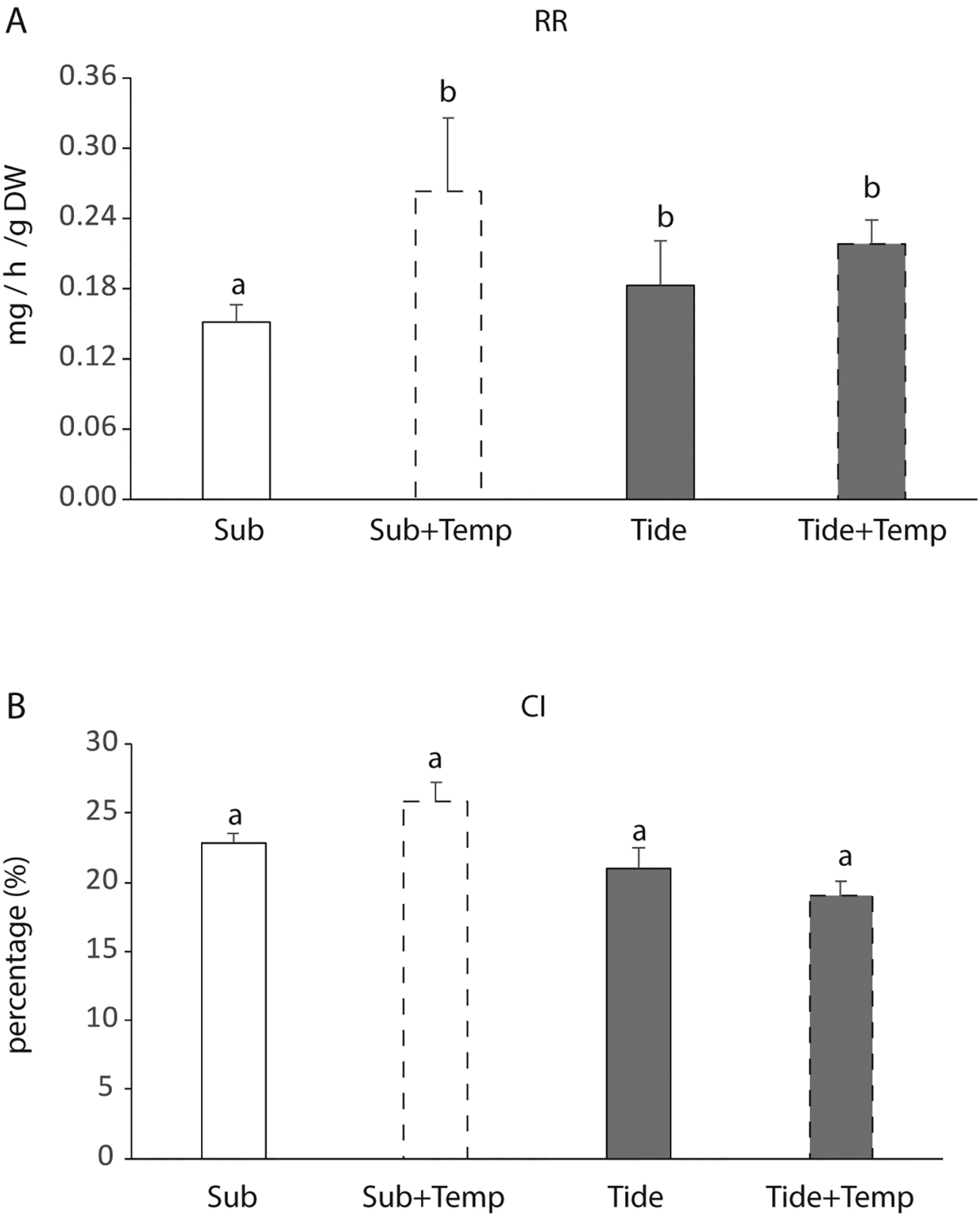
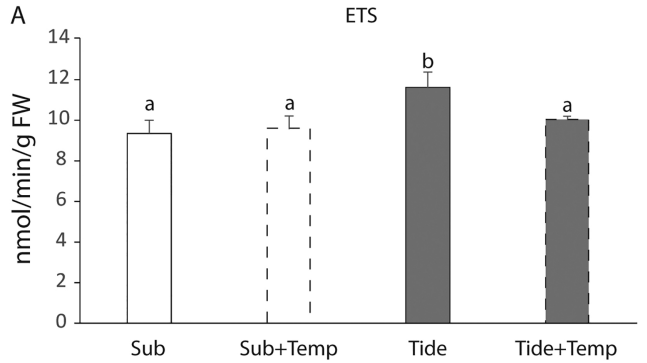
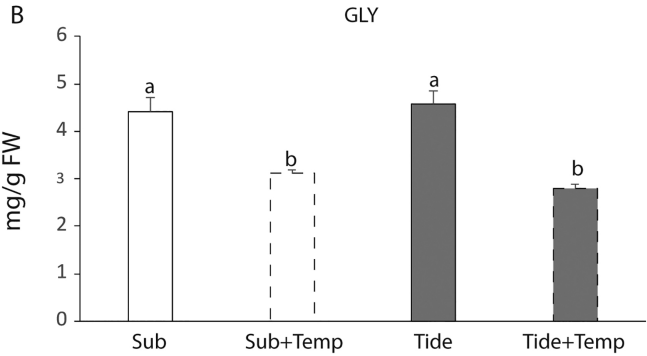

A
RR
mg / h /g DW
0.36
0.30
0.24
0.18
0.12
0.06
0.00
a
b
b
b
Sub
Sub+Temp
Tide
Tide+Temp
B
CI
percentage (%)
30
25
20
15
10
5
0
a
a
a
a
Sub
Sub+Temp
Tide
Tide+Temp

Figure 1


A
ETS
nmol/min/g FW
14
12
10
8
6
4
2
0
a
a
b
a
Sub
Sub+Temp
Tide
Tide+Temp


B
GLY
mg/g FW
6
5
4
3
2
1
0
a
b
a
b
Sub
Sub+Temp
Tide
Tide+Temp

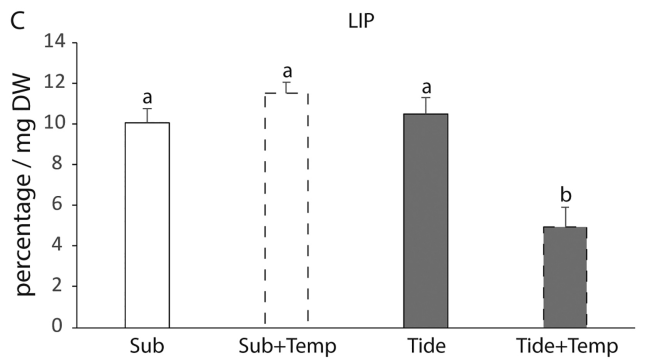
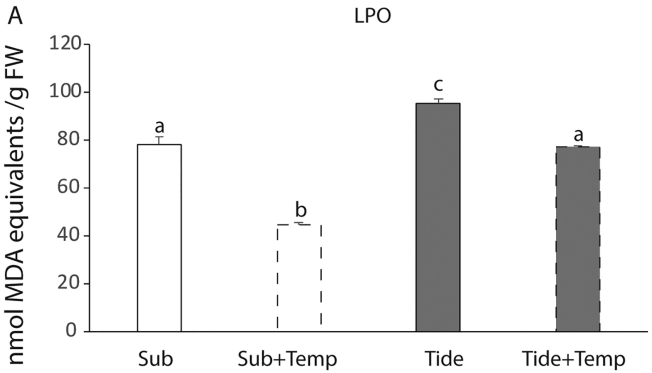
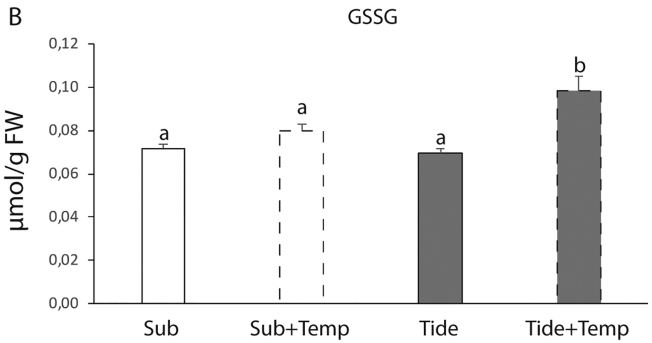
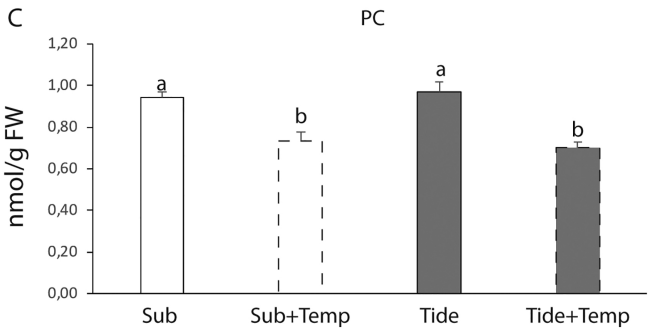

C
LIP
percentage / mg DW
14
12
10
8
6
4
2
0
a
a
a
b
Sub
Sub+Temp
Tide
Tide+Temp

Figure 2


A
LPO
nmol MDA equivalents /g FW
120
100
80
60
40
20
0
a
b
c
a
Sub
Sub+Temp
Tide
Tide+Temp


B
GSSG
µmol/g FW
0,12
0,10
0,08
0,06
0,04
0,02
0,00
a
a
a
b
Sub
Sub+Temp
Tide
Tide+Temp


C
PC
nmol/g FW
1,20
1,00
0,80
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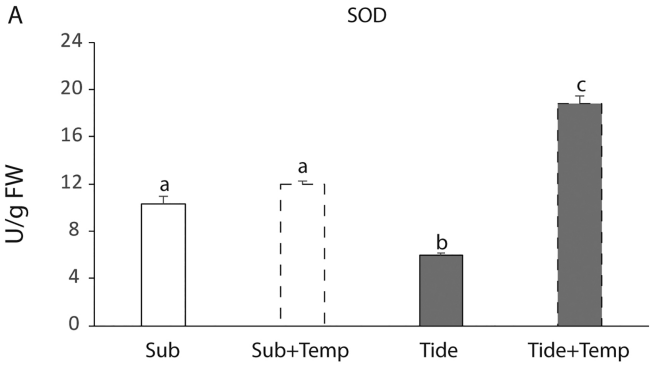
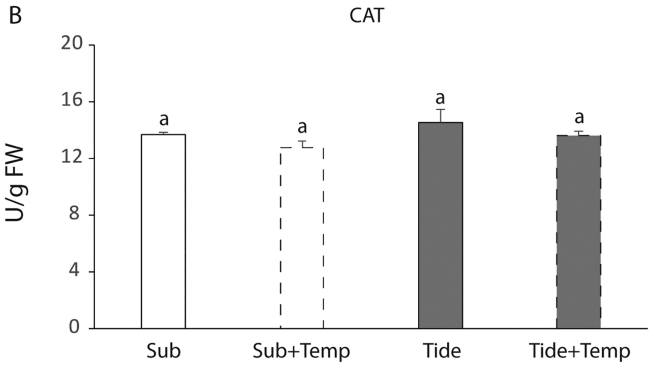
0,40
0,20
0,00
a
b
a
b
Sub
Sub+Temp
Tide
Tide+Temp

Figure 3


A
SOD
U/g FW
24
20
16
12
8
4
0
a
a
b
c
Sub
Sub+Temp
Tide
Tide+Temp


B
CAT
U/g FW
20
16
12
8
4
0
a
a
a
a
Sub
Sub+Temp
Tide
Tide+Temp

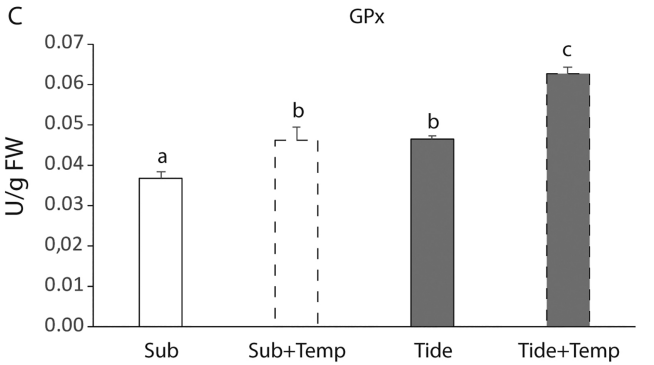

C
GPx
U/g FW
0.07
0.06
0.05
0.04
0.03
0,02
0.01
0.00
a
b
b
c
Sub
Sub+Temp
Tide
Tide+Temp

Figure 4