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Effects of seawater acidification and salinity alterations on metabolic, osmoregulation and oxidative stress markers in *Mytilus galloprovincialis*

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ABSTRACT

The impacts of seawater acidification and salinity shifts on metabolism, energy reserves, and oxidative status of mussels have been largely neglected. With the aim to increase the current knowledge for the mussel *Mytilus galloprovincialis* a 28-day chronic test was conducted during which mussels were exposed to two pH (7.8 and 7.3; both at control salinity 28) and three salinity (14, 28 and 35, at control pH, 7.8) levels. After exposure to different conditions, mussels electron transport system activity, energy reserves (protein and glycogen content) carbonic anhydrase activity, antioxidant defences and cellular damage were measured. Results obtained showed that mussels exposed to seawater acidification presented decreased metabolic capacity that may have induced lower energy expenditure (observed in higher glycogen, protein and lipids content at this condition). Low pH condition induced the increase of carbonic anhydrase activity that was related to acid-base balance, while no significant activation of antioxidant defence mechanisms was observed resulting in higher LPO. Regarding the impacts of salinity, the present study showed that at the highest salinity (35) mussels presented lower metabolic activity (also related to lower energetic expenditure) and an opposite response was observed at salinity 14. Carbonic anhydrase slightly increased at stressful salinity conditions, a mechanism of homeostasis maintenance. Lower metabolic activity at the highest salinity, probably related to valves closure, helped to mitigate the increase of LPO in this condition. At low salinity (14), despite an increase of antioxidant enzymes activity, LPO increased, probably as a result of ROS overproduction from higher electron transport system activity. The present findings demonstrated that *Mytilus galloprovincialis* oxidative status and metabolic capacity were negatively affected by low pH and salinity changes, with alterations that may lead to physiological impairments namely on mussels reproductive output, growth performance and resistance to disease, with ecological and economic implications.

Indicators: Physiological and biochemical changes in *Mytilus galloprovincialis* in response to low pH and salinity changes

1. Introduction

Due to human activities, atmospheric CO_2 partial pressure (pCO_2) is increasing, and is predicted to reach 500–1000 μatm by the end of this century (Caldeira and Wickett, 2003, 2005; IPCC, 2013; Orr et al., 2005; Raven et al., 2005). These changes have led to an increase of global mean temperature (a process called global warming) and to a decrease in both pH and the availability of carbonate ions in seawater (a phenomenon known as ocean acidification) (Caldeira and Wickett, 2003; Feely et al., 2009; Orr et al., 2005; Raven et al., 2005). As a result of global warming salinity shifts are also expected to occur since the atmospheric temperature rise is causing changes in the hydrological cycle at a global scale, with increases in precipitation at high latitudes and near the tropics, and decreases in the sub-tropical and mid-latitude regions (Fenoglio et al., 2010). As a consequence, many areas may experience frequent flood and drought events which lead to prolonged and more frequent periods of decreased or increased salinity, particularly in estuarine areas (Bussell et al., 2008). Additionally, the continuous release of CO_2 into the atmosphere has caused a decrease in ocean pH of approximately 0.1 pH units with respect to pre-industrial levels, and further decrease from 0.3 to 0.5 pH units are predicted to occur up to year 2100 (Caldeira and Wickett, 2003, 2005; IPCC, 2013; Orr et al., 2005; Raven et al., 2005).

Besides studies on climate change predictions, a growing body of

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evidence shows that seawater acidification and salinity alterations can impact aquatic species, with particular attention to calcifying organisms such as bivalves (e.g Beniash et al., 2010; Bressan et al., 2014; Dickinson et al., 2012; Velez et al., 2016). Despite a growing number of studies published on seawater acidification, that show impacts on bivalves physiological performance including feeding and excretion rates, growth and larval development, calcification, respiration and reproduction (e.g., Bressan et al., 2014; Dupont et al., 2010; Fernández-Reiriz et al., 2011; 2012; Gazeau et al., 2010; Kurihara, 2008; Michaelidis et al., 2005; Navarro et al., 2013; Sun et al., 2016; Wang et al., 2015; Xu et al., 2016), less information is available regarding alterations on bivalves oxidative status and metabolic capacity exposed to such scenarios (Hu et al., 2015; Matoo et al., 2013; Matozzo et al., 2013). Furthermore, few studies evaluated the effects of salinity on bivalves physiological (Dickinson et al., 2012; Kim et al., 2001; Hamer et al., 2008) and biochemical (e.g.: Bussel et al., 2008; Carregosa et al., 2014a, 2014b; Gonçalves et al., 2017; Hamer et al., 2008; Moreira et al., 2016a; Velez et al., 2016) performance.

Among marine bivalves, several mussel species are commonly used as bioindicators of environmental stressors since they present physiological and biochemical changes when exposed to inorganic and organic contaminants (among others Apeti et al., 2010; Filimonova et al., 2016; Lavradas et al., 2016; Milun et al., 2016; Signa et al., 2015), including *Mytilus galloprovincialis* (e.g. Cajaraville et al., 1996; Roberto et al., 2010; Rocha et al., 2016; Vlahogianni et al., 2007). Most recently, *M. galloprovincialis* has also been used as model organism to study climate change related stressors (Anestis et al., 2007; Bressan et al., 2014; Duarte et al., 2014; Fernández-Reiriz et al., 2012; Matozzo et al., 2013; Michaelidis et al., 2005; Range et al., 2012). However, no studies identified and compared the osmotic, metabolic and oxidative stress alterations induced in *M. galloprovincialis* under salinity alterations and seawater acidification. To enhance the current knowledge on this species performance under a changing environment, the present study aimed to evaluate the capacity of *M. galloprovincialis* as a bioindicator of salinity changes and seawater acidification. For this, mussels physiological and biochemical responses were identified, including metabolic capacity (electron transport system activity), energy reserves content (protein, glycogen and lipid), osmoregulation capacity (carbonic anhydrase activity), cellular damage (lipid peroxidation levels) and antioxidant capacity (superoxide dismutase and catalase activity), after mussels chronic exposure (28 days) to different salinity (14, 28, 35) and pH (7.8 and 7.3) levels.

2. Materials and methods

2.1. Study organisms and experimental setup

Mussels were collected in September 2015 in the Ílhavo channel, the most narrow and short main channel of the Ria de Aveiro lagoon (Northwest coast of Portugal), with a length of ca. 15 km. Previous studies demonstrated low metal contamination levels in this channel (Freitas et al., 2014; Velez et al., 2015).

After collection mussels were transferred to the laboratory, where they were acclimated and depurated for 15 days prior to exposure. During this period, mussels were kept in artificial seawater (Tropic Marin Reef Mix) prepared at salinity 28 ± 1, pH 7.8, temperature maintained at 17 ± 1 °C, 12 light: 12 dark photoperiod and continuous aeration. Animals were fed two times per week with Algamac Protein Plus (150.000 cells/animal).

After this period organisms were distributed in different aquaria to test different salinities (14, 28, 35) at control pH (7.8) and different pH levels (7.8 and 7.3) at control salinity (28). The following conditions were used for exposures: salinity 14 (pH 7.8), salinity 28 (control salinity, pH 7.8), salinity 35 (pH 7.8), pH 7.3 (control salinity). For each condition 3 replicates were used, with 5 organisms per replicate (15 organisms per condition). For each replicate 1L of artificial seawater per individual was used. Organisms were exposed to each condition for 28 days. During the experimental period dissolved oxygen concentration was monitored in all aquaria and animals were checked for mortality every day. Aquaria were maintained at constant temperature (17 ± 1 °C), 12 light: 12 dark photoperiod and continuous aeration. Two times per week, animals were fed with Algamac Protein Plus (150.000 cells/animal), seawater was renewed every week, and pH and salinity levels re-established. Dead organisms were removed when identified. After exposure (28 days), surviving organisms were frozen until biochemical and physiological analysis.

For pH acclimation, organisms were submitted to a gradual pH decrease (0.2 values per day) to reach pH 7.3 and maintained at salinity 28 ± 1. The remaining organisms were maintained at pH 7.8 and salinity 28 ± 1. To reach the salinity levels of 14 and 35, mussels under pH 7.8 were exposed every 2–3 days to different salinities, which were gradually lowered or increased in 2–3 values to reach the test levels. Mussels exposed to different salinity conditions were maintained under pH 7.8 (considered as control pH).

Low pH was obtained by directly diffusing CO_2 into aquaria. Individual aquarium pH levels were continuously monitored and controlled using a pH Stat system (Aquamedic AT Controller) and crosschecked with independent probes (Hanna Instruments). pH Stat system probes were calibrated using NIST buffers (NBS scale). Seawater pH was crosschecked against an independent probe (Hanna Instruments) at least twice a week, and the pH Stat computer reset to match the independent probes pH if needed. Lowered pH was set to 7.3, to give a 0.5 pH unit reduction relative to control (pH 7.8). Control pH was considered as the average pH measured at the sampling area (7.7–7.9) and a pH decrease of 0.5 units as predicted for the end of the twenty first century (Raven et al., 2005). Tested pH values are within naturally occurring range in temperate estuarine systems (Ringwood and Keppler, 2002; Cochran and Burnett, 1996; Ramos et al., 2006; Ansari and Gill, 2013). Testing conditions gave pCO2 values ranging from ~940 (pH 7.8) to ~3700 µatm pCO_2 (pH 7.3), within values that occur in coastal habitats (Cochran and Burnett, 1996; Melzner et al., 2012). Salinity 28 was used as control taking into account the salinity at the sampling area. Salinities tested (14 and 35) were selected to resemble extreme weather events of rain and drought periods, namely in the study area (Dias et al., 1999; Dias and Lopes, 2006; Génio et al., 2008; Vaz and Dias, 2008).

Water samples were collected from pH test aquaria (conditions: pH 7.8 and salinity 28; pH 7.3 and salinity 28), prior to water renewal, and used to determine Total alkalinity (TA) by potentiometric titration (Gran, 1952). Calculated TA values and measured parameters (tem-

Table 1
Carbonate system physicochemical parameters for pH experiments (mean ± SD). Measured pH, and determined total alkalinity (A_t) from weekly water sampling (Temperature 17.1 °C ± 0.75 and salinity 28.4 ± 0.5). 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 ().

	pH	A_t (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	ΩCal	ΩAra
pH 7.8	7.86 ± 0.06	2271 ± 127	940 ± 149	2081 ± 126	78.3 ± 10.0	1.95 ± 0.26	1.2 ± 0.14
pH 7.3	7.34 ± 0.02	2495 ± 92	3645 ± 218	2429 ± 90	27.2 ± 1.8	0.68 ± 0.04	0.43 ± 0.03