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Monograph

Ocean warming lead to heat shock protein expression and decrease in the feeding rate of the Patagonian sea star *Anasterias minuta*

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ABSTRACT

Climate change has consequences over marine organisms and their behavior. Echinoderms have been found to be negatively affected on righting time, survival, feeding rate or growth by high temperatures, low salinity or their interaction. We analyzed the effects of temperature and salinity fluctuation under a climate change scenario on feeding rate, righting time and heat shock proteins (Hsp70) expression in the sea star *Anasterias minuta*. Our results showed negative effects of increased temperature in the feeding rate of *A. minuta*. In addition, Hsp70 expression in gonads showed an upregulation after water temperature increase in normal salinity conditions. Regarding the righting activity, we detected a variable pattern among treatments with significant statistical differences in the interaction of temperature and salinity, and in time intervals measured. Variations in sea stars feeding ecology could produce direct and cascading effects driving ecological shifts as changes in species composition of the coastal Patagonian ecosystems. Investigating the effect of sea stars on intertidal and subtidal assemblages in a climate change scenario is of great importance to provide valuable information for an adequate management of intertidal habitats, which have been little studied in Atlantic Patagonia.

1. Introduction

Global warming, as result of anthropogenic activities, is emerging as an important factor changing ocean heat, setting stress on marine species and ecosystems (Intergovernmental Panel on Climate Change – IPCC, 2013, 2014, 2021). Increasing temperature – projecting to rise over 4 °C by 2100 based on the extreme IPCC scenario RCP8.5 – would result in boost infection intensities and drive on mass mortalities of species (Hughes, 1994; Bates et al., 2009; Cole et al., 2016; Harianto et al., 2018). Oceans are also under an irreversible trajectory of acidification (i.e., decreases in pH and carbonate-ion concentration, see Caldeira and Wickett, 2003, 2005; Bednarek et al., 2021). Seawater pH is expected to decrease between 0.14 and 0.32 units by the year 2100 under different climate scenarios (IPCC, 2014). This reduction in carbonate availability will negatively affect skeleton formation in marine

calcareous organisms (Gooding et al., 2009; Dupont et al., 2010; Byrne et al., 2013; Keppel et al., 2015; Randazzo, 2021). Furthermore, sea surface salinity is likely to become more saline in subtropical areas, while intermediate waters formed at higher latitude will become fresher under a climate change scenario (Boyer et al., 2005; Terray et al., 2012; IPCC, 2013). The effects of low salinity over species can reduce the amount of energy intake and the efficiency of energy utilization (Shirley and Stickle, 1982; Forcucci and Lawrence, 1986; see Russell, 2013 for review).

Echinoderms are poikilothermic and stenohaline, formed by a calcareous endoskeleton (Chen and Chen, 1993). Although some species can set tissue hydration by isosmotic intracellular regulation (see Diehl and Lawrence, 1984; Forcucci and Lawrence, 1986; Stickle and Diehl, 1987), changes in salinity and temperature can affect their behavior (Stickle and Diehl, 1987; Sanford, 2002; Gooding et al., 2009; Sherman,

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2015). Previous works in echinoderms have found negative effects on righting time, survival, feeding rate or growth by high temperatures (Bates et al., 2009; Byrne et al., 2009, 2013; Ardor Bellucci and Smith, 2019), low salinity (Forcucci and Lawrence, 1986; Kashenko, 2003; Held and Harley, 2009) or their interaction (Chen and Chen, 1993; Watts and Lawrence, 1990; Balogh and Byrne, 2021). The synergistic effect of the combination of high temperature and low salinity decrease organisms' activity and may increase the energy required for maintenance, affecting the ability of asteroids to feed and development (Forcucci and Lawrence, 1986; Chen and Chen, 1993; Balogh and Byrne, 2021). A molecular marker of the response of organisms to increase temperature and other stress conditions is the highly conserved heat shock protein 70 (Hsp70) which serve as biomarkers of environmental stress (Matranga et al., 2005; Pinsino and Matranga, 2015; Gao et al., 2019). Particularly Hsp70 family have an important role as cellular chaperon against stress (Fink, 1999) and present one of the higher rates of synthesis in ectotherms organisms (Tomanek and Somero, 2000).

Rocky intertidal is characterized by being exposed to a wide range of stressors such as ultraviolet radiation, temperature, and strong wind (Raffaelli and Hawkins, 2012). The oral brooding sea star *A. minuta* Perrier, 1875 (see Hernández and Tablado, 1985; Brogger et al., 2013) is an abundant species in rocky intertidal along Atlantic Patagonian coast which feeds on a wide range of prey, predominantly on the abundant purple mussel *Perumytilus purpuratus* and the snail *Tegula patagonica* (Gil and Zaixso, 2008; Arribas et al., 2016, 2017). Gonadal maturation occurs in summer and females spawn only a small number of eggs (Gil et al., 2011; Pérez et al., 2015; Arribas et al., 2017), and does not feed during the brooding period (Gil and Zaixso, 2007; Pérez et al., 2015).

The aim of the present study was to investigate the effects of temperature and salinity fluctuation under a climate change scenario expected by 2100 (IPCC, 2013, 2014) over the feeding rate, the righting time and the Hsp70 expression on the intertidal sea star *A. minuta*. We predict that an increase in temperature coupled with a decrease in salinity would result in a significantly lower feeding rate and righting time, and an increase in Hsp70 expression.

2. Materials and methods

2.1. Study area and sampling design

Individuals of the sea star *A. minuta* were collected at Punta Colorada (41°42' S – 65°1' W), south of San Matías Gulf, Atlantic Patagonia. Punta Colorada rocky outcrop belongs to a group of volcanic and marine sediments with large pyroclastic contribution (Kokot et al., 2004). Sea stars ($n = 24$, mean sea star arm $R = 25.18 \pm 2.18$ mm, and biomass = 5.40 ± 1.32 g) were collected from low rocky intertidal, in early Autumn of 2017 before their brooding season. The southern region of San Matías Gulf is characterized by an average sea surface temperature (SST) of 16.0 ± 2.5 °C (Williams et al., 2013). In Winter, sea temperature varies between 10 and 11 °C, while Summer presents temperatures between 16 and 18 °C (Orrego, 2020). Values of seasonally satellite-derived SST from MODIS Aqua around 2 km from the coast of Punta Colorada for the period 2003 to 2015 are 17.01 ± 0.48 °C for Summer, 15.80 ± 0.37 °C for Autumn, 11.47 ± 0.17 °C for Winter, and 12.04 ± 0.35 °C for Spring (Bigatti, unpublished data). Waters of San Matías Gulf exhibit high salinity, with values over 34.0 throughout the year. In Autumn and Winter the salinity range between 33.9 and 34.1, and in Spring and Summer between 33.9 and 34.0 (Lucas et al., 2005).

Only normal-looking sea stars (not regenerating arms) were collected. Sea stars were transferred to the laboratory and held in 50 l filtered seawater aquaria containers at collection temperature and salinity during two weeks for acclimation. During this period sea stars were starved.

The experiment on seawater temperature and salinity was run over 6 weeks. Sea stars were exposed to two different temperatures and salinities. Temperature was warmed to the experimental level at a rate of 1 °C

per day, and salinity was diminished approximately 1.25 per day. Salinity survival tolerance in sea star *A. minuta* was tested previously with a short pilot experiment with three different salinities (35, 30 and 20, $n = 10$ by treatment) during two weeks to decide salinity level in temperature-salinity experiment under climate change scenario. Most of the sea stars died when exposed at salinity of 20 (unpublished data). Sea stars were randomly assigned to 1.5 l containers with experimental temperature and salinity. Temperature and salinity were recorded three times per week in the containers, pH levels of water were checked weekly (Milwaukee pH 55) and water exchanged as needed. The photoperiod was 12:12 h.

Individual 1.5 l plastic containers were partially submerged in four 60 l water baths with thermostatically controlled heaters and chillers to maintain constant temperatures. Two treatments were used: control temperature (15 °C), representing nearly the average temperature in austral Autumn at the collection site (14.98 ± 0.93 °C) based on field data collected and satellite images (extracted mean Autumn temperature from 2003 to 2017), and high temperature (19 °C), representing control temperature plus 4 °C, the average global increase predicted for 2100 by the IPCC RCP8.5 scenario. Experimental salinity levels were achieved by adding freshwater on containers. Two levels were tested: a control salinity of 35 (average Autumn salinity at the collection site 35.56 ± 0.43) and a decreased salinity of 30. Six containers were randomly assigned to each of the four treatment combinations (15T:35S, 15T:30S, 19T:35S, 19T:30S), giving a total of 24 containers (2 temperature levels \times 2 salinity levels \times 6 containers per temperature-salinity treatment). Each container held one sea star. The pH level of water remained constant in all containers (7.87 ± 0.05) during the experiment. We defined our system with 24 sea stars based on similar experiment studies on echinoderms' feeding rate, righting time, and Hsp70 expression (Lawrence, 1973; Lares and McClintock, 1991; Holm et al., 2008; Peck et al., 2008; Keppel et al., 2015).

2.2. Sea stars response variables

Sea stars size and wet weight were measured at the beginning of the experiment. Size was measured as the longest arm length R with Vernier calipers (± 0.01 mm). Wet weight was obtained drying animals with paper towels and placed on a digital scale (± 0.1 g). Animals were returned to containers immediately after measurements. Sea stars were fed *ad libitum* with tiny mussels (*Perumytilus purpuratus*, shell length 10–15 mm). Feeding rate was calculated as the number of mussels consumed daily *per sea star*.

The activity of all sea stars was recorded at four times intervals (*i.e.*, day 14, day 28, day 34 and day 42) over the feeding experiment (see Ellington and Lawrence, 1974; Stickle and Diehl, 1987; Watts and Lawrence, 1990). The righting activity coefficient (RAC) was used to indicate the functional well-being of all individuals and was calculated as the ratio of 1000 divided by the time in seconds required for individuals placed on their aboral surface to right themselves on a horizontal surface (Stickle and Diehl, 1987). Individuals that did not initiate a righting response within ten minutes were assigned a value of 0 (Watts and Lawrence, 1990).

2.3. Quantification of heat shock protein 70

After 6 weeks, sea stars ($n = 24$) were opened and gonads removed to analyze Hsp70 proteins. Western blots of Hsp70 expression were previously evaluated in gonad tissue from sea urchins (Nguyen et al., 2013; Harianto et al., 2018), stating that gonads are ease of sampling and ensuring sufficient obtained protein. Frozen tissues were homogenized in ice-cold TEDGS 10% buffer (50 mM Tris pH = 7.4, 7.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.25 M sucrose), including protease inhibitors. Proteins were quantified by Lowry method (Lowry et al., 1951), and equivalent amounts of protein (100 μ g) from lysates tissue were separated on discontinuous polyacrylamide gels and detected by

Western Blot (Giulianelli et al., 2020). Membranes were probed with Hsp70 (sc-33,575 rabbit-polyclonal 1/600) or β -Actin (sc-47,778 mouse-monoclonal 1/600) commercial antibodies (Santa Cruz Biotechnology), and then incubated with peroxidase-conjugated secondary antibodies (Vector Laboratories 1/4000) diluted in TBS-T (Tris 20 mM, NaCl 150 mM, Tween 20 0.1%). The luminescent signal was generated by ECL method and the blots were exposed to a medical X-ray film (AGFA) (Nieto Vilela, 2020). Each sea star gonad was evaluated twice for Hsp70 and β -Actin expression. Band intensity was determined by densitometry using ImageJ software (Schneider et al., 2012).

2.4. Statistical analyses

A two-way ANOVA was used to determine the effects of temperature and salinity on sea star feeding rate, and a repeated measures ANOVA on sea stars righting activity coefficient (RAC). The statistical package STATISTICA 7.0 was used for ANOVA analysis. Initial sea star arm length and wet weight at the beginning of the experiment were not significantly different between any treatment combinations ($p > 0.05$, sqrt ($x + 1$) transformation was applied to wet weight to achieve assumptions, data not shown). Data were tested for normality with the Shapiro–Wilk test and for homoscedasticity with Cochran's C-test. Post-hoc among treatment means were compared with Tukey HSD tests.

In Hsp70 protein levels determination, the Hsp70 optical density was normalized by a housekeeping protein band (β -Actin) per sample. A 'common sample' was used to control variations in optical density and background noise between gels (Lima et al., 2016). Then, normalized values (Hsp70/ β -Actin) were quantified relative to the optical density of the 'common sample' bands previously assessed for each gel. To test differences in the average of Hsp70 expression between the temperature and salinity experimental conditions, a factorial ANOVA was carried on. Ln transformation was applied to achieve ANOVA assumptions.

3. Results

From 24 sea stars analyzed, all remained alive until the end of the experiment, although on 19T:35S treatment two sea stars presented open areas with injured tissue over their aboral region. Feeding rate of sea stars was higher in the control treatment (15T:35S) but significantly decreased with the increased temperature (Table 1, Fig. 1). The lower feeding rate was found in the 19T:35S treatment, although no significant interaction was found. Salinity changes did not show effect on the feeding rate of *A. minuta* (Table 1).

Righting activity (RAC) of *A. minuta* showed different trends among experiment treatments (Fig. 2), with significant statistical differences in the interaction of temperature and salinity, and in time intervals measured (Table 2). The RAC of individuals exposed to high temperature and low salinity treatment (19T:30S) at day 14 were higher than in all other combinations. Low salinity at high temperature seems to increase the righting activity of sea star individuals in comparison with treatment at the same temperature but at control salinity (19T:35S), which showed the lowest RAC values throughout the entire period sampled (Fig. 2).

The two-factor analysis of variance showed a significant interaction

Table 1

Two-way Factorial ANOVA testing feeding rate (mussel seastar⁻¹ day⁻¹) under future climate change predictions to increased temperature (control temperature = 15 °C, high temperature = 19 °C) and low salinity (control salinity = 35, low salinity = 30). * $p < 0.001$.

	df	MS	F
Temperature	1	0.83	25.92*
Salinity	1	0.004	0.13
Temp x Salin	1	0.02	0.70
Error	20	0.03	

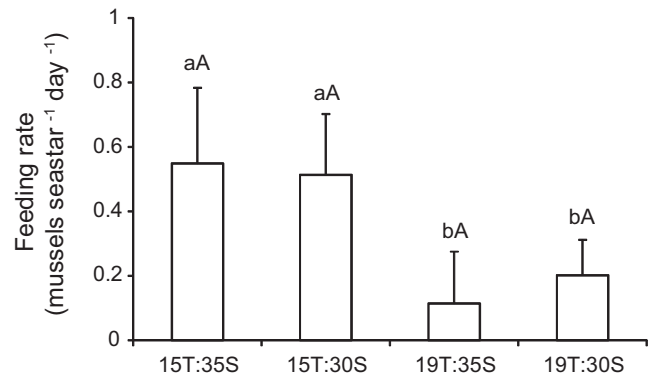


Fig. 1. Response of *Anasterias minuta* (mean \pm SD) to increased temperature (T, control temperature = 15 °C, high temperature = 19 °C) and decreased salinity (S, control salinity = 35, low salinity = 30) in the feeding rate of mussels. Different lower case letters above the bars indicate significant temperature differences ($p < 0.001$), and same capital letters above the bars means not significant salinity differences.

between temperature and salinity conditions in Hsp70 expression from gonads of *A. minuta*. Hsp70 expression showed an upregulation after water temperature increase in normal salinity condition (Fig. 3, Table 3). Probably higher levels of Hsp70 were expressed at high temperature in response to greater levels of stress, although low salinity at high temperature seems to alleviate the negative effects of high temperature.

4. Discussion

Our results showed negative effects of increased temperature over feeding rate of the sea star *A. minuta*, as well as an interaction of increased temperature and control salinity in righting activity (RAC), and in energy investment of heat shock Hsp70 defense in gonads.

The RAC of individuals showed a variable pattern among treatments displaying an abruptly fell at the end of the experiment, except for control treatment. The combination of increased temperature and control salinity seems to have negative effects in RAC. The righting activity coefficient is an important measure that indicates the health condition of individuals (Watts and Lawrence, 1990; Ardor Bellucci and Smith, 2019) and although individuals continued to function with an increase temperature or decrease salinity, lowest activity can affect the survival of *A. minuta* in long-term. The exposure to high temperature at lower salinity seems to reduce the negative effects of increased temperature, showing a compensatory acclimation in RAC response to low salinity conditions. Echinoderms present intracellular isosmotic regulation (Stickle and Diehl, 1987; Watts and Lawrence, 1990), and the physiological functioning and metabolic rate of individuals of *A. minuta* when exposed to stress conditions suggest some capacity for acclimatization to future elevated temperatures and reductions of salinity in righting activity.

Concerning sea water temperature, the experimental increase of 4 °C predicted by IPCC to the year 2100 in an extreme RCP 8.5 scenario (IPCC, 2014; Keppel et al., 2015; Cole et al., 2016), showed negative effects in the feeding ecology of the sea star *A. minuta*. Previous works have indicated the consequences of changes in ocean temperature over sea stars population (Watts and Lawrence, 1990; Stanwell-Smith and Peck, 1998; Sanford, 2002; Byrne et al., 2013; Keppel et al., 2015). The feeding decreased over *A. minuta* population by increased temperature revealed a lack in the acclimatization capacity to increased temperature, which could rise metabolic rates and have physiological consequences that might affect the reproduction and survival of individuals. As predator species, changes in feeding by *A. minuta* in response to habitat warming can lead to modify the predator-prey interaction and impact

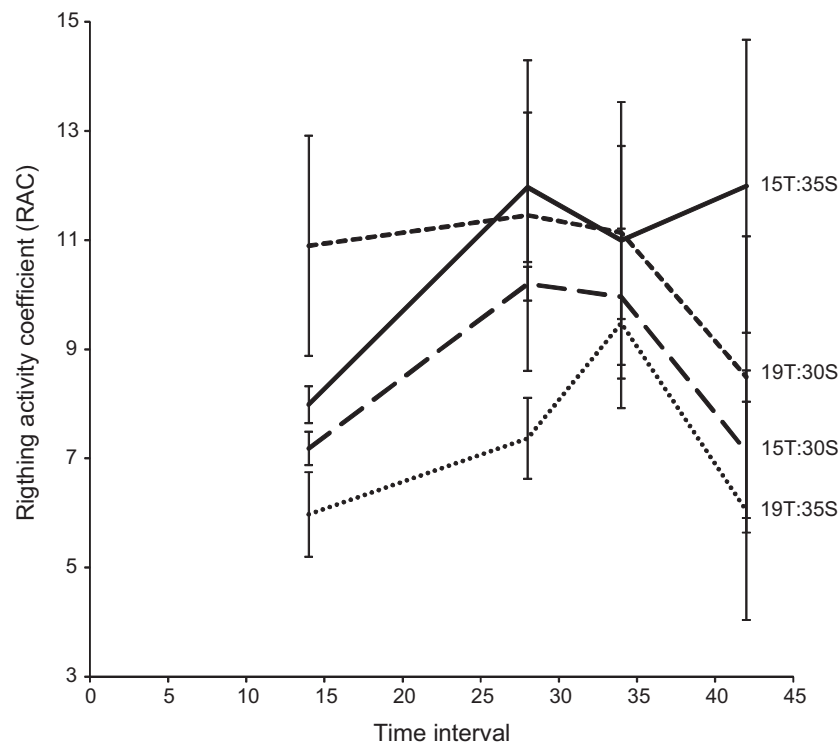


Fig. 2. Effects of temperature (T, control temperature = 15 °C, high temperature = 19 °C) and salinity (S, control salinity = 35, low salinity = 30) combinations on the righting activity coefficient (RAC, mean \pm SE) of *Anasterias minuta* ($n = 6$ individuals) at four times intervals (*i.e.*, day 14, day 28, day 34 and day 42).

Table 2

Repeated measures ANOVA testing righting time (RAC) at four times intervals (*i.e.*, day 14, day 28, day 34 and day 42) under future climate change predictions to increased temperature (control temperature = 15 °C, high temperature = 19 °C) and low salinity (control salinity = 35, low salinity = 30). * $p < 0.05$.

	df	MS	F
Temperature	1	16.19	0.46
Salinity	1	8.09	0.23
Temp x Salin	1	174.81	4.92*
Error	20	35.50	
Time	3	36.38	2.93*
Time x Temp	3	12.24	0.98
Time x Salin	3	11.58	0.93
Time x Temp x Salin	3	5.67	0.45
Error	60	12.43	

the structure of benthic marine communities (Watts and Lawrence, 1990; Gil and Zaixso, 2008; Keppel et al., 2015; Arribas et al., 2017), especially in intertidal environments where species are living close to thermotolerance thresholds (Christensen et al., 2011).

Conserved protein Hsp70 has been described as good indicators of stress in echinoderms in response to various environmental conditions (Pinsino et al., 2007; Holm et al., 2008; Pinsino and Matranga, 2015). The biosynthesis and chaperoning activities of Hsps are energetically costly, suggesting a tradeoff between thermal tolerance and use of metabolic energy (Somero, 2002; Pinsino et al., 2007; Holm et al., 2008). Our results showed an interaction of temperature and salinity in the expression of Hsp70. When sea stars were maintained in control seawater salinity and temperature increased 4 °C (*i.e.*, treatment 19T:35S), Hsp70 expression increased 47.74%, indicating that this combination is a clear stressor in *A. minuta*. High levels of heat shock proteins in *A. minuta* in this experimental condition could be related with the role of chaperones preventing proteins irreversible aggregation or restoration of their native conformation of unfolded states (Ponomarenko et al., 2013). The sea star *A. minuta* is close to its northern

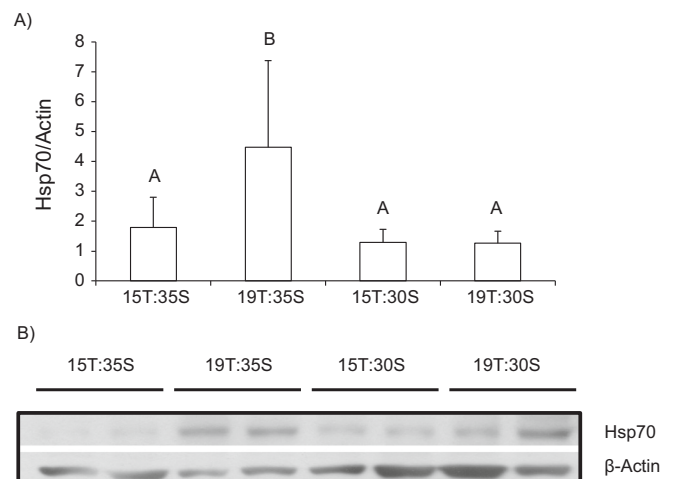


Fig. 3. A) Hsp70 expression in gonads of *Anasterias minuta* (mean \pm SD) to increased temperature (T, control temperature = 15 °C, high temperature = 19 °C) and decreased salinity (S, control salinity = 35, low salinity = 30), B) Representative Western blot showing Hsp70 expression in total extracts from gonads of *A. minuta* after treatments. β -Actin was used as a loading control. Different capital letters above the bars means significant differences ($p < 0.05$) among treatments.

distribution limit (Gil and Zaixso, 2007, 2008), living in intertidal zones or tide pools, where salinity can increase due to evaporation or decrease from rainfall (Vidolin et al., 2002). In the study area, the sea temperature during Summer can reach values close to the experimental conditions (Orrego, 2020), for which *A. minuta* could be living in stressful conditions during those months, and therefore an increase of Hsp70 expression is expected. Ecological tolerance to external stressors is a species-specific trait, nevertheless tolerance to low salinity was also described in other echinoderms (Dong et al., 2008; see Table 3.2 in

Table 3

Two-way Factorial ANOVA testing Hsp70 expression in *Anasterias minuta* under future climate change predictions to increased temperature (control temperature = 15 °C, high temperature = 19 °C) and decreased salinity (control salinity = 35, low salinity = 30). Ln transformation was applied to Hsp70 values. * $p < 0.05$ ** $p < 0.01$.

	df	MS	F
Temperature	1	0.25	4.07
Salinity	1	0.78	12.48**
Temp x Salin	1	0.41	6.46*
Error	20	0.06	

Russell, 2013). A previous study has shown that some echinoderms can temporally regulate the osmolarity of the coelomic fluids modifying the permeability of the body wall (Vidolin et al., 2002), which could be occurring in the sea star *A. minuta* in treatment 19T:30S, since heat shock protein expression did not show significant differences with control temperature and salinity. Furthermore, Meng et al. (2011) found that after 24-h exposure the down-regulation of Hsp70 in the sea cucumber *Apostichopus japonicus* was correlated with a depression of metabolism at low salinity, suggesting decreased capability of physiological regulation, reaching levels similar to those in the control after 72-h. Our experimental results suggest that *A. minuta* present a wide ecological tolerance to temperature and salinity, therefore, the lack of significant differences of Hsp70 expression found in *A. minuta* under low salinity exposure could be related with their tolerance, but also with the experimental exposure time. While future experiments are needed, *A. minuta* could have more chances of maintaining the population in near future climatic change condition, although an increase of 4 °C could demand more energy investment in thermal defense of *A. minuta* population which could drive ecological effects altering the intertidal communities' composition of Atlantic Patagonia.

Intertidal species as *A. minuta* which presents brooding reproductive strategy with low dispersal ability (Hernandez and Tablado, 1985; Salvat, 1985) can be more susceptible to anthropogenic impacts as climatic change (Poulin and Féral, 1996; Pearse et al., 2009). The interaction between Patagonian harsh environment and near future warming conditions can increase the metabolic rate of *A. minuta*, spend more energy on maintenance and be unavailable for growth or reproduction (Christensen et al., 2011; Ardor Bellucci and Smith, 2019). Our results showed that climate warming will negatively affects *A. minuta* feeding rate, which could exceed their physiological tolerances and consequently have implications for the persistence of their populations, although RAC and Hsp70 expression suggest a wide ecological tolerance to external stressors at low salinity, and we emphasize the importance of addressing this topic in a specific-species way since climate warming effects act differently among species (Gooding et al., 2009). This work predicts direct and cascading effects driving ecological shifts as changes in species composition on the Patagonian coastal habitats and provides useful information for the conservation and management of intertidal habitats poorly studied in Atlantic Patagonia.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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