

Heat-shock response in Patagonian gastropods

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Abstract: The physiological process to counteract environmental stress are extremely diverse in marine species that inhabit intertidal zones. Ecological approaches have demonstrated a variety of adaptations among narrowly distributed species. The present study is focused on physiological responses of two key marine gastropods from Atlantic Patagonia living in one of the most stressful intertidal rocky shores of the world: *Tegula patagonica* and *Trophon geversianus*. We evaluated the body temperature and its heat-shock response by Hsp70 expression. Our results suggest that *T. geversianus*, inhabiting the mid-intertidal zone and exposed for longer to aerial conditions, presents better physiological and behavioral adaptations to temperature than *T. patagonica*, living in the low intertidal with less physical stress. *Tegula patagonica* seems to have an inefficient thermal defense, an inability to upregulate Hsp70 expression against water temperature increase. Contrariwise, a significant upregulation of Hsp70 was registered in *T. geversianus* added to a protective behavior of hiding during periods of high temperature. These results will be useful as a reference for monitoring sentinel species to assess real-time changes in coastal intertidal communities due to global warming.

Key words: Thermal stress, strategies, rocky shores, Hsp70.

Resumen: Respuesta de choque térmico en gasterópodos Patagónicos. Los procesos fisiológicos para contrarrestar el estrés ambiental en las especies marinas que habitan las zonas intermareales son extremadamente diversos. Los enfoques ecológicos han demostrado una variedad de adaptaciones entre especies de distribución limitada. El presente estudio se focaliza en las respuestas fisiológicas de dos gasterópodos marinos clave de la Patagonia atlántica que viven en una de las costas rocosas intermareales más estresantes del mundo: *Tegula patagonica* y *Trophon geversianus*. Evaluamos la temperatura corporal y su respuesta al choque térmico mediante la expresión de Hsp70. Nuestros resultados sugieren que *T. geversianus*, habitante del intermareal medio y expuesto por más tiempo a las condiciones aéreas, presenta mejores adaptaciones a la temperatura en comparación a *T. patagonica*, que habita en el intermareal bajo con menor estrés físico. *Tegula patagonica* parece tener una defensa térmica ineficiente, incapaz de incrementar la expresión de Hsp70 frente al aumento de la temperatura del agua. Por el contrario, se registró un aumento significativo de Hsp70 en *T. geversianus* sumado al comportamiento defensivo de esconderse durante los períodos de alta temperatura. Estos resultados serán útiles como referencia para monitorear especies centinela y evaluar cambios en tiempo real en las comunidades intermareales costeras debido al calentamiento global.

Palabras clave: Estrés térmico, estrategias, intermareales rocosos, Hsp70

INTRODUCTION

The rocky intertidal communities, modeled by the interaction between biotic and abiotic pressures (Silliman *et al.*, 2011; Menge & Branch,

2001; Bertness *et al.*, 2006; Silliman *et al.*, 2011; Rechimont *et al.*, 2013), undergo a gradient of physical factors between marine to terrestrial conditions (Palomo *et al.*, 2019) determined by the height of the tides (Denny & Wethey, 2001).

Northern Patagonia rocky intertidal habitats are considered within the world's most stressful, particularly because of the high desiccation rates (Bertness *et al.*, 2006). These shores, subjected to the unobstructed and persistent dry winds of "The Roaring 40 s" (Crespi *et al.*, 2018), present a low annual rainfall (180 mm/yr) and low humidity (40%) (Bertness *et al.*, 2006). In Patagonia, tides are semi-diurnal, with amplitudes of more than 8 m, reaching 12.5 m in some sites, exposing the intertidal for hours (Boraso de Zaixso & Zaixso, 2015). Hence as a result of the individual adaptations to these abiotic forces, the individuals present a specific position along the intertidal (biotic zonation) that determines the abundance, diversity, biomass, and species richness (Menge & Branch, 2001; Raffaelli & Hawkins, 2012).

Thermal tolerance is decisive in intertidal habitat selection (Somero, 2002). Ectotherms can be particularly susceptible to temperature variations (Porter & Gates, 1969), highlighting the importance of strategies to avoid desiccation. In this sense, sun-exposed and shaded microhabitat selection is extremely important for some species in terms of their distributional limits (Tomanek & Sanford, 2003; Pöhlmann *et al.*, 2011). One important mechanism against a variety of stress conditions during tidal emersions, such as temperature and oxygen deficiency, is the heat-shock response. It describes the activation of heat-shock proteins (Hsp), which act as chaperones stabilizing and preventing cytotoxic protein aggregates (Lindquist, 1986; Tomanek & Sanford, 2003; Jenó & Brokordt, 2014). Also, Hsp expression is a physiological defense indicator against endogenous proteins denaturation (Feder & Hofmann, 1999). Mollusks, being ectotherms, are excellent models for studying thermal tolerance and monitoring changes in environmental temperature. The expression of Hsp, under the action of different stressors, has been studied in several mollusk species such as *Ostrea edulis* (Piano *et al.*, 2005), *Placopecten magellanicus*, *Argopecten irradians* (Brun *et al.*, 2008), *Mytilus galloprovincialis* (Franzellitti & Fabbri, 2005), *Concholepas sp.* (Roco, 2010), *Haliotis tuberculata* (Farcy *et al.*, 2007) and *Tegula sp.* (Tomanek & Somero, 1999), observing an increase in its synthesis under heat stress conditions.

The mollusks *Tegula patagonica* (d'Orbigny, 1835) and *Trochodon geversianus* (Pallas, 1776) are abundant organisms that inhabit rocky areas along the Argentine coasts. *Tegula patagonica* is distributed from southern Brazil (23°03' S; 43°21' W) to Tierra del Fuego (54°48'30" S;

68°18'30" W) province in Argentina (Pastorino, 1994), mainly inhabiting the shallow subtidal and the low intertidal. On the other hand, *T. geversianus* is recorded from the province of Buenos Aires (38°00' S; 57°32' W) to the Burdwood bank (54°30' S; 60°30' W) in the Namuncurá Reserve in the southern Atlantic Ocean (Griffin & Pastorino, 2005; Pastorino, 2005) and up to 42° latitude south in the Pacific Ocean, from mid intertidal to shallow subtidal. Over the lower tides in the rocky shores, *T. patagonica* is a dominant herbivorous, essential in the ecosystem dynamics (Miloslavich *et al.*, 2016; Avaca *et al.*, 2021; Nieto-Vilela *et al.*, 2021), who scrapes the biofouling adhered to laminar algae (Teso *et al.*, 2009). In the mid intertidal areas, *T. geversianus* is one of the key predators in the community (Andrade & Ríos, 2007), being mussels their principal resource (Rechimont *et al.*, 2013). Both gastropods are distributed on different intertidal levels (microhabitats) of the same rocky shore, while *T. patagonica* inhabits the low zone (Rechimont *et al.*, 2013) and remains motionless during exposure to air, *T. geversianus* inhabits the mid intertidal level, seeking shelter in crevices during air exposure.

There is extensive evidence about stressors in Patagonian intertidal habitats (Bazterrica *et al.*, 2007; Bertness *et al.*, 2006; Silliman *et al.*, 2011). However, we have poor information about the strategies that organisms have to deal with them. The present study is intended to understand the responses of *T. patagonica* and *T. geversianus* to heat stress, as an input to field monitoring in a climate change scenario. Understanding the dynamic among heat stress and the organism responses can provide baseline information to monitor the impact of climatic change on South Atlantic rocky shores. We measured the heat-shock response, by assessing Hsp70 expression in the foot and gonad tissues of both rocky intertidal species.

MATERIAL AND METHODS

Sampling site

Punta Loma, a Protected Area (42°48' S; 64°53' W) near Puerto Madryn, Patagonia Argentina (Fig. 1), presents semidiurnal tides. The intertidal zone is exposed to terrestrial conditions for around 6 hours each tide cycle. The low, middle, and high intertidal levels are exposed up to 2, 4 and 6 hours, respectively (Boraso de Zaixso & Zaixso, 2015). Air temperature presents an annual mean of 13.4 °C with an annual

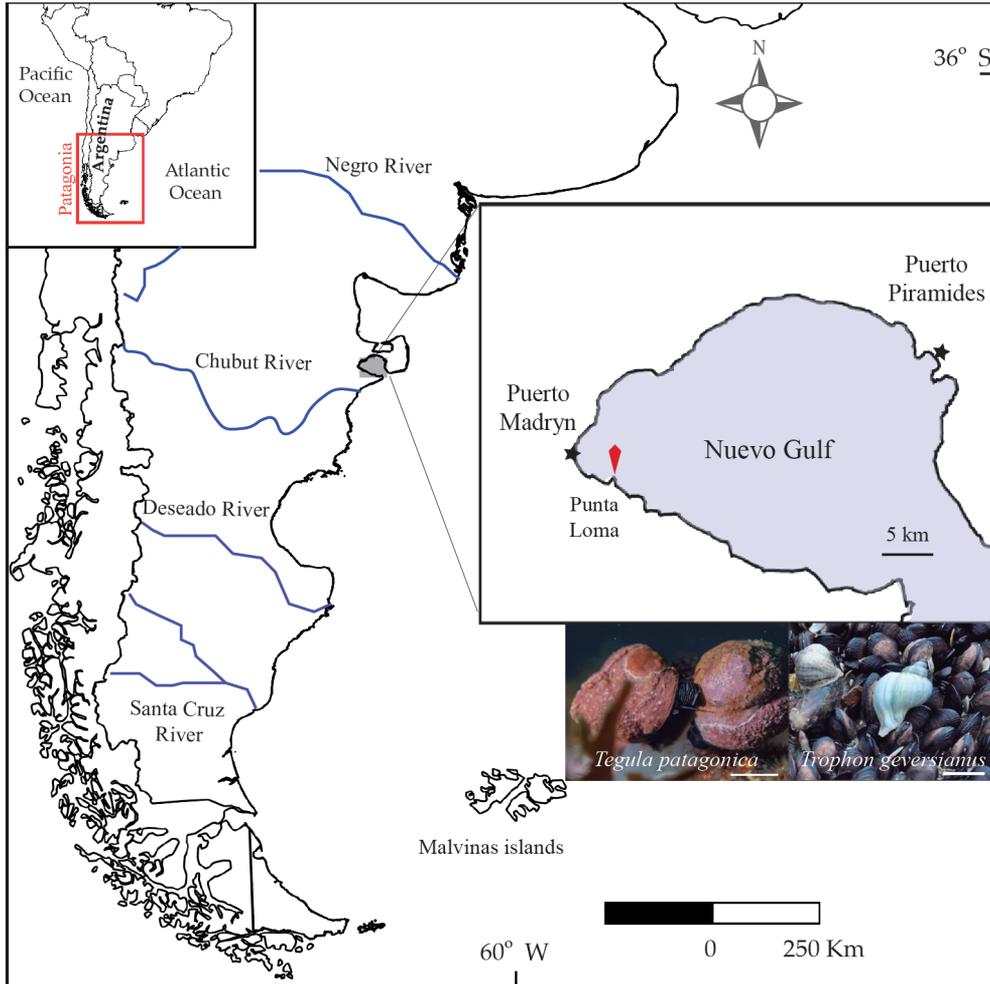


Fig. 1. Sampling site: Punta Loma, Chubut, Argentina ($42^{\circ}48'S$, $64^{\circ}53'W$). Scale bar 1 cm.

amplitude reaching 40°C and daily variations of up to 30°C (Rechimont *et al.*, 2013). The yearly mean wind speed registered is 15 ± 2.1 km/h, with extreme historical records higher than 90 km/h (EMA, Last visit 2019). The sea surface temperature presents an annual mean of around 13.5°C , with maximum values of 20°C at the end of the summer, and a minimum of 8°C during the spring (Dellatorre *et al.*, 2012).

To characterize the variation of air temperature in the intertidal, a digital temperature recorder was located in the middle zone, collecting data continuously between the summer seasons of 2018 and 2019 with a 60 minute interval and a 0.01°C resolution. To verify if the air temperature recorded influences the shell surface temperature of *T. patagonica* and *T. geversianus* gastropods, thermal images ($n=18$, nine pictures

of a single individual of each species) were taken using a FLIR C2 thermal camera, in one extreme event with temperatures higher than 30°C (Supplementary Figure 1). The images were taken over randomly visualized adults, following the published mature sizes (Cumplido *et al.*, 2010; Nieto-Vilela 2014). The images were then analyzed with the FLIR Tools software registering, in each image, the mean temperature in the same defined body area.

Thermal stress experiments

Hsp70 expression was assessed in adults of *T. patagonica* ($n=10$) and *T. geversianus* ($n=10$), collected during summer 2018 from the low and mid intertidal levels. Snails were acclimated for 10 days at 12°C in aquaria within a temperature-controlled room under a 12:12 h photoper-

riod. Biofilm was used for feeding *T. patagonica*, and scorched mussels (*Perumytilus purpuratus* and *Brachidontes rodriguezii*) for *T. geversianus*. Aquaria were controlled daily, regarding animal conditions, feeding rate, water pH, temperature, salinity and oxygen levels. After the acclimatizing period, animals were randomly exposed to two experimental temperatures in seawater 200 ml jars (Tomanek & Somero, 2000): the control treatment T1=14 °C (similar to the annual average water temperature in the site 13.5 °C), and T2=20 °C, (resembling the maximum temperature recorded in the water of the study site, <http://www.hidro.gov.ar/>; Acker & Leptoukh, 2007). Experimental temperatures were increased by 1 °C every 1 h up to 14 °C or 20 °C respectively, adapting previous methodologies (Tomanek & Somero, 1999; Tomanek & Zuzow, 2010). After 48 h of treatment, each individual was immediately frozen at -80 °C until further processing.

Cell line

Human T47D cells were obtained from ATCC and maintained in DMEM/F12 without phenol red (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin with 10% fetal bovine serum (Gibco).

Hsp70 determination

Total cell extracts from T47D cells were prepared by using RIPA lysis buffer including protease inhibitors (Giulianelli *et al.*, 2019). Two tissues with different exposure to temperature and clear heat-shock response were selected: foot, the more exposed tissue and gonad, the less exposed tissue in the same stage of maturation (Lima *et al.*, 2016; Smolina *et al.*, 2016; Nieto-Vilela 2020; Sukhan *et al.*, 2021). Around 20 mg of tissue were dissected from each animal and homogenized in ice-cold TEDGS 10% lysis buffer (50mM Tris pH=7.4, 7.5mM EDTA, 0.5mM dithiothreitol, 10% glycerol, 0.25M sucrose), including protease inhibitors. Total protein concentration was determined by Lowry method (Lowry *et al.*, 1951). Equivalent amounts of protein (100 µg) from tissue lysates were separated on discontinuous polyacrylamide gels and detected by Western Blot (Giulianelli *et al.*, 2020). Briefly, protein samples were boiled for 5 min in 4X Laemmli buffer (250mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 8% beta-mercaptoethanol, 0.02% bromophenol blue) and protein separation was performed by electrophoresis on SDS-polyacrylamide gels (5% for stacking and 10% for the resolving gels, respectively, using the Mini-

PROTEAN systems, BioRad). To allow quantitative comparison among samples from different gels, we prepared a 'standard sample' by mixing aliquots from all tissue lysates (Lima *et al.*, 2016). The human T47D cell line was used as a positive control. Proteins were transferred to 0.45 µm nitrocellulose membranes (Santa Cruz Biotechnology). After transfer, membranes were blocked by 0.5% nonfat dried milk in TBS-T solution and then incubated with Hsp70 (sc-33575, 1:600) or β-Actin (sc-47778, 1:600) antibodies (Santa Cruz Biotechnology) overnight at 4 °C. The primary Hsp70 antibody recognizes both the cognate (Hsc70) and inducible (Hsp70) forms of mammalian Hsp70 with demonstrated cross-reactivity with Hsp70 present in other mollusk species (Long *et al.*, 2015; Arribas *et al.*, 2021). Membranes were then washed with TBS-T solution, and incubated with peroxidase-conjugated secondary antibodies (Vector Laboratories, 1:4000). The luminescent signal was generated by a luminol-based method (Sigma), and the blots were exposed to a medical X-ray film (AGFA). Finally, the band intensities were determined by densitometry using ImageJ software (Schneider *et al.*, 2012). Hsp70 levels in each sample were normalized related to β-Actin (loading control) expression (Nakano & Iwama, 2002; Evans & Somero, 2010; Choi *et al.*, 2018; Giulianelli *et al.*, 2020). Then, normalized values were quantified relative to the optical density of the standard sample band previously assessed for each gel.

Statistical analysis

Student *t* test was used to compare the differences in the body temperatures and Hsp70 expression. In all graphs, the mean±SEM is shown. Significant differences between groups are indicated with asterisks (**p < 0.01; ***p < 0.001).

RESULTS

The annual air mean temperature in the intertidal for 2018 and 2019 was 13.99±4.79 °C with oscillations of 41.5 °C (Fig. 2A). The highest value registered was 36.3 °C in January, and the minimum was -4.7 °C in August. The highest air thermal amplitude recorded during the same day was more than 20 °C in February. Moreover, during the summer (January-March) there was 32 °C of thermal variation. In addition, the monthly and seasonal mean temperatures recorded for each year were included as Supplementary Tables 1 and 2.

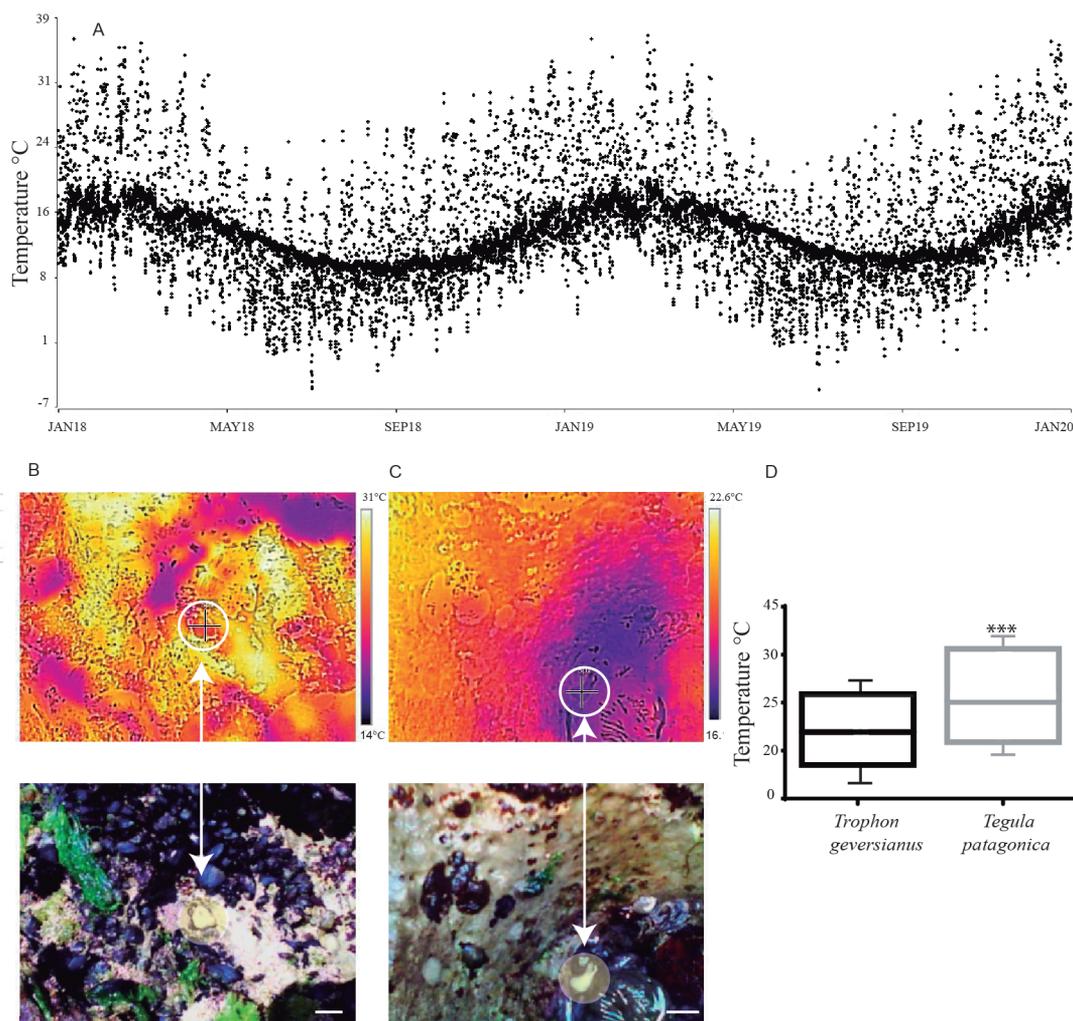


Fig. 2. Annual air temperature in the rocky intertidal of Punta Loma recorded during 2018 and 2019 and body temperatures of *T. patagonica* and *T. geversianus* after thermal images analysis. (A) Hourly temperature registered by thermal recorder placed in the middle intertidal level. The oscillation curve formed by the high density of points represents the time when the tide was high. (B) and (C) Thermal images (top) and associated digital pictures (bottom) of *T. patagonica* (scale bar: 1cm) and *T. geversianus* (scale bar: 2.5 cm), respectively, obtained at the middle intertidal level of Punta Loma area. (D) Average body temperature recorded for each species. ***, $p < 0.001$.

The shell surface temperature that each gastropod species undergoes was always lower than the air temperature. In the field *T. patagonica* were found exposed to the sun, whereas *T. geversianus* were always found in shaded crevices (Fig. 2 B and C). A higher body temperature was observed in *T. patagonica* in comparison with *T. geversianus* (Fig. 2D).

Thermal induction of heat-shock protein expression

Hsp70 was detected in gonads and the foot of

T. patagonica and *T. geversianus*. Immunoblots showed a single protein band at approximately 70 kDa, with a migration pattern similar to that of the human Hsp70 isoform (Fig. 3). Hsp70 was upregulated, approximately 3.8-fold in gonads and 2.2-fold in foot in *T. geversianus* snails exposed to 20 °C for 48 h, relative to the control expression (14 °C; Fig. 3A). No differences in Hsp70 expression were found in *T. patagonica* gonads or foot after water temperature increase (Fig. 3B). These results indicate a different dy-

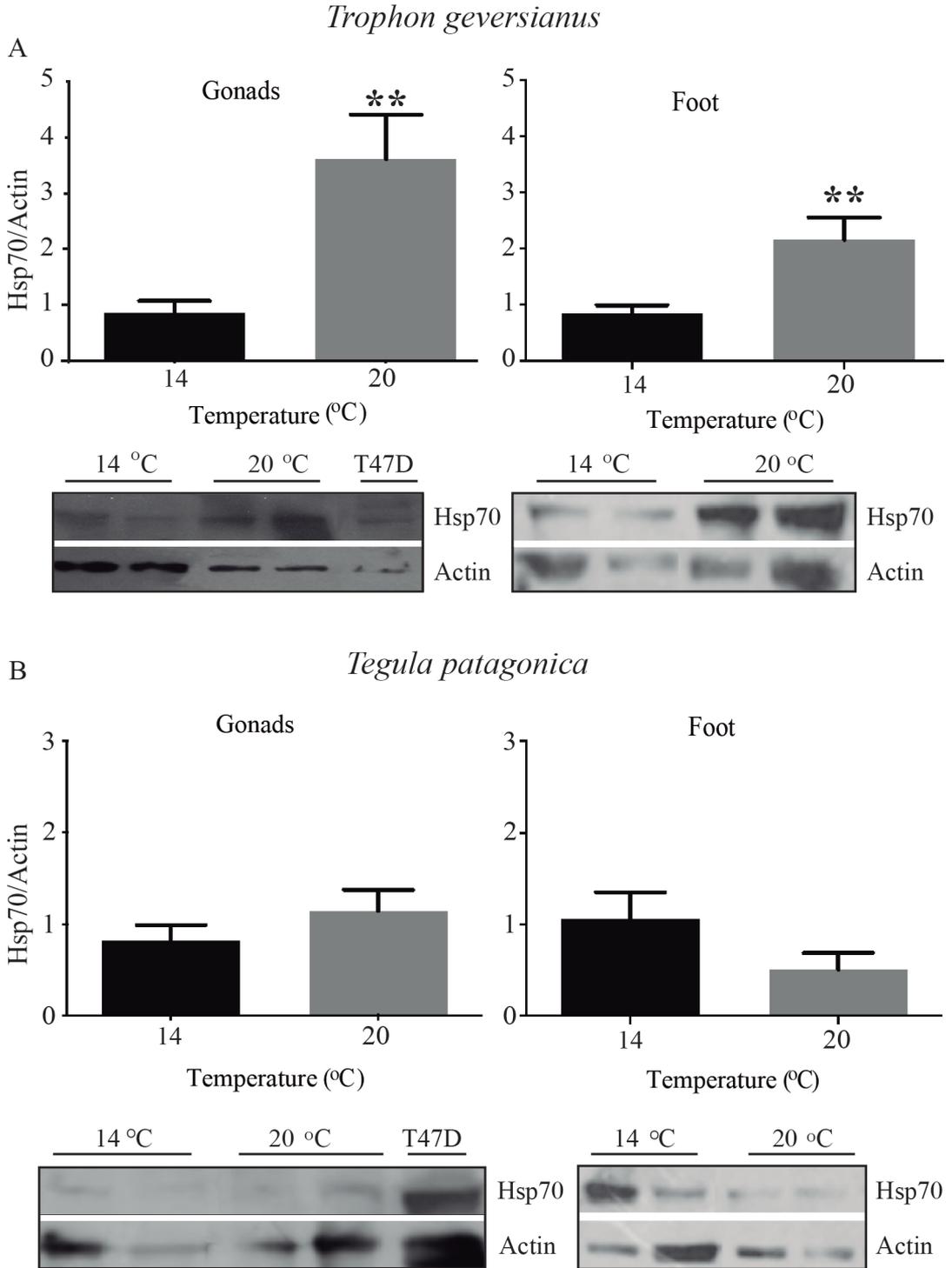


Fig. 3. Effect of temperature on Hsp70 expression of *T. geversianus* and *T. patagonica*. *T. geversianus* (A) and *T. patagonica* (B) western blot of Hsp70 expression from total extracts of gonads (left) and foot (right) after 48 h of thermal stress. The band intensity ratios of Hsp70 expression relative to β -Actin were plotted. Human T47D cell extracts were used as positive control. A representative western blot using two individual samples for each temperature treatment is shown. **, $p < 0.01$.

namic in the Hsp70 expression in response to the water temperature increase in our experimental setting between both studied species.

DISCUSSION

Our work is a first approach to study the physiological response to temperature in two widely distributed intertidal gastropods from Atlantic Patagonian rocky shores, where a different sensitivity is shown in species inhabiting distances less than a meter.

Temperature fluctuation registered throughout the year and even during the same day reveals the importance of thermal regulation investment in intertidal inhabitants. Air temperature, which is a crucial factor for ectotherms physiology (Hofmann, 1999), would seem to affect the shell surface temperature of both species in different ways. *Trophon geversianus* and *T. patagonica* could be exposed to thermal variations of more than 20 °C during the same day. Thermal imaging taken during an extreme heat event showed a difference in body temperature between both gastropod species, suggesting that *T. patagonica* might be more affected by high temperatures than *T. geversianus*. Future contributions will corroborate whether it is possible to use thermal imaging as a rapid indicator for tissue damage or heat stress before physiological analysis of biochemical indicators or even lethal response.

A common strategy in sessile invertebrates to stay wet during low tides is to tightly close their shells (Pöhlmann et al., 2011). While for mobile invertebrates, achieving protected microenvironments is a well-documented strategy to avoid desiccation (Bertness et al., 2001). Nevertheless, in the present study, we found that *T. geversianus* remains hidden in crevices during the hottest periods, whereas *T. patagonica* remains exposed. In this case, the use of microhabitats for sun protection maintained lower body temperatures in *T. geversianus*, and could be described as an effective strategy to avoid desiccation.

In terms of physiological response to water temperature increase, our experimental results showed basal levels of Hsp70 expression in *T. geversianus*, which were upregulated when exposure temperature reached 20 °C, independently of the examined tissue. Conversely, *T. patagonica* was unable to increase Hsp70 synthesis after 48 h at 20 °C. Studies made with congeners of *Tegula* on different intertidal levels showed that *T. funebris* was able to activate and complete the heat-shock response faster than its conge-

ner from the low-intertidal to subtidal species, *T. brunnea* (Tomanek & Somero, 2000). This fast reaction was described as a response for mitigating the unpredictable physical conditions in the intertidal against periods of heat stress. In the theory of “preparatory defense”, described for congeners of *Lottia* by Dong et al. (2008), the fully sun-exposed species (*L. scabra* and *L. austrodigitalis*) in the high intertidal zone, exhibited high constitutive levels of Hsp70, while only *L. austrodigitalis* exhibited a high inducible synthesis of Hsp70 at extreme temperature. The species that inhabit the lowest intertidal zone (*L. scutum* and *L. pelta*) showed low levels of Hsp70, being still able to respond to heat stress by increasing the synthesis of Hsp70. Furthermore, these results are in line with those found in the dogwhelk *Nucella canaliculata*, where the subpopulations exposed to more severe environmental conditions showed a pattern of inducible Hsp70 (Sorte & Hofmann, 2004). Our results suggest that the muricid *T. geversianus*, inhabiting the middle intertidal and exposed longer to aerial conditions at low tides (Boraso de Zaixso & Zaixso, 2015), might have a successful heat stress defense strategy, maintaining a constitutive level of Hsp70 and responding to water temperature increase by Hsp70 upregulation. Contrarywise, given that *T. patagonica* is very common in shallow waters, up to 30 m depth, and it is scarce in the low intertidal (Rechimont et al., 2013), it would indicate that the species is more adapted to subtidal stability than intertidal habits. The *T. patagonica* population exposed to the current experimental conditions did not show an effective thermal defense by Hsp70 upregulation, suggesting a greater influence by the environmental temperature.

The heat-stress response evoked by *T. geversianus* was evident at behavioral level, remaining hidden in crevices during high air temperature events, and also at physiological level increasing Hsp70 expression. Even though *T. patagonica* remains exposed during low tides with high body temperatures, we could not find a physiological heat-stress response.

Our results are reference information that could be useful for future monitoring of global warming effects on marine biota, highlighting the importance of analyzing behavioral and physiological sentinels. Future experiments would help us to thoroughly explore the strategies of this keystone species. Keeping in mind the ecological importance of both species studied in this work, *T. patagonica* as a leading herbivorous and

T. geversianus as a key predator in the community, inhabiting the subtidal and the low intertidal level, we proposed both species as sentinels of climate changes monitoring in Southwestern Atlantic coasts.

DECLARATION OF COMPETING INTEREST

We have no competing interests.

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