Thermal tolerance of the crab *Pachygrapsus marmoratus*: intraspecific differences at a physiological (CTMax) and molecular level (Hsp70)

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Abstract Temperature is one of the most important variables influencing organisms, especially in the intertidal zone. This work aimed to test physiological and molecular intraspecific differences in thermal tolerance of the crab *Pachygrapsus marmoratus* (Fabricius, 1787). The comparisons made focused on sex, size, and habitat (estuary and coast) differences. The physiological parameter was upper thermal limit, tested via the critical thermal maximum (CTMax) and the molecular parameter was total heat shock protein 70 (Hsp70 and Hsp70 plus Hsc70) production, quantified via an enzyme-linked immunosorbent assay. Results showed that CTMax values and Hsp70 production are higher in females probably due to different microhabitat use and potentially due to different hormonal regulation in males and females. Among females, non-reproducing ones showed a higher CTMax value, but no differences were found in Hsp70, even though reproducing females showed higher variability

in Hsp70 amounts. As reproduction takes up a lot of energy, its allocation for other activities, including stress responses, is lower. Juveniles also showed higher CTMax and Hsp70 expression because they occur in greater shore heights and ageing leads to alterations in protein synthesis. Comparing estuarine and coastal crabs, no differences were found in CTMax but coastal crabs produce more Hsp70 than estuarine crabs because they occur in drier and hotter areas than estuarine ones, which occur in moister environments. This work shows the importance of addressing intraspecific differences in the stress response at different organizational levels. This study shows that these differences are key factors in stress research, climate research, and environmental monitoring.

Keywords CTMax · Hsp70 · Intraspecific differences · Crabs · Sex · Size · Habitat

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Introduction

Temperature is one of the main abiotic factors influencing living things. Marine organisms, mostly ectotherms, are especially affected by this variable (Stillman and Somero 1996) and its rate of change in time and space. Temperature exerts its effects at different levels of organization for instance molecular, biochemical, physiological, and behavioral (Mora and Ospina 2001; Hochachka and Somero 2002; Dent and Lutterschmidt 2003). It also affects community interactions (Yamane and Gilman 2009) and ecosystem structure (Glynn 1988). Thermal stress leads to changes in energy allocation for the organism's activities such as growth, reproduction and foraging, with consequences in performance and fitness. Thus, reproductive rate, recruitment, mortality and population size and distribution are

dependent on temperature (e.g., Kröncke et al. 1998; Perry et al. 2005; Pörtner et al. 2008), setting ecological patterns in the marine environment.

The intertidal zone and its inhabiting communities have served as models in climate studies (see Helmuth et al. 2006; Yamane and Gilman 2009). Organisms living in greater vertical shore heights have evolved specific adaptations that allow them to cope with environmental stress due to exposure to terrestrial conditions (Stillman 2002). This makes intertidal organisms great models in studies of stress physiology, ecology, and evolution.

Physical factors play an important role in the intertidal habitat, particularly temperature, solar radiation, tidal regime, wave energy, substrate, and salinity (Dethier and Schoch 2000). It is a highly variable environment with steep temperature gradients and the stress experienced by organisms depends on the timing of the low tide, cloud cover, zonation pattern of the species, and available microhabitats (Hofmann 1999). Due to the tidal cycle and hence the regime of immersion/emersion, the intertidal fauna can undergo changes of more than 20°C (Tomanek 2010), which partially controls zonation patterns along with other abiotic and biotic factors.

Thus, environmental tolerance limits and the species' competitive and predatory interactions with other species play an important role in the vertical distribution of intertidal and supratidal fauna (e.g., Paine 1974; Stillman and Somero 2000; Flores and Paula 2001). The tolerance window for each species is described as a favorable range of an environmental factor; this range includes an optimal zone and a suboptimal zone. This zone begins at a temperature where the maximum of oxygen delivery cannot be increased further and aerobic scope starts to decrease (Fry 1971; Brett and Groves 1979). This temperature was defined as "pejus temperature" by Frederich and Pörtner (2000). When environmental factors fluctuate above or below the suboptimal zone, performance of the species is negatively affected and it can only survive for a limited period of time. This implies habitat selection through behavioral thermoregulation in ectotherms, which is very important for the species' fitness and survival since it maximizes the aerobic metabolic scope for activity (Lagerspetz and Vainio 2006).

Within each species, environment (e.g., estuary/coast), sex, and size can be important determinants influencing the tolerance to environmental variables (e.g., Sørensen et al. 2003). There is contradictory evidence on intraspecific differences in thermal tolerance in ectotherms. Some authors did not find any differences (e.g., Du et al. 2000; Badillo et al. 2002) while others did (e.g., Gaston and Spicer 1998; Strange et al. 2002; Nakajima et al. 2009). There is a need to deepen intraspecific studies in order to understand how species react to thermal stress. Knowing these differences enables us to comprehend population changes and thus

ecological patterns and potential impacts of climate change on communities and ecosystems. The mechanisms that confer thermal tolerance to individuals might differ according not only to the physiology of males/females, juveniles, and adults but also to the type of environment and latitude. One of these mechanisms is the production of heat shock proteins (HSPs).

Heat shock proteins are an important component of the heat shock response (Yamashita 2010) and play a relevant role on the cellular defense against proteotoxic stress. These proteins act as chaperones, stabilizing both denatured polypeptides and nascent proteins, preventing the occurrence of cytotoxic aggregations (Moseley 1997; Fink 1999). Additionally, they have other functions in the targeting of proteins for degradation (Feder and Hofmann 1999), in DNA repairing processes (Zou et al. 1998), in protein translocation between cellular organelles (Hartl 1996; Fink 1999), and in immune responses (Bachelet et al. 1998). HSPs are a ubiquitous and highly conservative group of proteins (Feder and Hofmann 1999) with a constitutive or induced expression. Therefore, they are important in cell functioning and protection during both regular and stressful conditions (Currie et al. 1999; Kregel 2002).

Within HSPs, the most studied group has been the 70 kDa. This family of proteins is both constitutively expressed and highly sensitive to temperature although its production can also be triggered by other factors (see Iwama et al. 1998; Feder and Hofmann 1999). Hsp70 has been linked to thermal tolerance in animal cells (e.g., Bährndorff et al. 2009), and along with other HSPs, it has been used not only as a biochemical indicator for the degree of protein unfolding in the cell but also as an indirect measure of protein damage (e.g., Hofmann 2005; Tomanek and Zuzow 2010; Tomanek 2010, 2011). Thus, it is useful as a biomarker in stress studies, especially thermal stress. Furthermore, HSP expression may be seen as a mechanism with a selective value contributing to the organism's and species' success across an environmental gradient (Hofmann 2005). Thus, HSPs can be seen as adaptations maintained via natural selection, although this requires intraspecific variation and effects on individual fitness (Feder and Hofmann 1999). This shows how relevant it is to study intraspecific differences in thermal tolerance and mechanisms of resistance. Studies in several populations and species have shown that there is a great variation in HSP expression patterns, mainly in three types of categories: as a function of thermal history, correlating to microhabitat, and between species (Hofmann et al. 2002). These types of studies have been performed in several marine organisms such as fish (e.g., Dietz and Somero 1992; Norris and Hightower 2002; Buckley and Hofmann 2002; Fangue et al. 2006), bivalves (e.g., Chapple et al. 1998; Buckley et al. 2001; Helmuth and Hofmann 2001; Encomio and Chu 2005), snails (e.g.,

Tomanek and Somero 1999, 2002; Tomanek and Sanford 2003; Clark and Peck 2009), and crustaceans (e.g., Botton et al. 2006; Kelley et al. 2011). Since most of the studies on intraspecific differences in thermal tolerance focus on latitudinal, intertidal distribution, and seasonal comparisons (see Hofmann 1999), it is important to approach intraspecific differences that have been less explored in marine organisms, especially sex and size differences. These studies enable a better understanding of the ecological and evolutionary significance of the components of the heat stress response.

The aim of this study was to test intraspecific differences on upper thermal limits and Hsp70 production, measuring both constitutive (Hsc70) and inducible (Hsp70) forms, in order to improve the understanding of how temperature affects organisms and species and consequently have a broader understanding of how population parameters are affected (reproductive success, recruitment, mortality, and population size/distribution). This is important given the current climate change scenarios and consequent ongoing changes in the ecosystems. More specifically, we tested the influence of sex, size and habitat (estuary or coast) (1) on the critical thermal maximum (CTMax) and (2) on the production of Hsp70 (constitutive and inducible).

Materials and methods

Study area and sampling method

The sampling was performed in a coastal rocky intertidal zone (Cabo Raso, Cascais) and in an estuarine rocky intertidal zone (Sado estuary), Portugal, at an approximate latitude of 38°42' and 38°28' N (Northeast Atlantic), respectively.

This study focused on the crab *Pachygrapsus marmoratus* (Fabricius, 1787) because it is abundant, easily captured, easily maintained, and a relevant species in the rocky intertidal ecosystem. It is a supratidal species although it can explore the whole intertidal range (Flores and Paula 2001). It has a temperate/subtropical distribution ranging from Northern Europe to North Africa. Females caught with eggs were considered reproductive females and the rest were considered non-reproductive females. The juveniles tested were smaller versions of the adults; they have the same anatomy and morphology. None of the organisms studied were molting.

The crabs were caught by hand during the summer (July 2010). The mean estuarine water temperature in the sampling site was 24°C. Spatial variation of estuarine temperature in July also occurs, as it varies from 25°C at the upper estuary to 20°C at the lower part of the estuary (Coutinho

2003) (see [Electronic supplementary material](#)). Seasonal variations in estuarine water temperature are around 17.4°C. Water temperature varied from a minimum of 10°C in the winter to a maximum of 27.4°C in the summer. The mean air temperatures range from 6.4°C in the coldest month to 29.1°C in the hottest month (Coutinho 2003).

The coastal water temperature is usually in the range of 15–18°C during the summer months. However, during July 2010, it reached 23°C (MOHID database). Seasonal variations in coastal water temperature are around 5°C. Intertidal pools reached higher mean water temperatures of approximately 24–26°C. Atmospheric temperatures, recorded every half an hour, for the coastal intertidal zone in July 2010 were also obtained from open access MOHID database, which can be consulted at www.mohid.com. These values were used to calculate the mean±SD of daily mean temperatures (from sunrise 7 a.m. to sunset 9 p.m.), which was 23.4±3.5°C. During July 2010, the maximum temperature recorded in this area was 36.6°C and the minimum was 17.2°C. Daily variations in temperature were between 3.1 and 14.5°C during the day for July. Seasonal variations were around 25°C.

Since mean water and mean atmospheric temperatures were really close, we chose 24°C as the control temperature.

Thermal tolerance method

Thermal tolerance was determined using the dynamic method described in Mora and Ospina (2001). The parameter measured was the Critical Thermal Maximum (CTMax given in degrees Celsius), which is defined as the “arithmetic mean of the collective thermal points at which the end-point is reached” (Mora and Ospina 2001). This end-point is defined as the loss of equilibrium.

After being captured, the organisms were transported to the laboratory and placed in a re-circulating system with 70 L aquaria of aerated sea water, a constant temperature of 24°C and a salinity of 35‰. The dissolved O₂ level of the water varied between 95 and 100 %. The crabs acclimated for two weeks, being fed ad libitum twice a day. They were starved for 24 h before the experiments. To determine the CTMax, the organisms were subjected to a thermostated bath. During the experiment, animals were exposed to a constant rate of water-temperature increase of 1°C h⁻¹, and observed continuously, until they reached the end-point. Haemolymph samples from ten individuals were taken every 2°C until the end-point was reached. The total number of individuals was 70).

The temperature at which each animal reached its end-point was measured with a digital thermometer, registered and then CTMax and its standard deviation were calculated. The experiments were carried out in shaded day light (15 L; 09D). To prevent any additional

handling stress, the total length of all individuals was measured at the end of each trial using a slide caliper ruler.

Hsp70 extraction and quantification

The haemolymph was chosen as target for analysis since it is easily collected using a syringe while sampling muscle tissues (common approach is HSP studies) in juveniles is very difficult due to the organism's size. Nonetheless, haemolymph (including plasma and/or hemocytes) is often chosen as target in stress research using invertebrate organisms as models (e.g., Joy and Gopinathan 1995; Guo et al. 2004; Cellura et al. 2007; De la Veja et al. 2007; Díaz et al. 2010; Chen et al. 2010; Liu et al. 2011). Haemolymph samples were centrifuged for 5 min at 16,000×g and then diluted 1:100 in 0.05 M carbonate–bicarbonate buffer (Sigma-Aldrich, St. Louis, MO). Hsp70 in plasma was quantified using an enzyme-linked immunosorbent assay (ELISA) (Njemini et al. 2005) with 96-well microplates (Nunc-Roskilde, Denmark). Either ELISA or western blot can be successfully employed in HSP quantification (Brun et al. 2008). Three replicates of 50 µL were taken from each diluted sample, transferred to the microplate wells and incubated overnight at 4°C. The microplate was washed (three times) in PBS 0.05 % Tween-20 and then blocked by adding 200 µL of 1 % bovine serum albumin (BSA; Sigma-Aldrich). The microplate was incubated at 37°C for 90 min. After microplate washing, the primary antibody (anti-Hsp70/Hsc70, Acris, San Diego, CA), diluted to 0.5 µg/mL in 1 % BSA, was added to the microplate wells (50 µL each). Then the microplates were incubated for 90 min at 37°C. After another washing stage, the secondary antibody (anti-mouse IgG, fab specific, and alkaline phosphatase conjugate; Sigma-Aldrich) was diluted (1 µg/mL in 1 % BSA) and added (50 µL) to each well followed by incubation at 37°C for 90 min. After the washing stage, 100 µL of substrate (SIGMA FAST™ *p*-nitrophenyl Phosphate Tablets, Sigma-Aldrich) was added to each well and incubated for 30 min at room temperature. Fifty microliters of stop solution (3 N NaOH) was added to each well and the absorbance was read in a 96-well microplate reader at 405 nm (BIO-RAD, Benchmark, El Cajon, CA). For quantification purposes, a calibration curve was constructed using serial dilutions of purified Hsp70 active protein (Acris) to give a range from 0 ng to 2,000 ng/mL.

For normalization purposes, the Bradford Assay was used to quantify the total amount of protein in each sample (Bradford 1976). The analysis was carried out in 96-well

microplates (Nunc-Roskilde) by adding 200 µL of Bradford reagent in each well and 10 µL of each sample or standards. After 10 min of reaction, the absorbance was read at 595 nm in a microplate reader (BIO-RAD, Benchmark). A calibration curve was constructed using BSA standards.

Since the antibody detects both Hsc70 and Hsp70, these results account for both forms. In cells, the constitutive form Hsc70 remains unchanged (e.g., LeBlanc et al. 2011) or is only slightly upregulated (up to 2-fold) in certain tissues (e.g., Liu et al. 2004; Rendell et al. 2006) whereas the inducible form Hsp70, is highly upregulated from low basal levels (Deane and Woo 2005). Thus, as both Hsp70 and Hsc70 confer thermal tolerance (e.g., Fanguie et al. 2006), accounting for both forms of Hsp70 is a better predictor of thermal tolerance (Sorte and Hofmann 2005). The use of antibodies that detect both isoforms has already been carried out for HSP90 (e.g., LeBlanc et al. 2011).

Criteria of selection of crabs for each comparison

In the comparison between males and females, we used fully differentiated adult male and female crabs from the coast and estuary. In the comparison between juveniles and adults we used a mixed population of male and female crabs from both the estuary and coast. In both cases, a similar number of male and female crabs from the estuary and from the coast were used, so that no habitat effects would influence the results. In the comparison between estuarine and coastal crabs we used a mixed population of males, females, juveniles, and adults.

Data analysis

Data obtained for CTMax were analyzed through Student's *t* tests or Mann–Whitney tests ($\alpha=0.05$) depending on the normality of the data (Shapiro–Wilk's test) and homocedasticity (Levene's test).

Hsp70 data were analyzed via two-way ANOVAs ($\alpha=0.05$) followed by Tukey post hocs. The factors were temperature combined with sex, habitat, and size. Results for females with eggs and females without eggs were compared through a Student's *t* test.

Results

Significant differences were found for all of the comparisons carried out with CTMax data except for habitat comparison. Females showed higher CTMax values than males (*p* value=0.01, Fig. 1a) and among females, the ones which were reproducing showed a lower CTMax value (*p* value=0.02, Fig. 1b). Comparing different sized individuals, results showed that juveniles had a higher CTMax value than adult

individuals (p value=0.03, Fig. 1c). Finally, estuarine and coastal crabs showed no significant differences in CTMax values (p value=0.07, Fig. 1d).

Two-way ANOVA results showed significant differences in Hsp70 for all comparisons (Table 1). Not only is Hsp70 production altered by increasing temperature but also females (Fig. 2a), juveniles (Fig. 2b) and coastal (Fig. 2c) crabs showed higher levels of Hsp70 than males, adults and estuarine crabs. The t-test performed to compare Hsp70 levels in reproducing females and non-reproducing females showed no significant differences ($p=0.798$). Reproducing females showed a much higher standard deviation than non-reproducing females.

Discussion

The exposure of *P. marmoratus* to increasing temperature led to different magnitudes of stress responses both physiologically and at a molecular level according to sex, size and habitat (estuary or coast). It was found that females had a higher CTMax value and higher Hsp70 production than males. Higher tolerance for females when exposed to stress has also been found in other ectotherms (e.g., Mills and Fish 1980; Afonso et al. 2003; Winne and Keck 2005; Nakajima et al. 2009; Mikulski et al. 2011).

According to Øverli et al. (2006), sex differences can occur in the response to stressful situations. Although the authors focused on behavioral differences, they do mention that these can be associated with distinct physiological profiles and down-regulation of certain neuroendocrine responses. Pottinger et al. (1996) and Knowlton and Sun (2001) have shown that organismal and cellular stress responses are linked to sex steroids in vertebrates. The authors found that estrogen (17β -estradiol) and progesterone activate Heat Shock Factor 1 (transcription factor) and upregulate hsp72; testosterone showed no effect on HSP levels. Additionally, Janz et al. (1997) showed that increased Hsp70 mRNA levels were associated with plasma 17β -estradiol in fish. Even though the neuroendocrine system in invertebrates differs from that of vertebrates, equivalent hormones may be playing similar roles in crustaceans. Moreover, Verslycke et al. (2002) mention studies that have detected vertebrate-type steroids such as 17 -estradiol, testosterone and progesterone in malacostracan crustaceans, suggesting that these compounds have a functional role in crustaceans. Although we have not quantified any hormones in this study and further research is needed to clarify hormone regulation of Hsp70 expression in crustaceans, our results suggest a neuroendocrine influence on HSP expression. If so,

Fig. 1 CTMax results for **a** females and males (p value=0.01), **b** reproducing females and non-reproducing females (p value=0.02), **c** juveniles and adults (p value=0.03), and **d** estuarine and coastal crabs

(p value=0.07). Groups with a significantly higher CTMax value are tagged with an asterisk

Table 1 *Pachygrapsus marmoratus*: results for Hsp70 comparisons (sex, habitat, and size)

Hsp70—ANOVA results	
	<i>p</i> value
Males versus females	0.000
Temperature (°C)	
Sex	0.016
Temperature (°C)×sex	0.003
Juveniles versus adults	0.000
Temperature (°C)	
Size	0.000
Temperature (°C)×size	0.000
Estuarine versus coastal	0.000
Temperature (°C)	
Habitat	0.000
Temperature (°C)×habitat	0.000

p values are reported for each factor (first and second lines of each section) and for the interaction effect of both factors (third line of each section)

males and females may be using different strategies to withstand stressful conditions.

Nevertheless, different tolerances and cellular responses might also be due to different microhabitat use by males and females. This has been shown for intertidal invertebrates (Hofmann 1999) and crustaceans such as *Daphnia* (Mikulski et al. 2011). In fact, the spatial strategy of *P. marmoratus* differs between females and males. Cannicci et al. (1999) showed that the large males were more concentrated in the sublittoral fringe, while both small males and females were confined to the eulittoral and littoral fringe. Therefore, if adult males mostly occur in the sublittoral fringe, they are less frequently exposed to extreme heat and desiccation and thus they reduce their molecular defenses. As females occur in greater shore heights, where they are exposed to terrestrial conditions and a greater variability of the environmental factors, they showed higher CTMax values and a greater magnitude of the molecular response. Combining our study with other scientific papers reveals that sexual dimorphism in stress tolerance and Hsp70 production seems to occur across several taxa (e.g., Mills and Fish 1980; Afonso et al. 2003; Winne and Keck 2005; Nakajima et al. 2009; Mikulski et al. 2011).

Fig. 2 Heat shock protein 70 (Hsc70+Hsp70; in μg Hsp70/ μg total protein) results for **a** females and males, **b** juveniles and adults, and **c** estuarine and coastal crabs at increasing temperatures. Females (p value=0.016), juveniles (p value=0.000), and coastal crabs (p value=0.000) have significantly higher amounts of total Hsp70. Note that *y*-axis values differ between graph (a) and graphs (b) and (c). In (a), we evaluated only adults which were fully sexually differentiated. As shown, adults produce less amounts of Hsp70, thus the different *y*-axis values when comparing to (b) and (c). Induction profiles are also different between graph 2a and 2b,c due to the same reason. Comparing graphs (a) and (b) (the adult induction profile) you will see that these are quite similar. Asterisks mark significant differences in Hsp70 expression between temperature groups, when comparing to controls (24°C). Stars mark significant differences in Hsp70 expression between sexes, habitats, and sizes

Moreover, crustacean females make greater investments in reproduction than males so higher tolerances could improve their fitness and allow them to explore microhabitats

suitable for their progeny's growth and survival. When comparing reproducing females to non-reproducing ones, the results showed that non-reproducing females had a greater CTMax value but no differences were found for Hsp70 production. However, reproducing females showed higher standard deviations in the production of Hsp70, showing greater variability in the response compared with non-reproducing females. When organisms enter the reproductive season, gonad growth, gamete maturation, and vitellus production lead to a high energetic cost. Furthermore, the maintenance, aerating, and cleaning of the egg mass (Baeza and Fernández 2002) also lead to a high energetic expenditure. Given that most of the energy is allocated to reproductive processes, the energy left for stress responses is lower, leading to an inferior CTMax value. Thermal stress provokes energy-demanding responses (Somero 2002; Sorte and Hofmann 2005; Tomanek and Zuzow 2010) which decrease reproductive capacity. In fact, high levels of HSP lead to a decrease in fecundity and reproductive capacity (Krebs and Loeschke 1994; Silbermann and Tatar 2000). The higher standard deviation in Hsp70 production in reproducing females shows that molecular stress responses during this phase are extremely variable and probably depend on individual energy status and health.

Results for different sized individuals showed that juveniles have a higher CTMax value and higher Hsp70 induction than adults, which is in accordance with other studies performed on ectotherms (e.g., Mundahl and Benton 1990; Winne and Keck 2005). One can argue that Hsp70 differences between juveniles and adults might be due to different growth rates. As juveniles have higher growth rates, a higher quantity of Hsp70 would be necessary in order to fold the higher amount of nascent proteins. However, this difference would only be accountable in controls (24°C). As heat stress is applied, the much higher induction seen in juveniles is due to heat stress and not higher growth rates. Considering that smaller *P. marmoratus* (both males and females) concentrate in warmer stretches such as the eulittoral and littoral fringe (see Cannicci et al. 1999), they experience more extreme conditions so they need higher amounts of HSPs to deal with greater amounts of protein damage caused by elevated temperatures. Furthermore, males go through an ontogenetic shift in microhabitat use and as adults they prefer the sublittoral fringe. Therefore, this male pattern may be reflected in the results for adult organisms (lower CTMax and lower HSP amounts). Our results are in accordance with the statement by Sørensen et al. (2003): "occupation of different environments for different life stages might select for life-stage-specific HSP expression and resistance". In addition, it is known that HSP expression declines with age (Hall et al. 2000; Snoeckx et al. 2001; Kregel 2002). Ageing leads to alterations in the gene transcription, mRNA translation and protein degradation

(Kukreja et al. 1994). More specifically, genetic expression in response to stress is altered so younger cohorts have a high HSP production while in older ones this begins to decrease (Kregel 2002).

Finally, when comparing *P. marmoratus* from the estuary with those from the coast, there were no differences in CTMax values. However, coastal crabs produce higher amounts of Hsp70. It was expected that estuarine crabs would have a slightly higher CTMax value not only because estuarine ecosystems are warmer and more variable than coastal ecosystems but also because individual thermal history is believed to cause irreversible changes in thermal tolerance (Shaefer and Ryan 2006). Nonetheless, the lack of difference in CTMax values suggests limited acclimatory plasticity (in accordance with Stillman and Somero 2000; Tomanek 2010) related to different habitats and no local adaptation in upper tolerance limits. There is mixed evidence in relation to intraspecific differences in ectothermic organisms coming from different environments (e.g., MacIsaac et al. 1985; Smale and Rabeni 1995; Lohr et al. 1996; Strange et al. 2002; Winne and Keck 2005; Fangue et al. 2006; Kelley et al. 2011). It is possible that intraspecific differences due to latitude or habitat depend on how steep the thermal gradient is, leading or not to local adaptation of thermal tolerance. Although local adaptation does not seem to be occurring in the physiological parameter CTMax, results at the molecular level suggest the opposite. It was found that coastal crabs had higher production of Hsp70 suggesting that these may be more exposed to thermal damage than estuarine crabs. The vertical zonation of this species is not well known in estuarine shores; however our observation of the study site was that this species occurred, under rocks in very moist environments and closer to the water than on the coast. On the coast individuals were clearly supratidal, occurring under rocks which were very dry and hot, and predominantly exposed to terrestrial conditions. This is in accordance with the Hsp70 results, which revealed that individuals from the coast have the ability to produce much more Hsp70 than those from the estuary.

Conclusions

The current work showed that *P. marmoratus* responds differently to increasing temperatures depending on sex, size, and habitat. These findings demonstrate the importance of studying intraspecific differences on the stress response. Also, the present study provides insight into the importance of future studies that address hormonal regulation of HSPs in marine organisms.

Considering that organisms inhabiting intertidal zones have thermal limits close to current maximal habitat temperatures (Stillman and Somero 2000; Stillman 2002), the

ecological and evolutionary consequences of upper thermal tolerance and its plasticity are significant (Stillman 2002). Our results show that analysis of thermal tolerance and potential climate change effects on populations and ecosystems should take these intraspecific differences into account. They provide new information on how vulnerable populations are and how different cohorts and sexes may react to the same environmental changes. Understanding patterns within a species can provide additional insights into the nature of adaptive variation in thermal tolerance (see Somero 2002; Fangue et al. 2006) and improve conservation-related activities. Additionally, HSPs have been widely used as biomarkers for environmental monitoring so future studies should consider these factors (sex, size, and habitat) in order to get accurate and reliable results.

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