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Variations in physiological responses to thermal stress in congeneric limpets in the Mediterranean Sea

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ABSTRACT

Cardiac activity (Arrhenius breakpoint temperatures and Q10 relationships) and heat shock response (*hsp70* expression) were measured in the congeneric limpets *Patella rustica*, *P. caerulea* and *P. ulyssiponensis* in order to test the relationship between their vertical distribution and physiological thermal tolerance. These species exhibit different vertical distributions along Mediterranean shores and despite the narrow tidal range in the Mediterranean, they experience different environmental conditions and consequently had specific thermal windows. Cardiac activity of the upper zoned *P. rustica* was maintained at higher temperatures than its mid- or low shore counterparts, *P. caerulea* and *P. ulyssiponensis*. *P. rustica* had the highest Arrhenius breakpoint temperature (37.9 ± 2.1 °C, mean ± SD), followed by *P. caerulea* (35.9 ± 2.6 °C), and finally the low-zoned *P. ulyssiponensis* (32.2 ± 2.3 °C). The same pattern was found for Q10 relationships. Expression of *hsp70* increased at 34 °C and kept increasing with temperature in *P. rustica*. In *P. caerulea*, expression reached a maximum at 36 °C and decreased at 38 °C, suggesting that *hsp70* expression in *P. rustica* provides a more efficient defence against thermal stress than in *P. caerulea*. As summer environmental temperatures in the Mediterranean regularly reach 35 to 38 °C, performances of these limpets suggest that they are already living at the edges of their thermal window, and further temperature changes may have large-scale consequences for these keystone species.

1. Introduction

Intertidal invertebrates are ectotherms, their body temperatures being driven by external climatic factors (Helmuth et al., 2002, 2006). As such, the thermal environment species experience is probably the most important external factor affecting their survival and performance (Somero, 2002, 2005). Due to the dynamic physical environment imposed by the rise and fall of the tides, many intertidal species are assumed to be living close to their thermal limits (Helmuth, 2002; Somero, 2010) and measuring an organism's thermal performance is, therefore, crucial to an understanding of how species are adapted to their present day environments (Hochachka and Somero, 2002).

Traditionally, the upper limits of species on rocky shores are assumed to be set by tolerance to abiotic, environmental conditions, especially thermal stress, resulting from increased duration of emersion high on the shore (Davies, 1969, 1970; Foster, 1971). As a result, species inhabit different vertical heights according to their thermal niches, which are set by tidal cycles and local weather conditions (Helmuth et al., 2002). This gradient is clearly defined on shores with large tidal ranges, where clear delimitation between species can be observed (often termed species 'zonation' patterns). These differences in species' vertical distribution patterns are mirrored by their thermal tolerance; as has been demonstrated in congeneric species of Porcelain crabs (*Petrolisthes* species, Stillman and Somero, 1996); topshells (*Tegula* species, Tomanek and Somero, 1999, 2000; reviewed in Somero, 2010) and limpet species (*Cellana* species, Dong and Williams, 2011). In general, species living higher on the shore show a variety of behavioural (Garrity, 1984; Williams and Morritt, 1995) and physiological adaptations (see Somero, 2010 for review) which allow them to survive in thermally stressful environments as compared to their lower shore, less tolerant, counterparts.

On shores with smaller tidal ranges, such as the Mediterranean, the distribution of species is compressed into a narrow range and species live in close proximity over the tidal gradient. Despite this, limpets

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such as *Patella rustica* Linnaeus, 1758, *P. caerulea* Linnaeus, 1758 and *P. ulyssiponensis* Gmelin, 1791 still inhabit different vertical heights: *P. rustica* occurring in the upper intertidal, *P. caerulea* being dominant in the lower mid-littoral and *P. ulyssiponensis* inhabiting the lower shore (Davies, 1969; Mauro et al., 2003; Šimunović, 1995). As a result, these congeneric species will experience different environmental conditions, including variation in the levels and duration of thermal stress (see Stillman and Somero, 1996; Tomanek and Somero, 1999) over a very narrow vertical range. The Mediterranean intertidal zone is highly stressful for organisms, as maximum habitat temperatures (*sensu* Somero, 2010) can reach 45–50 °C in the supratidal and 35–38 °C during summer midday low tides in the intertidal (Sarà et al., 2014). Since habitat conditions mediate larger-scale climate effects, adjacent congeners living at different vertical heights are likely to be adapted to different thermal regimes. To investigate the influence of temperature variation on these congeneric limpets, we measured the break point in their heart beat rate (HBR), expressed as the Arrhenius Breakpoint Temperatures (ABT, which can represent the limit of metabolic functioning of animals, see Dong and Williams, 2011; Somero, 2005; Stillman and Somero, 1996) and the gene expression of the molecular chaperone *hsp70*. The ABT of an organism is often used to define the species-specific critical thermal limits (Stillman, 2003) whereas the synthesis of inducible *hsps*, which is initiated only above a certain temperature threshold, can be used as a reliable indicator that animals are experiencing stressful conditions (Feder and Hofmann, 1999). Accordingly, intertidal animals with different thermal tolerance show variable expression patterns of *hsps* when thermally stressed (Dong and Williams, 2011; Dong et al., 2008; Tomanek and Somero, 2000).

Numerous studies have shown that different expression patterns of both *hsps* and HBR are related with the microhabitats that intertidal animals inhabit in areas with large tidal ranges (Chelazzi et al., 2001; Dong and Williams, 2011; Dong et al., 2008; Tomanek and Somero, 2000; Williams et al., 2011). However, little is known about thermal adaptation of intertidal species over the tidal gradient in the Mediterranean Sea. Although this narrow gradient may result in species having overlapping thermal niches, each species is assumed to be adapted to specific thermal windows, and the borders of individual windows should be denoted by the limits of species' acclimatization capacities (Pörtner, 2012). Measurement of HBR and *hsps* can, therefore, help define the thermal niches of the three Mediterranean *Patella* species and presents an opportunity to test the relationship between species' vertical distribution and physiological thermal tolerance over a severe, narrow environmental gradient. To achieve this, we analyzed abrupt exposure and short-term responses of limpets to changes in temperature, with the overall hypothesis that the responses between these congeners should vary in relation to their vertical position, and that the high shore *P. rustica* will be more tolerant to thermal stress compared to its low and mid zoned congeners.

2. Materials and methods

2.1. Study area and limpet collection

Limpets, *P. rustica* (shell length 25.3 ± 2.1 mm, mean ± SD), *P. caerulea* (25.3 ± 2.6 mm), and *P. ulyssiponensis* (25.9 ± 3.2 mm) were collected in December 2011 from the shores at Altavilla Milicia (38°01′56.89″ N, 13°35′03.18″ E) and Addaura (38°11′46.06″ N, 13°20′20.15″ E), on the north west Sicily coast. During this period, air temperature ranged from 8.0 to 22.4 °C (16.6 ± 3.5 °C, mean ± SD), water temperature from 15.5 to 20.1 °C (16.4 ± 0.5°C) and tidal amplitude ranged from −0.1 to +0.6 m (Italian Institute of Environmental Research, ISPRA, http://www.mareografico.it). *P. rustica* was collected in the upper intertidal (~0.5 m above the mean-lower-low-water (MLLW) level, Sarà et al., 2014) while *P. caerulea* and *P. ulyssiponensis* were collected from the mid and low intertidal (between 0.4 and 0.1 m above the MLLW, respectively).

Limpets were collected on the ebbing tide and so were assumed to have been actively feeding before collection and were about to become inactive prior to emersion (see Williams et al., 2005). Individuals were immediately placed in Petri dishes and kept moist with seawater during transportation (<1 h) to the Laboratory of Experimental Ecology at the University of Palermo. In the laboratory, limpets in their Petri dishes were placed into a tank with seawater spray at room temperature (20 °C) for 1 h to allow them to regain mantle water, discard metabolic wastes and recover from stress due to transportation (see Williams et al., 2005). During this period limpets did not feed and settled onto the Petri dishes, after which they were immediately used for experiments. The ABT experiment consisted of five replicate sessions on different days; in each session three individuals (of each species) were collected from the shore and tested on that day. In the case of *hsp* analysis all limpets were collected and tested on the same day.

2.2. Arrhenius breakpoint temperature (ABT) measurements

Heart rates were measured using the non-invasive technique developed by Depledge and Andersen (1990), modified by Chelazzi et al. (1999) and improved by Burnett et al. (2013). The shell of each limpet was cleaned and individuals were placed on a Petri dish with the side wall removed to allow drainage. An infrared sensor was glued onto the limpet shell (Loctite Super, Italy) in a position directly over the heart. The sensor consisted of an infrared-light-emitting diode from which the signal was filtered, amplified (AMP-3, Newshift Lda, Portugal) and recorded using a portable Oscilloscope (PicoScope 2203). Heart rate traces could subsequently be viewed and analyzed using a PicoScope software (version 6.6.13.15, see Burnett et al., 2013 for examples). Animals were then returned, in their Petri dishes, back to the seawater spray for 1 h to recover from handling.

Arrhenius breakpoint or Arrhenius break temperatures (ABT, the temperature above which cardiac activity drops off dramatically; Stillman and Somero, 1996) of the three species, were then determined. Each limpet in their Petri dish was placed into a beaker (d = 45 mm, h = 70 mm) in air, which was then partly immersed in a programmable water bath (PID system, MPM Instruments s.r.l., type M428-BM) at 20 °C. Animals were allowed 15 min to acclimatize, during which the temperature inside the water bath (and subsequently of the air in the beaker) was raised to 23 °C, and then temperature was continuously increased at a rate that mimicked an emersion period in the natural environment (~3 °C per 15 min; Sarà et al., 2014) and monitored until regular heart beat was lost (see De Pirro et al., 2001). To ensure that temperatures were stable, temperature inside the beaker and bath water temperature were recorded every minute using data loggers (iButton Inc, ±0.5 °C). In general, limpet body temperatures are within a degree of air and/or substrate temperature (Denny and Harley, 2006; Denny et al., 2006). Using a similar methodology, limpet body temperatures were found to typically be within ±0.2 °C of the vial surface temperatures (KA Villarta, unpublished data), so it is assumed that there is a strong relationship between measured temperature and limpet body temperature. At the end of the experiment, each limpet was measured (length, ±0.1 mm) and weighed (±0.001 g).

Real time heart rates (beats per second) of five individuals of each species were recorded every 5 min. To estimate heart rate, at least five heart beat cycles were counted during three continuous oscilloscope frames of 10 s each, within each 5 minute recording. Individual average heart rates from the three counts were computed and transformed to the natural logarithm of beats per second for Arrhenius plots. Arrhenius break temperatures were determined for each individual by generating the two intersecting linear regressions that best fitted the data, and calculated from the intersection of these two lines (see Dahlhoff and Somero, 1993; Stillman and Somero, 1996). To test potential differences in temperature sensitivity, Q10 relationships (Van't Hoff, 1884) were calculated in the range of 23 to 33 °C for all three species.

2.3. Hsp70 expression

To determine the expression of *hsp70* under different thermal regimes 25 *P. rustica* and 25 *P. caerulea* were exposed to different temperatures and different durations of specific temperatures. *Hsp70* levels could not be measured for *P. ulyssiponensis* due to inclement weather conditions preventing collection of enough animals. We mimicked a possible exposure scenario (Denny et al., 2006) where limpet's body temperature rises during emersion to a maximum level, after which the animal would be immersed by the rising tide or splashed by waves. Limpets were randomly taken from the holding tank in seawater spray (20 °C), measured (length ± 0.1 mm), wet weighed (±0.001 g), placed on individual Petri dishes and left again in seawater spray at 20 °C to recover from handling. After 1 h each limpet was put into a beaker, in air, which was immersed in the programmable water bath with an initial temperature of 20 °C (see above). Five non-heated (held only in seawater spray at 20 °C) limpets of each species were used as controls. The temperature in the water bath was increased at a rate of 2 °C per 15 min to reach 38 °C and maintained at 38 °C for 2 h (8 °C per 1 h, as used by Denny et al., 2006; Dong et al., 2008). Temperatures in the beaker and in the water bath were recorded every minute as above. At 20 °C, 34 °C, 36 °C and 38 °C (two time points at 38 °C: after 60 and 120 min duration, to simulate different emersion periods) five individuals of each species were randomly collected and placed under seawater spray (20 °C) for 2 h to allow expression of *hsp70* (as described in Dong and Williams, 2011; Dong et al., 2008). After 2 h, individuals were removed, rapidly dissected, and the foot muscle was macerated in an Eppendorf tube and stored in RNA*later* (Sigma-Aldrich) prior to estimation of *hsp* expression.

Total RNA was isolated from ~20 mg of foot tissue by TrizolReagent (Invitrogen, USA). A sample of 1 μg of total RNA was used as the template for synthesis of the first strand of cDNA using PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). Four degenerated primer pairs: SeaActinF and SeaActinR (Clark et al., 2008, Table 1), CAL5 and CAL6 (Jennings and Etter, 2011), BtubF1 and ABtub4r (Einax and Voigt, 2003), NS4 and AB1 (Lin et al., 2013) were used to amplify the *β-actin*, *calmodulin*, *beta-tubulin* and *18S ribosomal RNA* gene; and two degenerated primers, dAIHSP70F and dAIHSP70R, were used to amplify the *hsp70* gene (Song et al., 2006, Table 1). A 488 bp partial *β-actin* gene (GenBank accession nos. KF494231, and KF494235), a 258 bp *calmodulin* gene (GenBank accession no. KF494234), a 1212 bp *beta-tubulin* gene (GenBank accession nos. KF494230, and KF494234), a 879 bp *18S ribosomal RNA* gene (GenBank accession nos. KF494227, and KF494228) and a 626 bp partial *hsp70* gene (GenBank accession nos. KF494229, and KF494232) were amplified from the limpets. Comparison of the similarity of the sequences using a BLAST search in GenBank confirmed that the heat shock protein genes amplified from the two limpets were inducible isoforms of *hsp70*.

The expression of *hsp70* was determined by using real-time quantitative PCR with primers qrHSP70F, qrHSP70R, qcHSP70F and qcHSP70R (Table 1), which were designed based on the partial *hsp70* gene. The reference genes were selected from *18S ribosomal RNA*, *β-actin*, *β-tubulin* and *calmodulin* using GeNorm Algorithm (Primer Design, Ltd., Southampton University, UK) as described by Etschmann et al. (2006). GeNorm is a bioinformatics tool designed to rank candidate reference genes by using a normalization factor calculated on the basis of the geometric mean of the expression levels of the candidate reference genes in an array of representative samples. For *P. rustica* and *P. caerulea* the expression stability measures (M values) of *18S ribosomal RNA*, *β-actin*, *β-tubulin* and *calmodulin* were 2.449, 1.775, 1.775 and 2.553 and 1.416, 0.733, 0.733 and 1.046 respectively, when all genes were included in the calculation of M. Based on its low M values, partial sequences of the *β-actin* gene were selected as reference housekeeping genes to normalize the level of *hsp 70* expression, and the partial *β-actin* gene, amplified using the primers qactionF and qactionR, was selected as an internal control.

Real-time PCR conditions were the same for all set of primers and was carried out on a ABI 7500 Real-Time PCR System (Applied Biosystems, USA) in a 20-μl reaction volume containing 10 μl of 2 × FastStart Universal SYBR Green Master (Roche, Swiss), 0.8 μl of each primer (10 nmol μl^{-1}), 1 μl of cDNA template and 7.4 μl of RNase-free water. PCR conditions were as follows: 50 °C 2 min; 95 °C 10 min; 40 cycles of 95 °C 20 s; 55 °C 20 s and 72 °C 40 s with a final dissociation curve step. All samples were measured in triplicate. Ct (dR) values were analyzed using the ABI 7500 System Software (Applied Biosystems, USA). The abundance of *hsp70* mRNA was computed as the relative expression ratios (RU) of the *hsp70* compared to the *β-actin* between the control and the treated samples as described by Pfaffl (2001).

Table 1
Sequences of primers used for gene clone, their amplicon size and real-time PCR efficiency in *Patella rustica* and *P. caerulea*.

Primer name	Primer Sequences (5′–3′)	Amplicon size (bp)	PCR efficiency	Source
Gene clone				
SeaActinF	ACCGACTACYTSAKKAAGATCCT	488		Clark et al., 2008
SeaActinR	GAVGCVAGGATGGAGCCRCC			
dAIHSP70F	CAGGAATTCAARCGYAAACAC	626		Song et al., 2006
dAIHSP70R	TTGGTCATKGCTCGYTCTCC			
CAL5	TTYGACAAGGAYGGHGATGG	258		Jennings and Etter, 2011
CAL6	TCGGCGGCACTGATGAANCCGTTNCCGTC			
BtubF1	CAGGCYGGNCAGTGYGGHAACCAGATTGG	1212		Einax and Voigt, 2003
ABtub4r	GCYTCNGTGAARTCCATYTCGTCCAT			
NS4	CTTCCGTCAATTCCTTTAAG	879		Lin et al., 2013
AB1	GGAGGATTAGGGTCCGATTCC			
Real-time PCR analysis				
qactinF	ATATCAACATCGCACTTCAT	87	1.95, *P. rustica*	Self-designed
qactinR	ACTCTTCCAACCTTCCTT		2.05, *P. caerulea*	Self-designed
qrHSP70F	TTATTGGTGGATGTAGCC	123	1.86	Self-designed
qrHSP70R	AGCATAAGTTGTGAATATCTG			Self-designed
qcHSP70F	AACATCGCAGATATTCACAAC	75	1.86	Self-designed
qcHSP70R	GCTCGCTCTCCTTCATAG			Self-designed
qCALF	AACCATTACAACCAAGGA	178	1.972, *P. rustica*	Self-designed
qCALR	TTCTTCTTCACTGTCTGT		2.001, *P. caerulea*	Self-designed
q18SF	ATGGAATAATGGAATAGGA	180	1.955, *P. rustica*	Self-designed
q18SR	TTCGTTCTTGACTAATGA		1.950, *P. caerulea*	Self-designed
qBTUBF	TTCTGTTCTTGATGTTGT	138	2.050, *P. rustica*	Self-designed
qBTUBR	GGATATTCTTCACGGATT		1.953, *P. caerulea*	Self-designed

2.4. Statistical analysis

Data were analyzed using STATISTICA (STATSOFT) and PRIMER (PRIMER-E Ltd). The assumption of homoscedasticity of variances was tested using Levene's test. To compare differences in ABTs and Q10 relationships between the three species, one-way ANOVA was performed followed by Tukey *post hoc* comparisons. For the analysis of *hsp70* mRNA expression, data were log transformed and differences in *hsp70* mRNA between different temperatures and species analyzed using two factor PERMANOVA (fixed factors: species and temperature) using an unbalanced design as *hsp70* could not be measured in some replicates due to technical difficulties. After PERMANOVA, *post hoc* pairwise tests were used to determine differences for each temperature and species. Because of the small permutation number in pairwise tests, *P*-values were calculated using Monte-Carlo simulation with a critical probability value set at 0.05.

3. Results

3.1. Arrhenius break temperatures

The three species exhibited consistent differences in heart beat rates, which were not related to size differences between the three species, as all experimental animals were of similar weight (2.5 ± 1 g, mean ± SD). Individual cardiac performance could be divided into two phases with increasing temperatures (Fig. 1). All three species in the first phase (23 to 33 °C) had a regular increase of heart beat frequencies as described in the Arrhenius curve (Fig. 1). In this phase, metabolic rates increased with temperature and this temperature range was used to calculate Q10 relationships. In the second phase (33 to 45 °C), after temperature increased continuously, all three species showed a steady phase, after which heart rates decreased considerably (Fig. 1). Arrhenius plots of *Patella ulyssiponensis* heart rates were qualitatively different from the other species. The heart rates of *P. rustica* and *P. caerulea* decreased gradually from their ABT point, while that of *P. ulyssiponensis* showed a three phase pattern, with a levelling-off in heart rate in the mid phase, after which the heart rate decreased more abruptly (see Fig. 2 for examples of changes in the heart beat traces for all three species). In general, *P. caerulea* had consistently faster heart rates throughout the experiment (2.4 ± 0.4 Hz, mean ± SD) than *P. rustica* (2.1 ± 0.4 Hz), while *P. ulyssiponensis* had relatively slow heart rates (1.9 ± 0.3 Hz, Fig. 2). Based on two-phase regression, ABTs for the three species were all significantly different (Tukey test following ANOVA, $F_{(2,12)} = 7.58$, $P = 0.007$) with *P. rustica* having the highest ABT (37.9 ± 2.1 °C, mean ± SD), followed by *P. caerulea* (35.9 ± 2.6 °C), and finally the low-zoned *P. ulyssiponensis* (32.2 ± 2.3 °C). The same pattern was found for Q10 relationships, which also differed between the three species (Tukey test following ANOVA, $F_{(2,12)} = 12.79$, $P = 0.001$). *P. rustica* had the highest Q10 value (1.9 ± 0.1, mean ± SD), which was similar to *P. caerulea* (1.8 ± 0.2), whereas the Q10 of both species differed from *P. ulyssiponensis* which had the lowest metabolic increase (1.3 ± 0.2) in the range from 23° to 33 °C.

3.2. Hsp70 expression

P. rustica and *P. caerulea* showed different expression patterns of inducible *hsp70* mRNA (Fig. 3). Both species had similar, low levels of *hsp70* mRNA at 20 °C, which was the control temperature at the beginning of the experiment. After high temperature exposure, however, there were significant differences in *hsp70* mRNA levels among different temperatures and species (Table 2). In general, *hsp70* levels of *P. rustica* were greater than *P. caerulea,* especially at higher temperatures (Table 2, Fig. 3). *Hsp70* levels of *P. rustica* varied with temperature with upregulation occurring at 34 °C (~36% RU), which continued at 38 °C after 60 min (~143% RU) and 120 min of exposure (~194% RU). In *P. caerulea*, however, levels of *hsp70* mRNA (Fig. 3) were relatively low at 34 °C (~4% RU), but increased to reach maximum levels at 36 °C (~71% RU), and remained at similar, but lower levels at 38 °C (both after 60 min and 120 min exposure).

4. Discussion

There were clear differences in both ABT and *hsp* expression in the Mediterranean limpets which were associated with their vertical distribution. The higher shore *P. rustica* was able to tolerate higher temperatures than the two lower shore species, with Arrhenius breakpoint temperature being ~2 or 5 °C higher than those of *P. caerulea* or *P. ulyssiponensis,* respectively. This suggests that *P. rustica* individuals should be able to maintain cardiac activity and normal oxygen supply at very high ambient temperatures, even above 37 °C. Since rock temperatures on the Mediterranean shores are often above 37 °C during midday at low tide in the summer (Sarà et al., 2014), the proximity of maximal habitat temperature and upper thermal tolerance limits of *P. rustica* suggests that this species is actually living close to its thermal limits and would face increased risk from further warming of environmental temperatures (Artale et al., 2010; Wethey et al., 2011).

P. caerulea had faster heart rates as compared to *P. rustica* or *P. ulyssiponensis.* Heart rate is assumed to be a reliable indicator of metabolic rate in limpets (Chelazzi et al., 2001; Marshall and McQuaid, 1992; Santini et al., 1999), suggesting that *P. caerulea* has a higher

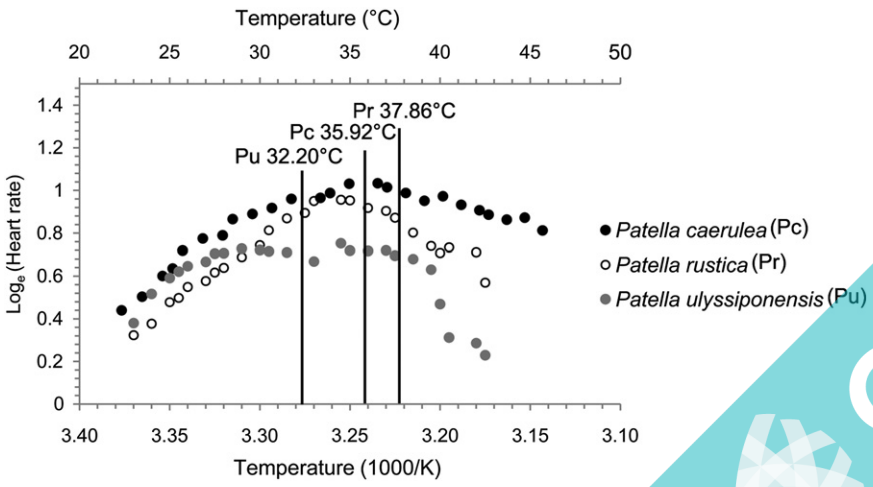

Fig. 1. Arrhenius plots and break temperatures (ABT) of heart rates for *Patella rustica*, *P. caerulea* and *P. ulyssiponensis* ($N = 5$).

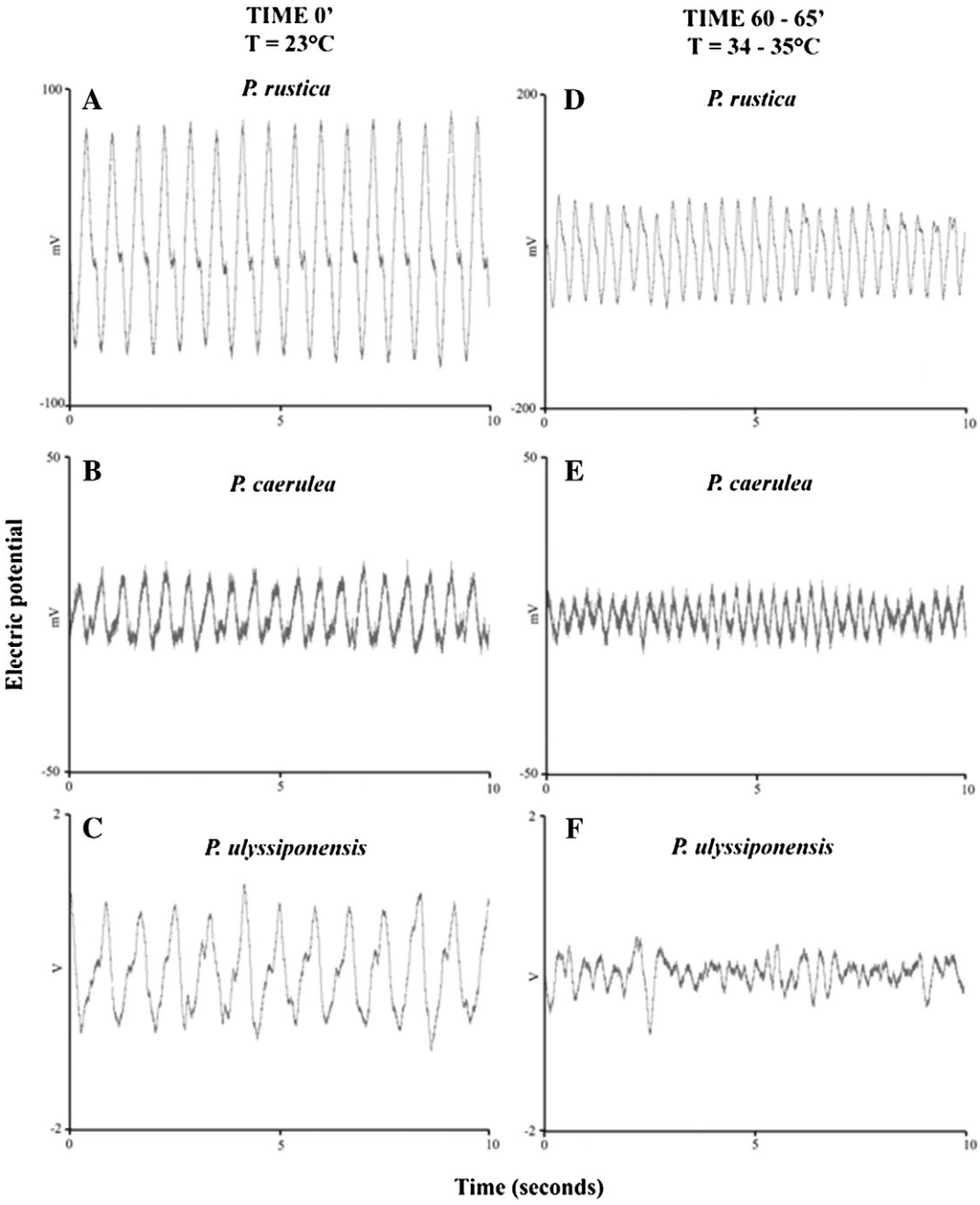

Fig. 2. Representative examples of variation in the heart beat traces recorded at the beginning (23 °C) of the experiment for *Patella rustica* (A), *P. caerulea* (B) and *P. ulyssiponensis* (C) and traces recorded after 60 or 65 min of constant heating (34–35 °C) for *Patella rustica* (D), *P. caerulea* (E) and *P. ulyssiponensis* (F). Time scale is 10 s and the scale of the *y*-axes varies depending on species.

metabolism than the other two species. Previous studies have shown that, in general, lower shore limpets have higher metabolic rates (Chelazzi et al., 2001; Dong and Williams, 2011) than their higher shore counterparts. A lower metabolic rate in high shore animals has been interpreted as a mechanism to cope with more variable environmental conditions (Branch, 1981; Chelazzi et al., 2001; De Pirro et al., 1999; Marshall et al., 2011). In contrast, the lower zoned *P. ulyssiponensis*, which is usually confined to the sublittoral fringe (Boaventura et al., 2002; Sella et al., 1993), appears to be an exception to this generality, as this species has a very low metabolism (low heart rates) and the lowest Q10 relationship, but appears very sensitive to any variation in temperature; for example *P. ulyssiponensis* showed clear signs of bradycardia during exposure to elevated temperatures. This partially supports the findings of De Pirro et al. (1999) who showed that when exposed to salinity extremes, the main response of *P. caerulea* was an initial increase in heart rate followed by decrease in cardiac activity, whereas *P. ulyssiponensis* exhibited bradycardia as a consequence of short term exposure to changes in salinity. Such findings support the idea that bradycardic patterns in response to stress might be adaptively used by limpets as a form of physiological isolation from stressful external conditions to reduce blood flow through the gills or pedal sinus as proposed by Chelazzi et al. (1999) and De Pirro et al. (2001).

Previous studies on Mediterranean limpets demonstrated that the functional traits of the three studied species (e.g. foraging activities, Della Santina and Chelazzi, 1991; Della Santina et al., 1993; physiological adaptations, such as energetic resources, Santini and Chelazzi, 1995; respiration rates, Bannister and McQuaid, 1974; and cardiac responses to different salinities or to copper pollution, De Pirro et al., 1999,

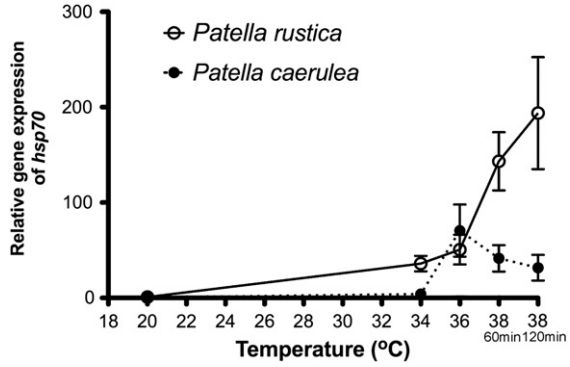

Fig. 3. *Hsp70* levels (relative units, RU %) in *Patella rustica* ($N = 25$) and *P. caerulea* ($N = 20$) after exposure to different temperature regimes (20, 34, 36 °C) and different durations at 38 °C (60 min and 120 min).

2001) are substantially different and related to their position on the shore, even though they can be found living within a few cm of each other. Santini and Chelazzi (1995) suggested that *P. rustica* has a more efficient mechanism of energy allocation during unfavourable conditions, resulting in a lower metabolism than *P. caerulea*. Such an 'energy conservation' strategy has been suggested to be important for high shore species such as *P. rustica* (Sokolova and Pörtner, 2003), which are resource limited due to prolonged emersion periods. By contrast, lower shore species, such as *P. caerulea*, are proposed to adopt a more 'exploitative strategy', exploiting resources and increasing metabolic rates with increasing temperatures (Sokolova and Pörtner, 2003).

Evolutionary adaptation to specific thermal niches has also resulted in species' specific capacities for passive heat resistance and consequently, different threshold temperatures for the induction of heat shock proteins (see Hochachka and Somero, 2002). More cold-adapted and/or lower shore species generally show upregulation of *hsps* at lower temperatures than warm-adapted and/or high shore counterparts, as shown in *Tegula* snails (Tomanek and Somero, 1999). In the high shore littorinid, *Echinolittorina malaccana*, Marshall et al. (2011) demonstrated that these snails exploit a strategy of metabolic depression when thermally stressed, with the onset of the heat shock response only occurring close to their breakpoint temperatures, after which aerobic scope becomes constrained and performance declines. These findings support results from this study, underlining the fact that upregulation of *hsps* is closely related with the thermal tolerance limits of *P. caerulea* and *P. rustica*. Upregulation of *hsp70* occurs in *P. rustica* at 34 °C, but increased at 38 °C and kept increasing after prolonged exposure to the same temperature. Upregulation of the heat shock response in *P. caerulea*, however, reaches a maximum level at 36 °C. Hence, the onset of the heat shock response in both species is closely related with their thermal tolerance limits as represented by Arrhenius breakpoint temperature. The Arrhenius breakpoint temperature may in fact be correlated to the critical temperature, after which metabolism changes from being aerobic to anaerobic (Pörtner, 2012). The present study tested the response of the three Mediterranean intertidal limpets under abrupt experimental thermal exposure, similar to conditions experienced in the last decade in the Southern Mediterranean during heat waves (Cerrano and Bavestrello, 2009). Such events do not allow individuals the necessary time to acclimate their physiological and subcellular responses, and under such conditions, individuals may easily exceed their energetic limits (Abele, 2012).

Mediterranean tides are small in range and even over these small tidal gradient Mediterranean limpets still show thermal adaptations to specific, but perhaps more narrow thermal windows, which results in a very small optimal range of environmental conditions for these species (see Tewksbury et al., 2008). Maintenance of a narrow thermal window will minimize energetic costs which would be associated with species exhibiting thermal plasticity and having more wide thermal windows (Somero, 2002). The physiological mechanisms that allow species to extend their thermal windows are energetically demanding, and this cost is usually met at the expense of other critical functions such as growth and reproduction (Hofmann and Todgham, 2010; Sarà et al., 2013). There are, however, costs associated with having more narrow thermal windows (as suggested for tropical species where thermal stress is consistently high; Tewksbury et al., 2008), as this lack of plasticity or capacity to tolerate wide thermal ranges could make Mediterranean intertidal organisms even more sensitive to increasing temperatures than corresponding species living along oceanic coasts worldwide where tidal amplitude is greater (e.g. Petes et al., 2007). Failure of heart function in the Mediterranean limpets, for example, is very close to their upper thermal limits, and may become a weak link in denoting species' thermal tolerance, and consequently, could be a key determinant of their geographic range. Mediterranean limpets, therefore, already exist on the edges of their thermal tolerance windows, and any change in temperature, regardless of how small, is likely to have detrimental consequences to these species, with consequent impacts on shore community structure and functioning.

Table 2
Two factor PERMANOVA to investigate variation in *hsp70* gene expressions of the limpets *Patella rustica* ($N = 25$) and *P. caerulea* ($N = 20$; five replicates were not successfully analyzed due to technical difficulties) under different temperature treatments, followed by *post hoc* pairwise tests.

Source	df	SS	MS	pseudo-F	*P*
Species	1	2.7621	2.7621	27.879	0.001
Temperature	4	29.0510	7.2626	73.303	0.001
Species × temperature	4	1.6462	0.4116	4.154	0.011
Residual	35	3.4677	0.0091		
Total	44	38.0280			
Temperature					
20 °C *P. caerulea* = *P. rustica* 34 °C *P. caerulea* < *P. rustica* 36 °C *P. caerulea* = *P. rustica* 38 °C 60 min *P. caerulea* < *P. rustica* 38 °C 120 min *P. caerulea* < *P. rustica*					
Species					
P. rustica	20 °C < 34 °C = 36 °C < 38 °C 60 min = 38 °C 120 min				
P. caerulea	20 °C < 34 °C < 38 °C 120 min = 38 °C 60 min = 36 °C				

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