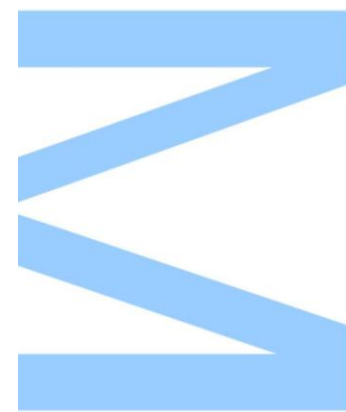


Does thermal microhabitat variability modulate thermal stress responses? A study focusing on the physiology and behavior of *Patella vulgata*



Catarina Cunha Moreira

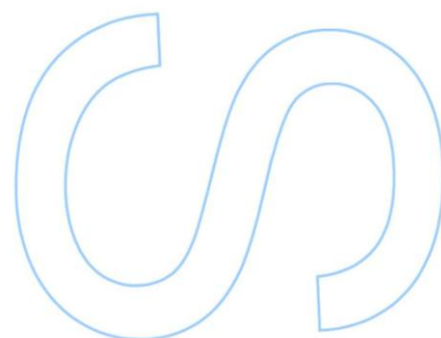
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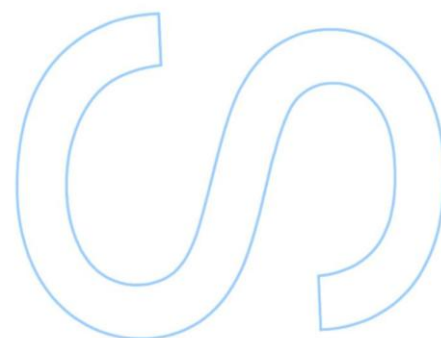
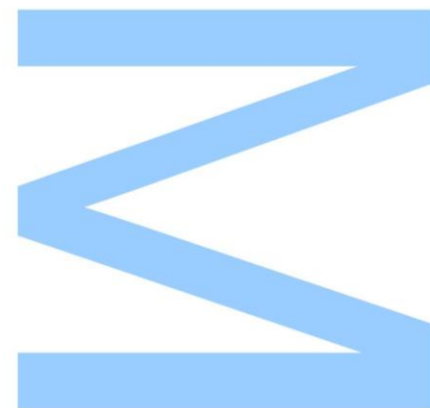


Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto,

____/____/____



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À minha família, em especial aos meus pais, irmão e avós pela presença e apoio incondicional porque sem isso tudo o resto seria impossível.

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Summary

Global warming is being intensively studied and temperature is considered one of the most important abiotic stress factors. The rocky intertidal, one of the most thermally complex environments on Earth, is inhabited by marine organisms which are periodically exposed to stressful terrestrial conditions during emersion. Therefore the intertidal is ideal to study the thermal response of organisms. Although this ecosystem harbours numerous microhabitats, the thermal variability between sun-exposed and shaded microhabitats has already been described. Our study uses robotlimpets and thermal images to confirm that sun-exposed microhabitats are consistently hotter than shaded microhabitats. The mechanism that translates thermal stress into differential physiological and behavioral performance and fitness is poorly understood. Even though several physiologic studies have been carried out on intertidal organisms, most are not based on actual thermal data collected on the field. Our study aimed at studying the cardiac response and behavior of individuals from shaded and sun-exposed microhabitats, using a realistic heat stress treatment (peaking at 40 °C). The difference on cardiac response between microhabitats was non-significant, although there was a higher proportion of individuals from shaded microhabitats having a cardiac failure than individuals from sun-exposed microhabitats. The behavior response showed a decrease on the proportion of individuals moving with the increase of temperature but an increase of the mushrooming behavior after being exposed to the maximum temperature. This highlights the importance of realistic studies to avoid under- or over-estimating the organism's responses to thermal stress.

Key words: *Patella vulgata*, thermal stress, cardiac activity, behavior, realistic studies.

Resumo

O estudo do aquecimento global tem sido bastante aprofundado e a temperatura é considerada um dos stresses abióticos mais importantes. O intertidal rochoso, um dos ecossistemas mais complexos do planeta a nível térmico, é habitado por organismos que estão expostos periodicamente a condições terrestres stressantes durante a emersão. Daí o intertidal ser ideal para o estudo da resposta térmica dos organismos. Apesar deste ecossistema ter bastantes microhabitats, a variabilidade térmica entre os microhabitats expostos ao sol e os microhabitats na sombra já foi descrita. Este estudo usa robolapas e imagens térmicas para confirmar que os microhabitats expostos ao sol são mais quentes que os da sombra. O mecanismo que traduz o stress térmico a uma performance fisiológica e comportamental é pouco conhecido. Apesar de muitos estudos fisiológicos terem sido elaborados com organismos intertidais, a maioria não é baseada em dados térmicos recolhidos no campo. Este estudo tem como objetivo estudar a resposta cardíaca e comportamental de indivíduos de microhabitats expostos ao sol e microhabitats na sombra, usando um stress térmico realista (com um máximo de 40 °C). A diferença da resposta cardíaca entre microhabitats não foi significativa, no entanto uma maior proporção de indivíduos provenientes de microhabitats na sombra tiveram um falha cardíaca comparativamente aos provenientes de microhabitats expostos ao sol. A nível comportamental, se por um lado houve uma diminuição na proporção de indivíduos em movimento com o aumento da temperatura, por outro lado houve um aumento do comportamento de “mushrooming” após estarem expostos à temperatura máxima. Este estudo destaca a importância de estudos realistas de maneira a evitar a sub- ou sobre- estimação da resposta térmica dos organismos.

Palavras-chaves: *Patella vulgata*, stress térmico, atividade cardíaca, comportamento, estudos realistas.

Table of Contents

1. Introduction	10
2. Materials and Methods.....	12
2.1. Field measurements of <i>P. vulgata</i> body temperatures	12
2.2. Collection of individuals.....	13
2.3. Experimental Set-up	13
2.4. Heart rate measurements	13
2.5. Behavior analyses.....	15
2.6. Statistical analyses	15
3. Results.....	15
3.1. Robolimpets.....	15
3.2. Thermal images	18
3.3. Heart Beat measurements	18
3.4. Behavior.....	19
4. Discussion.....	20
5. Bibliographic References.....	23

List of Figures

Fig.1. Data from robolimpets in shaded (blue) and sun-exposed (red) microhabitats. (a) Daily cumulative heat of shaded (238.05 ± 7.71 °C, mean \pm SE) and sun-exposed (292.88 ± 12.69 °C, mean \pm SE); (b) Maximum temperature of shaded (15.09 ± 0.23 °C, mean \pm SE) and sun-exposed (22.65 ± 0.83 °C, mean \pm SE); (c) Heating rate of shaded (0.65 ± 0.04 °C/h, mean \pm SE) and sun-exposed (2.63 ± 0.22 °C/h, mean \pm SE); (d) Cooling rate of shaded (0.80 ± 0.07 °C/h, mean \pm SE) and sun-exposed (2.73 ± 0.28 °C/h, mean \pm SE).

Fig.2. Body temperature obtaining from thermal images of individuals from shaded (blue) (27.46 ± 0.37 °C, mean \pm SE, n = 283) and sun-exposed (red) microhabitats (15.05 ± 0.27 °C, mean \pm SE, n= 317).

Fig.3. Data obtained from the cardiac activity of individuals from shaded (blue) and sun-exposed (red) microhabitats during the simulated thermal stress. (a) Arrhenius break temperatures (ABT) of heart rates of individuals from shaded (27.60 ± 1.24 °C, mean \pm SE) and sun-exposed microhabitats (29.38 ± 0.94 °C, mean \pm SE) were not significantly different (ANOVA, P = 0.399, df = 11, n = 6 for each species); (b) Proportion of individuals reaching ABT from shaded (75.00 %, n=16) and sun-exposed microhabitats (53.8 %, n=26) were significantly different (Chi-square test, P = 0.002).

Fig.4. Behavior of individuals from shaded (blue) and sun-exposed (red) microhabitats during three phases of the simulated thermal stress: Before (B), on the temperature peak (P) and after (A). (a) Proportion of individuals from shaded (B: 1.96 ± 0.86 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) and sun-exposed microhabitats (B: 2.26 ± 0.67 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) performing rotation; (b) Proportion of individuals from shaded (B: 22.56 ± 6.15 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) and sun-exposed microhabitats (B: 18.19 ± 8.68 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) performing displacement; (c) Proportion of individuals from shaded (B: 6.80 ± 4.40 ; P: 10.00 ± 5.96 ; A: 9.64 ± 3.49 , mean \pm SE) and sun-exposed microhabitats (B: 3.57 ± 3.19 ; P: 6.67 ± 5.96 ; A: 14.44 ± 5.63 , mean \pm SE) performing mushrooming.

List of Abbreviations

ABT : Arrhenius brake temperature

HSP: Heat shock protein

IR: Infra-red

1. Introduction

Earth has undergone many major climatic changes over the course of history, including glaciations and warm periods. However, over the past 250 years, there has been an anthropogenically-induced increase in the frequency of extreme climatic events worldwide, such as heat waves and storms (Coumou and Rahmstorf, 2012). The current rate of warming exceeds anything recorded over the past 10,000 years (Marcott *et al.*, 2013) and has led to unprecedented sea level change and sea ice loss over the past several decades (IPCC, 2007). In the last three decades, 71% of the world's coastlines have been significantly warming (Lima and Wethey, 2012). Climate change is currently considered one of the major threats upon biodiversity and the sustainability of both terrestrial and marine systems (Burrows *et al.*, 2011; Sala *et al.*, 2000). One of the main concerns is the ability of species to adapt to these rapid changes, especially since distribution shifts and extinctions have already been observed (IPCC, 2007). Many of the recent climate-change induced distribution shifts have been related to changes in the availability of specific thermal habitats (Helmuth *et al.*, 2006; Bennie *et al.*, 2008). Temperature is considered as one of the most important abiotic factor since it affects biological processes at all levels of biological organization (Pörtner *et al.*, 2006). For instance, thermal stress has been shown to cause a decrease in growth rates (Somero, 2002, 2010; Woodin *et al.*, 2013) and aerobic capacity (Pörtner, 2010), a reduction in body size (Gardner *et al.*, 2011), and an increase of oxygen consumption rates (Sinclair *et al.*, 2006).

A challenge faced by researchers nowadays is to determine which species are more vulnerable to climate change, and in particular to rising temperatures, in order to anticipate the ecological consequences of distribution changes. In this respect, knowledge on the physiological and behavioral responses of ectothermic species to heat stress is critical as their temperature is less independent from environmental conditions, and thus, they have higher probabilities of being affected. Extreme and highly heterogeneous ecosystems such as intertidal habitats are excellent and complex test bed systems since their inhabitants are periodically exposed to stressful abiotic factors (e.g. desiccation) during low tide. Therefore, it is not unreasonable to assume that they are already at or close to their thermal tolerance (Pincebourde *et al.*, 2008). Intertidal species are thus expected to respond rapidly to climate changes and could provide information on how other species will respond to the future climate. Due to the physical complexity of most rocky intertidal ecosystems, they harbor numerous microhabitats (Seabra *et al.*, 2011; Lathlean and Minchinton, 2012). The

characterization of such microhabitats offers major opportunities to understand the mechanisms driving individual thermal stress. Recent studies by Seabra *et al.* (2011) and Lima *et al.* (2015) focused on the importance of solar exposition on the patterns of intertidal thermal stress. Seabra *et al.* (2011) concluded that differences in temperature between sun-exposed and shaded microhabitats were larger than the variability associated with seasons or even with shore level. Lima *et al.* (2015) took a step further, and showed that these thermal differences imply differential physiological performance in the limpet *Patella vulgata* – individuals collected from sun-exposed microhabitats were consistently more stressed than those ones collected from shaded microhabitats. These differences were attributed to a combination of factors, from the metabolic cost of replacing heat damaged proteins to the heat-shock response itself.

Several studies have been performed relating the physiological response of intertidal organisms to thermal stress. Besides microhabitat (e.g., Gedan *et al.* 2011), other environmental factors such as seasonality (e.g., Chapperon *et al.* submitted) and the cumulative stress caused by increasing water and air temperature (Seabra *et al.*, in press) have also been demonstrated to impact physiology at different levels. Additionally, species-specific traits, such as physiological tolerance, (e.g., Dong *et al.*, 2008), mobility (Huey *et al.*, 1989; Chapperon and Seuront, 2011b; Marshall *et al.*, 2013), behavior (e.g., Chapperon and Seuront, 2011a) or the ability to acclimate (e.g., Meng *et al.*, 2009) have also been shown to modulate the thermal response. Although these studies have been informative, the conditions simulated in the laboratory were in most cases, not very realistic (e.g., warming was frequently simulated in water baths instead of using infra-red heating, the thermal ramps lacked realism, but see Seabra *et al.*, in press). Consequently, physiological responses to climatic variation and change may still be far from understood.

The aim of this study was to determine if *Patella vulgata* collected from sun-exposed and shaded microhabitats showed different capacities to cope with thermal stress. We hypothesized that individuals from sun-exposed are able to acclimate during thermal stress. We used a four year long dataset on high resolution body temperature data collected with robolimpets (Lima and Wetthey, 2009) over the majority of the distribution range of *Patella vulgata* to obtain realistic temperature profiles for our experiments. Additionally, robolimpets together with thermal imaging, which has been demonstrated to be a powerful method for determining the body temperature of intertidal organisms (Lima and Wetthey, 2009; Lathlean and Seuront, 2014), were used for the characterization of microhabitats in the field. Both methods have been proved useful in

identifying microhabitat thermal heterogeneity to determine individual thermal stress (Lima and Wetthey, 2009; Lathlean and Seuront, 2014). Based on high resolution temperature data from robolimpets we simulated a low tide during one of the hottest days recorded (i.e., corresponding to the 95 percentile of the whole dataset). We used heart failure as a proxy for thermal stress and we monitored behavior during the heating experiment. Specifically, we expected that, in comparison with individuals from shaded microhabitats, animals from sun-exposed microhabitats would show less cardiac failure and less changes in behavior in face of thermal stress. We expect that the present work will present both a better understanding of the thermal characteristics of microhabitats and of the stress mechanisms that drive ecological changes at the individual level.

2. Materials and Methods

2.1. Field measurements of *P. vulgata* body temperatures

To characterize the thermal history of *Patella vulgata in situ* in both microhabitats, a total of 4 robolimpets (two per microhabitat) (Lima and Wetthey, 2009) were deployed on the rocky shore of Le Dellec, Plouzané (48°21'09"N, 4°34'01"W) for a total of 21 days, during May 2015. Robolimpets consist of a lithium battery connected to a reprogrammed microchip that can measure temperature and store data (iButton DS1922L, Dallas semiconductor). They are inserted into a *Patella spp.* shell and isolated from the seawater with a plastic resin. There are two seawater-corrosion-resistant external electric contacts made of constantan that allow to program and collect data without opening the robolimpet (Lima and Wetthey, 2009). Robolimpets were deployed in sun exposed (South facing) and shaded (North-facing) rock walls to capture the temperature differences associated with differences in solar exposure between these two microhabitats. A quick setting resin (Z-spar, Kop-Coat Inc., Pittsburgh, Pennsylvania, USA) was used to attach the loggers to the rock.

Robolimpets were programmed in the field using an USB cable to connect the device to a laptop. They were programmed to record temperatures with a 30 minute interval and a resolution of 0.0625 °C. On each microhabitat, data from both robolimpet replicates was averaged. Since our study focused on emersion temperature profiles, all data except for the diurnal low tide was rejected from further analysis. The compilation of the temperature profiles of the diurnal low tide allowed us to calculate the maximum

temperature, the cooling and heating rates as well as the daily cumulative heat for this rocky shore.

For comparison purposes, thermal pictures of individuals from both microhabitats were taken with a thermal camera (Optris PI 200) during midday low tide of the 19 of March and 8 and 12 of April. The thermal sensitivity of the thermal camera is 0.2 °C at 30 °C, and the temperature measurement accuracy is 2% or 2 °C, whichever is greater. Emissivity value considered was 0.95 (Chapperon, personal comm.). Data of each day was pooled in order to measure the average of individual's body temperature for each microhabitat.

2.2. Collection of individuals

A week prior to experiments, 48 individuals of *Patella vulgata* with maximum length of 30-35cm were collected from sun exposed (n=24) and shaded (n=24) microhabitats at Le Dellec, brought back to the laboratory within an hour and then labeled and placed into the experimental aquarium. Each experiment lasted three days and was repeated once in order to verify results and minimize technical errors, so in total 96 individuals were collected (48 from each microhabitat).

2.3. Experimental Set-up

The aquarium system consisted of an open-water circuit for which seawater was provided by a pumping station (Sainte-Anne de Portzic) nearby the laboratory and field site (< 4 km). A custom-made controller using the Arduino system, which is an open-source electronic prototyping platform. This allowed us to program and simulate field conditions through the control of temperature (by using IR lights), tides and visible light. Temperature was controlled using robolimpets as feedback sensors, ensuring that the thermal conditions mimicked those registered in the field. Acclimation lasted three days, and consisted of a sequence of high and low tides, also mimicking the natural tidal cycle. Temperatures during acclimation were kept at low-stress levels: 15 °C during high tide and 16 °C during low tide, which match the average minimum temperatures normally recorded at this rocky shore (Chapperon, personal comm.). The experiment consisted of exposing limpets during emersion to a single stress event of 40 °C with realistic heating and cooling rates (6 and 10 °C/h, respectively), determined by averaging 4 year data collected by robolimpets throughout the distributional range of *P. vulgata* (Lima and Wetthey, 2009), followed by one day of recover.

2.4. Heart rate measurements

The day prior to the simulated thermal stress, sixteen of the twenty four individuals were fitted with an infra-red (IR) heart beat sensor in order to examine their physiological response to heat stress during the following three days of experiment. The heart rate measurements were done using a modified version of the system described by Burnett *et al.* (2013). The system is completely automatic and features a series of IR sensors, a signal amplifying circuit, a real-time clock, and is able to autonomously log up to eight channels simultaneously. Each individual's heart was logged at a frequency of 1/20 Hz for one minute every 30 minutes. The IR sensors combine an IR emitter and an IR detector. To record the heart rate of a limpet, the sensor is glued with cyanoacrilate cement to the exoskeleton, at a approximate location over the animal's heart. In this position, the IR light can pass through the shell and illuminate the heart and nearby circulatory vessels. The IR light reflections vary with the changes in the shape or volume of the circulatory structures during a heart contraction and that information is received by the IR detector and processed by the software.

All recordings with undetectable heartbeats were discarded. Thus, we kept 27% of individuals from sun-exposed and 16% from shaded microhabitats. Because each animal has its characteristic basal frequency (i.e., the frequency when at rest and without the interference of external stressing factors), absolute differences among the cardiac frequencies of several animals do not necessarily mean that their metabolic performance is different. To overcome this issue, cardiac frequencies recorded throughout the experiment were normalized separately for each individual. This was done by dividing each individual's cardiac frequency by its average heartbeat frequency during the last day of acclimation.

Using only the data from the simulated thermal stress event, the normal logarithm (\ln) transformation of the normalized heart rate was plotted against the inversion of temperature in Kelvin, in order to determine the Arrhenius break temperature (ABT), which is the thermal limit of cardiac function (Stillman and Somero 1996). For individuals for which this limit was reached, the ABT was calculated using regression analyses to generate the best fit line on both sides of a putative break point. Some individuals withstand the entire thermal stress treatment without showing any evidence for cardiac failure or loss of performance (thus, for these animals ABTs could not be computed). We computed the percentage of individuals that did not show signals of cardiac failure and used that value as an indication of the overall resilience of the sampled pool.

2.5. Behavior analyses

During the simulated thermal stress event of all experiments, behavior of limpets was recorded using a GoPro Hero 3+. The behavior was classified into two categories: immobile and mobile. The mobile category was subdivided into three sub-categories: displacement- when the limpets moved from one position to another; rotation - when limpet rotated; and mushrooming behavior – when limpets raised its shell repeatedly. Individuals that were out of the camera's view were not accounted for. The proportion of individuals showing the three different types of behaviors was calculated for three phases of the experiment: before the temperature peak, at the peak (40 °C), and after the peak.

2.6. Statistical analyses

Data from robolimpets and thermal imaging was analyzed for each microhabitat, the average and standard error were calculated and plotted.

The effects of temperature on heart rate were assessed in two different ways: (1) a one-way ANOVA was performed using the values of ABT of individuals from both microhabitats to determine if they were statistically different; and (2) a Chi-square test was performed using the proportion of the occurrence of ABTs to determine if there were differences in the cardiac response of individuals from different microhabitats.

The behavior data was assessed for each microhabitat distinguishing the three different types of behavior: rotation, displacement and mushrooming. The proportion of individuals performing each behavior in each step of the temperature profile (before the temperature peak, on the temperature peak and after) was plotted.

All statistical analyses were carried out using Microsoft Office Excel 2007.

3. Results

3.1. Robolimpets

Analysis of the temperature data collected from the two robolimpets deployed for 21 days at Le Dellec in each microhabitat allowed us to distinguish them according to their solar exposure.

The thermal profile of both microhabitats was characterized by plotting the daily cumulative heat (Fig. 1.a), the maximum temperature (Fig. 1.b), heating rates (Fig. 1.c) and cooling rates (Fig. 1. d). All these analyses showed that sun-exposed microhabitats are hotter and have higher heating and cooling rates than shaded microhabitats. The difference in maximum temperatures between microhabitats was 8 °C and the difference in daily cumulative heat between microhabitats was 55 °C. The heating and cooling rates were substantially higher in sun-exposed microhabitats when compared with their shaded counterparts. Heating rates were four times higher and cooling rates were three times higher (Fig 1c and d).

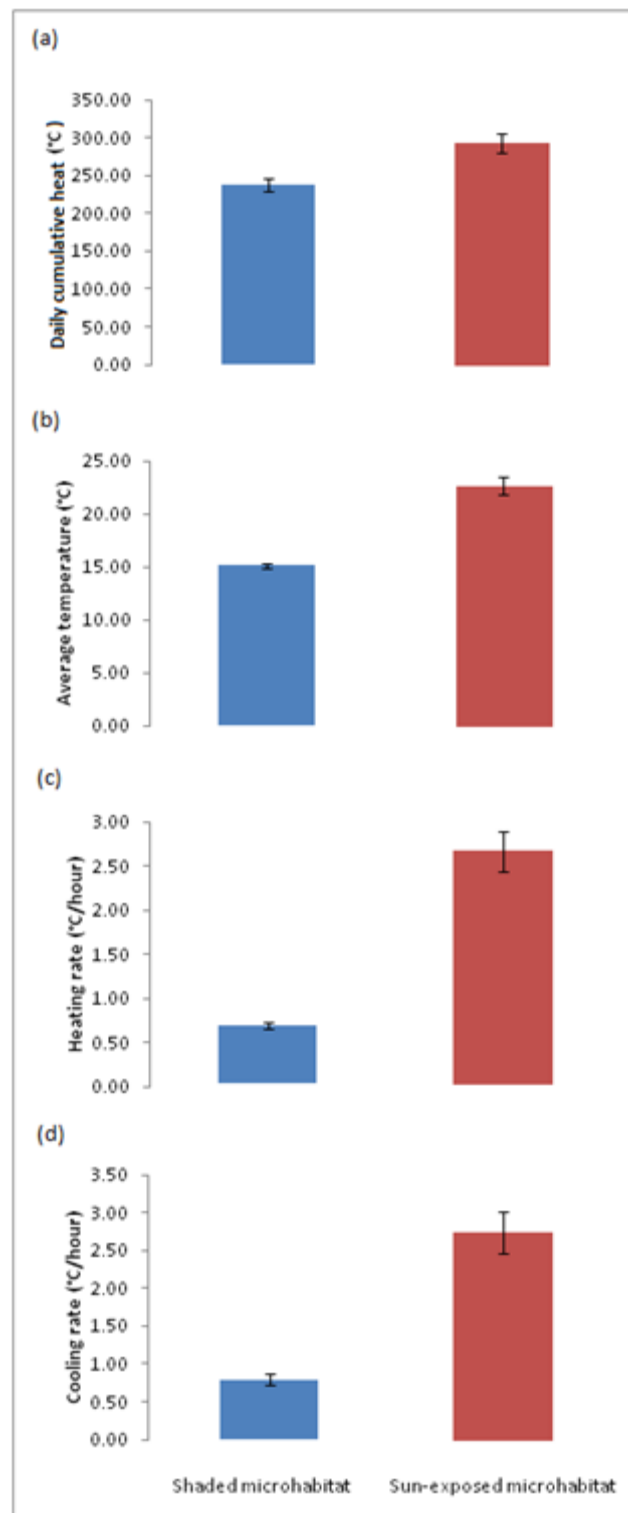


Fig.1. Data from robolimpets in shaded (blue) and sun-exposed (red) microhabitats. (a) Daily cumulative heat of shaded (238.05 ± 7.71 °C, mean \pm SE) and sun-exposed (292.88 ± 12.69 °C, mean \pm SE); (b) Maximum temperature of shaded (15.09 ± 0.23 °C, mean \pm SE) and sun-exposed (22.65 ± 0.83 °C, mean \pm SE); (c) Heating rate of shaded (0.65 ± 0.04 °C/h, mean \pm SE) and sun-exposed (2.63 ± 0.22 °C/h, mean \pm SE); (d) Cooling rate of shaded (0.80 ± 0.07 °C/h, mean \pm SE) and sun-exposed (2.73 ± 0.28 °C/h, mean \pm SE).

3.2. Thermal images

Thermal images of a total of 283 and 317 limpets from shaded and sun-exposed microhabitats, respectively, were taken in three different days in order to assess the body temperature of *P. vulgata*.

Thermal imaging confirmed that sun-exposed microhabitats are hotter than shaded microhabitats. Temperature from individuals inhabiting sun-exposed microhabitats was, in average, 1.82 times higher than the body temperature of individuals living in shaded microhabitats (Fig.2).

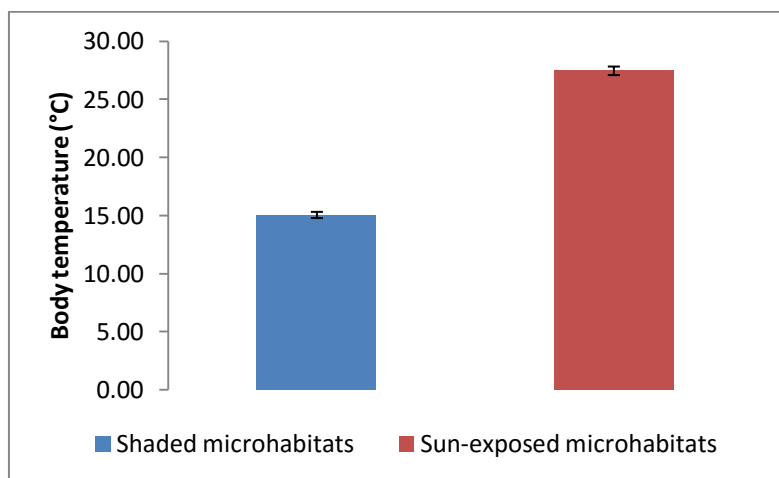


Fig.2. Body temperature obtaining from thermal images of individuals from shaded (blue) (27.46 ± 0.37 °C, mean \pm SE, n= 283) and sun-exposed (red) microhabitats (15.05 ± 0.27 °C, mean \pm SE, n= 317).

3.3. Heart Beat measurements

The cardiac activity of a total of 26 individuals from sun exposed microhabitats and 16 individuals from shaded microhabitats was assessed.

A total of six individuals from each microhabitat reached the ABT. The value of ABT in sun-exposed and shaded microhabitats was 29.4 and 27.6 °C, respectively (Fig.3a), but they were not statistically different ($p = 0.399$, $df = 11$, ANOVA). The proportion of occurrence of ABT in shaded microhabitats was 20% higher than in sun-exposed microhabitats ($p = 0.002$, chi-square test) (Fig.3b).

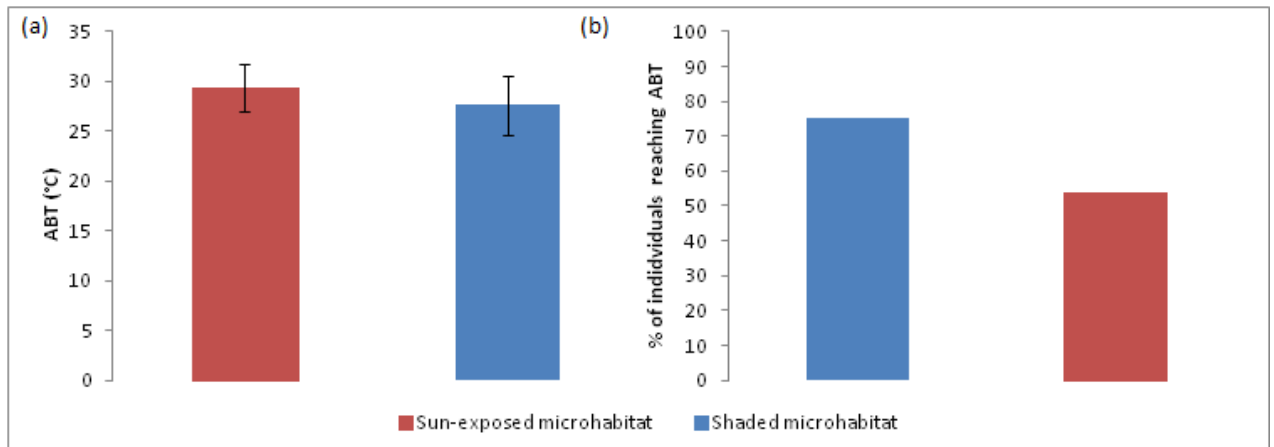


Fig.3. Data obtained from the cardiac activity of individuals from shaded (blue) and sun-exposed (red) microhabitats during the simulated thermal stress. (a) Arrhenius break temperatures (ABT) of heart rates of individuals from shaded (27.60 ± 1.24 °C, mean \pm SE) and sun-exposed microhabitats (29.38 ± 0.94 °C, mean \pm SE) were not significantly different (ANOVA, $P = 0.399$, $df = 11$, $n = 6$ for each species); (b) Proportion of individuals reaching ABT from shaded (75.00 %, $n=16$) and sun-exposed microhabitats (53.8 %, $n=26$) were significantly different (Chi-square test, $P = 0.002$).

3.4. Behavior

After removing all individuals that were not present, a total of 26 individuals from shaded microhabitats and 19 from sun-exposed microhabitats were considered for the behavior analyses.

While before the temperature peak the behavior was mostly displacement and a higher proportion of individuals from shaded microhabitats were active, on the peak and after the peak, all the individuals exclusively performed mushrooming. Individuals from sun-exposed started to be more active after the peak than individuals from the shaded microhabitat (Fig.4 a-c).

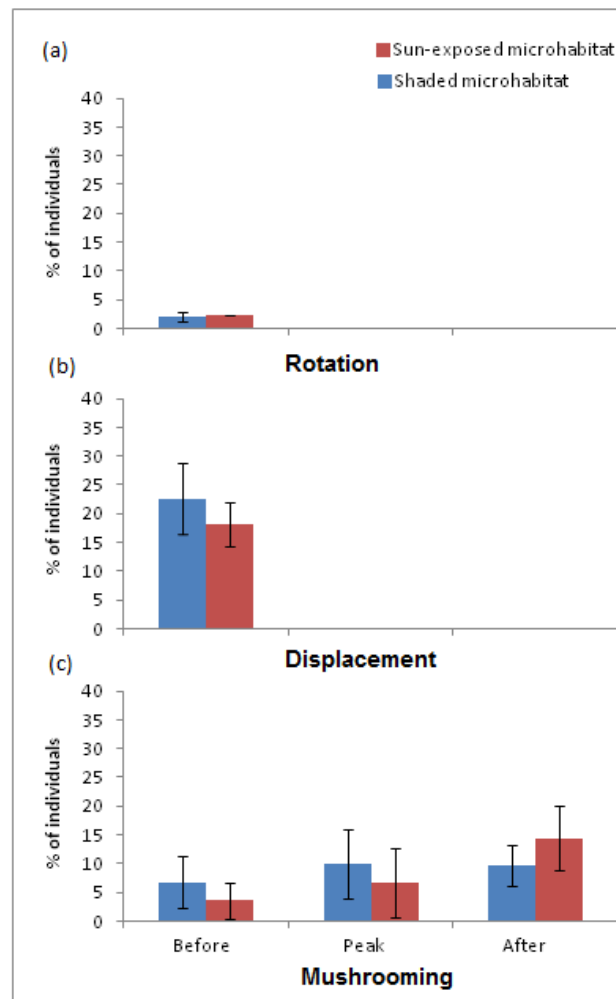


Fig.4. Behavior of individuals from shaded (blue) and sun-exposed (red) microhabitats during three phases of the simulated thermal stress: Before (B), on the temperature peak (P) and after (A). (a) Proportion of individuals from shaded (B: 1.96 ± 0.86 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) and sun-exposed microhabitats (B: 2.26 ± 0.67 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) performing rotation; (b) Proportion of individuals from shaded (B: 22.56 ± 6.15 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) and sun-exposed microhabitats (B: 18.19 ± 8.68 ; P: 0.00 ± 0.00 ; A: 0.00 ± 0.00 , mean \pm SE) performing displacement; (c) Proportion of individuals from shaded (6.80 ± 4.40 ; P: 10.00 ± 5.96 ; A: 9.64 ± 3.49 , mean \pm SE) and sun-exposed microhabitats (B: 3.57 ± 3.19 ; P: 6.67 ± 5.96 ; A: 14.44 ± 5.63 , mean \pm SE) performing mushrooming.

4. Discussion

This study aimed at determining the ability of individuals from different microhabitats to cope with thermal stress. Using robolimpets and thermal imaging, both microhabitats were characterized showing that sun-exposed microhabitats are consistently hotter than shaded microhabitats. Although there were differences in the physiological (cardiac activity) response to acute stress by *Patella vulgata* originating from different habitats, there were no differences in the behavior.

Microhabitat variability in thermal stress

Data from present work confirm that *Patella vulgata* experience different thermal regimes according to their position in the shore. Observed temperatures in shaded and sun exposed microhabitats were different, especially regarding heating and cooling rates. Previous studies already showed differences in temperature from high to low shore as well as at the microhabitat level, between crevices and flat rocks. Furthermore, thermal stress experienced by organisms was shown to be higher in sun-exposed than in shaded microhabitats (Stirling 1982, Tomanek and Somero 1999, Stillman and Somero 1996, Davenport and Davenport 2005, Stenseng *et al.* 2005, Nguyen *et al.* 2011, Prusina *et al.* 2014, Chapperon *et al.* submitted). The present data unequivocally demonstrate that the maximum temperature (a proxy for chronic stress), the daily cumulative heat (a proxy for the degree of acute stress) and the heating and cooling rates of sun exposed and shaded microhabitats, are consistently higher in sun exposed habitats. The observed differences are likely to be responsible for the higher expression of heat shock proteins by *P. vulgata* from sun-exposed microhabitats described for Atlantic coast of Europe (see Lima *et al.*, 2015).

Physiological response to thermal stress

Experimental temperature profiles based on realistic heating and cooling rates allowed the determination of the proportion of individuals from different microhabitats that are able to cope with thermal stress.

However, a significantly higher proportion of individuals from shaded microhabitats reached Arrhenius breaking point temperature (ABT), the value of ABT were not significantly different from individuals from sun-exposed microhabitats. Still, these results seem to indicate that sun-exposed individuals are more acclimated and therefore are likely to better cope with acute heat stress than individuals from shaded microhabitats. Since intertidal organisms are daily exposed to stressful conditions and consequently are close to their thermal limit, an increase of temperature can lead to high mortalities rates as reported before (see Harley, 2008). Furthermore, these results indicate a potential for acclimation what can also suggest that individuals from sun-exposed microhabitats, which are already exposed to higher temperatures than individuals from shaded microhabitats, have the capacity to cope with an acute stress, as also have been studied (Chapperon *et al.* submitted).

Behavioral response to thermal stress

Short-term acclimation and variability in heat stress are likely to be translated in different behavioral patterns. We hypothesize that a behavior such as mushrooming (shell raising), which has an important role in evaporative cooling (Denny and Harley, 2006) and in reducing thermal stress (Williams *et al.*, 2005), should be more frequent in individuals less acclimated to high temperatures. This behavior is considered a short-term and a high-risk strategy since when raising their shell organisms lose water and therefore may become unable to maintain adhesion to the rock surface (Williams *et al.*, 2005). Individuals that need to perform this behavior are more likely to not survive in extreme events.

Our results show that at the beginning of the experiment a higher proportion of individuals were moving around when the rock of the aquarium was still wet. At this point the temperature was not restricting their behavior. With the increase of temperature and consequently the increase of heart rate all individuals changed their behavior. A low proportion of moving individuals on the temperature peak and after the peak is possibly due to some of them reaching the ABT. When the temperature reached its maximum, all individuals changed their behavior to mushrooming. Since a 40°C day is not common at the location where individuals were collected, it is not surprising that a high proportion of individuals would perform this behavior. There was a higher proportion of individuals from sun-exposed microhabitats performing mushrooming, although its significance cannot be assessed.

Further prospects

With ongoing climate change there is a major concern to understand the thermal stress mechanisms. The importance of performing realistic studies depends on having confidence to really understand these mechanisms. Therefore, studies that are not realistic can be misleading and may provide erroneous results. Even though our study used realistic heating and cooling rates, we acknowledge that the way we computed them (i.e., pooling temperature data from most of the distribution range of the species) may not be the most appropriated for the population that was used in the experimental trials. This study would undoubtedly benefit from temporal, and, especially, from spatial replication. It would be interesting to compare the responses of populations from the middle of the distribution range with those from the range edges, especially since recent studies have found that this species displays regional variations in thermal stress response (measured via Hsp expression, see Lima *et al.*, 2015). Other

molecular tools, such as transcriptomic analysis could also help us to understand the mechanisms driving the thermal response of this species.

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