

UNIVERSIDADE ESTADUAL PAULISTA - UNESP  
INSTITUTO DE BIOCÊNCIAS DE BOTUCATU  
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - ZOOLOGIA

PRISCILA GRANADO

**Resposta de organismos do entremarés ao aumento da temperatura: abordagem fisiológica e comportamental em uma perspectiva de aquecimento global**

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Botucatu-SP

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Tese apresentada ao Programa de Pós Graduação em Ciências Biológicas (Zoologia), do Instituto de Biociências de Botucatu- Universidade Estadual Paulista, como parte dos requisitos para a obtenção do título de Doutora.

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a mulher mais forte que conheci.

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## **Apresentação**

Os ambientes de entremarés como manguezais e costões rochosos apresentam diversidade de organismos, grande quantidade de nutrientes e controles bióticos eficientes sendo importantes economicamente e ecologicamente (Christofolletti et al. 2010; Alongi 2015; Nie et al. 2016). Esses ambientes do entremarés são influenciados diariamente por fatores abióticos como salinidade, ação das ondas, da maré e temperatura (McLusky & Elliot, 2004; Burrows et al. 2008; Firth & Williams, 2009; Dong et al. 2015).

Dentre os fatores abióticos que atuam nos ambientes de entremarés, a temperatura tem sido extensivamente estudada, pois tem efeitos na fisiologia, comportamento e sobrevivência de organismos ectotérmicos, que têm suas funções vitais diretamente dependentes da temperatura ambiental (Helmuth et al. 2006; Paganini et al. 2014; Kordas et al. 2015; Helmuth et al. 2016). Além disso, estamos vivenciando um período de mudanças do clima e o aumento da temperatura é considerado um dos primeiros efeitos dessas mudanças (Cox et al. 2000; IPCC 2013). Dada a importância ecológica e econômica dos ambientes de entremarés e o atual contexto de aquecimento global, torna-se imperativo estudar os efeitos do aumento da temperatura nos organismos ectotérmicos que ocupam esses ambientes.

O aumento da temperatura acarreta consequências fisiológicas, pois aumenta a demanda metabólica de organismos ectotérmicos (Fusi et al. 2016). Ainda, enzimas e proteínas de membrana têm suas atividades diretamente relacionadas ao aumento da temperatura influenciando também nas respostas fisiológicas desses organismos (Angilletta Jr. 2009; Madeira et al. 2014). Dessa forma, organismos ectotérmicos apresentam respostas fisiológicas para mitigar ou cessar os efeitos de temperaturas elevadas (Allen et al. 2012; Madeira et al. 2014; Portner et al. 2017).

Além de consequências fisiológicas, o aumento da temperatura também tem consequências no comportamento alimentar desses organismos (Pellan et al. 2016). O aumento da demanda metabólica e o processo digestivo mais rápido em temperaturas elevadas fazem com que os organismos apresentem uma seleção diferencial de itens alimentares, de acordo com seus conteúdos energéticos (Griffen et al. 2015).

Neste trabalho, para avaliação das consequências fisiológicas e comportamentais do aumento da temperatura, escolhemos organismos reconhecidamente importantes para estruturação de comunidades de costões rochosos e manguezais. Entendemos que o aquecimento global afetando essas espécies, também afetará toda a comunidade desses ambientes. Para respostas fisiológicas e comportamentais ao aumento da temperatura de organismos de costões rochosos escolhemos o caranguejo *Pachygrapsus transversus* (Gibbes, 1850). Esta espécie é abundante em costões rochosos brasileiros e tem hábito alimentar onívoro, em que se alimenta de organismos autotróficos e tecido animal (Flores & Negreiros-Fransozo 1999). Sua presença influencia a diversidade e abundância de organismos em costões rochosos (Christofolletti et al. 2010). Para respostas fisiológicas de organismos de manguezais frente ao aumento da temperatura, escolhemos as espécies *Parasesarma bidens* (De Haan, 1835) (para nova classificação veja Shahdi & Schubart 2017) e *Metopograpsus frontalis* (Miers, 1880). Essas espécies são conhecidas por exercerem influência na cadeia alimentar de manguezais asiáticos. *Metopograpsus frontalis* é generalista, utiliza uma variedade de micro habitats, como manguezais e bancos de lama, e está, portanto, envolvido em várias cadeias alimentares (Poon et al. 2010). *Parasesarma bidens* consome principalmente folhas em decomposição e os fragmentos não digeridos retornam à superfície do sedimento quando liberam suas fezes (Lee, 1998). Essas duas espécies consomem materiais vegetais mortos, tornando-os importantes na cadeia alimentar detritívora, além de



consumir algas e sedimentos inorgânicos, que possuem alta densidade de bactérias e diatomáceas (Fratini et al. 2000; Skov & Hartnoll, 2002).

Baseando-se no exposto acima, a presente tese foi dividida em três capítulos: O **capítulo 1** foi realizado durante o período de doutorado sanduíche, em Hong Kong. Este capítulo utilizou como organismos modelo as espécies de caranguejos *Parasesarma bidens* e *Metopograpsus frontalis*. Essas espécies ocupam diferentes regiões no manguezal, com diferentes graus de exposição à temperatura. Assim, o objetivo desse capítulo foi avaliar os efeitos da temperatura nas respostas fisiológicas dessas espécies. O **capítulo 2** foi realizado com *P. transversus*, espécie de caranguejo abundante em costões rochosos brasileiros. Teve como objetivo avaliar respostas fisiológicas dessa espécie ao aumento da temperatura. Por fim, o **capítulo 3** foi realizado também com a espécie *P. transversus* e o objetivo foi avaliar a seleção de itens alimentares desse caranguejo quando exposto a diferentes temperaturas.

## Capítulo 1

### **Physiological performance of *Parasesarma bidens* and *Metopograpsus frontalis* at elevated temperatures**

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We intent to submit this manuscript to Marine Ecology Progress Series and it is in their formatting requirements. Further details see: <http://www.intres.com/journals/meps/meps-home/>

## ABSTRACT

Temperature is one of the main abiotic factors that affects the physiology of organisms in intertidal environments such as mangroves. This factor can vary in the vertical gradient with the presence or not of vegetation and, considering the current scenario, can vary with the climatic changes. Here our objective was to evaluate the effects of high temperatures on physiological parameters of crabs of the species *Parasesarma bidens* and *Metopograpsus frontalis*. *P. bidens* presented lower Arrhenius break temperature and higher desiccation rate. In the same way showed higher concentration of ions in hemolymph when exposed to longer periods at high temperatures and higher righting response time at longer periods. *M. frontalis*, on the other hand, was more stable in relation to these physiological parameters. Thus, *P. bidens* was more sensitive than *M. frontalis* to the increase in temperature. *P. bidens* lives in the upper mangrove areas, associated with vegetation and due to this is accustomed to milder temperatures and less time of exposure to desiccation. *M. frontalis* lives in the most exposed and devoid of vegetation, in addition to inhabiting the region closest to the water line. Therefore, we attribute our results to the different distribution of these organisms along the vertical gradient of the mangrove.

**Keywords:** climate change; heat stress; mangroves; Sesarmidae; Grapsidae

## INTRODUCTION

Temperature and its effects in physiology, behaviour and survival of organisms has been the goal of many works (Helmuth et al. 2006; Paganini et al. 2014; Kordas et al. 2015; Helmuth et al. 2016). Practically all vital processes of organisms are affected in some way by temperature, because its affect enzymes actions, cellular membrane structure and oxygen limitation (Angilletta Jr. 2006; Ohlberger 2013). Therefore, to evaluate the temperature effects it becomes important considering that is one of the first effects of the current climate change scenario (Cox et al. 2000; IPCC 2013). Among the organisms affected by temperature we have ectotherm intertidal organisms. They are susceptible to temperature variations because they have body temperatures controlled by the environmental temperature, yet they are exposed a seasonal and daily temperature changes, this fact influencing physiological parameters (Helmuth et al. 2016; Fusi et al. 2016; Portner et al. 2017).

These parameters such as cardiac performance (Dong & Williams 2011), hemolymph osmolality (Madeira et al. 2014), desiccation (Levinton et al. 2015) and righting response time (Nobbs & Blamires 2017) have been accurate responses for physiological analysis of the effect of temperature increase on intertidal organisms. When temperature exceeds the pejus temperature ( $T_p$ , limiting temperature for optimal oxygenation of hemolymph), there is a decrease in oxygen supply to aerobic respiration due to the limited capacity of the circulatory and ventilatory system to supply the oxygen demand for the organism (Dong & Williams 2011). Thus, cardiac performance and the determination of the Arrhenius break temperature (ABT) are sensitive parameters to estimate the critical temperatures of the organisms (Burnett et al. 2013; Dong et al. 2017). Hemolymph osmolality is an important factor to be evaluated as it measures the total amount of ions in the hemolymph. The control of these ions, to

maintain homeostasis, is also temperature dependent, since the transport of ions is made by proteins, which have their activities directly influenced by temperature (Angilletta Jr. 2009; Madeira et al. 2014). In the same way, righting response time is correlated with metabolic and thermal stress in an intertidal species, since the temperature increases ATP synthesis and this fact is correlated with decrease in responsiveness of the organisms by directing their energies to ATP production (Frederich et al. 2009). Another useful parameter to determine the vulnerability of organisms to temperature is the rate of water loss of organisms over time, simulating when exposed to high temperatures at low tide and being one of the most limiting physical factors for intertidal species (Levinton et al. 2015).

Cardiac performance, hemolymph osmolality, desiccation and righting response time in organisms are influenced by temperature in different ways depending on the vertical distribution of the species in an intertidal environment (Chappuis et al. 2014). One fact that influences is the presence of the vegetation cover along the vertical gradient. Species living in regions with vegetative cover maintains milder internal temperatures and, consequently, they have less thermal stress than species living in exposed regions (Nobbs & Blamires 2017).

Considering these aspects, in this work we studied ectothermal organisms from mangrove. In Hong Kong mangroves we found the crabs species *Metopograpsus frontalis* Mier (Grapsidae) and *Parasesarma bidens* De Haan (Sesarmidae). They present different distributions, being *M. frontalis* found in mangrove fringes and boulder areas and *P. bidens* in interior of the mangroves (Lee 1998; Poon et al. 2010). These species are known to exert influence on the mangrove food chain. *M. frontalis* is a generalist, uses a range of microhabitats such as mangrove and mudflats and is therefore involved in several food chains (Poon et al. 2010). *Parasesarma bidens* mostly

consumes decomposing leaves and undigested fragments often return to the surface of the sediment when they release their feces (Lee 1998). Thus, they have the possibility to re-ingest this partially degraded food increasing the efficiency of the detritivorous chain (Skov & Hartnoll 2002). These two species consume dead plant materials, making them important in the detritus feed chain, besides consuming algae and inorganic sediment, which has high density of bacteria and diatoms (Fratini et al. 2000, Skov & Hartnoll 2002). Thus, *M. frontalis* and *P. bidens* can regulate epifauna and meiofauna colonization as well as the production of microphytobenthos (Poon et al. 2010). Considering that the species in question occupy different regions in the mangrove, our hypothesis is that *M. frontalis*, living in more exposed regions, is more resistant to high temperatures. Otherwise, *P. bidens*, living in more sheltered regions, is more vulnerable to rising temperatures. Therefore, considering that these species of crabs have great ecological importance and live in different regions in the mangroves with different degrees of exposure to temperature, our objective was to evaluate the physiological performance that both present to the increase in temperature.

## MATERIAL AND METHODS

### *Sample collection and model species*

*Parasesarma bidens* and *Metopograpsus frontalis* were collected in Three Fathoms Cove, located in the eastern coast of Hong Kong (Fig. 1). Crabs were collected by hand during low tides and the carapace width for *Metopograpsus frontalis* was 19-25 mm and for *Parasesarma bidens* was 15-20 mm. These sizes corresponding the dominant adult sizes for these species (Poon et al. 2004). After collected, crabs were maintained separated by species, in aquariums containing sediment, food, sea water and rocks for to simulate their natural environment.



**Figure 1-** Hong Kong. Local of collection of organisms: Three Fathoms Cove

#### *Heart beat analysis*

The individuals of *P. bidens* (CW:  $18.52 \pm 2.74$  mm) and *M. frontalis* (CW:  $21.92 \pm 1.29$  mm) underwent temperature stress when they were placed in a water bath. The equipment started with a minimum temperature of 25 °C, and then increase by an average of  $3^{\circ}\text{C h}^{-1}$ , average temperature rise in Hong Kong mangroves, reaching a maximum temperature of 50°C. The organisms were placed in containers that had an opening in the lid for the output of the heart rate sensor. Heart rate sensors were fixed on the dorsal region (pericardial cavity) of the crabs species and temperature sensors, connected in a thermometer (Lutron TM-946) were fixed on gill chamber. Then the organisms were put into contact (in air) with the water in the water bath (Grant GP200, UK) so that they experienced the rising air temperatures in a controlled manner. The variation of the heartbeat was transmitted through the sensors and the reading taken by the computer program PicoScope 6 (see Burnett et al. 2013). This methodology detected if there was variation in heartbeat of organisms with increasing temperature (see Williams et al. 2011). Data were analysed to determine changes in the heart frequency

and break points (ABT) in cardiac function to determine the thermal performance of the species.

#### *Dehydration experiments, righting response time and hemolymph osmolality*

##### Before experiments

Individuals males and females of *P. bidens* (CW:  $17.89 \pm 2.02$  mm; n = 45) and *M. frontalis* (CW:  $22.61 \pm 2.14$  mm; n = 45) were kept individually and fasting in containers with sea water for 48 hours. Thus, they can release their feces, with this factor do not influencing in weight (for dehydration experiment) and they remained hydrated.

##### Dehydration experiments

Before the experiment, organisms were put an aquarium with absorbent tissue for one hour for we do not overestimate the water loss. The experiments were carry on an air circulation oven, in three different experimental temperatures (Hong Kong observatory): 26 °C (average daily over the 10-year period in Hong Kong), 30 °C (intermediate temperature between 26 °C and 35 °C) and 35 °C (average daily maximum in the 10-year period in Hong Kong) and the relative humidity in an oven ranged to 30-60%. The water loss rate was measured by weight loss (Levinton et al. 2015) of organisms in each 30 minutes over 2 hours (0 minutes, 30 minutes, 60 minutes and 120 minutes).

##### Righting response time and hemolymph osmolality

With the same crabs, after weighting them, we did righting response time experiment. We put crabs with dorsal side upward in a trail and calculated, with stopwatch, how long they took to return to their normal position (Nobbs & Blamires 2017a). Then, we withdraw hemolymph in a last pair of pereopods using a syringe. Hemolymph samples were frozen in -4 °C for posterior osmolality analysis.



Before analysis, hemolymph samples were thawed at room temperature, then put in centrifuge (rotation of 4000 rpm per 10 minutes). The osmolality was measured using osmometer (Vapor pressure osmometer, Vapro® 5520) and we obtained values in milliosmols per kilogram (mOsm  $\text{k}^{-1}$ ).

### *Data Analysis*

#### Heart rate measurements

Data for cardiac performance curves for both species were fit with Beta equation (see Shi et al. 2015). Differences in ABT between individuals of the two species were calculated using nonlinear regression model.

#### Desiccation, Osmolality and righting response time

To test the effects of temperature and time in desiccation, osmolality and righting response time we first tested homoscedasticity of the data using Levene's test. When data were not homoscedastic, we made the LOG ( $X + 1$ ) transformation for ANOVA assumptions. Analysis of variance (ANOVA) was used, comparing fixed factors temperature (3 levels: 26 °C, 30 °C and 35 °C) and time (5 levels: 0, 30, 60, 90 and 120 minutes) and LSD *post hoc* analysis for factors that were significant.

We considered statistical significance when  $P < 0.05$ .

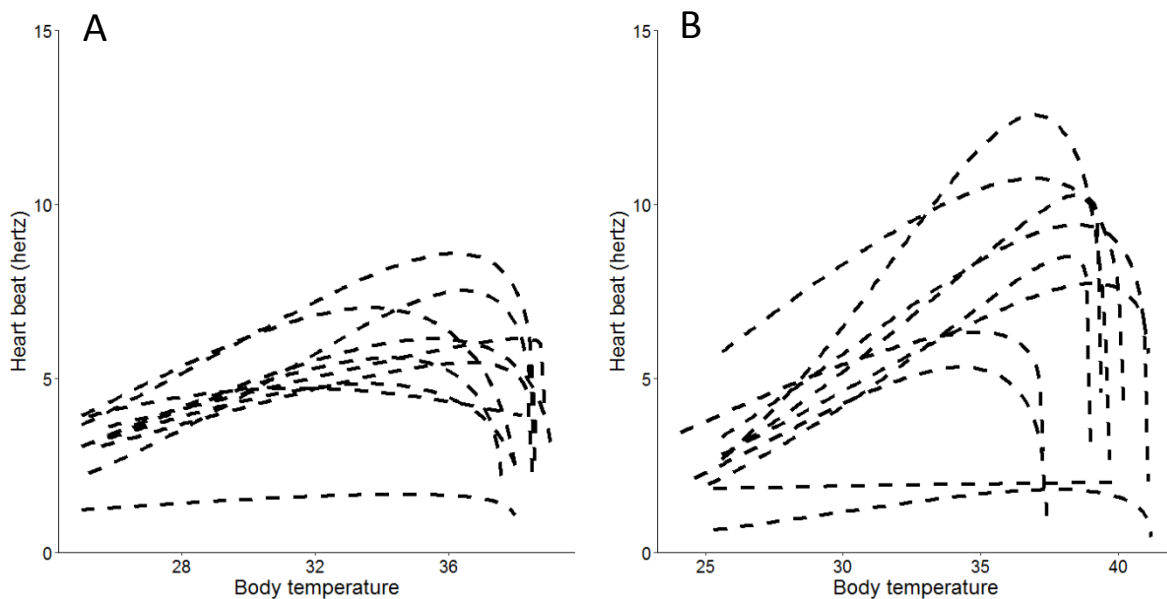
## **RESULTS**

*Parasesarma bidens* and *Metopograpsus frontalis* showed a significant difference in a physiological performance when subjected to high temperatures. First, the heart rate

curves will be shown, where we can know which ABT for the species in question and secondly the data of desiccation, osmolality and response time.

a) Heart beat performances

Individuals of *P. bidens* showed a consistent pattern of heart rate curves, with a mean thermal resistance of approximately 36 °C (ABT mean =  $36.29 \pm 0.918271$  °C) (Fig. 2A) and died at the mean temperature of  $38.35 \pm 0.46$ °C. Individuals of *M. frontalis* presented more variable heartbeats curves, which can be noticed by the different amplitudes of the curves for the different individuals and presented thermal resistance of approximately 38 °C (ABT mean =  $37.92 \pm 1.1612$  °C) (Fig. 2B ) and died at the mean temperature of  $39, 63 \pm 1.40$  °C.



**Figure 2-** Cardiac performance curves for (A) *Parasesarma bidens* and (B) *Metopograpsus frontalis*.

b) Dehydration

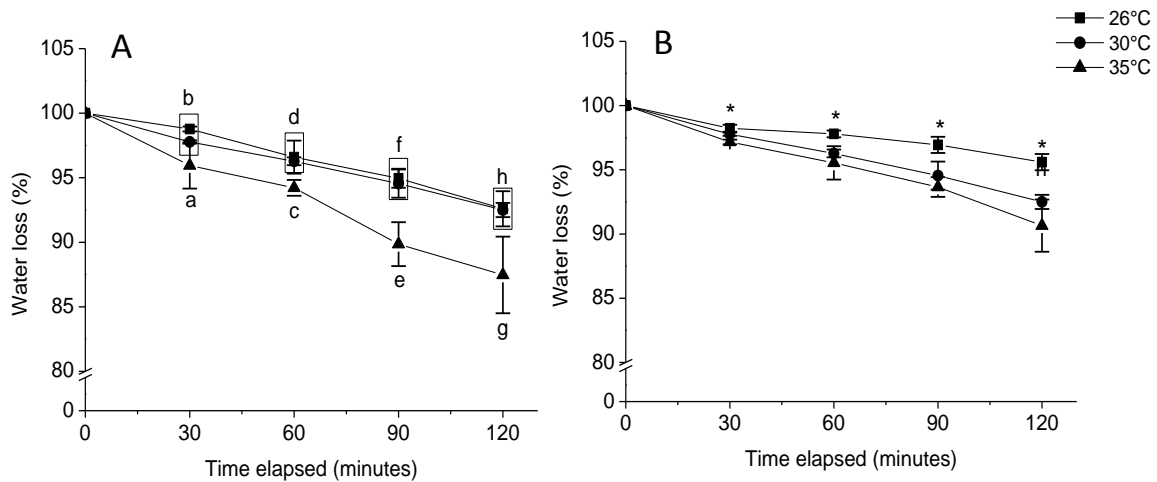
First, we tested the humidity in different temperatures to evaluate if would have significant differences among temperatures. There was no significant differences of humidity among temperatures (One-way ANOVA,  $F_{2,12} = 2.31$ ;  $P > 0.05$ ) (Table 1).

**Table 1-** The humidity of circulation oven in experimental temperatures. One-way ANOVA ( $P > 0.05$ ).

Treatment	df	MS	F	P
Temperature	2	111.27	2.3164	0.14
Error	12	48.03		

For dehydration, temperature (Factorial ANOVA  $F_{2,24} = 35.362$ ,  $P < 0.05$ ) and time (Factorial ANOVA  $F_{3,24} = 58.753$ ;  $P < 0.05$ ) influenced the desiccation of *P. bidens*. There were no significant differences in desiccation between temperatures of 26 °C and 30 °C, but there were differences between all the different times at these temperatures, showing that organisms lose more water over time independent of temperature. The organisms exposed to the temperature of 35 °C lost more water at all times when compared to the organisms of the treatments of 26 °C and 30 °C independent of the time elapsed of these treatments (Fig. 3A).

For *M. frontalis*, the factors temperature (Factorial ANOVA  $F_{2,24} = 44.4$ ,  $P < 0.05$ ) and time (Factorial ANOVA  $F_{3,24} = 65.6$ ;  $P < 0.05$ ) influenced the desiccation of the individuals. For this species there were differences between all temperatures and times. Organisms lost more water at higher temperatures and during the time they were exposed to desiccation (Fig. 3B).

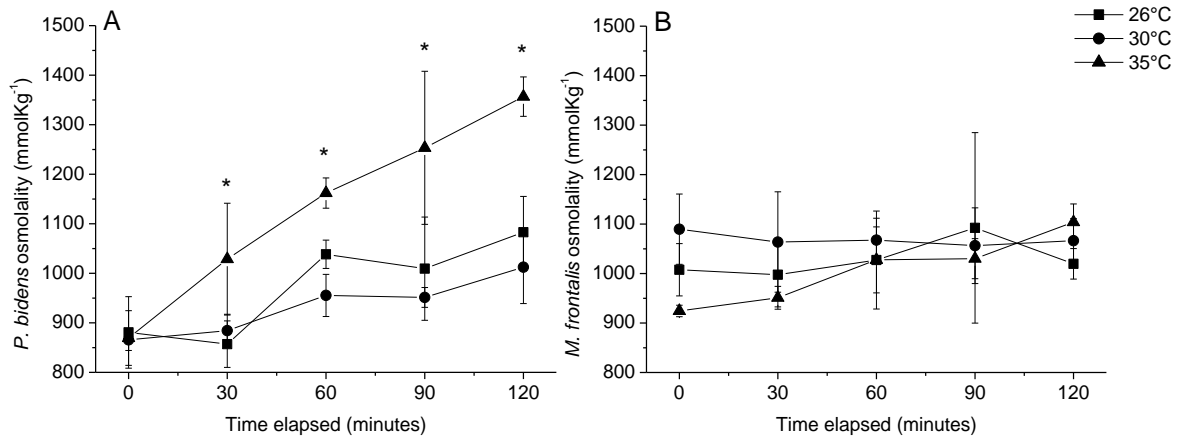


**Figure 3-** Water loss of the two species over time. (A) *Parasesarma bidens* water loss; (B) *Metopograpsus frontalis* water loss. Error bars indicates standard deviation, boxes indicates similarity between temperatures, different letters indicates statistical differences between temperatures and time ( $P<0.05$ ) and asterisks indicates all temperatures and times differed ( $P<0.05$ ).

### c) Osmolality

For *P. bidens* there was a significant difference between the experimental temperatures (Factorial ANOVA  $F_{2,30} = 33.39$ ;  $P < 0.05$ ), with an increase in the osmolality of the organisms at temperature of 35 °C and in organisms with a longer time of exposure to desiccation (Factorial ANOVA  $F_{4,30} = 23.11$ ;  $P < 0.05$ ) (Fig. 4A).

Individuals of *M. frontalis* showed no difference in hemolymph osmolality at temperatures of 26 °C, 30 °C and 35 °C (Factorial ANOVA  $F_{2,30} = 2.8$ ;  $P > 0.05$ ) nor did they differ between the different times (Factorial ANOVA  $F_{4,30} = 1.4$ ,  $P > 0.05$ ) (Fig. 4B).

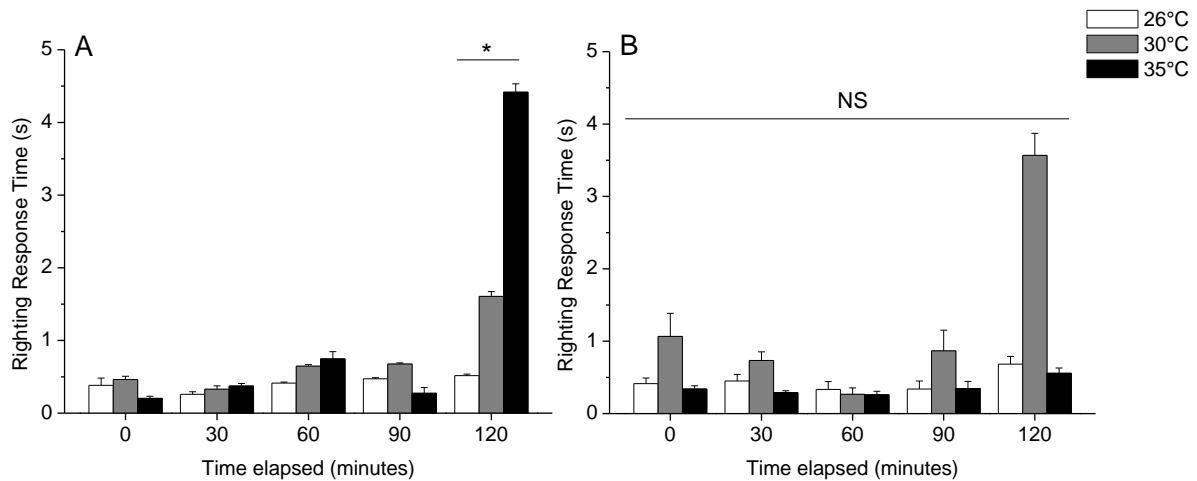


**Figure 4-** The relationship among hemolymph osmolality, time elapsed and temperature between crab species. (A) *Parasesarma bidens* hemolymph osmolality, (B) *Metopograpsus frontalis* hemolymph osmolality. Error bars indicates standard deviation and asteriks indicates significant difference among temperatures in the same time ( $P < 0.05$ ).

#### d) Righting response time

For *P. bidens* in a righting response time, there was a significant difference among time, especially in 120 minutes (Factorial ANOVA  $F_{4,30}=4.93$ ,  $P < 0.05$ ). Individuals of *P. bidens* took much longer time to return to their normal positions at 120 minutes of experiment (Fig. 5A) independent of temperature.

Organisms of *M. frontalis* did not show difference about righting response time at different temperatures (Factorial ANOVA  $F_{2,30}=1.13$ ,  $P > 0.05$ ) and experimental times ( $F_{4,30}=1.10$ ,  $P > 0.05$ ) (Fig. 5B).



**Figure 5-** Righting response time depending on temperature and time elapsed for *Parasesarma bidens* (A) and *Metopograpsus frontalis* (B). Error bars indicates standard errors, asterisk indicates statistical difference among elapsed time ( $P < 0.05$ ) and NS means not significant statistic difference between elapsed time and temperatures.

## DISCUSSION

*Parasesarma bidens* and *Metopograpsus frontalis* showed a significant difference in a physiological performance when subjected to high temperatures. The differences found are related to the physiological limitations of the species that are tied to the different areas that the crabs occupy in mangrove.

The heart beat experiment shows that there is a variation of the heart beat at high temperatures in *P. bidens* and *M. frontalis*. We used thermal performance curves relating the frequency of heart rate with increasing temperature. *P. bidens* showed to be more sensitive to temperature increase with lower ABT and lethal temperature than *M. frontalis*. Our findings are in agreement with the distribution of these organisms seen in the field. *P. bidens* is found in more sheltered regions, where mangrove vegetation is present and little exposed to the direct action of elevated temperatures (Lee 1998; Fratini et al. 2000; Poon et al. 2010). This environment is more vegetated, with milder temperatures and not exposing the crab to prolonged periods of high temperatures and

direct dehydration (Poon et al. 2010; Marshall et al. 2015). *M. frontalis* is found in the most exposed areas of the intertidal environment, which allows the selection of organisms more resistant to temperature and are also close to the water line, reducing the time of exposure to high temperatures (Lee 1998; Fratini et al. 2000; Poon et al. 2010) because when tide rises first reaches the region where *M. frontalis* inhabits.

We found differences between the two species in resistance to dehydration as well. Our data show that *P. bidens* lost more water over time at a temperature of 35 °C. From this result we suggest that besides of area that they occupy influencing their physiological responses, *P. bidens* avoids long periods of exposure to high temperatures because it may require excess energy to maintain metabolic activities that is higher in elevated temperatures (Fusi et al. 2015). We can infer this also based on heart rate data where 35 °C is a temperature close to the limiting temperature for *P. bidens*. For *M. frontalis* dehydration, the results showed significant differences in water loss over time at all temperatures tested. This species, living closer to the waterline, has shorter periods of exposure to desiccation (Vermeiren et al. 2015). And because it lives close to the water line, it usually feeds on moist sediments, recovering lost water during the periods of exposure (Poon et al. 2010; Vermeiren et al. 2015).

The hemolymph osmolality experiment showed variation for *P. bidens*, but *M. frontalis* presented stable osmolality at all temperatures and over time. The environmental temperature influences osmoregulation in crustaceans (Dobrzycka-Kraheil et al. 2014). The different capacities of intertidal organisms to regulate and support abiotic stress conditions such as temperature and desiccation are directly linked to their different thermal tolerances and vertical distributions in these environments (Wolcott 1973). *P. bidens* showed a significant increase in hemolymph osmolality at 35 °C at all times considered indicates that this specie was most sensitive at that temperature which is close

to its ABT temperature (approximately 36 °C). Other studies also showed that prolonged periods of exposure to desiccation lead to large water loss in upper intertidal organisms and, thereby, increase the osmotic concentration of body fluids (Williams et al. 2011; Wong et al. 2014). Moreover, the increase in osmolality at 35 °C in *P. bidens* may be associated with a compensatory mechanism of  $\text{Na}^+ / \text{K}^+$  atpase, the most important enzyme in the regulation of ions in crustaceans (Henry et al. 2012), where this enzyme may have worked at its maximum capacity to osmoregulate, since the organism was close to its limiting temperature (36 °C); thus also increasing osmolality. *M. frontalis* showed no increase in hemolymph osmolality at any of the experimental temperatures and times corroborating, once again, with the results found for its ABT. The temperatures used in the experiment to evaluate the osmolality were not enough limiting for this species, since it has a limiting temperature of around 38 °C. In addition, we suggest that the water loss from *M. frontalis* is not sufficient to cause increased osmolality (Williams et al. 2011). From this fact, we also verified that the process of osmoregulation besides depending on the environmental conditions can be specie-specific (Romano & Zeng 2012).

For the righting response time, we verified that only *P. bidens* showed sensitivity to the time that was exposed during the experiment. This specie when placed with the dorsal side of the carapace down, took longer time to return to its normal position and *M. frontalis*, on the other hand, showed no difference in its response time. Our results corroborate with the results found for *Tubuca flamulla* and *Tubuca elegans* (Nobbs & Blamires 2017). Crabs living in more sheltered regions, when placed in more exposed regions, with higher temperatures, present greater righting response time than those that live naturally in exposed regions (Nobbs & Blamires 2017). Besides that our results show that *P. bidens* is more sensitive to high temperatures and dehydration than *M. frontalis*. Thus, we suggest that exposure to these stressful conditions is more energy costly for *P.*



*bidens* and this fact reflects in their higher righting response time, since the higher demand for ATP production decreases responsiveness (Frederich et al. 2009).

In summary, we conducted a series of experiments that allowed us to evaluate the influence of temperature on the physiological performance of intertidal organisms. These crabs species perform important functions and they are distributed vertically in different regions in mangroves. From our results, we can infer that the temperature influences physiological parameters of both species as heart rate, dehydration, hemolymph osmolality and righting response time. Yet, we suggest that the differential distribution of *P. bidens* and *M. frontalis* in a vertical gradient in this environment is important and influences directly in a physiological responses.

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## Capítulo 2

### **Temperature affects *Pachygrapsus transversus* physiology: a global warming perspective**

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## ABSTRACT

Ectothermal intertidal organisms are exposed daily to tidal variation and temperature and the consequences of global warming tend to be drastic for these organisms, living in this environment with varying conditions. Here we evaluated physiological responses of *Pachygrapsus transversus* in different temperatures, submersion and emersion condition. Oxygen consumption and ammonia concentration were higher at 35 °C in the submersion condition. In emersion condition, *P. transversus* presented a lower index of the perigastric organ and lower concentration of glucose in hemolymph at 35 °C. For dehydration responses they showed great water loss at the highest temperatures and over time and water loss was higher for small crabs. Thus, *P. transversus* presented physiological responses for all parameters evaluated. Variations in environmental conditions such as tide and temperature increase the metabolic demand and the use of energy reserves of *P. transversus*.

**Keywords:** physiology performance; rocky shore; climate change

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## INTRODUCTION

Ectothermic intertidal organisms are insert in environments with daily strong physical variations (Williams et al. 2008; Dong et al. 2015; Pörtner et al. 2017). In response, these organisms developed mechanisms to mitigate the extreme effects of daily physical fluctuation. Thus, these organisms are extensively used in physiological and behavioral studies as models (Marshall et al. 2011; Fusi et al. 2016). Among the physical conditions that vary daily and influence the physiological responses of these organisms are tide and temperature. Both abiotic factors varies throughout the day with periods of low and high tides and acts differently depending of zonation in the intertidal environment (Chappuis et al. 2014). The effects of temperature can become more dramatic as we are experiencing global warming. The average air temperature is expected to rise by as much as 4.8 °C by the end of the 21st century (IPCC 2013).

Species of crustaceans are among the intertidal organisms that undergo the influence of tide and temperature (Flores et al. 2007; Fusi et al. 2015; Levinton et al. 2015). These animals can show differential patterns of water loss as physiological response. They may lose more or less water depending on the tide, where dehydration is greatest in periods of emersion and these organisms are exposed to higher temperatures (Allen et al. 2012).

The exposure to high temperatures and tidal variations also has the consequence of metabolic increase in crustaceans (Madeira et al. 2014; Fusi et al. 2016). Thus, these organisms demand higher production of ATP (Adenosine triphosphate) to obtain energy for vital functions (Fusi et al. 2016). Among physiological process involved in ATP production oxygen consumption and excretion of ammonia is important indexes. Oxygen consumption provides energy for the formation of ATP in a respiration process (Fusi et al. 2016) and excretion of ammonia is result of the amino acid deamination



process, which generates more ATP (Ganser et al. 2015; Nie et al. 2017). The concentration of glucose in the hemolymph is also a useful index for the increase in an energy demand because glucose is the main energy substrate, thus is fundamental in ATP production (Lorenzon et al. 2007). In crustaceans the energy reserve is the perigastric organ (see new classification in Cervellione et al. 2017). The energy reserve is spent to supply higher metabolic demand and support ATP production (Vinagre & Chung 2016), then the perigastric organ index may be informative about physiological responses in stress situations.

Considering the above context, in this work we study physiological responses to temperature increase in the periods of emersion and submersion of the crab *Pachygrapsus transversus* (Gibbes, 1850). It is an intertidal ectothermal organism abundant in brazilian rocky shores, have omnivorous feeding habits and is important in community structure because their presence influence the diversity and abundance of organisms in rocky shores (Flores & Negreiros-Fransozo 1999; Christofolletti et al. 2010). Our hypotheses are that both temperature and immersion and submersion influence the physiological responses of *P. transversus*. Thus, the aim of present study is to test physiological responses as oxygen consumption and ammonia concentration considering the period of submersion of the organisms. And, considering the period of emersion, we tested glucose concentration in hemolymph, perigastric organ index and water loss rate.

## **MATERIALS AND METHODS**

### *Study area, organism collection and experimental temperatures*

In order to determine the sizes of *P. transversus* that would be used in this work, we sampled with the objective of obtaining the population structure of these organisms

in Baixada Santista. Individuals were sampled manually in low tide at night when the crabs are more active (Arab et al. 2015), at Itanhaém (24° 10 '59 "S 46° 47' 20" W), São Vicente (23° 57 '47 "S 46° 23' 31" W) and Guarujá (23° 59'35 "S 46 ° 15'23" W) in May 2016, being the result of two sampling per locality in one week (N = 2 per locality;  $\Sigma$  of sampling = 6). All the organisms found were collected by the capture effort method and had the sex identified and the carapace width measured. After identification and measurement, the organisms were released in the same rocky shore that were captured.

For the dehydration experiment the organisms were sampled in São Vicente. We obtained 15 small crabs (carapace width ranging from 5.1 to 7 mm) and 15 large crabs (carapace width ranging from 13.1 to 15 mm). We choose these sizes, based on population structure data (Figures 1 and 2) because the size class from 5.1 to 7 mm was the most abundant among the organisms considered juveniles (small) and the size class 13.1 to 15 mm the most abundant among organisms considered adult (large) (see figure 2). All the organisms used were intact with all pereopods and chelipods.

For the physiological experiments (oxygen consumption, perigastric organ index, glucose and ammonia concentration) the collection was performed in Itanhaém. The crabs had carapace widths ranging between 13.1 and 17 mm. We collected only males aiming to eliminate the gender variable for these responses.

Experiments that follow were done at air temperatures of 24 °C, 35 °C and 40 °C. The air temperatures used were established based on data from SIMCOSTA (Brazilian Coast Monitoring System) and included a mean of temperatures obtained from September 2014 to March 2016 for São Paulo State. The temperature of 24 °C is the average temperature of the air temperature, 35 °C is the highest temperature recorded in this period and the temperature of 40 °C was determined by considering the

temperature of 35 °C (highest temperature found in the data set) and adding 5 °C, the worst scenario of IPCC by the end of this century (IPCC 2013).

### *Physiological parameters*

To evaluate the physiological parameters such as oxygen consumption, glucose concentration in hemolymph, perigastric organ index and ammonia concentration, organisms (N = 12 for each temperature) were submitted to the three experimental temperatures of 24 °C, 35 °C and 40 °C (fixed, 3 levels).

To obtain these responses, the crabs were allowed to acclimate for 72 hours without tide simulation, at 24 °C and mussels were offered as food during acclimatization. After acclimatization, the crabs were arranged in aquariums 30x12x20 cm, with hide of 2.5 cm diameter, marble slabs with 12x9 cm approximately and sea water. The hiding place and the marble slabs allowed the organism not to be in contact with the water during the whole experiment time. The aquariums with the organisms were then put in germination chambers (FANEM Mod. 347 CDG) to control experimental temperatures. The experiment lasted for 72 hours and throughout that time the crabs were constantly aerated and fragments of mussels were offered as food (approximately 0.050 grams) alternately. Only in the 24 hours before the end of the experiment the organisms were fasted so that the feed did not interfere with the physiological responses. In this experiment we did not simulate tide, because the tide simulation would interfere with temperature, our predictor variable.

The experiments of physiological responses that follow were performed only with crabs exposed to temperatures of 24 °C and 35 °C, as all crabs exposed at 40 °C in germination chambers died with 24 hours of experiment.

### *High tide responses*

The experiments carried out to evaluate the physiological responses of *P. transversus* when high tide were oxygen consumption and excretion of ammonia. For these experiments, we used respirometric chambers filled with sea water. They were then placed in a water bath at temperatures of 24 °C and 35 °C so that the temperature of the chambers was homogenized and then we put the crabs inside of chambers that experienced oxygen consumption under water, as in high tide. First, they were left acclimatizing for 30 minutes with aeration, after acclimatization we measured the initial oxygen concentration with oximeter (YSI mod.53). After removal of the aeration, the chambers were closed and the crabs remained in chambers for 1 hour. After 1 hour, we measured again the oxygen concentration (Augusto & Masui 2014). The oxygen consumption is measured by the difference in initial and final oxygen concentration in the chamber (Augusto & Masui 2014).

For ammonia testing, we removed water samples from the respirometric chambers of each individual and samples were frozen for further analysis. The concentration of ammonia was determined by calorimetry (Koroleff, 1983), where the samples were read at 630 nm in a spectrophotometer.

#### *Low tide responses*

After measuring oxygen consumption and ammonia concentration, using the same crabs, approximately 20 µL of hemolymph was withdrawn with a syringe from the last pair of pereopods of the crabs and then these samples were frozen for further analysis of the glucose concentration in hemolymph. To evaluate glucose concentration, the samples were thawed at room temperature and we used the glucose oxidation procedure using a commercial reagent kit (Glicose Liquiform Ref. 133,

LabTestDiagnóstica, Brazil). The samples were then read at 505 nm in a spectrophotometer.

After removal of hemolymph, the organisms were euthanized by low temperature exposure (- 4 °C). To obtain the perigastric organ index, we dissected the crabs and removed the perigastric organ. This organ and the bodies of the crabs were weighed to obtain the wet weight. They were then placed in an air circulation oven for 48 hours at 60°C and weighed again to obtain the dry weight. The formula of the calculation of perigastric organ index:  $(W_{do}/W_{db}) \times 100$ , where  $W_{do}$  is dry weight of organ and  $W_{db}$  is dry weight of body.

#### *Dehydration experiment*

For this experiment, we verified water loss of small crabs and large crabs by weight loss at three different temperatures (24 °C, 35 °C, 40 °C, 3 levels fixed) over time (0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes, 9 levels fixed).

Before the start of the experiment the organisms were left individually and fasted for 48 hours in containers containing only sea water to eliminate their feces and then the stomach contents would not influence the weight and they would remain hydrated. After acclimatization, the organisms were placed in aquariums with absorbent paper for one hour, so they could stay dry in the experiment and there was no overestimation of their weight losses in the initial minutes of the experiment.

Then the organisms were again individualized in enumerated plastic containers and we measured the initial weights of all. After this procedure, the organisms were placed in an air circulation oven where we controlled the experimental temperatures. Every 15 minutes the organisms were removed and weighed for to check the weight loss, which would indicate the loss of water from the crabs (Levinton et al. 2015).

### *Statistical analyses*

For distribution of size classes we used descriptive statistic to obtain the size classes and the most common size class in population.

For dehydration and physiological responses data, we first tested the data for homoscedasticity using the Levene's test. Once confirmed the homoscedasticity, for dehydration experiment we used repeated measures ANOVA where our fixed factors were temperature (3 levels: 24 °C, 35 °C and 40 °C), size (2 levels: large and small;) and time (8 levels: 15, 30, 45, 60, 75, 90, 105, 120). We used post-hoc Fisher LSD test and considered statistical significance when  $p < 0.05$ .

To evaluate oxygen consumption, perigastric organ index, glucose and ammonia concentration at temperatures of 24 °C and 35 °C, we used t- student test for homocedastic data and Mann-Whitney for non-homocedastic data. We considered statistical significant when  $p < 0.05$ .

### *Ethical note*

This study was performed according to Brazilian law where we had license to collect the organisms from Authorization and Information System in Biodiversity (SISBio; protocol number 47474).

## **RESULTS**

### *Population structure of *Pachygrapsus transversus* in Baixada Santista*

We present the histograms for each locality in which the organisms were collected (Figure 1) and then the histogram encompassing the three localities (Figure 2). We did a descriptive analysis of the data from collection in Baixada Santista, where a

total of 1943 animals were examined, and the most common size (mode) were 13.1 mm carapace width. The collected organisms ranged between 3.1 and 23 mm of carapace width (Figure 2). The most abundant size class of large organisms was 13.1 to 15 mm carapace width (Figure 2). Among the organisms considered the minor the sizes varied from 3.1 to 7 mm in carapace width.

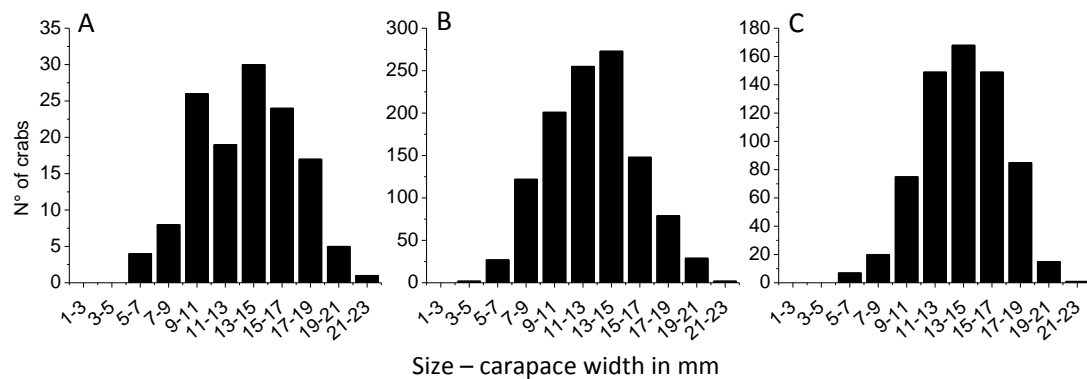


Figure 1: Histogram with size classes (carapace width in mm) of *Pachygrapsus transversus* in (A) São Vicente; (B) Itanhaém and (C) Guarujá.

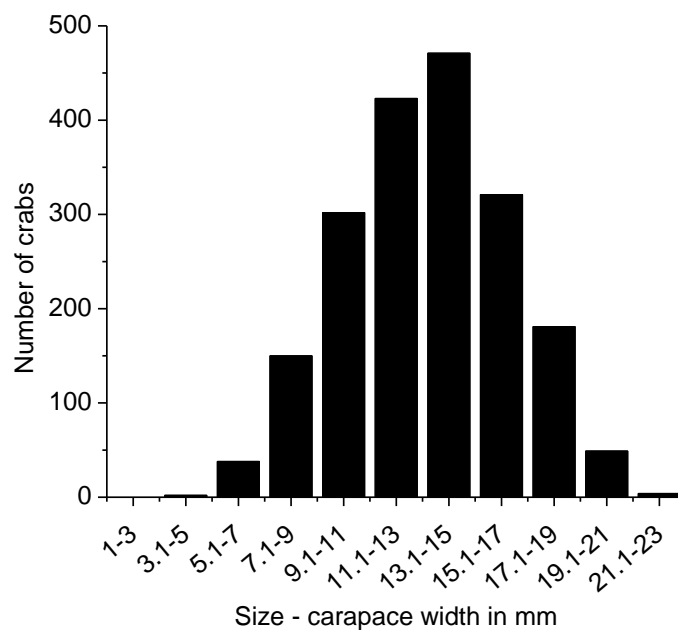


Figure 2: Histogram with size classes (carapace width in mm) of *Pachygrapsus transversus* considering three populations: Guarujá, Itanhaém and São Vicente.

### *Survival*

For physiological responses (oxygen consumption, ammonia concentration, glucose and perigastric organ index), where organisms were exposed to experimental conditions for 72 hours, all organisms exposed at 40 °C died with 24 hours of experimentation.

### *High tide*

#### *Oxygen consumption and ammonia concentration*

*P. transversus* has been shown to be sensitive to high temperatures at high tide showing a higher rate of oxygen consumption at 35 °C, when comparing consumption at different temperatures (Figure 3, t-test = -2.1, df = 22, p = 0.04). The same pattern was observed for ammonia concentration that increased significantly in 35 °C (Figure 4, U = 24, Z = -2.77, p = 0.005).

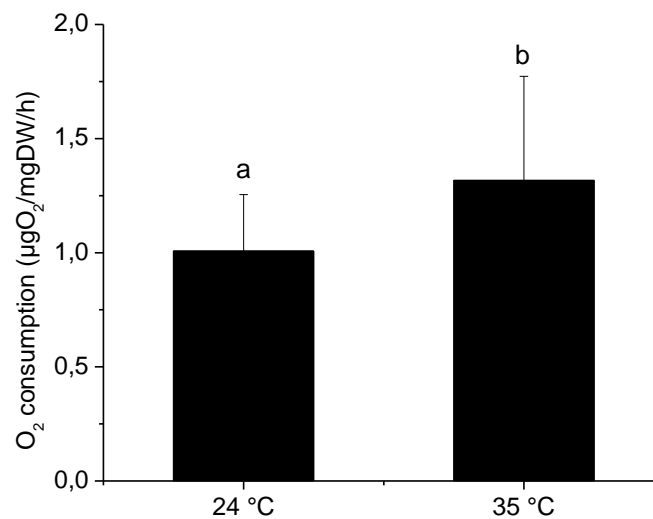


Figure 3: Oxygen consumption of *Pachygrapsus transversus* in 24 °C and 35 °C. Error bars indicates standard deviation and different letters means statistical difference (p < 0.05).



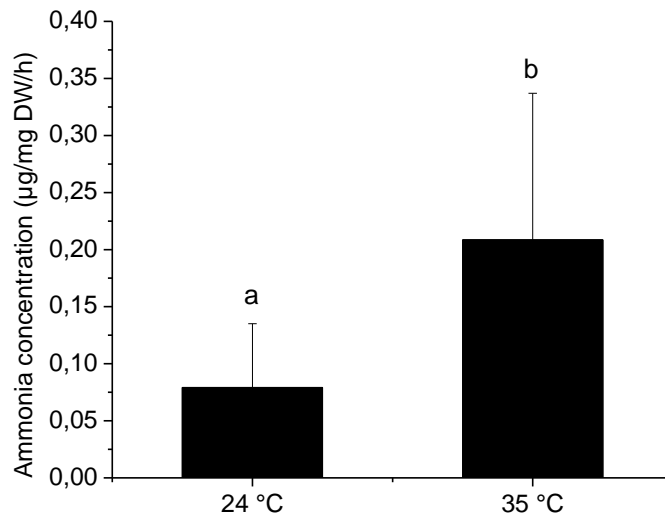


Figure 4: Ammonia concentration of *Pachygrapsus transversus* in 24 °C and 35 °C. Error bars indicates standard deviation and different letters means statistical difference ( $p < 0.05$ ).

#### *Low tide*

#### *Glucose concentration, perigastric organ index and dehydration experiment*

High temperature in the low tide also proved to be stressful for *P. transversus*. Organisms showed a higher concentration of glucose in the hemolymph at 24 °C than at 35 °C (Figure 5, t-test = 2.84, df = 22,  $p = 0.009$ ). The same pattern was observed for perigastric organ index, that also showed a difference where it was higher at 24 °C (Figure 6, t-test = 3.49, df = 22,  $p = 0.002$ ).

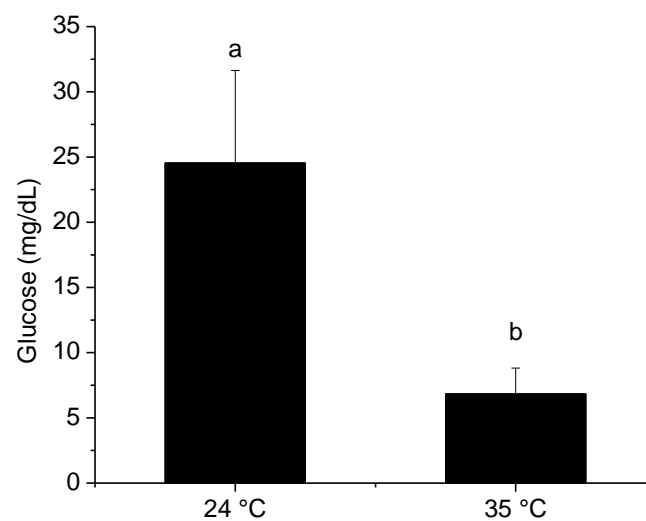


Figure 5: Glucose concentration in hemolymph of *Pachygrapsus transversus* in 24 °C and 35°C. Error bars indicates standard error and different letters means statistical difference ( $p < 0.05$ ).

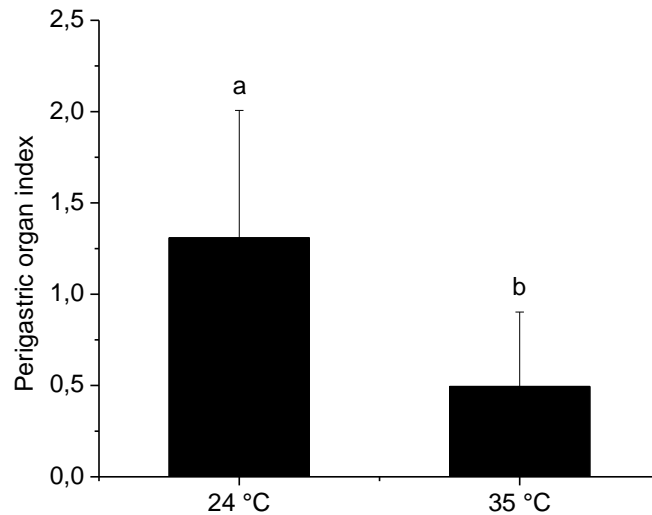


Figure 6: Perigastric organ index of *Pachygrapsus transversus* in 24 °C and 35 °C. Error bars indicates standard deviation and different letters means statistical difference ( $p < 0.05$ ).

For results of dehydration experiments (Figures 7A and 7B) we obtained a significant interaction among the factors temperature, size and time (ANOVA repeated measures:  $F_{14,588} = 4.66$ ;  $p < 0.0001$ ). Small crabs showed greater water loss at all temperatures and over time when compared to large crabs (Figure 7A and 7B). Large crabs presented significant water loss only at 40 °C after 30 minutes and when compared to the 24 °C temperature. For the small crabs there was significant water loss over time when all temperatures were compared.

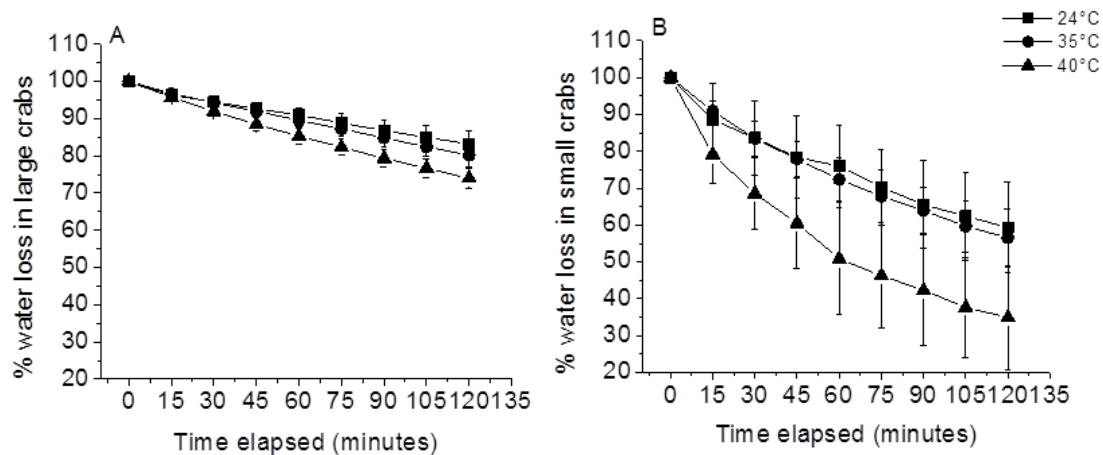


Figure 7: Pattern of water loss in large (A) and small (B) *Pachygrapsus transversus* over time in 24°C, 35°C and 40°C. Error bars indicates standard deviation.

## DISCUSSION

Our results demonstrated that exposure to high temperature and to dehydration influenced the physiological responses. When *P. transversus* are immersed there is an increase in oxygen consumption and ammonia excretion. When emerging, elevated temperature provides decreased glucose concentration and perigastric organ content, besides increased water loss over time of exposure.

As intertidal organism, *P. transversus* is subject to the temperature and tide variation. The physiological responses of intertidal organisms when submerged are different from when they are emerged (Bjelde and Todgham 2013). Considering the time of submersion, our results for oxygen consumption corroborates with that found for *Pachygrapsus marmoratus* in which it also increases its oxygen uptake when under water at high temperature (Fusi et al. 2016). Thus, the metabolism of *P. transversus* in water is temperature dependent. This can be evidenced by increased oxygen consumption at 35 °C that is higher because of increased metabolic demand (Fusi et al. 2016). High temperatures in the water propel the displacement of *P. marmoratus* to the terrestrial environment, where the metabolic cost (e.g. ability to supply oxygen to all organism's tissues) at high temperatures will be lower, since the oxygen consumption in the air for this species remained constant (Fusi et al. 2016). Therefore, *P. transversus* increased oxygen consumption at 35 °C in submersed condition, suggests that this specie may to explore terrestrial environment evidencing to be well adapted to the bimodal way of life. Considering submersed condition, results of oxygen consumption and ammonia concentration are related. The increase of the ammonia concentration at 35 °C is related to increase in oxygen consumption and the energy metabolic demand at high temperature (Barbieri et al. 2016; Nie et al. 2017). Increasing metabolic demand provides increased amino acid deamination by converting proteins from the organism's

stock into  $\text{NH}_4^+$  (ammonium ions) (Ganser et al. 2015). Ammonium ions will be released and the carbon resulting from the deamination process will be used in the energy production process (Forsberg and Summerfelt 1992; Ganser et al. 2015). Thus, ammonia is also an indicative of thermal stress.

When in emerged condition, these organisms undergo the effect of temperature and desiccation. High temperatures at low tide also affect the glucose concentration in the hemolymph and perigastric organ index in *P. transversus*. Both are influenced by temperature, because high temperatures increase the energy demand by the organism for vital process as respiration, locomotion and digestion (Allen et al. 2012; Rho & Lee 2017; Portner et al. 2017). The first substance to be released is glucose, since it is the main energy source for ATP production when oxygen availability at high temperatures decreases (Lorenzon et al. 2007). The perigastric index and the glucose concentration decreased by 35 °C indicates that much of the energy reserve of *P. transversus* was used at this temperature. Our results corroborate with those found in Foucreau et al. (2014), where the rate of glucose in gammarids also decreased at extreme temperatures suggesting that glucose rate may be an important indicator of thermal tolerance of ectothermal organisms. The results from Madeira et al. (2014) for *Pachygrapsus marmoratus*, the same genus of the organism from this work, showed that the critical thermal maximum (CTmax) of *P. marmoratus* is approximately 36 °C, close to the 35 °C temperature of our work. Thus, our results suggest that 35 °C begins to be a limiting temperature for *P. transversus* and that at temperatures above that can be fatal, as observed in our work where all organisms submitted to 40 °C died with 24 hours of experiment. Perigastric organ index decreased at 35 °C as well. This result indicates lipolysis of perigastric organ on this stress situation (Vinagre & Chung 2016). Being

this organ the mainly responsible for the energy storage in crustaceans (Cervellione et al. 2017), its lowest index indicates high-energy requirement at 35 °C.

Exposure to high temperatures at low tide causes loss of water and physiological adaptations by these organisms. In our dehydration experiment our variable response was the loss of water at different experimental temperatures over time. Small crabs have lost much more water at all temperatures and over time than large crabs. For large crabs the critical temperature was 40°C and from 30 minutes exposure. Our results demonstrate that volume surface ratio is determinant for these crabs and influences physiology and behavior. Larger crabs are larger in volume, therefore lose less water over time than small crabs when exposed to desiccation (Pellegrino 1984; Levinton et al. 2015). Our results corroborate with those found for fiddler crabs, in which small crabs lose more water than large crabs (Allen et al. 2012). Thus, we infer that physiological consequences are also similar between crab species, which small crabs reaches limiting levels of physiological performance (e.g. maximum aerobic speed) faster than large crabs (Allen et al. 2012). Water loss is one of the means for crabs to control their body temperatures (Allen et al. 2012). In the field, the greater or less loss of water by these organisms can also be controlled by their behaviors, since the great loss of water can lead them to death (Cannicci et al. 1999). Then, we suggest that minor individuals, for losing more water in a shorter exposure period, are almost always associated with macroalgae, mussel banks and barnacles in an attempt to avoid further water loss. Instead, larger individuals are found feeding and exploring the environment even in bare rock (personal observation).

In summary, our study demonstrates that the increase in temperature will directly influence the physiological responses of *P. transversus* when exposed to both low and high tides. The results indicate that *P. transversus* is well adapted to intertidal

environment with physiological adaptations to cope the elevated temperatures increasing their energy demand and presenting differential water loss.

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### Capítulo 3

#### **How can temperature rise change food intake of omnivorous organisms? Trials from the rocky shore crab *Pachygrapsus transversus***

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## ABSTRACT

The temperature increase influences the feeding behavior of ectothermic omnivorous organisms. High temperatures can lead to differential consumption of food items depending on their constitution and rate of absorption by the organism. Here we investigate the effect of temperature increase on feeding of *Pachygrapsus transversus*, abundant omnivorous crab in rocky shores. For this, experiments were conducted without and with choice in temperatures of 24 °C, 35 °C and 40 °C. *Pachygrapsus transversus* fed more at elevated temperatures when only macroalgae were available. When there were only mussels as food item, there was no difference in feed rate between temperatures. When given the choice they fed more mussels regardless of temperature. Moreover, the analysis of the stomach contents allowed us to infer about its digestion in which macroalgae have faster digestion and absorption by the organism at high temperatures when compared to the mussel. Macroalgae contains lower energy content and high absorption rates for the organism that is potentiated when it is at high temperatures. Thus, it is necessary to eat more and frequently. Mussel contains higher energy content and lower rate of absorption by the organism, thus, it does not present difference in consumption between temperatures. When the choice is available, consumption is not influenced by temperature and *P. transversus* feeds more mussels because of its nutritional characteristics, but also because this crab is selective, feeding more on animal matter when available. Therefore, we found evidence that *P. transversus* is well adapted for omnivore feeding behavior even in an elevated temperatures.

**Keywords:** Global warming; feeding behavior; community structure; rocky shore

## INTRODUCTION

The increase in temperature is one of the first consequences of climate change (Cox et al., 2000; IPCC, 2013). Ectothermal organisms are the most vulnerable to these changes due to the dependence of environmental temperature on regulating body temperature (Angilletta, 2006). In this sense, the temperature acts regulating physiological and behavioral processes in these organisms (Pellan et al., 2016; Pörtner et al., 2017). Among the behaviors of ectothermic organisms affected by temperature we have the feeding behavior. Temperature, influencing metabolic processes, can direct the differential choice of food items according to their energy values and absorption rates (Rho and Lee 2017).

It is important to understand the differential selection of nutrients in the context of temperature increase especially when we consider omnivorous ectothermic organisms. This food strategy consists of the ingestion of food of animal and autotrophic organisms and can have impacts on the survival, reproduction and growth of species (Buck et al., 2003). For example, a diet rich in animal tissue enhances the growth of *Armases* sp. (Buck et al., 2003) and improves reproductive efforts of *Aratus pisonii* (Riley et al., 2014). Thus, the temperature influencing the selection of a particular type of food item, will be directly influencing the fitness of the species. Yet, some species of omnivorous organisms are known to influence the structure of community (Christofoletti et al. 2010; Griffen et al. 2015; Carreira et al. 2016). Thus, their consumption will determine the abundance and diversity of the species of a given environment because the higher consumption of one item will favor another (Behrens and Lafferty, 2007; Christofoletti et al., 2010; Carreira et al., 2016).

In general, elevated temperatures induces higher food intake (Zhang et al., 2016). This is due to an increase in the metabolic demand expressed, for example, in the increase of the rate of oxygen consumption (Fusi et al., 2016). Some studies show that the increase in temperature leads to omnivorous increase the consumption of autotrophic food items (Boersma et al., 2016; Rho and Lee, 2017). These foods contain high rates of carbohydrates that are rapidly absorbed and are an immediate source of energy, which is beneficial to the organism since there is an increase in metabolic demand at high temperatures (Griffen et al., 2015; Boersma et al., 2016). Results from other studies show that there is a higher consumption of animal tissue in higher temperatures, with high concentration of proteins, due to the fact that the energy content is higher and absorption slower, causing the organism not to need to feed several times (Schmitz et al., 2016). This ambiguous scenario about the consequences of increasing temperature on the feeding of omnivorous organisms show us that the relationship can be complex and the understanding about it can help us to predict future consequences of global warming on species and the structuring of communities.

Among ectothermic and omnivorous organisms we have the rocky shore crab *Pachygrapsus transversus*. This crab is widely distributed in the tropical and subtropical southwest Atlantic of Brazil (Flores and Negreiros-Fransozo, 1999). It is characterized by having small size and moving quickly. It feeds on animals and macroalgae and their consumption can determine the abundance and richness of species in communities of rocky shores (Christofolletti et al., 2010). In addition, organisms living in this environment are exposed daily to temperature variations (Little et al., 2009). Therefore, *P. transversus* is an appropriate organism model to study the consequences of temperature increase in the feeding behavior of ectothermic organisms.

Considering the context presented above, our hypotheses were that *P. transversus* feeds more at elevated temperatures regardless of the food item and when given the possibility of selection, it presents differential feed behavior, feeding more of animal tissue at elevated temperatures. Therefore, the objectives of our work were to evaluate if *P. transversus* feeds more at elevated temperatures and if shows differential feed behavior by animal tissue or macroalgae when exposed to high temperatures. Experiments were carried out at three experimental temperatures: 24 °C, 35 °C and 40 °C in treatments with no choice, where only one food item was offered; and with choice, where it was offered macroalgae and animal tissue at the same time for each temperature.

## **MATERIAL AND METHODS**

### *Study area and organisms*

*P. transversus* and food items were collected on the rocky shore of Sonho Beach, Itanhaém, São Paulo, Brazil (24°10'59"S 46°47'20"W), where the crabs and food items are abundant. Only male crabs with all appendages were collected by hand during the low tide period. Crabs were selected with carapace widths between 13 and 18 mm, corresponding to the most abundant sizes of adult organisms for this species (Granado et al. capítulo 2).

After being collected, the crabs were kept individually in containers containing hiding place (diameter of 2.5 cm), sea water and constant aeration. The crabs were fasted for 48 hours to make sure there was no food in their stomach and this would influence their behavior when in experiment.

### *Food behavior experiment*

To evaluate if *P. transversus* feeds more at elevated temperatures and if they consume more food of animal matter than macroalgae in higher temperatures we had three trials for each three temperatures (fixed factor, 3 levels: 24 °C, 35 °C and 40 °C). First, we offer the macroalgae item *Ulva fasciata* (fixed factor; N = 10) and the mussel *Perna perna* (fixed factor; N = 10) separately to evaluate if *P. transversus* feed more at high temperatures (no choice experiments). Then, we conduct the third trial to compare if crabs feed more animal matter or macroalgae, offering both food items simultaneously (multiple-choice experiments, fixed factors, 2 levels: macroalgae and mussel; N = 5 per food item). Our response variables were the amount consumed of each food item, which could be verified through the difference between initial and final weight of the items and, for the multiple choice treatment, besides the weight of the food we also verified the stomach contents of the organisms.

The food items offered had approximately 2 grams of weight and the mussel was offered out of the shell. Both conditions are not those found in the field, however we think it was appropriate to offer the same weight of food items, since the consumption rate was calculate comparing the food items consumed. And the fact of offering the mussel outside the shell was to eliminate the size of the shell as a variable and influencing the consumption of that item.

The air temperatures used were established based on data from SIMCOSTA (Brazilian Coast Monitoring System) and included a mean of temperatures obtained from September 2014 to March 2016 for São Paulo State. The temperature of 24 °C is the average temperature of the air temperature, 35 °C is the highest temperature recorded in this period and the temperature of 40 °C was determined by considering the temperature of 35 °C (highest temperature found in the data set) and adding 5°C, the worst scenario of IPCC by the end of this century (IPCC 2013). The experiments were



conducted in germination chambers (FANEM Mod. 347 CDG) that allowed temperature control.

Plastic containers had a thin layer of sea water, allowed to crabs to maintain hydrated, and with constant aeration and in the center of container we make available a PVC shelter (2.5 cm diameter). The crabs were placed in the containers and allowed to acclimate at different temperatures for 1 hour. After that, food items were inserted and the experiment lasted 12 hours. In this experiment we did not simulate tide, as this factor could alter the experimental temperatures. At the end of the experiment, the organisms were removed and placed in plastic bags and immediately frozen for further analysis of stomach contents and remained food items were weighed.

In order to ensure that weight loss of food items was due only to crab consumption, since they lose weight naturally, we conducted an experiment with food items only at the experimental temperatures. For this experiment, approximately 2 grams of each food item were placed in containers ( $N = 10$  each food item) containing sea water. This experiment also lasted for 12 hours and at the end the food was weighed. With the weight of food items a correction index was made, based on the formula  $(\bar{M} C_f/C_i)$  where we have the mean ( $\bar{M}$ ) of the ratio between the final weight ( $C_f$ ) and initial weight ( $C_i$ ) of food items when tested without crab. Obtained this index, the calculation of what was consumed by the crab was based on the following formula:  $((W_i \times \bar{M} C_f/C_i) - W_f)$ ; where  $W_i$  e  $W_f$  are the initial and final weights, respectively, of the food items that were made available to the crab (Cronin and Hay, 1996).

As the process of absorption of food items is different and this has implications on the rate of consumption, in the multiple choice treatment we also analyze the stomach contents of the organisms. The organisms had their carapace opened

immediately after the removal of the freezer. In this way, complete withdraw of the stomach contents was easier. Stomach contents were spread and the stomach washed with distilled water on a plate. Then, 5 drops of toluidine blue solution were used to stain cells containing cellulose, which allows better differentiation between macroalgae and mussel fragments (Poon et al., 2010). Fragments of food items were counted by spreading the contents through a plate containing 100 squares and counted and identified by stereomicroscope (Zeiss Discovery V8). The abundance of food items was calculated using the formula: (total number of squares filled by food item being analyzed / total number of squares filled by food) x 100 (Poon et al., 2010).

To validate this methodology, we made an additional experiment in which we collect the crabs in the field and left them (N = 10) 48 hours fasting in containers with water and constant aeration. Then, we froze them and analyzed their stomach contents in the same way as above. The percentage of filling the stomachs of these crabs was 1.33% and allowed us to conclude that 48 hours of fasting was enough for our analyses.

#### *Data analysis*

For experiments in which there was no choice we did one-way ANOVA comparing the amount of food items ingested (in grams) at the three different experimental temperatures. Two-way ANOVA was used for the multiple-choice experiments, comparing temperature and food item (mussel and macroalgae) in which our response variables were the amount of food items ingested (in grams) and the abundance of macroalgae and mussels in the stomach of the crabs.

Data homoscedasticity was tested with Levene's test for ANOVA assumptions. When data were not homocedastic, we made the LOG (X + 1) transformation and we

used Fisher LSD test for *post hoc* tests. We considered statistical differences when  $P < 0.05$ .

#### *Ethical note*

This study was performed according to Brazilian law where we had license to collect the organisms from Authorization and Information System in Biodiversity (SISBio; protocol number 47474).

## RESULTS

Our results demonstrate that the crabs consumed more macroalgae at temperatures of 35 °C and 40 °C than 24 °C. (Two-way ANOVA  $F_{2,27} = 4.14$ ,  $P = 0.027$ ; Fig. 1A). While for mussels we had no difference of crab consumption among the three temperatures (Two-way ANOVA  $F_{2,27} = 0.88$ ,  $P = 0.42$ , Fig. 1B).

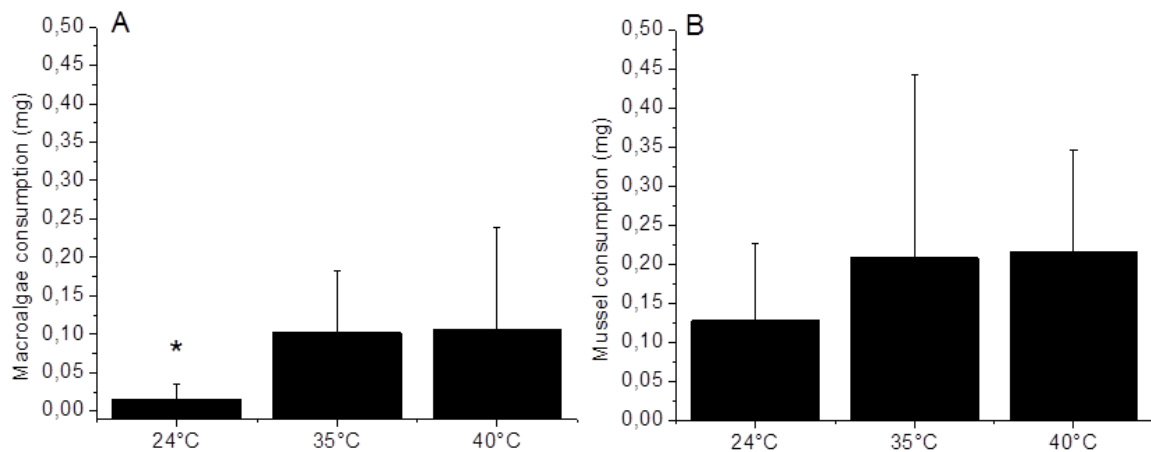


Figure 1: Quantity of macroalgae (A) and mussel (B) consumed by *Pachygrapsus transversus* in treatments with no choice. Error bars indicates standard deviation and asterisks indicates statistical difference ( $P < 0.05$ )

For the multiple-choice experiment, the amount of food items consumed (in grams) was different only in the food item, this means that crabs eat more mussels regardless of temperatures (Table 1 and Fig. 2).

Table 1: Effect of temperature on the consumption of *Pachygrapsus transversus* for different food items. Two-way ANOVA,  $P < 0.05$  only for food item.

Treatment	df	MS	F	P
Temperature	2	0.002428	1.23356	0.309068
Food item	1	0.020812	10.57291	0.003389
Temperature*Food item	2	0.006363	3.23257	0.057134
Error	24	0.001968		

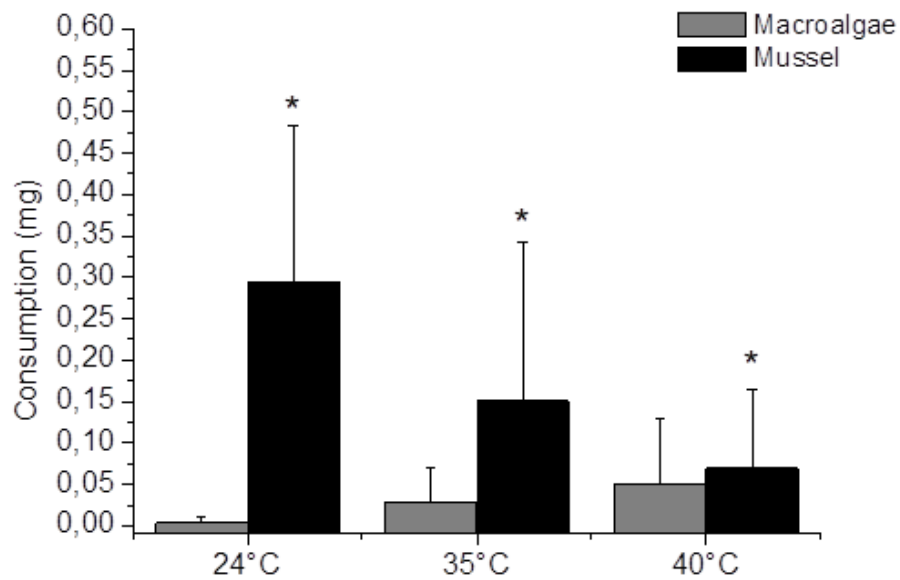


Figure 2: Quantity of food item consumed by *Pachygrapsus transversus* in a multiple choice experiment. Error bars indicates standard deviation and asterisks indicates statistical differences between food items ( $P < 0.05$ ).

For multiple choice experiment where the variable response was abundance of food items in the stomach, there was an intersection between the temperature and food items (Two-way ANOVA  $F_{2,54} = 11.24$ ,  $P < 0.001$ ). Thus, there was a higher abundance of macroalgae fragments at temperatures of 24 °C and 35 °C, while at 40 °C there were more mussel fragments (Fig 3).

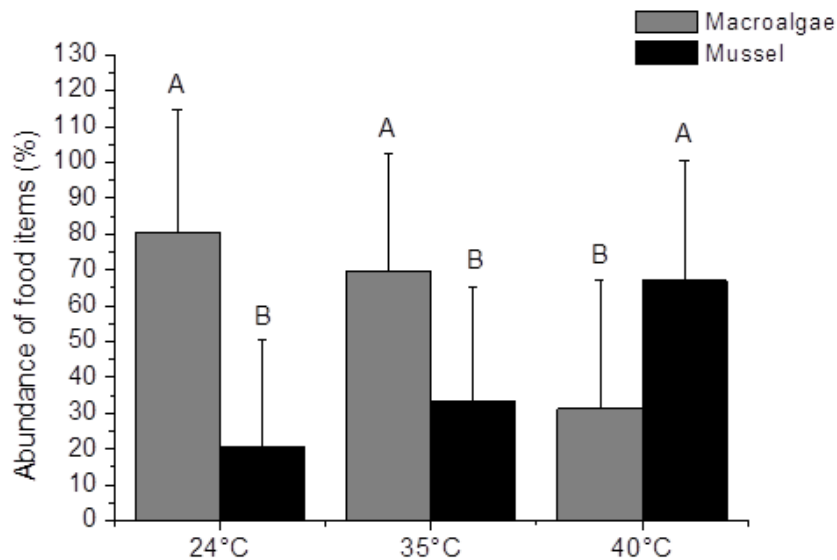


Figure 3: Abundance of food items in a stomach of *Pachygrapsus transversus*. Error bars indicates standard deviation, different letters indicates statistical differences between treatments and food items ( $P < 0.05$ ).

## DISCUSSION

In our work, we demonstrated that elevated temperatures influences *P. transversus* feeding behavior when they have only one food item available. This crab eats more at elevated temperatures, depending on the food item. The consumption rate of a specific food item is related to food item constitution. In addition, the increased intake of a food item may be related to the rate of absorption of this item by the organism, where foods that are absorbed faster, require higher consumption and more often. We also demonstrated that, when given the choice, *P. transversus* confirms its selective feeding behavior where it feeds more animal tissue independent of temperature.

In this study in treatments with no choice, our results confirm that *P. transversus* eats more macroalgae at elevated temperatures, while there was no difference in the amount of mussels ingested at the three temperatures. High temperatures favor higher food intake due to the increase in the energy demand of the ectothermic organisms

(Pellan et al., 2016). The highest intake of macroalgae may be a metabolic compensation of crabs to the increase in energy costs caused by temperature (Erickson et al., 2008; Riley et al., 2014). In addition, the species of macroalgae we used, *Ulva fasciata*, contains high concentrations of carbohydrates in its composition (De Pádua et al., 2004). Carbohydrates are considered to be an immediate and less expensive source of energy for organisms (Wang et al., 2016), which may also justify the higher consumption of macroalgae at 35 °C and 40 °C, since there is increased metabolism in ectothermic organisms at elevated temperatures (Fusi et al., 2016). *Pachygrapsus transversus* showed no difference in the rate of mussel consumption when this item was offered alone at the three experimental temperatures. Animal tissue has a higher energy content (Griffen et al., 2015), suggesting that the organism does not need to increase its consumption even at high temperatures. In addition, the rate of absorption of animal tissue is lower because digestion is slower and more costly for the organism (Griffen et al., 2015) given these characteristics, even with increased metabolic demand at elevated temperatures, crab need not increase the rate of consumption of animal tissue.

The consumption of *P. transversus* in the multiple choice treatment was not related to temperature because they eat more mussels at all experimental temperatures. This result corroborates that found in Christofolletti et al. (2010), in which *P. transversus* exhibits selective feeding behavior for animal prey. This selective feeding behavior becomes temperature not a determining factor in the food selection of this crab. In addition, the consumption of proteins entails metabolic benefits for organisms. It was verified for other species of omnivorous crabs that higher protein consumption provided higher survival and growth of the animal (Buck et al., 2003; Riley et al., 2014). Yet, we suggest for this treatment the need to increase the number of samples of multiple choice treatment and consequent reassessment of these results, since we had a

marginal interaction between temperature and the quantity of each food item ingested (see table 1). This may help us to elucidate feed behavior of *P. transversus* in elevated temperatures.

Considering the multiple choice treatment to know the digestion of different food items by the crabs at different temperatures, we also used as a response variable the percentage of food items contained in the stomach of *P. transversus*. For this response variable we obtained a greater amount of mussel fragments at 40 °C, whereas at 24 °C and 35 °C we obtained more macroalgae fragments. These results are complementary from those found for consumption measured in milligrams. Considering this variable response (consumption measured in milligrams) we verified that the organisms consumed more mussels at all temperatures and, therefore, we expected to find more mussel fragments in the stomachs of the organisms exposed to the three experimental temperatures. More fragments of mussels in stomach only at 40 °C was a physiological consequence, being a result of a faster digestive process at high temperatures, where the digestion of macroalgae was faster than the animal tissue.

The digestive process has been faster at elevated temperatures corroborates with the results found for other ectothermal organisms such as fish (Miegel et al., 2010) and insects (Rho and Lee, 2017). High temperatures enhance the action of digestive enzymes, which have their activity peaks close to 40 °C (Freese et al., 2012), accelerating the process and promoting the rapid passage of the digestive content (Miegel et al., 2010; Rho and Lee 2017). Carbohydrates, contained in *Ulva fasciata*, have high digestibility and high absorption rates by the organisms (Campaña-Torres et al., 2008; Griffen et al., 2015), which explains that we did not find fragments of macroalgae abundant in the stomach of *P. transversus* exposed at 40 °C. This result also helps us to corroborate the higher consumption of macroalgae at elevated temperatures

in treatment without choice. Due to the high rate of digestion and absorption of this food item by the organism, it needs to eat more frequently and quantity. Now, if we consider the great abundance of mussels at 40 °C, this result is due to the slower digestion and lower absorption efficiency of the animal tissue, which is in agreement with the one found for *Hemigrapsus sanguineus*, a species of crab also omnivorous (Griffen et al., 2015). The high temperatures decrease the efficiency in the digestion of proteins in ectothermic organisms. This fact is due to the exponential increase in oxygen consumption according to the temperature increase by shifting the energy of the organism to this process (Lemoine and Shantz, 2016). For more robust evaluation of the abundance of the stomach contents we also suggest an additional experiment in which the order of intake of food items at different temperatures be evaluated. Thus, we suggest that food items that are consumed first will have less abundance in the stomach as they will already be more advanced in the digestive process.

In summary, the increase in temperature influences in a quantity of food eaten by *P. transversus* depending of food as well as in the digestive process. Also, we showed that temperature does not influence the selective feeding behavior of *P. transversus*, where it selects animal matter independent of temperature. Thus, we showed that *P. transversus* is a specie well adapted to its omnivorous feeding behavior even under stressful conditions such as elevated temperatures. Therefore, we suggest further studies considering the temperature variable influencing other vital aspects to these organisms, such as physiological responses. We emphasize the importance of these studies considering the current global warming context and the ecological importance of this specie.



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### **Considerações finais**

De acordo com o presente trabalho organismos de manguezal e costão rochoso se mostraram sensíveis e apresentaram respostas fisiológicas e comportamentais quando expostos a altas temperaturas. No entanto, demonstraram ser adaptados ao ambiente que vivem. Inferimos que a distribuição desses organismos ao longo do gradiente vertical do ambiente do entremarés influencia nas respostas fisiológicas apresentadas. Além disso, esses organismos possuem adaptações fisiológicas para manter as condições vitais e suprir energeticamente todo o corpo quando sob o efeito estressante de altas temperaturas. Também apresentaram comportamento alimentar diferencial em temperaturas elevadas, em que este fator influencia na quantidade ingerida do item alimentar e no processo digestivo.

Dessa forma, verificamos que o aquecimento global irá influenciar diretamente a fisiologia e comportamento de organismos ectotérmicos. As respostas fisiológicas e comportamentais desses animais se complementam e tornam-se interessantes para construção de um cenário abrangente das consequências das mudanças do clima no ambiente do entremarés. Considerando estudos futuros, a avaliação dos impactos de outros fatores como diminuição do pH, elevação do nível do mar e eventos extremos (tempestades, por exemplo) também se faz importante, visto que mudanças do clima englobam também esses fatores e não somente o aumento da temperatura.

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