UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - ZOOLOGIA

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Silas Candido Principe de Souza

Orientadora: Profª Drª Tânia Marcia Costa

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DIFFERENTIAL EFFECTS OF WATER LOSS AND TEMPERATURE INCREASE IN THE PHYSIOLOGY OF *FIDDLER CRABS* FROM DISTINCT HABITATS

Silas Candido Principe de Souza

Orientadora: Profª Drª Tânia Marcia Costa

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Zoologia), do Instituto de Biociências de Botucatu - Unesp, como parte dos requisitos para a obtenção do título de Mestre.

Botucatu - SP

2017

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Souza, Silas Candido Principe de.
Differential effects of water loss and temperature increase in the physiology of fiddler crabs from distinct habitats / Silas Candido Principe de Souza. - Botucatu, 2017

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Tânia Marcia Costa
Capes: 20502001

1. Caranguejo. 2. Dessecação. 2. Habitat (Ecologia). 3. Fisiologia. 4. Mudanças climáticas. 5. Altas temperaturas - Pesquisa.

Palavras-chave: Dessecação; Ecologia térmica; Fisiologia térmica; *Leptuca thayeri*; *Minuca rapax*.

Agradecimentos

Agradeço primeiramente a Deus pela oportunidade de fazer o mestrado e por todas as experiências que pude vivenciar neste período.

Agradeço imensamente à minha amada esposa, Josi, por todo o apoio, paciência, carinho e até mesmo ajuda nas coletas.

Da mesma forma, agradeço à minha família pela ajuda, incentivo e compreensão ao longo desses anos de estudo.

Agradeço aos meus colegas do LABECOM, em especial ao Juan, Fernando, Alexandre e Renan pela ajuda nas coletas e experimentos.

Agradeço aos meus colegas da UFSCar Sorocaba por toda a troca de experiências e colaboração nesse período, em especial à Heidi e ao Fernando pela ajuda com as análises fisiológicas.

Também agradeço aos professores Dr. John McNamara (USP Ribeirão) e Drª Cleoni Santos (UFSCar Sorocaba) pelo auxílio com as análises fisiológicas.

Agradeço ao Departamento de Biologia da UFSCar Sorocaba pelos afastamentos concedidos para realização de experimentos e disciplinas.

Agradeço ao programa de Pós-graduação em Ciências Biológicas (Zoologia) do IBB-Unesp pela oportunidade de realizar o mestrado junto ao programa.

Faço um agradecimento especial à Profª Drª Alessandra Augusto pelo apoio, colaboração e parceria nesse trabalho. Ainda que não tenha sido possível tornar a co-orientação oficial, esse trabalho não teria sido concluído sem sua valiosa ajuda. Obrigado.

Agradeço à minha orientadora Profª Drª Tânia, pela orientação nesses últimos anos, desde a graduação até o mestrado. Foram muitas as conversas e aprendizados que construíram minha formação. Obrigado em especial pela paciência.

Sumário

** Dissertação elaborada segundo as normas da revista científica "Journal of Thermal Biology".*

Versão resumida em português

Diferentes efeitos da perda de água e do aumento de temperatura na fisiologia de *fiddler crabs* de distintos habitats.

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Resumo

A temperatura é uma das principais restrições ambientais à distribuição dos organismos, afetando a fisiologia e sobrevivência. Organismos que habitam a zona do entremarés estão constantemente expostos à variação da temperatura e, com as mudanças climáticas, esses organismos devem enfrentar condições diferentes, que incluem temperaturas mais elevadas, levando a maiores taxas de perda de água por evaporação e, consequentemente, redução do desempenho ou mortalidade. Neste estudo, testamos os efeitos da dessecação em duas espécies de caranguejos violinistas (*Leptuca thayeri* e *Minuca rapax*) que ocupam habitats distintos em relação à cobertura da vegetação e posição no entremarés e, portanto, podem responder de forma diferente ao estresse por dessecação e ao aumento da temperatura. *Leptuca thayeri*, que é restrita à zona intermediária do entremarés, é mais sensível à dessecação do que *M. rapax*, uma espécie generalista, com maiores taxas de dessecação e mortalidade quando expostas à dessecação por 120 minutos. Além disso, em comparação com *M. rapax*, *L. thayeri* possui uma carapaça mais permeável. Também avaliamos se o aumento de temperatura pode causar alterações fisiológicas na espécie mais restrita *L. thayeri*, tendo acesso a alimento e à água. Uma elevação de temperatura de 10 ° C e 20 ° C durante 72 h não causou mortalidade em *L. thayeri* nem mudanças na concentração de glicose e proteína na hemolinfa. No entanto, as temperaturas mais altas aumentaram os níveis de lactato desidrogenase, e houve alterações na osmolalidade da hemolinfa e grau de hidratação muscular na temperatura intermediária, sugerindo que a essa temperatura essa espécie tenha melhores capacidades osmoreguladoras. Nossos resultados mostram que espécies de diferentes habitats respondem de forma diferente à dessecação devido a adaptações morfológicas, e espécies de zonas de maré baixa e áreas sombreadas podem enfrentar os efeitos nocivos das mudanças climáticas de forma mais aguda.

Palavras-chave: ecologia térmica; Fisiologia térmica; dessecação; *Leptuca thayeri*; *Minuca rapax*

Introdução

A temperatura é uma das principais restrições do ambiente à vida, pois afeta reações bioquímicas com consequentes efeitos na fisiologia, alimentação, crescimento e reprodução (por exemplo, Weinstein, 1998; Ruscoe et al., 2004; Allen et al., 2012). Embora a temperatura da água do mar seja relativamente estável ao longo do dia, os organismos do entremarés estão continuamente expostos à variação de temperatura durante a maré baixa (Helmuth et al., 2006; Schneider, 2008; Somero, 2002). Além disso, neste período, os organismos podem enfrentar a dessecação devido à exposição ao ar (Allen et al., 2012; Chapman and Underwood, 1996; Miller et al., 2009; Thurman, 1998). A perda de água pode causar a mortalidade de indivíduos (por exemplo, Turra e Denadai, 2001) ou reduzir seu desempenho, diminuindo as oportunidades de reprodução e forrageamento (Allen et al., 2012; Pincebourde et al., 2008). Os organismos que habitam diferentes áreas do entremarés, por exemplo, desenvolveram adaptações fisiológicas de acordo com as condições ecológicas do habitat (por exemplo, Somero, 2002; Prusina et al., 2014; Wong et al., 2014; Nobbs e Blamires, 2017).

As mudanças climáticas aumentarão a temperatura em algumas regiões do mundo e potencialmente alterarão os padrões de distribuição de espécies (IPCC, 2014; Wilson et al., 2005), e os ectotérmicos serão especialmente prejudicados (Kingsolver et al., 2013; Paaijmans et al., 2013; Sunday et al., 2011). À medida que a temperatura muda, as espécies devem apresentar respostas diferentes de acordo com adaptações morfológicas e comportamentais (Eshky et al., 1995; Herreid, 1969a; Huey et al., 2012; Levinton et al., 2015; Thurman, 1998; Yoder et al., 2005), além de respostas fisiológicas (Levinton et al., 2015). Os *fiddler crabs* ou caranguejos violinistas (Decapoda: Ocypodidae) são um excelente modelo experimental para estudar os efeitos da mudança de temperatura e exposição ao ar. Esses caranguejos ocorrem em ambientes estuarinos de zonas tropicais e temperadas (Crane, 1975) e têm sua distribuição global controlada principalmente pela temperatura (Levinton e Mackie, 2013). Localmente, muitos fatores abióticos e ambientais podem influenciar sua distribuição (por exemplo, tamanho de grão do sedimento, conteúdo orgânico, cobertura de vegetação, Nobbs 2003, Thurman et al., 2013, Mokhtari et al., 2015, Checon e Costa, 2017) e existem espécies de áreas arenosas, secas e expostas (Rabalais e Cameron, 1985; Thurman et al., 2013) até áreas sombreadas e lamosas (Thurman et al., 2013). Devido à sua natureza semi-terrestre, os *fiddler crabs* permanecem parte do dia expostos a diferentes temperaturas e níveis de dessecação do ar (Thurman, 1998; Yoder et al., 2005), mas as respostas fisiológicas à temperatura podem variar entre as espécies (Thurman, 1998) . Esses organismos desempenham um papel fundamental nos ambientes estuarinos, principalmente por sua função como engenheiros do ecossistema, afetando a ciclagem de nutrientes e os parâmetros químicos dos sedimentos (Kristensen e Alongi, 2006; Kristensen et al., 2008). Assim, mudanças na

distribuição e abundância desse grupo podem afetar a estrutura e o comportamento das comunidades (Citadin et al., 2016).

Nosso objetivo foi comparar a resposta de dessecação em espécies de *fiddler crabs* que habitam áreas de manguezal com diferentes graus de exposição à radiação solar: *Leptuca thayeri*, que vive em áreas lamosas e vegetadas na zona intermediária do entremarés, e *Minuca rapax*, cujo habitat varia de áreas lamosas sombreadas até áreas arenosas expostas à luz solar, sendo uma espécie mais generalista que ocupa a zona superior do entremarés ou o supralitoral. Alguns estudos recentes sobre a ecologia dessas espécies foram feitos (por exemplo, Gusmão-Junior et al., 2012; Cuellar-Gempeler e Munguia, 2013; Costa e Soares-Gomes, 2015; Capparelli et al., 2016), mas pouco é conhecido sobre os efeitos da dessecação e aumento de temperatura em sua fisiologia. Embora ambas as espécies coexistam nos estuários brasileiros (Thurman et al., 2013, Checon e Costa, 2017), nossa hipótese é de que *L. thayeri* deveria ser mais sensível à dessecação do que *M. rapax*, já que o primeiro está atualmente restrito a áreas lamosas e sombreadas, o que teria implicações para sua distribuição em um cenário de aumento de temperatura. Como a dessecação é apenas um dos efeitos da elevação da temperatura, também testamos as respostas fisiológicas (níveis de glicose, LDH e proteínas na hemolinfa, osmolalidade da hemolinfa e grau de hidratação do músculo) e a capacidade de sobrevivência de *L. thayeri*, uma espécie mais restrita, a temperaturas mais altas. Isso foi feito expondo os organismos a três tratamentos diferentes de temperatura (25°C – controle, 35°C e 45°C) em estufas. Compreender os diferentes efeitos da dessecação e da temperatura em *fiddler crabs* de áreas com distintos graus de exposição à luz solar pode ajudar a prever os efeitos das mudanças climáticas na distribuição desse grupo.

Conclusões

A exposição ao ar representa um grande desafio para os organismos do entremarés, pois leva à perda de água e mudanças no metabolismo (Allen et al., 2012; Chapman e Underwood, 1996; Miller et al., 2009). Espera-se que as espécies que habitam áreas expostas e vivam nas zonas superiores do entremarés lidem de melhor maneira à exposição ao ar, pois apresentam adaptações a este ambiente severo (por exemplo, Thurman, 1998; Prusina et al., 2014; Wong et al., 2014). Em nosso estudo, a espécie mais restrita *L. thayeri*, que habita áreas lamosas e vegetadas, apresentou maior taxa de perda de água do que *M. rapax*, que geralmente é distribuída em áreas mais expostas na zona superior do entremarés. Além disso, nossos resultados confirmam que o controle de perda de água pode ser crucial para a sobrevivência desses organismos, pois *L. thayeri* apresentou maior mortalidade em relação a *M. rapax*, evidenciado por diferenças na permeabilidade da carapaça, com *M. rapax* tendo menor permeabilidade na carapaça. No entanto, o aumento da temperatura não foi crítico para *L. thayeri*, pois não houve mortalidade durante a exposição a temperaturas

mais elevadas com água e alimentos disponíveis, embora tenha havido respostas fisiológicas a esse aumento. Estes resultados mostram que os organismos podem responder de forma diferente à dessecação e à elevação da temperatura.

Com as mudança climáticas, os organismos serão expostos à diferentes temperaturas da média atual (IPCC, 2014, Parmesão e Yohe, 2003) e, embora fisiologicamente, L. *thayeri* não é criticamente afetado pelo aumento de temperatura, espera-se que esta espécie experimente mudanças na sua distribuição e abundância, especialmente com modificações na cobertura da vegetação que podem levar a uma maior exposição à dessecação. O mesmo se espera que aconteça com outras espécies como *L. thayeri* atualmente restritas a às áreas sombreadas e lamosas do manguezal, enquanto espécies generalistas como *M. rapax* poderão invadir novos habitats. Com as mudanças distribucionais causadas pelas mudanças climáticas, é possível prever um aumento da abundância e densidade de algumas espécies, o que certamente afetará o funcionamento da comunidade e a ecologia dos manguezais.

Versão completa em inglês submetida na revista "Journal of Thermal Biology"

Differential effects of water loss and temperature increase in the physiology of fiddler crabs from distinct habitats.

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Abstract

Temperature is one of the main environmental constraints to organism distribution, affecting both physiology and survival. Organisms that inhabit the intertidal zone are constantly exposed to temperature variation and, with climate change, those organisms should face different conditions which include higher temperatures, leading to higher rates of water loss through evaporation and then fitness reduction or mortality. In this study we tested the effects of desiccation in two fiddler crabs species (*Leptuca thayeri* and *Minuca rapax*) that occupy distinct habitats in regard to vegetation cover and position on the intertidal zone and thus may respond differently to desiccation stress and increased temperature. *Leptuca thayeri*, which is restricted to the mid-tide zone, was more sensitive to desiccation than *M. rapax*, a generalist species, having higher desiccation and mortality rates when exposed to desiccation for 120 minutes. Also, compared to *M. rapax*, *L. thayeri* has a more permeable carapace. We also assessed if a temperature increase could cause physiological changes in the restricted species *L. thayeri*, while having access to both food and water. A temperature elevation of 10 °C and 20 °C for 72 h caused no mortality in *L. thayeri* nor changes in hemolymph glucose and protein concentration. However, higher temperatures increased hemolymph lactate dehydrogenase levels, and there were changes in hemolymph osmolality and muscle hydration in the intermediate temperature, suggesting that at this temperature this species has better osmoregulatory capacities. Our results show that species from different habitats respond differently to desiccation due to morphological adaptations, and species from lower tidal zones and shaded areas may face the harmful effects of climate change

more acutely.

Keywords: thermal ecology; thermal physiology; desiccation; *Leptuca thayeri*; *Minuca rapax*

1. Introduction

Temperature is one of the main environmental constraints to life as it affects biochemical reactions with consequent effects on physiology, feeding, growth and reproduction (e.g. Weinstein, 1998; Ruscoe et al., 2004; Allen et al., 2012). Although sea water temperature is relatively stable along the day, intertidal organisms are continually exposed to temperature variation during low tide (Helmuth et al., 2006; Schneider, 2008; Somero, 2002). Also, in this period organisms may face desiccation due to air exposure (Allen et al., 2012; Chapman and Underwood, 1996; Miller et al., 2009; Thurman, 1998). Water loss may cause the mortality of individuals (e.g. Turra and Denadai, 2001) or reduce their performance, decreasing opportunities for reproduction and foraging (Allen et al., 2012; Pincebourde et al., 2008). Organisms that inhabit different areas of the intertidal zone, for instance, developed physiological adaptations according to ecological conditions of the habitat (e.g. Somero, 2002; Prusina et al., 2014; Wong et al., 2014; Nobbs and Blamires, 2017).

Climate change will increase temperature in some regions of the world and will potentially alter patterns of species distribution (IPCC, 2014; Wilson et al., 2005), with ectotherms being specially harmed (Kingsolver et al., 2013; Paaijmans et al., 2013; Sunday et al., 2011). As temperature changes, species should exhibit different responses according to morphological and behavioral adaptations (Eshky et al., 1995; Herreid, 1969a; Huey et al., 2012; Levinton et al., 2015; Thurman, 1998; Yoder et al., 2005) in addition to physiological responses (Levinton et al., 2015). Integument permeability of intertidal crabs regulates water loss and thermoregulation, affecting their capacity to live in harsh environments (Herreid, 1969a; Thurman, 1998). Many species also have the ability to construct burrows to rehydrate and use as refuge from higher temperatures (Levinton et al., 2015; Powers and Cole, 1976), but with the cost of fewer opportunities to forage and reproduce (Allen and Levinton, 2014). When facing acute temperature stress, metabolism usually increases (e.g. Resgalla et al., 2007) and energy is mobilized. In crustaceans, glucose is the main energy provider for activities such as foraging and combat (Briffa and Elwood, 2001; Full and Herreid II, 1984; Matsumasa and Murai, 2005). Blood levels of glucose are controlled by the hyperglycemic hormone (CHH) and studies showed that CHH levels increase during thermal stress (Wilcockson et al., 2002; Zou et al., 2003). Also, levels of lactate dehydrogenase (LDH) in the hemolymph may increase during temperature stress due to higher energetic demand. In that case, LDH will convert pyruvate into lactate, an anaerobic path of energy production. Hemolymph osmolality increases as the

organism loses water, creating an unfavorable condition (Warburg and Goldenberg, 1984). With this, individuals mobilize water from muscular cells to get back into homeostasis, altering muscle hydration degree and consequently their metabolism (Pierce, 1982).

The fiddler crab (Decapoda: Ocypodidae) is an excellent experimental model to study the effects of temperature change and air exposure. These crabs occur in estuarine environments of tropical and temperate zones (Crane, 1975) and have their global distribution controlled mainly by temperature (Levinton and Mackie, 2013). Locally, many abiotic and environmental factors may influence their distribution (e.g. sediment grain size, organic content, vegetation cover; Nobbs 2003; Thurman et al., 2013; Mokhtari et al., 2015; Checon and Costa, 2017) and there are species ranging from dry, exposed sandy areas (Rabalais and Cameron, 1985; Thurman et al., 2013) to shaded, muddy areas (Thurman et al., 2013). Due to their semi-terrestrial nature, fiddler crabs stay part of the day exposed to different temperatures and air desiccation levels (Thurman, 1998; Yoder et al., 2005), but physiological responses to temperature may vary between species (Thurman, 1998). These organisms play a fundamental role on estuarine environments, mainly by their function as ecosystem engineers, affecting nutrient cycling and chemical parameters from the sediment (Kristensen and Alongi, 2006; Kristensen et al., 2008). Thus, changes in fiddler crab distribution and abundance could affect structure and behavior of communities (Citadin et al., 2016).

Our aim was to compare the desiccation response in species of fiddler crabs inhabiting mangrove areas with different degrees of sun exposure: *Leptuca thayeri*, which lives in muddy and vegetated areas in the mid-tide zone, and *Minuca rapax*, whose habitat varies from shaded muddy areas to sun exposed sandy areas, being a more generalist species that occupies the upper or supratidal zone. Some recent studies regarding the ecology of these species have been done (e.g. Gusmão-Junior et al., 2012; Cuellar-Gempeler and Munguia, 2013; Costa and Soares-Gomes, 2015; Capparelli et al., 2016), but less is known about the effects of desiccation and temperature increase on their physiology. Although both species coexist in Brazilian estuaries (Thurman et al., 2013; Checon and Costa, 2017), our hypothesis was that *L. thayeri* should be more sensitive to desiccation than *M. rapax* as the former is currently restricted to muddy and shaded areas, which will have implications for its distribution in a scenario of temperature increase. As desiccation is only one of the effects of temperature elevation we also tested the physiological responses and survivability of *L. thayeri,* a restricted species, to higher temperatures. Understanding the differential effects of desiccation and temperature in fiddler crabs from areas with different degrees of exposure to sunlight could help forecast the effects of climate change on this group's distribution.

2. Material and methods

2.1 Study organism

Minuca rapax is distributed from Florida to southeast Brazil, a generalist fiddler crab species inhabiting sandy to muddy areas (Melo, 1996; Thurman et al., 2013). Additionally, it is considered one of the crustaceans with wider osmotic regulation capacity, tolerating a wide range of salinities (Thurman et al., 2010, 2013; Zanders and Rojas, 1996). This characteristic makes *M. rapax* an excellent model for physiological experimentation. In studies of temperature and desiccation, *M. rapax* showed high resistance when compared to other fiddler crabs (Thurman, 1998). *Leptuca thayeri* is another common species of fiddler crab in mangroves from the east Atlantic coast. This species shows a more restrict distribution in the mangrove when compared to *M. rapax*, inhabiting muddy and shaded areas rich in organic matter (Gusmão-Junior et al. 2012; Thurman et al., 2013). Both species are common and abundant inhabitants of Brazilian mangroves (Checon and Costa, 2017; Thurman et al., 2013). As other fiddler crabs, these species build burrows and return periodically to them during the low tide to rehydrate (Crane, 1975).

Animals were collected in mangrove areas from the cities of Praia Grande (*L. thayeri* and *M. rapax* - 23°59' S; 46°24' W), and Peruíbe (*L. thayeri* - 24°26' S; 47°05' W), on the southern coast of São Paulo state, Brazil. Sampling occurred on the low tide in the months of October and November of 2016 and crabs were sampled by burrow excavation. Carapace width (CW) was measured with a caliper and only adult males of *L. thayeri* with CW between 16 and 25 mm (Farias et al., 2014) and *M. rapax* with CW between 17 mm and 22 mm (Castiglioni and Negreiros-Fransozo, 2006) were collected. Only males in the intermolt with the major claw present and non-regenerated were used in this study. After collection, crabs were transported to the laboratory in plastic buckets with sediment from their original area to avoid stress. Individuals collected from Peruíbe were used in an increased temperature experiment and those from Praia Grande were used in desiccation, rehydration and carapace permeability experiments. Some *L. thayeri* individuals from Praia Grande (n=6) were used to complement the experiment of temperature elevation as some animals died prior to experimentation. In this case, crabs were randomly divided and each treatment received two individuals. This procedure was done to avoid any possible effect of population differences.

2.2 Desiccation in *Leptuca thayeri* and *Minuca rapax*

The rate of water loss over time varies between fiddler crab species and is an indicative of their resistance to air exposure during low tide periods (Levinton et al., 2015; Thurman, 1998). Our aim was to evaluate if the response to desiccation differs in species from areas with varied degrees of exposure to sunlight and vegetation cover. For that, 39 individuals (each) of *L. thayeri* and *M. rapax*, with CW between 16 and 21

146 mm and 17 and 22 mm, respectively, were used in this experiment. We chose loss of
147 water over time as a desiccation indicator.

148 In the laboratory, crabs were cleaned by removing all sediment attached to their body
149 and were then acclimated for 48 h in aquariums with seawater (salinity of 25 PSU,
150 which corresponds to high tide salinity in the area, i.e. the moment when animals are
151 submerged) with constant aeration and ambient temperature (~25 °C). Individuals
152 were not covered by water so they still had access to the air. Each aquarium received
153 ca. 12 crabs separated by glass in groups of ca. six individuals to decrease the
154 possibility of combat and stress. Crabs were then dried using absorbent paper in a
155 standardized way and placed in previously weighed containers individually. Containers
156 with individuals were weighed again in a precision balance (0.001 g - Shimadzu, Model
157 AY220) to obtain weight and then put in a drying chamber (Marconi, Brazil, Model
158 MA035) set to 30 °C (±1 °C). This value was chosen because air temperatures on non
159 vegetated areas reach up to 28 °C (based on previous unpublished data), and sediment
160 temperature can be several degrees higher than in the air (Levinton et al., 2015). Also,
161 this parameter enabled comparisons to other works on fiddler crab thermal ecology,
162 which commonly use this specific temperature (e.g. Herreid, 1969b; Allen et al., 2012;
163 Levinton et al., 2015). Relative air humidity inside the chamber measured in conditions
164 exactly the same as in this experiment showed 24.15±0.55%.

165 Crabs of both species were simultaneously exposed to desiccation for 120 minutes as
166 previous experiments showed that longer exposure could cause mortality of all
167 organisms and would not allow us to evaluate differences in resistance (see also
168 Levinton et al., 2015). During this period, each individual was weighed as previously
169 after 15, 30, 60, 90 and 120 min of exposure. Weight values were then used to assess
170 water loss over time. These intervals were chosen based on previous experiments and
171 published works (Levinton et al., 2015) which used intervals of 15 min.

172 Desiccation data was analyzed using a Repeated Measures GLM, comparing the loss of
173 weight (water) over time between the two species, using size (carapace width in mm)
174 as a covariable (n=39 for each species). Mortality was compared between species using
175 a chi-squared test.

176 2.3 Rehydration in *Leptuca thayeri* and *Minuca rapax*

177 The study of rehydration capacity in these organisms enables us to understand the role
178 of the burrow on fiddler crab physiology (Levinton et al., 2015). In normal situations,
179 fiddler crabs constantly return to the burrow to rehydrate (Christy, 1982; Crane, 1975),
180 with direct water contact being the only way to accomplish that (Yoder et al., 2005). To
181 evaluate the capacity of rehydration in *L. thayeri* and *M. rapax* after desiccation stress,
182 we used 19 individuals of each species. Immediately after 120 min of desiccation
183 (experiment 1) organisms were individually submerged in 150 ml of seawater (25 PSU)

and kept in ambient temperature (~25°C) for 120 min. Crabs were then removed from the water, dried with absorbent paper and weighed as previously described. In this experiment, crabs were not weighed in the same intervals as the previous experiment as this would be a physiological extreme and uncommon stressful situation (Levinton et al., 2015). Initial weight (before the desiccation experiment) and final weight (after 120 min of rehydration) were correlated in both species using Pearson's Linear Correlation.

2.4 Carapace permeability in *Leptuca thayeri* and *Minuca rapax*

The crab carapace is essential for desiccation control (Herreid, 1969a) and, in some species, may stop up to 60% of water loss through the body (Thurman, 1998). Thus, species may have different carapace permeability, especially when inhabiting distinct intertidal regions, like *L. thayeri* and *M. rapax*. To evaluate carapace permeability in both species, individuals (six *L. thayeri* and six *M. rapax*) were euthanized by freezing (-16 °C) and a section of the dorsal carapace was dissected using lab scissors. The section was cleaned by removing any adhered tissue, and was then dried with absorbent paper. This section was then glued to the inlet of a plastic (polypropylene) microtube (0.5 ml) with cyanoacrylate and the space between carapace and tube was sealed using epoxy to avoid any loss of water other than through the carapace. The carapace was glued to the outside border of the tube. Inlet inside diameter was 6.95 mm and outside diameter was 9.59 mm. A 0.2 ml physiological (Ringer) solution for fiddler crabs (in mM: 525 NaCl, 13.3 KCl, 12.4 $CaCl_2 * 2H_2O$, 24.8 $MgCl_2 * 6H_2O$; Thurman, 1998) was added to the tube through a hole made with a hot needle and was then sealed with parafilm (Bemis Flexible Packaging, USA). Tubes were put in the drying chamber at 30 °C with the carapace turned down, so to make contact with the liquid, and left for one hour to reach temperature equilibrium. After that, tubes were weighed in a precision balance and then submitted to one hour of desiccation in the drying chamber at 30 °C with the same conditions. The same was done with open tubes to evaluate fluid evaporation. Previous experiments showed that evaporation in this kind of experiment is constant over time as there is no regulatory compensation (e.g. Thurman, 1998). Evaporation area corresponded to 0.38 cm^2. We compared evaporation rate per hour per cm^2 in the two species using Student's t-test, after homogeneity and normality were confirmed.

2.5 Effect of temperature elevation in *Leptuca thayeri* physiology and survival

As *L. thayeri* demonstrated less resistance to desiccation, we evaluated the effect of a temperature increase in its physiology and survival. Our hypothesis, considering that *L. thayeri* is more sensitive to desiccation, is that a temperature elevation could cause sub-lethal effects on its physiology and, in extreme cases, mortality. In order to assess this hypothesis we chose mortality, hemolymph glucose concentration, protein concentration, LDH levels, osmolality and major claw tissue hydration. Glucose is the

main provider for ATP production through glycolysis and is directly related to energy mobilization for individual activities (Full and Herreid II, 1984). Proteins also serve as energy reserves for crustaceans and changes in concentration happen due to higher energy demands. Besides that, protein levels change due to the mobilization of free amino acids to regulate cellular volume. LDH levels change by both an increase on energy demand (leading to the conversion of pyruvate into lactate) and stress conditions. Osmolality, on the other hand, changes according to the hydration state of the organism, and ionic mobilization compensates physiological changes (Warburg and Goldenberg, 1984; Thurman, 1998). In the same way, water can be displaced from cells to the hemolymph in response to osmolality increase, with tissue hydration consequently being reduced (Pierce, 1982).

Experiments were conducted in chambers with heating and 12 h light cycle. Chambers measured 1.75X0.5X0.5m and were constructed using wood for the structure and black polyethylene hard plastic as insulant. The heater was positioned at the center of the chamber and, to guarantee temperature homogeneity inside it, a circulation system was constructed using fans. These fans provided a low circulation so it would not interfere on evaporation rates but was enough to homogenize the air inside the chamber. Three experimental chambers were set at the same time, each for an experimental group, with 13 replicates in each one: control temperature (25 °C), 35 °C and 45 °C. We chose 25 °C as the control temperature as this is the summer mean air temperature for the *L. thayeri* area of collection, i.e. a vegetated area (24.83±1.95; mode=26.1; Pardo et al., unpub. data). Temperature was controlled using a thermostat (1.5 °C variation) and monitored using a digital thermometer. Measurements of humidity during the experiments were not possible due to technical limitations but measurements in the chamber in conditions exactly the same as in the experiment showed that humidity levels were 30.55±1.51 (45 °C); 35.57±3.62 (35 °C) and 39.24±6.08 (25 °C).

Before the experiment, crabs were kept in terrariums with moist sediment from the collection area (~25 °C) for 48 h. Animals had CW between 20 mm and 25 mm. Means with SD for each treatment were: 25 °C - 22.86±1.58, 35 °C - 21.48±1.41 and 45 °C - 22.27±1.45. Each chamber received 13 2 l plastic containers (175x132x115 mm) with moist muddy sediment from the occurrence area of *L. thayeri* (approximately 9 cm of sediment). Each container received one male *L. thayeri* adult and was then closed with a polyester mesh. After closed, crabs were acclimated for 24 h inside the chamber, still in ambient temperature (~25 °C). Following this period, crabs were exposed for 72 h to temperature treatments. To guarantee that there were not any effects of microclimatic variation inside the chamber, containers were rotated every 24 h. During the experiment organisms had access to both water and food (in the sediment) and could construct burrows. We chose 72 h of exposure because our aim was to test the effects of temperature elevation during longer exposures. Although this situation is not

completely real as tides were not simulated, this approach was adopted as it mimics the low tide period, when organisms are exposed to wider climatic variation.

After 72 h, crabs were cleaned, dried with absorbent paper and weighed. Organisms were then anesthetized with ice and a hemolymph sample was withdrawn from the ventral hemocoel of each crab using a 1 ml tuberculin syringe through a puncture on the fifth pereiopod. The sample was put in 0.5 ml microtubes and then frozen for later analysis of glucose and osmolality as it would not be possible to analyze all samples immediately at the end of the experiment. After hemolymph was sampled, crabs were euthanized (exposure to low temperature (-16°C) for 2 min) for evaluation of tissue hydration of the major claw.

A commercial kit of colorimetric analysis (Glicose Liquiform Ref. 133, LabTest Diagnóstica, Brazil) was used to assess hemolymph glucose concentration. The sample was centrifuged for 4 min in 3000 rpm to separate any residual tissue and then 10 µl of sample were put in a 1.5 ml tube. After that, each tube received 1 ml of the commercial reagent and was incubated for 10 min at 37 °C. Samples were read in a spectrophotometer in the 505 nm band. Osmolality analyses were performed with a 10 µl sample using a vapor pressure osmometer (Wescor 5500). Protein concentration was assessed using the method described by Bradford (1976) and LDH levels were assessed by the method described by Bergmeyer (1974).

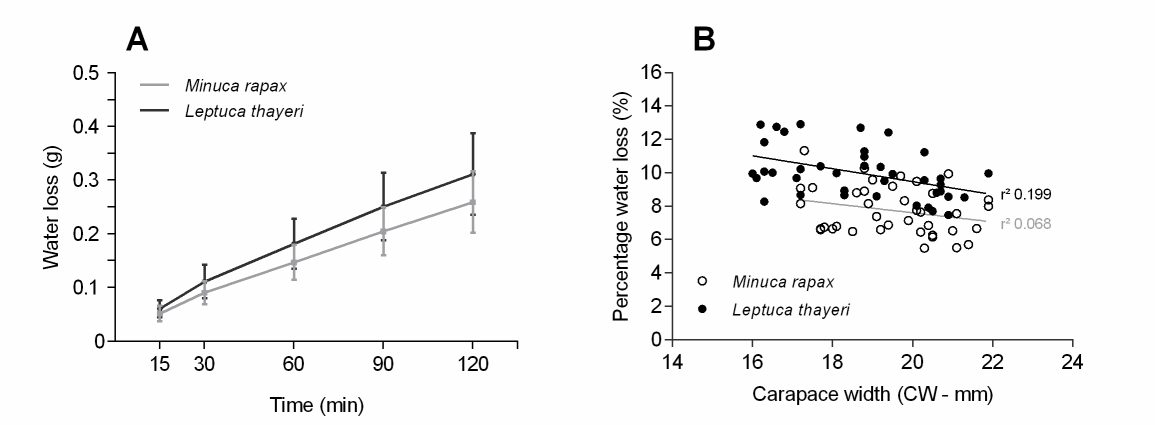
To obtain tissue hydration values, the major claw was removed and a portion of muscle was dissected with a forceps and put in a previously weighed aluminum foil. We chose the muscle of the claw because it is easy to obtain and because osmolality alterations should reflect changes to the muscle hydration degree of the body. Excess of muscle humidity was removed with absorbent paper and the sample was weighed in a precision balance (0.001 g). Samples were dried for 72 h in the drying chamber set to 65 °C and then reweighed. The difference between humid and dry weight corresponded to tissue hydration (in percentage).

All data (glucose, osmolality, protein, LDH and tissue hydration) were analyzed individually using an ANOVA (One-Way Analyses of Variance) comparing the results of each variable between the three treatments (25 °C, 35 °C and 45 °C) followed by Tukey's (for glucose) and LSD (for LDH) post hoc tests. Glucose data were log transformed to obtain normality. Both glucose and osmolality outliers were removed from the statistical analysis using the methodology of Wilkinson et al. (1996). Homogeneity was not achieved in proteins concentration, osmolality and tissue hydration so we used the Brown-Forsythe test, with Games-Howell as post-hoc. We had no mortality at the end of the experiment, so no further analysis was performed.

299 3. Results

300 3.1 Desiccation in *Leptuca thayeri* and *Minuca rapax*

301 *Leptuca thayeri* showed a higher rate of water loss than *M. rapax* ($F_{1,75}$=56.516,
302 p<0.001) (Fig. 1A). After 120 min, total water loss in *L. thayeri* was 0.31±0.08 g
303 (9.72±1.53% from starting value), while in *M. rapax* it was 0.26±0.06 g (7.62±1.44%
304 from starting value). Both species had a significant loss over time ($F_{1,83}$=62.284,
305 p<0.001). There was also a significant influence of size (CW in mm) on water loss
306 (figure 1B), with larger animals losing less water over time ($F_{1,83}$=109.583, p<0.001).



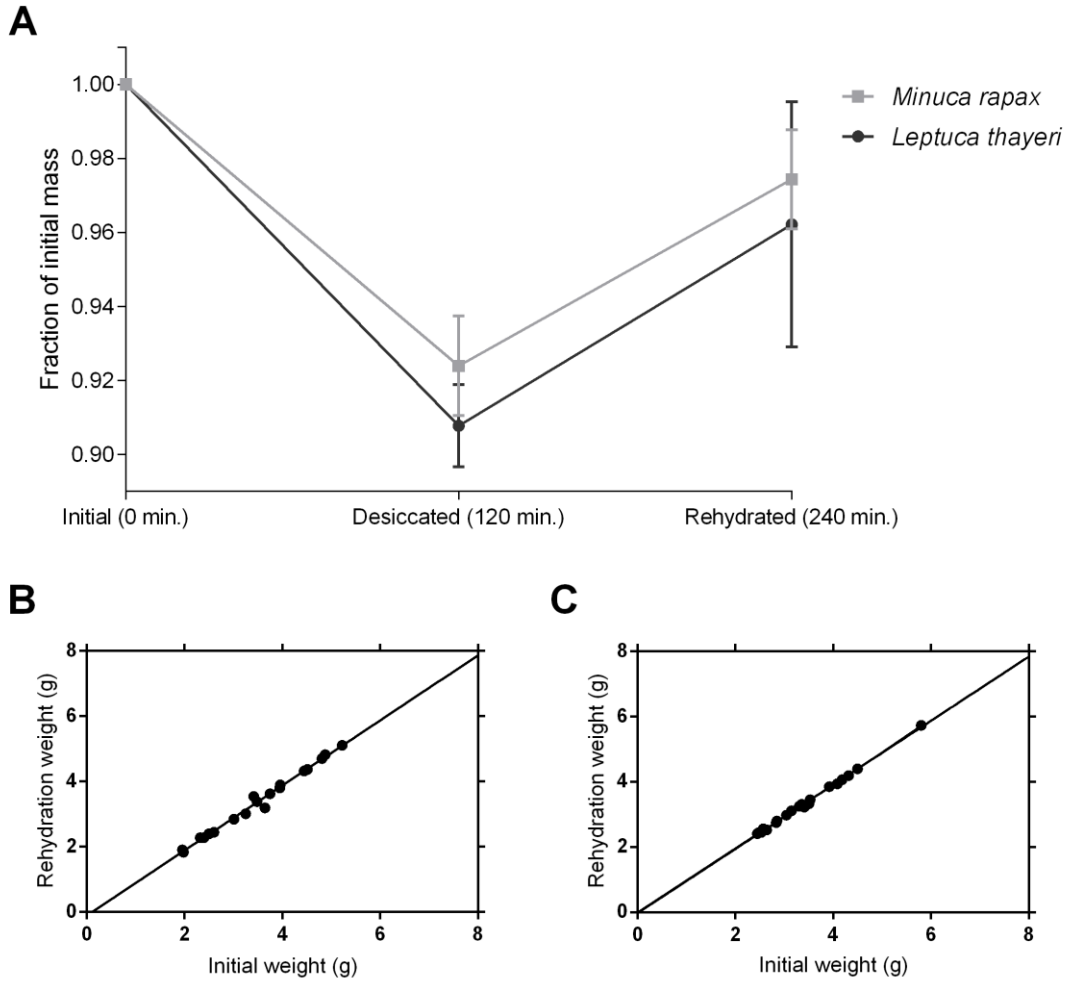
307

308 **Fig. 1. Water loss in *Minuca rapax* and *Leptuca thayeri*.** (A) Loss of water in *M. rapax* and *L. thayeri* as a function of
309 desiccation time. Bars represent standard deviation. (B) Total loss of water as a function of size (CW in mm). *M.*
310 *rapax*, closed circles; *L. thayeri*, open circles; black line is regression for *M. rapax* and grey line is regression for *L.*
311 *thayeri*.

312 Mortality was significantly higher in *L. thayeri* than in *M. rapax* after 120 min of
313 desiccation (X2(1)=10.12, p<0.005). 11 *L. thayeri* individuals died (28.2%) and one *M.*
314 *rapax* individual died (2.6%).

315 3.2 Rehydration in *Leptuca thayeri* and *Minuca rapax*

316 Both *M. rapax* and *L. thayeri* were able to recover almost totally their initial weight
317 after 120 min of rehydration (Fig. 2A). The correlation between initial weight and
318 weight after rehydration was significantly positive for both species (respectively:
319 r=0.999, N=20, p<0.001; r=0.995, N=19, p<0.001) (Fig. 2B and C).



320

321 **Fig. 2. Rehydration in *Minuca rapax* and *Leptuca thayeri*.** (A) Mean fraction (±SD) of initial mass in three time
322 intervals: Initial (time 0); Desiccated (after 120 minutes of desiccation); and Rehydrated (after 120 minutes of
323 rehydration). (B) Weight after rehydration as a function of initial weight in *L. thayeri*. (C) Weight after rehydration as
324 a function of initial weight in *M. rapax*.

325 3.3 Carapace permeability

326 *Leptuca thayeri* presented higher water loss through the carapace when compared to
327 *M. rapax* ($t(10)=2.87$ $p<0.05$). Water loss in *L. thayeri* was 7.02±3.19 mg/h/cm² and in
328 *M. rapax* it was 3.07±1.07 mg/h/cm². In both species the carapace prevented more
329 than 80% of water loss when compared to the control treatment (liquid evaporation
330 only - 36.84±2.63 mg/h/cm² (n=3)).

331 3.4 Effects of temperature increase in *Leptuca thayeri* physiology and survivability

332 There was no mortality in *L. thayeri* due to temperature increase in any of the
333 treatments. Also, there was no effect on hemolymph glucose levels (N=13; $F_{2,36}=1.581$,
334 $p>0.1$) and proteins levels ($F_{2,14.07}=1.280$, $p>0.1$) (Fig. 3). However, there were effects
335 on LDH levels ($F_{2,25}=5.627$, $p<0.01$; 25 °C, N=7; 35 °C, N=11; 45 °C, N=10) with the mean
336 and higher temperatures (35 °C and 45 °C) presenting higher levels of LDH compared

to the lower one (25 °C) (46.62±55.66 UI/mg of protein; $p<0.05$ for both comparisons). Levels of LDH for 45 °C (178.94±146.11 UI/mg of protein) were similar to those from 35 °C (254.17±141.07 UI/mg of protein; $p>0.1$).

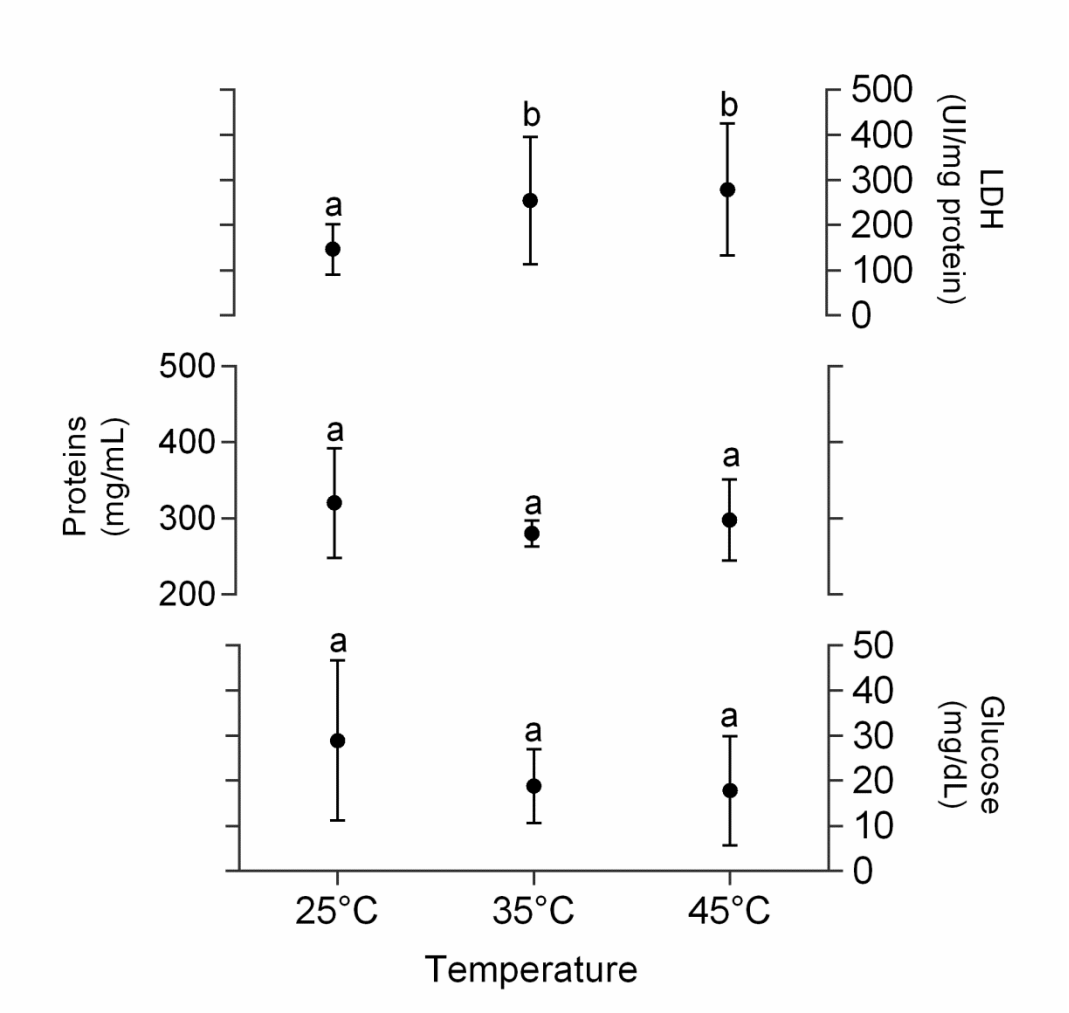
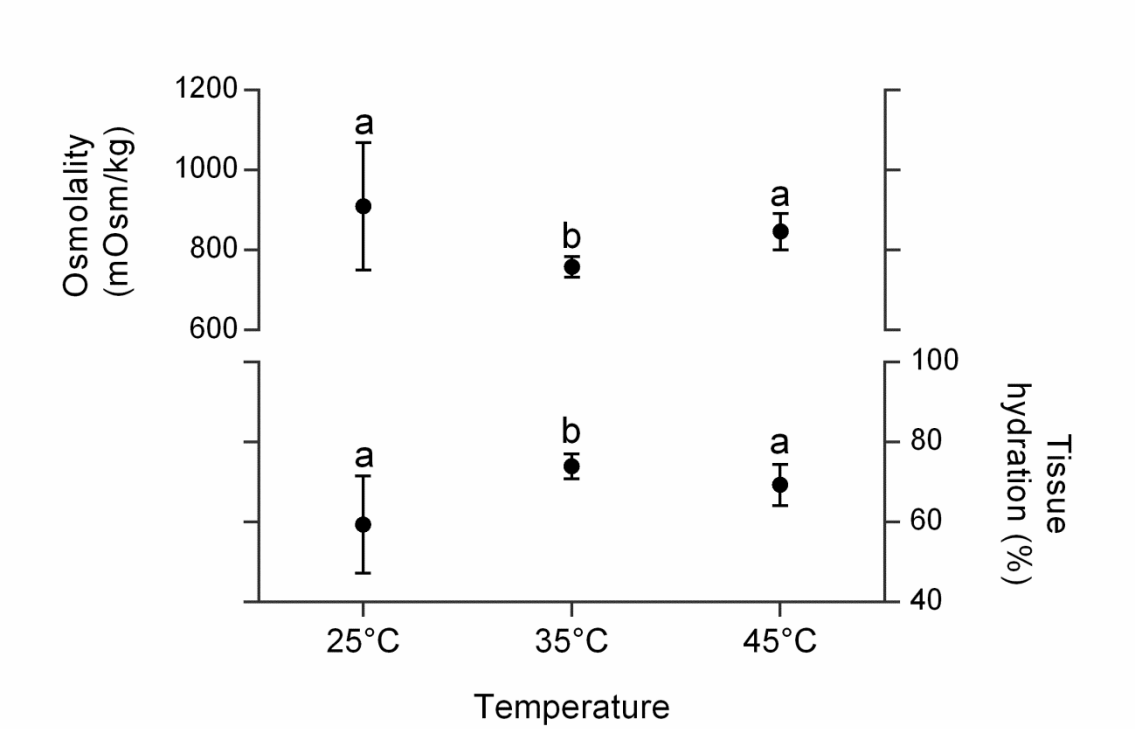


Fig. 3. Responses on glucose, protein and LDH levels on hemolymph of *Leptuca thayeri* exposed to different temperatures. Mean levels of lactate dehydrogenase (top), protein concentration (center) and glucose (bottom). Bars are standard deviation. Different letters represent significant differences between temperature treatments ($p<0.05$).

There were significant differences among temperatures for both osmolality ($F_{2,14.74}=7.887$, $p<0.01$) and major claw tissue hydration ($F_{2,17.96}=11.739$, $p<0.01$) (Fig. 4). In both cases differences occurred between 35 °C and the two other treatments (25 °C and 45 °C). Osmolality levels of organisms submitted to 35 °C were lower (mean 758.42±25.11; N=12) than the others (25 °C mean: 910.23±159.27, N=13, $p<0.01$; 45 °C mean: 846.58±46.18, N=12; $p<0.001$). On the other hand, tissue hydration was higher at 35 °C (73.94±3.08%) than in the other treatments (25 °C mean: 59.41±12.12, N=13, $p<0.005$; 45 °C mean: 69.29±5.15, N=13; $p<0.005$).



353

354 **Fig. 4 Hemolymph osmolality and tissue hydration degree of *Leptuca thayeri* exposed to different temperatures.**
355 Mean levels of hemolymph osmolality (top) and major claw tissue hydration (bottom). Bars are standard deviation.
356 Different letters represent significant differences between temperature treatments ($p<0.05$).

357

358 ## 4. Discussion

359 Exposure to air poses a great challenge to intertidal organisms as it leads to water loss
360 and changes in the metabolism (Allen et al., 2012; Chapman and Underwood, 1996;
361 Miller et al., 2009). Species that inhabit exposed areas and live in the upper tidal zone
362 are expected to better cope with air exposure, as they have adaptations to this harsh
363 environment (e.g. Thurman, 1998; Prusina et al., 2014; Wong et al., 2014). In our
364 study, the restricted species *L. thayeri*, which inhabits muddy and vegetated areas,
365 showed a higher rate of water loss than *M. rapax*, which is usually distributed in more
366 exposed areas in the upper tidal zone. Also, our results confirm that water loss control
367 can be crucial for survivability, as *L. thayeri* showed higher mortality compared to *M.*
368 *rapax*, evidenced by differences on carapace permeability, with *M. rapax* having the
369 less permeable one. However, temperature increase alone was not critical for *L.*
370 *thayeri*, as there was no mortality during exposure to higher temperatures when water
371 and food were available, although there were physiological responses to such increase.
372 These results show that organisms can respond differently to desiccation and
373 temperature elevation.

374 Water loss may cause mortality of organisms in the intertidal zone or reduce their
375 performance, as water is essential to body functioning (Allen et al., 2012; Turra and

Denadai, 2001). With dehydration, metabolism and branchial respiration are compromised, hemolymph osmolality increases and activities are limited (Foster, 1971; McMahon, 1990; Somero, 2002; Williams et al., 2011; Wong et al., 2014). Fiddler crabs are semi-terrestrial and many live in dry and exposed environments, with morphological and behavioral characteristics that enable them to deal with desiccation (Thurman, 1998; Thurman et al., 2013; Yoder et al., 2007, 2005). *Minuca rapax* lost less water when compared to *L. thayeri* and this may help explain its distribution in estuaries. *Minuca rapax* is a generalist species which inhabits sandy and exposed to muddy and shaded areas (Thurman et al., 2013). Higher resistance to desiccation in *M. rapax* may contribute to enhanced flexibility when exploring a variety of habitats. An example of such capability is in the population found by Magalhães and Costa (2007) in the inland state of Minas Gerais, Brazil, more than 200 km away from their original coastal area. This population was not replenished by reproduction, but established itself in a river margin after escaping from an ornamental breeder and even exhibited complex behaviors. In a scenario of climate change the resistance to desiccation in *M. rapax* could have implications for its distribution.

In contrast, *L. thayeri* usually inhabits the mid-tidal zone and, being restricted to muddy and shaded areas (Thurman et al., 2013). Also, it is often found in mangrove forests in areas rich in organic matter (Gusmão-Junior et al., 2012; Checon and Costa, 2017). Many studies showed that species living in the lower tidal zone areas are less resistant to temperature change, and this may be the same for *L. thayeri*. For example, Wong et al. (2014) found that the higher shore barnacle *Tetraclita japonica* had greater tolerance than the low-subtidal *Megabalanus volcano*. The same was found by Prusina et al. (2014) in three species of limpets, the one higher up on the shore the most tolerant. *Minuca rapax* may have adaptations to deal with the higher temperature in the upper tidal zone and this ensured the survivability of organisms after dehydration stress in our experiments. *Leptuca thayeri* had a high mortality rate which was probably due to severe dehydration. Also, for both species there is a straight correlation between crab size and rate of water loss. Larger individuals have a smaller percentage of water loss when compared to small ones. Evaporative water loss can be higher in smaller individuals than in larger ones due to differences in the surface/volume relation, with smaller animals not having as high as a volume than larger ones. Studies showed that larger males are capable of spending more time mating and foraging and exploring dryer areas of the mangrove as they are more resistant to desiccation (e.g. Allen and Levinton, 2014; Levinton et al., 2015).

Differential resistance to water loss and temperature elevation between species from the upper and lower tidal zone are linked to morphological, physiological and behavioral adaptations (Prusina et al., 2014; Stillman and Somero, 1996; Wong et al., 2014). Our study evidences morphological differences between species as water loss through the carapace was lower over time in *M. rapax* than in *L. thayeri*. *Minuca rapax*

values were comparable to those of Thurman (1998). Carapace-related water loss may account for more than 50% of such loss in crabs and morphological adaptations in the integument are crucial for their semi-terrestrial life (Herreid, 1969a, 1969b; Thurman, 1998). In our case the carapace prevented more than 80% of water loss. As evaporative cooling is the main way crabs control temperature (Allen et al., 2012; Thurman, 1998) water loss is essential to regulate body temperature (Darnell et al., 2015; Thurman, 1998; Yoder et al., 2007) but the strategy of losing more or less water depends on species morphology as extreme loss of water may lead to death (Thurman, 1998; Yoder et al., 2007). *Leptuca subcylindrica*, the most terrestrial fiddler crab of south Texas, for example, loses more water than its counterparts (Thurman, 1998). This species has a large branchial chamber that stores water (Rabalais and Cameron, 1985), and this is believed to allow the crab to lose more water through evaporation, keeping its temperature low, but still have water to basic functions like respiration (Thurman, 1998). Also, species adapted to terrestrial habitats have more complex respiratory mechanisms that maximize their respiration on land with less water involved. This is in accordance with Paoli et al. (2015) who found that *Gelasimus vocans* (=*Uca vocans*) has a complex branchiostegal lung which maximizes respiration in terrestrial habitats. Other terrestrial species of fiddler crabs must have even more complex structures than *G. vocans*, which is associated with marine habitats. Thus, there is the possibility that there are differences not accounted for on this study between branchial chambers and their associated structures in *M. rapax* and *L. thayeri* that may also lead to different desiccation rates, but this remains to be studied.

Fiddler crabs build burrows, and by constantly returning to them, desiccation and temperature can be controlled (Levinton et al., 2015) as burrow temperature can be several degrees lower than the outside according to its depth (Powers and Cole, 1976). Also, semi terrestrial crabs assimilate interstitial water from the soil (Crane, 1975; Thompson et al., 1989) and the burrow plays an important role in the process of rehydration as it concentrates water in its bottom (Christy, 1982; Crane, 1975). Our results show that both species can regain water in a similar way, almost completely restoring lost water due to desiccation after 2 h. This implies that, although species dehydrate differentially, they can compensate equally by returning to the burrow, which may be due to other morphological differences than carapace permeability. However, returning to the burrow leaves the crab less time to forage and reproduce. In that way, if a species is more resistant to water loss it will likely make fewer trips back to the burrow and will have more time to spend with other activities. Taking into account the limitations in our study, like the differences between the experimental chamber and the environment, and that in the field organisms return periodically to the burrow, it is possible to infer that the differences on habitat preference for the studied species may be in part due to differential water loss and carapace permeability. Studies on crabs returning to the burrow according to temperature are still lacking and may help to advance this topic.

One of the expected effects of increasing temperature is an increase in metabolic rates (e.g. Resgalla, 2007; Matsumasa and Murai, 2005). In *L. thayeri*, the intermediate and higher temperatures (35 and 45 °C) increased LDH levels in comparison to the control temperature (25 °C). LDH results may indicate an increase in energy demand at higher temperatures, complemented by anaerobic metabolism (Thébault, 1984). Changes in hemolymph osmolality and muscle hydration showed a different pattern from those. Hemolymph osmolality was lowered and degree of muscle hydration increased only in animals exposed to 35 °C. Although the mean temperature for the *L. thayeri* area of occurrence used here is around 25 °C (Pardo et al., unpub. data) it is possible that 35 °C constitutes an optimum temperature to the functioning of enzymes related to osmoregulation and thus is more adequate to the secretion of salts (Freire et al. 2008; Janas and Spicer, 2008; Lucu, 1993). Coherently, the highest temperature (45 °C) reduced salt secretion capacity. Elevated temperatures may affect the viscosity of biological membranes, internalizing or exposing channels and pumps responsible for the transport of ions, thus altering membrane permeability (Novo et al., 2005; Pruitt, 1990). Opposite results of osmolality and muscular hydration degree are consistent because water is mobilized from cells to cope with higher ionic concentration on the hemolymph (Pierce, 1982). Although LDH activity increased at 35 °C and 45 °C evidencing increased energy demand, neither glucose nor protein levels were altered by temperature increase. Glucose may not have been used as the main metabolic substrate to deal with this stress, and the organism may have altered its activity pattern or channeled a lesser percentage of ingested energy into growth, molt and gonadal development (Su et al., 2010; Vernberg, 1998). It is worthy of mention that values of glucose in the control group had higher variability. Although this is not uncommon for this species (e.g. Matsumasa and Murai, 2005), a larger sampling could maybe reduce that variability. Proteins are involved in many physiological activities of the organism, but it is possible that here the use of free amino acids as metabolic substrate was not altered and/or that amino acids were not utilized to control cellular volume, i.e. to cope with osmolality changes (Augusto e Valenti, 2016; Faria et al 2011).Thus, temperature elevation increased only LDH and this may be an adaptation to higher temperatures as energy is produced by anaerobic metabolism. Also, the higher capacity of salt secretion at 35 °C suggests that this species may not necessarily inhabit the optimal habitat concerning osmoregulation. This is not surprising as this species occurs in the northern region of Brazil where air temperatures may be much higher than 25 °C (Thurman et al. 2013). Future studies about thermal limits and thermal preference on *L. thayeri* are necessary to advance on this topic.

5. Conclusions

Environmental stressors play a fundamental role in controlling distribution and

abundance of fiddler crabs (Nobbs and Blamires, 2017) and intertidal organisms in general (e.g. Prusina et al., 2014; Wong et al., 2014). It is clear from our results that the restricted *L. thayeri* has higher rates of water loss compared to the generalist *M. rapax,* what is partially due to morphological adaptations like a less permeable carapace in *M. rapax*. With climate change, organisms will be exposed to different temperatures from the current average (IPCC, 2014; Parmesan and Yohe, 2003), and although physiologically *L. thayeri* is not critically affected by an increase in temperature, this species is expected to experience changes in its distribution and abundance, especially with modifications to vegetation cover that may lead to more desiccation exposure. The same is expected to happen with other species like *L. thayeri* that are currently restricted to wetter mud and shaded areas of the mangrove, while generalist species like *M. rapax* may invade new habitats. With such distributional shifts caused by climate change it is possible to forecast an increase in abundance and density of some species, which will certainly affect community functioning and the ecology of mangroves.

Acknowledgements

We thank Dr. John C. McNamara, Dr. Cleoni S. Carvalho and Msc. Heidi S. M. Utsunomiya for the help with physiological analyses. We are also grateful to Farias-Pardo, J.C.F., De Grande, F.R. and Carvalho, R. for the help with animal collection and experiments. This work was supported by the São Paulo Research Foundation (FAPESP) financial aid granted to TM Costa (grant numbers 2015/50300-6). Tania Costa was supported by the Brazilian National Research Council (CNPq).

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