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High thermal tolerance does not protect from chronic warming – A multiple end-point approach using a tropical gastropod, *Stramonita haemastoma*

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ABSTRACT

Animal physiology and ecology are affected by increasing environmental temperatures, and this is particularly relevant in the tropics, where organisms are already living on the warm edge of their thermal windows. Here, we present data on sub-lethal effects of temperature (using molecular biomarkers), thermal tolerance, warming safety margins and body size shifts of a gastropod (*Stramonita haemastoma*) from tropical rocky shores, under an experimental setup of a climate warming scenario. Heat shock response, protein damage, antioxidant activity and lipid damage were all evaluated once a week during one month of exposure at a control temperature, and at an experimental temperature of plus 3 °C. Significant increase of heat shock protein response, lipid peroxides and catalase at the elevated temperature suggest the activation of cytoprotective pathways as response to an increased thermal load. Duration of exposure also had a significant influence in the animals' responses, since whole body thermal tolerance only showed acclimation potential in the short-term, but not in the long-term. Thermal safety margin was low for this species, suggesting a narrow ability to tolerate further warming. Smaller body sizes were observed in specimens exposed to increased temperature, suggesting the occurrence of slower growth and possible changes in energy metabolism. Hence, enduring thermal stress, as predicted if present day warming trends are not reversed, may compromise populations of tropical marine snails.

1. Introduction

Predicting how organisms and populations respond to climate warming is a foremost concern of global change biologists (Marshall et al., 2015). Rising sea surface temperature (SST) is one of the principal stress factors influencing the physiology, ecology and evolution of marine organisms (Díaz et al., 2015; Nguyen et al., 2011). Extinction risk analyses and models suggest that communities and populations from coastal regions in the tropics are particularly vulnerable to climate warming (Finnegan et al., 2015). In fact, there is significant empirical evidence that marine animals in tropical areas are increasingly susceptible to environmental warming because they have evolved in a relatively stable and non-seasonal environment and the current temperatures they encounter are already close to their thermal limits (Deutsch et al., 2008; Huey et al., 2009; Tewksbury et al., 2008). These limits may be surpassed very soon considering the global average temperature has already risen ca. 0.74 °C over the past century (1906–2005) and is expected to increase between 1.4 °C and 5.8 °C over the remainder of this century (Hartmann et al., 2013; IPCC, 2013).

Shallow water and intertidal ecosystems are prone to enormous thermal variability in time and space (Helmuth and Hofmann, 2001), and therefore ectotherms inhabiting these ecosystems constitute important models to address the impacts of climate change. Since these organisms live at the interface of land and sea, they are subjected both to fluctuating air and aquatic conditions (Helmuth et al., 2010; Rubal et al., 2013). Given the temporal heterogeneity in the velocity of temperature change in such environments (including short-time scales due to tidal cycle and diurnal and nocturnal variations as well as long time-scales due to climate related gradual increase in SST) (Lima and Wethey, 2012), remarkably different scenarios may arise, and selection associated with temperature extremes is expected to play a major role on the thermal biology of species inhabiting these ecosystems (Marshall et al., 2015). Therefore, the physiological responses of such species to physical stress are unique, revealing specific adaptive responses and strategies to deal with changing environmental temperatures (Helmuth and Hofmann, 2001; Somero, 2002). Among invertebrates, gastropods

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in particular are an ideal group to study. They are accessible, numerous, slow-moving, easy to collect and suitable for experimental manipulation (Hammond and Synnot, 1994). Additionally, as an extremely diversified group (Schilthuizen and Rutjes, 2001) with a variety of clades prevailing in harsh intertidal conditions, including tropical rocky shores (Flores Valero et al., 2015; Garrity, 1984), they are also excellent sentinel candidates, prone to noticeable die-off under extreme thermal conditions (Williams and Morritt, 1995).

Vulnerability to thermal stress has been suggested to be associated with the aerobic capacity of the animal, thermal stress unbalancing the oxygen supply versus demand (Deschaseaux et al., 2010). Thus, thermoregulation mechanisms constitute a crucial strategy to ameliorate the impact of stressful temperatures (Harley et al., 2009; Madeira et al., 2012; Pörtner and Farrell, 2008). In particular, the most important mechanisms involved in the maintenance of physiological homeostasis within the temperature range of a species are heat shock proteins, the anti-oxidative system and the mechanisms of alterations of general energetic metabolism (Axenov-Gribanov et al., 2014; Cooke et al., 2014; Farrell, 2009; Feder and Hofmann, 1999; Pörtner and Farrell, 2008; Somero, 2010). These defense mechanisms allow an organism to overcome the negative consequences of thermal stress, including the accumulation of free radicals, protein degradation and energy depletion (Abele et al., 2002; Hofmann and Somero, 1995; Tomanek, 2010). In this sense, studying the activation of cellular and biochemical stress responses can be a critical point for defining the thermal window and thermal tolerance of a species (Deschaseaux et al., 2010). These biological responses can be used as stress biomarkers providing early warning signals of environmental deterioration and indicating occurrences of adverse ecological consequences (Wu et al., 2005). It is crucial that under such context, biomarkers' initial induction, maximum induction, adaptation and recovery are fully studied and understood (Wu et al., 2005) so that they can be effectively applied in bio-monitoring programs at field sites.

The main aim of the present study was to investigate the thermal physiology of a tropical Atlantic rocky reef gastropod species, *Stramonita haemastoma*, throughout one month. This species is widely distributed throughout tropical areas in the Southeast Pacific, Atlantic Ocean and the Mediterranean (Houart and Gofas, 2009). It inhabits all kinds of hard substrates (Sept, 2016) and it is commonly found in shallow subtidal waters and inside tide pools during low tide, making it an ideal species to study the impact of temperature change in physiology. In particular, the objectives of the present study were to: i) assess sub-lethal effects of increased temperature at a molecular and cellular level by analyzing biomarkers of biological effects (heat shock protein 70 kDa – Hsp70, ubiquitin – Ub, catalase – CAT, lipid peroxides – LPO, glutathione-S-transferase – GST, superoxide dismutase – SOD and acetylcholinesterase – AChE) in foot muscle tissue; ii) estimate the upper thermal limits (Critical Thermal Maximum – CTMax), thermal safety margins (warming tolerance) and acclimation capacity of thermal tolerance of this species, as well as its intraspecific variation, under control (mean summer water temperature at the collection site and time – 29 °C) and warming scenarios (future mean summer water temperature – 32 °C); iii) analyze body size shifts along time under the different temperature conditions and iv) evaluate the dynamics of time-scales of the responses in a context of constant thermal fluctuations (i.e. rocky intertidal) vs gradual temperature increase due to climate change.

2. Materials and methods

2.1. Ethical statement

This study complied with all the ethical guidelines for animal experimentation, namely Portuguese and Brazilian legislation (Decreto-Lei n° 113/2013 and Lei n° 11.794/2008, respectively), and experimental protocols were approved by Direcção Geral de Alimentação e Veterinária (Portuguese licensing body, authorization document 0421/000/000/2013) and by Comissão de Ética no Uso de Animais (USP – Ribeirão Preto, Brazilian licensing body, authorization document 13.1.981.53.7). The study design followed ARRIVE guidelines and three authors have level C FELASA certification (Federation of European Laboratory Animal Science Associations).

2.2. Test species, specimen collection and acclimation procedure

Gastropod mollusk *Stramonita haemastoma* (Linnaeus 1767, Muricidae) was selected as biological model. Snails were sampled from mid to low tide pools (< 2 m from the water line and < 0.5 m in elevation over basaltic rock formations) in the rocky platform zone of Barequeçaba Beach, São Sebastião, located in the southeastern coast of Brazil (23°49′42″S, 45°26′29″W). The local thermal environment was assessed during 3 months of the warm season (December 2015 to February 2016) by i) analyzing data from the Brazilian National Meteorology Institute (www.inmet.gov.br) for air temperature and ii) collecting in situ data with Hobo V2 probes (continuous sampling over 56 days at 2 h intervals) for tide pool temperature (eight probes in eight replicate pools sampled) and for subtidal water temperature (two probes, ~1 m depth). Adult animals were collected by hand (total n = 120, mean ± SD total length = 3.54 ± 0.76 cm, mean ± SD total weight = 9.32 ± 5.35 g) and placed in plastic containers with aerated sea water. The total number of animals collected was higher than the determined sample sizes, allowing for accidental death during transport and acclimation to captivity conditions. Animals were taken to experimental facilities at the CEBIMar – Universidade de São Paulo (São Sebastião), and allowed to acclimate at 29.0 °C ± 0.5 °C for two weeks. They were fed once a day ad libitum with frozen shrimp (Costa Sul Congelados®).

2.3. Experimental design

Chronic heat stress experiments were performed following a scenario of increased summer SSTs in rocky shore subtidal shallow waters, where *S. haemastoma* seeks thermal refugia during high summer temperatures. Gastropods were subjected to either one of the following temperature treatments for one month: i) current mean summer water temperature at the collection site/time (control, 29 °C) and ii) future mean summer water temperature (32 °C) according to IPCC regional projections of the SST anomaly for the tropical/subtropical Atlantic (+3 °C at the end of this century in the RCP8.5 scenario (IPCC, 2013)) (see Fig. ESM1 in Supplementary material). The experimental trials in captivity were performed in two separate semi-open aquaria systems of 200 L each. Each of the systems consisted of 2 replicate glass tanks (25 × 25 × 25 cm), one seawater deposit, one sump and a UV filter. Specimens were randomly allocated into tanks (n = 30 individuals.tank^{-1}), which were then randomly attributed to control and temperature treatment (2 replicate tanks per temperature). Temperature treatment of 32 °C was attained by gradually raising water temperature from 29 °C at a rate of 0.10 °C.h^{-1} during two days. All tanks were filled with clean aerated seawater (95–100% oxygen saturation) and salinity 35. Live rocks were used as environmental enrichment. Temperatures were maintained for 28 days using thermostats (Eheim® Jager Heater 150W, Germany). Experiments were carried out in shaded day light (14L:10D).

2.4. Molecular biomarkers

Molecular biomarkers have been unanimously adopted as early warning signals of individuals' physiological stress (Wu et al., 2005). They are defined as any measurable molecular response (Colin et al., 2015) and have significantly contributed to the mechanistic understanding of how stressors affect organisms (Kidd et al., 2007). Timing of samplings for biomarker analyses was chosen based on OECD