Marine Environmental Research 141 (2018) 148–158

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Thermal stress and energy metabolism in two circumtropical decapod crustaceans: Responses to acute temperature events

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ARTICLE INFO

Keywords:
Decapod crustaceans
Marine heat waves
Thermal stress
Integrated biomarker index
Energy reserves
Tropical Indo-Pacific

ABSTRACT

Extreme events associated with global warming, such as ocean heat waves, can have contrasting fitness consequences for different species, thereby modifying the structure and composition of marine communities. Here, we examined the effects of a laboratory simulated heat wave on the physiology and performance of two Indo-Pacific crustacean species: the shrimp *Rhynchocinetes durbanensis* and the hermit crab *Calcinus laevimanus*. We exposed the crustaceans to a control temperature or to a +5 °C temperature (25 °C vs 30 °C) for two consecutive weeks, and weekly analyzed protective proteins, antioxidant activity, and lipid peroxides in muscle and visceral mass. Fulton's K, total protein, %C, and C:N molar ratio of muscle tissue were also analyzed at the end of the experiment. Results showed that 1) the most responsive tissues were the muscle in the shrimp species and the visceral mass in the hermit crab species; 2) biomarker responses in both species occurred mostly after 7 days of exposure; 3) temperature stress led to an increase in biomarker levels; 4) highest biomarker fold-changes were detected in protective chaperones and antioxidants superoxide dismutase and glutathione-S-transferase; 4) integrated biomarker indices suggested poorer health status in individuals subjected to the heat wave; 5) performance changes at the organism level were only detected in *R. durbanensis*; and 6) mortality rates of both species remained unchanged with the heat wave. Finally, we concluded that these species are capable of physiological adjustments in response to rapid environmental changes, which ultimately confers them with enough thermal tolerance to withstand this simulated heat wave without major consequences for fitness.

1. Introduction

A body of evidence is building up concerning global increases in extreme weather and climatic events (EWCEs), including the occurrence of heat waves. Global future projections show that warmer and more frequent days above 35 °C (maximum air temperature), as well as increased frequency of heat waves, have a 90%–99% probability to occur (IPCC, 2014). Maximum daily temperatures have risen 2.3 °C worldwide between 1981 and 2000, and projections estimate an additional 3.5 °C for 2081–2100 (Kharin et al., 2007). The intensity and duration of these extreme events are also expected to increase as global warming continues (Goodess, 2012). Because EWCE events are key drivers of marine biodiversity patterns (Wernberg et al., 2016), addressing how such episodic events affect species performance is critical to further develop predictive models of species distribution and go beyond their current basis on gradual warming trends (Ummenhofer and Meehl, 2017).

The Indo-Pacific region has been ground of some record-breaking heat waves in a very recent past (2013–2015). For instance, SST (sea surface temperature) data showed anomalies exceeding three standard deviations in consecutive years in the north Pacific (Di Lorenzo and Mantua, 2016). In 2015–2016, the Indian and Pacific oceans experienced extremely high tropical SSTs associated to an El Niño event, which resulted in the third massive coral bleaching event recorded in history, spanning from Australia to Southeast Asia (NOAA, 2015). In the Western Indian ocean, mainly along the African coastal line, the magnitude of heat waves has also increased since 1996 in temperature, days, and extension area (Ceccherini et al., 2017). Finally, warm pools in the Indian and Pacific oceans are also expanding, with areas between 29 °C and 30 °C showing the largest increase rates at interdecadal timescales (Lin et al., 2011, 2013).

Marine organisms exposed to extreme or unusual environmental

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https://doi.org/10.1016/j.marenvres.2018.08.015
Received 18 April 2018; Received in revised form 22 August 2018; Accepted 25 August 2018
Available online 27 August 2018
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List of abbreviations:

Abs	Absorbance
AChE	Acetylcholinesterase
ACTI	Acetylthiocholine iodide
ANOVA	Analysis Of Variance
AOX	Antioxidant enzymes
ARRIVE	Animal Research: Reporting In Vivo Experiments
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
C:N	Carbon:Nitrogen
CAT	Catalase
CNDB	1-chloro- 2,4-dinitrobenzene
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EWCE	Extreme Weather and Climatic Events
GST	Glutathione-S-Transferase
HRP	Horseradish peroxidase
HSD	Honest Significant Difference
Hsp	Heat Shock Protein
IBR	Integrated Biomarker Response index
LPO	Lipid Peroxides
MDA	Malondialdehyde
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NAPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NBT	Nitroblue Tetrazolium
PBS	Phosphate Buffer Saline
PCA	Principal Components Analysis
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SST	Sea Surface Temperature
TBARS	Thiobarbituric Acid Reactive Substances
XOD	Xanthine Oxidase

changes can have different responses, resulting in varying outcomes for their populations. If environmental changes are physiologically tolerable (i.e. animals have the ability to adjust their physiology to maintain homeostasis), individual acclimation and/or adaptation over generations can occur. If environmental changes are physiologically intolerable, migration or death might follow (González-Ortegón et al., 2013), with consequent re-distribution of species. Ectothermic animals are especially vulnerable to extreme temperatures and may therefore experience severe performance decrements during a heat wave event. Nevertheless, even ectotherms are able to modulate metabolic mechanisms and behavior to some extent to maintain physiological functions, cell survival, and avoid organ failure (Feder and Hofmann, 1999; Logan and Somero, 2011; Madeira et al., 2016).

One of the main effects of increased temperature at a cellular level is membrane leakiness, which results in higher ATP demand and exacerbates the use and need for ATP under heat stress. Ultimately, this leads to a mismatch between the oxygen demand and supply system (Pörtner and Knust, 2007), which decreases the efficiency of aerobic metabolism. The higher production of reactive oxygen species (ROS) in such conditions exacerbates cellular oxidative stress, leading to macromolecular damage, e.g. to protein structure (Hofmann and Somero, 1995; Kregel, 2002), and DNA (Kültz, 2004). When the damage extension rises above a certain threshold, cell apoptosis and tissue necrosis can occur (Mukherjee, 2008; Madeira et al., 2014b). As a consequence, organisms may down-regulate their aerobic energy metabolism whereas other metabolic pathways are activated to respond to increased oxidative stress, or organisms may switch metabolic energy fuels (Tomanek, 2014). Specifically, cytoprotective pathways include: 1) management of damaged/denatured molecules, through increased production of heat shock proteins and proteolysis via the ubiquitin-proteasome pathway (Hofmann and Somero, 1995; Halpin et al., 2002; Tomanek and Somero, 2002; Hofmann, 2005; Madeira et al., 2012), 2) regulation of oxidative stress and detoxification of toxic byproducts by increasing antioxidant enzyme activity (with ROS scavenging function) (Lushchak, 2011; Madeira et al., 2013, 2014a; 2016; Vinagre et al., 2014), 3) shifting from pro-oxidant NADH producing and oxidizing pathways to antioxidant NADPH producing and consuming pathways (Wegener, 1988; Alberts et al., 2002; Tomanek, 2014).

In this study, we explored the physiological responses of two Indo-Pacific crustacean species: the camel shrimp, *Rhynchocinetes durbanensis*, and the orange and black hermit crab, *Calcinus laevimanus*. In order to determine their vulnerability to heat wave events and its relation to molecular pathways of cytoprotection and acclimation, we evaluated these species' thermal sensitivities, their ability to sustain performance, and survive periods of severe environmental stress. Decapod crustacean species are important components of circumtropical coastal habitats, not only in terms of diversity and biomass but also as key organisms in the trophic dynamics and nutrient cycling of these systems (Abele, 1974; Grilo et al., 2011; Pachelle et al., 2016). If heat waves affect these organisms' health, growth, or survival, there may be unforeseen functional consequences for coastal populations and communities. In particular, we aimed to: 1) compare these species' thermal stress responses at two ecologically relevant experimental temperatures (control and heat wave simulation), using a set of selected thermal, oxidative, neurotoxicity, and energy metabolism biomarkers; 2) perform a comprehensive and comparative animal health assessment between control and heat wave conditions, based on integrated biomarker indices to understand the role of thermal induced stress in physiological or health impairments; and 3) analyze each species performance at control and extreme temperature by measuring body condition and mortality.

2. Materials and methods

2.1. Test organisms and acclimation procedure

Two circumtropical crustacean species commonly found in the Indo-Pacific region were used in this study: the camel shrimp *Rhynchocinetes durbanensis* (Gordon, 1936), and the orange and black hermit crab *Calcinus laevimanus* (Randall, 1840). Both species inhabit reef areas, either in biogenic (coral) reefs or rocky platforms. *R. durbanensis* is a subtidal species, living at depths from 2 to 40 m (Weinberg, 2005), whereas *C. laevimanus* is both intertidal and subtidal, and can be found at any depth from 0 to 95 m (Markham, 2003). Adult specimens (n = 30 individuals for each species; 17 males and 13 females of *R. durbanensis,* whereas for *Calcinus laevimanus* gender determination was not possible) were obtained from Opérculo, Lda® ornamental aquaculture company (Guincho, Portugal). The use of captive-bred animals is justified based on the necessity of having knowledge and control on the animals' thermal histories, thereby eliminating carry over effects that may occur when early larvae or juvenile stages are exposed to varying thermal conditions. Upon arrival at the laboratory, animals were placed in one indoor re-circulating water system (total volume of 2000 L), comprised of four 70 L polyvinyl tanks supplied with aerated sea water, one sump, an external skimmer, and an UV filter. Crustaceans were haphazardly distributed across tanks (n = 7–8 individuals of each species per tank) and allowed to acclimate at 25.0 ± 0.5 °C for 2 weeks.

2.2. Experimental design and sampling

All experimental procedures were approved by Direcção Geral de Alimentação e Veterinária (DGAV) following ethical guidelines in national and international legislation. Study design followed ARRIVE guidelines and recommendations by the Federation of European Laboratory Animal Science Associations (see Festing and Altman, 2002; Hau and Schapiro, 2010). Common-garden experiments are the gold-standard design to separate environmental/laboratory controlled effects from possible differentiation among populations and animals of a single species (Gaitán-Espitia et al., 2017). Therefore, the experiments consisted of two temperature treatments using common garden conditions: 25.0 °C ± 0.5 °C (control – temperature at which the animals were reared, corresponding to mean temperatures in the Indo-Pacific region outside warm-pool areas, as shown by satellite data in www.seatemperature.org) and 30.0 °C ± 0.5 °C (simulating a +5.0 °C heat wave event), with the duration of two weeks and samplings at 0, 7, and 14 days. The duration of the experiment was based on the following rationale: present duration of heat waves in the Indo-Pacific is on average 5–7 days, but time-series trends show an increase in heat wave duration of about 1.3 days.decade^{-1} from 1961 to 2013, and if this trend is to be continued, future heat waves by the end of this century are expected to last for up to two weeks (Zinke et al., 2015; Rohini et al., 2016; Ceccherini et al., 2017). To simulate the heat wave, we used a stepwise method to increase temperature in the treatment group. At day zero, temperature was increased from 25.0 °C to 27.0 °C. After 24 h at 27.0 °C, temperature was increased further up to 30.0 °C and then kept for two consecutive weeks. Then, on the last day, temperatures were lowered again to 25.0 °C. Animals in the control treatment were kept at 25.0 °C throughout the 14-day period. This procedure follows Collier and Waycott (2014) and Arambourou and Stoks (2015) for heat wave simulation experiments. Each treatment had 2 replicate tanks (35 × 35 × 55 cm each), with 7–8 individuals of each species per tank. All tanks were provided with a thermostat heater (ELITE 200W). To keep environmental parameters constant throughout the experiment, a monitoring scheme was employed. Each tank was provided with a Petco thermometer with suction cup to continuously monitor temperature. Other parameters, such as salinity (kept at 35), pH (kept at 8 ± 0.01), ammonia (kept below 0.25 mg L^{-1}), and nitrites (kept under 0.3 mg L^{-1}), were monitored twice a week using a hand-held refractometer (Atago, Japan), a digital pH probe (model HI9025, Hanna Instruments, USA), and Tetra Test Kits (Tetra Ammonia Test Kit and Tetra Nitrites Test Kit, USA), respectively. Animals were fed twice a day with fresh food (mixture of shrimp and mussels in proportion 1:1) and dry food (Tropical Super Spirulina Forte Mini Granulat), except in sampling days as animals were not fed in the prior 24 h. Environmental enrichment consisted of adding sand, live rocks, and seaweed *Caulerpa taxifolia* to the tanks. Mortality was recorded every day in each tank. At each sampling day, 5 animals of each species from each treatment were chosen randomly (n = 5 individuals × 3 timepoints × 2 temperatures = 30), sacrificed by longitudinal transection, and then measured and weighed. Internal organs (muscle and visceral mass) were extracted and frozen at −80 °C until further analyses.

2.3. Biochemical biomarkers

2.3.1. Total protein extraction and quantification

Tissue samples from muscle and visceral mass (~200 mg) were homogenized in 1 mL phosphate buffered saline (PBS) solution (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) using a Tissue Master 125 homogenizer (Omni International, Kennesaw, USA) on ice-cold conditions. Homogenates were then centrifuged at 4 °C for 15 min at 10,000 × g. The supernatant was collected, transferred to new microtubes (1.5 ml) and frozen immediately (−80 °C). Aliquots from these homogenates were then used in all biomarker procedures.

Total protein determination was performed according to Bradford (1976): Bradford reagent (Comassie Blue G250, methanol, phosphoric acid, distilled water) was added to three replicates of each sample in a 96-well microplate. Absorbance was read at 595 nm in a microplate reader (BIO-RAD, Benchmark, USA). BSA standards were used for a calibration curve (0–4 mg).

2.3.2. Thermal stress proteins quantification

Heat shock protein 70 (Hsp70) and total ubiquitin (Ub) were quantified using an indirect Enzyme Linked Immunosorbent assay (ELISA) and a direct ELISA (respectively, and following Madeira et al., 2014a). Briefly, in both procedures, samples (three replicates of each) were incubated in 96-well plates overnight at 4 °C. On the next day, microplates were washed in PBS 0.05% Tween-20 and blocked with 1% BSA (Sigma-Aldrich, USA) in PBS and incubated at 37 °C for 90 min. Microplates were then washed again with PBS. The primary antibodies (anti-Hsp70/Hsc70, Acris, USA, for Hsp70 and Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA, for total ubiquitin) were then added to the samples and microplates were again incubated for 90 min at 37 °C. After another washing stage:

a) For Hsp quantification, the secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was added to each well followed by incubation at 37 °C for 90 min. After the washing stage, the substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma- Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Stop solution (3 N NaOH) was added to each well and the absorbance was read at 405 nm.
b) For ubiquitin quantification, the substrate (TMB/E, Temecula California, Merck Millipore) was added to each well and incubated for 30 min at room temperature. Stop solution (1 N HCl) was added to each well and the absorbance was read at 415 nm.

For quantification purposes, calibration curves were constructed using serial dilutions of purified Hsp70 active protein (Acris, USA) and of purified ubiquitin (UbpBio, E−1100, USA), respectively, to give a range from 0 to 2 μg mL^{-1} of protein.

2.3.3. Oxidative stress enzymes

The enzymatic assay of catalase (CAT; Enzyme Comission number - EC 1.11.1.6) was carried out according to procedures described elsewhere (Aebi, 1983; Beers and Sizer, 1952; Li and Schellhorn, 2007). Samples were placed in microplates' wells, followed by assay buffer (100 mM potassium phosphate), pure methanol and 0.035 M hydrogen peroxide, and incubated for 20 min in the shaker. Potassium hydroxide 10 M and Purpald 34.2 mM were then added and samples were left to incubate again for another 10 min in the shaker. Activity from a standard bovine catalase solution of 1523.6 U mL^{-1} was used as a positive control. Formaldehyde standards were used to produce a calibration curve. Catalase activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C.

The enzymatic assay of glutathione-S-transferase (GST) activity (EC 2.5.1.18) was adapted from Habig et al. (1974) and optimized for 96-well microplates. After placing the samples in the microplates' wells, a reagent mix containing: 200 mM reduced L-glutathione, 100 mM CDNB (1-chloro- 2,4-dinitrobenzene) and buffer Dulbecco (Sigma Aldrich, USA) was added to each sample. The substrate CDNB was used to react with the enzyme. After reading the absorbance at 340 nm GST activity was calculated using a molar extinction coefficient for CDNB of 5.3 mM (mM^{-1}. cm^{-1}) after correction for the microplate's wells path length.

The enzymatic assay of superoxide dismutase (SOD) activity (EC 1.15.1.1), using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), was carried out according to Sun et al. (1988). Briefly, samples

were placed in the microplates' wells and a reagent mix containing EDTA 3 mM, xanthine 3 mM, NBT 0.75 mM and XOD 100 µm was added to each well. Negative controls of mix without sample were also included. After reading the absorbance at 560 nm, SOD activity was calculated using the equation for the % inhibition:

$$\frac{Abs560/\min\ negative\ control\ - Abs560/\min\ sample}{Abs560/\min\ negative\ control} \times 100$$

2.3.4. Oxidative damage products – lipid peroxidation

The lipid peroxides (LPO) assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978). Monobasic sodium phosphate buffer (50 mM) was added to each sample, followed by SDS 8.1%, trichloroacetic acid (20%, pH = 3.5), and thiobarbituric acid (1%). Milli-Q grade ultrapure water was added to each mixture and microtubes were then put in a vortex for 30 s and incubated in boiling water for 10 min. To stop the reaction, microtubes were placed on ice for 10 min. Duplicates of the supernatant of each reaction were put into a 96-well microplate and absorbance was read at 530 nm. To quantify the lipid peroxides, an eight-point calibration curve (0–0.3 M TBARS) was calculated using malondialdehyde-bis(dimethylacetal) (MDA) standards (Merck Millipore, Portugal).

2.3.5. Nervous system enzyme

Enzymatic assay of acetylcholinesterase (AChE; EC 3.1.1.7) was performed using thiol quantification based on Ellman's method (Ellman et al., 1961). A reagent mix containing sodium phosphate buffer 50 mM (pH 8.0), 1 mM Ellman's reagent (DTNB – 5,5′-dithiobis-(2-nitrobenzoic acid)) in 50 mM phosphate buffer and 75 mM ACTI (acetylthiocholine iodide) in phosphate buffer 50 mM was added to each sample in a 96-well microplate. Negative controls were included. Absorbance was read at 415 nm each minute for 10 min. AChE activity was calculated using a molar extinction coefficient for DTNB of 0.00781εmM.

2.3.6. Energy biomarkers

The C:N (carbon:nitrogen) molar ratio was used as a proxy for changes in energy metabolism/reserves, and % C (carbon) was used as a proxy for lipid reserves (following Sterner and Elser, 2002). Muscle pellets were lyophilized (by freeze-drying in vacuum) and grounded to a fine homogeneous powder. Samples of ~0.5 mg were loaded into tin cups and analyzed using an Elementar Isoprime continuous-flow mass spectrometer (GV Instruments) coupled to a vario PYRO cube elemental analyser (Elementar, Hanau, Germany). Reference materials (acetanilide; Stable Isotope Research Facility, Indiana University, USA) were assayed at the beginning of each run and after every 10 samples. Additionally, total protein (as described previously) was also analyzed as a proxy of energy reserves.

2.4. Data analysis

Stress biomarker data were assessed for normality through Shapiro-Wilk's test, homoscedasticity through Levene's test and outliers were assessed graphically. When assumptions (normal distribution and homoscedasticity) were not met, either a data transformation was performed or a non-parametric test was used. Factorial ANOVAs and post hoc Tukey HSD tests (whenever the null hypotheses were rejected) were carried out to detect: 1) which biomarkers (thermal, oxidative or neurotoxic stress) contributed to differentiate between temperature treatments (25 °C vs 30 °C) and exposure times (T0, T7, T14); 2) differences between biomarker levels throughout the experiment at different temperatures; and 3) differences in the responses between females *vs* males and interactions between sex and temperature (only for *R. durbanensis*).

In order to integrate results from these biomarkers and understand global/general responses, the Integrated Biomarker Response (IBR) index was calculated according to Beliaeff and Burgeot (2002). In brief, IBR was calculated by summing up triangular star plot areas calculated for each two neighbouring data. The general mean (m) and the standard deviation (s) of all data (including all sampling times) for a given biomarker was calculated, followed by a standardization to obtain Y, where $Y = (X - m)/s$, and X is the mean value for the biomarker at a given time. Then Z was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponding respectively to an inhibition or a stimulation. The score (S) was calculated by $S = Z + |Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated Y in a given biomarker at all measurements made. Star plots were then used to display Score results (S) and to calculate the integrated biomarker response (IBR) as:

$$IBR = \sum_{t=1}^{n} Ai$$

$$Ai = \frac{Si}{2} \sin\beta (Si \cos\beta + S_{i+1} \sin\beta)$$

$$\beta = \tan^{-1}\left(\frac{S_{i+1} \sin\alpha}{S_i - S_{i+1} \cos\alpha}\right)$$

where Si and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot; Ai corresponds to the area the connecting two scores; n the number of biomarkers used for calculations; and $\alpha = 2\pi/n$. Lower or higher index core values can be translated into the impact of high temperature on organisms: higher index core values are indicative of a poorer health status (stressed organisms). IBR values were used to compare intensity of responses between the two species and between tissues in each species. Principal Component Analysis (PCA) was also carried out for each species to detect biomarkers that correlate with temperature, time, and tissue, and that contribute to explain the variance in the dataset.

To calculate body condition of the animals, Fulton's K was determined from the morphometric data as follows:

$$K = 100 M_t / L_t^3$$

where M_t is the total wet mass (mg) and L_t is the total length (mm) (Ricker, 1975). Student t-tests were employed to detect differences in condition index, as well as energy reserves (total protein, %C, and C:N molar ratio) between treatments at the end of the heat wave event simulation. Relationship between %C and LPO extension at 30 °C in the muscle was also tested by Pearson correlation in both species. No statistical tests were applied to mortality because there were no dead specimens to report for both species. All statistical analyses were performed using the software Statistica v8 (StatSoft Inc., USA). All results were considered to be statistically significant at p-value < 0.05.

3. Results

3.1. Thermal, oxidative and neurotoxic stress biomarkers

Three out of seven biomarkers here tested were affected by temperature in the muscle of *R. durbanensis* (Hsp70, CAT, GST), while none of the biomarkers showed significant responses in the same tissue in *C. laevimanus* (Table 1). On the other hand, while only two out of six biomarkers responded in *R. durbanensis* visceral mass (Hsp70, Ub), five out of six biomarkers responded in this tissue in *C. laevimanus* (all except for SOD, Table 2). Time-dependent differences between temperatures (tested as temperature-time interactions) showed identical results when testing temperature alone. The exception was *R. durbanensis* visceral mass, where Hsp70 did not show significant interactive differences despite differences in temperature alone, and GST that did not show differences when testing temperature alone, but recorded significant changes when testing interactions with time (Table 2).

While levels of Hsp70 (protein unfolding biomarker) increased after 7 days of thermal challenge in both species (3.2-fold in muscle in *R.*

Table 1
Factorial ANOVAs results in muscle tissue of a) *Rhynchocinetes durbanensis* and b) *Calcinus laevimanus*. Effect of temperature (25 °C vs 30 °C) and its interaction with time (T0, T7, T14) on biomarker levels (thermal, oxidative and neurotoxic stress). Significant results (p-value < 0.05) are presented in bold.

	a)				b)			
	Temperature		Temp × Time		Temperature		Temp × Time	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Hsp70	**4.88**	**0.036**	**14.03**	**< 0.001**	0.40	0.528	0.72	0.493
Ub	0.66	0.423	1.00	0.382	0.01	0.967	0.01	0.992
CAT	**12.51**	**0.001**	**6.10**	**0.007**	0.02	0.869	0.01	0.993
LPO	0.00	1.00	0.07	0.929	0.00	0.984	0.00	0.999
GST	**5.35**	**0.029**	**13.33**	**< 0.001**	0.04	0.829	0.12	0.879
SOD	0.47	0.496	0.14	0.868	0.63	0.432	0.22	0.800
AChE	3.42	0.076	1.21	0.315	0.18	0.671	0.46	0.632

durbanensis and 1.3-fold in visceral mass in *C. laevimanus*, Fig. 1A, D), Ub (protein denaturation biomarker) showed significant increases both after 7 (1.6-fold and 2.3-fold for each species, Fig. 1C and D, respectively) and 14 days (2.6-fold only in *C. laevimanus*, Fig. 1D) of thermal challenge, but these changes were only observed in the crustaceans' visceral mass. Focusing on AOX (antioxidant enzymes), GST, and SOD showed significant increases at T7 in *R. durbanensis* (0.6-fold and 2.8-fold, in muscle and visceral mass, respectively, Fig. 1A, C), while CAT showed a significant decrease in the muscle of this species (−0.7-fold, Fig. 1A), also at T7. AOX enzymes in *C. laevimanus* only showed significant time-dependent changes with temperature in visceral mass (Fig. 1D). SOD increased significantly after 7 days (2.0-fold), while GST increased only after 14 days (5.3-fold). CAT showed a significant increase in fold-change at all timepoints (2.9-fold in T7 and 2.8-fold in T14). Considering oxidation products arising from oxidative stress, LPO only showed a significant fold-change (4.2-fold) in T7 in *C. laevimanus*'s visceral mass. No significant changes were detected for AChE (neurotoxic stress marker).

Regarding variations in the responses to thermal stress between females and males (*R. durbanensis* only), ANOVA results only showed significant differences between sexes in the % of SOD inhibition (F = 3.356, p-value = 0.042). All the other biomarkers analyzed were not affected by gender. Interactions between sex and temperature tested by Wilk's test were non-significant.

In summary, *R. durbanensis* showed: 1) a pattern of biomarker increase after 7 days of exposure to a higher temperature, but no response was detected after 14 days of exposure (in none of the tissues) – different biomarkers responded in each tissue, but the timing of responses was similar; 2) more biomarkers showing significant changes in muscle tissue when compared to visceral mass, although the magnitude of fold-changes was higher in the latter; 3) Hsp70 in the muscle and SOD in visceral mass were the biomarkers with highest fold-changes; and 4) significant biomarker fold-change variation (between −0.7-fold and 3.2-fold). Finally, *C. laevimanus* results showed that: 1) muscle was irresponsive to the thermal challenge, whereas the visceral mass showed consistent biomarker significant increases after being exposed to a higher temperature (both after 7 and 14 days); 2) biomarkers Ub and CAT responded in all timepoints tested; and 3) all biomarkers that responded significantly showed positive fold-changes (that varied from 2.0-fold to 5.3-fold), and the highest fold-change was detected in GST.

3.2. Integrated biomarker responses (IBR) index

IBRs were first individually calculated for each tissue type and each timepoint (for both species, Fig. 2). For *R. durbanensis*, results showed that index values varied along time in muscle tissue, being higher at 30 °C in earlier timepoints (T0 and T7), and then decreasing at T14, giving overall mean IBR values that are very similar between temperatures. For *C. laevimanus*, IBR values in muscle tissue were similar at all timepoints (Fig. 2B). In visceral mass of the two tested species, however, IBR index core values were consistently higher at 30 °C across timepoints (except for T14 in *R. durbanensis*, Fig. 2A), and mean IBR from the whole experiment trial duration was also higher at heat wave temperature when compared to control temperature in both species.

Star plots for biomarker responses (in all tissues and sampling times) showed that graph areas were higher at 30 °C when compared to 25 °C for both species (Fig. 3). This suggests consistently higher scores at 30 °C for both species, translating into higher index core values at this temperature and, therefore, stress and poorer health status during the heat wave simulation.

PCAs (Fig. 3) showed that biomarkers mostly correlated to temperature were Hsp70 and GST in *R. durbanensis* and LPO and CAT in *C. laevimanus*, whereas CAT and SOD, and SOD and Ub were mostly correlated to tissue type in *R. durbanensis* and *C. laevimanus*, respectively. No particular biomarker was associated with time in both species analyzed. The cumulative amount of variance explained (Fig. 4) was 83.87% in the shrimp species and 81.30% in the hermit crab species. In Fig. 4A, a separation between tissues emerges along factor 1 for *R. durbanensis*, while in Fig. 4B, a separation among temperature groups emerges along factor 1 in visceral mass of *C. laevimanus*.

3.3. Energy metabolism and performance

Condition estimates and energy marker results showed that higher temperature only affected *R. durbanensis*, namely in Fulton' K (increased from 25 °C to 30 °C) and %C (decreased in muscle from 25 °C to 30 °C). No significant differences were found for *C. laevimanus* in any of the parameters here tested (Table 3). Significant Pearson correlations were observed between %C and LPO extension at 30 °C in the muscle for both species (positive for *R. durbanensis*: R = 0.615, p-value = 0.015; and negative for *C. laevimanus*: R = −0.657, p-value = 0.039). Mortality was 0% for both species in both temperature treatments.

Table 2
Factorial ANOVAs results in visceral mass tissue of a) *Rhynchocinetes durbanensis* and b) *Calcinus laevimanus*. Effect of temperature (25 °C vs 30 °C) and its interaction with time (T0, T7, T14) on biomarker levels (thermal and oxidative stress). Significant results (p-value < 0.05) are presented in bold.

	a)				b)			
	Temperature		Temp × Time		Temperature		Temp × Time	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Hsp70	**4.92**	**0.036**	2.48	0.104	**5.34**	**0.029**	**4.59**	**0.020**
Ub	**4.34**	**0.048**	**5.66**	**0.009**	**18.30**	**< 0.001**	**4.58**	**0.020**
CAT	0.54	0.468	2.94	0.071	**36.71**	**< 0.001**	**9.28**	**0.001**
LPO	0.73	0.398	0.67	0.516	**11.68**	**0.002**	**3.25**	**0.056**
GST	0.954	0.338	**3.76**	**0.037**	**20.92**	**< 0.001**	**12.05**	**< 0.001**
SOD	3.18	0.086	2.75	0.083	3.02	0.094	1.47	0.249

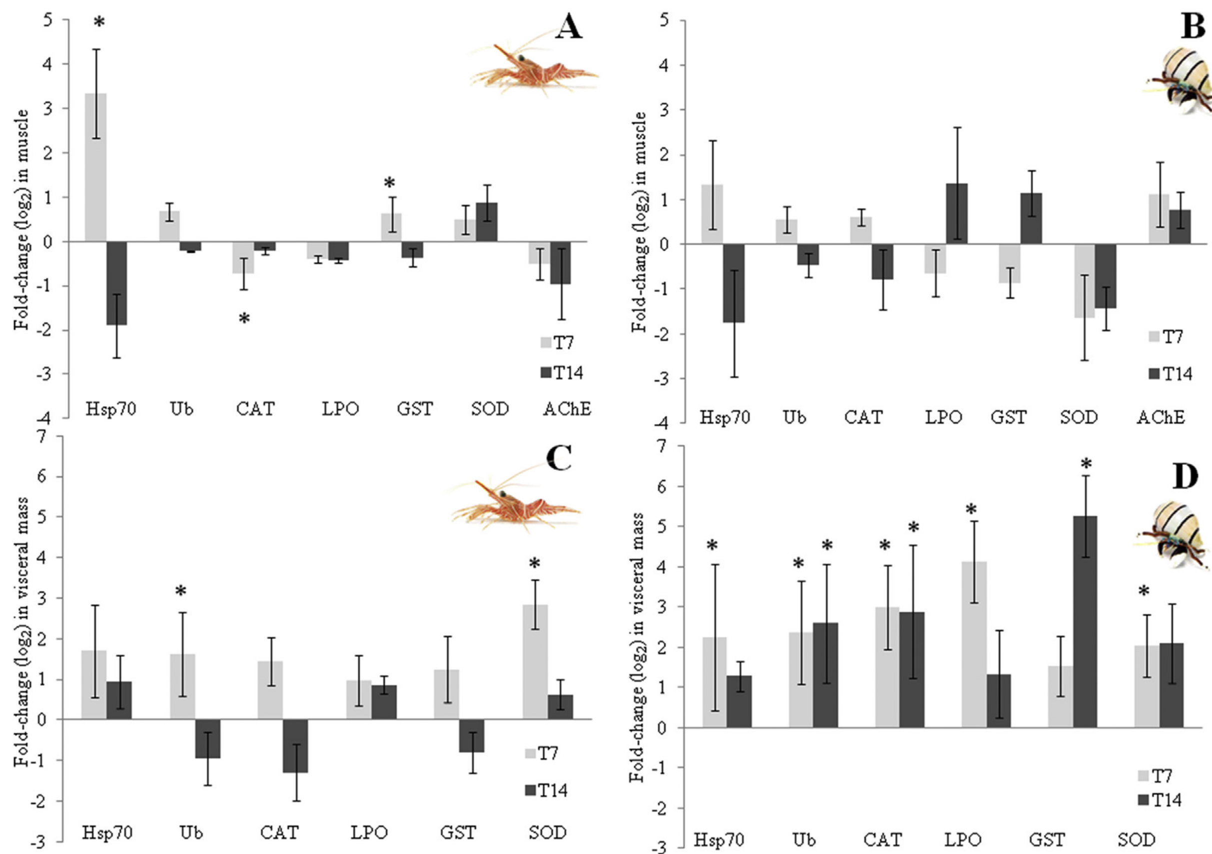

Fig. 1. Biomarker log_2 fold-change after 7 and 14 days of exposure to heat wave simulation: A) *Rhynchocinetes durbanensis* muscle, B) *Calcinus laevimanus* muscle, C) *R. durbanensis* visceral mass, D) *C. laevimanus* visceral mass. Significant differences from control (T0, fold-change 0) are presented with an asterisk (*, p-value < 0.05; see also fig. SM1-SM4 and tables SM1-SM2 in supplemental material for original biomarker data).

4. Discussion

Temperature affects organisms at different biological organization levels, ranging from molecular to whole-body (Mora and Ospína, 2001; Dent and Lutterschmidt, 2003). In this study, we showed for both crustacean species that temperature effects were mainly detected in the amount of protective proteins and enzyme activity levels. Performance consequences were only observed in *R. durbanensis*, particularly in condition and energy reserves. The general pattern of biomarker variation here analyzed showed that: 1) different species and different tissues display different thermal sensitivities, 2) biomarker changes occurred at similar timings for both species, and 3) biomarkers with significant log_2 fold-changes showed mild to moderate (Hsp70, CAT, GST) or severe stress (Ub and LPO) in cells (cell stress degree as in Logan and Somero, 2011). All these observations suggest that an increase of +5 °C during two consecutive weeks had a significant quantifiable impact in these species' physiology. Heat waves are generally associated to an increase in animals' metabolic activity (Arambourou and Stoks, 2015), which is mostly associated with increased respiration rates as a consequence of extra maintenance, repair, and protein production (Feder and Hofmann, 1999; Tomanek, 2010). This ultimately suggests that organisms need to adjust their metabolic rates during heat stress in order to balance tissue oxygenation and energy production/expenditure (McElroy et al., 2012). The high magnitude of fold-changes detected in Hsp70 in the muscle and SOD in visceral mass for *R. durbanensis* after 7 days suggests that increased temperature caused protein unfolding and cytotoxic aggregations (Yamashita et al., 2010; Madeira et al., 2015a) in muscle cells, and a higher flux of superoxide radical arising from oxidative stress (Rosa et al., 2014) in visceral mass. *C. laevimanus* showed highest order of magnitude changes in GST (at 14 days) in the visceral mass, suggesting enzyme activity to detoxify toxic lipid peroxides that arose (at 7 days) from oxidative damage to cell membranes (Madeira et al., 2013; Vinagre et al., 2014). Such responses to a thermal challenge have also been documented in many other crustacean species (e.g. caridean shrimps: *Palaemon* spp. Allan et al., 2006; González-Ortegón et al., 2013; Madeira et al., 2015b; Magozzi and Calosi, 2015, and *Crangon crangon* Reiser et al., 2014; white shrimp *Litopenaeus vannamei* González et al., 2010; lobster *Homarus americanus* Chang, 2005; and grapsidae crab *Cyclograpsus cinereus* Lardies et al., 2011). Mean IBR values were always higher at 30 °C when compared to 25 °C (except for muscle in *C. laevimanus*), indicating that animals exposed to the heat wave were in poorer health status (i.e., stressed) when compared to control conditions.

This heat wave simulation seemed to be perceived differently by each species. Neither species showed changes in mortality levels, suggesting that the 5 °C temperature increase over two consecutive weeks was not an extremely stressful one. However, the imposed temperature did show a significant influence in performance parameters of *R. durbanensis*. Although Fulton's K increased during the heat wave, %C levels decreased, indicating a process of depletion of lipid reserves. Other studies have also shown that thermal stress (either warm or cold) can cause lowered energy stores (Klepsatel et al., 2016; Wen et al., 2017), even if growth increases (Arambourou and Stoks, 2015). Another aspect of the results was the significant correlation obtained between %C and LPO levels – the amount of stored lipids seems to be a key aspect in the extension of lipid peroxidation experienced by the animals under heat stress. However, opposite correlations were observed for each species (positive for *R. durbanensis* and negative for *C. laevimanus*), which makes it difficult to hypothesize which pathways are at work. We suggest two possibilities: 1) a positive correlation suggests that lipid rich tissues suffer proportionally from lipid oxidation leading to

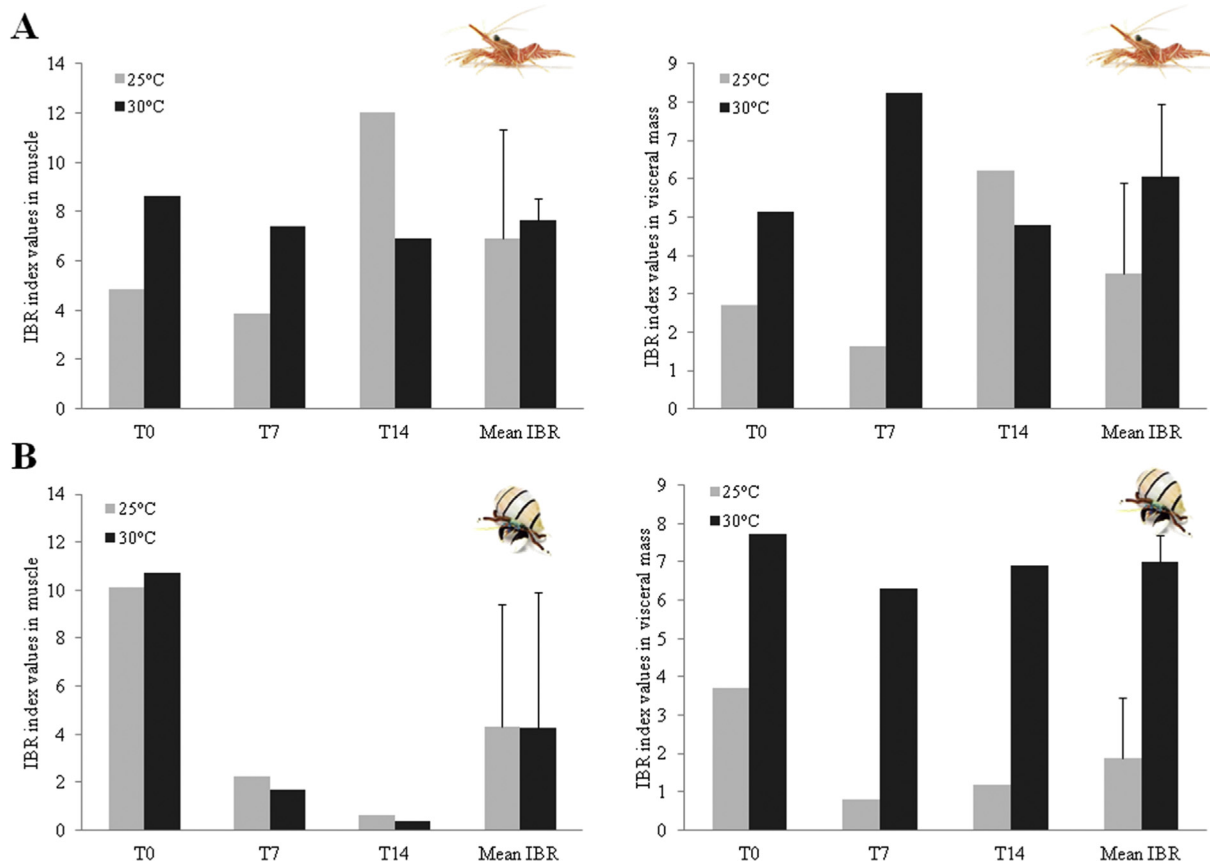

Fig. 2. Integrated biomarker response index (IBR) after experimental trials at 25 °C and 30 °C in each timepoint sampled, and mean + SD of all timepoints in muscle and visceral mass of A) *Rhynchocinetes durbanensis* and B) *Calcinus laevimanus*.

increased accumulation of LPOs, whereas 2) a negative correlation suggests that lipid rich tissues have more energy available to direct towards cytoprotective pathways, therefore avoiding cellular damage and leading to low levels of LPOs. However, this is an issue that requires further research before any conclusions can be drawn.

All of these differences in heat wave responses detected between species from the same reef environment can be associated with 1) differences in the kinetics of responses, 2) differences in temperature sensitivity, and 3) differences in the habitats they explore (and consequently in life histories and activity levels). For instance, the shrimp *R. durbanensis* is a strictly subtidal animal, while the hermit crab *C. laevimanus* inhabits both intertidal and subtidal areas, which means that hermit crabs are more versatile and are commonly used to explore steep thermal gradients (Helmuth and Hofmann, 2001; Somero, 2002; Madeira et al., 2015b). This is a very significant feature in intertidal reefs where there are topographically complex microhabitats including rock pools, crevices, boulders, and emergent platforms (Lathlean et al., 2017). Consequently, hermit crabs' responses may be more plastic, mediated by internal changes that can include, but are not limited to, higher basal biomarker levels and inducible responses (Madeira et al., 2014a, 2017b), sooner activation of energy saving cellular pathways (e.g. metabolic depression) (Madeira et al., 2015b), and changes in mitochondrial densities and in the oxygen supply system (e.g. hemolymph hematocrit) (Abele et al., 2002; Verberk et al., 2016). All of these possible responses allow hermit crabs to optimize metabolism in varying thermal windows. Therefore, higher acclimation to temperature variation could be expected for *C. laevimanus* when compared to *R. durbanensis*.

The absence of mortality for both species during the heat wave here simulated can be associated with several reasons. It is possible that the thermal challenge was not intense or prolonged enough to cause mortality, even though this was the first time that these animals were exposed to 30 °C (both crustaceans were bred, reared, and maintained at 25 °C). Previous studies have shown that animals have the ability to withstand temporal spikes in temperature (Arambourou and Stoks, 2015) until a certain physiological threshold is achieved, above which growth is severely affected and mortality levels can rise up to 100% of the population. Additionally 1) the previous generations may have originated from strains resistant to temperature variation, depending on the original broodstock thermal history; 2) high acclimation to captivity conditions may limit stress response patterns (Madeira et al., 2017a) (as domestication affects thermal physiology and the more generations have been kept in captivity, the stronger this effect may be due to inbreeding and lower genetic diversity); 3) both species are native to reef environments that naturally experience dynamic temperature variations over short periods, even at subtidal depths (Leichter et al., 2006). For instance, it is known that sub-surface temperatures can vary as a result of diurnal warming in shallow waters up to 10 m depth (Brown, 1997; Craig et al., 2001), and due to tidal forcing and internal waves at greater depths (20–30 m) (Lee et al., 1999). As the high specific heat and thermal conductivity of water ensure that marine ectotherms will have body temperatures equivalent to that of their surroundings (Feder and Hofmann, 1999), then inhabitants of these environments are likely adapted to the kind of thermal challenge tested here.

When species are geographically distributed across temperature gradients that are greater than projected changes due to climate warming, those particular species may be genetically wired for thermal acclimation and adaptation (Munday et al., 2008, 2012). This seems to be the case for the species here studied as their natural distributions are broad - from eastern Africa, to India, Southeast Asia and Western Pacific islands (*R. durbanensis*, Grave, 2010; *C. laevimanus*, Lemaitre and McLaughlin, 2017). Also, SST variability exhibited along the coast lines

Fig. 3. Integrated biomarker response index (IBR) and visual representation of variables in the principal components analysis carried out for each species A) *Rhynchocinetes durbanensis* and B) *Calcinus laevimanus* exposed to 25 °C and 30 °C, considering all sampling times (0, 7 and 14 days) and both tissues (muscle and visceral mass).

of the Indo-Pacific region where these species inhabit ranges from about 22 °C to 31 °C. Interestingly, the capacity to acclimate to altered conditions in such cases is likely to be similar among most of the natural populations of those species along latitudinal gradients, if there are no restrictions to gene flow, as was observed for the crab *Petrolisthes violaceus* along the Pacific coast of Chile (Gaitán-Espitia et al., 2017). This means that even tank-raised species may share this capacity with their wild counterparts, inherited from the original broodstocks.

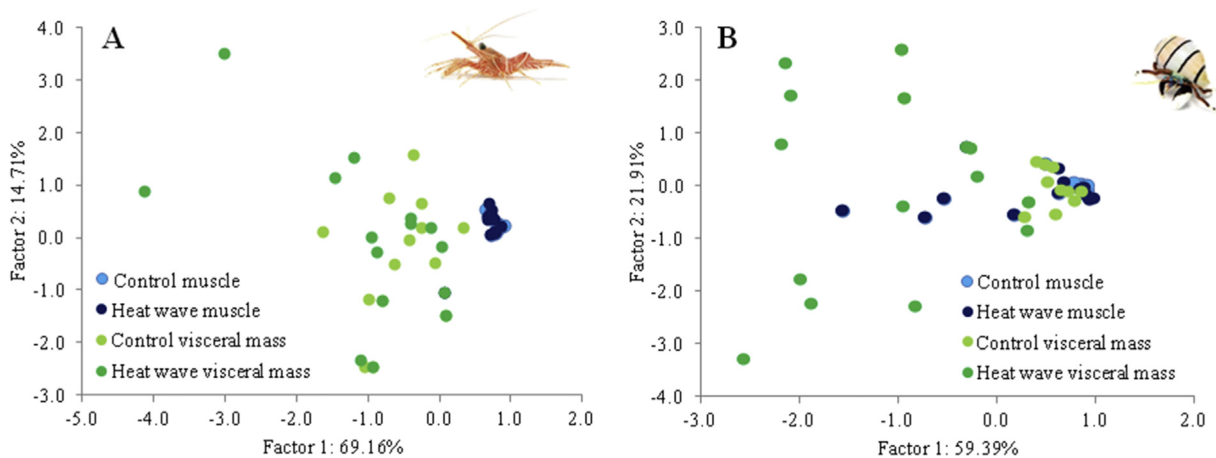

Fig. 4. Factor 1 and factor 2 of the principal components analysis carried out for both tissues (muscle and visceral mass) in A) *R. durbanensis* and B) *C. laevimanus* exposed to 25 °C (control) and 30 °C (heat wave).

Table 3
Student t-tests performed on condition parameters after exposure to a 2-week heat wave simulation experiment. Significant results (p-value < 0.05) are presented in bold.

	Fulton's K		Total protein		%C		C:N molar ratio	
	t	*p-value*	*t*	*p-value*	*t*	*p-value*	*t*	*p-value*
R. durbanensis	**−2.513**	**0.017**	0.527	0.601	**2.497**	**0.018**	0.474	0.638
C. laevimanus	0.806	0.908	−0.966	0.437	−1.682	0.994	0.411	0.613

5. Conclusions

The main conclusions to retrieve from this study are that both animal species displayed a plastic response under heat wave conditions with no mortality, suggesting that the physiological adjustments they employed conferred them thermal tolerance. The chosen approach highlights the mechanistic background for thermal stress and/or acclimation responses of the species tested while yielding reliable results for thermal stress monitoring purposes. The calculated indices have shown to be intuitive, easy to understand and to communicate results. It is expected that they can be used to identify vulnerable species and populations, and categorize their susceptibility to extreme thermal events when combined with whole body performance measures. On the other hand, their integrative nature can lead to loosing details between the different processes and components of responses at variable levels of biological complexity (i.e., macromolecular damage, ROS scavenging, accumulation of energy reserves and whole-body performance) during the analysis. By further optimizing such tools and methodologies, we can then implement them in field studies with natural populations and fill the knowledge gap on tropical thermal stress biology by placing physiological information in a larger ecological context. This provides a first step on improving ecological risk assessment of vulnerable populations and it will allow the implementation of better conservation measures in already fragile tropical communities.

Financial disclosure

This study had the support of the Portuguese Foundation for Science and Technology (FCT) through the WarmingWebs project (PTDC/MAR-EST/2141/2012), the "Investigador FCT" position granted to C.V. and the strategic projects UID/MAR/04292/2013 granted to MARE and UID/Multi/04378/2013 granted to UCIBIO. C.M. was funded by a PhD grant (SFRH/BD/92975/2013), and M.C.L. by a post-doc grant (SFRH/BPD/115298/2016), all granted by FCT. The funding institution had no involvement in the study design and data collection, in the writing of the report or in the decision to submit the article for publication.

Declarations of interest

None.

Author contributions

C.M. performed the experimental trials, laboratory and statistical analyses and wrote the paper, M.C.L. performed laboratory and statistical analyses and supervised procedures for the determination of energy reserves; MSD optimized all biomarker methods and supervised all laboratorial analyses; HNC revised the statistical analyses performed; MSD, HNC and CV designed the study, supervised the work at all stages, and provided all means necessary to the accomplishment of the tasks; all authors contributed to the interpretation and discussion of results as well as to outline the first draft of the manuscript. The final version of the paper was revised and approved by all authors.

Acknowledgements

Authors thank Aquário Vasco da Gama (public aquarium) for providing live rock and seaweeds, and thank everyone involved in maintenance of the experimental tanks.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.marenvres.2018.08.015.

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