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RESEARCH ARTICLE

Thermal adaptation in the intertidal snail *Echinolittorina malaccana* contradicts current theory by revealing the crucial roles of resting metabolism

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SUMMARY

Contemporary theory for thermal adaptation of ectothermic metazoans focuses on the maximization of energy gain and performance (locomotion and foraging). Little consideration is given to the selection for mechanisms that minimize resting energy loss in organisms whose energy gain is severely constrained. We tested a hypothetical framework for thermal performance of locomotor activity (a proxy for energy gain) and resting metabolism (a proxy for energy loss) in energetically compromised snails in the littoral fringe zone, comparing this with existing theory. In contrast to theory, the thermal ranges and optima for locomotor performance and metabolic performance of *Echinolittorina malaccana* are mismatched, and energy gain is only possible at relatively cool temperatures. To overcome thermal and temporal constraints on energy gain while experiencing high body temperatures (23–50°C), these snails depress resting metabolism between 35 and 46°C (thermally insensitive zone). The resulting bimodal relationship for metabolism against temperature contrasts with the unimodal or exponential relationships of most ectotherms. Elevation of metabolism above the breakpoint temperature for thermal insensitivity (46°C) coincides with the induction of a heat shock response, and has implications for energy expenditure and natural selection. Time-dependent mortality is initiated at this breakpoint temperature, suggesting a threshold above which the rate of energy demand exceeds the capacity for cellular energy generation (rate of ATP turnover). Mortality in a thermal range that elevates rather than limits aerobic metabolism contrasts with the hypothesis that cellular oxygen deficiency underlies temperature-related mortality. The findings of this study point to the need to incorporate aspects of resting metabolism and energy conservation into theories of thermal adaptation.

Key words: gastropod, hypometabolism, *Littorinidae*, metabolic theory of ecology, oxygen consumption.

INTRODUCTION

Considerable advances have been made in our theoretical analyses of the thermal adaptation of ectothermic animals (Angilletta, 2009; Pörtner, 2010). There is thus the need to test the rigor of new theoretical perspectives (often based on few model species) by empirical observations for animals living in extreme and intermediate environments, such as marine intertidal environments. However, studies using air-breathing, intertidal ectotherms are complicated by the fact that theoretical developments for terrestrial and marine animals have historically followed different directions (Angilletta, 2009; Pörtner, 2010). The focus for marine animals has been largely on adaptive mechanisms at the cellular and systemic levels (Hochachka and Somero, 2002; Somero, 2002; Somero, 2005; Pörtner, 2002; Pörtner, 2010; Pörtner and Knust, 2007), whereas that for terrestrial organisms has mainly concerned the performance of fitness-enhancing attributes, including growth and reproduction (Huey and Stevenson, 1979; Huey and Kingsolver, 1989; Gilchrist, 1995; Angilletta, 2009). Nonetheless, commonality between the two perspectives is seen in the concept of thermal optimality of performance (Huey and Stevenson, 1979; Pörtner and Knust, 2007; Pörtner, 2010). Evolutionary selection is believed to underlie the maximization of energy gain (energy transfer from environment to organism) over an optimal thermal range (performance breadth) through thermally maximizing the locomotor performance that underlies foraging and feeding (Huey and Stevenson, 1979; Pörtner and Knust, 2007; Pörtner, 2010). This implies that periods of inactivity, when energy gain is zero, make no contribution to evolutionary fitness (see Gilchrist, 1995; Gilchrist, 2000; Gillooly et al., 2001). Reduced metabolism in aestivating or hibernating animals is typically interpreted as a means of extending the rest period and improving survival under adverse environmental conditions, rather than improving fitness by reducing energy loss (Brown et al., 2004). Further commonality between the terrestrial and marine perspectives is the assumption that thermal habitat is behaviourally selected on the basis of its potential to maximize performance (Angilletta, 2009). Remarkably, these two concepts seem inapplicable to snails living in the littoral fringe zones of rocky shores. First, their occupation of this extreme environment is driven by intense biological interaction (competition and predation) lower on the shore and an ability to withstand physiological stress during emersion (Garrity, 1984), rather than by performance-enhancing thermal habitat selection. Second, as a consequence of their distribution, littoral-fringe snails experience lifelong temporal and thermal constraints on foraging and energy gain, suggesting that

their thermal evolution may be more closely linked to mechanisms that conserve energy reserves during rest than those that maximize energy gain during activity (Marshall and McQuaid, 2011).

A major difference in the approaches generally followed by terrestrial and marine researchers that confounds comparison of either terrestrial or marine species with air-breathing marine fringe snails concerns metabolic performance. For example, models for terrestrial ectothermic organisms assume that metabolic rate is exponentially related to temperature (Gillooly et al., 2001; Dillon et al., 2010), and do not include 'metabolic performance' with more tangible fitness performance measures such as growth, reproduction or survivorship (Huey and Stevenson, 1979; Angilletta, 2009). In contrast, contemporary marine models explicitly address the matching of optimal thermal relationships for metabolic performance with other performance measures (Pörtner, 2002; Pörtner, 2006; Pörtner and Knust, 2007). This approach is presumably based on the reasoning that the capacity for cellular energy generation (metabolic capacity) within a given time at a particular temperature underlies all higher-order organismal functions, including energy gain (feeding) and fitness-enhancing growth and reproduction (Pörtner and Knust, 2007). However, although the thermal ranges and breadths for metabolic performance and other performance traits are closely matched in most marine ectotherms (Pörtner and Knust, 2007; Pörtner, 2010), metabolic and locomotor performances are thermally mismatched in tropical littoral-fringe snails, as they only become active and feed when cooled during immersion or wetting. Consequently, maximal locomotor performance (P_{max}) is limited by the reduced underlying metabolism at these relatively low temperatures (see hypothetical model, Fig. 1). Additionally, because immersion or wetting is infrequent in fringe snails, sometimes occurring only seasonally (D.J.M., unpublished), feeding and energy gain are severely constrained. To compensate for these energetic constraints, combined with the demands of high resting temperatures, intertidal snails depress cellular metabolism below the standard level (Guppy and Withers, 1999; Sokolova and Pörtner, 2001; Sokolova and Pörtner, 2003; Storey and Storey, 2004; Marshall and McQuaid, 2011). In some marine invertebrates, energy is conserved by a neutral or negative relationship between metabolism and temperature (Newell, 1969; Brown and Da Silva, 1979; Newell and Branch, 1980; Boutet et al., 2009; Marshall and McQuaid, 1992; Marshall and McQuaid, 2011), in striking contrast to the positive relationship proposed for all ectotherms in accordance with fundamental thermodynamics (Fig. 1) (Gillooly et al., 2001; Dillon et al., 2010). Furthermore, depressed, temperature-insensitive metabolism implies a relationship between energy gain and resting energy loss that is fundamentally different from that predicted by theoretical contexts for thermal adaptation.

Here we present a thermal framework for energy gain (locomotor performance) and resting energy loss (metabolic performance) in tropical littoral-fringe snails (Fig. 1). We compare this hypothesis with contemporary hypotheses for ectothermic animals, and validate it using *Echinolittorina malaccana*. In doing so, we describe a zone of energy-conserving, thermally insensitive metabolism well below the critical temperature. Because this metabolism is sometimes negatively related to temperature (Marshall and McQuaid, 2011), we also refer to thermonegative metabolism. To improve understanding of the adaptive significance of this metabolism, we explore the following questions: (1) how often do temperatures naturally rise above the upper threshold for thermally insensitive metabolism (hereafter, the thermally insensitive breakpoint temperature, T_{BP}), (2) what underlies metabolic elevation above the T_{BP} (is this solely due to thermodynamics or does it incorporate a heat shock response), and (3) does sustained maximal metabolism due to continuous exposure to above-T_{BP} temperatures that are well below the acute lethal temperature have survival consequences? Addressing these questions should provide insight into the conflicting need of these snails to conserve energy at high temperatures while at the same time generating energy in support of a heat shock response (see Somero, 2002; Kültz, 2005).

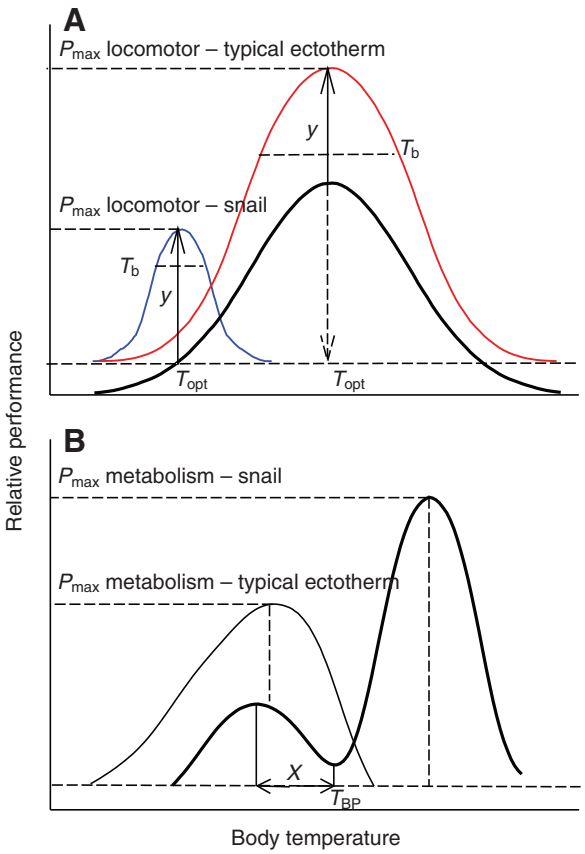

Fig. 1. (A) Conceptual Gaussian models showing performance against temperature for locomotor activity of a typical ectotherm (red line) and a tropical littoral-fringe snail (blue line), and the 'general' metabolism for both kinds of animal (thick black line). The thermal ranges and breadths (T_b) for locomotor and metabolic performance overlap in most ectotherms (Pörtner, 2010), whereas locomotor performance in tropical littoral-fringe snails is restricted to cooler temperatures below those maximizing metabolism. Maximal locomotor performance (P_{max}) of the snails is limited by the reduced metabolic capacity at these temperatures (y). Relative values for performance of locomotor activity and metabolism and for thermal ranges of both the snail and the typical ectotherm are arbitrary. T_{opt}, thermal optimum. An assumption of the models is that performance curves for resting and active metabolism have similar shapes. (B) A model for metabolic performance relative to temperature of a typical ectotherm (thin line) and a littoral-fringe snail (thick line), showing metabolic depression and bimodality (see Marshall and McQuaid, 2011). The snails are adapted to exposure of much high temperatures than most ectotherms. x represents the temperature zone for thermonegative metabolism, and T_{BP} is the thermal breakpoint at which this is limited.

MATERIALS AND METHODS

Echinolittorina malaccana (Philippi 1847) (formerly *Nodilittorina trochoides*) is broadly distributed across the West Indo Pacific region (Reid, 2007). These snails have separate sexes, feed on microalgae and show determinate growth. Their thermal habitat is aseasonal

(Marshall et al., 2010) and they exhibit behavioural isolation that allows them to withstand more than 50 days of continuous exposure to air by withdrawing into the shell (D.J.M., unpublished).

Metabolic performance

Aerobic metabolic performance was assessed from oxygen consumption rate and heart rate. Anaerobic metabolism of littorinid snails in air, when oxygen is not limited, is negligible (Sokolova and Pörtner, 2001; Sokolova and Pörtner, 2003). Mature snails (shell length=7–9 mm) were collected from an artificial seawall at Jerudong, Brunei Darussalam (BN; 4°32′N; 114°43′E; April–July 2009), or from a natural rocky shore at Shek O, Hong Kong (HK; 22°13′43N, 114°15′22E; July 2009). In the laboratory, snails were washed in filtered seawater, allowed to emerge from their shells and attach to lidded plastic Petri dishes. To compare the effects of the duration of aestivation, experiments were performed on field fresh snails (FF snails, which had actively fed within 6 h of collection) and snails that were kept in ambient air at 30°C for 4 to 7 days after collection (7d snails). Prior to experiments, FF snails were exposed to fan-blown air to induce behavioural isolation and dry the shells (2 h, 30°C, Memmert UFE 500, Schwabach, Germany) (Marshall and McQuaid, 2011). Respiratory rates were determined for BN/FF (N=10 replicates), BN/7d (N=9) and HK/7d snails (N=6), and heart rates for BN/FF snails (N=30 individuals). Replicates for the respiratory rate experiments comprised 10 to 14 individuals (0.220–0.310 g wet mass replicate^{-1}), whereas individuals were the replicates in heart rate experiments.

Respirometry was performed in dry air (0% saturation), controlled by activated silica gel filling approximately 40% of the respirometer. Thermally insensitive metabolic depression characterizes snails isolated inside their shells or emerged from their shells, in both dry and moist air (Marshall and McQuaid, 2011). Respirometers consisted of wide-neck McCarthy bottles (7 ml) with rubber-washered screw lids fitted with fine electrodes (MI-730 Micro-Oxygen Electrode, Microelectrodes, Inc., Bedford, NH, USA). The electrodes were coupled to adapters (MI O2-ADPT, Microelectrodes, Inc.) and a data acquisition system (PowerLab/4SP, ADInstruments, Castle Hill, Australia). Electrodes were periodically calibrated in N_2 and humid air according to manufacturer's recommendations, and drift for blanks containing no snails was always <2% over 12 h at 40°C. At the start of an experiment, respirometers containing snails were bagged and placed in a Grant programmable water bath (GP200, Cambridge, UK) set to 30°C. After recordings had stabilized to ambient pressure at 30°C (approximately 10 min), the upper scale was set to 21% oxygen (air saturation) and respirometer caps were tightened. P_{O_2} (% air saturation) was recorded at a rate of 40 Hz, and the mean value was logged every 1 min. Heating was controlled at 0.2°C min^{-1} between 30 and 60°C after an initial 20 min at 30°C. Temperature within a blank respirometer in the water bath was recorded every 1 min using a Fluke digital recording thermometer and a fine K-type thermocouple (Cromega, Stamford, CT, USA). At the end of each experiment, the air volume within the experimental respirometer was determined by weighing the water that displaced the free space within it (approximately 4.5 ml), and the wet mass of the snails was determined. Snails were placed in 7% nitric acid until the shells had completely dissolved (within 1 h) (see McMahon et al., 1995), after which the tissue was washed in distilled water, blotted dry and weighed. Oxygen consumption was determined every 1 min from the difference in the change in P_{O_2} (measured as $\%O_2$) for the experimental respirometer and the blank respirometer. Percentage oxygen was converted to μmol O_2 using the ideal gas law equation to give μmol O_2 g^{-1} wet mass (see Sokolova and Pörtner, 2003).

Because active ventilation is prevented in air-exposed snails withdrawn into their shells, heart function closely reflects respiratory and aerobic metabolic rate (Marshall et al., 2010; Marshall and McQuaid, 2011). Furthermore, unlike respiratory rate, heart rate (f_H) can easily be continuously determined under variable heating and cooling. We therefore determined f_H under constant heating (for comparison with respiratory rate), and under changing and stable temperatures that resemble natural situations. Optoelectronic (infrared) reflective sensors (CNY70, Vishay Semiconductors, Shelton, CT, USA) were adhered to the shells of snails near the mantle cavity (using BluTac, Bostick). Signals from the sensors were amplified and filtered with a custom-built bridge amplifier and digitally logged (PowerLab/4SP, Chart 5, ADInstruments). Sampling rate was set at 40 Hz and the amplitude varied between 40 and 1000 mV. Data were smoothed [triangular (Bartlett)] before analysis.

In the constant heating experiments, f_H (beats min^{-1}) was logged (N=10 snails) every 1 min during heating at a rate of 0.25°C min^{-1} between 30 and 60°C. Experimental temperature was controlled using a Grant GP200 programmable water bath. The response of f_H to variable heating and cooling conditions was determined using a programmable Peltier-cooled Memmert (IPP400) incubator. Two cycling regimes of increasing, decreasing and stable temperatures were used. The upper temperature of one regime was 40°C and that of the other was 50°C. The ramping (R) protocol was: R1, 26–30°C (30 min); R2, stable 30°C (120 min); R3, 30–40°C (30 min) or 30–50°C (60 min); R4, stable 40 or 50°C (240 min); and R5, 40–30°C (30 min) or 50–30°C (60 min). The temperature between cycles was allowed to decline to 25°C. Snails (N=10 for each regime) were exposed to three cycles totalling more than 1200 min (20 h) per experiment. All snails recovered within 24 h (at 30°C) after the experiment. Data were standardized to account for individual differences by dividing the f_H of each individual by the maximum f_H of the same animal. The mean standard f_H for each minute for all individuals in each cycling experiment was plotted. Temperature was recorded every 1 min from a mimic snail (a silicone-filled shell, see below) next to the live one.

Breakpoint temperatures for the physiological rates were determined from Arrhenius plots (ln rate against $1/T$, where T is temperature in Kelvin, K) using piecewise least-square linear regressions with a quasi-Newton fitting method (STATISTICA 6.1, StatSoft, Tulsa, OK, USA). Three breakpoint temperatures were determined: (1) the temperature that initiated thermally insensitive metabolism (T_{BP1}, from the range of 32–39°C), (2) the upper limit for thermally insensitive metabolism (T_{BP}, thermally insensitive breakpoint temperature, >40–52°C, or 36–48°C for f_H), and (3) the Arrhenius breakpoint temperature (T_{AB}), above which rate declines with further heating (using the range 49–58°C, or 49–56°C for f_H). Several different ranges were explored and that in which the variance best explained the breakpoint (usually by >90% for all cases in the group) was used. Apparent activation energies (E_a) for respiratory rate were calculated according to Sokolova and Pörtner (Sokolova and Pörtner, 2003) for the ranges 40–45 and 46–51°C, which represent metabolic rates before and after the T_{BP}, respectively. Calculations were based on slopes (slope=$-E_a/2.303R$, where R is the universal gas constant) of Arrhenius plots [$\ln V_{O_2}$ against $1/T$, where T is temperature (in K) and V_{O_2} is the rate of O_2 uptake] determined by least-square linear regressions for BN/FF snails (STATISTICA 6.1). Q_{10} was calculated for the same thermal ranges.

Locomotor performance

We determined the effect of temperature on crawling rate and the thermal limit for crawling. Twenty BN/FF snails (collected in December 2010) were placed in droplets of water on a slide warmer (Fisher Scientific, Pittsburgh, PA, USA) set to one of the following surface temperatures: 25.2, 33.4, 36.5, 38.5, 40.5, 41.3, 42.6 or 43.5°C (all ±0.5°C). Surface temperature and operative body temperature within a mimic snail glued to the surface of the warmer were determined using Fluke digital recording thermometers and fine thermocouples. The number of snails that crawled within 10 min of being placed on the warmer was recorded, and crawling rate was calculated from the length of the mucus trail produced within 30 s. Thermal optimality for locomotor performance was determined from a Gaussian model: $y=a\exp\{-0.5[(x-x_0)/b]^2\}$, where x_0 is the peak (Sigma Plot 8, Systat Software, San Jose, CA, USA). The upper physiological limit to movement was determined from the heat coma temperature (T_{HC}), at which foot neuromuscular functioning is impaired and adhesion capacity is lost (McMahon, 1990). FF snails (N=30) were placed individually in seawater-filled test tubes (50 ml) and allowed to adhere to the glass for 30 min at 30°C in a Grant water bath. The bath temperature was then increased manually by 1°C every 10 min when snail adhesion was assessed.

Field body temperature

Field body temperature variation in Brunei Darussalam was assessed using a mimic snail (see Denny et al., 2006; Miller et al., 2009; Marshall et al., 2010). Temperatures were recorded from inside a silicone-filled shell (7 cm long) that was glued flat onto a light sandstone rock, placed horizontally to maximize sunlight exposure during continuous aerial emersion (no tidal wetting). The mimic was fitted with a fine K-type thermocouple (Cromega). Data were recorded using Fluke 54 Series II digital thermometers and downloaded to Fluke Viewforms. Temperatures were logged every 1 or 10 min for 6 h in the daytime (to capture the daily maximum temperatures) or for 24 h periods (to determine diurnal variation). Recordings were made on a total of 57 days in December 2006, April 2008, September–October 2009 and June 2011. Monthly maximum air temperatures recorded at the government weather station in Brunei Darussalam (approximately 4 km from the study site) over 33 months (2006, 2008, 2009) showed little seasonality in air temperature variation, though cloud cover and solar radiation probably vary between monsoon and inter-monsoon seasons (data courtesy of the Meteorological Service, Ministry of Communications) (Marshall et al., 2010). Model snail temperatures correlated with the temperatures inside live snails after 20 s of insertion of a thermocouple through the aperture (N=60, P<0.001).

Heat shock response

Heat shock response (HSR) was assessed from Hsp70 production (Tomanek and Somero, 1999; Tomanek, 2002; Dahlhoff, 2004). Snails were collected from Shek O and transported to the Swire Institute of Marine Science of The University of Hong Kong within 30 min (July 2009). In the laboratory they were rinsed in filtered seawater (30 psu), dried and kept at 27°C for 48 h. Snails were put into small vials (10 in each vial), which were placed into an oven (Yue Fat Engineering and Oven Works Co., Hong Kong) for thermal stress exposure. Temperature in the vials was measured using a thermometer (K/L Tm903A, LuTron, Taiwan), and was increased from 30 to 50°C at a rate of 5°C h^{-1}. When the temperature reached 50°C, it was increased to 57°C at a rate of 2°C h^{-1}. One randomly selected vial was taken out of the oven at each of the following temperatures: 32, 35, 40, 45, 47, 49, 51, 53, 55 and 57°C. The vial was then returned to 28°C seawater for 2 h and then frozen at –80°C for later use. Foot muscle of the snails was homogenized in 300 µl of homogenization buffer [50 mmol l^{-1} Tris-HCl, pH 8.0; 150 mmol l^{-1} NaCl; 150 mmol l^{-1} DL-Dithiothreitol (DTT)]. Protease inhibitors were added following the protocol of the manufacturer (Complete-Mini, Roche, Mannheim, Germany). The samples were homogenized for 20 s using a homogenizer (IKA, Wilmington, NC, USA), the homogenate was then centrifuged at 10,000***g*** for 15 min and the supernatant was transferred to a new tube. Protein concentration of the samples was determined using the 2D Quant kit (Amersham Biosciences, Little Chalfont, UK). Samples were stored at –80°C for further analysis. After boiling at 100°C for 5 min with loading buffer (120 mmol l^{-1} Tris, 2.17 mol l^{-1} glycerol, 138.7 mmol l^{-1} sodium dodecyl sulfate (SDS), 200 mmol l^{-1} DTT), equal amounts of proteins (15 µg) were loaded in each lane and electrophoresed on polyacrylamide gels (4.5% stacking gel, 10% resolving gel). After electrophoresis, proteins were transferred onto nitrocellulose membranes (0.2 µm, Bio-Rad) using the semi-dry transfer method. Transfer was carried out at 0.8 mA cm^{-2} for 2 h with a transfer buffer containing 28.8 g l^{-1} glycine, 6 g l^{-1} Tris and 1.5 g l^{-1} SDS, and 40% methanol. Following transfer, the membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS)/0.5% Tween-20 (25 mmol l^{-1} Tris-HCl and 150 mmol l^{-1} NaCl), and then incubated overnight with the primary antibody solution of mouse anti-Hsp70 monoclonal antibody (MA3-007, Affinity Bioreagents, Golden, CO, USA) diluted 1:2000. After being washed three times with TBS/0.1 Tween-20, the membrane was incubated for 1 h with the secondary antibody [HRP-rabbit anti-mouse (H+L), ZYMED® Laboratories, Invitrogen, South San Francisco, CA, USA] diluted 1:5000. This antibody can detect heat shock proteins in the 70 kDa gene family, including Hsp70, Hsc70 and GRP78 and, following heat shock, Hsp72. After washing in TBS/0.1 Tween-20, the western blot was developed using enhanced chemoluminescence detection (Amersham Biosciences) and exposed to X-ray film (Kodak, Rochester, NY, USA). The films were scanned and bands quantified using Image J (version 1.38). Samples were diluted to ensure that the band intensities fell within the linear range of the detection system. Hsp70 levels were expressed as values relative to the levels of a standard sample (relative unit, RU) which was taken from an individual heated at 35°C. The difference of Hsp70 among different temperatures was analyzed using one-way ANOVA followed by *post hoc* Duncan's multiple range tests using SPSS 13.0.

Thermal tolerance

The effect of chronic high temperature exposure on recovery time and mortality was determined using BN/FF snails (collected in December 2010). Survival of 24 or 48 h exposure to 40, 42, 44, 46, 48, 50 or 52°C was assessed from three groups of 10 snails. Snails were held in air in test tubes placed in a Grant water bath at constant temperature (±0.1°C) and ambient humidity. Survival was assessed from the ability of snails to right, extend their foot and reattach to a moistened plastic Petri dish at 25°C within 24 h after the experiment. Recovery time was determined after different exposure periods at 48 and 50°C. Twenty snails each were exposed to 2, 4, 6, 8 or 10 h at each temperature, and recovery (attachment) was determined at 1 h intervals for up to 8 h.

RESULTS

Metabolic performance

The relationship for respiratory rate against temperature of *E. malaccana* was characterized by a zone of thermal insensitivity (Fig. 2). Mean upper limits of this zone (T_{BP}) were 45.8°C for BN/FF

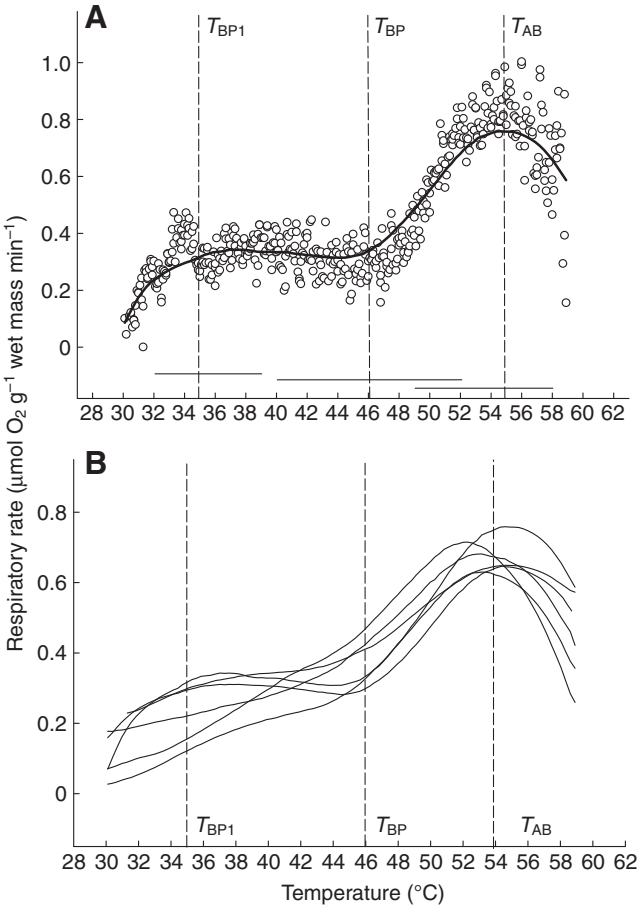

Fig. 2. (A) Relationship between respiratory rate and temperature in *Echinolittorina malaccana* (BN/FF) snails under continuous heating. A Loess curve is fitted to data recorded every 1 min. (B) Loess curves of six representative experiments to show the level of variability among experiments. T_{BP1} (temperature at which thermally insensitive metabolism is initiated), T_{BP} and T_{AB} (Arrhenius breakpoint temperature) are indicated in figures. Lower bars in A show the ranges used to determine breakpoints.

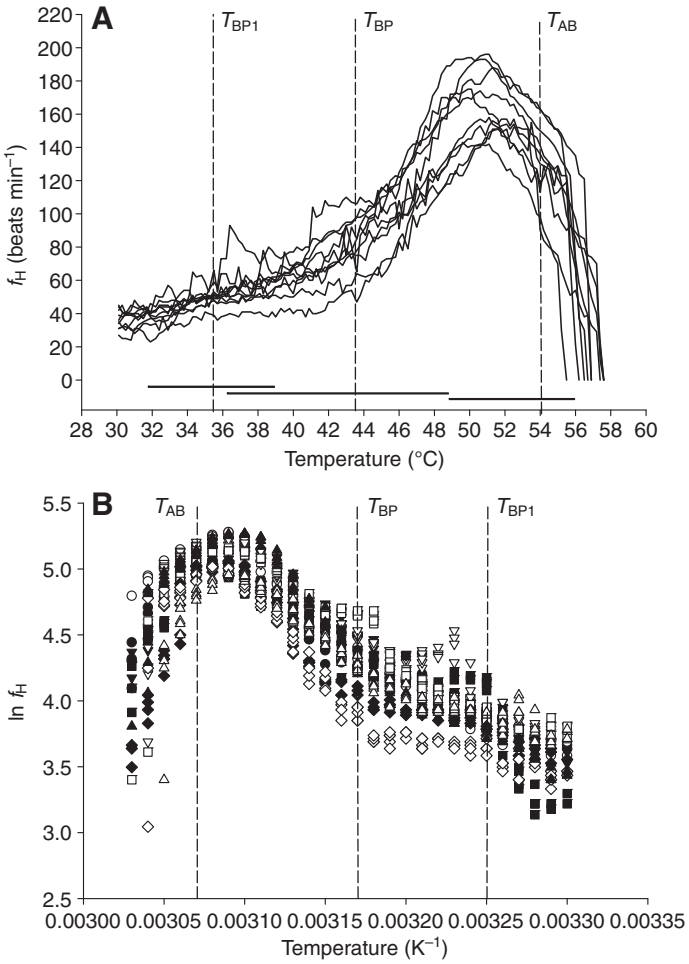

Fig. 3. (A) Plots for heart rate (f_H) against temperature for 10 individual *E. malaccana* snails under constant heating. (B) Arrhenius plots of data shown in A that were used to calculate breakpoint temperatures (symbols indicate different individuals). The various thermal breakpoints for f_H performance (T_{BP1}, T_{BP} and T_{AB}) are indicated in both figures. Lower bars in A show ranges used to determine breakpoints.

and HK/7d snails and 45.9°C for BN/7d snails, and were not significantly different (ANOVA, $F_{2,22}$=1.8, P=0.19; Cochran's C=0.61). Given the similarity of this limit for the populations and aestivation treatments, only BN/FF snails were used in further analyses. The mean lower limit for the thermally insensitive zone for respiration (T_{BP1}) of BN/FF snails was 35.4°C, and the mean Arrhenius breakpoint temperature (T_{AB}) for their respiration was 53.7°C (Fig. 2B). The E_a for BN/FF snails below the T_{BP} was 0.39±0.14 eV (mean ± 1 s.e.m.) and that above the T_{BP} was 1.38±0.14 eV (P<0.001, Cochran's C=0.48). An E_a >0.75 eV suggests that factors other than a thermodynamic effect contribute to metabolic rate (Gillooly et al., 2001). The mean Q_{10} was 1.4 below the T_{BP} and 3.5 above the T_{BP}.

Under constant heating, the pattern of heart rate performance was similar to that of respiratory rate. There was distinctive flattening of the curve in the mid-thermal range (Fig. 3). Means (±1 s.e.m.) for each of the three breakpoints were: T_{BP1}=35.5±0.35, T_{BP}=43.6±0.33 and T_{AB}=54±0.15°C. The slightly lower T_{BP} for heart rate compared with that of metabolic rate might be explained by the fact that other factors are involved in oxygen delivery (stroke volume and blood pigment function).

In the temperature cycling experiments (Fig. 4), exposure to a stable 40 or 50°C led to f_H depression; f_H at 40°C fell well below that at 30°C, though this was not true at 50°C. During cooling, mean f_H recovered initially, but with further cooling, it either stabilized (40°C experiment) or became temperature sensitive (50°C experiment, Fig. 4). Rapid switching between thermal sensitivity and thermal insensitivity of metabolism has, however, previously been found in some individuals experiencing 40°C thermal cycles (see Marshall and McQuaid, 2011). Heating always increased f_H in the 50°C experiment, but sometimes caused f_H depression in the 40°C experiment (Fig. 4). f_H depression below 30°C at a stable 40°C raises a caution against interpretations based on constant heating protocols (see above, the respiratory rate experiments of this study). Because the highest daily body temperatures in the field are fairly stable, thermonegative physiological performance is likely to be induced during most emersion periods, even in non-aestivating snails (Fig. 4A) (Marshall and McQuaid, 2011).

Locomotor performance

Less than 50% of the snails crawled at any temperature, and all crawling stopped at a body temperature of 41.5°C (surface

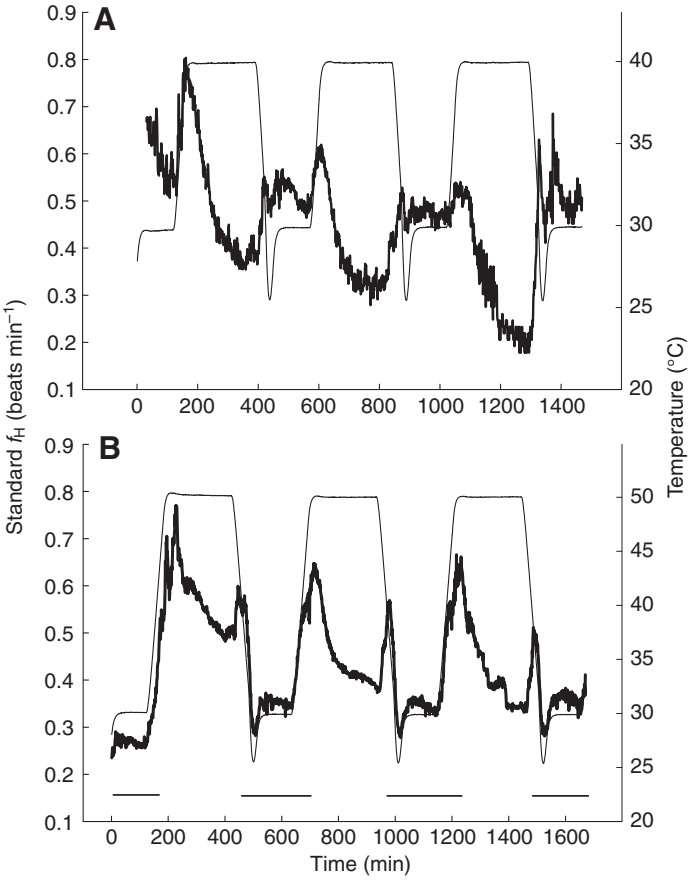

Fig. 4. Temporal change in mean standardized f_H of *E. malaccana* (N=10 individuals per cycling experiment) when temperature is cycled between 25°C and (A) 40°C or (B) 50°C. Thick line indicates f_H and thin line indicates temperature. Lower bars indicate times and temperatures for thermally sensitive heart function.

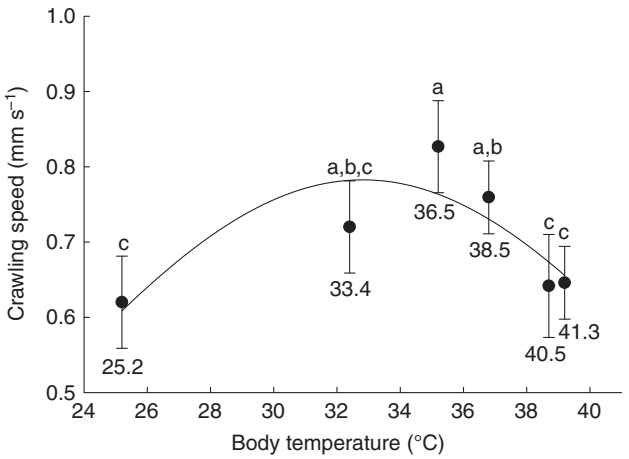

Fig. 5. Crawling speed of *E. malaccana* in relation to body temperature (mean ± 1 s.e.m.). Different letters indicate significantly different means (P<0.05). Values below the data points indicate the temperature of the surface on which the snail crawled (±0.3°C). A Gaussian model is shown fitted to the data.

temperature of 43.5°C). Above this temperature snails could move their shell and foot but not crawl forwards; some elevated their shells in an apparent thermoregulatory posture. Maximal crawling rate occurred at a body temperature of 35.2°C (surface temperature of 36.5°C; Fig. 5), though the thermal peak determined from a fitted Gaussian model (P=0.16) was 32.8°C (P<0.001). The mean (±s.e.m.) heat coma temperature (T_{HC}, the thermal limit for all locomotor activity) was 46.1±0.5°C. Assuming a Gaussian relationship for locomotor performance with a lower thermal limit at the minimum body temperature experienced (23°C) and an upper limit at the T_{HC}, then physiological performance is maximized at 34.5°C (the midpoint for 23–46.1°C), which is slightly above the observed thermal optimum for crawling. This temperature corresponds with the lower thermal limit for thermally insensitive metabolism.

Field body temperature

During aerial exposure, snails are heated in the morning and the highest daily temperatures, which are reached around noon, stabilize for a few hours (Fig. 6A). The overall highest temperature recorded was 49.6°C, and the mean (±s.d.) daily maximum was 44.5±3.3°C (N=51). The lowest minimum temperature recorded was 23°C. Diurnal cycling during a 9 day continuous recording period in April 2007, an inter-monsoon period when cloud cover is lowest, is shown in Fig. 6B. Body temperatures were below 30°C for approximately 70% of the time (Fig. 6C). Above 30°C, snails experience similar periods of exposure to each one degree interval (approximately 2% of the time per degree), but temperatures only rose above 46°C (the T_{BP}) for 1.56% of the total time (Fig. 6C).

Heat shock response

Using the antibody MA3-007, one Hsp70 band could be clearly identified. At 32°C, the level of Hsp70 was low at ~0.3 relative units (RU) (Fig. 7). When temperature was elevated from 35 to 47°C, Hsp70 levels were very stable at 0.7–1.0 RU and significantly higher than those at 32°C (ANOVA, $F_{9,39}$=19.483, P<0.001). The thermal range for this stable higher level of Hsp70 matches the metabolic thermally insensitive zone, suggesting thermoprotection in aestivating snails over a broad thermal range. At 49°C, the Hsp70 level had increased dramatically to ~1.5 RU. Further heating above this temperature caused Hsp70 levels to decrease. There were no significant differences between Hsp70 levels at 32°C and those at 53, 55 or 57°C (Duncan's multiple range tests). On the basis of the same thermal patterning for metabolism of BN and HK snails, we assume similarity in the Hsp70 expression for these populations.

Thermal tolerance

No mortality was observed after 24 h of exposure to temperatures between 42 and 46°C, whereas significant mortality occurred at temperatures above 46°C (χ^2=19.2, d.f.=1, P<0.01); 48 h of exposure showed a similar pattern, but with slight mortality at 46°C (χ^2=31.2, d.f.=1, P<0.01, for mortality frequencies at 46 and 48°C; Fig. 8A). Mortality at temperatures between 46 and 50°C was time dependent. Whereas full recovery was observed after 4 h of exposure to 50°C, 90% of the snails died from 10 h of exposure to this temperature (χ^2=16.2, d.f.=1, P<0.01; Fig. 8C). Recovery following non-lethal temperature exposure (10 h or less at 48°C) was also time dependent, generally taking longer after longer exposure (Fig. 8B). For example, full recovery after 4 h of exposure to 48°C was achieved within 2 h, but only after 4 h following exposure to this temperature for 10 h (Fig. 8B). By comparison, full recovery following 4 h of exposure to 50°C (only 2°C higher) took 8 h. These experiments suggest that recovery time may be a good indicator of the energetic deficit incurred during high temperature exposure, assuming that metabolism is maximal during recovery at 25°C.

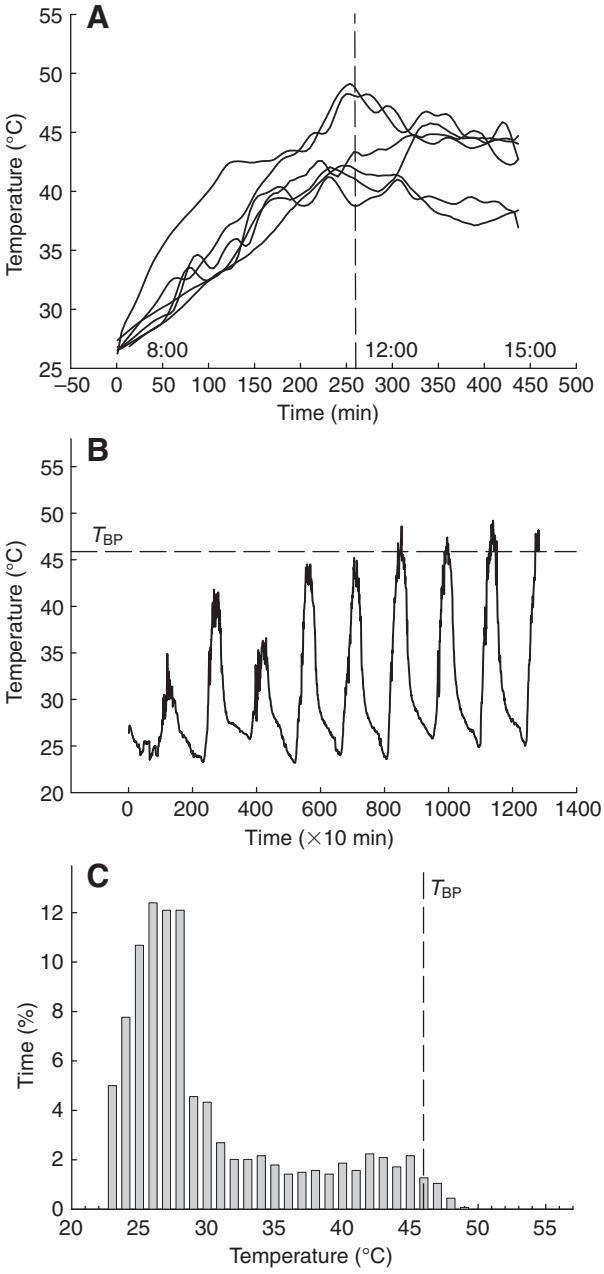

Fig. 6. Continuous recordings of body temperature of *E. malaccana* in the field. (A) One-minute, Loess-smoothed data for 6 days recorded between 08:00 and 15:00 h, showing flattening of curves after 12:00 h (dashed line) when temperatures are highest (June 2011). (B) Recordings for 9 days (April 2007). (C) Analysis of data in B, showing the time exposed to each 1°C interval. T_{BP} is shown.

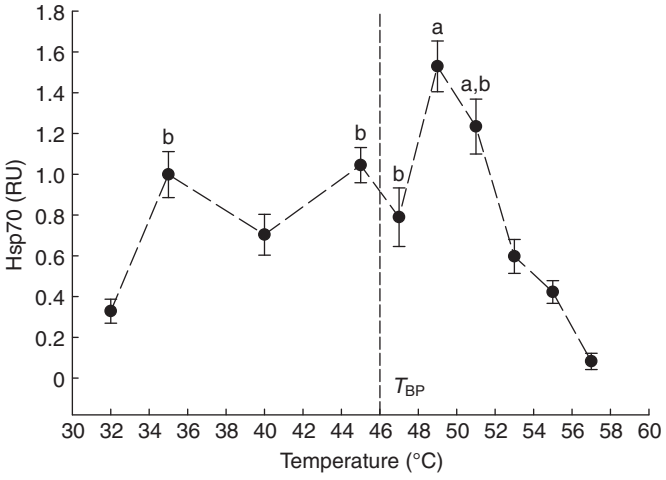

Fig. 7. Relationship between Hsp70 concentration (mean ± 1 s.e.m.) and temperature. Temperature was increased from 30 to 50°C at 5°C h^{-1}. At 50°C, temperature was increased to 57°C at 2°C h^{-1}. Lowercase 'a' indicates the higher values of Hsp70 and different letters indicate significantly different means (P<0.05). T_{BP} is shown.

DISCUSSION

Optimal performance curves are traditionally used to explain the evolution of thermal sensitivity in ectotherms (Huey and Stevenson, 1979; Angilletta et al., 2003; Angilletta et al., 2010; Angilletta, 2009). However, metabolism is not considered as a performance trait in most models for terrestrial ectotherms (Angilletta, 2009), but rather is described as relating exponentially to temperature according to basic thermodynamic principles (Gillooly et al., 2001; Dillon et al., 2010). For water-breathing marine organisms, metabolic performance has an optimal relationship with temperature, underlying locomotor and feeding performance, and thus fitness-enhancing energy intake (Pörtner, 2006; Pörtner, 2010; Pörtner and Knust, 2007). However, the situation seems entirely different for air-breathing marine littoral-fringe snails. In comparison to many oceanic ectotherms that seldom experience acute thermal fluctuations of more than a couple of degrees, eurythermal littoral-fringe snails can experience daily temperature fluctuations of approximately 27°C (Fig. 6). Furthermore, although feeding in many oceanic ectotherms is not constrained by temperature or time outside regular diurnal patterns that may include vertical migration, feeding in littoral-fringe snails is limited to unpredictable immersion in relatively cool seawater or when splashed. In contrast to contemporary models for most ectotherms, which suggest similar thermal ranges for different performance measures such as metabolism and growth (Pörtner, 2006; Pörtner, 2010; Pörtner and Knust, 2007), and in accordance with our prediction (Fig. 1), the thermal ranges and breadths for locomotion and metabolism in *E. malaccana* are grossly dissimilar: locomotor performance was maximized at approximately 35°C and limited at 47°C, whereas metabolism was maximized at 54°C and limited at 56°C. Such mismatching implies an intrinsic reduction in maximal locomotor performance for energy gain (P_{max}), as P_{max} occurs at a much lower temperature than that which maximizes the underlying cellular energy generation (see Fig. 1A) (see Pörtner, 2006). As in other marine intertidal gastropods, thermal and temporal constraints on energetic gain in *E. malaccana* are offset by temperature-insensitive metabolism (Newell, 1969; Brown and Da Silva, 1979; Newell and Branch, 1980; Marshall and McQuaid, 1992). This metabolism is induced in *E. malaccana* between 35 and 46°C (Figs 1 and 2), with the degree of thermal sensitivity being dependent on the state of aestivation and the rate of heating (Figs 1–3) (Marshall and McQuaid, 2011). Typically, their physiological performance becomes negatively related to temperature within this thermal zone in aestivating snails and under stable temperatures. Because higher daily temperatures become relatively stable (Fig. 6A), such thermonegative metabolism should commonly prevail naturally, even in non-aestivating snails during regular tidal cycling. The general metabolic performance of these snails can therefore be described as bimodally related to temperature, further supporting our hypothesis (Fig. 1) and contrasting with the optimal or exponential relationships

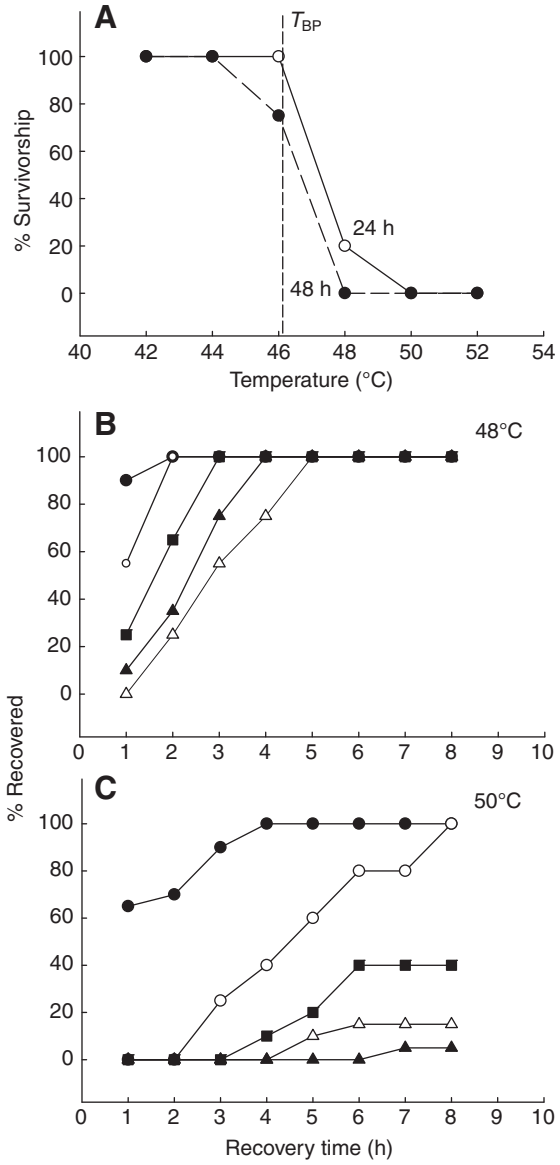

Fig. 8. (A) Survival of thermal exposure between 42 and 52°C for 24 or 48 h in *E. malaccana* (N=30 for each treatment). T_{BP} is shown. (B) Level of recovery relative to recovery time, following exposure to 48°C or (C) 50°C (N=20 for each symbol, for each temperature). Symbols refer to different exposure times (hours) for each temperature as follows: filled circle, 2; open circle, 4; filled square, 6; open triangle, 8; filled triangle, 10.

of most ectotherms (Gillooly et al., 2001; Pörtner, 2006; Angilletta, 2009; Dillon et al., 2010). The cellular processes underlying thermally insensitive metabolism are probably not unlike those triggering metabolic depression (see Guppy and Withers, 1999; Storey and Storey, 2004). Thermally insensitive metabolism and its adaptive significance raise several questions, including: (1) what mechanisms underlie elevation of metabolism above the upper thermal threshold for thermally insensitive metabolism (T_{BP}); (2) how often are transgressions of the T_{BP} experienced naturally; and (3) what are the implications of such transgressions?

Rocky-shore organisms are exposed to highly variable and unpredictable temperatures, which can sometimes cause mortality (Garrity, 1984; Williams and Morritt, 1995; Harley, 2008; Garrabou et al., 2009). Consequently, they have evolved costly HSRs, involving complex, tiered, cellular processes (Kültz, 2005; Logan and Somero, 2011), including the upregulation of heat shock proteins (Hofmann and Somero, 1996; Feder and Hofmann, 1999; Somero, 2002; Dahlhoff, 2004). The HSR in *E. malaccana* is maximized above the T_{BP}, when metabolic depression is abandoned. The E_a above the T_{BP} exceeded that purely due to a thermodynamic effect (Gillooly et al., 2001), suggesting the occurrence of an additional metabolic cost to support the HSR. The need for additional energy generation above the T_{BP} was further reflected in the heart rate data, particularly by the generally elevated cardiac performance at 50°C (Fig. 4). The thermal threshold for a HSR usually varies in relation to vertical position on the shore (Tomanek and Somero, 1999; Tomanek, 2002), but it is less clear whether the frequency of HSRs is similarly affected (Dong et al., 2008). In any event, lower-shore species seem better able to deal with a heat-shock-induced cost than higher-shore species, because more frequent emersion provides greater opportunities to feed (Hofmann and Somero, 1996). The infrequency and unpredictability of energy gain in littoral-fringe snails could be expected to drive the selection of behavioural and physiological mechanisms that limit costly HSRs.

The adaptive significance of energy-conserving physiology involving thermally insensitive metabolism and limiting HSRs is revealed by how closely these physiological attributes match the naturally experienced thermal regime. Although *E. malaccana* can survive acute heating to 56°C (Cleland and McMahon, 1986; Marshall et al., 2010), the maximum temperature experienced in the field was 49.6°C and thermal transgressions of the T_{BP} seem infrequent, occurring on only 1.56% of 66 days (Fig. 6). Although the situation will of course vary with latitude, orientation of the shoreline and the thermal properties of the rock from which heat is gained (Denny et al., 2006; Miller et al., 2009; Marshall et al., 2010), these field data certainly suggest evolutionary selection to extend the T_{BP} to near the upper limit of the thermal niche. Furthermore, the biophysical model used in these temperature recordings does not account for the ability of these snails to regulate body temperature behaviourally (Muñoz et al., 2005; Marshall et al., 2010). Behavioural thermoregulation could keep body temperatures within the thermally insensitive zone, thus limiting the induction of HSRs (D.J.M., unpublished).

These findings highlight the ecological and evolutionary significance of the T_{BP}, suggesting how fitness (growth and fecundity) could benefit from conserving energy, but energetics are also central to current theory for thermal tolerance (Pörtner, 2002). Functioning at the molecular and cellular levels must be thermally limited through protein denaturation, etc., but the theory on heat resistance in marine organisms focuses on the limitation of performance at the systemic level (Pörtner, 2002; Pörtner, 2010). Heat-related mortality in water-breathing marine ectotherms arises from progressive incapacity of the oxygen delivery system (Pörtner, 2002; Pörtner, 2010). Impairment of oxygen delivery commences at the 'pejus' limit (a temperature typically higher than that which maximizes metabolic performance) and continues to the critical thermal limit (CT_{max}). Consequently, above the critical limit, mortality is time dependent and related to thermoprotective capacity (Pörtner, 2002; Pörtner, 2010). Several basic physiological differences suggest that this model may not apply to air-breathing littoral-fringe snails. These include the capacity of these snails to depress metabolic and oxygen demand, and to prevent active ventilation when aestivating under high temperatures. Furthermore, time-dependent mortality was initiated around the T_{BP} (46°C), which is well below the thermal limit to aerobic performance (56°C) (Marshall et al., 2010) and below the temperature that maximizes metabolism (T_{AB}=54°C). This means that an energetic

deficit arises over a thermal range that elevates rather than depresses aerobic metabolism, as would be predicted by the oxygen limitation model (Pörtner, 2002). This therefore suggests that temperature-related mortality between the T_{BP} and the T_{AB} is caused by an inability to generate energy dynamically at a rate that meets the cellular demand. Because the T_{BP} coincides with the thermal limit for locomotor performance and is the thermal threshold for time-dependent mortality, it probably holds similar adaptive significance to *E. malaccana* snails as the CT_{max} of most other ectotherms, even though it is well below the acute lethal temperature in the case of these snails.

In conclusion, although contemporary theory for thermal adaptation considers only the consequences of locomotor performance, organisms in which locomotor performance and thus energy gain are severely constrained are more likely to improve fitness by reducing resting energetic losses relative to thermal variation. We show that *E. malaccana* snails actively depress metabolism in response to heating (thermonegative metabolism), a physiological anomaly that is seemingly driven by their need to conserve energy at high temperatures to offset lifelong constraints on energy gain. The case for these snails points to the need to broaden the context of thermal adaptation theory (Huey and Kingsolver, 1989; Gilchrist, 1995; Gilchrist, 2000; Gillooly et al., 2001; Pörtner, 2010) to include the concept of resting metabolic performance.

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