

## SHORT COMMUNICATION

# Substantial heat tolerance acclimation capacity in tropical thermophilic snails, but to what benefit?

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## ABSTRACT

The theory for thermal acclimation of ectotherms suggests that (1) heat tolerance is traded off for thermal acclimation in thermophilic species and that (2) plasticity is constrained in tropically distributed ectotherms, which commonly experience relatively thermally stable environments. We observed substantial heat tolerance plasticity in a test of this theory using tropical, thermophilic marine intertidal snails that inhabit extremely hot and highly variable thermal environments. The implication of these results is that plasticity selection is largely driven by habitat temperature conditions irrespective of basal heat tolerance or latitude. However, heat tolerance of field-fresh snails was comparable with that of laboratory warm-acclimated snails, suggesting that snails in the field may often be unable to improve heat hardening with further environmental warming. These findings suggest that field referencing is crucial to using laboratory-measured acclimation capacity when inferring climate warming vulnerability in ectotherms, and overall they question how well current thermal biology theory predicts the outcomes of global change in intertidal environments.

**KEY WORDS:** Climate change, Gastropods, Heat stress, Latitudinal gradients, Thermal adaptation

## INTRODUCTION

The capacity of individual ectotherms to resist climate warming depends upon the plasticity of their heat tolerance. Because tropical animals are thought to have relatively inflexible heat tolerance limits, they are often predicted to be vulnerable to climate warming (Stillman, 2003; Ghilambor et al., 2006; Somero, 2010; Overgaard et al., 2011; Gunderson and Stillman, 2015). However, these conclusions are based on taxa that do not encompass the full range of physiological and ecological diversities in the tropics. In particular, a core assumption of many studies is that tropical environments are both hot and (relatively) thermally stable (Deutsch et al., 2008). This combination is incongruent with selection for phenotypic plasticity, which may account for the paradigm of inflexible heat tolerance in the tropics, which we term the tropical acclimatory limitation (TAL) hypothesis. However, tropical marine intertidal habitats can be both extremely hot and highly thermally variable (Helmuth and Hofmann, 2001).

Because of inherent biophysical constraints on high temperature physiology, plasticity of ectotherm heat tolerance is predicted to be

negatively related to basal thermal tolerance (Gunderson and Stillman, 2015; Somero et al., 2017). Under this acclimatory trade-off hypothesis (ATO), species trade off increased basal tolerance for reduced plasticity. Thus, species near the middle of the metazoan heat tolerance spectrum should exhibit greater plasticity than thermophilic species near the spectrum's upper end. However, empirical work has focused mainly on species with critical thermal maxima ( $CT_{max}$ )  $<50^{\circ}C$ , without incorporating data for highly thermotolerant species (Gunderson and Stillman, 2015).

We tested both hypotheses using a tropical gastropod snail that experiences highly variable and extreme temperatures and is ranked among the most heat-resistant metazoans (Liao et al., 2017; Somero et al., 2017). *Echinolittorina malaccana* (Philippi 1847; Gastropoda) inhabits the tropical high rocky shore zone, and displays complex physiology to withstand air temperatures exceeding  $56^{\circ}C$  (Marshall and McQuaid, 2011; Marshall et al., 2011; Liao et al., 2017). Despite the recent discovery of several heat-related molecular innovations (Liao et al., 2017), heat tolerance plasticity of this species has not previously been explored. Because of heterogeneity across their vertical and microhabitat distributions (Helmuth and Hofmann, 2001; Marshall et al., 2013; Fig. S1), different *E. malaccana* individuals on the same shoreline can experience vastly different thermal regimes. Moreover, the thermal regime that an individual snail experiences can shift drastically within the time frame of a single tidal cycle; after tidal wetting, whether a snail rests in the shade or sun is unpredictable because snails abruptly halt to avoid the desiccation risk of moving over dry rock surfaces (Marshall et al., 2010, 2013; Monaco et al., 2017; Fig. S1). Contrary to the hypotheses tested, we found that heat tolerance of laboratory-acclimated *E. malaccana* snails is highly plastic. However, the benefit of this plasticity is unclear, as field heat tolerance levels were close to the upper end of the species' capacity. This implies that even a high degree of plasticity of tropical species living in thermally heterogeneous environments will not necessarily provide the thermal safety margin required in the face of climate warming.

## MATERIALS AND METHODS

We measured tolerance of acute heating in field-fresh snails and snails laboratory acclimated to warm or cool temperatures. Snails (7–9 mm) were collected while awash and feeding from their lowest intertidal distribution (1.8–2 m chart datum) on a rocky shore seawall at Pantai Tungku, Brunei Darussalam ( $4.974^{\circ}N$ ,  $114.867^{\circ}E$ ; October 2017 to April 2018). In the laboratory, snails were rinsed in fresh seawater (start of acclimation) or dried in blown air ( $30^{\circ}C$  for 20 min; Memmert UFE 500, Schwabach, Germany) to inactivate them for field-fresh determinations. Heat tolerance experiments for the field-fresh snails began within 12 h of collection (Fig. S1).

We cool acclimated snails in air at  $22$ – $23^{\circ}C$ , which approximates the coolest conditions experienced by tropical *Echinolittorina* populations (Marshall et al., 2010; Monaco et al., 2017; Fig. S1).

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We warm acclimated snails to a daily thermal cycle from 25 to 45°C in a Memmert Peltier-cooled (IPP400) incubator, to mimic sun-exposed, high-shore conditions (Fig. S1). These acclimations proceeded for 10 days, with snails (unfed and resting) immersed each day in flowing seawater for 5 min, to simulate tidal wetting, to maintain full hydration and to prevent deep aestivation (Marshall and McQuaid, 2011). Because these snails are adapted to withstand month-long continuous air exposure and because they undergo temperature-independent metabolic rate depression up to 45°C, we assumed no interactive energetic effect between lack of feeding and temperature for the different acclimation treatments (Marshall and McQuaid, 2011; Marshall et al., 2011). The laboratory temperature regimes were pragmatically selected to produce the greatest differential in response, based on pilot studies that tested effects of nutritive and hydration states, thermal conditions and acclimation duration on heat hardening of this species. In the field, cooler daily regimes are relatively thermally stable, whereas hotter regimes exhibit greater thermal fluctuation (Fig. S1).

Two measures of heat tolerance were used: median lethal temperature following acute heat exposure ( $LT_{50}$ ) and cardiac flat-line temperature (FLT), the temperature at which the heart fails during acute heating (Marshall et al., 2011). Note that the better-known critical thermal maximum ( $CT_{max}$ ) is inappropriate for measuring heat tolerance in these snails, because desiccation-related inactivity precedes thermal stress (Marshall and McQuaid, 2011; Marshall et al., 2013, 2015; Monaco et al., 2017). For both assays, snails were placed in dry 50 ml glass tubes in a programmable bath (Grant TXF200, Cambridge, UK) and equilibrated at 30°C for 10 min before being heated at 0.25°C min<sup>-1</sup>, a realistic heating rate for tropical marine intertidal situations (Marshall et al., 2010, 2013; Monaco et al., 2017, Fig. S1). To maintain water bath temperature stability during the  $LT_{50}$  experiment, heating rate was slowed to 0.12°C min<sup>-1</sup> between 50 and 60°C. Temperature inside the test tubes was recorded every minute using calibrated K-type

thermocouples connected to a TC-08 interface and Picolog software (Pico Technology, Cambridge, UK).

For  $LT_{50}$  determination, groups of 15 snails were randomly removed from the bath at 1°C intervals from 55 to 59°C, and allowed to recover at 28°C in wetted plastic Petri dishes. Overall, we used 15 snails per temperature per trial, for a total of 18 trials (6 each for condition). Snails that emerged from their shells, extended their foot and remained attached to the surface after 12 h were scored as alive; alive but unattached snails, which are ecologically non-functional and eventually die, were scored dead. The effect of acclimation on  $LT_{50}$  was statistically compared with generalized linear models, using a binomial distribution with logit-link function (Statistica v12, StatSoft, Tulsa, OK, USA). Logistic regressions were plotted with Sigmaplot v14 (Systat Software, Inc., San Jose, CA, USA). The procedure for determining cardiac activity and FLT is described in detail elsewhere; this refers to the 0.25°C temperature band in which the last detectable heart pulse falls, during experimental warming (Marshall and McQuaid, 2011; Marshall et al., 2011; Fig. S2). FLTs were compared among treatments with one-way ANOVA (Statistica v12). For each assay, we determined absolute ( $\Omega$ ) and relative acclimation potentials (acclimation response ratio or ARR), which respectively refer to the change in heat tolerance and this change as a ratio of the change in body temperature (see Gunderson and Stillman, 2015).

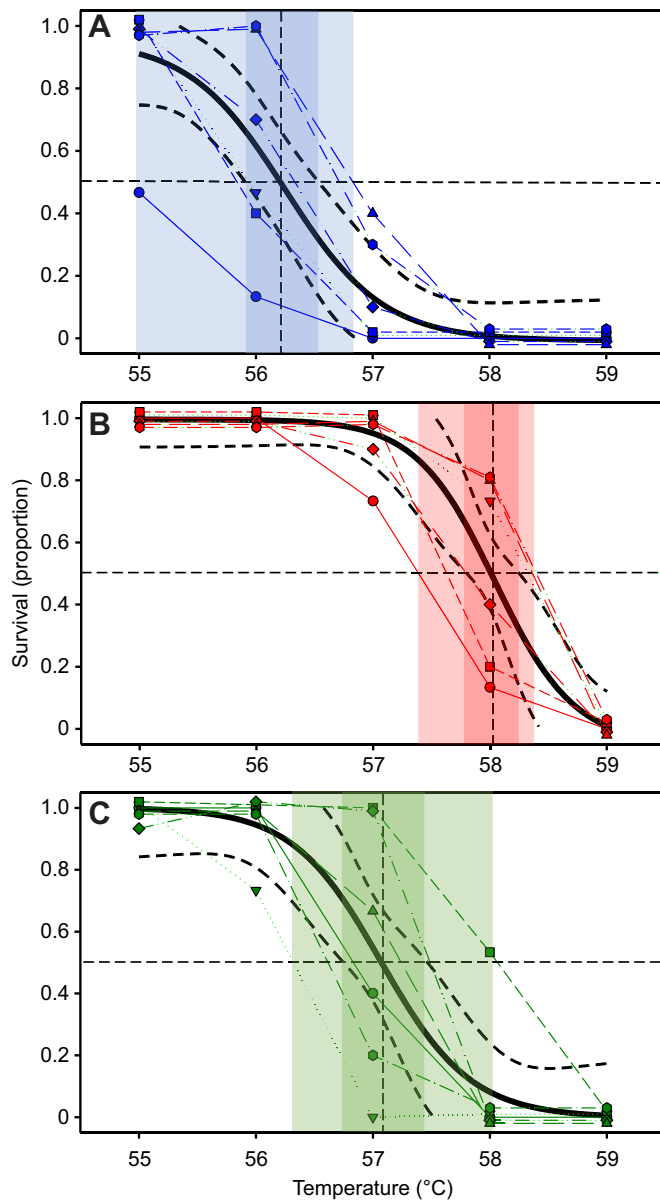
## RESULTS AND DISCUSSION

Comparisons of cool and warm laboratory-acclimated *E. malaccana* snails revealed  $\Omega$  and ARR of ~2°C and ~0.2, respectively, for both heat tolerance assays (Table 1).  $LT_{50}$  following acute heating increased significantly from 56.2°C in cool-acclimated snails to 58.0°C in warm-acclimated snails, and the mean cardiac FLT of individual snails shifted from 55.7 to 57.6°C with warm acclimation (Figs 1 and 2, Table 1). These potentials compare favourably with the plasticity of inherently less

**Table 1. Statistics for acute heat tolerance and acclimatory potential for cool- and warm-acclimated and field-fresh *Echinolittorina malaccana* snails**

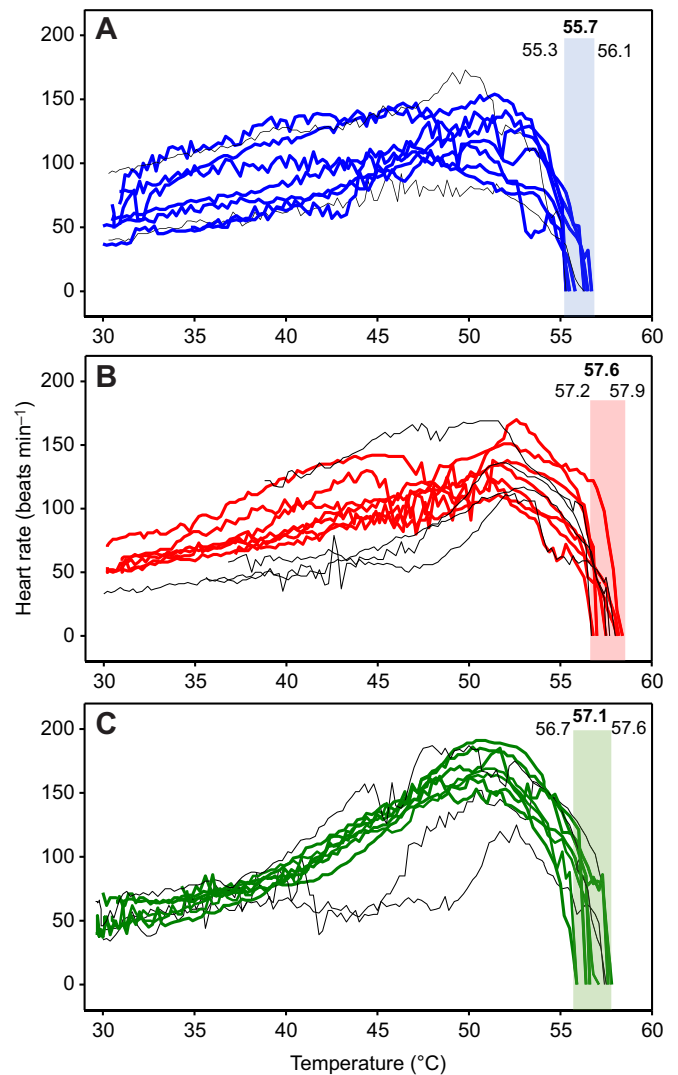
Heat tolerance				
$LT_{50}$	Value	95% CI	<i>n</i> (trials)	
Cool	56.2	55.9–56.5	6	
Warm	58.0	57.8–58.3	6	
Field	57.1	56.7–57.4	6	
FLT	Mean	95% CI	<i>n</i> (individuals)	
Cool	55.7	55.3–56.1	12	
Warm	57.6	57.2–57.9	14	
Field	57.1	56.7–57.6	8	
Comparisons and acclimation potentials				
$LT_{50}$	$\Omega$ (°C)	Wald statistic	<i>P</i> -value	ARR
Warm versus cool	1.8	90.23	<0.001	0.20
Warm versus field	0.9	55.56	<0.001	0.47
Cool versus field	0.9	66.51	<0.001	0.13
Average				<b>0.27</b>
FLT	$\Omega$ (°C)	<i>F</i> -value	<i>P</i> -value	ARR
Warm versus cool	1.9	29.05	<0.0001	0.22
Warm versus field	0.5	29.05	<0.25	0.26
Cool versus field	1.4	29.05	<0.001	0.2
Average				0.23

Median lethal temperature following acute heat exposure ( $LT_{50}$ ) values were determined from logistic regressions. Flat-line temperatures (FLT) were determined from the last heart pulse under constant warming. Acclimation response ratios (AARs) were calculated from the mean exposure temperatures (Fig. S1).  $\Omega$ , absolute acclimation potential. CI, confidence interval. Comparative tests involved either generalized linear models or one-way ANOVA (see Materials and Methods).



**Fig. 1. Survival of acute heating by laboratory-acclimated and field-fresh *Echinolittorina malaccana* snails.** Logistic regressions based on the combined data for each condition are shown as solid black lines, with associated 95% confidence intervals shown as dashed black regressions. Plots of the six individual trials (based on 75 snails each) are shown for each acclimation condition in coloured lines: (A) cool acclimation; (B) warm acclimation; (C) field fresh. Vertical dashed lines indicate median lethal temperature following acute heat exposure ( $LT_{50}$ ) values, dark bands indicate 95% confidence intervals, and light bands indicate the range of responses (Table 1).

heat tolerant, temperate populations/species (Gunderson and Stillman, 2015), thus contradicting both the TAL and ATO hypotheses (Stillman, 2003; Ghalambor et al., 2006; Somero, 2010; Overgaard et al., 2011; Gunderson and Stillman, 2015). Coupled with snail exposure to spatially and temporally highly variable heating (Fig. S1), these potentials advocate habitat thermal heterogeneity as the primary driver of rapid reversible heat-hardening selection in thermophilic and tropical animals (Chevin and Hoffmann, 2017). Variable temperature exposures of *E. malaccana* snails within a tidal–daily time frame develop from the complexity of the emersion–submersion thermal regimes,



**Fig. 2. Heart rate–temperature curves and flat-line temperatures (FLT) of individual snails during acute heating following the various acclimation conditions.** Data are shown for cool acclimation (A), warm acclimation (B) and field-fresh (C) conditions. Means  $\pm$  95% confidence intervals for FLT values are indicated at the top of each coloured bar; coloured bars represent ranges. Traces were drawn down to FLT, determined from the last heartbeat. Outlier responses are indicated with thin black lines, but were used to calculate mean FLT (see Fig. S2).

inactivity behaviour to limited desiccation risk and the unpredictability of heat loading at resting sites during emersion (Marshall et al., 2013, 2015; Monaco et al., 2017; Fig. S1). Whereas acute heating above  $\sim 45^{\circ}\text{C}$  induces a heat-shock response in *E. malaccana* (Marshall et al., 2011; Han et al., 2019), the loss of heat hardening in cooler regimes should also be adaptive (Hoffmann et al., 2003), considering the extension of the geographical distribution of this species into cool temperate China (Reid, 2007). Furthermore, the scenario presented here for littorinid snails does not necessarily generalize taxonomically across co-habiting intertidal animals. For example, behaviourally thermoregulating crabs living side by side with these snails always shelter under rocks, and thus always experience relatively stable and cool temperatures, have reduced heat tolerance and have reduced acclimation potential (Stillman, 2003; Somero, 2010).

Comparisons between laboratory-acclimated and field-acclimated snails in this study allude to the importance of field

referencing when interpreting acclimation capacity.  $LT_{50}$  values for field-fresh snails were significantly greater than those for cool-acclimated snails, and lower than those of warm-acclimated snails, although individual measures (ranges) strongly overlapped with those of warm-acclimated snails (Figs 1 and 2, Table 1). FLT values of field-fresh snails, however, were not significantly different from those of warm-acclimated snails (Figs 1 and 2, Table 1). Thus, despite substantial capacity for acclimatory adjustment, the field-fresh snails were sometimes nearly completely acclimatized, such that improvement of hardening with further environmental warming may often be limited. Notably, snails occupying hotter habitats on the rocky shore seem less likely to further adjust tolerance and are thus under a greater future warming threat. The overlap between field measurements and the most extreme tolerance we could elicit in the laboratory indicates a need for field referencing when using acclimation potentials ( $\Omega$  and ARR) in climate warming vulnerability assessments, as the acclimatory response may already be fully expressed by individuals in nature.

Most studies relating ectothermic physiological responses with latitude consider marine, terrestrial and freshwater ectotherms distinctly, without considering intertidal organisms that span habitats. Whereas physical, ecological and behavioural phenomena can buffer or obscure latitudinal–thermal relationships in intertidal systems (Helmuth et al., 2006; Jurgens and Gaylord, 2018), there are also cases at all latitudes where these organisms are exposed to exceptional water and thermal stresses. Including these outliers, as we have here, contradicts the widely held expectations about tropical species lacking plasticity and basal heat tolerance constraining plasticity. However, integration with field studies shows that these individuals may nevertheless be under risk from increasingly warming environments, suggesting that theory cannot yet use thermal biology to predict the outcomes of global change in marine intertidal environments.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.J.M.; Methodology: D.J.M., B.J.S., Y.D.; Software: D.J.M.; Formal analysis: D.J.M., A.B., N.M.; Investigation: D.J.M., A.B., N.M.; Resources: D.J.M., A.B., N.M.; Data curation: D.J.M., A.B., N.M.; Writing - original draft: D.J.M., B.J.S., Y.D.; Writing - review & editing: D.J.M., A.B., N.M., B.J.S., Y.D.; Visualization: D.J.M., A.B., N.M.; Supervision: D.J.M.; Project administration: D.J.M.

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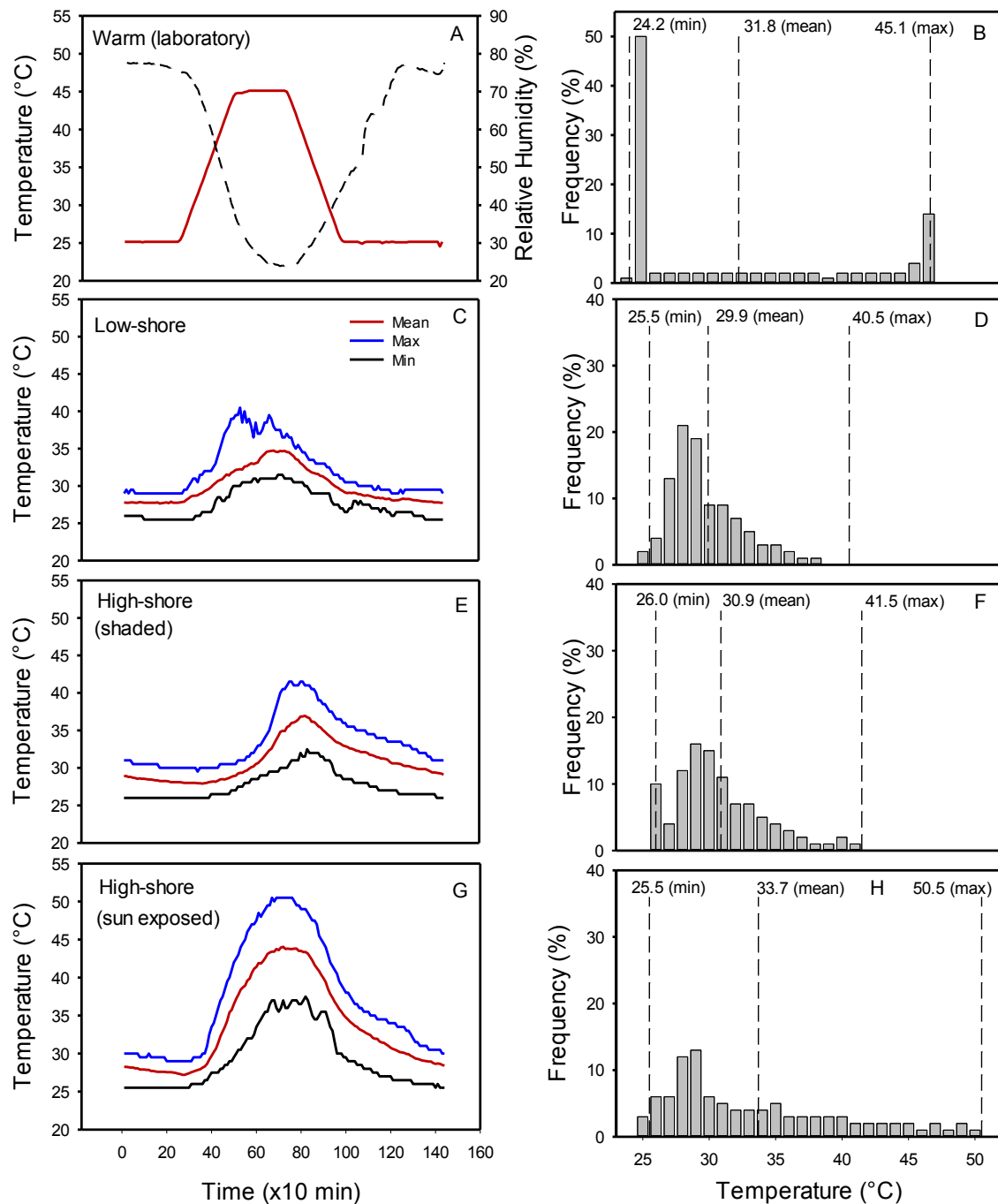
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#### Supplementary information

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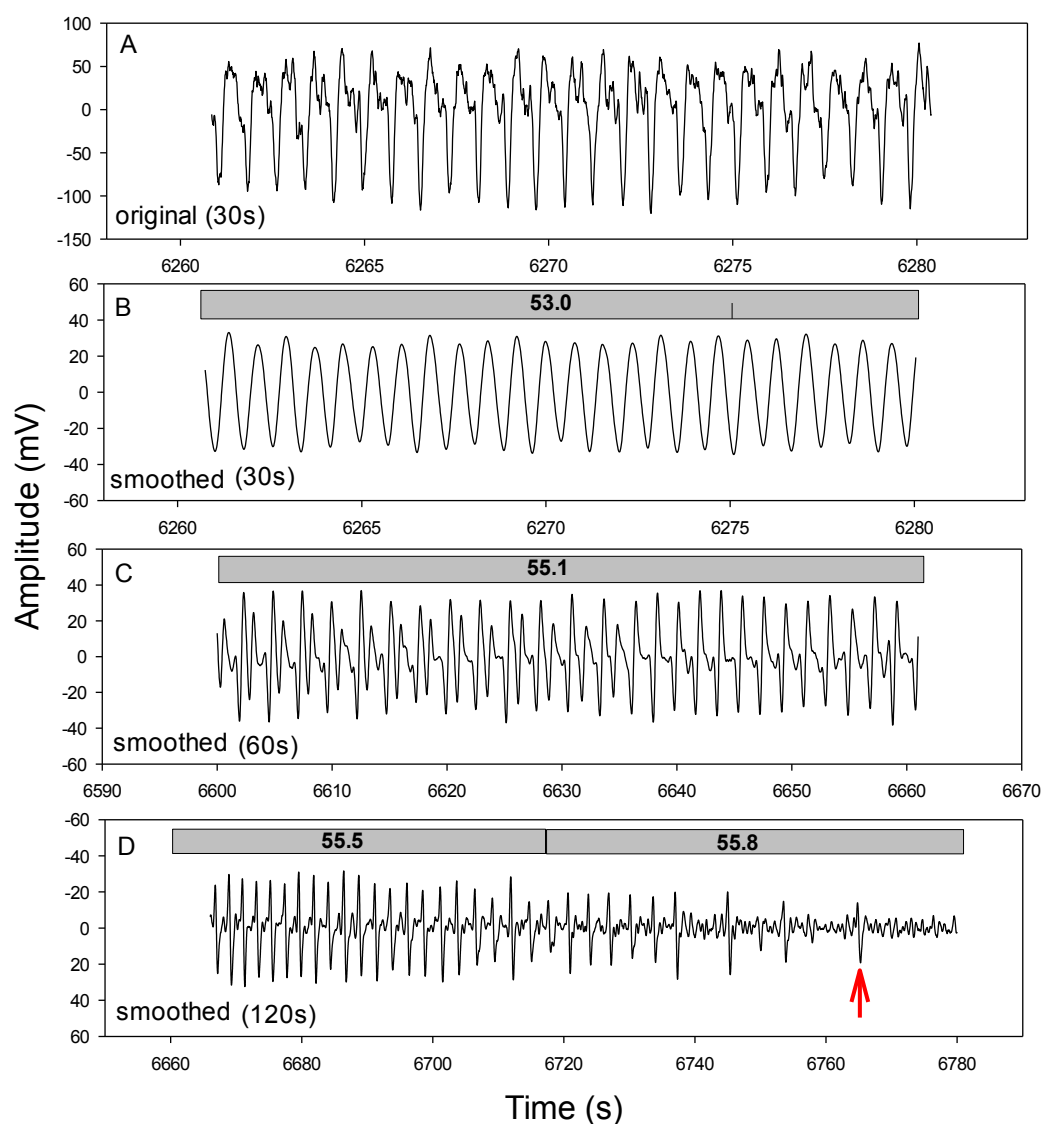
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**Figure S1. Daily temperature regimes and thermal frequencies.** Figures show warm-acclimated treatment (A and B) and temperatures of field habitats (low-shore, C and D; high-shore shade, E and F, and high-shore sun, G and H). All data were collected using DS1923-F5# Hygrochron loggers for 10-14 days. Field temperatures were recorded between 15 and 29 August 2017 at a nearby station (4.969°N, 114.858°E) and not at the same locality where experimental snails were collected. Temperatures for the stable cool-acclimated treatment are not shown in the figure (mean = 22.9, max = 23.6 and min = 22.1°C). The daily fluctuating warm-acclimated temperature profile (A) approximates the high-shore sun-exposed mean (G), whereas the

stable cool-acclimated treatment approximates the minimum high-shore-shaded habitat (E) during the coolest months of the year.

Local snails may experience any of the above daily field temperature regimes. Regime changes may take place following wetting, when individual snails may rest in the sun or the shade, depending on the desiccation risk associated with shade-seeking activity. Thus air-exposed individuals may settle and glue their shells to surfaces that become much hotter (direct solar exposure, G) or much cooler (low-shore or high shore shade, C and E) than the surfaces occupied before tidal wetting and activity. Notably, resting/aestivating individuals in the high-shore may remain in the same position for months with no tidal submersion.



**Figure S2. Cardiac traces showing show determination of FLT.** X-axis represents time (s) since the start of the experiment, and Y-axis the the millivolt amplitude. Original (A) and smoothed traces (Triangular Bartlett window smoothing for 50 samples; B) at 53.0°C. (C) and (D) show smoothed traces for the last three minutes of the recording, with associated temperatures (gray bars) and the recoding periods. The red arrow indicates the last pulse within the FLT time band of 55.8°C. In some cases the last beat was less clear, but FLT could always be assessed from loss of periodicity within a one-minute (0.25 °C) band. Heart beat was recorded using infrared optoelectronic sensors (Vishay Semiconductors, CNY70), a custom-built pre-amplifier, and an ADInstruments PowerLab 4/SP (Marshall and McQuaid, 2011; Marshall et al., 2011).