Untangling the roles of microclimate, behaviour and physiological polymorphism in governing vulnerability of intertidal snails to heat stress

Yun-wei Dong, Xiao-xu Li, Francis M. P. Choi, George N. Somero and Brian Helmuth

1. Introduction

Forecasting the responses of plants and animals to anticipated novel climate conditions is now a major focus of climate change research [1,2]. A key aspect of this effort is the elucidation of mechanisms that determine organisms' thermal sensitivities and their capacities for coping with rising temperatures. When combined with information on current and future environmental conditions, such information can provide a foundation for predicting effects of climate change on biogeographic patterning. An increasing number of studies have pointed to the likely (but often ignored) importance of small-scale processes that may cumulatively interact to drive emergent patterns at much larger scales. For example, recent studies have pointed to the role that thermal refugia—microhabitats where microclimatic conditions permit survival during extreme events—may play [3,4], and the key importance of behaviour in accessing these microhabitats [5,6]. Authors have also highlighted the potential consequences of ignoring biochemical and physiological variability among conspecific organisms [7] and, further, have
pointed to the importance of considering not only mortality but also changes in cumulative physiological performance in response to environmental change [2,8,9]. Despite these advances, it is very rare for studies to incorporate these considerations simultaneously under field conditions, especially over large geographical scales and among multiple species.

Vulnerability to present-day and future increases in temperature is often quantified using a thermal safety margin (TSM) metric (electronic supplementary material, figure S1), defined as the difference between an organism's critical thermal maximum and the highest temperature that an organism is likely to experience in nature [10]. The use of TSMs has sparked a wide-ranging discussion, for example, comparing the TSMs of tropical versus temperate organisms [5,10], the relative importance of average temperatures versus variability in temperature [11], and the role of behavior in allowing organisms to avoid lethal temperatures through behavioral thermoregulation [5,12]. TSMs have also contributed to studies exploring the potential for populations with limited genetic dispersal (such as brooding organisms) to adapt to local environmental conditions [13]. These studies implicitly rely on a quantitative comparison of what realized (operational) temperatures of organisms are in the field, and the lethal limits of the studied organisms.

Several factors make this comparison a more complex endeavor than is generally recognized (e.g. [14]). First, geographical patterns in environmental drivers can exhibit large-scale mosaics that do not conform to latitudinal gradients [15,16]. Second, even within a population, organisms can experience highly variable temperatures due to microhabitat variation, especially between sun-exposed and shaded microhabitats [6,17,18]. Mobile species can also exhibit behavioral thermoregulation [4,19], moving among microhabitats in anticipation of impending extreme events [20,21]. For example, during hot periods, animals may seek refuge in cooler, shaded areas [4,5,17], or, in intertidal systems, can migrate lower down the shore [22]. As a result of a combination of these factors, body temperatures of mobile species are often substantially different from the temperature of the surrounding air or substrate [16,23], which has prompted calls to forecast species range edges as a function of large-scale environmental conditions, but on conditions in refugia available to each species of interest [6,18,24].

Physiological variation (polymorphism) among conspecifics at a single site, and populations of a species in different environments, also requires deeper understanding for predicting the effects of climate change. Such variations in physiological responses are rarely incorporated in models of species’ range limits, which often apply a single TSM value for that species’ critical lethal maximum across its entire range [5] (but see [25]). Even in cases where behavioral thermoregulation and microhabitat variation are included, inter-individual variability in thermal sensitivity is generally not accounted for, due to difficulties in quantifying processes at these small spatial and temporal scales (e.g. [26]). We, therefore, have a rather poor understanding of how a failure to simultaneously consider microhabitat heterogeneity, behavioral thermoregulation [27], physiological polymorphism [7], and environmental predictability [28] affects forecasts of the ecological impacts of climate change [29].

Another gap in our understanding of thermal physiology concerns TSMs based on sublethal, but physiologically consequential effects of warming. Conventionally, TSMs have focused on lethal effects, but sublethal stress that impairs physiological function will also influence the success of organisms facing rising temperatures. The cumulative effects of prolonged exposure to sublethal conditions such as these can lead to reproductive failure and contribute to mortality in the face of other biotic and abiotic stressors [29].

Here, we use heart rate as an index of thermal tolerance and as an indicator for determining when sublethal physiological stress occurs. In general, heart rate increases with temperature until the Arrhenius breakpoint temperature (ABT) is reached (electronic supplementary material, figure S1), after which heart rate decreases rapidly and anaerobic metabolism is activated [30]. In all intertidal mollusks examined to date, recovery from exposure to the ABT is possible, although ‘ecological death’ can occur when animals are unable to avoid predators due to decreased movement or maintain their position on the rocky surface [31–33]. When temperature further increases to the flat line temperature (FLT), where heart-beat ceases, death ensues [34]. TSM values were, therefore, calculated from FLT and ABT (TSM_{FLT} and TSM_{ABT}) to indicate the effects of lethal and sublethal thermal stress, respectively (electronic supplementary material, figure S1).

Rocky intertidal habitats are a model system for exploring the ecological consequences of thermal stress [17,35–37], as thermal environments are extremely dynamic and variable, which can result in organism body temperatures well above air temperatures when in direct sunlight [17,23]. Physiological responses of species to thermal stress are also highly variable among populations [38,39]. Whereas relatively few studies have measured intra-population physiological polymorphisms in intertidal species, the few studies that exist also suggest high inter-individual variability in physiological responses to thermal stress [40–42].

In this study, we use a combination of empirical measurements and theoretical models to examine links among (i) patterns of heterogeneity in a thermal landscape [43,44], (ii) the variation in thermal tolerance (physiological polymorphisms) among individuals, populations, and species, (iii) the predictability of extreme events, and, ultimately, (iv) the probabilities of encountering physiologically damaging or lethal temperatures in different microhabitats and sites.

2. Material and methods

(a) Animal collection and acclimation

We used three species of slow-moving intertidal snails as a model system (electronic supplementary material, figure S2): (i) Littorina sinensis, a species widely distributed along the Chinese coastline, (ii) Littoraria breviscula, a northern species with its current southern distribution limit in Xiamen, where the population has been observed to disappear during the summer presumably due to high temperatures, and (iii) Nerita yoldii, a southern species with its northern distribution limit located at Zhashan. In the last three decades, a northward shift of the distribution of N. yoldii to the middle of the Yangtze River Delta has been observed. Following collection, animals were randomly allocated into plastic containers at fixed densities and acclimated at 16 C under identical conditions for 6–282 days, a period likely to be sufficient to eliminate past thermal history [34].

(b) Operative temperature estimations

Body temperatures of snails were estimated to examine the potential range of microclimatic conditions within each site and across
the eight study sites (five sites for N. yoldii). Within each site, body temperature estimations in two types of microhabitats were calculated to reveal the maximum thermal range snails may experience: one representing fully shaded refugia where temperatures would be lowest, and the other in full sunlight, where animal temperatures would be maximal on cloud-free days (see electronic supplementary material for further details). The goal of these models was not to calculate the probability distribution function of temperatures as a function of microhabitat types; rather it was intended to bracket the range of temperatures in the coolest and warmest microclimates at each location. All body temperature estimations were calculated using a heat budget model [45] modified for snails from a model originally developed for mussels [46]. The model was tailored by changing the shape and size of the animal, and the amount of contact with the substratum to be specific for each snail species. Environmental variables used in the heat budget model were hourly air temperature, wind speed, and solar radiation from 1983 to 2013. Data were downloaded from the pixel closest to each study site from the National Centers for Environmental Prediction Climate Forecast System Reanalysis (CFSR), which provides hourly data with a spatial resolution (pixel size) of 0.5° × 0.5° (approx. 38 km) [47]. Body temperature predictions for each species at each site were summarized as the maximum temperature observed over the 30-year period as well as the 95th percentile of modelled temperatures.

(c) Cardiac performance measurements

A non-invasive method was used to measure individual heart rates [48]. Experimental temperatures were increased from 16°C (acclimation temperature) at a rate of 6°C h⁻¹ in air until a temperature was reached where the heart rate fell to zero (FLT). The infrared heartbeat signal was amplified (AMP03, Newshift, Leiria, Portugal), filtered, smoothed, and recorded with a Powerlab unit (4/30, ADInstruments, March-Hugstetten, Germany) or PicoScope (2204, Pico Technology, Cambridgeshire, UK). Data were viewed and analysed using LabChart (v. 7.2) for Powerlab measurements (electronic supplementary material, figure S4), and PicoScope (v. 6.10.6.1) for PicoScope data. ABTs were calculated by linear regression using data that lay either above or below the temperature at which the highest heart rate occurred (electronic supplementary material, figure S1), using Origin version 9.0 (OriginLab Corp., MA, USA). FLT was the temperature at which cessation of heartbeat was first observed.

(d) Thermal safety margin and temporal autocorrelation analysis

TSMs for sublethal (TSM_{ABT}) and lethal (TSM_{FLT}) effects were calculated at each site for snails in shaded and full sun-exposed microhabitats by comparing body temperature against measured ABTs and FLTs for individuals from each population of the three species.

The number of days when temperatures exceeded ABTs and FLTs (stressful periods) was noted, as well as the average return time of events within the same year. To examine the temporal patterns of stressful body temperature at each site, we conducted a temporal autocorrelation analysis [16]. Autocorrelation represents the degree to which a data point in a times series is influenced by its own historical values, i.e. the degree to which environmental conditions on one day are diagnostic of future environmental conditions at different lag times. We calculated autocorrelation in maximum daily predicted body temperatures for each species at each site for lag periods ranging from 1 to 14 days.

(e) Statistical analyses

Differences in ABT and FLT between sites and species were analysed using two-way analysis of variance (ANOVA) (fixed factors, species, and sites) with Tukey’s post hoc analysis for significant factors using SPSS 22 (SPSS, Chicago, IL, USA).

Cardiac performance curves were generated by fitting data with an exponentially modified Gaussian (EMG) [49]. Variance of cardiac performance curves between individuals was calculated with generalized additive modelling (GAM). Individual variance was estimated by comparing the cardiac performance curve of a single individual and that of its population. EMG and generated additive mix modelling (GAMM) were carried out in R (v. 3.2.2) with the packages mgcv [50] and nlmixr [51]. The same method was used for analysing the variance among different populations within the same species, using the overall performance curve for all populations as a comparison.

3. Results

Our empirical and modelling results demonstrate significant variation among species, conspecifics, and habitats in thermal stress relationships. We first present data on cardiac performance and, then, integrate these data with field temperature measurements and model-derived values for thermal conditions found in microhabitats from the different latitudinal regions in which the species occur.

(a) Differences in cardiac performance among individuals and populations

All individuals shared a broadly similar pattern of heart rate response when temperature increased from 16°C to the FLT (figure 1; electronic supplementary material, figure S3). All species showed high thermal resistance, as indicated by FLTs that frequently exceeded 50°C (figure 1; electronic supplementary material, figure S3). The ABT and FLT values all exhibited high variations among different populations (electronic supplementary material, figure S4), and there were also significant inter-individual variations within the same population (e.g. the L. sinensis population in Xiamen, figure 1a; electronic supplementary material, table S1). Because of the high intra-population variability in cardiac performance, we analysed data using both a two-way ANOVA and a GAM. Two-way ANOVA was applied to analyse the difference in thermal limits (ABT and FLT) among different populations, and GAM was applied to analyse the difference of the whole cardiac performance curves among different populations. There was no significant difference in ABT among different populations of any species (two-way ANOVA, p = 0.273), but a significant difference for FLT was found between Yangguangdao and Xiamen (Tukey’s post hoc analysis, p = 0.017; electronic supplementary material, figure S4). Among the three species, the ABT of L. brevicula was higher than the other two species (Tukey’s post hoc analysis, p < 0.001), but there was no significant difference in FLT among the three species (two-way ANOVA, p = 0.128). The difference between FLT and ABT of L. brevicula was significantly lower than for the other two species (Tukey’s post hoc analysis, p < 0.001; figure 2). An F-test of GAM of heart rate to temperature results indicated that the cardiac thermal performance curves differed among different populations of the same species (electronic supplementary material, table S2; L. sinensis, figure 1b, L. brevicula and N. yoldii, electronic supplementary material, figure S3).
(b) Thermal limits in a heterogeneous thermal environment

The thermal microhabitats and, therefore, the predicted snail body temperatures were, as expected, markedly different between sun-exposed and shaded microhabitats. Maximum body temperatures in the sun-exposed microhabitats were at least 10°C higher than those in shaded microhabitats for all three species (electronic supplementary material, table S3). Differences in shell size, in comparison, were predicted to lead to small (generally less than 1°C) differences in $T_b$ among the three species (electronic supplementary material, table S3). Inter-individual variation in thermal tolerance and thermal environmental heterogeneity led to large differences in TSMs for both physiological impairment (TSMABT) and death (TSMFLT) between sun-exposed and shaded microhabitats (figure 3). In sun-exposed microhabitats, temperatures exceeded ABTs for most individuals in all populations at least once in the last 30-year period (figure 3c), and a few individuals at some sites were predicted to have body temperatures that exceeded the FLT. By contrast, most individuals could maintain normal cardiac function in the shaded microhabitats, where heating to the ABT was infrequent and temperatures never reached the FLT (figure 3b/3d).

However, sites also varied in the autocorrelation (predictability) of extreme events. Daily body temperatures were more predictable (greater temporal autocorrelation) in the shaded than in the sun-exposed microhabitats across all sites (figure 4), which is not surprising, given that shaded temperatures are primarily driven by air temperature and sun-exposed temperatures are driven by multiple environmental factors [45]. For all three species, body temperatures in the sun were least temporally autocorrelated at Xiamen (XM, 24.43° N), followed by Lvsi (LS, 32.11° N) and Wenzhou (WZ, 27.86° N).

**Figure 1.** High inter-individual, inter-population, and interspecific variability in cardiac performance curves. Heart rate versus temperature curves were generated using an EMG model for individuals exposed to increasing temperature at a rate of 6°C$h^{-1}$ from 16°C to the FLT. (a) Variation within a population: dashed lines depict individual heart rates and the solid line depicts the curve for all individuals of *L. sinensis* from the Xiamen population (n = 10). (b) Variation among populations: dashed lines depict heart rate curves generated for each population and the solid line depicts the curve for all individuals of *L. sinensis* from all populations (n = 70). (c) Interspecific comparisons.

**Figure 2.** Difference between the FLT and ABT of *L. sinensis*, *L. brevicula*, *N. yoldii*. The difference between FLT and ABT in *L. brevicula*, a northern species, is significantly lower than those of *L. sinensis*, a widespread species, and *N. yoldii*, a southern species. Box plot explanation: upper horizontal line of box, 75th centile; lower horizontal line, 25th centile; horizontal bar within box, median; upper horizontal bar outside box, 90th centile; lower bar outside box, 10th centile. The boxes with different letters represent significant differences.
Variation in thermal safety margins with latitude

TSM values were calculated for both FLT and ABT in sun-exposed (TSM_{FLT_SUN} and TSM_{ABT_SUN}) and shaded microhabitats (TSM_{FLT_SHADED} and TSM_{ABT_SHADED}) at all sites (figure 5; electronic supplementary material, figure S5). The TSM values varied in a nonlinear fashion with latitude. In sun-exposed sites, the southernmost (Xiamen) populations of all species lived close to their upper thermal limits as defined by the FLT (figure 5a; electronic supplementary material, table S4). Some mid-latitude populations, including Yangguangdao (YGD, 32.52° N), Zhendongzha (ZDZ, 34.20° N), Lianyungang (LYG, 34.76° N), and especially Lvsi, were even more challenged by thermal stress. Predicted maximum body temperatures in sun-exposed microhabitats were frequently much higher than the FLTs of all the three species at Lvsi (electronic supplementary material, table S4).

In shaded microhabitats, the maximum body temperature was below the FLT for all individuals at all sites, and was also below the ABT at most sites, except the Lvsi population (figure 5b). For most individuals in all populations, maximum body temperatures in the sun-exposed microhabitats were higher than their ABTs, so physiological impairment in the absence of behavioural thermoregulation is likely if animals remained in these microhabitats.

4. Discussion

Microhabitat heterogeneity, behavioural selection, and physiological polymorphism in thermal sensitivity can interact to establish widely different degrees of realized thermal stress over distances of centimetres within the rocky intertidal zone [18,52]. This small-scale variation in temperature provides a spectrum of thermal environments that can provide refugia to avoid lethal or sublethal heat stress [18]. The ability of an organism to benefit from those refugia depends on environmental factors [53], the rate at which it can move [54], its size relative to the microhabitat [21], and the frequency at which the organism can access shelter during unpredictable extreme conditions [55]. Thus, the ‘grain size’ of the thermal environment will vary among interacting species [21]. For example, a shaded microhabitat suitable for a snail may be too small to provide any benefit to a large animal such as a seastar [20,22]; and some species, such as chitons, with more flexible shell plates, can use habitats which are unavailable to fixed shell gastropods [22]. In the present study, shaded microhabitats provided a relatively benign thermal environment that is predicted to allow snails to maintain aerobic metabolism ($T_b < ABT$) and survive ($T_b < FLT$) compared with sun-exposed microhabitats.

However, even though all sites in this study presented snails with thermal refugia, temporal autocorrelation varied markedly among sites. Importantly, the three sites with the most extreme temperatures (Lvsi, Xiamen, and Wenzhou) also had the lowest thermal predictability. This suggests that although behavioural thermoregulation and physiological acclimatization could potentially be critical methods of avoiding lethal temperatures [5], the unpredictability of extreme conditions may promote variation in the strategies which
these animals adopt. While remaining in shaded microhabitats would seem to be a thermally beneficial strategy, most intertidal gastropods partition their behaviour to leave these habitats to forage on open rock surfaces, which provide more productive foraging areas than the smaller, shaded microhabitats [53,56–59], before returning after feeding to occupy refugia during the emersion period. Small-scale (i.e. between days) variability in duration of feeding and inactivity as a result of changing environmental conditions has been demonstrated for a number of intertidal species, suggesting these animals are highly plastic in their responses to daily environmental changes [53,60,61].

**Figure 4.** Body temperature autocorrelation of the three gastropod snails, *L. sinensis*, *L. brevicula*, and *N. yoldii* along the Chinese coastline in (a) shaded habitats and (b) sun-exposed habitats. Colour variation describes the correlation strength with dark red representing a perfect correlation (1) and dark blue a weak correlation (0.3).
This study also documented high physiological variation among individuals within and between populations, an aspect of population-level vulnerability that is often not considered when predicting the effects of temperature on species function and survival. The large inter-individual variations in ABT and FLT show that vulnerability to heat stress differs markedly among individuals at a single site. While it is not known if the variation observed reflects genetic variation or different states of acclimatization among individuals [38], the long (60–282 days) acclimation period prior to experimentation is likely to have eliminated effects of acclimatization and past thermal history [34]. Under field conditions, however, acclimatization could lead to variation in ABT and FLT. Such a strategy would be beneficial only in cases where extreme temperatures were preceded by days with moderately hot conditions, i.e. there was some degree of temporal autocorrelation in environmental conditions. In studies of intertidal turban snails (genus Chlorostoma), Stenseng et al. [34] found that laboratory acclimation could modify ABT by as much as 6.6°C, but FLT changed by no more than 1.1°C. Thus, TSMABT appears to be more physiologically plastic than TSMFLT [34], a trend which was confirmed for the three species in the present study.

An in situ survey of expression of genes related to the heat shock response and energy regulation in an intertidal limpet, Cellana toreuma, showed that the amplitudes of gene expression could differ among individuals by more than 1 000-fold at the same site and same date [40]. Though the inter-individual variation of physiological responses has received relatively little attention for evaluating the ecological impacts of climate change, the variation of thermal tolerance, not just the mean tolerance for all individuals, can lead to wide variation in metabolic status and survival [7]. In the present study, some individuals in the same population had relatively high thermal limits (both ABT and FLT) compared with conspecifics and could survive at the maximum habitat temperature, which was higher than the average thermal limit of the population. For intertidal invertebrates that are usually r-strategists, with high reproductive and larval dispersal capability, partial survival of the existing population is important for the maintenance of the local population [62], especially in the face of climate change. While not conclusive, our results suggest the benefit of high standing genetic variation, which is thought to confer population-level resistance to extreme events [2,63].

Studies of sublethal heating on intertidal invertebrates have shed light on the types of physiological changes that occur at temperatures near the ABT and on the amounts of inter-individual variation in physiological response. The importance of temperature sensitivity of cardiac function in setting thermal limits varies among taxa [2,35,37]. Collapse of cardiac function can cause death in marine crustaceans and, once the body temperature exceeds ABT, heart function cannot recover from heat stress [64,65]. By contrast, some gastropods can survive at temperatures several degrees higher than their ABTs [34], at least for a limited time. This finding suggests that adaptations of gastropods to limited oxygen supply (i.e. their abilities to rely on anaerobic pathways of ATP production) are important for their survival when cardiac function (oxygen delivery) is reduced at temperatures above the ABT. Even among gastropods, differences in ability to rely on anaerobic metabolism may exist. In this study, the difference between FLT and ABT of L. brevicula is smaller than those of the other two species, indicating that L. brevicula might have relatively limited ability to rely on anaerobic metabolism to cope with thermal stress.

Figure 5. TSM of three gastropods with latitude. Solid lines show linear regressions for TSM with latitude and dotted lines show 95% CIs. TSM was calculated for the FLT (a,b) and the ABT (c,d) in sun-exposed (a,c) and shaded habitats (b,d). (Online version in colour.)
Finally, the thermal sensitivity of intertidal species along the Chinese coastline shows a highly nonlinear relationship with latitude; the most thermally vulnerable population is found in the mid-latitude regions of the coastline. In China, these ‘hot spots’ of thermal stress, as indicated by TSMs, thus do not lie at low latitude, more tropical areas, but rather in mid-latitude sites such as the Yangtze River Delta (32°–34° N). The frequency of extremely hot habitats in this region may, under future conditions, create a biogeographic barrier that impedes the migration of some intertidal species northward along the Chinese coastline. Such a barrier could prevent the predicted general poleward shift of species described for other coastlines [66], and lead to a thermal mosaic more akin to that demonstrated for the west coast of North America [67]. Notably, the relative vulnerability among sites differed among the three species. Whereas Littorina and Littoraria were predicted to experience highest levels of stress at the Lvsi site, Nerita was predicted to exceed its ABT most frequently at the Xiamen site. While such interspecific differences in geographical patterns of thermal stress have been suggested [68], this is the first demonstration of this phenomenon in the field. Critically, these patterns were only recognizable given the approach taken here, which quantified intra- and interspecific variability in physiological vulnerability, body temperatures, and microhabitat influence. These results thus strongly caution against the simple use of a single metric for any of these components of vulnerability when assessing the likely geographical impacts of climate change, and argue for the inclusion of mechanistic detail at small scales when making predictions over large latitudinal gradients.

Data accessibility. All data associated with this paper can be found at the designated Dryad depository (http://dx.doi.org/10.5061/dryad.5vn5n) [69].


Competing interests. We declare we have no competing interests.

Funding. This work was supported by grants from National Natural Science Foundation of China (41476115), National Basic Research Program of China (2013CB956504), Program for New Century Excellent Talents of Ministry of Education, China, the State Key Laboratory of Marine Environmental Science, and Nature Science funds for Distinguished Young Scholars of Fujian Province, China (2017J07003).

References


65. Frederich M, Portner HO. 2000 Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, Maja squinado.


