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High thermal stress responses of *Echinolittorina* snails at their range edge predict population vulnerability to future warming

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Abstract

Populations at the edge of their species' distribution ranges are typically living at the physiological extreme of the environmental conditions they can tolerate. As a species' response to global change is likely to be largely determined by its physiological performance, subsequent changes in environmental conditions can profoundly influence populations at range edges, resulting in range extensions or retractions. To understand the differential physiological performance among populations at their distribution range edge and center, we measured levels of mRNA for *heat shock protein 70* (*hsp70*) as an indicator of temperature sensitivity in two high-shore littorinid snails, *Echinolittorina malaccana* and *E. radiata*, between 1°N to 36°N along the NW Pacific coast. These *Echinolittorina* snails are extremely heat-tolerant and frequently experience environmental temperatures in excess of 55°C when emersed. It was assumed that animals exhibiting high temperature sensitivity will synthesize higher levels of mRNA, which will thus lead to higher energetic costs for thermal defense. Populations showed significant geographic variation in temperature sensitivity along their range. Snails at the northern range edge of *E. malaccana* and southern range edge of *E. radiata* exhibited higher levels of *hsp70* expression than individuals collected from populations at the center of their respective ranges. The high levels of *hsp70* mRNA in populations at the edge of a species' distribution range may serve as an adaptive response to locally stressful thermal environments, suggesting populations at the edge of their distribution range are potentially more sensitive to future global warming.

Key words: biogeography, global change, heat shock response, physiological stress, distribution range

Introduction

Rising atmospheric carbon dioxide levels have led to changes in global temperature, climate in general and seawater chemistry resulting in subsequent impacts on species' distribution patterns (Hofmann & Todgham, 2010; Parmesan, 2003). Variation in environmental conditions resulting from climate change has, for example, been shown to result in broad biogeographic shifts of species, such as range retraction or expansion (Perry *et al*., 2005; Poloczanska *et al.*, 2013), as well as localized changes in species which are distributed in spatially patchy habitats (Somero, 2012). As many species occur over wide latitudinal or altitudinal gradients, populations in different locations of a species' distribution range may experience very different environmental challenges (Buckley & Huey, 2016; Mathiasen & Premoli, 2016; Vergeer & Kunin, 2013). Typically, populations at the edge of a species' range are located at the extreme of the environmental stress gradient that the species can tolerate (Gaston, 2009; Mägi *et al*., 2011; Sexton *et al*., 2009). Such populations are, therefore, assumed to be constrained by current environmental conditions and subsequently are predicted to be more sensitive to future climate change (Sagarin & Somero, 2006; Sorte & Hofmann, 2004). Understanding the divergent responses between different populations along their distributional range is, therefore, important to inform predictions of the ecological impacts of climate change.

Physiological adaptation is a key response for species to cope with environmental variations, and the physiological responses of different populations are closely related to their local environments (Helmuth, 2009; Seabra *et al*., 2015; Somero *et al*., 2016). The heat shock response (HSR), which is highly conserved across almost all taxa (Feder & Hofmann, 1999; Somero *et al.*, 2017), is a homeostatic response that maintains the correct protein-folding environment in the cell (Guisbert *et al*. 2008) and, as such, heat shock proteins (Hsps) are considered to be one of the most important cellular defense mechanisms against environmental stresses (Sørensen *et al*., 2003; Tomanek, 2010). As a consequence, Hsp expression is believed to reflect both the environmental variability associated with different microhabitats and its interaction with large-scale environmental gradients across the whole distribution range of a species (Dutton & Hofmann, 2009; Lima *et al*., 2016; Sagarin & Somero, 2006, Wang *et al*., 2018). Environmental stress is also an evolutionary driver for local adaptation in natural populations (Kawecki & Ebert, 2004). Generally, natural selection acts on the HSR in different populations by changing the expression of *hsp* genes, rather than by gene mutations that affect the properties of the heat shock proteins themselves (Bettencourt *et al*., 2002; Dutton & Hofmann, 2009; Sagarin & Somero, 2006; Sorte & Hofmann, 2004). As a result, geographic variation of expression of Hsps as well as plasticity of expression are both heritable (Somero *et al.*, 2017), and consequently, divergent HSRs to local thermal environments have been identified in various marine invertebrates (Gleason and Burton, 201*5;* Schoville *et al*., 2012), showing the evolutionary adaptation of the HSR to local environmental conditions (Tedeschi *et al*., 2016) and its suitability to be used as a measure of thermal sensitivity (Tomanek & Somero, 1999a).

Rocky shores are one of the most physically challenging habitats on earth, both on a local scale (as a result of tidal changes; wave action etc. Helmuth and Hofmann, 2001; Little *et al*., 2009) but also with highly variable thermal environments over biogeographic scales (Harley, 2008; Helmuth *et al*. 2002). Activation of the HSR within the typical range of body temperatures organisms experience is part of the strategy of intertidal species to cope with thermal stress. Distinct strategies of Hsp70 expression can be employed by intertidal species occupying different heights along the intertidal zone. High-intertidal species, for example, tend to have higher constitutive levels of Hsp70 than low- and mid-intertidal species. By contrast, lower-occurring species maintain low levels of Hsp70, but are capable of rapidly inducing high levels of synthesis when exposed to heat stress (Dong *et al*., 2008; Nakano & Iwama, 2002). Different populations of widely distributed species, thus, can face divergent thermal regimes at different locations along their distribution range (Helmuth *et al.* 2002, Dong *et al*. 2017). As a result, persistent regional differences in tidal regimes, climate and other environmental factors will act as selective forces to influence the physiology of intertidal species which span broad latitudinal ranges. Upper limits of the temperature range of Hsp synthesis are close to the highest body temperatures that these organisms experience under natural conditions (Sorte & Hofmann, 2005; Tomanek & Somero, 1999; Tomanek, 2002). Consequently, Hsp synthesis may be a thermally sensitive weak-link that contributes to setting species' thermal tolerance limits (Somero, 2004). The HSR is thus a convenient biochemical indicator to assess levels of physiological stress among populations along their distribution range (Dutton & Hofmann, 2009; Sagarin & Somero, 2006; Sorte & Hofmann, 2004, Stillman & Tagmount, 2009).

At present, many intertidal organisms experience temperatures at or above their tolerance limits during low tides in stressful seasons (Wolcott, 1973; Williams 1994; Williams *et al.*, 2005; Somero, 2012; Zhang *et al*., 2016). High shore species suffer from the most extreme thermal stress and, as a result, have been postulated to be especially vulnerable to climate warming (Tomanek & Somero, 1999b; Somero, 2012). The littorinid snails, *Echinolittorina malaccana* and *E. radiata* are widely distributed along the NW Pacific (NWP) coast, with representatives of the two species covering over 50 degrees of latitude from 5°S to 45°N-(Fig. 1, Reid, 2007). Living on the high shore, littorinid snails experience long emersion periods when environmental (=rock surface) temperatures can exceed 60 °C (Williams, 1994; Marshall *et al*., 2011; Seuront & Ng, 2016). The thermal environment of intertidal species along the NWP coastline is extremely variable among different locations (Dong *et al*., 2015), and shows a highly non-linear relationship with latitude (Helmuth *et al*., 2002; Lima *et al*., 2016; Dong *et al*., 2017). Different populations of *Echinolittorina* snails, therefore, face extreme and divergent thermal stresses along their distribution ranges.

Using specifically designed primers, we can precisely detect the mRNA levels of inducible isoforms of Hsp70 (*hsp70*), which is an important biochemical indicator to assess levels of physiological stress. Up-regulation of *hsp70* in response to thermal stress has been well documented in a variety of marine taxa (bivalves, gastropods and shrimps; Clark et al., 2008; Cottin et al., 2010; Giomi *et al*., 2016; Han *et al*., 2013; Prusina *et al*., 2014). In a previous study, we found that Hsp70 protein levels significantly increased in response to thermal stress in *Echinolittorina malaccana* (Marshall *et al*., 2011) which supports the use of up-regulation of *hsp70* mRNA to predict the production of Hsp70 protein in *Echinolittorina* snails, and the positive relationship between levels of *hsp70* mRNA and Hsp70 protein. The present study was, therefore, designed to highlight the importance of estimating population-specific physiological performance when evaluating and predicting the ecological impacts of climate change on species with wide geographic distributions. Specifically, we investigated variation in heat shock response using levels of mRNA for *hsp70* to test the hypothesis that populations at the range edge of the two *Echinolittorina* snails would be more sensitive to thermal stress than those located towards the center of their distribution.

Materials and Methods

Expression *of heat shock protein* in field-acclimatized populations across the species' biogeographic ranges

The latitudinal distribution of *Echinolittorina malaccana* ranges from India through Southeast Asia and into China, whilst *E. radiata* is a more northern species and ranges from Japan to Vietnam (Reid, 2007, Fig. 1). Based on the distribution of the two species in different ecoregions and previous phylogeographic studies (Reid, 2007; Spalding et al., 2007; Dong *et al*., 2012; Wang *et al*., 2015, 2016), the range center and edge of each species were classified. In the present study, the distribution center for *E. malaccana* includes the ecoregions of the Java Sea, Malacca Strait, Gulf of Thailand, Southern Vietnam and Gulf of Tonkin, and its northern distribution range includes the two north most ecoregions (Fig. 1); whilst for *E. radiata*, its distribution center includes the two ecoregions in the East China sea and Yellow Sea, and its southern distribution edge include two south most ecoregions (Fig. 1).

To measure expression of *hsp 70* under natural conditions (field-acclimated), ten locations along the species' ranges were selected, including for *E. malaccana* populations: Sanya (18.17°N), Da Nang (16.07°N), Si Chang (13.15°N), Sepang (2.59°N) and Singapore (1.29°N) which were determined as central populations, while Keelung (25.14°N), Xiamen (24.42°N) and Hong Kong (22.21°N) were determined as northern edge populations; and for *E. radiata*: Qingdao (36.05°N), Zhoushan (29.94°N) and Keelung (25.14°N) which were determined as central populations, while Xiamen (24.42°N) and Hong Kong (22.21°N) populations were determined as southern edge populations (Fig. 1). Daily maximal air temperature (2012-2014) for each site was download from NASA and analyzed in R using *ncdf4* package (Bosilovich *et al*., 2015). The thermal environment along the Northwestern Pacific coastline shows a highly non-linear relationship with latitude (Fig. S1), with highest air temperatures at Si Chang (36.78°C) and Zhoushan (36.87°C). The highest temperatures of the year, which represent the most stressful season, however, occur in July to September for each location (Fig. S1). To avoid the confounding effects of seasonal acclimatization on *hsp70* expression levels from animals sampled from the shore (Buckley *et al*., 2001; Sagarin and Somero, 2006), samples were collected during the season of highest environmental temperatures (the most thermally stressful = hot season) from July to September in 2012-2014 (Table 1). To minimize the effects variation of *hsp70* levels across tidal cycles (Gracey *et al*., 2008) and the interaction of stress from temperature and rainfall (Dong *et al*., 2014), 15-20 individuals were sampled from two semi-exposed rocky shores sites (sites A and B, separated by at least 30 meters and distant from obvious anthropogenic influences) in each location (except Keelung) during low spring tides on days with no rain. On collection, body temperatures were immediately measured by inserting a thermocouple past the operculum onto the foot (K-type thermocouple, Lutron thermometer ±0.1°C) and then shells were immediately cracked and immersed in RNAlater (Life Technologies, Carlsbad, CA, USA). All samples were sent to the State Key Laboratory of Marine Environmental Science (MEL), Xiamen University and kept at -80 °C until analysis.

Laboratory assessment of heat shock protein gene expression

To determine whether there are divergent heat shock responses among populations, snails were collected from Xiamen (*Echinolittorina malaccana* and *E. radiata*), Hong

Kong (*E. malaccana* and *E. radiata*) and Sanya (*E. malaccana*). At each location, 150 individuals (7-8 mm maximal shell length) were collected from the two sites and taken back to the laboratory during the most stressful season (as above). In the laboratory, animals were sprayed with filtered seawater and kept at 28 °C for three days for short-term acclimation to reduce the effects of recent thermal history. Five snails were put into small vials in air (Φ = 22 mm, height = 35 mm), which were placed into a Grant water bath (Grant Instruments, Cambridge, UK). Temperature within a blank vial in the water bath was recorded every 1 min using a Fluke digital recording thermometer and a fine K-type thermocouple (Lutron, Taiwan). Temperatures were increased from 28 to 50 °C at a rate of 5°C/hr as described in Marshall *et al* (2011). When the temperature reached 50 °C, it was increased to 57 °C at a rate of 2°C/hr. Three randomly chosen vials were removed at 35, 40, 45, 47, 49, 51, 53, 55 and 57 °C (Σn =9 temperatures x 3 replicates = 27). After heating, the animals in each vial were returned to 28°C seawater for 2hrs, and then placed in RNAlater after cracking their shells. Samples collected at Hong Kong and Sanya were treated at The Swire Institute of Marine Science (SWIMS), The University of Hong Kong; while samples collected at Xiamen were treated at MEL, Xiamen University. All samples were sent to MEL to analyze gene expression of *hsp70*.

Quantitative analysis of Hsp70 mRNA

Total RNA was isolated from ~30 mg of foot tissue from each individual using an EastepTM Universal RNA Extraction Kit (Promega, Madison, WI, USA) and quantified using a NanoDrop ND-1000 photometer (Thermo Fisher Scientific, Waltham, MA, USA). A sample of 0.5 μg of total RNA was used as the template for synthesis of the first strand of cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Otsu, Shiga, Japan). The real-time PCR Primers were designed based on the partial sequences of four genes which cloned from *E. malaccana*: *hsp 70* (GenBank accession No. KR082142), *beta-actin* (GenBank accession No. KR082141), *beta-tubulin* (GenBank accession No. KR082140) and *elongation factor 1-gamma* (GenBank accession No. KR082139) using Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA, USA). The efficiency of each primer set for *E malaccana* and *E. radiata* was determined by real-time PCR with an appropriate dilution series of cDNA prior to sample analyses. The sequences of primers, the product length and the efficiency for each species are shown in Table S1. Real-time PCR was carried out on a CFX96 Touch Real-Time PCR System (Bio Rad, Hercules, CA, USA) in a 20-μl reaction volume containing 10 μl of 2× master mix (DyNAmo Flash SYBR Green qPCR Kit, Thermo Fisher Scientific, Waltham, MA, USA), 1 μl of each primer (10 nmol μl-1), 1 μl of cDNA template and 7 μl of RNase-free water. PCR conditions were as follows: 95 °C 7 min; 40 cycles of 95 °C 20 s, 60 °C 1 min; and a final melt curve step. All samples were measured in triplicate.

Data processing and analysis

Hsp70 mRNA relative expression data were analyzed using Bio-Rad CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA). Three genes (*beta-actin*, *beta-tubulin* and *elongation factor 1-gamma*) were selected as the reference genes. One field sample, collected at Da Nang, was randomly selected as the reference sample.

For all analyses, generalized linear models (GLM) were performed using a gamma error distribution, following Friedman *et al*. (2010). To illustrate the latitudinal pattern of *hsp70* expression levels, latitude was the explanatory variable. Residuals from the GLM varied between sites in some locations, indicating potential spatial variations at a smaller scale than latitude ($P > 0.05$ in all cases except for Da Nang and Sepang for *E. malaccana*, and Xiamen and Qingdao for *E. radiata*, where P ranged from 0.03 to < 0.001). Data from the two sites at each location, however, were pooled as there were too few levels to accurately estimate random (i.e. between sites) effects (Bolker 2015).

To investigate if the temperature sensitivity of *hsp70* expression (change in *hsp70* expression per unit change in body temperature) varied within the littorinids' range, distribution location (range edge, RE or range center, RC), body temperature, and their interactions were used as explanatory variables. Such a geographical pattern was further analyzed by testing if the temperature sensitivity of *hsp70* expression in both *Echinolittorina malaccana* and *E. radiata* varied with latitude within their respective RE or RC with, again, body temperature, latitude and their interactions as explanatory variables.

In the laboratory assessment, *hsp70* mRNA production in both *E. malaccana* and *E. radiata* was investigated to test if *hsp70* expression differed between temperatures, locations, and their interactions. Data from the two sites at each location were again pooled (see above) as residuals from the GLM generally did not vary between sites ($P > 0.05$ in all cases except in Xiamen for *E. radiata*, where $P = 0.02$). All tests were performed with the base package and *car* (Fox *et al*., 2013) package in R (version 3.3.1, R Core Team, 2016).

Results

Body temperature and corresponding *hsp70* levels for each individual are reported in Table S1. On a broad geographical scale, *hsp70* expression levels showed clear latitudinal patterns, increasing with latitude in *Echinolittorina malaccana* ($t_{235} = 6.18$, $P < 0.001$) and decreasing in *E. radiata* ($t_{169} = -4.84$, $P < 0.001$, Fig. 2). Levels of *hsp70* expression also showed a positive relationship with field body temperature in both snails (Table 2).

When populations were divided according to their location into range edge (RE) and range center (RC) groups, *hsp70* mRNA levels increased with field body temperature in both RE and RC for *E. malaccana* (Table 2, Fig. 3a). In the *E. malaccana* RC group, *hsp70* expression of snails increased with field body temperature ($\chi^2_1 = 4.370$, $P = 0.037$) but varied among different locations ($\chi^2_4 = 31.750$, $P < 0.001$). Similarly, *hsp70* expression of snails increased with field body temperature ($\chi^2_1 = 34.474$, $P < 0.001$) and varied among different locations ($\chi^2_2 = 8.294$, $P = 0.016$) in the *E. malaccana* RE group. The temperature sensitivity (changes in *hsp70* expression per unit change in body temperature) was higher in RE than RC (RE: $t_{83} = 6.077$, $P < 0.001$; RC: $t_{138} = 2.099$, $P = 0.038$).

*Hsp*70 mRNA levels also increased with field body temperature in the RE but not in the RC group for *E. radiata*, (Table 2, Fig. 3b). In contrast to *E. malaccana*, in the *E. radiata* RC group, *hsp70* expression did not change with field body temperature ($\chi^2_1 = 0.229$, $P = 0.632$) but, similar to *E. malaccana,* did vary among different locations ($\chi^2_2 = 42.982$, $P < 0.001$). In the *E. radiata* RE group, however, *hsp70* levels increased with field body temperature ($\chi^2_1 = 42.951$, $P < 0.001$) and varied among locations ($\chi^2_1 = 9.806$, $P = 0.002$), as with *E. malaccana*, suggesting that temperature sensitivity of *hsp70* expression was higher at the distribution RE for both species. The mean *hsp70* expression level was also higher at the RE for both *E. malaccana* (*t* =-8.418, df=98.658, *P* < 0.001) and *E. radiata* (*t* = -3.610, df = 88.549, *P* < 0.001, Fig. 4).

In the laboratory assessments, *hsp70* expression increased with increasing snail body temperature (Table 3, Fig. 5). Relative *hsp70* expressions in *E. malaccana* from Hong Kong and Sanya were higher than those of Xiamen individuals when body temperatures were higher than 47 °C (Fig. 5). The levels of *hsp70* in *E. radiata* from Hong Kong were higher than those of snails from Xiamen at body temperatures less than 45 °C, but were similar or slightly higher at body temperatures higher than 45 °C (Table 3).

Discussion

Individuals collected from populations at the northern range of *Echinolittorina malaccana* and the southern range of *E. radiata* had higher *hsp70* levels than individuals collected from the respective species' range centers. The temperature sensitivity of *hsp70* expression was also dramatically higher in the range edge populations than in those at the species' range centers. Whilst heat shock proteins can effectively repair heat-induced protein damage, energy expenditure will be required at several stages in the HSR (Somero, 2002). The strong HSR recorded for populations at the species' range edges, therefore, implies an important diversion of energy allocation, which ultimately may influence ecological fitness.

CHECK letter for changes Echinolittorina snails can depress their resting metabolism within a thermally insensitive range (approximately 35-46°C) while experiencing high temperatures, which enhances their thermal tolerance (Marshall *et al.*, 2010; Marshall *et al.*, 2011). Within this thermal insensitive range, metabolic rate is delinked from increasing temperature, which is in contrast to the predictions of the UTD model (universal temperature-dependence model; Clarke, 2004). When temperature exceeds the upper thermal threshold for thermal insensitivity (approximately 46 °C), however, the metabolism of snails is elevated to meet the energy demands of maintenance at these temperatures (Marshall *et al.*, 2011). Thus, metabolic rate returns to be positively correlated with temperature. Metabolic depression may, therefore, influence the production of Hsp70. over a broad thermal range. Marshall and co-workers, for example, showed that levels of Hsp70 initially increased with increasing temperature, but then reached a plateau during the thermally insensitive range, but finally increased when temperature exceeded the upper limit of the thermally insensitive temperature (Marshall et al., 2011, Fig. 7). As a consequence of this metabolic depression, these snails can save energy when in their thermally insensitive zone, which may allow them to conserve energy for increased production of Hsp70 at higher temperature, leading to enhanced thermal tolerance. The structural and functional cellular modifications associated with this depression are, however, still unclear, and the role of latent mRNA remains to be clarified. Snails are killed, however, when they are exposed to temperatures beyond their thermal insensitivity threshold for long durations, suggesting a threshold above which the rate of energy demand exceeds the capacity for cellular energy generation. The time to gain energy is severely constrained on the shore to periods when awash by the rising or falling tide. When resources are limiting, any investment in a specific trait must carry a cost that is expressed in trade-offs with other traits (Araújo *et al*., 2015). A trade-off between the production of defense metabolites and growth has, for example, been clearly demonstrated in ectothermic animals, plants and algae (Clobert *et al*., 2000; Sparkman & Palacios, 2009; Dworjanyn *et* *al*., 2006; Nylund *et al*., 2013). The fitness of range edge populations, therefore, is likely to be reduced by shifting investment from growth, storage, reproduction to maintenance (Jump & Woodward, 2003; Viejo *et al*. 2011; Sokolova *et al*., 2012), which would increase the vulnerability of such populations to future changes in climate.

Stronger stress responses at a species' distribution range edge have rarely been recorded in rocky shore species due to the complex mosaic of thermal environments experienced in the intertidal zone (Helmuth *et al*., 2002). Sagarin & Somero (2006), for instance, showed that the patterns of expression of Hsp70 in the mussel, *Mytilus californianus,* and whelk, *Nucella ostrina,* were complex and more consistent with the surrounding, local, environmental conditions than with broader latitudinal effects. Lima *et al* (2016) also found that Hsp70 levels in the limpet, *Patella vulgata,* were tightly associated with immediate microtopography rather than with shore latitude. In the present study, however, the *hsp70* expression levels in *E. malaccana* and *E. radiata* showed clear latitudinal patterns at the broad geographical scale, suggesting that strong latitudinal gradients in environmental stressors can drive divergent responses among populations throughout their geographic ranges. Similarly, Sorte & Hofmann (2004) found that the dogwhelk, *Nucella canaliculata,* living near their southern range edge also had higher levels of Hsp70 than those near the center of their range. Pearson and co-worker (2009) also found that expression of heat shock genes was higher at the same temperature in range edge populations as compared to populations closer to the center of the range of the alga, *Fucus serratus*.

The vertical distribution (Ferreira *et al*. 2014), ability to acclimate (Stillman 2003) or mobility (which allows species to use behavioural thermoregulation, Marshall *et al*., 2013; Ng *et al.* 2017) may explain species-specific differences in stress response at species' range edge and centers. It has been hypothesized that under future global warming, extinction risks are generally expected to be highest at species' range edges for organisms that exhibit differential stress responses and stronger responses at distribution range edges (Sexton *et al*., 2009). In contrast, mortality will be more likely at some 'hot spots' for some intertidal organisms which don't exhibit linear stress response along the latitude (Sagarin & Somero, 2006). Thus, species-specific differences in individual performance at range edges and range centers are important to consider when predicting the impacts of climate change on the potential distribution range shifts of intertidal organisms.

Temperature is a main inducing factor for the expression of *hsp* genes. In the present study, there was no significant variation in the body temperatures of *Echinolittorina radiata* individuals between range edge and center populations. Thus, the higher *hsp70* levels of *E. radiata* were mainly the result of higher temperature sensitivity of HSR of the range edge populations. In the case of *E. malaccana*, the body temperatures of samples collected from the range edge populations were relatively higher than those from the range center which, together with their stronger thermal sensitivity, should contribute to the higher *hsp70* levels recorded at the range edge.

The higher temperature sensitivity of *hsp70* expression may be caused by plasticity and/or evolutionary adaptation to the local thermal environment at the edge of the species' distribution range. Range edge populations are assumed to be physiologically constrained (Sorte & Hofmann, 2004), and thus to suffer selective pressures to develop novel strategies for persistence and adaptation at these challenging environments to avoid population extinction (Araújo *et al*. 2015). In the laboratory assessments, the expression patterns of *hsp70* in response to heat stress also showed significant variations among different populations, suggesting plasticity and/or local adaptation of HSR in response to heat stress. *hsp70* was more readily induced in RC populations (Sanya) than RE populations (Hong Kong and Xiamen). It is likely that thermal regimes in RE locations may have already induced higher levels of Hsps in the cell which would be sufficient to tolerate mild temperature stress (Buckley *et al*., 2001). Local adaptation can result in resident genotypes that have a higher fitness in their native habitat than foreign genotypes from more distant populations (Kawecki and Ebert, 2004; Sanford and Kelly, 2011). Given the present approach, we are unable to determine whether the higher temperature sensitivity of *hsp70* expression between the range edge and center populations was genetically fixed. If, however, the variation of HSR between the range edge and the center populations was due to local adaptation, any immigration from the range center to the range edge will be unlikely to be successful, because the range center populations will lack the ability to achieve the required higher *hsp70* levels in response to thermal stress experienced at the range edge.

Lastly, we recognize that the use of a single stress indicator, *hsp70* mRNA, provides only a partial image of heat stress in field populations. Different stress-related genes have different onset temperatures for expression, so including a battery of stress-responsive genes in future studies would provide further insights into the variation among populations in capacities for responding to heat stress. Another focus of future studies could involve time-dependent changes in mRNAs of stress-related proteins, as turnover of mRNA, including *hsp70* mRNA, is rapid and, therefore, the time between field heat stress and sampling of specimens can be critical in experimental design (see Gracey *et al*., 2008).

Obtaining samples across a time period that spans the full tidal cycle would be a logical and insightful next step in such analyses.

Conclusions

The populations that inhabit the edge of a species' distributional range are likely to be critically important in determining a species' response to ongoing climate change. Using *hsp70* expression as an indicator, our results showed significant geographical variation in physiological response of two high shore *Echinolittorina* snails, suggesting the adaptive differentiation of populations to local environmental conditions. The temperature sensitivity of *hsp70* expression was higher in range edge than range center populations, and the individuals collected at the northern range edge of *E. malaccana* and southern range edge of *E. radiata* had higher *hsp70* expression than individuals collected from their respective range center. The high inducible *hsp70* levels in the range edge populations likely serve as an adaptive response to the stressful thermal environment at the expense of energy allocation to other fitness traits, and as such populations living at their species' range edges are likely to be more vulnerable to future changes in environmental conditions.

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Author Contributions

Y.-W.D and G.A.W designed the study, and G.-D.H, S.R.C, M.G, B.K.K.C, K.A.A.A, N.H and J.W performed the research. G.-D.H and T.Y.H analyzed the data, and G.-D.H, Y.-W.D and G.A.W wrote the paper.

Conflict of Interest

The authors have no conflict of interest to declare.

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Tables

Table 1. Geographic locations, range distribution of species' populations and sampling dates for *E. malaccana* and *E. radiata*. The ecoregions of different population located were showed as Spalding et al. (2007) described.

Locations	Ecoregion	Distribution: Range edge/center (RE/RC)	Sampling dates	Latitude
Echinolittorina malaccana				
Keelung, Taiwan	52	RE	July 3-4, 2012	25.14° N
Xiamen, Mainland China	113	RE	September 11, 2012	24.42° N
Hong Kong	113	RE	August 23-24, 2012	22.21° N
Sanya, Mainland China	112	RC	August 20, 2013	18.17° N
Da Nang, Vietnam	112	RC	September 12, 2013	16.07° N
Si Chang Island, Thailand	115	RC	September, 2014	13.15° N
Sepang, Malaysia	117	RC	September 10, 2014	2.59° N
Singapore	117	RC	September, 2014	1.29° N
Locations		Range distribution	Sampling dates	Latitude
Echinolittorina radiata				
Qingdao, Mainland China	50	RC	August 30, 2012	36.05° N
Zhoushan, Mainland China	52	RC	August 17, 2012	29.94° N
Keelung, Taiwan	52	RC	July 3-4, 2012	25.14° N
Xiamen, Mainland China	113	RE	September 11, 2012	24.42° N
Hong Kong	113	RE	August 23-24, 2012	22.21° N

Table 2. Analysis of deviance for GLM with gamma error distribution, to investigate the effects of distribution ranges (range edge, RE or range center RC) and on-shore body temperature on the relative *hsp70* expressions in *E. malaccana* and *E. radiata*. n = 148 for *E. malaccana* RC and n = 89 RE, while n = 100 for *E. radiata* RC and n = 71 RE.

E. malaccana			
Source of variation	χ^2	df	*P*
Distribution range	11.411	1	< 0.001
Body temperature	32.699	1	< 0.001
Distribution range × Body temperature	0.011	1	0.915
E. radiata			
Source of variation	χ^2	df	*P*
Distribution range	5.436	1	0.020
Body temperature	16.591	1	< 0.001
Distribution range × Body temperature	7.331	1	0.007

Table 3. Analysis of deviance for GLM with gamma error distribution, to investigate the effects of locations (Hong Kong (HK), Sanya (SY) and Xiamen (XM)) and treatment temperature on the relative *hsp70* expressions in *E. malaccana* and *E radiata*. n = 5 – 6 for both *E. malaccana* and *E radiata*. Significant interactions were investigated further using GLM at the respective body temperatures.

	E. malaccana			*E. radiata*		
Source of variation	χ^2	df	*P*	χ^2	df	*P*
Location	73.2	2	< 0.001	84.9	1	< 0.001
Treatment temperature	326.4	8	< 0.001	611.3	8	< 0.001
Location × Treatment temperature	145.2	16	< 0.001	105.5	8	< 0.001

Interactions between Location and Treatment temperature

Treatment temperature(°C)	*E. malaccana*	*E. radiata*
35	HK < SY < XM	HK < XM
40	XM = HK = SY	HK < XM
45	XM = HK = SY	HK < XM
47	XM = HK < SY	HK = XM
49	XM < HK < SY	HK = XM
51	XM < HK < SY	XM < HK
53	XM < HK = SY	XM = HK
55	XM < HK = SY	XM = HK
57	XM < HK = SY	XM < HK

Figures and captions

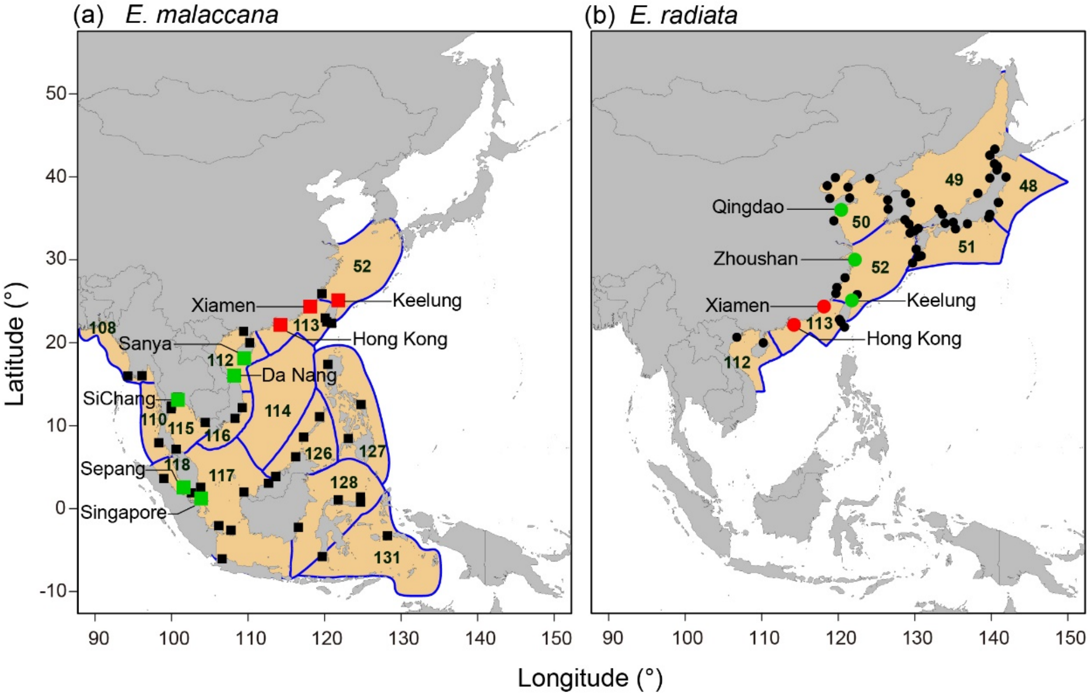

Figure 1. Map of the sampling locations for *E. malaccana* (a) and *E. radiata* (b) (for further details, see Table 1). Black squares and dots represent the distribution locations for *E. malaccana* and *E. radiata*, respectively (Reid 2007). Green symbols are sampling locations for populations at the species' range center, while the red symbols are sampling locations of populations at the species' range edge. The Arabic numerals represent the marine ecoregions where the snails distribute: 48, Northeastern Honshu; 49, Sea of Japan; 50, Yellow Sea; 51, Central Kuroshio Current; 52, East China Sea; 108, North Bay of Bengal; 110, Andaman Sea Coral Coast; 112, Gulf of Tonkin; 113, Southern China; 114, South China Sea Oceanic Islands; 115, Gulf of Thailand; 116, Southern Vietnam; 117, Java Sea; 118, Malacca Strait; 126, Palawan; 127, Eastern Philippines; 128, Sulawesi Sea; 131, Banda Sea (Spalding *et al.*, 2007).

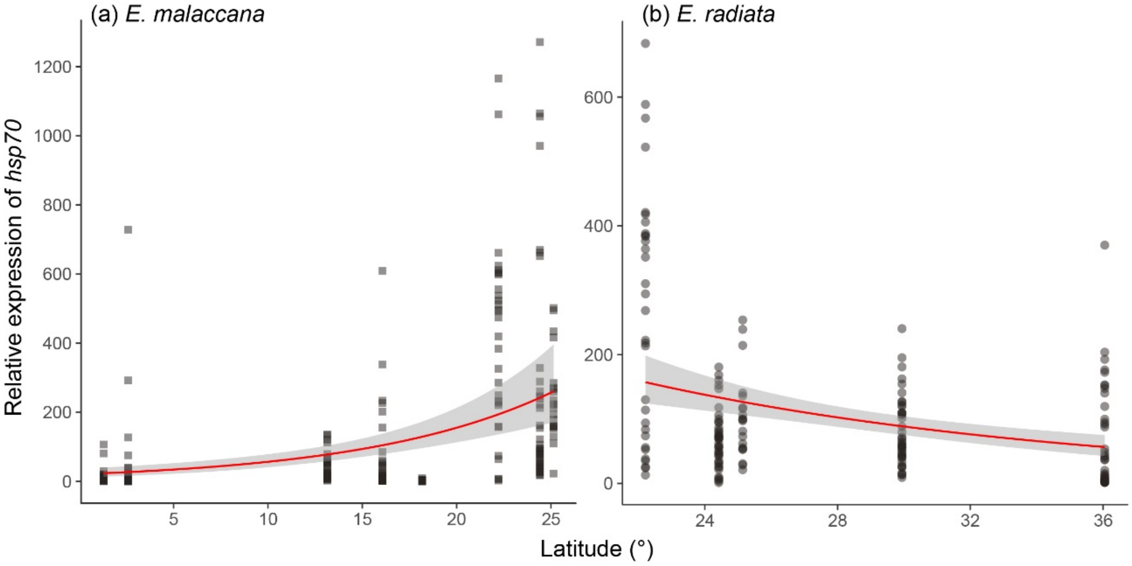

Figure 2. Relative expression of *hsp70* from individuals collected on the shore. The scatter diagrams show latitudinal patterns of *hsp70* levels of *E. malaccana* (a) and *E. radiata* (b) along the Northwestern Pacific coast. The red solid lines represent the generalized linear model regression quantile estimate for the latitude as a function of the body temperature or *hsp70* levels. The grey areas represent 95% confidence intervals.

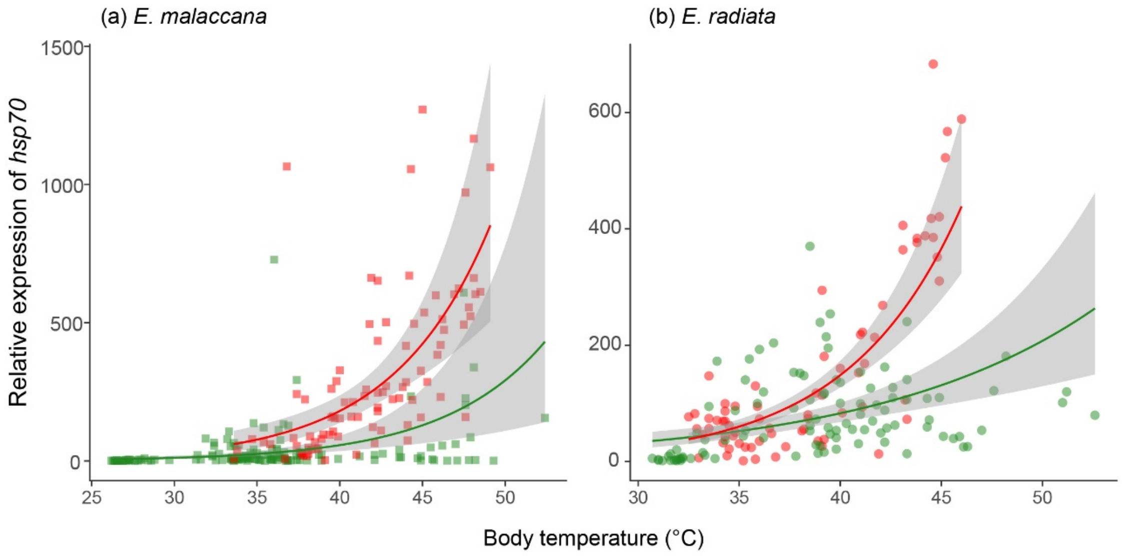

Figure 3. On-shore relative *hsp70* expression of (a) *E. malaccana* and (b) *E. radiata* at various body temperatures. Lines represent best-fitting GLM models with gamma error distribution, while shaded regions represent 95% confidence intervals. Red lines and points represent data from snails at their range edge, while green lines and points represent snails at their range center. n = 148 for *E. malaccana* RC and n = 89 RE, while n = 100 for *E. radiata* RC and n = 71 RE.

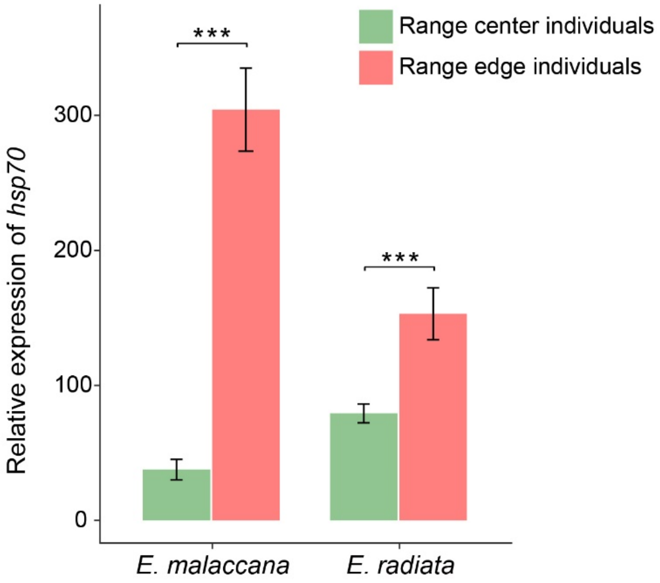

Figure 4. The mean *hsp70* levels of individuals collected from populations at the range edge and range center (mean ± SEM). * indicates significant differences ($P < 0.001$ for both *E. malaccana* and *E. radiata*) between treatments (see text for further details).

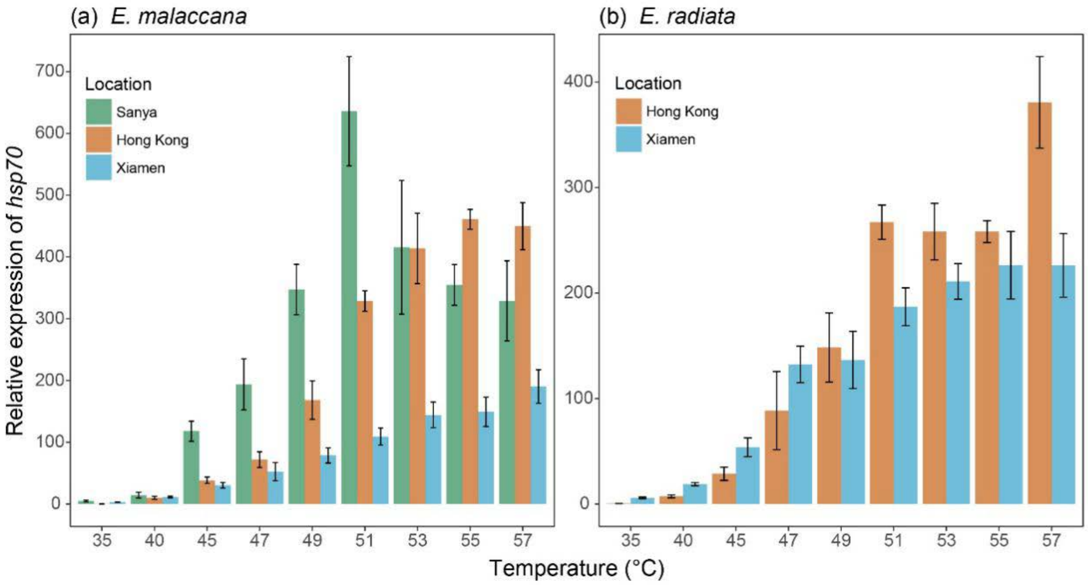

Figure 5. Relative expression of *hsp70* in response to experimental temperatures at 35, 40 45, 47, 49, 51, 53, 55, 57°C (mean ± SEM). Transcript patterns were shown for *E. malaccana* (a) and *E. radiata* (b) populations sampled in Xiamen, Hong and Sanya.

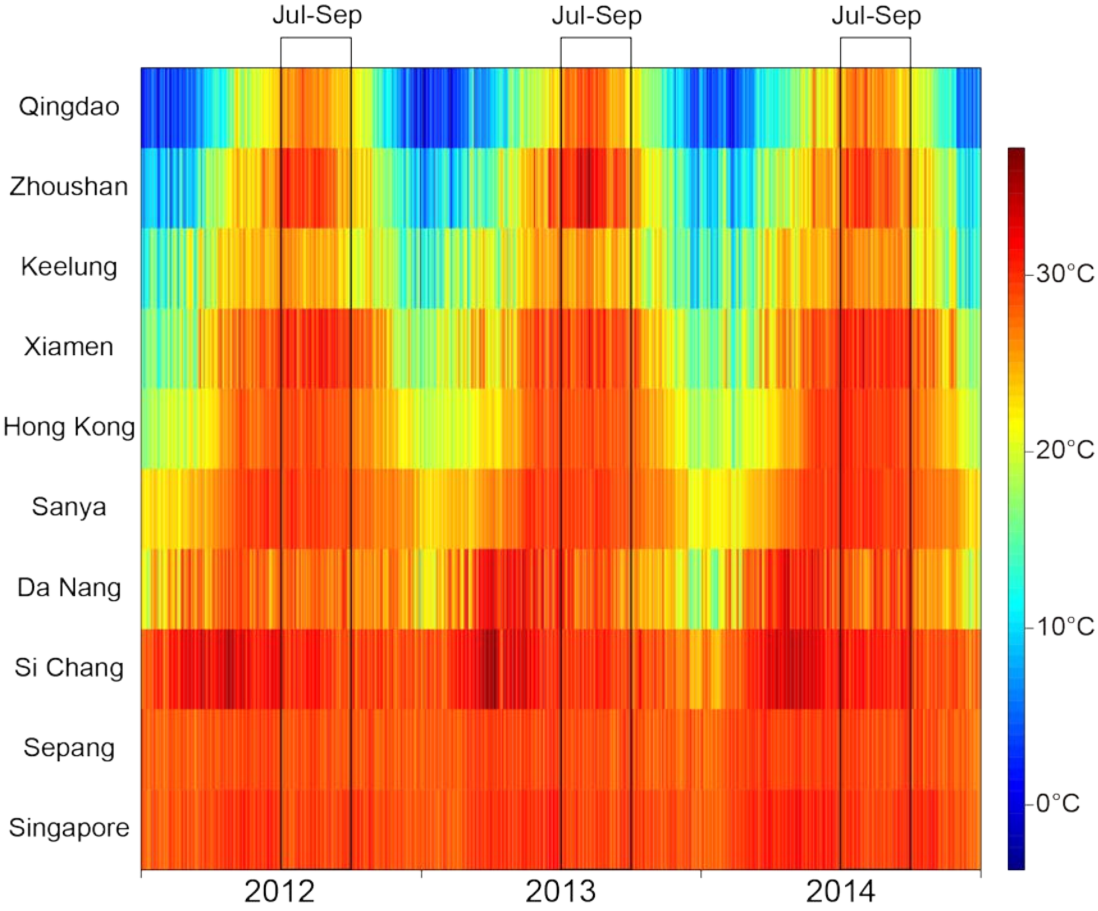

Figure S1. Color-coded charts showing daily maximal air temperatures for each sampling location.