

Latitudinal variability of physiological responses to heat stress of the intertidal limpet *Cellana toreuma* along the Asian coast

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ABSTRACT: Potential future distribution shifts of intertidal invertebrates along the Asian coast in the face of global change remain unclear. Integrative study that comprise environmental temperature monitoring and comparative physiological study of thermal adaptation among different geographical populations of species are important to identify population-related differences in thermal ecology that could affect the persistence of species in their present distribution ranges. In the present study, *in situ* operative temperatures were recorded continuously for 5 mo from August 2011 to January 2012, and measurements of lethal temperature, cardiac performance and gene expression were carried out in 3 geographical populations of the limpet *Cellana toreuma* (tropical shore: Sichang Island, Thailand; subtropical shore: Nanding Island, Zhangzhou, China; temperate shore: Dagong Island, Qingdao, China). Compared to limpets on the tropical shore in Sichang Island with high mean temperature (28.28°C) and narrow thermal range (19.13 to 46.56°C), limpets on the subtropical shore in Nanding Island have to cope with a thermal environment characterized by a wider temperature range (6.42 to 44.98°C). The absence of differences in lethal temperature (LT₅₀) of limpets from all the locations indicates that limpets currently suffer from intensive heat stress across their biogeographic range. Although the populations lacked differences in upper thermal limit, variations were noted among populations in transcriptional responses in genes linked to energy metabolism. Limpets on the subtropical shore in Nanding Island live closer to their upper thermal limits and thus will be very sensitive to future temperature increases.

KEY WORDS: Biogeography · Limpet · Body temperature · Physiological response · Rocky intertidal zone

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INTRODUCTION

Temperature is one of the major factors controlling species distribution and abundance (Mayhew et al. 2008). Global warming, the primary consequence of climate change (Cox et al. 2000), is confronting most organisms with a changing thermal environment (Root et al. 2003, Cheung et al. 2013). Physiological responses and their plasticity are crucial for animals in coping with the changing thermal environment (Marshall et al. 2011, Pörtner 2012) and are closely

related to organisms' distribution and biogeography (Pörtner & Knust 2007, Pörtner & Farrell 2008, Eliason et al. 2011). Based on analyses of physiological performances and environmental temperature monitoring, it is feasible to investigate the ecological impacts of thermal stress and to predict the 'winners' and 'losers' in the face of future climate change (Stillman & Somero 2000, Somero 2011, 2012).

The rocky intertidal shore is one of the harshest environments on Earth. Intertidal species commonly live close to their upper thermal limits (Branch 1981,

Stillman & Tagmount 2009) and may be especially vulnerable to climate change (Mieszkowska et al. 2006, Hawkins et al. 2008). Extensive studies have shown that temperature is the most important factor determining the vertical distribution and biogeography of intertidal species (Wolcott 1973, Blanchette et al. 2008), and long-term observation data have shown a poleward shift in the biogeographic ranges of many intertidal species (Barry et al. 1995, Southward et al. 2004).

Upper thermal limits among different populations of a species are different, and these variations are usually not linearly related to the latitude (Fusi et al. 2014, Mislán et al. 2014). For example, some high-latitude populations have higher upper-limit thermal tolerance than low-latitude populations (Kuo & Sanford 2009, Mislán et al. 2014). The upper thermal limit of intertidal organisms is closely related to the local thermal environment resulting from both temperature and the tidal cycle (Helmuth et al. 2002, 2014, Somero 2010). For evaluating the potential impacts of temperature increase on biogeographic patterning and predicting future population dynamics, it is important to study thermal tolerance and related physiological responses in different geographical populations (Pörtner & Knust 2007).

The Asia-Pacific region with its high biodiversity is one of the most vulnerable regions to global warming (IPCC 2013, Sun et al. 2014). Intertidal animals in this region suffer extremely intensive heat stress in summer, which has induced large-scale mortality of some intertidal species (Lewis 1954, Firth & Williams 2009, Dong et al. 2014). Limpets are important in the intertidal ecosystem in their role as grazers (Branch 1981, Jenkins & Hartnoll 2001, Jenkins et al. 2005). During emersion at low tide in summer, limpets frequently suffer from heat stress (Williams & Morritt 1995). With temperature increases due to global warming, warm-adapted limpets have begun to replace cold-adapted species (Mieszkowska et al. 2006, Dong & Somero 2009). In addition to the direct impacts of climate change on the limpets' distribution and abundance, climate change can also affect limpet grazing (Moore et al. 2007) and interspecific competition (Firth et al. 2009), consequently affecting the structure and functioning of rocky shore ecosystems. *Ceclana* limpets are common species along the Asian coast and are widely distributed from Indonesia to the Bering Sea (Nakano & Ozawa 2007). In the present study, 3 populations of *Cellana toreuma* from China or Thailand (temperate: Dagong island, Qingdao, Northern China; subtropical: Nanding Island, Zhangzhou, Southern China; tropical: Sichang Is-

land, Thailand) were selected to investigate the sensitivity of different populations to heat stress. Median lethal temperature (LT_{50}) and Arrhenius break temperature (ABT) for cardiac performance were measured to investigate the difference of upper thermal limit among different populations, and the expression of genes involved in different cellular processes were quantified to understand the inter-population difference of physiological adaptations at the transcriptional level. Of particular interest was the potential effect of a latitudinal cline in environmental thermal heterogeneity on the sensitivities of the 3 populations to high temperature stress.

MATERIALS AND METHODS

Measurement of operative temperature

The operative temperatures of limpets were measured at the upper vertical distribution limits of the species using Robolimpets, following a method by Lima & Wetthey (2009) with minor modification. The Robolimpets were assembled using shells of *C. toreuma*. Before deployment, all Robolimpets were calibrated using a thermometer (Fluke 54II, Fluke, WA, USA). In the present study, the operative temperatures of limpets were monitored on Dagong Island, Qingdao, China (35° 58' N, 120° 30' E), Nanding Island, Fujian (24° 09' N, 117° 59' E), and Sichang Island, Thailand (13° 08' N, 100° 48' E). In Dagong Island, tides are regular semidiurnal tides, and the maximum and average tidal ranges are 4.8 and 2.8 m, respectively. At Nanding Island, tides are irregular semidiurnal tides, and the maximum and average tidal ranges are 5.2 and 2.5 m, respectively (Hu & Gu 1989). At Sichang Island, the study area experiences mixed tides with a maximum tidal range of 3.6 m. Based on the records of the local meteorological observatories, the daily average air temperatures at Dagong Island were 3–10, 17–25, 18–24 and 10–12°C in spring (January to March), summer (April to June), autumn (July to September) and winter (October to December) of 2013, respectively. The daily average air temperatures at Nanding Island were 14–21, 23–31, 23–30 and 12–21°C in the spring, summer, autumn and winter of 2013, respectively. The average air average temperatures at Sichang Island between March 2008 and February 2009 were 30.9, 28.5 and 23.0°C in summer (March to May), rainy season (June to October) and winter (November to February), respectively (Sujitra et al. 2010). At the 3 islands, there are natural rocky shores, and *C.*

toreuma is a dominant grazer on the shore. At each site, 3 Robolimpets each were deployed on a semi-wave-exposed and a sun-exposed shore. Operative temperature recordings were made every 30 min (Sichang Island) or 60 min (Dagong Island and Nanding Island) for 5 mo from August 2011 to January 2012.

Animal collection and acclimation

From April to August 2013, controlled laboratory experiments were carried out at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand, and at Xiamen University and Ocean University of China, China. All operations were performed using a standard protocol by the same group of investigators. Limpets were randomly collected along a ~500 m shoreline in each location. After collection, limpets were transported back to the laboratory within 2 h and acclimated for 7 d at 2 temperatures (25°C, the highest temperature frequency at Dagong Island and Nanding Island, and 30°C, the highest temperature frequency at Sichang Island). Special aquaria were designed for limpet acclimation in all the locations. In each aquarium, an artificial rock (60 cm length × 30 cm width) was placed on a plastic shelf in 20 l fresh seawater; so that it was emersed. Seawater was aerated continuously and exchanged daily. In each location, 600 limpets were randomly allocated into 6 aquaria, and ~100 limpets were reared on the rock in each aquarium (with a density of ~1 limpet per 20 cm²). Seawater spray was provided every 12 h. The rock temperatures (25 or 30°C) were controlled by incandescent lights and an air conditioner. Body temperatures of limpets were recorded every 10 min using a thermometer (Fluke 54II). No food was provided over the 7 d period for acclimation. After acclimation, animals were randomly selected for lethal temperature measurement, heart-rate measurement and gene-expression quantification.

Lethal temperature measurement

The shell lengths of limpets were measured using a caliper (Guilin Guanglu Measuring Instrument Co., Guilin, Guangxi, China). Limpets had a shell length of 21.8 ± 3.6 mm, a width of 16.3 ± 2.9 mm and a height of 4.5 ± 1.1 mm (mean ± standard deviation). At each site and for each acclimation treatment, 500 individuals were randomly selected and placed on petri dishes to settle down. There were 10 individuals

in each petri dish ($\varnothing = 20$ cm). When the LT₅₀ measurement was carried out, 15 individuals at each acclimation temperature (25°C or 30°C) were randomly collected and were given 2 h seawater spray. These individuals were regarded as unheated controls. Other limpets in the petri dishes were immersed in a water bath, allowing the substratum temperature in the petri dish to be increased at a rate of $\sim 0.1^\circ\text{C min}^{-1}$ to simulate thermal stress on the natural rocky shore. The water bath was controlled manually. A fine thermal couple was inserted between the foot muscle and petri dish for real-time body temperature measurement using a thermometer (Fluke 54II). At designated temperatures (25°C acclimated animals: 30, 36, 38, 40, 42 and 44°C for Sichang Island, and 30, 32, 34, 36, 38, 40, 42 and 44°C for Dagong Island and Nanding Island; 30°C acclimated animals: 36, 38, 40, 42 and 44°C for Sichang Island, and 32, 34, 36, 38, 40, 42 and 44°C for Dagong Island and Nanding Island), 3 groups (10 individuals in each group) were randomly collected for determination of survival. After the heat exposures, these animals were given a 2 h seawater spray. Survival was then assessed by touching the foot using a dissecting needle. If the foot withdrawal response did not occur, the limpet was regarded as dead. The mortalities were recorded, and the median lethal temperature (LT₅₀) was calculated with probit analysis (Finney 1971). The surviving animals were dissected immediately, and foot muscles were stored in RNA_{later}® RNA stabilization reagent (Qiagen) for subsequent use in gene-expression quantification.

Because of high mortality (>90%) of the limpets acclimated at 25°C in the Sichang population, the lethal temperature could not be measured for this group of specimens. The cause of the high mortality in this group is unclear, but is possibly due to the poor adaptive capability to acute temperature decrease.

Cardiac performance

Randomly selected individuals (n = 3 to 13) were placed on petri dishes to settle (5 individuals in each petri dish), and the substrata of the petri dishes were then heated at a rate of $\sim 0.1^\circ\text{C per min}$ using a water bath as described above. Heart beat rates of limpets were measured during heating using a non-invasive method (Chelazzi et al. 2001, Dong & Williams 2011). The heartbeat was detected by means of an infrared sensor fixed with Blue-Tac (Bostik, Staffordshire, UK) to the limpet shell at a position above the heart. Variations in the infrared heartbeat signal were amplified, filtered and recorded using an infrared signal ampli-

fier (AMP03, Newshift, Leiria, Portugal) and Powerlab (4/30, ADInstruments, March-Hugstetten, Germany). Data were viewed and analyzed using Chart (v. 5.0). The ABT for cardiac performance, the temperature at which the heart rate decreases dramatically with progressive heating, was determined using piecewise linear regression in SigmaPlot for Windows v. 10.0 (Systat Software).

There are 2 major haplotypes of cytochrome oxidase subunit I (COI) in the Dagong Island population, as a previous study mentioned (Dong et al. 2012). Fifteen limpets in each acclimation temperature in Dagong Island population were dissected after heart-rate measurement for COI sequencing using LCO 1490 and HCO2198 primers (Folmer et al. 1994). PCRs were conducted in a 25 μ l reaction volume containing 2.5 μ l of 10 \times buffer (Mg²⁺ Plus), 2 μ l of 2.5 mM dNTPs, 1 μ l of each 10 mM primer, 0.25 μ l (1.25 U) of Taq DNA polymerase and 200 ng DNA template. Amplification was initiated with denaturing at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, annealing at 40°C for 1 min and 72°C for 1 min and then a final extension at 72°C for 10 min. PCR products of the COI gene were sent to a commercial company for sequencing (Invitrogen Biotechnology, Shanghai, China).

Gene expression quantification

To analyze the transcriptional response of limpets, a real-time PCR array system was developed based on our transcriptomics results with *C. toreuma* (S. Zhang, G. D. Han & Y. W. Dong unpubl. data). In total, 21 genes were selected to investigate the physiological responses to thermal stress (Table S1). These genes, which encode molecular chaperones, antioxidant enzymes, metabolic regulators, metabolic enzymes and caspases, were sensitive to thermal stress, and expression of these genes was different between unheated and heated limpets based on the previous transcriptomic study. Three genes, including 18S rRNA, β -actin and β -tubulin, were designated as housekeeping genes and used to normalize expression of the other genes. Three biological replicates were measured in each treatment (locations \times acclimation \times heat-shock temperature).

Total RNA was isolated from ~50 mg of foot muscle using Trizol Reagent (Invitrogen). The first strand of cDNA was synthesized using total RNA as a template. Reverse transcriptase (RT) reactions were performed using a PrimeScript™ RT reagent kit with gDNA Eraser (TAKARA). PCR was carried out in a Bio Rad CFX96 PCR System (Bio Rad) in a 20 μ l re-

action volume containing 10 μ l of 2 \times Master mix (DyNAmo Flash SYBR Green qPCR Kit, Thermo Scientific), 0.8 μ l of each primer (10 nmol μ l⁻¹), 1 μ l of cDNA template and 7.4 μ l of RNase-free water. The PCR conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 20 s and 60°C for 1 min with a final dissociation curve step. All samples were measured in triplicate as technical replicates. Relative quantity (Δ Cq) was chosen to calculate the gene-expression levels of samples using the Bio-Rad CFX Manager 3.1 software.

Statistical analyses

The frequencies of operative temperature were fitted in a Gaussian distribution (sum of 2 Gaussian distributions) using Prism 6.0 (GraphPad Software). Temperature coefficients (Q_{10}) were calculated with heart-rate data from temperatures at which the experiment started (25 or 30°C) to the temperature at which the maximum heart rate was reached with the equation

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{10 / (T_2 - T_1)}$$

where R is the heart rate, and T is the temperature (Kelvins). The differences in Q_{10} among different populations were analyzed using 1-way ANOVA with Duncan's post hoc analysis using the SPSS 17.0 for Windows statistical package. The differences in LT_{50} s and ABTs between different locations and acclimation temperatures were analyzed using 2-way ANOVA with Duncan's post hoc analysis. The difference between mean values of thermal limits (ABTs and LT_{50} s) and maximum habitat temperatures (MHT) was calculated to identify the relationship between MHTs and thermal limits in different locations. The differences in gene expression between different groups (locations \times acclimation temperature \times heat-shock temperatures) were analyzed using 3-way ANOVA with Duncan's post hoc analysis. To find out which individuals and genes could group together, a hierarchical clustering algorithm was generated with a Euclidean distance similarity metric and Centroid linkage method after log-transformation of all gene expression using Cluster 3.0 (Eisen et al. 1998, de Hoon et al. 2004; University of Tokyo, Human Genome Center). The correlations between heat-shock temperature and gene expression were analyzed using Spearman analysis with Bonferroni correction. To illustrate the relative contribution of experimental variables (i.e. locations and acclimations) to the variation in gene ex-

pression, principal components analysis (PCA) was applied for a dimensionality reduction of gene-expression data using SPSS 17.0.

RESULTS

Latitudinal variations of thermal environments

The operative temperature data showed that thermal regimes in the 3 locations were different at the limpets' upper vertical distribution limit (Fig. 1). On Sichang Island, Thailand, the operative temperatures were relatively high and stable, and the mean temperature was 28.28°C (standard deviation: $\pm 3.20^\circ\text{C}$) from August 2011 to January 2012. During the same period, temperatures on Nanding Island, Zhangzhou, and Dagong Island, Qingdao, were more variable. The standard deviations in Nanding Island and Dagong Island were 6.98°C and 5.67°C, respectively. The maximum temperatures on Sichang Island (46.56°C) and Nanding Island (44.98°C) were similar and were higher than that on Dagong Island (39.41°C). The minimum temperature on Dagong Island was -0.55°C .

Heart rates and lethal temperatures

Because there are 2 major haplotypes (using COI as a biomarker) in the Qingdao region (Dong et al. 2012), 15 individuals in each acclimation temperature in Dagong Island were selected for heart-rate measurement to compare the difference in cardiac function between the 2 haplotypes. However, there was no difference in ABT between the 2 haplotypes, so all heart-rate data of the 2 haplotypes were merged for further analysis.

Limpets from Nanding Island showed a higher maximum heart rate (~ 245 beats min^{-1}) compared to those from Dagong Island and Sichang Island (~ 200 beats min^{-1}). Despite this population difference, there were no significant differences in maximum heart rates between the 2 thermal acclimation groups from the same location (Table 1). Temperature coefficients (Q_{10}) were calculated with heart-rate data from temperatures at which the experiment started (25 or 30°C) to the temperature at which the maximum heart rate was reached. After 1 wk of acclimation at 25°C, there was no significant difference in Q_{10} among the 3 populations ($F_{(2,18)} = 0.507$, $p = 0.612$). However, limpets in Nanding Island exhibited higher Q_{10} values than those from the other 2 loca-

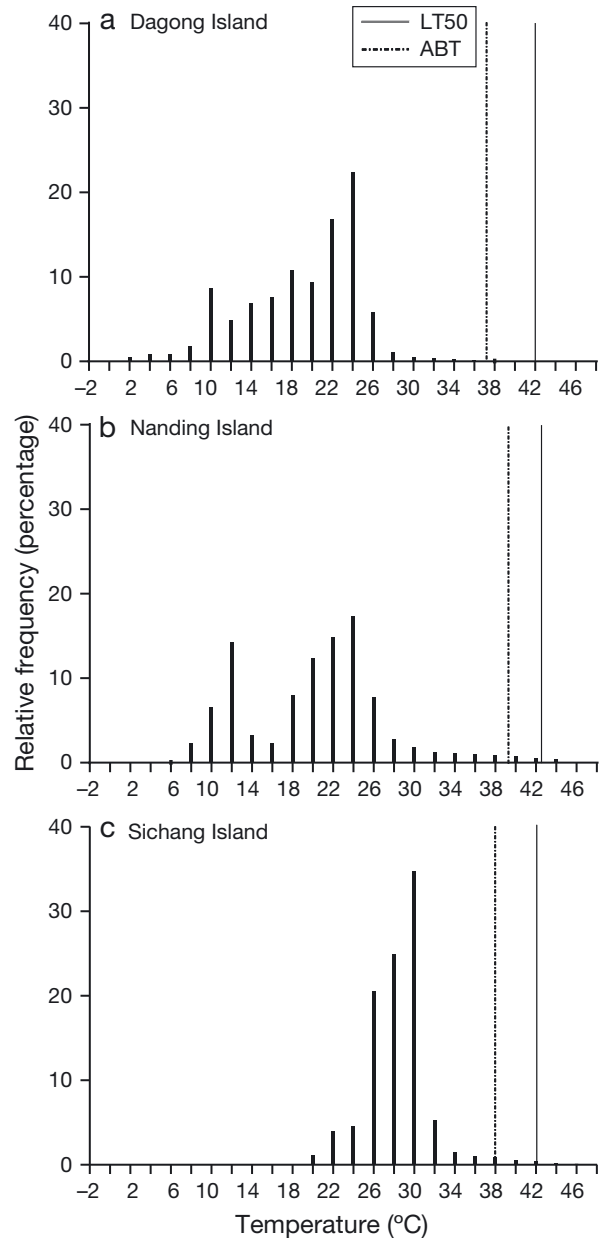


Fig. 1. Relative frequencies of different operative temperatures in (a) Dagong Island, Qingdao, China ($35^\circ 58' \text{N}$, $120^\circ 30' \text{E}$), (b) Nanding Island, Zhangzhou, China ($24^\circ 09' \text{N}$, $117^\circ 59' \text{E}$) and (c) Sichang Island, Thailand ($13^\circ 08' \text{N}$, $100^\circ 48' \text{E}$). The dashed line and solid line represent the LT_{50} and ABT of limpets, respectively

tions after being acclimated at 30°C for 1 wk ($F_{(2,17)} = 5.942$, $p = 0.011$).

In all locations, limpets showed high upper thermal tolerance limits (LT_{50} : approximately 41.29 to 43.36°C; Fig. 2a). There were marginally insignificant differences related to location or acclimation temperature based on the 2-way ANOVA results (among location: $F_{(2,15)} = 4.000$, $p = 0.053$; between acclimation

Table 1. Maximum heart rates (mean ± SD) and temperature coefficients of *Cellana* limpets from the 3 study locations. Temperature coefficients (Q_{10}) were calculated with heart rate data from temperatures at which the experiment started (25 or 30°C) to the temperature at which the maximum heart rate was reached

Acclimation temperature	Dagong Island	Nanding Island	Sichang Island
Maximum heart rate (beat min⁻¹)			
25°C	190.0 ± 20.2 (N = 13)	245.1 ± 1.3 (N = 3)	197.0 ± 7.6 (N = 3)
30°C	204.2 ± 17.5 (N = 12)	245.4 ± 29.0 (N = 4)	193.5 ± 15.8 (N = 4)
Temperature coefficient (Q_{10})			
25°C	1.527 ± 0.197	1.639 ± 0.099	1.560 ± 0.037
30°C	1.403 ± 0.154	1.785 ± 0.331	1.470 ± 0.116

temperature: $F_{(1,15)} = 4.246$, $p = 0.066$), and limpets from Nanding Island had slightly higher LT_{50} values than those from Dagong Island and Sichang Island.

The ABT of heart rate differed among locations (Fig. 2b). Two-way ANOVA analysis showed that

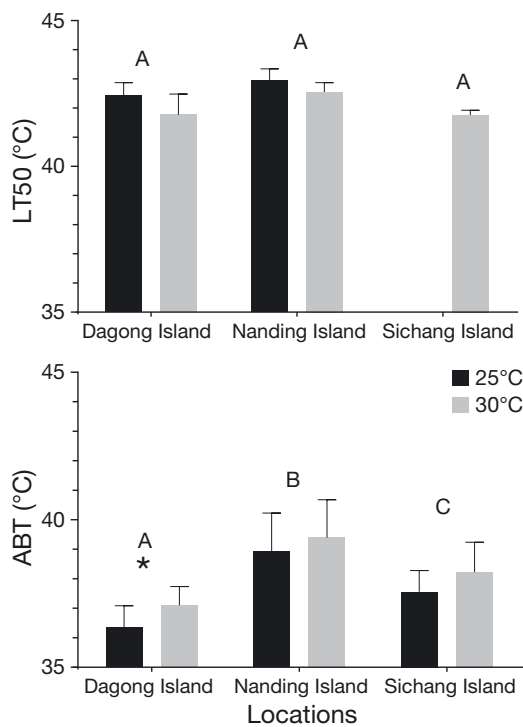


Fig. 2. Lethal temperatures (LT_{50}) and Arrhenius breakpoint temperatures of heart rate (ABT) of limpets acclimated at 25 and 30°C from Dagong Island, Nanding Island and Sichang Island. Different letters indicate where there were significant differences among locations. Limpets were heated at a rate of ~0.1°C per min using a water bath to simulate thermal stress on the natural rocky shore. Mortality was checked after 2 h recovery in seawater. Asterisk indicates statistical differences in ABTs between 2 acclimation temperatures in the same location ($F_{(1,40)} = 5.388$, $p = 0.026$)

ABTs of limpets in Nanding Island were significantly higher than those of limpets from Dagong Island and Sichang Island (among location, $F_{(2,40)} = 22.256$, $p < 0.001$). When acclimated at 30°C, limpets acquired higher ABTs than those of limpets acclimated at 25°C ($F_{(1,40)} = 5.388$, $p = 0.026$).

The relationship between thermal limits (ABT and LT_{50}) and maximum habitat temperatures (MHT) was also analyzed to evaluate how close the animals live to their thermal limits (Fig. 3). At all locations,

MHTs exceeded limpets' ABTs. At Dagong Island and Sichang Island, MHTs were higher than LT_{50} s measured using our heat-ramp protocol. However, MHT was lower than LT_{50} in the Dagong Island population.

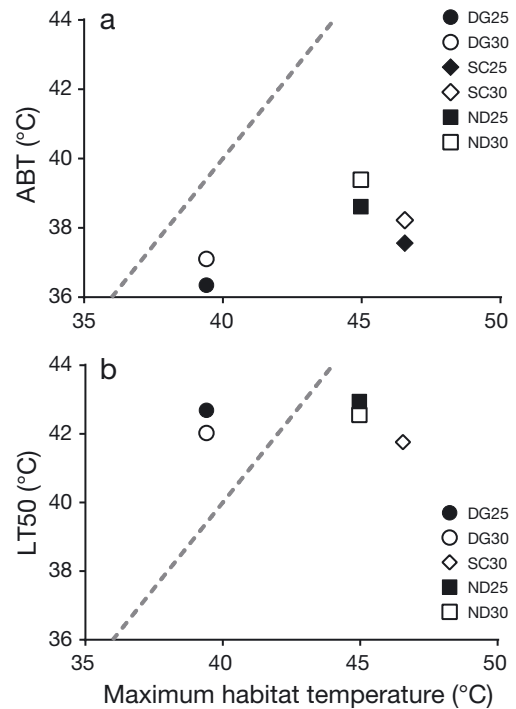


Fig. 3. (a) Relationship between Arrhenius breakpoint temperatures of heart rate (ABT) and maximum habitat temperature (MHT), where the dashed line represents equality between ABT and MHT, and (b) the relationship between lethal temperature (LT_{50}) and MHT, where the dashed line represents equality between LT_{50} and MHT. DG25: Dagong Island population acclimated at 25°C; DG30: Dagong Island population acclimated at 30°C; ND25: Nanding Island population acclimated at 25°C; ND30: Nanding Island population acclimated at 30°C; SC25: Sichang Island population acclimated at 25°C; SC30: Sichang Island population acclimated at 30°C

Gene expression

The mRNA levels of 21 genes involved in different metabolic pathways were detected using a real time PCR array. The amplification efficiencies for all genes were 1.887 to 2.119, indicating that the primers and reagent concentrations were appropriate for all genes (Table S1 in the Supplement at www.int-res.com/articles/suppl/m529p107_supp.pdf).

Cluster analysis results showed that limpets could be divided into 3 groups. One group (Group I) included individuals from Dagong Island that were heated at 30 to 44°C. Group II included several individuals from Dagong Island, most individuals from Sichang Island and some individuals from Nanding Island at low heat-shock temperature. Group III included individuals from Nanding Island at high heat-shock temperatures and some individuals from Sichang Island at high heat-shock temperatures (Fig. 4). Animals acclimated at different temperatures could not be separated by cluster analysis.

Cluster analysis results also showed that the 21 genes we quantified in the present study could be allocated into 3 groups. The first group included caspases, *cat-1*, *ampk α* , *axin*, *sirt1*, *hmgb1* and *ldh*, the second group included *hsc70*, *hsp22*, *hsp70*, *hsp60*, *hsp90*, *grp94* and *gapdh*, and the third group included *pfk*, *pk*, *ldh*, *sirt5* and *sod* (Fig. 4). Five genes (*pfk*, *pk*, *ldh*, *sirt5* and *sod*) were clustered together, and the expressions of these genes were significantly different among locations (*pfk*: $F_{(2,217)} = 49.554$, $p < 0.001$; *pk*: $F_{(2,217)} = 117.977$, $p < 0.001$; *ldh*: $F_{(2,217)} = 293.210$, $p < 0.001$; *sirt5*: $F_{(2,217)} = 889.347$, $p < 0.001$; *sod*: $F_{(2,217)} = 1084.775$, $p < 0.001$; Fig. 5). Duncan's post hoc analysis results showed that expression levels of the 5 genes in limpets from Nanding Island were significantly higher than those from Dagong Island ($p < 0.05$). Except *pfk*, levels of the other 4 genes in Sichang Island were significantly higher than those in Dagong Island ($p < 0.05$).

For PCA analysis, 2 components were extracted, and those 2 components accounted for 52.05% of the total variance. The PCA results showed that individuals from Dagong Island were relatively distinct from limpets from Sichang Island and Nanding Island (Fig. 6).

The levels of expression of genes encoding molecular chaperones, including Hsps, HMGB1 (a DNA chaperone; Table S1) and GPR94, were sensitive to temperature change at the 2 acclimation temperatures and 3 locations. Spearman correlation analysis showed that the correlation coefficients between all the molecular chaperons and heat-shock tempera-

tures were statistically significant (Table S2). Except *hmgb1*, levels of all molecular chaperons were positively correlated with each other.

The expressions of genes involving energy metabolism regulation, glycolysis and the Krebs cycle were variable in different treatments and locations. The expression levels of *pk*, *ldh* and *axin* were positively correlated to heat-shock temperatures (Table S3). Genes encoding enzymes involved in glycolysis (*pfk*, *gapdh*, *pk* and *ldh*) were positively correlated to each other. Expression levels of the gene encoding IDH, an important enzyme in the Krebs cycle, were positively correlated to all genes encoding enzymes involved in glycolysis except *ldh*. The levels of *axin*, which encodes the important energy metabolism regulator AXIN, were positively correlated to the levels of *pfk*, *gapdh*, *icd*, *ampk α* , *sirt1* and *sirt5*. The expression levels of the gene encoding SIRT5, another important metabolic sensor, were positively correlated with levels of all genes involved in glycolysis (*gapdh*, *pk* and *ldh*).

The expression of genes encoding superoxide dismutase (SOD) and catalase, enzymes linked to breakdown of reactive oxygen species, were not statistically correlated to heat shock temperatures, but there was a significant correlation between *sod* and *cat-1* expressions (Table S4). There was no statistically significant correlation between heat-shock temperature and *casp-8*. However, the correlations were significant between heat-shock temperatures and the other 2 caspase genes (*casp-3* and *casp-7*). The expression levels among the 3 caspase genes were positively correlated to each other (Table S5).

DISCUSSION

Latitudinal variations of upper thermal limits

The lethal temperatures of *Cellana toreuma* measured using a laboratory heat-ramp protocol were similar among the 3 geographical populations. The relative invariance in the upper thermal limits of ectotherms at large geographical scales suggests that limpets live in regions where ambient temperatures often approach their upper limits (Chown & Gaston 2008). Based on high-resolution temperature records, the maximum habitat temperature (MHT) in all 3 locations exceeded limpets' ABT and at Nanding Island and Sichang MHT could exceed limpets' LT₅₀ (Figs. 1 & 3). These results indicate that the limpet *C. toreuma* suffers from intensive heat stress in its natural distribution range. In the present study, the mor-

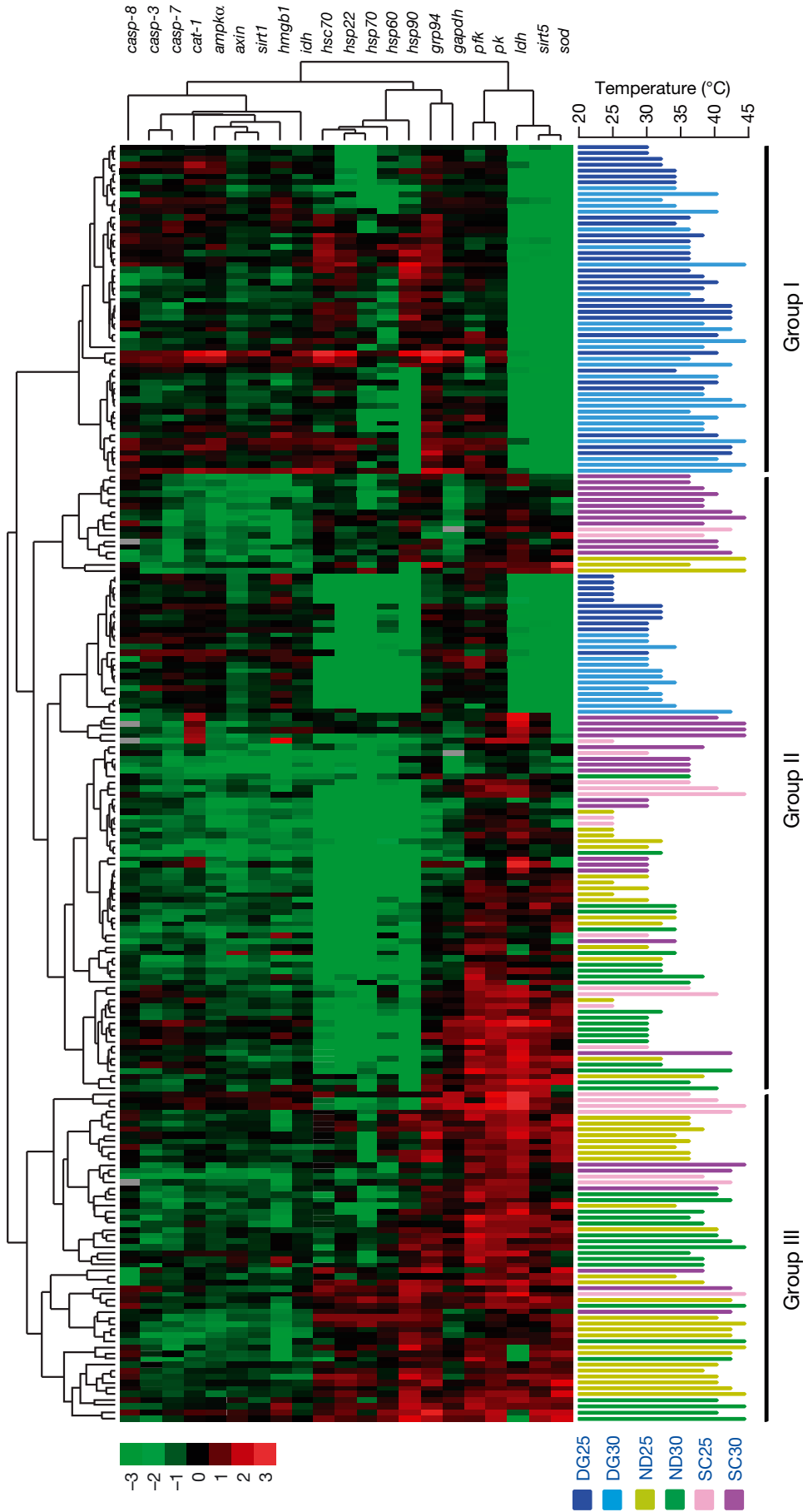


Fig. 4. Heat map of the normalized expression (log-transformed data) of limpets in different locations and acclimation temperatures. Individuals are clustered using a hierarchical clustering algorithm, which identified 3 major clusters: Group I, Group II and Group III. The color scale bar indicates log-transformed data, with green, red and black indicating down-regulation, up-regulation and no change, respectively. See Fig. 3 for abbreviations. The bars labelled 'temperature' represent the heat shock temperatures of individual limpets

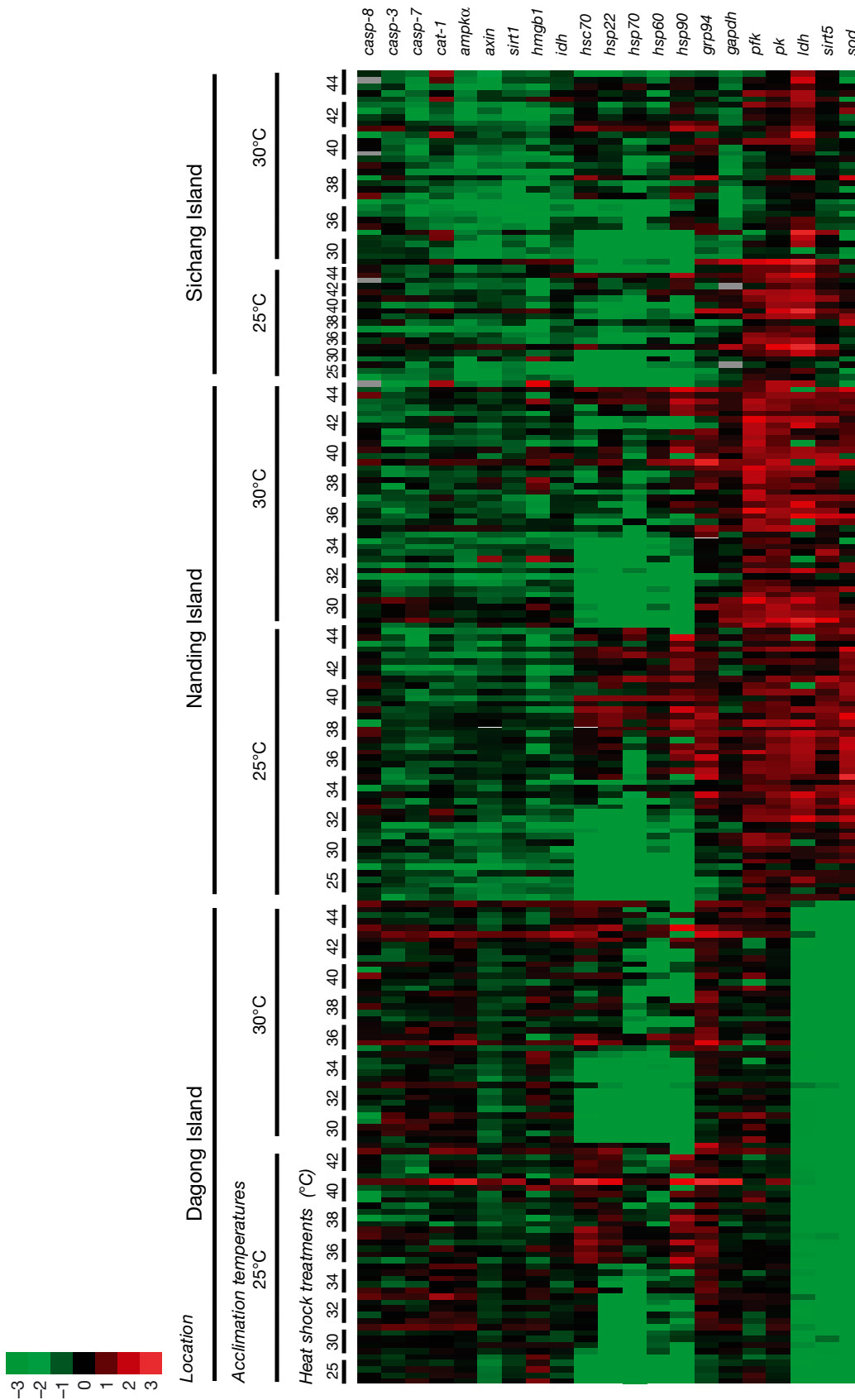


Fig. 5. Heat map of the normalized expression (log-transformed data) to show the expression patterns of 21 genes in different locations and acclimation temperatures. Individuals are clustered using a hierarchical clustering algorithm. The color scale bar indicates log-transformed data, with green, red and black indicating down-regulation, up-regulation and no change, respectively. The heat shock treatments show the temperature to which limpets were exposed

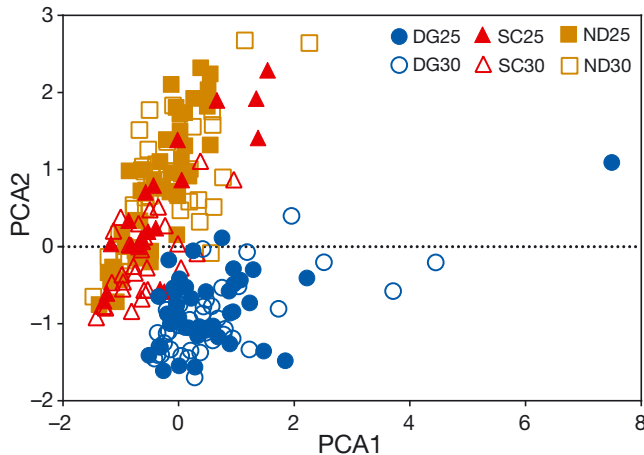


Fig. 6. Principal components analysis of expression levels of 21 genes of limpets from Dagong Island (circles), Nanding Island (triangles) and Sichang Island (squares) that were acclimated at 2 temperatures (25 and 30°C) and then heat shocked at different temperatures. Two components were extracted, and those 2 components accounted for 52.05 % of the total variance

tality was checked after 2 h recovery in seawater. Previous studies found that the mortality following heat stress increased after 24 to 48 h recovery (Logan et al. 2012, Dowd & Somero 2013). Therefore, the mortality after heat shock in the present study might be underestimated, and limpets in the 3 locations might have differences in mortality after a longer recovery.

The actual severity of *in situ* stress and the extent of mortality from high temperature will be a consequence of several factors, including heating rate, time of exposure to extremes of heat and frequency of occurrence of high temperatures over consecutive days (Hochachka & Somero 2002). In addition to the environmental factors, acclimation and local adaptation can also affect organisms' thermal tolerance (Stillman & Somero 2000, Kuo & Sanford 2009, Palumbi et al. 2014). For example, the tropical reef fish *Acanthochromis polyacanthus* is highly sensitive to small increases in water temperature but can rapidly acclimate over multiple generations. This study revealed the importance of transgenerational acclimation as a mechanism for coping with rapid climate change (Donelson et al. 2012). Intertidal snails *Nucella canaliculata* from 7 sites along the west coast of United States that were reared through 2 generations in a common garden experiment showed clear local adaptation of thermal tolerance traits (Kuo & Sanford 2009). Further studies should be carried out to investigate effects of these factors on limpets' upper thermal limits.

After 1 wk of acclimation at different temperatures (25 and 30°C), there were no significant differences in LT_{50} s between 2 acclimation treatments (Fig. 2), indicating that limpets living in highly variable environments may have limited ability for further increasing their tolerance of rising temperatures (Tomanek 2008, 2010). However, the acclimation duration was relatively short in the present study. If limpets were acclimated at different temperatures for a longer period, they might show phenotype plasticity of LT_{50} .

The latitudinal variability and plasticity of cardiac function of limpets provide insights into heat stress and metabolism. The ABT of the Nanding Island population was significantly higher than those of limpets at Dagong Island and Sichang Island. The ABT values of *C. toreuma* in Hong Kong were about 41°C (Dong & Williams 2011). These results indicate that ABTs of *C. toreuma* in Southern China are higher than those in Thailand and northern China, and the absence of plasticity of cardiac function in Nanding Island and Sichang Island populations indicates that limpets in these 2 locations suffer from intensive heat stress and have a relatively poor capability for coping with the future temperature increase.

Latitudinal variations of transcriptional responses

Though post-transcriptional mechanisms (selective translation of mRNAs, post-translational modification of proteins and actions of small molecule regulators of enzyme activity) are important for thermal adaptations, comparative analyses of variations in mRNA level can also provide useful information for investigating physiological responses to heat stress (Place et al. 2008, Stillman & Tagmount 2009, Lockwood et al. 2010, Palumbi et al. 2014). Based on their expression patterns, in the present study, the 21 genes were allocated into 3 groups, and their expression patterns were population-specific (Fig. 4), indicating that some genes and related cellular processes are more sensitive to thermal stress and the physiological adaptations to high temperature might be different among the 3 populations. The expression patterns of these genes were different among the 3 populations, and limpets in Dagong Island were relatively isolated from the other 2 populations (Fig. 6), indicating distinct transcriptional responses to thermal stress of the temperate population.

Genes encoding heat-shock proteins, including *hsp22*, *hsp60*, *hsp70*, *hsc70* and *hsp90*, were sensitive to heat stress in all limpets, and their expression pat-

terns were similar in different populations (Fig. 5). The high sensitivity of these genes suggests that all limpet populations have effective heat-shock responses at the transcriptional level. The upregulation of heat-shock proteins in limpets has also been discovered at the post-transcriptional level in previous studies (Sanders et al. 1991, Dong et al. 2008, Dong & Williams 2011). Due to the important roles of heat-shock proteins in protecting cells from heat stresses as molecular chaperones (Feder & Hofmann 1999, Tomanek 2010), heat-shock proteins are widely regarded as useful biomarkers indicating organisms' thermal tolerance (Clark & Peck 2009). In the present study, the significant upregulation of *hsps* in limpets from the 3 sites further confirms the existence of effective cellular defenses to highly variable thermal environments for intertidal organisms. The temperatures of maximal induction (T_{peak}) of *hsps* in limpets from all 3 sites were around 40°C. The similar T_{peak} values of *hsps* at the 3 sites indicate that limpets from different sites along a latitudinal gradient in the Asian coast share similar capabilities for protein stability maintenance and provide a reasonable partial explanation for the similar LT_{50} s of limpets in different locations.

A group of genes (*sirt5*, *pfk*, *pk*, *ldh* and *sod*) showed low levels of expression in Dagong Island compared to those in Nanding Island and Sichang Island, and this result provides a chance to investigate the latitudinal variations of physiological responses of intertidal limpets to heat stress. NAD-dependent deacetylase, sirtuin 5, is up-regulated in response to caloric restriction and is controlled by the cellular NADH: NAD⁺ (Cantó et al. 2009, Houtkooper et al. 2012, Tomanek 2012). Some metabolic processes, including fatty acid metabolism, gluconeogenesis and the Krebs cycle, are regulated by deacetylation activity of sirtuins (Houtkooper et al. 2012). Sirtuin 5 is also involved in heat-shock responses (Tomanek 2012). The expression levels of *pfk*, *pk* and *ldh* are also maintained at low levels in limpets from Dagong Island. As 2 rate-limiting enzymes, PFK and PK regulate the overall activity of glycolysis. PFK catalyzes the important first committed step of glycolysis, the conversion of fructose 6-phosphate and ATP to fructose 1,6-bisphosphate (Fru-1,6-P₂) and ADP, and PK activity is activated by Fru-1,6-P₂. LDH catalyzes the conversion of pyruvate to lactate under conditions of low cellular oxygen availability. These cellular processes are critical for cellular energy supply, and modulating activities of these rate-limiting enzymes can be used by intertidal animals to acclimatize to different thermal environ-

ments (Lesser & Kruse 2004, Tomanek & Zuzow 2010). The relatively low levels of *sod* expression of limpets from Dagong Island may be due to the low production of reactive oxygen species, which is closely related to an organism's rate of metabolism (Abele et al. 2002). The low mRNA levels of these genes in limpets from Dagong Island suggest that the energetic metabolism of limpets from the temperate area is relatively low compared to those from the subtropical area. Compared to limpets on Nanding Island, the low heart rates of the Dagong Island population at the high temperature also support this speculation (Table 1).

Potential population dynamics of rocky intertidal species

The operative temperature data and physiological data are useful for analyzing how closely organisms live to their upper thermal limits and predicting which population is more vulnerable in the face of global warming. Compared to limpets on the tropical shore with a narrow temperature range (Fig. 1), limpets on the subtropical shore have to cope with a thermal environment with a wider temperature range and frequent extremely high temperature events under the impact of the West Pacific subtropical high in summer (Huang et al. 2007). Similar to what Helmuth et al. (2002) found regarding the mosaic pattern of thermal environment along the West Coast of the United States, our results indicate that heat stress along the Asian coast is not linearly dependent on latitude, and limpets on the subtropical shore face conditions as stressful as those on the tropical shore. Based on the operative temperature data and the upper thermal limits of different populations (Fig. 3), the present study also indicates that limpets in Nanding Island live closer to their upper thermal limits and possibly will be more sensitive to future temperature increase. Based on high-resolution coastal sea surface temperature records, the warming rates in the East China Sea were higher than those in the South China Sea and Yellow Sea since the 1960s (Bao & Ren 2014). Therefore, it is reasonable to speculate that the stressful thermal environment on some subtropical shores will potentially impede the poleward distribution shift of tropical limpet populations.

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