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RESEARCH ARTICLE

Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion

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SUMMARY

The effects of heat stress on organisms are manifested at the levels of organ function, metabolic activity, protein stability and gene expression. Here, we examined effects of high temperature on the intertidal limpet *Cellana toreuma* to determine how the temperatures at which (1) organ failure (cardiac function), (2) irreversible protein damage (carbonylation) and (3) expression of genes encoding proteins involved in molecular chaperoning (*hsp70* and *hsp90*) and metabolic regulation (*ampk* and *sirt1*) occur compare with field temperatures, which commonly exceed 30°C and can reach 46°C. Heart failure, indexed by the Arrhenius break temperature, occurred at 34.3°C. Protein carbonylation rose significantly at 38°C. Genes for heat shock proteins HSP70 (*hsp70*) and HSP90 (*hsp90*), for two subunits of AMP-activated protein kinase (AMPK) (*ampkα* and *ampkβ*) and for histone/protein deacetylase SIRT1 (*sirt1*) all showed increased expression at 30°C. Temperatures of maximal expression differed among genes, as did temperatures at which upregulation ceased. Expression patterns for *ampk* and *sirt1* indicate that heat stress influenced cellular energy homeostasis; above ~30°C, upregulation of ATP-generating pathways is suggested by elevated expression of genes for *ampk*; an altered balance between reliance on carbohydrate and lipid fuels is indicated by changes in expression of *sirt1*. These results show that *C. toreuma* commonly experiences temperatures that induce expression of genes associated with the stress response (*hsp70* and *hsp90*) and regulation of energy metabolism (*ampk* and *sirt1*). At high temperatures, there is likely to be a shift away from anabolic processes such as growth to catabolic processes, to provide energy for coping with stress-induced damage, notably to proteins.

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INTRODUCTION

Intertidal animals frequently encounter extreme thermal stress during aerial emersion (Helmuth and Hofmann, 2001; Gilman et al., 2006; Helmuth et al., 2006a; Miller et al., 2009), and temperature is regarded as one of the most important factors determining their zonation patterns (Wolcott, 1973; Hochachka and Somero 2002; Davenport and Davenport, 2005). Three decades of high-resolution data show that 71% of the world's coastlines are significantly warming, and extremely hot days are becoming more common in 38% of coastal areas (Lima and Wethey, 2012). Moreover, the mosaic patterns of thermal stress on rocky shores along a latitudinal gradient make some shores more thermally stressful than expected based strictly on latitude, such that local populations are more vulnerable to climate change (Helmuth et al., 2002; Helmuth et al., 2006b). Consequently, significant changes in intertidal communities have been recorded, based on long-term *in situ* observations, during the past several decades (Barry et al., 1995; Southward et al., 1995).

Physiological adaptation is one of the main response options for organisms facing global change (Hofmann and Todgham, 2010), and the potential for acclimatization and genetic adaptation will determine 'winners' and 'losers' in the future (Hochachka and Somero, 2002; Somero, 2011). As an evolutionary response to local environmental conditions (Eliason et al., 2011), the physiological performance of ectothermic organisms is sensitive to environmental temperature variation and closely relates to an organism's thermal tolerance (Hochachka and Somero, 2002; Pörtner et al., 2006; Clark et al., 2008a). Mechanistically, the thermal-tolerance windows of many marine ectothermic organisms are determined by the effects of temperature change on metabolism, notably on the ability to maintain an adequate aerobic scope at elevated temperatures (Pörtner et al., 2006; Pörtner, 2010).

To cope with the harsh thermal environment they face, intertidal animals have developed diverse physiological adaptations for sustaining metabolism and for directing energy toward repair of thermally induced damage (Tomanek and Helmuth, 2002; Hofmann and Todgham, 2010). Upregulation of stress proteins occurs as a time-limited cellular defense against thermal stress in almost all intertidal organisms to maintain protein homeostasis (Sanders et al., 1991; Tomanek and Somero, 1999; Tomanek and Somero, 2002; Dong et al., 2008; Clark et al., 2008b; Tomanek, 2010; Dong and Williams, 2011). However, upregulation of heat-shock proteins (HSPs) requires energy (Sørensen et al., 2003; Tomanek and

Zuzow, 2010), and to compensate for the elevated cellular energy demands, energy allocation is shifted from growth and reproduction to the stabilization and restoration of protein structures and functions (Sokolova et al., 2012). Damage to proteins at high temperature can also result from oxidative stress from reactive oxygen species that cause non-reversible, covalent modifications of proteins. One result of protein oxidation is an increase in the number of protein carbonyl groups (Lushchak and Bagnyukova, 2006). Quantification of carbonyl groups is commonly used as a biomarker of irreversible oxidative damage to proteins (Dalle-Donne et al., 2003).

Shortages of energy may be of pivotal importance in setting thermal tolerance limits under conditions of chronic heat stress. Because metabolic rates are limited, stress-induced changes in the energy budget that lead to reallocation of energy from anabolic processes such as growth to repair of damage are closely related to ecological fitness. Discovering biomarkers that provide insight into the energy balance of an organism is thus important to understanding the ecological physiology of heat stress. Such metabolism-related biomarkers can be used to determine the conditions under which metabolic transitions between anabolic and damage-repair processes occur and thus to predict ecological consequences of stress exposure (Sokolova et al., 2012).

AMP-activated protein kinase (AMPK), a metabolic sensor of the AMP/ATP ratio (Hardie and Sakamoto, 2006), has proven to be a good cellular indicator of the transition into the pejus temperature range during heat stress in intertidal crabs (Frederich et al., 2009). The histone/protein deacetylase SIRT1 is a fuel-sensing molecule that has coexisted with AMPK in cells throughout evolution (Ruderman et al., 2010). SIRT1 regulates fat and glucose metabolism in response to physiological changes in energy levels, thereby acting as a crucial regulator of the network that controls energy homeostasis (Cantó et al., 2009; Houtkooper et al., 2012) (Fig. 1). The induction of SIRT1 occurs during low energy status and repression occurs during energy excess status. The close relationship between AMPK and SIRT1 and energy expenditure make them potentially useful biomarkers for gauging cellular energy homeostasis and elucidating metabolically related thermal tolerance. Because activation of AMPK and SIRT1 can indicate the switching on of catabolic pathways and the switching off of anabolic pathways (Cantó et al., 2009), the upregulation of these two metabolic sensors also indicates that more energy is being allocated to maintenance and less energy to growth, storage and reproduction. These changes in energy allocation are closely related to the ecological fitness of a population. SIRT1 also plays an important role in the regulation of activity of heat shock factor 1 (HSF1); SIRT1 can prolong HSF1 binding to the heat shock promoter by maintain HSF1 in a deacetylated, DNA-binding competent state (Westerheide et al., 2009).

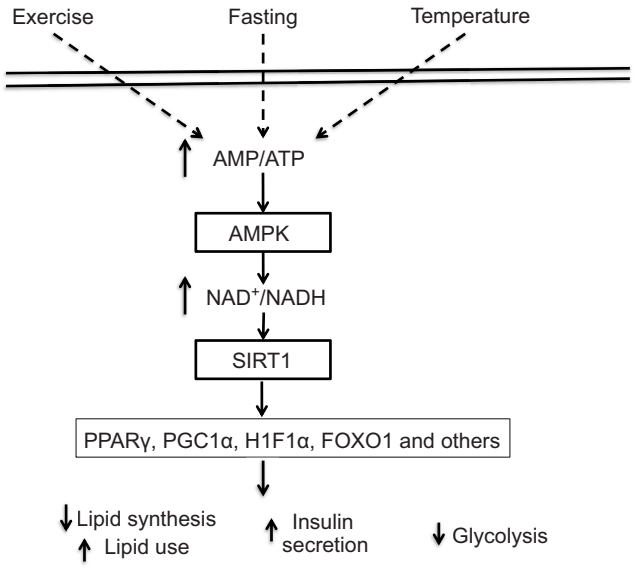

Fig. 1. Scheme showing the convergent actions of AMPK and SIRT1. Factors such as exercise, fasting and temperature can lead to increase of the AMP/ATP ratio, and then trigger the upregulation of AMPK. The activation of AMPK can induce an increase of the NAD^+/NADH ratio, which activates SIRT1. The impact of AMPK and SIRT1 on peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), forkhead box O (FOXO) and other transcriptional regulators will then affect carbohydrate and lipid metabolism (Cantó et al., 2009).

In this multi-level study, we examined the effects of high temperature on the intertidal limpet *Cellana toreuma* (Reeve 1854) to determine how the temperatures at which (1) cardiac failure, (2) irreversible protein damage (indexed by carbonylation) and (3) expression of genes for proteins involved in protein repair (molecular chaperoning: *hsp70* and *hsp90*) and metabolic regulation (*ampk* and *sirt1*) occur compare with field temperatures for this species. This eurythermal species is widely distributed in the Indo-Pacific (Dong et al., 2012) and it naturally experiences large-scale mortality during hot summer periods (Williams and Morritt, 1995; Firth and Williams, 2009). It therefore is an appropriate study species for examining the questions listed above and for developing an understanding of how further changes in temperature associated with climate change may jeopardize its persistence in its current habitat.

MATERIALS AND METHODS

In situ temperature measurement

The use of Robolimpets has been shown to be an effective method of measuring the body temperature of limpets (Lima and Wethey, 2009). In this method, a biomimetic data logger consists of a micro-logger inserted into the shell of a limpet from which soft tissues have been removed. In the present study, the operative body temperatures of limpets were estimated using Robolimpets at a field site on Nanding Island, Fujian, China (24°09′N, 117°59′E). A total of 16 Robolimpets were deployed in a semi-wave-exposed shore between ~1.0 and 4.0 m above chart datum (CD; the level of water that charted depths displayed on a nautical chart are measured from) on both south-facing and west-facing rocky surfaces. *Cellana toreuma* is known to migrate vertically up and down the shore during a tidal cycle within the tidal range used for the deployment of the Robolimpets (Y.-w.D. and G.-d.H., unpublished data). Operative temperature recordings were made every 30 min during the hottest time of the year (between 28 August and 26 September 2011).

Limpet collection and temperature treatment

A total of 66 limpets (body length, 1.5±0.2 cm) were collected from Nanding Island, and were immediately transported back to an indoor aquarium at the State Key Laboratory of Marine Environmental Science, Xiamen University (24°26′N, 118°05′E). The limpets were kept under conditions of seawater spray, and immersed under water for 12 h every day. After 1 week of acclimation at ~20°C, 12 limpets (N=4 groups of three limpets pooled together) were selected as controls (no heat), while the others were used in heat exposure experiments. Randomly selected individuals were placed on an artificial rock to settle and were then heated at a rate of ~0.1°C min^{-1} to 40°C, using four 1000 W incandescent lights. Following this ramped heating, limpets were kept at 40°C for 1 h. These temperature

conditions are consistent with those experienced by this species on natural rocky shores. Thermocouples were inserted into the shells for continuous recording, and body temperature of live limpets was recorded every minute using a thermometer (Fluke 54II, Fluke, WA, USA). Heart rates of limpets were measured during heating and at a constant 40°C. When target temperatures (30, 32, 34, 36, 38, 40 and 40°C for 1 h) were reached, nine limpets (N=3 groups of three limpets pooled together) were immediately collected and dissected for the determination of protein carbonyl levels and gene expression.

As *C. toreuma* is not a protected species, and collections were only made from public access areas, no specific permits were required to either collect this species from these locations or perform these activities.

Cardiac performance

Heart rates were measured using a non-invasive method (Chelazzi et al., 2001; Dong and 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 light-dependent current produced by the heartbeat were amplified, filtered and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria, Portugal) and Powerlab AD converter (4/30, ADInstruments, March-Hugstetten, Germany). Data were viewed and analyzed using Chart (version 5.0). The Arrhenius break temperature (ABT) for cardiac performance, the temperature at which the heart rate decreases dramatically with progressive heating, was determined using a regression analysis method that generates the best fit line on either side of a putative break point for the relationship of ln-transformed heart rate against temperature (see Stillman and Somero, 1996).

Protein carbonyl groups

The abundance of protein carbonyl groups was determined using OxyBlot Oxidized Protein Detection Kit (Millipore, Billerica, MA, USA). Approximately 50 mg of foot muscle in each specimen was homogenized with 400 µl lysis buffer. After centrifugation (12,000 ***g***, 10 min, 4°C), the supernatants were transferred to new tubes and protein concentration was determined using the Bradford protein assay (Bradford, 1976). Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the manufacturer's instructions. Abundance of carbonyl protein groups was analyzed by quantitative densitometry using ImageJ software (Abràmoff et al., 2004).

Gene cloning and expression

Total RNA was isolated from ~50 mg of foot muscle using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). 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, Shiga, Japan).

To obtain sequences of target genes from *C. toreuma*, PCR was used to amplify partial sequences with degenerate primers (the sequences of primers used in this study are given in the supplementary material Table S1), and then the full-length cDNAs were obtained using the rapid amplification of cDNA ends (RACE) protocol with the 3′-Full RACE Core Set and 5′-Full RACE Kit (Takara, Shiga, Japan).

The reference genes were selected from *18S rRNA*, *β-actin*, *β-tubulin* and *calmodulin* using GeNorm Algorithm (Primer Design, Southampton University, Highfield Campus, Southampton, Hants, UK) as described by Etschmann et al. (Etschmann et al., 2006). GeNorm is a bioinformatics tool designed to rank candidate reference genes using a normalization factor calculated on the basis of the geometric mean of the expression levels of the candidate reference genes in an array of representative samples. The expression stability measures (M-values) of *18S rRNA*, *β-actin*, *β-tubulin* and *calmodulin* are 1.135, 1.008, 1.100 and 1.008 when all genes were included in the calculation of M. Therefore, based on its low M-value of 1.008, a partial sequence of the *β-actin* gene was selected as a reference housekeeping gene to normalize the level of expression. The levels of *hsp70*, *hsp90*, *ampkα*, *ampkβ* and *sirt1* expression were quantified using real-time quantitative PCR with primers designed from the sequences obtained as described above (GenBank accession nos: *hsp70*, JX69849; *hsp90*, JX69850; *ampkα*, JX69847; *ampkβ*, JX69848; *sirt1*, JX69851). PCR was carried out in an ABI 7500 Real-Time PCR System (Applied Biosystems, Bedford, MA, USA) in a 20 µl reaction volume containing 10 µl of 2× FastStart DNA Universal SYBR Green Master (Roche, Grenzach-Wyhlen, Germany), 0.8 µl of each primer (10 nmol µl^{-1}), 1 µl of cDNA template and 7.4 µl of RNase-free water. The PCR conditions were as follows: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 20 s; 59°C, 20 s; and 72°C for 40 s with a final dissociation curve step. All samples were measured in triplicate. Cycle threshold (C_T) values were analyzed using the ABI 7500 System Software (Applied Biosystems, Bedford, MA, USA). The expression of *hsp70*, *hsp90*, *ampkα*, *ampkβ* and *sirt1* mRNA for the various heat treatments was determined relative to the value of β-actin for experimental *versus* control treatments.

Statistical analysis

The differences in expression of genes were analyzed using the SPSS 17.0 for Windows statistical package. Data were logarithmically transformed, and then tested for homogeneity of variance using Levene's test. Differences in *hsp70*, *hsp90*, *ampkα*, *ampkβ* and *sirt1* expression levels among different temperatures were determined using one-way ANOVA followed by *post hoc* Duncan's multiple range tests. The correlation among *hsp70*, *hsp90*, *ampkα*, *ampkβ* and *sirt1* expression levels was analyzed using Spearman correlation analysis. Differences were considered significant at P<0.05. The hierarchical clustering algorithm was performed using the Euclidean distance similarity metric after log transformation and the centroid linkage method. Analysis was conducted in the Cluster 3.0 (University of Tokyo, Human Genome Center).

RESULTS

In situ temperature measurements

Of the 16 Robolimpets deployed, 13 produced reliable data. Thermal regimes of the Robolimpets differed among locations (south- and west-facing slopes) and for the different tidal heights (Fig. 2). Limpets on the south-facing shore typically experienced higher temperatures. With the exception of data-loggers on the west-facing low shore (1.0 m above CD), those at all the other locations recorded temperatures above 34°C. At all south-facing locations (Fig. 3) and west-facing locations (supplementary material Fig. S1) >2.0 m above CD, data loggers recorded temperatures above 38°C. In both the south- and west-facing high shore (>3.5 m above CD), temperatures occasionally reached 46–48°C. The relative frequencies of the temperatures experienced were always strongly skewed to the right, with the median being either 24 or 26°C for all Robolimpets, regardless of slope face or vertical height on the shore (Fig. 3). The period of exposure to higher temperatures generally increased with increasing vertical height.

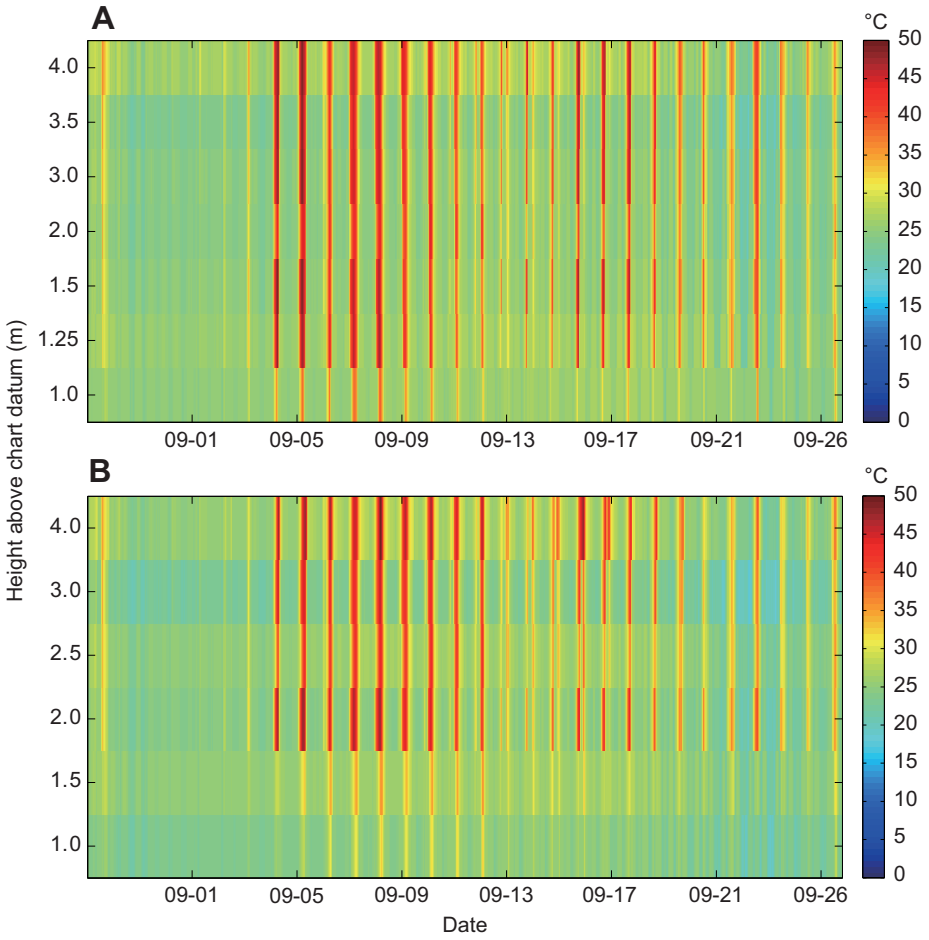

Fig. 2. Colour-coded charts showing relationships for operative temperature, tidal height and time for (A) a relatively extreme south-facing rocky slope and (B) a relatively benign west-facing slope at the study site. Data were collected every 30 min between 28 August and 26 September 2011, over a tidal range of ~1.0–4.0 m above chart datum. Colours represent the temperatures shown in the scale bar next to the charts.

Cardiac performance

Heart beat frequency increased from ~1.5 Hz at 21°C to an average and absolute maximum frequency, respectively, of ~3 and 3.7 Hz at ~34°C (Fig. 4). There was a marked decline in heart rate during heating above this temperature, and the heart rate continued to fall when limpets were kept at 40°C for 1 h. When data were linearized and regressions were plotted (for all heart rates of the three individuals explored), the ABT was found to be 34.3±3.1°C.

Protein carbonyl groups

Four main bands of carbonylated proteins, including two near 40 kDa, one near 30 kDa and one near 25 kDa, were detected at all temperatures and showed the greatest abundance at elevated temperatures (supplementary material Fig. S2). One-way ANOVA showed that high temperature enhanced the levels of protein carbonyl groups ($F_{6,21}$=4.533, P=0.008). The level of carbonyl groups reached a maximum at 38°C, which was significantly higher than that at 22°C (*post hoc* Duncan's multiple range test; Fig. 5).

HSP gene expression

The expression of the genes encoding two molecular size classes of HSPs, HSP70 and HSP90, was upregulated at high temperatures (Fig. 5). The expression of *hsp70* at high temperatures was over 500 times greater than that at the lowest temperature investigated. One-way ANOVA showed significant differences among the temperature treatments ($F_{7,24}$=12.255, P<0.0001), with the *hsp70* expression between 30 and 40°C being greater than that at 22°C. The temperature at which *hsp70* was expressed initially (T_{on}) was 30°C, and the temperature at which *hsp70* was expressed maximally (T_{max}) was 32°C. However, there was no significant difference between the expression of this gene at 32°C and at higher temperatures, which precluded the determination of the temperature at which expression ceased (T_{off}).

The pattern of expression of *hsp90* was similar to that of *hsp70*, but the magnitude of upregulation was lower (10- to 30-fold). One-way ANOVA confirmed significant differences in the expression of this gene under different temperature treatments ($F_{7,24}$=3.512, P=0.016). The T_{on} and T_{max} for *hsp90* were 30 and 34°C, respectively, and the temperature at which upregulation of gene expression ceased was ~40°C (Fig. 5).

ampk expression

Upregulation of *ampkα* and *ampkβ* occurred at high temperatures (Fig. 6). One-way ANOVA showed that there were significant differences in the expression of both *ampkα* ($F_{7,24}$=3.001, P=0.030) and *ampkβ* ($F_{7,24}$=4.170, P=0.008) at different temperatures. The two subunits of *ampk*, the catalytic subunit and one of two regulatory subunits, shared similar expression patterns against temperature; the values of T_{on}, T_{max} and T_{off} of the two genes were 30, 30 and 36°C, respectively.

sirt1 expression

When temperature was increased from 22 to 40°C, the levels of *sirt1* mRNA increased initially and then decreased to the control level (Fig. 6). The levels of *sirt1* expression at 30, 32 and 34°C were significantly higher than those at other temperatures (*post hoc* Duncan's multiple range tests, $F_{7,24}$=3.264, P=0.022). The values

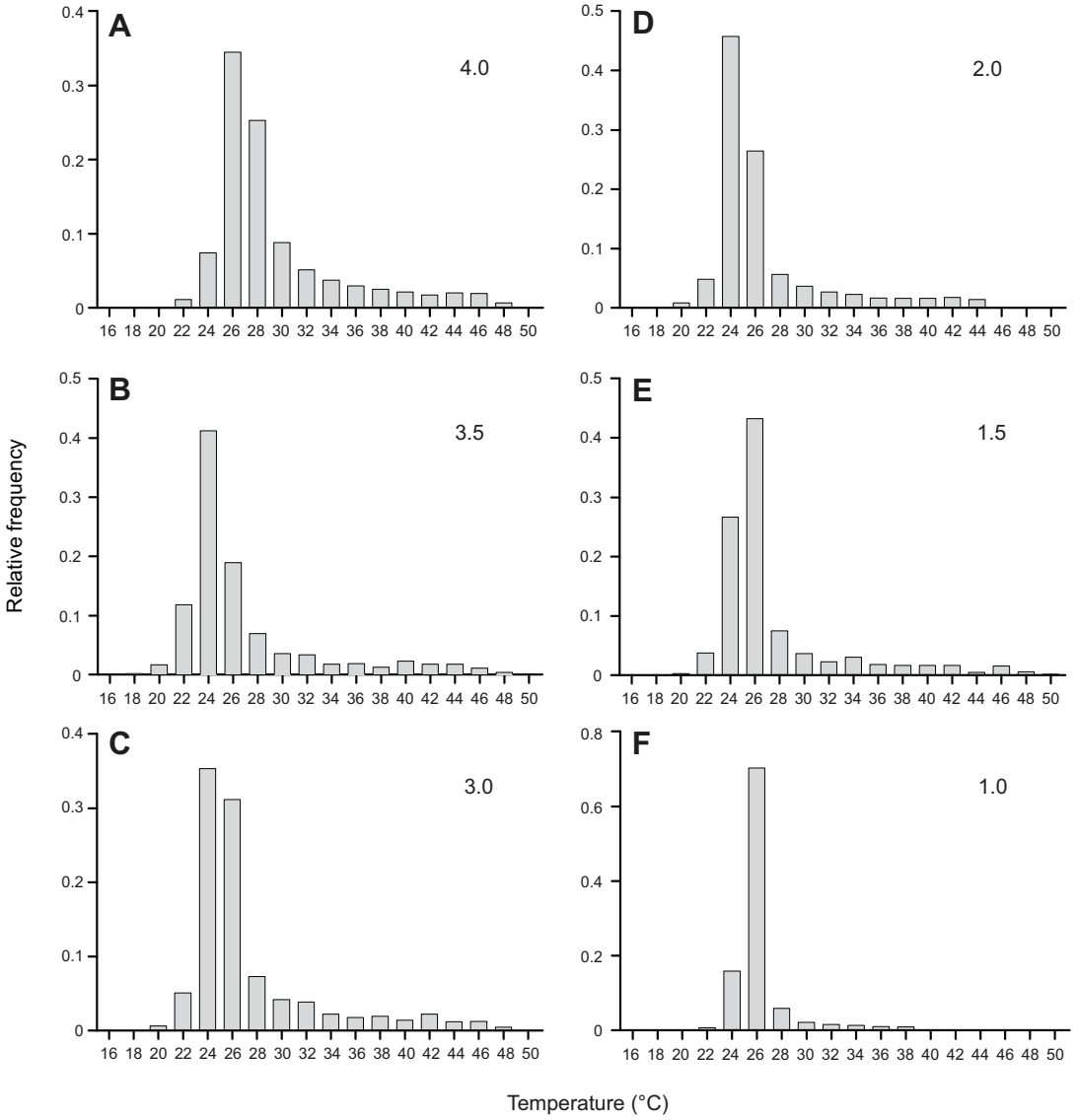

Fig. 3. Relative frequencies of the operative temperature of the limpet *Cellana toreuma* based on the data shown in Fig. 2, for each tidal level (A–F show 4.0. 3.0, 2.5, 2.0, 1.5 and 1.0 m above chart datum) investigated on the south-facing rocky surfaces.

of T_{on}, T_{max} and T_{off} for *sirt1* expression were 30, 32 and 36°C, respectively.

Parallel and specific expression among genes

Spearman correlation analysis revealed that the correlation between the three metabolic sensors (*ampkα*, *ampkβ* and *sirt1*) was statistically significant ($P<0.001$, Table 1), and there was a significant ($P<0.001$) positive correlation between *hsp70* and *hsp90* expressions. The correlations between metabolic sensors and HSP mRNA were also statistically significant except that between *hsp70* and *ampkβ* ($P=0.175$).

The gene expression responses are summarized in the dendrogram showing the clustering of individuals in different temperature treatments based on the gene expression pattern (Fig. 7). Individuals are clustered using a hierarchical clustering algorithm, which identified two major clusters. The first cluster comprised the four individuals maintained at ~20°C where low expression existed in all five genes. The second cluster included individuals with significant upregulation of *hsp70*. Within the second cluster, upregulation of all five genes occurred in all animals at 30, 32, 34 and 36°C. However, in most animals exposed to 38, 40 and 40°C for 1 h, there was lack of the upregulation of *ampkα*, *ampkβ* and *sirt1*.

DISCUSSION

Biomarkers of thermal stress in an intertidal limpet

Whole-organism-level uptake and delivery of oxygen, which involves ventilation and perfusion (cardiac function) of the respiratory surfaces, is important for the maintenance of cellular-level aerobic functioning, and is thus fundamental to moderating physiological responses of intertidal organisms to thermal stress (Pörtner, 2010; Sokolova et al., 2012; Somero, 2012). An organism's thermal tolerance is often closely related to the capacity for oxygen delivery to the cells (Pörtner et al., 2006), and in air-exposed gastropods, in which ventilation is limited, this capacity is largely sustained by cardiac function. In the present study, the heart rate of *C. toreuma* increased with increasing temperature, and then decreased sharply when temperature rose above the ABT (34.3°C). Although a higher ABT of 41.8°C has been reported for a population of this species from Hong Kong (Dong and Williams, 2011), the Hong Kong and Fujian populations are known to be genetically similar, suggesting that the ABT differences relate to differences in thermal histories and different thermal acclimatory effects among the populations (Somero, 2002; Stillman, 2002; Stenseng et al., 2005; Pörtner, 2010; Somero, 2010).

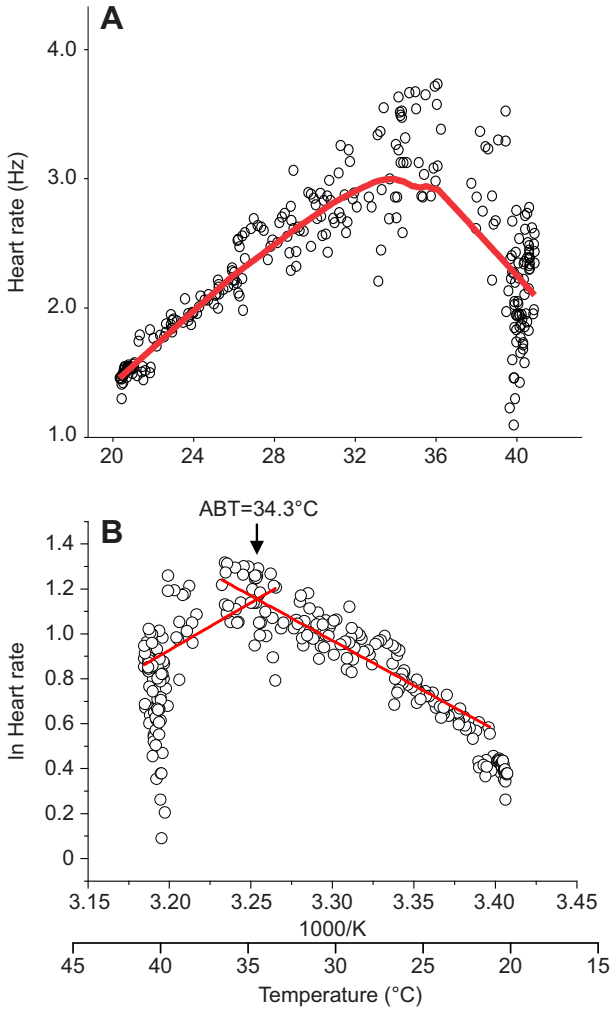

Fig. 4. (A) Relationship between heart rate and temperature of the limpet *Cellana toreuma* under continuous heating. A Loess curve is fitted to the data recorded every 1 min (one individual as example). (B) Determination of the Arrhenius break temperature (ABT) for the study population of *Cellana toreuma* (one individual as example). An increase in temperature above the ABT (34.3±3.1°C) results in a decline in heart rate (*N*=3).

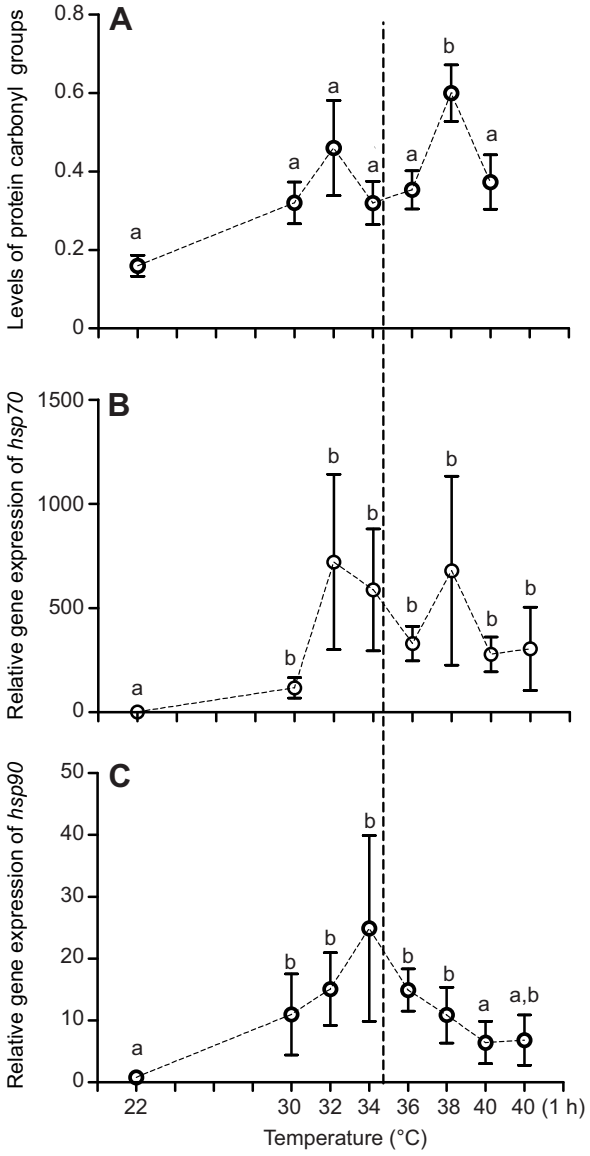

Fig. 5. Levels of (A) protein carbonyl groups, (B) *hsp70* and (C) *hsp90* mRNA in *Cellana toreuma* exposed to temperatures of 22, 30, 32, 34, 36, 38 or 40°C during heating, and after 1 h at constant 40°C. Values are means ± 1 s.d.; *N*=4 at 22°C and *N*=3 for other temperature treatments. The vertical dashed line indicates the ABT of *C. toreuma*. Means with different letters are significantly different (one-way ANOVA followed by Duncan's multiple range test, $P<0.05$).

The ABT for heart function also represents the thermal limit for survival of some intertidal invertebrates (crustaceans) upon acute heating [critical thermal maximum (Somero, 2002; Stillman, 2002)], but this is generally not the case for intertidal gastropods. Rather, in these gastropods the ABT occurs at a much lower temperature than the flatline temperature (FLT_{hot}) at which heart function ceases and which corresponds with the upper lethal temperature (Stenseng et al., 2005). The thermal range between the ABT and the FLT_{hot} can be highly variable among species and populations in relation to thermal history and with respect to different degrees of plasticity of these traits, as ABT is usually more strongly influenced by thermal acclimation than is FLT_{hot} (Stenseng et al., 2005). The present study reports ABT and FLT_{hot} values of ~34°C and ~40°C, respectively, whereas in the Hong Kong population of *C. toreuma* studied, heat coma temperature, ABT and FLT_{hot} were found to be ~40°C (Ng, 2007), 41.8°C (Dong and Williams, 2011) and ~44°C (Y.-w.D., unpublished data), respectively.

The upregulation of stress protein is an effective but energy-consuming way of combating thermal stress (Sørensen et al., 2003; Tomanek and Zuzow, 2010). The dramatic upregulation of *hsp*s, especially *hsp70* (~1000-fold upregulation) in *C. toreuma* indicates the important defensive role against thermal stress of HSPs in this species. Although there was no significant upregulation of the inducible paralogue (isoform) of HSP70 in the Hong Kong population between 28 and 40°C (Dong and Williams, 2011), such upregulation is indicated by the high level expression of *hsp70* in the present study. The difference in pattern of *hsp70* expression between the studies could again be due to the differences in acclimation or acclimatization temperature.

Protein carbonyl groups are considered as biomarkers of oxidative stress; an increasing amount of side-chain carbonylation indicates an imbalance toward the pro-oxidant side of pro-oxidant/antioxidant homeostasis (Stadtman and Levine, 2003;

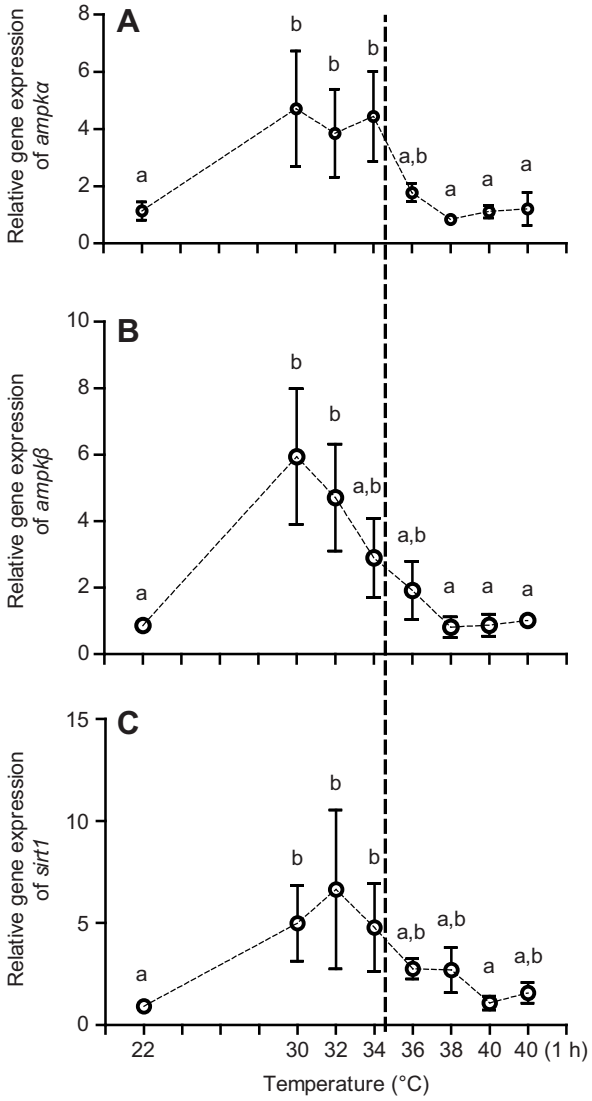

Fig. 6. Levels of (A) *ampkα*, (B) *ampkβ* and (C) *sirt1* mRNA in *Cellana toreuma* exposed to temperatures of 22, 30, 32, 34, 36, 38 or 40°C during heating, and after 1 h at constant 40°C. Values are means ± 1 s.d.; *N*=4 at 22°C and *N*=3 for other temperature treatments. The vertical dashed line indicates the ABT of *C. toreuma*. Means with different letters are significantly different (one-way ANOVA followed by Duncan's multiple range test, $P<0.05$).

Dalle-Donne et al., 2003). In the present study, the maximum level of protein carbonyl groups occurred at 38°C, a temperature that was somewhat higher than the temperatures at which the highest levels of expression of HSP genes occurred, especially *hsp90*. These results suggest that HSPs could stabilize proteins or transfer irreversibly damaged protein to the proteolytic machinery after carbonylation. When temperature increased to 38°C, however, expression of genes encoding HSPs showed a pattern of decrease. If this transcriptional pattern were a reflection of the concentrations and activities of HSPs, then HSPs would no longer be able to effectively maintain the stabilization of proteins or assist the degradation of denatured proteins at these high temperatures.

Like HSPs, the induction of AMPK is also sensitive to thermal stress. The genes *ampkα* and *ampkβ* were initially and maximally expressed at 30°C. Frederich et al. (Frederich et al., 2009) suggest that AMPK might represent an earlier indicator of temperature stress than HSP70 in rock crabs. The results of the present study support this idea in the case of *C. toreuma*, as the maximal expression of both *ampkα* and *ampkβ* (T_{max}) occurred at 30°C, compared with that of *hsp70* and *hsp90* at 32 and 34°C, respectively. The upper temperature limiting further upregulation of *ampk* (T_{off}) was also lower than that of *hsp70* (>40°C) and *hsp90* (40°C). *Sirt1* had an expression pattern similar to that of *ampk*, and showed a narrower temperature range of increased expression (30–36°C) relative to the genes for HSPs (Fig. 8).

Table 1. Spearman correlation coefficients between *ampkα*, *ampkβ*, *hsp70*, *hsp90* and *sirt1*

		ampkα	*ampkβ*	*hsp70*	*hsp90*	*sirt1*
ampkα	Coefficient	1.000	0.731	0.440	0.585	0.815
	P (two-tailed)		0.000	0.028	0.002	0.000
	N	25	24	25	25	24
ampkβ	Coefficient	0.731	1.000	0.286	0.490	0.797
	P (two-tailed)	0.000		0.175	0.015	0.000
	N	24	25	24	24	23
hsp70	Coefficient	0.440	0.286	1.000	0.847	0.600
	P (two-tailed)	0.028	0.175		0.000	0.002
	N	25	24	25	25	24
hsp90	Coefficient	0.585	0.490	0.847	1.000	0.717
	P (two-tailed)	0.002	0.015	0.000		0.000
	N	25	24	25	25	24
sirt1	Coefficient	0.815	0.797	0.600	0.717	1.000
	P (two-tailed)	0.000	0.000	0.002	0.000	
	N	24	23	24	24	25

Ecological significance of biomarkers

Recent studies for intertidal animals concerning transcriptomic and proteomic responses to stress have shown the induction of energy metabolism genes in response to heat stress, including in mussels (*Mytilus*) (Connor and Gracey, 2011; Gracey et al., 2008; Lockwood et al., 2010; Place et al., 2008), the porcelain crab *Petrolisthes cinctipes* (Stillman and Tagmount, 2009) and the fish *Gillichthys mirabilis* (Logan and Somero, 2010). Concordant with our findings, these studies showed that expression profiles of genes encoding proteins of energy metabolism were closely related to profiles of genes encoding HSPs. Thus, heat stress could have linked effects on expression of stress-related proteins, which commonly require ATP for their functions, and enzymes involved in energy metabolism (ATP generation).

Studies of AMPK and SIRT1 are likely to provide insights into organismal energy expenditure and assist understanding of fitness in different thermal habitats. In the present study, the low levels of *ampk* and *sirt1* expression at 22°C suggest that the limpets were in a balanced energy state, at which ATP demand matches ATP production within the cell. The upregulation of expression of these genes at high temperatures suggests that *ampkα*, *ampkβ* and *sirt1* could be appropriate markers of the limpet's metabolic status under moderate thermal stress. Furthermore, the significant upregulation of *ampkα* and *ampkβ* when the temperature rose to and above 30°C is consistent with a loss of energy homeostasis indicated by a higher ADP/ATP ratio (see Bergeron et al., 1999; Sambandam and Lopaschuk, 2003; Hardie and Sakamoto, 2006). This metabolic state seemingly favours upregulation of *sirt1*, leading to a shift in biochemical pathways towards an increase in catabolic metabolism (Houtkooper et al., 2012). These changes in expression of *ampk* and *sirt1* biomarkers are consistent with more energy being allocated

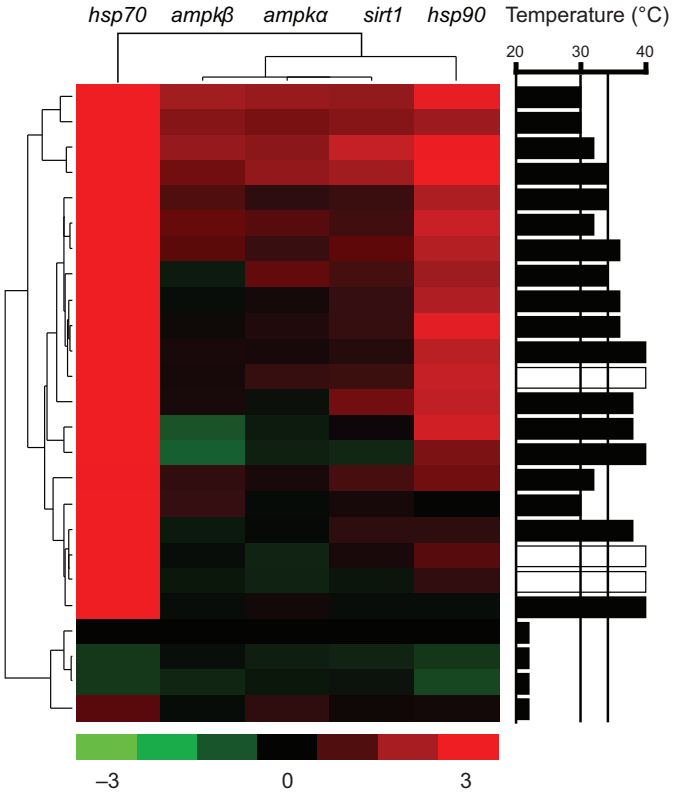

Fig. 7. Heat map of the normalized expression (log-transformed data) of individuals treated at different temperatures. The dendrogram shows the clustering of individuals in different temperature treatments. Individuals are clustered using a hierarchical clustering algorithm, which identified two major clusters (see Results, 'Parallel and specific expression among genes', for details). The colour scale bar indicates log-transformed data, with green indicating downregulation, red upregulation and black no change compared with the median of the control samples. *N*=4 at 22°C and *N*=3 for other temperature treatments. Body temperature data are shown to the right, and the open rectangles represent the treatment of 40°C for 1 h.

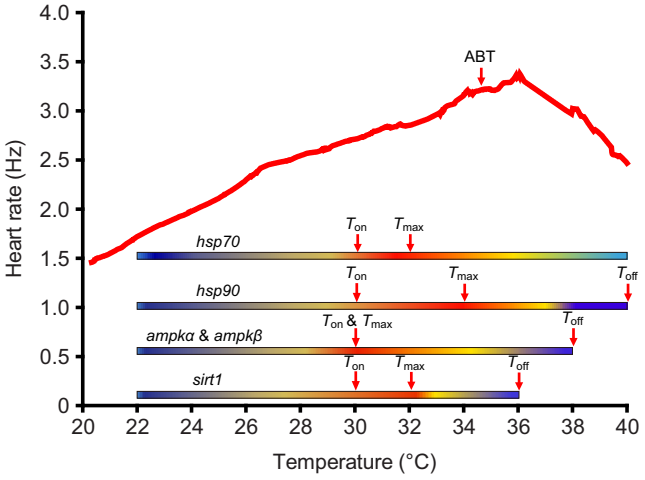

Fig. 8. Relationship between temperature, heart rate and the expression of molecular markers in *Cellana toreuma*. T_{on}, T_{max} and T_{off} represent, respectively, the temperatures at which the genes are initially expressed, maximally expressed or no longer upregulated during heating. The colour scale shows the expression levels of genes. Red and blue indicate the highest and the lowest levels of mRNA, respectively. Heart rate is given as a mean curve for three individuals; ABT is indicated.

to maintenance and damage repair under heat stress (e.g. for synthesis and function of HSPs) and a diversion of energy allocation away from growth and reproduction.

Previous work shows that the downregulation of SIRT1, which coincides with the loss of upregulation of *sirt1* mRNA, may accelerate the attenuation of a heat shock response [see description in Westerheide et al. (Westerheide et al., 2009)]. Protein acetylation has been shown to control wide-ranging metabolic changes (Wang et al., 2010; Zhao et al., 2010), and the proteomic responses to heat stress of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* suggest that sirtuin (sirtuin 5) is a possible regulator of heat stress metabolic changes (Tomanek and Zuzow, 2010; Tomanek, 2012). We therefore suggest that the physiological performance and related energy homeostasis of the subtropical population of *C. toreuma* should be potentially threatened when temperatures rise beyond 30°C for fairly long periods during each tidal cycle in the hottest months. Furthermore, these results provide a reasonable explanation for the vertical distribution of this species and its large-scale mortality in summer on tropical rocky shores. These results also indicate that the two metabolic sensors can be regarded as linkages between cellular metabolism, physiological performance and ecological fitness. A full interpretation of the energetic homeostasis over the period of a tidal cycle and of the impact of the energy cost of heat stress, however, requires data for energy gain during immersion and feeding.

In situ temperature, distribution on the shore and climate change

This study presents the first data set for environmental temperature data for the mid-intertidal limpet *C. toreuma* inhabiting a sub-tropical rocky shore in China. These limpets may experience large diurnal and seasonal temperature variation, depending on tidal height, weather conditions, and rock topography and slope (Fig. 3). During the hottest season (28 August to 26 September 2011), the body temperature of limpets found at most tidal heights frequently exceed 30°C (the temperature that causes disruption of energy homeostasis) and even 34°C (the ABT), and sometimes exceeded the heat coma temperature [~40°C (Ng, 2007)]. Even when considering the potential effects of behaviour in alleviating thermal stress, it is common to find *C. toreuma* at tidal heights between ~1.0 and 3.0 m above CD during low tide (Y.-w.D. and G.-d.H., unpublished), suggesting that many individuals may suffer frequent and extreme thermal stress in summer, and may be living very close to their thermal limit for survival and reproduction. In the context of climate warming, limpets in the subtropical rocky shore will have to invest more energy for maintenance, which should result in less energy being available for growth and reproduction, placing a constraint on ecological fitness and affecting population dynamics.

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AUTHOR CONTRIBUTIONS

Y.-w.D., D.J.M., and C.-h.K. designed the experiments. G.-d.H. and S.Z. performed the experiments. Y.-w.D. and G.-d.H drafted and revised the article.

COMPETING INTERESTS

No competing interests declared.

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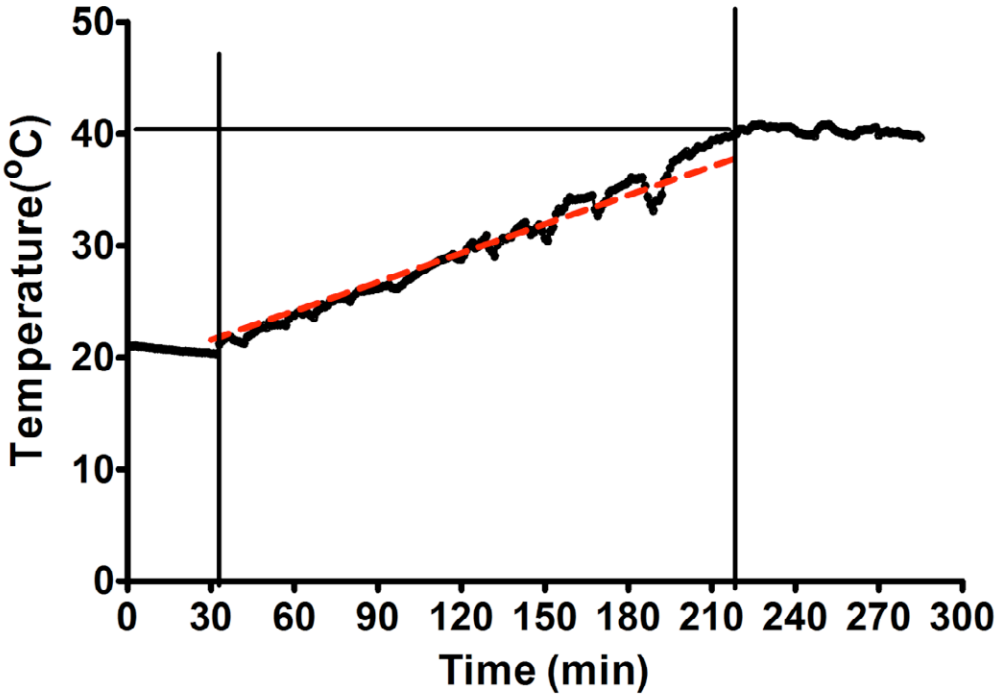

Fig. S1. Relative frequencies of the operative temperature of the limpet *Cellana toreuma* based on the data shown in Fig. 2, for each tidal level (A–F show 4.0. 3.5, 3.0, 2.0, 1.5 and 1.0 m above chart datum) investigated and the west-facing rocky surfaces.

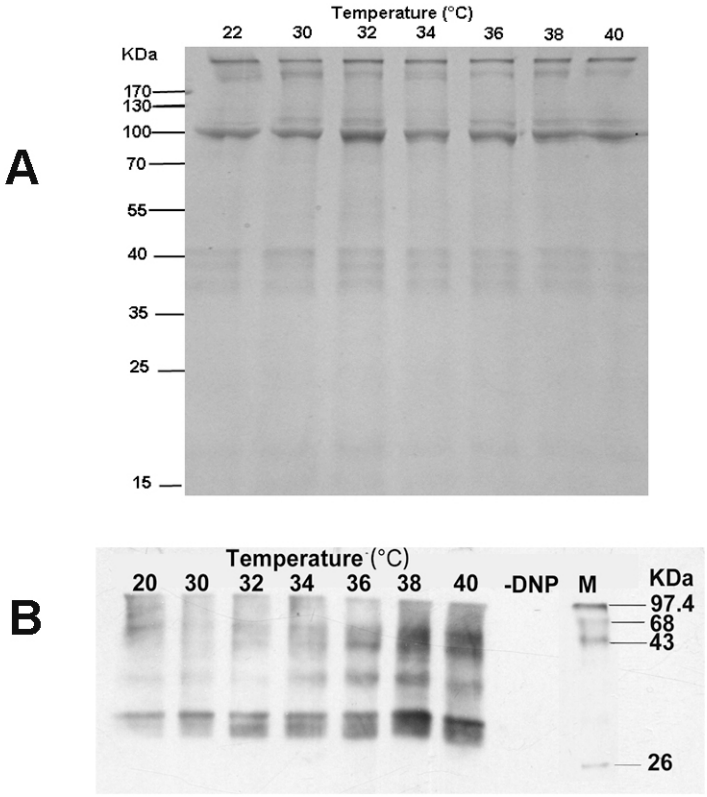

Fig. S2. Protein carbonyl groups derivatized with 2.4-dinitrophenylhydrazine (DNPH) and detected by (A) Coomassie Blue and (B) western blotting. The lane of '-DNP' indicates that the sample was incubated with only 2.5 mol l^{-1} HCl (no DNPH) as a negative control.

Table S1. Primers used for gene clone and real-time PCR amplification

Primer	Sequence	Objectives	Sources
SeaactinF	ACCGACTACYTSAKKAAGATCCT	partial *β-actin* sequence	Clark, 2008.
SeaactinR	GAVGCVAGGATGGAGCCRCC		
dAIHSP70F	CAGGAATTCAARCGYAAACAC	partial *hsp70* sequence	Song, 2006.
dAIHSP70R	TTGGTCATKGCTCGYTCTCC		
HSP90F1	GDGTGTTYATCATGGACAAYTGTGA	partial *hsp90* sequence	Gao, 2007.
HSP90R1	TTCATGATYCTYTCCATGTTDGC		
AMPKαF	AAYCTBAARCTHTTYMGWCA	partial *ampkα* sequence	Self-design
AMPKαR	GCATARTTWGGNGADCCACA		
AMPKβF	GAYTTTGARGTDTTTSADGC	partial *ampkβ* sequence	Self-design
AMPKβR	GTNGCRCTDARHACCATKAC		
SIRT1F	GATCATGGTGCTGACCGGNGCNGGNT	partial *sirt1* sequence	Self-design
SIRT1R	CACCTGCTCCAGGGTGTCDATRTTYTG		
3R1HSP70	CCAAGCCGAATACAAATC	3’ sequence clone of *hsp70*	Self-design
3R2HSP70	CTGCTGCTGCCTTGGCTTAT		
5R1HSP70	TCTGTGGGTTCAACGCTA	5’ sequence clone of *hsp70*	Self-design
5R2HSP70	TGTTTCCCTGGTCGTTGG		
3R1HSP90	GAAGATTGCCGAGTTGCT	3’ sequence clone of *hsp90*	Self-design
3R2HSP90	AGTGAGAAAGCGAGGGTT		
5R1HSP90	CTCAGCAACTCGGCAATC	5’ sequence clone of *hsp90*	Self-design
5R2HSP90	CAACTCCATCGCCTTCTT		
3R1AMPKα	TGCATGATGGCGAGTTTT	3’ sequence clone of *ampkα*	Self-design
3R2AMPKα	TTACGCGGCACCAGAAGT		
5R1AMPKα	AATTAGGTGAACCACAGC	5’ sequence clone of *ampkα*	Self-design
5R2AMPKα	ATGCATCATGTTTGACAGACCT		
3R1AMPKβ	GAGCCATCTTTATTACCTG	3’ clone sequence of *ampkβ*	Self-design
3R2AMPKβ	GTTGAATCATCTGTATGCCTTGT		
5R1AMPKβ	AAGGCATACAGATGATTC	5’ sequence clone of *ampkβ*	Self-design
5R2AMPKβ	AGGTAATAAAGATGGCTC		
3R1SIRT1	CGACTTTCCTAATCTACC	3’ clone sequence of *sirt1*	Self-design
3R2SIRT1	TTCTGTATCTTGTGGTATA		
qActinF	GAAGGATGGCTGGAACAA	Real-time primer for *β-actin*	Self-design
qActinR	CCGAGACATCAAGGAGAAG		
qAMPKαF	CGATTGTAGATGTAGATGTTGT	Real-time primer for *ampkα*	Self-design
qAMPKαR	GCCATTCTCTGATTGTCTAT		
qAMPKβF	AATGTGAATAGTGAACCGATA	Real-time primer for *ampkβ*	Self-design
qAMPKβR	AGCATAACAGCAGAACTC		
qHSP70F	AATATAAGGAAGAGGACGAGAG	Real-time primer for *hsp70*	Self-design
qHSP70R	TATCAGCCAGAGCATTAGC		
qSIRT1F	GCTGCTGATAAGGATGAG	Real-time primer for *sirt1*	Self-design
qSIRT1R	TACATTGGCTGGAAGAGA		
qHSP90F	ATGATCGGTCAGTTTGGTGT	Real-time primer for *hsp90*	Self-design
qHSP90R	AGTTGGGTTGGTCAGGTGT		

q18S-F	GCGATAACCTTGATGTAT	Real-time primer for *18*	Self-design
q18S-R	ACGAAATATTCTGGCTAC		
qBTUB-F	GAAGTTGATGAACAGATG	Real-time primer for *β-tublin*	Self-design
qBTUB-R	AGATTCTCTTGAACAGTT		
qCAL-F	ACGGTAATGGTACAATAG	Real-time primer for *calmodulin*	Self-design
qCAL-R	TCATCTCATCTACTTCCT		

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