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ORIGINAL PAPER

# Comparative transcriptomics across populations offers new insights into the evolution of thermal resistance in marine snails

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**Abstract** Adaptation to thermal conditions in intertidal ectotherms involves tolerating and surviving frequent and often extreme variations in environmental temperatures. Modulation of gene expression plays an important role in the adaptive evolution of thermal tolerance. To understand such patterns, we investigated the thermal tolerance among three Asian populations (from Xiamen, Hong Kong, and Singapore) of the marine littorinid snail *Echinolittorina malaccana* and examined gene expression profiles in these populations before, during, and after heat stress using an Illumina RNA-sequencing platform. Analysis of transcriptomic changes between different conditions revealed that a proportion of the differentially expressed genes showed similar expression profiles across all three populations, including many classic molecular chaperones such as *HSP70* and *HSP90* that may constitute the core of the thermal stress response machinery in marine snails. Meanwhile, population-specific transcriptomic responses to heat stress were also detected. A few genes in particular showed more analogous expression profiles in the more thermally tolerant Hong Kong and Singapore populations, and these genes are likely to contribute, at least in part, to the enhanced thermal tolerance of these populations. We argue that repression of a subset of metabolic genes including several cytochrome P450 gene family members, to minimize energy expenditure and control reactive oxygen species turnover, may underpin the enhanced thermal resistance in these two populations. As such, these findings offer new insights into how marine snails cope with thermal stress and their potential evolutionary trajectory toward adapting to a warming climate.

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## Introduction

Understanding the genetic basis of resistance to environmental stresses in natural populations is of fundamental importance for predicting how organisms may respond to anthropogenic disturbances, including global climate change (Hoffmann and Sgro 2011; Evans and Hofmann 2012; Franks and Hoffmann 2012). While our knowledge of this subject for terrestrial organisms is well developed with increasing research efforts, analogous information is yet to reach the same level in the marine realm (Doney et al. 2012). In particular, the genetic basis of how various taxa in the rocky intertidal zone cope with environmental stressors and adapt to environmental changes remains poorly understood (Somero 2012).

The rocky intertidal zone is one of the most variable and dynamic habitats on earth (Gracey et al. 2008). Organisms inhabiting this challenging habitat face severe selection pressure from both abiotic and biotic factors (Helmuth et al. 2002). Specifically, intertidal species are constantly exposed to a range of environmental stressors, including great variation in temperature, pH, dissolved oxygen, and availability of nutrients (Helmuth and Hofmann 2001; Place et al. 2012). As a consequence of long-term selection, these organisms may display pronounced phenotypic plasticity and evolutionary adaptation (Sanford and Kelly 2011; Somero 2012), and thus serve as tractable model systems to explore the molecular basis of adaptation to environmental stress (Somero 2002; Kelly et al. 2012).

Thermal tolerance can be altered through physiological acclimation, evolutionary adaptation, or a combination of both. Acclimation and adaptation to thermal stress, including temperature extremes, involves functionally tuning a diverse range of molecules, cell types, and organs to respond to thermal challenges (Kassahn et al. 2009; Somero 2012). These processes involve the modulation of gene expression, the primary mechanism through which information encoded in the genome is converted into physiological phenotypes (Storey et al. 2007). Thus, identification of the key gene regulatory programs associated with resistance to thermal stress can provide considerable insights into the molecular mechanisms underlying thermal acclimation and adaptation (Swindell et al. 2007). RNA sequencing has been applied to identify genes that exhibit transcriptional plasticity in response to various stressors in a wide range of taxa (Schoville et al. 2012; Barshis et al. 2013; Evans et al. 2013; Dunning et al. 2014; Gleason and Burton 2015). Genes showing differential expression between stressful and benign conditions are assumed to play important roles in relevant stress regulatory networks (de Nadal et al. 2011; Pespeni et al. 2013). However, the often extremely large number of candidate genes identified by this approach makes interpretation of their practical significance difficult (Swindell et al. 2007).

Recently, an increasing number of studies have stressed that both neutral and selective forces shape the evolution of gene regulation (Rifkin et al. 2003; Gilad et al. 2006; Whitehead and Crawford 2006; Romero et al. 2012). Transcriptional plasticity alone is, thus, a poor indicator of a gene's importance for fitness (Gibney et al. 2013). Nonetheless, since the genes that can affect survival of individuals under thermal stress may display differential expression between thermally tolerant and susceptible populations, comparative studies of gene expression regulation in conspecifics showing divergent thermal tolerances offer an opportunity to pinpoint the gene regulatory pathways associated with enhanced fitness under stress (Schoville et al. 2012; Whitehead 2012). Such an integrative physiological and transcriptomic approach can allow us to gain important insights into the mechanistic linkages between gene expression, biochemical pathways, and organ/system function that underlie adaptation and acclimation to environmental stresses (Scott and Brix 2013).

The marine snail, *Echinolittorina malaccana* (Gastropoda, Littorinidae), is an ideal model organism to investigate how gene expression is differentially regulated to enable thermal adaptation in intertidal ectotherms. This species is commonly found in the upper intertidal/supratidal zone along the coast of the South China Sea, where it experiences high levels of thermal stress (Marshall et al. 2011). The distribution range of this species covers more than 25° of latitude, from the temperate to equatorial zones (Reid et al. 2006), and the thermal tolerance and physiological response to heat stress of this species have been documented in Brunei and Hong Kong (Marshall et al. 2011; Li 2012). The transcriptome of this species has also been sequenced, with preliminary data on genes showing transcriptional responses to thermal stress in whole body tissues (Wang et al. 2014). Despite these efforts, critical information is lacking with regard to if, or how, the capacity of thermal resistance diverges among natural populations across the geographic range of *E. malaccana*, and how gene expression is differentially regulated among the populations.

The current study integrates the assessment of gene expression divergence and thermal tolerance variation in three different populations (from Xiamen, Hong Kong, and Singapore) of *E. malaccana*. Based on a mitochondrial DNA marker (COI gene), the Xiamen and Singapore populations were previously found to be genetically homogenous, while the Hong Kong population displayed weak but significant differentiation from the above two populations (Wang 2014). We postulate that adaptation or acclimation to local thermal regimes can lead to divergence among populations in physiological traits that dictate thermal tolerance, which will be manifested as differentiation in transcriptional regulation. To explore how gene regulation varies between populations, we measured the capacity of thermal tolerance in the three snail populations, investigated their dynamic transcriptomic profiles including both constitutive and stress-induced genome-wide gene expression scenarios, and examined the correlation between patterns of gene expression divergence and population variation in thermal tolerance.

## Materials and methods

### Sample collection

Mature snails (7–9 mm shell length) were collected from the high intertidal zone in Singapore (1.39N, 103.99E),

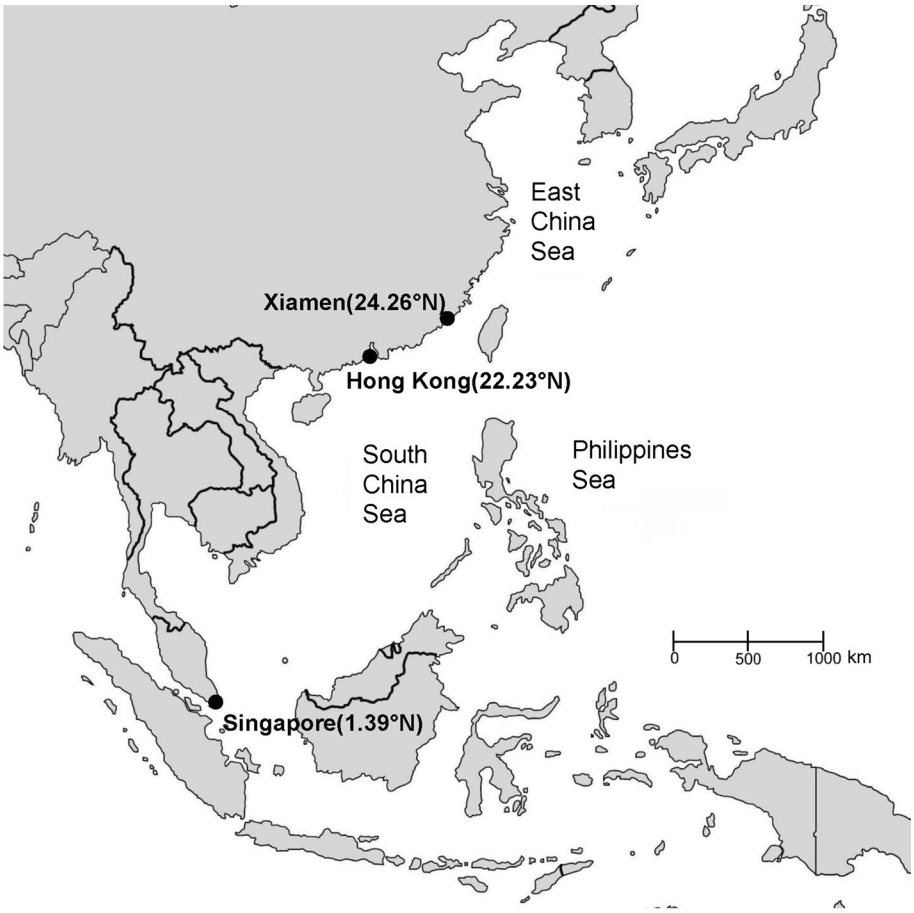


**Fig. 1** Sampling localities of *E. malaccana*. GPS parameters for the three sampling localities (*filled circles*) are shown

Hong Kong (22.23N, 114.26E), and Xiamen (24.26N, 118.08E) during the most stressful season in each locality (July–September, 2013, Fig. 1). Snails were placed dry in plastic bottles and air transported to Hong Kong within 2 days of collection. The snails were first maintained in separate tanks at room temperature (sprayed with seawater ~25 °C) for 3 days prior to experiments to allow the snails to recover and minimize the confounding effects of transportation. Three days between collection and experimentation may be too short a time to eliminate the effects of past thermal history on the snails. However, our previous trials showed that this rapid transfer and treatment does not impact the thermal responses of the snails (Cartwright and Williams unpublished data), and results obtained in the experiments, therefore, reflect the differential responses of the snails to thermal stress.

### Determination of thermal tolerance

Lethal temperature (LT50), as defined as the temperature at which 50 % of a population dies, is commonly used as a proxy for thermal tolerance in intertidal species (Stirling 1982). Thermal tolerance levels of the three populations were determined by measuring LT50 values based on Stirling's (1982) method with some modifications (see Li 2012). Briefly, randomly selected active snails were placed, in air, in plastic vials with each vial containing five individuals. Vials were loosely capped and ~90 % immersed in a water bath (Grant GP200, programmable water bath) at 25 °C. Temperature was increased at 1 °C increments over 5-min intervals to 60 °C at a rate of 0.2 °C per minute which mimics the rate of temperature change on Hong Kong shores (Cartwright and Williams 2014). Based on data in Li (2012), starting at 51 °C, two randomly selected vials for each population were removed at 1 °C incremental intervals. Vials were allowed to cool to ambient temperature, and then, snails were placed in Petri dishes with ambient seawater (25 °C) to allow recovery. After 12 h, snails with their foot extended and attached to the substratum, or that responded to pricking, were scored as alive. LT50 values were calculated using GraphPad Prism. Experiments were repeated three times, to derive three separate LT50 values per population, and LT50 values were compared between populations using one-way ANOVA and Duncan's multiple range test in SPSS 19.0.

### Heat stress experiments and tissue dissection

For each population, samples for RNA sequencing were collected at three phases, before (control), during (heat stress), and after heat stress (recovery), with two biological replicates at each phase. Due to the small size of the snails, combined mantle tissues from six individuals were used as a pooled sample for RNA extraction. The mantle was selected in the study as it is an important tissue protecting the visceral mass and secreting the shell, and is one of the tissues exposed to change in environmental temperature. With two replicates per treatment, between-individual variability in gene expression could not be fully assessed. Our experimental design still, however, enabled us to capture the general gene expression profiles at different phases in the three populations. At the beginning of the experiment, 12 randomly selected snails from each population were collected to prepare two control samples. To induce heat stress, the other animals were placed in plastic vials (ten in each vial) partially immersed in a water bath as described above. A thermometer (K/L Tm903A, LuTron, Taiwan) was attached in close proximity to the snails in each vial to monitor the air temperature inside the vials. Following the protocol in a previous study by Marshall et al. (2011), the heat ramp was controlled to begin at room temperature (i.e., 25 °C) and increase at a rate of 5 °C/h until the temperature reached 45 °C. This temperature was chosen because it is an ecologically relevant temperature frequently experienced by the snails on tropical rocky shores and can incur significant physiological stress (see Marshall et al. 2011; Li 2012). None of the snails died during the exposure showing that this temperature was not lethal to the snails during the experimental period. The snails were kept at 45 °C for 1 h to further induce transcriptomic responses. At this time, 12 individuals from two different vials were randomly selected, their shells cracked open, and their soft bodies immediately transferred to RNAlater® solution (Life Technologies). The temperature was then lowered at the same rate until it reached room temperature and the snails were allowed to recover for 2 h before the tissues from a further 12 randomly chosen individuals were sampled. All animals were then dissected under a stereomicroscope to remove the mantle tissues.

### RNA extraction, library construction, and RNA sequencing

RNA was extracted from the tissues using TRIzol Reagent (Life Technologies) and purified with RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA quality and concentration were then determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). After quality control, a total of 18 RNA samples (three populations, three phases, and two replicates at each phase) were subjected to standard library construction procedures of the Illumina RNA-sequencing platform. In brief, paired-end libraries with an approximate average insert size of 200 bp were constructed by BGI, Shenzhen, using the TruSeq RNA Sample Prep Kit (Illumina, CA). The concentration and size of the sequencing libraries were further checked on an Agilent 2100 Bioanalyzer (Agilent Technologies). Qualified libraries were then separately barcoded and sequenced using an Illumina HiSeq 2000.

### Reads mapping, gene expression comparison, and bioinformatic analysis

Raw sequencing reads were first preprocessed by BGI to trim flanking adaptors and remove contamination or low-quality reads. High-quality reads for each sample were then aligned to a previously constructed reference transcriptome (Wang et al. 2014) using Bowtie-1.0.0 (Langmead et al. 2009), and the reads count for each gene calculated using RSEM-1.2.3 (Li and Dewey 2011).

Differentially expressed transcripts between each pair of the samples (Table S1) were identified using the EdgeR Bioconductor package (Robinson et al. 2010). The raw count data were used to calculate FPKM (fragments per kilobase transcript per million reads mapped) values and further normalized before being used for generating heat maps and other plots. Transcripts that showed significantly large expression variation between the two replicates ($|\log_2(\text{fold-change})| \geq 2$) were not included in the analysis, as genes that tend to show low expression variation between replicates are probably more robust to environmental differences (Romero et al. 2012) and variation in expression of these genes between populations and treatments is more likely to reflect real physiological differences. This conservative approach might result in loss of certain information on transcriptomic changes, but was adopted to overcome the limitation of having only two replicates, and it enabled us to exclude potential noisy genes and, therefore, focus on the most reliable patterns of gene expression regulation. Also, transcripts showing low expression levels (reads count <10) were not included in subsequent analysis given that expression differences in these rare transcripts could not be analyzed with statistical confidence.

Following the filtering process, transcriptomic responses to heat stress were firstly analyzed in the three populations separately, as heat-shock response is an important aspect of thermal adaptation and merits elaboration. Then, to investigate the dynamic changes in gene expression across time points and populations (i.e., not only responses to heat stress but also constitutive gene expression differences between localities, recovery vs. control, recovery vs. heat stressed, etc.), transcripts identified as most differentially expressed in the pairwise comparisons (Table

S1, $\log_2$(fold-change) $\geq$ 2, FDR $\leq$ 0.001) were further extracted and clustered according to their dynamic expression profiles across all the samples using the "hcluster" function in the R software 3.1.0 (R Development Core Team 2014). Hierarchical clustering of genes was performed based on the Euclidean correlation dissimilarity matrix and the complete linkage method. Heat maps depicting profiles of gene expression across populations were plotted using the "gplots" package (Warnes et al. 2014) in the R software. To enable a simplified and straightforward illustration of the dynamic gene expression profiles, average expression values of the two replicates at each time point for each population were used to plot gene expression changes for each cluster of genes. Enrichment of gene ontology (GO) terms in different clusters of genes was analyzed using Fisher's exact test with multiple testing correction of FDR by Blast2GO (Conesa et al. 2005).

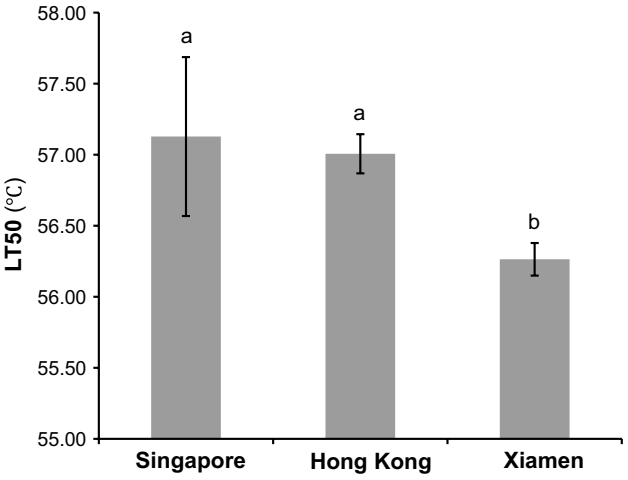


**Fig. 2** Lethal temperature 50 (LT50, mean ± SD) values of three *E. malaccana* populations. *Different letters* indicate significant differences between populations (one-way ANOVA followed by Duncan's multiple range test, $P < 0.05$)

## Results

### Variation in thermal tolerance of the three populations

The average LT50 values were 56.26 ± 0.12 (SD, $n = 3$), 57.01 ± 0.14, and 57.13 ± 0.56 °C for the Xiamen, Hong Kong, and Singapore populations, respectively (Fig. 2). The values for the Singapore and Hong Kong populations were not significantly different, but both were significantly higher than the Xiamen population (one-way ANOVA, $P = 0.04$, followed by Duncan's multiple range test, Fig. 2).

### Sequencing statistics

The amount of clean reads for the samples ranged from 23.90 to 39.93 million, corresponding to a total of 2.39 and 3.99 Gb clean bases, respectively (Table S2). The Q20 value was above 97 % for all the samples, and GC content varied from 45.31 to 47.93 %. All sequencing reads have been deposited in NCBI Sequence Read Archive (Accession Number: SRP071352).

### Comparative transcriptomics across populations

Prior to the analysis of gene expression variation, genes showing large transcriptional variation between replicates or genes with extremely low expression levels were removed. After the filtering process, a total of 13,380 protein-coding transcripts were included in subsequent analysis.

Focusing on genes that showed the largest magnitude of transcriptional changes ($\log_2$(fold-change) $\geq$ 2, FDR $\leq$ 0.001), 143, 146, and 108 genes were either highly induced or repressed in response to heat stress in the Xiamen, Hong Kong, and Singapore populations, respectively (Fig. 3). The vast majority of genes showing prominent differential expression were actually up-regulated upon thermal stress, while a very small proportion were down-regulated (Fig. 3a).

Assessment of the overlap of most strongly up- and down-regulated genes among the three populations (Fig. 3b, c) showed that although many genes (72 induced and two repressed) were found to be regulated in the same fashion upon thermal stress across the three populations, some genes exhibited transcriptional responses in only two populations but not the third one, and each population also possessed some unique differentially expressed genes.

As transcriptomic responses to heat stress only provide a partial view of divergence in gene regulatory programs, and population specificity in the responses makes the functional relevance of differentially expressed genes ambiguous, we further conducted pairwise comparisons of gene expression variation between all the samples (Table S1), including analysis of constitutive gene expression differences between populations. A total of 865 genes were detected as differentially expressed in at least one pairwise comparison across the control, heat stress, and recovery samples in the three populations. The differentially expressed genes were heuristically clustered into 17 groups based on their expression profiles among the three populations (Fig. 4). For these genes, the dynamic gene expression profiles across three time points in all the three populations were further examined to detect any common or unique patterns. The expression profiles for several distinctive clusters of genes are elaborated below, while information on other clusters is provided in supplementary files (Figure S1, Table S3).

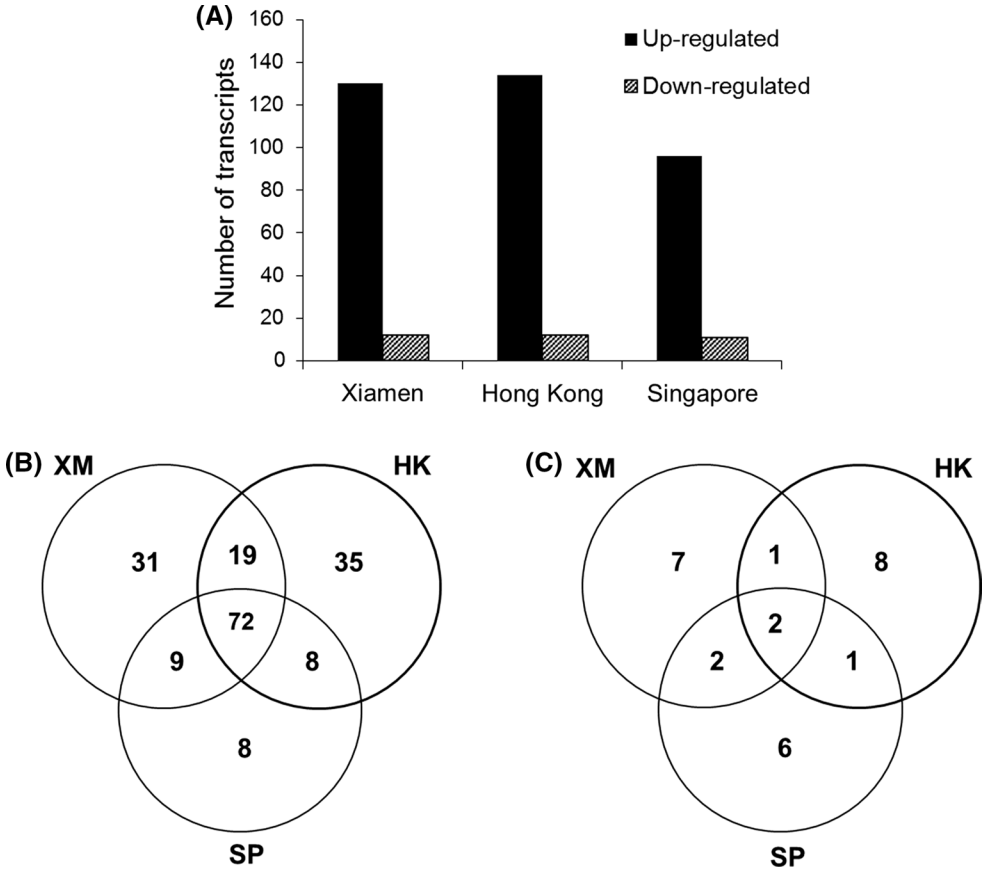


**Fig. 3** Summary of most strongly regulated genes in response to thermal stress in the Xiamen (XM), Hong Kong (HK), and Singapore (SP) populations. **a** Number of transcripts responsive to thermal stress; **b** overlap of stress-induced genes; **c** overlap of stress-repressed genes

*Analogous gene expression profiles in three populations*

About half of the differentially expressed genes exhibited similar expression levels and responses to heat stress among the three populations. The majority of these genes were expressed at low levels in benign conditions, but induced by different magnitudes upon heat stress (clusters 1, 2, 3, and 16, Fig. 4). In the recovery phase, expression of these genes was slightly reduced but still maintained at a high level. Genes in cluster 1 exhibited the largest magnitude of induction, and 17 of the 21 genes in this cluster were heat-shock protein (HSP)-related genes (Table S3), such as *HSP70* and *HSP90*. In comparison, genes in the other clusters sharing similar expression patterns across populations were not dominated by HSPs and encompassed a wider range of genes, including several isoforms of hypoxia up-regulated protein 1 (HYOU1), cathepsin L (Cpl), cAMP-responsive element-binding protein-like 2 (Crebl2) and caseinolytic peptidase B protein homolog (ClpB). There were also groups of genes that were slightly down-regulated upon heat stress in all the three populations (clusters 9 and 11, Fig. 4). Interestingly, after heat stress, genes in cluster 9 promptly had their expression elevated back to the control levels (Fig. 5e). The 49 members in this cluster included several well-studied genes such as G-protein pathway suppressor 2 (GPS2), cyclic AMP-dependent transcription factor ATF-4 (ATF4), and CCAAT/enhancer-binding protein (CEBPB).

Analysis of the enrichment of GO terms for genes in all seven clusters showing similar expression profiles across populations (Fig. 5) showed that unfolded protein binding was the most overrepresented term in the molecular function category, and protein folding, response to stress, oxidation-dependent protein catabolic process, response to abiotic stimulus, and response to hypoxia were on the top of the most overrepresented terms in the biological process category (Table 1).

*Distinct gene expression profiles in the least tolerant Xiamen population*

There were 33 genes that displayed more similar expression profiles in the Hong Kong and Singapore populations but diverged in the Xiamen population (cluster 12, Fig. 4). These genes were generally maintained at lower expression levels throughout the experiment in both the Hong Kong and Singapore populations (Fig. 6). Expression of these genes was, however, elevated in the Xiamen population constitutively, upon heat stress and during recovery. Although there was one gene that deviated from the common trend, genes

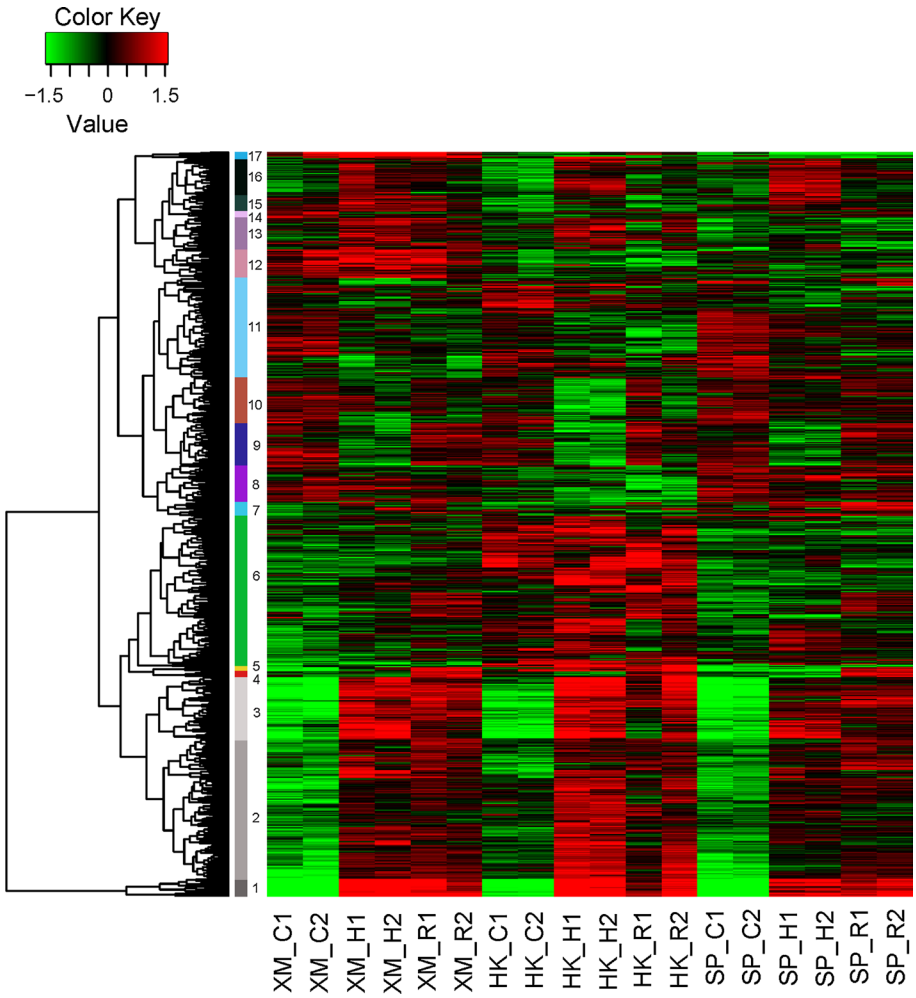


**Fig. 4** Heat map showing gene expression profiles across time points and populations. *Each line* represents a unique gene, and *each column* represents a unique sample. Relative gene expression levels are signified by *different colors*. Genes are divided into 17 clusters according to their dynamic expression profiles in the three populations. From *left* to *right*, the first six columns are for the Xiamen population, the next six columns are for the Hong Kong population, and the last six are for the Singapore population. For each population, the *first two columns* represent samples before heat stress (C1, C2), the *middle two* represent samples during heat stress (H1, H2), and the *last two* represent samples after heat stress (R1, R2)

in this cluster generally did not show drastic responses to thermal stress, and their expression levels were not significantly altered over the three phases (Fig. 6). Genes in this category included many metabolic enzymes, such as cytochrome P450 3A13 (CYP3A13), cytochrome P450 3A21 (CYP3A21), cytochrome P450 3A41 (CYP3A41A), cytochrome P450 4B1 (CYP4B1), cytochrome P450 2J6 (CYP2J6), glutathione S-transferase (GST), glutamine synthetase, and sorbitol dehydrogenase (Sord).

In terms of GO enrichment, only two terms under the category of molecular function, monooxygenase activity and oxidoreductase activity, were significantly overrepresented (Table 2).

### *Gene expression profiles more similar between the Xiamen and Singapore populations*

A total of 270 genes showed more similar expression profiles in the Xiamen and Singapore populations that were distinct from the Hong Kong population (clusters 6, 8, and 10, Fig. 4). Genes in cluster 6, for example, showed higher expression levels in the Hong Kong population as compared with the other two populations, over all the three phases (Fig. 7a). Ninety-five genes (clusters 8 and 10) also exhibited lower expression levels in the Hong Kong population (Fig. 7b, c), including many genes with essential cellular functions, such as eukaryotic translation elongation factor 2 (EEF2), NAD-dependent protein deacylase sirtuin-5 (SIRT5), superoxide dismutase, and collagen alpha-1(I) chain (COL1A1). Analysis of GO enrichment suggested that no specific terms were enriched. Using the same approach, no genes were found which were unique for the Singapore population and showed similar expression profiles in the other two populations.

## Discussion

Using LT50 value as a proxy, populations of the marine snail *E. malaccana* from Singapore and Hong Kong were found to exhibit similar levels of thermal tolerance, and both were more thermally tolerant than snails from

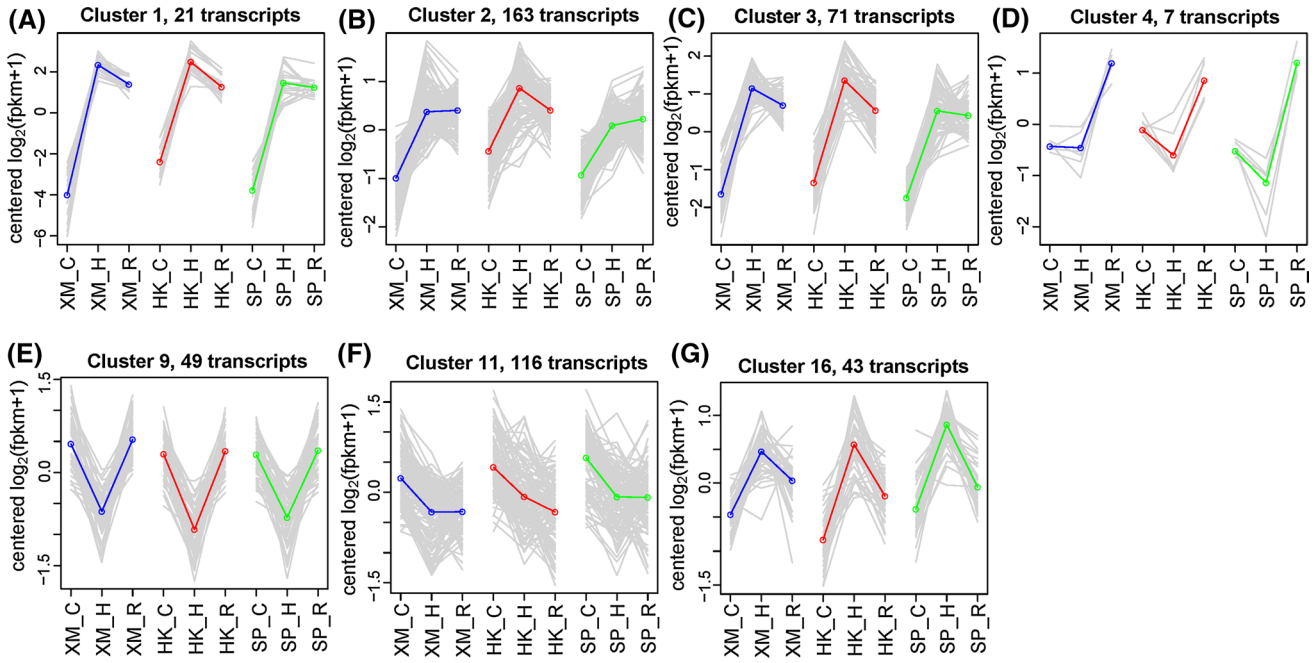


**Fig. 5** Genes showing analogous expression profiles in the three populations. Dynamic gene expression profiles (*gray area*) are depicted based on relative gene expression levels of individual genes at the three sampling points. Median values are used to generate representative trend lines for the Xiamen (*blue line*), Hong Kong (*red line*), and Singapore (*green line*) populations. Genes in these clusters show analogous expression profiles across populations, both in terms of expression levels and responses to heat stress. In each graph, the labels on the *x* axis from *left* to *right* designate samples (*C* control, *H* heat stress, *R* recovery) from Xiamen, Hong Kong, and Singapore

Xiamen. Given that Xiamen and Hong Kong are geographically much closer to each other while Singapore is more distant from both these locations, this pattern is not intuitive. Xiamen and Hong Kong experience similar, monsoonal, subtropical climates with the monthly average minimum–maximum temperature being 10–17 °C in January (the coolest month) and 25–32 °C in August (the hottest month) for Xiamen, and 12–18 °C in January and 26–32 °C in August for Hong Kong (data from 2000 to 2012; www.worldweatheronline.com). In comparison, Singapore has a tropical rainforest climate and has a much more stable, hot, and humid climate, with monthly average minimum–maximum temperature being 24–30 °C in January and 25–32 °C in September. The fact that geographically closer populations do not exhibit more similar physiologies, as compared to a more distant population, implies that thermal tolerance does not simply follow a latitudinal cline and latitude alone may be a poor indicator of local thermal conditions (Helmuth et al. 2006). Microhabitat differences in temperature profiles may play a greater role in shaping the capacity of thermal tolerance in intertidal organisms than latitudinal location (Helmuth et al. 2006; Kuo and Sanford 2009; Lee and Boulding 2010), thus collecting environmentally relevant data, including temperature profiles at microhabitat scales, may contribute toward a more environmentally realistic explanation of observed patterns of species' thermal tolerance.

Upon heat stress, only a small number of genes displayed major changes in expression levels. The small number of strongly regulated genes may be due to the stringent criterion we applied to remove genes showing large variation between duplicates, which may have filtered out more transcriptionally variable genes that also play adaptive roles in thermal tolerance. Moreover, it is noteworthy that most of the genes responsive to heat stress were up-regulated, and large-scale transcriptional shutdown was not observed. This response suggests that when the animals are exposed to moderate thermal stress, they do not mount a transcriptome-wide repression (which may be very costly) and only induce genes essential to address immediate damage.

Our relatively conservative approach enabled us to focus on the most prominent patterns of gene expression divergence among populations and to draw conclusions with reasonable confidence. Through analysis of the dynamic gene expression changes among all the samples, several groups of genes with conserved expression patterns were found across populations either constitutively, or upon thermal stress, or during recovery. Pronounced divergence was also observed in the transcriptomic profiles of the three snail populations. Such prominent patterns of gene regulation

**Table 1** GO enrichment in genes showing common expression profiles across three populations

| GO-ID | Term | Category | FDR | *P* value |
|---|---|---|---|---|
| GO:0051082 | Unfolded protein binding | F | 1.14E−14 | 9.32E−19 |
| GO:0006457 | Protein folding | P | 2.72E−13 | 4.47E−17 |
| GO:0006950 | Response to stress | P | 4.19E−06 | 1.03E−09 |
| GO:0070407 | Oxidation-dependent protein catabolic process | P | 5.77E−04 | 2.37E−07 |
| GO:0070361 | Mitochondrial light-strand promoter antisense binding | F | 5.77E−04 | 2.37E−07 |
| GO:0009628 | Response to abiotic stimulus | P | 8.62E−04 | 4.24E−07 |
| GO:0001666 | Response to hypoxia | P | 1.01E−03 | 5.79E−07 |
| GO:0036293 | Response to decreased oxygen levels | P | 1.09E−03 | 7.19E−07 |
| GO:0004176 | ATP-dependent peptidase activity | F | 1.11E−03 | 8.18E−07 |
| GO:0070482 | Response to oxygen levels | P | 1.20E−03 | 9.84E−07 |
| GO:0005832 | Chaperonin-containing T-complex | C | 1.79E−03 | 1.62E−06 |
| GO:0001932 | Regulation of protein phosphorylation | P | 2.85E−03 | 2.80E−06 |
| GO:0044267 | Cellular protein metabolic process | P | 3.57E−03 | 3.81E−06 |
| GO:0006986 | Response to unfolded protein | P | 1.11E−02 | 1.37E−05 |
| GO:0009408 | Response to heat | P | 1.11E−02 | 1.37E−05 |
| GO:0006515 | Misfolded or incompletely synthesized protein catabolic process | P | 1.11E−02 | 1.46E−05 |
| GO:0042325 | Regulation of phosphorylation | P | 1.28E−02 | 1.86E−05 |
| GO:0035966 | Response to topologically incorrect protein | P | 1.28E−02 | 1.89E−05 |
| GO:0033554 | Cellular response to stress | P | 1.31E−02 | 2.05E−05 |
| GO:0007006 | Mitochondrial membrane organization | P | 1.44E−02 | 2.36E−05 |
| GO:0060561 | Apoptotic process involved in morphogenesis | P | 1.85E−02 | 3.19E−05 |
| GO:0043281 | Regulation of cysteine-type endopeptidase activity involved in apoptotic process | P | 2.01E−02 | 3.63E−05 |
| GO:2000116 | Regulation of cysteine-type endopeptidase activity | P | 2.89E−02 | 5.46E−05 |
| GO:0007005 | Mitochondrion organization | P | 3.00E−02 | 5.92E−05 |
| GO:0090005 | Negative regulation of establishment of protein localization to plasma membrane | P | 3.07E−02 | 6.30E−05 |
| GO:0019538 | Protein metabolic process | P | 3.39E−02 | 7.23E−05 |
| GO:0043154 | Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process | P | 3.91E−02 | 8.68E−05 |
| GO:0010623 | Developmental programmed cell death | P | 4.22E−02 | 9.78E−05 |
| GO:0051246 | Regulation of protein metabolic process | P | 4.22E−02 | 1.00E−04 |
| GO:0031072 | Heat-shock protein binding | F | 4.26E−02 | 1.05E−04 |
| GO:0007021 | Tubulin complex assembly | P | 4.29E−02 | 1.09E−04 |
| GO:0006916 | Negative regulation of apoptotic process | P | 4.34E−02 | 1.14E−04 |
| GO:2000117 | Negative regulation of cysteine-type endopeptidase activity | P | 4.37E−02 | 1.18E−04 |
| GO:0031399 | Regulation of protein modification process | P | 4.69E−02 | 1.39E−04 |
| GO:0000166 | Nucleotide binding | F | 4.69E−02 | 1.68E−04 |
| GO:0051403 | Stress-activated MAPK cascade | P | 4.69E−02 | 1.72E−04 |
| GO:0043067 | Regulation of programmed cell death | P | 4.69E−02 | 1.79E−04 |
| GO:0042645 | Mitochondrial nucleoid | C | 4.69E−02 | 1.92E−04 |
| GO:0009266 | Response to temperature stimulus | P | 4.69E−02 | 1.96E−04 |
| GO:1901265 | Nucleoside phosphate binding | F | 4.69E−02 | 2.05E−04 |
| GO:0036094 | Small molecule binding | F | 4.69E−02 | 2.12E−04 |
| GO:0052548 | Regulation of endopeptidase activity | P | 4.69E−02 | 2.18E−04 |
| GO:0071780 | Mitotic cell cycle checkpoint | P | 4.69E−02 | 2.21E−04 |
| GO:0007008 | Outer mitochondrial membrane organization | P | 4.69E−02 | 2.21E−04 |
| GO:0051880 | G-quadruplex DNA binding | F | 4.69E−02 | 2.21E−04 |
| GO:0045040 | Protein import into mitochondrial outer membrane | P | 4.69E−02 | 2.21E−04 |
| GO:0051434 | BH3 domain binding | F | 4.69E−02 | 2.21E−04 |
| GO:0051400 | BH domain binding | F | 4.69E−02 | 2.21E−04 |

**Table 1** continued

| GO-ID | Term | Category | FDR | *P* value |
|---|---|---|---|---|
| GO:0070513 | Death domain binding | F | 4.69E−02 | 2.21E−04 |
| GO:0070364 | Mitochondrial heavy-strand promoter sense binding | F | 4.69E−02 | 2.21E−04 |
| GO:0070363 | Mitochondrial light-strand promoter sense binding | F | 4.69E−02 | 2.21E−04 |
| GO:0070362 | Mitochondrial heavy-strand promoter antisense binding | F | 4.69E−02 | 2.21E−04 |
| GO:0047837 | D-xylose 1-dehydrogenase (NADP+) activity | F | 4.69E−02 | 2.21E−04 |
| GO:0070182 | DNA polymerase binding | F | 4.69E−02 | 2.21E−04 |
| GO:0008634 | Negative regulation of survival gene product expression | P | 4.69E−02 | 2.21E−04 |
| GO:0097136 | Bcl-2 family protein complex | C | 4.69E−02 | 2.21E−04 |
| GO:0043069 | Negative regulation of programmed cell death | P | 4.69E−02 | 2.23E−04 |
| GO:0031098 | Stress-activated protein kinase signaling cascade | P | 4.69E−02 | 2.23E−04 |

*F* molecular function, *P* biological process, *C* cellular component

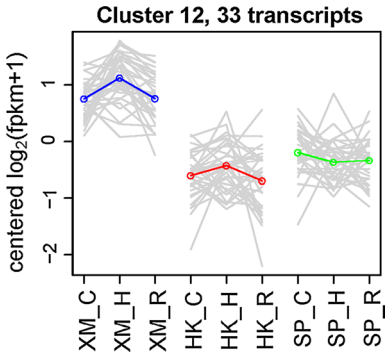


**Fig. 6** Genes showing more analogous expression profiles in the Hong Kong and Singapore populations. See legend of Fig. 5 for explanation. Genes in this cluster display more similar expression profiles in the thermally tolerant Hong Kong and Singapore populations, both in terms of expression levels and reaction to heat stress

**Table 2** GO enrichment in genes showing more analogous expression profiles in the Hong Kong and Singapore populations

| GO-ID | Term | Category | FDR | *P* value |
|---|---|---|---|---|
| GO:0004497 | Monooxygenase activity | F | 1.97E−02 | 2.90E−06 |
| GO:0016491 | Oxidoreductase activity | F | 1.97E−02 | 3.25E−06 |

*F* molecular function

are, therefore, likely to play important roles in the evolution of thermal tolerance among the snail populations.

## Common gene expression profiles: the core transcriptional regulation

A large proportion of the identified genes displayed conserved expression profiles across populations, in terms of both expression levels and reaction to thermal stress. These conserved genes probably represent components of the ancestral gene "toolkit" of marine snails that is essential for tolerance of thermal stress. As such, these genes may constitute the backbone of the snail stress response network that cannot accommodate much transcriptional deviation between populations, and regulation for these genes may have been fixed by evolutionary constraints (Gilad et al. 2006; Romero et al. 2012), which would make these genes interesting candidates to examine in other species.

Many HSP-related genes were found in this category, and protein folding was detected as the most overrepresented GO term. The parallel induction of these genes among populations underscores their functional significance and supports the concept that HSPs are the most important group of genes required for coping with thermal stress (Richter et al. 2010; Chen et al. 2011). Many studies have shown that both the direction and magnitude of regulation of HSPs tend to be conserved among populations (Feder and Hofmann 1999; Richter et al. 2010). There are also cases, however, where variation in the constitutive expression levels of HSPs, or the magnitude of HSP induction, contributes to population divergence in thermal tolerance. Barshis et al. (2014), for example, found that the "front-loading," or elevated constitutive expression levels of 60 genes including *HSP70* underlined the enhanced thermal tolerance in the coral, *Acropora hyacinthus*. Schoville et al. (2012) also reported that in populations of the intertidal copepod, *Tigriopus californicus,* along the coast of California, the southern population (San Diego) which had higher thermal tolerance also exhibited significantly elevated levels of HSP induction than its northern counterpart (Santa Cruz). Similar patterns were not, however, found in *E. malaccana*, as the HSPs showed comparable constitutive expression and were induced by similar amplitudes across the three populations.

Previous researchers have argued that the heat-shock response may involve the regulation of many genes other

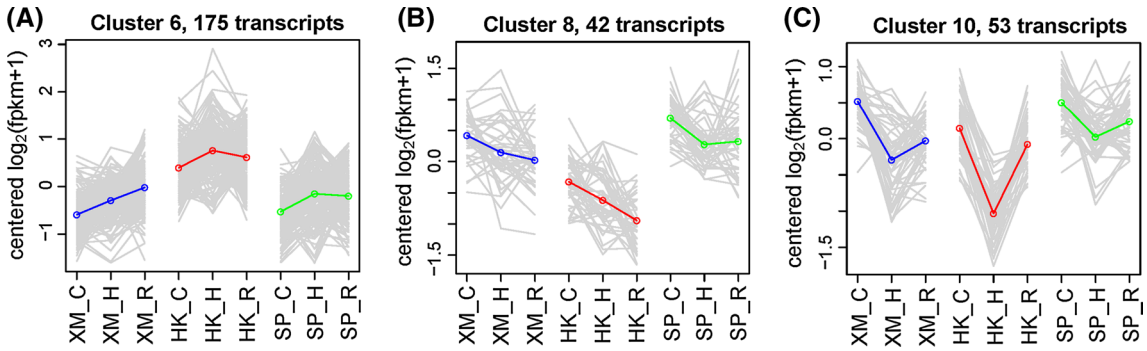


**Fig. 7** Genes showing more analogous expression profiles in the Xiamen and Singapore populations. See legends of Fig. 5 for explanation. Genes in these clusters exhibit more similar expression profiles in the Xiamen and Singapore populations

than those encoding HSPs (Teranishi and Stillman 2007). This assumption also appears to hold true for *E. malaccana*, as genes aside from molecular chaperones were also differentially expressed upon thermal stress and a diverse range of significantly enriched GO terms were identified from these genes. Under the biological process category, a number of apoptosis-related terms were found on the list, such as "negative regulation of cysteine-type endopeptidase activity involved in apoptotic process" and "negative regulation of programmed cell death" (Table 1). Accumulation of misfolded proteins can trigger cellular stress responses, which may lead to cell cycle arrest and programmed cell death (Walter and Ron 2011), and induction of genes involved in the "negative regulation" of apoptosis-related pathways in the mantle tissue may reflect an intention to inhibit apoptotic programs. This response may be induced when the snail is facing short-term thermal stress that is not lethal, and the snail may, therefore, prioritize the utilization of other strategies, such as HSP-dependent protein repair/rescue mechanism and proteolysis, to stabilize or remove damaged proteins, instead of immediately mounting a broad-scale cell death program which is energetically costly and has long-term consequences (Shore et al. 2011). It is also likely that only when these "rescue efforts" cannot recover cellular homeostasis that the apoptotic programs will become dominant so as to counter thermal stress (Richter et al. 2010; Jager et al. 2012). Further experiments to test these hypotheses where the effects of long-term thermal challenge or more severe stress are examined could prove insightful in understanding the different levels of organismal responses to varying degrees of thermal stress.

There were also several signaling pathway-related terms on the list of significantly enriched GO terms, including "stress-activated MAPK cascade" and "stress-activated protein kinase signaling cascade." It is known that stress-induced signaling pathways orchestrate various facets of cellular stress response (Kourtis and Tavernarakis 2011). Enrichment of genes in these specific signaling pathways in the mantle tissue indicates their prime importance in the heat stress response network of *E. malaccana*. How these genes and associated pathways participate in cellular stress responses and how they contribute to thermal resilience are interesting questions for the future.

## Patterns corresponding with physiological traits may unravel the genetic basis of enhanced thermal resistance

The Singapore and Hong Kong populations of *E. malaccana* exhibited similar levels of thermal tolerance, which were significantly higher than the Xiamen population. Corresponding with the inter-population variation in thermal tolerance, a small subset of genes showing more similar expression profiles in the two more thermally tolerant populations, but a distinct profile in the least tolerant population, was identified. It is likely that these genes are regulated in a similar manner so as to enhance the capacities of thermal resistance in the more tolerant populations. That is, regulation of these genes may contribute, at least in part, to the evolution of enhanced thermal tolerance in the Singapore and Hong Kong populations.

The fact that there are relatively few genes showing this pattern suggests that the differential regulation of a small subset of genes could play an adaptive role in the micro-evolutionary divergence in thermal tolerance of these populations. As expression patterns of these genes were closely associated with population differences in thermal tolerance, regulation of these genes is likely to have evolved under directional selection. This evolutionary force has been widely detected in other taxa such as in the fish, *Fundulus heteroclitus,* where divergence in gene expression does not accord with genetic relatedness but is associated with phenotypic traits (Whitehead and Crawford 2006).

A closer look at the identities of genes showing similar expression profiles in the more thermally tolerant

populations revealed that most of these genes were associated with metabolic processes, and expression of these genes was obviously higher in the Xiamen population either constitutively, or upon heat stress, or during recovery. Although expression of these genes slightly increased in the Xiamen population upon thermal stress, these genes generally did not significantly respond to thermal stress. If these genes are indeed underlying the observed divergence in thermal tolerance, this finding lends support to a previous study on yeast which discovered that the genes required for thermal stress survival do not, necessarily, exhibit obvious transcriptional plasticity (Gibney et al. 2013).

Genes with similar expression profiles in the more thermally tolerant populations comprise many metabolic enzymes, including several members of the cytochrome P450 gene family. Monooxygenase activity and oxidoreductase activity are further identified as the only two enriched molecular function terms. If higher expression levels of these metabolic genes reflect higher magnitudes of metabolic activities, it seems that elevated metabolism is somehow associated with the lower thermal tolerance of the Xiamen population. The exact evolutionary drivers of this upgraded metabolism in the Xiamen population and its ecological significance in the natural habitat are unknown, but other related studies on littorinids suggest that when energy gain is extremely limited, the inability to sufficiently reduce energy consumption could be the culprit of the reduced fitness (Sokolova 2013). Littorinids can induce a state of metabolic depression when exposed to stressful conditions such as thermal stress (Marshall et al. 2011; Marshall and McQuaid 2011). During this estivation period, the snails are immobile and nutrient intake is almost suspended, resulting in extremely limited, if any, energy gain (Marshall and McQuaid 2011; Marshall et al. 2011). Consequently, depressing metabolism to a substantial extent and saving energy is a crucial strategy to survive thermal stress (Storey and Storey 2012; Sokolova 2013; Jeno and Brokordt 2014). Higher magnitudes of metabolic activities, as evidenced by higher expression levels of the metabolic genes in the Xiamen population, could lead to more severe energy deficiency and reduced fitness under thermal stress, and may be associated with reduced thermal tolerance of this population.

Another potential explanation for the lower thermal tolerance of the Xiamen population is an increased production of reactive oxygen species (ROS) (Dickinson and Chang 2011). ROS are produced during many metabolic processes and are important for signaling transduction and cellular activity (Santos et al. 2009; Dickinson and Chang 2011). However, when production of ROS is increased, and there is an imbalance between pro-oxidants and antioxidants, the organism experiences oxidative stress (Madeira et al. 2014). Repressing these metabolic genes and fine-tuning of associated metabolic processes to minimize energy consumption and better control ROS turnover may be a physiological regulatory mechanism that confers enhanced thermal resistance to the snails.

Given the predicted scenario of global climate change, intertidal ectotherms such as *E. malaccana* are among the most susceptible taxa that will be exposed to frequent and more extreme thermal stress such as heat waves and high temperatures (Helmuth and Hofmann 2001; Helmuth et al. 2002). To develop physiological adaptation to a warming climate, these metabolic genes are likely to be targeted by natural selection. Structural mutation of these genes, or regulatory variation such as epigenetic modification resulting in down-regulation of these genes, may represent important aspects of the potential evolutionary strategy to enhance the capacity for thermal tolerance (Reusch 2014). These genes, therefore, can be used as biomarkers to track organismal responses to environmental thermal stress and help to assess the evolutionary potential of marine snails in a changing climate.

### Patterns not associated with the capacity for thermal resistance may result from neutral processes

Recent perspectives on gene evolution have stressed that regulation of gene expression is affected by both neutral and selective forces, suggesting that not all genes exhibiting differential expression patterns between populations or taxa have functional significance (Whitehead and Crawford 2006; Storey et al. 2007; Bergmann et al. 2010). In the present study, it was found that the transcriptional profile of several groups of genes (e.g., Fig. 7) was not correlated with the thermal tolerance of snails from the three populations. Although these genes displayed similar expression profiles in the Xiamen and Singapore populations (Fig. 7), thermal tolerance was significantly different between these two populations. Also, although expression profiles of these genes were quite different between the Hong Kong and Singapore populations, these two populations exhibited similar levels of thermal tolerance.

Genes whose expression changes do not incur significant difference in fitness may evolve with little or no selection pressure so that regulatory variation for these genes is primarily controlled by neutral processes such as genetic drift and gene flow (Oleksiak et al. 2002; Whitehead and Crawford 2006; Romero et al. 2012). These genes, therefore, tend to display more similar expression patterns in genetically closer lineages but diverge among distant lineages (Oleksiak et al. 2002). In a concomitant study, based on a mitochondrial DNA marker (COI gene), it was found that the Xiamen and Singapore populations are genetically homogenous ($F_{st} = 0.00241$) while the Hong Kong population displays weak, yet significant, differentiation to the other populations ($F_{st} = 0.04189$ and 0.04036 vs. the Xiamen and Singapore populations, respectively) (Wang 2014). In line with this

finding, genes in this category were more similarly regulated in the Xiamen and Singapore populations, but displayed relatively unique expression profiles in the Hong Kong population. While the data on population genetic relatedness of the three populations need to be further corroborated, it is likely that this gene set is enriched for genes that do not have significant contributions to thermal resistance so that their expression variation follows genetic divergence.

## Conclusions

Populations of the marine snail, *E. malaccana,* exhibited divergent capacities of thermal resistance, with the Singapore and Hong Kong populations showing significantly higher thermal tolerance than their Xiamen counterpart. Genes in the mantle tissue were identified that may constitute the backbone of the snail thermal stress response network, as well as genes that might dictate population divergence in thermal tolerance. Importantly, repression of a small subset of metabolic genes and fine-tuning of associated metabolic processes, probably to minimize energy expenditure and tightly control ROS turnover, indicate a possible physiological mechanism behind the enhanced thermal resistance. Future studies are required to elucidate how regulation of these candidate genes contributes to phenotypic differences, which will enable a holistic understanding of the evolution of thermal resistance in marine snails. In addition, our study highlights the importance of taking into account the correlation of differential gene expression with phenotypic traits across multiple populations in comparative transcriptomic studies. The comparative approach we employed here has the potential of being applied to studies of other types of environmental stresses and in a wider range of taxa, including a large number of non-model organisms of substantial ecological importance.

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**Compliance with ethical standards**

**Conflict of interest** The authors have no conflict of interest.

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