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RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*

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Abstract

To investigate the role of gene expression in adaptation of marine ectotherms to different temperatures, we examined the transcriptome-wide thermal stress response in geographically separated populations of the intertidal snail *Chlorostoma funebralis*. Snails from two southern (heat tolerant) and two northern (heat sensitive) populations were acclimated to a common thermal environment, exposed to an environmentally relevant thermal stress and analysed using RNA-seq. Pooling across all populations revealed 306 genes with differential expression between control and heat-stressed samples, including 163 significantly upregulated and 143 significantly downregulated genes. When considered separately, regional differences in response were widely apparent. Heat shock proteins (Hsps) were upregulated in both regions, but the magnitude of response was significantly greater in northern populations for most Hsp70s, while the southern populations showed greater upregulation for approximately half of the Hsp40s. Of 177 stress-responsive genes in northern populations, 55 responded to heat stress only in northern populations. Several molecular chaperones and antioxidant genes that were not differentially expressed in southern populations showed higher expression under control conditions compared with northern populations. This suggests that evolution of elevated expression of these genes under benign conditions preadapts the southern populations to frequent heat stress and contributes to their higher thermal tolerance. These results indicate that evolution has resulted in different transcriptome responses across populations, including upregulation of genes in response to stress and preadaptation of genes in anticipation of stress (based on evolutionary history of frequent heat exposure). The relative importance of the two mechanisms differs among gene families and among populations.

Keywords: ecological genomics, local adaptation, mollusc, RNA-seq, rocky intertidal, thermal tolerance

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Introduction

Latitudinal gradients in environmental stressors (Helmuth *et al.* 2002, 2006; Schoch *et al.* 2006; Lathlean *et al.* 2014) may result in adaptive divergence among populations. Consequently, when assessing the potential impact of global climate change, it is important to determine whether geographically distinct conspecific populations vary in thermal tolerance. Where such differences occur, insight into the underlying mechanisms is key to better predict how species will respond to global warming; whether plasticity or local adaptation is responsible for population-specific thermal tolerances will affect populations' vulnerability to local extinction (Sanford & Kelly 2011). Although numerous studies have examined the role of transcriptional phenotypic plasticity on temperature response in marine organisms

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(Sorte & Hofmann 2004; Zippay & Hofmann 2010; Jimenez-Melero *et al.* 2012; Massamba-N'Siala *et al.* 2012; Smith *et al.* 2013), few have investigated genetically based population differences in thermal tolerance (but see Kuo & Sanford 2009; Willett 2010; Schoville *et al.* 2012; Gleason & Burton 2013).

Within marine environments, some of the most challenging physical conditions occur in the rocky intertidal zone (Raffaelli & Hawkins 1996; Menge & Branch 2001). Recent studies have found that many intertidal species already live near the upper edge of their thermal limits (Tomanek & Somero 1999; Stillman 2003; Somero 2010; Tomanek & Zuzow 2010) and thus are particularly susceptible to rising temperatures worldwide. Here, we focus on conspecific populations experiencing a latitudinal gradient in thermal environments and assess the potential role of population differences in gene expression in local adaptation to thermal stress.

Changes in gene expression play an important role in physiological resilience to thermal stress (Hofmann 2005; Teranishi & Stillman 2007; Gracey *et al.* 2008; Place *et al.* 2008; Dutton & Hofmann 2009; Lockwood *et al.* 2010). Recent evidence in intertidal limpets, mussels and periwinkle snails suggests that concentration increases of molecular chaperones such as heat shock proteins (Hsps) can repair heat-induced cellular damage (Bedulina *et al.* 2010; Dong *et al.* 2010; Lesser *et al.* 2010; Wang *et al.* 2014). Thermal tolerance in other marine molluscs such as abalone has been linked to the upregulation of antioxidant genes (Abele *et al.* 1998; De Zoysa *et al.* 2009). Alternatively, some organisms use a preemptive strategy. Tomanek & Somero (1999) and Dong *et al.* (2008) found that high intertidal species of the snail genus *Chlorostoma* and the limpet genus *Lottia*, respectively, employ such a 'preparative defense', having higher constitutive levels of Hsp70 than low intertidal congeners; conversely, the low intertidal species showed induction of Hsp70 expression when exposed to modest elevation of temperatures that did not elicit response from the high intertidal forms. Barshis *et al.* (2013) suggested higher expression of Hsps and antioxidants under control conditions, or 'frontloading', is what enables corals to maintain physiological resilience. While this strategy may contribute to thermal tolerance, it also incurs potential costs (Sanchez *et al.* 1992; Heckathorn *et al.* 1996). For instance due to greater energy expenditure, this approach could ultimately reduce the organism's fecundity, growth and survival, especially if these 'frontloaded', or preadapted, genes are overexpressed when they are not needed (Sokolova *et al.* 2012; Gao *et al.* 2014). Although it is understood that these processes of upregulation and preadaptation are not necessarily mutually exclusive, whether both pathways are involved in the evolution of population adaptations to local thermal environments remains largely unexplored.

We focus on the effects of emersion-associated heat stress on the mid-intertidal snail *Chlorostoma* (formerly *Tegula*) *funebralis*. *Chlorostoma funebralis* is found in rocky habitat along the Pacific coast of North America from Vancouver Island, British Columbia to Baja California, Mexico (Abbott & Haderlie 1980; Sagarin & Gaines 2002). Across this large latitudinal range, populations experience significantly different maximum, minimum, and average air and water temperatures (National Oceanographic Data Center, NOAA Satellite and Information Service). Gleason & Burton (2013) demonstrated that northern and southern California populations differ significantly in thermal tolerance, with three southern populations consistently suffering significantly lower mortality and recovering significantly more quickly following heat stress compared with three northern populations. However, the molecular mechanisms underlying these differential tolerances have not been investigated.

This study's objective was to assess the role of differential gene expression on the ecological and evolutionary adaptation of *C. funebralis* to regional differences in thermal environments. Following common-garden acclimation, we exposed two southern and two northern California populations of *C. funebralis* to a heat stress simulating low tide conditions and then performed RNA-seq to gain a detailed snapshot of transcriptome-wide gene expression before and after thermal stress.

Methods

Collection, animal maintenance and assay preparation

Small to medium-sized *Chlorostoma funebralis* adults (15–20 mm in shell diameter) were collected in the winter of 2013 from two northern California sites: Slide Ranch (SR), Marin Co. (37°52′N, 122°35′W) and Pigeon Point (PP), San Mateo Co. (37°11′N, 122°23′W) and from two southern California sites: Aliso Beach (AB), Orange Co. (33°30′N, 117°45′W) and La Jolla (LJ), San Diego Co. (32°52′N, 117°15′W; Fig. 1). Snails were transported to Scripps Institution of Oceanography (SIO) within 24 h of collection.

At SIO, snails were maintained in flow-through running seawater aquaria and regularly fed freshly collected kelp, *Macrocytis pyrifera*. To eliminate confounding effects due to previous environmental differences, snails were common-garden acclimated for 3 weeks in ambient temperature seawater (~15 °C) in a constant environment (i.e. there was no simulated tidal cycling). All aquaria were filled with water to within a couple inches of the top of the tank, so snails were able

Fig. 1 *Chlorostoma funebralis* collecting sites.

to choose whether they were submerged or emersed in the small space above the water line. Twenty-four hours prior to all treatments, individuals were put in weighted 'underwater cages' and kept constantly immersed in seawater without kelp to normalize aerial exposure and feeding status.

Heat stress experiment

Animals and equipment were prepared for each experiment per methods in Gleason & Burton (2013). At the start of each experiment, treatment animals were placed in a temperature-programmable incubator (Thermo Precision Model 818) and air temperature was increased by 3 °C every half hour (starting at 15 °C) to simulate a natural rate of heating in the intertidal (Tomanek & Somero 1999). This gradual increase was continued until the target temperature of 34 °C was reached; the incubator stayed at this target temperature for the remaining 2 h and 20 min of the experiment. Each heat stress lasted a total of 5.5 h, which is an estimate of a typical low tide period for *C. funebralis* in the intertidal (Pentcheff, WWW Tide and Current Predictor). Control animals were exposed to 15 °C in air in the same incubator for 5.5 h. The temperature inside the incubator during all experiments was monitored with a HOBO Pendant Temperature Data Logger (Onset Computer Corporation, Cape Cod, MA, USA; Gleason & Burton 2013). Immediately after heat stress or control exposure, all individuals were frozen in liquid nitrogen and stored at −80 °C. Two biological replicates of both heat stress and control conditions were performed for each population.

RNA preparation and sequencing

The shell of each frozen animal was cracked with a vise and removed. Animals were sexed via visual examination of the gonads (Ortíz-Ordoñez *et al.* 2009), and each individual was ground to a fine powder with a mortar and pestle. Liquid nitrogen was poured on the powder intermittently to ensure it did not thaw. For each animal, total RNA was extracted from 0.5 g of whole animal frozen tissue powder with TRIzol reagent (Sigma) using the manufacturer's protocol modified for samples with high protein, fat, polysaccharide and proteoglycan content. As tissue from the whole animal was used rather than a single organ or tissue, it is worth noting that possible weight differences in individual organs and/or tissues could potentially affect the number of transcripts ultimately obtained. Total RNA from eight individuals for each treatment (four males and four females) was quantified by UV–Vis spectroscopy (Nanodrop) and a pooled sample (8 μg from each animal) was DNase-treated and cleaned with the Qiagen RNeasy Mini Kit (Qiagen, MD, USA). In total, we prepared 16 different pooled samples (four populations × two treatments × two replicates). An Agilent 2100 BioAnalyzer was used for final RNA quantification before the samples were sent to the sequencing facility. Small fragment cDNA library construction (including bar coding) and 100 bp paired-end Illumina HiSeq 2000 sequencing were performed by Cofactor Genomics (St. Louis, MO, USA; one LJ control and treatment, one PP control and treatment) or BGI (Hong Kong, China; all other samples). One microgram of total RNA was used to construct each RNA-seq library with average insert sizes of 250–300 bp using the Truseq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) following manufacturer's recommendations. The four Cofactor Genomics samples were run in a single lane (along with samples for other projects). The 12 BGI samples were sequenced across two lanes (six populations per lane). All sequencing generated unstranded data.

De novo transcriptome assembly and annotation

Sequences were trimmed for ambiguity (a maximum of two ambiguous nucleotides were allowed) and quality (low-quality bases were removed, using a quality cut-off of 20), and reads shorter than 60 bp were discarded. Cleaned sequences were assembled using CLC GENOMICS WORKBENCH 5.1 (CLC Bio) with a minimal read length fraction of 0.5 and a similarity parameter of 0.9. Separate *de novo* transcriptome assemblies constructed for

each of the four populations, pooling the data from control and treatment samples, had higher N50 values and mean contig (transcript sequences assembled from overlapping reads) lengths than an assembly constructed from all populations. These higher quality (Kumar & Blaxter 2010; Schliesky *et al.* 2012; O'Neil & Emrich 2013) population-specific assemblies were used for subsequent analyses. Only contigs with a minimum length of 100 bp and supported by at least 10× coverage were retained in the assemblies. CAP3, a sequence assembly program, was run twice on each assembly to reduce redundancy (Huang & Madan 1999).

Before annotation, a four-way reciprocal blast was performed to ensure the population-specific assemblies were comparable. To assess orthology among contigs of the four populations, we used NCBI's scripts to create BLAST-searchable databases with each set of contigs and then performed BLASTN searches between each possible pair of data sets. We only kept pairs of sequences that were each other's best hit, using the criteria E $\leq 10^{-20}$ (Schoville *et al.* 2012), generating 1:1:1:1 orthologous sets. In addition, to ensure contigs were the same length in all assemblies, the four sequences for each ortholog were aligned using CLUSTAL OMEGA v1.2.0 (Sievers *et al.* 2011) and trimmed to the shortest sequence length using TRIMAL v1.4 (Capella-Gutierrez *et al.* 2009).

BLAST2GO was then used to annotate each contig of the assemblies. Each assembly was blasted (BLASTX) to NCBI's nonredundant (nr) database and Swiss Prot and blasted (BLASTN) to NCBI's nr database and EST_others database with a set *e*-value of 1.0E-3. The highest scoring blast hit was used to assign a gene name to each contig. Gene Ontology (GO) (Ashburner *et al.* 2000) terms were retrieved at an *e*-value threshold of 1.0E-6 for contigs with a positive BLAST hit.

Mapping and identification of differentially expressed genes

CLC GENOMICS WORKBENCH (version 6.0.4) was used to map trimmed reads to the assembly of each respective population (read mapping parameters: minimum fraction length of read overlap = 0.8, minimum sequence similarity = 0.95). Only uniquely mapped reads were retained for further analysis. Read counts were analysed using the package DESEQ (Anders & Huber 2010) in the statistical environment R (www.CRAN.R-project.org; R Development Core Team 2008). Reads were normalized for library size, and low expression (average normalized expression <5) contigs were excluded from analyses to avoid potential artefacts caused by sequencing and/or assembly errors. The false discovery rate (FDR) was controlled at 5% according to the method of Benjamini and Hochberg (Benjamini & Hochberg 1995; p.adjust in R). Previous phenotypic work has shown that southern populations (including AB and LJ) have higher thermal tolerance than northern populations (including SR and PP), but there is no significant difference among the populations within each region (Gleason & Burton 2013). Thus, to test for regional differences in gene expression, the data from SR and PP were treated as biological replicates for the northern region and AB and LJ were treated as replicates for the southern region. Four pairwise comparisons were performed to examine differences in gene expression in response to temperature: (i) all control vs. all thermal stress samples, (ii) northern control vs. northern thermal stress samples, (iii) southern control vs. southern thermal stress samples and (iv) northern controls vs. southern controls. These respective analyses examine genes (i) differentially expressed (DE) as part of a general heat stress response in *C. funebralis*, (ii) DE after heat stress in the north, (iii) DE after heat stress in the south, and (iv) DE between north and south under control (unstressed) conditions.

Fisher's tests and multivariate analysis

After identifying which genes were DE between control and treatment conditions in the respective northern and southern populations, Fisher's exact tests were performed on normalized read counts in R to determine whether specific genes were significantly differentially expressed (SDE) between the two regions following heat stress (i.e. does the magnitude of change in expression in response to stress differ between regions?).

To identify multivariate patterns in the RNA-seq data, the package FACTOMINE (Le *et al.* 2008) in R was used to perform principal component analyses (PCA) on the normalized expression values of all samples. FACTOMINE was also used to identify individual genes whose expression was significantly positively or negatively correlated with the dimensions of the PCA. Enrichment analyses were then performed in BLAST2GO to detect any functional GO categories that were overrepresented in the significantly correlated genes. Statistical significance for any enriched GO terms was assessed using Fisher's exact tests.

Constitutive gene expression

We tested for regional differences in baseline expression ('frontloading' as discussed in Barshis *et al.* 2013, here termed 'preadaption') that might contribute to differences in thermal tolerance. Genes were classified as preadapted if they showed reduced magnitude of response in one region compared with the other (measured as the ratio of fold change following heat stress) and

showed higher constitutive expression in this same region (measured as the ratio of control expression).

Results

Illumina sequencing, de novo assembly and RNA-seq mapping

RNA sequence data from this study have been submitted to the NCBI Gene Expression Omnibus under Accession no. GSE57142. Paired-end 100 bp Illumina sequencing resulted in ~49–72 million reads per sample (Table 1). Following *de novo* transcriptome assembly, 15 903 contigs were identified as orthologs based on reciprocal BLAST among population-specific transcriptomes. These contigs formed the reference transcriptomes used in all analyses.

Shared response to thermal stress

Following the 5.5-h heat stress, changes in gene expression were observed in both geographic regions, as visible in the PCA in Fig. 2A,B. Comparing control and thermal stress treatments, 306, 177 and 143 DE genes were found for all samples (pooled across populations), for northern populations, and for southern populations, respectively (5% FDR correction; Fig. 3). GO term classification of the genes significantly DE after heat stress for all samples pooled across populations identified the highest number of genes relating to (i) apoptotic process and signalling (16 contigs), (ii) response to unfolded proteins (13 contigs), (iii) ubiquitination and catabolism (11 contigs), (iv) the inflammatory response (five contigs) and (v) oxidoreductase activity (five contigs).

Table 1 Number of total raw reads obtained following Illumina Hi-Seq 2000 100 bp paired-end sequencing and number of reads mapped to the respective *de novo* transcriptome assembly for each of the 16 control and heat stressed samples used in this study

Sample	Number raw reads	Number mapped reads
SR Control 1	49 965 768	18 237 160
SR Control 2	58 730 072	20 958 377
PP Control 1	51 564 472	21 096 946
PP Control 2	60 115 720	17 594 009
AB Control 1	63 272 810	22 447 739
AB Control 2	54 233 246	19 259 255
LJ Control 1	61 727 292	18 291 063
LJ Control 2	50 127 096	23 320 565
SR Treatment 1	66 764 680	23 443 519
SR Treatment 2	57 004 890	20 037 374
PP Treatment 1	56 612 840	20 266 641
PP Treatment 2	56 735 442	19 767 039
AB Treatment 1	72 598 522	25 814 068
AB Treatment 2	51 246 952	18 484 362
LJ Treatment 1	58 309 644	26 103 215
LJ Treatment 2	71 534 982	24 080 728

AB, Aliso Beach; LJ, La Jolla; PP, Pigeon Point; SR, Slide Ranch.

The genes DE after heat stress in the northern populations were compared to those DE in the southern populations, and we identified 122 overlapping genes (Fig. 3). Eighty-six were upregulated, and 36 were downregulated. Among these 122 genes, the three most highly upregulated were all heat shock proteins. The most significantly downregulated gene in control-stress animal comparisons was not annotated, but the fifth (~33 fold reduction) was a receptor-type tyrosine-protein phosphatase T with putative roles in regulating cellular processes such as cell growth, differentiation and the mitotic cycle (Walton & Dixon 1993). However, although these 122 genes were significantly DE in both regions, the magnitude of upregulation and downregulation was often different between regions (see Region-specific response to temperature below). Moreover, the northern populations showed 23.8% more DE genes than the southern populations (177 vs. 143, Fig. 3).

Region-specific response to temperature

PC1 of the PCA (Fig. 2A) clearly separates the replicates that were sequenced by different commercial facilities (see Study limitations: replication and variance below). Correlation analysis between PC1 and expression of each contig in the PCA revealed two mitochondrial respiratory chain genes, cytochrome *c* oxidase subunit VIIa polypeptide 2 and mitochondrial ATP synthase f chain, show a highly significant ($P < 0.0001$) positive correlation of 0.99. Why this difference occurred is unknown, but it can be considered a 'batch effect' and removed from further consideration by focusing on the other components of the PCA. The patterns of gene expression projected on PC2 and PC3 are consistent among replicates and reveal that the northern and southern populations' responses to heat stress differ in multivariate space (Fig. 2B; Table S2, Supporting information). While northern treatment samples showed the most positive loadings on PC3, southern treatment samples showed the most negative loadings on PC2. Genes significantly positively correlated with PC3 were functionally enriched in responses to unfolded proteins and response to stress, suggesting northern treatment samples are most highly correlated with these stress response GO categories. Conversely, genes whose expression was significantly negatively correlated with

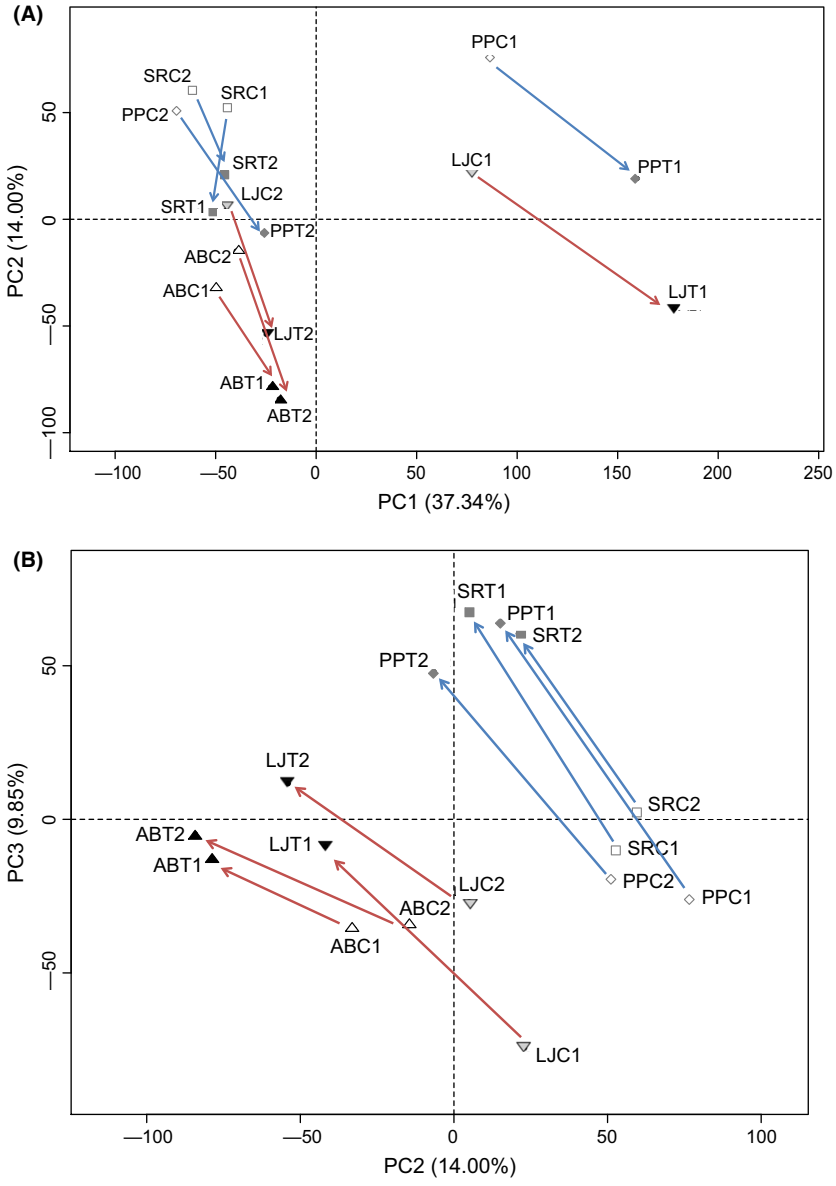

Fig. 2 Principal component analyses (PCA) dimensions 1 and 2 (A) and 2 and 3 (B) of expression values for all 15 903 contigs in each population's reference assembly. The numbers in parentheses indicate the proportion of variance explained by that PCA dimension. Open symbols denote control samples and filled symbols denote treatments. Grey symbols (both open and filled) represent northern populations (SR, Slide Ranch; PP, Pigeon Point), and black symbols represent southern populations (AB, Aliso Beach; LJ, La Jolla). Arrows show the trajectory from control to thermal stress for each replicate and are added for clarity. Red arrows indicate southern populations, and blue arrows indicate northern populations. PCA was computed in R using the FactoMine package.

PC2 were enriched in ATP binding and DNA-dependent regulation of transcription; southern treatment samples are most highly correlated with the expression of genes with these unique GO terms. Moreover, seven of the 42 genes significantly negatively correlated with PC2 function in cell cycle progression.

Because the PCA revealed that four samples suffered from batch effects, preliminary differential expression analyses were performed with both the full data set and with the batch effect samples removed. Removing these four samples increased the number of DE genes (by reducing the variance in the data set), but did not substantially affect the conclusions of the analysis. We have chosen to be conservative, and all subsequent results and discussion refer to the full data set. Twenty-one genes responded to heat stress in southern populations but not in northern ones (Fig. 3, Table S1, Supporting information). One of these 21 genes is a dnaj class molecular chaperone. Another gene, mitosis inhibitor protein kinase wee1, which is a negative regulator of the G2 to M transition (Rhind & Russell 2001), was only significantly downregulated in southern populations. In northern populations, 55 genes significantly responded to heat stress but did not change expression levels in southern populations (Fig. 3, Table S1, Supporting information). These genes include an Hsp60 and an Hsp of unknown molecular weight. Although both regions use Hsps as a defence against thermal damage, the particular genes that respond appear to be region specific (Table 2).

Even those genes that significantly responded to treatment in both regions did not always change

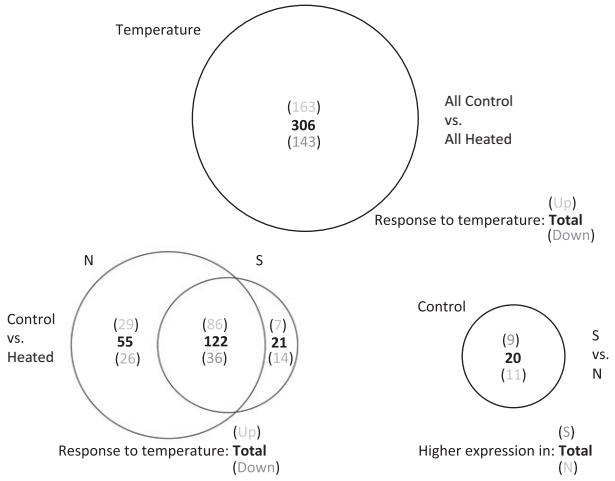

Fig. 3 Venn diagram showing the number of differentially expressed genes identified during analysis based on temperature, within-region temperature response, and between-region constitutive differences. Bold numbers indicate totals and respective shades of grey indicate upregulated vs. downregulated or higher expression in southern (S) vs. northern (N) regions, respectively.

expression levels to the same magnitude. Of 122 genes that changed expression in both regions, 40 (32.8%) were SDE, meaning they were significantly more upregulated or downregulated in one region compared with the other (Fisher's exact test, Table S1, Supporting information). Genes that were significantly more upregulated in northern populations compared with southern populations included ten heat shock proteins (Table 2), four regulators of apoptosis and dual specificity protein phosphatase 10, which negatively regulates MAPK proteins associated with cellular proliferation and differentiation (Patterson *et al.* 2009). Notably, there were no genes significantly more upregulated in southern populations compared with northern ones.

Region-specific response to temperature: Hsps

The expression of 17 Hsps (and five of the six annotated Hsp70s) was significantly positively correlated with PC3 of the PCA, and northern treatment samples showed the most positive loadings on PC3 (Fig. 2B). Nineteen of the 33 annotated Hsps (60.6%) were SDE in northern compared with southern populations (Table 2). Moreover, the direction of these significant differences (i.e. more upregulated in northern or southern populations) was unique to the particular Hsp gene family. For instance, four of the six (66.7%) annotated Hsp70s showed a significantly higher fold change after heat stress in northern vs. southern populations. In contrast, nine of the 15 (60%) dnaj (Hsp40) genes were more highly expressed in the southern populations.

Hsp paralog gene expression

Hsp paralogs show markedly varied expression patterns, even within a single region (Table 2). Not only do these genes show different levels of expression under control conditions, but they are also upregulated to varying degrees following thermal stress. Six genes annotated as Hsp70 paralogs showed highly variable responses to heat stress, with upregulation ranging from 1.4-fold (Hsp70 #6) to 2243-fold (Hsp70 #1) in northern populations, and from 1.5-fold (Hsp70 #6) to 68-fold (Hsp70 #3) in southern populations. Dnaj genes also show a wide range of expression. The fold change of these genes following heat stress ranges from 0.9 (dnaj homolog subfamily c member 13) to 162 (dnaj class molecular chaperone) in the northern populations and from 0.98 (dnaj homolog subfamily c member 13) to 800 (dnaj class molecular chaperone) in the southern populations.

Region-specific constitutive expression

Unstressed control snails from the northern and southern populations differed significantly in gene expression across 20 genes (11 higher in northern populations and nine higher in southern populations, Fig. 3, Table 3). Northern control samples had higher transcript abundances in 31 GO categories, including proteolysis. Southern control samples had higher transcript abundances in five GO categories, including metal ion binding and endodeoxyribonuclease activity.

Overall, there were 2024 genes with a lower magnitude of upregulation (measured as fold change following heat stress) in northern populations compared with southern populations, and 1082 of these (53.5%; Chi-squared test; NS) also showed higher expression in northern vs. southern control samples. Eight hundred and thirty-one of 1856 genes (44.8%; Chi-squared test; NS) with a lesser magnitude of downregulation in northern populations also show lower control expression compared with southern controls. Conversely, there were 2697 genes with a reduced magnitude of upregulation in southern populations compared with northern populations, and 1683 of these (62.4%; Chi-squared test; $P < 0.0001$) showed higher expression in southern vs. northern control samples (Fig. 4). Similarly, 1762 of 2639 genes (66.8%; Chi-squared test; $P < 0.0001$) with a reduced magnitude of downregulation in southern populations show lower control expression levels for these genes compared with northern controls. In sum, for genes with reduced stress response in southern populations, it often appears that control expression has evolved to levels that preadapt the animals to the frequent heat stress they experience and therefore reduce

Table 2 The average normalized expression values of all 33 heat shock proteins identified in the reference assemblies, divided into four categories: (i) differentially expressed (DE) following heat stress in both regions, (ii) DE only in the north, (iii) DE only in the south and (iv) DE in neither region. Fisher's *P*-values in bold indicate genes showing significantly different expression following heat stress in northern vs. southern populations. Values in categories (ii) and (iii) are averages ±1 SEM

Contig ID	North control	North treatment	South control	South treatment	Fisher's *P*-value
DE in both					
Small heat shock protein #1	1261	49 857	899	36 864	0.42
Small heat shock protein #2	10 846	218 533	10 456	181 527	**<2.2e-16**
Small heat shock protein 26	144	81 074	134	38 487	**3.52E-08**
Heat shock protein #1	155	212 458	159	139 741	**1.04E-04**
Dnak protein	0	92.5	0	36.6	1
Heat shock protein 70 #1	203	456 072	536	361 842	**<2.2e-16**
Heat shock protein 70 #2	43 980	196 794	44 706	178 039	**<2.2e-16**
Heat shock protein 70 #3	904	78 530	864	58 692	**3.24E-07**
Heat shock protein 70 #4	1275	11 388	1306	8521	**7.66E-14**
Heat shock protein 70 #5	243	158 050	208	118 989	0.18
Heat shock protein 90	6886	109 494	7436	74 370	**<2.2e-16**
Dnaj homolog subfamily a member 1	539	11 248	586	8763	**6.36E-08**
Dnaj homolog subfamily b member 1	1449	270 952	1459	225 394	**<2.2e-16**
HSPa (heat shock 70 kDa) binding cytoplasmic cochaperone 1	201	24 051	278	17 867	**2.21E-11**
Stress-induced phosphoprotein 1 (Hsc70/Hsp90 organizing protein)	4.4	96	2.5	100	0.72
DE in N only					
Heat shock protein 60	777 ± 181	2737 ± 460	1154 ± 141	2623 ± 373	**3.18E-16**
Heat shock protein #2	1176 ± 109	3175 ± 470	1291 ± 76.6	2741 ± 469	**6.02E-07**
Dnaj homolog subfamily a member 2	2545 ± 449	6508 ± 1022	2344 ± 253	5895 ± 1204	0.62
DE in S only					
Dnaj class molecular chaperone	7.4 ± 7.1	865 ± 538	0 ± 0	172 ± 79.9	0.37
DE in neither					
Heat shock factor-binding protein 1-like	2818	3611	2943	3222	**1.17E-05**
Heat shock protein 70 #6	423	596	528	827	0.22
Activator of 90 kDa heat shock protein ATPase homolog 1-like	74.1	1380	94.5	1086	**2.96E-03**
Dnaj homolog subfamily b member	706	646	476	613	**3.22E-05**
Dnaj homolog subfamily b member 11-like	291	298	392	350	0.23
Dnaj homolog subfamily b member 13-like	446	424	451	431	0.96
Dnaj homolog subfamily c member 2 isoform 2	536	612	844	1130	**0.033**
Dnaj homolog subfamily c member 3-like	229	316	180	276	0.44
Dnaj homolog subfamily c member 5	233	189	229	223	0.2
Dnaj homolog subfamily c member 9	439	440	644	878	**3.47E-04**
Dnaj homolog subfamily c member 11-like	196	203	248	275	0.64
Dnaj homolog subfamily c member 13	1141	999	1304	1031	0.092
Dnaj-like protein subfamily c member 14	1039	889	963	1033	**4.42E-04**
Dnaj homolog subfamily c member 16-like	52.1	121	48.5	131	0.64

the required magnitude of the acute stress response. We note that although these results clearly implicate 'preadaptation' in the southern populations, the null expectation of 50% of genes with reduced up- (or down-) regulation in southern populations also showing higher (or lower) constitutive expression in the south puts large numbers of genes in this category, so the analysis does not directly determine which genes are preadapted.

Contigs with higher constitutive expression under control conditions and lower response to heat stress in southern populations include Hsp 60, Hsp70 #2 and #4, Hsp90 and heat shock factor-binding protein 1 like. It is worth noting that no Hsps showed lower constitutive expression under control conditions and a lower response to heat stress in southern compared with northern populations. Contigs with lower constitutive expression and reduced response to heat stress in

Table 3 The average normalized expression values and Gene Ontology (GO) category or categories of the 20 genes that differed significantly in expression between northern and southern populations under control conditions. The 'Type' column indicates the three domains of the GO categories, where C = cellular component (the parts of a cell or its extracellular environment), F = molecular function (the elemental activities of a gene at the molecular level) and P = biological process (operations or sets of molecular events). If a gene did not have any GO categories available, this column was left blank

Contig ID	GO category	Type	North control	South control	Fisher's *P*-value
Higher in N Control					
NA			145	4	1.60E-04
NA			53	6	2.80E-02
Failed axon connections homolog isoform x2			184	25	5.10E-04
NA			140	23	2.20E-02
Hypothetical protein	Proteolysis	P	211	40	1.80E-04
BRAFLDRAFT 120079	Peptidase activity; aminopeptidase activity	F			
NA			103	20	1.80E-03
NA			82	18	8.40E-03
Hypothetical protein BRAFLDRAFT 110510			159	39	4.00E-03
Fibroblast growth factor receptor 2	Exocrine system development; protein autophosphorylation; branching involved in prostate gland morphogenesis; embryonic cranial skeleton morphogenesis; regulation of cell differentiation; immune system process; positive regulation of cell proliferation; lung development; developmental growth; forebrain development; muscle structure development; negative regulation of cellular process; positive regulation of phospholipase activity; regulation of multicellular organismal process; heart development; anatomical structure formation involved in morphogenesis; peptidyl-tyrosine phosphorylation; positive regulation of MAPK cascade; fibroblast growth factor receptor signalling pathway; cell development	P	106	27	2.00E-02
NA	Fibroblast growth factor binding; protein homodimerization activity; fibroblast growth factor-activated receptor activity	F	817	291	1.50E-02
NA	Cell cortex; cell surface; nucleus; extracellular matrix; integral to plasma membrane	C	890	354	2.10E-02
Higher in S control					
mynd finger	Metal ion binding	F	259	384	2.20E-02
NA			64	192	2.00E-02
Deoxyribonuclease tatdn2-like	Endodeoxyribonuclease activity, producing 5′-phosphomonoesters	F	64	196	1.60E-02
	Nucleic acid phosphodiester bond hydrolysis	P			
	Cellular component	C			

Table 3 *Continued*

Contig ID	GO category	Type	North control	South control	Fisher's *P*-value
Sialate o-acetylesterase			2235	6956	6.50E-04
NA			67	231	2.10E-03
PREDICTED: uncharacterized protein LOC101847681 isoform X2			1345	5492	5.50E-05
RNA-binding protein musashi like protein 1			32	131	1.40E-02
Vitellogenin			7246	48 039	2.30E-02
fp1 mytga ame: adhesive plaque matrix protein ame; foot protein 1 ame	Extracellular region	C	7	63	1.40E-02

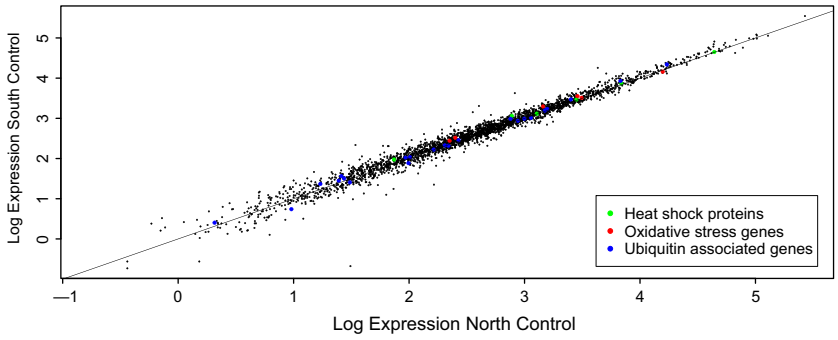

Fig. 4 Scatterplot of the 2697 genes with lower upregulation after heat stress in southern vs. northern populations. One thousand six hundred and eighty-three genes above the diagonal 1:1 line have higher control expression in southern populations, and 1014 genes below the 1:1 line have higher control expression in northern populations. Expression values are normalized for library size and contig length and are log-transformed. Closed coloured circles represent specific groups of classified genes (seven ubiquitin-associated, one oxidative stress, and 0 heat shock proteins below the 1:1 line, and 16 ubiquitin-associated, five oxidative stress, and six heat-shock proteins above the 1:1 line).

southern populations include several cytochrome p450s and multiple ankyrin repeat containing proteins (involved in the initiation of the immune response).

Discussion

We utilized RNA-seq to investigate the potential contribution of differential gene expression to varying thermal tolerance across geographically distinct populations of the marine ectotherm *Chlorostoma funebralis*. Our main finding is that thermally tolerant southern California *C. funebralis* individuals appear to utilize both preadaptation of gene expression levels prior to thermal stress exposure and upregulation of genes after exposure to cope with heat stress.

Constitutive expression

Higher constitutive expression for some genes in southern vs. northern populations may represent a degree of 'preadaptation' that could serve as a preparative defence against frequent heat stress events, conferring higher thermal tolerance to the southern populations. In this study, 13 of the 29 genes that were significantly upregulated only in northern populations following heat stress were apparently preadapted in the southern populations (Table 4). Rather than utilizing acute responsiveness, we propose that because of this preadaptation, these 13 genes did not need to be significantly upregulated by the southern populations following heat stress. One of these preadapted genes is Hsp60; Hsps are discussed in further detail below (see Differential upregulation of Hsps).

Concordant with other studies (e.g. Barshis *et al.* 2013), several genes besides Hsps were also preadapted. Notably, of the 1683 total preadapted genes, 16 (seven e3 ubiquitin ligases, three ubiquitin carboxyl-terminal hydrolases, two poly-ubiquitin proteins, two ubiquitin-like proteins, a ubiquitin domain containing protein, and a ubiquitin conjugating enzyme) function in the

Table 4 The average normalized expression values and Gene Ontology (GO) category or categories of the 29 genes that were upregulated following heat stress only in northern populations. Rows in bold indicate genes that were more highly expressed under control conditions in southern populations compared with northern populations. The 'Type' column indicates the three domains of the GO categories, where C = cellular component (the parts of a cell or its extracellular environment), F = molecular function (the elemental activities of a gene at the molecular level) and P = biological process (operations or sets of molecular events). If a gene did not have any GO categories available, this column was left blank

Contig ID	GO category	Type	North control	North treatment	South control	South treatment
NA			120	356.1	92.2	176.5
Tyrosine recombinase-like			314.1	1332.6	300.4	870
Hypothetical protein CGI 10012932			27	89.9	21.9	50.1
NA			1633.9	4773.1	1472.9	3080.6
NA			**77.8**	**602.3**	**87.7**	**490.4**
NA			54.1	189.2	52	131.2
Transcription intermediary factor 1-alpha-like	Metal ion binding; zinc ion binding	F	**591.1**	**1474**	**673**	**1301.1**
	Intracellular	C				
Heat shock protein #2	M band; striated muscle dense body	C	**1176.3**	**3175.3**	**1290.7**	**2741.4**
NA			**10.3**	**103.2**	**12**	**94.8**
Unknown, partial			12899.8	36453.3	11983.6	27532.5
klf534-like protein	Metal ion binding; nucleic acid binding	F	**7648.9**	**19466.9**	**8861.5**	**18918.4**
	Biological process	P				
Protein fam46c-like isoform x1			**1641.3**	**3765.7**	**1926.9**	**3726.2**
e3 ubiquitin-protein ligase topors-like			**93.3**	**296.5**	**104.8**	**285.2**
Suppressor of g2 allele of skp1 homolog	Intracellular part	C	**475**	**1351.7**	**483.8**	**1186.9**
NA			2891.3	7527.8	2540.9	5738.1
Zinc finger protein c3h1 type-like 1	Metal ion binding	F	6364.8	16230.1	6316.5	14087.2
vwka dicdi ame: alpha-protein kinase vwka ame: von willebrand factor a alpha-kinase			285.4	1304.5	211.7	858.3
dnaj homolog subfamily a member 2	Protein binding; ion binding	F	2544.9	6508.4	2344.1	5895
Protein btg2	Negative regulation of translation; protein binding; neuron projection development; negative regulation of cell proliferation; positive regulation of nuclear-transcribed mRNA poly(A) tail shortening; DNA repair	P	37328.6	162068.5	35348.7	151987.5
Multiple epidermal growth factor-like domains 11			163.2	4418.4	124.4	4059.7
NA			42.4	147.5	18.8	80.3
Perlucin 5			130.6	312.4	91.8	279.6
NA			1.3	180.1	0.7	195.4
NA			3.4	43.9	2.5	75.5
NA			**0.6**	**72.7**	**2.3**	**49.3**
Tyrosine recombinase	DNA integration; DNA recombination	P	**49.4**	**178.9**	**77.2**	**122.6**
	DNA binding	F				

Table 4 *Continued*

Contig ID	GO category	Type	North control	North treatment	South control	South treatment
NA			**10.7**	**51.4**	**13.5**	**29**
Hypothetical protein TcasGA2 TC005055			**50**	**263.6**	**55.1**	**176**
Heat shock protein 60	Cytoplasm	C	**776.7**	**2737.4**	**1154.2**	**2622.9**
	ATP binding	F				
	Protein refolding	P				

ubiquitin stress response pathway, in which proteins irreversibly damaged by heat stress are degraded (Parag *et al.* 1987). For example, e3 ubiquitin protein ligases transfer ubiquitin to damaged proteins and hence target them for degradation by the proteasome (Ardley & Robinson 2005).

We also found southern populations show higher constitutive expression and less upregulation following heat stress of several antioxidant genes. Increased temperature can result in oxidative stress (Abele *et al.* 2002; Heise *et al.* 2003; Abele & Puntarulo 2004; Yang *et al.* 2010; Cui *et al.* 2011); thus, mitigating oxidative stress by increasing synthesis of antioxidants can increase thermal tolerance (Dilly *et al.* 2012). For instance, preadaptation of the antioxidant gene peroxidasin has been suggested to confer thermal tolerance in the coral *Acropora hyacinthus* (Barshis *et al.* 2013). In this study, we found evidence that southern populations of *C. funebralis* may also utilize this strategy: four components of the antioxidant defence system (Cadenas 1989; Cox *et al.* 2009; Murphy 2012; Mailloux *et al.* 2013), superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin and peroxiredoxin 6 (Prdx6), showed higher constitutive expression in southern populations and less upregulation following heat stress compared with northern populations (see Table S3, Supporting information for exact expression values). SOD catalyses the dismutation of superoxide into oxygen and hydrogen peroxide (McCord & Fridovich 1988), while GPx catalyses the reduction of hydroperoxides (Meister & Anderson 1983). The expression of GPx is an indicator of intracellular H_2O_2 levels (De Zoysa *et al.* 2009). Thus, the higher constitutive expression of this gene in southern populations suggests these individuals experience oxidative stress more often during 'baseline' conditions, while the higher upregulation of this gene in northern populations indicates these individuals possess more reactive oxygen species and potentially more oxidative damage following heat stress compared with southern populations. The thioredoxin–peroxiredoxin system has been suggested to scavenge up to 90% of the hydrogen peroxide in mitochondria (Cox *et al.* 2009). Thioredoxins are antioxidants that facilitate the reductions of other proteins by cysteine thiol-disulphide exchange (Holmgren 1989), and Prdx6 reduces hydrogen peroxide and short chain organic, fatty acid and phospholipid hydroperoxides. Studies in mice suggest Prdx6 may protect against oxidative injury; overexpression of Prdx6 protects against oxidative stress and the toxicity of hyperoxia, whereas knockout mice showed increased oxidative stress, apoptosis, lipopolysaccharide-induced acute lung injury and an increased sensitivity to hyperoxia (Manevich & Fisher 2005; Yang *et al.* 2011). Similarly, higher constitutive expression of Prdx6 in southern vs. northern populations could provide increased protection against thermally induced oxidative stress in southern populations. Moreover, previous proteomic work in *Mytilus* suggests oxidative stress is a costressor of environmental stresses besides heat, such as osmotic and pH (Tomanek 2012). Further work is needed to determine whether the population-specific expression of oxidative stress genes such as SOD, GPx, thioredoxin and Prdx6 observed under thermal stress in this study may also play a role in differential tolerance of *C. funebralis* populations to other abiotic stressors.

Differential gene expression as a result of thermal tolerance

Differential gene expression may result in regional differences in thermal tolerance. Alternatively, the unique transcriptome response to heat stress in southern populations may reflect that these more thermally tolerant individuals experience lower levels of physiological stress than northern populations given the same thermal event (Barshis *et al.* 2013); the unique transcriptome response may be a consequence of the southern populations' higher heat tolerance. Some of these uniquely expressed genes with reduced upregulation following heat stress in southern populations include Hsp70 #4, von Willebrand factor, an apoptosis inhibitor and a gene involved in activation of the MEK/ERK signalling pathway during the innate immune response and in apoptosis regulation. Previous work has also shown

that expression of von Willebrand factor, a multimeric glycoprotein, correlates with levels of thermally induced intracellular stress in heat-tolerant and heat-sensitive populations of the coral *A. hyacinthus* (Barshis *et al.* 2013). Further work is needed to determine whether expression of these genes with higher response following heat stress in the less thermally tolerant northern populations might indicate levels of physiological damage following heat stress in other marine invertebrates as well.

Several negative regulators of entry into the cell cycle are either significantly more highly upregulated in northern vs. southern populations (dual specificity protein phosphatase 10) or are only significantly downregulated in southern populations (mitosis inhibitor protein kinase wee1). Moreover, seven of the 42 genes significantly negatively correlated with PC2 (southern treatment samples are most highly correlated with the expression of these genes; see Table S2, Supporting information) are involved in normal progression through the cell cycle. In stressful conditions, cells must prevent the initiation of DNA replication and cell division in favour of cytoprotective functions (Jonas *et al.* 2013), and cells with damaged DNA must be prevented from entering the next cell cycle (Lee *et al.* 2009). We thus hypothesize that the expression pattern of these genes indicates that as a result of their higher thermal tolerance, cells of southern populations are able to continue normal progression through the cell cycle, while the cells of the less thermally tolerant northern populations undergo some degree of cell cycle arrest as a result of stressful conditions and the higher upregulation of Hsps. For instance in bacteria, a heat shock-induced depletion of available Hsp70 or merely the presence of unfolded proteins leads to cell cycle arrest (Jonas *et al.* 2013), and upregulation of Hsps in insects is thought to play a functional role in maintaining the cell cycle arrest characteristic of diapause (Tammariello & Denlinger 1998; Storey & Storey 2000). How these expression differences in cell cycle regulation genes might affect cell proliferation and growth in populations of *C. funebralis* with varying thermal tolerance merits further study.

Differential upregulation of Hsps

More than half of the Hsps identified in this study were SDE in northern compared with southern animals following heat stress. However, whether these expression differences were a consequence or a cause of higher thermal tolerance in the southern populations appears to depend on the Hsp family. All but one of the annotated Hsp70s were more highly upregulated in northern compared with southern populations. The expression of these same five Hsp70s was also significantly positively correlated with PC3 of the PCA, and northern treatment samples show the most positive loadings on PC3. This could likely be a result of southern populations having a higher thermal tolerance, indicating they do not incur as much damage following heat stress. The observed differences in Hsp expression between *C. funebralis* populations appear to parallel the differences in response among different *Chlorostoma* and *Lottia* species (Tomanek & Somero 1999; Dong *et al.* 2008), with the north–south geographic cline in thermal stress seen in *Chlorostoma* populations replacing the low to high intertidal thermal gradient in *Chlorostoma* and *Lottia* species. This Hsp expression pattern has been observed in corals as well; Barshis *et al.* (2013) found that less heat-tolerant *Acropora* populations showed a higher fold change in several small Hsps compared with more heat-tolerant corals following heat stress. However, this pattern contrasts with that observed in the tide pool copepod *Tigriopus californicus*, where a more thermally tolerant population upregulated several Hsp70 paralogs to a greater extent than a less tolerant population (Schoville *et al.* 2012).

The dnaj, or Hsp40, gene family showed the opposite pattern. Nine of the 15 annotated dnaj contigs were more highly expressed in the southern compared with the northern populations following heat stress. We hypothesize that in contrast to the Hsp70 expression pattern, which may be a result of the southern populations' higher thermal tolerance, this Hsp40 expression pattern is actually contributing to the southern population's higher thermal tolerance. The primary function of dnaj is to act as a cochaperone for Hsp70. Not only does dnaj increase Hsp70s hydrolysis rate of ATP (Malyshev 2013), but because dnaj has several variable domains that can interact with different substrate proteins, its binding to Hsp70 allows Hsp70 itself to bind to a broader spectrum of protein substrates than it could on its own (Misselwitz *et al.* 1998). In other words, Hsp40 substantially expands the field of activity of Hsp70 (Genevaux *et al.* 2007). As a result of this interaction, it has been proposed that Hsp70 and Hsp40 might contribute to the increase in protein thermostability and to the accelerated recovery from protein damage in thermotolerant cells (Gebauer *et al.* 1997; Terada *et al.* 1997; Bimston *et al.* 1998). Because Hsp40 seems to enhance the overall chaperone efficiency and ability of Hsp70, the higher dnaj expression in southern populations could be partially responsible for their higher thermal tolerance. This higher Hsp40 expression combined with a reasonable level of constitutive Hsp70 expression (Table 2) could also negate the need for southern populations to upregulate Hsp70s as drastically as northern populations do. Similar findings have been reported in

other thermally tolerant populations of mice, silk worms and *Drosophila* as well, with dnaj expression being higher in the heat-tolerant individuals (Velu *et al.* 2008; Carmel *et al.* 2011; Islam *et al.* 2013).

Finally, it is worth noting that previous work on *Chlorostoma* congeners presents evidence that *C. funebralis* might be at the edge of its thermal limits: the temperature range of Hsp synthesis is close to the upper body temperature of the animals (Tomanek & Somero 1999). Our results indicate that thermal stress is indeed an important force driving local adaptation in gene expression. For instance, the fact that the expression of molecular chaperones such as Hsps differs between northern and southern populations suggests that environmental stress (and heat stress in particular) plays a significant role in how these populations evolve.

Hsp paralogs and their unique responses to heat stress

One important advantage of RNA-seq over microarrays (and most protein-based analysis) is the ability to distinguish paralogs among a gene family. In this study, we identified 15 dnaj (Hsp40) paralogs, two small heat shock proteins, two 'heat shock proteins' and six Hsp70s. Expression patterns even within a particular Hsp family vary widely, indicating different members of the same gene family have unique functions (or no function) in the heat shock response. This is important to keep in mind when comparing this study to previous work examining Hsp protein expression. Our data generally agree with previous results that found less thermally tolerant *Chlorostoma* congeners occupying lower tidal habitats show higher Hsp protein expression following heat stress than congeners found higher in the intertidal zone (Tomanek & Somero 1999, 2000; Tomanek 2001, 2002; Tomanek & Sanford 2003). However, these previous studies failed to distinguish the expression patterns of several of the paralogs we now know exist in *C. funebralis*.

Best practices for RNA-seq: sequencing depth and methodology reporting

The Encyclopedia of DNA Elements (ENCODE) Project has put together a list of best practices for RNA-seq that seeks to provide the community with standards and guidelines for constructing 'reference quality' transcriptome measurements (The ENCODE Consortium). However, it is important to note that different study aims will require appropriate adjustments to these standards. For instance, the goal of this study was to evaluate the similarity and differences between the transcriptional profiles of our heat stress and control samples of each population; therefore, extensive sequencing was not necessary, and only modest depths of sequencing were required. ENCODE's recommendation for this modest sequencing depth is 30M paired-end reads of length >30NT, of which 20–25M are mappable to the known transcriptome. Each of our samples had between 50 and 72M paired-end reads of 100 bp length, and approximately 20M or more for each sample were mapped. Thus, our sequencing depth was adequate given our study's unique goals. (For the exact number of raw reads and reads mapped for each sample, please see Table 1).

In addition, many variations in RNA-seq methodology are used (in transcriptome assembly, read mapping, etc.), and thus, it is important to report the details of such analyses. Along these lines, we compared our methods to recent RNAseq studies in *Molecular Ecology* (Burke & Strand 2014; Mojib *et al.* 2014; Petek *et al.* 2014; Westram *et al.* 2014) and both our methodology itself and the reporting of our methods in the manuscript reaches, and in some instances exceeds, the standards set by these recent publications.

Study limitations: replication and variance

The original experimental design of this study was intended to assess patterns of gene expression both within and between geographic regions, with replicated control and experimental treatments for two northern and two southern populations. Unfortunately, data analyses revealed unexpectedly high variance between biological replicates analysed by different commercial vendors. The replicates for the northern PP and the southern LJ populations were obtained from separate experiments and sequenced by two different sequencing facilities, while the replicates for the northern SR and the southern AB populations were obtained from the same experiment and sequenced by the same facility. Library preparation for RNA-seq can be bias prone and is the main cause of discrepancy in repeated gene expression measurements (Cai *et al.* 2012; van Dijk *et al.* 2014); batch effects from day-to-day sample processing can further contribute to variance between replicates (Leek *et al.* 2010; Taub *et al.* 2010; Lauss *et al.* 2013; Head *et al.* 2014). The PCA clearly resolved the differences between sequencing facilities on the PC1 axis, and subsequent correlation analysis indicated the expression of two mitochondrial respiratory chain genes appears to contribute to the batch effects. Once this variance is accounted for, the patterns of gene expression projected on PC2 and PC3 show remarkable consistency among replicates. Furthermore, removal of these four 'batch effect' samples did not substantially affect the region-specific differential or constitutive expression analyses (data not shown). Although examination of

population differences within geographic regions remains an important goal, here we opt for a more conservative regionally based analysis consistent with the known phenotypic differences between geographic regions (Gleason & Burton 2013).

Conclusions

This study suggests thermally tolerant populations of ectothermic marine organisms, such as southern California *Chlorostoma funebralis* individuals, may employ at least two different gene regulation strategies to cope with heat stress: upregulation of genes in response to stress and preadaptation of genes in anticipation of stress (based on evolutionary history of frequent heat exposure). The relative importance of the two mechanisms differs among gene families and among populations, presumably reflecting the cost/benefit of the two strategies given the physiological role of the specific genes and the ecological differences among populations. Overall, our results provide further insight into the transcriptomic mechanisms that may contribute to heat tolerance in organisms frequently exposed to thermal extremes.

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L.U.G. and R.S.B. designed research, L.U.G. performed experiments and analysed data, and L.U.G. and R.S.B. wrote the manuscript.

Data accessibility

RNA-seq raw sequence reads and normalized expression values for each gene are available through the NCBI Gene Expression Omnibus under Accession no. GSE57142. The normalized expression values of all genes DE between control and heat stress conditions are listed in Table S1 (Supporting information), and the contig identity and Gene Ontology (GO) category of all genes with a significant correlation of greater than 0.9 or less than −0.9 between their normalized expression values and Dimension 2 or 3 of the PCA in Fig. 2B are listed in Table S2 (Supporting information). The normalized expression values of the four oxidative stress genes that showed higher constitutive expression and less upregulation after heat stress in southern vs. northern populations are listed in Table S3 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 The normalized expression values of genes differentially expressed between control and heat stress conditions, divided into three categories: (i) differentially expressed (DE) following heat stress in both regions, (ii) DE only in the north, and (iii) DE only in the south.

Table S2 The contig identity and Gene Ontology (GO) category or categories of all genes with a significant correlation of greater than 0.9 or less than −0.9 between their normalized expression values and Dimension 2 or 3 of the PCA in Fig. 2B.

Table S3 The normalized expression values of the four oxidative stress genes that showed higher constitutive expression and less upregulation after heat stress in southern populations compared to northern populations.