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Regional patterns of thermal stress and constitutive gene expression in the marine snail *Chlorostoma funebralis* in northern and southern California

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ABSTRACT: Southern California (USA) populations of the intertidal snail *Chlorostoma funebralis* occupy warmer climates than northern California populations, and southern populations are more thermally tolerant and have unique transcriptomic responses to heat stress compared to northern populations. To investigate how climate affects body temperature patterns for *C. funebralis*, iButton temperature loggers encased in empty *C. funebralis* shells (robosnails) were deployed at 3 northern and 3 southern California sites for 1.5 mo in the late summer and early fall of 2014, typically when maximum annual temperatures are reached. Measurements revealed that southern, thermally tolerant populations experienced higher average daily maximum and absolute maximum temperatures than northern, less tolerant populations, and that robosnails in southern, but not northern, California exceeded temperatures that cause 100% mortality. Similarly, the probability of a site reaching 27°C, the temperature that induces the heat shock response in *C. funebralis*, was 3 times higher at the southern compared to the northern sites. To determine whether these exposures to stressful temperatures are related to gene expression differences, we then tested for a correlation between the probability of reaching 27°C and the constitutive (non-induced) expression of genes previously implicated as pre-adapted in southern California populations. We identified 222 genes (including 14 involved in ubiquitin protein degradation, a response to heat stress) with a significant correlation. The results demonstrate how combining *in situ* temperature and transcriptome data can increase our understanding of thermal adaptation and better inform predictions regarding the impact of future climate change.

KEY WORDS: Body temperature · Pre-adaptation · Heat stress · Rocky intertidal · Mollusk · Gene expression

INTRODUCTION

Temperature affects almost all aspects of organismal physiology, from survival and physiological performance to species' geographic distributions (Orton 1929, Hutchins 1947, Vernberg 1962, Somero 2005). Temperature plays an especially important role in the rocky intertidal zone, the dynamic interface between marine and terrestrial environments. In these habitats, temperature affects species distributions in 2 different dimensions: across horizontal latitudinal gradients and across vertical tidal heights (Davenport & Davenport 2005). For instance, upper zonation limits are often set by thermal stress related to aerial exposure during low tide (Connell 1972). Moreover, variation across intertidal habitats is often used as a model for how temperature affects geographic ranges, and it is increasingly thought that the intertidal can also serve as a model for effects of climate change on species' distributions (Fields et al. 1993, Southward et al. 1995, Sagarin et al. 1999, Helmuth et al. 2006).

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However, despite the overarching effects of temperature on organismal physiology and its relevance to climate change, our knowledge about how climate patterns affect *in situ* body temperatures of organisms is often limited (Helmuth & Hofmann 2001, Helmuth et al. 2006). While we have a basic understanding of how climate changes over large geographic scales (Stenseth et al. 2003), it is known that body temperatures are often very different from the surrounding air or substrate (Porter & Gates 1969, Stevenson 1985, Huey et al. 1989, Gilman et al. 2006). Consequently, 2 organisms exposed to the same climate can experience very different body temperatures and levels of physiological stress (Helmuth et al. 2006). Thus, relying on climate for estimates of thermal exposure is inadequate (Lathlean et al. 2011).

As an alternative to climate approximations, previous work has shown that using biomimetic devices gives good estimates of actual body temperatures animals experience in the field. For instance, waterproof loggers, named robolimpets, were deployed to measure temperatures of intertidal limpets *Tectura persona* at Friday Harbor, Washington, USA. These measurements were found to closely match the temperature trajectories, warming and cooling rates, and daily temperature maxima and minima of live limpets at the same intertidal locations (Lima & Wethey 2009). Moreover, the authors noted that the same method they used to build robolimpets could be used to create a variety of loggers mimicking different intertidal animals such as dogwhelks, topshells, and other marine mollusks.

In this study, we focused on quantifying body temperature in the intertidal snail *Chlorostoma* (formerly *Tegula*) *funebralis* using biomimetic loggers (robosnails). *C. funebralis* has the widest distribution of the 5 species in its genus (Bouchet 2013), and can be found along the Pacific coast of North America from Vancouver Island, British Columbia to Baja California, Mexico (Abbott & Haderlie 1980, Sagarin & Gaines 2002). Across this range, previous phenotypic, transcriptomic, and genomic work has shown that northern and southern California *C. funebralis* populations are locally adapted to heat stress (Gleason & Burton 2013, 2015, 2016). While double-digest restriction site-associated DNA (ddRAD) sequencing provided no genome-wide evidence for population structure, outlier analysis identified candidates under positive selection that do show regional differentiation between northern and southern California; several of these outliers are involved in responses to environmental stress such as heat (Gleason & Burton 2016). Moreover, southern, more thermally tolerant populations (Gleason & Burton 2013) use a unique gene expression strategy of pre-adaptation to cope with frequent thermal stress (Gleason & Burton 2015). Pre-adaptation is a 'preparative defense' in which populations that are frequently exposed to heat stress have evolved high constitutive expression levels of genes such as heat shock proteins. This is a result of natural selection in southern populations, where individuals with higher constitutive expression have been favored because they have higher fitness (higher survivorship/reproduction) than low expression individuals in the southern thermal environment. This pre-adaptation of gene expression suggests that southern populations experience heat stress more frequently than northern populations, are under stronger selective pressure, and hence have evolved a pre-emptive or 'frontloading' strategy (Barshis et al. 2013). However, to validate this hypothesis, rigorous quantification of northern and southern California *C. funebralis* body temperatures and the selective forces driving local adaptation to heat stress in these regional populations is necessary. Previous work examined patterns of body temperature for *C. funebralis* and its congener *C. brunnea*, but only at a single site in Monterey, California (Tomanek & Somero 1999). In order to gain further insight into the local adaptation to heat stress that has already been documented in *C. funebralis* (Gleason & Burton 2013, 2015, 2016), we examined body temperatures experienced by this species at geographically separated sites along the California coast.

This study had 2 main objectives: to investigate regional patterns of thermal stress and to determine whether these regional differences significantly correlated with the expression of pre-adapted, or constitutively expressed, genes. To determine how variations in climate across latitude affect spatial and temporal patterns of body temperature in *C. funebralis*, we deployed 2 robosnails (iButton temperature loggers inside empty *C. funebralis* shells) at each of 3 northern and 3 southern California sites. The magnitude and frequency of heat stress events at each of these sites was then quantified to examine how the environmental selective forces driving local adaptation to heat stress differ in northern and southern California *C. funebralis* populations. To better understand evolutionary adaptations to these differences in thermal stress exposure between the 2 populations, we also integrated these *in situ* body temperature estimates with previously obtained transcriptome data (Gleason & Burton 2015) and identified significant correlations between thermal regime and the expression of specific genes.

MATERIALS AND METHODS

Temperature instrumentation and deployment

To create robosnails, iButton data loggers (Maxim Integrated) set to record temperature once every hour were placed inside empty *Chlorostoma funebralis* shells and sealed with silicon aquarium sealant (Marineland). Two robosnail loggers were then deployed at each of 6 field sites; 3 in northern California: Slide Ranch (SR; 37° 52′ N, 122° 35′ W) in Marin County, and Pescadero (PES; 37° 15′ N, 122° 24′ W) and Pigeon Point (PP; 37° 11′ N, 122° 23′ W) in San Mateo Co., and 3 in southern California: Aliso Beach (AB; 33° 30′ N, 117° 45′ W) in Orange Co., and La Jolla (LJ; 32° 52′ N, 117° 15′ W) and Bird Rock (BR; 32° 48′ N, 117° 15′ W) in San Diego Co. (Fig. 1). To deploy the data loggers, robosnails were attached at midday to the horizontal surface of rocks next to live, sun-exposed *C. funebralis* individuals in the intertidal using Z-spar epoxy (A-788 Splash Zone Compound; Lima & Wethey 2009). This placement ensured that temperatures were recorded from habitat that *C. funebralis* individuals utilize. Previous work has shown that *C. funebralis* individuals move about 1 m d^{-1}, and remain within the same 1 to 2 m in the intertidal for months (Frank 1975). Moreover, some of this behavior is cold-biased, in which individuals are thought to seek out rock crevices (Tepler et al. 2011, Tomanek & Somero 1999). Although these movements can result in the experience of different (likely cooler) microhabitats (Byers 1983), this particular study was interested in differences in extreme temperature that live *C. funebralis* individuals at various geographic sites experience. In other words, during the time period of this study (42 to 48 d, see below), the robosnails were placed next to individuals that were likely to remain within 2 m of that site, and due to their cold-biased behavior, none of the movements of the live animals were likely to expose them to warmer microhabitats. Thus, *C. funebralis*' relatively limited movement patterns do not prevent an investigation of extreme temperature differences among sites using stationary robosnails.

At each site, one logger was placed at ~ +1.0 m above mean lower low water (MLLW) and one was placed at ~ +1.5 m; both heights are well within the vertical zonation limits of *C. funebralis* at northern (PP: −0.3 to +1.8 m above MLLW) and southern (LJ: +0.4 to +2.0 m above MLLW) California sites (authors' unpubl. data). These tidal heights for logger deployment were chosen to represent the middle to upper portion of the range for both geographic regions, in order to increase our understanding of the thermal extremes these populations experience (we chose not to deploy mimics at the very upper portion of the tidal range, i.e. +1.8 to +2.0 m above MLLW, because although *C. funebralis* were found in the field at these heights, the density of individuals was low).

Fig. 1. Deployment sites of *Chlorostoma funebralis* iButton data loggers (robosnails) along the California coast

Loggers were deployed for 42 to 48 d during August to October 2014. A longer duration would have been preferred, but deployment time was limited due to loss of instrumentation. Nevertheless, this particular time period was chosen for deployment because it generally encompasses the warmest time of the year for northern and southern California (www.nodc.noaa.gov). In fact, the robosnails recorded some periods of high temperatures in southern California that exceed the temperature that causes mortality (see 'Results' below), thereby producing a valuable dataset that allowed us to investigate thermal extremes that have survival consequences for individuals at the different geographic sites.

Temporal patterns: data summary

Daily data from the 2 iButtons at each site were used to calculate the maximal changes in body temperature during a tidal cycle, the overall average, and the average daily maxima and minima for the

entire recording period at each site. From these data, we identified the absolute highest and lowest temperatures at each site during the recording period. We also calculated the percentage of time spent at or above 27°C. The 27°C threshold was chosen as a cutoff because it is the temperature that was sufficient to induce production of heat shock proteins (Hsps) in *C. funebralis* individuals from Pacific Grove, California (Tomanek & Somero 1999). While these Pacific Grove animals can be considered representative of the northern California *C. funebralis* individuals examined in this current study, no work to date has explicitly investigated the induction temperature (T_{on}) for Hsp expression in any other *C. funebralis* populations. Given this data gap, we chose a single temperature of 27°C for both geographic regions for several reasons. First, previous phenotypic and transcriptomic work has suggested that most of the differences in response to heat stress between northern and southern California individuals result from variation in the degree of the response, not in the temperature that first induces it (Gleason & Burton 2013, 2015). Second, Tomanek & Somero (1999) found no differences in the induction temperature of Hsps following acclimation changes comparable to temperature differences between northern and southern California in any of the 4 *Chlorostoma* species examined. Finally, in populations of killifish that, like *C. funebralis*, show differences in thermal tolerance between northern and southern populations, the majority of inducible Hsp genes investigated showed no differences in T_{on} between geographic regions (Fangue et al. 2006). In sum, the available evidence indicates that the general pattern of heat shock response induction is likely similar in northern and southern California *C. funebralis* populations; thus we used the same temperature (27°C) for both geographic regions.

In addition, we also calculated the percentage of time spent at or above 38 and 41°C. Based on previous phenotypic data (Gleason & Burton 2013), 38°C is the threshold at which mortality following 5.5 h of heat stress first occurs for both northern and southern populations (on average 15 and 2% mortality, respectively), and 41°C is the threshold at which there is 100% mortality for both northern and southern populations (all 6 populations tested) following heat stress. As discussed above, previous studies have documented differences in thermal tolerance, loci under positive selection, and transcriptomic responses to heat stress between northern and southern California populations (Gleason & Burton 2013, 2015, 2016). For this study, although we analyzed all data by treating each of the 6 geographic sites as an independent data point, we also discuss regional differences between northern and southern sites to gain further insight into the patterns of *in situ* body temperature that could be contributing to these already established differences. For these regional analyses, SR, PES, and PP are considered northern California sites and AB, LJ, and BR are considered southern California sites.

Temporal patterns: survival distribution functions

As mentioned above, previous data indicate that exposure to temperatures above 27°C is sufficient to induce production of Hsps in *C. funebralis* (Tomanek & Somero 1999), so we used this temperature to compare the potential for thermally-induced stress among sites. Using the 'HistogramTools' package (Stokely 2013) in R (R Development Core Team 2008), we calculated a survival distribution function, $1 - f(x)$, where $f(x)$ is the cumulative distribution function. From this, we obtained the probability of observing a daily maximum above 27°C for each of the 6 geographic sites.

Probability of encountering heat stress and constitutive level gene expression

Previous research has demonstrated that southern California *C. funebralis* populations use a strategy of pre-adaptation of gene expression levels prior to thermal stress exposure to cope with heat stress. However, although this previous work clearly implicated pre-adaptation (high level of constitutive, or non-induced, gene expression evolved in response to frequent stress exposure) is occurring in the southern California populations, the particular analysis used put large numbers of genes into this category, and thus did not directly determine which genes are pre-adapted (Gleason & Burton 2015). To gain a better understanding of which of these potentially pre-adapted genes are specifically responding to the frequent heat stress that southern populations experience, we used Pearson's correlation analyses in R to determine whether the constitutive level expression of these pre-adapted genes was significantly associated with the probability of reaching 27°C (the temperature that induces the heat shock response) at each individual geographic site. For each of the 1683 genes whose pattern of expression generally suggested it was pre-adapted in southern populations in

Gleason & Burton (2015), a correlation analysis was performed on each of the 4 respective populations, comparing constitutive gene expression (normalized expression values) in SR and PP (northern populations) and AB and LJ (southern populations) to the probability of observing a daily maximum above 27°C. For complete experimental details explaining how the transcriptome data for these 4 populations were obtained, see Gleason & Burton (2015). For the correlation analyses, 2 biological gene expression replicates for each of the 4 populations were treated as separate data points, resulting in 8 total data points for each gene tested. Note that in this previous study, only data from 2 northern and 2 southern California populations were obtained. Thus, the northern population PES and the southern population BR were not included in this correlation analysis because there was no available transcriptome data for these 2 populations.

To evaluate how robust this correlation analysis is to small changes in the temperature threshold values, the probability of observing a daily maximum above 25, 28, and 30°C was also calculated as described above (see 'Temporal patterns: survival distribution functions') and Pearson's correlation analyses in R were used to determine whether the genes whose constitutive level expression was significantly correlated with the probability of reaching 27°C were also significantly correlated with the probability of reaching 25, 28, and 30°C.

Furthermore, additional analyses to better understand the implications of choosing a single threshold temperature for individuals from both geographic regions were also performed. To examine the effect of southern California individuals potentially having a different heat shock induction temperature compared to northern California individuals, we calculated the probability of observing a daily maximum above 26 and 28°C and performed Pearson's correlation analyses in R as described above using both slightly lower (26°C) and slightly higher (28°C) heat shock induction temperatures for the southern and northern populations.

Spatial patterns: thermal stress and latitude

To determine if there is a significant relationship between thermal extremes experienced at each site and that site's geographic location, linear regressions were run in R comparing measurements of chronic and acute stress to latitude. Chronic stress was calculated as the average daily maximum (ADM, the average of all daily high temperatures) at each of the 6 sites. Acute stress was calculated as the 99th percentile of all temperatures recorded at each site. Geographic coordinates for each site were obtained from Google Earth.

RESULTS

Summarizing temporal patterns in extreme temperatures

Study sites in southern California reached higher temperatures than those in the north (Fig. 2). Shapiro-Wilk normality tests revealed that the temperature data for the various metrics were not normally distributed, so nonparametric tests and associated post hoc analyses were used to compare differences among sites. Estimated body temperatures for both regions varied with the daily tidal rhythm, with maximal changes in body temperature during a tidal cycle being significantly different among the 6 sites (Kruskal-Wallis test, $p < 0.0001$). Post hoc analyses identified a significant difference between sites SR and AB (Studentized range Kruskal-Wallis post hoc test, $p = 0.012$; Table S1 in the Supplement at www.int-res.com/articles/suppl/m556p143_supp.xlsx); in addition, the northern site PP was significantly different from all other sites except SR (Studentized range Kruskal-Wallis post hoc tests; Table S1). The 6 sites also had significantly different average daily maximum temperatures (Kruskal-Wallis test, $p < 0.0001$). Pairwise comparisons revealed that the northern site PP was significantly different from each of the other 5 sites (Studentized range Kruskal-Wallis post hoc tests; Table S2 in the Supplement). The other 2 northern sites (SR and PES) were significantly different from each of the 3 southern sites, but none of the 3 southern sites were significantly different from each other (Studentized range Kruskal-Wallis post hoc tests; Table S2). In terms of individual populations, LJ in the south had the highest average daily maximum (32.5°C) and absolute maximum (44°C), while PP in the north had the lowest average daily maximum (19.5°C) and absolute maximum (27°C; Table 1). Moreover, it is worth noting that no northern populations spent any amount of time at or above 38°C. In contrast, all southern populations spent at least some fraction of time at or above 38°C, with LJ spending 2.2% of the recorded time at or above 38°C, and 1.1% of the recorded time at or above 41°C. These measures of absolute maximum temperature are especially noteworthy given that acute

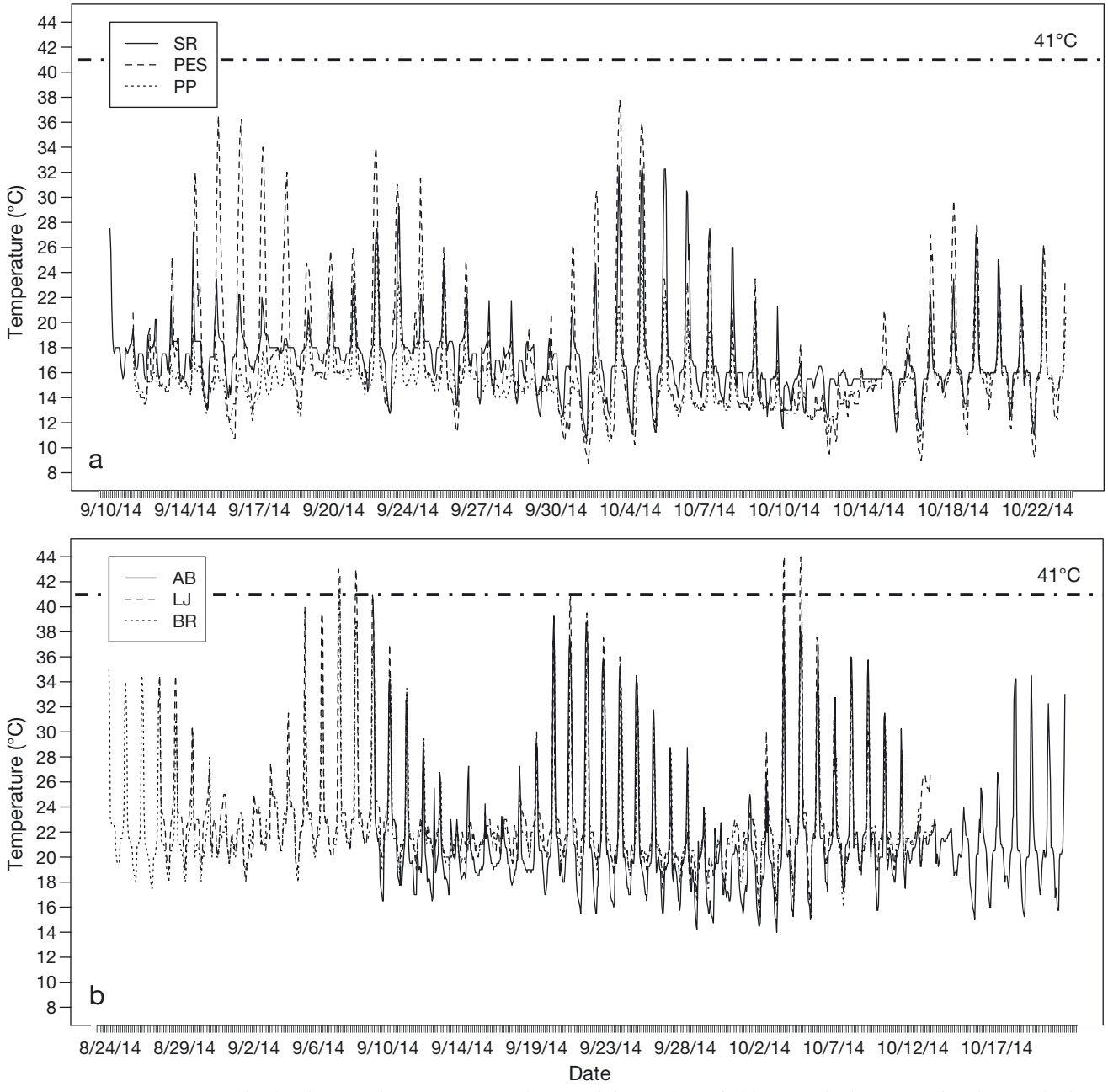

Fig. 2. Temperature profiles for the 6 study sites in (a) northern and (b) southern California. The line at 41°C indicates the heat stress temperature that causes 100% mortality in the laboratory for *Chlorostoma funebralis* snails collected from all 6 sites. SR: Slide Ranch; PES: Pescadero; PP: Pigeon Point; AB: Aliso Beach; LJ: La Jolla; BR: Bird Rock. Dates are mm/dd/yy

thermal tolerance may contribute more to survival than response to chronic temperature exposures (Angilletta 2009).

Temporal patterns: survival distribution functions

The probability of exposure to a temperature above 27°C (at which induction of the heat-shock response is likely) showed a clear latitudinal pattern (Fig. 3). Northern California sites had an average probability of 0.038 of reaching this temperature (SR: $p = 0.032$; PES: $p = 0.077$; PP: $p = 0.005$), while southern sites had an average probability of 0.120 (AB: $p = 0.118$; LJ: $p = 0.126$; BR: $p = 0.114$), which is more than 3 times higher than in the north. In terms of individual sites, LJ had the highest probability of reaching 27°C ($p = 0.126$), and PP had the lowest ($p = 0.005$).

Table 1. Average (±1 SD) temperature data collected by iButton data loggers (robosnails) at the 6 study sites, and percent of time logged above critical temperature thresholds

Location	Average temp. (°C)	Daily average minimum (°C)	Daily average maximum (°C)	Absolute minimum (°C)	Absolute maximum (°C)	% Time ≥ 27°C	% Time ≥ 38°C	% Time ≥ 41°C
North								
Slide Ranch	17.1 ± 3.0	13.8 ± 2.1	23.1 ± 4.5	10.8	32.5	2.1	0.0	0.0
Pescadero	16.7 ± 4.9	12.7 ± 2.0	25.3 ± 6.2	8.8	37.8	5.1	0.0	0.0
Pigeon Point	15.2 ± 2.2	13.0 ± 1.4	19.5 ± 3.5	10.3	27.0	0.1	0.0	0.0
South								
Aliso Beach	21.0 ± 4.5	17.1 ± 1.6	30.4 ± 5.7	14.0	40.5	10.0	0.7	0.0
La Jolla	22.7 ± 4.3	19.1 ± 1.4	32.5 ± 7.0	15.0	44.0	10.1	2.2	1.1
Bird Rock	22.3 ± 3.7	18.9 ± 1.5	30.0 ± 5.4	15.0	41.5	9.1	0.6	0.08

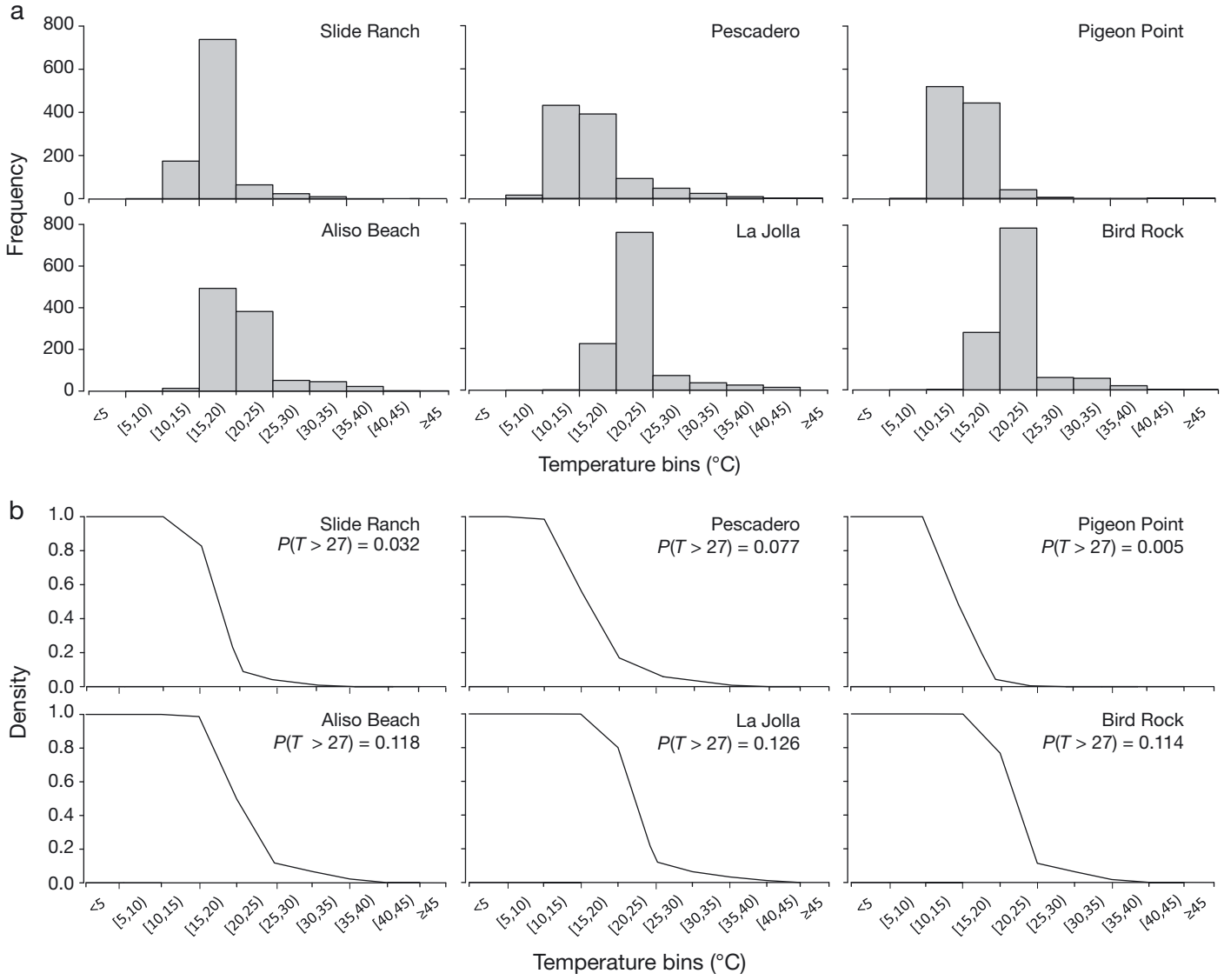

Fig. 3. (a) Frequency distributions and (b) survival functions for daily maximum body temperature of *Chlorostoma funebralis* (estimated from robosnails) at all 6 sites ranging from northernmost (top left) to southernmost (bottom right) (see Fig. 1 for locations). Temperatures for all plots are arranged in 5°C bins; histograms in (a) represent the number of occurrences of temperatures in those bins in the time period. $P(T > 27)$ in each plot in (b) represents the probability that any given temperature will exceed 27°C

Probability of encountering heat stress and constitutive level gene expression

Correlation analyses identified 222 genes (13.2% of the genes tested) whose constitutive levels of expression were significantly associated ($p < 0.05$) with the probability of reaching 27°C (the temperature that induces the heat shock response in *C. funebralis*) (Table S3 in the Supplement). These 222 genes include 14 linked to the ubiquitin pathway, 12 involved in DNA damage response and nucleotide repair, 10 related to calcium binding, 4 negative regulators of apoptosis, 4 associated with response to heat or stress in general, and 3 molecular chaperones/proteins that bind to unfolded proteins (Table 2, Fig. 4). All 6 of these gene categories are significantly overrepresented (chi-square test with Yates correction, $p < 0.014$ for all 6 categories) compared to the original transcriptome assembly from Gleason & Burton (2015) (Table 3). Three genes had p-values less than 0.001: ruvb-like 1, which is involved in cellular response to DNA damage stimulus (Jha & Dutta 2009), and troponin c type 2 and phospholipase a2 isozyme pa4f, which are both associated with calcium ion binding (Gahlmann & Kedes 1990, Pan et al. 2001).

Additional correlation analyses using different threshold temperatures (either using this same or slightly different temperatures for the 2 different geographic regions) resulted in correlations very similar to the 27°C analysis described above. Changing the temperature threshold for both geographic regions had little effect; of the 222 genes whose expression is significantly correlated with the probability of reaching 27°C, 86.2% showed significant correlation with a 25°C threshold, 99.5% with a 28°C threshold and 97.2% with a 30°C threshold. None of these proportions of the 1683 genes tested overall for significant correlation with constitutive gene expression is significantly different from the 222 genes identified in the 27°C analysis. Moreover, even in the 25°C threshold analysis, 7 of the 9 gene categories that are enriched in the 27°C analysis are also significantly enriched at $p < 0.05$, and 8 of 9 are significantly enriched at $p < 0.01$. Overall, these analyses indicate that the conclusions of this study are robust to small changes in the temperature threshold values.

Analyses using both slightly lower and slightly higher heat shock induction temperatures for the southern compared to the northern populations also resulted in correlations very similar to the 27°C analysis. When a correlation analysis was run using the probability of reaching a temperature of 27°C for the 2 northern populations but 28°C for the 2 southern populations, 100% of the genes identified as having a significant correlation between their constitutive level of expression and the probability of reaching 27°C in all 4 populations were also significantly correlated in this tiered analysis (data not shown). Similarly, a correlation analysis using 27°C for the northern populations but 26°C for the southern populations resulted in 97.7% of the genes with a significant correlation when 27°C was used as the threshold temperature for both regions still being significant in the tiered analysis (data not shown). Thus, although further work is needed to confirm that northern and southern California individuals do indeed have the same heat shock induction temperature, slight differences between the 2 geographic regions are not expected to alter the results of this study.

Spatial patterns: thermal stress and latitude

Acute and chronic temperature exposures for all 6 geographic sites are shown in Fig. 5. Similar to the summary extreme temperatures calculated above, the southern site LJ had the highest chronic (32.5°C) and acute (40.9°C) temperature exposures, while the northern site PP had the lowest chronic (19.5°C) and acute (23.7°C) temperature exposures. Overall, across the 6 sites, thermal extremes in chronic stress temperature measurements, and to a lesser extent acute stress temperature measurements, showed a latitudinal pattern. Chronic stress significantly negatively correlated with latitude, with an adjusted r^2 of 0.78 and a p-value of 0.013 (Fig. 6). Acute stress showed a marginally significant negative relationship with latitude, with an adjusted r^2 of 0.56 and a p-value of 0.054 (Fig. 7).

Because the northern site PP showed signs of being an outlier (its average daily maximum was significantly lower than all other sites; see 'Summarizing temporal patterns in extreme temperatures' above), this site was removed from the dataset and the linear regression analyses with latitude were repeated. With this reduced dataset (2 northern and 3 southern sites), the correlation between acute stress and latitude remained marginally significant (adjusted $r^2 = 0.60$, $p = 0.078$; data not shown), and the correlation between chronic stress and latitude was still significant (adjusted $r^2 = 0.90$, $p = 0.009$; data not shown). Thus, although PP significantly differs from the other 5 sites, these results confirm that this site is not biasing the data and creating false strong correlations with latitude.

Table 2. Genes whose constitutive level expression is significantly correlated (Pearson correlation analyses) with the probability of reaching 27°C in *Chlorostoma funebralis*, and whose function relates to ubiquitin activity, DNA damage response and nucleotide repair, calcium ion binding, negative regulation of apoptosis, response to heat, or unfolded protein binding. For a full list of all 222 genes whose constitutive expression was significantly correlated with the probability of reaching 27°C, see Table S3 in the Supplement at www.int-res.com/articles/suppl/m556p143_supp.xlsx. All gene expression data are from Gleason & Burton (2015)

Contig name	Gene annotation	p-value	Gene function
SR_contig_16137	Ruvb-like 1	<0.001	DNA repair; cellular response to DNA damage stimulus
SR_contig_2105	Troponin c type 2	<0.001	Calcium ion binding
SR_contig_355	Phospholipase a2 isozyme pa4	<0.001	Calcium ion binding
SR_contig_2352	C-binding protein	0.0013	Calcium ion binding
SR_contig_33767	Comm domain-containing protein 5	0.0029	May modulate activity of cullin-RING E3 ubiquitin ligase (CRL) complexes
SR_contig_70219	DNA repair and recombination protein rad54-like	0.0045	DNA repair
SR_contig_21389	RNA-binding protein 4	0.0048	Negative regulation of translation in response to stress; stress-activated MAPK cascade
SR_contig_6111	Ubiquitin carboxyl-terminal hydrolase 16	0.0061	Protein ubiquitination
SR_contig_912	T-complex protein 1 subunit theta-like	0.0084	Molecular chaperone
SR_contig_7767	T-complex protein 1 subunit epsilon-like	0.0085	Unfolded protein binding
SR_contig_49706	DNA mismatch repair protein	0.0092	DNA mismatch repair
SR_contig_51672	DNA repair endonuclease xpf	0.010	Damaged DNA binding; nucleotide excision repair; DNA damage removal
SR_contig_24497	Low-density lipoprotein receptor	0.011	Calcium ion binding
SR_contig_37624	Suppressor of cytokine signaling 5	0.011	Protein ubiquitination; may be a substrate-recognition component of a SCF-like ECS (Elongin BC-CUL2/S-SOCS-box protein) E3 ubiquitin-protein ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins
SR_contig_2464	Nucleoporin nup188 homolog	0.011	Regulation of cellular response to heat; regulation of HSF1 mediated heat shock response
SR_contig_81428	Chromatin assembly factor 1 subunit b	0.012	Unfolded protein binding; DNA repair
SR_contig_65692	Tyrosyl-DNA phosphodiesterase 1	0.015	Hydrolyzes 3′-phosphoglycolates on protruding 3′ ends on DNA double-strand breaks due to DNA damage by radiation and free radicals
SR_contig_4549	Activating signal cointegrator 1 complex subunit 2	0.017	DNA repair; DNA dealkylation involved in DNA repair
SR_contig_17445	Endonuclease iii-like protein 1	0.018	Base excision repair
SR_contig_3212	Von willebrand factor type egf and pentraxin domain-containing protein 1	0.018	Calcium ion binding
SR_contig_73036	Ankyrin repeat protein	0.019	Ubiquitin ligase complex; protein ubiquitination
SR_contig_12161	Lipoxygenase homology domain-containing protein 1	0.019	Calcium ion transmembrane transport; calcium channel activity
SR_contig_16669	Phospholipase a-2-activating protein	0.023	Involved in maintenance of ubiquitin levels
SR_contig_13590	Vitellogenin receptor	0.023	Calcium ion binding
SR_contig_46837	Hypothetical protein CGI 10003222	0.027	Calcium ion binding
SR_contig_212341	DNA excision repair protein ercc-8	0.031	Proteasomal ubiquitin-dependent protein catabolic process; response to oxidative stress; positive regulation of DNA repair
SR_contig_36429	DNA-directed RNA polymerase ii subunit rpb2	0.032	DNA repair; nucleotide excision repair
SR_contig_1664	F-box lrr-repeat protein 4	0.033	Ubiquitin dependent protein catabolic process; ubiquitin ligase process
SR_contig_2292	Puromycin-sensitive aminopeptidase	0.033	Protein polyubiquitination
SR_contig_9246	F-box lrr-repeat protein 17	0.034	Substrate-recognition component of the SCF (SKP1-CUL1-F-box protein)-type E3 ubiquitin ligase complex
SR_contig_4629	20 kda calcium-binding protein (antigen sm20)	0.035	Calcium ion binding
SR_contig_15577	Mitochondrial carrier homolog 2	0.038	Regulation of mitochondrial membrane permeability involved in apoptotic process

(Table continued on next page)

Table 2 (continued)

Contig name	Gene annotation	p-value	Gene function
SR_contig_8098	Cullin-associated nedd8-dissociated protein 1	0.039	Cullin-RING ubiquitin ligase complex
SR_contig_5836	Ubiquitin-like modifier-activating enzyme 1	0.039	Catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation through the ubiquitin-proteasome system; essential for timely DNA repair; cellular response to DNA damage stimulus
SR_contig_152	Neurogenic locus notch homolog protein 2-like	0.040	Calcium ion binding; negative regulation of apoptotic activity
SR_contig_33458	Nuclear pore complex protein nup205	0.042	Cellular response to heat
SR_contig_52972	26S protease regulatory subunit 7	0.042	Negative regulation of apoptotic process; DNA damage response; ATP-dependent degradation of ubiquitinated proteins
SR_contig_4356	Nuclear pore complex protein nup98-nup96	0.044	Cellular response to heat
SR_contig_4090	26S proteasome non-atpase regulatory subunit 6-like	0.046	Proteasomal ubiquitin-dependent protein catabolic process
SR_contig_50295	Atpase family aaa domain-containing protein 3-a-like	0.046	Negative regulation of apoptotic process
SR_contig_17808	Squamous cell carcinoma antigen recognized by t-cells 3-like	0.047	Ubiquitin-specific protease binding
SR_contig_31746	Baculoviral iap repeat-containing 5	0.048	Negative regulation of apoptotic process

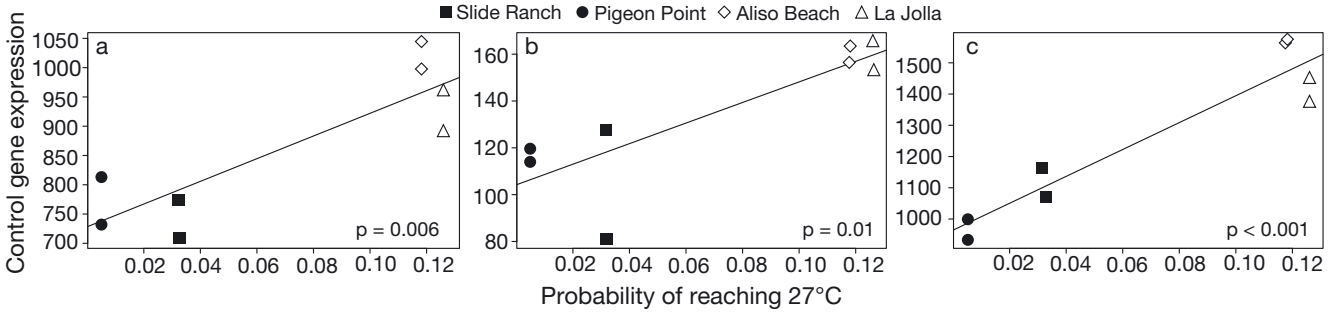

Fig. 4. Scatter plots for genes representative of the 3 most common stress response functions in *Chlorostoma funebralis* with significant correlation (Pearson correlation analyses) between constitutive level gene expression (normalized expression values) and the probability of reaching 27°C. Representative genes (and their respective functions) include (a) ubiquitin carboxyl-terminal hydrolase 16 (ubiquitin protein degradation pathway), (b) DNA repair endonuclease xpf (cellular response to DNA damage stimulus), and (c) troponin c type 2 (calcium ion binding). All gene expression data are from Gleason & Burton (2015). Filled symbols: northern populations; open symbols: southern populations

Table 3. Gene function categories significantly enriched (chi-square test with Yates correction) in the 222 genes whose constitutive expression was significantly correlated with the probability of reaching 27°C, the temperature that induces the heat shock response in the intertidal snail *Chlorostoma funebralis*

Gene function category	No. of genes	Enrichment p-value
Ubiquitin pathway	14	<0.0001
DNA damage response and nucleotide repair	12	<0.0001
DNA binding	12	0.0014
Calcium binding	10	0.0119
Translation and translation initiation	5	<0.0001
Negative regulators of apoptosis	4	0.0014
Response to heat or general stress	4	0.0003
Ubiquinone related	4	<0.0001
Molecular chaperones and proteins that bind to unfolded proteins	3	<0.0001

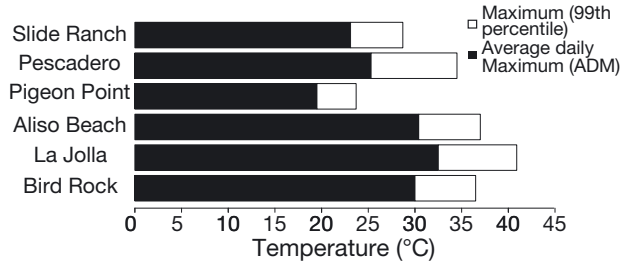

Fig. 5. 'Acute' (99th percentile of temperatures) and 'chronic' high-temperature (peak average daily maximum) exposures for *Chlorostoma funebralis* calculated at each site. Sites are arranged from north to south (see Fig. 1 for locations)

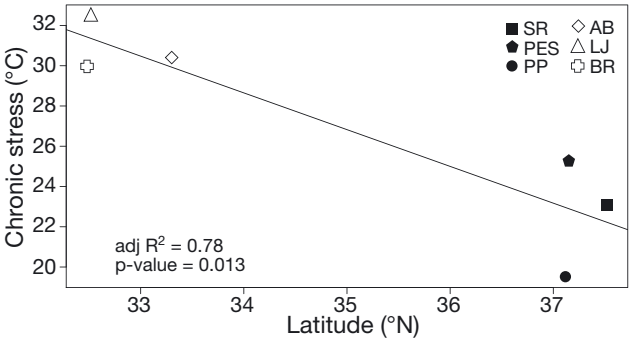

Fig. 6. Linear regression of chronic stress temperature measurements and latitude. Filled symbols: northern *Chlorostoma funebralis* populations; open symbols: southern populations. (SR: Slide Ranch; PES: Pescadero; PP: Pigeon Point; AB: Aliso Beach; LJ: La Jolla; BR: Bird Rock)

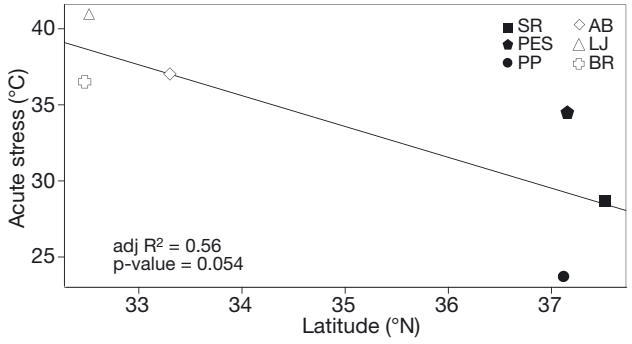

Fig. 7. Linear regression of acute stress temperature measurements and latitude. Filled symbols: northern *Chlorostoma funebralis* populations; open symbols: southern populations. (SR: Slide Ranch; PES: Pescadero; PP: Pigeon Point; AB: Aliso Beach; LJ: La Jolla; BR: Bird Rock)

DISCUSSION

We utilized iButton temperature loggers imbedded in snail shells (robosnails) to record estimates of body temperature for *Chlorostoma funebralis* individuals in northern and southern California. Robosnails deployed at southern California sites, where snails are more thermally tolerant and suffer lower mortality following heat stress (Gleason & Burton 2013), generally experienced higher acute and chronic thermal stress exposures, were 3 times as likely as northern robosnails to reach temperatures that induce the heat shock response in live *C. funebralis* individuals, and were regularly exposed to temperature extremes that are known to cause mortality in the laboratory. Furthermore, we identified 222 genes whose constitutive level of expression is significantly correlated with the probability of reaching body temperatures that induce the heat shock response. Previous genomic work has identified outlier loci involved in response to heat stress that are putatively under divergent selection and that show regional differentiation between northern and southern California populations (Gleason & Burton 2016). Together with these findings, the *in situ* temperature data from this study suggest that the environmental selective forces driving local adaptation to heat stress differ in northern and southern California *C. funebralis* populations, and that the exposure to higher temperatures and thus stronger selection for thermal tolerance in the southern populations could be causing evolutionary change. In particular, we identified genes associated with several distinct aspects of the cellular stress response that could be responding to these differences in thermal selective pressure.

Genes correlated with *in situ* thermal stress: unique insight into local adaptation

Previous studies have identified phenotypic, genomic, and transcriptomic evidence for local adaptation to heat stress in northern and southern California *C. funebralis* populations (Gleason & Burton 2013, 2015, 2016). For instance, ddRAD sequencing identified F_{ST} outlier loci as candidates for positive selection; these loci, involved in response to heat stress, show regional divergence between northern and southern California (Gleason & Burton 2016). In addition, RNA sequencing revealed unique transcriptome-wide gene expression in northern (thermally sensitive) and southern (thermally tolerant) California populations, both before and after heat stress (Gleason & Burton 2015). While that study identified a pattern of pre-adaptation (e.g. elevated constitutive expression under benign conditions with reduced up-regulation following stress), the analysis was based on the fact that a greater number of loci showed the expression than expected by chance —

the particular genes that were pre-adapted were not identified. Here, we re-examined the transcriptome data by correlating these results with *in situ* body temperature estimates from robosnails in order to pinpoint the individual pre-adapted genes that may be contributing to local adaptation in *C. funebralis.* Constitutive expression of genes in a thermally tolerant population is not unique to *C. funebralis*, as this pattern has also been observed in corals and seagrasses (Barshis et al. 2013, Kenkel et al. 2013, Franssen et al. 2014, reviewed in DeBiasse & Kelly 2016). However, to our knowledge this is the first study to investigate the molecular basis of local adaptation to heat stress by explicitly connecting the driving factor for ecological divergence (*in situ* temperature differences) with the expression levels of individual genes that are likely responding to this regional variation. By doing so, we identify the genes that are at higher expression levels at geographic sites more frequently experiencing *in situ* thermal stress, and therefore detect candidate loci that can be subjected to functional tests in future studies (Barreto et al. 2015).

Genes correlated with thermal stress exposure: calcium-binding

Of the genes whose constitutive level of expression was found to significantly correlate with the probability of reaching a temperature that induces the heat shock response, 10 were involved in calcium ion binding (including troponin c type 2, phospholipase a2 isozyme pa4, c-binding protein, and low density lipoprotein receptor). Calcium is one of the most important small molecule secondary messengers in eukaryotes, and has been shown to play an important role in several different signal transduction pathways (Berridge 2008). Exposure to different stress conditions results in changes in free cytosolic Ca^{2+} levels (Wang et al. 2009, Kader & Lindberg 2010), and calcium is involved in the response to heat stress in plants (Reddy et al. 2011, Qin et al. 2008, Larkindale & Knight 2002), human lung cancer cells (Chang et al. 2006), and marine invertebrates. For instance, in corals (DeSalvo et al. 2008) and sea squirts (Serafini et al. 2011) the response to heat stress involves calcium-binding genes and proteins, respectively. This current study is one of only a handful (and the first in mollusks) to suggest a role for expression of calcium-related genes in the response to heat stress in marine organisms. The possibility that the differential thermal tolerance of northern and southern California populations of *C. funebralis* is mediated by the secondary messenger calcium is an interesting hypothesis that merits further study.

Genes correlated with thermal stress exposure: ubiquitin protein degradation

In addition to calcium-binding genes, the constitutive expression of 14 genes associated with the ubiquitin stress response pathway, in which proteins irreversibly damaged by heat stress are degraded (Parag et al. 1987), was also significantly correlated with the probability of reaching a temperature that induces the heat shock response. These 14 genes include ubiquitin carboxyl-terminal hydrolase 16, an ankyrin repeat protein, several f-box lrr repeat proteins (4 and 17), and several 26S proteasome regulatory subunits (6 and 7). Thus, at moderate levels of heat stress in *C. funebralis*, it seems proteolysis is initiated to remove proteins that cannot be rescued by chaperones (Hofmann & Somero 1995, Logan & Somero 2011), which in turn minimizes the amount of unfolded proteins by tagging them for degradation (Dahlhoff 2004). The thermal environment affects ubiquitin expression levels in other marine organisms as well. Notably, similar patterns of constitutive ubiquitin expression have been observed in eurythermal gobies, with fish showing higher constitutive levels of expression for the gene ubiquitin with warmer steady-state acclimation temperatures (Logan & Somero 2010, 2011). Additional studies have examined the downstream effects of ubiquitin gene expression; for instance, heat-stressed mussels, *Mytilus trossulus*, found in high-intertidal habitats contained significantly higher levels of ubiquitinated proteins than subtidal conspecifics (Hofmann & Somero 1995). However, it is important to note that these 2 previous studies focused on phenotypically plastic gene expression and protein levels in response to heat stress, compared to our study which looked at genetically-based transcriptomic responses following common garden acclimation. Overall, the identification of these 14 pre-adapted (high constitutive expression) ubiquitin genes with significant correlation with the probability of reaching body temperatures that induce heat shock response indicates that, under control conditions in southern California populations, selection favors constitutive expression of genes to cope with moderate levels of thermal stress that cannot be dealt with by molecular chaperones alone.

Genes correlated with thermal stress exposure: DNA damage response and repair

Following severe thermal stress, basic activities like cell proliferation may cease due to cellular damage to DNA. If this damage is not repaired, apoptosis may occur (Lesser 2006). Thus, genes responsible for sensing and repairing DNA damage can be considered 'severe heat related genes' (Maor-Landaw et al. 2014). We identified 12 pre-adapted genes involved in DNA damage response and nucleotide repair whose constitutive expression was significantly correlated with the probability of reaching 27°C. Specifically, we identified 2 genes (DNA repair endonuclease xpf and tyrosyl-DNA phosphodiesterase 1) that are involved in DNA double strand break (DSB) repair. DSBs are thought to be the most serious form of DNA damage because they can impede transcription, replication, and chromosome segregation (Nitiss 1998); thus, the fact that these 2 DSB repair genes were found to correlate with the chances of reaching a heat-stress inducing temperature suggests that *C. funebralis* individuals from northern and southern California require different levels of DNA repair due to their unique thermal environments.

DNA damage has also been shown to be a consequence of heat stress in other marine mollusks, such as *Mytilus* mussels. In *Mytilus galloprovincialis*, DNA damage was detected in mantle and gill tissue following heat stress (Koutsogiannaki et al. 2014), and heat stress led to significant double and single stranded breaks in DNA in *M. californianus* and *M. galloprovincialis* hemocytes (Yao & Somero 2012). Moreover, there were interspecific differences in DSBs following heat stress, with lower amounts of DNA damage occurring in the more heat tolerant *M. galloprovincialis*, suggesting that this more heat tolerant species can maintain its DNA stability across a larger range of temperatures compared to its more heat sensitive congener, *M. californianus*. It was hypothesized that these interspecific differences may reflect variations not only in levels of DNA damage but also in DNA repair, with the more tolerant species possessing a stronger repair ability. Similarly, higher constitutive expression of DNA damage repair genes in southern versus northern California *C. funebralis* populations could indicate that the more thermally tolerant southern populations have a stronger DNA repair ability. This differential expression of DNA repair genes could play an important role in the region-specific thermal tolerance of northern and southern *California* individuals; thus, the DNA damage response in *C. funebralis* merits further study. For instance, the actual amount of DNA damage that occurs in northern and southern California individuals following heat stress could be quantified to examine the functional consequences of differences in DNA repair gene expression.

Physiological implications of geographic variation in thermal stress

The gene expression data discussed above suggest that the strategy of pre-adaptation (a pre-emptive strategy or preparative defense in which there are high constitutive levels of stress response genes versus up-regulation once the heat stress has already occurred; Gleason & Burton 2015) is likely beneficial in southern, but not northern, California *C. funebralis* populations because individuals in southern California are 3 times as likely to reach body temperatures that induce the heat shock response. However, this induction of the heat shock response is energetically costly (Sanchez et al. 1992, Heckathorn et al. 1996). Energy is required to make Hsps (Hochachka & Somero 2002), and Hsps require the hydrolysis of ATP to refold damaged proteins (Mayer & Bukau 2005). Moreover, overexpression of Hsps can actually decrease fitness (Feder et al. 1992, Krebs & Loeschcke 1994), in part due to the preferential production of Hsps over other proteins at high temperatures. For these reasons, inter-population variation in the frequency of heat shock response induction could be associated with differences in how temperature affects northern and southern California populations' energy budgets (Tomanek & Somero 1999), with southern populations incurring a much higher energy cost. Similar patterns to those observed in northern and southern populations of *C. funebralis* described in this study have also been observed in *Chlorostoma* congeners, with species that occupy higher intertidal heights (and are thus exposed to higher temperatures) inducing the heat shock response more often than congeners living lower in the intertidal. In that study, as in ours, the temperature differences between *Chlorostoma* species were verified with *in situ* body temperature measurements. Overall, the data suggest that differences in body temperature and thus induction of the heat shock response between northern and southern California populations have consequences for evolutionary selective pressure on physiological energy budgets.

Thermal extremes and mortality events

Our data indicate that southern, but not northern, field sites reached temperatures (38°C and above) that cause mortality in all 6 populations, at least in controlled laboratory experiments (Gleason & Burton 2013). This finding suggests that southern, more thermally tolerant populations are more vulnerable to climate change, given that climate change will likely alter the probability of extreme events (Parmesan et al. 2000). Previous work has demonstrated that, somewhat unexpectedly, more warm-adapted animals may be less able to respond to climate change than more cold-adapted animals because the warm-adapted animals are already closer to their upper thermal limit (Stillman 2003, Somero 2010, Tomanek 2010). This study similarly demonstrates that northern populations on average can survive temperatures roughly 8°C higher than the average acute stress exposure they experience in the field (29°C; Gleason & Burton 2013). Conversely, southern populations suffer 100% mortality at 41°C, a temperature that 2 of the southern California sites, LJ and BR, exceeded on several occasions throughout the data record. This result is also consistent with a previous study that investigated the thermal limits of heart function in *Chlorostoma* congeners; Stenseng et al. (2005) found that *C. funebralis* can encounter body temperatures in the field in southern California that exceed its flatline temperature, the temperature at which the heart stops beating upon heating. Thus, although southern populations have a higher baseline thermal tolerance than northern populations (Gleason & Burton 2013), it appears that southern populations will not be able to cope with temperature increases without suffering substantial, if not complete, mortality. These population-specific responses to thermal stress could have a large effect on future local extinctions and geographic range shifts for *C. funebralis.* Ultimately, the significance of mortality events will depend on larval supply (Caley et al. 1996, Colson & Hughes 2004, Liu et al. 2011), and whether the spatial extent of mortality exceeds the maximum dispersal of *C. funebralis* larva. Previous dispersal simulations based on the 5 to 13 d planktonic larval duration for *C. funebralis* (Moran 1997) suggest that larvae released from the 6 geographic sites used in this study disperse between populations within northern and within southern California, but there was no evidence for direct gene flow between the 2 geographic regions (Gleason & Burton 2016). Thus, further work is needed to better understand the amount of gene flow that is occurring among the sites between northern and southern California.

Thermal stress and latitude

This study found a significant relationship between chronic stress exposure and latitude along the California coast, in contrast to previous work that found a 'mosaic' of thermal stress exposure and no correlations with latitude at sites along the western coast of North America from Washington down to southern California (Helmuth et al. 2002, 2006). This discrepancy demonstrates that it is important to consider the particular geographic location of the sites being examined when performing such studies. For instance, despite the overall lack of correlations with latitude, Helmuth et al. (2002) found that at the mid-intertidal range, the cumulative hours of summer midday aerial exposure for sites within California comparable to our study (Point Reyes down to Santa Barbara) do show a latitudinal trend, with the more southern sites experiencing almost 80 h of exposure, compared to only 60 h for northern California sites. Thus, when examining differences between northern and southern California sites such as those used in this study, thermal stress may correlate with latitude. However, when including sites further north along the Pacific coast of the United States (for instance comparing sites from Washington to sites in southern California as in Helmuth's studies), the pattern of thermal stress may be dominated by mosaic 'hotspots' that do not correspond with latitude across this broader scale.

Robosnail limitations and caveats

Although the techniques used were appropriate for the goals of this study (see 'Materials and methods' above), the approach of estimating *in situ* body temperatures from robosnail measurements has several limitations that are worth noting. For instance, because we had only 2 loggers deployed at each of the 6 sites, we were not able to examine spatial variability among individuals within a geographic site. However, studies have demonstrated that variation over small scales can be equivalent to mean differences that occur over much larger scales (e.g. Bartlett & Gates 1967, Elvin & Gonor 1979, Miller et al. 2009, Denny et al. 2011, Seabra et al. 2011, Pincebourde & Woods 2012). Thus, it is important to keep in mind that our data provide general extreme temperature estimates for each site, without taking into account these individual differences that can occur across different microhabitats within a site (Lima & Wethey 2009).

Similarly, it is important to note that the body temperature patterns described here may be specific to the organism and to the intertidal height at which they were measured. Because *C. funebralis* is a dark-shelled organism, the patterns of body temperature across latitude from this study may not apply to other species in this intertidal habitat that lack such a morphological feature, or whose shell color more closely matches that of the substrate (Helmuth et al. 2002, Lima & Wethey 2009). Moreover, because tidal height can affect regional temperature patterns (Helmuth et al. 2006), the body temperatures obtained here from +1.0 and +1.5 m above MLLW may be expected to differ greatly from similar measurements obtained from the upper and lower intertidal habitats.

In addition, this approach also has some inherent limitations due to the fixed nature of the robosnails. While we specifically deployed the temperature loggers at sun-exposed local sites right next to live *C. funebralis* individuals in the middle of the day, the robosnails cannot mimic any movements of live animals. For instance, a similar study using robolimpets found that several of the live limpets neighboring the robolimpets moved up to 45 cm away from the loggers during the first tide cycle period following deployment (Lima & Wethey 2009). Furthermore, it is known that some *C. funebralis* individuals exhibit a 'cold-biased' behavioral response that may guide snails to refuges in shaded cracks and crevices (Tomanek & Somero 1999, Tepler et al. 2011), habitats in which they are somewhat buffered from desiccation (Marchetti & Geller 1987). The robosnails used in this study were not mobile, and thus cannot capture any of the variation in body temperatures that such behavior would cause. However, it is worth noting that these movements of live *C. funebralis* will likely result in individuals occupying cooler microhabitats, while the main goal of this study was to investigate variation in extreme temperatures individuals at different geographic sites experience.

CONCLUSIONS

In situ temperatures differ between northern and southern California *Chlorostoma funebralis* populations in both the frequency and magnitude of extreme thermal stress exposures. Southern, more thermally tolerant, populations are 3 times as likely as northern populations to reach temperatures that induce the heat shock response in the field, and they even experience temperatures that cause mortality. Thus, southern populations are under stronger selective pressure to cope with heat stress. These results are in agreement with a previous study that identified outlier loci (involved in response to heat stress) putatively under divergent selection between northern and southern populations (Gleason & Burton 2016). Overall, our results provide insight into why the gene expression strategy of pre-adaptation (high constitutive expression levels) is likely beneficial in southern, but not northern populations, while also demonstrating that combining *in situ* temperature measurements with transcriptome data provides unique insight into the genes that may underlie local adaptation to differences in thermal stress. Ultimately, the data obtained in this study can be used to better inform predictions regarding how rocky intertidal invertebrates such as *C. funebralis* will respond to future climate change.

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