Ecologically relevant temperature ramping rates enhance the protective heat shock response in an intertidal ectotherm

Keywords: life history; thermal stress; hardening; heat shock response; heating rate; copepod

What is already known

The geographic and ecological distribution of intertidal organisms is strongly affected by temperature extremes, which vary daily across latitudes and habitats. Although the thermal tolerances of many intertidal ectotherms have been studied in order to help predict adaptive responses to a changing climate, the importance of different rates of diel temperature change in determining thermal tolerance remains relatively understudied. The heat shock response is known to provide an important protective mechanism for organisms, and understanding how it responds to both temperature extremes and rates of temperature change continues to be the focus of ongoing research.

What this study adds

We performed a comparison of gradual versus abrupt thermal exposures in an intertidal ectotherm and found that the more gradual exposure resulted in higher thermal tolerance and reduced effects on developmental rate compared to the abrupt exposure. Because we also found heat shock protein (HSP) genes were upregulated to a greater extent in the gradual exposure than in the abrupt exposure, and upregulation began prior to the peak temperature, we conclude that a slower heating rate is necessary for organisms to adequately mitigate the costs of thermal stress via the heat shock response. These findings show the importance of ecologically relevant thermal exposures for a complete understanding of the heat shock response.

Abstract

Thermal stress experiments are essential for understanding organisms' thermal limits and the physiological processes that contribute to establishing those limits. Experiments typically employ either an abrupt transfer to near-lethal temperatures or a gradually increasing thermal exposure. In the current study, we used three populations of the intertidal copepod *Tigriopus californicus* that are known to differ in upper thermal tolerance to investigate the effects of gradual versus abrupt thermal exposures on survivorship, developmental time, and heat shock protein gene expression. Developmental rate of nauplii was unaffected following the gradual exposure, whereas developmental time slowed by ~2 days (~20%) following an abrupt exposure. The gradual exposure also improved survivorship in comparison to the abrupt exposure. Furthermore, the heat shock protein genes *hsp70* and *hspb1* showed greater upregulation during the gradual thermal exposure compared to the abrupt exposure. Though the differences in response to each thermal regime varied in magnitude among the different populations, the types of responses were very similar (i.e. following the gradual exposure survivorship increased, developmental time showed no effect, and heat shock protein gene upregulation during the exposure increased). Therefore, the enhanced protective effect of the heat shock response during gradual exposures appears to be conserved within the species despite population-level differences in thermal tolerance. Thus, an ecologically relevant thermal exposure likely enables improved cellular protective mechanisms by allowing for an effective and timely heat shock response, which plays a role in mitigating the effects of thermal stress and thereby enhances tolerance to elevated temperatures.

Introduction

Effects of temperature occur at all levels of biological organization, from the molecular level to whole ecosystems, and these effects are thought to play a major role in establishing the biogeographic distributions of organisms, particularly ectotherms (Dahlhoff and Somero 1993; Somero 2005, 2012; Fangue et al. 2009; Sunday et al. 2012). Recently, it has been demonstrated that occasional exposure to extreme temperatures, or increased variation in temperature, may play a bigger role in organisms' adaptive responses to stress than rising average temperatures (Clusella-Trullas et al. 2011; Vasseur et al. 2014). Experiments examining thermal stress can provide insight into the physiological mechanisms underlying whole-organism thermal limits to acute temperature change, as well as long-term consequences of acute stress, such as changes in growth, reproduction, and mortality (Pörtner et al. 2006). These effects are typically viewed as tradeoffs: in order for organisms to survive a stressor, they must allocate energy away from non-essential processes (such as growth and reproduction) and toward physiological maintenance measures, such as the induction of the heat shock response (Angilletta et al. 2003).

Because of their extremely variable habitats, rocky intertidal organisms are commonly used in studies of thermal stress. These studies frequently employ gradual thermal ramping methods to mimic the warming of a tidepool (Denny et al. 2006; Tomanek and Zuzow 2010; Kelly et al. 2011; Paganini et al. 2014; Bjelde et al. 2015; Gleason and Burton 2015; Jimenez et al. 2016). Abrupt thermal exposures, sometimes referred to as plunging or static assays, are less commonly used in studies of intertidal organisms but can still provide valuable insights into physiological responses and are often used to observe specific processes (such as the heat shock response) or outcomes (such as survivorship) at temperatures near an organism's lethal limit (Dong et al. 2010; Willett 2010; Schoville et al. 2012; Fields et al. 2016; Giomi et al. 2016; Kelly

et al. 2017; Vergara-Amado et al. 2017). However, although there are studies and reviews comparing gradual and abrupt thermal exposures for terrestrial ectotherms (reviewed in Terblanche et al. 2011), this comparison has not, to our knowledge, been made in detail for an intertidal species.

In this study, we directly compare the effects of an abrupt versus gradual thermal exposure on life history traits and heat shock protein gene expression in the copepod *Tigriopus californicus,* a common inhabitant of high rocky tidepools along the west coast of North America (Dethier 1980; Ganz and Burton 1995). *T. californicus* populations are adapted to their local habitats, with southern populations showing higher heat tolerance than their northern conspecifics (Willett 2010; Kelly et al. 2011; Schoville et al. 2012; Pereira et al. 2014; Tangwancharoen and Burton 2014). To date, most studies of *T. californicus* have used abrupt exposure (i.e., rapid increase in temperature, typically for one hour) to study thermal stress responses. Gradual ramping exposures (i.e., slowly increasing temperature over the course of several hours or more) have occasionally been used in this species (e.g., Kelly et al. 2012), but there have been no direct comparisons of the two protocols. The habitat of *T. californicus* makes understanding the consequences of rates of temperature change particularly relevant: because it is found only in shallow splash pools in the high intertidal, it experiences more extreme and more rapid temperature fluctuations than species inhabiting the lower intertidal where wave action can provide quick respite from the heat.

Here we developed a gradually increasing thermal exposure that is similar to the conditions observed in the natural tidepool habitats of *T. californicus*. We chose three populations of *T. californicus* from California to compare: San Diego (SD) is the farthest south and most heat tolerant, Abalone Cove (AB, Los Angeles County) has a mid-level heat tolerance,

and Santa Cruz (SCN) is the most northern and least heat tolerant population (Willett 2010; Tangwancharoen and Burton 2014). We compared the effects of abrupt versus gradual exposures on developmental rate and survivorship among all populations. Furthermore, *T. californicus* has been shown to upregulate heat shock proteins (HSPs) in response to thermal stress, especially in thermally tolerant populations (Schoville et al. 2012; Lima and Willett 2017), so to clarify some of the mechanisms potentially underlying the life history effects we chose three key HSP genes involved in heat shock response of *T. californicus—hsp60, hsp70,* and *hspb1* (Arya et al. 2007; Schoville et al. 2012; Barreto et al. 2015)—and examined their expression following both types of exposures.

Based on previous research, the expected outcome of a comparison of slowly ramping thermal exposures versus abrupt exposures is unclear. One possible outcome is that gradual exposure to an extreme but sublethal temperature will result in more detrimental effects on life history traits and reduced thermal limits due to the relatively longer duration of thermal stress ("heat load") relative to an abrupt exposure (see Pörtner 2010). This possibility is supported by a number of studies (Terblanche et al. 2007; Chown et al. 2009; Mitchell and Hoffmann 2010) that found that slower ramping rates during gradual thermal exposures led to lower thermal limits and poorer tolerance. These negative effects of ramping may be amplified in the more thermally sensitive northern population, SCN (i.e. we might see a bigger difference in the lethal temperature between the abrupt and gradual exposures for SCN than for AB or SD). An alternative outcome, that a gradual thermal exposure can yield higher thermal tolerance due to enhanced HSP expression, has also found some support (Chidawanyika and Terblanche 2011; Bahar et al. 2013). Because of the much longer duration of thermal stress in the gradual exposure, it remains to be seen whether higher upregulation of heat shock proteins is enough to

mitigate adverse effects of thermal stress. The effect of HSP gene upregulation may again be especially apparent in the northern thermally sensitive population, which does not upregulate heat shock proteins as highly as the southern populations (Schoville et al. 2012).

Materials and Methods

Culturing conditions

Copepods were collected from high rocky tidepools at three sites in California, USA: "SD" from Ocean Beach, San Diego County (32° 45' N, 117° 15' W), "AB" from Abalone Cove, Los Angeles County (33° 44' N, 118° 22' W), and "SCN" from Santa Cruz County (36° 56' N, 122° 02' W). Animals were held in 400-ml beakers in 250 ml of 0.4 μm filtered seawater (35 ppt) and fed ground TetraVeggie algae wafers. Prior to use in thermal stress or gene regulation experiments, populations were maintained under laboratory conditions at 20 °C with a 12-h light/dark cycle for at least one full generation (~1 month).

Development of gradually increasing thermal exposure

To determine an approximate temperature ramping rate for the gradual exposure, iButton temperature loggers (Maxim Integrated) were used to monitor field conditions within tidepools. Loggers were coated with Plasti Dip rubber coating (Performix) and secured with marine epoxy (Splash Zone) in tidepools containing *T. californicus.* Loggers were placed out of direct sunlight and submerged to monitor habitat utilized by the animals. Two loggers were deployed at the SD site to test variation among pools. Loggers measured temperature every 20 minutes for 7 days (Figure 1). Although the temperature loggers were placed in only one of the collection sites for a relatively short period of time, the goal of the deployment was to determine rates of temperature

change in copepod-inhabited pools on hot sunny days when maximum thermal stress might be experienced.

The rate of heating during the day in SD *T. californicus* tidepools averaged 0.033 °C per minute (ranging from 0.029 °C min^{-1} to 0.039 °C min-1. From these data, we developed a gradual thermal ramping exposure to approximate this rate of observed temperature increase: in a thermal cycler we raised the temperature 2 °C every 40 min (for an average increase of 0.05 °C min^{-1}). So that both abrupt and gradual thermal regimes exposed animals to the same maximum temperature for the same duration, the maximum temperature was held for 1-h in both treatments. For the abrupt thermal regime, we measured the rate of heating of 1 ml seawater in a microcentrifuge tube and found that it took approximately 3 minutes to reach our maximum exposure temperature for a heating rate of 5 °C min^{-1}, approximately 100-fold faster than the gradual thermal exposure.

Survivorship assay

For each population, six replicate groups of ten adult animals were exposed to each (abrupt or gradual) thermal exposure. The abrupt exposure consisted of placing 1.5 ml microcentrofuge tubes containing 10 copepods in 1 ml seawater into a circulating water bath set to 36 °C. Animals were left in the water bath for 1-h then placed back into 20 °C incubators. For the gradual thermal exposure, groups of 10 copepods were placed in 0.3 ml of seawater in 0.5 ml microcentrifuge tubes and a thermal cycler was used to increase temperature in 2 °C increments every 40 min (as described above) starting at 20 °C until 36 °C was reached. The peak temperature was held for 1-h, then temperature was decreased back to 20 °C in 8 °C increments every 40 min (Fig. 2). Following treatment, all animals were placed into 6-well plates (10

animals per well) with fresh filtered seawater and food. Survivorship was counted 3 days post exposure (Tangwancharoen & Burton 2014). A generalized linear model was fit to the data using a binomial distribution, and significance was assessed by ANOVA.

Developmental rate

Gravid female copepods have external egg sacs that change in color from green to red as they develop. Red egg sacs were removed from gravid copepods and left to hatch overnight in separate wells of a 12-well plate in a 20 °C incubator. Individual broods of nauplii were split between two treatments: half were heat-treated and half were kept as controls (i.e., maintained at 20 °C). The number of broods used for the gradual exposure were SD: 7, AB: 9, and SCN: 9. The number of broods used for the abrupt exposure were SD: 13, AB: 5, and SCN: 8. Broods were exposed to the corresponding thermal exposures as described in the "Survivorship assay" section above. Following treatment, nauplii were fed and checked daily for metamorphosis into the first copepodid stage (CI). The date of the first appearance of CI copepodids was recorded for each group (Tangwancharoen and Burton 2014), and a two-way ANOVA followed by a Sidak's multiple comparisons test was used to assess variation in developmental rate among the treatments.

Heat shock protein (HSP) gene expression

Groups of 50 adult animals were heat stressed using the abrupt or gradual thermal exposures while control groups were was kept at 20 °C (3 groups per treatment). Because all animals from SCN died in the 36 °C abrupt exposure, we used 35 °C as the peak temperature for all gene expression studies in the SCN population and 36 °C for both SD and AB. Total RNA

was extracted immediately following the heat treatment (no recovery time) or from control groups. Additionally, we examined the time course of gene regulation during the gradual thermal exposure using three groups of 30 animals compared to a control. Groups were removed after reaching a moderate temperature (28 °C for SD and AB and 27 °C for SCN), a stressful temperature (34 °C for SD and AB and 33 °C for SCN), and finally after completing their 1-h exposure at the peak temperature (36 °C or 35 °C).

In both experiments, RNA was extracted using Trizol and cDNA was synthesized from 200 ng RNA using the High Capacity RNA-to-cDNA kit, which employs random octamers and oligo-dT primers (Applied Biosystems). cDNA was then diluted to 3 ng μl^{-1} (RNA equivalent) before adding to a 15 µl reaction containing 9 ng template, 1X iTaq Universal SYBR Green supermix (Bio-Rad), and 0.35µM of each primer (sequences and GenBank accession numbers listed in Table 1). Reactions were run on a Stratagene MX3000P (Agilent) system with a denaturation at 95 °C for 2 min, and 40 cycles of 95 °C for 10 s and 59 °C for 20 s, followed by a melting dissociation curve step. Relative expression of each gene was assessed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Fold change estimates were normalized using the geometric average of *myosin* and *GAPDH* genes, which have been identified as suitable reference genes for *T. californicus* qPCR following thermal stress (Schoville et al. 2012; Barreto et al. 2015).

For each of three target HSP genes (*hsp60, hsp70,* and *hspb1*), log fold change (with 95% confidence interval) was calculated comparing either the gradual or abrupt thermal exposure to a control group. Significance was assessed using a randomized block ANOVA followed by Sidak's multiple comparisons test between treatments for each population. Similarly, for the analysis of gene regulation during the abrupt exposure, log fold change (with 95% confidence

interval) was calculated comparing three temperatures during the gradual ramp (27 °C for SCN and 28 °C for SD/AB; 33 and 34°C; and 35/36 °C) to a control group. Significance was assessed using a randomized block ANOVA followed by Tukey's multiple comparisons test between treatments for each population.

Results

Survivorship

In all populations, survivorship was significantly higher following the gradual exposure than it was following the abrupt exposure (Fig. 3). Two generalized linear models with binomial distribution were compared using a likelihood ratio test: one with thermal exposure type and population both as main effects, and one with an added interaction between the two terms. Since inclusion of the interaction term did not improve the fit of the model, the simpler model with no interaction term was chosen. An ANOVA showed a significant effect of thermal exposure type ($X^2_{1,30}$= 15.40, $P < 0.0001$) but not of population, though population had a nearly significant effect ($X^2_{2, 30} = 5.72$, $P = 0.0573$).

Developmental rate

The differential effects of the abrupt versus gradual thermal exposure compared to controls on developmental rate were striking. For the abrupt exposure (Fig. 4A), a two-way ANOVA showed a significant effect of population ($F_{2, 46} = 3.546$, $P = 0.0370$) and exposure ($F_{1, 46} = 33.05$, $P <0.0001$) but no significant interaction ($F_{2, 46} = 1.424$, $P = 0.2511$). Post-hoc tests found a significantly slower developmental rate following heat stress compared to control in SD ($P = 0.0200$), AB ($P = 0.0113$), and SCN ($P = 0.0003$). The average delay in development was

about 2 days, slowing from 7 days in the control groups to about 9 days after the abrupt exposure. In contrast, there was no significant effect of exposure ($F_{1, 44} = 3.025$, $P = 0.0890$) or population ($F_{2, 44} = 0.2436$, $P = 0.7849$) on developmental rate in the animals that experienced the gradual thermal exposure (Fig. 4B).

Heat shock protein gene expression

In comparisons of the relative expression of three HSP genes (*hsp60, hsp70,* and *hspb1*) following either an abrupt or a gradual thermal exposure, the gradual exposure led to higher upregulation than the abrupt exposure in most comparisons for *hsp70* and *hspb1* (Fig. 5). There was a significant interaction between population and thermal exposure for all three genes (*hsp60*: $F_{2,6,3} = 11.22$, $P = 0.0094$; *hsp70*: $F_{2,6,3} = 11.69$, $P = 0.0085$; *hspb1*: $F_{2,6,3} = 25$, $P = 0.1926$). *Hsp60* did not show strong changes in expression in any of the populations or treatments, but was upregulated to a greater extent by the abrupt exposure than the gradual exposure in the SD population only (SD: $P = 0.0141$; AB: $P = 0.5511$; SCN: $P = 0.4367$). For *hsp70,* upregulation of gene expression was greater after the gradual thermal exposure than the abrupt exposure in AB ($P = 0.0101$) and SCN ($P = 0.0056$), but not in SD ($P = 0.7782$). The gradual exposure significantly increased upregulation of *hspb1* in comparison to the abrupt exposure in all three populations (SD and AB: $P < 0.0001$; SCN: $P = 0.0003$).

Hsp70 and *hspb1* also showed upregulation during a gradual thermal exposure when we measured expression at three temperature/time points during the gradual exposure (Fig. 6). *Hsp70* showed a significant effect of temperature but not of population, nor did it show an interaction (temperature: $F_{2,12,3} = 63.21$, $P < 0.0001$; population: $F_{2,6,3} = 1.511$, $P = 0.2941$; interaction: $F_{4,12,3} = 0.4696$, $P = 0.7572$). *Hspb1* showed a significant effect of temperature,

population, and interaction (temperature: $F_{2,12,3} = 58.33$, $P < 0.0001$; population: $F_{2,6,3} = 19.39$, $P = 0.0024$; interaction: $F_{4,12,3} = 3.366$, $P = 0.0457$). In the post-hoc analysis, the same pattern was observed for both genes in all three populations: there was significant upregulation between the low and middle temperature (*hsp70,* SD*:* $P = 0.0001$; *hsp70,* AB: $P = 0.0002$; *hsp70,* SCN: $P = 0.0013$; *hspb1,* SD*:* $P = 0.0137$; *hspb1,* AB: $P < 0.0001$; *hspb1,* SCN: $P = 0.0004$), as well as between the low and high temperature (*hsp70,* SD*:* $P = 0.0003$; *hsp70,* AB: $P = 0.0004$; *hsp70,* SCN: $P = 0.0003$; *hspb1,* SD*:* $P = 0.0341$; *hspb1,* AB: $P < 0.0001$; *hspb1,* SCN: $P = 0.0003$), but not a significant difference between the middle and high temperature (*hsp70,* SD*:* $P = 0.7974$; *hsp70,* AB: $P = 0.8483$; *hsp70,* SCN: $P = 0.6486$; *hspb1,* SD*:* $P = 0.8689$; *hspb1,* AB: $P = 0.3639$; *hspb1,* SCN: $P = 0.9964$).

Discussion

In this study, we directly compared effects of an abrupt thermal exposure versus a gradual ramping exposure on *T. californicus*. Animals that experienced the gradual exposure experienced high temperatures for substantially longer than those that experienced the abrupt temperature change, since the duration of exposure to the maximum temperature was 1-h in both regimes, but the relatively slow increase in temperature for the gradual exposure meant that copepods also experienced relatively high temperatures during the ramping phase. Despite this increased exposure to high temperature, the gradual exposure proved to be less stressful. Survivorship of animals that experienced the gradual exposure was much higher than those that experienced the abrupt treatment. Higher heat tolerance during a gradual thermal ramp, often measured via knockdown temperature, has been seen in some studies (e.g., Chidawanyika and Terblanche 2011; Bahar et al. 2013), but often slowly ramped groups show either lower tolerance

than abruptly stressed animals or no difference (Terblanche et al. 2007; Mitchell and Hoffmann 2010; Overgaard et al. 2012; Nguyen et al. 2014). In contrast to knockdown assays, we propose that measuring survivorship after thermal stress exposure is more informative because it integrates the compounding effects that heat exposure can have over time.

Remarkably, there was no effect of the gradual exposure on developmental rate, compared to a ~2 day (~20%) slower development of abruptly exposed copepods. Though the effects of thermal stress on developmental rate have been tested in many organisms, increased heat exposure often causes developmental rate to speed up (Bermudes and Ritar 2008; Roberts et al. 2012; Runcie et al. 2012), while slower developmental rate, as seen here following abrupt stress, is not common (Sgrò et al. 2010; Tangwancharoen and Burton 2014). To our knowledge there are no studies comparing the effects of gradual and abrupt thermal exposure on development, though there is evidence that cycling between control and stressful temperatures can significantly delay development or slow growth rates (Sgrò et al. 2010; Kingsolver and Woods 2016). One possibility that could contribute to these observed differences is the higher heat load experienced by the gradually exposed copepods. However, the life history data presented in the current study suggest that even when the time at high temperatures is longer, gradually ramping up to those temperatures over the course of hours at ecologically relevant rates of change makes them markedly less stressful for both larval and adult stages.

Although the gradual exposure had less impact on life history parameters than the abrupt exposure, gradually increasing temperature resulted in greater upregulation of heat shock protein genes, an observation consistent with previous studies (McMillan et al. 2005; Sobek et al. 2011; Sørensen et al. 2013). Though the heat shock response is often cited as evidence for increased cellular stress and therefore increased damage (Terblanche et al. 2011; Sørensen et al. 2013), the

opposite has previously been found in *T. californicus*: more thermally tolerant populations show higher upregulation of heat shock proteins (Schoville et al. 2012). This pattern fits with our observations of higher upregulation of selected heat shock proteins during and following the gradual exposure compared to the abrupt exposure and correspondingly higher survivorship and no change to developmental rate in gradually exposed copepods. There is evidently a mechanism for limiting damage that may be time-sensitive so that it cannot act quickly enough to be effective during an abrupt stress but will protect the animals when temperature increases gradually; our data suggest that the heat shock response is an excellent candidate process to underlie these effects. The high temperatures used in this study could potentially alter enough proteins into non-efficient conformation states that heat shock proteins cannot “keep up” during abrupt stress, whereas gradual exposure may allow HSPs to reach higher levels of upregulation during the critical period of high temperature stress (as the qPCR data suggests) such that the negative effects of unfolded proteins are mitigated.

A well-known phenomenon in studies of thermal stress is "hardening," characterized by a plastic response of animals that are able to better survive near-lethal temperatures following prior exposure to a stressful temperature (Bowler 2005; Hoffmann et al. 2013). Hardening commonly refers to a sublethal stress performed well in advance of the second more extreme stress, often 24 hours or more, followed by a recovery period (Loeschcke et al. 1994; Pasparakis et al. 2016). A long recovery period may not be required, as several studies have found evidence for rapid hardening, where exposure to a cold or warm temperature stress as soon as 2 hours prior to the next stress is enough to increase an organism’s survival (Lee et al. 1987, 2006; Dahlgaard et al. 1998). Hardening, especially in the short term, can be linked to the upregulation of key heat shock proteins in a variety of ectotherms (Sconzo et al. 1986; Dahlgaard et al. 1998; Bahrndorff

et al. 2009; Benoit et al. 2011; Hu et al. 2014; Giomi et al. 2016). This appears to be an adaptive mechanism to protect against thermal stress in variable environments similar to what we see during the gradual exposure where maximal upregulation of heat shock proteins during ramping occurred prior to the peak temperature and remained highly upregulated. Thus, the benefits of gradual versus abrupt exposure that we observed (i.e., life history effects) may be a result of a similar frontloading or hardening-like effect despite appearing methodologically different (similarly proposed in Sgrò et al. 2010).

Though all organisms do not share the responses to gradual thermal exposure we observed in *T. californicus*, there are some valuable insights provided by this study that may be widely applicable. First, if the upregulation of key heat shock proteins is indeed responsible for limiting the negative consequences of heat stress, then varying the speed of the ramping rate could have significant effects at the organismal level. The ramping rate we employ (~0.05 °C min^{-1}) is one of the slower rates in the literature (Sgrò et al. 2010; Terblanche et al. 2011; Sørensen et al. 2013) but is consistent with rates of temperature change we observed in natural tidepools. Given that we observed high upregulation of heat shock protein genes well below lethal temperatures, the slow rate of temperature increase appears to have a protective effect. In fact, Sørensen et al. (2013) similarly found that a slower ramping (0.06 °C min^{-1} compared to 0.1 °C min^{-1}) caused higher upregulation of heat shock proteins in *Drosophila melanogaster*. Because the rate we used is closer to the warming rate measured in tidepools, our results may be more informative about the true thermal limits and responses of ectotherms than those of studies that use faster ramping (although faster ramping may be appropriate for intertidal species exposed to air at low tide). Second, we saw consistent developmental rate and HSP responses among the three populations tested following the gradual exposure. As stated previously, these

populations vary widely in response to acute heat stress (Willett 2010; Tangwancharoen and Burton 2014), and they continue to maintain population differences in survivorship in the present study. However, it is notable that we did not see a higher degree of mortality in the more thermally sensitive SCN population, but rather a higher degree of protection, with SCN showing the highest change in survivorship between the abrupt and gradual exposures. The similarity of the effects on each population's life history and gene expression suggests that our findings could apply widely to marine (if not also terrestrial) ectotherms.

The findings of this study indicate that the use of ecologically relevant thermal exposures is essential for a complete understanding of organismal response to thermal stress: all populations of *T. californicus* survived to higher temperatures in our study than had been observed previously. This necessitates a re-defining of the thermal limits of *T. californicus* populations and a reconsideration for how thermal tolerance assays are conducted in this species. Though abrupt exposure thermal assays can still be valuable for answering mechanistic questions regarding variation in thermal tolerance, gradual exposure assays may be able to place laboratory studies in a context more relevant to the organisms in their natural habitats, and to understand how organisms have evolved in response to local thermal regimes. Critically, our results emphasize that ecologically relevant thermal exposures may reveal that organisms can be more resilient than previous studies have suggested.

Literature Cited

Angilletta M.J., R.S. Wilson, C.A. Navas, and R.S. James. 2003. Tradeoffs and the evolution of thermal reaction norms. Trends Ecol Evol.

Arya R., M. Mallik, and S.C. Lakhotia. 2007. Heat shock genes - Integrating cell survival and death. J Biosci.

Bahar M.H., D. Hegedus, J. Soroka, C. Coutu, D. Bekkaoui, and L. Dosdall. 2013. Survival and Hsp70 gene expression in *Plutella xylostella* and its larval parasitoid *Diadegma insulare* varied between slowly ramping and abrupt extreme temperature regimes. PLoS One 8.

Bahrndorff S., J. Mariën, V. Loeschcke, and J. Ellers. 2009. Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. Funct Ecol 23:233–239.

Barreto F.S., S.D. Schoville, and R.S. Burton. 2015. Reverse genetics in the tide pool: Knock-down of target gene expression via RNA interference in the copepod *Tigriopus californicus*. Mol Ecol Resour 15:868–879.

Benoit J.B., G. Lopez-Martinez, K.R. Patrick, Z.P. Phillips, T.B. Krause, and D.L. Denlinger. 2011. Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. Proc Natl Acad Sci 108:8026–8029.

Bermudes M. and A.J. Ritar. 2008. Response of early stage spiny lobster *Jasus edwardsii* phyllosoma larvae to changes in temperature and photoperiod. Aquaculture 281:63–69.

Bjelde B.E., N.A. Miller, J.H. Stillman, and A.E. Todgham. 2015. The role of oxygen in determining upper thermal limits in *Lottia digitalis* under air exposure and submersion. Physiol Biochem Zool 88:483–493.

Bowler K. 2005. Acclimation, heat shock and hardening. J Therm Biol.

Chidawanyika F. and J.S. Terblanche. 2011. Rapid thermal responses and thermal tolerance in adult codling moth Cydia pomonella (Lepidoptera : Tortricidae). J Insect Physiol 57:108–117.

Chown S.L., K.R. Jumbam, J.G. Sørensen, and J.S. Terblanche. 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Funct Ecol 23:133–140.

Clusella-Trullas S., T.M. Blackburn, and S.L. Chown. 2011. Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. Am Nat 177:738–751.

Dahlgaard J., V. Loeschcke, P. Michalak, and J. Justesen. 1998. Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. Funct Ecol 12:786–793.

Dahlhoff E. and G.N. Somero. 1993. Effects of temperature on mitochondria from abalone (genus: *Haliotis*): Adaptive plasticity and its limits. J Exp Biol 185:151–168.

Denny M.W., L.P. Miller, and C.D.G. Harley. 2006. Thermal stress on intertidal limpets: long-term hindcasts and lethal limits. J Exp Biol 209:2420–2431.

Dethier M.N. 1980. Tidepools as refuges: Predation and the limits of the harpacticoid copepod *Tigriopus californicus* (Baker). J Exp Mar Bio Ecol 42:99–111.

Dong Y., T. Ji, X. Meng, S. Dong, and W. Sun. 2010. Difference in Thermotolerance Between Green and Red Color Variants of the Japanese Sea Cucumber , Apostichopus japonicus Selenka : Hsp70 and Heat-Hardening Effect 87–94.

Fangue N.A., J.G. Richards, and P.M. Schulte. 2009. Do mitochondrial properties explain

intraspecific variation in thermal tolerance? J Exp Biol 212:514–522.
Fields P.A., E.M. Burmester, K.M. Cox, and K.R. Karch. 2016. Rapid proteomic responses to a near-lethal heat stress in the salt marsh mussel *Geukensia demissa*. J Exp Biol 219:2673–2686.
Ganz H.H. and R.S. Burton. 1995. Genetic differentiation and reproductive incompatibility among Baja California populations of the copepod *Tigriopus californicus*. Mar Biol 123:821–827.
Giomi F., C. Mandaglio, M. Ganmanee, G.-D. Han, Y.-W. Dong, G.A. Williams, and G. Sara. 2016. The importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster. J Exp Biol 219:686–694.
Gleason L.U. and R.S. Burton. 2015. RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*. Mol Ecol 24:610–627.
Hoffmann A.A., S.L. Chown, and S. Clusella-Trullas. 2013. Upper thermal limits in terrestrial ectotherms: How constrained are they? Funct Ecol 27:934–949.
Hu J. tao, B. Chen, and Z. hong Li. 2014. Thermal plasticity is related to the hardening response of heat shock protein expression in two *Bactrocera* fruit flies. J Insect Physiol 67:105–113.
Jimenez A.G., S. Alves, J. Dallmer, E. Njoo, S. Roa, and W.W. Dowd. 2016. Acclimation to elevated emersion temperature has no effect on susceptibility to acute, heat-induced lipid peroxidation in an intertidal mussel *(Mytilus californianus)*. Mar Biol 163:1–10.
Kelly M.W., M.S. Pankey, M.B. DeBiasse, and D.C. Plachetzki. 2017. Adaptation to heat stress reduces phenotypic and transcriptional plasticity in a marine copepod. Funct Ecol 31:398–406.
Kelly M.W., E. Sanford, and R.K. Grosberg. 2011. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. Proc R Soc B Biol Sci 279:349–356.
Kingsolver J.G. and H.A. Woods. 2016. Beyond thermal performance curves: Modeling time-dependent effects of thermal stress on ectotherm growth rates. Am Nat 187:283–294.
Lee R.E., C.P. Chen, and D.L. Denlinger. 1987. A rapid cold-hardening process in insects. Science 238:1415–7.
Lee R.E., K. Damodaran, S.X. Yi, and G.A. Lorigan. 2006. Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. Cryobiology 52:459–463.
Lima T.G. and C.S. Willett. 2017. Locally adapted populations of a copepod can evolve different gene expression patterns under the same environmental pressures. Ecol Evol 1–14.
Loeschcke V., R.A. Krebs, and J.S.F. Barker. 1994. Genetic variation for resistance and acclimation to high temperature stress in *Drosophila buzzatii*. Biol J Linn Soc 52:83–92.
McMillan D.M., S.L. Fearnley, N.E. Rank, and E.P. Dahlhoff. 2005. Natural temperature variation affects larval survival, development and Hsp70 expression in a leaf beetle. Funct Ecol 19:844–852.
Mitchell K.A. and A.A. Hoffmann. 2010. Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. Funct Ecol 24:694–700.
Nguyen C., H. Bahar, G. Baker, and N.R. Andrew. 2014. Thermal tolerance limits of diamondback moth in ramping and plunging assays. PLoS One 9.
Overgaard J., T.N. Kristensen, and J.G. Sørensen. 2012. Validity of thermal ramping assays used to assess thermal tolerance in arthropods. PLoS One 7:1–7.
Paganini A.W., N.A. Miller, and J.H. Stillman. 2014. Temperature and acidification variability reduce physiological performance in the intertidal zone porcelain crab *Petrolisthes cinctipes*. J Exp Biol 217:3974–3980.

Pasparakis C., B.E. Davis, and A.E. Todgham. 2016. Role of sequential low-tide-period conditions on the thermal physiology of summer and winter laboratory-acclimated fingered limpets, *Lottia digitalis*. Mar Biol 163:1–17.

Pereira R.J., F.S. Barreto, and R.S. Burton. 2014. Ecological novelty by hybridization: Experimental evidence for increased thermal tolerance by transgressive segregation in *Tigriopus californicus*. Evolution (N Y) 68:204–215.

Pörtner H.-O. 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213:881–893.

Pörtner H.O., A.F. Bennett, F. Bozinovic, A. Clarke, M.A. Lardies, M. Lucassen, B. Pelster, et al. 2006. Trade-offs in thermal adaptation: The need for a molecular to ecological integration. Physiol Biochem Zool 79:295–313.

Roberts S.D., C.D. Dixon, and L. Andreacchio. 2012. Temperature dependent larval duration and survival of the western king prawn, *Penaeus (Melicertus) latisulcatus* Kishinouye, from Spencer Gulf, South Australia. J Exp Mar Bio Ecol 411:14–22.

Runcie D.E., D.A. Garfield, C.C. Babbitt, J.A. Wygoda, S. Mukherjee, and G.A. Wray. 2012. Genetics of gene expression responses to temperature stress in a sea urchin gene network. Mol Ecol 21:4547–4562.

Schmittgen T.D. and K.J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108.

Schoville S.D., F.S. Barreto, G.W. Moy, A. Wolff, and R.S. Burton. 2012. Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. BMC Evol Biol 12:170.

Sconzo G., M. Roccheri, M. La Rosa, D. Oliva, A. Abrignani, and G. Giudice. 1986. Acquisition of thermotolerance in sea urchin embryos correlates with the synthesis and age of the heat shock proteins. Cell Differ 19:173–177.

Sgrò C.M., J. Overgaard, T.N. Kristensen, K.A. Mitchell, F.E. Cockerell, and A.A. Hoffmann. 2010. A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of *Drosophila melanogaster* from Eastern Australia. J Evol Biol 23:2484–2493.

Sobek S., A. Rajamohan, D. Dillon, R.C. Cumming, and B.J. Sinclair. 2011. High temperature tolerance and thermal plasticity in emerald ash borer *Agrilus planipennis*. Agric For Entomol 13:333–340.

Somero G.N. 2005. Linking biogeography to physiology: Evolutionary and acclimatory adjustments of thermal limits. Front Zool 2:1.

Somero G.N. 2012. The Physiology of Global Change: Linking Patterns to Mechanisms. Ann Rev Mar Sci 4:39–61.

Sørensen J.G., V. Loeschcke, and T.N. Kristensen. 2013. Cellular damage as induced by high temperature is dependent on rate of temperature change - investigating consequences of ramping rates on molecular and organismal phenotypes in *Drosophila melanogaster*. J Exp Biol 216:809–814.

Sunday J.M., A.E. Bates, and N.K. Dulvy. 2012. Thermal tolerance and the global redistribution of animals. Nat Clim Chang 2:686–690.

Tangwancharoen S. and R.S. Burton. 2014. Early life stages are not always the most sensitive: Heat stress responses in the copepod *Tigriopus californicus*. Mar Ecol Prog Ser 517:75–83.

Terblanche J.S., J.A. Deere, S. Clusella-Trullas, C. Janion, and S.L. Chown. 2007. Critical

thermal limits depend on methodological context. Proc R Soc B Biol Sci 274:2935–2943.
Terblanche J.S., A.A. Hoffmann, K.A. Mitchell, L. Rako, P.C. le Roux, and S.L. Chown. 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. J Exp Biol 214:3713–3725.
Tomanek L. and M.J. Zuzow. 2010. The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. J Exp Biol 213:3559–3574.
Vasseur D.A., J.P. Delong, B. Gilbert, H.S. Greig, C.D.G. Harley, K.S. Mccann, V. Savage, et al. 2014. Increased temperature variation poses a greater risk to species than climate warming Increased temperature variation poses a greater risk to species than climate warming.
Vergara-Amado J., A.X. Silva, C. Manzi, R.F. Nespolo, and L. Cárdenas. 2017. Differential expression of stress candidate genes for thermal tolerance in the sea urchin *Loxechinus albus*. J Therm Biol 68:104–109.
Willett C.S. 2010. Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. Evolution (N Y) 64:2521–2534.

Table 1. Primer sequences for *Tigriopus californicus* genes used in qPCR (Barreto *et al.* 2015).

Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Product size**
hsp60	GAGATGTTGATTGGCGTGGAC	CACATCTTGGACGAGTTTGGC	189
hsp70	CTGGATTGATGCTCTTGTTCA	CTCTGTGCCGACCTTTTCC	184
hsp beta-1	CGATTTTCATCTGGGTCTCAA	TTGAAGAACTCCTCCGCTGT	175
myosin	GTGTCGCAAAAGCAAATGAC	GAACCTCAACCTCCTCCTCA	154
GAPDH	GGAGGAGGGGATGATGTTTT	CAACCACGAGCAATACGAGA	226

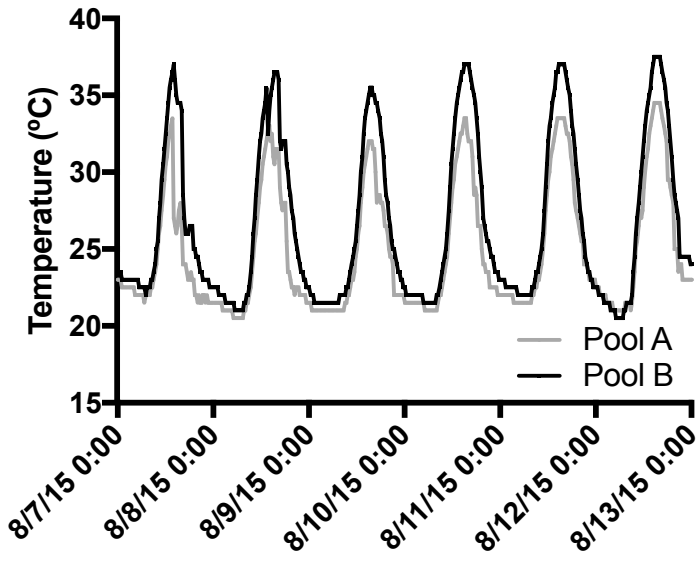

Figure 1. iButton Data Logger temperature measurements. iButton loggers were placed in two separate pools at the SD location. Temperature was recorded every 20 minutes for one week in August 2015.

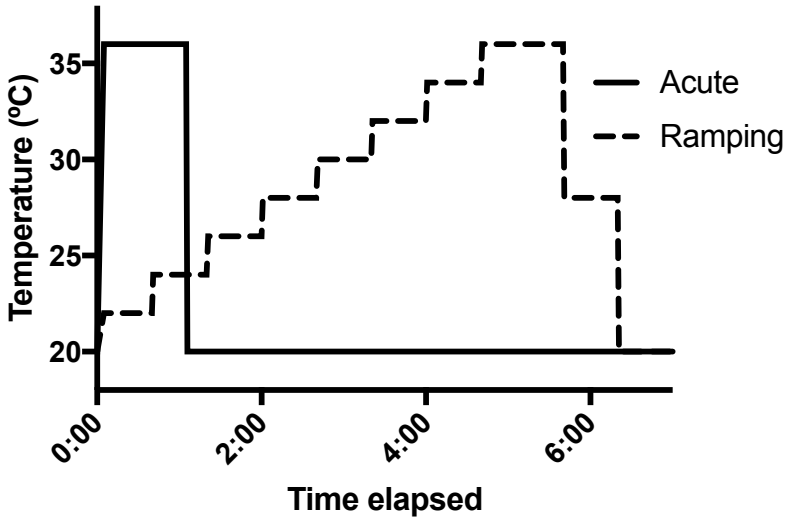

Figure 2. Acute vs. ramping thermal stress protocol. Acute stress, represented by the solid line, places animals directly into a water bath set to the given temperature (in this case 36 ºC). Due to the small volume of the tubes into which copepods are placed, water in the tube reaches the high temperature within a minute. Ramping stress, represented by the dashed line, involves placing animals in microcentrifuge tubes into a thermal cycler, which raises the temperature by 2 ºC every 40min, and for 1h at the peak temperature. They are then quickly ramped back down by 8 ºC every 40min.

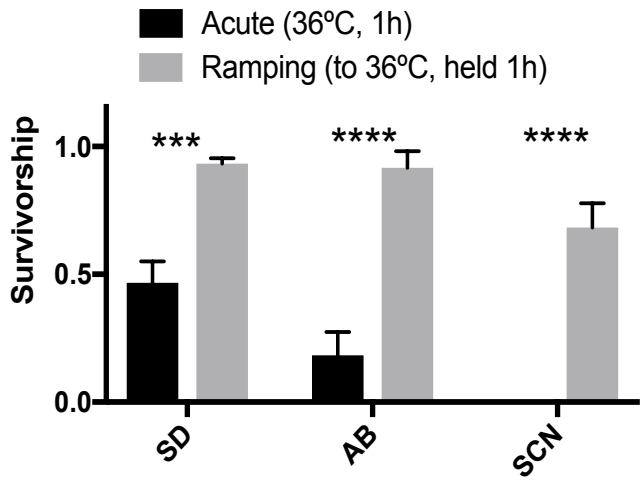

Figure 3. Survivorship (±1 SE) following acute and ramping thermal stress. Six tests with ten adult animals each were performed for each treatment. A two-way ANOVA showed a significant effect of population ($F_{2,30}$= 13.37) and treatment ($F_{1, 30}$ = 122.1). A Sidak's multiple comparisons test found a significant difference between survivorship following acute and ramping stress in SD ($p = 0.0001$), AB ($p< 0.0001$), and SCN ($p< 0.0001$).

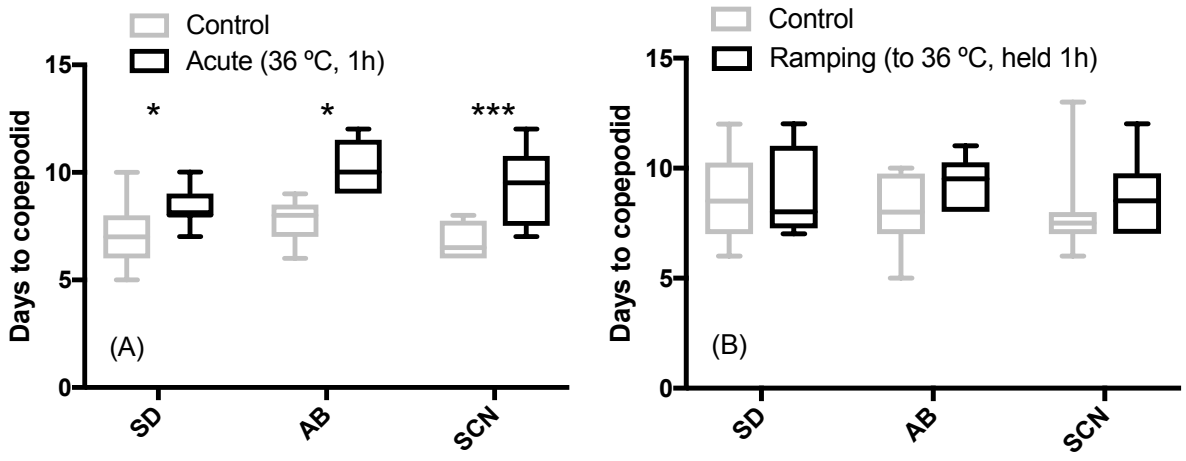

Figure 4. Effects of differing heat stress protocols on developmental rate of nauplii. Broods were split into control and heat stress, either acute (A) or ramping (B). Days to reach first copepodid were counted. For the acute stress, a two-way ANOVA showed a significant effect of population ($F_{3, 46} = 2.863$) and treatment ($F_{1, 46} = 16.31$). A multiple comparisons test found a significantly slower developmental rate following heat stress compared to control in SD ($p = 0.0266$), AB ($p = 0.0150$), and SCN ($p = 0.0005$). No significant difference in developmental rate was found between ramping heat stress and control ($F_{1, 28} = 0.1894$, $p = 0.67$).

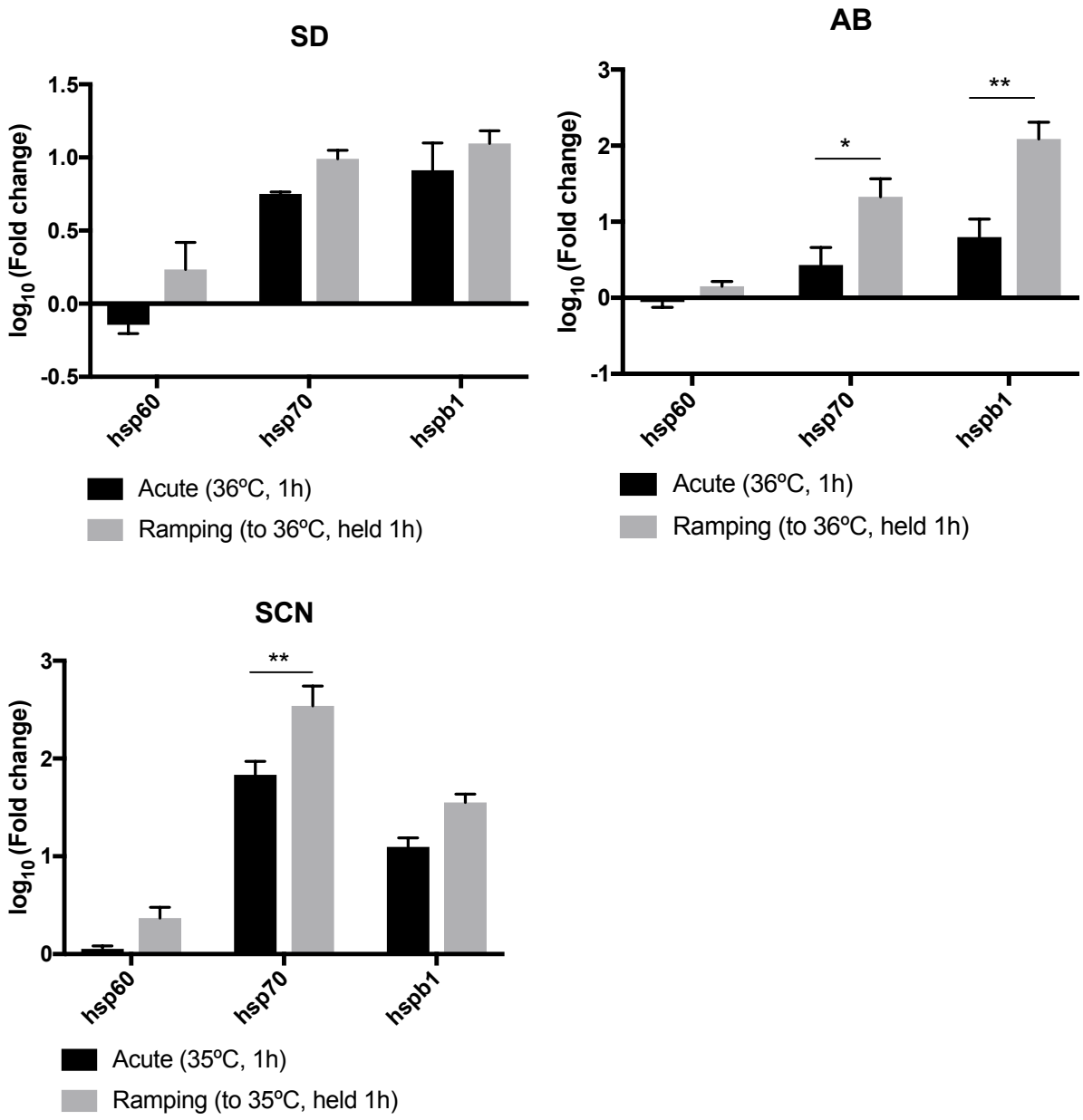

Figure 5. Gene expression following thermal stress. For each of three genes, log fold change (±1 SE) was calculated comparing either ramping or acute stress (up to 1 ºC below the population's lethal limit: 35 ºC for SCN and 36 ºC for SD and AB) to a control group. In all cases ramping appeared to produce higher upregulation than acute stress. Significance was assessed using ANOVA followed by Sidak's multiple comparisons test; the effect of treatment was significant for all populations (SD: $F_{1,12,3} = 7.636$, $P = 0.0172$; AB: $F_{1,12,3} = 25.58$, $P = 0.0003$; SCN: $F_{1,12,3}$

= 24.45, $P = 0.0003$). Ramping caused significantly higher upregulation for *hsp70 and hspb1* in AB ($P = 0.0192$ and 0.0015). In SCN, *hsp70* was significantly upregulated following ramping ($P = 0.0044$) and *hspb1* was marginally non-significant ($P = 0.0641$).

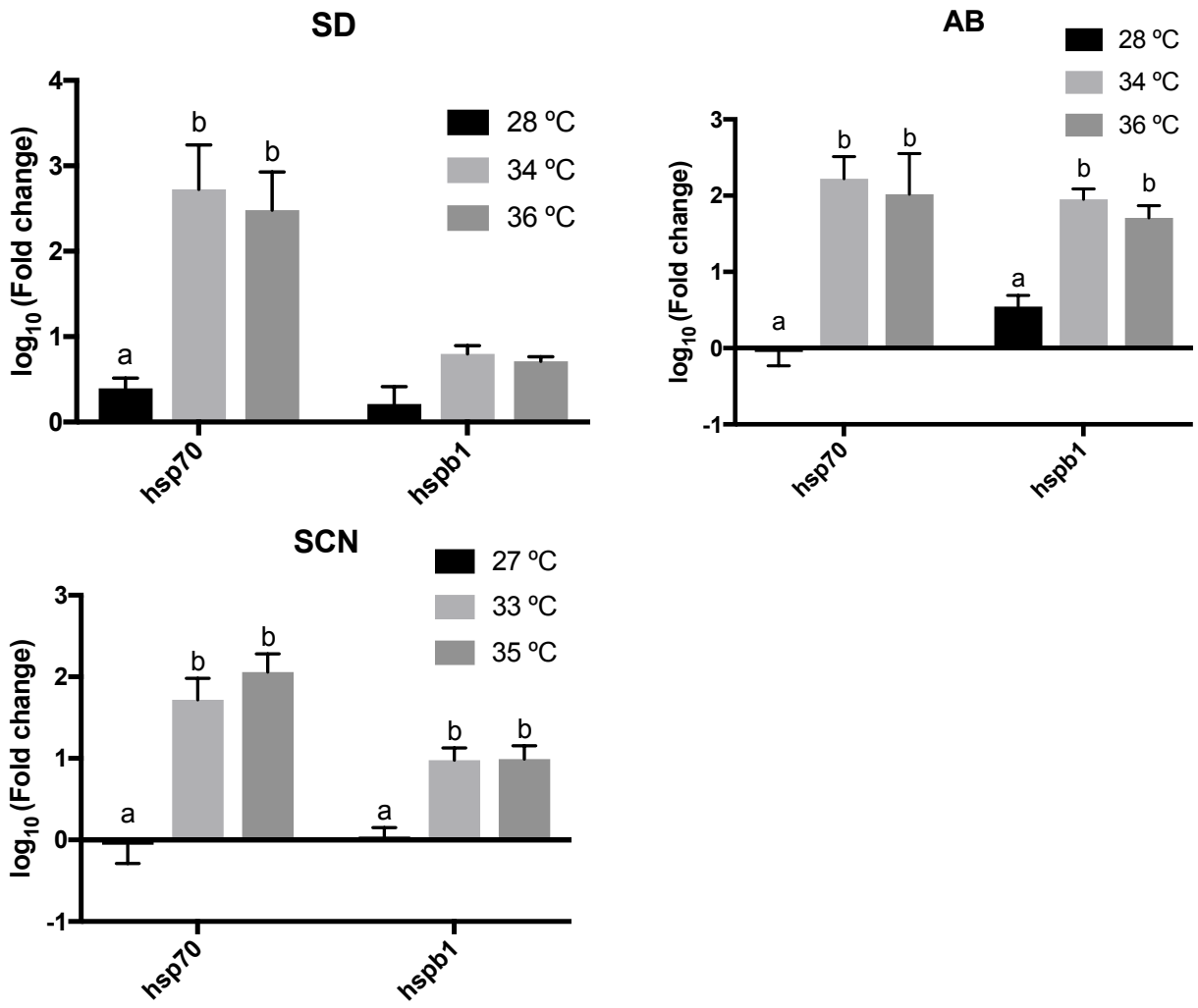

Figure 6. Gene expression during ramping stress. For each of three genes, log fold change (±1 SE) was calculated comparing three temperatures during ramping stress (27 ºC for SCN and 28 ºC for SD/AB; 33 and 34ºC; and 35/36 ºC) to a control group. In each population there was significantly higher upregulation of at least one of the genes from the low (27/28 ºC) to the higher temperature (33/34 ºC). Significance was assessed using ANOVA followed by Tukey's HSD; the effect of temperature was significant for all populations (SD: $F_{2,12,3} = 14.36$, $P = 0.0007$; AB: $F_{2,12,3} = 25.57$, $P < 0.0001$; SCN: $F_{2,12,3} = 36.03$, $P < 0.0001$). In SD, 34 and 36 ºC caused significant upregulation of *hsp70* from 28 ºC ($P = 0.0004$ and 0.0009). The same was true

in the AB population for both *hsp70* ($P = 0.0003$ and 0.0006) and *hspb1* ($P = 0.0102$ and 0.0310) and in the SCN population (*hsp70:* $P = 0.0001$ and <0.0001; *hspb1:* $P = 0.0136$ and 0.0125).