Modulation of the heat shock response is associated with acclimation to novel temperatures but not adaptation to climatic variation in the ants *Aphaenogaster picea* and *A. rudis*

Sara Helms Cahan^a, Andrew D. Nguyen^a, John Stanton-Geddes^a, Clint A. Penick^b,
Yainna Hernáiz-Hernández^a, Bernice B. DeMarco^c, Nicholas J. Gotelli^a

^a Department of Biology, University of Vermont, Burlington, VT 05405 USA

^b Department of Applied Ecology and Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC 27695 USA

^c Department of Entomology, Smithsonian Institution, Washington DC 20013 USA.

Corresponding author: Sara Helms Cahan, scahan@uvm.edu

Abstract

Ecological diversification into thermally divergent habitats can push species toward their physiological limits, requiring them to accommodate temperature extremes through plastic or evolutionary changes that increase persistence under the local thermal regime. One way to withstand thermal stress is to increase production of heat shock proteins, either by maintaining higher baseline abundance within cells or by increasing the magnitude of induction in response to heat stress. We evaluated whether environmental variation was associated with expression of three heat shock protein genes in two closely-related species of woodland ant, *Aphaenogaster picea* and *A. rudis*. We compared adult workers from colonies collected from 25 sites across their geographic ranges. Colonies were maintained at two different laboratory temperatures, and tested for the independent effects of environment, phylogeny, and acclimation temperature on baseline and heat-induced gene expression. The annual maximum temperature at each collection site (Tmax) was not a significant predictor of either baseline expression or magnitude of induction of any of the heat shock protein genes tested. A phylogenetic effect was detected only for basal expression of *Hsp40*, which was lower in the most southern populations of *A. rudis* and higher in a mid-range population of possible hybrid ancestry. In contrast, a higher acclimation temperature significantly increased baseline expression of *Hsc70-4*, and increased induction of *Hsp40* and *Hsp83*. Thus, physiological acclimation to temperature variation appears to involve modulation of the heat shock response, whereas other mechanisms are likely to be responsible for evolutionary shifts in thermal performance associated with large-scale climate gradients.

63 Key Words: Heat shock proteins, Ants, Heat Shock Response, Hsc70-4, Hsp70, Hsp90,
64 Hsp40

65

66 Abbreviations:

67 Hsp, heat shock protein

68 *Hsp*, heat shock protein gene

69 CSR, cellular stress response

70 Tmax, annual maximal temperature at the site of collection (Bioclim variable 5)

71

Introduction

Temperature is a primary determinant of the geographic distributions of species and an important selective force shaping organismal phenotypes (Cahill et al., 2014; Diamond et al., 2012; Sunday et al., 2012). For small-bodied ectotherms such as insects, whose body temperatures track external temperatures, maintaining a suitable internal temperature can be challenging in the face of environmental variation (Stevenson 1985). Excessively high temperatures can lead to protein denaturation, loss of membrane integrity, neuronal failure, and death (White et al., 2007). To cope with such stressors, organisms can behaviorally avoid thermal extremes or deploy a number of physiological response mechanisms that prevent or repair heat-related damage (Feder and Hoffmann, 1999). One of the oldest and most conserved is the Cellular Stress Response (CSR), an inducible reaction to perturbation and cellular damage characterized by production of heat shock proteins (Hsps) — chaperone molecules that protect other proteins from damage, refold those that have denatured, and help to dispose of irreversibly damaged proteins (Lindquist, 1986; Parsell and Lindquist, 1993).

Because stress response mechanisms such as the CSR are structurally and metabolically costly (Fitzgerald-Dehoog et al., 2012; Hoekstra and Montooth, 2013), the extent to which they are employed is expected to track the risk of exposure to temperature extremes. Individuals may respond plastically to changes in temperature over time, enhancing Hsp production when acclimated to conditions in which they are more likely to encounter stress (Fader et al., 1994; Hu et al., 2014). When thermal regimes vary across space, such as along latitudinal or altitudinal gradients, divergent

95 selection pressures acting on populations at different locations may promote evolutionary
96 changes instead of or in addition to an acclimation response (Addo-Bediako et al., 2000;
97 Sheth and Angert, 2014). In more extreme environments, populations are likely to
98 frequently face conditions at or beyond their physiological limits, selecting for increased
99 constitutive expression or enhanced inducibility of chaperone molecules (Barshis et al.,
100 2013; Bettencourt and Feder, 2001). Under milder conditions, however, the cost of
101 maintaining a response mechanism may lead to reduction or loss of the capacity to
102 respond to temperature extremes (Silbermann and Tatar, 2000). Moreover, if increasing
103 cold-tolerance comes at the expense of heat-tolerance (Rodriguez-Verdugo et al., 2014),
104 the entire thermal niche may be forced downward as populations approach their lower
105 thermal range limit (Addo-Bediako et al., 2000; Anderson et al., 2003).

106 Determining whether variation in thermal physiology is plastic or evolved is critical
107 for estimating the thermal safety margin of populations facing future changes in climate
108 (Sawall et al., 2015). If physiological coping mechanisms have been evolutionarily lost at
109 the local level, populations may have significantly lower resilience than that suggested by
110 the thermal range across which the species as a whole occurs (Martinez et al., 2015; Rojo
111 et al., 2015). Yet most studies of natural systems investigating evolutionary adaptation of
112 the CSR have compared only a small number of populations or species from discrete
113 habitat types (e.g., Koenigstein et al., 2013; Palumbi et al., 2014; Tedeschi et al., 2016).
114 Variation in Hsp expression patterns across continuous gradients in temperature have
115 been less frequently investigated (Mizrahi et al., 2015; Schoville et al., 2012).

116 The ant genus *Aphaenogaster* is common in deciduous forests in eastern North
117 America (Lubertazzi, 2012). The eastern clade of this genus, which includes 13

morphologically delimited species, underwent a recent radiation within the last 5my associated with post-glacial range expansion (Demarco and Cognato, 2016). Despite their recent genetic divergence, however, species of *Aphaenogaster* occupy a wide range of thermal environments, with mean annual temperature varying from 5 to 22°C. Environmental temperature is correlated with species occurrence at regional biogeographic scales and along local elevational gradients (Warren and Chick, 2013). Evidence from the warm-edge boundary of the geographic range of *A. rudis* suggests that it is sensitive to upper thermal extremes: in a controlled field experiment, the incidence of *A. rudis* declined in heated environmental chambers (Pelini et al., 2014, Diamond et al., 2016).

In this study, we compared the expression patterns of heat shock proteins in two widespread species of *Aphaenogaster*, the cool-climate *A. picea* and the warm-climate *A. rudis*, whose geographic ranges overlap broadly in the southern and central Appalachian Mountains of the eastern United States. We collected colonies of the two species from 25 sites along a latitudinal transect, spanning an 8.3°C gradient in maximal annual temperature (Table S1). To evaluate the acclimation response in addition to population/species-level evolutionary divergence in the CSR, we maintained colonies from these sites at two different common-garden rearing temperatures. Workers were assessed for baseline Hsp expression and induction in response to static sublethal heat shock for three stress-inducible heat shock protein genes whose expression patterns are associated with variation in thermal tolerance in ants: *Hsp40*, *Hsc70-4 h2* (one of two heat-inducible genes in the Hsp70 gene family in ants), and *Hsp83* (Gehring and Wehner, 1995; Nguyen et al., 2016).

Methods

Field collections and lab acclimation

Aphaenogaster colonies were collected in the summer of 2013 at 25 sites along the eastern seaboard of the US (Fig. 1A; Table S1). Whole colonies were collected by overturning rocks and breaking open downed twigs and logs to locate colonies, and aspirating into a collection chamber the queen and as many individual adults and brood of all life stages as could be collected in ~5 minutes. Voucher specimens were pinned for each colony and identified to species by a single taxonomic expert (B. DeMarco).

Morphological criteria used to identify *A. picea* include a blackish-brown body, medium-length spines (two-thirds of the length of the thoracic declivity) and lighter color of the last four antennal segments. In contrast, *A. rudis* is characterized by a reddish-brown body, short spines (less than half the length of the thoracic declivity) and uniformly colored antennal segments. All taxonomic identifications were made without knowledge of the collection locale.

One to four colonies per site were established between July 9 and July 29, 2013 in common-garden laboratory conditions at North Carolina State University. All existing eggs, larvae and pupae were removed from each colony to prevent mixing of field and laboratory-reared individuals in gene-expression experiments, and the remaining adults were housed in plastic nest boxes with a plaster floor in which two 16 × 150mm test tubes partially filled with water that was plugged by a cotton ball were provided as brood chambers. The plaster in each nest was moistened daily with distilled water to

maintain humidity. We fed colonies an artificial diet designed specifically for ants (Bhatkar and Whitcomb, 1970) that we changed 3 times per week and supplemented with freeze-killed beetle larvae (*Zophobas morio*) and vials containing 20% sucrose solution.

One to two replicate colonies from each site were assigned randomly to low rearing-temperature (20°C) and high rearing-temperature (26°C) treatments; sites from which only a single colony was available were assigned randomly to a single temperature treatment. Thirty-three colonies were reared at 20° C and 24 colonies were reared at 26° C, for a total of 57 colonies. The lower of these temperatures represents a mid-point value of the mid-summer nest temperatures likely to be encountered across the collection sites based on field measurements (17-23°C, C. Penick, unpublished field measurements); both rearing temperatures are well below the threshold temperature at which any of Hsp genes assayed is activated in response to acute heat stress in adult *A. picea* workers (35°C; Nguyen et al., 2016).

Genotyping by double digested restriction site-associated DNA sequencing (ddRADseq)

To control for the effect of shared ancestry on phenotypic trait values, we conducted SNP discovery and genotyping using double-digest RADseq (ddRADseq), and used the resulting SNP genotypes to reconstruct genetic relationships among the experimental colonies. Genomic DNA was extracted from tissue of a single worker from each colony with the Qiagen DNAeasy kit according to the manufacturer's instructions. Ant tissue was homogenized prior to extraction with ~20 1.4mm zirconium silicate beads in 200ul chilled ATL buffer for three minutes in a Next Advance Bullet blender spinning

at maximum speed. A single worker sample of *A. fulva* was also extracted as an outgroup.

Samples were genotyped in pooled sets of 48 individually-barcoded ddRADseq libraries constructed from 100-200ng of genomic DNA per individual following the protocol of Recknagel et al. (2013). Briefly, samples were double-digested with the restriction enzymes NlaIII and MluCI at 37°C for three hours, purified using a 1.5X concentration of AMPure purification beads (Beckman Coulter Life Sciences, USA), and quantified with a Qubit analyzer. The purified samples were ligated to barcoded P1 and universal P2 adaptors, and sample concentrations were normalized by pooling 40ng of each sample into a single library. Three hundred microliters of the pooled library was purified with a 1X AMPure bead purification eluted into 30ul of Qiagen AE buffer. The ligated fragments were amplified in seven 20ul PCR reactions containing approximately 20ng of DNA with the PhusionTaq PCR kit. The appropriate number of PCR cycles was determined empirically by comparing amplification intensities of 11, 13 and 15 cycles; the final library was constructed using 13 cycles. The combined PCR reactions were pooled and purified with 1.5X AMPure beads into a final volume of 30ul. Fragments 300-400bp in total length were size-selected from a 1.5% agarose gel and extracted with the QIEX II gel extraction kit. Library size range and quality were verified on a Bioanalyzer and with kapa qPCR. Each library was single-end sequenced in a single HiSeq 2000 rapid-run lane at the University of Vermont Advanced Genome Technologies Core facility, yielding approximately 2.5 million reads per sample.

Sequences were demultiplexed using the program *sabre* (<https://github.com/najoshi/sabre>), allowing for up to a single base pair mismatch in the

barcoding sequence, and the restriction site sequence was trimmed. The total length of all sequences was trimmed to 90bp with the fastx_trimmer tool in the FASTX Toolkit v. 0.0.14, and low-quality reads, defined as those whose quality score dropped below 10 at any point along the sequence, were excluded from downstream analysis with the fastq_trimmer tool.

Because there is no sequenced genome available for the genus *Aphaenogaster* or closely related ant genera, we used a reference set of five samples from across the geographic extent of the transect to identify a repeatable subset of loci exhibiting Mendelian genotypes, which was then used as a reference against which the complete sample set was mapped and genotyped. The reference sample sequences were assembled into homologous tags using the denovo.pl pipeline in STACKS. We retained those tags for which a) there were from zero to three SNPs present across the five samples, b) all five samples contained one or more reads at the tag, c) all SNPs were biallelic, and d) all samples contained no more than two alternate haplotypes. The consensus sequences of the 61,518 retained tags were assembled into a FASTA reference file, and the quality-filtered sequence reads of all samples including the reference samples were mapped against the reference with Bowtie. SNP genotypes were identified by assembling the mapped reads into stacks using the ref_map.pl pipeline in STACKS with a minimum of two reads to call a genotype. For each sample, the SNP genotypes across tags were concatenated into a single pseudo-sequence that was used for all downstream biogeographic analyses.

Heat shock treatment

Workers from all colonies were subjected to static heat shock on September 3-4 2013, 5-8 weeks after establishment of field-collected colonies in common-garden conditions. At this stage, the first cohort of laboratory-produced progeny had not yet eclosed as adults, so all experimental animals had experienced at least some exposure to field conditions as adults prior to common-garden acclimation.

For each colony, two groups of four workers were sampled haphazardly from the colony. Each group was placed into a single 45mm screw-cap glass vial and fully submerged in either a baseline temperature (25 °C) or a sublethal heat shock (37°C) circulating water bath treatment. After 1 hour, the ants were flash-frozen in liquid nitrogen and stored at -80 °C for gene expression quantification.

Quantifying Hsp gene expression

RNA was isolated from each set of four pooled ants using the RNeasy micro kit (Qiagen, USA) following the manufacturer's instructions. 50ng of RNA was converted to cDNA using the High Capacity cDNA reverse transcription kit (Life Technologies, USA). We quantified gene expression using quantitative PCR for three heat shock protein genes (*Hsp83*, *Hsc70-4 h2*, and *Hsp40*; Table 1) and one house-keeping gene (18s rRNA) whose standard deviation in expression was confirmed to be below 2 (Dheda et al., 2004), run in duplicates on an ABI StepOnePlus Real-Time PCR machine. Each reaction was comprised of 2ng of template cDNA, 500 nM of total primers, and 10uL of Power SYBR® Green Master Mix (Life Technologies, USA), with a cycling program of heating at 95 °C for 10 minutes, and then 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds,

and acquisition of fluorescence at 70 °C for 60 seconds. We performed a melt-curve analysis and sequenced a subset of samples to verify specificity.

Gene expression was quantified using the $\Delta\Delta$ CT method (Livak and Schmittgen, 2001). For basal expression, we used the mean CT values across all samples for each Hsp and housekeeping gene treated at 25 °C as the reference to calculate relative quantity for each sample. To quantify induction of each gene, we calculated fold-increase as the CT values at 37 °C relative to CT values at 25 °C for each colony.

Statistical Analyses

We determined the effects of shared ancestry, thermal niche, and acclimation temperature on baseline expression and fold-induction of each of the three heat shock protein genes with linear regressions performed in R. Phylogenetic relationships were estimated with RaxML 8 (Stamatakis, 2014) following a GTR+Gamma substitution model; group support was evaluated with 100 fast bootstrap replicates. To convert phylogenetic relationships into continuous variables suitable for a regression model, we decomposed the phylogenetic distances with a principal coordinate analysis with the *ape* statistical package (Paradis et al., 2004), which produces orthogonal eigenvectors that capture different nodes across the tree (Fig. S1; Diniz et al., 1998). The first three eigenvectors corresponded to the three deepest nodes with 100% bootstrap support (Fig. 1); all three were included in the full models, but because Axis 1 was highly correlated with Tmax (correlation coefficient = 0.836), and therefore collinearity could have prevented detection of an effect of environment, we also conducted the analyses without Axis 1. The results were identical, and only the full model results are presented here.

Because the heat shock response is expected to impact fitness most strongly during temperature extremes, we used annual thermal maximum (Tmax) at the collection site as a proxy of field thermal conditions expected to impose selective pressure on expression parameters. Substituting mean annual temperature (MAT) for Tmax produced identical statistical results. Tmax values were extracted from the bioclim database (<http://www.worldclim.org/bioclim>). To avoid over-fitting, we used forward selection of variables based on AIC.

Results

A total of 80,847 SNPs from 42,655 unique tags were discovered across the set of 57 ingroup colonies and the outgroup (*A. fulva*). As expected from morphological identification, the majority of the ingroup colonies separated with 100% bootstrap support into the two nominal species with overlapping geographic ranges: *A. picea* (n=42 colonies), which was the predominant species at northern latitudes and extended as far south as North Carolina at higher elevations, and *A. rudis* (n=12), whose range extended from the southern edge of Pennsylvania to Georgia (Fig. 1). Two well-supported clades within *A. rudis* associated with geography were evident in the SNP-based phylogenetic reconstruction: the southern clade included all samples from the two southernmost sites in Georgia and central North Carolina, whereas the northern clade encompassed the remaining northern and western sites in Pennsylvania, North Carolina and Tennessee.

The southern clade included one sample from North Carolina (UN3) identified morphologically as *A. carolinensis*. A third clade basal to both species was comprised of two samples from site NK in central Pennsylvania as well as a single sample from site

RW in upstate New York; two of these samples (NK 1 & NK2) were morphologically intermediate between *A. picea* and *A. rudis*, with the darker body color characteristic of *A. picea* but lacking pale antennal tips. The third specimen (RW1) was identified as *A. picea*. Principal Coordinate analysis produced three eigenvectors, representing 55.4% of the total genetic variation, corresponding to the nodes splitting *A. picea* from *A. rudis* (axis 1), the basal clade (axis 2), and the north-south split within *A. rudis* (axis 3; Fig. 1B).

All three heat shock protein genes (*Hsp83*, *Hsc70-4*, and *Hsp40*) were successfully amplified by qPCR in all samples. Induction under heat shock varied significantly among the three genes, with a 50.2-fold mean increase in expression of *Hsc70-4*, while *Hsp83* and *Hsp40* were induced 11.2 and 10.5-fold, respectively (ANOVA; $F_{2,165}=102.3$; $p < 0.001$). Although the collection sites varied in Tmax from 25-33°C, there was no relationship between Tmax and either basal expression or extent of induction for any of the genes (Table 2, Fig. 2). Phylogeny was associated with Hsp gene expression only for *Hsp40*, for which basal expression was significantly lower in the southern clade of *A. rudis* than in all other clades, while the basal clade was significantly higher overall but did not differ significantly from either *A. picea* or the northern clade of *A. rudis* in post-hoc pairwise comparisons (Fig. 3).

Laboratory acclimation temperature, in contrast, had multiple significant effects on gene expression patterns (Table 2). Basal expression of *Hsc70-4* was significantly higher when colonies were reared at 26°C than at 20°C (Linear regression, $t_{55} = 6.75$, $P < 0.001$, Fig. 4C), and higher temperature increased the magnitude of induction in response to heat shock in *Hsp 40* ($t_{53} = 2.53$, $P < 0.05$) and *Hsp83* ($t_{55} = 2.10$, $P < 0.05$; Fig. 4F).

Discussion

Aphaenogaster picea and *A. rudis* occupy broad but predominantly non-overlapping thermal environments along the eastern seaboard of North America. In areas of overlap, the two species differ by 2°C in critical thermal maxima and minima (Warren and Chick, 2013), suggesting that selection on thermal limits may be driving evolutionary divergence in the heat shock response. Despite this variation, however, we found little evidence of evolved divergence in expression profiles of three heat shock proteins. Instead, Hsp gene expression was phenotypically plastic: when reared at very warm temperatures, both species increased baseline and induced expression of interacting genes of the response pathway.

Comparative data from other taxa have shown an association between environmental temperature and either higher constitutive expression of Hsp genes (Bedulina et al., 2010; Li et al., 2014; Mizrahi et al., 2016; Shatilina et al., 2011) or a stronger induced response (Chandrakanth et al., 2015; Dong and Williams, 2011; Haguenaue et al., 2013; Li et al., 2014; Schoville et al., 2012; Shatilina et al., 2011; Tedeschi et al., 2016; Yu et al., 2012). The comprehensive sampling in this study of populations experiencing a wide range of T_{max} values should have provided ample power to detect a cline in gene expression if it were present, but T_{max} was not related to either constitutive or induced expression in any of the three Hsp genes (Fig. 2).

If *A. picea* and *A. rudis* have diverged in their upper thermal limits, why is this not reflected in Hsp expression? It is possible that the difference is produced by aspects of the heat shock response other than those measured in this study; response to heat stress

and damage is a dynamic process, and the static heat shock treatment used here cannot reveal variation in other important parameters such as temperature of activation, speed of upregulation, or duration of maximal response, all of which are known to vary among species as a function of thermal tolerance (Feder and Hofmann, 1999).

It is also possible that the heat shock response may not be the most important factor conferring the specific type of thermal tolerance quantified in previous studies. The Critical Thermal maximum (CT_{max}) can be measured in a number of different ways that are often not strongly correlated with one another, suggesting different underlying mechanisms (Blackburn et al., 2014; Terblanche et al., 2011). Previous comparisons between *Aphaenogaster* species have used a rapid-heating protocol (1°C min⁻¹; Warren and Chick, 2013); activation of the Hsp response requires time for mRNA production and protein synthesis, so it is perhaps not surprising that it would not underlie tolerance in our static heat shock assay (Sorensen et al., 2013). Moreover, rapid heating mimics the acute stressors likely to be encountered by foraging workers, which tend to be older and more resource-starved (Dussutour et al., 2016) and may be limited in their ability to mount a robust response (Verbeke et al., 2001). Thus, lower-cost structural adaptations that reduce susceptibility rather than enhanced damage repair may be selected for as the primary mechanism of acute stress resistance in ground-foraging ants.

The heat shock response may be more important under slow-heating or extended sub-lethal conditions, such as might be encountered over the course of an entire day or during a longer period of high temperature (Overgaard et al., 2012; Sorensen et al., 2013). In social insects such as ants, the subterranean nest may act as an effective buffer to these types of temperature extremes, weakening selection on Hsp-mediated thermal

limits. Both *A. picea* and *A. rudis* make their nests in rotting logs and underground, and colonies regularly move their nests short distances in response to temperature and humidity changes. Preliminary data suggest that CT_{max} measured with a slow-ramp protocol (0.1°C min⁻¹) in fact does not vary across this set of species and populations (A. Nguyen, unpublished data).

It is important to note that environment and phylogeny at least partially covary: thermal variation is arrayed along a North-South axis, leading to greater geographic distance and potential for genetic isolation between more thermally divergent populations. Because *A. picea* occupies significantly cooler habitats than does *A. rudis*, and the two clades of *A. rudis* correspond to geographically northern (and cooler) and southern (warmer) regions, adaptive responses to temperature associated with these historical shifts into novel thermal niches are more likely to be captured by their corresponding phylogenetic axes than by T_{max}. Although most of the Hsp expression properties showed no phylogenetic signal, the basal level of *Hsp40* varied significantly along two of the three phylogenetic axes.

Interestingly, however, this effect was in the opposite direction than that expected from environmental selection: the most southern clade of *A. rudis*, which experiences the warmest temperatures, had significantly lower *Hsp40* basal expression than the more northern clades. This pattern of basal expression matches the results of a much more phylogenetically distant comparison between warm- and cool-climate ants (Nguyen et al. 2016). Reduction in *Hsp40* production in the southern *A. rudis* clade may be indicative of a shift in investment to alternate, non-Hsp mediated mechanisms of resistance to chronically extreme temperatures. Although evolutionary enhancement of the heat shock

response in more thermally tolerant species has been documented in a number of systems, the most common pattern associated with adaptation to higher temperatures is an upward shift of the Hsp activation temperature, suggesting that in such species the enzymes that Hsps protect are less susceptible to damage (Gehring and Wehner, 1995; Hofmann and Somero, 1996; Mizrahi et al., 2012; Tomanek and Somero, 1999).

Similarly, some comparative studies have shown reduced, rather than enhanced, Hsp activity associated with more extreme environments (Bedulina et al., 2013; Bedulina et al., 2010; Hu et al., 2014; Narum et al., 2013). Production and maintenance of a standing stock of Hsp proteins are likely to be costly, both because of direct effects of cytoplasmic Hsp chaperones on cellular functions (Krebs and Feder, 1997, 1998b) and because of energetic costs that reduce available resources (Krebs and Bettencourt, 1999; Krebs and Feder, 1998a; Silbermann and Tatar, 2000; Sorensen et al., 1999). In ectotherms such as ants, increasing temperature accelerates both growth rate and metabolism, so the heat shock response is likely to become more costly at the same time as it is triggered more frequently and thus may not be the most efficient resistance mechanism under more extreme conditions (Stanton-Geddes et al., 2016).

In contrast to the limited evolved responses to climatic conditions, higher acclimation temperature significantly increased both baseline expression and magnitude of induction in two interacting Hsp genes: baseline levels of *Hsc70-4* were elevated, while induction increased in *Hsp40*. Although Hsp70 can interact directly with denatured proteins, its intrinsic ATPase activity is low, and complex formation with targets is substantially enhanced by Hsp40 (Fan et al., 2003; Laufen et al., 1999). Because these two proteins directly interact, complementary modifications of their expression may

improve the sensitivity and speed of response in acclimated individuals without overly retarding normal metabolic processes. Heat shock proteins are produced relatively rapidly in response to stressors, on the order of minutes (Lindquist, 1980), but producing both proteins following exposure to a stressor may lead to a significantly slower response than a strategy of having one member of the complex available to be recruited immediately upon production of its partner. Hsp40 also may be activated at lower temperatures than Hsp70 (Nguyen et al. 2016), so by making Hsp40 the limiting protein in the pathway, the overall response can be triggered at lower temperatures in advance of significant damage.

There is abundant evidence that the heat shock response responds dynamically to environmental change over multiple time scales, in line with the predictions of the Beneficial Acclimation Hypothesis for individual traits (Huey et al., 1999; Woods and Harrison, 2002). Rapid, short-term exposure to a stressor leads to a hardening response, in which protective upregulation of Hsp genes temporarily extends upper thermal limits in the face of subsequent heat shocks (Krebs and Loeschcke, 1994). Chronic exposure to sublethal temperatures also typically leads to enhanced Hsp activity, which can occur seasonally (Dietz and Somero, 1992; Fader et al., 1994; Hamdoun et al., 2003; Hofmann and Somero, 1996; Roberts et al., 1997) or in response to short-term rearing conditions (Karl et al., 2008; Kristensen et al., 2016; Li et al., 2012; Lucentini et al., 2002).

Although neither of the temperatures to which colonies were exposed in this study were likely to be directly stressful to adult workers (Nguyen et al. 2016), if nest and external temperatures are correlated, workers leaving for foraging trips may benefit from using even modestly elevated nest temperatures as a cue to anticipate stressful external

conditions. Alternatively, worker responsiveness could be non-adaptive in adults but carried over from mechanisms used in the larval stage, when individuals are relatively immobile and can be faced with chronic thermal stress (Sambucetti et al., 2013).

Over the next 50-70 years, northeastern deciduous forest habitats are projected to increase in mean annual temperatures by 4-8°C (Kunkel et al., 2013), pushing resident species toward their physiological limits. The results of this study reveal a similar capacity to plastically adjust the heat shock response to elevated temperatures across the latitudinal range of both *A. picea* and *A. rudis*. This suggests that, at least in the short term, they may possess some resiliency to the direct impacts of temperature increases throughout their ranges. Reliance on the heat shock response, however, is likely to be accompanied by increasing costs that will disproportionately affect cooler-climate species; indeed, the elevational boundary between *A. picea* and *A. rudis* in the Smoky Mountains is already moving upwards (Warren and Chick, 2013), while *A. rudis* is being displaced in warmer areas by a more thermally tolerant species, *Crematogaster lineolata* (Resasco et al., 2014). Understanding how stress-related costs affect growth rates and competitive ability will be important for predicting their ability to persist in a changing landscape.

Acknowledgements

We thank Joseph Karlik and Mary Vincent for assistance in collecting and rearing colonies for the duration of this study, as well as Lacy Chick, Mike Herrmann, Jackie Fitzgerald, Katie Miller, and Aaron Ellison for additional help collecting *Aphaenogaster*

colonies. We also thank Janet Shurtleff and Carole Saravitz at the Phytotron facility at NC State University. This work was supported by the National Science Foundation [DEB-1136644].

All data, protocols and statistical analyses are publically available on Zenodo:

<https://zenodo.org/record/164922#.WCCPfvSrEud>, doi:10.5281/zenodo.164922

References

- Addo-Bediako, A., Chown, S.L., Gaston, K.J., 2000. Thermal tolerance, climatic variability and latitude. *P Roy Soc B-Biol Sci* 267, 739-745.
- Anderson, A.R., Collinge, J.E., Hoffmann, A.A., Kellett, M., McKechnie, S.W., 2003. Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsp-omega* gene in *Drosophila melanogaster*. *Heredity* 90, 195-202.
- Barshis, D.J., Ladner, J.T., Oliver, T.A., Seneca, F.O., Traylor-Knowles, N., Palumbi, S.R., 2013. Genomic basis for coral resilience to climate change. *P Natl Acad Sci USA* 110, 1387-1392.
- Bedulina, D.S., Evgen'ev, M.B., Timofeyev, M.A., Protopopova, M.V., Garbuz, D.G., Pavlichenko, V.V., Luckenbach, T., Shatilina, Z.M., Axenov-Gribanov, D.V., Gurkov, A.N., Sokolova, I.M., Zatsepina, O.G., 2013. Expression patterns and organization of the *hsp70* genes correlate with thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal. *Mol Ecol* 22, 1416-1430.
- Bedulina, D.S., Zimmer, M., Timofeyev, M.A., 2010. Sub-littoral and supra-littoral amphipods respond differently to acute thermal stress. *Comp Biochem Phys B* 155, 413-418.
- Bettencourt, B.R., Feder, M.E., 2001. *Hsp70* duplication in the *Drosophila melanogaster* species group: How and when did two become five? *Mol Biol Evol* 18, 1272-1282.
- Bhatkar, A., Whitcomb, W.H., 1970. Artificial diet for rearing various species of ants. *Florida Entomologist* 53, 229-232.
- Blackburn, S., van Heerwaarden, B., Kellermann, V., Sgro, C.M., 2014. Evolutionary capacity of upper thermal limits: beyond single trait assessments. *J Exp Biol* 217, 1918-1924.
- Cahill, A.E., Aiello-Lammens, M.E., Fisher-Reid, M.C., Hua, X., Karanewsky, C.J., Ryu, H.Y., Sbeglia, G.C., Spagnolo, F., Waldron, J.B., Wiens, J.J., 2014. Causes of warm-edge range limits: systematic review, proximate factors and implications for climate change. *J Biogeogr* 41, 429-442.
- Chandrakanth, N., Ponnuvel, K.M., Moorthy, S.M., Sasibhushan, S., Sivaprasad, V., 2015. Analysis of transcripts of heat shock protein genes in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Eur J Entomol* 112, 676-687.
- DeMarco, B.B., Cognato, A.I., 2016. A multiple-gene phylogeny reveals polyphyly among eastern North American *Aphaenogaster* species. *Zoologica Scripta*.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G., Zumla, A., 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 37, 112-+.

Diamond, S.E., Sorger, D.M., Huler, J., Pelini, S.L., Del Toro, I., Hirsch, C., Oberg, E., Dunn, R.R., 2012. Who likes it hot? A global analysis of the climatic, ecological, and evolutionary determinants of warming tolerance in ants. *Global Change Biol* 18, 448-456.

Diamond, S.E., Nichols, L.M., Pelini, S.L., Penick, C.A., Barber, G.W., Cahan, S.H., Dunn, R.R., Ellison, A.M., Sanders, N.J. and Gotelli, N.J., 2016. Climatic warming destabilizes forest ant communities. *Science Advances* 2:e1600842.

Dietz, T.J., Somero, G.N., 1992. The threshold induction temperature of the 90-Kda heat-shock protein is subject to acclimatization in eurythermal Goby fishes (Genus *Gillichthys*). *P Natl Acad Sci USA* 89, 3389-3393.

Diniz, J.A.F., De Sant'ana, C.E.R., Bini, L.M., 1998. An eigenvector method for estimating phylogenetic inertia. *Evolution* 52, 1247-1262.

Dong, Y.W., Williams, G.A., 2011. Variations in cardiac performance and heat shock protein expression to thermal stress in two differently zoned limpets on a tropical rocky shore. *Mar Biol* 158, 1223-1231.

Dussutour, A., Poissonnier, L.A., Buhl, J., Simpson, S.J., 2016. Resistance to nutritional stress in ants: when being fat is advantageous. *J Exp Biol* 219, 824-833.

Fader, S.C., Yu, Z.M., Spotila, J.R., 1994. Seasonal-variation in heats-shock proteins (Hsp70) in stream fish under natural conditions. *J Therm Biol* 19, 335-341.

Fan, C.Y., Lee, S., Cyr, D.M., 2003. Mechanisms for regulation of Hsp70 function by Hsp40. *Cell Stress Chaperon* 8, 309-316.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu Rev Physiol* 61, 243-282.

Fitzgerald-Dehoog, L., Browning, J., Allen, B.J., 2012. Food and heat stress in the California mussel: Evidence for an energetic trade-off between survival and growth. *Biol Bull-Us* 223, 205-216.

Gehring, W.J., Wehner, R., 1995. Heat-shock protein-synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara Desert. *P Natl Acad Sci USA* 92, 2994-2998.

Haguenauer, A., Zuberer, F., Ledoux, J.B., Aurelle, D., 2013. Adaptive abilities of the Mediterranean red coral *Corallium rubrum* in a heterogeneous and changing environment: from population to functional genetics. *J Exp Mar Biol Ecol* 449, 349-357.

Hamdoun, A.M., Cheney, D.P., Cherr, G.N., 2003. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): Implications for thermal limits and induction of thermal tolerance. *Biol Bull-Us* 205, 160-169.

Hoekstra, L.A., Montooth, K.L., 2013. Inducing extra copies of the Hsp70 gene in *Drosophila melanogaster* increases energetic demand. *Bmc Evol Biol* 13.

Hofmann, G.E., Somero, G.N., 1996. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: Evidence from the heat shock response and protein ubiquitination. *Mar Biol* 126, 65-75.

- Hu, J.T., Chen, B., Li, Z.H., 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.
- Huey, R.B., Berrigan, D., Gilchrist, G.W., Herron, J.C., 1999. Testing the adaptive significance of acclimation: A strong inference approach. *Am Zool* 39, 323-336.
- Karl, I., Janowitz, S.A., Fischer, K., 2008. Altitudinal life-history variation and thermal adaptation in the copper butterfly *Lycaena tityrus*. *Oikos* 117, 778-788.
- Koenigstein, S., Pohlmann, K., Held, C., Abele, D., 2013. Ecological comparison of cellular stress responses among populations - normalizing RT-qPCR values to investigate differential environmental adaptations. *Bmc Ecol* 13.
- Krebs, R.A., Bettencourt, B.R., 1999. Evolution of thermotolerance and variation in the heat shock protein, Hsp70. *Am Zool* 39, 910-919.
- Krebs, R.A., Feder, M.E., 1997. Deleterious consequences of Hsp70 overexpression in *Drosophila melanogaster* larvae. *Cell Stress Chaperon* 2, 60-71.
- Krebs, R.A., Feder, M.E., 1998a. Experimental manipulation of the cost of thermal acclimation in *Drosophila melanogaster*. *Biol J Linn Soc* 63, 593-601.
- Krebs, R.A., Feder, M.E., 1998b. Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *J Insect Physiol* 44, 1091-1101.
- Krebs, R.A., Loeschcke, V., 1994. Effects of exposure to short-term heat-stress on fitness components in *Drosophila melanogaster*. *J Evolution Biol* 7, 39-49.
- Kristensen, T.N., Kjeldal, H., Schou, M.F., Nielsen, J.L., 2016. Proteomic data reveal a physiological basis for costs and benefits associated with thermal acclimation. *J Exp Biol* 219, 969-976.
- Kunkel, K.E., Stevens, L.E., Stevens, S.E., Sun, L., Janssen, E., Wuebbles, D., Rennells, J., DeGaetano, A., Dobson, J.G., 2013. Regional climate trends and scenarios for the U.S. National Climate Assessment Part I: Climate of the Northeast U.S. NOAA Technical Report NESDIS.
- Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., Bukau, B., 1999. Mechanism of regulation of Hsp70 chaperones by DnaJ cochaperones. *P Natl Acad Sci USA* 96, 5452-5457.
- Li, J.Q., He, Q.G., Sun, H., Liu, X., 2012. Acclimation-dependent expression of heat shock protein 70 in Pacific abalone (*Haliotis discus hannai* Ino) and its acute response to thermal exposure. *Chin J Oceanol Limn* 30, 146-151.
- Li, Q.R., Xiao, Y., Wu, F.Q., Ye, M.Q., Luo, G.Q., Xing, D.X., Li, L., Yang, Q., 2014. Analysis of midgut gene expression profiles from different silkworm varieties after exposure to high temperature. *Gene* 549, 85-96.
- Lindquist, S., 1980. Translational efficiency of heat-induced messages in *Drosophila melanogaster* cells. *J Mol Biol* 137, 151-158.
- Lindquist, S., 1986. The heat-shock response. *Annu Rev Biochem* 55, 1151-1191.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25, 402-408.
- Lubertazzi, D., 2012. The biology and natural history of *Aphaenogaster rudis*. *Psyche* Article ID 752815.

- Lucentini, L., Lorenzoni, M., Panara, F., Mearelli, M., 2002. Effects of short- and long-term thermal stress in perch (*Perca fluviatilis*) determined through fluctuating asymmetry and HSP70 expression. *Ital J Zool* 69, 13-17.
- Martinez, B., Arenas, F., Trilla, A., Viejo, R.M., Carreno, F., 2015. Combining physiological threshold knowledge to species distribution models is key to improving forecasts of the future niche for macroalgae. *Global Change Biol* 21, 1422-1433.
- Mizrahi, T., Goldenberg, S., Heller, J., Arad, Z., 2015. Natural variation in resistance to desiccation and heat shock protein expression in the land snail *Theba pisana* along a climatic gradient. *Physiol Biochem Zool* 88, 66-80.
- Mizrahi, T., Goldenberg, S., Heller, J., Arad, Z., 2016. Geographic variation in thermal tolerance and strategies of heat shock protein expression in the land snail *Theba pisana* in relation to genetic structure. *Cell Stress Chaperon* 21, 219-238.
- Mizrahi, T., Heller, J., Goldenberg, S., Arad, Z., 2012. The heat shock response in congeneric land snails (*Sphincterochila*) from different habitats. *Cell Stress Chaperon* 17, 639-645.
- Narum, S.R., Campbell, N.R., Meyer, K.A., Miller, M.R., Hardy, R.W., 2013. Thermal adaptation and acclimation of ectotherms from differing aquatic climates. *Mol Ecol* 22, 3090-3097.
- Nguyen, A.D., Gotelli, N.J., Cahan, S.H., 2016. The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *Bmc Evol Biol* 16.
- Overgaard, J., Kristensen, T.N., Sorensen, J.G., 2012. Validity of thermal ramping assays used to assess thermal tolerance in Arthropods. *Plos One* 7.
- Palumbi, S.R., Barshis, D.J., Traylor-Knowles, N., Bay, R.A., 2014. Mechanisms of reef coral resistance to future climate change. *Science* 344, 895-898.
- Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* 20, 289-290.
- Parsell, D.A., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance - degradation and reactivation of damaged proteins. *Annu Rev Genet* 27, 437-496.
- Pelini, S.L., Diamond, S.E., Nichols, L.M., Stuble, K.L., Ellison, A.M., Sanders, N.J., Dunn, R.R., Gotelli, N.J., 2014. Geographic differences in effects of experimental warming on ant species diversity and community composition. *Ecosphere* 5.
- Recknagel, H., Elmer, K.R., Meyer, A., 2013. A hybrid genetic linkage map of two ecologically and morphologically divergent Midas cichlid fishes (*Amphilophus spp.*) obtained by massively parallel DNA sequencing (ddRADSeq). *G3-Genes Genom Genet* 3, 65-74.
- Resasco, J., Pelini, S.L., Stuble, K.L., Sanders, N.J., Dunn, R.R., Diamond, S.E., Ellison, A.M., Gotelli, N.J., Levey, D.J., 2014. Using historical and experimental data to reveal warming effects on ant assemblages. *Plos One* 9.
- Roberts, D.A., Hofmann, G.E., Somero, G.N., 1997. Heat-shock protein expression in *Mytilus californianus*: Acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *Biol Bull-US* 192, 309-320.
- Rodriguez-Verdugo, A., Carrillo-Cisneros, D., Gonzalez-Gonzalez, A., Gaut, B.S., Bennett, A.F., 2014. Different tradeoffs result from alternate genetic adaptations to a common environment. *P Natl Acad Sci USA* 111, 12121-12126.

- Rojo, C., Martinez-Ruiz, C., Carraminana, M., Rodrigo, M.A., 2015. Foreseeable global warming will differentially affect *Chara vulgaris* populations from different altitudes. *Aquat Bot* 122, 20-26.
- Sambucetti, P., Scannapieco, A.C., Loeschcke, V., Norry, F.M., 2013. Heat-stress survival in the pre-adult stage of the life cycle in an intercontinental set of recombinant inbred lines of *Drosophila melanogaster*. *J Exp Biol* 216, 2953-2959.
- Sawall, Y., Al-Sofyani, A., Hohn, S., Banguera-Hinestroza, E., Voolstra, C.R., Wahl, M., 2015. Extensive phenotypic plasticity of a Red Sea coral over a strong latitudinal temperature gradient suggests limited acclimatization potential to warming. *Sci Rep-Uk* 5.
- Schoville, S.D., Barreto, F.S., Moy, G.W., Wolff, A., Burton, R.S., 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.
- Shatilina, Z.M., Riss, H.W., Protopopova, M.V., Trippe, M., Meyer, E.I., Pavlichenko, V.V., Bedulina, D.S., Axenov-Gribanov, D.V., Timofeyev, M.A., 2011. The role of the heat shock proteins (HSP70 and sHSP) in the thermotolerance of freshwater amphipods from contrasting habitats. *J Therm Biol* 36, 142-149.
- Sheth, S.N., Angert, A.L., 2014. The evolution of environmental tolerance and range size: A comparison of geographically restricted and widespread *Mimulus*. *Evolution* 68, 2917-2931.
- Silbermann, R., Tatar, M., 2000. Reproductive costs of heat shock protein in transgenic *Drosophila melanogaster*. *Evolution* 54, 2038-2045.
- Sorensen, J.G., Loeschcke, V., Kristensen, T.N., 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.
- Sorensen, J.G., Michalak, P., Justesen, J., Loeschcke, V., 1999. Expression of the heat-shock protein HSP70 in *Drosophila buzzatii* lines selected for thermal resistance. *Hereditas* 131, 155-164.
- Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312-1313.
- Stanton-Geddes, J., Nguyen, A., Chick, L., Vincent, J., Vangala, M., Dunn, R.R., Ellison, A.M., Sanders, N.J., Gotelli, N.J., Cahan, S.H., 2016. Thermal reactionomes reveal divergent responses to thermal extremes in warm and cool-climate ant species. *Bmc Genomics* 17.
- Sunday, J.M., Bates, A.E., Dulvy, N.K., 2012. Thermal tolerance and the global redistribution of animals. *Nat Clim Change* 2, 686-690.
- Tedeschi, J.N., Kennington, W.J., Tomkins, J.L., Berry, O., Whiting, S., Meekan, M.G., Mitchell, N.J., 2016. Heritable variation in heat shock gene expression: a potential mechanism for adaptation to thermal stress in embryos of sea turtles. *P Roy Soc B-Biol Sci* 283.
- Terblanche, J.S., Hoffmann, A.A., Mitchell, K.A., Rako, L., le Roux, P.C., Chown, S.L., 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. *J Exp Biol* 214, 3713-3725.

- Tomanek, L., Somero, G.N., 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: Implications for limits of thermotolerance and biogeography. *J Exp Biol* 202, 2925-2936.
- Verbeke, P., Fonager, J., Clark, B.F.C., Rattan, S.I.S., 2001. Heat shock response and ageing: Mechanisms and applications. *Cell Biol Int* 25, 845-857.
- Warren, R.J., Chick, L., 2013. Upward ant distribution shift corresponds with minimum, not maximum, temperature tolerance. *Global Change Biol* 19, 2082-2088.
- White, M.G., Luca, L.E., Nonner, D., Saleh, O., Hu, B., Barrett, E.F., Barrett, J.N., 2007. Cellular mechanisms of neuronal damage from hyperthermia. *Prog Brain Res* 162, 347-371.
- Woods, H.A., Harrison, J.F., 2002. Interpreting rejections of the beneficial acclimation hypothesis: When is physiological plasticity adaptive? *Evolution* 56, 1863-1866.
- Yu, H., Wan, F.H., Guo, J.Y., 2012. Different thermal tolerance and hsp gene expression in invasive and indigenous sibling species of *Bemisia tabaci*. *Biol Invasions* 14, 1587-1595.

Tables

Table 1. Primers used for qPCR.

Gene	Direction	5'-3' sequence	Amplicon Length (bps)
<i>Hsp83</i>	Forward	AATTCGATGGAAARCAGYTGG	99
	Reverse	AAYTTGGCYTTGTCYTCCTC	
<i>Hsc70-4</i>	Forward	GCGATYGARAAATCTACVGGC	124
	Reverse	TGYTCRTCYTCCGATCGGTA	
<i>Hsp40</i>	Forward	AAAGATCGYGCYCARGATCC	100
	Reverse	GCYCGTCTRCATATYTTTCATC	
<i>18s rRNA</i>	Forward	CTCTTTCTTGATTCCGGTGGGTG	100
	Reverse	TTAGCAGGCTAGAGTCTCGTTC	

Table 2. Summary table of significant predictors from linear regression models for relative basal expression ($\Delta\Delta CT$ relative to grand mean) and fold-induction in response to heat shock ($\Delta\Delta CT$ relative to 25°C) of Hsp83, Hsc70-4, and Hsp40. Significance level is reported only for those factors included in the final model after forward AIC model selection. Axes 1,2 and 3 represent the first three axes of phylogenetic variation from PCoA decomposition (see Fig. 1 for nodes captured by each axis).

Gene	Expression Type	Predictor				
		Rearing Temperature	Tmax	Axis 1	Axis 2	Axis 3
<i>Hsp83</i>	Basal			ns		ns
	Induction	+				
<i>Hsc70-4</i>	Basal	+++				
	Induction	ns				
<i>Hsp40</i>	Basal	ns		ns	+	+++
	Induction	+			ns	

ns = not significant, ⁺ p < 0.05, ⁺⁺ p < 0.01, ⁺⁺⁺ p < 0.001.

Figure Legends

Figure 1. Map of collection sites of *Aphaenogaster picea* and *A. rudis*, with the topology recovered from a ddRADseq-based Kimura 2-parameter NJ reconstruction of genetic relationships among colonies. Only bootstrap values of 100 are shown. Color codes of field sites correspond to clade membership of colonies collected at the site: *A. picea* (blue circles), *A. rudis* (northern clade = light orange triangles, southern clade = dark orange squares), and a basal clade with intermediate morphological characters (gray diamonds). Dark gray shaded ovals indicate the nodes captured by the first three principal coordinate axes of genetic variation.

Figure 2. Relationship between maximal annual temperature of the site of field collection and basal expression relative to the grand mean ($\Delta\Delta CT$) or fold induction in response to one hour of heat shock at 37°C for *Hsp83* (A,B), *Hsc70-4* (C,D) and *Hsp40* (E,F). Points were fitted with spline curves with 95% confidence intervals indicated by the red line and shading, respectively.

Figure 3. Box plot of relative *Hsp40* basal expression ($\Delta\Delta CT$) across the four clades differentiated by PCA analysis; axes 2 and 3 were significant in the full model. Letters indicate significantly different groupings as indicated by Tukey's post-hoc pairwise comparisons.

Figure 4. Effect of rearing temperature on relative basal expression and induction in response to heat shock for *Hsp83* (A,B), *Hsc70-4 h2* (C,D) and *Hsp40* (E,F). P-values indicate the level of significance of rearing temperature from regression analysis.

Fig. S1 PCoA scatterplots of the first three axes of variation in the *Aphaenogaster* SNP-based genetic distance matrix. A) Axis 1 versus axis 2, which differentiate the *A. picea*-*A. rudis* (axis 1) and intermediate basal clade (axis 2) clusters. B) Axis 2 versus axis 3, which differentiates the southern and northern clades of *A. rudis*.

752
753

754
755

756
757

758