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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*

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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.

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1. 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 Hofmann, 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 selection pressures acting on populations at different locations may promote evolutionary changes instead of or in addition to an acclimation response (Addo-Bediako et al., 2000; Sheth

Abbreviations: Hsp, heat shock protein; *Hsp*, heat shock protein gene; CSR, cellular stress response; Tmax, annual maximal temperature at the site of collection (Bioclim variable 5).

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and Angert, 2014). In more extreme environments, populations are likely to frequently face conditions at or beyond their physiological limits, selecting for increased constitutive expression or enhanced inducibility of chaperone molecules (Barshis et al., 2013; Bettencourt and Feder, 2001). Under milder conditions, however, the cost of maintaining a response mechanism may lead to reduction or loss of the capacity to respond to temperature extremes (Silbermann and Tatar, 2000). Moreover, if increasing cold-tolerance comes at the expense of heat-tolerance (Rodriguez-Verdugo et al., 2014), the entire thermal niche may be forced downward as populations approach their lower thermal range limit (Addo-Bediako et al., 2000; Anderson et al., 2003).

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

The ant genus *Aphaenogaster* is common in deciduous forests in eastern North 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).

2. Methods

2.1. 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 min. 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 × 150 mm 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).

2.2. 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 1.4 mm zirconium silicate beads in 200 μl 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 to 200 ng of genomic DNA per individual following the protocol of Recknagel et al. (2013). Briefly, samples were double-digested with the restriction enzymes *Nla*III and MluCI at 37 °C for three hours, purified using a 1.5× 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 40 ng of each sample into a single library. Three hundred microliters of the pooled library was purified with a 1× AMPure bead purification eluted into 30ul of Qiagen AE buffer. The ligated fragments were amplified in seven 20ul PCR reactions containing approximately 20 ng 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 1.5× AMPure beads into a final volume of 30 μl. Fragments 300–400 bp in total length were size-selected from a 1.5% agarose gel and extracted with the QIEX II gel extraction kit.

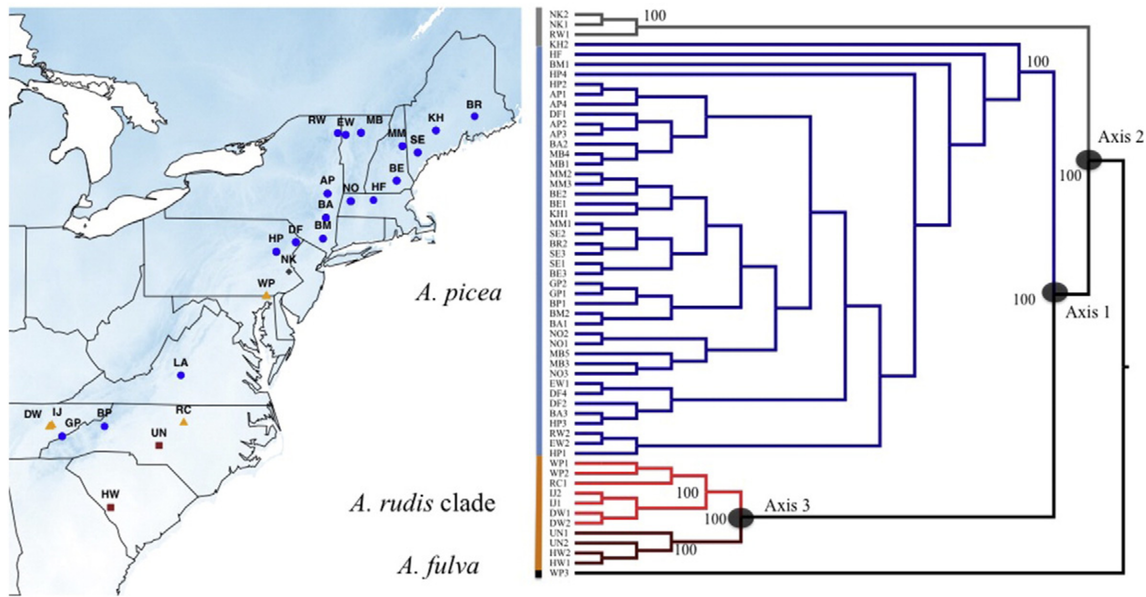

Fig. 1. Map of collection sites of *Aphaenogaster picea* and *A. rudis*, with the topology recovered from a ddRADseq-based Kimura 2-parameter NJ reconstruction of genetic relationships among colonies. Only bootstrap values of 100 are shown. Color codes of field sites correspond to clade membership of 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.

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 90 bp 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.

2.3. 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 enclosed 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 45 mm 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 h, the ants were flash-frozen in liquid nitrogen and stored at −80 °C for gene expression quantification.

2.4. Quantifying Hsp gene expression

RNA was isolated from each set of four pooled ants using the RNeazy micro kit (Qiagen, USA) following the manufacturer's instructions. 50 ng 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 (18 s 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 2 ng of template cDNA, 500 nM of total primers, and 10 μl of Power SYBR® Green Master Mix (Life Technologies, USA), with a cycling program of heating at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and acquisition of fluorescence at 70 °C for 60 s. We performed a melt-curve analysis and sequenced a subset of samples to verify specificity.

Table 1
Primers used for qPCR.

Gene	Direction	5′–3′ sequence	Amplicon length (bps)
Hsp83	Forward	AATTCGATGGAAARCAGYTGG	
	Reverse	AAYTTGGCYTTGTCYTCCTC	99
Hsc70-4	Forward	GCGATYGARAAATCTACVGGC	
	Reverse	TGYTCRTCYTCCGATCGGTA	124
Hsp40	Forward	AAAGATCGYGCYCARGATCC	
	Reverse	GCYCGTCTRCATATYTTCATC	100
18s rRNA	Forward	CTCTTTCTTGATTCGGTGGGTG	
	Reverse	TTAGCAGGCTAGAGTCTCGTTC	100

Gene expression was quantified using the ΔΔCT 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.

2.5. 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.

3. 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 (NK1 & 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 to 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).

4. 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; Haguenauer 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 Tmax values should have provided ample power to detect a cline in gene expression if it were present, but Tmax 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).

Table 2
Summary table of significant predictors from linear regression models for relative basal expression (ΔΔCT relative to grand mean) and fold-induction in response to heat shock (ΔΔCT 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
Hsp83	Basal			ns		ns
	Induction	+				
Hsc70-4	Basal	+++				
	Induction	ns				
Hsp40	Basal	ns		ns	+	+++
	Induction	+			ns	

ns = not significant, $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$.

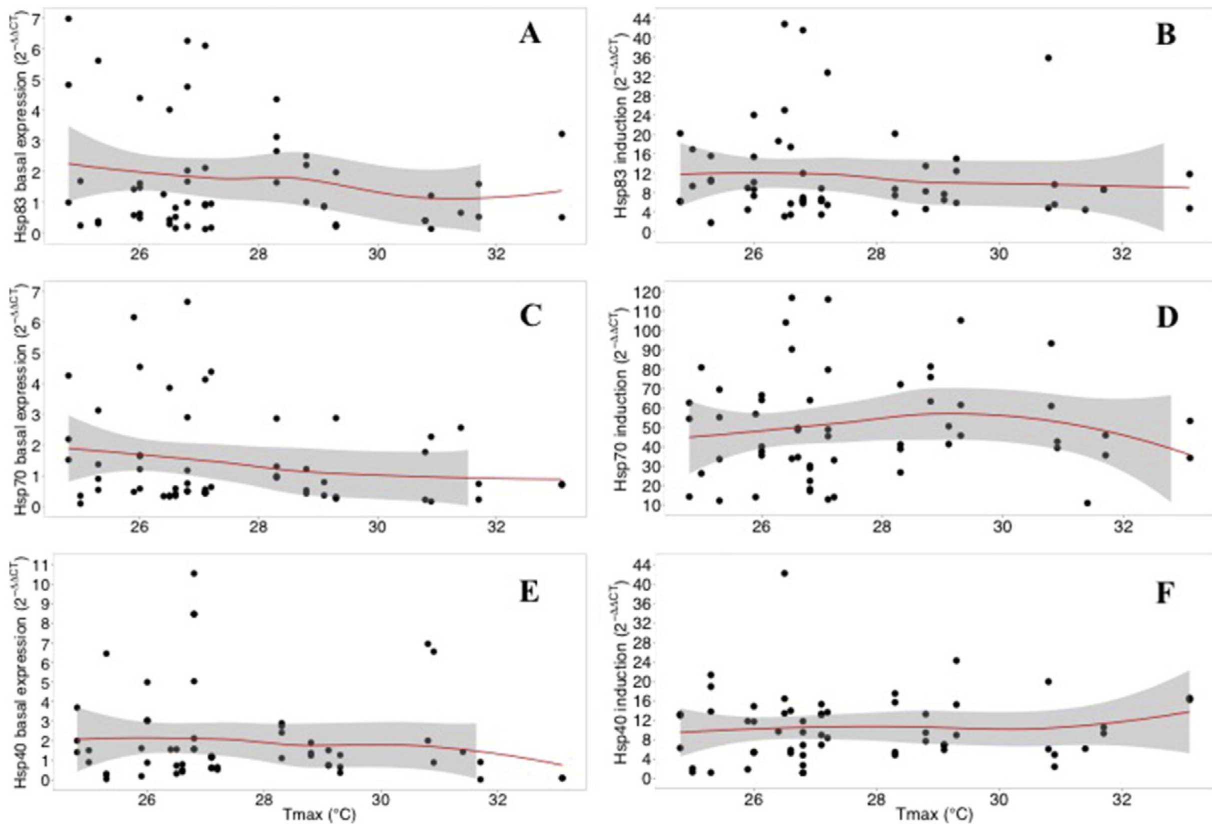

Fig. 2. Relationship between maximal annual temperature of the site of field collection and basal expression relative to the grand mean (ΔΔCT) or fold induction in response to one hour of heat shock at 37 °C for Hsp83 (A, B), Hsc70-4 (C, D) and Hsp40 (E, F). Points were fitted with spline curves with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

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 (CTmax) 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.

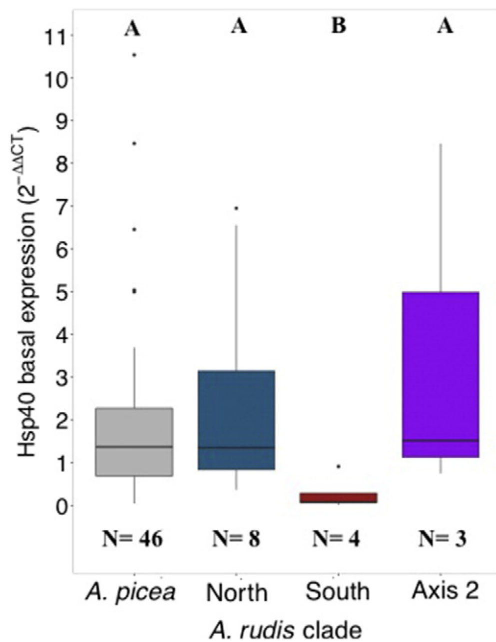

Fig. 3. Box plot of relative Hsp40 basal expression (ΔΔCT) across the four clades differentiated by PCA analysis; axes 2 and 3 were significant in the full model. Letters indicate significantly different groupings as indicated by Tukey's post hoc pairwise comparisons.

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 CTmax 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)

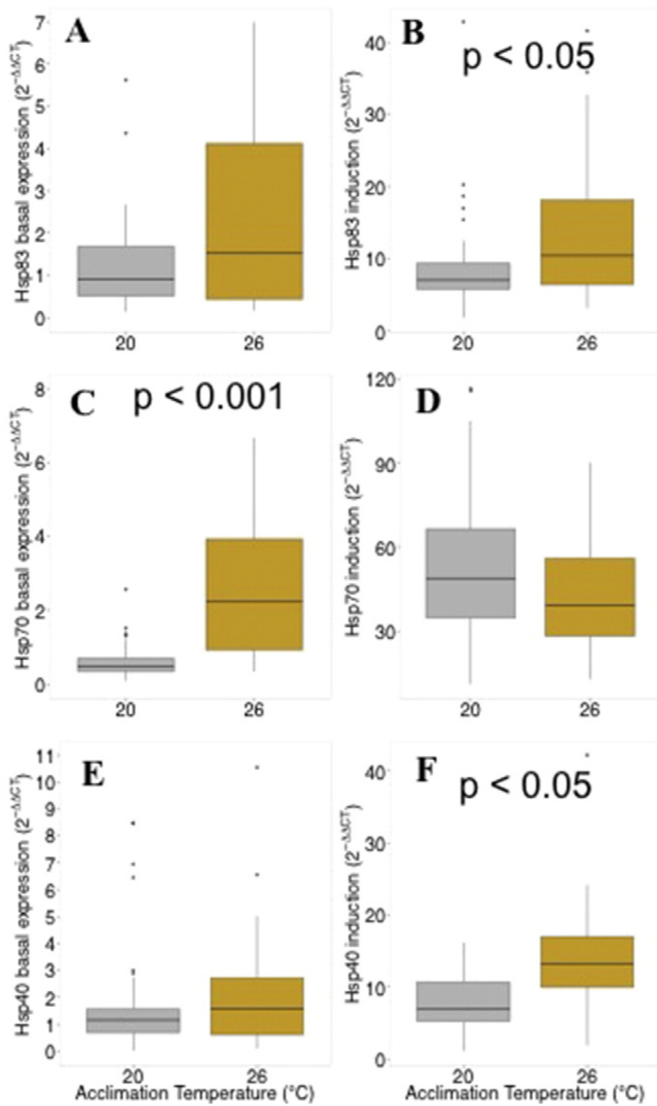

Fig. 4. Effect of rearing temperature on relative basal expression and induction in response to heat shock for *Hsp83* (A, B), *Hsc70-4 h2* (C, D) and *Hsp40* (E, F). P-values indicate the level of significance of rearing temperature from regression analysis.

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 Tmax. 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.

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