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Evolutionary Innovations In Ants To Thermally Stressful Environments

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EVOLUTIONARY INNOVATIONS IN ANTS TO THERMALLY STRESSFUL
ENVIRONMENTS

A Dissertation Presented

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Andrew D. Nguyen

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ABSTRACT

Temperature is a fundamental environmental force shaping species abundance and distributions through its effects on biochemical reaction rates, metabolism, activity, and reproduction. In light of future climate shifts, mainly driven by temperature increases, how will organisms persist in warmer environments? One molecular mechanism that may play an important role in coping with heat stress is the heat shock response (HSR), which protects against molecular damage. To prevent and repair protein damage specifically, Hsps activate and become up-regulated. However, the functional diversity and relevance of heat shock proteins (Hsps) in extending upper thermal limits in taxonomic groups outside marine and model systems is poorly understood. Ants are a good system to understand the physiological mechanisms for coping with heat stress because they have successfully diversified into thermally stressful environments. To identify and characterize the functional diversity of Hsps in ants, I surveyed Hsp orthologues from published ant genomes to test for signatures of positive selection and to reconstruct their evolutionary history. Within Hymenoptera, ants utilize unique sets of Hsps for the HSR. Stabilizing selection was the prevailing force among Hsp orthologues, suggesting that protein activity is conserved. At the same time, regulatory regions (promoters) governing transcriptional up-regulation diversified: species differ in the number and location of heat shock elements (HSEs). Therefore, Hsp expression patterns may be a target for selection in warm environments. I tested whether Hsp expression corresponded with variation in upper thermal limits in forest ant species within the genus *Aphaenogaster*. Whole colonies were collected throughout the eastern United States and were lab acclimated. There was a positive relationship between upper thermal limits (Critical Thermal maxima, CTmax) and local temperature extremes. Upper thermal limits were also higher in ant species that lived in open habitats (shrub-oak and long-leaf pine savannah) than species occupying closed habitats (deciduous forest). Ant species with higher CTmax expressed Hsps more slowly, at higher temperatures, and at higher maximum levels than those with low CTmax. Because Hsps sense and repair molecular damage, these results suggest the proteomes of open relative to closed canopy forests are more stable. Although deciduous forest ant species may be buffered from temperature stress, it is likely that temperature interacts with other environmental stressors such as water and nutrient availability that may impact upper thermal limits. I measured the influence of dehydration and nutrition stress on upper thermal limits of forest ants from a single population. Ants that were initially starved were much less thermally tolerant than controls and ants that were initially desiccated. Because ants are likely to experience similar combination of stressors in the wild, upper thermal limits may be severely overestimated in single factor experiments. Therefore, realistic forecasting models need to consider multiple environmental stressors. Overall, adaptive tuning of Hsp expression that reflects better protection and tolerance of protein unfolding may have facilitated ant diversification into warm environments. However, additional stressors and mechanisms may constrain the evolution of upper thermal limits.

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CHAPTER 1: INTRODUCTION

Climate change will alter the environment for many species from ancestral

conditions (Diffenbaugh and Field 2013). Precipitation regimes will become more

variable (Knapp et al. 2008) and temperature will increase both in mean and extremes

(Diffenbaugh and Field 2013). Temperature shifts represent one of the most notable

threats to species persistence because it influences all levels of biological organization

such as biochemical reaction rates, activity, and reproduction (Kingsolver 2009; Dell et

al. 2011). Therefore, elevating temperatures can expose individuals to temperatures

outside of their optimum, especially for ectothermic species whose internal body

temperature is generally closely tied to environmental temperatures (Kingsolver 2009). In

turn, the thermal sensitivity of ectotherms sets the limits to their abundance and

geographical distribution (Slatyer et al. 2013; Lancaster et al. 2016) and the offsets from

species' thermal optimum could lead to contractions at the warm edge of their range

(Chen et al. 2011; Wiens 2016). In order to survive pending temperature shifts, species

will need to respond by tracking suitable thermal habitat (Chen et al. 2011), or stay in

place through acclimation (plasticity) and/or evolutionary adaptation (Moritz and Agudo

2013).

Physiological adjustments to thermal extremes may be evolutionarily

constrained (Hoffmann et al. 2013). At broad taxonomic scales, upper thermal limits

exhibit no relationship with latitude in terrestrial ectotherms, but lower thermal limits

negatively correlate with latitude (Addo-Bediako et al. 2000). Furthermore, lower

thermal limits are more plastic than upper thermal limits (Gunderson and Stillman 2015).

The lack of clinal variation in upper thermal limits across ectotherms generally may be

due to relaxed selection, and/or be reflective of phylogenetic inertia that impedes

response to selection for each species within a clade (Kellerman et al. 2012). Relaxed

selection may be due to species relying on behavioral mechanisms that mitigate the

effects of temperature stress (Kearney et al. 2009). However, at finer taxonomic scales,

local populations and species groups do show clinal variation in upper thermal limits

(Calosi et al. 2010, Sgrò et al. 2010). Clinal relationships suggest populations or species

are locally adapted across the climate gradient and that populations have historically been

able to respond to selection (Hoffmann and Sgrò 2011). Although there has been no

formal comparison of the adaptive potential between upper and lower thermal limits, both

traits respond equally to artificial selection in lab derived fruit flies (Bubliy and Loeschke

2005). However, the heritability of upper thermal limits can be low (van Heerwaarden

and Sgrò 2013) or absent (Mitchell and Hoffmann 2010), depending on whether upper

thermal limits were measured under rapid or slow heating regimes. Regardless of metric,

selection may have eroded standing genetic variation when populations or species are

locally adapted to a climate gradient.

Part of the difficulty in extending upper thermal limits compared to lower

thermal limits is reflected by the relationship between organismal performance and

temperature, known classically as the thermal performance curve (Huey and Stevenson

1979). Thermal performance curves have a characteristic asymmetric shape: performance

gradually elevates from the critical thermal minimum (CTmin) to an optimum (Topt) as

temperatures increase, but beyond the peak, performance drops drastically (Huey and Stevenson 1979; Kingsolver 2009). The rapid drop in performance from the thermal optimum towards the critical thermal maxima or CTmax, in part, is related to the deleterious effects of extremely high temperatures (Richter et al. 2010).

High temperature impairs performance from the whole organism to the molecular level (Dell et al. 2011). The critical hallmark of CTmax is loss of motor function and is accompanied by large-scale tissue damage (Krebs and Feder 1997) including brain and muscle, measured as cell death. At the cellular level, heat shock decreases the firing of action potentials (Miller and Stillman 2012) and disrupts ion balance (O' Sullivan et al. 2016) which is needed to maintain electrochemical potential across cell membranes. Within cells, heat shock causes fragmentation of the endoplasmic reticulum and golgi apparatus (Welch and Suhan 1985). Cytoskeletal networks collapse (Toivola et al. 2010), thus hindering translation and cellular transportation. At the molecular level, macromolecules become damaged as well (Kültz 2005). For example, heat shock introduces double stranded breaks in DNA (Kantidze et al. 2016) and proteins unfold and lose biological activity (Somero 1995; Dill et al. 2011).

Species can cope with thermal damage through the evolutionarily conserved heat shock response (HSR; Lindquist 1988). The HSR involves a molecular cascade that halts the expression of catalytic enzymes and simultaneously up-regulates stress proteins (Sørensen et al. 2005). The first glimpse of the HSR was observed in chromatin puffs within salivary cells of fruit flies that were heat shocked, indicating open transcriptionally active regions of chromatin (Ritossa 1962). Later, Ashburner et al. (1979) pinpointed the

genes that become rapidly up-regulated within chromatin puffs known as heat shock proteins (Hsps). Hsps mediate the HSR by refolding damaged proteins (Lindquist 1988; Richter et al. 2010) or facilitating their degradation (Qian et al. 2006). In fact, Hsps may respond to other, non temperature stressors if protein folding is perturbed (Morris et al. 2013).

Hsps themselves are highly diverse sets of multigene families (Fink 1999; Lindquist 1988). Within arthropods, there are generally five broad gene families that are named after their molecular weight: Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (sHsps; Richter et al. 2010). Hsp90, Hsp70 and Hsp40 form a refolding network under heat shock (Richter et al. 2010). Briefly, Hsp70 can refold proteins through ATP hydrolysis, which can be stimulated by the J-domain of Hsp40 (Qiu et al. 2006). Although Hsp70 and Hsp40 can refold proteins independently, non-native proteins can be refolded cooperatively with Hsp90 through co-chaperones containing TPR domain motifs (Ile-Glu-Glu-Val-Asp) which allow Hsp70 and Hsp90 to interact (Taipale et al. 2010). If proteins are irreversibly damaged, they can be cleared by Hsp70-bound substrates and ubiquitinated. This subsequently leads to degradation by the proteasome (Qian et al. 2006).

Given the ability for Hsps to sense and repair protein damage (Craig and Gross 1991), they should be a major target of selection in thermally stressful environments (Moseley 1997; Sørensen et al. 2003). In fact, the expression patterns of Hsps are known to differ among divergent populations and species in two general non-mutually exclusive strategies that depend on thermal experience (Feder and Hofmann 1999). Encountering

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constant thermal stress selects for consistently elevated levels of Hsp expression and

confers protection from protein damage, while encountering variable thermal extremes

selects for induction of Hsps and confers tolerance to protein damage (Feder and

Hofmann 1999; Sørensen et al. 2003; Barshis et al. 2013). For example, limpets that face

constant solar radiation in the intertidal invest in higher baseline Hsp expression across a

wide temperature gradient, while limpets that face less solar radiation induce Hsps under

higher temperatures (Dong et al. 2008). Although protection and tolerance strategies have

been well documented (Feder and Hofmann 1999; Sørensen et al. 2003), the relative

importance of protection and tolerance mediated by Hsps associated with divergence in

upper thermal limits is poorly understood. One study (Bedulina et al. 2013) suggests that

there may be a trade-off between these two strategies because baseline Hsp expression is

negatively correlated with Hsp induction. More studies are needed to determine whether

trade-offs between constitutive and induced Hsp expression relate to adaptive divergence

of thermal limits.

Ants are a good system to study the mechanisms of temperature adaptation.

They represent a diverse taxonomic group that has been able to meet the challenge of

variable environments (Sanders et al. 2007; Dunn et al. 2009) and have colonized all

continents except Antarctica over the past 140-180 million years (Moreau et al. 2006;

Brady et al. 2006). Because ants have radiated across the globe, they likely have evolved

innovations in coping with and responding to extreme thermal environments. In fact, the

diversity of upper thermal limits is reflected in the different biomes they inhabit

(Diamond et al. 2012). For example, ants within the genus *Cataglyphis*, which inhabit

sub-saharan Africa, are among the most thermally tolerant ant species (Gehring and

Wehner 1995), and have evolved long legs to reduce thermal stress from the surface soil

layer in the desert environment (Sommer and Wehner 2012). In contrast, *Prenolepis*

imparis, also known as the winter ant, is known to forage under snowy conditions

(Tschinkel 1987). Although the ecology and evolution of ants is generally well studied,

the molecular players underlying physiological mechanisms that contribute to phenotypic

divergence in upper thermal limits is lacking, outside of a few studies. *Cataglyphis* ants

have high baseline HSP70 expression under higher temperatures than a temperate genus

(*Formica*), suggesting they protect against thermal damage (Gehring and Wehner 1995).

However, the functional diversity of Hsps outside Hsp70 is poorly understood.

In the following dissertation chapters, I seek to understand how ant species cope

with thermally variable environments. Because Hsps are likely targets of selection in

thermally extreme environments, I first characterized the functional diversity of Hsps in

Hymenoptera. Next, to determine the evolutionary importance of Hsps, I tested how

different aspects of Hsp gene expression explain variation in upper thermal limits.

Because the variation in thermal limits in nature is complex and interacts with other

stressors, I tested the effects of desiccation or starvation on upper thermal limits.

Works Cited

Addo-Bediako A, Chown SL, Gaston KJ (2000) Thermal tolerance, climatic variability
    and latitude. *Proceedings of the Royal Society of London B: Biological Sciences*,
    267, 739–745.
Ashburner M, Bonner JJ (1979) The induction of gene activity in Drosophila by heat
    shock. *Cell*, **17**, 241–254.

Barshis DJ, Ladner JT, Oliver TA *et al.* (2013) Genomic basis for coral resilience to climate change. *Proceedings of the National Academy of Sciences*, **110**, 1387–1392.

Bedulina DS, Evgen'ev MB, Timofeyev MA *et al.* (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. *Molecular Ecology*, **22**, 1416–1430.

Brady SG, Schultz TR, Fisher BL, Ward PS (2006) Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proceedings of the National Academy of Sciences*, **103**, 18172–18177.

Bubliy OA, Loeschcke V (2005) Correlated responses to selection for stress resistance and longevity in a laboratory population of Drosophila melanogaster. *Journal of Evolutionary Biology*, **18**, 789–803.

Calosi P, Bilton DT, Spicer JI, Votier SC, Atfield A (2010) What determines a species' geographical range? Thermal biology and latitudinal range size relationships in European diving beetles (Coleoptera: Dytiscidae). *Journal of Animal Ecology*, **79**, 194–204.

Chen I-C, Hill JK, Ohlemüller R, Roy DB, Thomas CD (2011) Rapid Range Shifts of Species Associated with High Levels of Climate Warming. *Science*, **333**, 1024–1026.

Craig EA, Gross CA (1991) Is hsp70 the cellular thermometer? *Trends in Biochemical Sciences*, **16**, 135–140.

Dell AI, Pawar S, Savage VM (2011) Systematic variation in the temperature dependence of physiological and ecological traits. *Proceedings of the National Academy of Sciences*, **108**, 10591–10596.

Diamond SE, Sorger DM, Hulcr J *et al.* (2012) Who likes it hot? A global analysis of the climatic, ecological, and evolutionary determinants of warming tolerance in ants. *Global Change Biology*, **18**, 448–456.

Diffenbaugh NS, Field CB (2013) Changes in Ecologically Critical Terrestrial Climate Conditions. *Science*, **341**, 486–492.

Dong Y, Miller LP, Sanders JG, Somero GN (2008) Heat-Shock Protein 70 (Hsp70) Expression in Four Limpets of the Genus Lottia: Interspecific Variation in Constitutive and Inducible Synthesis Correlates With in situ Exposure to Heat Stress. *The Biological Bulletin*, **215**, 173–181.

Dunn RR, Agosti D, Andersen AN *et al.* (2009) Climatic drivers of hemispheric asymmetry in global patterns of ant species richness. *Ecology Letters*, **12**, 324–333.

Feder ME, Hofmann GE (1999) HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. *Annual Review of Physiology*, **61**, 243–282.

Fink AL (1999) Chaperone-Mediated Protein Folding. *Physiological Reviews*, **79**, 425–449.

Gehring WJ, Wehner R (1995) Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara desert. *Proceedings of the National Academy of Sciences*, **92**, 2994–2998.

Gunderson AR, Stillman JH (2015) Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**, 20150401.

van Heerwaarden B, Sgrò CM (2013) Multivariate analysis of adaptive capacity for upper thermal limits in *Drosophila simulans*. *Journal of Evolutionary Biology*, **26**, 800–809.

Hoffmann AA, Chown SL, Clusella-Trullas S (2013) Upper thermal limits in terrestrial ectotherms: how constrained are they? *Functional Ecology*, **27**, 934–949.

Hoffmann AA, Sgrò CM (2011) Climate change and evolutionary adaptation. *Nature*, **470**, 479–485.

Huey RB, Stevenson RD (1979) Integrating Thermal Physiology and Ecology of Ectotherms: A Discussion of Approaches. *American Zoologist*, **19**, 357–366.

Kantidze OL, Velichko AK, Luzhin AV, Razin SV (2016) Heat Stress-Induced DNA Damage. *Acta Naturae*, **8**, 75–78.

Kearney M, Shine R, Porter WP (2009) The potential for behavioral thermoregulation to buffer "cold-blooded" animals against climate warming. *Proceedings of the National Academy of Sciences*, **106**, 3835–3840.

Kellermann V, Overgaard J, Hoffmann AA *et al.* (2012) Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences*, **109**, 16228–16233.

Kingsolver JG (2009) The Well-Temperatured Biologist. *The American Naturalist*, **174**, 755–768.

Knapp AK, Beier C, Briske DD *et al.* (2008) Consequences of More Extreme Precipitation Regimes for Terrestrial Ecosystems. *BioScience*, **58**, 811–821.

Krebs RA, Feder ME (1997) Tissue-specific variation in Hsp70 expression and thermal damage in Drosophila melanogaster larvae. *The Journal of Experimental Biology*, **200**, 2007–2015.

Kültz D (2005) Molecular and Evolutionary Basis of the Cellular Stress Response. *Annual Review of Physiology*, **67**, 225–257.

Lancaster LT (2016) Widespread range expansions shape latitudinal variation in insect thermal limits. *Nature Climate Change*, **6**, 618–621.

Lindquist S, Craig EA (1988) The Heat-Shock Proteins. *Annual Review of Genetics*, **22**, 631–677.

Miller NA, Stillman JH (2012) Neural Thermal Performance in Porcelain Crabs, Genus Petrolisthes. *Physiological and Biochemical Zoology*, **85**, 29–39.

Mitchell KA, Hoffmann AA (2010) Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in Drosophila. *Functional Ecology*, **24**, 694–700.

Moreau CS, Bell CD, Vila R, Archibald SB, Pierce NE (2006) Phylogeny of the Ants: Diversification in the Age of Angiosperms. *Science*, **312**, 101–104.

Moritz C, Agudo R (2013) The Future of Species Under Climate Change: Resilience or Decline? *Science*, **341**, 504–508.

Morris JP, Thatje S, Hauton C (2013) The use of stress-70 proteins in physiology: a re-appraisal. *Molecular Ecology*, **22**, 1494–1502.

Moseley PL (1997) Heat shock proteins and heat adaptation of the whole organism. *Journal of Applied Physiology*, **83**, 1413–1417.

O'Sullivan JDB, MacMillan HA, Overgaard J (2016) Heat stress is associated with disruption of ion balance in the migratory locust, Locusta migratoria. *Journal of Thermal Biology*.

Qian S-B, McDonough H, Boellmann F, Cyr DM, Patterson C (2006) CHIP-mediated stress recovery by sequential ubiquitination of substrates and Hsp70. *Nature*, **440**, 551–555.

Qiu X-B, Shao Y-M, Miao S, Wang L (2006) The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and Molecular Life Sciences CMLS*, **63**, 2560–2570.

Richter K, Haslbeck M, Buchner J (2010) The Heat Shock Response: Life on the Verge of Death. *Molecular Cell*, **40**, 253–266.

Ritossa F (1962) A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia*, **18**, 571–573.

Sanders NJ, Lessard J-P, Fitzpatrick MC, Dunn RR (2007) Temperature, but not productivity or geometry, predicts elevational diversity gradients in ants across spatial grains. *Global Ecology and Biogeography*, **16**, 640–649.

Sgrò CM, Overgaard J, Kristensen TN *et al.* (2010) A comprehensive assessment of geographic variation in heat tolerance and hardening capacity in populations of Drosophila melanogaster from eastern Australia. *Journal of Evolutionary Biology*, **23**, 2484–2493.

Slatyer RA, Hirst M, Sexton JP (2013) Niche breadth predicts geographical range size: a general ecological pattern. *Ecology Letters*, **16**, 1104–1114.

Sommer S, Wehner R (2012) Leg allometry in ants: Extreme long-leggedness in thermophilic species. *Arthropod Structure & Development*, **41**, 71–77.

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, **6**, 1025–1037.

Sørensen JG, Nielsen MM, Kruh?ffer M, Justesen J, Loeschcke V (2005) Full genome gene expression analysis of the heat stress response in Drosophila melanogaster. *Cell Stress & Chaperones*, **10**, 312–328.

Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature Reviews Molecular Cell Biology*, **11**, 515–528.

Toivola DM, Strnad P, Habtezion A, Omary MB (2010) Intermediate filaments take the heat as stress proteins. *Trends in Cell Biology*, **20**, 79–91.

Tschinkel WR (1987) Seasonal life history and nest architecture of a winter-active ant,Prenolepis imparis. *Insectes Sociaux*, **34**, 143–164.

Welch WJ, Suhan JP (1985) Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and

nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. *The Journal of Cell Biology*, **101**, 1198–1211.

Wiens JJ (2016) Climate-Related Local Extinctions Are Already Widespread among Plant and Animal Species. *PLOS Biology*, **14**, e2001104.

CHAPTER 2: THE EVOLUTION OF HEAT SHOCK PROTEIN SEQUENCES, CIS-REGULATORY ELEMENTS, AND EXPRESSION PROFILES IN THE EUSOCIAL HYMENOPTERA

Abstract

Background

The eusocial Hymenoptera have radiated across a wide range of thermal environments, exposing them to significant physiological stressors. We reconstructed the evolutionary history of three families of Heat Shock Proteins (Hsp90, Hsp70, Hsp40), the primary molecular chaperones protecting against thermal damage, across 12 Hymenopteran species and four other insect orders. We also predicted and tested for thermal inducibility of eight Hsps from the presence of cis-regulatory heat shock elements (HSEs). We tested whether Hsp induction patterns in ants were associated with different thermal environments.

Results

We found evidence for duplications, losses, and cis-regulatory changes in two of the three gene families. One member of the Hsp90 gene family, *hsp83*, duplicated basally in the Hymenoptera, with shifts in HSE motifs in the novel copy. Both copies were retained in bees, but ants retained only the novel HSE copy. For Hsp70, Hymenoptera lack the primary heat-inducible orthologue from *Drosophila melanogaster* and instead induce the cognate form, *hsc70-4*, which also underwent an early duplication. Episodic diversifying selection was detected along the branch predating the duplication of *hsc70-4* and continued along one of the paralogue branches after duplication. Four out of eight Hsp

genes were heat-inducible and matched the predictions based on presence of conserved

HSEs. For the inducible homologues, the more thermally tolerant species,

Pogonomyrmex barbatus, had greater Hsp basal expression and induction in response to

heat stress than did the less thermally tolerant species, *Aphaenogaster picea*.

Furthermore, there was no trade-off between basal expression and induction.

Conclusions

Our results highlight the unique evolutionary history of Hsps in eusocial Hymenoptera,

which has been shaped by gains, losses, and changes in cis-regulation. Ants, and most

likely other Hymenoptera, utilize lineage-specific heat inducible Hsps, whose expression

patterns are associated with adaptive variation in thermal tolerance between two ant

species. Collectively, our analyses suggest that Hsp sequence and expression patterns

may reflect the forces of selection acting on thermal tolerance in ants and other social

Hymenoptera.

**Keywords: Heat shock proteins, Heat shock response, Heat shock elements,
Thermal tolerance, Gene expression, Cis-regulation, Comparative genomics**

Background

Heat stress causes proteins to lose stability, misfold, and form aggregates, which

can impair function and reduce organismal fitness [1-4]. To cope with macromolecular

damage, the heat shock response (HSR) transcriptionally up-regulates thermally

responsive genes such as heat shock proteins (Hsps), which maintain proteostasis by

refolding or degrading denatured proteins and preventing aggregations [1,2,5]. Hsps are a

set of highly conserved molecular chaperone proteins of diverse multigene families, named after their molecular weight (Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps) [6,7].

Although Hsps as a group are highly conserved, diversity within each Hsp gene family reflects evolutionary gains and losses of gene copies [8,9]. Each Hsp protein family includes paralogues localized to different subcellular compartments (cytosol, endoplasmic reticulum, or mitochondria) that participate in housekeeping functions and/or respond to environmental stress [10-12]. For heat-inducible forms, the transcribed heat shock factors (HSF), bind to cis-regulatory elements known as heat shock elements (HSEs) and up-regulate Hsp transcription [13-15]. Patterns of variation in Hsp gene expression among taxa include expansion of additional Hsp genes [16] and shifts in the arrangement and position of HSE elements [14,17,18]. Among taxa, both the level of constitutive expression and the magnitude of Hsp induction are associated with adaptive variation in upper thermal limits [19-22]. Gene structure may also play a role in Hsp expression, but has not been well-studied. For example, genes with introns allow for more mRNA accumulation than do intronless genes [23-25].

The eusocial Hymenoptera (wasps, ants, and bees) occupy diverse thermal environments from low to high latitudes [26] and elevations [27-28], suggesting that temperature may have played an important selective role in their evolution [29]. Species employ a variety of behavioral [30,31] and physiological strategies [32] to reduce individual and colony-level exposure to thermal stress. However, individual foragers that leave the nest each day and immobile brood that develop in the nest are likely to encounter sufficiently high temperatures to trigger the HSR [33,34]. Although key

members of Hsp90 and Hsp70 have been identified in a few species of Hymenoptera [33-36], the diversity, functional properties, and regulation of molecular chaperones underlying adaptive variation in Hymenopteran thermal tolerance are poorly understood.

In this study, we evaluated the diversity and evolutionary history of Hsps across 12 species of Hymenoptera and five outgroup species (*Culex quinquefasciatus, Drosophila melanogaster, Bombyx mori*, *Tribolium castaneum*, *Acyrthosiphon pisum*) spanning four insect orders. We analyzed recently published genomes of multiple species of ants [37-42], bees (*Apis* [43] and *Bombus* [44]), and the jewel wasp (*Nasonia vitripennis* [45]) to identify orthologues within each major Hsp gene family and to characterize the upstream regulatory motifs governing their transcription (HSEs). We reconstructed molecular evolutionary relationships within each Hsp multigene family to identify evolutionary gains and losses and tested for positive or purifying selection for each homologous Hsp among lineages and across sites. To characterize the evolution of cis-regulation and identify Hsps involved in the HSR, we identified cis-regulatory HSEs within the promoter region for each homologous Hsp. We then tested whether HSE presence and configuration successfully predicted inducibility in two species of ants that experience different thermal environments: the hot-climate *Pogonomyrmex barbatus,* which inhabits deserts of the southwestern United States [46], and the cool-climate *Aphaenogaster picea*, which inhabits temperate deciduous forests of the eastern United States [47]. We found that ants, and probably other Hymenoptera, harbor unique, lineage-specific sets of heat inducible Hsps that were shaped by evolutionary gains, losses, and

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shifts in cis-regulation. Expression patterns of these heat-inducible Hsps reflect adaptive variation in thermal tolerance between *P. barbatus* and *A. picea*.

Results

Identification of conserved Hsp and cis-regulatory HSEs

We recovered conserved Hsps from all of the major gene families (Hsp90, Hsp70, Hsp60, Hsp40, small Hsps; Table 1). Three paralogues within the Hsp90 gene family (*trap1*, *gp93*, and *hsp83*) were found across all surveyed insects. We recovered 5 of the 6 *Drosophila melanogaster* Hsp70 homologues (CG2918, *hsc70-3* (BIP), *hsc70-4*, *hsc70-5*, and *hsp70CB*; Table 1) for Hymenoptera. With the exception of *Nasonia vitripennis*, the Hymenopteran taxa all lacked the heat-inducible orthologue *hsp70* (Table 1). For all species, we recovered two paralogues of Hsp60 (Table 1). Hsp40 gene families are one of the most diverse Hsps, but we narrowed our search to *DnaJ-1*, which is the known heat-inducible paralogue of *D. melanogaster* (Table 1). We did not recover a *DnaJ-1* paralogue from any of the insects surveyed and found the best BLAST match to be *D. melanogaster CG5001* (Table 1). Forward BLAST searching for *D. melanogaster* sHsps (*hsp22*, *hsp23*, *hsp26*, *hsp27*) yielded no reciprocal BLAST hits; instead, the closest match was *lethal 2 essential for life* (*l(2)efl)*, for which there were 3-9 copies in the Hymenoptera, and 1-17 copies in other members of the outgroup (Table 1).

Of the Hsp homologues, eight were quantifiable by qPCR and were subsequently searched for cis-regulatory HSEs (Table 1, indicated with asterisks). Local alignment of the promoter regions of *hsp83*, *hsc70-4* (h1 and h2), and *hsp40* across species indicated conserved location, conformation, and arrangement of cis-regulatory HSEs (Figures 1-3),

whereas *hsc70-3* (BIP), *hsc70-5*, *hsp60*, and *l(2)efl* had less conserved HSEs (Additional files 4-6: Figures S1-S3; data not shown for *l(2)efl*). For Hsps with conserved HSEs, 193 HSE motifs were annotated, including 114 head types ('nGAAn') and 79 tail types ('nTTCn'; Figures 1-3). Across all sampled insects, we found no consistent preference for head or tail motifs in *hsp83* (exact binomial test, p=0.055), significant preference for the head motif in *hsc70-4* (p<0.001), and significant preference for the tail motif in *hsp40* (p<0.05).

Heat shock protein (Hsp) and cis-regulatory heat shock element (HSE) evolution:

Hsp83

Phylogenetic reconstruction of *hsp83* revealed multiple duplications and losses in both the outgroup and Hymenoptera (Figure 1). An early duplication event in a common ancestor of the Hymenoptera generated two paralogues of *hsp83* (h1 and h2 in Figure 1). Although both paralogues are present in bees and wasps, only one paralogue (h2) exists in ants, indicating a secondary loss. A second duplication of the h2 orthologue occurred in *Linepithema humile.* Selection analysis along the length of the gene sequence indicated that most sites (608/714 and 625/714, Single likelihood ancestor counting (SLAC) and Relative effects likelihood (REL) analyses, respectively, Table 2) identified purifying selection; there was no evidence for episodic diversifying selection in branches leading to Hymenopteran paralogues (Branch-REL, p> 0.5; Figure 1).

In spite of overall sequence conservation, Hymenopteran *hsp83* h2 differs in genomic structure and cis-regulation from Hymenopteran *hsp83* h1 and from outgroup

species in three ways. First, Hymenopteran *hsp83* h1 and most outgroup species completely lack introns, whereas *hsp83* h2 has two introns; *Apis mellifera hsp83* h1 is the exception, with one intron in *hsp83* h1 (Additional file 7: Figure S4). Second, Hymenopteran *hsp83* h2 has a split HSE arrangement (4-6 and 3 HSE motifs), whereas both *hsp83* Hymenopteran h1 and the outgroup have a contiguous HSE arrangement (6-9 HSE motif length) at the proximal end of the molecule (30-100 bps upstream TSS; Figure 1). Third, there is a preference in head-type motifs only in Hymenopteran *hsp83* h2 (Fisher's Exact Test, p <0.001; Figure 1).

Hsc70-4

Phylogenetic reconstruction of *hsc70-4* indicates multiple duplication events both within species (*C. quinquefasciatus* and *A. pisum*) and in a common ancestor of the Hymenoptera, leading to two paralogues (h1 and h2; Figure 2). Each paralogue forms a strongly supported clade, with the exception of the two *Bombus* species, in which the h1 paralogue is nested within the h1 clade but the second copy does not group with either Hymenopteran paralogue (Figure 2). There is evidence of episodic diversifying selection along the branch preceding the *hsc70-4* duplication in the Hymenoptera and also in the Hymenopteran *hsc70-4* h2 lineage (Branch-REL, p <0.001 in both cases; Figure 2), even though most individual sites (608/710 and 610/710, SLAC and REL analyses, respectively) were under purifying selection (Table 2).

Hymenopteran *hsc70-4* differs in genomic structure and cis-regulatory HSEs from that of *D. melanogaster*. The orthologue of *hsc70-4* in *D. melanogaster* lacks introns and cis-regulatory HSEs (Additional file 8: Figure S5; Figure 2). In contrast, Hymenopteran

hsc70-4 h1 has one intron, with the exception of *N. vitripennis,* which has two introns.

Hymenopteran *hsc70-4* h2 also has two introns, with the exception of *Bombus*

(Additional file 8: Figure S5). Compared to the *hsc70-4* in members of the outgroup

(Figure 2, *right*), both Hymenopteran *hsc70-4* paralogues showed high diversification in

cis-regulatory HSEs, particularly at the more distal positions (>120bps upstream TSS).

At the proximal position (30-115 bps upstream TSS), however, HSEs of Hymenopteran

hsc70-4 aligned locally with the inducible *D. melanogaster hsp70* gene (data not shown).

Hsp40

Both sequence and copy number of *hsp40* were phylogenetically conserved across

all insect species (Figure 3). Most sites were under purifying selection (Table 2), and

there was no evidence of episodic diversifying selection along branches leading to the

Hymenoptera (Figure 3). Cis-regulatory HSEs of *hsp40* were concentrated in one

conserved proximal block of 3-7 HSE subunits that were located 35-100 bps upstream of

the TSS, although in *D. melanogaster* HSEs were located 255-285 bps upstream (Figure

3). However, the genetic structure appears less conserved, ranging from zero to three

introns (Additional file 9: Figure S6).

Inducible Hsp expression

We tested whether the presence or absence of conserved cis-regulatory HSEs

successfully predicted Hsp gene induction in response to experimental heat shock. The

four Hsp genes with conserved HSEs were all significantly up-regulated in response to

increasing temperature treatments (*hsp83* ($F_{5,12}$=8.48; p<0.01), *hsc70-4* h1 ($F_{5,12}$=3.74;

p<0.05), *hsc70-4* h2 ($F_{5,12}$=10.6; p<0.001), and *hsp40* ($F_{5,12}$=6.97, p<0.01); Figure 4A-D).

The other four Hsps, which lacked conserved HSEs, were not significantly up-regulated after heat shock (*hsc70-5* ($F_{5,12}$=2.17; p=0.13), *hsc70-3* ($F_{5,12}$=1.91; p=0.17), *hsp60* ($F_{5,12}$=2.86; p=0.063), and *l(2)efl* ($F_{5,12}$=0.223; p=0.946); Figure 5A-D).

Species comparisons

We then tested whether variation in thermal tolerances between two ant species was accompanied by changes in Hsp inducibility. The median lethal temperature 50 (LT_{50}) of the warm-climate *P. barbatus* (median LT_{50}=46.9 °C) was significantly higher than the LT50 of the cool-climate *A. picea* (median LT_{50}= 38.78 °C; generalized linear model (GLM) with a binomial response variable: influence of species, p<0.001; Additional file 10: Figure S7). These survivorship differences were matched by patterns of Hsp gene expression: *P. barbatus* shifted its expression profile toward higher temperatures than did *A. picea* for all inducible Hsps (Figure 4A-D). For *hsp83*, *hsc70-4 h1*, and *hsc70-4* h2, *P. barbatus* showed peak expression at 43°C, whereas *A. picea* showed peak expression at 35-38.5°C (Figure 4A-C). For *hsp40*, peak expression was 40°C and 35°C for *P. barbatus* and *A. picea*, respectively (Figure 4D). *P. barbatus* exhibited significantly higher constitutive expression of *hsc70-4* h1 (ANOVA, $F_{1,5}$=87.8, p<0.01) and *l(2)efl* ($F_{1,5}$=6.92, p<0.05), and significantly lower constitutive expression of *hsc70-3* ($F_{1,5}$=596, p<0.01), *hsc70-5* ($F_{1,5}$=24.3, p<0.001), and *hsp60* ($F_{1,5}$=31.2, p<0.01) than did *A. picea* (Figure 6). Among the inducible Hsps, there was a positive relationship between relative basal expression levels and relative inducibility (linear regression, r^2= 0.918, p<0.05; Figure 7).

Discussion

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Molecular characterization of Hymenopteran Hsps reveals a number of functionally important differences in identity, amino acid sequence, and regulation of chaperone proteins relative to *Drosophila* (Table 2, Figures 1-2). Both *hsp83* and *hsc70-4* display Hymenoptera-specific gains and losses, resulting in unique sets of homologues. Although most codons exhibited purifying selection (Table 1), instances of positive selection along branches leading to and within the Hymenoptera (Figures 1-2, left) suggest novel chaperoning activity [48]. This sequence divergence, coupled with cis-regulatory HSE distribution and expression patterns (Figures 1-2, right; Figures 4-5), suggests that although there is substantial conservation of ancestral inducibility, the HSR response in Hymenoptera has been additionally augmented by expansion and subfunctionalization of novel gene duplicates that are activated by thermal stress.

As in other taxonomic groups, cytoplasmic Hsps mainly mediate the HSR in Hymenoptera (Figures 4-5), whereas mitochondrial and ER-localizing forms of Hsp70 [9,49] and Hsp90 appear to play little role [50,51, but see 52]. The set of inducible Hsps identified likely interact with one another to protect and refold denatured proteins. Upon protein denaturation, Hsp40 delivers unfolded proteins to Hsp70, and the two together mediate refolding through cycles of substrate binding and release driven by ATP binding and hydrolysis [53]. Despite their interdependence, however, the extent of functional diversification of *hsc70-4* and *hsp40* differed substantially (Figures 1-3). *Hsc70-4* showed the most dramatic variation, with the primary inducible member *hsp70* in *Drosophila* completely lost in Hymenoptera, which instead induces two *hsc70-4* paralogues that vary in both fold-increase in response to heat stress (Figures 2,4). The

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insect-wide phylogeny of *hsc70-4* also contains gene duplications in other taxa, suggesting that in general, *hsc70-4*s have undergone multiple evolutionary gains, losses and functional shifts. For example, *Culex quinquefasciatus* has two paralogues and appears to have gained cis-regulatory HSEs in one paralogue (Figure 2), suggesting that one copy is heat-inducible and the other serves housekeeping functions.

Among different species of *Drosophila*, the primacy of *hsp70*'s role in the heat shock response is achieved through an evolutionary increase in copy number from 2 to 5 [16,20]. We detected minimal copy number variation in either *hsp83* or *hsc70*, although it is important to note that lack of detailed manual annotation of non-*Drosophila* genomes may make it difficult to detect highly similar copies should they be present. Nevertheless, our results suggest that Hymenopteran (and other insect) Hsps may have evolved the heat shock response primarily through expansions in cis-regulatory HSEs across the entire promoter region, along with transcriptional enhancement associated with introns that are lacking in *D. melanogaster* (Additional files 7-8: Figures S4-5)[23-25,54-55].

For *hsp83*, we found two paralogues in bees and wasps, one with an ancestral contiguous arrangement of HSEs, and one with a derived split arrangement similar to that of *Drosophila hsp70*. This split arrangement has reduced cooperative binding of HSF trimers, leading to lower basal expression and higher inducibility than in the more contiguous motif of *hsp83*[14,56,57]. The presence of two differentially regulated paralogues may reflect novel functionalization in *hsp83* to provide both basal and inducible Hsp expression. Foraging bees are known to super-heat thoracic muscles prior

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and during flight, which necessitates both constitutive and inducible chaperoning activity [32,34]. Transcriptomic screens in *Apis mellifera* have found weak support for Hsp90 up-regulation in foraging relative to nurse bees, but more detailed and precise quantification of each paralogue will determine whether they have subfunctionalized into constitutive and inducible roles [58]. In contrast, except for the nuptial flight of males and queens, worker ants are flightless, which may explain the secondary loss of the ancestral paralogue but the retention of the more inducible form.

In contrast to *hsc70* and *hsp83, hsp40* was much more conserved. There was a single gene copy per taxon in which most sites were under purifying selection, suggesting that their co-chaperoning activity has been retained across insects. In particular, the conserved J domain of Hsp40 stimulates the ATPase domain of Hsp70 proteins. Across the insect *hsp40* phylogeny, HSE configuration remained conserved for all but *D. melanogaster*, whose primary motif was further from the transcriptional start site (Figure 3). Although the Hsp40 gene family is one of the most diverse molecular chaperones, we captured the paralogue that participates in the HSR because it was significantly up-regulated in response to heat stress. Interestingly, *hsp40* in *P. barbatus* peaked in up-regulation at a less extreme temperature than did the other Hsp proteins (Figure 4D). Early expression of *hsp40* may enhance chaperoning activity by binding to existing and accumulating pools of *hsc70* and also by providing crosstalk with Hsp90-mediated chaperoning [59].

Comparisons of two ant species that experience very different thermal ranges revealed correlated shifts in both the basal expression and inducibility of Hsps that reflect

the higher and more frequent thermal stress expected in extreme habitats (Figure 7, Additional file 10: Figure S7). Workers of the harvester ant *P. barbatus* forage in extreme desert heat [60,61] and may be more reliant on both constitutive and inducible mechanisms to cope with thermal stress than workers of *A. picea*, which are more temperature sensitive and occur in thermally buffered mesic deciduous forest [47,62]. The gene expression responses of *P. barbatus* and *A. picea* are consistent with previous work comparing two hot-desert ant species of *Cataglyphis* with the cool woodland ant *Formica polyctena* [33]. In that study, HSP70 (*hsc70-4*) basal expression and induction were higher in *Cataglyphis*, although alternative paralogues were not fully distinguished. Although Hsp chaperoning activity expends energy (ATP), there may not be trade-offs between continual and maximum induction of Hsps because investment in the HSR is less costly than the loss of biochemical activity from protein denaturation [4,63,64]. In addition, the HSR in *P. barbatus* in this study was shifted upward by ~5-7°C (Figure 4), suggesting underlying differences in overall proteome stability that permit *P. barbatus* to tolerate significantly higher temperatures than *A. picea* (Additional file 10: Figure S7).

Conclusions

Our study represents the most comprehensive survey to date of Hsp sequence and cis-regulatory evolution for insects. Hymenoptera have unique Hsp evolutionary histories shaped by processes such as of gains, losses, and changes in cis-regulation. Based on the presence of conserved cis-regulatory elements (HSEs), we reliably predicted the heat inducible Hsps that are critical for mounting the HSR in ants, suggesting that the ancestral inducibility has been retained. We uncovered greater

diversity in the number, arrangement, and location of cis-regulatory HSEs in

Hymenoptera for two major Hsp genes (*hsp83* and *hsc70-4*), suggesting that the HSR is

mediated through changes in cis-regulation as opposed to increasing gene copy number.

Furthermore, Hsp expression patterns were associated with the thermal limits of two ant

species that inhabit different thermal environments. Collectively, our analyses suggest

Hsp sequence and expression patterns may reflect the forces of selection acting on

thermal tolerance in ants and other social Hymenoptera.

Methods

Phylogenetic Reconstruction

To reconstruct the evolutionary relationships of heat shock proteins, we identified

orthologous Hsps in 17 insect species representing five insect orders using the well-

characterized Hsps of *Drosophila melanogaster* as a reference (Additional file 1: Table

S1). Reciprocal best BLAST (blastp) searches (e-value < 1E-10, and top bit score) were

used to identify annotated orthologues of the known *D. melanogaster* paralogues with an

ant-specific genome database (http://antgenomes.org/, [65]) as well as with the NCBI

non-redundant protein and nucleotide databases (Additional file 1: Table S1). To find

unannotated sequences, we queried *D. melanogaster* orthologues with tblastn to each

insect species' genome. To identify additional homologues not found with BLAST, we

employed a similar search with Hmmer 3.0 [66]. We used *Drosophila melanogaster*

transcripts to search (*hmmsearch*) each individual genome and identified orthologues

based on e-value < 1E-10 and top bit score. HMMER searches recovered 9 additional

copies from 2 genes (*gp93* and *hsp70*) for *Culex quinquefasciatus*. Identified nucleotide

sequences were translation-aligned with MAFFT using default settings [67] to identify

homologous codons for subsequent selection analyses and the resultant alignment was

translated for phylogenetic reconstruction. We reconstructed gene relationships of

homologous Hsps with PhyML [68], and bootstrap support was estimated for 1,000

replicate searches utilizing an amino acid substitution model inferred from Prottest3 [69].

Similar phylogenetic relationships were recovered with a Bayesian analysis using

MrBayes [70] (data not shown).

Tests of Selection

Selection at the protein-coding level was quantified as the ratio of the

nonsynonymous substitution rate to the synonymous substitution rate ($\omega = d_N/d_S$); $\omega > 1$

indicates positive selection, whereas $\omega < 1$ indicates purifying selection, and $\omega = 1$ is

indicates neutral evolution [71]. For each homologous Hsp, we tested for selection at

individual codons as well as across the phylogeny using the HyPhy package [72] on the

web-interface Datamonkey (http://www.datamonkey.org).

We identified individual codon sites for positive selection using Single-

Likelihood Ancestor Counting (SLAC), Random Effects Likelihood (REL), and Fixed

Effects Likelihood [71]. SLAC calculates the number of observed and expected d_N and d_S

rates and conservatively estimates ω using a recommended cutoff of p=0.1 [73]. The REL

method is an extension of analyses in PAML [71] that allows d_N and d_S to vary across

sites and uses a Bayes factor (>50) to assess selection [73]. FEL estimates d_N and d_S from

the codon substitution model and implements a likelihood ratio to evaluate significance

using a recommended cutoff of p=0.1 [73].

In addition to testing for selection at sites along the gene, we tested for changes in selective pressures across the reconstructed amino acid phylogeny, which might indicate evolutionary shifts in gene function. Episodic diversifying selection was assessed using branch-REL and MEME [74,75]: branch-site REL detects episodic diversifying selection for individual lineages [74], whereas MEME is an extension of FEL that detects episodic diversifying evolution by allowing ω to vary across branches and sites [75].

Identification of genomic structure and cis-regulatory Heat Shock Elements (HSE)

Identification of genomic structure and cis-regulatory HSEs was performed for Hsps that were detectable by qPCR (for methods, see Quantitative real time PCR). We mapped transcripts to their respective genomic regions in Geneious Pro 6.1 [76] and identified exons and introns, making further manual alignments by hand when necessary. The transcriptional start site (TSS) was predicted using Neural Network Promoter Predictor (NNPP)[77]. Previous chip-seq experiments in *D. melanogaster* revealed that HSF binds primarily to Hsp promoters within 1250 bps of the TSS [78]; sequences were trimmed to this length and locally aligned to identify orthologous HSEs.

To identify cis-regulatory HSEs, we followed a modified search procedure adapted from Tian et al. (2010) [17]. Promoter sequences were searched for the putative HSE motif (head conformation = nGAAnnTTCnnGAAn or tail conformation = nTTCnnGAAnnTTCn) [79], allowing for a 2 base-pair mismatch from the preferred sequence. HSE motifs were then manually screened and annotated by the number and type of subunit occupying the distal end (subunits beginning with 'nGAAn' or 'nTTCn' refer to the head or tail conformation, respectively). Mismatches occurring at critical sites

for HSF binding (G in 2nd position of head conformation, C in 4th position of tail conformation) [80] were discarded, unless motifs were interior to a HSE with 3 or more subunits, known as a gapped HSE [17].

A final screen was employed to quantify the binding strength of each HSE subunit. Briefly, a WebLogos [81] was generated for head and tail types recovered from the search. Bit scores for the preferred base at each of the 5 possible positions in a subunit were summed; the match between the individual subunits and the preferred subunit was expressed as the ratio of the summed observed bit score over the preferred bit score. Subunits with scores less than 0.5 were discarded unless flanked with subunits with scores greater than 0.5, again indicating a 'gapped' HSE. 253 out of 1753 total HSEs were retained after screening (Additional file 2: Table S2).

Field collections and lab rearing

Hsp induction was quantified in workers sampled from lab-acclimated colonies of *Pogonomyrmex barbatus* and *Aphaenogaster picea*. Three *Pogonomyrmex barbatus* colonies were reared from queens collected following a mating flight at the Welder Wildlife Foundation in Sinton Co., Texas (28.10837 °N 97.42265 °W) in June 2007. Colonies were maintained in an environmental room at the University of Vermont, Department of Biology, with a 12:12 light dark light cycle at 30°C in $17 \times 12 \times 6$ cm plastic nest boxes provided with three 16×150mm disposable glass test tubes in which water was supplied behind a cotton stopper as a nest site. Each week, colonies were fed 2 mealworms (*Tribolium molitor*) and an *ad libitum* seed mixture composed of oat bran, wheat germ, millet, thistle seeds, and quinoa.

Eight colonies of *A. picea* were collected in May and June 2012 from black spruce forest adjacent to Molly Bog (44.508611°N, 72.702222°W), located near Stowe, Vermont. Entire live colonies containing 500-1000 workers, brood, and queen were excavated from the leaf litter. Colonies were maintained for 1 month in the laboratory at 25°C +/- 1°C with 12 hour light/dark cycles in a 7 x 3 ¼ x 1 ¾ inch plastic nest box covered with red cellophane and connected to an open plastic foraging arena filled with ~1cm sand and lined with Insect-a-slip (BioQuip) to prevent escape. 1-3 cotton-plugged water tubes (16 ×150 mm) were provided in the nest box for each colony to maintain humidity. Approximately 200 μl of 20% honey water and one bisected mealworm were provided in each foraging arena every 3 days.

Thermal Tolerance Assays

Acute upper thermal limits in both species were determined by quantifying a LT_{50} temperature, defined as the temperature at which a one-hour exposure produced 50% worker mortality after 3 days of recovery using the *dose.p* function in the MASS package within R (version 3.2.0)[82]. Ants were exposed to six different temperature regimes (30, 35, 40, , 42, 45, 46 °C for *P. barbatus* and 25, 30, 35, 36.5, 38.5 40°C for *A. picea*). Temperature treatments were applied by confining 10-13 nest-mate workers together in a 5mL screw-cap glass vial and submerging the vial in a pre-set Thermo Neslab EX17 heating water bath for 1 hour. Temperature inside the vials was monitored with a temperature probe inserted in an empty 5 mL glass vial submerged in the water bath simultaneously. After the application of temperature treatment, ten ants were allowed to recover for survival counts in a 16×150mm cotton-plugged water tube. For each

treatment, three ants per colony from four of the eight *A. picea* colonies and the three *P. barbatus* colonies were flash frozen and stored at -80°C for gene expression analyses.

Quantitative real time PCR

RNA was isolated from flash-frozen ants with RNAzol (Molecular Research Center, Inc., USA) and then purified with the RNeazy micro kit (QIAGEN, USA) for downstream gene expression quantification. Flash-frozen ants from each temperature treatment were pooled and homogenized in a Bullet Blender (Next Advance Inc., USA) homogenizer at top speed (10) with 1.4mm zirconium silicate grinding beads (Quackenbush Co., Inc., USA) and 500 uL of RNAzol buffer (Molecular Research Center, Inc., USA) for 3 minutes. Following the manufacturer's instructions for RNAzol, RNA samples were resuspended in 100 uL of water and subsequently purified with Rneasy micro kit with DNAse I (Qiagen, USA) treatment on the micro column to remove genomic DNA contamination. RNA was quantified with Nanodrop spectrophotometry; all sample 260/280 ratios were between 2.0-2.2, indicating acceptable RNA quality. mRNA was reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (ABI, USA).

To detect specific heat shock proteins, primers were designed for a whole suite of genes for each gene family (Table 1, Additional file 3: Table S3). Table 1 highlights (in *) working primer sets. Quantitative PCR was performed on an ABI StepOnePlus Real-Time PCR system. Reactions were performed in 20 μl volumes with 2 ng of template cDNA, 500 nM total primer, and 10 μl of Power SYBR® Green Master Mix (Life Technologies, USA). Cycling conditions consisted of an initial 95°C incubation for 2 min

and then 40 cycles of 95°C for 15 s, with 55°C annealing and extension for 60 s.

Following amplification, melt curve analyses confirmed the presence of a single

amplicon. All gene products from preliminary experiments were sequenced for

verification of specific gene amplification.

Gene expression fold changes were calculated relative to rearing temperatures

using the ΔΔ CT method [83] after empirically determining ~100% primer efficiencies

for each primer set (Additional file 3: Table S3). The set of housekeeping genes for

normalization was determined with Normfinder [84], which calculated the relative

stability of four housekeeping genes (18s rRNA, GAPDH, β-actin, and Ef1β) and selected

the most stable genes across samples. For *A. picea*, 18s rRNA and β-actin were most

stable (0.20 stability), whereas 18s rRNA and GAPDH (0.25 stability) were the most

stable for *P. barbatus*. For cross-species comparisons, 18s rRNA and β-actin were the

most stable (0.05 stability). Differences in HSP up-regulation across temperature

treatments were determined with a one-way Analysis of Variance (ANOVA) in which

fold expression values were log_{10} transformed to meet assumptions of normality.

Significant up-regulation relative to controls was identified with *post hoc* Tukey tests.

Additional files

The data sets supporting the results of this article are included within the article and its

additional files.

Additional file 1: Table S1. Nucleotide sequences used to characterize the molecular

evolution of heat shock proteins.

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Additional file 2: Table S2. Sequence annotations (position, length, arrangement) of cis-regulatory HSEs for each HSP gene and across all species screened.

Additional file 3: Table S3. Primer sets for qPCR including housekeeping and heat shock genes.

Additional file 4: Figure S1. Maximum likelihood phylogeny of *Hsp60* (mitochondrial form) for 17 species of insects (rooted on *A. pisum*) using a JTT amino acid substitution model and 1,000 bootstraps replicates.

Additional file 5: Figure S2. Maximum likelihood phylogeny of *hsc70-3* (BIP) for 17 species of insects (rooted on *A. pisum*) using a JTT amino acid substitution model and 1,000 bootstrap replicates.

Additional file 6: Figure S3. Maximum likelihood phylogeny of *hsc70-5* for 17 species of insects (rooted on *A. pisum*) using a JTT amino acid substitution model and 1,000 bootstrap replicates.

Additional file 7: Figure S4. Local alignment of the genomic region of orthologous *hsp83* from 17 insect species spanning 5 insect Orders.

Additional file 8: Figure S5. Local alignment of the genomic region of orthologous *hsc70-4* from 17 insect species spanning 5 insect Orders.

Additional file 9: Figure S6. Local alignment of the genomic region of orthologous *hsp40* from 17 insect species spanning 5 insect Orders.

Additional file 10: Figure S7. Percent survival (+/1 SD) of *Aphaenogaster picea* and *P. barbatus* (right panel) from heat shock treatments at different temperature treatments.

Abbreviations

ANOVA: Analysis of Variance; Hsp: Heat shock protein; HSR: Heat shock response; HSE: Heat shock element; HSF: Heat shock factor; qPCR: quantitative polymerase chain reaction

Competing interests

The authors declare that they have no competing interests.

Authors' Contributions

ADN, SHC, NJG conceived and designed the study. ADN conducted the phylogenetic reconstruction, tests for selection, thermal tolerance assays, qPCR, and wrote the manuscript. SHC and NJG assisted with the statistical analyses and writing of the manuscript. All three authors have read and approved the submitted manuscript.

References

1. Lindquist S, Craig EA: **The Heat-Shock Proteins**. *Annual Review of Genetics* 1988, **22**:631–677.

2. Feder ME, Hofmann GE: **HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology**. *Annual Review of Physiology* 1999, **61**:243–282.

3. Kültz D: **Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function**. *J Exp Biol* 2003, **206**:3119–3124.

4. Geiler-Samerotte KA, Dion MF, Budnik BA, Wang SM, Hartl DL, Drummond DA: **Misfolded proteins impose a dosage-dependent fitness cost and trigger a cytosolic unfolded protein response in yeast**. *Proceedings of the National Academy of Sciences* 2011, **108**:680–685.

5. Hartl FU, Bracher A, Hayer-Hartl M: **Molecular chaperones in protein folding and proteostasis**. *Nature* 2011, **475**:324–332.

6. Ashburner M, Bonner JJ: **The induction of gene activity in drosophila by heat shock**. *Cell* 1979, **17**:241–254.

7. Richter K, Haslbeck M, Buchner J: **The Heat Shock Response: Life on the Verge of Death**. *Molecular Cell* 2010, **40**:253–266.

8. Nei M, Rooney AP: **Concerted and Birth-and-Death Evolution of Multigene Families***. *Annual Review of Genetics* 2005, **39**:121–152.

9. Krenek S, Schlegel M, Berendonk TU: **Convergent evolution of heat-inducibility during subfunctionalization of the Hsp70 gene family**. *BMC Evolutionary Biology* 2013, **13**:49.

10. Fink AL: **Chaperone-Mediated Protein Folding**. *Physiological Reviews* 1999, **79**:425–449.

11. Johnson JL: **Evolution and function of diverse Hsp90 homologs and cochaperone proteins**. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2012, **1823**:607–613. [*Heat Shock Protein 90 (Hsp90)*]

12. Morris JP, Thatje S, Hauton C: **The use of stress-70 proteins in physiology: a re-appraisal**. *Molecular Ecology* 2013, **22**:1494–1502.

13. Bonner JJ, Ballou C, Fackenthal DL: **Interactions between DNA-bound trimers of the yeast heat shock factor**. *Mol Cell Biol* 1994, **14**:501–508.

14. Fernandes M, Xiao H, Lis JT: **Binding of heat shock factor to and transcriptional activation of heat shock genes in Drosophila.** *Nucleic Acids Res* 1995, **23**:4799–4804.

15. Santoro N, Johansson N, Thiele DJ: **Heat Shock Element Architecture Is an Important Determinant in the Temperature and Transactivation Domain Requirements for Heat Shock Transcription Factor**. *Mol Cell Biol* 1998, **18**:6340–6352.

16. Bettencourt BR, Feder ME: **Hsp70 Duplication in the Drosophila melanogaster Species Group: How and When Did Two Become Five?** *Mol Biol Evol* 2001, **18**:1272–1282.

17. Tian S, Haney RA, Feder ME: **Phylogeny Disambiguates the Evolution of Heat-Shock cis-Regulatory Elements in Drosophila**. *PLoS ONE* 2010, **5**:e10669.

18. Astakhova LN, Zatsepina OG, Przhiboro AA, Evgen'ev MB, Garbuz DG: **Novel arrangement and comparative analysis of hsp90 family genes in three thermotolerant species of Stratiomyidae (Diptera)**. *Insect Molecular Biology* 2013, **22**:284–296.

19. Krebs RA, Feder ME: **Natural Variation in the Expression of the Heat-Shock Protein HSP70 in a Population of Drosophila melanogaster and Its Correlation with Tolerance of Ecologically Relevant Thermal Stress**. *Evolution* 1997, **51**:173–179.

20. Hoffmann AA, Sørensen JG, Loeschcke V: **Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches**. *Journal of Thermal Biology* 2003, **28**:175–216.

21. Dong Y, Miller LP, Sanders JG, Somero GN: **Heat-Shock Protein 70 (Hsp70) Expression in Four Limpets of the Genus Lottia: Interspecific Variation in**

Constitutive and Inducible Synthesis Correlates With in situ Exposure to Heat Stress. *Biol Bull* 2008, **215**:173–181.

22. Carmel J, Rashkovetsky E, Nevo E, Korol A: **Differential Expression of Small Heat Shock Protein Genes Hsp23 and Hsp40, and heat shock gene Hsr-omega in Fruit Flies (Drosophila melanogaster) along a Microclimatic Gradient**. *J Hered* 2011, **102**:593–603.

23. Buchman AR, Berg P: **Comparison of intron-dependent and intron-independent gene expression.** *Mol Cell Biol* 1988, **8**:4395–4405.

24. Nott A, Meislin SH, Moore MJ: **A quantitative analysis of intron effects on mammalian gene expression**. *RNA* 2003, **9**:607–617.

25. Le Hir H, Nott A, Moore MJ: **How introns influence and enhance eukaryotic gene expression**. *Trends in Biochemical Sciences* 2003, **28**:215–220.

26. Dunn RR, Agosti D, Andersen AN, Arnan X, Bruhl CA, Cerdá X, et al.: **Climatic drivers of hemispheric asymmetry in global patterns of ant species richness**. *Ecology Letters* 2009, **12**:324–333.

27. Sanders NJ, Lessard J-P, Fitzpatrick MC, Dunn RR: **Temperature, but not productivity or geometry, predicts elevational diversity gradients in ants across spatial grains**. *Global Ecology and Biogeography* 2007, **16**:640–649.

28. Munyai TC, Foord SH: **Temporal Patterns of Ant Diversity across a Mountain with Climatically Contrasting Aspects in the Tropics of Africa**. *PLoS ONE* 2015, **10**:e0122035.

29. Kaspari M, Clay NA, Lucas J, Yanoviak SP, Kay A: **Thermal adaptation generates a diversity of thermal limits in a rainforest ant community**. *Glob Change Biol* 2015, **21**:1092–1102.

30. Traniello JFA, Fujita MS, Bowen RV: **Ant foraging behavior: ambient temperature influences prey selection**. *Behav Ecol Sociobiol* 1984, **15**:65–68.

31. Penick CA, Tschinkel WR: **Thermoregulatory brood transport in the fire ant, Solenopsis invicta**. *Insect Soc* 2008, **55**:176–182.

32. Heinrich B: **Mechanisms of Body-Temperature Regulation in Honeybees, Apis Mellifera: I. Regulation of Head Temperature**. *J Exp Biol* 1980, **85**:61–72.

33. Gehring WJ, Wehner R: **Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara desert.** *PNAS* 1995, **92**:2994–2998.

34. Elekonich MM: **Extreme thermotolerance and behavioral induction of 70-kDa heat shock proteins and their encoding genes in honey bees**. *Cell Stress and Chaperones* 2009, **14**:219–226.

35. Xu PJ, Xiao JH, Xia QY, Murphy B, Huang DW: **Apis mellifera has two isoforms of cytoplasmic HSP90**. *Insect Molecular Biology* 2010, **19**:593–597.

36. Wang H, Li K, Zhu J-Y, Fang Q, Ye G-Y, Wang H, Li K, Zhu J-Y: **CLONING AND EXPRESSION PATTERN OF HEAT SHOCK PROTEIN GENES FROM THE ENDOPARASITOID WASP, Pteromalus puparum IN RESPONSE TO ENVIRONMENTAL STRESSES**. *Archives of Insect Biochemistry and Physiology* 2012, **79**:247–263.

37. Bonasio R, Zhang G, Ye C, Mutti NS, Fang X, Qin N, et al.: **Genomic Comparison of the Ants Camponotus floridanus and Harpegnathos saltator**. *Science* 2010, **329**:1068–1071.

38. Nygaard S, Zhang G, Schiøtt M, Li C, Wurm Y, Hu H, et al.: **The genome of the leaf-cutting ant Acromyrmex echinatior suggests key adaptations to advanced social life and fungus farming**. *Genome Res* 2011, **21**:1339–1348.

39. Smith CD, Zimin A, Holt C, Abouheif E, Benton R, Cash E, et al.: **Draft genome of the globally widespread and invasive Argentine ant (Linepithema humile)**. *PNAS* 2011, **108**:5673–5678.

40. Smith CR, Smith CD, Robertson HM, Helmkampf M, Zimin A, Yandell M, et al.: **Draft genome of the red harvester ant Pogonomyrmex barbatus**. *PNAS* 2011, **108**:5667–5672.

41. Suen G, Teiling C, Li L, Holt C, Abouheif E, Bornberg-Bauer E, et al.: **The Genome Sequence of the Leaf-Cutter Ant Atta cephalotes Reveals Insights into Its Obligate Symbiotic Lifestyle**. *PLoS Genet* 2011, **7**:e1002007.

42. Wurm Y, Wang J, Riba-Grognuz O, Corona M, Nygaard S, Hunt BG, et al.: **The genome of the fire ant Solenopsis invicta**. *PNAS* 2011, **108**:5679–5684.

43. The Honeybee Genome Sequencing Consortium: **Insights into social insects from the genome of the honeybee Apis mellifera**. *Nature* 2006, **443**:931–949.

44. Sadd BM, Barribeau SM, Bloch G, Graaf DC de, Dearden P, Elsik CG, et al.: **The genomes of two key bumblebee species with primitive eusocial organization**. *Genome Biology* 2015, **16**:76.

45. Werren JH, Richards S, Desjardins CA, Niehuis O, Gadau J, Colbourne JK: **Functional and Evolutionary Insights from the Genomes of Three Parasitoid Nasonia Species**. *Science* 2010, **327**:343–348.

46. Johnson RA: **Habitat segregation based on soil texture and body size in the seed-harvester ants Pogonomyrmex rugosus and P. barbatus**. *Ecological Entomology* 2000, **25**:403–412.

47. Lubertazzi D: **The Biology and Natural History of *Aphaenogaster rudis***. *Psyche: A Journal of Entomology* 2012, **2012**.

48. Carretero-Paulet L, Albert VA, Fares MA: **Molecular Evolutionary Mechanisms Driving Functional Diversification of the HSP90A Family of Heat Shock Proteins in Eukaryotes**. *Mol Biol Evol* 2013, **30**:2035–2043.

49. Gross TL, Myles KM, Adelman ZN: **Identification and Characterization of Heat Shock 70 Genes in Aedes aegypti (Diptera: Culicidae)**. *Journal of Medical Entomology* 2009, **46**:496–504.

50. Sørensen JG, Nielsen MM, Kruhøffer M, Justesen J, Loeschcke V: **Full genome gene expression analysis of the heat stress response in Drosophila melanogaster**. *Cell Stress Chaperones* 2005, **10**:312–328.

51. Bettencourt BR, Hogan CC, Nimali M, Drohan BW: **Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in Drosophila melanogaster but does not compensate for loss of thermotolerance in Hsp70 null flies**. *BMC Biology* 2008, **6**:5.

52. Clark MS, Thorne MAS, Toullec J-Y, Meng Y, Guan LL, Peck LS, Moore S: **Antarctic Krill 454 Pyrosequencing Reveals Chaperone and Stress Transcriptome**. *PLoS ONE* 2011, **6**:e15919.

53. Qiu X-B, Shao Y-M, Miao S, Wang L: **The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones**. *Cell Mol Life Sci* 2006, **63**:2560–2570.

54. Garbuz DG, Molodtsov VB, Velikodvorskaia VV, Evgen'ev MB, Zatsepina OG: **Evolution of the Response to Heat Shock in Genus Drosophila**. *Russian Journal of Genetics* 2002, **38**:925–936.

55. Garbuz D, Evgenev MB, Feder ME, Zatsepina OG: **Evolution of thermotolerance and the heat-shock response: evidence from inter/intraspecific comparison and**

interspecific hybridization in the virilis species group of Drosophila. I. Thermal phenotype. *J Exp Biol* 2003, **206**:2399–2408.

56. Xiao H, Lis JT: **Heat shock and developmental regulation of the Drosophila melanogaster hsp83 gene.** *Mol Cell Biol* 1989, **9**:1746–1753.

57. Xiao H, Perisic O, Lis JT: **Cooperative binding of drosophila heat shock factor to arrays of a conserved 5 bp unit**. *Cell* 1991, **64**:585–593.

58. Whitfield CW, Cziko A-M, Robinson GE: **Gene Expression Profiles in the Brain Predict Behavior in Individual Honey Bees**. *Science* 2003, **302**:296–299.

59. Brychzy A, Rein T, Winklhofer KF, Hartl FU, Young JC, Obermann WMJ: **Cofactor Tpr2 combines two TPR domains and a J domain to regulate the Hsp70/Hsp90 chaperone system**. *The EMBO Journal* 2003, **22**:3613–3623.

60. Gordon DM: **The rewards of restraint in the collective regulation of foraging by harvester ant colonies**. *Nature* 2013, **498**:91–93.

61. Helms KR, Vinson B: **Surface activity of native ants co-occurring with the red imported fire ant, Solenopsis invicta (Hymenoptera: Formicidae)**. *Southwestern Entomologist* 2005, **30**:223-237.

62. Pelini SL, Diamond SE, MacLean H, Ellison AM, Gotelli NJ, Sanders NJ, Dunn RR: **Common garden experiments reveal uncommon responses across temperatures, locations, and species of ants**. *Ecology and Evolution* 2012, **2**:3009–3015.

63. Krebs RA, Loeschcke V: **Costs and Benefits of Activation of the Heat-Shock Response in Drosophila melanogaster**. *Functional Ecology* 1994, **8**:730–737.

64. Sørensen JG, Kristensen TN, Loeschcke V: **The evolutionary and ecological role of heat shock proteins**. *Ecology Letters* 2003, **6**:1025–1037.

65. Wurm Y, Uva P, Ricci F, Wang J, Jemielity S, Iseli C, Falquet L, Keller L: **Fourmidable: a database for ant genomics**. *BMC Genomics* 2009, **10**:5.

66. Mistry J, Finn RD, Eddy SR, Bateman A, Punta M: **Challenges in homology search: HMMER3 and convergent evolution of coiled-coil regions**. *Nucl Acids Res* 2013, **41**:e121–e121.

67. Katoh K, Misawa K, Kuma K, Miyata T: **MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform**. *Nucl Acids Res* 2002, **30**:3059–3066.

68. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O: **New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0**. *Syst Biol* 2010, **59**:307–321.

69. Darriba D, Taboada GL, Doallo R, Posada D: **ProtTest 3: fast selection of best-fit models of protein evolution**. *Bioinformatics* 2011, **27**:1164–1165.

70. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP: **MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space**. *Syst Biol* 2012, **61**:539–542.

71. Yang Z: **Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution.** *Mol Biol Evol* 1998, **15**:568–573.

72. Pond SLK, Frost SDW: **Datamonkey: rapid detection of selective pressure on individual sites of codon alignments**. *Bioinformatics* 2005, **21**:2531–2533.

73. Pond SLK, Frost SDW: **Not So Different After All: A Comparison of Methods for Detecting Amino Acid Sites Under Selection**. *Mol Biol Evol* 2005, **22**:1208–1222.

74. Pond SLK, Murrell B, Fourment M, Frost SDW, Delport W, Scheffler K: **A Random Effects Branch-Site Model for Detecting Episodic Diversifying Selection**. *Mol Biol Evol* 2011, **28**:3033–3043.

75. Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Kosakovsky Pond SL: **Detecting Individual Sites Subject to Episodic Diversifying Selection**. *PLoS Genet* 2012, **8**:e1002764.

76. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A: **Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data**. *Bioinformatics* 2012, **28**:1647–1649.

77. Reese MG: **Application of a time-delay neural network to promoter annotation in the Drosophila melanogaster genome**. *Computers & Chemistry* 2001, **26**:51–56.

78. Gonsalves SE, Moses AM, Razak Z, Robert F, Westwood JT: **Whole-Genome Analysis Reveals That Active Heat Shock Factor Binding Sites Are Mostly Associated with Non-Heat Shock Genes in Drosophila melanogaster**. *PLoS ONE* 2011, **6**:e15934.

79. Perisic O, Xiao H, Lis JT: **Stable binding of Drosophila heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit**. *Cell* 1989, **59**:797–806.

80. Fernandes M, Xiao H, Lis JT: **Fine structure analyses of the Drosophila and Saccharomyces heat shock factor--heat shock element interactions.** *Nucleic Acids Res* 1994, **22**:167–173.

81. Schneider TD, Stephens RM: **Sequence logos: a new way to display consensus sequences.** *Nucleic Acids Res* 1990, **18**:6097–6100.

82. R Development Core Team. **R: A Language and Environment for Statistical Computing. Vienna, Austria.** 2010. http://www.R- project.org/. Accessed 10 January 2014.

83. Livak KJ, Schmittgen TD: **Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method**. *Methods* 2001, **25**:402–408.

84. Andersen CL, Jensen JL, Ørntoft TF: **Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets**. *Cancer Res* 2004, **64**:5245–5250.

Figure and Figure Legends
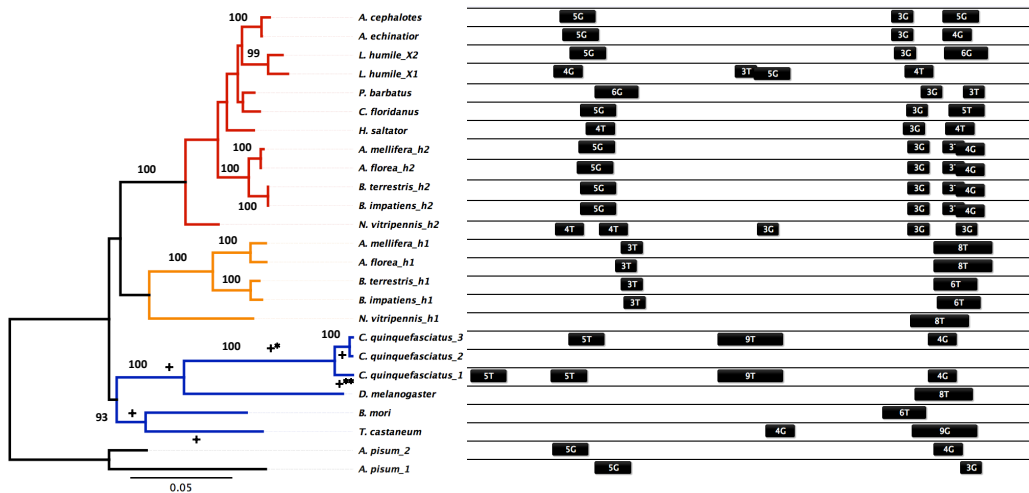
Figure 1. Evolutionary gains and losses in *hsp83* within Hymenoptera, followed by diversification in cis-regulatory HSEs. Relationships of homologous *hsp83* were reconstructed with PhyML for 17 insect species (rooted with *A. pisum*) using a JTT substitution model with 1,000 bootstrap replicates (> 90 bootstrap support indicated; left). Branches of the outgroup taxa are colored in blue and black, while well-supported paralogues of Hymenopteran branches are colored in orange (h1) and red (h2). Statistically significant episodic diversifying selection using

Branch-Rel is indicated along the branch (+ corresponds to p<0.05; * = p<0.01; ** = p<0.001).

Cis-regulatory HSEs in the promoter region spanning 400 bps from the transcription start site

(TSS; right) are mapped onto the phylogeny and are annotated by their length and motif type.
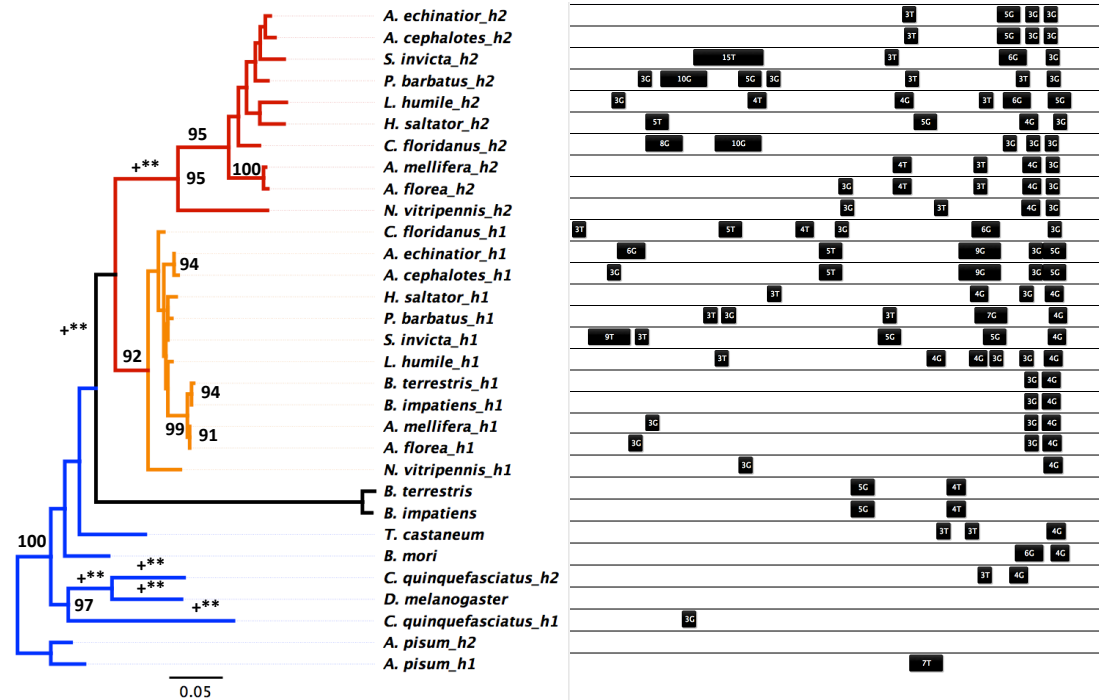
Figure 2. Evolutionary conservation of two copies of *hsc70-4* within Hymenoptera, but both copies harbor an extraordinary amount of diversity in cis-regulatory HSEs. Relationships of homologous *hsc70-4* were reconstructed with PhyML for 17 insect species (rooted on *A. pisum*) using a JTT substitution model with 1,000 bootstrap replicates (> 90 bootstrap support indicated; left). Branches of the outgroup taxa are colored in blue, while well-supported paralogues of Hymenopteran branches are colored in orange (h1) and red (h2). Statistically significant episodes of positive selection identified with Branch-Rel are indicated along the branch(+ corresponds to p<0.05; * = p<0.01; ** = p<0.001). Cis-regulatory HSE elements in the promoter region spanning 570 bps from the transcription start site (TSS; right side) are mapped onto the phylogeny and are annotated by their length and motif type.
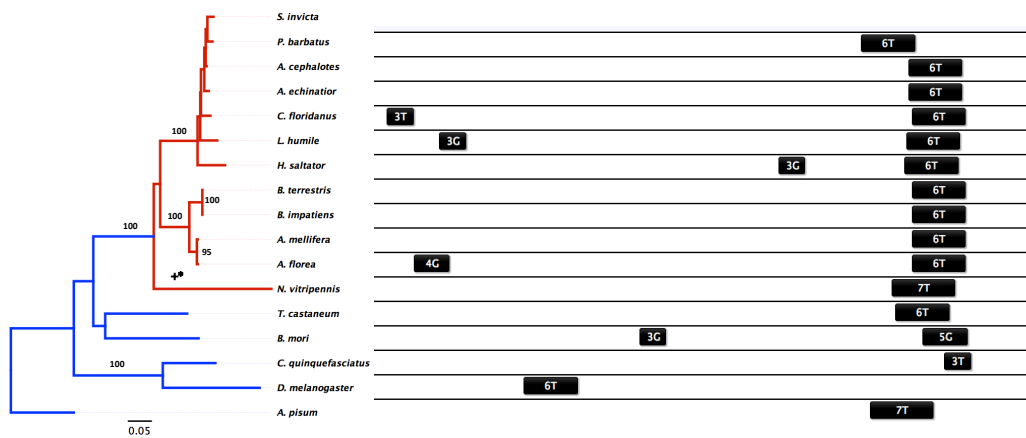
Figure 3. Evolutionary conservation of *hsp40* copy number and cis-regulatory HSEs.

Relationships of homologous hsp40 were reconstructed with PhyML for 17 insect species (rooted on A. pisum) using a JTT substitution model with 1,000 bootstrap replicates (> 90 support indicated). The outgroup and Hymenopteran branches are indicated in blue and red, respectively. Statistically significant episodes of positive selection using Branch-Rel are indicated along the branch (+ corresponds to p<0.05; * = p<0.01; ** = p<0.001). Cis-regulatory HSE elements in the promoter region spanning 370 bps from the transcription start site (TSS; right side) are mapped onto the phylogeny and are annotated by their length and motif type. *S. invicta* did not provide enough sequence information for the identification of cis-regulatory HSEs.
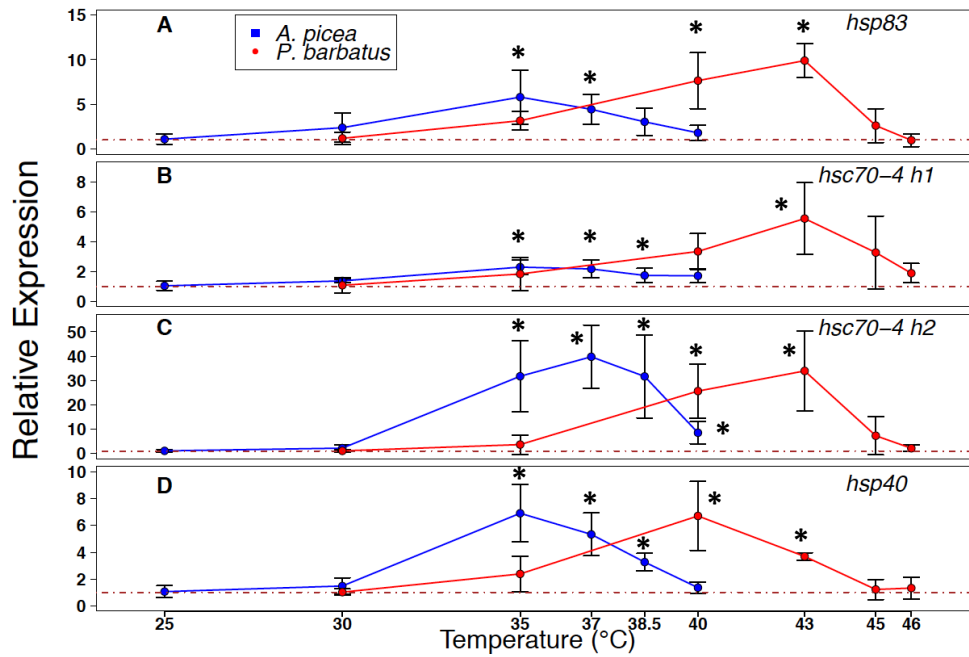
Figure 4. Relative fold increase in gene expression (+/- SD) for inducible HSPs in *A. picea* and *P. barbatus* across different temperature treatment. Relative values were normalized to the 18s rRNA and β-actin, 18s rRNA and *GAPDH* in *A. picea* (N=4 per treatment) and *P. barbatus* (N=3 per treatment), respectively. Significant up-regulation from 25°C (*A. picea*) and 30°C (*P. barbatus*) is denoted by '*' from *post hoc* Tukey tests (p<0.05).
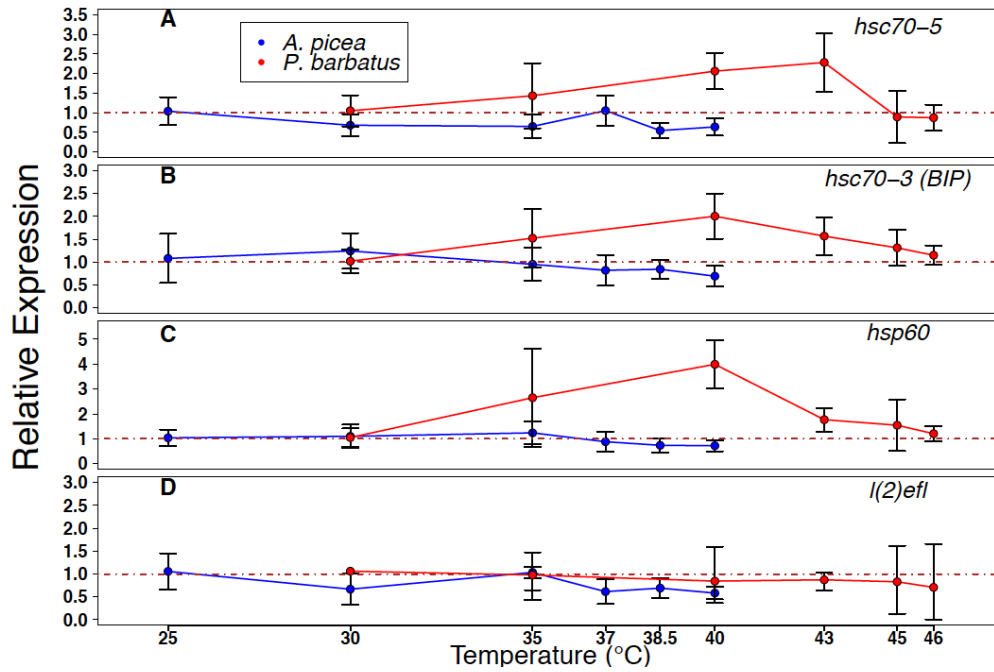
Figure 5. Relative fold change in gene expression (+/- SD) for non-inducible HSPs in *A. picea* and *P. barbatus* across different temperature treatment. Relative expression were normalized to the 18s rRNA and β-actin and 18s rRNA and GAPDH for *A. picea* (N= 4 per treatment) and *P. barbatus* (N= 3 per treatment), respectively.
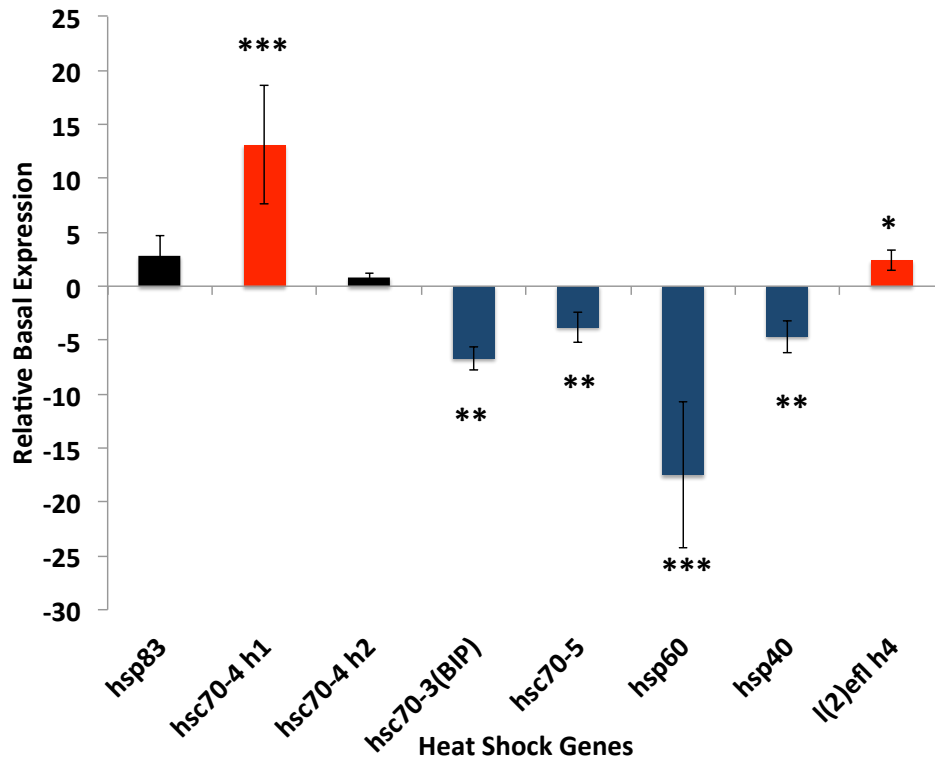
Fig 6. Relative basal heat shock gene (target) expression (+/- SD) between *P. barbatus*(N=3) and *A. picea* (N=4). Relative gene expression was normalized with the geometric mean of 18s rRNA and β-actin as the calibrator (*= p<0.05;** = p<0.01; *** = p < 0.001 levels of significance) and fold change was calculated as *P. barbatus* relative to *A. picea* was calculated as follows: $2^{Target(Pbar-Apic)}/2^{Calibrator(Pbar-Apic)}$(Pbar= *P. barbatus*, Apic= *A. picea*). -1 was divided by values less than one to calculate negative relative basal expression. Significant up-regulation in *P. barbatus* and *A. picea* are colored in red and blue, respectively.
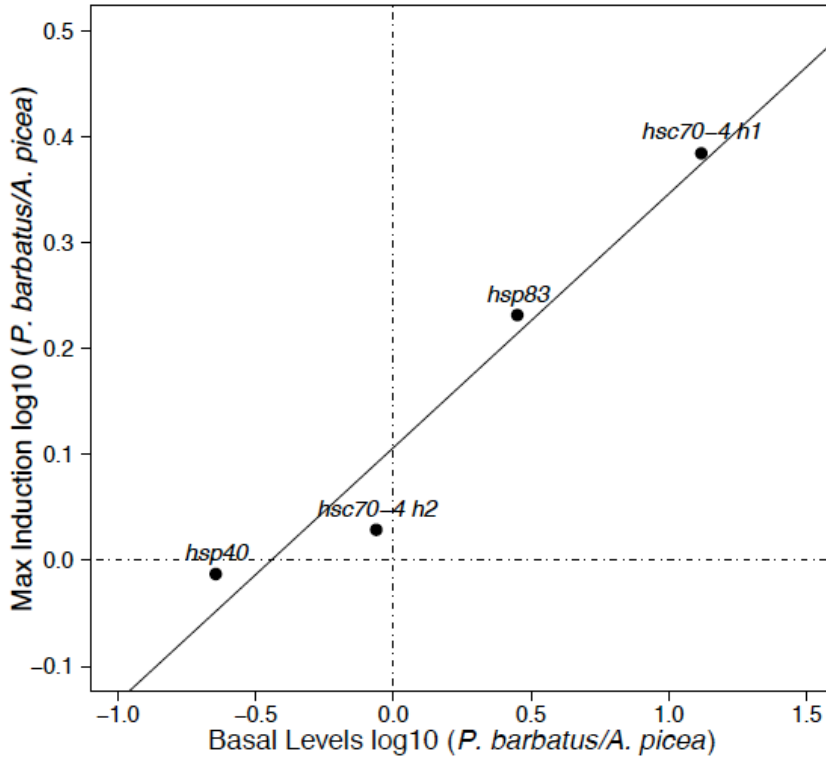
Figure 7. The positive relationship between the log ratios of basal expression levels (*P. barbatus*/*A. picea*) at rearing temperatures and max induction (β_1 slope=0.2398, r^2=0.918, p<0.05).

Tables and Figures

Table 1. Summary of orthologous HSPs from the combination of reciprocal BLAST and HMMER searches using *D. melanogaster* as the reference.

| Gene Family | Gene | Outgroup | | | | Hymenoptera | | |
		C. quinque.	*T. castaneum*	*B. mori*	*A. pisum*	Ants	Bees	*N. vitripennis*
Hsp90	*trap1*	1	1	1	1	1	1	1
	gp93	3	1	1	1	1	1	1
	hsp83	3	3	1	2	1-2*	2	2
Hsp70	*CG2918*	1	1	1	3	1	1	1
	hsc70-3 (BIP)	1	1	1	1	1*	1	1
	hsc70-4	2	1	1	2	2*	2	2
	hsc70-5	1	1	1	1	1*	1	1

	hsp70	6	1	2	3	0	0	1
	hsp70CB	1	1	1	1	1	1	1
Hsp60	tcp-1	1	1	1	1	1	1	1
	hsp60	1	1	1	1	1*	1	1
Hsp40	CG5001	1	1	1	1	1*	1	1
	hsp23	0	0	0	0	0	0	0
	hsp24	0	0	0	0	0	0	0
small Hsps	hsp26	0	0	0	0	0	0	0
	hsp27	0	0	0	0	0	0	0
	l(2)efl	8	10	17	1	3-6*	4-9	7

Each entry is the number of orthologous HSPs detected. The astericks (*) indicate orthologues that were detectable by qPCR. For *l(2)efl*, only one paralogue was detectable by qPCR. *C. quinque = Culex quinquefasciatus, T. castaneum = Tribolium castaneum. B. mori = Bombyx mori, A. pisum = Acyrthosiphon pisum, N. vitripennis = Nasonia vitripennis*. See text for further details of ants and bees used for analysis.

Table 2. Summary of selection analyses for three HSP genes.

Gene	N	Codons	Global ω		ω-/ω+		
			SLAC	REL	SLAC	FEL	MEME
hsp83	25	714	0.0603	0.071	608/0	625/0	NA/1
hsc70-4	31	710	0.0549	0.051	608/0	610/0	NA/7
hsp40	17	384	0.1147	0.100	253/0	284/0	NA/1

For each gene, the number of sequences and number of codons were used for detecting positive selection. The mean global ω is shown for SLAC and REL methods. The number of sites that are negatively or positively selected are shown under ω -/ ω + for SLAC, FEL. P-values were set to default (p=0.1) for SLAC, FEL, REL. MEME provides evidence for episodic positive selection at individual branches and sites (p<0.01) and the number of negatively selected sites are non applicable (NA).

CHAPTER 3: CORRELATED CHANGES IN HEAT SHOCK PROTEIN GENE EXPRESSION PROFILES PREDICT UPPER THERMAL LIMITS IN EASTERN FOREST ANTS

3.1 Abstract

Colonization of thermally stressful environments increases the risk of protein denaturation under high temperatures. Adaptation to thermal stress may involve the action of heat shock proteins, which can pre-emptively protect against unfolding or respond to protein damage. The extent to which protection, sensing, and tolerance mechanisms underlie physiological limits in natural systems is poorly understood. We evaluated variation in upper thermal limits (CTmax) across a clade of eight species of ant in the genus *Aphaenogaster,* whose geographical ranges in the eastern the US encompass a 4°C maximum temperature gradient and two distinct forest types. Workers acclimated to common-garden conditions were assayed for CTmax with a slow-ramping protocol, which provided sufficient time for the heat shock response to be elicited. We used a function-valued trait approach to model the expression dynamics of three heat shock protein genes (*hsp83, hsp70, hsp40*) to characterize baseline expression, the onset and rate of Hsp gene up-regulation, and the magnitude of induction in response to thermal stress. CTmax varied in accordance with both maximal environmental temperature and habitat type. An evolutionary transition from closed-canopy deciduous forests into open-canopy pine forests is inferred to have occurred once, coinciding with a 1.5°C increase in CTmax. Shifts in Hsp gene expression explained 40% of the total variation in CTmax

across the group; notably, all of the shifts involved dynamics of gene induction rather than baseline expression. The strongest shifts were toward enhanced maximal induction to response in thermal extremes and delayed activation of the heat shock response consistent with stress tolerance, with a smaller contribution of pre-emptive protective induction under moderate stress conditions. The same correlated suite of changes was associated with both adaptation to thermal conditions and colonization of open pine forests, suggesting common regulatory control. Collectively, extension of CTmax involves adaptive, coordinated shifts of Hsp gene expression profiles to maintain protein homeostasis under temperature challenge and may have facilitated diversification of forest ants into divergent thermal environments. These results lay out the expectation for the types of evolved mechanisms required for surviving a warmer world in the future.

3.2 Introduction

Clades of ectotherms that have diversified into thermally divergent environments provide exemplar test cases for understanding the types of adaptations required to colonize novel ecological niches (Huey and Stevenson 1979; Hunter 1998). Ectothermic species must meet the challenge of maintaining performance (growth, activity, and reproduction) in the face of shifts in internal body temperatures across latitude, along elevation, among different habitats and within a growing season (Angiletta 2009; Scheffers et al. 2014). When operative body temperatures reach upper thermal limits, organismal performances and fitnesses degrade due to heat stress (Kingsolver

2009; Geiler-Samerotte et al. 2011). High temperatures induce macromolecular damage (Richter et al. 2010), including loss of biochemical activity associated with protein denaturation (Somero 1995; Fields 2001). Therefore, adaptation to warm temperatures should involve physiological mechanisms to cope with protein unfolding (Stillman and Somero 2001; Somero 2011; Fields et al. 2015).

One of the primary mechanisms to cope with heat-induced protein unfolding is the heat shock response (HSR) (Lindquist 1988; Kültz 2005), a series of intracellular responses that collectively operate to maintain protein homeostasis. Genes with catalytic activity are quickly down-regulated (Sørensen et al. 2005) to reduce anabolism, while heat shock proteins (Hsps) become rapidly up-regulated in response to temperature-mediated protein unfolding (Moseley 1997; Lindquist 1988; Craig and Gross 1991). Hsps are ancient and evolutionarily conserved proteins that recognize exposed hydrophobic regions of unfolded proteins and refold them (Fink 1999) or facilitate their degradation (Qian et al. 2006) with the expenditure of ATP.

Given the ability for Hsps to sense and repair protein unfolding, their expression patterns may be targets for selection in populations or species that vary in thermally different environments (Moseley 1997; Feder and Hofmann 1999; Sørensen et al. 2003). Species can utilize Hsps to maintain protein homeostasis with two general, non-mutually exclusive strategies. Constitutive or induced Hsp expression under benign or low ends of the temperature gradient can pre-emptively prevent unfolding when organisms face high temperatures (Carmel et al. 2011; Bedulina et al. 2013; Dong et al. 2008). However, constitutive Hsp expression is costly and diverts energy resources away from growth

(Krebs and Loeschcke 1994; Hoekstra and Montooth 2013). Additionally, species can invest in inducing Hsp expression towards higher levels to tolerate heat stress (Feder and Hofmann 1999; Sørensen et al. 2003). Finally, because Hsps act as a sensor of perturbations to temperature (Craig and Gross 1991), the onset of expression should match the stability of the proteome (Dahlhoff and Rank 2000; Tomanek and Somero 1999; Tomanek and Zuzow 2010; Willot et al. 2017). The extent to which natural populations have utilized each of these elements during thermal niche expansion is currently unclear, and may depend on the type of environment and the evolutionary timescale being considered (Somero 2011; Garbuz and Evgen'ev 2017).

Capturing dynamics of the heat shock response representing protection, sensing, and tolerance requires characterization of Hsp expression profiles over a range of temperatures, represented as a reaction norm or function-valued trait (Stinchcombe et al. 2012). Hsp gene expression usually follows a non-linear, logistic function (Kingsolver & Woods 2016); the parameters of this function can capture distinct types of evolutionary modification to extend upper thermal limits. The evolution of higher upper thermal limits via protective Hsp production can occur through higher basal Hsp expression (constitutive protection; Fig. 1*A*) or increasing Hsp induction under sub-stressful conditions, leading to a more graded activation response during the up-regulation phase of the curve (induced protection; Fig. 1*B*). In contrast, increases in the inflection point indicates later onset of protein damage (stress resistance; Fig. 1*C*). If thermal tolerance is achieved via greater responsiveness to protein damage, this should be reflected in higher peak expression levels (enhanced response, Fig. 1*D*). These four mechanisms are not

mutually exclusive, and may evolve in concert or independently as lineages encounter and adapt to novel thermal conditions.

Ants are ubiquitous and ecologically important members of terrestrial ecosystems on every continent except Antarctica (Wilson and Hölldobler 2005). Thermal niche segregation is an important determinant of competitive overlap within ant communities (Albrecht and Gotelli 2001; Wittman et al. 2010), while the biogeography of individual ant genera indicates substantial thermal niche diversification associated with post-glacial dispersal and local adaptation, making them an attractive system to investigate the evolution of upper thermal limits (Diamond et al. 2012; Warren and Chick 2013; Kaspari et al. 2015; Penick et al. 2017). In this study, we evaluated the extent and mechanisms of physiological adaptation to the local thermal environment in a clade of ground-dwelling ants within the genus *Aphaenogaster* that experience distinct thermal regimes along the Eastern United States (DeMarco and Cognato 2016). *Aphaenogaster* species occur across a climate gradient from Florida to Maine (Fig S1) and two forest types, deciduous forests and flatwoods that differ in canopy cover (Fig S2). We characterized the upper thermal limits (CTmax) of workers under common garden conditions collected from colonies along a ~15.6 degree latitudinal transect to investigate the influence of phylogenetic history and local environmental selective pressures on the evolution of CTmax evolution. To determine the relative contributions of protection, response and tolerance mechanisms to variation in upper thermal limits, we tested the extent to which CTmax among colonies is explained by variation in basal expression, rate of expression increase, inflection point temperature, or peak expression level of three Hsp

genes known to be heat-inducible in ants, i.e., *hsp83*, *hsp70*, and *hsp40* (Nguyen et al.
2016).

3.3 Methods

3.3.1 Sampling and Lab-acclimating Forest Ants of Eastern USA

Eight ant species within the genus *Aphaenogaster* were collected along a latitudinal

transect from Florida to Vermont for a total of 30 sites (Fig. S1). Ants were collected

across two broad habitat types: flat woods and deciduous forests. Flat woods forests are

typically dominated by grasses and have low canopy cover; deciduous forests are

dominated by broadleaf trees and have high canopy cover (Fig. S2). In the summer of

2014 and 2015, whole ant colonies including larvae, pupae, workers, and queens (when

possible) were excavated through a combination of digging and sifting. Ants were

maintained under stable lab conditions at the University of Vermont in an

environmentally controlled room at 25 °C with 12:12 hour light:dark cycles. Ant colonies

were housed in insect-a-slip lined 22 × 16 cm plastic containers with 160 × 40 mm glass

water tubes to satisfy humidity requirements and sandy terrain. Colonies were fed

bisected mealworms and 100 uL of 20% v/v honey water three times a week. Vouchers

were collected for species level identification and deposited in the Zadock Thompson

Zoological Collections at the University of Vermont. Colonies represented in the

outgroup were *Messor pergandei*. In total, 100 ant colonies were acclimated for at least

one month prior to measuring upper thermal limits. Colonies were lab-acclimated for at

least one month prior to any heat treatments.

3.3.2 Reconstructing Evolutionary History

Phylogenetic relationships were constructed based off of a character SNPs matrix. SNPs were identified with double digested restriction site-associated DNA sequencing (ddRADseq). Genomic DNA was isolated from a single ant for each representative colony with the Qiagen DNAeasy kit, following the manufacturer's instructions. 100-200ng of DNA for each sample was digested with two restriction enzymes: NlaIII and MluCl at 37 °C for three hours. To enable multiplex sequencing, unique P1 barcode adapters and universal P2 adaptors were ligated with the digested DNA and 40 ng of each individual workers was pooled into a single library for 48 individuals at a time. The ligated fragments were amplified in 20 uL reactions for 13 cycles, purified again with 1.5X AMPure beads, and resuspended in 30 uL. The library preparations were electrophoresed on a 1.5% agaraose gel and to size select 300-400 bp fragments with a QIEX II gel extraction kit. Sizing was verified on a Bioanalyzer and kappa qPCR. Finally, the library was single-end sequenced for 2.5 million reads per sample spanning 3 lanes on a HiSeq 2000 rapid run at the University of Vermont Advanced Genome Technologies Core (VACC) facility.

Sequences were de-multiplexed with sabre (https://github.com/najoshi/sabre). Sabre allowed for up to one base pair mismatch and trimmed restriction enzyme sites. Total sequence length was trimmed to 90 bps and low quality scores (< 10) were excluded in downstream phylogenetic analyses. A de novo assembly of six representative samples from different *Aphaenogaster* species (*A. picea* - HF3, *A. floridana* - Fbragg2, *A.*

ashmeadi - KH4, *A. lamellidens* - Duke6, *A. miamiana* - Ala2, *A. rudis* - Lex19) was

constructed in STACKS for reference mapping because no sequenced genome was

available. Using the denovo.pl pipeline in STACKS, tags were retained under the

following criteria: 1) 0-3 SNPs were present in the representative samples, 2) all reads

contained one or more reads per tag, 3) SNPs were biallelic, and 4) samples contained no

more than two alternative haplotypes. Using Bowtie, all samples (100 colonies) were

mapped to the reference consensus tag sequences. SNPs were identified by assembling

mapped reads into STACKS using ref_map.pl function.

To create the character matrix for phylogenetic inference, we concatenated

SNPs across tags into a single sequence. Phylogenetic relationships were reconstructed

based on the resultant supergene 174,000 SNP character matrix. The SNP matrix was

analyzed in a maximum likelihood framework using RaxML 8 (Stamatakis 2014) with a

GTR+gamma substitution model and group support was evaluated with 100 fast

bootstrap replicates. There was no reliable SNP data for samples LPR4, Bing, and CJ10,

but species identities between ddRAD-seq data morphology were highly concordant, and

these samples were inserted as polytomies within their species group for subsequent trait

analyses.

3.3.3 Measuring Upper Thermal Limits

Upper thermal limits (CTmax) were determined using a slow automatic ramp method of

0.1°C/min (Terblanche et al. 2011) in a circulating waterbath (PolyScience, USA). For

each colony, four individuals from each colony were placed into 160×40 mm glass tubes

and randomly placed into the water bath preset to 25 °C for 5 minute incubation prior to

heat treatment. An internal probe tracked temperatures within a separate glass tube and then the water bath ramped at a rate of 0.1 °C/min. CTmax was determined as the temperature in which ants lost the ability to self-right (Terblanche et al. 2011). The colony level CTmax was taken as the average of 3-4 individual ants.

3.3.4 Measuring the Heat Shock Response

Reaction norms for Hsp gene expression was quantified across 6-10 temperature points spanning 25-41°C under the same ramping procedure to determine CTmax. To control for the effects of time, we included two additional time matched controls at the middle and end of heat shock treatment. For each colony, four ants were placed into 8-12 tubes and placed into a circulating water bath randomly. For each temperature point, 2 ants/tube were flash frozen and stored at -80 °C. The remaining two tubes were incubated at 25°C separately and sampled in the middle time point when the temperature in the water bath reached 31.5C and then again during last time point (41°C) in order to determine changes in housekeeping gene expression.

First, RNA was isolated by homogenizing whole ant bodies in 1.4 mm zirconium silica beads with 350 uL of lysis buffer (RLT) in a bullet blender (Next Advance Inc., USA) at top speed (10) for 2 minutes. RNA was isolated from the lysate with the RNeazy micro kit (Qiagen, USA) and DNAse I treatment for 30 minutes to diminish the contribution of genomic DNA. The isolated RNA (50 ng) was reverse transcribed into cDNA in 20 uL reactions with a High Capacity cDNA Reverse Transcription kit (ABI, USA) and diluted 1:20 (0.125 ng/uL). Next, the expression of 18s rRNA (house keeping gene), and 3 Hsps: *hsp83*, *hsc70-4* h2, and *hsp40,* were quantified

with qPCR with an ABI StepOnePlus Real-Time PCR system. Each temperature point and colony were ran in duplicates. Reactions consisted of 1x Power SYBR green (5uL), 100-250nM of each primer (0.1-0.25 uL each), nuclease-free water (0.5 uL) and 0.5 ng (4 uL) of template cDNA, for a total volume of 10 uL. PCR conditions involved an initial 95 C incubation for 2 minutes, and then 40 cycles of 55-60 °C for annealing and 70 °C elongation and fluorescence acquisition, followed by a melt curve analysis to check for specificity of amplification. Samples that had non-specific amplification were excluded. Specificity was further confirmed by sequencing a representative amplicon for each gene and each species. CT values were extracted from a standardized threshold value. Basal gene expression was calculated for each Hsp as ΔCT (CT_{Hsp}-$CT_{18srRNA}$) at rearing temperature 25 °C. Gene induction of each Hsp was quantified using the $\Delta\Delta CT$ method (Livak and Schmittgen 2001), where 18s rRNA served as the internal reference. To check for the stability of 18s rRNA for each colony, we regressed the temperature with 18s rRNA CT and utilized a Benjamini-Hochberg to correct for multiple testing. Only one colony (GB33-1) displayed a significant relationship between ΔCT 18s rRNA and temperature, but excluding this colony did not qualitatively alter results.

Two types of curves were fitted to the non-linear expression profiles of each Hsp: Gaussian (Kingsolver et al. 2013) and modified Boltzmann function. Preliminary curve fitting revealed that the Boltzmann function was a better fit (Figure 6), as determined by AIC (data not shown). Furthermore, because basal expression did not correlate with CTmax (Figure 4), we fitted the Boltzmann with minimum value as 1, to which expression values in terms of fold induction were compared to at 25 °C.

$$1 \; | \; \frac{max \quad 1}{1 \; | \; e^{\frac{Tm \; T}{a}}}$$

We used the nls() function to estimate the expression rate (slope parameter, a), inflection

point (Tm), and max expression value (max) for the temperature points (T). We started

values with 1, 35, and 50 for a, Tm, and max, respectively. In total, we quantified

reaction norms for 49 colonies of *hsp83*, 49 colonies of *hsp70*, and 47 colonies of *hsp40*.

3.3.5 Statistics

All statistical analyses were performed in R (version 3.2.2; R Development Core

Team, 2010).). The evolutionary history of CTmax and habitat type was estimated with

an ancestral trait reconstruction with fast estimation ML and a Bayseian MCMC

approach, respectively (phytools package; Revell 2012). A phylogenetic generalized least

squares (PGLS from the CAIC package; Orme et al. 2009) model was used to determine

the effect of habitat type and local temperature extremes (Tmax) on CTmax, while

accounting for phylogenetic relationships and under the assumption of Brownian motion.

Variance partitioning was used to estimate the amount of variation attributable to the

unique phylogeny component, unique local environment component, and the shared

effects of phylogeny and local environment, also known as phylogenetically structured

environmental variation (Desdevises et al. 2003). We first decomposed the branch

lengths (ultrametricized to ensure equal variances of the phylogeny with a principal

coordinate analysis (PCOA) to produce orthogonal eigenvectors with each eigenvector

representing the nodes of the phylogeny. Second, the local environment was represented

by habitat type and local Tmax (bioclim, http://www.worldclim.org/bioclim). Lastly,

using the *varpart()* function (Vegan package), variance in CTmax was partitioned into

phylogeny, phylogeny independent of environment, local environment, and local environment independent of phylogeny. Significance of each variance component was determined with a redundancy analysis (RDA), except for the shared effects of phylogeny and local environment, which is indirectly estimated.

To determine the role of Hsp reaction norm parameters (basal expression, slope, Tm, max expression), we regressed each parameter against CTmax with linear regressions. To identify the effect of local environment on Hsp reaction norm parameters, we tested the effects of habitat type, Tmax, and their interaction with ANCOVA models. We also performed a principal component analysis (PCA) on all 12 Hsp parameters to capture the correlation between these variables and reduce the number of variables. The subsequent principal components (PC) were used in regression analyses to test the relationship with CTmax.

3.4 Results

3.4.1 *CTmax in* Aphaenogaster *is associated with thermal selective regime*

We found a total of eight *Aphaenogaster* species from Florida to Maine, with most of the species occurring below Pennsylvania and a single species, *A. picea,* occurring north of Pennsylvania (Fig. S1). Two species, *A. floridana* and *A. ashmeadi,* were found in flatwoods habitat, while *A. rudis*, *A. picea, A. fulva, A. tenneseensis, A. miamiana* and *A. lamellidens* were found in deciduous forests (Fig. 2*A*). Across all samples, CTmax varied by 6.5°C, from 36.8-43.3°C, slightly lower than the 8.4°C difference in the mean warmest temperature of the warmest month (Tmax) across these sites (24.9-33.3°C, Fig. 2*B*). When including species as a random effect, CTmax was

significantly positively related to Tmax (β=0.014± 0.006; t=2.17, p <0.001; Fig 2B), and

colonies in flat woods had higher CTmax than colonies in deciduous forests (β=1.05±

0.379; t=2.76, p <0.05). However, these relationships were masked when accounting for

common ancestry from phylogenetic analyses (PGLS; $F_{2,5}$=0.47, p=0.25; Fig 2*A*).

Variance partitioning of CTmax revealed a large amount of overlapping variance (43%)

between common ancestry (phylogeny) and local environment (Fig. 2*C*), while the

separate effects of each component were significant (phylogeny: RDA, $F_{9,90}$=10.83,

p<0.005; local environment: RDA, $F_{3,96}$=25.03, p<0.005), but their unique components

were not (phylogeny independent from local environment: RDA, $F_{9,87}$=1.84,p=0.09; local

environment independent of phylogeny: RDA, $F_{3,87}$=1.84, p=0.61; Fig. 2*C*). Ancestral

trait reconstruction indicated that deciduous forest of intermediate temperature was the

most likely habitat of the most recent common ancestor of *Aphaenogaster* in the eastern

United States (Fig. 3). There was a single evolutionary transition into flat woods (*A.*

floridana + A. ashmeadi) accompanied by a 1.25°C upward shift in CTmax, as well as a

single colonization of northern latitudes (*A. picea*) associated with a ~0.75°C reduction in

CTmax (Fig. 3).

3.4.2 *Variation in CTmax is explained by Hsp gene expression patterns*

Altogether, *hsp83/70/40* gene expression explained 40% of the variation in

CTmax (Table S3). There was no significant relationship between basal expression and

CTmax for any of the three Hsps (Fig. 4*A,E,I*). However, all three of the induction

parameters varied with CTmax for at least one Hsp gene (Table S1). Colonies with

higher CTmax had slower rates of increase in expression (slope parameter) of *hsp70* (Fig. 4*F*) and *hsp40* (Fig. 4*J*), while the inflection point (Tm) for all three genes was higher in colonies with higher CTmax (Tm; Fig. 4 *C,D,K*). Maximal expression values also increased with CTmax for all three Hsp genes (Fig. 4 *D,H,C*).

3.4.3 *Correlated changes in Hsp reaction norms underlie adaptation to habitat*

Despite the latitudinal gradient in air temperature, there was no relationship between any Hsp gene expression parameter and local temperature extremes (Tmax). In contrast, there was a significant difference in Hsp gene expression parameters between colonies from flat woods vs. deciduous forests (Figs. 5, S3, S5, Table S2). For *hsp83*, colonies occupying open habitats had higher inflection-point temperatures by 2.16°C (Tm, β=2.166 ± 0.583; t=3.703 p <0.001, Fig. 5*B*) and a 4.72 fold higher maximum expression value (β=4.720 ± 1.551; t=3.044, p <0.005; Fig. 5*C*). For *hsp70*, colonies occupying open habitats had slower expression rates (slope parameter; β=0.495 ± 0.176; t=2.807, p <0.01, Fig. 5*E*), higher inflection-point temperatures by 1.37°C (Tm; β=1.373 ± 0.337; t=4.073, p <0.001, Fig. 5*F*) and ~20 fold higher maximum expression (β=20.357± 4.864; t=4.185, p <0.001; Fig. 5*G*). For *hsp40*, colonies in flat woods initiated expression 2.47°C higher than deciduous forest colonies (Tm, β=2.469 ± 0.666; t=3.705, p <0.001; Fig. 5*J*). The sigmoidal Hsp gene induction curve reveals several differences in the total reaction norm when parameters were visualized together (Fig. 5 *D, H, L*).

3.5 Discussion

Colonization of thermally stressful environments depends on the ability to behaviorally and physiologically cope with temperature stress (Somero 2002; Huey et al. 2012). The results of this study suggest that physiological adaptation to upper thermal extremes has played an important role in facilitating niche expansion in the genus *Aphaenogaster* in the Eastern United States. High maximal environmental temperature and the transition from closed canopy forests (deciduous) to open canopy flatwoods were both accompanied by an increase in upper thermal limits (CTmax; Fig 2, S2). Broadly within ants, divergence in upper thermal limits is associated with habitat, particularly between open habitats and forests for ground dwelling ants (Oberg et al. 2011; Diamond et al. 2012). Our study shows that even within the broadly defined forest category, different forest types with significant differences in canopy cover can produce divergence in upper thermal limits and the heat shock response (HSR).

Forest environments generate considerable heterogeneity in thermal microclimates that influences the thermal experiences of ectotherms (Scheffers et al. 2014). Deciduous forests are significantly more buffered from thermal extremes than flat woods due to higher canopy cover (Fig. S2), especially over the growing season where ant foraging activity is the highest. These species are known to utilize behavioral thermoregulation such as migrating to new nesting locations when colonies experience unsuitable environments and workers may maintain optimal body temperatures by avoiding solar radiation in the leaf litter, also known as the Bogert effect (Bogert 1945; Smallwood 1982; Diamond et al. 2016). The effectiveness of the Bogert effect depends

on the spatial availability of microrefugia (Sears et al. 2016). Deciduous forests may not completely buffer thermal extremes across large climate gradients because we found that every 0.014°C increase in Tmax led to a 1°C increase in CTmax across *Aphaenogaster*'s range. However, this clinal relationship was mostly driven by the most northern species, *A. picea*, whose CTmax had the steepest cline with Tmax. The historical northward expansion in *A. picea* as glaciers receded after the last ice age might have led to the reduction of upper thermal limits in favor of coping with cold extremes. *Aphaenogaster* species in deciduous forest likely rely on a combination of behavioral and physiological mechanisms to cope with heat stress.

In contrast to deciduous forest species, flat woods species had higher CTmax than local extreme temperatures would predict. Due to low canopy cover in flat woods habitats (Fig. S2), ant foragers are expected to encounter inescapable thermal extremes as solar radiation superheats the soil to higher temperatures than ambient (Porter and Gates 1969; Angiletta 2009). Furthermore, flatwoods species (*A. floridana* and *A. ashmeadi*) nest near the surface (13-92 cm in depth; Tschinkel 2011) and are not known to migrate at all. Because thermal stress may be difficult to escape, these species should rely more on physiological mechanisms to respond to temperature challenges. The increase in upper thermal limits associated with canopy cover is analogous to habitat shifts for species threatened by deforestation and urbanization (Alkama and Cescatti 2016), whereby the subsequent loss of microrefugia may impose greater selection for physiological mechanisms to cope with heat stress.

The transition into flat woods and increase in CTmax was accompanied by dynamic non-linear changes in the heat shock response, mainly in Hsp expression profiles (Fig. 5D,H,L). Between forest types, we found a ~10-fold increase in Hsp expression at 35 °C in flatwoods species when considering the rate of the Hsp induction alone (Fig. 5E), supporting the protective action of Hsps through a graded inducible response (Figs. 1B, 5E). However, gene expression differences at 35 °C disappear when integrating all parameters because species in deciduous forests induce Hsps at a lower temperature (Tm). The differences in Tm reflect resistance to temperature stress and suggest that species in open canopy forests may have a more stable proteome. Beyond Tm and at higher ends of the temperature gradient, slower expression rate resulted in lower expression (4 fold at 39°C, Fig. 5E) for flatwoods species just before reaching maximum expression levels (Fig 1). Flatwoods species with higher CTmax appear to have compensated by simultaneously elevating maximum expression by as much as 20 fold to add greater thermal damage repair at lethal temperatures, supporting greater thermal tolerance. The function-valued trait approach (Fig. 1,6) highlights the strength of gene expression profiling over a wide temperature gradient to capture the kinetics of gene expression. We found that protection, sensing, and tolerance mechanisms by Hsps predicts variation (40%) in upper thermal limits.

The overall correlated responses in Hsp induction parameters are consistent with the thermostat model (Craig and Gross 1991) for sensing and responding to heat stress. The thermostat model predicts that Hsp expression patterns should match the level of heat stress (Craig and Gross 1991), especially because the production and utilization of

Hsps requires energy (Hoekstra and Montooth 2013). All three Hsps possess

evolutionarily conserved heat shock elements (HSE) in the promoter regions of ant Hsp

genes (Nguyen et al. 2016), indicating that transcription is activated in a temperature-

dependent manner (Fernandes et al. 1995). The common regulatory control of Hsps may

be dialed to facilitate evolutionary transitions for species into different thermal

environments (Tomanek 2010).

Hsp induction rather than constitutive protection is more important for niche expansion in

forest ants (Fig. 3, 5). Protection by Hsps is typically achieved by constitutive Hsp

expression at the expense of induction (Dong et al. 2008; Bedulina et al. 2013; Porcelli et

al. 2015). However, the shift between induced to basal Hsp expression may not be

universal. In particular, temperate forests are highly seasonal and should select for

differences in phenotypic plasticity, i.e., the ability to adjust the expression of Hsps in

response to environmental changes. Forest ants can alter Hsp basal expression and

induction under different rearing temperatures with no evolved differences between

species (Helms Cahan et al. 2017), suggesting that organisms can seasonally acclimatize

their Hsp gene expression (Dietz and Somero 2004; Banerjee et al. 2014). In contrast, our

study reveals adaptive differences mainly in Hsp induction with no detectable differences

in basal expression, suggesting that selection may be greater in short term temperature

increases than longer term, seasonal variation. Therefore, Hsp patterns of expression are

largely influenced and depend on the selective thermal regime (Tomanek 2010).

Conclusion

The combinatorial and correlated changes in the stress response are the types of adapted shifts needed under impending anthropogenic-mediated global changes (Somero 2011; Diamond and Martin 2016). Habitat type is a critical feature of the landscape that dictates the thermal experiences of ectotherms (Scheffers et al. 2014). Therefore, habitat deforestation, urbanization, and climate change will pose a new and major challenge for ectotherms as the environment shifts away from ancestral conditions to which populations are locally adapted (Bonan and Gordon 2008; Diffenbaugh and Field 2013; Diamond et al. 2015; Alkama and Cescatti 2016). In particular, deforestation and subsequent urbanization act as heat islands (Oke 1982; Arnfield 2003), and heat bouts are projected to increase under climate change (Diffenbaugh and Field 2013). Ectotherms can respond to changing conditions through active Hsp mediated repair and overall stress resistance (Somero 2011). Further adaptive modifications in the heat shock response will depend on the adaptive potential of Hsp expression (Huey et al. 2012; Tedeschi et al. 2016; Diamond 2016). Currently, some ants are able to capitalize on anthropogenic change (Angiletta et al. 2007; Diamond et al. 2017), but the mechanisms by which they thrive in urban environments and how commonly species can exploit warm extremes across the whole clade of ants will provide insights on the susceptibility or resiliency to environmental change.

3.6 Works Cited

Angilletta MJ Jr, Wilson RS, Niehaus AC *et al.* (2007) Urban Physiology: City Ants
Possess High Heat Tolerance. *PLoS ONE*, **2**, e258.

Arnfield AJ (2003) Two decades of urban climate research: a review of turbulence,
exchanges of energy and water, and the urban heat island. *International Journal
of Climatology*, **23**, 1–26.

Banerjee D, Upadhyay RC, Chaudhary UB *et al.* (2014) Seasonal variation in expression
pattern of genes under HSP70: Seasonal variation in expression pattern of genes
under HSP70 family in heat-and cold-adapted goats (Capra hircus). *Cell Stress
& Chaperones*, **19**, 401–408.

Bedulina DS, Evgen'ev MB, Timofeyev MA *et al.* (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. *Molecular Ecology*, **22**, 1416–1430.

Bogert CM (1949) Thermoregulation in Reptiles, A Factor in Evolution. *Evolution*, **3**,
195–211.

Bonan GB (2008) Forests and Climate Change: Forcings, Feedbacks, and the Climate

    Benefits of Forests. *Science*, **320**, 1444–1449.

Carmel J, Rashkovetsky E, Nevo E, Korol A (2011) Differential Expression of Small

    Heat Shock Protein Genes Hsp23 and Hsp40, and heat shock gene Hsr-omega in

    Fruit Flies (Drosophila melanogaster) along a Microclimatic Gradient. *Journal*

    of Heredity, **102**, 593–603.

Cavicchi S, Guerra D, Torre VL, Huey RB (1995) Chromosomal Analysis of Heat-Shock

    Tolerance in Drosophila melanogaster Evolving at Different Temperatures in the

    Laboratory. *Evolution*, **49**, 676–684.

Craig EA, Gross CA (1991) Is hsp70 the cellular thermometer? *Trends in Biochemical*

    Sciences, **16**, 135–140.

Dahlhoff EP, Rank NE (2000) Functional and physiological consequences of genetic

    variation at phosphoglucose isomerase: Heat shock protein expression is related

    to enzyme genotype in a montane beetle. *Proceedings of the National Academy*

    of Sciences, **97**, 10056–10061.

DeMarco BB, Cognato AI (2016) A multiple-gene phylogeny reveals polyphyly among eastern North American *Aphaenogaster* species (Hymenoptera: Formicidae). *Zoologica Scripta*, n/a-n/a.

Desdevises Y, Legendre P, Azouzi L, Morand S (2003) Quantifying Phylogenetically Structured Environmental Variation. *Evolution*, **57**, 2647–2652.

Diamond SE (2016) Evolutionary potential of upper thermal tolerance: biogeographic patterns and expectations under climate change. *Annals of the New York Academy of Sciences*, n/a-n/a.

Diamond SE, Chick L, Perez A, Strickler SA, Martin RA Rapid evolution of ant thermal tolerance across an urban-rural temperature cline. *Biological Journal of the Linnean Society*.

Diamond SE, Dunn RR, Frank SD, Haddad NM, Martin RA (2015) Shared and unique responses of insects to the interaction of urbanization and background climate. *Current Opinion in Insect Science*, **11**, 71–77.

Diamond SE, Martin RA (2016) The interplay between plasticity and evolution in response to human-induced environmental change. *F1000Research*, **5**, 2835.

Diamond SE, Sorger DM, Hulcr J *et al.* (2012) Who likes it hot? A global analysis of the

      climatic, ecological, and evolutionary determinants of warming tolerance in

      ants. *Global Change Biology*, **18**, 448–456.

Dietz TJ, Somero GN (1992) The threshold induction temperature of the 90-kDa heat

      shock protein is subject to acclimatization in eurythermal goby   fishes

(genus Gillichthys). *Proceedings of the National Academy of Sciences of   the

United States of America*, **89**, 3389–3393.

Diffenbaugh NS, Field CB (2013) Changes in Ecologically Critical Terrestrial Climate

      Conditions. *Science*, **341**, 486–492.

Dong Y, Miller LP, Sanders JG, Somero GN (2008) Heat-Shock Protein 70 (Hsp70)

      Expression in Four Limpets of the Genus Lottia: Interspecific Variation in

      Constitutive and Inducible Synthesis Correlates With in situ Exposure to Heat

      Stress. *The Biological Bulletin*, **215**, 173–181.

Feder ME, Hofmann GE (1999) HEAT-SHOCK PROTEINS, MOLECULAR

      CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and

      Ecological Physiology. *Annual Review of Physiology*, **61**, 243–282.

Fields PA (2001) Review: Protein function at thermal extremes: balancing stability and
flexibility. *Comparative Biochemistry and Physiology Part A: Molecular &
Integrative Physiology*, **129**, 417–431.

Fields PA, Dong Y, Meng X, Somero GN (2015) Adaptations of protein structure and
function to temperature: there is more than one way to "skin a cat." *The Journal
of Experimental Biology*, **218**, 1801–1811.

Fink AL (1999) Chaperone-Mediated Protein Folding. *Physiological Reviews*, **79**, 425–
449.

Garbuz DG, Evgen'ev MB (2017) The evolution of heat shock genes and expression
patterns of heat shock proteins in the species from temperature contrasting
habitats. *Russian Journal of Genetics*, **53**, 21–38.

Geiler-Samerotte KA, Dion MF, Budnik BA *et al.* (2011) Misfolded proteins impose a
dosage-dependent fitness cost and trigger a cytosolic unfolded protein response
in yeast. *Proceedings of the National Academy of Sciences*, **108**, 680–685.

Helms Cahan S, Nguyen AD, Stanton-Geddes J *et al.* (2017) 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. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **204**, 113–120.

Hoekstra LA, Montooth KL (2013) Inducing extra copies of the Hsp70 gene in Drosophila melanogaster increases energetic demand. *BMC Evolutionary Biology*, **13**, 68.

Huey RB (1974) Behavioral Thermoregulation in Lizards: Importance of Associated Costs. *Science*, **184**, 1001–1003.

Huey RB, Kearney MR, Krockenberger A *et al.* (2012) Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 1665–1679.

Huey RB, Kingsolver JG (1993) Evolution of Resistance to High Temperature in Ectotherms. *The American Naturalist*, **142**, S21–S46.

Hunter JP (1998) Key innovations and the ecology of macroevolution. *Trends in Ecology & Evolution*, **13**, 31–36.

Kaspari M, Clay NA, Lucas J, Yanoviak SP, Kay A (2015) Thermal adaptation generates a diversity of thermal limits in a rainforest ant community. *Global Change Biology*, **21**, 1092–1102.

Kellermann V, Overgaard J, Hoffmann AA *et al.* (2012) Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences*, **109**, 16228–16233.

Kingsolver JG (2009) The Well-Temperatured Biologist. *The American Naturalist*, **174**, 755–768.

Kingsolver JG, Woods HA, Kearney M, Michalakis Y (2016) Beyond thermal performance curves: modeling time-dependent effects of thermal stress on ectotherm growth rates. *The American Naturalist*, **187**, 283–294.

Krebs RA, Loeschcke V (1994) Costs and Benefits of Activation of the Heat-Shock Response in Drosophila melanogaster. *Functional Ecology*, **8**, 730–737.

Kültz D (2005) Molecular and Evolutionary Basis of the Cellular Stress Response. *Annual Review of Physiology*, **67**, 225–257.

Leuenberger P, Ganscha S, Kahraman A *et al.* (2017) Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. *Science*, **355**, eaai7825.

Lindquist S, Craig EA (1988) The Heat-Shock Proteins. *Annual Review of Genetics*, **22**, 631–677.

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, **25**, 402–408.

Lockwood BL, Sanders JG, Somero GN (2010) Transcriptomic responses to heat stress in invasive and native blue mussels (genus Mytilus): molecular correlates of invasive success. *The Journal of Experimental Biology*, **213**, 3548–3558.

Moseley PL (1997) Heat shock proteins and heat adaptation of the whole organism. *Journal of Applied Physiology*, **83**, 1413–1417.

Nguyen AD, Gotelli NJ, Cahan SH (2016) The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC Evolutionary Biology*, **16**, 15.

Oke TR (1982) The energetic basis of the urban heat island. *Quarterly Journal of the Royal Meteorological Society*, **108**, 1–24.

Penick CA, Diamond SE, Sanders NJ, Dunn RR (2017) Beyond thermal limits: comprehensive metrics of performance identify key axes of thermal adaptation in ants. *Functional Ecology*, n/a-n/a.

Porcelli D, Butlin RK, Gaston KJ, Joly D, Snook RR (2015) The environmental genomics of metazoan thermal adaptation. *Heredity*, **114**, 502–514.

Porter WP, Gates DM (1969) Thermodynamic Equilibria of Animals with Environment. *Ecological Monographs*, **39**, 227–244.

Richter K, Haslbeck M, Buchner J (2010) The Heat Shock Response: Life on the Verge of Death. *Molecular Cell*, **40**, 253–266.

Scheffers BR, Edwards DP, Diesmos A, Williams SE, Evans TA (2014) Microhabitats reduce animal's exposure to climate extremes. *Global Change Biology*, **20**, 495–503.

Sikkink KL, Reynolds RM, Ituarte CM, Cresko WA, Phillips PC (2014) Rapid Evolution of Phenotypic Plasticity and Shifting Thresholds of Genetic Assimilation in the

Nematode Caenorhabditis remanei. *G3: Genes|Genomes|Genetics*, **4**, 1103–
1112.

Smallwood J (1982) The Effect of Shade and Competition on Emigration Rate in the Ant
Aphaenogaster Rudis. *Ecology*, **63**, 124–134.

Somero GN (1995) Proteins and Temperature. *Annual Review of Physiology*, **57**, 43–68.

Somero GN (2004) Adaptation of enzymes to temperature: searching for basic
"strategies." *Comparative Biochemistry and Physiology Part B: Biochemistry
and Molecular Biology*, **139**, 321–333.

Somero GN (2011) Comparative physiology: a "crystal ball" for predicting consequences
of global change. *American Journal of Physiology - Regulatory, Integrative and
Comparative Physiology*, **301**, R1–R14.

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role
of heat shock proteins. *Ecology Letters*, **6**, 1025–1037.

Sørensen JG, Nielsen MM, Kruh?ffer M, Justesen J, Loeschcke V (2005) Full genome
gene expression analysis of the heat stress response in Drosophila melanogaster.
Cell Stress & Chaperones, **10**, 312–328.

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 *et al.* (2016) Thermal reactionomes reveal divergent responses to thermal extremes in warm and cool-climate ant species. *BMC Genomics*, **17**, 171.

Stillman JH, Somero GN (2001) A comparative analysis of the evolutionary patterning and mechanistic bases of lactate dehydrogenase thermal stability in porcelain crabs, genus Petrolisthes. *Journal of Experimental Biology*, **204**, 767–776.

Stinchcombe JR, Kirkpatrick M (2012) Genetics and evolution of function-valued traits: understanding environmentally responsive phenotypes. *Trends in Ecology & Evolution*, **27**, 637–647.

Sunday JM, Bates AE, Kearney MR *et al.* (2014) Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proceedings of the National Academy of Sciences*, **111**, 5610–5615.

Tedeschi JN, Kennington WJ, Tomkins JL *et al.* (2016) Heritable variation in heat shock
   gene expression: a potential mechanism for adaptation to thermal stress in
   embryos of sea turtles. *Proc. R. Soc. B*, **283**, 20152320.

Terblanche JS, Hoffmann AA, Mitchell KA *et al.* (2011) Ecologically relevant measures
   of tolerance to potentially lethal temperatures. *The Journal of Experimental
   Biology*, **214**, 3713–3725.

Tomanek L, Somero GN (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. *The Journal of Experimental Biology*, **202**, 2925–2936.

Tomanek L, Zuzow MJ (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. *The Journal of
   Experimental Biology*, **213**, 3559–3574.

Tschinkel WR (2011) The Nest Architecture of Three Species of North Florida
   Aphaenogaster Ants. *Journal of Insect Science*, **11**, 1–30.

Willot Q, Gueydan C, Aron S (2017) Proteome stability, heat hardening, and heat-shock

protein expression profiles in Cataglyphis desert ants. *Journal of Experimental*

Biology, jeb.154161.

Wilson EO, Hölldobler B (2005) The rise of the ants: A phylogenetic and ecological

explanation. *Proceedings of the National Academy of Sciences of the United*

States of America, **102**, 7411–7414.

Wittman SE, Sanders NJ, Ellison AM *et al.* (2010) Species interactions and thermal

constraints on ant community structure. *Oikos*, **119**, 551–559.

3.7 Tables

Table S1 : Individual linear regression models to determine the relationship between

CTmax and parameter estimates of Hsp expression profiles

Gene	Parameter	df	Estimate	SE	t-value	p-value	R^2
hsp83	basal	1,47	-0.10	0.12	-0.86	0.393	0.00
	slope	1,47	0.25	0.14	1.90	0.062	0.05
	Tm	1,47	0.23	0.06	4.00	<0.001	0.24
	max	1,47	0.07	0.03	3.00	<0.005	0.15
hsp70	basal	1,47	0.04	0.15	0.30	0.76	0.00
	slope	1,47	0.90	0.20	4.35	<0.001	0.27
	Tm	1,47	0.49	0.09	5.40	<0.001	0.37
	max	1,47	0.03	0.01	4.92	<0.001	0.32
hsp40	basal	1,45	-0.10	0.12	-0.88	0.38	0.00
	slope	1,45	0.38	0.14	2.60	<0.05	0.11
	Tm	1,45	0.26	0.05	4.80	<0.001	0.33
	max	1,45	0.06	0.02	2.50	<0.05	0.10

Table S2 : Summary of output from multiple linear regression models of reaction norm parameters for three Hsps.

Gene	Response	Predictor	Estimate	SE	t value	p value
hsp83	basal	Tmax	-0.003	0.007	-0.377	0.708
		habitat	-0.480	0.363	-1.324	0.191
	slope	Tmax	0.008	0.006	1.316	0.195
		habitat	0.302	0.324	0.933	0.356
	Tm	Tmax	0.011	0.011	0.955	0.345
		habitat	2.166	0.585	3.703	<0.001
	max	Tmax	-0.003	0.030	-0.091	0.928
		habitat	4.720	1.551	3.044	<0.005
hsp70	basal	Tmax	0.010	0.006	0.870	0.390
		habitat	-0.120	0.365	-0.340	0.740
	slope	Tmax	0.002	0.003	0.611	0.544
		habitat	0.495	0.176	2.807	<0.01
	Tm	Tmax	0.004	0.007	0.575	0.568
		habitat	1.373	0.337	4.073	<0.001
	max	Tmax	0.134	0.094	1.416	0.164
		habitat	20.357	4.864	4.185	<0.001
hsp40	basal	Tmax	0.005	0.009	0.516	0.609
		habitat	-0.382	0.509	-0.750	0.458
	slope	Tmax	0.006	0.006	0.932	0.356
		habitat	0.413	0.315	1.313	0.196
	Tm	Tmax	-0.012	0.013	-0.911	0.367
		habitat	2.469	0.666	3.705	<0.001
	max	Tmax	-0.001	0.037	-0.026	0.980
		habitat	3.649	1.897	1.924	0.061

Table S3 : Principal component analysis (PCA) of four different gene expression parameters for three different Hsp genes. The four parameters were first standardized such that the mean equals zero and variance equals one prior to PCA. The variance for each principal component (PC) are indicated in the first row and the second row indicates the cumulative variance explained with increasing PCs. The rest of the rows indicate the loading patterns for each variable in the PCA.

|	PC1	PC2	PC3	PC4
Variance	39.07	15.61	9.89	8.58
Cumulative Variance	39.07	54.67	64.56	73.14
hsp70 basal expression	-0.073	-0.596	0.071	-0.224
hsp83 basal expression	-0.023	-0.593	-0.008	0.098
hsp40 basal expression	-0.023	0.008	0.461	0.803
hsp70 max expression	-0.321	-0.16	0.404	-0.273
hsp70 slope parameter	-0.28	-0.286	0.217	0.189
hsp70 Tm	-0.374	0.157	0.226	-0.133
hsp40 max expression	-0.35	-0.082	-0.324	0.129
hsp40 slope parameter	-0.292	-0.149	-0.524	0.171
hsp40 Tm	-0.368	0.063	-0.26	0.149
hsp83 max expression	-0.35	0.057	0.153	-0.213
hsp83 slope parameter	-0.29	0.171	-0.145	0.186
hsp83 Tm	-0.351	0.31	0.171	-0.143

3.8 Figures
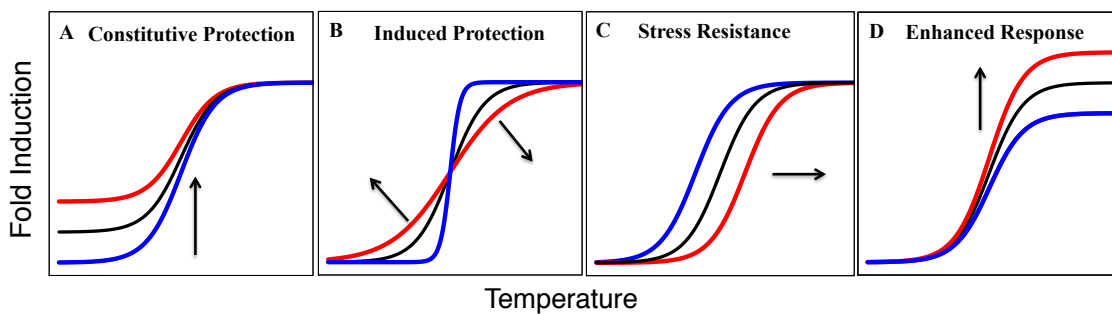
Figure 1 : Non-mutually exclusive predictions of shifts in the reaction norms of Hsp gene expression related to variation in upper thermal limits. Species with higher upper thermal limits may invest in constitutive protection by elevating basal expression (a) or invest in induced protection by altering the rate of induction (b) or resist stress by having a higher inflection point of expression (c), or have an enhanced response by inducing Hsps at higher maximum expression (d).
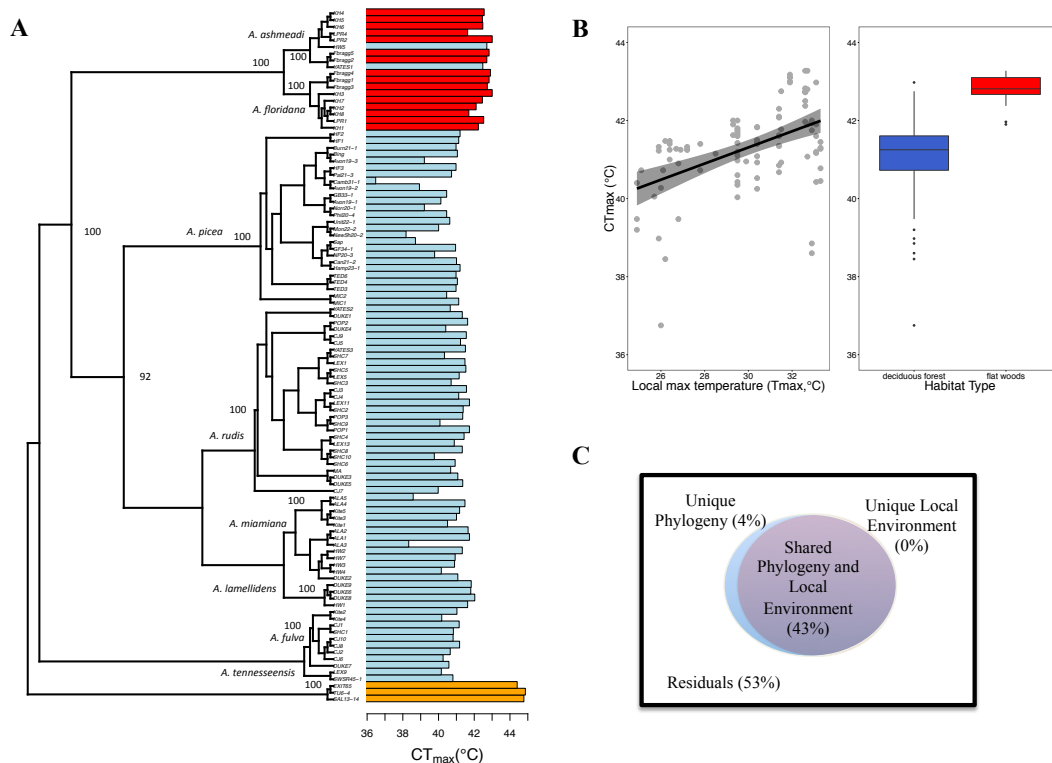
Figure 2 : Influence of shared ancestry and local thermal environment on upper thermal limits (CTmax). CTmax is mapped onto the phylogenetic relationships among colonies and species with bootstrap support > 90 (A). Phylogenetic relationships were estimated in RAxML, using a 174,000 SNP character matrix and GTR+ gamma substitution model rooted on *Messor pergandei*. CTmax was estimated with a slow ramping protocol (0.1°C/min) and barplots are colored coded by forest habitat type (blue = deciduous forest, red = flat woods [shrub-oak or long-leaf pine savannah], orange = desert). The relationships between environmental conditions (Tmax and forest type) and CTmax (B) in the absence of phylogenetic effects. Variance partitioning of CTmax into phylogenic (blue), local environmental (red), and overlap variance components (purple; C).
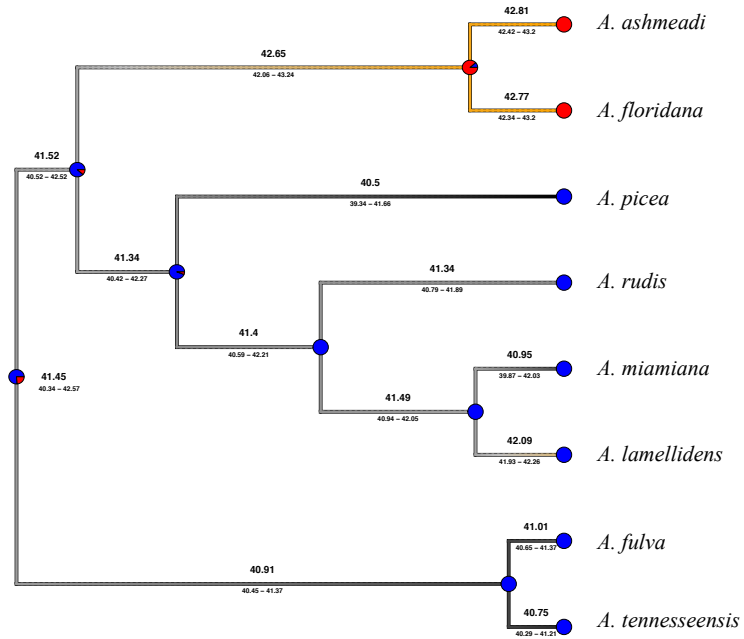
Figure 3 : Ancestral state reconstruction of CTmax (branches) and forest type (nodes) onto the species level phylogeny of the genus *Aphaenogaster*. Ancestral states for CTmax was estimated with fast estimation ML (Revell 2012) and ancestral states for habitat type (DF = blue, FW= red) was estimated with a Bayesian MCMC approach (phytools package; Revell 2012).
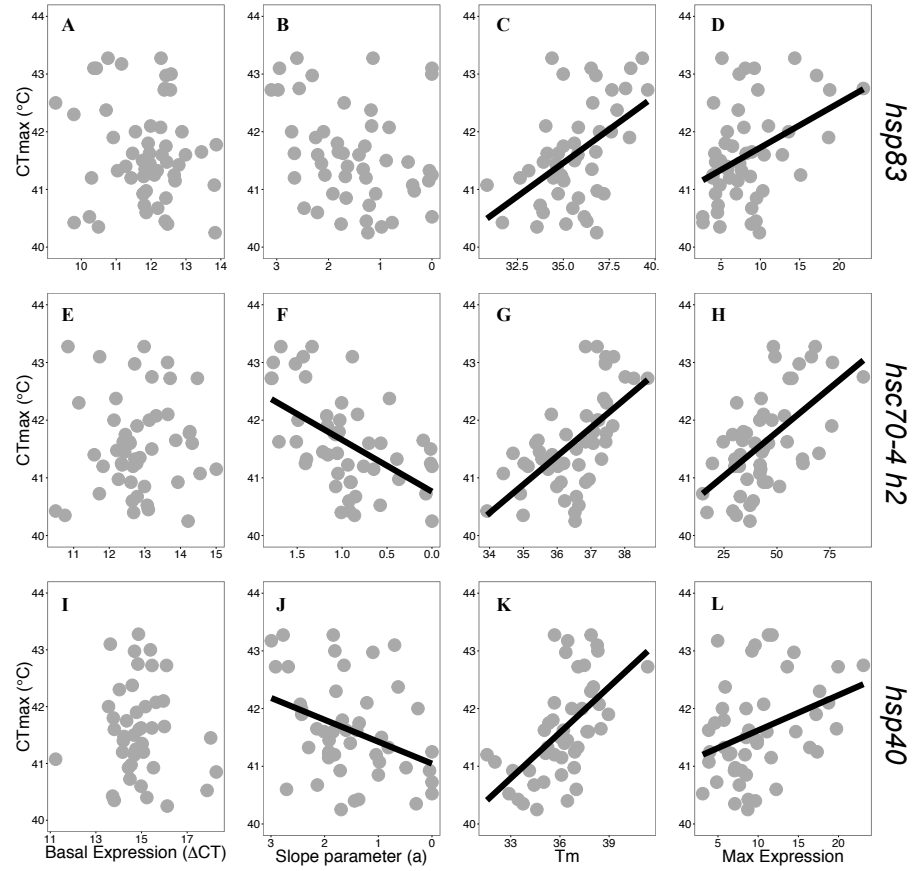
Figure 4 : The relationship between CTmax with different gene expression parameters (basal expression, slope parameter, Tm, and Max expression) of three Hsps (*hsp83: A-D*, *hsp70: E-H*, *hsp40: I-L*). Basal expression is calculated as ΔCT (CT$_{Hsp}$ - CT$_{18s\ rRNA}$). The axis of the slope parameter was reversed to reflect increasing expression rate.
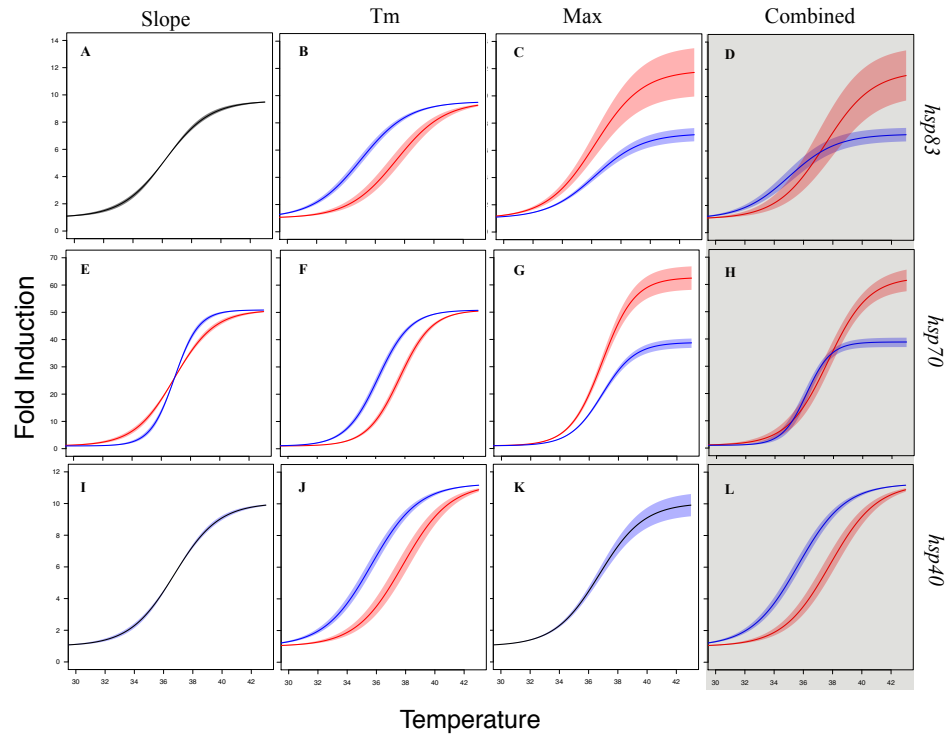
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Figure 5 : Adaptive shifts in the reaction norms of Hsp fold induction ($2^{-\Delta\Delta CT}$, Livak and Schmittgen 2001) between deciduous forest (blue) and flat woods species (red). Six out of 12 parameters show adaptive differences (basal patterns not shown). Differences among forest type for each parameter are shown with two curves (B, C, D, E, F, G, H, J, L) while holding all other parameters constant (average), otherwise, one reaction norm is shown to indicate no differences (A,I,K). The last column (D, H, L) shows the combination of parameters in one composite curve that were different among forest types. The standard errors are shown as shaded regions.

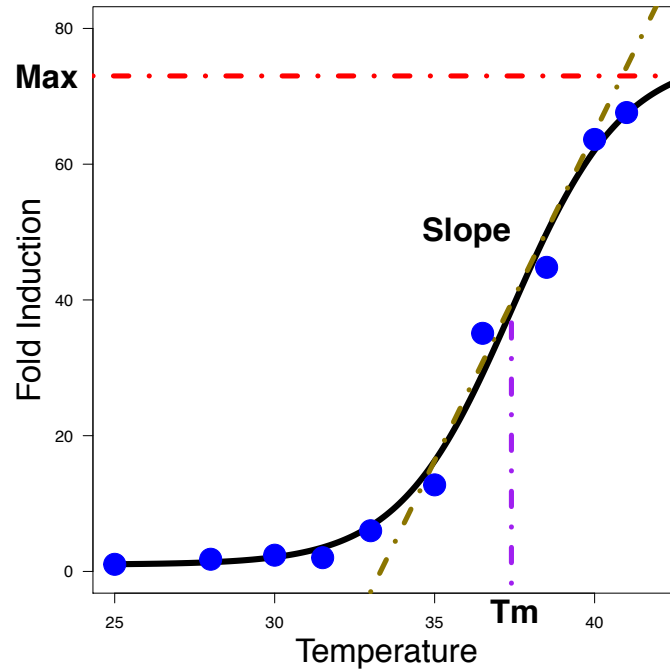**Figure 6 : Example of a reaction norm of Hsp gene fold induction as a function of temperature for a representative colony (blue points). The reaction norm is fitted with a Boltzmann function:**

$$1 + \frac{max-1}{1+e^{\frac{Tm-x}{a}}}$$ **(black line) that parameterizes the slope parameter (gold dot-dash line), inflection point (purple dot-dash line), and max fold induction (red dot-dash line).**
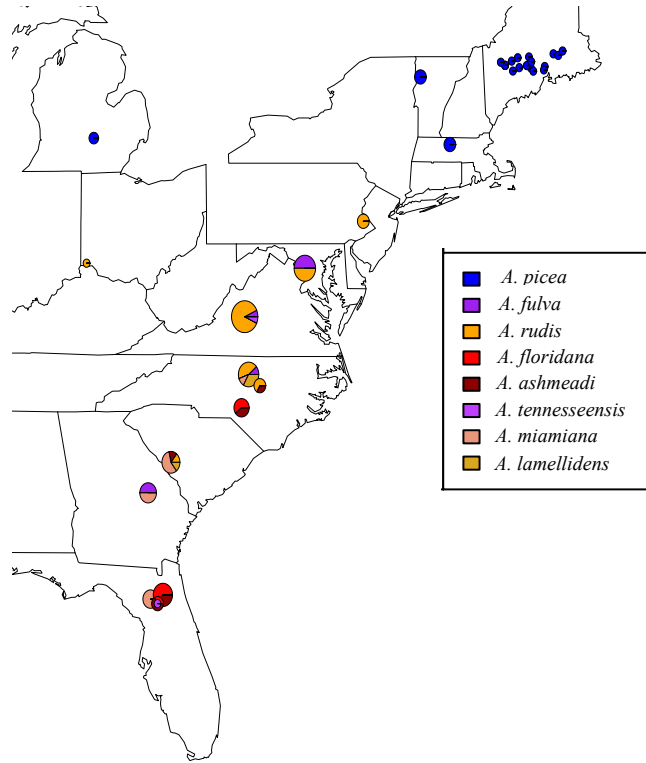
Figure S1 : Map of 33 collection sites for different species of *Aphaenogaster* across the Eastern United States. Each site shows a pie chart of the proportions of species and the size is scaled by the number of colonies.
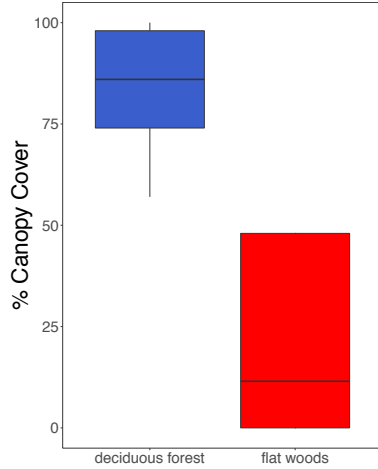
Figure S2 : The difference in % canopy cover between deciduous forests and flat woods. % canopy cover data were extracted from the National Land Cover Database 2011 (NLCD2011, https://catalog.data.gov/dataset/nlcd-2011-percent-tree-canopy-cartographic) at 5 meter resolution.
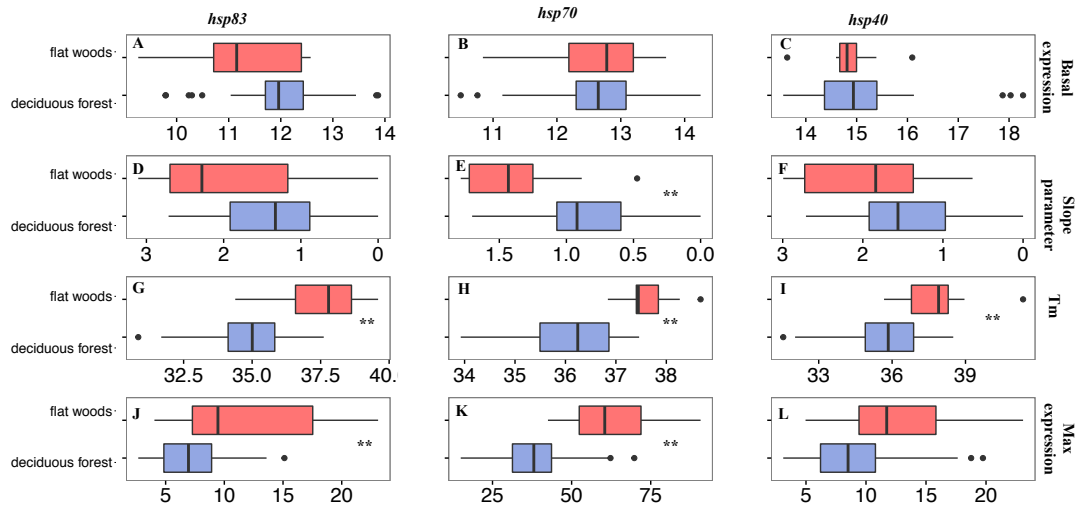
Figure S3 : Differences in each of the three Hsp gene expression parameters between species from different habitat types.

Figure S4 : The negative relationship between PC1 scores and CTmax. PC1 scores represent the correlational structure of Hsp gene induction parameters.

Figure S5 : The difference in PC1 scores between two broad forest types. PC1 scores represent the correlational structure of Hsp gene induction parameters.

CHAPTER 4: EFFECTS OF DESICCATION AND STARVATION ON THERMAL TOLERANCE AND THE HEAT SHOCK RESPONSE IN FOREST ANTS

Abstract

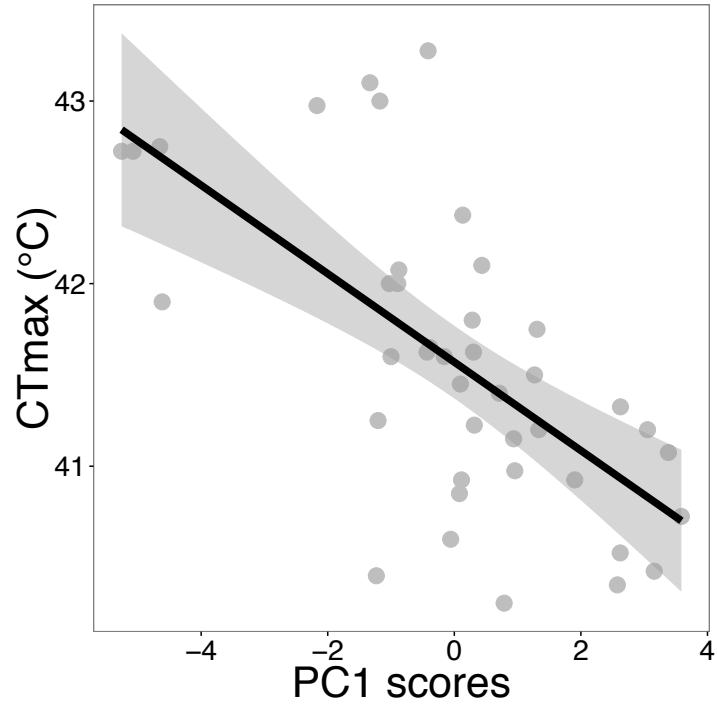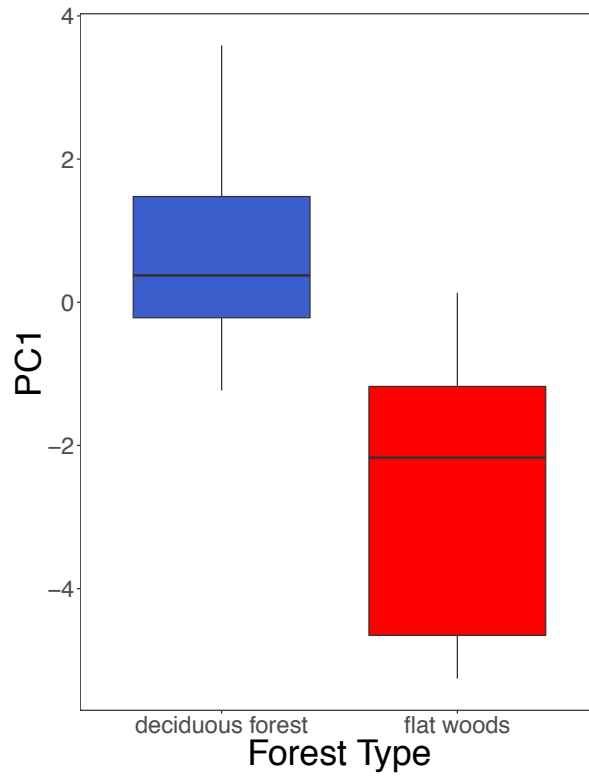Temperature increases associated with global climate change are likely to be accompanied by additional environmental stressors such as desiccation and food limitation, which may alter how temperature impacts organismal performance. To investigate how interactions between stressors influence thermal tolerance in the common forest ant, *Aphaenogaster picea*, we compared the thermal resistance of workers to heat shock with and without pre-exposure to desiccation or starvation stress. Knockdown (KD) time at 40.5°C of desiccated ants was reduced 6% compared to controls, although longer exposure to desiccation did not further reduce thermal tolerance. Starvation, in contrast, had an increasingly severe effect on thermal tolerance: at 21 days, average KD time of starved ants was reduced by 65% compared to controls. To test whether reduction in thermal tolerance results from impairment of the heat shock response, we measured basal gene expression and transcriptional induction of two heat shock proteins (*hsp70* and *hsp40*) in treated and control ants. We found no evidence that either stressor impairs the Hsp response: both desiccation and starvation slightly increased basal Hsp expression under severe stress conditions and did not affect the magnitude of induction under heat shock. These results suggest that the co-occurrence of multiple environmental stressors predicted by climate change models may make populations more vulnerable to future warming than is suggested by the results of single-factor heating experiments.

Keywords: Ants, Heat Shock Response, Desiccation, Heat Shock Proteins, Starvation, Thermal Tolerance

Abbreviations:

KD   Knockdown

HSR   Heat shock response

Hsp   Heat shock protein gene

hsp70	Heat shock protein 70 gene
hsp40	Heat shock protein 40 gene
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
Ef1β	Elongation factor 1 Beta
RT-qPCR	Real-time quantitative polymerase chain reaction
GLM	Generalized Linear Model
ANOVA	Analysis of Variance
LT_{50}	Median Lethal Time

Introduction

Temperature increases are projected to alter species distributions and abundances, particularly for ectotherms (Diffenbaugh and Field 2013). Whether a population is resilient in the face of higher temperatures is likely to depend on the temperature differential between the local thermal environment and the organism's critical thermal maximum, known as the thermal safety margin (Deutsch et al. 2008). Species with smaller thermal safety margins are predicted to be at risk of population declines as they approach or exceed their upper thermal limits (Deutsch et al. 2008, Diamond et al. 2012; Clusella-Trullas et al. 2011; Kellermann et al. 2012). In contrast, species with larger thermal safety margins may benefit from additional warming, because they typically operate at sub-optimal temperatures and would thus be shifted up their performance curve toward optimal operating body temperature (Deutsch et al. 2008; Diamond et al. 2012; Clusella-Trullas et al. 2011; Kellermann et al. 2012). However, taxa with larger thermal safety margins generally occupy locations with high temperature variation and extreme high temperatures may cause overheating (Kingsolver et al. 2013). Additionally, species that overwinter may be at risk of mortality during the winter months because warming can impact the microclimate and expose quiescent organisms to higher temperatures (Williams et al. 2015).

Critical thermal limits may also vary with environmental context, enhancing or reducing the thermal safety margin (Cahill et al. 2013; Chahal and Dev 2013; Duffy et al. 2015). Although thermal tolerances are typically measured in animals maintained under ideal conditions, extreme heat is projected to co-occur with reduced precipitation (Mueller and Seneviratne 2012), which may result in species

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simultaneously encountering both thermal and desiccation stress. Furthermore, temperature may act indirectly through shifts in prey availability or interspecific competition, potentially leading to nutritional stress (Araújo and Luoto2007).

The combined effect of multiple environmental stressors ultimately depends on the underlying molecular pathways used to combat their effects (Sinclair et al. 2013). If different stressors activate the same response pathways, exposure to one stressor can enhance resistance to another in a cross-protective manner (cross-tolerance; Todgham and Stillman 2013; MacMillan et al. 2009). In Antarctic midges, for example, desiccation provided cross-protection against heat stress (Benoit et al. 2009). One molecular pathway likely to show a generalized response is the heat shock response (HSR), which senses and repairs protein damage (Richter et al. 2010). However, if different stressors activate distinct molecular pathways, exposure to one may have no effect on response to the other, or may even decrease tolerance (cross-susceptibility) due to the energetic demands of responding to multiple stressors simultaneously (Sinclair et al. 2013; Todgham and Stillman 2013). In fruit flies, desiccation stress reduced upper thermal limits across a broad range of sub-lethal temperatures (Da Lage et al. 1989). Similarly, starvation has been found to either have no effect (Bubliy et al. 2012b) or a cross-susceptibility effect on thermal tolerance (Floyd 1985).

Ants are a good system to explore the impact of different stressors on thermal limits because they have colonized and inhabit diverse environments (Moreau and Bell 2013; Economo et al. 2015). Many species have a broad geographical range and are exposed to considerable environmental variation (Sanders et al. 2007; Dunn et al. 2009; Kaspari et al. 2015). Foraging activity is sensitive to temperature (Albrecht and Gotelli 2001; Wittman et al. 2009), soil moisture (Gordon 2013), available resources (Stuble et al. 2013), and species interactions (Rodriguez-Cabal et al. 2012) that altogether impact food intake for the whole colony. Ants are experimentally tractable for studies of physiological studies in response to multiple environmental conditions. Although ants likely face multiple stressors, we have very little understanding of how these additional sources of environmental stress such as desiccation and starvation are likely to impact thermal tolerance.

In this study, we tested how desiccation and nutritional stressors affect thermal tolerance in a common forest ant, *Aphaenogaster picea.* In a static heat-shock experiment, we compared knock-down (KD) times of workers maintained in control conditions to those exposed to either desiccation or starvation stress at progressive levels of severity. To determine whether changes in thermal tolerances were due to repression or enhancement of the heat shock response (HSR), we quantified baseline and transcriptional activation of two representative genes: *hsp70* and *hsp40*. We found that desiccation and starvation did not alter the HSR, but both diminished thermal limits across all levels of severity.

Materials and Methods

Natural History of Aphaenogaster picea

Aphaenogaster picea is a ground-dwelling species that occurs in mesic deciduous forests in the eastern United States from the high elevations of Virginia to Maine (DeMarco and Cognato 2015). Across their distribution, mean annual temperature ranges from 5- 14 °C, but leaf litter temperatures in the summer can be as high as 40 °C, while below-ground temperatures may remain at 20 °C (Lubertazzi 2012). Colonies are comprised of roughly 180-1,000 individuals that nest within the soil and coarse woody debris (Lubertazzi 2012). Foragers collect and disperse seeds containing eliaosomes (Warren et al. 2011), which provide the colony with a nutritional benefit (Morales and Heithaus 1998; Clark and King 2012).

Over the last 40 years, elevational limits have shifted upwards at the warm edge of their geographical range, suggesting that contemporary environmental change may already be affecting local populations (Warren and Chick 2013). Seed collection and dispersal by *A. picea* are sensitive to soil surface temperatures (Warren et al. 2011; Stuble et al. 2014) and soil moisture (Warren et al. 2010). Increasing temperatures have also led a phenological mismatch with ant-dispersed seed plants, as well as increased competitive pressure from more thermophilic native and invasive ant species (Bewick et al. 2014; Warren and Bradford 2014).

Field Collections and Rearing Ant Colonies

Whole queenright *A. picea* colonies consisting of eggs, larvae, pupae, and adult ants were collected from coarse woody debris and leaf litter between June-July 2013 at East Woods (N 44.440 W 73.197) located near the University of Vermont, in South Burlington, Vermont. Colonies were maintained

in the laboratory at 25°C constant temperature in 22 × 16 cm plastic containers lined on the sides with Insect-a-slip (Bioquip) to prevent escape, and filled to a depth of 50 mm with sand. We supplied each colony with 2 test-tubes (160 x 140 mm) half filled with water that were plugged with a cotton wad. The saturated cotton provided water and ants nested in the opening of the tube. Colonies were fed approximately 100 μL of 20% honey water and one bisected mealworm three times a week.

Stress Pre-treatment: Desiccation

To determine the effect of desiccation on thermal tolerance, we placed sets of 10 adult nestmate workers either into a 15 mL conical tube filled at the 7 mL mark with desiccant (10% relative humidity) separated by cotton to avoid contact, or a 15 mL conical tube half-filled with water capped with a cotton plug. To characterize the time-course of survival under continuous desiccation stress, we recorded survival status every half hour over an eight-hour period for 4 colony-level replicates. Based on the survival analysis, we chose three desiccation time points to represent mild, moderate, and severe sub-lethal desiccation stress and repeated the treatments for a new set of colonies (Fig 1a.). For each desiccation and control treatment and across each time point, we treated and then sampled 10 ants per colony for thermal tolerance assays (see below) with 7 colony-level replicates; for 5 of these colonies, an additional 4 ants for each treatment group were sampled for gene expression. To estimate the extent of desiccation, we measured for each set of 4 ants, the pooled initial wet weight (W_i), pooled final wet weight (W_f), and pooled final dry weight (D_f) to calculate the % initial water content as follows:

$$\% \text{ Initial Water Content} = 100 - \left(\frac{W_i - W_f}{W_i - D_f}\right) * 100$$

Stress Pre-treatment: Starvation

In order to determine the effect of starvation on thermal tolerance, we established 2 dietary treatments (starved and fed) for 5 time points spread over three weeks (day 1, 3, 7, 14, 21). Forty ants were randomly assigned to each dietary treatment and time point. Starved ants were reared in a cotton-plugged water tube with no access to food. Dead ants were removed daily to prevent cannibalism. Control ants were reared in an identical rearing tube but with access to 20% honey water and meal worms every two days. We tracked survival at each time point. At each time point, we used 10 ants from each treatment to assay for

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thermal tolerance with a total of 10 colony-level replicates and 4 ants per treatment group for gene

expression analyses for 5 of the colonies. To quantify condition, we measured the pooled dry mass (to the

nearest 0.01 mg) of ants after thermal tolerance experiments (see below). To control for ant size, we

regressed the mean head widths (mm) against mean dry mass and used the standardized residual (mean = 0

and variance = 1) as a measure of size-corrected dry mass. Head width was measured as the maximum

distance in mm (to the nearest 0.01) between the eyes using ImageJ software.

Measuring Thermal Tolerance

We used a static heat-shock protocol (Terblanche et al. 2011) to avoid the confounding issue of

ongoing desiccation associated with a slow ramping protocol (Rezende et al. 2011). Preliminary trials

revealed that 40.5 °C yielded KD times under an hour and that ants are able to recover from and survive

for at least a few days. For each set of 10 nest-mate workers associated with each time point and treatment

(see pre-treatments above), pairs of randomly selected workers were placed in five separate 5mL glass

screw-cap vials. Three of the five vials were heat shocked by fully submerging the vial at 40.5 °C in a pre-

set Thermo Neslab EX17 heating water bath, while the remaining two vials were simultaneously held at

room temperature (25 °C). Heat shocked ants were observed continuously at a temporal resolution of

roughly 10 seconds until KD, defined as loss of activity (Terblanche et al. 2011). To avoid bias, we

measured KD times without prior knowledge of the treatment groups.

Measuring the HSR

For the subset of colonies that we sampled to measure the HSR, the ants were exposed to identical

heat-shock and control conditions as those in the thermal tolerance assay, but were removed at 25 minutes

and flash-frozen in liquid nitrogen and stored at -80 °C. Ants were sampled regardless of KD status and

preliminary analyses showed that ants were able to induce hsp70 and hsp40 at the 25 minute mark.

For each gene expression sample, two of the four flash-frozen ants were pooled and

homogenized in a bullet blender homogenizer (Next Advance Inc., USA) at top speed (10) with 1.4 mm

zirconium silicate beads (Quackenbush Co., Inc, USA). RNA was isolated with RNAzol (Molecular

Research, USA) and then purified with the RNeazy Micro Kit (Qiagen, USA), both following the

manufacturers' instructions. 100 ng of RNA was converted to cDNA with the High-Capacity cDNA

Reverse Transcription Kit (Life Technologies, USA) following the manufacturer's instructions.

The gene expression patterns of *hsp70* (*hsc70-4* h2 orthologue) and *hsp40*were quantified using

previously developed primers (Nguyen et al. 2016) with RT-qPCR on a StepOnePlus instrument (Applied

Biosystems, USA). Each sample was run in triplicate in 20 μL reactions comprised of 2 ng of template,

250nM of forward primer and of 250nM reverse primer, and 1x Power SYBR® PCR master mix (Life

Technologies, USA). Reactions were incubated at 95°C for 2 minutes and then underwent 40 cycles of 95

°C for 15 seconds followed by 60°C for 60 seconds. Amplicon specificity was assessed with a melt-curve

analysis. We used the geometric mean of *ef1β* and *gapdh*as house keeping genes, which had the lowest

measure of variation according to NormFinder (stability = 0.23; Andersen et al. 2004).We used $2^{-\Delta\Delta CT}$ as

the measure of basal gene expression and fold induction under heat shock (Livak and Schmittgen 2001).

For basal gene expression, $2^{-\Delta\Delta CT}$ was calculated relative to colony-matched controls (water-plugged

treatment or fed treatment). Fold induction of heat-shocked ants was calculated relative to room

temperature (25°C) colony- matched controls.

Statistical Analyses

All statistical analyses were performed in R (version 3.2; R Core Team, 2016). In

all of our statistical analyses, colony was treated as an independent block for estimating treatment effects;

including colony as a random effect achieved similar results and we present only the findings from fixed

effects models. Survival was analyzed with a GLM, which fits a logistic relationship between the

proportion of individuals surviving and time (hours or days). Lethal time at 50% (LT_{50}) was estimated from

GLM-fitted models with the *dose.p()* function in the MASS package. We determined the effect of time,

treatment, and time × treatment interaction on KD time or Hsp gene fold induction with an ANCOVA. To

avoid over-fitting statistical models, we used a backwards AIC selection criterion with the *stepAIC()*

function in the MASS package (Venables and Ripley 2002). In models of *Hsp* expression (basal and

induction), values were log10-transformed to meet the assumptions of normality. To determine significant

differences in Hsp basal gene expression between pre-treatments (desiccation or starvation) with controls

for each time point, we used a one-sample t-test to test for significant differences from zero.

Results

Effect of Desiccation on Thermal Tolerance and HSR

Ants in the desiccation treatment experienced 100% mortality after 8 hours ($LT_{50}= 5.7 \pm 0.1$ hours; GLM: Desiccation z=-1.23), during which time there was no mortality of control ants (Fig. 1a). The onset of mortality for desiccated ants was at ~ 4 hours (Fig. 1a). Although water content of ants in both treatments decreased through time, (ANOVA, $F_{1,77}= 20.73$, p <0.001), desiccated ants had lower water content than controls ($F_{1,77}= 20.09$, p <0.001; Fig. 1b), with most water loss occurring in the first hour. With heat shock, the KD time of desiccated ants was reduced within the first hour by 6% compared to controls ($F_{1,58}=25.21$, p <0.001; Table 1) and did not decrease further with through time (Fig. 1c).

Pooled among times, desiccated and control treated ants did not differ in overall basal expression of *hsp70* (one sample t-test, t= 1.03, df = 12, p =0.32) or *hsp40* (t= 0.41,df = 12, p =0.69). When time points were analyzed separately, desiccated ants also did not differ significantly from controls in *hsp70* basal gene expression (Fig. 2a), but desiccated ants had 1.3-fold higher *hsp40* basal expression at the 3.5 h (one-sample t-test, t= 4.54, df=4, p<0.05, Fig. 2b). In response to heat shock, desiccated and control ants induced *hsp70* and *hsp40* to similar levels (Fig. 2,b,d; Table 1).

Effect of Starvation on Thermal Tolerance and HSR

Starved ants died ($LT_{50}= 24.3 \pm 1.1$ days) sooner than control ants ($LT_{50}= 35.5 \pm 3.9$ days; GLM: Dietary treatment z = -3.138, p <0.001; Fig. 3a). Residual dry mass decreased more rapidly in starved than control treated ants (Treatment × Time interaction, $F_{1,59}=4.18$, p < 0.05, Fig. 3b). By day 21, starved ants declined in residual dry mass to 83.5% of controls (Fig. 3b). KD time decreased significantly with progressive starvation (Treatment × Time interaction, $F_{1,45}=38.68$, p < 0.001, Fig. 3c, Table 1). By day 21, KD time of starved ants declined to 35% of the controls (Fig. 3c).

Starved and control treated ants did not differ overall in basal expression of *hsp70* (one sample t-test, t= -0.34, df = 23, p =0.73) and *hsp40* (t= 0.92 ,df = 23, p =0.36). When analyzed separately by day, however, starved ants significantly down-regulated *hsp70* 1.66-fold on day 3 (t= -4.08, df =4, p < 0.05), but up-regulated it 1.4-fold on day 21 (t = 4.27, df =4 , p <0.05; Fig. 4a). Starved ants up-regulated h*sp40* 2-fold more than controls on day 1 (t = 4.24, df =4, p <0.05; Fig. 4c). Starved and control ants induced *hsp70*

and *hsp40 (*$F_{1,28}$=0.00, p = 0.98; Fig. 4b,d*)* to similar levels, but the induction of hsp70 declined with time in both groups (ANCOVA, $F_{4,28}$=8.01, p < 0.001; Fig. 4b).

Discussion

Environmental stressors often act in concert, either increasing tolerance through cross-protection or decreasing tolerance through cross-susceptibility (Todgham and Stillman 2013). In this study, we found support for cross-susceptibility of thermal limits to desiccation and starvation (Fig. 1c, Fig. 3c). The magnitude of reduced thermal tolerance reflected additive and interactive effects of desiccation and starvation on survival (Fig.1a, Fig. 3a) and physiological condition (Fig. 1b, Fig. 3b). The effect of desiccation stress was relatively mild, but starvation produced a marked effect over time, decreasing KD time by over 50% under severe stress conditions. If such stressors are experienced in the field, the thermal safety margin of this species under projected climate change scenarios for the northeastern US may be significantly smaller than that predicted by temperature alone (Clusella-Trullas et al. 2011).

The cross-effects of desiccation stress on thermal tolerance may depend on the timing and magnitude of stressors applied (Kingsolver and Woods 2016; Gunderson et al. 2016). Simultaneous application of drying and warming often diminish thermal tolerances (Maynard Smith 1957; Da Lage et al. 1989; Holmstrup et al. 2002; Bubliy et al. 2012a), and we also found cross-susceptibility between desiccation and rapidly-applied heat under sequential application of stressors. However, under slow heating protocols, desiccation has not been found to depress thermal tolerance (Terblanche et al. 2011). In fact, with sufficient recovery time between these two stressors (Bubliy et al. 2012b) or slow application of desiccation (Benoit et al. 2010), desiccation conferred cross-tolerance against heat stress.

Dehydration can either enhance or inhibit thermal defense mechanisms (Benoit et al. 2009; Bubliy et al. 2012b). Protein denaturation resulting from any type of perturbation, including desiccation stress, elicits the HSR (Kültz 2003, Kültz 2005) by rapidly up-regulating Hsps (Craig and Gross 1991; Hayward et al. 2004; Morris et al. 2013; Mizrahi et al. 2010). Hsps that remain induced after the stressor subsides offer a short-term "hardening" effect that can increase survival in the face of subsequent exposure to the same stressor (Cavicchi et al. 1995), and can also potentially cross-protect against other stressors (Bubliy et al. 2012b). Consistent with hardening, we found that desiccation increased basal expression of

*hsp40*at the highest exposure (Fig. 2c). Despite this effect, however, increased *hsp40* expression did not result in enhanced upper thermal limits.

In comparison to desiccation, starvation imposed greater cross-susceptibility to thermal damage. Consistent with previous work (Bubliy et al. 2012b; Overgaard et al. 2012), thermal tolerance was not affected by mild starvation, but with increasing exposure time, thermal tolerance declined 65% compared to fed workers of the same age (Fig. 3b). Because thermal tolerance is typically estimated for lab-acclimated organisms provided with *ad libitum* food, our result suggests that such experiments may substantially overestimate thermal tolerance expressed by individuals in the field (Tagliarolo and McQuaid 2016). Such an effect may be particularly ecologically relevant in ants because foraging outside the nest is performed primarily by the oldest and most resource-depleted individuals (Howard and Tschinkel 1998; Tschinkel 1998; Tripet and Nonacs 2004; Dussutour et al. 2016).

Thermal defenses are metabolically expensive (Bettencourt et al. 2008; Hoekstra and Montooth 2013), leading to the expectation that HSR activation would lessen as internal energy reserves become depleted. In both dietary groups, both *hsp70* gene fold induction and KD time declined over the course of the experiment, potentially reflecting decreased ability to mount a sufficient response as individuals aged (Bowler and Terblanche 2008). However, starvation was associated with transient increases in basal Hsp gene expression, and starved and fed ants invested similarly in Hsp gene up-regulation in response to heat stress (Fig. 4), suggesting that allocation of energy to protein protection is not impacted under low-resource conditions. As with desiccation, starvation-induced increases in basal Hsp gene expression at early and late time points were not associated with increases in KD time (Fig. 4a,c). It is possible that other molecular pathways that contribute to coping with stress that were not measured here, such as damage repair, redox regulation, and energy metabolism, are depressed by starvation and outweigh the slight increase in the Hsp response (Zinke et al. 2002; Kültz 2005).

Taken together, the results of this study suggest that single-stressor assays may not be a reliable method for estimating thermal tolerance, and thus the capacity to withstand additional warming. Future climate change is likely to impose simultaneous combinations of environmental stressors such as temperature, desiccation, and starvation. Each of these is likely to impose stress on individual and colony-

level performance and elicit physiological defenses; however, in addition to their independent effects, their interaction has the potential further reduce temperature tolerances. To improve species forecasts, models of physiological responses to climate change should account for these diverse sources of stress (Terblanche et al. 2007).

Conflict of interest   No competing interest declared.

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References

Albrecht M, Gotelli NJ (2001) Spatial and temporal niche partitioning in grassland ants. Oecologia 126:134–141. doi: 10.1007/s004420000494

Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. Cancer Res 64:5245–5250. doi: 10.1158/0008-5472.CAN-04-0496

Araújo MB, Luoto M (2007) The importance of biotic interactions for modelling species distributions under climate change. Global Ecology and Biogeography 16:743–753.doi: 10.1111/j.1466-8238.2007.00359.x

Benoit JB, Lopez-Martinez G, Elnitsky MA, et al (2009) Dehydration-induced cross tolerance of Belgicaantarctica larvae to cold and heat is facilitated by trehalose accumulation. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 152:518–523. doi: 10.1016/j.cbpa.2008.12.009

Benoit JB, Lopez-Martinez G, Phillips ZP, et al (2010) Heat shock proteins contribute to mosquito dehydration tolerance. Journal of Insect Physiology 56:151–156.doi:10.1016/j.jinsphys.2009.09.012

Bettencourt BR, Hogan CC, Nimali M, Drohan BW (2008) Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in Drosophila melanogaster but does not compensate for loss of thermotolerance in Hsp70 null flies. BMC Biology 6:5. doi: 10.1186/1741-7007-6-5

Bewick S, Stuble KL, Lessard J-P, Dunn RR, Adler FR, Sanders NJ (2014) Predicting future coexistence in a north American ant community. Ecology and Evolution 4:1804-1819. doi: 10.1002/ece3.1048

Bowler K, Terblanche JS (2008) Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? Biological Reviews 83:339–355.doi: 10.1111/j.1469-185X.2008.00046.x

Bubliy OA, Kristensen TN, Kellermann V, Loeschcke V (2012a) Humidity affects genetic architecture of heat resistance in Drosophila melanogaster. Journal of Evolutionary Biology 25:1180–1188.doi: 10.1111/j.1420-9101.2012.02506.x

Bubliy OA, Kristensen TN, Kellermann V, Loeschcke V (2012b) Plastic responses to four environmental stresses and cross-resistance in a laboratory population of Drosophila melanogaster. Functional Ecology 26:245–253.doi: 10.1111/j.1365-2435.2011.01928.x

Cahill AE, Aiello-Lammens ME, Fisher-Reid MC, et al (2013) How does climate change cause extinction? Proceedings of the Royal Society of London B: Biological Sciences 280:20121890. doi: 10.1098/rspb.2012.1890

Cavicchi S, Guerra D, Torre VL, Huey RB (1995) Chromosomal Analysis of Heat-Shock Tolerance in Drosophila melanogaster Evolving at Different Temperatures in the Laboratory. Evolution 49:676–684.doi: 10.2307/2410321

Chahal J, Dev K (2013) Shifting clinal patterns of stress resistance traits in Drosophila ananassae. EvolEcol 27:333–351.doi: 10.1007/s10682-012-9599-6

Clark RE, King JR (2012) The Ant, Aphaenogaster picea, Benefits From Plant Elaiosomes When Insect Prey is Scarce. Environmental Entomology 41:1405–1408.doi: 10.1603/EN12131

Clusella-Trullas S, Blackburn TM, Chown SL, et al (2011) Climatic Predictors of Temperature Performance Curve Parameters in Ectotherms Imply Complex Responses to Climate Change. The American Naturalist 177:738–751.doi: 10.1086/660021

Craig EA, Gross CA (1991) Is hsp70 the cellular thermometer? Trends in Biochemical Sciences 16:135–140.doi:10.1016/0968-0004(91)90055-Z

Da Lage J-L, Capy P, David J-R (1989) Starvation and desiccation tolerance in Drosophila melanogaster adults: Effects of environmental temperature. Journal of Insect Physiology 35:453–457.doi: 10.1016/0022-1910(89)90051-6

Demarco BB, Cognato AI (2015) Phylogenetic Analysis of Aphaenogaster Supports the Resurrection of Novomessor (Hymenoptera: Formicidae). Annals of the Entomological Society of America sau013.doi:10.1093/aesa/sau013

Deutsch CA, Tewksbury JJ, Huey RB, et al (2008) Impacts of climate warming on terrestrial ectotherms across latitude. PNAS 105:6668–6672.doi: 10.1073/pnas.0709472105

Diamond SE, Sorger DM, Hulcr J, et al (2012) Who likes it hot? A global analysis of the climatic, ecological, and evolutionary determinants of warming tolerance in ants.Glob Change Biol 18:448–456.doi:10.1111/j.1365-2486.2011.02542.x

Diffenbaugh NS, Field CB (2013) Changes in Ecologically Critical Terrestrial Climate Conditions. Science 341:486–492.doi: 10.1126/science.1237123

Duffy GA, Coetzee BW, Janion-Scheepers C, Chown SL (2015) Microclimate-based macrophysiology: implications for insects in a warming world. Current Opinion in Insect Science 11:84–89. doi: 10.1016/j.cois.2015.09.013

Dunn RR, Agosti D, Andersen AN, et al (2009) Climatic drivers of hemispheric asymmetry in global patterns of ant species richness. Ecology Letters 12:324–333. doi: 10.1111/j.1461-0248.2009.01291.x

Dussutour A, Poissonnier L-A, Buhl J, Simpson SJ (2016) Resistance to nutritional stress in ants: when being fat is advantageous. Journal of Experimental Biology 219:824–833.doi:10.1242/jeb.136234

Economo EP, Klimov P, Sarnat EM, et al (2015) Global phylogenetic structure of the hyperdiverse ant genus Pheidole reveals the repeated evolution of macroecological patterns. Proceedings of the Royal Society of London B: Biological Sciences 282:20141416. doi: 10.1098/rspb.2014.1416

Floyd RB (1985) Effects of Photoperiod and Starvation on the Temperature Tolerance of Larvae of the Giant Toad, Bufo marinus. Copeia 1985:625–631. doi: 10.2307/1444753

Gordon DM (2013) The rewards of restraint in the collective regulation of foraging by harvester ant colonies. Nature 498:91–93. doi: 10.1038/nature12137

Gunderson AR, Armstrong EJ, Stillman JH (2016) Multiple Stressors in a Changing World: The Need for an Improved Perspective on Physiological Responses to the Dynamic Marine Environment. Annual Review of Marine Science 8:357–378. doi: 10.1146/annurev-marine-122414-033953

Hayward SAL, Rinehart JP, Denlinger DL (2004) Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae. Journal of Experimental Biology 207:963–971.doi:10.1242/jeb.00842

Hoekstra LA, Montooth KL (2013) Inducing extra copies of the Hsp70 gene in Drosophila melanogaster increases   energetic demand. BMC Evolutionary Biology 13:68. doi: 10.1186/1471-2148-13-68

Holmstrup M, Hedlund K, Boriss H (2002) Drought acclimation and lipid composition in Folsomia candida:implications for cold shock, heat shock and acute desiccation stress. Journal of Insect Physiology 48:961– 970.doi: 10.1016/S0022-1910(02)00175-0

Howard DF, Tschinkel WR (1980) The effect of colony size and starvation on food flow in the fire ant, Solenopsis invicta (Hymenoptera: Formicidae). Behav Ecol Sociobiol 7:293–300. doi: 10.1007/BF00300670

Kaspari M, Clay NA, Lucas J, et al (2015) Thermal adaptation generates a diversity of thermal limits in a rainforest ant community. Glob Change Biol 21:1092–1102. doi: 10.1111/gcb.12750

Kellermann V, Overgaard J, Hoffmann AA, et al (2012) Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. PNAS 109:16228–16233.doi: 10.1073/pnas.1207553109

Kingsolver JG, Diamond SE, Buckley LB (2013) Heat stress and the fitness consequences of climate change for terrestrial ectotherms. FunctEcol 27:1415–1423.doi: 10.1111/1365-2435.12145

Kingsolver JG, Woods HA, Kearney M, Michalakis Y (2016) Beyond thermal performance curves: modeling time-dependent effects of thermal stress on ectotherm growth rates. The American Naturalist 187:283–294.

Kültz D (2003) Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. J Exp Biol 206:3119–3124.doi: 10.1242/jeb.00549

Kültz D (2005) Molecular and Evolutionary Basis of the Cellular Stress Response. Annual Review of Physiology 67:225–257.doi: 10.1146/annurev.physiol.67.040403.103635

Lubertazzi D (2012) The Biology and Natural History of *Aphaenogaster rudis*. Psyche: A Journal of Entomology.doi: 10.1155/2012/752815

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2-\Delta\Delta CT$ Method.Methods 25:402–408.doi: 10.1006/meth.2001.1262

MacMillan HA, Walsh JP, Sinclair BJ (2009) The effects of selection for cold tolerance on cross-tolerance to other environmental stressors in Drosophila melanogaster. Insect Science 16:263–276.doi: 10.1111/j.1744-7917.2009.01251.x

Mizrahi T, Heller J, Goldenberg S, Arad Z (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. Cell Stress and Chaperones 15:351–363. doi: 10.1007/s12192-009-0150-9

Morales MA, Heithaus ER (1998) FOOD FROM SEED-DISPERSAL MUTUALISM SHIFTS SEX RATIOS IN COLONIES OF THE ANT APHAENOGASTER RUDIS. Ecology 79:734–739. doi: 10.1890/0012-9658(1998)079[0734:FFSDMS]2.0.CO;2

Moreau CS, Bell CD (2013) Testing the Museum Versus Cradle Tropical Biological Diversity Hypothesis: Phylogeny, Diversification, and Ancestral Biogeographic Range Evolution of the Ants. Evolution 67:2240– 2257. doi: 10.1111/evo.12105

Morris JP, Thatje S, Hauton C (2013) The use of stress-70 proteins in physiology: a re-appraisal. Molecular Ecology 22:1494–1502.doi: 10.1111/mec.12216

Mueller B, Seneviratne SI (2012) Hot days induced by precipitation deficits at the global scale.PNAS 109:12398–12403.doi: 10.1073/pnas.1204330109

Nguyen AD, Gotelli NJ, Cahan SH (2016) The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. BMC Evolutionary Biology 16:15.doi: 10.1186/s12862-015-0573-0

Overgaard J, Kristensen TN, Sørensen JG (2012) Validity of Thermal Ramping Assays Used to Assess Thermal Tolerance in Arthropods. PLoS ONE 7:e32758.doi: 10.1371/journal.pone.0032758

Rodriguez-Cabal MA, Stuble KL, Guénard B, et al (2012) Disruption of ant-seed dispersal mutualisms by the invasive Asian needle ant (Pachycondyla chinensis). Biol Invasions 14:557–565. doi: 10.1007/s10530-011-0097-5

Rezende EL, Tejedo M, Santos M (2011) Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. Functional Ecology 25:111–121.doi: 10.1111/j.1365-2435.2010.01778.x

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Richter K, Haslbeck M, Buchner J (2010) The Heat Shock Response: Life on the Verge of Death. Molecular Cell 40:253–266.doi: 10.1016/j.molcel.2010.10.006

Sanders NJ, Lessard J-P, Fitzpatrick MC, Dunn RR (2007) Temperature, but not productivity or geometry, predicts elevational diversity gradients in ants across spatial grains. Global Ecology and Biogeography 16:640–649. doi: 10.1111/j.1466-8238.2007.00316.x

Smith JM (1957) Temperature Tolerance and Acclimatization in Drosophila Subobscura. J ExpBiol 34:85–96.

Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA (2013) Cross-tolerance and Cross-talk in the Cold: Relating Low Temperatures to Desiccation and Immune Stress in Insects. Integr Comp Biol ict004. doi: 10.1093/icb/ict004

Stuble KL, Pelini SL, Diamond SE, et al (2013) Foraging by forest ants under experimental climatic warming: a test at two sites. Ecol Evol 3:482–491. doi: 10.1002/ece3.473

Stuble KL, Patterson CM, Rodriguez-Cabal MA, et al (2014) Ant-mediated seed dispersal in a warmed world. PeerJ. doi: 10.7717/peerj.286

Tagliarolo M, McQuaid CD (2016) Field Measurements Indicate Unexpected, Serious Underestimation of Mussel Heart Rates and Thermal Tolerance by Laboratory Studies. PLOS ONE 11:e0146341. doi:10.1371/journal.pone.0146341

Terblanche JS, Deere JA, Clusella-Trullas S, et al (2007) Critical thermal limits depend on methodological context. Proceedings of the Royal Society of London B: Biological Sciences 274:2935–2943. doi: 10.1098/rspb.2007.0985

Terblanche JS, Hoffmann AA, Mitchell KA, et al (2011) Ecologically relevant measures of tolerance to potentially lethal temperatures. J ExpBiol 214:3713–3725.doi: 10.1242/jeb.061283

Tripet F, Nonacs P (2004) Foraging for Work and Age-Based Polyethism: The Roles of Age and Previous Experience on Task Choice in Ants. Ethology 110:863–877.doi: 10.1111/j.1439-0310.2004.01023.x

Todgham AE, Stillman JH (2013) Physiological Responses to Shifts in Multiple Environmental Stressors: Relevance in a Changing World. Integr Comp Biol 53:539–544. doi: 10.1093/icb/ict086

Tschinkel WR Sociometry and sociogenesis of colonies of the harvester ant, Pogonomyrmex badius: worker characteristics in relation to colony size and season. Insectessoc 45:385–410.doi: 10.1007/s000400050097

Venables, W. N. & Ripley, B. D. (2002) Modern Applied Statistics with S. Fourth Edition.Springer, New York.ISBN 0-387-95457-0

Warren RJ, Bahn V, Bradford MA (2011) Temperature cues phenological synchrony in ant-mediated seed dispersal. Global Change Biology 17:2444–2454.doi: 10.1111/j.1365-2486.2010.02386.x

Warren RJ, Bradfor MA (2014) Mutualism fails when climate response differs between interacting species. Global Change Biology 20:466-474. doi:10.1111/gcb.12407

Warren RJ, Chick L (2013) Upward ant distribution shift corresponds with minimum, not maximum, temperature tolerance. Global Change Biology 19:2082–2088.doi: 10.1111/gcb.12169

Warren RJ, Giladi I, Bradford MA (2010) Ant-mediated seed dispersal does not facilitate niche expansion. Journal of Ecology 98:1178–1185.doi: 10.1111/j.1365-2745.2010.01694.x

Williams CM, Henry HAL, Sinclair BJ (2015) Cold truths: how winter drives responses of terrestrial organisms to climate change. Biol Rev 90:214–235. doi: 10.1111/brv.12105

Wittman SE, Sanders NJ, Ellison AM, et al (2010) Species interactions and thermal constraints on ant community structure. Oikos 119:551–559. doi: 10.1111/j.1600-0706.2009.17792.x

Zinke I, Schütz CS, Katzenberger JD, et al (2002) Nutrient control of gene expression in Drosophila: microarray analysis of starvation and sugar-dependent response. The EMBO Journal 21:6162–6173.doi:10.1093/emboj/cdf600

Tables and Figures

Table 1 Summary of statistical analyses for survival, thermal tolerance(KD time), condition (Water content, Dry Mass), and heat shock response (Hsp70 and Hsp40 expression).

Experiment	Sources of Variation	Survival	KD-time	Water Content	Dry Mass	Hsp70 Basal	Hsp70 Induction	Hsp40 Basal	Hsp40 Induction
Desiccation	Colony	++							ns
	Treatment (Desiccated/Control)	+++	+++	+++				+	ns
	Time (hours)	+++		+++				+	ns
	Treatment x Time								ns
Starvation	Colony	+	+++				ns		ns
	Treatment (Starved/Control)	++	+++		+++	+	ns	+	
	Time (Days)	+++	+++		ns	+	+++	+	
	Treatment x Time		+++		+		ns		

Note- '+': p < 0.05; "++": p < 0.01; "+++": p < 0.001; ns = not significant. Empty cells represent predictors that were not retained from AIC model selection.
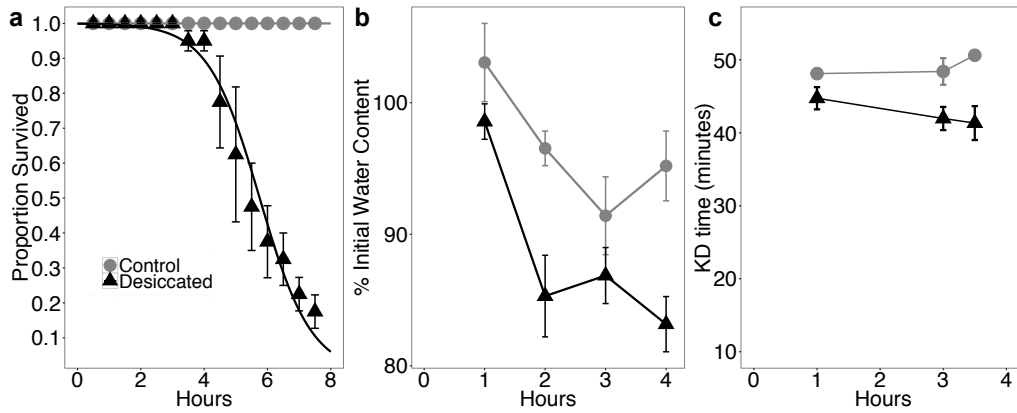

Fig. 1 Effect of desiccation (black triangles) on (**a**) survival, (**b**) initial water content, and (**c**) thermal tolerance relative to control ants (gray circles; n = 7 colony replicates per time point and treatment). Survival under desiccation stress was determined over an 8 hour period and water content was measured from 1 to 4 hours; we selected hours 1, 3, and 3.5 for subsequent static heat shock treatment (40.5 °C)and error bars represent ± 1 standard error of the mean.
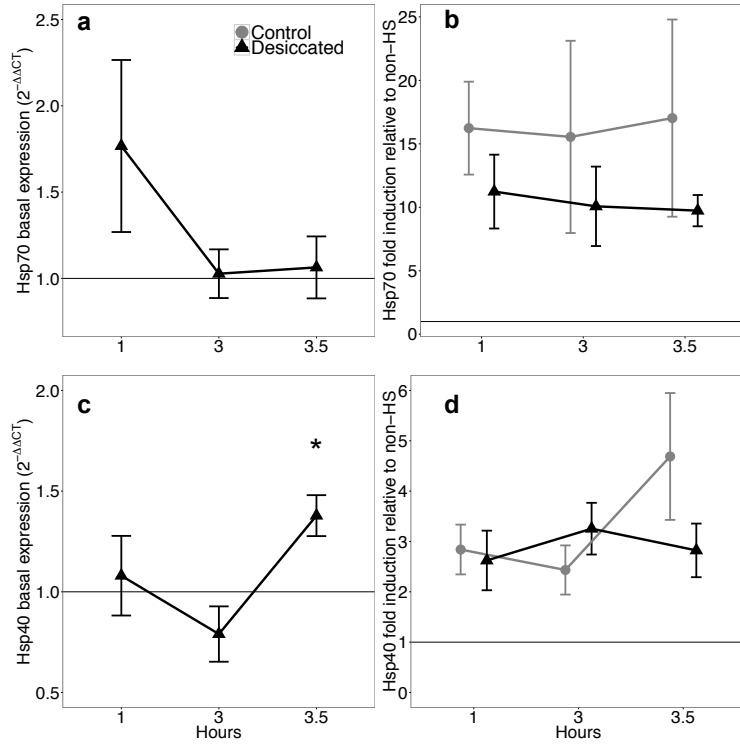
Fig. 2 Effect of desiccation (shaded) on *hsp70* and *hsp40*. Panels **a, c** show basal gene expression; panels **b** and **d** show the extent of fold induction in response to heat shock relative to control ants (white). Basal gene expression and Hsp fold induction were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). For each treatment and time point, there were 4-5 colony-level replicates and error bars represent $\pm$ 1 standard error of the mean.
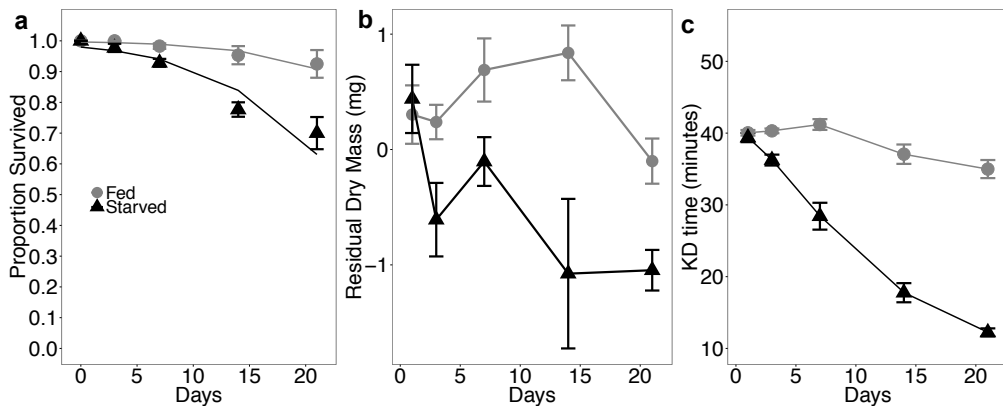

Fig. 3 Effect of starvation (black triangles) on (**a**) survival, (**b**) residual dry mass, and (**c**) thermal tolerance relative to control ants (gray circles). Survival under starvation stress was determined over 21 days and we

selected 1, 3, 7, 14, 21 day time points for subsequent static heat shock treatment (40.5 °C) and measured

residual dry mass. Residual dry mass represents the size corrected dry mass, which was obtained by

extracting the residuals from a linear regression between dry mass and head width. Residual dry mass was

standardized such that the mean = 0 and variance = 1. For each treatment and time point, there were 10

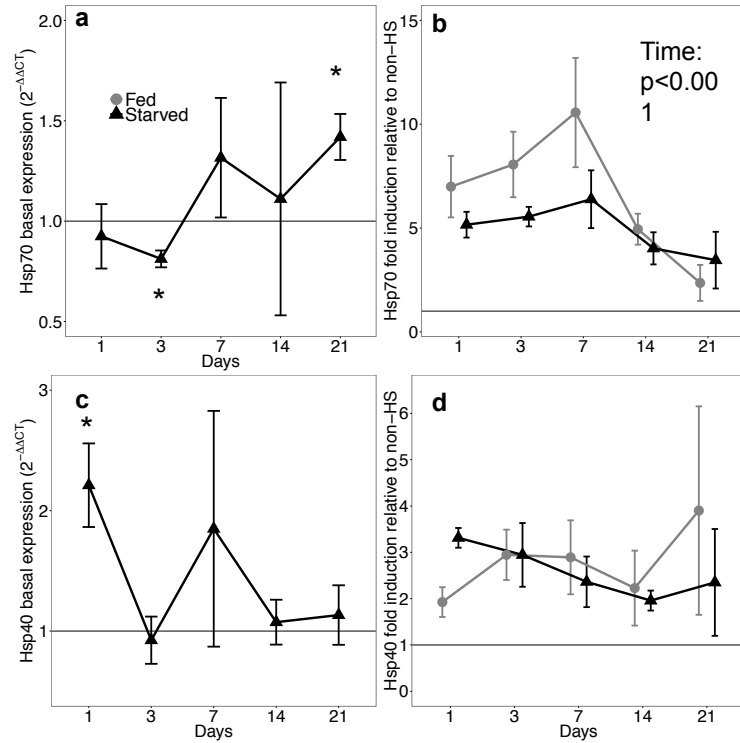colony-level replicates and error bars represent ± 1 standard error of the mean.

Fig. 4 Effect of starvation (shaded) on *hsp70* and *hsp40*. Panels **a** and **c** show basal gene expression; panels

b and d show the extent of fold induction relative to control ants (white). Basal gene expression and Hsp

fold induction were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). For each treatment

and time point, there were 4-5 colony level replicates and error bars represent± 1 standard error of the

mean.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation integrated different levels of biological organization to understand how organisms can survive and colonize different thermal environments. The first chapter characterized the functional diversity of Hsps, ants and other hymenoptera. Hymenoptera respond to heat stress by utilizing a different Hsp70 orthologue from fruit flies. Although evolutionary gains and losses of gene copies produce unique homologues, Hsps still retain the ability to respond to temperature which was associated with the presence of HSE in their promoters in ants. HSEs were mostly conserved in the proximal end of the promoter: we found more diversity towards the distal end of the promoter, whose function in transcriptional activation should be explored.

The HSF-HSE transcriptional cascade in the HSR represents an evolutionarily conserved mechanism for transcriptional regulation. While HSEs were largely conserved in *hsp83*, and *hsp40*, HSEs within the promoter region of *hsc70-4* were much more diverse in arrangement and location, particularly at the more distal end of the promoter. The proximal HSEs are critical for transcriptional activation and the more distal HSEs can also interact with RNA polymerase if the promoter bends (Tian et al. 2010). The condition to which HSF-HSE binds to locations within the genome is unknown in Hymenoptera. In fruit flies, the chromatin landscape determines the affinity for HSF to bind HSEs, whereby hallmarks of more open chromatin facilitate binding (Guertin and Lis 2010). Because the chromatin state might be critical for Hsp induction through HSF-

HSE binding, it is possible that the thermal experiences of queens may be passed on to workers to produce correlated transgenerational epigenetic inheritance of Hsp expression profiles. To functionally test the effect of number and position of previously surveyed HSEs, a combination of chromatin immunoprecipitation (ChIP)-seq experiments and gene reporter gene assays will be required under different contexts. For example, gene reporter assays will determine whether distally located HSEs can activate transcription and ChIP-seq sampling of histone markers and HSFs between queens and workers among colonies will determine whether HSF-HSE binding is transgenerationally inherited.

Patterns of Hsp induction itself explained 40% of the variation in phenotypic divergence in upper thermal limits within forest ant species of the genus *Aphaenogaster*. *Aphaenogaster* species occupying flat woods had higher CTmax than species in deciduous forests. Because flat woods forests have lower canopy cover than deciduous forest, foraging ants are likely to experience more thermal extremes from solar radiation, which selects for greater ability to withstand heat stress. Withstanding heat stress is likely due to a better ability to mitigate protein unfolding through Hsp chaperoning and also greater resistance to protein unfolding. The dynamics of how the whole proteome unfolds in response to temperature is the next step to understanding stress resistance.

There are multiple strategies for organisms to evolve a more stable proteome. Increasing individual protein stability can involve very few amino acid changes (Lockwood and Somero 2012), but these changes need to be distributed across the whole proteome. Empirical measurements of whole proteome stability are rare, but a study by Leuenberger et al. (2017) finds that thermophilic bacteria (*Thermus thermophilus*) have

higher average proteome stability than *Escherichia coli*, yeast, and humans. However, shifting proteome stability through amino acid changes may not be easily achievable for closely related taxa with very little time to evolve stabilizing amino acids across the whole proteome. Alternatively, species can enhance proteome stability through protein-protein interactions such as post-translational modifications (Storey and Wu 2013). For example, transfer of ubiquitin onto a protein marks it for degradation through the proteasome, but this strategy costs energy (Golberg 2003). Degrading thermally labile proteins would enrich for more thermally stable proteins. Accumulating protective osmolytes can non-specifically increase protein stability across the whole proteome (Hottiger et al. 1994) through the action of a few enzymes (De Virgilio et al. 1990; Teets et al. 2013) and may be a less costly strategy. The relative contribution of amino acid sequence variation, protein-protein interactions, and osmolytes that explains variation in proteome stability needs to be tested in a comparative design through multiplex proteomic (Savitski et al. 2014; Leuenberger et al. 2017) and metabolomic experiments (Malmendal et al. 2006). I predict that shifting osmolyte concentrations to confer stress resistance may be the largest contributor to differences in proteome stability and upper thermal limits within a rapidly diversifying clade.

However, the effectiveness of osmolyte shifts contributing to proteome stability may be influenced by the presence of other stressors. For example, trehalose is a sugar that stabilize proteins under heat stress (Eleutherio et al. 1993) and it also protects against extreme cold and desiccation (Elbein et al. 2003). Under desiccation, trehalose solvates phospholipids in place of water and stabilizes cell membranes (Hazel and Eugene 1990).

The availability of trehalose depends on nutritional status (Elbein et al. 2003) and

standing pools will deplete under starvation. Therefore, the protective benefits of

trehalose under heat stress will potentially be less effective with each additional stressor

organisms face.

Ectotherms in natural populations experience a combination of stressors, which

may enhance or diminish the thermal performance of foragers. We found that forest ants

were cross-susceptible to heat stress when they previously encountered desiccation and

starvation. Whether cross-susceptibility was mediated by trehalose could be tested in the

future. How general cross-susceptibility is across ants and other ectotherms as a whole

will give more accurate predictions on how species will respond to future environmental

change.

Works Cited

De Virgilio C, Simmen U, Hottiger T, Boiler T, Wiemken A (1990) Heat shock induces enzymes of trehalose metabolism, trehalose accumulation, and thermotolerance in Schizosaccharomyces pombe, even in the presence of cycloheximide. *FEBS Letters*, **273**, 107–110.

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology*, **13**, 17R–27R.

Eleutherio ECA, Araujo PS, Panek AD (1993) Protective Role of Trehalose during Heat Stress in Saccharomyces cerevisiae. *Cryobiology*, **30**, 591–596.

Goldberg AL (2003) Protein degradation and protection against misfolded or damaged proteins. *Nature*, **426**, 895–899.

Gonsalves SE, Moses AM, Razak Z, Robert F, Westwood JT (2011) Whole-Genome Analysis Reveals That Active Heat Shock Factor Binding Sites Are Mostly Associated with Non-Heat Shock Genes in Drosophila melanogaster. *PLoS ONE*, **6**, e15934.

Guertin MJ, Lis JT (2010) Chromatin Landscape Dictates HSF Binding to Target DNA Elements. *PLoS Genet*, **6**, e1001114.

Hazel JR, Eugene Williams E (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research*, **29**, 167–227.

Hottiger T, De Virgilio C, Hall MN, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. *European Journal of Biochemistry*, **219**, 187–193.

Leuenberger P, Ganscha S, Kahraman A *et al.* (2017) Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. *Science*, **355**, eaai7825.

Lockwood BL, Somero GN (2012) Functional Determinants of Temperature Adaptation in Enzymes of Cold- versus Warm-Adapted Mussels (Genus Mytilus). *Molecular Biology and Evolution*, **29**, 3061–3070.

Malmendal A, Overgaard J, Bundy JG *et al.* (2006) Metabolomic profiling of heat stress: hardening and recovery of homeostasis in Drosophila. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, **291**, R205–R212.

Savitski MM, Reinhard FBM, Franken H *et al.* (2014) Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science*, **346**, 1255784.

Smith CR, Anderson KE, Tillberg CV, Gadau J, Suarez AV (2008) Caste Determination in a Polymorphic Social Insect: Nutritional, Social, and Genetic Factors. *The American Naturalist*, **172**, 497–507.

Storey KB, Wu C-W (2013) Stress response and adaptation: A new molecular toolkit for the 21st century. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **165**, 417–428.

Teets NM, Kawarasaki Y, Jr REL, Denlinger DL (2013) Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, Belgica antarctica. *Journal of Comparative Physiology B*, **183**, 189–201.

Tian S, Haney RA, Feder ME (2010) Phylogeny Disambiguates the Evolution of Heat-Shock cis-Regulatory Elements in Drosophila. *PLoS ONE*, **5**, e10669.

Tomanek L (2010) Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. *The Journal of Experimental Biology*, **213**, 971–979.