Effects of experimental warming on the common woodland ant

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*Introduction*

Models ofclimate change predict global temperature increases for terrestrial ecosystems. Tropical species at low latitude live near their optimal temperature and can tolerate only relatively narrow temperature fluctuations, making them more susceptible to warming than northern species (Deutsch et al. 2008). Species experiencing temperatures above their optimum for too long may avoid unfavorable conditions by migrating to cooler regions or adapting to new warm conditions, but whether either of these is possible depends on the connectivity of the landscape and the severity of thermal stress experienced (Walther et al. 2002). Ecotherms, which acquire heat from their external environment, are particularly vulnerable because ambient temperature strongly influences their activity and reproduction (Deutsch et al. 2008). Current predictions of species response to climate change depend on measures of their lethal thermal limits, but organisms experience costs to their physiological functions far before reaching their ecological death (Evans & Hoffman 2012). For better predictions on how rising temperatures will impact species, a more accurate proxy for assessing sub-lethal stress must be identified.

Unfavorable temperature imposes costs by damaging macromolecules such as proteins and nucleic acids, leading to loss in biochemical reactions and a subsequent decline in performance; organisms combat these forces of sub-lethal stress by utilizing the cellular stress response (CSR) (Kültz 2005, Morris et al. 2013). When environmental factors − such as temperature − inflict protein damage, the CSR induces transcription of molecular chaperones to assist with the refolding or denaturing of unfolded proteins. Heat shock proteins (HSPs) are a universal group of molecular chaperones, classified into groups by their molecular weights, that function with different co-chaperones to cope with heat stress (Li & Srivastava 2004).

*Hsp70* is one of the most studied because of their stress-induced response from unfavorable temperature and involvement with different co-chaperones to determine functionality and the final product of a damaged protein (Morris et al. 2013; Lanneau et al., 2010; Cassel et al. 2010; Liberek et al. 2008; Fan et al. 2003; Hartl 1996). To avoid immediate degradation or aggregation of damaged client proteins, *hsp40* co-chaperones bind to a client protein and deliver it to a matching *hsp70*/ATP complex to determine the specialized function and location of the unfolded protein (Fan et al. 2003). *Hsp40* chaperones then assist with ATP hydrolysis creating a stable *hsp40*/ *hsp70*/damaged protein complex holding the unfolded protein in a stable environment until its outcome is known to prevent aggregation of unfolded proteins (Hartl 1996). ADP dissociation releases the unfolded protein allowing it to rebind with *hsp40*, aggregate in the cell, or undergo a refolding mechanism with assistance of other chaperone proteins.

Anther HSP co-chaperone which assists in deciding what to do with an unfolded protein is *hsp90* (gene name *hsp83*). Once *hsp83* binds damaged client proteins it can connect with *hsp70* to signal further chaperones completing a protein-refolding pathway (Hartl 1996). If a client protein is too damaged to refold, *hsp83* can directly signal ubiquitination pathways to avoid aggregation and further sub-lethal stress (Liberek et al. 2008). The inducible nature of *hsp70* in response to unfavorable temperature and the assistance provided from its co-chaperones to stabilize, denature, or refold damaged proteins makes these three heat shock proteins, *hsp70, 40*, and *83*, the best potential proxy for sub-lethal stress invoked by unfavorable temperature. To determine if HSPs can serve as a proxy for sub-lethal stress, temperatures were experimentally manipulated at a northern site (Harvard Forest in Massachusetts) and a southern site (Duke Forest in North Carolina). Twelve experimental open top warming chambers were established at both sites warming ambient temperature of the chambers by blowing warm air from ranges of 0.5° to 5.5°C.

Ectotherms, such as ants, may be most impacted by warmer temperatures because many of their physiological functions are influenced by ambient temperature. Ants provide key ecosystem services such as seed-dispersal and soil turnover, so increased temperatures forcing a change in abundance or foraging trends may result in negative cascading effects for the ecosystem (Stuble et al. 2013). The common woodland ant genus, *Aphaenogaster,* are a good model to study species responses to climate change because they occupy temperate deciduous forests from Maine to Florida, where they experience very diverse thermal environments (Warren & Chick 2013). The upper lethal limits of *Aphaenogaster* populations increase with decreasing latitude, but not as fast as environmental temperatures, suggesting that southern populations survive closer to their lethal limit and may face greater heat stress than northern populations (Diamond et al. 2013). Previous experiments revealed that ant colonies from southern sites display larger decreases in worker abundance under elevated temperatures compared to those from northern forests (Diamond et al. 2013), suggesting they may be more negatively impacted by warmer temperatures; however, a better environmental monitor is needed to directly measure levels of sub-lethal stress in order to inform predictions of how this species will react to warming.

My overall objective is to assess species response to heat in wild populations of the woodland ant genus *Aphaenogaster* using HSPs as a proxy. From reviewing the literature assessing ectotherms thermal performance and *Aphaenogaster’s* response to warming, I predict HSP expression will increase with experimental temperature for colonies at the southern site. However, HSP expression in the northern ants will remain relatively constant across temperatures because they survive below their optimal temperatures (Deutsch et al. 2008; Diamond et al. 2013). This would match previous behavioral data, where southern ants showed symptoms of stress when exposed to warming chambers, while northern forest ants did not show a decrease in abundance or performance in these warmer conditions (Diamond et al. 2013). A positive correlation between temperature experienced at the time of capture and the amount of HSP transcription will indicate the degree to which these ants are stressed and confirm that HSPs can serve as a good proxy for sub-lethal stress.

*Materials and Methods*

**Field Collections**

The experiment took place at a southern site (Duke Forest (DF) in Durham, North Carolina) and a northern site (Harvard Forest (HF) in Petersham, Massachusetts) to assess the potential impacts of warming on *Aphaenogaster* from their northern and southern boundaries. Twelve experimental open top warming chambers were established at both sites. Ambient temperature was increased for nine of the chambers from 0.5° to 5.5°, while three control chambers blew ambient air into the plot (see *Pelini et al., 2011* for a detailed description of the chambers). Samples were chosen on a relatively “hot” and “cool” day in the summers of 2013 and 2014 in order to capture as wide a temperature range as possible (DF sample dates: 7/2/2013; 9/10/203; 7/17/2014; 9/11/2014)(HF sample dates: 6/26/2013; 8/21/2013). By baiting them with food, three foraging workers could be selected at random and flash frozen immediately in liquid nitrogen. Three replicate samples were collected from each chamber. To quantify temperatures the ants were experiencing at the time of collection, four ground temperature measurements were made for each bait collection with an infrared thermometer. In total 262 samples (164 DF, 98HF) were sent to the University of Vermont and stored at -80° C until HSP quantification began.

**Measuring Environmental Stress**

To determine if southern ants experience more thermal stress under warmer conditions than those in the north, *hsp*40, *hsp*70, and *hsp*80 gene expression was quantified from *Aphaenogaster* at the southern and northern site (Primers used= *hsp*40: 541\_641, *hsp*70: 1468\_1592, *hsp*83: 1583\_1682).HSP quantification began by extraction and purification of mRNA from each sample using the RNeazy micro kit (QIAGEN, USA). Frozen ants were homogenized in a Bullet blender (Next Advance Inc., USA) for two minuets at top speed (10) in 1.4mm zirconium silicate grinding beads (Quackenbush Co., Inc., USA) and 500 uL of RNAzol buffer (Molecular Research Center, Inc., USA). Following the manufacturer’s instructions, RNA samples were resuspended in 100 uL of water and purified using the RNeazy micro kit with DNAse I (QIAGEN, USA) treatment to remove any DNA contamination. RNA concentration was verified using Qubit Fluorometric Quantitation (Invitrogen, USA) and samples were converted to 50ng of cDNA following instructions from the (find out name kit). Quantifications of the HSP DNA segments were obtained through quantitative polymerase chain reaction (qPCR) using ABI StepOnePlus Real-Time PCR system. Reactions took place in 10 uL volume with 1ng of cDNA, 250 nM total primer, and 5 μL of Power SYBR® Green Master Mix (Life Technologies, USA). Cycling conditions began at an initial 95**°**C incubation for 2 min followed by 40 cycles of 95**°**C for 15 seconds, with 60**°**C annealing and extension for 60 seconds. Melt curve analyses following amplification validated the presence of a single amplicon and any nonspecific samples were removed. Given the cycle threshold values via qPCR, relative gene expression fold change was calculated using the ΔΔCT method relative to the housekeeping gene, *18s rRNA,* once 100% primer efficiency was ensured (Livak & Schmittgen 2001). (18s *rRNA* used for preliminary data, currently testing actin, GAPDH, and EF1B for stability comparison). HSP expression levels were compared to local ground temperature experienced at the time of sampling to see if HSPs can serve as a proxy for sub-lethal stress, as well as the extent to which elevated temperature increases stress levels at the two sites.

**Statistical Analysis**

Using the 84 Duke Forest samples and the 48 Harvard forest samples with complete gene expression data, ANCOVAs were ran for each gene to identify any relationship or interaction between the predictors, site and temperature, and the response, HSP gene expression. Values were log transformed and met the assumptions of normality.

*Results*

Out of the total 229 samples successfully sent to the University of Vermont (161 DF, 68HF), RNA extraction, cDNA conversion, and gene expression data has been completed for 185 samples (118 DF, 65 HF). Similar results were seen for the relative gene expression of each HSP at both locations. *Hsp40* relative expression was positively correlated to the ground temperature the ants were experiencing at the time of collection in both Duke and Harvard Forest (Fig. 1a: site p-value= 2.85e-06\*\*\*; local temperature p-value= 0.00347\*\*; R2=0.2004). *Hsp70* displayed a similar positive correlation increasing relative expression with local ground temperature (Fig. 1b: site p-value= 7.58e-06\*\*\*; local temperature p-value= 1.32e-06\*\*\*; R2=0.2659). Lastly, hsp83 also showed a positive correlation increasing relative expression with temperature. (Fig. 1c: site p-value= 1.37e-11\*\*\*; local temperature p-value= 6.04e-15\*\*\*; R2=0.5111).

1. ***Hsp40***



1. ***Hsp70***

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1. ***Hsp*83**

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**Figure 1**. Relative gene expression fold change level relative to the local ground temperature each ant was experiencing at the time of collection for *hsp40* (a),

*hsp70* (b), and *hsp83* (c). Red dots represent Duke Forest and blue dots represent Harvard Forest

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