Title: Common woodland ants are stressed under experimental warming

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**Ask sara and nick where this should be submitted and then look up that format and change ms accordingly**

**Abstract**

Temperature increases due to climate change will impose costs for many ectotherm species by elevating macromolecular damage. Yet, we know very little about the extent to which costs will manifest under future warming. We took advantage of a southern and northern experimental warming site to quantify protein damage using a panel of 3 heat shock proteins for a keystone mutualist, *Aphaenogaster picea.* At both locations ants were collected from heating twelve chambers that increased ambient ground temperature from 0°C to 5.5°C to determine the relationship between Hsp gene expression and local warming temperatures. For…hsp83, hsp70, hsp40 we found…

**Introduction (redo this…write an outline first)**

Models ofclimate change predict global temperature increases for terrestrial ecosystems (Deutsch et al. 2008). Expand the effects of climate change. Pose problem: we’re not sure how warming will really affect species…we only have data from CTmax…but we know they can experience costs way before

Experimental warming studies are good way to study..

Our overall objective was to assess the potential impacts of warming in wild populations of the woodland ant genus *Aphaenogaster* using Hsps as a proxy for…..

You need a hypothesis before a prediction

We predict Hsp expression will increase with experimental temperature for colonies at the southern site (Duke Forest, North Carolina), their narrow range of thermal tolerance will cause southern populations to survive closer to their critical thermal maxima and experience more sub-lethal stress from warmer temperature (Deutsch et al. 2008; Diamond et al. 2013). However, we predict Hsp expression in *Aphaenogaster* from the northern site (Harvard Forest, Massachusetts) will remain relatively constant across temperatures because they survive below their optimal temperature, so an increase in temperature may push colonies closer to their optimum reducing levels of sub-lethal stress (Deutsch et al. 2008; Diamond et al. 2013). A positive correlation between temperature experienced at the time of capture and the amount of Hsp transcription will indicate an increase in protein damage and the degree to which these ants are stressed when exposed to warmer temperature supporting the use of Hsps as a proxy and environmental monitor for sub-lethal thermal stress.

**Materials and Methods**

*Experimental warming sites and chambers*

We collected ants from two experimental warming sites, Duke Forest (DF), Durham, North Carolina and Harvard Forest (HF), Petersham, Massachusetts, where twelve experimental open-top warming chambers were established in 2010 (Pelini et al. 2011). Nine warming chambers increased ambient temperature by blowing warmed air from 0.5°C, 1.5°C, and 5.5°C, directed towards the ground with three chambers at each temperature setting. Three control chambers blew ambient air into the plot and three chamberless control plots were established (see *Pelini et al., 2011* for a detailed description of the chambers).

Samples were chosen on a relatively “hot” and “cool” day in the summer of 2013 at HF and both 2013 and 2014 at DF in order to capture as wide a temperature range as possible (See Table 1 for sampling dates). Eight artificial next boxes arrayed in pairs in each cardinal direction were placed approximately one meter apart in each chamber. Bait cards holding crumbled pecan sandwiches (cite?) were placed between each set of nest boxes to sample foraging workers outside the nest box begin exposed to the warmed ground temperature. Three random foragers from each bait station were selected at random and grouped together to be immediately flash frozen 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 (company,country). Samples were stored at -80° C until Hsp mRNA quantification.

*Quantifying Hsp Gene Expression*

We quantified *hsp40*, *hsp70*, and *hsp83* gene expression fold change relative to the stable housekeeping gene, 18s *rRNA,* from *Aphaenogaster* samples collected at the southern (DF) and northern (HF) warming chamber sites (See Table 2 for genes tested and primers used). Total mRNA from each sample was extracted and purified using the RNeazy micro kit (QIAGEN, USA). Each sample containing three frozen ants was homogenized in a Bullet blender (Next Advance Inc., USA) for two minutes at top speed (10) in 1.4mm zirconium silicate grinding beads (Quackenbush Co., Inc., USA) and 350 uL of RLT buffer (QIAGEN, USA). RNA samples were treated with DNAse I (QIAGEN, USA) to remove DNA contamination and purified following the manufacturer’s instructions. RNA concentration was verified using Qubit Fluorometric Quantitation (Invitrogen, USA) and RNA integrity was tested using a NanoDrop Bioanalyzer (ThermoScientific, USA) . Samples were converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM). Abundance of each Hsp gene and the housekeeping gene *18S* was quantified with quantitative polymerase chain reaction (qPCR) using the 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 followed by 60 seconds at 70°C to obtain the Sybr Green florescence reading. Melt curve analyses following amplification validated the presence of a single amplicon and samples displaying nonspecific amplicons were manually checked and removed. Hsp gene expression was calculated relative to the housekeeping gene *18s* using the ΔΔCT method (Livak & Schmittgen 2001).

*Statistical Analysis*

We tested the effects of local ground temperature, site, and interaction effects between site and temperature on relative Hsp gene expression fold change with a linear regression stepwise analysis using backwards selection. To control for RNA quality, RIN values were added as a covariate. Hsp relative gene expression values were log transformed to meet assumptions of normality. For graphing, did we plot log10 expression?

**Results**

Out of the total 231 samples (164 DF, 68HF), RNA extraction, cDNA conversion, and gene expression analyses have been completed for 187 samples (119 DF, 68 HF). A significant main site effect was identified between *hsp40, hsp70,* and *hsp83* relative expression and the ground temperature ants were experiencing at the time of collection indicating Hsp expression was up regulated in response to warming at both Duke and Harvard Forest, but mean Hsp expression was significantly higher in *Aphaenogaster* from Duke Forest (Fig. 1a: *hsp40*; df = 186, site *P* < 0.0001\*\*\*, temperature, *P* = 0.001\*\*, *R2*=0.20). (Fig. 1b: *hsp70*; df = 186, site *P* < 0.0001\*\*\*, local temperature *P* < 0.0001\*\*\*, R2=0.27). (Fig. 1c: *hsp83*; df = 186, site *P* < 0.0001\*\*\*, local temperature *P* < 0.0001\*\*\*, R2=0.51).

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.

**Discussion**

Experimental warming does appear to increase expression of the thermally stress induced protein, *hsp70,* and two of its corresponding co-chaperons, *hsp40* and *hsp83* (Fig. 1). Unlike our predictions of observing a site by temperature interaction, *Aphaenogaster* experiencing higher ground temperature display a greater relative expression of all three Hsps at both Harvard and Duke Forest. Although *Aphaenogaster* in northern forests may survive further from their maximum thermal limits (Diamond et al. 2013),these results suggest both locations experience sub-lethal stress as temperature increases, not just populations found at lower latitude with narrow ranges of thermal tolerance.

While *Aphaenogaster* colonies at both sites indicated the presence of sub-lethal stress, Hsp expression was found to be significantly higher in Duke Forest implying foragers from the southern site are primed to up-regulate Hsp expression quicker than those at Harvard forest. (Because they’ve already been experiencing warm temps? Because they are experiencing a higher degree of stress? More literature searching and examples).

Gene expression values were calculated using the ΔΔCT method comparing CT values of each HSP gene to a stable housekeeping gene. 18S rRNA was used as our housekeeping gene but did not display stable expression across temperatures at Harvard Forest (Fig. 1d). We are currently conducting qPCR testing three additional genes (actin, EF1B, and GAPDH) for stability to see which will serve as the best housekeeping gene. Although 18S expression in Harvard Forest could be affecting the observed relationships, a more stable housekeeping gene would increase Hsp expression values contradicting previous behavioral data recorded from Harvard Forest to an even greater extent. (examples)

To increase the accuracy of our results RIN values will be found for each sample, supplementary data from remaining samples will be added, quantified Hsp samples will be sequenced, and qPCR will be conducted using two additional genes (actin and GAPDH) to identify the most stable housekeeping gene. Added molecular data will increase the accuracy of our data providing further validity to our results.

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**Supplemental**

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| --- | --- | --- | --- | --- | --- | --- |
| **Collection date** | 7/2/2013 | 9/10/2013 | 7/17/2014 | 9/11/2014 | 6/26/2013 | 8/21/2013 |
| **Location** | Duke | Duke | Duke | Duke | Harvard | Harvard |
| **Number of samples collected** | 45 | 41 | 45 (not yet in data set) | 33 | 33 | 35 |

**Table 1.** Sampling dates at Duke and Harvard Forest and the number of samples collected each day

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Gene** | *hsp70* | *hsp83* | *hsp40* | *18s rRNA* | *actin* | *GAPDH* |
| **Forward Primer** | 1468 | 1583 | 541 | 328 |  |  |
| **Reverse Primer** | 1592 | 1682 | 641 | 427 |  |  |

**Table 2**. Genes tested and primers used.

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