Andrew Nguyen
Thesis proposal

Title: Molecular basis of thermal adaptation within the genus *Aphaenogaster*

Specific Aims

Rapid changes in the environment accompanying climate change will impose increasing stress on species. Organisms cope with the negative fitness consequences of stress via the cellular stress response (CSR), which senses and repairs macromolecular damage through the action of chaperone proteins such as the heat shock proteins (HSPs). Although the basic CSR response is one of the most highly conserved mechanisms across the tree of life, thermal tolerance varies widely among species and is often correlated with local environmental conditions. Clinal variation in thermal tolerance among populations of a species suggests adaptive modification of the CSR over evolutionary time. However, the molecular mechanisms underlying variation in thermal tolerance are poorly understood among broader non-model taxonomic groups. I propose to study the evolutionary forces that shape the CSR for a widespread North American ant genus, *Aphaenogaster*. This genus is ideal for studying adaptive changes in the CSR because species occupy diverse thermal regimes, from deserts to northern wet forests; thus, they likely display interspecific variation in both the extent and mechanisms of thermal tolerance. Drawing techniques and tools from multiple disciplines such as molecular biology, ecology, and evolution, I will address four questions: **1) What is the evolutionary history of the major families of heat shock proteins in ants that respond to thermal stress? 2) What is the relationship between thermal environment and physiological tolerance to temperature in *Aphaenogaster*? 3) What molecular mechanisms underlie adaptive variation in thermal tolerance across species? 4) Are the same mechanisms important for resistance to different types of stressors predicted to increase with future shifts in climate?** This study will provide insights into the evolutionary history and functional significance of adaptive molecular processes that may allow better prediction of species responses to climate change.

Background

Climate change is predicted to alter species abundance and distributions through rapid changes in the environment (IPCC 2007). Future climate models project increases in both average and extreme temperatures, the magnitudes of which will be greater across increasing latitudes and greater for terrestrial ecosystems vs. aquatic ecosystems (IPCC 2007; Diffenbaugh & Field 2013). Terrestrial ectotherms may be particularly susceptible to these increases in climate warming given that their performance (activity, growth, reproduction) is coupled to ambient temperature. Upper thermal limits of performance or critical thermal maxima (CTmax) is highest at low latitudes and lowest towards the poles (Addo-Bediako et al. 2000; Sunday et al. 2010). Given this clinal variation, tropical species may face greater population declines than temperate species because any additional warming will more rapidly shift performance towards their CTmax (Deutsch et al. 2008; Clusella-Trullas et al. 2011; Diamond et al. 2012; Kingsolver et al. 2013). However, these projected outcomes assume no compensatory mechanisms that ultimately determine resiliency or vulnerability to future climate warming such as migration,

acclimation, and adaptation (Deutsch et al. 2008; Sunday et al. 2012; Hoffmann et al. 2013).

Adaptive modification of a conserved physiological mechanism, known as the cellular stress response (CSR), may confer resilience to climate shifts by providing resistance to temperature stress (Sørensen 2003; Somero 2010; Geiler-Samerotte et al. 2010). The CSR senses and repairs temperature induced macromolecular damage to DNA, lipids, and proteins by up- and down-regulating a cascade of genes (Kultz 2003; Kultz 2005; Sørensen et al. 2005). Sets of multigene families known heat shock proteins (HSPs) mediate the CSR by refolding proteins and restoring biological activity (Fink 1999, Kultz 2003). Upon irreversible protein damage, HSPs interact with proteolytic pathways to degrade proteins (Somero 2011). Both protein refolding and degradation by HSPs maintain homeostasis in the face of thermal threats (Lindquist 1988).

The patterns of HSP induction are an important target of temperature adaptation (Feder & Hofmann 1999; Garbuz et al. 2003; Sørensen 2003). However, previous studies associating adaptive variation in CTmax with HSP expression have mixed results. In *Drosophila*, some studies have related greater shifts in upper thermal limits with higher initial investment (elevated basal HSP expression) or higher induction levels (max HSP expression), but there are instances where both mechanisms are utilized (Krebs et al. 1997; Garbuz et al. 2003; Carmel et al. 2011). These types of mechanisms are not universal and there are cases where HSP expression patterns do not play an apparent role in conferring thermal tolerance (Jensen et al. 2009; Calabria et al. 2012).

Adaptive variation in HSP expression may be missed due to experimental approach. Past studies often employ an ANOVA type of experimental design where there are few temperature categories although temperature is more continuous in nature (Gomulkiewicz & Kirkpatrick 1992; Stanton-Geddes et al., unpublished). The thermal reaction norm, or continuous measurements of expression over a range of temperatures (Angilletta 2009), can reveal adaptive shifts in the CSR other than basal and max HSP expression levels (Dietz & Somero 1992; Stanton-Geddes et al., unpublished). For example, populations or species can vary in threshold values (Hofmann & Somero 1996; Tomanek & Somero 2000) or expression inflection points, representing the onset of sublethal stress and transcriptional rate, respectively (Fig. 3). The extent to which these mechanisms are utilized for stress resistance in non-model terrestrial organisms remains unexplored (Evans & Hofmann 2012; Morris et al. 2013).

Ant species within the genus *Aphaenogaster* provide an ideal and important system for studying species responses to climate change because they occupy diverse habitats, are numerically abundant, and provide key ecosystem services such as nutrient recycling and seed dispersal (Lubertazzi 2012; Ness et al. 2009; Warren et al. 2011). Therefore, systematic extirpation of populations across species may have negative cascading effects on ecosystems (Ness et al. 2009; Warren et al. 2011). The preservation of these ecosystem services depends on the extent of resiliency and vulnerability of *Aphaenogaster* species to climate shifts (Somero 2010; Evans & Hoffmann 2012; Warren & Chick 2013). Because they experience a wide range of temperatures (Caroll 1975; Fig. 1) and display interspecific variation in thermal tolerance (Warren & Chick 2013), *Aphaenogaster* species have the potential to reveal novel molecular mechanisms of stress resistance within a comparative framework.

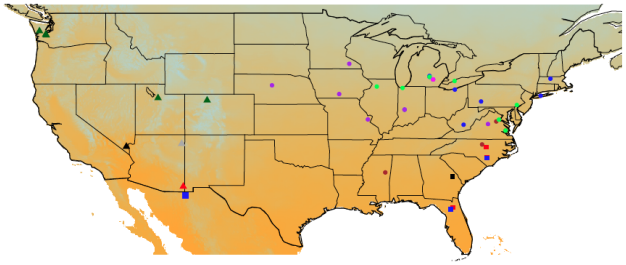

Figure 1. Map of Aphaenogaster species found in temperate areas of the United States. Species are represented by different color symbols. Local thermal regimes are shown as the color gradient from blue to orange.

Research Approach

My overall research goal is to understand how upper thermal limits and underlying molecular mechanisms relate to or are shaped by local thermal environments across species. Initially, to determine important molecular mechanisms underlying thermal tolerance for populations and species, I will identify the HSPs that are likely targets of temperature adaptation (Q1). Next, I will establish the relationship between local thermal environments to upper thermal limits of ants within the genus *Aphaenogaster* in a common garden experiment (Q2), and then determine the amount of variation in thermal tolerance attributable to HSP expression across the same set of species (Q3). To overcome the issue of statistical non-independence when comparing traits across species, I will statistically control for evolutionary history using independent contrasts (Felsenstein 1985). Finally, species are expected to experience several challenges accompanying climate change. To understand the full impact of future climate change, I will determine the effect of multiple stressors (desiccation and starvation) on thermal tolerance and underlying molecular mechanisms (HSP expression) in detailed physiological experiments for a single species (Q4).

1) What is the evolutionary history of the major families of heat shock proteins in ants that respond to thermal stress?

I expect *a priori* that one of the major targets of temperature adaptation in ants are shifts in heat shock protein (HSPs) expression profiles given their role in the cellular stress response and positive relationship with thermal tolerance (Krebs & Feder 1997). However, precisely identifying HSPs that underlie thermal tolerance is difficult because HSPs consist of several multigene families of molecular chaperones (HSP90, HSP70, HSP60, HSP40, small HSPs) that often undergo birth and death evolutionary processes (Nei & Rooney 2005; Bettencourt & Feder 2001) and homologues participate in maintaining proteostasis under non-stressed and stressed conditions (Richter 2012). HSP orthologues that function under stressed conditions are rapidly induced when cis-regulatory elements (heat shock elements, HSE) are bound by heat shock factor (HSF) and interact with RNA polymerase (Fernandes et al. 1995; Tian et al. 2013). The core set of stress inducible HSPs have yet to be discovered in ants. Therefore, I will identify thermally responsive HSPs in ants by characterizing the evolutionary history, selective forces, and stress inducibility of ant orthologues in all the major HSP families.

To pinpoint thermally responsive HSP orthologues, I will reconstruct the evolutionary relationships of HSP by screening across insect genomes for which there is protein coding and cis-regulatory sequence information. Phylogenetic relationships of the major HSP families (HSP90, HSP70, HSP60, HSP40, sHSPs) will be reconstructed for 17 insect species spanning 5 Orders. Within the promoter region, HSEs will be identified bioinformatically and mapped back to the phylogeny in order to predict inducibility among orthologues. I predict that the orthologous HSPs with cis-regulatory HSEs to be heat inducible and little to no induction in orthologous HSPs absent of cis-regulatory HSEs. Selection will be determined across protein coding sites and along branches using the traditional metric of d_N/d_S, whereby $d_N/d_S>1$ would indicate positive selection (Yang 1998). Given that these proteins are evolutionary and functionally conserved, purifying selection may be the prevailing force, whereas changes in cis-regulatory sequences correspond to changes in expression.

To match predictions of inducibility and determine whether HSP expression matches the patterns of variation in upper thermal limits, I will compare HSP gene expression profiles over a range of temperatures for a cool-adapted species, *Aphaenogaster picea*, and a warm-adapted species, *Pogonomyrmex barbatus*. The more thermally tolerant species, *P. barbatus*, is expected to have higher basal and/or max gene expression.

2) What is the relationship between thermal environment and physiological tolerance to temperature in *Aphaenogaster*?

A few species of *Aphaenogaster* inhabiting deciduous forest vary in thermal tolerance (CTmax) across latitude (Warren & Chick 2013). However, *Aphaenogaster* occupy diverse habitats in temperate regions within the United States such as flat woods type of forests, suggesting more variation may exist in upper thermal limits within the genus (Caroll 1975). To uncover more variation in upper thermal limits and its potential relationship with the thermal environment, roughly 10-20 species of *Aphaenogaster* will be sampled haphazardly from 10-15 sites along a south to north transect (Florida to Vermont; Fig. 2). With the help of expert experience and prior knowledge of species locales, I will collect colonies within diverse microhabitats such as the soil, decaying logs, and trees from flat woods to deciduous forests. Up to 80-100 colonies in total from different species will be reared under common garden conditions (25°C) to isolate genetic influences on thermal traits. Upper thermal limits will be determined as the temperature at which ants lose motor function, or CTmax (Lutterschmidt & Hutchison, 1997), by slowly heating ants starting at 25°C in an automatic slow ramping procedure of 0.1°C/min (adapted from Oveergaard et al. 2012).

To identify whether species thermal limits have adapted to the local thermal environment, climatic variables accessed from the publicly available Worldclim database will be regressed against independent contrasts of CTmax. Independent contrasts rely on accurate species relationships, which will be determined through phylogenetic reconstruction. Phylogenetic markers will be identified by haphazardly sampling single nucleotide polymorphisms (SNPs) along the genome by sequencing DNA libraries subjected to endonuclease cleavage with unique identifiers (double digest RAD-seq). Identified SNPS will be concatenated into a supermatrix for phylogenetic reconstruction using RAxML. To determine the evolutionary directionality of thermal traits, closely

related species *Pogonomyrmex barbatus* and *Veromessor pergandei* will serve as outgroups.

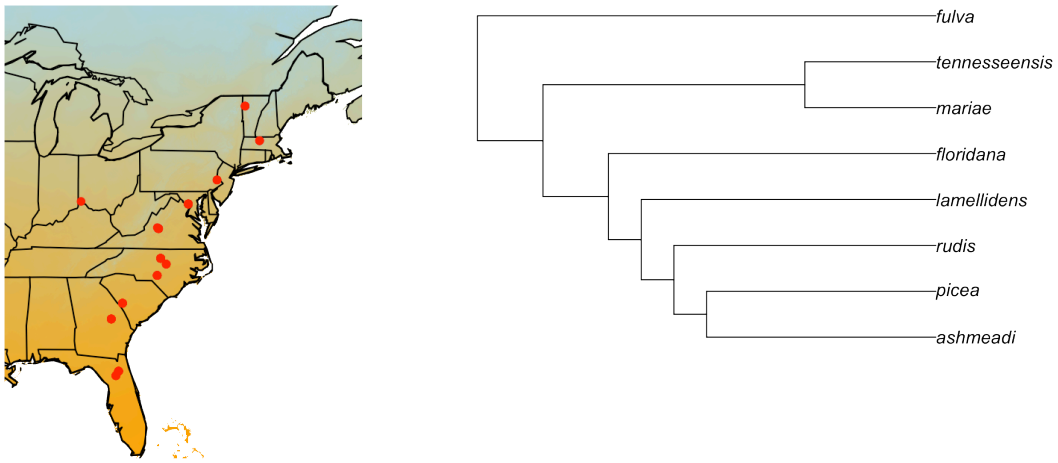

Figure 2. *Left:* Proposed samplings of sites in a South-North transect. Local thermal temperatures are shaded in a blue-orange gradient. *Right:* Simplified phylogeny for species expected to be collected. *A. rudis* is in a species complex ranging between 3-5 species but are represented once.

3) What molecular mechanisms underlie adaptive variation in thermal tolerance across species?

Given the identification of thermally responsive HSPs that are likely targets of temperature adaptation for ants (Q1) and the adaptive variation in upper thermal limits in *Aphaenogaster* (Q2), I will investigate molecular mechanisms of upper thermal limits by determining the amount of variation in CTmax that can be explained by the thermal reaction norm for HSP expression. In a slow ramping procedure (same as Q2 above), preliminary quantification of HSP gene expression using quantitative PCR shows a sigmoidal shape curve as temperature increases (Fig. 3). This curve not only shows typical investment (basal expression) and max induction (max expression), but also the onset (threshold value) and rate (inflection point) of HSP transcription.

Across species, more thermally tolerant species are hypothesized to have evolved shifts in HSP expression norm of reactions in conjunction with increased thermal limits. One or more combinations of curve properties are predicted to significantly explain variation in CTmax. For example, more thermally tolerant species may invest more in the initial CSR (basal) and/or possess greater CSR (max) but also experience stress at higher temperatures (threshold value) and or have greater rates of transcription (inflection point), which may be reflective of overall greater proteome stability and inducibility, respectively (Fig. 3; Table 1). For each colony, reaction norms of HSP expression will be quantified with quantitative PCR (qPCR) on ten samples, continuously sampled from a slow heat ramp treatment. To identify important molecular mechanisms that underlie adaptive variation in thermal tolerance, I will associate HSP expression patterns with CTmax across species in a regression analysis with independent contrasts of trait values.

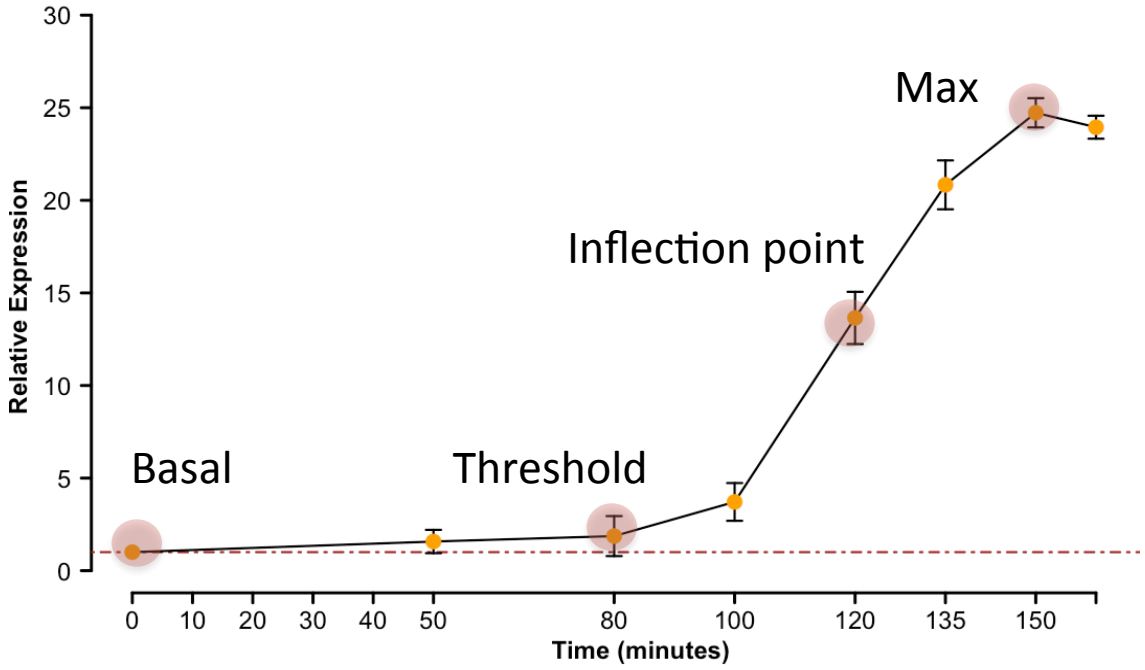

Figure 3. Preliminary reaction norm of HSP70 expression shows a sigmoidal shaped curve.

Table 1. Predictions of HSP expression patterns that may underlie thermal tolerance across species.

Shifts in HSP expression	Molecular mechanism
Basal expression	**Greater initial investment**
Threshold value	**Greater stress resistance; enhanced proteome stability**
Inflection point	**Greater induction rates; enhanced proteome stability**
Max expression	**Greater overall induction**

4) Are the same mechanisms important for resistance to different types of stressors predicted to increase with future shifts in climate?

Aphaenogaster may simultaneously experience multiple environmental stressors that accompany climate change whose impact on thermal tolerance and underlying mechanisms in the CSR have not been fully assessed. Responding to stressors in concert may confer resistance with one another in the short term, but decreases in organismal performance over extended exposure (Bubliy 2012). For example, a single species, *A. picea,* may experience desiccation and heat stress simultaneously because the number of hot days is predicted to increase with more dry conditions (increase in surface moisture deficits) along the eastern United States (Mueller & Seneviratne 2012). Warming has also been suggested to cause phenological mismatches between *A. picea* and the eliaosome containing seeds they disperse, as well as shifts in foraging time of day which together may limit resources and potentially resulting in starvation stress (Warren et al. 2011; Stuble et al. 2013; Stuble et al. 2014). The effects of either desiccation or starvation on

thermal tolerance and how underlying CSR participates in the patterns of stress resistance are not known in *Aphaenogaster*.

In order to determine the effect of multiple stressors on thermal tolerance and underlying molecular mechanisms, ants pre-treated with either desiccation or starvation will be assayed for thermal tolerance and quantified for HSP gene expression. The relationship between thermal limits and underlying molecular mechanisms will determine whether HSP expression are a generalizable mechanism for resistance against stressors brought about by future climate shifts. Upper thermal limits will be quantified as the time in which motor function is lost, or knockdown time (KD-time), when subjected to a static 40°C heat shock across 5-10 colony level replicates from sub-lethal to lethal time points. Over the short term, there may be a cross-resistant effect where prior exposure to a sub-lethal stressor (desiccation or starvation) confers greater KD-time and elevates higher HSP induction, indicating potential thermal buffering from future warming. Over extended exposures, there may be trade-offs in coping with different stressors, which result in additive or synergistically lower KD-time and HSP induction, perhaps due to energy constraints (Hoekstra & Montooth 2013).

Intellectual Merit

All levels of biological activity from molecular components to whole organism performance are shaped by temperature. A rapidly warming world will alter species abundance and distribution, but the extent and magnitude of how species will respond is not completely clear and requires integrative approach to provide novel solutions. My thesis utilizes an interdisciplinary approach from molecular biology to ecology and evolution to illuminate the molecular basis of thermal tolerance that allow for better predictions to species resilience and vulnerabilities to future climate shift. To aid in this endeavor, all protocols, gene expression data and scripts will be publicly available through the Harvard Forest Data Archive and personal website and ensures open access and reproducibility in analyses.

Broader Impacts

Given the importance ants in terrestrial ecosystems, they are a prime example for illustrating how organisms are impacted by anthropogenic driven climate change. I plan to focus on public outreach through an ant-oriented website that will distill and communicate scientific discoveries about insects and climate change.

The interdisciplinary nature of my project enables me to mentor undergraduate students that expose them to a wide range of topics in biology. I have currently mentored five undergraduate students who have participated in field collections and lab work. I plan to mentor additional students to explore more physiological, morphological and behavioral mechanisms of thermal tolerance, which are important for species to mitigate and cope with the effects of stress.

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