Behavioral Responses of Ants to Climate Warming: Insights from Field and Laboratory Studies Across Three Species from Shenandoah to Panama

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Abstract of Dissertation

Behavioral Responses of Ants to Climate Warming:
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The impacts of climate change have long been documented from ecosystems to the organismal level in a wide variety of taxa. Large-scale population declines, shifts in community composition, phenological mismatches, and range expansions or contractions have been observed. Temperature also has physiological impacts on individual organisms. However, the first responses of animals to stressful conditions are often behavioral, and studying these behavioral changes can help predict future large-scale ecological patterns. Insects are particularly vulnerable to temperature changes because they are ectotherms. Social insects have both individual and collective behavioral mechanisms of coping with temperature stress. Therefore, it is vital to understand how social insects will respond individually, as well as at the colony level. I studied the behavioral response of ants to climate warming, as they are abundant in diverse habitats and provide vital ecosystem services. Although physiological thermal limits are a valuable tool for predicting future responses, ants can modulate their behavior with slight temperature increases. Therefore, studying the behavioral response to sub-lethal temperature stress is essential.

In the first two chapters, I studied the *Aphaenogaster* ants, which are keystone seed dispersers in the forests of the Eastern United States. They provide an opportunity to study two closely related species: *A. rudis*, the warm-tolerant low-elevation species, and *A. picea*, the cold-tolerant high-elevation species. The low-elevation species is expanding its range into higher elevations as they become warmer, displacing *A. picea*. I studied

their activity and foraging efficiency across natural temperature variation along an elevation gradient and found that *A. rudis* is more active and efficient than *A. picea*. I then examined their activity, foraging, and reproductive output at sublethal temperatures under controlled lab conditions and found that with a 3°C rise, both species decrease activity, foraging, and reproductive output.

The third chapter addresses these questions within a tropical arboreal ant system of the acacia-ant mutualism. Tropical arboreal ants are even more vulnerable because they are exposed to extremely heated surfaces in the canopy and operate at temperatures close to their critical thermal maxima. *Pseudomyrmex spinicola* ants are mutualists of the plant *Vachellia collinsii*; the plant provides them with food and shelter, while the ants aggressively defend the plant from herbivores and competing vegetation. With an in-situ warming experiment, I studied the activity and defense behavior of the ants and the herbivory experienced by the plant. I found that the ants reduced patrolling and defense of the plant in the heated treatment, and are already experiencing temperatures near their critical thermal maxima.

Together, I employed three different methodological approaches: natural temperature variation along elevation, temperature manipulation in the lab, and in-situ warming, to study the behavioral response of ants to climate warming. My research demonstrates that ants adjust their behavior in response to small temperature increases, which can aid in predicting changes in the ecosystem services these ants provide.

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Dissertation Introduction

The latest Intergovernmental Panel on Climate Change (IPCC) report shows a rise in global surface temperature of about 1.1°C during 2011-2020 compared to 1850-1900, with a much larger increase of about 1.59°C on land surface. Apart from average temperatures, there are regional changes in precipitation and increasing frequencies of extreme weather events such as heatwaves (Calvin et al. 2023). The impacts of climate change are already detectable in animals (Parmesan and Yohe 2003; Parmesan 2006; Calvin et al. 2023). Ectotherms are particularly susceptible to environmental changes because they depend on the ambient environment to regulate their body temperature. This reliance makes them vulnerable to fluctuations in their surroundings, potentially impacting their metabolic processes, activity levels, and overall survival (Kingsolver et al. 2013; Paaijmans et al. 2013). Declines in vertebrate ectotherms, such as reptiles and amphibians, have been partly attributed to climate change, along with other environmental stressors, such as pollution, disease, and habitat loss. Climate change often worsens the impact of these stressors (Bickford et al. 2010; Rohr and Palmer 2013; Hayden Bofill and Blom 2024). Freshwater invertebrates also exhibit shifts in life history traits, such as development times, fecundity, and survival, due to climate change-induced fluctuations in salinity and hydroperiod (Stoks et al. 2014; Niedrist and Füreder 2023). Terrestrial invertebrate ectotherms, such as insects, have been reported to experience population declines, range shifts, and changes in community composition (Menke et al. 2014; Pureswaran et al. 2018; Halsch et al. 2021; McCain and Garfinkel 2021; Pardee et al. 2022; Dalton et al. 2023; Harvey et al. 2023). Ectotherms have a typical thermal performance curve where performance increases till temperatures reach the optimum,

where they reach peak fitness. At temperatures higher than the optimum, performance drops sharply until animals reach the maximum critical thermal limit (CT_{max}), at which point they lose muscle control (Parr and Bishop 2022). The lower end of temperatures where they lose muscle control is the minimum critical thermal limit (CT_{min}). Together, the critical limits determine the range of temperatures where the organism can remain active.

Under realistic climate warming scenarios, temperatures may not reach the thermal maxima of some species. Nevertheless, temperature increases of just a few degrees can be stressful, often inducing insects to modulate their behavior to escape from this thermal stress. These behavioral changes can ultimately lead to the large-scale ecological patterns mentioned above. For example, changes in nest site preferences can eventually lead to range shifts, and changes in activity patterns can cause phenological mismatches with interacting species that respond differently or respond to cues other than temperature. This is particularly interesting in social insects (e.g., ants, social bees, etc.), which exhibit both individual and collective thermoregulatory behaviors (Jones and Oldroyd 2006). Individuals can modulate their behaviors, such as foraging times and activity, to avoid extreme heat stress (Spicer et al. 2017; Villalta et al. 2020; Blanchard et al. 2021; Bujan and Yanoviak 2022). The reproductive queens and developing larvae are typically in a sheltered nest, often underground, that may be buffered from temperature fluctuations; typically, only non-reproductive foragers are exposed to temperature extremes. This leads to another advantage for social insects: the colony can survive even if a few individuals are lost to heat stress. Because social insects have replaceable workers, they could risk foraging at suboptimal temperatures to reduce the negative

impacts of competition and predation (Cerdá et al. 1998; Menzel and Feldmeyer 2021; Nascimento et al. 2022). Thus, their collective behavior and social structure might provide social insects with a buffer against the extreme effects of thermal stress, compared to solitary insects, in which individual death causes immediate reproductive failure. On the other hand, social insects have lower effective population sizes that limit their adaptability (Fisher et al. 2019). They could also have polymorphic workers where each type has a specialized role in the colony, and the loss of one type is not immediately replaceable. Thus, social insects respond to climate change both at the individual and colony levels, making it challenging to predict responses for a wide range of species and across large geographical scales.

Understanding the behavioral response is especially crucial in social insects such as ants. Ants dominate terrestrial ecosystems in terms of abundance, biomass, and diversity and occupy diverse ecological regions (Hölldobler and Wilson 1990; Grimaldi and Engel 2005; Schultheiss et al. 2022). They perform essential ecological functions, such as seed dispersal, pest control, and detritus clearing (Del Toro et al. 2012; Parr and Bishop 2022). Through competition and predation, they exert a top-down influence on the invertebrate community within a habitat, modifying its structure, food webs, and the ecosystem services they offer (Parr 2008; Parr et al. 2016). Ants exhibit individual and colony-level thermoregulatory behaviors that could help them cope with thermal stress (Perez and Aron 2020). For instance, individual workers can change their foraging window in response to temperature (Stuble et al. 2013; Andrew et al. 2013; Spicer et al. 2017; Jayatilaka et al. 2018; Villalta et al. 2020; Blanchard et al. 2021). At the colony level, they adapt the nest architecture in response to temperature, like building deeper

nests in the soil (García Ibarra et al. 2024). They can also move the larvae around the nest to achieve optimal rearing conditions (Tschinkel 1987; Roces and Núñez 1989; Penick and Tschinkel 2008; Falibene et al. 2016). Their small bodies and inability to fly subject them to unique microclimatic conditions by exposing them to the high-temperature boundary layer of heated surfaces (Kaspari et al. 2015; Stark et al. 2017). Practically, compared to flying social insects such as bees and wasps, it is easier to monitor entire colonies and manipulate the temperatures that the ants experience.

Tropical ants are even more vulnerable, especially those that nest or forage in the canopy. Temperate animals have broader thermal tolerance than tropical ones because they evolved in environments with larger seasonal temperature fluctuations (Janzen 1967; Sunday et al. 2014; Dewenter et al. 2024). Consequently, tropical species face higher heat stress risks due to their narrower thermal ranges, with ambient temperatures often at or near their critical thermal maxima (Deutsch et al. 2008; Nascimento et al. 2022; Parr and Bishop 2022). Arboreality adds another layer of vulnerability because the temperature fluctuates more drastically compared to below the ground. Though increasing air temperatures may not directly endanger these ants, fluctuations in cloud cover and periods of intense heat can expose the canopy to elevated surface temperatures, causing behavioral shifts in the ants (Kaspari et al. 2015; Perez and Aron 2020; Nascimento et al. 2022). Thus, it is crucial to understand whether and how canopy-dwelling species can cope with increasing thermal fluctuations.

Arboreal ants frequently participate in defense and mutualisms, and rising temperatures can disrupt these species interactions as the participating species respond differently to heat stress. Some ant-plant mutualisms are predicted to destabilize under

future climate change due to shifts in the services provided by a partner or due to switching partners (Barton and Ives 2014; Dejean et al. 2022; Youngsteadt et al. 2024; Câmara et al. 2024). Temperature can also change the participating species in a mutualism, which can skew the services provided (Fitzpatrick et al. 2014). Obligate mutualisms are especially vulnerable because the effects on one partner can seriously impact the other and potentially cause species loss. These mutualisms often support other specialist and generalist species. Therefore, disruptions in mutualisms not only impact the involved species but also other related species, leading to cascading effects within the ecosystem.

In the first chapter, I studied two closely related *Aphaenogaster* ants along an elevation gradient in Shenandoah National Park, Virginia, US. Warren and Chick (2013) have reported that the low-elevation *A. rudis* is expanding its range into the higher elevations and displacing the high-elevation *A. picea* in the Southern Appalachian Mountains. I surveyed the abundance of the two species from 500m elevation to 1100m elevation. I measured foraging activity and efficiency of both species at different times of day, as well as nest temperatures in conjunction with activity data at six sites along the elevation gradient. I predicted that overall, *A. rudis* would be more active and efficient than *A. picea*, especially in the afternoon when temperatures are warmer. I predicted that *A. picea* would exhibit lower activity and display a bimodal peak in activity during the cooler times of the day, specifically in the morning and evening. I also measured the critical thermal limits from populations at different elevations and predicted that the minimum critical thermal limit would change with elevation, as in Warren & Chick (2013).

In the second chapter, I exposed colonies of *A. rudis* and *A. picea* to two artificial temperature treatments in the lab: 25°C, which served as a control temperature presumably near their physiological optimum temperature, and 28°C, which is at the higher end of the range of temperatures experienced by these ants in Shenandoah and Rock Creek Park. I measured foraging activity, walking speed, and the distance covered by foragers without food. Next, I introduced food and measured the discovery time and estimated the colony-level foraging effort. I predicted that foraging performance would decline at 28°C due to heat stress. I predicted that *A. picea* would be more affected by increased temperature because they live in cooler habitats. In addition to foraging behavior, I also compared colony-level reproductive output and the aggressive response of individual ants when confronted with a heterospecific. I predicted that reproductive output would decline in the 28°C treatment if this temperature was stressful to ants. Alternatively, if 28°C is closer to their optimum temperature, reproductive output should increase. Again, *A. picea* would be more affected by increased temperature because they live in cooler habitats. For aggression, I predicted that *A. rudis* would be more aggressive than *A. picea*, which might explain their upward expansion into the range of *A. picea*.

In the third chapter, I examined the impact of elevated temperature on the behavior of the arboreal tropical ant *Pseudomyrmex spinicola* in Panama. These ants are mutualists of the acacia plant *Vachellia collinsii* (Fabaceae, Mimosoideae). The ants nest within the hollow thorns of the tree, feed on the extrafloral nectaries, and gather Beltian bodies from new leaves, which are rich in proteins and lipids, to nourish their larvae (Janzen 1966; Heil et al. 2004). They patrol the plant to aggressively attack and remove herbivores and clear other competing vegetation around the plant (Janzen 1966; Amador-

Vargas 2019; Amador-Vargas et al. 2021). I determined the behavioral response of *Pseudomyrmex spinicola* ants to naturally occurring and experimentally increased temperatures. I observed ant activity on plants in sunny and shaded locations during both the morning and afternoon. I then used plastic enclosures to experimentally raise temperatures on treatment plants, while similar enclosures with ventilation were used for control plants, which did not change the temperature. I predicted that activity and aggressive defense would decrease with the experimentally increased temperature. As a result of reduced defense, herbivory on the treatment host plants would increase, which would suggest a potential disruption to the mutualism mediated by temperature. I measured the CT_{max} of ants in the control and heat colonies to determine if they acclimate to increased temperatures and if they are experiencing temperatures close to their thermal tolerance limit.

Chapter 1: Activity and foraging behavior of *Aphaenogaster* ants along an elevational gradient

Abstract:

It is crucial to understand insect responses to climate change, the drivers of these responses and how climate change affects population and community structure. Social insects, especially ants, are dominant in terrestrial ecosystems and perform critical ecological functions. *Aphaenogaster* ants are keystone seed dispersers of spring ephemeral flowers in the forests of the Eastern United States. The lower elevation, warm-tolerant species *A. rudis* has been expanding its range into the higher elevations in the Southern Appalachian Mountains. This reported range expansion could displace the high-elevation, cold-tolerant species *A. picea*. To understand what role behavior plays in this upward elevation expansion, we studied these species along an elevation gradient in Shenandoah National Park and measured their activity and foraging efficiency. Surprisingly, we found a sudden change in species composition at around 700m, with lower sites exclusively populated by *A. rudis* and higher sites by *A. picea*. We found that *A. rudis* was more active and efficient than *A. picea*. The highest elevation site of *A. rudis* and the lowest of *A. picea* each maintained similar activity patterns, suggesting a competitive interaction when these two species occur in sympatry.

Introduction:

The impact of global climate change on animals is evident in population declines, range shifts, and changes in community structure (Parmesan and Yohe 2003). In ectotherms such as insects, body temperature is directly affected by the climate. Shifts in range, composition, abundance, and diversity in response to temperature fluctuations have been observed for a variety of insect species (Pureswaran et al. 2018; Halsch et al. 2021; McCain and Garfinkel 2021). Many insects can alter their behavior to cope with stressful temperatures (Kearney et al. 2009; Sunday et al. 2014). This includes changing activity levels, foraging times, and/or nest-site preferences to avoid exposure to stressful heat (Villalta et al. 2020; Blanchard et al. 2021; Bujan and Yanoviak 2022; Parr and Bishop 2022). Studying the behavioral and ecological adaptations to stressful temperatures can help us anticipate broader ecological changes and insects' ability to buffer temperature increases in the future (Youngsteadt et al. 2023).

Behavioral responses are especially crucial in social insects such as ants. Ants dominate terrestrial ecosystems in abundance, biomass, and diversity (Hölldobler and Wilson 1990; Grimaldi and Engel 2005). They perform essential ecological functions, such as seed dispersal, pest control, and detritus clearing (Del Toro et al. 2012; Parr and Bishop 2022). Through competition and predation, they have a top-down effect on the invertebrates of a habitat, altering community structure, food webs, and the ecosystem services provided (Parr 2008; Parr et al. 2016). Because they are social insects, ants exhibit individual and colony-level thermoregulatory behaviors that could help them cope with thermal stress (Perez and Aron 2020). For instance, individual workers can change their foraging window in response to temperature (Stuble et al. 2013; Andrew et al. 2013;

Spicer et al. 2017; Jayatilaka et al. 2018; Villalta et al. 2020; Blanchard et al. 2021). At the colony level, they can move the larvae to different microclimates in the nest to achieve optimal rearing conditions (Tschinkel 1987; Roces and Núñez 1989; Penick and Tschinkel 2008; Falibene et al. 2016). The reproductive queens and developing offspring are typically in a sheltered nest, often underground, that may be buffered from temperature fluctuations. Only non-reproductive foragers are exposed to temperature extremes. This leads to another advantage that social insects have: the colony can survive even if a few individuals are lost to heat stress. This could allow colonies to function in conditions with high desiccation risk because, unlike in a solitary insect, individual forager death would not preclude future reproduction. Thus, the colony can still function very close to its maximum thermal tolerance (Parr and Bishop 2022). Ants, like other ectotherms, have a typical thermal performance curve where performance usually improves with rising temperatures. Their metabolic rates increase, development times shorten, and they move faster in warmer conditions. They reach peak performance at an optimal temperature. When temperatures exceed this optimal point, however, performance drops sharply until reaching a critical thermal maximum (CT_{max}), beyond which increased temperature is lethal (Angilletta 2009; Parr and Bishop 2022). While behavioral modifications can buffer temperature change, behavior alone might not be adequate to combat future temperature warming.

Elevation gradients are a valuable tool for studying the effects of temperature, as there is a natural gradient from higher to lower temperatures with increasing elevation, while controlling for latitudinal variations. Several studies have examined patterns and drivers of ant diversity and abundance along elevation gradients in various geographic

regions (Sanders et al. 2003, 2007; Longino and Colwell 2011; Bishop et al. 2014; Szewczyk and McCain 2016; Subedi and Budha 2020; Chick et al. 2020). The distribution of ants along elevation gradients is mainly influenced by biotic interactions at lower elevations but by cold tolerance at higher elevations (Bishop et al. 2017; Chick et al. 2020). Villalta et al. (2020) studied the behavior of *Aphaenogaster iberica* populations along an elevation gradient in the Sierra Nevada mountains in Spain. They found that these ants maintain consistent foraging rates at different temperatures by adjusting their activity patterns at different elevations. Additionally, elevations with greater temperature variability had workers with a higher thermal tolerance (CT_{max}). Thus, elevation gradients are an excellent tool for examining behavioral changes resulting from small temperature differences within relatively small geographic areas.

Here we studied two species of *Aphaenogaster* ants along a 500m elevational gradient in Shenandoah National Park, USA. *Aphaenogaster* ants are widespread, generalist, and seed-dispersing ants found in the forests of the Eastern United States. *A. rudis* is a warm-tolerant species located at lower elevations, while *A. picea* is a cold-tolerant species found at higher elevations, and they often co-occur at intermediate elevations (Lubertazzi 2012; Warren and Chick 2013). Previous studies indicate that between 1974 and 2012, *A. rudis* expanded its range by 300m higher in the Southern Appalachian Mountains (Warren and Chick 2013). This suggests competitive dominance of *A. rudis,* but it is unclear what behavioral mechanisms underlie this shift, especially because *Aphaenogaster* ants are not particularly aggressive (Warren et al. 2020). We predicted that the range incursion of *A. rudis* into elevations previously occupied only by

A. picea may be mediated by *A. rudis* being better able to cope with the thermal stress of increased temperatures.

To explore these dynamics, we surveyed the abundance of the two species from 500m elevation to 1100m elevation. We selected six sites along the elevation gradient and measured foraging activity and efficiency of both species at different times of day, as well as nest temperatures in conjunction with activity data. We predicted that overall, *A. rudis* would be more active and efficient than *A. picea*, especially in the afternoon when temperatures are warmer. Additionally, we predicted that *A. picea* would exhibit lower activity and display a bimodal peak in activity during the cooler times of the day, specifically in the morning and evening. We also measured the foraging by the ant community in the leaf litter at each site by setting bait traps. Additionally, we measured the critical thermal limits from populations at different elevations and predicted that the minimum critical thermal limit would change with elevation, as in Warren & Chick (2013).

Methods:

Species and study sites:

Aphaenogaster ants are abundant in the hardwood forests of the Eastern United States. *A. rudis* and *A. picea* are monogamous and typically live in colonies of about 200-300 workers. They nest in high density in the soil underneath rocks, in fallen bark, or in leaf litter. During the summer, most ants are near the soil surface beneath the rock, in nests that are underneath rocks (the focus of this study), but nests can extend up to a few inches beneath the soil under the rock. These ants are generalist foragers in the leaf litter, collecting dead insects and plant material like seeds with elaisosomes. Workers forage individually, but some recruitment occurs, depending on the food source quality. The foraging range is small, typically less than a meter, but the high density of nests on the forest floor results in a large number of ants in the leaf litter. They quickly appear at baits and are relatively nonaggressive when defending food sources (Lubertazzi 2012).

This study was conducted in Shenandoah National Park during the summers of 2023 and 2024. Six sites were chosen in the park that had a high density of *Aphaenogaster* nests. The sites ranged from 500m to 1000m in elevation, at approximately 100m intervals. (500m site (487m): 38°53'01.7"N 78°12'32.1"W, 600m site (573m): 38°52'23.7"N 78°12'08.7"W, 670m site (670m): 38°51'00.4"N 78°12'20.9"W, 750m site (743m): 38°40′55.59″N 78°19′59.77″W, 900m site (927m): 38°32'04.2"N 78°25'14.8"W, 1000m site (1015m): 38°27'08.0"N 78°27'59.0"W.) Ten nests were selected at each site and marked with a flag. To standardize nest microhabitat location, only nests under medium-sized rocks were chosen for the study. The sites were

chosen to ensure that the understory was clear and the region was moderately rocky, allowing for observations of many colonies.

Temperature measurements:

Soil temperature was measured at three locations next to the rock every time before measuring ant activity. The average of the three was recorded as the nest entrance temperature. Temperature was measured with a handheld IR temperature gun (Etekcity Lasergrip 1260). We used a linear mixed-effect model to determine if nest temperatures were affected by elevation and time of day, with colony ID as a random factor. Two data loggers (Elitech GSP-6) were placed at each site to measure temperature and humidity continuously at 20-minute intervals from June to September 2024. One probe was placed on the ground, on top of the leaf litter, and one approximately 2 inches into the ground. We used a linear model to determine if below-ground temperatures were cooler than surface temperatures.

Percent abundance:

The abundance of *A. rudis* and *A. picea* was measured at the six sites during the summer of 2023. For each site, abundance was measured three times, with at least one month between measurements. Abundance was measured again at nine different sites throughout the elevation gradient in 2024. During each sampling, we flipped 40 haphazardly selected medium-sized rocks and collected two ants in a vial with 80% alcohol if there was a colony under a rock, following Warren et al. (2013). These ants were later identified to the species or genus level in the lab. Percent abundance was calculated as the percentage of a species out of the 40 rocks flipped at each site.

Nest activity:

Nest activity was measured monthly at each site from June to September 2024. During each sampling, activity for each colony was measured three times during the day: in the morning (between 8:00 and 10:00), afternoon (between 12:00 and 14:00), and evening (between 16:00 and 18:00). Since all the sites could not be sampled on the same day, we sampled two sites each day during three consecutive days. Ten nests were marked at each site, and the activity was measured by counting the number of ants entering and exiting the nest in a 5-minute interval. Since *Aphaenogaster* ants do not regurgitate food for nestmates, any food collected on the foraging trips can be seen in the mandibles of the ants. Hence, for ants entering the nest, the number of ants carrying something in their mandibles was also counted to measure foraging efficiency. Total activity is the sum of the number of ants seen entering and exiting the nest. Foraging efficiency is the proportion of ants carrying something in their mandibles out of the total ants seen entering the nest during each observation. *Aphaenogaster* ants relocate their nest often; hence, new colonies were marked at each elevation during each sampling, but the same colonies were observed throughout the day during each sampling.

Due to the apparent non-linear effect of temperature on activity, we used two separate generalized additive models (GAM) to determine the effect of temperature on total activity and foraging efficiency. Nest temperature was included as the non-linear term with species, elevation, and time of day as simple additive terms. A Gaussian distribution was used for the total activity model, and a quasibinomial distribution was used for the proportional foraging efficiency data.

To estimate the foraging activity of the ant community in the leaf litter at these sites, during each sampling at the marked nests, we set bait traps at the sites, but away

from the colonies under observation. Each bait consisted of a plain white 3" x 5" paper index card, on which one-half or a full mealworm (depending on size) was placed. The mealworm was cut to ensure it did not move off the bait card and to facilitate ant foraging. Additionally, 2-3 pinches of cracker crumbs (Ritz salt crackers) were scattered on the cards. Five bait cards were placed on the forest floor, at least 1m apart, typically arranged in a circular pattern to allow for simultaneous monitoring. Some leaf litter had to be cleared away to place the index card on flat ground and allow for better observation. To ensure consistent timing, the stopwatch was started midway through the placement of the baits, and the cards were observed for 20 minutes. During this period, we recorded the time at which ants arrived on the bait cards and noted whether the ants were *Aphaenogaster*. Different morphospecies were collected in alcohol vials for identification in the laboratory. If an ant of similar appearance had already been collected at that site during that trial, another was not collected. Additionally, some *Aphaenogaster* ants were collected to confirm if they were *A. rudis* or *A. picea*.

We analyzed variation in *Aphaenogaster* abundance on the bait cards using a negative binomial generalized linear model (GLM) to account for over-dispersed count data. Our predictor variables were elevation and time of day. We used another negative binomial GLM to model discovery time as the time it took for the first ant to arrive at a bait. Elevation, time of day, and the mean above-ground temperature recorded by our data loggers at the sites during sampling were the predictor variables.

Critical thermal limits:

The maximum critical thermal limit (CT_{max}) was measured for 10 *A. rudis* colonies and 10 *A. picea* colonies from across the elevation gradient. Three workers from

each colony were placed in individual straight-walled 2 ml microcentrifuge tubes. The cap was filled with modeling clay to prevent any thermal refuge for the ants. The tubes with ants were placed in a dry heat block (Benchmark Mini Dry Bath) and exposed to incremental temperature increases. The starting temperature was 36°C and increased by 2°C every 10 minutes. Every 10 minutes, the righting response of the ants was checked by gently flicking the tubes and noting if the ants could turn right side up. The temperature at which the ants lost their righting capability was recorded as the CT_{max}. The CT_{max} of each colony was determined by taking the average CT_{max} of the three workers. The measurements were taken on the same day the ants were collected. A linear mixed-effects model with colony ID as the random variable was used to determine whether CT_{max} changed with elevation or species and whether there was an interaction effect between species and elevation.

The minimum critical thermal limit (CT_{min}) was measured for 9 *A. rudis* colonies and 10 *A. picea* colonies from across the elevation gradient. CT_{min} was measured for four workers from each colony, and the average was considered the CT_{min} of the colony. Individual ants were placed in straight-walled 2 ml microcentrifuge tubes, with clay in the cap to prevent any thermal refuge. The ants were placed in a temperature-controlled ice bath (Quantum Northwest qEP-16 with a TC1 temperature controller), and the temperature was reduced sequentially. The starting temperature was 20°C, and the temperature was reduced by 2°C every 10 min. The ramping rate was 0.5°C/min, so it took 4 minutes to reach the next step of temperature, and then it was held constant for another 6 minutes. At every 2°C step, the righting response of the ants was checked, and the temperature at which the ants could not stand back up was measured as the CT_{min}. A

linear mixed-effects model with colony ID as the random variable was used to determine whether CT_{min} changed with elevation and between species, and whether there was an interaction between species and elevation.

All data analysis and visualization were performed in R (version 4.2.1).

Results:

Temperature variation:

Nest temperature decreased significantly with increasing elevation at the study sites. The three higher elevations (670m, 900m, and 1000m) had lower temperatures compared to the 500m elevation site (Linear mixed-effect model, Table 1.1, Fig. 1.1), confirming that there was a temperature gradient in the elevations we sampled. However, nest temperatures were not significantly lower at the 600m and 670m sites. Nest temperatures in the afternoon and evening were warmer than in the morning (Linear mixed-effect model, Table 1.1, Fig. 1.4). The temperature 2 inches below the ground was cooler and less variable than on the surface (LM, Estimate = -1.09, $p < 0.001$) (Above-ground: Mean ± SD = 20.49°C ± 4.054°C, Range = 7.6°C – 44.0°C; Below-ground: Mean ± SD = 19.4°C ± 2.946°C, Range = 11.7°C – 42.8°C).

Table 1.1: Summary of linear mixed effect model: Nest Temperature ~ Elevation + Time + (1 | Colony ID)

	Value	Std. Error	DF	t-value	p-value
Intercept	19.776039	0.5863189	281	33.72915	< 0.0001 *
Elevation 600	-0.579785	0.8194335	146	-0.70754	0.4804
Elevation 670	0.229911	0.9585408	146	0.23985	0.8108
Elevation 750	-2.367694	0.8119733	146	-2.91598	0.0041 *
Elevation 900	-2.651741	0.8056072	146	-3.29160	0.0012 *
Elevation 1000	-2.169604	0.8866539	146	-2.44696	0.0156 *
Time Afternoon	2.526401	0.1699972	281	14.86143	< 0.0001*
Time Evening	2.445126	0.1693038	281	14.44224	< 0.0001*

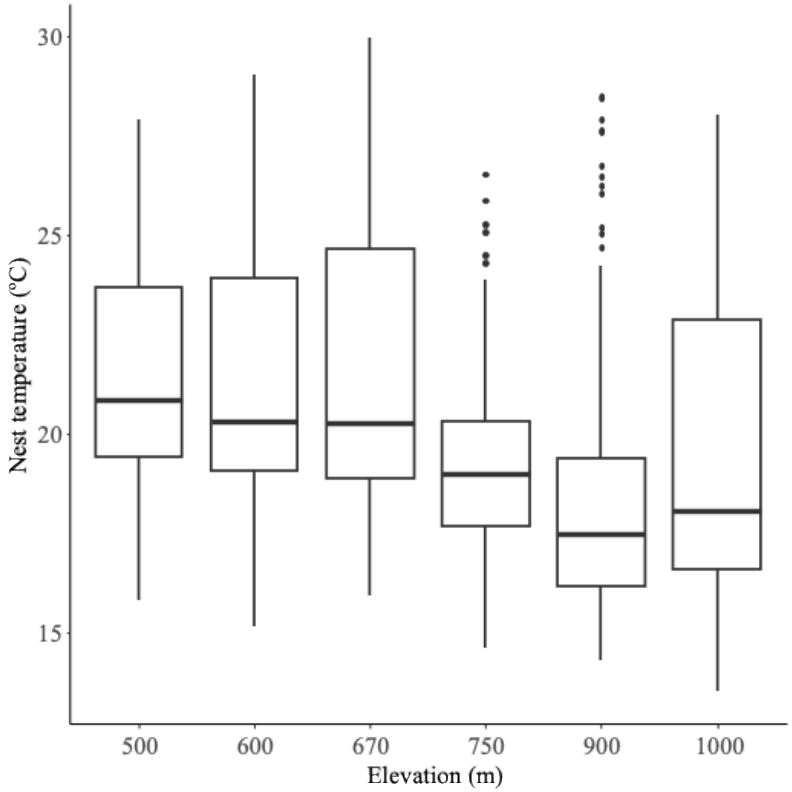

Figure 1.1. Nest temperatures at the six study sites: Compared to the lowest elevation, the three higher elevation sites had lower temperatures.

Percent abundance:

Contrary to our expectation, there was no overlap in the range of *A. rudis* and *A. picea.* Rather, there was a sharp change in the abundance of *A. rudis* and *A. picea* at about 700m of elevation (Fig. 1.2). Because of this, the three lower elevation sites of 500m, 600m, and 670m had only *A. rudis* colonies, and the three higher elevation sites of 750m, 900m, and 1000m had only *A. picea* colonies.

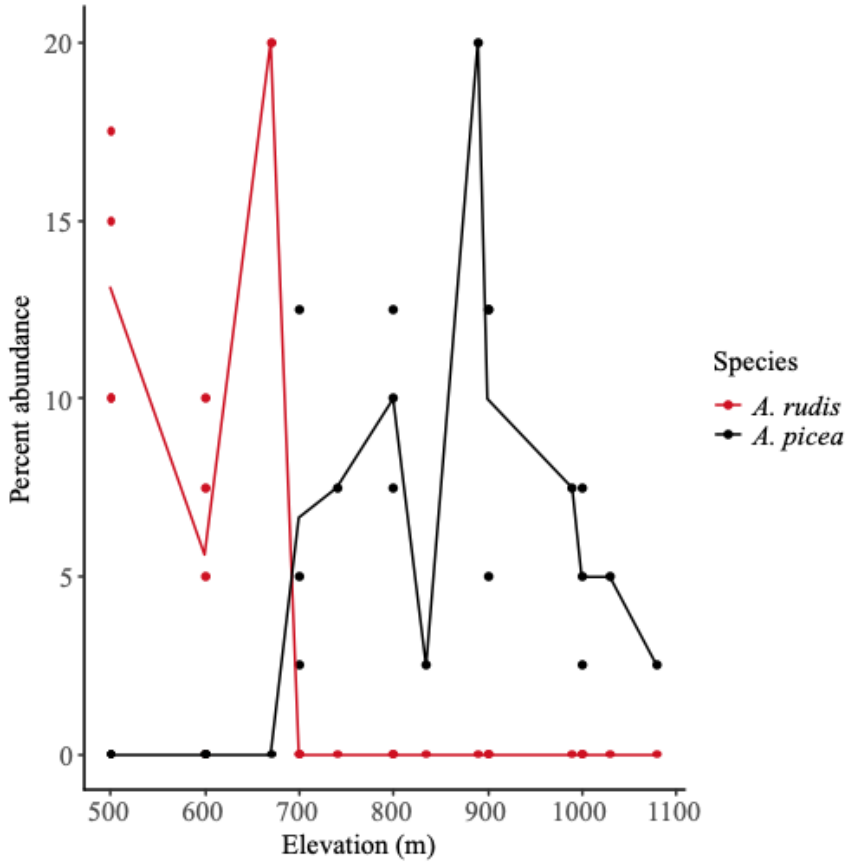

Figure 1.2. Percent abundance: Percent abundance of *A. rudis* and *A. picea* colonies through an elevation gradient, measured by counting the number of colonies found under 40 rocks during each sampling. The points represent data points, while the line joins the mean percentage for each species. *A. rudis* is shown in red and *A. picea* in black.

Activity:

Nest temperature had a non-linear effect on the activity of ant colonies with peak activity around 23°C and reduced activity at temperatures higher and lower than that (Generalized additive model, Table 1.2, Fig. 1.3). *A. rudis* was more active than *A. picea* (Generalized additive model, Table 1.2, Fig. 1.3). Overall, there was higher activity across both species in the morning than in the afternoon or evening (Generalized additive model, Table 1.2, Fig. 1.4). More specific patterns become apparent when examining activity at each site throughout the day (Fig. 1.4). At the lower elevation sites of 500m and 600m, activity reduces in the afternoon and evening as temperatures increase. At the

middle elevations of 670m and 750m, activity is bimodal, with activity higher in the morning and evenings. The lower afternoon activity corresponds to high afternoon nest temperatures. At the higher elevations of 900m and 1000m, activity remained low throughout the day, even as temperatures increased in the afternoon and evening.

Table 1.2. Summary of Generalized Additive model: Activity ~ s(Nest Temperature) + Species + Elevation + Time

Parametric terms:				
	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	4.742	0.316	15.028	< 0.001 *
Species A. picea	-0.824	0.250	-3.295	0.001 *
Elevation 600	-1.055	0.379	-2.782	0.006 *
Elevation 670	-0.563	0.442	-1.272	0.204
Elevation 750	0.283	0.241	1.177	0.240
Elevation 900	-0.585	0.242	-2.417	0.016 *
Elevation 1000	-0.522	0.273	-1.909	0.057
Time Afternoon	-1.455	0.301	-4.830	< 0.001 *
Time Evening	-1.687	0.3075	-5.487	< 0.001 *
Approximate significance of smooth terms:				
	edf	Ref df	F	p-value
s(Nest temperature)	2.567	9	4.661	< 0.001 *

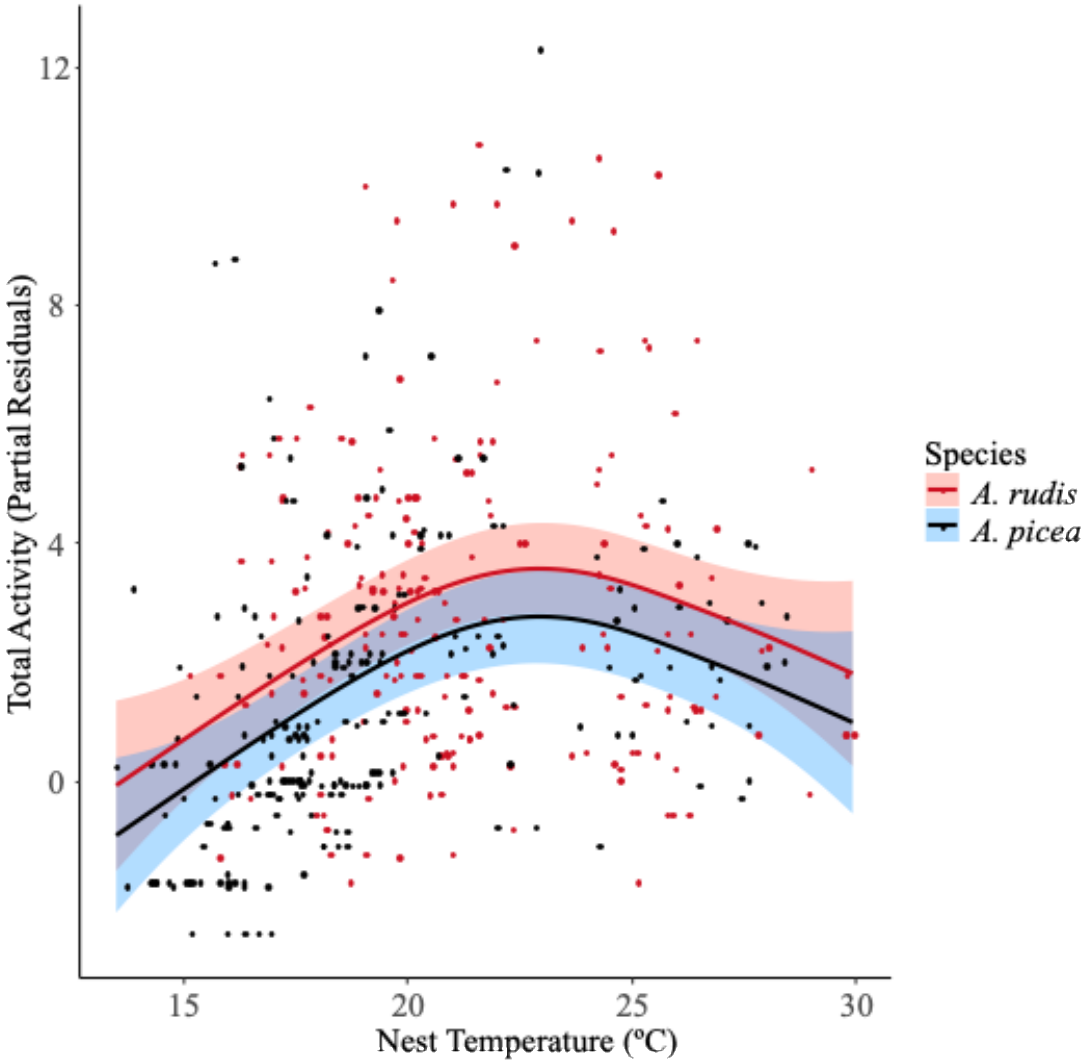

Figure 1.3. Change in activity with temperature: Activity was measured as the number of ants entering and exiting the nests. The effect of temperature on colony activity is non-linear. *A. rudis* is generally more active than *A. picea*. The lines indicate the conditional effect of temperature on activity while keeping other variables constant, the points indicate partial residuals, and the shaded region indicates the 95% CI band. *A. rudis* is shown in red and *A. picea* in black.

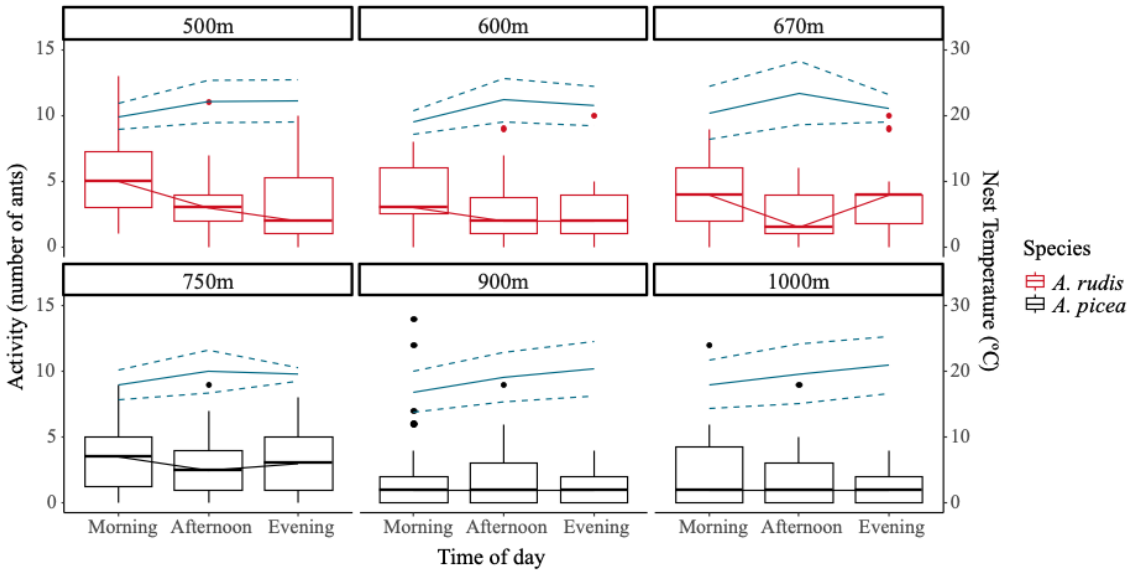

Figure 1.4. Change in activity through the day at the six different elevation sites: Activity generally reduces with increasing temperature except at the two highest elevations. The boxplots indicate colony activity (total number of ants exiting and entering the nest in 5 minutes). A line joins the median values of the boxplots. Red indicates *A. rudis* and black indicates *A. picea*. The corresponding nest temperature is shown in blue. The solid line shows mean nest temperatures, and the dotted lines show the band of standard deviation.

Efficiency:

Temperature also had a non-linear effect on foraging efficiency, with efficiency reduced at extremely low and high temperatures. *A. rudis* was more efficient than *A. picea*. Time of day did not affect foraging efficiency (Generalized additive model, Table 1.3, Fig. 1.5). Peak foraging efficiency was at about 21°C.

Table 1.3. Summary of Generalized Additive model: Foraging efficiency ~ s(Nest Temperature) + Species + Elevation + Time

Parametric terms:				
	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-1.094	0.259	-4.209	< 0.001 *
Species A. picea	-0.792	0.354	-2.234	0.026 *
Elevation 600	-0.401	0.317	-1.262	0.207
Elevation 670	-0.921	0.435	-2.117	0.034 *
Elevation 750	0.000	0.000	NA	NA

Elevation 900	0.298	0.399	0.746	0.455
Elevation 1000	0.728	0.408	-1.786	0.074
Time Afternoon	-0.451	0.283	-1.592	0.112
Time Evening	-0.211	0.269	-0.781	0.435
Approximate significance of smooth terms:				
	edf	Ref df	F	p-value
s(Nest temperature)	2.515	3.197	2.764	0.033 *

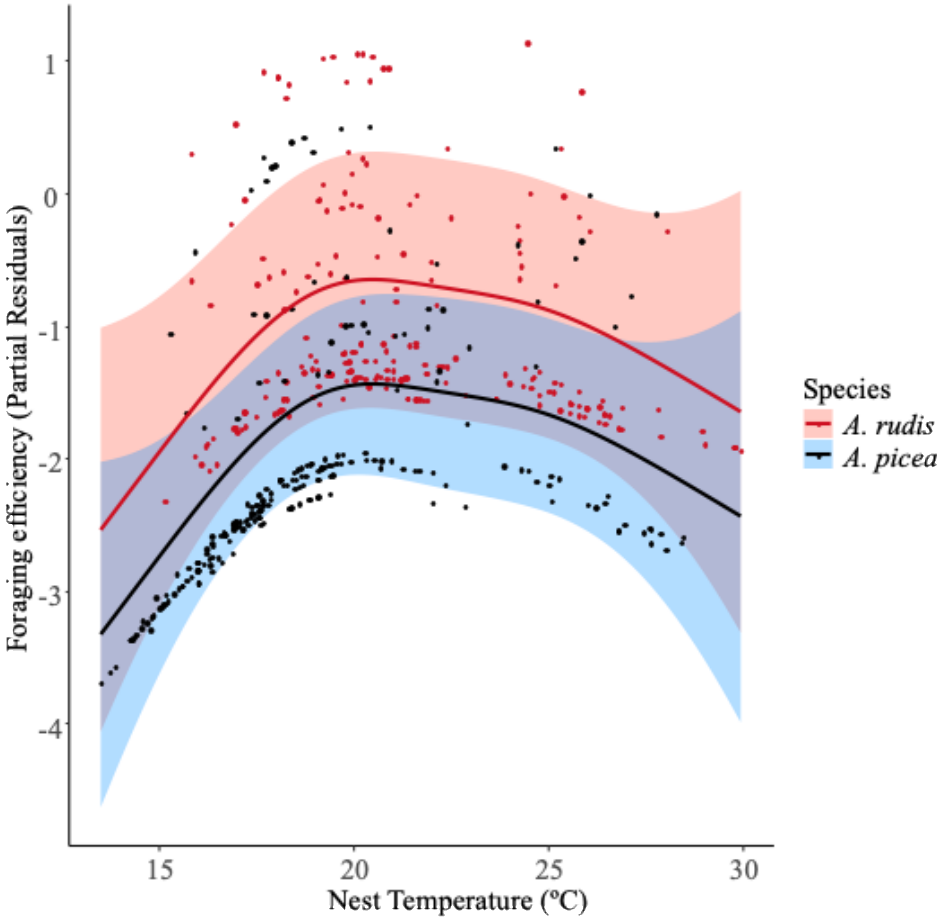

Figure 1.5. Change in foraging efficiency with temperature: Temperature has a non-linear effect on foraging efficiency. *A. rudis* is more efficient than *A. picea*. The lines show the conditional response of foraging efficiency to temperature while keeping the other variables constant in a generalized additive model. The points are partial residuals. *A rudis* is shown in red and *A. picea* in black.

The higher activity of *A. rudis* was also apparent in the abundance data at the baits. The abundance of *Aphaenogaster* ants at the baits decreased with increasing elevation (GLM: Estimate = -0.004, $p < 0.001$, Fig. 1.6a). The higher efficiency is further supported by the bait discovery times, as it took more time for the ants to find the baits with increasing elevation (GLM: Estimate = 0.002, $p = 0.032$, Fig. 1.6b). As expected, *Aphaenogaster* ants dominated the baits: 87.4% of all ants observed at the baits were *Aphaenogaster*. The diversity of ants observed at the baits decreased with elevation, with 100% of the ants seen at the highest elevation site being *Aphaenogaster*.

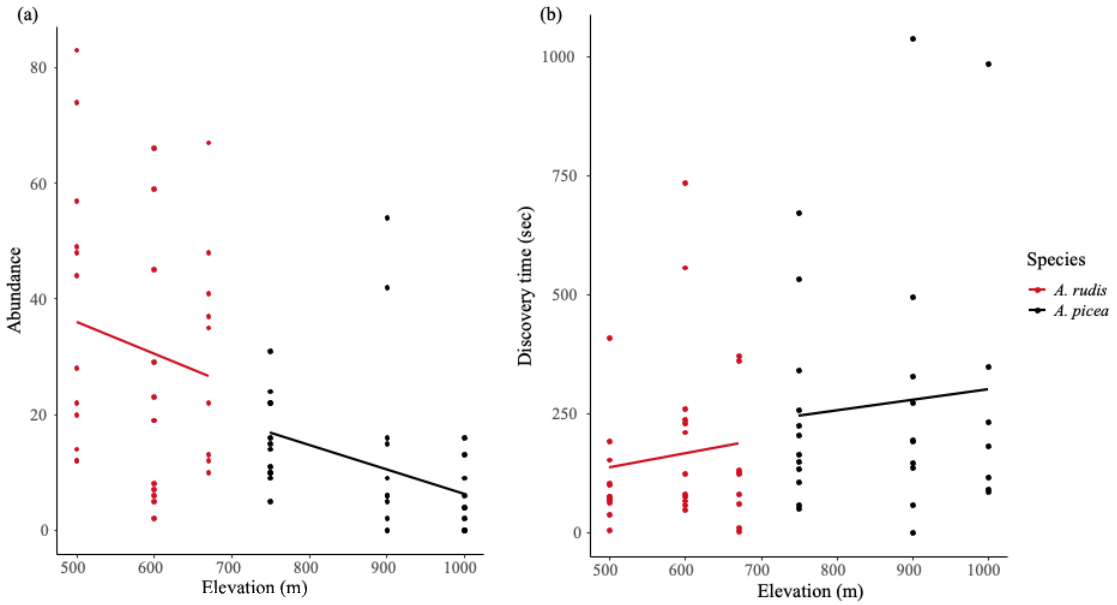

Figure 1.6. Activity at baits: (a) Abundance of Aphaenogaster ants at the baits reduces with increasing elevation. (b) The time it took for the first Aphaenogaster ant to show up at the bait increased with increasing elevation. Points show individual samplings, and the lines are plotted using the linear model smoothing function. Three lower elevations with *A. rudis* are in red and higher elevations with *A. picea* in black.

Critical thermal limits:

The maximum critical thermal limit (CT_{max}) for *A. rudis* and *A. picea* were 43.56°C ± 0.58°C and 43.57°C ± 0.44°C, respectively, while the minimum critical thermal limit (CT_{min}) was 4.4°C ± 0.4°C and 3.3°C ± 0.79°C, respectively. Colonies of

both species were sampled throughout the elevation gradient, and there was a marginally significant interaction between elevation and species for CT_{min}, indicating that the CT_{min} of *A. picea* reduces more with an increase in elevation, compared to *A. rudis* (LM: Estimate = -0.004, p = 0.054, Fig. 1.7b).

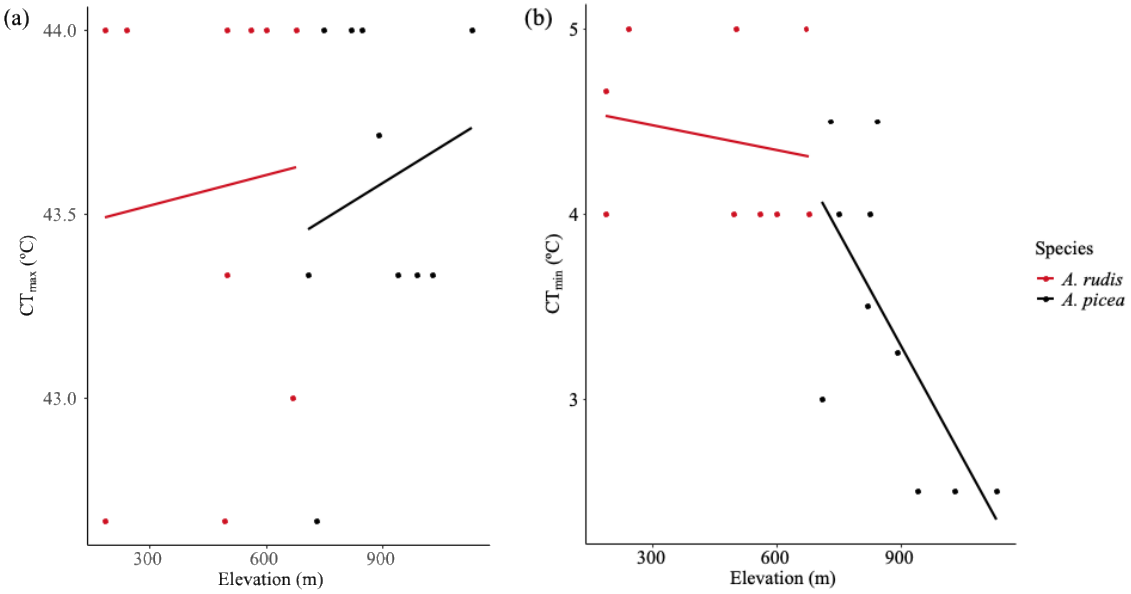

Figure 1.7. Critical thermal limits: Maximum (a) and minimum (b) critical thermal limits of *A. rudis* (red) and *A. picea* (black) along an elevation gradient. CT_{min} of *A. picea* reduces with an increase in elevation more so than *A. rudis*.

Discussion:

We studied the activity and foraging behavior of two *Aphaenogaster* ants along an elevation gradient in the Shenandoah National Park. Interestingly, we found a sharp boundary in the occurrence of *A. rudis* and *A. picea* across the elevation gradient. *A. rudis* dominated the sites below 700m of elevation, and *A. picea* dominated the sites beyond that, with no detectable overlap. This is different than the abundance pattern of the two species previously reported from the Southern Appalachian Mountains, USA (Warren and Chick 2013), where there is a zone of sympatry at middle elevations. We do not know why *A. rudis* and *A. picea* exhibit a zone of sympatric overlap in the Southern Appalachian Mountains but not in the Shenandoah area of the Appalachians.

Our results from different elevational populations prove insightful. We observed that *A. rudis,* the lower elevation species, was generally more active and more efficient in foraging than *A. picea*. Both activity and efficiency had a non-linear relationship with temperature, with a peak at an intermediate temperature of approximately 23°C. However, the interaction between species and temperature could not be tested because the two species' populations had non-overlapping elevational distributions. Our study was designed to study behavior and competition in the zone of sympatry, and compare that to results from the higher and lower allopatric populations. The sharp boundary in species distributions obviously precluded this.

The activity pattern at different elevations varied. It is notable that *A. rudis*, which is a warm-adapted species, reduced its activity when temperatures were warmer in the afternoon and evenings at the low elevation sites. Also, *A. rudis* from its highest elevation site exhibited a similar activity pattern to *A. picea* from its lowest elevation site. Both ant

species exhibited a bimodal activity pattern with reduced activity in the afternoon. *A. picea* had low activity throughout the day at our highest elevation site. This was also supported by our observations at baits conducted simultaneously with the nest activity sampling. The abundance of ants at the baits declined with increasing elevation, and the discovery time increased, indicating reduced foraging activity. A previous study at the same site as ours showed that despite the colder temperatures at higher elevations, *A. picea,* the high elevation species, had a longer activity season than did *A. rudis* (Meneses Ospina 2023). This was because *A. picea* was active earlier in the spring and later into the autumn than was *A. rudis.* This was interpreted as *A. picea* having a slower pace of life as an adaptation to its cooler climate (Meneses Ospina 2023). This is consistent with our data demonstrating lower activity levels, even at the same temperature, for *A. picea* relative to *A. rudis*. A study along an elevation gradient in the Sierra Nevada Mountains in Spain found that high-elevation *Aphaenogaster iberica* had higher metabolic rates, but this did not lead to a quick response in activity when temperatures were favorably warm (Shik et al. 2019).

As expected from a previous study, the population of *A. picea* in Shenandoah also showed a reduction in CT_{min} with increasing temperature, similar to Warren and Chick (2013). They also observed a slight reduction in cold tolerance of *A. rudis* with increasing elevation, which we did not observe in our study. This suggests that the CT_{min} determines the elevational range of the species rather than CT_{max}. This supports prior studies that showed that the elevational range of species is determined by their cold tolerance (Bishop et al. 2017; Chick et al. 2020). Our foraging data suggest that both species of ants avoid activity when the temperature is above approximately 25 degrees, but that *A. rudis,* the

low elevation species, is better able to exploit suitable temperature windows in the short term, even if *A. picea* may have a longer foraging season (Meneses Ospina 2023). Since these ants nest in the leaf litter shaded by the canopy, they are typically not exposed to extremely high temperatures. Thus, *A. rudis* can exploit the moderately warm periods. The low activity of *A. picea* at high elevations may not be directly detrimental, as there is also a lower diversity of ants competing for food resources at higher elevations. However, if there are sympatric populations of the two species in Shenandoah, then *A. rudis* might outcompete *A. picea* because of its higher activity. Additionally, they may be outcompeted by invasive ants such as *Pachycondyla chinensis,* which are known to displace populations of *Aphaenogaster* ants (Rodriguez-Cabal et al. 2012). We did not observe these ants at our baits; however, they have been reported primarily in the southeastern United States, with a few reports as far north as Virginia. The lack of sympatric populations to study means that we cannot explain mechanisms of direct or indirect competition that determine species range boundaries with any more precision. Therefore, a larger study covering more sites, especially at the extremes of ranges along with sympatric populations of the two species, would be beneficial.

Chapter 2: Behavioral response to temperature manipulation in two species of *Aphaenogaster* ants

Abstract:

Ectotherms, such as insects, rely on the environmental temperature to a greater degree than other animals. Social insects, like ants, are ecologically dominant species that respond to thermal stress at both the individual and colony levels. *Aphaenogaster* ants are common seed-dispersers in the forests of the Eastern United States. *A. rudis*, a warm-tolerant species found at low elevations, has been reported to replace *A. picea*, a cold-tolerant species found only in higher elevations. To understand how this range expansion is mediated by behavior, we studied the response of both species to a control and a 3°C warmer heat treatment. We found that both species are stressed at higher temperatures, which are at the upper end of the temperatures the species encounter in the wild. At these higher temperatures, colonies have fewer exploratory foragers, resulting in slower discovery time and fewer active foragers. They also have fewer pupae and reproductives at increased temperatures. We did not observe any effect of temperature on inter-species aggression. This shows that a temperature increase of a few degrees can alter the behavior of species and have colony-level fitness consequences, including reduced foraging and reproduction. Studying these behavior changes could help predict future large-scale ecological responses.

Introduction:

As global average temperatures increase, it is essential to study how these changes will affect animals. Ectotherms, including insects, risk a detrimental response to these rising temperatures. Thermal performance curves for terrestrial ectotherms indicate that performance usually improves with rising temperatures. Their metabolic rates increase, development times shorten, and they move faster in warmer conditions. They reach peak performance at an optimal temperature. When temperatures exceed this optimal point, however, performance drops sharply until reaching a critical thermal maximum, beyond which temperatures become lethal (Angilletta 2009; Parr and Bishop 2022). Thus, rising temperatures may benefit some insect species that are below their thermal optimum, but stress others that are already at or above their optimum temperature.

Insects can alter their behavior to cope with stressful temperatures (Kearney et al. 2009; Sunday et al. 2014). This includes changing activity levels, foraging times, and/or nest-site preferences to avoid exposure to stressful heat (Villalta et al. 2020; Blanchard et al. 2021; Bujan and Yanoviak 2022; Parr and Bishop 2022). Heat stress may also alter species ranges and ecological interactions. Shifts in species' range, composition, abundance, and diversity in response to fluctuations in temperature have been shown for numerous insect species (Parmesan and Yohe 2003; Pureswaran et al. 2018; McCain and Garfinkel 2021). Understanding the behavioral and ecological adaptations at stressful temperatures can help us anticipate such broader ecological changes in the future (Youngsteadt et al. 2023).

Behavioral responses to thermal stress are especially important in social insects such as ants. Ants dominate terrestrial ecosystems in abundance, biomass, and diversity (Hölldobler and Wilson 1990; Grimaldi and Engel 2005). Ants perform essential ecological functions, like seed dispersal, pest control, and detritus clearing (Del Toro et al. 2012; Parr and Bishop 2022). Through competition and predation, they have a top-down effect on the invertebrates of their habitat, altering community structure, food webs, and the ecosystem services provided (Parr 2008; Parr et al. 2016). Because they are social insects, ants exhibit individual and colony-level thermoregulatory behaviors that could help them cope with thermal stress (Perez and Aron 2020). For instance, individual workers can change their foraging window in response to temperature (Stuble et al. 2013; Andrew et al. 2013; Spicer et al. 2017; Jayatilaka et al. 2018; Villalta et al. 2020; Blanchard et al. 2021). At the colony level, they can move the larvae around the nest to avoid thermal stress and achieve optimal rearing conditions (Tschinkel 1987; Roces and Núñez 1989; Penick and Tschinkel 2008; Falibene et al. 2016). The reproductive queens and developing offspring are typically in a sheltered nest, often underground, that may be buffered from temperature fluctuations. Typically, only the non-reproductive foragers are exposed to temperature extremes, leading to another advantage that social insects have: the colony can survive even if a few individuals are lost to heat stress. This could allow colonies to function in conditions with high desiccation risk because, unlike in a solitary insect, individual forager death would not preclude future reproduction. Thus, the colony can still function very close to its maximum thermal tolerance (Parr and Bishop 2022).

Here, we use two species of *Aphaenogaster* ants to test the behavioral effects of a 3°C rise in external temperature. *Aphaenogaster* ants are widespread, generalist, and

seed-dispersing ants found in the forests of the Eastern United States. *A. rudis* is a warm-tolerant species located at lower elevations, while *A. picea* is a cold-tolerant species found at higher elevations (Lubertazzi 2012). Previous studies indicate that between 1974 and 2012, *A. rudis* expanded its range 300m higher in elevation in the Southern Appalachian Mountains (Warren and Chick 2013). This suggests competitive dominance of *A. rudis,* but it is unclear what behavioral mechanisms underlie this shift, and *Aphaenogaster* ants are not particularly aggressive (Warren et al. 2020). We predicted that the range incursion of *A. rudis* into elevations previously occupied only by *A. picea* may be mediated by being better able to cope with the thermal stress of increased temperatures.

To test this hypothesis, we studied colonies of *A. rudis* and *A. picea* at two experimental temperature treatments: 25°C, which served as a control temperature presumably near their physiological optimum temperature (Warren and Chick 2013; Penick et al. 2017), and 28°C, which is at the higher end of the range of temperatures experienced by these ants in Shenandoah and Rock Creek Park. We measured foraging activity, walking speed, and the distance covered by foragers without food. We then introduced food and measured the time until discovery and the total number of foragers at the food bait. We predicted that foraging performance would decrease at 28°C (relative to 25°C) because of heat stress. Alternatively, if colonies sacrificed foragers to maintain colony function, we predicted that there would be no difference between the treatments. Lastly, if increased temperature brought ants closer to their optimum activity levels, we predicted more foraging activity at 28°C, although this seemed unlikely given previous

research (Penick et al. 2017). We predicted that *A. picea* would be more affected by increased temperature because they live in cooler habitats.

In addition to foraging behavior, we also compared colony-level reproductive output and the aggressive response of individual ants when confronted with a heterospecific. We predicted that reproductive output would decline in the 28°C treatment if this temperature was stressful to ants (even if they compensated by working foragers harder). Alternatively, if 28°C is closer to their optimum temperature, reproductive output should increase. Again, we predicted that *A. picea* would be more affected by increased temperature because they live in cooler habitats. For aggression, we predicted that *A. rudis* would be more aggressive than *A. picea*, which might explain their upward expansion into the range of *A. picea* (but see Warren et al. (2020)).

Methods:

Colony collection and maintenance:

Established queenright *Aphaenogaster rudis* and *A. picea* colonies were collected from both Shenandoah National Park and Rock Creek Park and maintained in the lab in artificial nests. Colonies nesting in the soil underneath medium sized rocks were collected using aspirators. We first searched for the queen, and after confirming we had found her, collected all the workers possible. We waited a few minutes for any returning foragers or ants that had escaped when we lifted the rock to ensure we gathered as many workers as possible, along with all the brood in the nest. The typical colonies had about 100-200 workers. Colonies were collected during the summers of 2023-2025.

Experiments were conducted in the same year as the collection; colonies were not overwintered. Combining all years, 47 *A. rudis* and 48 *A. picea* colonies were collected. Not all colonies were used for all analyses, so individual sample sizes are reported for each experiment. The lab nests were constructed by setting dental plaster in a petri dish (9 cm diameter), covered by a lid and a black plastic plate to block light. The nest had a small opening on the side from which the ants could access the foraging area. There was high colony mortality in 2023 due to a lack of humidity inside the nest. To address this, in subsequent years, a plastic tube was inserted while setting the plaster, allowing us to add water without disturbing the colony and maintain its moisture. The nest was placed inside a plastic box (L: 32.8 cm x W: 19 cm x H: 11.4 cm) coated with Fluon. This box was the foraging area for the colony. The colonies were fed 30% w/v sugar solution and live mealworms every other day and had access to ad libitum water. All the colonies experienced a 12:12-hour light-dark cycle, with light from 0700 to 1900 and dark from

1900 to 0700. All colonies were kept in the same room, which had a temperature of 23°C and ~50% humidity.

Temperature treatments:

Colonies were maintained at two temperature treatments: 25°C and 28°C. The average daily temperatures in Rock Creek Park (USDA, NWCC report for Powder Mill Station in Maryland) and the lower elevations of Shenandoah National Park (Chapter 1) can reach 30°C, especially in July, which is the hottest month (Fig. 2.1A). The average daytime temperature measured at the nest entrance in Shenandoah National Park is 20.7°C ± 3.4°C, ranging from 13.5°C to 32.5°C (Fig. 2.1B). Thus, 25°C falls within the typical temperature range ants experience, while 28°C is at the higher end of the temperature range in their microhabitat. The plastic boxes were placed on a heating mat controlled by a thermostat. The temperature fluctuated in a diurnal rhythm to simulate lower nighttime temperatures in sync with the 12:12-hour light-dark cycle. Both treatments experienced a drop of 1.5°C at night at a rate of 0.1°C/min. We observed high mortality rates among colonies in 2023. In the field, *Aphaenogaster* nest in the soil and under leaves/fallen bark, which buffers extreme temperatures inside the nest (Duffy et al. 2015; Parr and Bishop 2022). Thus, the boxes were placed on the mats so that the Petri dish nest was off the mat, allowing only the foraging arena to receive the temperature treatment and to simulate natural foraging conditions.

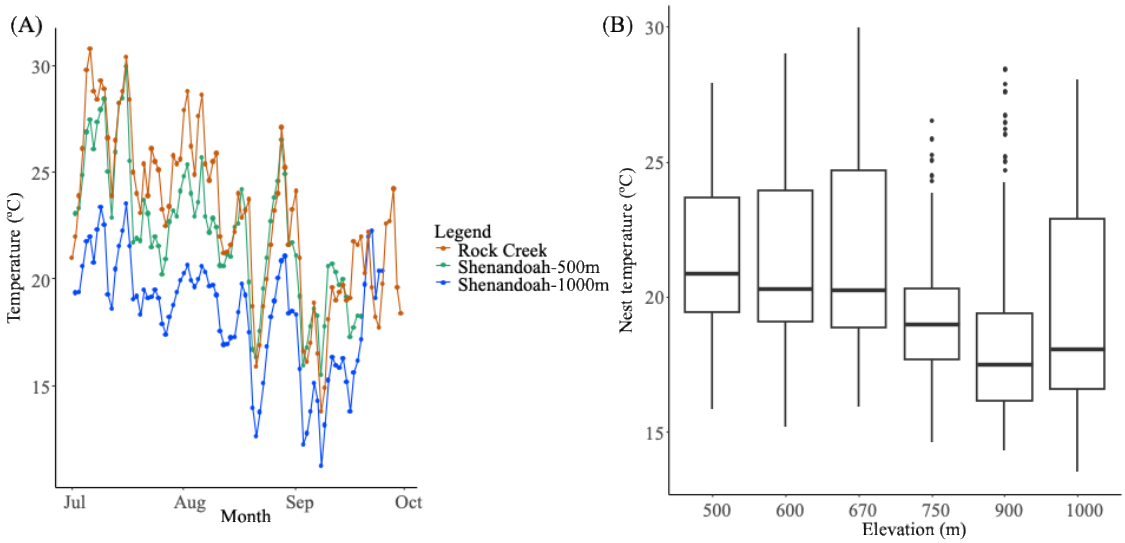

Figure 2.1. Temperature variation at the collection sites: (A) Air temperature data from Rock Creek Park (orange) and ground temperature data from two elevations at Shenandoah National Park; 500m in green and 1000m in blue. The lines indicate daily averages over 24 hours from July to September. (B) Daytime nest temperatures measured at different elevations in Shenandoah National Park (Chapter 1). The three lower elevations have *A. rudis,* while the three higher elevations have *A. picea*.

Foraging and discovery time:

We measured the time it took for the first ant in a colony to find a new food source, which we referred to as the discovery time. The colonies were starved for two days before any observations were made. A 50µl drop of 30% w/v sugar solution was placed in the foraging area, and we noted the time it took for the first ant to arrive and feed at the drop. The drop was placed at the farthest corner of the foraging arena from the nest (Approximately 16 cm from the nest entrance). Experiments were conducted between 11:00 and 14:00. Discovery time was measured three times, each on a different day, for each colony, and the mean discovery time was used for analysis. Discovery time was also measured through video taken for other experiments, as mentioned below. Discovery time was measured for 21 *A. rudis* colonies (11 at 25°C and 10 at 28°C) and 20 *A. picea* colonies (9 at 25°C and 11 at 28°C). The unequal sample sizes resulted from

the death of the queen in certain colonies during the experiment, which were discarded, and due to a few corrupted video files. A generalized linear mixed model (GLMM) with a Poisson distribution was used to analyze the effects of treatment and species, as well as the interaction between the two variables, on discovery time, with colony ID as the random factor.

To assess the colony-level foraging effort, we video-recorded the foraging arenas using GoPro cameras mounted approximately 60 cm above the arenas (GoPro 11 Black) for 2 to 3 hours. Once the first ant located the drop of sugar solution, we counted the number of ants at the drop every five minutes. We summed the number of ants from all observation periods to calculate the total number of ants observed at the drop, reported as "number of foragers". These data were recorded for 14 *A. rudis* colonies (8 at 25°C and 6 at 28°C) and 13 *A. picea* colonies (7 at 25°C and 6 at 28°C). The unequal sample sizes resulted from some queen mortality and a few corrupted videos. We used a generalized linear mixed model (GLMM) with a Poisson distribution to analyze the species and treatment effects, with colony ID being the random factor.

Exploration:

We counted the number of workers in the foraging arena before introducing the sugar drop (described above) as a measure of activity. These were the workers exploring outside the nest without a food reward present. Activity was measured for 21 *A. rudis* colonies (12 at 25°C and 10 at 28°C) and 20 *A. picea* colonies (11 at 25°C and 11 at 28°C). The unequal sample sizes resulted from queen mortality in some colonies, as colonies without queens were discarded from analysis. We used a generalized linear mixed model (GLMM) with a Poisson distribution to determine whether the Treatment,

Species, and their interaction influenced the activity of the ants, with colony ID as the random factor. A Spearman correlation test was performed between the number of ants exploring and discovery time, and between discovery time and the total foragers.

We tracked individual ants in videos of the foraging arena using the TrackMate plugin of Fiji software (Schindelin et al. 2012; Tinevez et al. 2017). A 20-second video clip recorded before the discovery time trials was utilized for this analysis. This allowed us to measure the ants' walking speed, and the total distance traveled while exploring the arena without a food incentive. All the ants present outside the nest in the foraging arena were tracked, and the mean speed and distance per colony were used for analysis. These data were recorded for 14 *A. rudis* colonies (8 at 25°C and 6 at 28°C) and 13 *A. picea* colonies (7 at 25°C and 6 at 28°C). None of the *A. rudis* colonies had any movement at the higher temperature before introducing the drop of sugar, so their speed and distance could not be measured. Due to this missing data, we only compared speed and distance travelled by *A. picea* at the two temperatures using a Mann-Whitney U Test. Three *A. picea* colonies at the higher temperature did not have any activity, so the sample size for the Mann-Whitney U test was small.

Reproductives:

To measure the colony's reproductive output, we counted the number of pupae and newly emerged queens and males. After being at the temperature treatments for three weeks, the number of late-stage pupae (identified by dark pigmentation in the eyes), winged queens and winged males were counted each week for 4 weeks. Only late-stage pupae were included in the count because they could be counted more reliably by eye pigmentation. The mean number of pupae and the maximum number of queens and males

observed were used for analysis. We used a linear mixed model (LMM) to analyze the difference in the mean number of pupae produced by colonies with treatment and species as predictor variables. We used a zero-inflated mixed model with a Poisson distribution to determine whether the number of queens produced by the colonies differed between the two treatments and the two species. We used a GLMM with a negative binomial distribution to account for overdispersion in the data and determine the effect of treatment and species on the number of males produced by the colonies. These three models used colony ID as the random factor.

Individual aggression:

To test the effect of temperature on ant aggression, we examined individual ants from both species subjected to the same temperature treatment. A forager from an *A. picea* colony and another from an *A. rudis* colony were placed in a box (L: 13 cm x W: 10 cm x H: 4 cm) coated with fluon. It had a Fluon-coated glass slide separation in the middle. The ants were positioned on either side of the partition and allowed to acclimate to the new environment for 5 minutes. After that, the partition was removed, and a 50 µL drop of a 30% sugar solution was placed in the center. We observed the interactions between the two ants for the next 10 minutes. This was done for 9 *A. rudis* colonies (4 at 25°C and 5 at 28°C) and 11 *A. picea* colonies (5 at 25°C and 6 at 28°C). Aggression trials were performed for all possible combinations of *A. rudis* and *A. picea* colonies at the same temperature treatment. In total, we performed 20 aggression trials at 25°C and 26 aggression trials at 28°C (could not complete all combinations due to queen mortality). A binomial regression mixed model was used to assess differences in aggression levels between species and treatments with colony ID as the random factor. The aggressive

interactions we recorded include extending mandibles, biting, curled gaster, and stinging. The non-aggressive interaction observed was antennating, and an avoidance behavior where the ant backed away rapidly after the interaction.

All data analyses and visualizations were performed in R version 4.2.1 (R Core Team 2022).

Results:

Foraging:

Both species took more time to find a new food source at the higher temperature treatment (GLMM: Estimate = 1.180, p = 0.035, Fig. 2.2). There was no difference between the two species (Estimate = 0.412, p = 0.473, Fig. 2.2). The interaction of species and treatment was also not significant (Estimate = -0.636, p = 0.427, Fig. 2.2). Both species also had a smaller total number of ants foraging at the higher temperature (GLMM: Estimate = -3.326, p < 0.001, Fig. 2.3). There were fewer total foragers for *A. picea* colonies than for *A. rudis* colonies (Estimate = -1.809, p = 0.045, Fig. 2.3). The trend was stronger in *A. rudis* as indicated by a significant interaction between species and temperature (Estimate = 3.01, p = 0.033, Fig. 2.3).

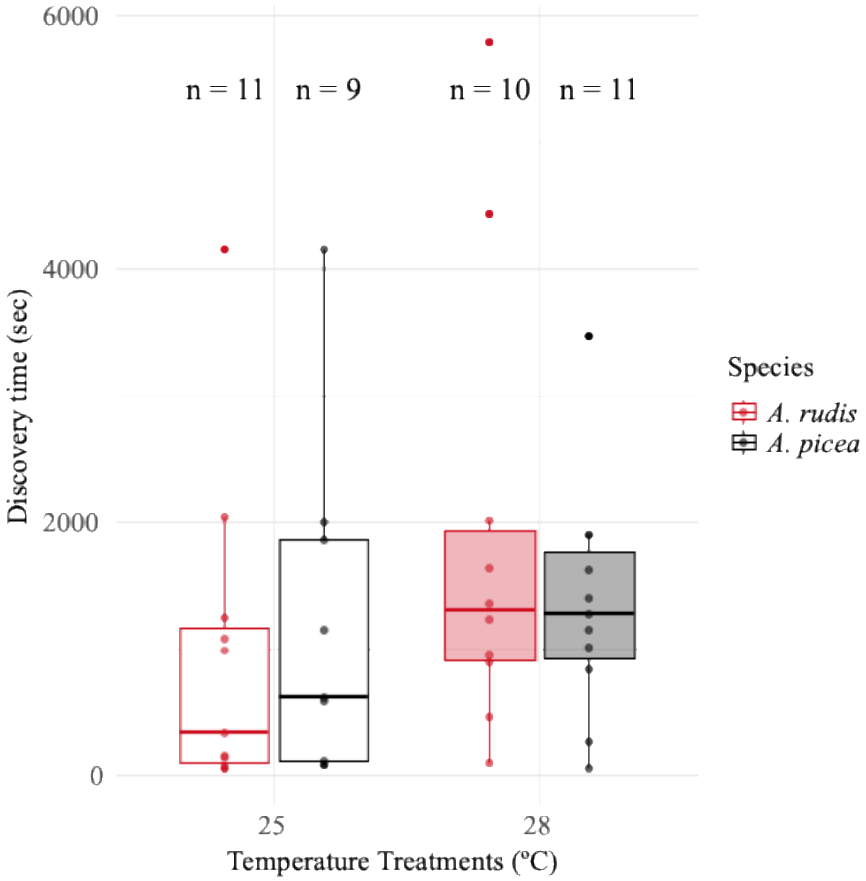

Figure 2.2. Discovery time: The boxplots show the distribution of discovery time for the two species at the two temperature treatments. The horizontal line within the boxplot is the median discovery time. The box limits indicate the interquartile range (IQR), and the whiskers indicate 1.5 times the IQR. The points show individual data points. Discovery time was higher for both species at the higher temperature. The numbers above the box plots indicate the number of colonies sampled. The boxes are filled in for the higher temperature treatment.

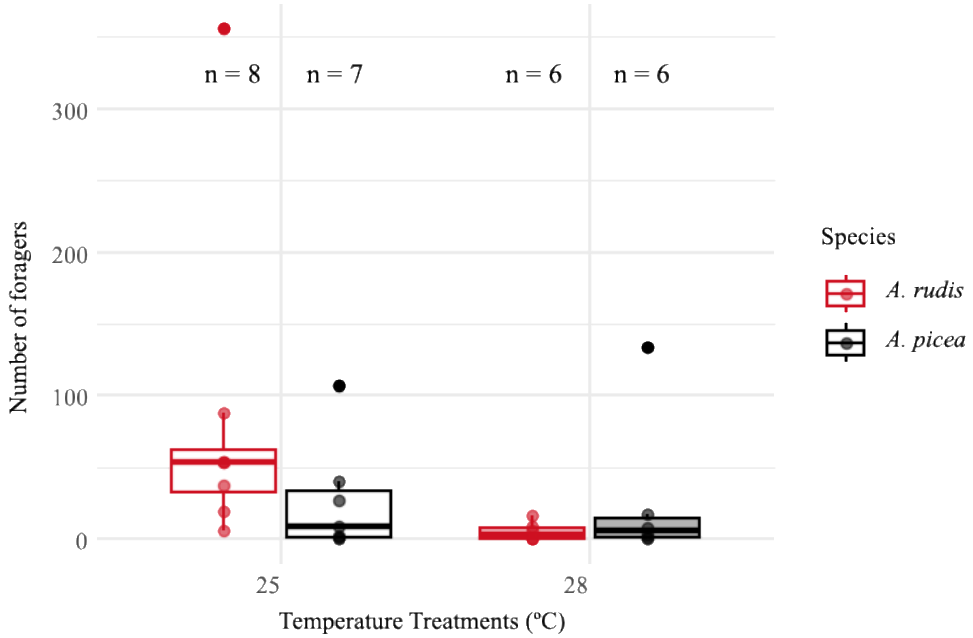

Figure 2.3. Total foragers: The boxplots show the distribution of the total number of foragers observed at a food source for the two species at the two temperature treatments. The horizontal line within the boxplot is the median number of foragers. The box limits indicate the interquartile range (IQR), and the whiskers indicate 1.5 times the IQR. The points show individual data points. Both species had fewer workers foraging at the higher temperature. *A. picea* had fewer foragers than *A. rudis*. A significant interaction effect indicates that *A. rudis* is affected more strongly than *A. picea*. The numbers above the box plots indicate the number of colonies sampled. The boxes are filled in for the higher temperature treatment.

Exploration:

The activity of ants, as measured by the number of ants exploring outside the nest without a food incentive, was less at the higher temperature (GLMM: Estimate = -1.232, p = 0.031, Fig. 2.4). There was no difference between the two species (Estimate = -0.483, p = 0.350, Fig. 2.4). The interaction between temperature and species was also not significant (Estimate = 1.129, p = 0.153, Fig. 2.4). There was a negative correlation

between the number of ants exploring and discovery time (rho = -0.484, p = 0.01) and between the discovery time and the total number of foragers (rho = -0.497, p = 0.008).

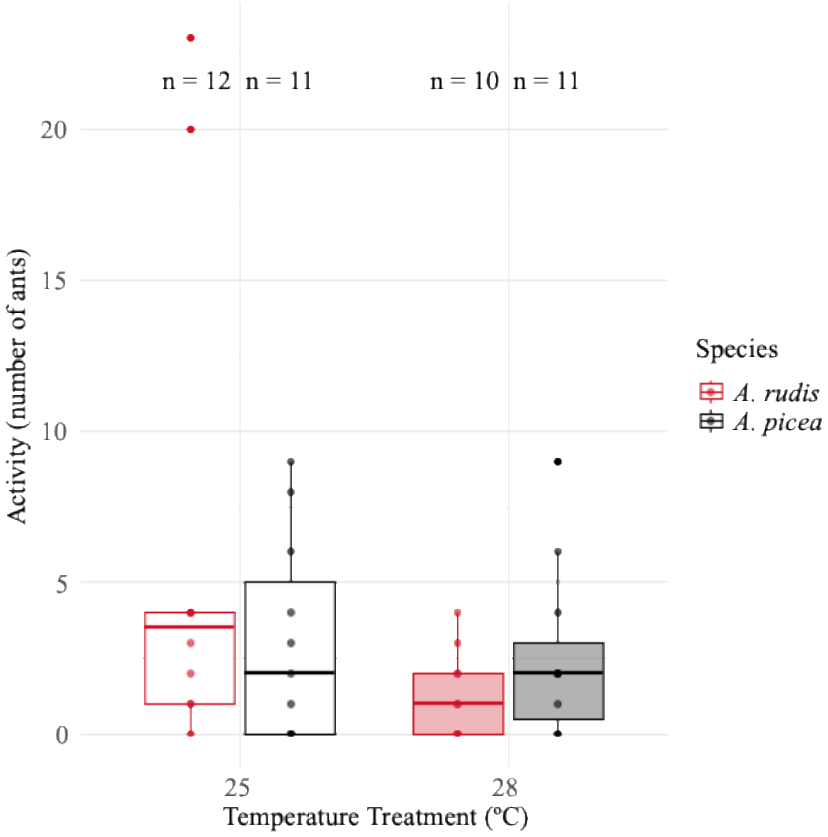

Figure 2.4. Exploratory activity: The boxplots show the exploratory activity of the two species at the two temperature treatments. This was measured as the number of ants active in the foraging area without a food reward present. The horizontal line within the boxplot is the median number of ants. The box limits indicate the interquartile range (IQR), and the whiskers indicate 1.5 times the IQR. The points show individual data points. Exploratory activity is reduced at the higher temperature. The numbers above the box plots indicate the number of colonies sampled, and the boxes are filled in for the higher temperature treatment.

For *A. picea*, walking speed (Mann-Whitney-U-Test: W = 9, p = 0.786, Fig. 2.5a), and distance travelled (Mann-Whitney-U-Test: W = 11, p = 0.393, Fig. 2.5b) were not significantly different between temperature treatments. The median walking speed at 25°C was 1.87 ± 0.365 mm/s, and at 28°C was 1.3 ± 0.621 mm/s. The median distance travelled at 25°C was 120.82 ± 36.104 mm, and at 28°C was 76.065 ± 15.411 mm. 6

colonies of *A. rudis* and 3 of *A. picea* in the 28°C treatment did not have any activity, so the sample sizes for the Mann-Whitney U test are reduced. Only colonies with activity are graphed in Figure 5.

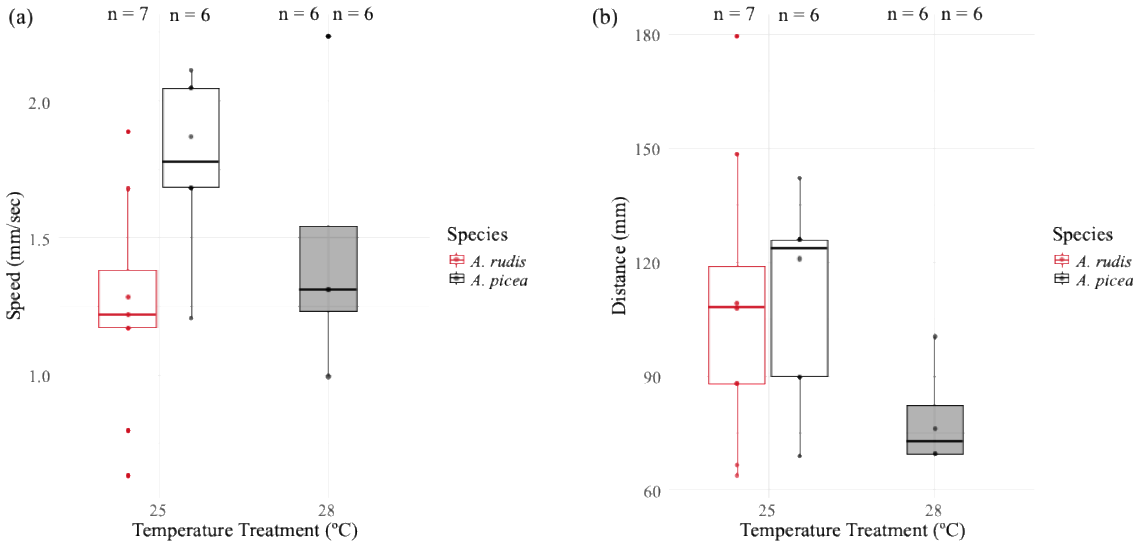

Figure 2.5. Ant walking speed and distance during exploratory activity: The boxplots show the walking speed (a) and distance travelled (b) for the two species at the two temperature treatments during exploratory activity. The horizontal line within the boxplot is the median value. The box limits indicate the interquartile range (IQR), and the whiskers indicate 1.5 times the IQR. The points show individual data points. *A. rudis* colonies at the higher temperature did not have any workers out in the foraging arena throughout our sampling. Thus, we could not measure the speed and distance travelled by the group. Although *A. picea* showed reduced speed and distance traveled at the higher temperature, the difference was not statistically significant, which could be attributed to our low sample size. The numbers above the box plots indicate the number of colonies sampled. However, since not all colonies showed measurable activity, some boxes have fewer data points than the number of colonies sampled. The boxes are filled in for the higher temperature treatment.

Reproductives:

Fewer pupae were produced by both species at 28°C compared to 25°C (LME: Estimate = -10.514, $p = 0.022$, Fig. 2.6). There was no difference between the two species (Estimate = -7.353, $p = 0.102$, Fig. 2.6). There were also fewer queens produced at the higher temperature treatment (Zero-inflated Poisson: Estimate = -0.662, $p = 0.015$, Fig. 2.6), and *A. rudis* produced fewer queens than *A. picea* (Estimate = -0.996, $p < 0.001$,

Fig. 2.6). The number of males produced at 28°C was marginally lower than at 25°C (GLMM: Estimate = -2.298, p = 0.090, Fig. 2.6), but was not different between the two species (Estimate = 1.011, p = 0.435, Fig. 2.6)

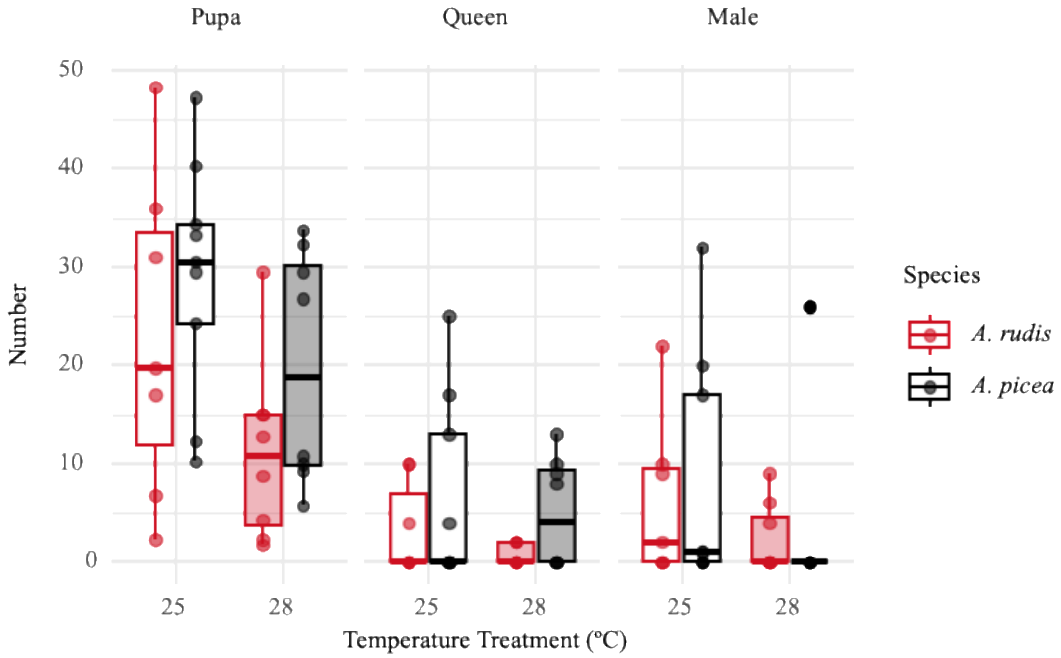

Figure 2.6. Reproductives: The boxplots indicate the number of pupae, queens, and males produced by the colonies of the two species at the two temperatures. The horizontal line within the boxplot is the median value. The box limits indicate the interquartile range (IQR), and the whiskers indicate 1.5 times the IQR. The points show individual data points. The boxes are shaded for the high-temperature treatment. Both species produced fewer last-stage pupae and queens at the higher temperature, while the effect on males was marginal.

Individual aggression:

There was no difference in aggressive, non-aggressive, or backing away behaviors between the two treatments (GLMM: Aggressive: Estimate = 0.127, p = 0.818; Non-aggressive: Estimate = -0.345, p = 0.387; Back away: Estimate = 0.998, p = 0.153, Fig. 2.7). There was considerable variation in the data, which could mask any patterns.

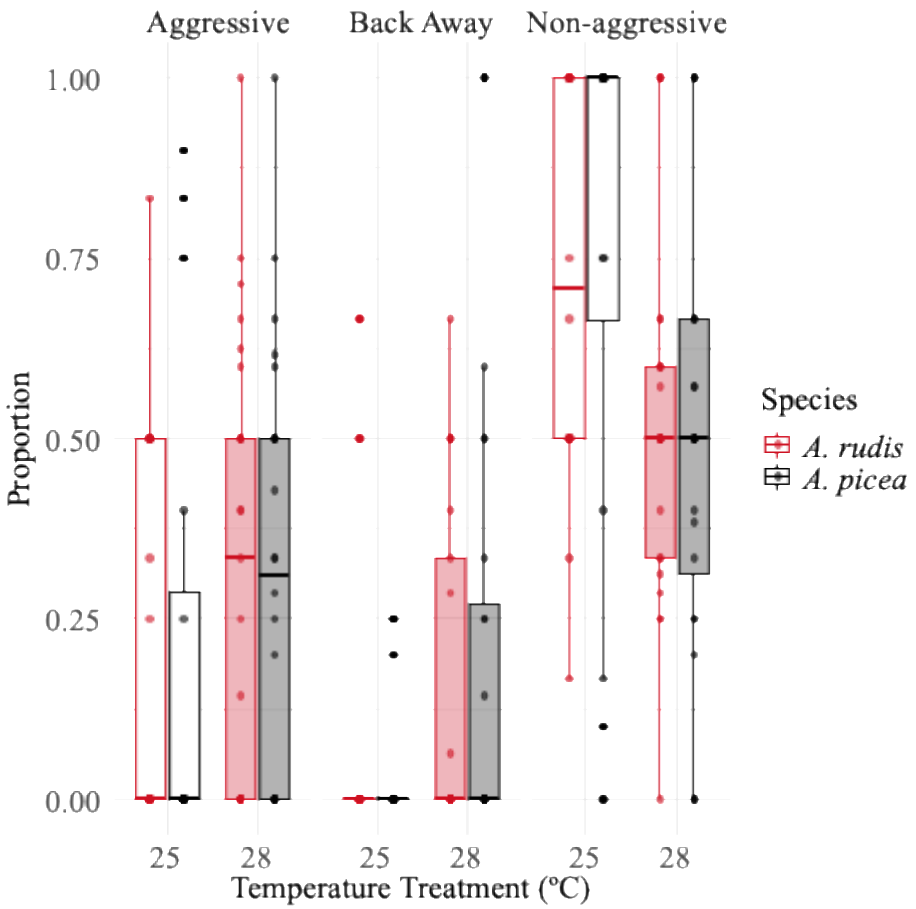

Figure 2.7. Aggression: The boxplots indicate the proportion of interactions between heterospecific ants at the same temperature that were aggressive, avoiding (back away), or non-aggressive. The lines within the boxplot show the median value, and the box limits show the inter-quartile range (IQR). The whiskers indicate the limits of 1.5 times the IQR. The points indicate individual data points. The boxes are filled in for the higher temperature treatment. There was no difference in aggressive behavior between the two treatments or between the two species.

Discussion:

We measured the performance of *A. rudis* and *A. picea* at two temperature treatments: 25°C and 28°C. Our predictions of reduced colony performance at 28°C were generally supported in both species, although there were some minor discrepancies that will be discussed below. This suggests that both species find 28°C stressful. The hypothesis that increased temperature would lead to increased activity was not supported. However, the prediction that *A. picea* would be more severely impacted by temperature stress was also not supported: both species were negatively affected. We found that the activity of both species was reduced at the higher temperature treatment. Each had fewer ants exploring for food at 28°C compared to 25°C, took more time to find a new food source, and had fewer workers foraging at the higher temperature. The slower discovery of food could be a result of fewer exploratory foragers, and such colonies also have fewer total foraging ants even in the presence of food. This indicates that both species are stressed at 28°C and likely do not expose foragers to increased risk to maintain foraging activity. *Aphaenogaster* are ecologically dominant, keystone, seed-dispersing ants in the hardwood forests of the Eastern United States, and are known to benefit numerous plants by transporting elaiosome-bearing seeds (Warren II and Giladi 2014). They are often the first ants to reach baits because of the large number of foragers on the forest floor resulting from a high nest density (Lubertazzi 2012). They are not very aggressive and are frequently driven away by other ants from food sources. Therefore, they depend on their quick food discovery times to obtain resources rather than asserting dominance at food sites. Higher temperatures can lessen their competitive edge by decreasing their

activity and foraging behavior, which can have broader ecological effects through the ecosystem services they offer.

The high-temperature treatment represents the upper end of the temperature range regularly experienced by these species in their natural habitat, which is the shaded leaf litter of a closed-canopy forest. Hence, even as daytime air temperatures exceed 30°C, the nest temperatures remain around 25°C, with a maximum of 32°C. The prolonged 12-hour exposure to 28°C in our experiment was likely more extreme than experiencing it for a few hours during the day, making it more stressful. It is, however, well within the thermal tolerance breadth of both species. Our previous studies indicate that the population of *A. rudis* in Shenandoah National Park has a CT_{min} of 4.4°C and a CT_{max} of 43.56°C, while *A. picea* has a CT_{min} of 3.3°C and a CT_{max} of 43.57°C. Thus, our results highlight the importance of studying the behavioral changes at small increments in temperature that are considered sub-lethal. The ecological effects of heat stress will become evident well before temperatures reach levels that are lethal to organisms.

Penick et al. (2017) also suggest a more comprehensive approach to studying the response of insects to temperature, rather than focusing solely on thermal limits. While they reported a correlation between thermal limits and the performance of a species at a particular temperature, they highlight that multiple thermally dependent traits, such as development time, activity, and mortality, interact to determine fitness. While comparing the performance of *A. rudis* to that of the highly temperature-tolerant species *Temnothorax curvispinosus*, they found that *A. rudis* exhibited increased mortality at 26°C and 29°C compared to 20°C and 23°C, while *T. curvispinosus* performed better at the higher temperatures. Stuble et al. (2013) manipulated temperatures for ant

communities in the forest over several years and found that *A. rudis* foraging activity did not change with temperature. However this is not directly comparable to our study because forager abundance was measured at baits located in forest patches that had been receiving the treatment for several years, while allowing for the movement of ants in and out of the experimental patches.

Temperature also affected the reproductive output of both species. Fewer pupae and new queens were produced at the higher temperature, and there was a marginal effect on the number of males as well. Fewer pupae could be either due to a slower egg-laying rate by the queen or due to egg/larval mortality. Penick et al. (2017) found increased egg mortality at 29°C in *A. rudis*, but did not find a reduction in the egg-laying rate of the queen. It is likely therefore that the fewer larvae are a result of egg mortality. Penick et al. (2017) highlight the importance of examining the effects of temperature on not just adults but also the immature stages. In our study, since only the foraging area and not the nests were exposed to the temperature treatments, the fewer mature larvae, as well as fewer queens and males, could be a result of reduced foraging at the higher temperature, leading to a lack of nutrients for the larvae and the development of reproductives. Temperature stress has been shown to affect reproductive output in other taxa. Higher temperature during overwintering increases mortality in solitary bees such as *Osmia bicornis*, and the bees that survive produce fewer offspring in the summer (Müller et al. 2025). Reproductive output also decreases in adults with exposure to drought conditions in subalpine bees (Wong et al. 2025). Reproductive output decreases indirectly through effects on the host plant in *Osmia lignaria* (Walters et al. 2024). Reduced reproductive output at high temperature has been reported in other insects (Campion et al. 2023; Ratz

et al. 2024; Martin et al. 2025) and has important consequences for pests and pest-management strategies (Liang et al. 2021; Hu et al. 2022; Weaving et al. 2024).

We did not observe any difference in the aggression of the two species, and it was not affected by temperature. The high variation indicates high individual and colony-level variability in aggression. In contrast, the ants *Prolasius advenus* (Barbieri et al. 2015) and *Tetramorium alpestre* (Krapf et al. 2023) exhibit increased aggression at higher temperatures. However, the cold-adapted winter ant *Prenolepis impairis* reduced aggression at higher temperatures (Barton and Ives 2014). The lack of aggressive behavior in *Aphaenogaster* could also explain the lack of a distinct pattern in aggression in our results (Warren et al. 2020). Thus, the range expansion of *A. rudis* may not be facilitated by aggressive behavior (Warren et al. 2020).

The results of our study on walking speed and distance traveled by exploratory individuals were inconclusive. The activity of colonies at the higher temperature treatment was so low that no *A. rudis* colonies were observed exploring during our video sampling, and only three colonies of *A. picea* had ants that were exploring. However, for *A. picea,* it seems that speed and distance traveled are lower at the higher temperature, but more data would be needed for any conclusive statements.

Overall, our results suggest that small temperature increments can have a profound impact on the behavior and ecology of ants. The effect is not always predictable: our prediction that the low-elevation, high-temperature-tolerant species would perform better than the high-elevation, low-temperature-tolerant species was not supported. Instead, both species performed worse at the higher temperature. As mentioned above, this could be because our prolonged exposure to high temperatures was

more severe than the periodic exposure in natural populations. Our data was inconclusive in determining the behavioral mechanisms underlying the upward shift of *A. rudis*. Gathering more long-term data, especially at the edges of the elevational range, would be beneficial. Understanding the mechanisms by which thermal stress reduces colony fitness is crucial, as they are not always predictable. Further research on how thermal stress affects immature stages and the resulting impact on adult behavior would help clarify the overall effects of thermal stress.

Considering the findings of the first two chapters together, *A. rudis* is better able to take advantage of moderately high temperatures, but both species reduce activity at extremely high temperatures. This likely has contributed to the upward expansion of *A. rudis* in the Southern Appalachian Mountains. Still, we do not know why there are no sympatric populations of the two species in Shenandoah. Further studies should examine how thermal stress affects queens, particularly during their colony-founding stage, to understand this strict separation between the two species. Since these species nest in leaf litter under a shaded canopy, they are not yet continuously exposed to extremely high temperatures, as in our treatment. However, the reduced activity and efficiency at increased temperatures not only directly affect their populations but can also make these ants vulnerable to invasive species that are known to displace *Aphaenogaster* ants, such as *Pachycondyla chinensis* (Rodriguez-Cabal et al. 2012), under future climate warming.

Chapter 3 Experimentally elevated temperatures reduce activity and host defense in the acacia ant *Pseudomyrmex spinicola*

Abstract:

The first response to a changing climate is often behavioral. The responses of social insects occur at both the individual and colony levels. Insects' behaviors to escape heat stress are especially important in tropical species because ambient temperatures are typically closer to physiological tolerance limits. We studied the effect of experimentally elevated temperatures on the tropical ant *Pseudomyrmex spinicola*, a mutualist of the bullhorn acacia plant, *Vachellia collinsii*. The plant provides shelter and food to the ants, and the ants defend the plant from herbivores. We performed in situ whole-plant warming experiments and compared ant behavior, physiology, and herbivory rates on heated and control plants. The ants were less active at experimentally elevated temperatures, which reduced their aggressive plant defense behavior. We measured the ants' maximum critical thermal limit and observed that they experienced temperatures close to their physiological tolerance. Despite the reduction in ant patrolling activity, we did not see increased herbivory in the experimentally heated treatment. Further long-term studies incorporating plant and herbivore responses to elevated temperatures should be pursued to examine whether this mutualism will persist with a changing climate.

Introduction:

Climate models predict that global temperatures will rise by 1.5°C by 2050 and between 2-4°C by the end of this century (Pachauri et al. 2015), and the impacts of climate change are already detectable in animals (Parmesan and Yohe 2003; Parmesan 2006). Ectotherms are especially vulnerable because they are unable to regulate their body temperature and are more susceptible to external environmental conditions (Kingsolver et al. 2013; Paaijmans et al. 2013). Declines in vertebrate ectotherms, such as reptiles and amphibians, have been partially attributed to climate change, along with other stressors, including pollution and habitat loss, which are often exacerbated by climate change (Bickford et al. 2010; Rohr and Palmer 2013; Hayden Bofill and Blom 2024). Freshwater invertebrates also exhibit shifts in life history traits, such as development times, fecundity, and survival, due to climate change-induced fluctuations in salinity and hydroperiod (Stoks et al. 2014; Niedrist and Füreder 2023). Terrestrial invertebrate ectotherms, such as insects, have also been reported to experience population declines, range shifts, and changes in community composition (Menke et al. 2014; Pureswaran et al. 2018; Halsch et al. 2021; McCain and Garfinkel 2021; Dalton et al. 2023; Harvey et al. 2023).

Ectotherms have a typical thermal performance curve where performance increases as temperatures increase until the optimum temperature, where they reach peak fitness. Performance then drops sharply with continued temperature increase until animals reach the maximum critical thermal limit (CT_{max}), at which point they lose muscle control (Parr and Bishop 2022). Temperate animals have wider thermal tolerance ranges than tropical animals because they have evolved in environments with larger

seasonal temperature variations (Janzen 1967; Sunday et al. 2010; Dewenter et al. 2024). This suggests that tropical species may be at greater risk from heat stress due to their narrow thermal tolerance ranges, meaning they are already experiencing temperatures close to their maximum critical thermal limit (Deutsch et al. 2008; Nascimento et al. 2022; Parr and Bishop 2022). Consequently, it seems likely that tropical insects will be more impacted by increased global temperatures.

Before temperatures reach the maximum tolerance level, insects can modulate their behavior to escape from thermal stress, which ultimately leads to the large-scale ecological patterns mentioned above. This is particularly interesting in social insects, which have collective thermoregulatory behaviors to maintain their nest temperature (Jones and Oldroyd 2006). Individuals can also modulate their behavior, such as foraging times and activity patterns, to avoid extreme heat stress (Spicer et al. 2017; Villalta et al. 2020; Blanchard et al. 2021; Bujan and Yanoviak 2022). For instance, warmer spring temperatures lead to increased foraging in the facultatively social bee *Halictus rubicundus*, which could result in a switch from solitary to social life histories (Schürch et al. 2016). Stuble et al. (2013) found a species-specific change in the foraging patterns of ants under experimental warming, where some species increased foraging while others reduced foraging at higher temperatures. At one of their study sites, these changes corresponded to their thermal limits such that more tolerant species foraged at higher temperatures. The same trend was not observed at another site, suggesting that the changes are not consistent between species and sites. However, because social insects have replaceable workers, they could risk foraging at suboptimal temperatures to avoid competition and predation (Cerdá et al. 1998; Menzel and Feldmeyer 2021; Nascimento

et al. 2022). Their collective behavior and social structure provide social insects with a buffer against the extreme effects of thermal stress, compared to solitary insects, in which individual death causes reproductive failure. Thus, the response of social insects to climate change occurs both at the individual and colony levels, making it challenging to predict responses for a wide range of species and across large geographical scales.

Ants are an excellent study organism for assessing the effects of temperature stress on colony behavior and fitness due to their ubiquitous presence in diverse ecological regions (Schultheiss et al. 2022), particularly in the tropics (Kaspari et al. 2000; Dantas and Fonseca 2023). Compared to flying social insects such as bees and wasps, it is easier to monitor entire colonies and manipulate the temperatures that the ants experience. They also experience unique microclimatic conditions, as their small bodies and inability to fly expose them to the high-temperature boundary layer of heated surfaces, especially in the canopy (Kaspari et al. 2015; Stark et al. 2017). While rising air temperatures may not be a direct threat to ants, changes in cloud cover and extreme heat days can expose the canopy to high surface temperatures, driving behavioral changes in the ants (Kaspari et al. 2015; Perez and Aron 2020; Nascimento et al. 2022). Arboreal nesting ants are exposed to more dramatic temperature fluctuations than ground-nesting species that can take refuge from thermal extremes in the leaf litter or underground. As a result, arboreal nesting species often have a higher maximum temperature at which they can function (CT_{max}) than ground-nesting species (Kaspari et al. 2015; Perez and Aron 2020; Nascimento et al. 2022). For example, *Acromyrmex subterraneus*, a tropical ground-dwelling ant, has a CT_{max} of 43.67 ± 0.6°C, whereas *Pseudomyrmex gracilis,* an arboreal ant from tropical forests, has a CT_{max} of 47.00 ± 0.9°C (Nascimento et al. 2022).

It is crucial to understand whether canopy-dwelling species can cope with increasing thermal fluctuations.

Arboreal ants frequently participate in defense mutualisms with plants, and rising temperatures can disrupt these species interactions as the participating species respond differently to heat stress. Some ant-plant mutualisms are predicted to destabilize under future climate change due to changes in the services provided by a partner or due to switching partners (Barton and Ives 2014; Dejean et al. 2022; Youngsteadt et al. 2024; Câmara et al. 2024). Temperature can also change the participating species in a mutualism, which can skew the services provided (Fitzpatrick et al. 2014). Obligate mutualisms are particularly vulnerable because the effects on one partner severely affect the other and can even lead to a loss of the species. Mutualisms often support other specialist or generalist species. For example, some birds preferentially nest in acacias defended by ants, potentially gaining protection from their own predators (Amador-Vargas and Porras-Brenes 2022; Lujan et al. 2023). Thus, disruptions in ant-plant mutualisms not only affect the participating species but also other associated species and can have cascading effects in the ecosystem.

We studied the effect of elevated temperature on the behavior of the arboreal tropical ant *Pseudomyrmex spinicola* to better understand the consequences of ongoing warming on social insect behavior. *Pseudomyrmex spinicola* is an obligate mutualist of *Vachellia collinsii* (Fabaceae, Mimosoideae) (Fig. 3.1a). The ants nest inside the hollow thorns of the tree, feed at the extrafloral nectaries, and collect Beltian bodies on new leaves, which contain proteins and lipids, to nourish their larvae (Janzen 1966; Heil et al. 2004). They patrol the plant to aggressively attack and remove herbivores and clear other

vegetation around the plant, thereby protecting their host from competitors (Janzen 1966; Amador-Vargas 2019; Amador-Vargas et al. 2021). The primary goal of this study was to determine the behavioral response of *Pseudomyrmex spinicola* ants to naturally occurring and experimentally increased temperatures. We observed ant activity on plants in sunny and shaded locations during both the morning and afternoon. We then used plastic enclosures to experimentally raise temperatures on treatment plants, while similar enclosures with ventilation were used for control plants, which did not experience temperature increases. We predicted that activity and aggressive defense would decrease with the experimentally increased temperature. We also predicted that, as a result of reduced defense, herbivory on the treatment host plants would increase. However, herbivory rates may also be affected by the response of the herbivores to increased temperatures. An increase in herbivory would suggest a potential disruption to the mutualism mediated by temperature. We measured the CT_{max} of ants in our study both before and after treatment. We predicted that CT_{max} would increase in treatment plants if the ants acclimate to the higher temperatures. Lastly, we measured the CT_{max} of ants in a nearby, hotter location, predicting that these ants would have a higher thermal limit than our study ants.

Methods:

Study site:

The study was conducted in the dry season, from February to April of 2024, in Parque Natural Metropolitano (PNM), Panama City, Panamá (8° 59' 37.8168" N, 79° 32' 46.698" W), a 232-ha tropical dry forest.

Focal species:

We sampled 33 *Vachellia collinsii* plants with established colonies of *Pseudomyrmex spinicola*. If the acacias were grouped within about 1m of each other with no other plants in between, they were considered to be in a patch. We selected only one plant from among them to ensure each acacia had a different *P. spinicola* colony because the same colony can colonize neighboring acacias and travel between them. Of all the plants studied, 10 plants were part of a larger patch (six control and four heat treatment plants); all the other plants were isolated from other acacias. All plants were less than 1.5 m in height to ensure that they fit in our greenhouse plastic enclosures. To measure the canopy cover, we took pictures of the canopy directly above each plant. Percent canopy cover was measured using ImageJ (Schindelin et al. 2012). Plants were assigned as control or heat treatment throughout all areas in the park to ensure that there was an even spread of both treatments in shaded and sunny areas. Of the 33 plants studied, 16 were controls and 17 were heated.

Enclosure setup:

We built open-top enclosures using greenhouse plastic (Torotrac, Panamá) and metal poles made from steel rebar (Marion et al. 1997; Werkmeister et al. 2022). Four poles were hammered into the ground around each plant. A galvanized wire was wound

around the poles to make a sturdy frame. The plastic sheet was then wrapped around and affixed to the poles, keeping a 10-15 cm buffer between the outermost leaves and the plastic. For the heat treatment plants, the lower edge of the plastic was flush with the ground and secured with stones to prevent any airflow through the bottom of the enclosure. To increase the heating effect, an A4-sized paper covered in black packing tape was placed inside the enclosure. This helped to absorb and radiate solar energy inside the enclosure (Figure 3.1c). For the control treatment, the lower edge of the plastic was just touching the ground, permitting airflow through the bottom of the enclosure. Also, large holes (approximately 20 x 30 cm) were cut in the plastic to facilitate airflow and prevent heating (Figure 3.1d). No black paper was placed in the control enclosures. This ensured that the ants, plants, and herbivores experienced similar exposure to the plastic enclosures, but that the control plants did not experience the rise in temperature from the experimental enclosures (see Results, below).

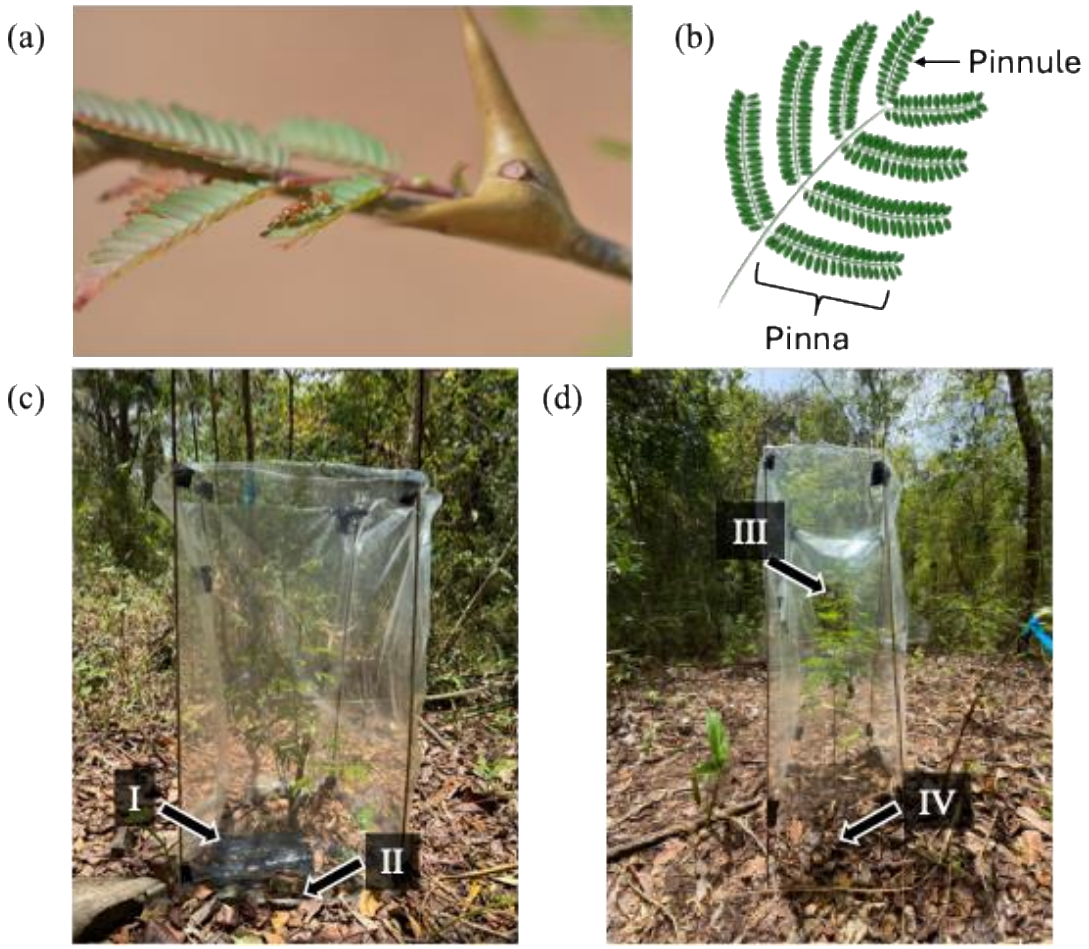

Figure 3.1. Focal organisms and experimental enclosures: (a) *Pseudomyrmex spinicola* worker patrolling a bull-horn acacia *Vachellia collinsii* plant (Photo credit: S. Amador). (b) Leaf terminology for a *V. collinsii* leaf. In-situ heating (c) and control (d) enclosures. Heating enclosures contained a black sheet of paper placed inside (I) and were secured to the ground with rocks (II). Control enclosures had holes in the plastic (III) and were not secured to the ground (IV). Enclosures were open from the top

Activity:

The activity of ants on the plants was measured on the branches and the leaves.

On the branches:

To measure activity on the branch, a point was marked on three branches of the plant growing in different directions from the main trunk. We selected branches with visible new leaf growth. The points were marked between two nodes with thorns, about

midway through the branch. The points were marked with a marker at least 24 hours before any observations to let the ants acclimate to the marker ink. These points were observed for 3 minutes, and the ants crossing the mark were counted. Average activity across the three branches was used for further analysis. Observations were conducted between 07:00 and 09:30 for "morning" measurements and between 12:00 and 15:00 for "afternoon" measurements.

Before setting up the enclosures, activity was measured twice in the morning, and twice in the afternoon, four weeks apart. Measurements from the two sets were pooled by taking the mean for each time interval because there was no difference in activity between the two sets. To analyze activity patterns before the enclosures were set up, data were square root transformed to satisfy assumptions for parametric statistical tests. One outlier colony was removed from all further analyses of ant activity. A linear mixed-effect model was used to check if the time of day and sun exposure affected ant activity, with colony ID as the random factor.

To test for changes in activity levels due to experimental warming, activity was measured again in both the morning and afternoon, one week after setting up the enclosures. We averaged all before treatment and all after treatment measurements for each plant. To analyze the change in activity after temperature manipulation, the difference in activity before and after placement of the enclosures was calculated for each colony, and a Mann-Whitney U test was used to determine a difference in activity change between control and heated plants following Fancher (2013).

<u>On the leaves:</u>

Ant activity on the leaves was only measured after the temperature manipulations with the enclosures. The number of ants on the leaf was counted for three leaves of each plant, in the morning and afternoon, and the total number of ants on the three leaves was used for analysis. To standardize the age of the leaves, we only chose a leaf located one node below a new growth for these observations. The number of ants on the top and bottom surfaces of the leaf was noted to determine if the ants take shelter on the bottom surface during high temperatures. We could not measure activity blind because we could see the kind of enclosure on each plant.

A zero-inflated Poisson regression model was used to determine if treatment, time of day, and sun exposure affected the number of ants patrolling the leaves.

Temperature measurements:

During each activity observation, we measured the temperature on the same branches using an infrared temperature gun (Etekcity Lasergrip). A linear model was used to determine the effect of sun exposure and time of day on the temperature recorded on branches. In addition, dataloggers were placed inside and outside of the enclosures on sunny days to measure the temperature change due to the enclosures (3 control and 8 heat). Temperature and humidity were measured every 10 minutes over 24 hours. All the probes were placed about 15 cm above the ground to measure the air temperatures. The difference between simultaneous inside and outside temperatures was calculated and used as the response variable. A linear mixed-effects model with Colony ID as the random factor was used to determine whether the enclosures altered the daytime temperature (between 07:00 and 19:00) inside the heat enclosures compared to the control enclosures. We also measured the temperature inside and outside the acacia thorns with a

thermocouple (Omega HH21 Microprocessor thermometer). The temperature inside was measured in brown thorns with ants inside to ensure that the thorns were neither old, brittle, and abandoned, nor very young and green. To avoid disturbing the colonies and because temperature manipulations were not necessary, we measured thorn temperatures on different acacias than the ones we selected for the study. The temperatures were measured for 13 thorns, each on a different acacia plant. The temperature outside was measured immediately next to the thorn. Both temperatures were recorded when there was no wind. A paired t-test was used to compare temperatures inside and outside the thorns.

Physiological tolerance:

To determine how *P. spinicola* acclimated to changing temperatures, three workers were collected from each colony before and three weeks after setting up the enclosures to measure their critical thermal maximum limit (CT_{max}). Actively patrolling individuals were collected from the plant between 09:00 and 10:00. The ants were placed in straight-walled 2 ml microcentrifuge tubes. The cap was filled with modeling clay to prevent any thermal refuge for the ants. The tubes with ants were placed in a dry heat block (Benchmark Mini Dry Bath) and exposed to incremental temperature increases. The starting temperature was 36°C and increased by 2°C every 10 minutes. Every 10 minutes, the righting response of the ants was checked by gently flicking the tubes and noting if the ants could get back up on their legs. The temperature at which the ants lost their righting capability was recorded as the CT_{max}. The CT_{max} of each colony was determined by taking the average CT_{max} of the three workers. The measurements were carried out on the same day that the ants were collected. We also measured CT_{max} for ten

newly emerged *P. spinicola* queens caught on young *V. collinsii* saplings in PNM. CT_{max} was also measured for ten colonies (three workers each) of *P. spinicola* from Bosque Seco Coronado, a dry forest in the Panama Oeste province of Panama that is hotter and drier than Panama City (Slot et al. 2021). A Mann-Whitney U test was used to determine if the change in CT_{max} post-treatment differed between the two treatments. Paired Wilcoxon tests were used to compare pre- and post-treatment CT_{max} for each treatment.

Defense:

To determine if the treatment affected the defensive behavior of the ants, we pinned a leaflet from a different plant, *Enterolobium cyclocarpum* (Fabaceae, Mimosoideae), at the base of the plant's trunk, approximately 5 cm below the first branch. Ants typically remove any competing vegetation from their host plant (Amador-Vargas 2019). Measuring the ants' defensive response to competing plants, rather than herbivorous insects, enabled us to measure the defensive response of the ants without the confounding factor of herbivorous insects' behavior. *E. cyclocarpum* belongs to the same family as the acacia plant and has bipinnate leaves, which enabled the standardization of the protocol. Fresh leaflets from the same compound leaf were used to test the response of all the colonies and control the properties of the foreign plant material, such as leaf age, thickness, and secondary metabolite composition. We recorded 1-minute videos to count the ants interacting with the foreign plant material. The ants respond instantaneously to any disturbance, and since they couldn't entirely remove the pinned leaflet over time, 1 minute was an appropriate amount of time to quantify their response. The videos were recorded in April between 08:00 and 09:00. We tested 29 of the 33 colonies (15 control and 14 heat). A generalized linear mixed model with Poisson

distribution was used to determine if the treatments affected the number of ants defending the plant from a foreign plant material, with colony ID as the random variable.

Herbivory:

We hypothesized that the heating treatment would influence the amount of herbivory that the plants experience. We marked three leaves, one node below a new growth, with a black marker. Pictures of the leaves were taken 2 weeks before setting up the enclosures and after 4 weeks of temperature treatments. Some marked leaves were lost during the experiment, so only the leaves that had both before and after pictures were used for the analysis. The pictures were analyzed using the software DotDotGoose (version 1.7.0) (Ersts Internet). Acacia leaves are compound leaves made of multiple pinnas, each having multiple pinnules (Fig. 3.1b). The number of missing pinnules, partially eaten pinnules, missing pinnae, and total pinnules was counted for each leaf. To minimize bias, the images were analyzed without knowing what treatment the plant received. The percent damage was calculated for each kind of damage as the proportion of missing or partially eaten pinnules out of the total number of pinnules for each leaf. The percent change was calculated as the difference in percent damage after treatment and the percent damage before. A Wilcoxon Rank Sum Test was used to compare the change in damage in the two treatments for each kind of damage.

R version 4.2.1 (R Core Team 2022) was used for all statistical analyses and visualizations.

Results:

Temperature variation:

Temperatures measured on the branches in the afternoon (between noon and 15:00) were about 9°C higher than temperatures on the branches in the morning (between 07:00 and 09:30; Estimate = 9.457, p = 0.001, Fig. 3.2a). The average temperature experienced by the ants was 32.93 ± 4.6°C. The maximum temperature recorded on the branch was 48°C.

The enclosures raised the daytime (between 07:00 and 19:00) temperatures inside the heat enclosures relative to the control enclosure (Linear mixed-effect model: Estimate = 1.247, p = 0.024, Fig. 3.2b). The mean difference between inside and outside daytime temperatures in the heat enclosures was 1.57°C ± 1.859°C, whereas that for control enclosures was 0.26°C ± 0.815°C.

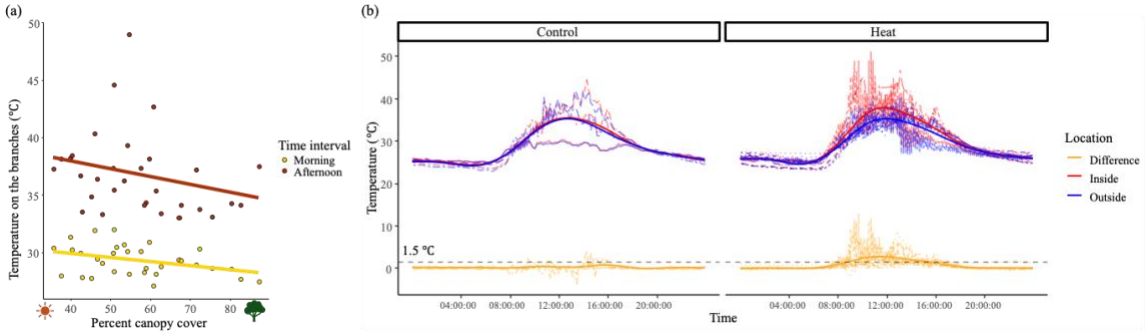

Figure 3.2. Temperature variations: (a) Variation in temperature measured on the branches before placement of the enclosures. Temperatures in the afternoon (red, between noon and 15:00) were about 9°C higher than temperatures in the morning (yellow, between 07:00 and 09:30). (b) Change in temperature due to enclosures. Two data loggers were placed inside and outside the enclosures to measure the temperature change due to the enclosures in both control (three) and heat (eight) enclosures. The red line indicates the temperature inside, the blue line indicates the temperature just outside the enclosures measured simultaneously, and the yellow line indicates the temperature difference. The dotted lines are for individual colonies, and the solid lines are the means. The heat enclosures raised the daytime temperature inside by ~1.5°C (horizontal dashed line)

Activity:

On the branches:

Ant activity was measured before setting up the enclosures to determine the change in activity in response to diurnal temperature variation and the sun exposure of the plant. The activity of ants patrolling the branches was lower on plants in direct sunlight compared to plants under the canopy (Linear mixed effect model: Estimate = -0.183, p = 0.009, Fig. 3.3). The overall activity of ants was marginally lower in the afternoon than in the mornings, but the difference is not statistically significant (Estimate = 0.209, p = 0.06, Fig. 3.3). A significant interaction between sun exposure and time of day shows reduced afternoon activity on sun-exposed trees (Estimate = -0.760, p = 0.011, Fig. 3.3). A significant interaction indicates that the change in activity was driven by temperature, rather than just a change in diurnal pattern due to the time of day. We also found that colonies in the heat treatment reduced activity compared to control colonies (Mann-Whitney-U Test: statistic = 185.5, p = 0.029, Fig. 3.4).

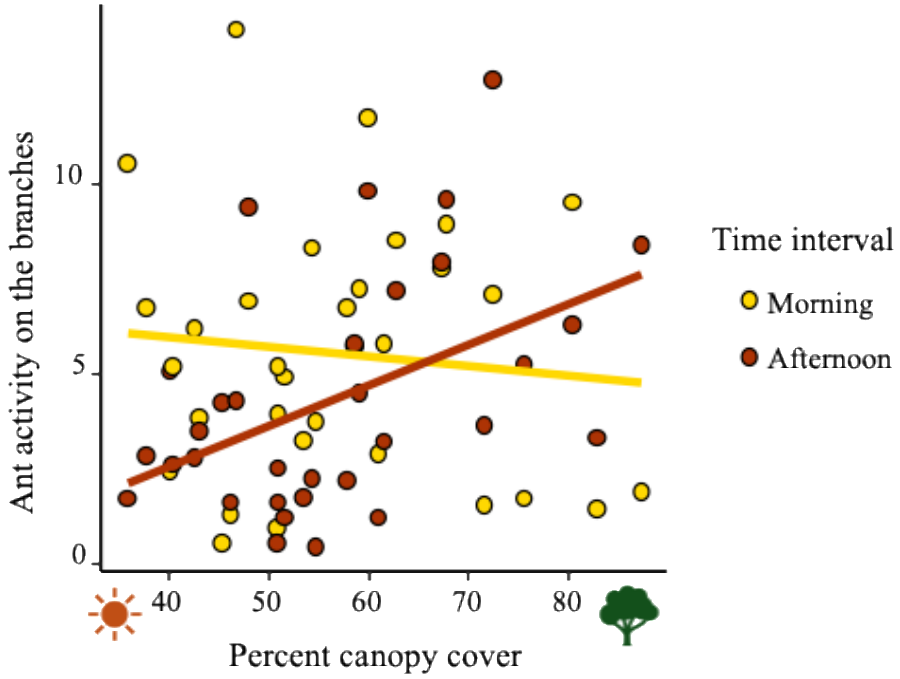

Figure 3.3. Ant activity before enclosures: Ant activity on the branches before the enclosures were set up. Yellow points are activity measurements in the morning, and the

red points indicate activity in the afternoon. The solid lines are plotted using a smoothing function with a linear model. Ants were less active in the afternoon than in the morning. A significant interaction indicates lower activity in the afternoons but on trees in less shade

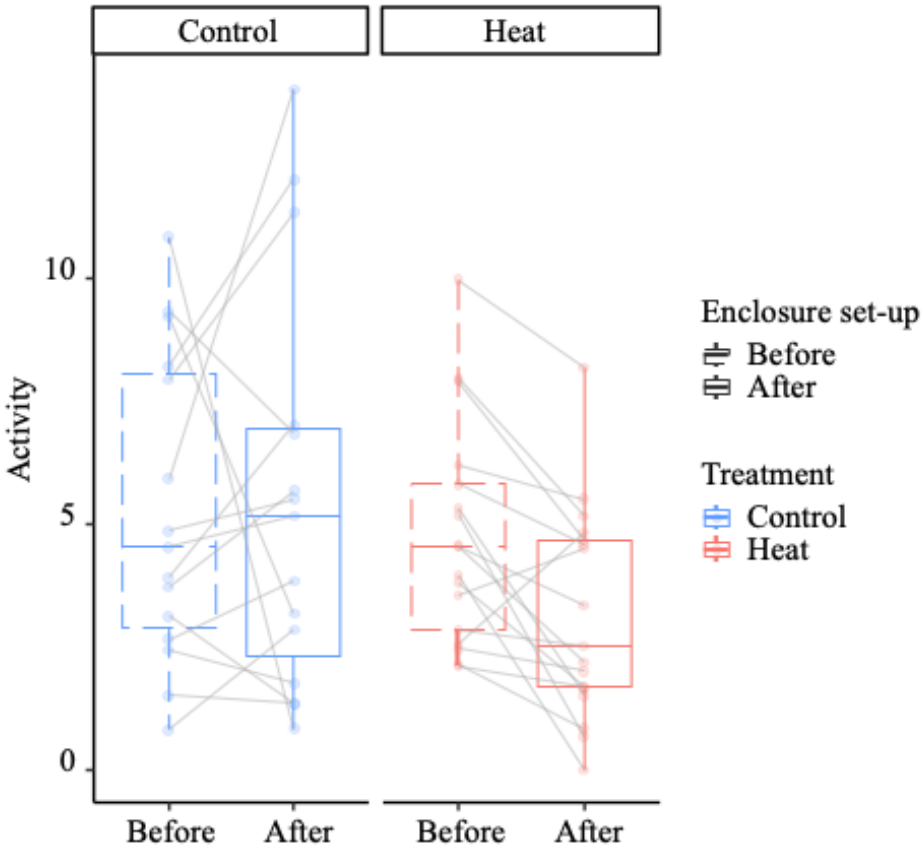

Figure 3.4. Change in ant activity after enclosure treatment: Ant activity in the control (blue) and heat (red) treatments before and after setting up the open-top chambers. The grey lines join values for the same colonies. Ant activity was reduced in plants that received the heating treatment compared to the controls

On the leaves:

Ant activity on the leaves is crucial to defending the plant against herbivores, so we counted the ants on the top and bottom surfaces of the leaves. Fewer ants patrolled the leaves in the afternoon (Zero-inflated Poisson Regression: Estimate = -0.427, p = 0.006, Fig. 3.5a) and on trees in the sun (Estimate = -0.408, p = 0.003, Fig. 3.5a). The temperature treatments did not affect ant activity on the leaves (Estimate = -0.002, p =

0.986). A significant interaction between time of day and sun exposure indicates that fewer ants are present in the afternoon on trees in the shade as compared to the morning, but trees in the sun have fewer ants throughout the day (Estimate = 0.477, p = 0.022, Fig. 3.5a). Similar to total ants on the leaf, the number of ants on the top surface of the leaf is lower in the afternoon (Zero-inflated Poisson Regression: Estimate = -0.760, p = 0.007, Fig. 3.5b) and lower on trees in the sun (Estimate = -0.716, p = 0.002, Fig. 3.5b). The interaction between time of day and sun exposure is significant (Estimate = 1.204, p = 0.002, Fig. 3.5b). In contrast, the number of ants on the bottom surface of the leaf did not vary with the time of day (Zero-inflated Poisson Regression: Estimate = 0.021, p = 0.918, Fig. 3.5c) or sun exposure (Estimate = -0.091, p = 0.630, Fig. 3.5c). From measuring ant activity on the bottom and top surfaces of the leaves, we found that the ants were avoiding direct sunlight by moving away from the exposed top of the leaf but not from the shaded bottom surface.

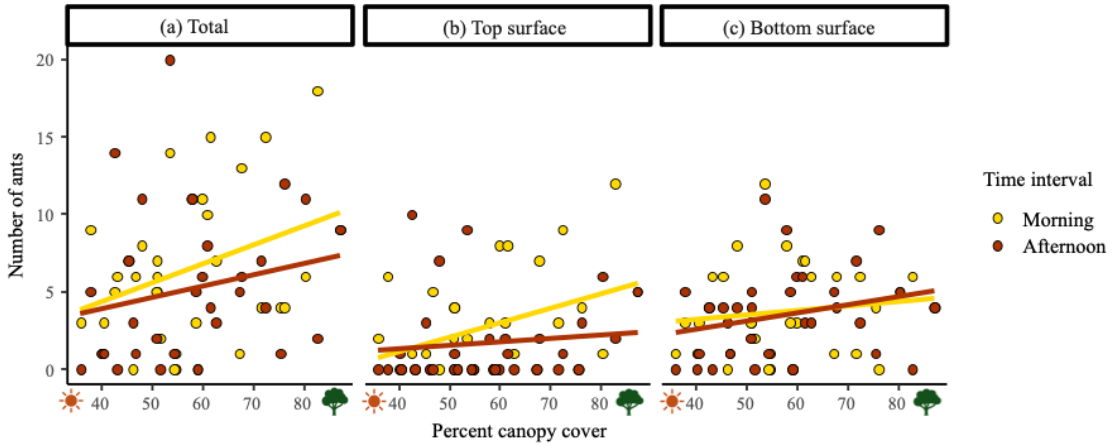

Figure 3.5. Ant activity on leaves: Activity on the leaves (a) Total number of ants patrolling the leaves (total on three leaves per tree). Fewer ants were present on the leaves in the afternoon and on trees in the sun. There was a significant interaction between time of day and exposure. (b) Ants on top of the leaf (total ants on three leaves per tree). Fewer ants were seen on top of the leaves in the afternoon and on trees in the sun. There was a significant interaction between the time of day and sun exposure. (c) Ants on the bottom surface of the leaf do not change with the time of day and sun exposure

Defense:

Changes in ant activity due to temperature variation affected the defensive behavior of the ants. As we expected, fewer ants were defensive against a pinned foreign plant material in the heated colonies than in the control colonies (Generalized linear mixed effect model: Estimate = -1.706, $p < 0.0001$, Fig. 3.6).

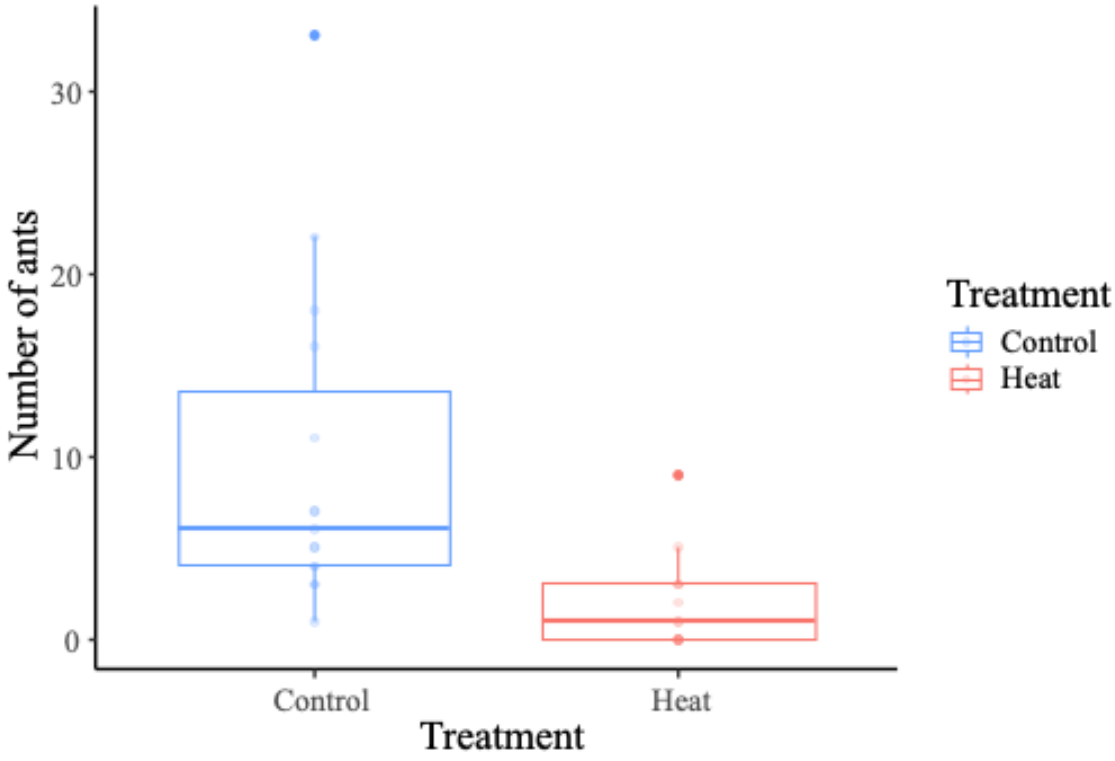

Figure 3.6. Defensive behavior: The number of ants that responded defensively to an external leaf was higher in control colonies than in colonies in the heat treatment

Physiological thermal tolerance:

The maximum critical thermal limit (CT_{max}) of *P. spinicola* was 46.5°C (mean = 46.485, SD = 1.465, n = 33 colonies (3 workers each)). Heated colonies showed no acclimation relative to controls. After being in the temperature treatments for three weeks, the change in CT_{max} for workers in heat colonies was not different from the control colonies (Mann-Whitney-U Test: statistic = 109.5, $p = 0.341$, Fig. 3.7a). For both

control and treatment colonies, the CT_{max} measured post-enclosures was higher than before, probably due to seasonal variation (Wilcoxon test: control: statistic = 15.5, p = 0.067; heat: statistic = 0, p = 0.001, Fig. 3.7a). Newly eclosed queens of *P. spinicola* from PNM had a CT_{max} of 48°C with no individual variation (mean = 48, SD = 0, n = 10, Fig. 3.7b). All individual *P. spinicola* workers from Coronado had a CTmax of 48°C; there was no individual variation (mean = 48, SD = 0, n = 10 colonies (3 workers each), Fig. 7b).

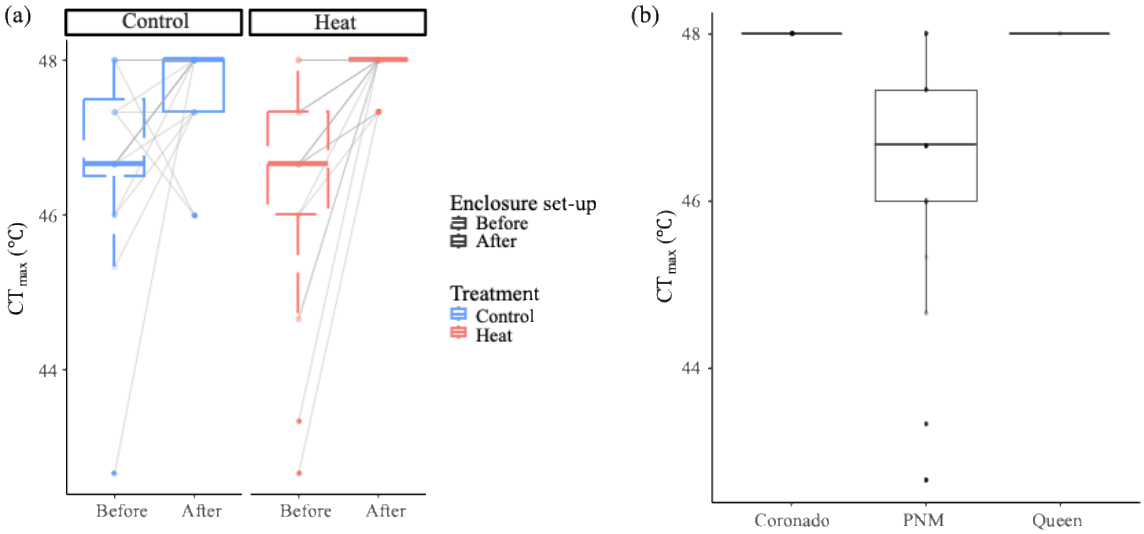

Figure 3.7. Maximum critical thermal limit (CT_{max}): (a) CT_{max} of *P. spinicola* colonies (3 workers per colony) in control and heat treatments before and three weeks after the treatments. The grey lines join values of the same colony. There is no difference in the change (after–before) of CT_{max} between control and heat colonies. (b) All ten Coronado colonies (3 workers per colony) had a CT_{max} of 48°C, with no variation. All 10 newly emerged queens had the same CT_{max} of 48°C as workers, with no variation

Herbivory:

Contrary to expectation, there was no change in the percent herbivory damage observed in the plants in control and heat treatments for any of the types of damages that we measured (Wilcoxon rank sum test for Missing pinna: W = 62, p = 0.816, missing pinnule: W = 80, p = 0.413, and partially eaten pinnules: W = 79, p = 0.442, Fig. 3.8).

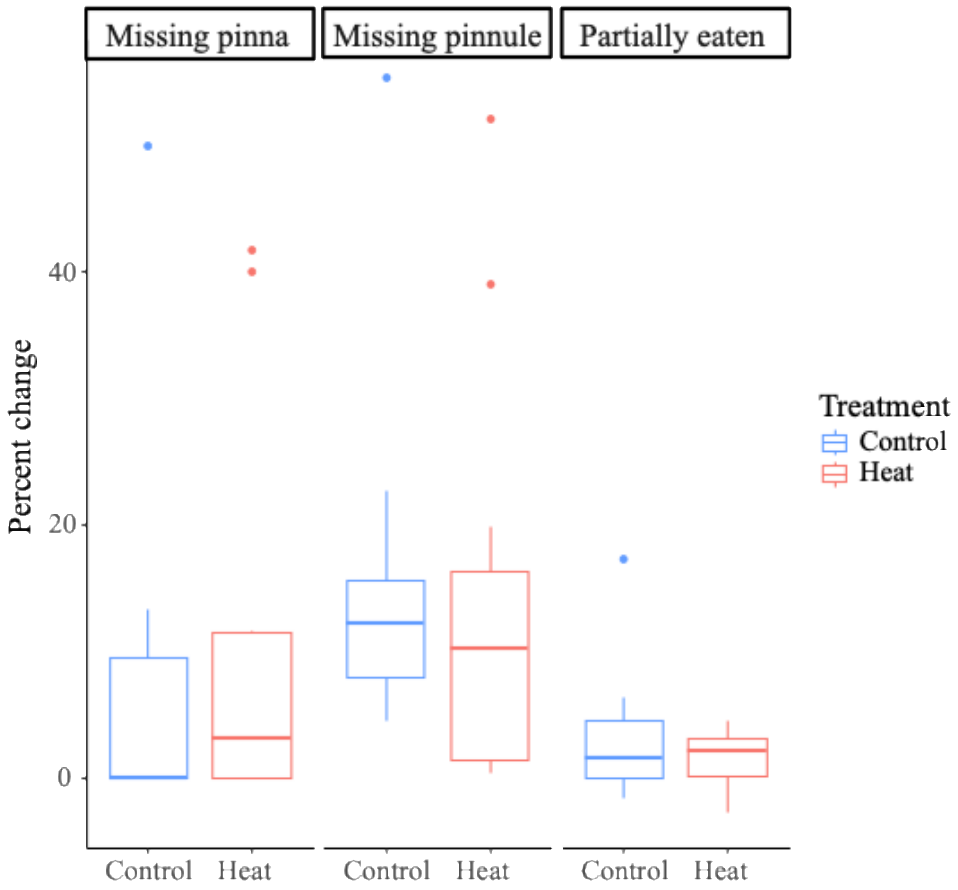

Figure 3.8. Herbivory damage: Change in the different levels of herbivory damage was not influenced by the temperature treatments

The temperature inside and outside the thorns:

The ants stayed inside the thorns when there was reduced activity on the branches and leaves. This protects them from direct sun exposure, but the temperature inside the thorns is 1.88 ± 1.2°C higher than outside the thorns (Paired T-test: t = -5.464, p = 0.0001, n = 13, Fig. 3.9).

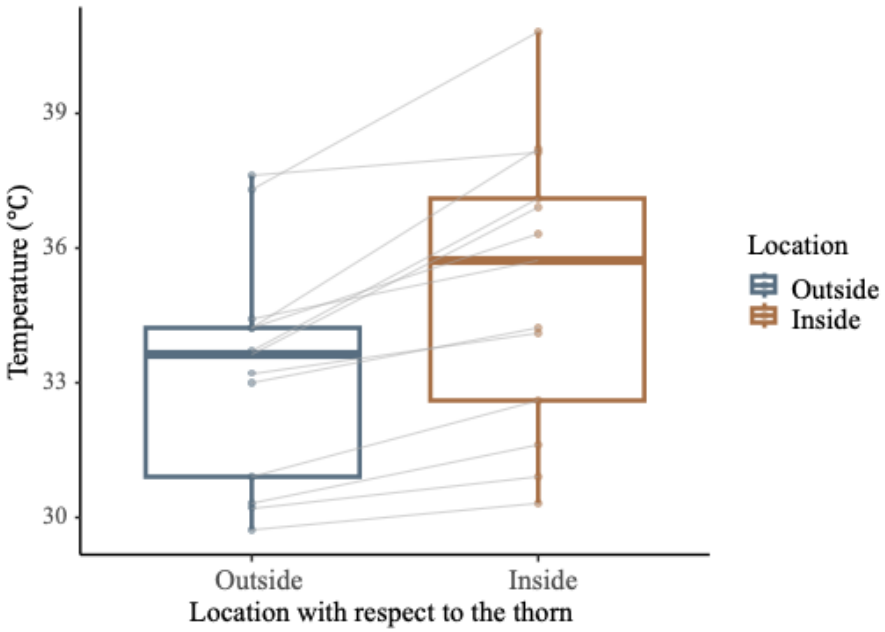

Figure 3.9. Temperature inside and outside the thorns: Temperature inside and outside the acacia thorns. It is hotter inside the thorns than the outside ambient temperature by 1.88 ± 1.2°C

Discussion:

Our observational and experimental data show that *Pseudomyrmex spinicola* ants modulate their behavior in response to heat. We observed lower ant activity on plants that are more exposed to sunlight, particularly during the afternoon. Although there is a large difference between morning and afternoon temperatures on all plants, activity is significantly lower on the plants in direct sunlight. This could be because ants are more exposed to direct sunlight and extremely heated surfaces on these plants. This significant interaction between the time of day and sun exposure on ant activity indicates that we did not merely observe a natural diurnal variation in activity, but demonstrated that the ants actively respond to temperatures on the plant. Furthermore, ants responded to our experimental manipulation of heat treatment by reducing overall activity compared to the control. Reduced activity in response to thermal stress has been observed in several other organisms. Roeder et al. (2022) observed the foraging activity of the red harvester ant *Pogonomyrmex barbatus* at different surface temperatures. They found that as temperatures increased, foraging distance remained unchanged, but the ants spent less time foraging by increasing their tempo. As the temperatures reached their critical thermal limit of 50°C, they stopped all foraging activity. Bumblebees also exhibit reduced foraging activity due to heat stress (Gérard et al. 2022b) and reduced learning with only short exposure to sub-lethal stressful temperatures (Gérard et al. 2022a). Hemberger et al. (2023) found that bumblebees reduced the number and duration of successful foraging visits under an experimental heat wave and were indirectly affected due to reduced nectar production. This does not only apply to small ectotherms: lizards also show behavioral thermal regulation by increased time spent in thermal refuges, which can have fitness

consequences, especially in older adults (Bestion et al. 2015; Díaz et al. 2022; Muñoz-Nolasco et al. 2023). Another study has reported behavioral plasticity in snakes that enables them to successfully avoid heat stress; however, this ability is diminished when coping with yearly temperature fluctuations (Aubret and Shine 2010). Thus, our study and studies in other taxa highlight the importance of studying the behavioral response to high temperatures, especially at sub-lethal stressful temperatures, which will help us determine if the physiological and behavioral responses are adequate to cope with climate change (Penick et al. 2017; Meza-Parral et al. 2020; Youngsteadt et al. 2023; White and Dillon 2023; Tatu et al. 2024).

The lower activity demonstrated in our study resulted in fewer ants trying to remove an *Enterolobium* leaflet from the acacia trunk. This reduced defensive behavior could impact how effectively the ants protect the acacia plant from herbivores and competing plants growing nearby. In other systems, the response of aggressive behavior to temperature varies. Barton and Ives (2014) studied an ant-aphid mutualism under experimental warming and found that at higher temperatures, the cold-tolerant winter ant *Prenolepis imparis* reduced its aggressive defense of aphids from their predators. Tanner (2009) showed that anaesthetization of individual ants by cooling can impact their subsequent behavior. They found increased aggression in *Formica xerophila* after a cooling treatment, but no effect in *Formica integroides*. Thus, behavioral response can be species-specific and should be examined on a case-by-case basis.

We compared herbivory rates in the two treatments to see if the reduced ant defense resulted in higher herbivory, but did not find any differences between the two treatments. Several factors could have influenced this. First, the enclosures that we placed

might have altered herbivore access to the plant. Second, herbivores are less abundant during the dry season, meaning that there may have been little herbivore pressure on the treatment leaves (Aide 1988, 1992; Aide and Angulo-Sandoval 1997; Richards and Windsor 2007). Third, we need a better understanding of the herbivore community on *Vachellia collinsii* and how they are responding to increased temperatures. The acacia plants have lost the ability to produce defensive secondary metabolites, so they are exposed to a wide range of potential generalist herbivores if not defended by the ants (Janzen 1966; Rosumek et al. 2009; Baer 2018; Amador-Vargas et al. 2021). With the ants present, some herbivores can eat the leaves, although there is scarce literature about these specific herbivores. We have observed from personal experience that insects, such as adult leaf beetles, fly in and out to eat the leaves, and caterpillars also chew on them. Some grasshoppers chew the leaves and jump out of the acacias if detected by the ants. Fourth, although we found that fewer ants patrolled the plant at higher temperatures, some ants were still present, which might be sufficient to deter any herbivores. If the herbivores also change their activity patterns in response to temperature, lower ant activity may not be detrimental to the plant. However, mutualistic ants not only defend against herbivores, but pathogens as well. The ants *Pseudomyrmex ferrugineus* are known to play a role in protecting their mutualistic partner plant, *Vachellia hindsii,* against pathogenic bacteria (González-Teuber et al. 2014). It also remains unclear how heat stress affects the tree's production of food rewards for the ants, which could indirectly influence herbivory. Depending on the severity of heat stress, nectar secretion may increase or decrease in Mediterranean plants (Takkis et al. 2018). Nectar concentration is also affected by temperature, with variable effects depending on the

species (McCombs et al. 2022; Plos et al. 2023). Thus, it would be beneficial to pursue additional research to examine the direct effects of warming on plants, herbivores, and pathogens, as well as the indirect effects on the acacia ant-plant system.

P. spinicola exhibits behavioral thermoregulation to escape high temperatures. They avoid direct sunlight in the afternoons while patrolling the leaves by moving away from the top surface but remaining active on the underside. Some of the acacia plants chosen for the experiment were part of a cluster occupied by the same colony, and the ants traveled between the plants within this cluster. Interestingly, we observed two instances when the ants completely abandoned the plant in the heat treatment and escaped to nearby acacia plant(s) in the patch that were not a part of the study. They did return to the treatment plant later, likely when the temperatures became suitable. This was not observed for any plants in the control treatment. Thus, if possible, they might evacuate the plant at high temperatures. Other tropical ants are known to exhibit the absconding behavior at high temperatures. Bujan et al. (2022) showed that tropical, canopy-dwelling ants abscond their nest under thermal stress. The temperature at which they abscond and whether they evacuate completely by carrying their brood, changes with species. The *Pseudomyrmex* ants in this study (which did not include *P. spinicola*) absconded from their nests at temperatures ranging from 45°C to 47°C, which is below the average critical limit for the genus, and abandoned their brood during the absconding process. Thus, we may have witnessed absconding by *P. spinicola*, but they return to the nest when conditions become favorable. Future studies could examine how thermal stress and potential absconding by adults affect the brood. Further investigation is also required to determine if the ants gradually shift their activity hours and possibly increase night-time

activity in response to high daytime temperatures. Previous studies have shown a mixed response, as some report a shift to nocturnality (Briese and Macauley 1980; Heatwole and Muir 1989; Cros et al. 1997), while others do not (Youngsteadt et al. 2023).

P. spinicola is already experiencing temperatures close to its critical thermal maximum (CT_{max}) of 46.5°C. The maximum CT_{max} measured was 48°C. The highest temperature recorded on the branch was also 48°C, and temperatures often exceeded 40°C in the afternoons. When we measured the temperatures inside the thorns, they were higher than the outside temperatures. The highest temperature recorded inside the thorn was 40.8°C. Thus, while the thorns do not provide a cooler environment, they at least protect the ants from exposure to direct sunlight. The change in CT_{max} was not different between control and heated colonies after they had been in the treatments for at least 3 weeks. Previous studies indicate that 3 weeks is long enough for ants to exhibit an acclimation response (Jumbam et al. 2008; Modlmeier et al. 2012; Coulin et al. 2019; Bujan et al. 2021). We also measured the CT_{max} of *P. spinicola* workers from Coronado. Coronado is hotter and drier than Parque Metropolitano, where the experiment was conducted. *P. spinicola* workers from Coronado all had a CT_{max} of 48°C, with no individual variation. These findings together indicate a limited acclimation ability in *P. spinicola* with a maximum thermal tolerance of 48°C, likely due to physiological or evolutionary constraints. Recently, a large meta-analysis of the plasticity of insect thermal limits found weak overall plasticity, with CT_{max} increasing by 0.9°C for every 1°C increase in acclimation temperature (Weaving et al. 2022). Another meta-analysis of ectotherms, including both invertebrates and vertebrates, found that terrestrial insects exhibited lower plasticity in thermal limits compared to aquatic taxa such as crustaceans

and fish (Gunderson and Stillman 2015). Based on their habitat-dependent patterns, they suggest that animals with higher behavioral plasticity in response to temperature exhibit lower plasticity in thermal limits. Our data aligns with these studies and further demonstrates that behavioral adaptations, such as those we observed, will be crucial for insects to cope with climate change.

We predicted that if the ants had an acclimation capability, ants in the heated treatment would have a higher CT_{max} than those in the control. Our data indicates a slight increase in CT_{max} in both treatments. Thus, temporal factors, apart from the treatment itself, may be influencing the CT_{max}, and seasonal variation could be a contributing factor. Seasonal variation has been observed in ants and other insects that experience a much greater temperature variability than the tropics (Bujan et al. 2020). These studies reported variation in CT_{max} of ants collected a few months apart. However, this is not directly comparable to the tropical ant community that experiences lower seasonal temperature variability than temperate regions. The average high temperature in Panama during the dry season (from February to April) is approximately 2°C higher than during the wet season, with April being the hottest month. The Smithsonian weather station at the study site records air temperatures every 15 minutes at a height of 68m (Paton 2025). This data indicates that the average temperatures in April (when CT_{max} post-enclosures were measured) were 0.7°C higher than the average temperatures in February (when CT_{max} pre-enclosures were measured). So, while there is some seasonal variation in temperatures between the wet and dry seasons, there is lower variation between the time points when CT_{max} was measured. However, we require more information from multiple time points throughout the year to draw any conclusive statements. CT_{max} is dependent on

several other factors, including food intake and temperature during the larval stage (Roeder et al. 2021). Thus, whether this seasonal variation is driven by temperature, humidity, or changes in food rewards by the plant is also not certain.

CT_{max} is often measured for workers, but there is limited information on the reproductives (Nascimento et al. 2022). The CT_{max} of queens and workers is the same for *Wasmannia auropunctata* (Coulin et al. 2019), whereas *Myrmecocystus romainei* workers have a higher CT_{max} than queens (Kay and Whitford 1978). Newly emerged queens from Parque Metropolitano also had a CT_{max} of 48°C. These newly emerged queens independently seek young, unoccupied acacia plants to establish a colony, exposing them to temperature variations as they find a suitable plant and raise the first brood of workers. Therefore, the thermal limits of the queens are crucial for successful colony founding and are likely directly influenced by climate warming. In the facultatively polygynous desert ant, *Pogonomyrmex californicus*, the number of queens affects the critical thermal limit of workers (Baudier et al. 2022). A larger number of founding queens results in higher CT_{max} of workers and greater variation in their CT_{max} compared to colonies founded by single queens. This suggests an advantage of polygyny in coping with thermal stress. The study emphasizes the importance of trait measurement for reproductives and the unique advantage for social insects compared to solitary insects.

Our study aimed to elucidate the impact of thermal stress on the behavior of the ant *Pseudomyrmex spinicola*, a mutualist of the acacia plant *Vachellia collinsii*. We demonstrate that the ants exhibit reduced activity and protection services in response to high temperatures and also exhibit some thermoregulatory behaviors to escape extreme heat. They are already functioning at temperatures close to their physiological tolerance

limit, and future climate warming will negatively impact the ants. This could destabilize the mutualism and have cascading effects on other associated organisms, such as herbivores, predators and parasites of these herbivores, ant predators, and even birds that selectively nest on the acacias. Also, while *P. spinicola* exclusively nests in *V. collinsii*; the plant can be inhabited by other ants that vary in the protection services they offer (Amador-Vargas et al. 2021). Thus, it would be beneficial to understand the response of these other ants to warming and whether warming could lead to a switch in the associated ant species. Further studies to examine the direct and indirect effects of temperature on the acacia plant and its herbivores will also help determine whether this mutualism will persist under a changing climate.

Dissertation conclusion

My research aimed to elucidate the effect of sub-lethal temperature increases on the behavior of ants. I employed three different methodological approaches in two study systems spanning the temperate and tropical regions. By surveying *Aphaenogaster* ants along an elevation gradient in Shenandoah National Park, I found that there is a strict boundary between the low-elevation *A. rudis* and the high-elevation *A. picea* at about 700m. There were no sites with sympatric populations of the two species. I also found that *A. rudis* is more active and has higher foraging efficiency than *A. picea*, which could explain its upward range expansion, but long-term data from Shenandoah National Park is required to determine the extent of upward range expansion by *A. rudis,* as has been reported in the Southern Appalachian Mountains. Exposure of the two species to artificial temperature manipulation in the lab revealed that both species are stressed at elevated temperatures of 28°C, which resulted in lower activity, slower food discovery, and fewer foragers participating in food retrieval compared to 25°C. This translated into the stressed colonies producing fewer larvae and new reproductives. 28°C is at the higher end of temperatures currently experienced by the species from where we sampled the colonies. Our prolonged exposure to the high temperature could have been more stressful to the colonies; however, it is well within the tolerance limit of the adults. Given that *Aphaenogaster* are keystone seed-dispersing ants, changes in activity patterns can have large-scale effects on the ecosystem. This research highlights that sub-lethal temperature warming can have profound effects on the behavior of ants, leading to colony-level effects on reproductive success. This has long-term implications due to the ecosystem services provided by these ants.

The tropical arboreal ant *Pseudomyrmex spinicola* also decreases the activity and aggressive defense of its host plant, *Vachellia collinsii,* at higher temperatures. The difference in daily activity patterns between plants in the shade and those with high sun exposure showed that the ants actively avoided patrolling the plants when exposed to high temperatures and direct sunlight. These ants have limited ability to acclimate their physiological tolerance range when exposed to high temperatures. Therefore, behavioral strategies to escape heat stress are even more important. The ants were observed to reduce activity at high temperatures and remain inside the plant's thorns. Although this does not shield them from high air temperatures, it offers protection from direct sunlight. While patrolling the leaves during the day, they move away from the exposed surface as temperatures peak and seek shelter on the shaded underside of the leaf. The temperatures measured on the branches approached and occasionally reached the ants' maximum thermal tolerance. The reduced defense response did not lead to increased herbivory on heated plants, but several factors, including the effects of temperature on herbivores and plants, still need investigation. This research further corroborates that tropical arboreal species are more vulnerable because they are approaching their thermal limits, and behavioral thermoregulatory mechanisms can help them avoid heat stress. However, this could also have cascading effects on the mutualism and other interacting species.

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