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ORIGINAL PA

E ects of cation and starvation on ther and the h ock response in forest a

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Temperature increases associ warmi change are likely to be accompanied b onal heat mental stressors such as desiccation and food limi- which may alter how temperature impacts organis- rformance. To investigate how interactions between ors influence thermal tolerance in the common for- nt, *Aphaenogaster picea*, we compared the therm ance of workers to heat shock with and without sure to desiccation or starvation stress. Knoc) time at 40.5 °C of desiccated ants was red pared to controls, although longer exposure ion did not further reduce thermal tolera n, in contrast, had an increasingly severe al tolerance: at 21 days, average KD tim as reduced by 65% compared to contro eduction in thermal tolerance results the heat-shock response, we measu sion and transcriptional induction teins (*hsp70* and *hsp40*) in trea found no evidence that eithe response: both desiccation a basal Hsp expression und did not a ect the magnit These results suggest ple environmental s models may mak

benefit from additional warming, because they typically operate at sub-optimal temperatures and would thus be shifted up their performance curve toward optimal operating body temperature (Deutsch et al. 2008; Diamond et al. 2012; Clusella-Trullas et al. 2011; Kellermann et al. 2012). However, taxa with larger thermal safety margins generally occupy locations with high-temperature variation and extreme high temperatures may cause overheating (Kingsolver et al. 2013). In addition, species that overwinter may be at risk of mortality during the winter months, because warming can impact the microclimate and expose quiescent organisms to higher temperatures (Williams et al. 2015).

Critical thermal limits may also vary with environmental context, enhancing or reducing the thermal safety margin (Cahill et al. 2013; Chahal and Dev 2013; Du y et al. 2015). Although thermal tolerances are typically measured in animals maintained under ideal conditions, extreme heat is projected to co-occur with reduced precipitation (Mueller and Seneviratne 2012), which may result in species simultaneously encountering both thermal and desiccation stresses. Furthermore, temperature may act indirectly through shifts in prey availability or interspecific competition, potentially leading to nutritional stress (Araújo and Luoto 2007).

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

Ants are a good system to explore the impact of di erent stressors on thermal limits, because they have colonized and inhabit diverse environments (Moreau and Bell 2013; Economo et al. 2015). Many species have a broad geographical range and are exposed to considerable environmental variation (Sanders et al. 2007; Dunn et al. 2009; Kaspari et al. 2015). Foraging activity is sensitive to temperature (Albrecht and Gotelli 2001; Wittman et al. 2010), soil moisture (Gordon 2013), available resources (Stuble et al. 2013), and species interactions (Rodriguez-Cabal et al. 2012) that altogether impact food intake for the whole colony. Ants are experimentally tractable for studies of physiological studies in response to multiple environmental conditions. Although ants likely face multiple stressors, we have very little understanding of how these additional sources of environmental stress such as desiccation and starvation are likely to impact thermal tolerance.

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

Materials and methods

Natural history of *Aphaenogaster picea*

Aphaenogaster picea is a ground-dwelling species that occurs in mesic deciduous forests in the eastern United States from the high elevations of Virginia to Maine (DeMarco and Cognato 2015). Across their distribution, mean annual temperature ranges from 5 to 14 °C, but leaf litter temperatures in the summer can be as high as 40 °C, while below-ground temperatures may remain at 20 °C (Lubertazzi 2012). Colonies are comprised of roughly 180–1000 individuals that nest within the soil and coarse woody debris (Lubertazzi 2012). Foragers collect and disperse seeds containing elaiosomes (Warren et al. 2011), which provide the colony with a nutritional benefit (Morales and Heithaus 1998; Clark and King 2012).

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

(mean = 0 and variance = 1) as a measure of size-corrected dry mass. Head width was measured as the maximum distance in mm (to the nearest 0.01) between the eyes using the ImageJ software.

Measuring thermal tolerance

We used a static heat-shock protocol (Terblanche et al. 2011) to avoid the confounding issue of ongoing desiccation associated with a slow ramping protocol (Rezende et al. 2011). Preliminary trials revealed that 40.5 °C yielded KD times under an hour and that ants are able to recover from and survive for at least a few days. For each set of ten nest-mate workers associated with each timepoint and treatment (see pre-treatments above), pairs of randomly selected workers were placed in five separate 5mL glass screw-cap vials. Three of the five vials were heat shocked by fully submerging the vial at 40.5 °C in a pre-set Thermo Neslab EX17 heating water bath, while the remaining two vials were simultaneously held at room temperature (25 °C). Heat-shocked ants were observed continuously at a temporal resolution of roughly 10 s until KD, defined as loss of activity (Terblanche et al. 2011). To avoid bias, we measured KD times without prior knowledge of the treatment groups.

Measuring the HSR

For the subset of colonies that we sampled to measure the HSR, the ants were exposed to identical heat-shock and control conditions as those in the thermal tolerance assay, but were removed at 25 min and flash-frozen in liquid nitrogen and stored at −80 °C. Ants were sampled regardless of KD status, and preliminary analyses showed that ants were able to induce *hsp70* and *hsp40* at the 25 min mark.

For each gene expression sample, two of the four flash-frozen ants were pooled and homogenized in a bullet blender homogenizer (Next Advance Inc., USA) at top speed (10) with 1.4 mm zirconium silicate beads (Quackenbush Co., Inc, USA). RNA was isolated with RNAzol (Molecular Research, USA) and then purified with the RNeazy Micro Kit (Qiagen, USA), both following the manufacturers' instructions. 100 ng of RNA was converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) following the manufacturer's instructions.

The gene expression patterns of *hsp70* (*hsc70-4* h2 orthologue) and *hsp40* were quantified using previously developed primers (Nguyen et al. 2016) with RT-qPCR on a StepOnePlus instrument (Applied Biosystems, USA). Each sample was run in triplicate in 20 µL reactions comprised of 2 ng of template, 250 nM of forward primer and of 250 nM reverse primer, and 1× Power SYBR® PCR master mix (Life Technologies, USA). Reactions were incubated at 95 °C for 2 min and then underwent 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s. Amplicon specificity was assessed with a melt-curve analysis. We used the geometric mean of *ef1β* and *gapdh*as house-keeping genes, which had the lowest measure of variation according to NormFinder (stability = 0.23; Andersen et al. 2004). We used $2^{-\Delta\Delta CT}$ as the measure of basal gene expression and fold induction under heat shock (Livak and Schmittgen 2001). For basal gene expression, $2^{-\Delta\Delta CT}$ was calculated relative to time and colony-matched controls (water-plugged treatment or fed treatment). Fold induction of heat-shocked ants was calculated relative to time and colony-matched controls (room temperature, 25 °C).

Statistical analyses

All statistical analyses were performed in R (version 3.2; R Core Team 2016). In all of our statistical analyses, colony was treated as an independent block for estimating treatment e ects; including colony as a random e ect achieved similar results and we present only the findings from fixed e ects models. Survival was analyzed with a GLM, which fits a logistic relationship between the proportion of individuals surviving and time (hours or days). Lethal time at 50% (LT_{50}) was estimated from GLM-fitted models with the *dose.p()* function in the MASS package. We determined the e ect of time, treatment, and time × treatment interaction on KD time or Hsp gene fold induction with an ANCOVA. To avoid over-fitting statistical models, we used a backwards AIC selection criterion with the *stepAIC()*

with most water loss occurring in the first hour. With heat shock, the KD time of desiccated ants was reduced within the first hour by 6% compared to controls ($F_{1,58}$ = 25.21, $p < 0.001$; Table 1) and did not decrease further with through time (Fig. 1

Fig. 2 E ect of desiccation (*dark gray*) on *hsp70* and *hsp40* gene expression. **a**, **c** Show basal gene expression between control and desiccation treatments. **b**, **d** Show the extent of fold induction of HS relative to non-HS ants between control and desiccation treatments. For each treatment and timepoint, there were 4–5 colony-level replicates and *error bars* represent ± 1 standard error of the mean. For basal gene expression, $2^{-\Delta\Delta CT}$ was calculated relative to time and colony-matched

(Terblanche et al. 2011). In fact, with su cient recovery time between these two stressors (Bubliy et al. 2012b) or slow application of desiccation (Benoit et al. 2010), desiccation conferred cross tolerance against heat stress.

Dehydration can either enhance or inhibit thermal

starvation was associated with transient increases in basal Hsp gene expression, and starved and fed ants invested similarly in Hsp gene up-regulation in response to heat stress (Fig. 4), suggesting that allocation of energy to protein protection is not impacted under low-resource conditions. As with desiccation, starvation-induced increases in basal Hsp gene expression at early and late timepoints were not associated with increases in KD time (Fig. 4a, c). It is possible that other molecular pathways that contribute to coping with stress that were not measured here, such as damage repair, redox regulation, and energy metabolism, are depressed by starvation and outweigh the slight increase in the Hsp response (Zinke et al. 2002; Kültz 2005).

Taken together, the results of this study suggest that single-stressor assays may not be a reliable method for estimating thermal tolerance, and thus the capacity to withstand additional warming. Future climate change is likely to impose simultaneous combinations of environmental stressors such as temperature, desiccation, and starvation. Each of these is likely to impose stress on individual and colony-level performance and elicit physiological defenses; however, in addition to their independent e ects, their interaction has the potential further reduce temperature tolerances. To improve species forecasts, models of physiological responses to climate change should account for these diverse sources of stress (Terblanche et al. 2007).

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Compliance with ethical standards

Conflict of interest No competing interest declared.

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