Remarkable insensitivity of acorn ant morphology to temperature decouples the evolution of physiological tolerance from body size under urban heat islands

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ABSTRACT

Environmental temperature can alter body size and thermal tolerance, yet the effects of temperature rise on the size-tolerance relationship remain unclear. Terrestrial ectotherms with larger body sizes typically exhibit greater tolerance of high (and low) temperatures. However, while warming tends to increase tolerance of high temperatures through phenotypic plasticity and evolutionary change, warming tends to decrease body size through these mechanisms and thus might indirectly contribute to worse tolerance of high temperatures. These contrasting effects of warming on body size, thermal tolerance, and their relationship are increasingly important in light of global climate change. Here, we used replicated urban heat islands to explore the size-tolerance relationship in response to warming. We performed a common garden experiment with a small acorn-dwelling ant species collected from urban and rural populations across three different cities and reared under five laboratory rearing temperatures from 21 to 29 °C. We found that acorn ant body size was remarkably insensitive to laboratory rearing temperature (ant workers exhibited no phenotypic plasticity in body size across rearing temperature) and among populations experiencing cooler rural versus warmer urban environmental temperatures (no evolved differences in body size between urban and rural populations). Further, this insensitivity of body size to temperature was highly consistent across each of the three cities we examined. Because body size was robust to temperature variation, previously described plastic and evolved shifts in heat (and cold) tolerance in acorn ant responses to urbanization were shown to be independent of shifts in body size. Indeed, genetic (colony-level) correlations between heat and cold tolerance traits and body size revealed no significant association between size and tolerance. Our results show how typical trait correlations, such as between size and thermal tolerance, might be decoupled as populations respond to contemporary environmental change.

1. Introduction

Human-induced changes in climate and land-use have increased global temperatures and led to widespread effects on plant and animal communities, yet we know little about the mechanisms that determine whether organisms will persist or perish in response to temperature changes (Chevin et al., 2010; Urban et al., 2016). Ectothermic species, particularly insects, appear to be especially vulnerable to rising temperatures; even within protected natural areas, declines in insect abundance approach or exceed 75% over the past several decades (Dirzo et al., 2014; Hallmann et al., 2017; Lister and Garcia, 2018). The ability to tolerate high temperatures is, unsurprisingly, a critical modulator of population persistence as the climate warms (Chown and Gaston, 2016). But what makes some organisms more heat-tolerant than others?

Although many traits contribute to greater heat tolerance (Williams et al., 2016), body size can be an important determinant of heat tolerance (Angilletta, 2009). However, the role of body size in shaping tolerance responses is complex: in aquatic systems, there is a negative

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association between body size and heat tolerance, which appears to be driven by gas exchange processes in large-bodied organisms (Verberk and Bilton, 2013). In terrestrial systems, the connection is less clear, though for some systems, a positive association between body size and heat tolerance appears to be driven by an increased ability to cope with cellular damage (Chown and Nicolson, 2004; Klok et al., 2004).

Cities, through the generation of urban heat island effects, provide warmed mesocosms (Diamond et al., 2015; Rivkin et al., 2019) where we can explore the relationship between body size and thermal tolerance. However, given the very recent origins of urban evolutionary ecology as a field (Rivkin et al., 2019), only a handful of studies have explored the size-tolerance relationship in cities. In water fleas (*Daphnia magna*), plastic and evolved shifts toward greater heat tolerance in cities are associated with smaller body size (Brans et al., 2017), as expected for aquatic systems. For terrestrial systems, synthetic research identified a general phenotypic trend toward smaller body size in cities in many taxa, albeit with several exceptions (Merckx et al., 2018). These patterns may be a result of plastic responses of body size under warm environmental temperatures (the 'temperature-size rule'; Atkinson, 1994) or evolved responses. Warmer temperatures may select for smaller adult body sizes through life history tradeoffs between development time and body size, and the benefits of rapid maturation in warm environments mediated through lifetime fecundity (Angilletta et al., 2004; Kingsolver and Huey, 2008). In any case, the differential effects of temperature on size and tolerance combined with the positive size-tolerance association sets up contrasting predictions regarding the effects of urban heat islands on body size, thermal tolerance and their relationship in terrestrial habitats: tolerance typically improves with larger body size, but cities tend to decrease body size.

Here we use acorn ants (*Temnothorax curvispinosus*), a terrestrial system for which we have demonstrated plastic and evolved shifts toward greater heat tolerance in cities (Diamond et al., 2018) to explore these contrasting predictions for thermal tolerance and size under temperature rise. We measured the body size of individual worker ants from urban and rural source populations across three cities (Cleveland, Cincinnati and Knoxville, USA). These ants were reared under five common garden rearing temperatures and phenotyped for heat and cold tolerance. For many terrestrial ectothermic species, there is a general pattern of greater thermal tolerance (heat and cold tolerance) with increasing body size (Angilletta, 2009); further, because heat and cold tolerance responses represent separate mechanisms (Chown and Nicolson, 2004), body size could be associated with these traits in different ways. In two of the three cities, Cleveland and Knoxville, we previously found evidence of plastic and evolved shifts toward greater heat tolerance and diminished cold tolerance in urban populations (Diamond et al., 2018, 2017). The third city, Cincinnati, showed no evolutionary differentiation in heat tolerance and an evolved reverse pattern for cold tolerance, where urban populations were more cold-tolerant than rural populations. This among-city variation of whether shifts in heat and cold tolerance went in expected directions (as in Cleveland and Knoxville) or unexpected directions (as in Cincinnati) provides an opportunity to explore the contrasting predictions for urban heat island effects on the relationship between thermal tolerance and body size.

In this study, we first asked how body size responded plastically to laboratory rearing temperature and how body size might evolve in response to urban heat islands. We then asked how body size was associated with heat and cold tolerance traits. If body size is independent of the heat and/or cold tolerance response, we expect urban heat islands to lead to smaller body size, via plastic and/or evolutionary responses to temperature rise, as is typical of terrestrial insects. However, if body size underlies a substantial portion of the tolerance response, we can make several alternative predictions: 1) the plastic effects of increased temperature leading to smaller body size should be accompanied by diminished heat and cold tolerance; the result for heat tolerance would be especially important in revealing a role for body size in tolerance, as heat tolerance is typically greater in warmer

environments; 2) the evolved shifts toward greater heat tolerance of urban populations in Cleveland and Knoxville should be accompanied by larger body size; and 3) the evolved shifts toward greater cold tolerance of urban populations in Cincinnati should be accompanied by larger body size.

2. Materials and methods

Acorn ants (*Temnothorax curvispinosus*) are a small, cavity-nesting species of ant. At maturity, colonies can contain approximately 200 workers and up to several queens (this species is facultatively polygynous; Stuart et al., 1993) that all reside within a single to several tree nuts (for example, oak and hickory), small twigs or other cavities (Herbers, 1989). Because of their unique nesting structure among the ants, entire colonies can be collected simply by bringing the acorn nest into the lab.

Acorn ant colony collections, common garden rearing and assessment of thermal tolerances were performed as part of a previously published study (Diamond et al., 2018). In the work presented here, we measured the body size of all worker ants that underwent these thermal tolerance assays and examined: 1) the plastic and evolved responses of body size to urban heat islands, and 2) the relationship between body size and thermal tolerance traits. We begin by briefly reviewing the procedures employed in the thermal tolerance study, and then describe the measurement and analysis of the body size data.

2.1. Ant colony collections and common garden experiment

We collected acorn ant (*T. curvispinosus*) colonies in the spring and early summer of 2016 from urban and rural populations across three cities including Cleveland, Ohio (42°N latitude), Cincinnati, Ohio (39°N latitude), and Knoxville, Tennessee (36°N latitude). We considered urban populations of acorn ants as those inhabiting sites with 40–60 percent developed impervious surface (ISA), and rural populations as those inhabiting sites with 0 percent ISA (Appendix A, Table A1). Acorn ant nest temperatures were elevated by one to several degrees Celsius in urban sites compared with rural sites across the three cities (1.61 °C for Cincinnati, 3.64 °C for Knoxville, and 4.45 °C for Cleveland).

Colonies were placed into one of five randomly assigned temperature treatments (21, 23, 25, 27, or 29 °C; Percival growth chambers, 36-VL), each with a long-day light cycle (14L:10D). This temperature range was selected to capture growing season temperatures across our three focal cities (Diamond et al., 2018, 2017). Ant colonies were housed individually within 120 mL plastic cups. Two 1.5 mL resource tubes, one with plain tap water and the other a 25% sucrose solution, were provided inside each cup along with dead meal worms supplied *ad libitum*. Each colony was reared at its treatment temperature for a minimum of 10 weeks. This design ensured complete turnover of workers within the colony, as worker lifespan is on average 30 days (Modlmeier et al., 2013) and worker development time from egg to callow worker ranges from 30 to 60 days over the temperature range used in our experiment, with several new eggs laid each day (Penick et al., 2017). Because we performed all thermal tolerance and body size trait measurements on these F1 offspring workers raised entirely within the laboratory environment, we were able to mitigate maternal effects and eliminate field developmental acclimation effects (also see Martin et al., 2019). This design allowed us to disentangle plastic effects of temperature (phenotypic differences among ants raised within the five laboratory rearing temperature treatments) from evolutionary divergence in response to urban heat islands (phenotypic differences stemming from genetic differences among ants from urban and rural source populations after rearing for a complete generation under common garden conditions).

2.2. Thermal tolerance

We assessed heat and cold tolerance of individual worker ants.

Because the assessment of thermal tolerance is destructive, only heat tolerance or cold tolerance could be assessed for an individual, though we tested a minimum of five workers for heat tolerance and another five workers for cold tolerance per colony. Our measures of heat and cold tolerance were the critical thermal maximum and minimum (CT_{max} and CT_{min}), respectively. We used a dynamic temperature ramping protocol of $1\,^{\circ}\text{C min}^{-1}$ with starting temperatures of $34\,^{\circ}\text{C}$ and $16\,^{\circ}\text{C}$ to assess CT_{max} and CT_{min}, respectively. Ants were placed individually into 1.5 mL Eppendorf tubes, and the temperature was manipulated using a dry block incubator (Boekel Scientific Tropicooler; the incubator is accurate to $0.1\,^{\circ}\text{C}$, though our estimates of thermal tolerance are on the order of $1\,^{\circ}\text{C}$ resolution). Ants were checked every minute for CT_{max} and CT_{min}, defined as the loss of muscular coordination (Lutterschmidt and Hutchison, 1997). This protocol allows for direct comparison of thermal tolerance values across different groups.

Although some have advocated for the use of surface temperature, rather than air temperature (as we used here) to estimate thermal tolerance values, as the choice of tolerance metric has been suggested to alter the size-tolerance relationship (Kaspari et al., 2015), we preferred air temperature for several reasons: 1) the goals of our study focused on disentangling plastic from evolved responses of body size and thermal tolerance to temperature, rather than assessing microclimatic effects on these traits; 2) our study was performed at the intraspecific level, and the effects of microclimate on the size-tolerance relationship are more likely to be important for interspecific comparisons where there is a greater range of variation in trait values; and 3) our focal species of acorn ants live in similar habitats (forest fragments versus intact forest) across our primary axis of interest, urban versus rural population comparisons.

2.3. Body size

All thermal tolerance assays were performed prior to the measurement of body size. Following thermal tolerance assays, ants were placed individually into 1.5 mL Eppendorf tubes filled with 95% ethanol. As our estimate of overall body size of individual workers, we used Weber's length (mesosomal length), a standard measure of ant body size (Brown, 1953). We measured Weber's length using a Leica M205-A dissecting microscope and the Leica Application Suite software (LAS version 4.4.0). Using the LAS software, we extended a digital caliper across each ant in profile from the basal angle of the metapleuron to the point where the pronotum meets the cervical shield, providing us with an estimate of Weber's length (in mm) for each individual worker. Weber's length was highly repeatable as indicated by the intra-class correlation coefficient; for a large sub-sample of the ants tested in our study, measurements of Weber's length repeated on the same individual showed that measurements were over 96% similar ($R = 0.961$, $F_{52,53} = 49.9$, $P < 0.0001$; Nakagawa and Schielzeth, 2010). We also explored whether the type of tolerance assessment individual ants underwent (CT_{max} or CT_{min}) affected the estimation of body size (Appendix A, A1. Effects of tolerance type on body size); because we did not detect a significant effect of tolerance type, we omitted this term in our subsequent analyses.

We obtained body size and thermal tolerance estimates for 4947 individual workers from 256 colonies (Appendix A, Table A2) across the three cities ($n_{Knoxville} = 81$; $n_{Cincinnati} = 84$; $n_{Cleveland} = 91$), two source populations ($n_{urban} = 123$, and $n_{rural} = 133$) and five rearing temperatures ($n_{21} = 52$; $n_{23} = 53$; $n_{25} = 50$; $n_{27} = 50$; $n_{29} = 51$; all sample sizes refer to the number of colonies).

2.4. Colony size

Colony size (the number of individuals per colony) can trade off with individual body size in ants (Hasegawa and Imai, 2012; Kaspari and Vargo, 1995; Shik, 2008). Specifically, colonies at the same developmental stage and under the same conditions, can invest in fewer individuals with larger body sizes or greater numbers of individuals with smaller body sizes. As a consequence, it is important to evaluate investment not only in individual worker body size, but also worker number. We performed two related assessments of colony size. First, we assessed whether field-caught colony size (the number of mature workers plus the number of immature workers, or 'brood') differed between our two source populations (urban versus rural) across each of the three cities. These data allowed us to determine whether there were initial differences in the field-caught colonies with respect to their investment in worker and brood number that could potentially influence investment in worker size over the course of the laboratory experiment. Second, we assessed whether investment in the number of individuals in the colony (the number of workers plus the number of brood) produced by the end of the 10-week laboratory acclimation period was related to colony mean worker body size, measured at the end of the laboratory experiment. Both field-caught and post-experiment colony size measurements were performed with the aid of a dissecting microscope (Leica M80) to count the total number of workers and brood present in a colony.

2.5. Statistical analyses

All analyses were performed using R 3.4.3 (R Core Team, 2018), and all models were checked for and conformed to assumptions of normality and homoscedasticity. First, we performed a suite of models aimed at understanding how body size responds plastically to rearing temperature and how it might evolve in response to urban heat island effects. Second, we examined the relationship between colony mean body size of workers and colony mean thermal tolerance (including both heat tolerance and cold tolerance). Finally, we examined whether there were tradeoffs between worker body size and colony size, *i.e.* the number of workers and brood produced over the course of the laboratory experiment.

To perform the first suite of models, we initially constructed a linear mixed effects model with Weber's length as the response variable, and source population (urban or rural), rearing temperature (21, 23, 25, 27, or $29\,^{\circ}\text{C}$), city (Cleveland, Cincinnati, or Knoxville), and the two- and three-way interactions among these variables as predictors. We included colony identity as a random effect to account for autocorrelation of workers from the same colony (all colonies contained a minimum of 10 workers for which body size data were available). Here and elsewhere, heterogeneous variance mixed effects models yielded qualitatively similar results as homogeneous variance models, so we reported results from the latter for simplicity. In the case of strong data separation among cities, and/or in the case of significant interactions with the city term, we constructed separate models for each of the three focal cities. These models shared the same general form as the cross-city model, except that the predictors only included source population, rearing temperature and their interaction (the response variable and random effects structure was kept the same). A significant effect of rearing temperature indicates a plastic response of body size to temperature. A significant effect of source population indicates an evolved response of body size to urban heat islands. A significant interaction between rearing temperature and source population indicates the plastic response to temperature has evolved between the urban and rural populations. Additionally, interactions with the city term indicate plastic and evolved responses to temperature differ among cities; additionally, cities might also exhibit mean differences in body size, *e.g.* across the latitudinal cline from Cleveland to Knoxville, as indicated by a significant main effect of city.

Our second suite of models was focused on whether shifts in body size were associated with shifts in thermal tolerance traits. We examined the correlation between colony-level mean body size and thermal tolerance traits (heat tolerance and cold tolerance). Because these traits are measured on F1 lab-reared offspring from the same colony, we can generally interpret these values as genetic correlations between size and tolerance traits. Owing to some marginal deviations from normality in the distributions of trait values, we performed Spearman's rank correlation analysis. We performed separate correlation analyses for each

combination of city and tolerance trait type (heat versus cold tolerance), resulting in six correlation analyses. Based on the results from our first suite of body size analyses, we pooled data across source population and across rearing temperature. We also performed individual-level analyses of the relationship between tolerance and body size on the same data subsets used in the colony-level analyses. We did these analyses because, regardless of whether these traits are genetically correlated at the colony level, we were additionally interested in whether larger body size conferred greater heat and cold tolerance at the individual level. To address this question, we constructed linear mixed effects models with heat and cold tolerance as functions of body size with colony identity as a random effect.

Finally, we constructed several linear models to assess whether field-caught colony sizes differed among urban and rural populations and whether post-experiment colony sizes traded off with worker body size. To assess whether field-caught urban and rural populations had different colony sizes, we fit linear models with the number of individuals (workers and brood) as the response and source population (urban or rural) as a categorical predictor. For consistency with our focal analyses on body size, we fit separate models for each city. We natural-log transformed the colony size variable to meet model assumptions of normality. To assess whether worker body size was influenced by investment in the number of individuals (workers and brood) over the course of the experiment, we fit linear models with mean colony worker body size (Weber's length) as the response and colony size (natural-log transformed) as a continuous predictor. Separate models were fit for each city.

3. Results

Our first suite of analyses asked whether body size exhibited plastic and evolved responses to urban heat islands across three urbanization gradients. We found that acorn ant body size was robust to laboratory rearing temperature, *i.e.*, we found no evidence of plastic responses of body size to rearing temperature in any of the three cities (Fig. 1, Tables 1,2). Further, we found no evidence of evolutionary differentiation in body size between urban and rural populations across the three cities, as the source population term was never significant (Fig. 1, Tables 1,2).

Acorn ant workers within the highest latitude sites, *i.e.,* urban and rural populations in Cleveland, Ohio, exhibited significantly larger body sizes than workers from the two lower latitude sites, Cincinnati, Ohio, and Knoxville, Tennessee, as evidenced by the significant city term (Table 1) and subsequent post-hoc analyses (Cincinnati – Cleveland: estimate -0.0232, SE $= 0.00404$, $t = -5.75$, $P < 0.0001$; Cleveland –

Table 1
Likelihood ratio test statistics and *P*-values for the significance of city, source population, rearing temperature, and the two- and three-way interactions of these terms on worker ant body size. Significant *P*-values at the 0.05 level are indicated in bold font.

Term	χ^2	P
City	57.6	**3.06E-13**
Source population	0.380	0.537
Rearing temperature	1.09	0.297
City × Source	2.30	0.316
City × Temperature	2.51	0.286
Source × Temperature	0.568	0.451
City × Source × Temperature	2.16	0.340

Table 2
Estimates, standard errors, likelihood ratio test statistics and *P*-values for the effects of rearing temperature, source population, and their interaction on worker ant body size. Models were performed separately for each city.

City	Term	Estimate	SE	χ^2	P
Cleveland	Rearing temperature	−0.000177	0.00124	0.300	0.584
	Source population	0.0184	0.0437	0.607	0.436
	Temperature × Source	−0.000581	0.00173	0.113	0.737
Cincinnati	Rearing temperature	0.00132	0.00160	2.51	0.113
	Source population	−0.0197	0.0583	1.18	0.278
	Temperature × Source	0.00108	0.00232	0.214	0.644
Knoxville	Rearing temperature	0.00205	0.00142	0.319	0.572
	Source population	0.0751	0.0528	0.721	0.396
	Temperature × Source	−0.00322	0.00211	2.34	0.126

Knoxville: estimate 0.0297, SE $= 0.00410$, $t = 7.25$, $P < 0.0001$; Cincinnati – Knoxville: estimate $= 0.00650$, SE $= 0.00417$, $t = 1.56$, $P = 0.266$).

Our second suite of analyses asked whether shifts in body size were related to shifts in thermal tolerance traits. Although we found no evidence that body sizes were altered by laboratory rearing temperature or evolutionary differentiation between urban and rural populations, we nonetheless explored whether colonies with larger body sizes were more heat tolerant or more cold tolerant as is typical of terrestrial insects. Spearman's rank correlations of colony mean body size and thermal tolerance revealed no significant relationships between these traits for any combination of tolerance trait type (CT_{max} or CT_{min}) or city (Fig. 2, Table 3). Our correlation analyses were pooled across the levels of rearing temperature and source population on the basis of non-significant effects from the above-described analyses of body size.
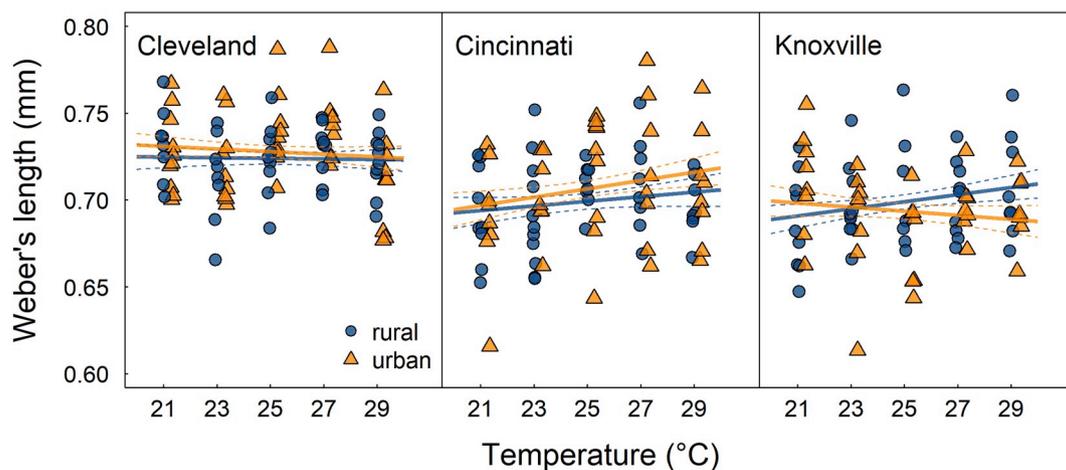

Fig. 1. Acorn ant worker body size (Weber's length, mm) as a function of laboratory rearing temperature (°C) for urban and rural source populations across the three focal cities (Cleveland, Cincinnati and Knoxville). Predicted values (solid lines) and standard errors (dashed lines) from linear mixed effects models, performed separately for each city. Points indicate colony mean Weber's length and are jittered within each combination of source population and temperature treatment.
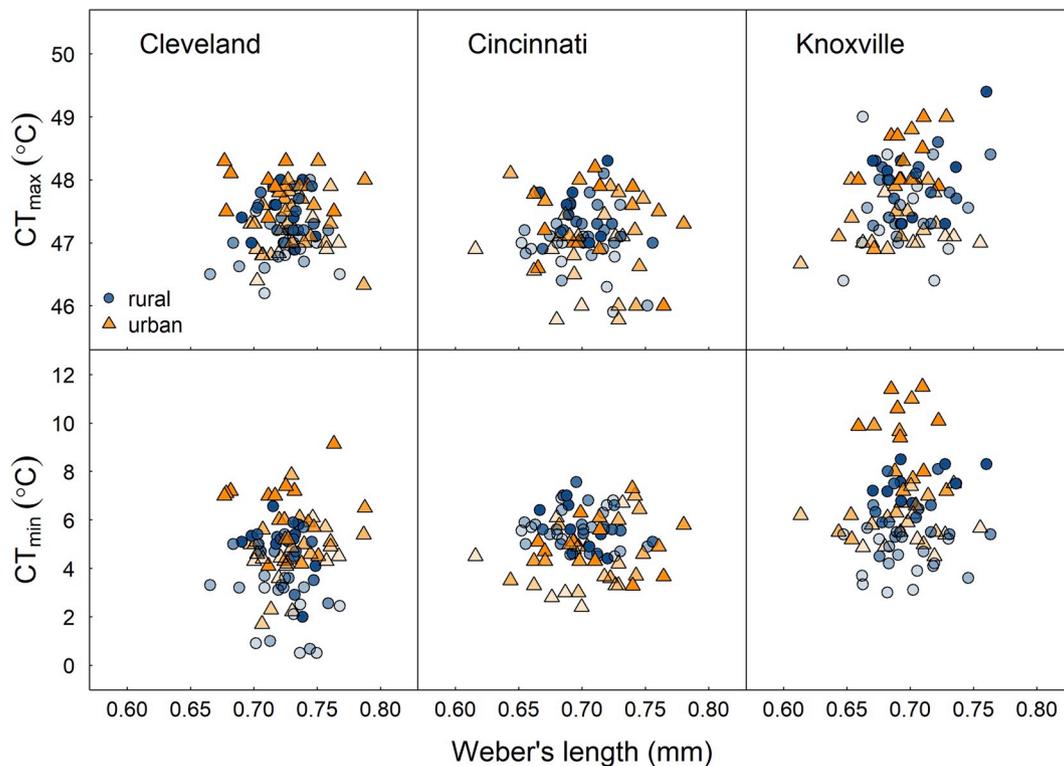
Fig. 2. Scatterplots of colony mean heat tolerance and cold tolerance (CT$_{max}$ and CT$_{min}$, °C) versus body size (Weber's length, mm) for urban and rural acorn ant source populations and the three focal cities (Cleveland, Cincinnati, and Knoxville). Points represent mean colony trait values. Shading of the points indicates the temperature treatments under which colonies were reared, from lower rearing temperatures (lighter shading) to higher rearing temperatures (darker shading).

Table 3
Spearman's rank correlations between body size (Weber's length) and thermal tolerance (both CT$_{max}$ and CT$_{min}$) for each of the three cities.

City	Traits	ρ	S	P
Cleveland	Weber's length CT$_{max}$	0.0347	121000	0.744
	Weber's length CT$_{min}$	−0.0296	129000	0.781
Cincinnati	Weber's length CT$_{max}$	−0.00354	99100	0.974
	Weber's length CT$_{min}$	−0.00974	99700	0.930
Knoxville	Weber's length CT$_{max}$	0.134	76700	0.235
	Weber's length CT$_{min}$	0.0937	80300	0.406

Because these trait values for tolerance and body size were derived from the same colony, our results do not support a genetic correlation between these traits. Analyses of individual-level tolerance and body size (rather than colony-level means) also indicated a lack of relationship between these traits (see Appendix A, Table A3).

Our final suite of analyses examined the potential for tradeoffs in investment. Specifically, we evaluated the potential for producing greater numbers of individual ants at the expense of smaller-sized individuals. However, we found no evidence of a tradeoff in the field-caught colonies. Urban and rural population ant colonies invested in similar numbers of individuals (Appendix A, Table A4). Nor did we find evidence of a tradeoff between colony size and worker body size in the post-experiment colonies. Investment in the number of ants within the colony over the course of the laboratory experiment was unrelated to the post-experiment body size of worker ants (Appendix A, Table A4).

4. Discussion

The direct effects of temperature on body size and the indirect effects of temperature-driven shifts in body size on other traits are cornerstones of evolutionary ecology (Angilletta, 2009; Chown and Nicolson, 2004). Rapidly expanding urban development over the past century provides

replicated experimental warming mesocosms (Rivkin et al., 2019) to explore variation in body size responses to temperature and the downstream consequences for size-related traits. In this study, we used acorn-dwelling ants that occur within nonurban forest habitat and urbanized forest fragments to explore the effects of urban heat islands on plasticity in body size and on evolutionary differentiation in body size between urban and rural populations. We then explored the correlation between body size and thermal tolerance traits. Surprisingly, we found that acorn ant body size was remarkably insensitive to temperature, both with respect to plastic effects of laboratory rearing temperature on body size and with respect to evolved differentiation between urban and rural populations. Because acorn ant body size was robust to temperature variation, we also found no evidence for the positive relationship between body size and heat- and cold-tolerance traits that is typical of many terrestrial insects including ants (*e.g.,* Baudier & O'Donnell, 2018). Our results demonstrate how suites of traits that are typically linked can exhibit differential responses to urban heat islands; specifically while heat and cold tolerance traits exhibit plastic and evolved responses to urbanization, body size does not.

Many ectothermic species exhibit a plastic response of smaller adult body size under warmer environmental temperatures (the 'temperature-size rule'; Atkinson, 1994; Angilletta and Dunham, 2003). Despite the high level of empirical support for this relationship, a considerable number of exceptions are known where body size is either insensitive to temperature variation or exhibits a reverse relationship such that organisms achieve larger body sizes in warmer temperatures (Angilletta and Dunham, 2003; Atkinson, 1995; Walters and Hassall, 2006). Some taxa appear to be especially prone to breaking the temperature-size rule (Walters and Hassall, 2006). However, it appears unlikely that the taxon of ants is a general exception to the temperature-size rule. For example, *Temnothorax nylanderi*, a closely related species to *Temnothorax curvispinosus*, followed the temperature-size rule when reared under common garden conditions (Molet et al., 2017). Exceptions can be induced by other environmental factors, such as resource quality (Diamond and

Kingsolver, 2009). Indeed, some studies of field-caught ants have shown a lack of relationship between environmental temperature and body size (*e.g.*, Nowrouzi et al., 2018). However, because the acorn ants were given unlimited access to a high-quality diet for a complete generation before the assessment of body size, it is unlikely that poor resource quality explains the lack of the temperature-size rule in our study (Fig. 1, Table 1).

Evolved shifts in body size in response to temperature are also apparent for many ectothermic species (Brans et al., 2017; Hall and Warner, 2017; Partridge et al., 1994), including several urban-specific study systems, although the direction of phenotypic change appears to be highly taxon- and habitat-specific (Brans et al., 2017; Hall and Warner, 2017). For example, water fleas have evolved smaller body sizes in cities (Brans et al., 2017), whereas urban grasshoppers and lizards have evolved larger body sizes (Hall and Warner, 2017; San Martin y Gomez and Van Dyck, 2012). Beyond these common garden studies, a recent synthesis of phenotypic shifts in body size found substantial support for urbanization-driven shifts in body size (Merckx et al., 2018), although this synthesis did not disentangle plastic from evolved responses. The general trend was toward smaller body size in cities, albeit with a number of taxonomic exceptions. Based on these widespread phenotypic shifts and growing evidence of evolutionary differentiation in body size across urbanization gradients, we expected acorn ant body size to evolve in response to urban heat islands. However, we found no evidence for evolutionary differentiation in acorn ant body size between any of the urban and rural populations from our three focal cities (Fig. 1, Table 2). We did find some evidence that body size evolved among the cities, as urban and rural Cleveland ants were significantly larger than those in the other two focal cities of Cincinnati and Knoxville (Fig. 1, Table 2). This result parallels biogeographical patterns of larger body sizes in cooler climates (Angilletta et al., 2004). However, because we found no evidence of evolved differentiation between Cincinnati and Knoxville, at a higher latitude and cooler climate versus lower latitude and warmer climate, respectively, it is difficult to say whether acorn ants follow a latitudinal cline in body size.

Because body size is sensitive to temperature, and because many other key life history and physiological traits are dependent upon size, it is important to consider the indirect effects of temperature on these traits as mediated by body size (Chown et al., 2002). In water fleas, urban heat islands lead to the evolution of higher heat tolerance, and these shifts are associated with the evolution of smaller body size (Brans et al., 2017). For aquatic ectotherms, the shift toward smaller body size appears to be a response to oxygen limitation and allows organisms to achieve high heat tolerance in warmer environments (Brans et al., 2017). By contrast, oxygen limitation of thermal tolerance is negligible in terrestrial ectotherms (Klok et al., 2004). For terrestrial ectotherms, larger body size is typically associated with a greater ability to tolerate heat and cold stress (*e.g.*, Scharf et al., 2014; Klockmann et al., 2017). Mechanistically, the greater thermal tolerances typically afforded by larger body sizes in these ectotherms are thought to arise from the mitigation of cellular damage caused by changes in membrane structure, protein denaturation, freezing of cellular water, and other detrimental microphysiological processes (Angilletta, 2009; Chown and Nicolson, 2004; Klok et al., 2004; Williams et al., 2016).

Yet, acorn ants do not meet these expectations. We found that both the greater heat tolerance and diminished cold tolerance of urban populations of acorn ants (Diamond et al., 2018, 2017) were independent of body size (Fig. 2, Table 3). By contrast, intraspecific comparative

studies in ants tend to support the positive size-tolerance relationship (Cerdá and Retana, 1997; Ribeiro et al., 2012; Baudier & O'Donnell, 2018; but see Esch et al., 2017; Nowrouzi et al., 2018), although interspecific comparative studies provide more mixed support (Baudier et al., 2015; Cerdá et al., 1998; Kaspari et al., 2015; Oberg et al., 2012; Verble-Pearson et al., 2015). However, each of these studies was performed on field-caught ants, making it difficult to separate plastic from evolved responses or to rule out potentially confounding factors that could influence the relationship between body size and thermal tolerance. Indeed, in ants, worker body size can trade off with colony size (Hasegawa and Imai, 2012), and this tradeoff can vary systematically with climate (Kaspari and Vargo, 1995). Thus a lack of relationship between tolerance and individual body size in some studies of field-caught ants could reflect investment along other axes, namely investment in colony size. Through our common garden experiment, we were able to directly evaluate this possibility. We found no evidence of a tradeoff between individual worker body size and colony size (Appendix A, Table A4).

Beyond a lack of phenotypic relationship between body size and thermal tolerance in acorn ants, we also found evidence that these traits are genetically uncorrelated (Fig. 2, Table 3). In some systems, such as seed beetles and termites, limitations on host resource size or nest size constrain the evolution of body size (Amarillo-Suárez and Fox, 2006; Matsuura, 2002). A similar scenario could be envisioned for acorn ants wherein the finite space of the acorn environment constrains the evolution of worker size. And because of the decoupling of size from tolerance, stabilizing selection on body size would not constrain directional selection and evolution of thermal tolerance traits in this system. In any case, these results leave open the question of how, in the absence of indirect effects of body size on thermal tolerance, sub-cellular mechanisms, such as increased production of heat shock proteins and differences in lipid content and cuticular thickness (Baudier et al., 2015), might contribute to the evolution of thermal tolerance in acorn ants.

Life history evolution and more recently, pace of life syndrome evolution, consider how multiple traits evolve in response to the environment (Réale et al., 2010). Our study shows how expected patterns of trait evolution can be decoupled in response to contemporary warming within cities; specifically, heat and cold tolerance in acorn ants evolve under urban warming, body size does not. Although few studies explore how suites of traits evolve in response to urbanization, recent work in water fleas found evidence for predicted pace of life syndrome evolution in cities: faster pace of life was accompanied by the evolution of suites of physiological traits for coping with stress in cities (Brans et al., 2018; Brans and De Meester, 2018). How typical suites of traits evolve over contemporary timescales in response to ever-increasing urban development, in particular whether these responses more often match or deviate from the predictions of evolutionary physiology and pace of life syndromes more generally, remains to be discovered.

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Declaration of competing interest

None.

Appendix A

A1. Effects of tolerance type on body size

To assess whether tolerance type was associated with worker ant body size (*i.e.*, whether size was assessed on an individual ant that underwent a

heat tolerance versus cold tolerance assessment), we constructed a linear mixed effects model for each individual city with Weber's length as the response variable, and tolerance type (*i.e.,* either CT_{max} or CT_{min}) as the predictor. We included colony identity as a random effect to account for autocorrelation of worker trait data from the same colony (all colonies contained a minimum of 10 workers for which body size data were available). Analysis of this model revealed no significant difference in Weber's length between tolerance types for any of the three focal cities (Cleveland: $\chi^2 = 2.12$, $P = 0.145$, Cincinnati: $\chi^2 = 0.855$, $P = 0.355$, Knoxville: $\chi^2 = 0.655$, $P = 0.419$).

Table A1

Acorn ant colony collection sites, including geographic coordinates (longitude and latitude), whether the colony was sourced from an urban or rural population, and the percent impervious surface area (ISA) of each site (calculated with a 120m buffer around each site).

City	Longitude	Latitude	Source population	ISA
Cleveland, Ohio	−81.4245	41.49842	Rural	0
	−81.6137	41.50897	Urban	49
	−81.5737	41.52751	Urban	44
	−81.3127	41.60880	Rural	0
Cincinnati, Ohio	−84.5148	39.11819	Urban	41
	−84.5527	39.15487	Urban	49
	−84.5061	39.16535	Urban	54
	−84.5605	39.16695	Rural	0
	−84.5702	39.17292	Rural	0
Knoxville, Tennessee	−83.9272	35.60687	Rural	0
	−83.6772	35.63531	Rural	0
	−83.9473	35.89117	Rural	0
	−83.9269	35.95279	Urban	39
	−83.9232	35.95765	Urban	61

Table A2

Individual and colony-level replication for each combination of city and source population.

City	Source population	Number of individuals	Number of colonies
Cleveland	Urban	890	46
	Rural	854	45
Cincinnati	Urban	775	40
	Rural	849	44
Knoxville	Urban	721	37
	Rural	858	44

Table A3

Estimates, standard errors, likelihood ratio test statistics and *P*-values for the effect of body size (Weber's length) on heat tolerance (CT_{max}) and cold tolerance (CT_{min}). Models were constructed at the level of the individual (with a random effect for colony identity) and were performed separately for each city. Note that qualitatively similar results were obtained when source population and rearing temperature were explicitly accounted for in these models versus when data were pooled across these variables.

City	Tolerance type	Estimate	SE	χ^2	P
Cleveland	CT_{max}	0.507	0.620	0.670	0.413
	CT_{min}	−1.47	0.864	2.87	0.0901
Cincinnati	CT_{max}	0.234	0.561	0.175	0.676
	CT_{min}	−1.18	0.958	1.52	0.217
Knoxville	CT_{max}	1.22	0.721	2.88	0.0895
	CT_{min}	0.845	0.929	0.828	0.363

Table A4

Results of linear models examining whether urban and rural populations differed in their initial colony size (number of workers plus brood), and whether the number of workers and brood produced over the course of the laboratory experiment traded off with worker body size. Separate models were performed for each city. *F*-statistics and *P*-values for each term are provided. Note that for models of post-experiment measurements, qualitatively similar results were obtained for the relationship between body size and colony size when source population and rearing temperature were explicitly accounted for in these models versus when data were pooled across these variables. Similar results were also obtained when field-caught (initial) colony size was subtracted from post-experiment (final) colony size as when final colony size was used alone, and when colony size was estimated as the number of mature workers only, excluding brood.

Sampling point	Response	Term	City	F	P
Field-caught measurements	Colony size (initial)	Source population	Cleveland	0.286	0.594
			Cincinnati	3.25	0.0743
			Knoxville	0.352	0.555
Post-experiment measurements	Colony mean Weber's length	Colony size (final)	Cleveland	0.0121	0.913
			Cincinnati	0.601	0.440
			Knoxville	0.0829	0.774

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