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# Keep your cool: Overwintering physiology in response to urbanization in the acorn ant, *Temnothorax curvispinosus*

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ABSTRACT

Winter presents a challenge for survival, yet temperate ectotherms have remarkable physiological adaptations to cope with low-temperature conditions. Under recent climate change, rather than strictly relaxing pressure on overwintering survival, warmer winters can instead disrupt these low-temperature trait-environment associations, with negative consequences for populations. While there is increasing evidence of physiological adaptation to contemporary warming during the growing season, the effects of winter warming on physiological traits are less clear. To address this knowledge gap, we performed a common garden experiment using relatively warm-adapted versus cold-adapted populations of the acorn ant, *Temnothorax curvispinosus,* sampled across an urban heat island gradient, to explore the effects of winter conditions on plasticity and evolution of physiological traits. We found no evidence of evolutionary divergence in chill coma recovery nor in metabolic rate at either of two test temperatures (4 and 10 °C). Although we found the expected plastic response of increased metabolic rate under the 10 °C acute test temperature as compared with the 4 °C test temperature, this plastic response, (*i.e.,* the acute thermal sensitivity of metabolic rate), was not different across populations. Surprisingly, we found that winter-acclimated urban ant populations exhibited higher heat tolerance compared with rural ant populations, and that the magnitude of divergence was comparable to that observed among growing-season acclimated ants. Finally, we found no evidence of differences between populations with respect to changes in colony size from the beginning to the end of the overwintering experiment. Together, these findings indicate that despite the evolution of higher heat tolerance that is often accompanied by losses in low-temperature tolerance, urban acorn ants have retained several components of low-temperature physiological performance when assessed under ecologically relevant overwintering conditions. Our study suggests the importance of measuring physiological traits under seasonally-relevant conditions to understand the causes and consequences of evolutionary responses to contemporary warming.

## 1. Introduction

Climate warming in winter can relax constraints on low-temperature tolerances and performance leading to population increases of terrestrial ectotherms (Biella et al. 2021; Deutsch et al. 2008). Yet despite these relaxed constraints, temperate insects are still vulnerable to negative effects of global climate change in winter ( Kingsolver et al. 2013; Sunday et al. 2011). For example, developmental acclimation under warmer temperatures and exposure to acute high temperature challenges can reduce insect cold tolerance and performance (Williams et al. 2015). These effects could cascade to population decline through exhaustion of metabolic reserves (Huey and Kingsolver, 2019; Sinclair, 2015) or increased mortality during exposure to recurrent cold snaps amidst warm temperature events (Marshall et al. 2020). The pattern of plastic responses of cold tolerance to warm temperatures are qualitatively similar to biogeographical temperature clines, likely as a result to some degree of evolutionary responses to temperature among terrestrial ectotherms (Addo-Bediako et al. 2000; Sunday et al. 2011). Specifically, the evolution of higher heat tolerance in warmer low-latitude or low-elevation environments is often accompanied by concurrent losses in cold tolerance. It remains an open question if contemporary evolutionary responses to increasing temperatures during global change result in these potentially maladaptive responses in winter physiology.

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Owing to their substantial warming effects over contemporary timescales and across small spatial distances, cities provide a venue to examine plastic and evolved effects of warming on overwintering physiology. When compared to non-urban (hereafter, 'rural') habitats, urban heat islands can serve as a model for future climate warming: *i.e.*, a space for time substitution; (Diamond and Martin, 2020; Lahr et al. 2018). Indeed, heat islands can cause shifts in thermal physiological traits of similar magnitude as trait responses to latitudinal and elevational biogeographic gradients (Diamond and Martin, 2021). In terms of low-temperature physiology, urban ectotherms generally exhibit lower performance than their rural counterparts. For instance, a meta-analysis of thermal responses to urban heat islands among multiple ectotherm systems found that the evolution of higher heat tolerance in urban populations is associated with evolutionary losses in cold tolerance (Diamond and Martin, 2021). Though this pattern of cold tolerance loss in response to heat islands is a frequent response, there are some exceptions (Angilletta et al. 2007; Warren II et al. 2018; Yilmaz et al. 2021). Importantly, however, these findings of low-temperature physiology across the urbanization gradient have been limited to tests done on warm-season acclimated organisms, so cold-season acclimated responses remain unknown.

Here, we examine the effects of contemporary warming on overwintering physiology using a population comparative approach between urban and rural populations of the acorn ant (*Temnothorax curvispinosus*). Acorn ants have adapted to the urban environment via the evolution of higher heat tolerances in response to the elevated temperatures of the urban heat island (Diamond et al. 2017; Martin et al. 2019; 2021). In turn, acorn ant low-temperature physiology has also evolved, with urban populations exhibiting diminished cold tolerance compared to rural populations (Diamond et al. 2017; Martin et al. 2019). Yet the evolutionary losses in low-temperature physiology might leave urban populations vulnerable to low-temperature events as urban populations transplanted to the rural environment experienced a winter bias in mortality (Martin et al. 2021). Further evidence of potentially maladaptive responses to urbanization comes from warm-season shifts in metabolic rate: urban ants express higher routine metabolic rates under non-stressful conditions than rural ants, though thermal sensitivity of metabolic rate was lower in urban ants (Chick et al. 2021). Whereas winter-biased mortality of urban ants transplanted to rural environments is putatively owed to their poorer cold tolerance and/or higher metabolic rates, these inferences rely on data from ants acclimated and reared under summer conditions.

The physiological traits of winter-acclimated *T. curvispinosus* have not been measured, and importantly, could differ from summer-acclimated trait values for several reasons. For example, traits might be costly to maintain when environments change to more relaxed conditions (Marshall et al. 2020; Sinclair, 2015). Alternatively, there might be possible constraints imposed by correlations with other traits important for adaptation to urban environments (Barley et al. 2021; Etterson and Shaw, 2001). In these contexts, the impacts of winter-acclimation effects on trait responses could play a critical role. To examine this, we winter-acclimated rural and urban *T. curvispinosus* colonies in a laboratory common garden experiment for a minimum of 10 weeks and then measured a suite of cold season thermal physiology traits including chill coma recovery time (CCRT), critical thermal maximum ($CT_{max}$), routine metabolic rate (RMR), and acute thermal sensitivity of metabolic rate (RMR $Q_{10}$). We also measured the change in colony size (number of workers) from the start to the end of the experimental period. In general, we expected that evolution to the urban habitat would result in diminished low-temperature performance in CCRT and metabolism (elevated mean RMR and higher RMR $Q_{10}$) and that colony size would be reduced by a greater amount in urban populations over winter compared to rural ants. Likewise, we expected that overwintering acclimation would reduce the magnitude of population divergence in $CT_{max}$.

## 2. Materials and methods

### 2.1. Study system

*Temnothorax curvispinosus* is a cavity-nesting ant found across North American temperate hardwood forests. Entire colonies of workers, brood (egg, larvae, pupae) and one or more queens, reside within cavities of hollow tree nuts (*e.g.,* acorns, hickories) within the leaf litter (Herbers, 1984). As this species overwinters aboveground, they make an excellent study system for examining the evolution of thermal physiology during cold seasons because they are much less buffered from seasonal temperatures than species nesting underground. Due to strong competition for nest sites between other cavity dwelling ants and social parasites (Herbers, 1989), remaining above ground over winter and being able to resume activity during warmer periods of the winter months may allow acorn ants greater success in securing new nest sites the following spring.

### 2.2. Collection locations

We collected 48 queenright (queen was present) colonies of *T. curvispinosus* from two rural and three urban sites in the Cleveland, Ohio, USA metropolitan area between September 9 and December 4, 2021. Sites were designated urban or rural based on percent developed Impervious Surface Area (ISA) from the 2019 National Land Cover Database (NLCD; Yang et al. 2018). To account for colony collection sites within the urban forest being relatively far from impervious structures but still within the influence of the urban heat island, we calculated mean ISA values with a 360 m buffer in R using the *arcgisbinding* and *raster* packages. Sites were binned as "urban" based on ISA values > 25% and "rural" with ISA < 5%. This pattern in ISA is a function of the fragmentation of forest habitats in the urban matrix where *T. curvispinosus* is found and the lack of colony presence in high (75–100%) and intermediate ISA locations (5–25% ISA; (Diamond et al. 2017). Prior work in the Cleveland metropolitan area (Diamond et al. 2018a) has shown a difference in growing season mean temperature of >4 °C between urban and rural sites, linking differences in ISA to temperature – a generalized pattern in most cities (Imhoff et al. 2010). Since colonies were collected across multiple months, we grouped them into three equal sized testing cohorts that were staggered to allow sufficient acclimation time for all colonies prior to physiological testing, though this led to variation in the total acclimation time.

### 2.3. Laboratory common garden experiment

Field collected urban and rural ant colonies were held in growth chambers (Percival Scientific, 36-VL) under a laboratory acclimation period of 10–15 weeks at 25 °C, a commonly experienced, non-stressful summer temperature at a 14:10 L:D light cycle, following Diamond et al. (2018a) This initial period was to ensure that a new lab reared generation of workers was produced to minimize environmental effects on physiological measurements in order to test for evidence of evolutionary divergence in overwintering physiology. Throughout the experiment, colonies were held in individual 118 mL plastic containers with empty 1.5 mL paper-covered cryo-tubes as artificial nesting sites and provided *ad libitum* with resource tubes of 25% w/w sugar water and aged tap water along with dried mealworms.

Following the acclimation period, we placed colonies in new growth chambers (MIR 154 PHCBI) and initiated a series of 6 weekly step-downs from fall temperatures (maximum: 20 °C, minimum: 2.5 °C) and light cycles (L:D) to overwintering conditions that matched the seasonal photoperiod at our site latitudes (Step 1: 20:17 °C | 13:11, Step 2: 15:13 °C |12:12, Step 3: 10:9 °C | 12:12, Step 4: 5:4 °C | 11:13, Step 5: 3.5:2.5 °C | 10:14, Step 6: 2.5:1.5 °C | 8:16; Table S1; Haatanen et al. 2015). Temperatures for each fall step-down were calculated as the mean temperature across the period of October to December 2019 while the winter acclimation temperature (Step 6) was the mean temperature across the period of January 1st to March 1st, 2020. Temperature data were measured at one of our rural sites from 1 cm below the soil surface using temperature loggers (iButton DS1921G-F5; Table S1; Figure S1; Figure S2). Fall step-downs were weekly adjustments from the mean fall temperature until reaching the overwintering temperature where we held all colonies for at least five weeks until conducting physiological assays (See Figure S5 for full timeline of rearing and physiological tests).

### 2.4. Colony demography

To determine if there were differences in colony worker number between urban and rural populations over the experiment, we conducted a census for each colony before and after the experiment. Following field collection, we counted the total number of worker ants to establish the initial colony size. Colonies were held at the winter temperature and photoperiod (2.5:1.5 °C | 8:16) for a total of 4 months over the experimental period before a final census of worker ants was conducted.

### 2.5. Physiological measurements

#### 2.5.1. Chill coma recovery time

Individual adult worker ants were tested for their chill coma recovery time (CCRT), a static measure of cold tolerance (Gibert and Huey, 2001; Macdonald et al. 2004) with time of recovery from cold shock as the response variable. CCRT was chosen over the dynamic ramping assessment of $CT_{min}$ since it is likely more biologically relevant in determining activity at colder temperatures (Sørensen et al. 2001) and because of difficulties in determining loss of muscular coordination at cold temperatures, as the ants exhibited very low baseline activity in the winter acclimation treatment. For logistical ease and to reduce acclimation to the testing environment we induced chill coma (loss of motor activity due to impaired neuromuscular function; Denlinger and Lee, 2010) at −10 °C on whole colonies of workers and then observed individuals for the time of recovery, determined by antennae or leg movement. The chill coma temperature was selected based on winter air and soil temperatures at our study sites (Figure S1.; Figure S3), similar tests of CCRT in congenerics (Modlmeier et al. 2012), related woodland ants (*Leptothorax* spp. Heinze et al. 1996; Heinze et al. 1998), and freeze susceptible insects in general (Turnock and Fields, 2005). To induce chill coma in whole colonies we transferred them into a fluon-lined petri dish (5 cm diameter) and then into a growth chamber (PHCBI MIR 154) set at −10 °C for 20 min. To determine CCRT at an ecologically relevant temperature, the colony was then moved into an ice filled cooler that matched their daytime winter rearing temperature (2.5 °C). We then monitored the whole colony using a digital camera (Canon Powershot G15) attached to a tripod. We measured the CCRT of 15 individual workers per colony unless the colony had insufficient numbers, in which case we measured all workers in the colony. We did not include any colony with fewer than six workers. Recording video of the whole colony allowed us to resolve each worker's recovery time to the second. CCRT assays were conducted two weeks following the metabolic rate tests (Figure S5).

#### 2.5.2. Routine metabolic rate and acute thermal sensitivity

After holding colonies for at least five weeks of winter conditions in the lab, we measured whole-colony routine metabolic rates of urban and rural colonies (*i.e.,* queen, workers, and brood) using flow-through respirometry (Lighton and Turner, 2004) at two acute test temperatures, 4 °C and 10 °C. We chose the 4 °C test temperature instead of the winter acclimation temperature (2.5 °C) following calibration tests that revealed low to no detectable differences between colony metabolic rate and the control chamber. The 10 °C test was used to simulate particularly warm winter day soil temperatures that occasionally occur in the field (Figure S1) and to detect differences in metabolic rate responses to the warmer temperature. Routine metabolic rate is considered in between a resting (representing zero activity) and active (representing maximum activity) metabolic rate and is used by ecologists as an index of energy expenditure in the field (Lighton, 2018; Steffensen, 2005). We chose to measure metabolic rate prior to the measures of $CT_{max}$ and CCRT as these later assays are sometimes lethal. We used a LICOR-7000 infrared $H_2O/CO_2$ gas analyzer that pushed ambient air through drierite and soda lime to scrub $H_2O$ and $CO_2$, respectively. The air was then split into a control line and an experimental line. In both lines, air was pushed through a flow control meter (ALICAT Scientific, MC-200SCCM). The control line led back to the $CO_2$ analyzer bypassing any colonies. The experimental line led to a flow control multiplexer (RM-8 Sable Systems) which diverted the air flow through chambers holding the colonies and then back to the analyzer. In addition to the colonies, the experimental line led to one empty respirometry chamber (control chamber) to account for background $CO_2$ leakage into the chambers during the experiment. Colonies were held within a dark environmental growth chamber (MIR 154 PHCBI) during recording. We used a constant flow rate of 30 mL/min, the minimum flow required to ensure complete air displacement of the container (30 mL) volume to avoid stagnation. We chose this minimum flow rate owing to the small organism size and low temperature at the time of recording to maximize the detection signal of metabolic rate. The flow controllers were calibrated using a gilibrator (Gilian Gilibrator-2 NIOSH) at both 4 and 10 °C.

We quantified metabolic rate at both temperatures for all colonies following Chick et al., (2021). In brief, the entire colony was transferred from their winter rearing temperatures to 30 mL glass respirometry chambers (RC Sable Systems International) connected to the multiplexer inside the growth chamber. We allowed colonies to acclimate for 30 min to stabilize the flow rate and establish a basal metabolic rate before measurement of $CO_2$ production began. $CO_2$ production was first measured at 4 °C for a 10 min interval with 5 min pauses in measurement between colonies so residual $CO_2$ could be cleared from the lines and colony chambers could be flushed of accumulated $CO_2$ during the acclimation period. $CO_2$ production was measured as the differential $CO_2$ concentration (ppm) between the experimental line (colony) and the control (blank) returning air line. Though the control chamber was empty (no colony), there were slight non-zero $CO_2$ levels detected due to sensor drift, and this value was subtracted from average colony $CO_2$ measures. Due to lower detection values at the 4 °C temperatures for smaller colonies, if metabolic rates were smaller or equal to the control chamber at the 4 °C test, we used the control chamber value for $CO_2$ as that colony's measure. The following week, the same colonies were tested at 10 °C following the same procedure to simulate a warmer winter day.

All metabolic rate data was processed through a UI – 3 data acquisition interface system and ExpeData software (Sable Systems International). We converted the $CO_2$ concentration values to the rate of $CO_2$ production ($VCO_2$ mL/min) by dividing the values by 1,000,000 to get the fractional value and then multiplied this by the flow rate to get the fractional flow rate. To find the least variable measure that most closely matched the routine metabolic rate for each colony, we used the last 5 min of data collection as it was the most stable reading (*i.e.,* slope closest to 0) value of the metabolic rate. Lastly, we used the metabolic rate measures over both temperatures to calculate the $Q_{10}$ coefficient: a unitless reaction rate factor defined as the increase in metabolic rate for every 10 °C increase in temperature and can be used to measure thermal sensitivity of metabolic rate between source populations.

#### 2.5.3. Critical thermal maximum

Because microclimate habitats in winter (*e.g.,* direct sunlight) include relatively warm temperatures (Stoutjesdijk, 1977) that are especially relevant for foraging ants, we measured the critical thermal maximum ($CT_{max}$) of mature workers using dynamic ramping temperature protocols (Diamond et al. 2018a; Terblanche et al. 2011). Two weeks after CCRT assays, 10 individual mature worker ants were collected from the winter chamber and placed in individual 1.5 mL tubes sealed with a cotton ball. The tubes were then transferred to a heat block (Boekel Tropicooler) at 22 °C and the temperature was increased by 0.5 °C per minute until the loss of muscular coordination was observed.

### 2.6. Statistical analyses

We used generalized linear mixed effects models to determine if urban and rural populations diverged in worker number, routine metabolic rate, acute thermal sensitivity of metabolic rate, CCRT, and $CT_{max}$ following winter acclimation. For each response, we fit separate generalized linear mixed-effects models using the *glmmTMB* function from the *glmmTMB* package (Brooks et al. 2017) and ran model diagnostics using the *simulateResiduals* function from the *DHARMa* package (Hartig and Lohse, 2022). In all models, we treated urbanization as the categorical predictor "source population". To account for variation in collection date for urban and rural colonies, we binned collection date into three factor levels (early, middle, late; Table S3) that correlated with ant testing cohorts and included it as a random effect in each model.

To test for the effect of source population on ending colony worker number over the winter period, we modeled colony number with census points as a two-level factor (beginning point and end point) and source population along with their interaction as predictors with a Poisson distribution and log link function. We included colony identity as a random effect since we sampled the same colonies at multiple time points. We also calculated the final colony worker number at the end of the experiment as a proportion of the starting colony worker number to account for differences in starting colony size. We modeled this proportion by source population using a beta binomial distribution and logit link function. For the CCRT model, we used a negative binomial distribution and log-link function and for the $CT_{max}$ model we used a Gaussian distribution and identity link function. For the CCRT and $CT_{max}$ models, we included colony identity as a random effect to control for non-independence of tested individuals from the same colony. For the routine metabolic rate and the acute thermal sensitivity metabolic rate models, we included colony mass as an additional fixed effect predictor since metabolic rate scales with mass (Speakman, 2005; West et al. 2002). In the routine metabolic rate model, we also included test temperature and its interaction with source population as predictors. Only colonies with at least six workers were included for physiological testing and of the 48 colonies, 41 were included in the CCRT and metabolic rate analyses and 38 were included for $CT_{max}$.

For each model, we determined significance of the predictors using log likelihood ratio tests using the *Anova* function with the Type = "III" sums of squares argument from the *car* package (Fox et al. 2022). For data visualization, we subsequently back transformed modeled results to the data scale using the *emmeans* function and package (Lenth et al. 2022). All statistical tests were conducted in R version 4.1.1 (R Core Team, 2021) with results reported to three significant digits.

## 3. Results

### 3.1. Colony demography

Following the four-month winter acclimation period, all acorn ant colonies declined in worker number (Table 1; Fig. 1A). As there was marginal (although statistically non-significant) evidence for an interaction of source population and census point (Table 1), we used post-hoc comparisons to test for differences in colony sizes between urban and rural colonies at each census point. The post-hoc analyses showed that field collected urban colonies initially contained more workers than rural ones (Urban initial number of workers – rural initial number of workers = 21.0, SE = ± 3.21, P < 0.0001; Table S2). This difference decreased to a large degree by the end of the experiment, but urban populations were still significantly larger (urban final number of workers – rural final number of workers = 2.88, SE = ± 1.16, P = 0.0148; Table S2). When we examined the remaining colony worker number as a proportion to account for these differences in starting colony number, our model showed no difference between urban and rural colonies (Table 1; Fig. 1B).

**Table 1**

Estimates and $\chi^2$ tests of significance for the generalized linear mixed effect models of source population on colony number and thermal performance: CCRT and $CT_{max}$. All estimates are shown on the natural log scale except for $CT_{max}$. Estimates and $\chi^2$ tests for metabolic rate include the main effect predictor of colony mass. Significant P values below the 0.05 level are shown in bold.

| Type of Response | Response variable | Term | Estimate | SE | $\chi^2$ | P |
|---|---|---|---|---|---|---|
| Colony demography | Colony size (number of workers at initial or final census points) | Source population | 0.226 | 0.268 | 0.710 | 0.399 |
| | | Time point | −1.31 | 0.0658 | 396 | < **0.001** |
| | | Source population* Time point | −0.172 | 0.0902 | 3.64 | 0.0563 |
| | Proportional change in number of workers (final/initial) | Source population | −0.0929 | 0.191 | 0.236 | 0.627 |
| Physiology | CCRT (seconds) | Source population | < 0.001 | 0.168 | 0.00 | 0.995 |
| | $CT_{max}$ (°C) | Source population | 1.28 | 0.512 | 6.24 | **0.0125** |
| | Acute routine metabolic rate | Colony mass | 0.0760 | 0.0984 | 59.7 | < **0.001** |
| | | Source population | 0.0217 | 0.0270 | 0.645 | 0.422 |
| | | Temperature | 0.210 | 0.0259 | 66.2 | < **0.001** |
| | | Source population* Temperature | −0.0179 | 0.0378 | 0.225 | 0.636 |
| | Acute thermal sensitivity of metabolic rate ($Q_{10}$) | Source population | 0.0967 | 0.100 | 0.92 | 0.337 |
| | | Colony mass | 0.0992 | 0.0456 | 4.73 | **0.0297** |

### 3.2. Chill coma recovery time

To determine if cold tolerance differed between urban and rural populations, we measured their chill coma recovery time (CCRT). We had expected urban ants to have a longer CCRT (*i.e.,* worse cold tolerance) than rural ants, however, we found no difference between urban and rural workers in recovery time with a high degree of variance among colonies (Table 1; Fig. 2; urban estimate = 577 s, SE = ± 72.6; rural estimate = 576 s, SE = ± 68.2).

### 3.3. Metabolic rate

We examined whole colony routine metabolic rate at two winter temperatures (4, 10 °C) to determine differences in acute metabolic rates and the acute thermal sensitivity of metabolic rate ($Q_{10}$) in urban and rural colonies. As expected, metabolic rate increased with colony mass (Table 1; Fig. 4; Fig. 5). However, we found no significant difference in routine metabolic rate between urban and rural colonies at either 4 or 10 °C (Table 1; Fig. 4). Similarly, we found no evidence for differences in acute thermal sensitivity of metabolic rate between urban and rural colonies (Table 1; Fig. 5).

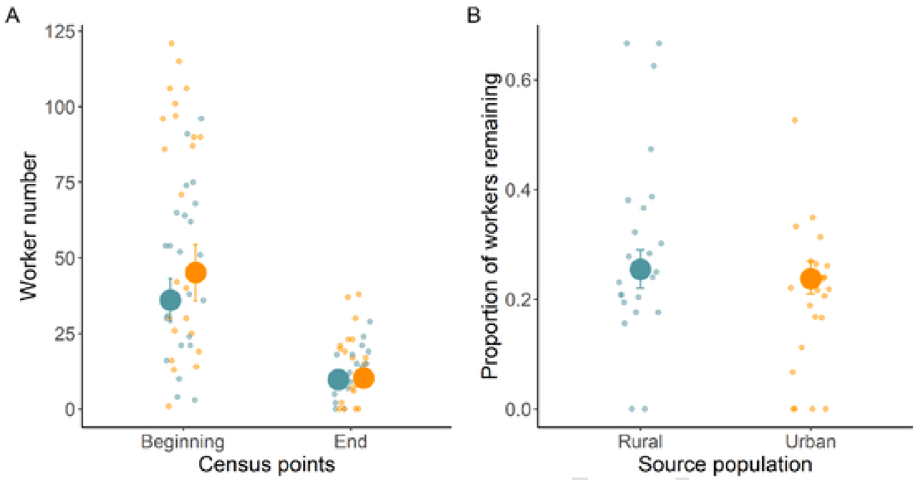

**Fig. 1.** Colony demographic responses to winter across urban and rural acorn ant populations. Large points show estimated means ± 1 SE from the corresponding statistical models (see main text section 2.6). A) Worker number at the initial and final censuses of the 4-month experiment across urban (orange symbols) and rural (blue symbols) populations back-transformed from the model to the data scale. Smaller points jittered behind the grand means display the raw data colony number of workers at each census point. B) Remaining number of workers as a proportion of the total population from urban (orange symbols) and rural (blue symbols) populations back-transformed from the model to the data scale. Smaller points jittered behind the estimated means display the raw data proportion of workers remaining. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

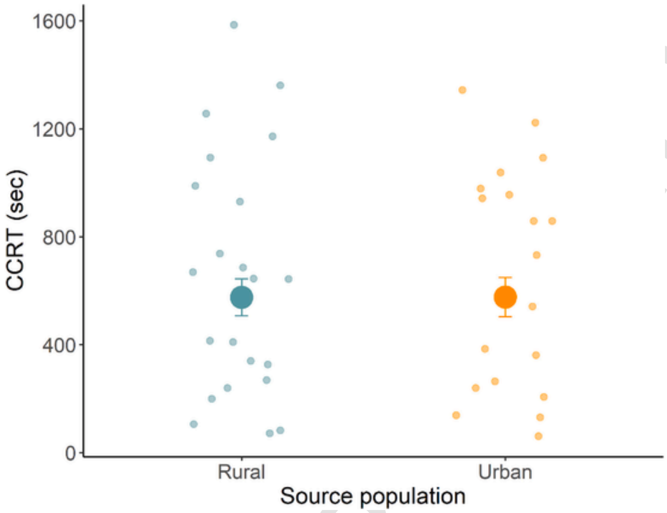

**Fig. 2.** Chill coma recovery time (CCRT; in seconds) following overwintering acclimation between urban and rural acorn ant colonies. Large points display the estimated means ± 1 SE, back-transformed from the model to the data scale, of recovery time from the corresponding statistical models (see main text section 2.6) from rural (blue) and urban (orange) colonies. Smaller points jittered behind the estimated means display the raw colony mean recovery times to show the variance across all colonies. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.4. *Critical thermal maximum*

To test if there were differences between urban and rural colonies in their upper thermal limits, we measured $CT_{max}$ of individual workers. We found that $CT_{max}$ of workers from urban collected colonies was significantly higher than workers from rural collected colonies (Table 1; Fig. 3; urban estimate = 46.2 °C, SE = ± 0.572, rural estimate = 44.9 °C, SE = ± 0.568).

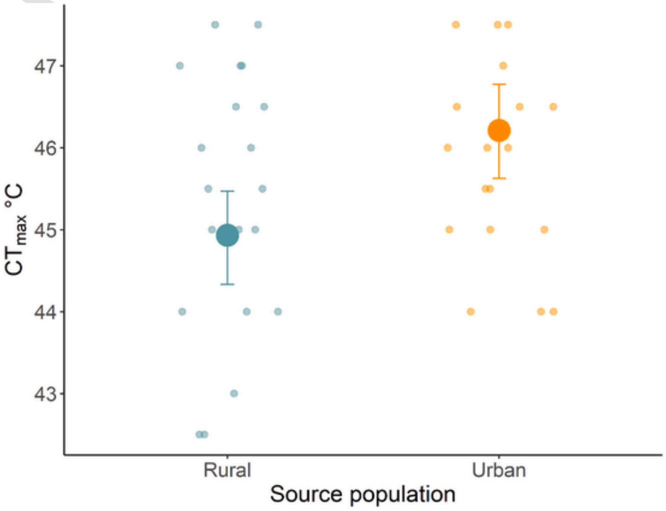

**Fig. 3.** Critical thermal maxima ($CT_{max}$ in °C) following overwintering acclimation between urban and rural acorn ant colonies. Large points display the estimated means ± 1 SE of $CT_{max}$ from the corresponding statistical models (see main text section 2.6) from rural (blue) and urban (orange) colonies. Smaller points jittered behind the estimated means display the raw colony mean $CT_{max}$ to show the variance across all colonies. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

## 4. Discussion

Determining the thermal physiology of winter-acclimated organisms is needed for assessing evolutionary responses to climate warming in winter where responses could be maladaptive and lead to population decline. Cities provide an experimental arena for exploring responses to contemporary warming and are thus important models for predicting broader thermal physiological responses to warming expected under future global change (Diamond and Martin, 2020). Whereas the evolution of thermal physiology in response to urban heat islands have generally followed biogeographic patterns of elevated heat tolerance with losses in cold tolerance (Diamond and Martin, 2021), we found that winter-acclimated urban ant colonies retain similar metabolic rates and cold

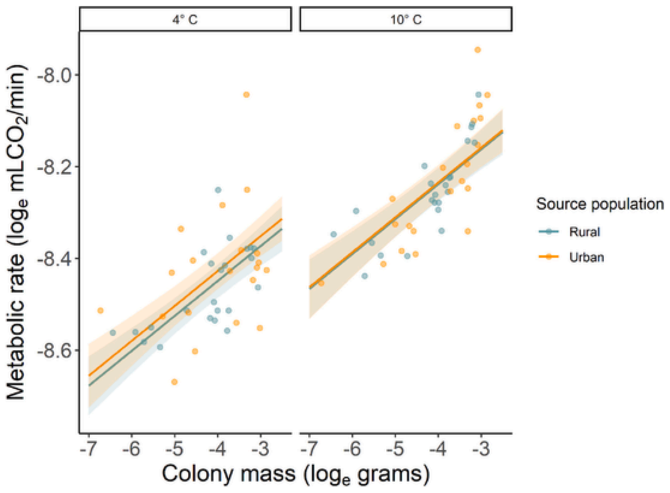

**Fig. 4.** Modeled estimates of natural log transformed mean whole colony metabolic rates by colony mass on the natural log scale for both 4 and 10 °C acute test temperatures. Rural colony trends are shown by the smoothed dark line in blue while urban colony trends are shown by the smoothed dark line in orange with shaded 95% confidence interval bands overlapping both lines in the figure. Points show the individual colony mean metabolic rates at each temperature for each source population (rural points in blue, urban points in orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

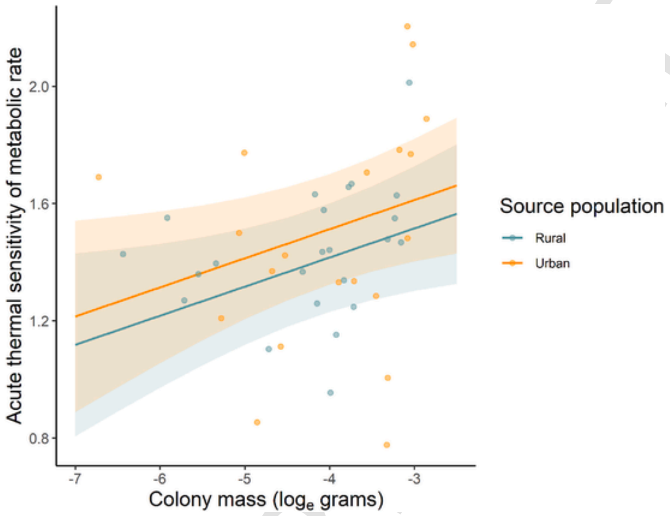

**Fig. 5.** Modeled estimates of acute thermal sensitivity of metabolic rate showing the reaction rate increase for every 10 °C increase in temperature ($Q_{10}$) as a function of colony mass in grams on the natural log scale. Rural colony estimates are shown by the smoothed dark line in blue while urban colony estimates are the smoothed dark line in orange with shaded 95% confidence interval bands overlapping both lines in the figure. Points show the individual colony reaction rate at each temperature for each source population (rural points in blue, urban points in orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tolerance performance as their rural counterparts but diverge in heat tolerance. Our findings suggest that the retention of similar low-temperature physiological performance as their rural counterparts is important for urban populations evolving to the urban habitat. More broadly, these results suggest that selection on thermal physiological traits may differ across seasons in response to contemporary warming.

What might account for the lack of divergence in cold tolerance between overwinter-acclimated ants? One answer could be our choice to investigate CCRT rather than CT$_{\min}$. We could not test directly for CT$_{\min}$ as winter-acclimated ants showed minimal to no movement making it impossible to assess the movement-based CT$_{\min}$ criterion of the loss of muscular coordination (Gibert and Huey, 2001; Terblanche et al. 2011). Although both CCRT and CT$_{\min}$ are biologically relevant to overwintering (Chown and Nicolson, 2004), they are underlain by different physiological mechanisms and functions (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). CCRT is driven by the ability to restore ion gradients across cellular membranes and upregulation of genes for cold injury repair (Koštál et al. 2007; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013) while CT$_{\min}$ is driven by the ability to avoid depolarization of muscle resting potential and allows insects to continue to maintain motor function at low temperatures (Findsen et al. 2014; Gibert and Huey, 2001). Thus, rapid CCRT is potentially critical in allowing overwintering organisms to take advantage of warm days to forage or to gain early access to nesting sites (Foitzik et al. 2004; Herbers, 1989). Low CT$_{\min}$ by contrast enables organisms to maintain ecological functions, such as foraging, during low temperatures. As such, our study cannot disentangle whether the lack of population divergence in CCRT among overwintered ants — despite evidence for divergence in CT$_{\min}$ among warm-season acclimated ants (Martin et al. 2019) — represents differences in the evolutionary response of these two traits between urban and rural populations or seasonal plasticity in response to overwintering conditions.

The genetic divergence in heat tolerance between urban and rural populations under common garden winter acclimation was similar in magnitude to that measured under growing season conditions (Diamond et al. 2017; Martin et al. 2019). Why was CT$_{\max}$ apparently unaffected by the laboratory overwintering acclimation? In insects, warmer developmental acclimation temperatures experienced across ontogeny are more strongly associated with adult CT$_{\max}$ than acclimation temperatures imposed solely at the adult stage (Kellermann et al. 2017; Kellermann and Sgrò, 2018; Slotsbo et al. 2016, but see Chown and Terblanche, 2006). This would suggest that the developmental rearing temperature might explain acorn ant worker CT$_{\max}$ to a greater degree than adult acclimation to the overwintering period. Our developmental rearing period temperature of 25 °C prior to overwintering is within the range of growing season temperature rearing regimes from previous work that showed similar CT$_{\max}$ differences between urban and rural acorn ants (Diamond et al. 2018a). Indeed, the overwinter-acclimated urban ants here had on average 1.3 °C higher CT$_{\max}$ than rural colonies which is consistent with prior findings (Range of 0.9–1.67 °C higher CT$_{\max}$ found in colonies reared under growing season conditions; Diamond et al. 2017; Martin et al. 2019) and is similar in magnitude of divergence across biogeographic clines in temperature (Diamond and Martin, 2021). The magnitude of this evolved increase in CT$_{\max}$ is ecologically important for *T. curvispinosus* to maintain activity levels during high temperature exposure (Pilakouta et al. 2020) and respond to rapid spatio-temporal temperature changes in the urban habitat (Diamond et al. 2018b). For example, high CT$_{\max}$ could allow ants to cope with rapid heat spikes during and especially as they emerge from the winter season (MacLean et al. 2017).

With respect to sub-lethal thermal performance, colder environments, such as the rural habitat in winter, could select for higher metabolic rates to enable growth and development under shorter growing seasons (Addo-Bediako et al. 2002; DeLong et al. 2018). Warmer winter conditions under urbanization, on the other hand, could select for lower metabolic rates that decrease the risk of metabolic reserve depletion (Moffett et al. 2018; Pilakouta et al. 2020). Our results did not support either of these arguments, however, as urban and rural colonies expressed similar mean metabolic rates and similar acute thermal sensitivity of metabolic rates. Prior research with acorn ants showed higher metabolic rate in urban ants compared with rural ants under a non-stressful test temperature (25 °C); urban ants also exhibited a lower acute thermal sensitivity of metabolic rate compared with rural ants when exposed to a high-temperature environment (38 °C) (Chick et al. 2021). However, clearly these tests were conducted at much warmer developmental acclimation and acute test temperatures compared with the ants in our overwintering study. Since temperature is predictive of metabolic rate and activity (Angilletta, 2009; Chown and Nicolson, 2004; MacLean et al. 2017), it is likely that under our cold season conditions the differences in metabolic rate were minimized simply due to low activity. Future research could investigate if a dampening of metabolic rate divergence in the overwintering period could mitigate potential fitness costs of elevated metabolism in the warmer urban winter habitat.

The lack of divergence across our low-temperature thermal physiological traits (*i.e.,* CCRT, metabolism) that we found could also be due to the smaller temperature difference across the urbanization gradient in winter compared to summer found in temperate U.S. cities (Imhoff et al. 2010). Variance in temperature in the urban habitat along with microclimatic differences between urban and rural sites, such as differences in snow cover (Groffman et al. 2001), could paradoxically lead to colder soil temperature extremes in urban areas, even if average air temperatures remain higher. This is supported by our soil level temperature data where there was greater diurnal and seasonal variance in temperature in the urban habitat and soil level average temperatures were at times lower than rural sites (Figure S1; Figure S2). This difference is likely due to smaller or more infrequent snowpack at urban habitats which can reduce soil level insulation and increase microsite temperature variability with consequences for urban populations (Mitrus, 2016; Thompson et al. 2016; Williams et al. 2015). Urban soil temperature profiles at the height that acorn ants would experience them are characterized by frequent short-term cold shocks, but warmer overall temperatures (Thompson et al. 2016; Figure S1), potentially providing an advantage to colonies that had retained a rapid CCRT like their rural counterparts.

The maintenance of higher heat tolerance in both the growing and winter seasons along with similar cold tolerance (CCRT) as rural populations could be beneficial for urban acorn ants in the warmer, but more variable, urban habitat. This range of adaptations may be favored under more variable conditions but may incur decreased optimal performance (*i.e.,* "A jack of all temperatures is a master of none"; Huey, 2010; Izem et al. 2005) or select for higher levels of plasticity that could be maladaptive (Diamond and Martin, 2020; Sgrò et al. 2016). Then again, it is possible that there is no trade off in thermal physiological traits; that is, there could be no cost associated with expressing similar cold tolerance in the warmer or more variable habitat. For instance, Sørensen et al. (2015) found no trade off in thermal tolerance traits in *Drosophila subobscura* populations in which acclimatization to the cold season did not carry a cost to development under warmer conditions in the spring. Further, El-Saadi et al. (2020) found that repeated warm periods in between cold stresses in *Drosophila melanogaster* improved CCRT resulting in greater survival and fecundity. Evidence of local adaptation to the warmer urban habitat (Martin et al. 2021; Yilmaz et al. 2022), however, suggests there might be such a cost to urban adaptation, that populations are not yet on a fitness peak, or that populations are still adapting to the relatively novel conditions of cities (Diamond et al. 2022). Regardless of the nature of the forces that shape overwintering physiology in acorn ants, the somewhat unexpected results of a lack of evolutionary divergence in low-temperature physiological traits and persistence of high-temperature trait divergence among overwintered acorn ants, underscores the importance of assaying traits under both winter and growing season conditions.

## CRediT authorship statement

**Eric G. Prileson:** Conceptualization, Investigation, Formal analysis, Visualization, Writing – Original draft preparation, and Writing – Reviewing and editing. **Ryan A. Martin:** Conceptualization, Formal analysis, Project administration, and Writing – Reviewing and editing. **Sarah E. Diamond:** Conceptualization, Investigation, Software, Formal Analysis, Resources, and Writing – Reviewing and editing. **Angie Lenard:** Investigation, Visualization, and Writing – Reviewing and editing. **Osmary A. Medina-Báez:** Investigation, Writing – Reviewing and editing. **Jordan Clark:** Investigation – data collection, and Writing – reviewing and editing. **Aaron R. Yilmaz** Investigation - data collection and Writing – reviewing and editing.

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## Declaration of competing interest

None.

## Data availability

Data are available from the Mendeley repository 10.17632/r5zp-phcnyv.3.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2023.103591.

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