Title:

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

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Abstract:

Winter presents an immense challenge for survival, yet temperate ectotherms have remarkable adaptations to physiologically compensate for these challenges. Such mechanisms are especially relevant under recent climate change where responses to warmer winters might negatively affect populations. While there is growing evidence of thermal physiological adaptations and population divergence in response to contemporary warming from the growing season, the impact of warming on overwintering ectotherms is less clear. To address this knowledge gap, we use a common garden experiment with urban and rural populations of the acorn ant, *Temnothorax curvispinosus,* to explore divergence in thermal physiological traits assessed following winter acclimation. Specifically, we use the thermal gradient of the urban heat island to assess population divergence in cold tolerance (chill coma recovery), metabolic rate, thermal sensitivity of metabolic rate, heat tolerance and colony demographics. Our results show there was no evidence of evolutionary divergence in low-temperature performance (chill coma recovery, metabolic rate at either of two test temperatures (4 and 10 °C) or acute thermal sensitivity of metabolic rate) among urban and rural colonies which differs from previous tests of warm-season acclimated ants. In contrast, we found winter-acclimated urban ants exhibited higher heat tolerance than rural ants with a magnitude of difference similar to growing season tests. These findings indicate that acclimation under overwintering conditions might dampen the expression of urban-rural population divergence in some thermal physiological traits and broadly suggest the potential importance of retaining at least some components of low-temperature physiology when evolving to urban heat islands and contemporary warming.

Keywords: cold tolerance, heat tolerance, urban heat island, contemporary evolution

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 & 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 apparently maladaptive responses in winter physiology.

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 & 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 & 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 & 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 & 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, we calculated mean ISA values with a 360 m buffer in R using the *arcgisbinding* and *raster* packages (ESRI, 2018). 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., 2018) has shown a difference in growing season mean temperature of > 4 °C between urban and rural sites, linking differences in ISA to temperature (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., (2018) This initial period was to ensure that a new lab reared generation of workers was produced to minimize environmental effects on physiological measurements. 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 A.1; Haatanen et al., 2015). We chose the stepdown temperatures based on seasonal and diurnal leaf litter temperatures measured at one of our rural sites using iButton temperature loggers (*Thermochron* | *Temperature Logging IButtons*, DS1921G-F5; Table A.1).

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 & 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 such as our ants that already have very low activity. 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 & Jr, 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 similar tests of CCRT in congenerics (Jacquier et al., 2022; Modlmeier et al., 2012), related woodland ants (*Leptothorax spp.* Heinze et al., 1996; Heinze et al., 1998), and freeze susceptible insects in general (Turnock & 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 minutes. 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.

2.5.2 Routine metabolic rate and acute thermal sensitivity

After holding colonies for four to six 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 & Turner, 2004) at two acute test temperatures, 4 °C and 10 °C. 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 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. 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 replacement 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 chamber. We allowed colonies to acclimate for 30 minutes 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 minute interval with five minute 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 average CO_2 concentration (ppm) from each colony over the 10 minute period compared to a control (blank) returning air flow tube. 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 five minutes 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. Due to lower detection values at low temperatures for smaller colonies, if metabolic rates were smaller or equal to the control at the 4 °C test, we used the control value for CO_2 as that colony's measure.

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., 2018; Terblanche et al., 2011). Briefly, 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 & 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 A.3) 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 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 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; Figure 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 A.2). 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 A.2). 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; Figure 1B).

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; Figure 2; urban estimate = 577 seconds, SE = ± 72.6; rural estimate = 576 seconds, 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; Figure 4; Figure 5). However, we found no significant difference in routine metabolic rate between urban and rural colonies at either 4 or 10 °C (Table 1; Figure 4). Similarly, we found no evidence for differences in acute thermal sensitivity of metabolic rate between urban and rural colonies (Table 1; Figure 5).

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; Figure 3; urban estimate = 46.2 °C, SE = ± 0.572, rural estimate = 44.9 °C, SE = ± 0.568).

Table 1. Estimates and χ^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 χ^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	χ^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**

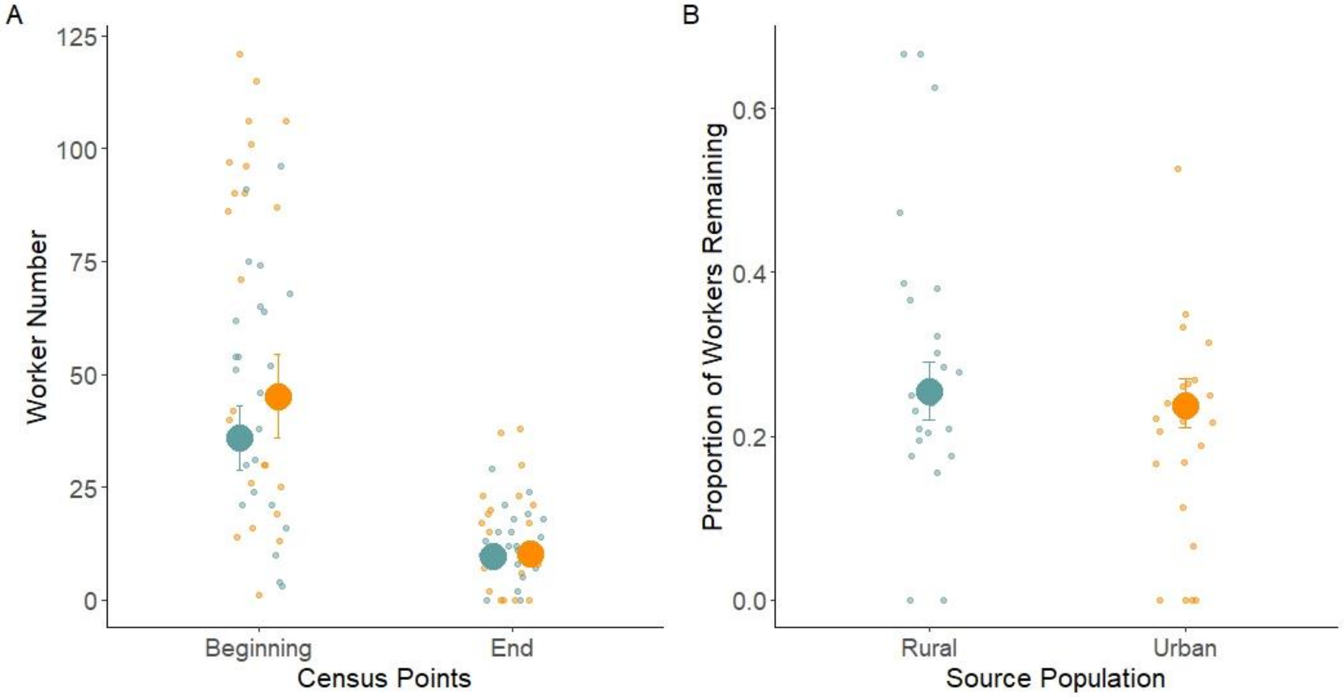

Figure 1. Colony demographic responses to winter across urban and rural acorn ant populations. Large points show estimated means ± 1 SE from the corresponding statistical models (see main text section 2.6). A) Worker number at the initial and final censuses of the 4-month experiment across urban (orange 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.

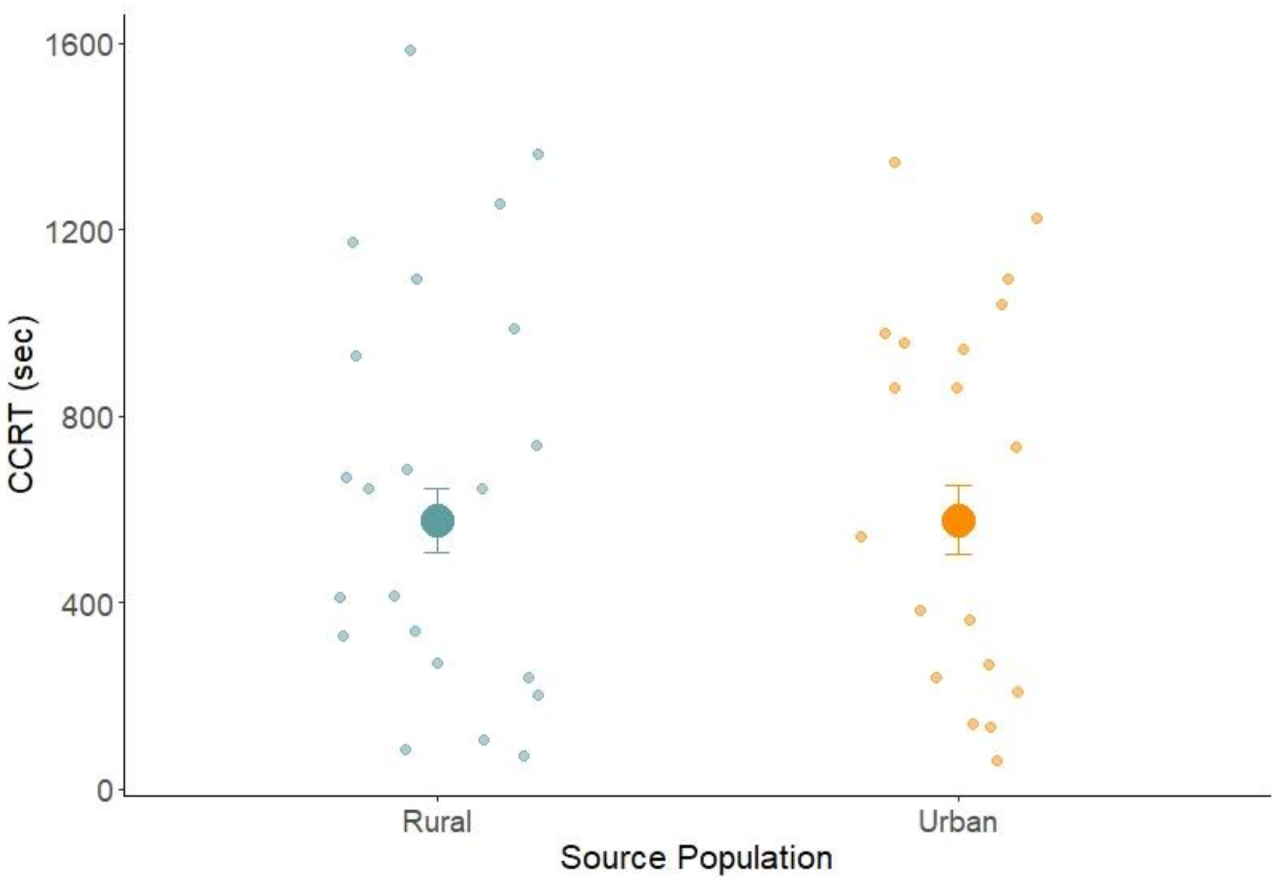

Figure 2. Chill coma recovery time (CCRT; in seconds) following overwintering acclimation between urban and rural acorn ant colonies. Large points display the estimated means ± 1 SE, back-transformed from the model to the data scale, of recovery time from the corresponding statistical models (see main 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.

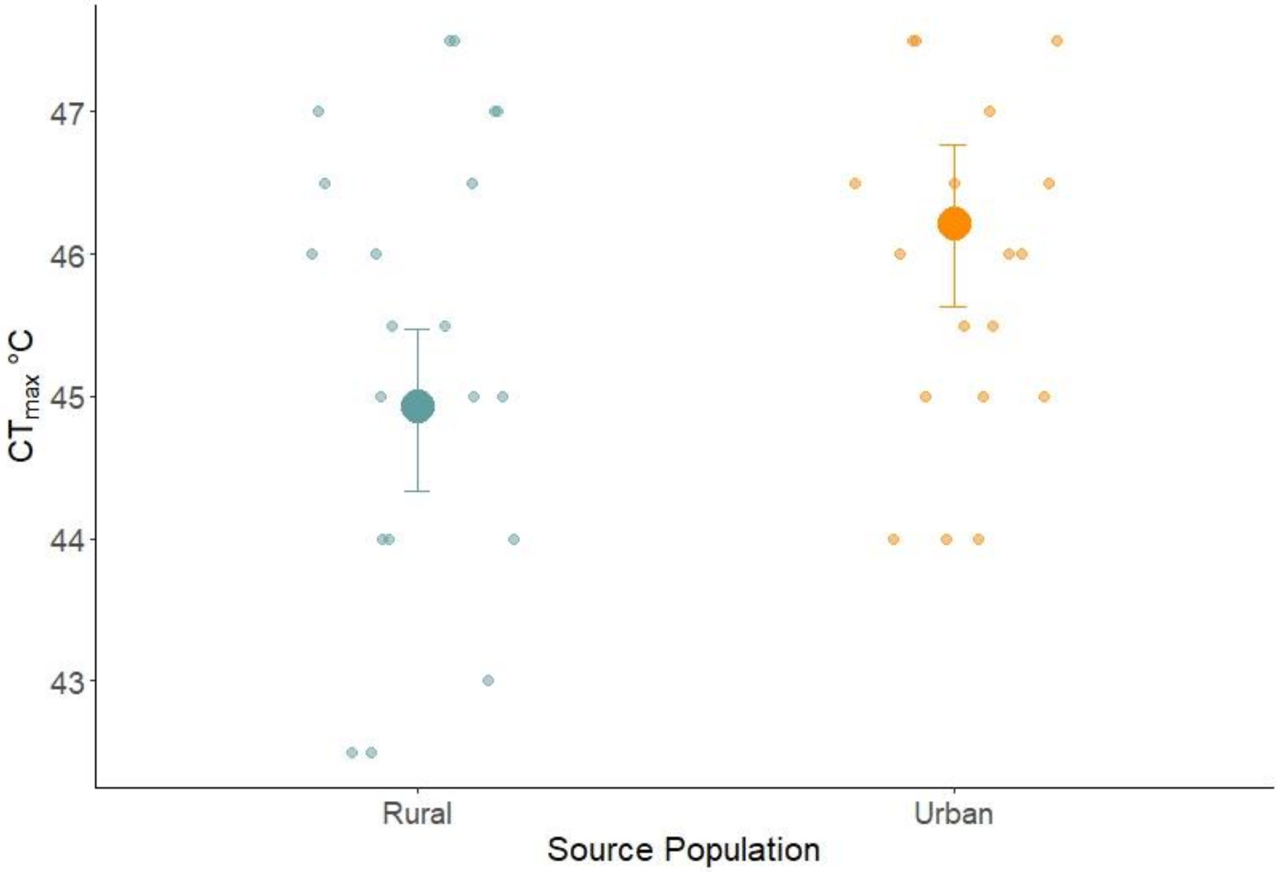

Figure 3. Critical thermal maxima (CT_{max} in °C) following overwintering acclimation between urban and rural acorn ant colonies. Large points display the estimated means ± 1 SE of CT_{max} from the corresponding statistical models (see main text section 2.6) from rural (blue) and urban (orange) colonies. Smaller points jittered behind the estimated means display the raw colony mean CT_{max} to show the variance across all colonies.

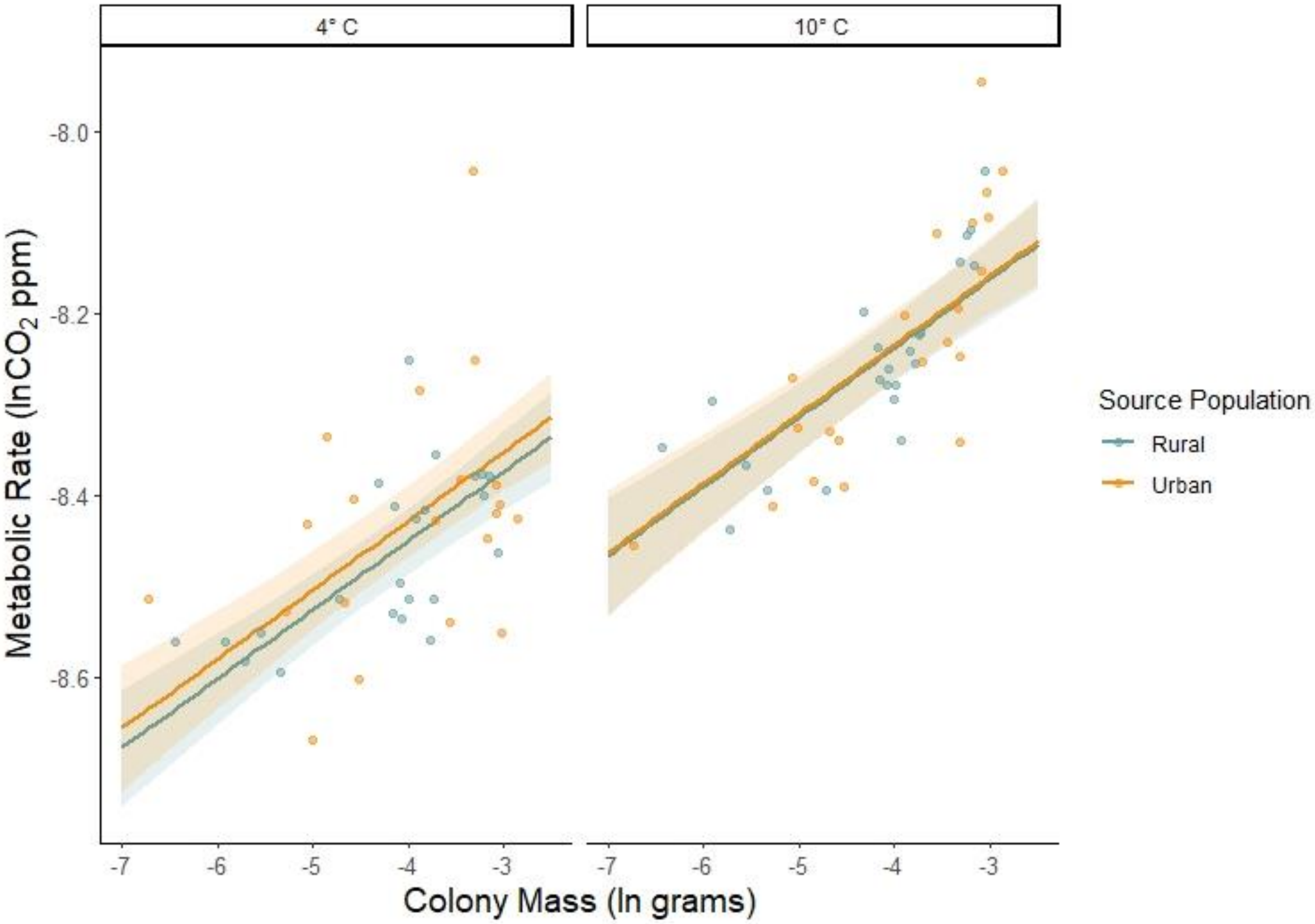

Figure 4. Modeled estimates of mean whole colony metabolic rates by colony mass on the natural log scale for both 4 and 10 °C acute test temperatures. Rural colony trends are shown by the smoothed dark line in blue while urban colony trends are shown by the smoothed dark line in orange with shaded 95% 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).

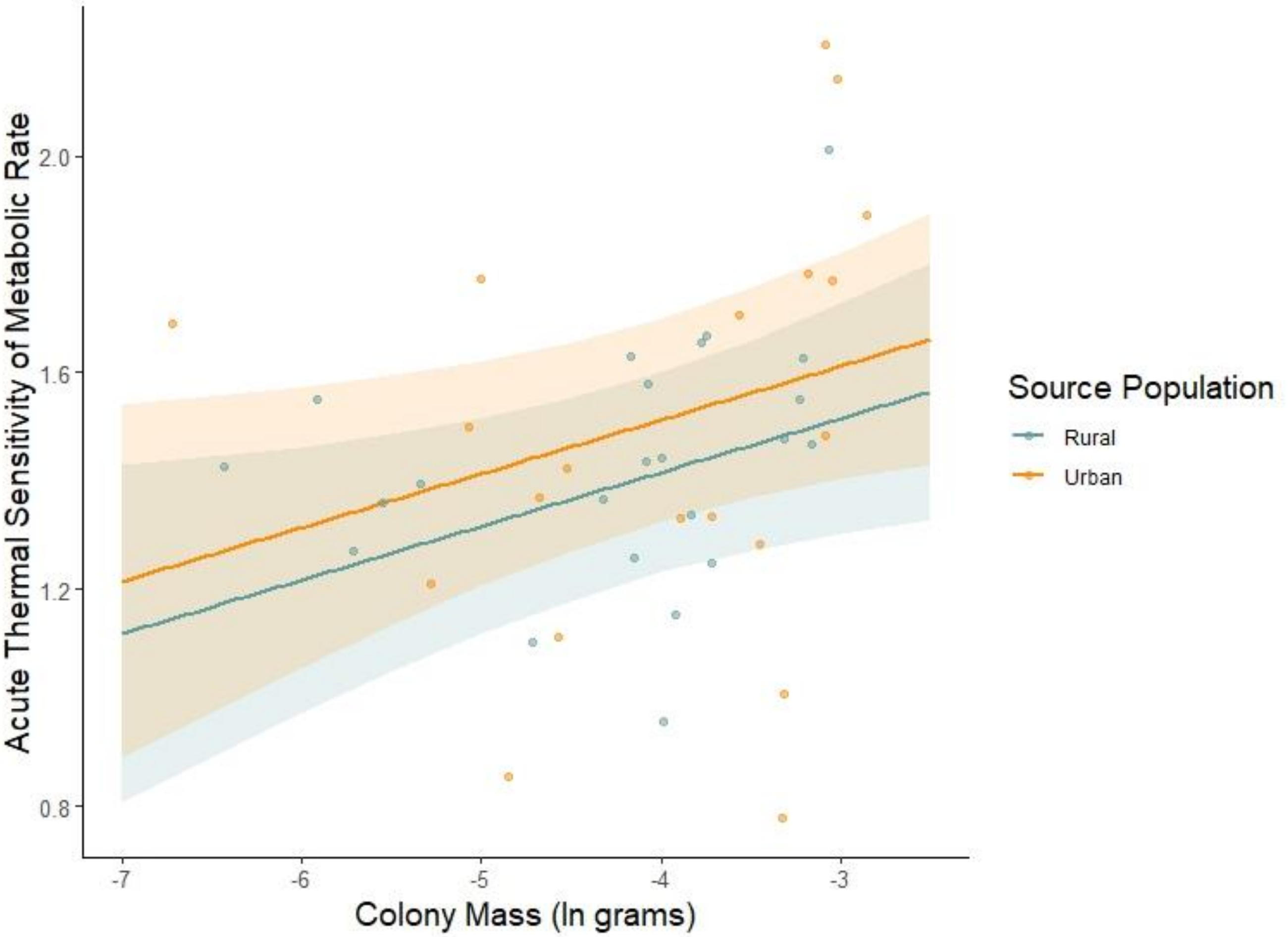

Figure 5. Modeled estimates of acute thermal sensitivity of metabolic rate showing the reaction rate increase for every 10 °C increase in temperature (Q_{10}) as a function of colony mass in grams on the natural log scale. Rural colony estimates are shown by the smoothed dark line in blue while urban 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).

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 & 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 & Martin, 2021), we found that winter-acclimated urban ant colonies retain similar metabolic rates and cold 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 and could not be assayed for the criterion for reaching the critical thermal minimum (Gibert & Huey, 2001; Terblanche et al., 2011). Although both CCRT and CT_{min} are biologically relevant to overwintering (Chown & Nicolson, 2004), they are underlain by different physiological mechanisms and functions (MacMillan & Sinclair, 2011; Overgaard & 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 & MacMillan, 2017; Teets & 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 & 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 divergence in CT_{min} among warm season acclimated ants, represent differences in the evolutionary response of these two traits between urban and rural populations versus seasonal plasticity in response to overwintering conditions.

If CT_{min} has diverged between urban and rural populations but CCRT has not, what might explain this? It is possible that selection is acting at different strengths on these traits across the urban and rural environments. For example, selection for lower CT_{min} could be relaxed in urban environments due to fewer low-temperature events and that winter acclimation can help to a degree with winter conditions that are relatively mild. This could explain the differences in mortality in the field experiment of urban ants transplanted to the rural habitat (Martin et al., 2021) where beneficial effects of acclimation were potentially overwhelmed by extreme temperature events that were likely much lower than laboratory conditions (Table A.1). Interestingly, previous studies with leaf cutter ants and worm lions across urbanization gradients also found no change in CCRT between urban and rural populations (Angilletta et al., 2007; Bar-Ziv & Scharf, 2018) which differed from general patterns in losses of CT_{min} in urban populations (Diamond & Martin, 2021).

On the other end of the thermal physiological spectrum, why was CT_{max} apparently unaffected by the laboratory overwintering acclimation, with similar magnitudes of divergence between urban and rural populations seen under warm rearing conditions alone (Diamond et al., 2017; Martin et al., 2019)? In insects, warmer developmental acclimation temperatures are more strongly correlated to adult CT_{max} than adult acclimation temperatures (Kellermann et al., 2017; Kellermann & Sgrò, 2018; Slotsbo et al., 2016, but see Chown & 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 at the high end of rearing regimes from previous work that showed similar CT_{max} differences between urban and rural acorn ants (Diamond et al., 2018). 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). That the warmer developmental temperature in this test likely contributed to lasting higher CT_{max} in urban acorn ants despite the overwintering period could mean that retaining a higher CT_{max} is beneficial in the warmer urban winter habitat.

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 thermal sensitivity of metabolic rates to temperature with no difference in average routine metabolic rates. Prior research with acorn ants had shown higher average metabolic rates for urban ants with lower thermal sensitivity than rural ants (Chick et al., 2021), but tests were done with ants reared under growing season conditions and conducted at much warmer temperatures. Since temperature is predictive of metabolic rate and thus activity (Angilletta, 2009; Chown & 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 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 cold-term shocks, but warmer overall temperatures (Thompson et al., 2016), 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 & 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 and had 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).

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Author Contributions:

EGP designed the study, completed experimental collections and measures, data analysis and visualization, and drafted the original manuscript. RAM designed and conceptualized the study and contributed formal analysis and helped review and edit the manuscript. SED helped design the study, assisted with collections and rearing of ants, provided R code for metabolic rate analyses, and provided edits to the manuscript. OMB assisted with metabolic rate methods and investigation along with edits to the manuscript. AL assisted with metabolic rate, statistical analysis approach and contributed to data visualization and edits to the manuscript. JLC assisted with colony caretaking, experimental measures, and manuscript edits.

Data Statement:

Data are available from the GitHub repository: https://github.com/eprileson/Acorn_Ant_OvW

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Appendix A:

Table A.1. Day: night temperatures and light cycles for acorn ant colonies across 6 weekly step downs from summer to winter temperatures. Temperatures for Fall step downs were derived from previously measured rural soil level data from one of the rural sites (CWRU farm). Temperatures for Fall were averaged from October to December 2019. Temperatures for winter were averaged from measures taken in January to March 2020 from 1 cm below the soil surface. ‘Start’ corresponds to the acclimation period temperature while ‘Step 6’ corresponds to the winter temperature and light:dark cycle.

	Start	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
Light Cycle (Light:Dark)	14:10	13:11	12:12	12:12	11:13	10:14	8:16
Temperature Day (C)	25	20	15	10	5	4	2.5
Temperature Night (C)	25	17	13	9	4	3.5	1.5

Table A.2. Estimated marginal means (emmeans) contrasts between census time points of urban and rural worker populations. Significant P values shown in bold.

Contrast	Estimate	SE	DF	T ratio	P value
Beginning Rural – End Rural	32.26	3.39	89	9.524	**<0.0001**
Beginning Rural – Beginning Urban	-20.97	3.21	89	-6.529	**<0.0001**
Beginning Rural – End Urban	29.38	3.25	89	9.050	**<0.0001**
End Rural – Beginning Urban	-53.22	5.35	89	-9.944	**<0.0001**
End Rural – End Urban	-2.88	1.16	89	-2.485	**0.0148**
Beginning Urban – End Urban	50.35	5.08	89	9.909	**<0.0001**

Table A.3. Acorn ant colony collection data including latitude and longitude coordinates of collection sites, beginning and final worker counts, and dates of collection, acclimation, and counts.

Colony ID	**Site**	**Latitude**	**Longitude**	**Collection_date**	**Test_cohort**	**Source.pop**	**Date_lab acclimated Beginning Count**	**Begin Worker Count**	**Final Worker Count**	**Date_ FinalCount**
8308	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	30	11	20220527
4717	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	52	13	20220527
7111	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	54	15	20220527
3456	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	46	12	20220527
9461	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	4	0	20220527
4523	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	54	11	20220527
7622	CWRU. Farm	41.4986	- 81.4227	20210913	Early	Rural	20210915	41	9	20220527
6143	CWRU. Farm	41.4986	- 81.4227	20210903	Early	Rural	20210908	36	7	20220527
5371	CWRU. Farm	41.4986	- 81.4227	20211004	Early	Rural	20211005	10	0	20220527
3309	CWRU. Farm	41.4986	- 81.4227	20211202	Late	Rural	20211202	21	14	20220709
7783	CWRU. Farm	41.4986	- 81.4227	20211204	Late	Rural	20211206	3	2	20220709
5980	CWRU. Farm	41.4986	- 81.4227	20211204	Late	Rural	20211206	31	10	20220709
3538	WR Land Conservancy	41.4533	-81.41314	20211111	Middle	Rural	20211111	24	5	20220527
1301	WR Land Conservancy	41.4533	-81.41314	20211111	Middle	Rural	20211111	64	10	20220527
7545	WR Land Conservancy	41.4533	-81.41314	20211112	Late	Rural	20211115	75	18	20220711
9753	WR Land Conservancy	41.4533	-81.41314	20211113	Late	Rural	20211115	16	10	20220711
6481	WR Land Conservancy	41.4533	-81.41314	20211116	Late	Rural	20211117	96	29	20220711
2158	WR Land Conservancy	41.4533	-81.41314	20211123	Late	Rural	20211123	74	21	20220711
8732	WR Land Conservancy	41.4533	-81.41314	20211123	Late	Rural	20211123	68	12	20220711
2504	WR Land Conservancy	41.4533	-81.41314	20211202	Late	Rural	20211202	65	15	20220711
3478	WR Land Conservancy	41.4533	-81.41314	20211202	Late	Rural	20211202	21	8	20220711
2906	WR Land Conservancy	41.4533	-81.41314	20211202	Late	Rural	20211202	91	19	20220711
1121	WR Land Conservancy	41.4533	-81.41314	20211202	Late	Rural	20211202	62	24	20220711
6892	WR Land Conservancy	41.4533	-81.41314	20211204	Late	Rural	20211204	38	18	20220711
3351	ForestHills	41.52108	-81.57834	20210912	Early	Urban	20210912	19	10	20220725
6951	ForestHills	41.52108	-81.57834	20210912	Early	Urban	20210912	14	0	20220527
8317	ForestHills	41.52108	-81.57834	20211001	Early	Urban	20211003	106	37	20220527
3630	ForestHills	41.52108	-81.57834	20211001	Early	Urban	20211003	121	38	20220527
5292	ForestHills	41.52108	-81.57834	20211018	Middle	Urban	20211019	86	19	20220527
6116	ForestHills	41.52108	-81.57834	20211018	Middle	Urban	20211019	40	10	20220527
3488	ForestHills	41.52108	-81.57834	20211118	Late	Urban	20211119	30	10	20220709
2702	ForestHills	41.52108	-81.57834	20211119	Late	Urban	20211120	115	30	20220709
2318	CWRU_SRes	41.50268	-81.59881	20210921	Early	Urban	20210922	30	2	20220527
1214	CWRU_SRes	41.50268	-81.59881	20210921	Early	Urban	20210922	16	0	20220527
9186	CWRU_SRes	41.50268	-81.59881	20210921	Early	Urban	20210922	26	7	20220527

6843	CWRU_SRes	41.50268	-81.59881	20211004	Early	Urban	20211005	87	23	20220527
5619	CWRU_SRes	41.50268	-81.59881	20211004	Early	Urban	20211005	1	0	20220527
9199	CWRU_SRes	41.50268	-81.59881	20211019	Middle	Urban	20211020	90	17	20220527
9212	CWRU_SRes	41.50268	-81.59881	20211101	Middle	Urban	20211102	25	6	20220527
1579	CWRU_SRes	41.50268	-81.59881	20211101	Middle	Urban	20211102	101	17	20220527
9335	Shaker_Doan	41.49269	-81.59221	20211022	Middle	Urban	20211025	90	15	20220527
2231	Shaker_Doan	41.49269	-81.59221	20211022	Middle	Urban	20211025	106	23	20220527
8293	Shaker_Doan	41.49269	-81.59221	20211025	Middle	Urban	20211025	97	20	20220527
6187	Shaker_Doan	41.49269	-81.59221	20211025	Middle	Urban	20211026	96	21	20220527
5260	Shaker_Doan	41.49269	-81.59221	20211025	Middle	Urban	20211026	13	0	20220527
5716	Shaker_Doan	41.49269	-81.59221	20211031	Middle	Urban	20211101	71	8	20220527
8462	Shaker_Doan	41.49269	-81.59221	20211031	Middle	Urban	20211101	42	10	20220527
6919	Shaker_Doan	41.49269	-81.59221	2021119	Late	Urban	20211120	16	0	20220620