

Evidence of plasticity, but not evolutionary divergence, in the thermal limits of a highly successful urban butterfly

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ABSTRACT

Despite the generally negative impact of urbanization on insect biodiversity, some insect species persist in urban habitats. Understanding the mechanisms underpinning the ability of insects to tolerate urban habitats is critical given the contribution of land-use change to the global insect decline. Compensatory mechanisms such as phenotypic plasticity and evolutionary change in thermal physiological traits could allow urban populations to persist under the altered thermal regimes of urban habitats. It is important to understand the contributions of plasticity and evolution to trait change along urbanization gradients as the two mechanisms operate under different constraints and timescales. Here, we examine the plastic and evolutionary responses of heat and cold tolerance (critical thermal maximum [CT_{max}] and critical thermal minimum [CT_{min}]) to warming among populations of the cabbage white butterfly, *Pieris rapae*, from urban and non-urban (rural) habitats using a two-temperature common garden experiment. Although we expected populations experiencing urban warming to exhibit greater CT_{max} and diminished CT_{min} through plastic and evolutionary mechanisms, our study revealed evidence only for plasticity in the expected direction of both thermal tolerance traits. We found no evidence of evolutionary divergence in either heat or cold tolerance, despite each trait showing evolutionary potential. Our results suggest that thermal tolerance plasticity contributes to urban persistence in this system. However, as the magnitude of the plastic response was low and comparable to other insect species, other compensatory mechanisms likely further underpin this species' success in urban habitats.

1. Introduction

Urbanization is a major contributor to the global insect decline because it drives local extirpations and shifts insect community composition (Fattorini, 2011; Sánchez-Bayo and Wyckhuys, 2019; Uhler et al., 2021). Yet, urban ecosystems are not devoid of insects, as some species cope with or even benefit from these altered landscapes (Hall et al., 2017; Theodorou et al., 2020). Variation in biodiversity trends across urban gradients suggest certain insect taxonomic groups, such as Lepidoptera, are particularly sensitive to urban pressures, while other taxonomic groups, such as Hymenoptera, are less impacted by or can even benefit from urban environments (Fenoglio et al., 2020; Perez and Diamond, 2019; Theodorou et al., 2020). Understanding the mechanisms contributing to urban tolerance is important to protect biodiversity and implement appropriate interventions in our increasingly urbanized world (Diamond et al., 2023).

Urban insect populations must deal with warmer temperatures and more variable maximum temperatures in cities (Manoli et al., 2019; Tam

et al., 2015), which may be particularly important in determining urban tolerance. Indeed, recent trait-based approaches highlight the importance of thermal traits for urban persistence. For example, grasshopper species with broader climatic niche breadths were less likely to go extinct and climatic niche breadth explained more variation in the probability of extinction than general habitat use and dispersal capability during urbanization in Rome, Italy (Ancillotto and Labadessa, 2023). Similarly, longer seasons of active flight (a potential proxy for high thermal flexibility) were associated with higher urban affinity scores among different butterfly species (Callaghan et al., 2021). Moreover, a recent meta-analysis of divergence in thermal tolerance traits across urbanization gradients found evidence of consistent shifts towards higher heat tolerance (and loss of cold tolerance) across a diverse range of ectothermic species (Diamond and Martin, 2021); yet, most of these studies were done at the phenotypic level, so the contributions of phenotypic plasticity and evolution to divergence are unknown.

As plasticity and evolution operate under different constraints and

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over different timescales, disentangling and understanding their contributions towards persistence in the face of environmental change is increasingly critical (Fox et al., 2019; Gibert et al., 2019; Kasada and Yoshida, 2020). It is therefore necessary to quantify the plastic and evolved components of thermal trait change across urbanization gradients to develop a mechanistic understanding of how species persist in urban habitats. Only a handful of species have been reared under common garden designs to be able to disentangle plastic from evolved responses, and thus shown to have a genetic component to shifts in thermal tolerance trait values between urban and rural populations (e. g., Brans and De Meester, 2018; Diamond et al., 2018; Merckx et al., 2023; Yilmaz et al., 2021). Therefore, it is still an important, open question of what mechanistically drives trait variation in response to urbanization. Evolved responses across urbanization gradients might be especially relevant with insects given their fast generations times (Thomas et al., 2010), but we currently lack a comprehensive understanding of how frequently this process occurs.

The cabbage white butterfly, *Pieris rapae*, is commonly present in urban habitats (Kuussaari et al., 2021; Matteson and Langellotto, 2012; Sobczyk et al., 2017) and displays an uncharacteristic preference for urban habitats relative to other butterfly species (i.e., has a high urban affinity; Callaghan et al., 2021); yet, it is unclear what mechanisms underlie its ability to withstand urban environments. *Pieris rapae* is a multivoltine butterfly with a near-global distribution (Ryan et al., 2019), suggesting the ability to acclimate to various climates through phenotypic plasticity and/or evolution. Temperature-induced plasticity and evolutionary divergence between populations have been documented in thermally relevant traits in *P. rapae* (Kingsolver et al., 2007; Li et al., 2020; Stoehr and Goux, 2008), but the contributions of these mechanisms to altering thermal physiology across urbanization gradients are unknown.

Here, we assess the contributions of evolution and plasticity towards differences in thermal tolerance traits across an urbanization gradient in *P. rapae* butterflies using a two-temperature, split-clutch common garden experiment. We quantified heat and cold tolerance of F1 generation adult cabbage white butterflies following developmental acclimation at a colder (20 °C) or warmer (30 °C) temperature. This experimental design allowed us to measure and disentangle the effects of phenotypic plasticity (i.e., a difference in thermal tolerance between temperature acclimation treatments) and evolutionary divergence in either trait means or the plastic response (i.e., a difference in thermal tolerance between population types [rural or urban] and a difference in how thermal tolerance responds to acclimation temperature between population types, respectively) in these traits. We also quantified evolutionary potential in heat and cold tolerance for rural and urban populations at each acclimation temperature by using variance partitioning to determine the genetic component of the total variance in thermal tolerance. We hypothesized *P. rapae* thermal tolerance traits would respond to the altered thermal environment of urban environments through plastic and evolutionary mechanisms, specifically predicting enhanced heat tolerance and diminished cold tolerance in urban populations. Although the capacity to increase heat tolerance via beneficial acclimation and/or thermal adaptation is perhaps the most directly relevant for understanding the success of *P. rapae* in cities (Diamond and Martin, 2020), the effects of warming on cold tolerance are also relevant. Cold tolerance is often diminished under warmer developmental acclimation temperatures in ectotherms (Gunderson and Stillman, 2015; Weaving et al., 2022), and the evolutionary loss of cold tolerance often accompanies the evolution of higher heat tolerance across urbanization gradients (Diamond and Martin, 2021). Because cities can generate complex, heterogeneous thermal landscapes over space and time, including the generation of urban cool islands over winter due to snow removal (Thompson et al., 2016), understanding capacities to maintain cold tolerance in spite of warming are relevant for understanding patterns of urban persistence.

2. Methods

2.1. Overview

We examined the temperature-induced plasticity and evolutionary divergence in the critical thermal limits (critical thermal maximum and critical thermal minimum, respectively CT_{max} and CT_{min}) of *P. rapae* across an urbanization gradient in Cleveland, OH. We used a two-temperature common garden experiment to examine differences in mean trait values and plasticity of thermal tolerance. We reared F1 offspring from rural and urban populations in a split-clutch design, which allowed us to compare the genetic component of thermal tolerance traits (i.e., determining evolutionary potential through the comparison of traits among full-siblings) across our two temperature treatments. We used a dynamic ramping assay to determine CT_{min} and CT_{max} (defined as loss of muscular coordination) for individual adult butterflies. We assessed plasticity, evolution, and evolution of plasticity at the level of population type (urban or rural).

2.2. Collection sites

We collected female *P. rapae* butterflies from three urban and three rural field sites around Cleveland, OH, USA (Table 1, Table S1). Environmental temperature data show a 2 °C difference in mean daily temperatures between our most urban and least urban sites and the expected pattern of night-time biased warming in urban areas (Table 1). However, temperature data was collected only at a subset of our six sites (Table 1), so we assigned sites to urbanization categories based on their level of impervious surface area (ISA). We used the NLCD percent impervious surface dataset, which provides ISA values at 30 m resolution (Dewitz and U.S. Geological Survey, 2021). We used the focal statistics tool in ArcMap (v 10.8.2) to determine the average ISA at a scale of 1 km² around the center of our sites because this scale encompassed the sampling boundaries at our sites and is more appropriate for a mobile species such as *P. rapae*. We categorized our three lowest ISA sites (<22 % ISA) as rural and our three highest ISA sites (>35 % ISA) as urban. Because the ISA values at our sites make up a continuous distribution (Table 1), we recognize that binning sites into two distinct categories could have limitations. Therefore, we retained the ISA value at 1 km² as a continuous variable to use in supplemental analyses.

Table 1

Site names, population type classifications, percent impervious surface area (ISA), daily mean, daily maximum, and daily minimum temperatures. ISA values are from the 2019 National Land Cover Database and processed to give an average for the surrounding 1 km² using the focal statistics tool in ArcMap. Daily temperatures (mean, max, and min) are average daily mean, daily maximum, and daily minimum temperature across the sampling period from 27 April 2022 to 27 August 2022 recorded with iButton temperature loggers (Thermochron, 0.5 °C resolution) approximately 1 m off the ground in open habitat. Temperatures were recorded at a subset of sites.

Site	Population type	ISA (%)	Daily mean (°C)	Daily max (°C)	Daily min (°C)
Case Western Reserve University	urban	60.68	21.8	29.6	15.7
Argonne Rd	urban	44.50	20.6	31.5	13.4
Acacia Reservation	urban	36.10	—	—	—
West Creek Reservation	rural	21.76	—	—	—
Bedford Reservation	rural	6.55	—	—	—
Squire Valleevue and Valley Ridge Farm	rural	2.75	19.8	29.3	11.7

2.3. Butterfly rearing

We captured gravid female *P. rapae* from our field sites between 24 April and 7 September 2022. Field-caught females were brought back to Case Western Reserve University and housed individually in mesh insect rearing cages (30 cm x 30 cm x 30 cm, Bug-Dorm 1) near bright, natural light at ambient room conditions. We provided each butterfly with two pieces of organic green cabbage as a laying substrate and a petri dish containing a sponge saturated in 10 % (v/v) honey solution. We checked cages for eggs daily. Upon finding eggs, we recorded the oviposition date and moved the cabbage into 118 mL cups. We placed egg cups inside temperature control growth chambers (Percival I36VLC8). While *P. rapae* can mate multiple times (mean 2.13 times; Svård and Wiklund, 1989), we treated eggs from the same female (hereafter ‘family’) as full siblings as there is strong sperm precedence in this species such that if a female mated more than once it is most likely one male fertilized all the eggs (Wedell and Cook, 1998); however, it is possible that some individuals in our study represent half-siblings. Families were split into constant temperature treatments of 20 °C and 30 °C with a 14:10 L:D cycle. We chose these temperatures because we were interested in capturing the thermal tolerance plasticity of *P. rapae* across the entirety of the flight period, and these temperatures represent the daily mean (19.8–21.8 °C) and daily maximum (29.3–31.5 °C) temperatures we measured across the Cleveland urbanization gradient (Table 1). We elected to use constant rearing temperatures because fluctuating rearing temperatures can differentially alter measurement of cold and heat tolerance and resistance depending on the mean temperature (i.e., Jensen’s inequality, reviewed in Colinet et al., 2015) and the focal trait (e.g., chill-coma recovery versus heat-knockdown, Fischer et al., 2011). When caterpillars hatched from eggs and reached the second instar life stage, we used a fine tip paintbrush to move them off the cabbage leaf and onto an artificial diet (recipe modified from Espeset et al., 2019). We placed up to a maximum of three individuals from the same family and egg date into a single 118 mL rearing cup. When an individual metamorphosed, we moved its pupa into a new cup with a piece of paper towel lining the bottom before placing it back in its developmental temperature treatment. Upon eclosion, we gave individual butterflies a unique identification code, determined sex, and recorded the date, population type, rearing temperature treatment, and family identification code. We created individual holding containers by perforating lids on 473 mL cups. We placed newly eclosed butterflies in these containers with a petri dish holding a sponge saturated in 10 % (v/v) honey water. After eclosion, all adult butterflies were removed from growth chambers and kept at common ambient room conditions in holding containers prior to undergoing thermal tolerance testing.

2.4. Thermal tolerance measurement

We assessed butterfly thermal tolerance through critical thermal minimum (CT_{min}) and critical thermal maximum (CT_{max}). We determined CT_{min} and CT_{max} by the loss of muscular coordination. CT_{min} and CT_{max} trials were conducted using an A40 ARCTIC series water bath with a SC150 circulator (Thermo Scientific). We assessed the CT_{min} of butterflies 24 hrs after eclosion (range: 12–72 hrs post-eclosion) and CT_{max} 24 hrs after CT_{min} (range: 12–96 hrs post-eclosion).

Body mass is often associated with tolerance traits in terrestrial ectotherms (Claunch et al., 2021; Gunderson, 2024; Rubalcaba and Olalla-Tárraga, 2020; Spicer et al., 2019), so we recorded butterfly mass (Sartorius MSE124S-100-DA; 0.0001 g precision) immediately before commencing the trial and placed each butterfly in a 100 mL container with a screw top lid. A maximum of five containers were placed in a foam float and were weighed down so that the containers were entirely submerged within the water bath. We acclimated butterflies for 15 min to the starting water bath temperature (15 °C for CT_{min} and 35 °C for CT_{max}). For both CT_{min} and CT_{max} trials we used a dynamic ramping protocol of 1 °C min⁻¹. After CT_{min} trials, we placed butterflies back into

their holding containers with fresh 10 % honey water to await CT_{max} testing on the following day. After CT_{max} trials, we placed individuals in glassine envelopes and into a freezer at –20 °C. Trials took place between 0700 and 1900hr from 26 May to 7 October 2022.

CT_{min} and CT_{max} trials used different methodology to assay for loss of muscular coordination. During CT_{min} trials individuals had overall low levels of activity due to low temperatures; therefore, starting at 10 °C, we inverted the containers to knock individuals prone. If individuals did not immediately get up from being prone, we gently tapped the containers to encourage movement. We removed individuals that remained prone from the water bath and confirmed that they did not get up within 30 s while the bath cooled to the next test temperature. Because we inverted the containers during CT_{min} trials, we sealed the rim of the screw top lid with parafilm to prevent water leakage into the containers. During CT_{max} trials individuals increased flight activity as temperature increased; therefore, we assessed if individuals that stopped flying and fell prone were able to get up from prone. Individuals that were unable to get up were marked as having reached their CT_{min} or CT_{max}. The recorded temperature is reflective of the container air temperature at CT_{min} or CT_{max}, but our measurement might overestimate butterfly body temperature at CT_{min} or CT_{max} given our fast ramp rate (1 °C min⁻¹) and the relatively large body mass of *P. rapae*.

While most individuals underwent both CT_{min} and CT_{max} trials, some butterflies died within the 24 hrs between CT_{min} and CT_{max} trials. When possible, we placed a different individual in the open spot for the CT_{max} trial. Therefore, our dataset contains a few individuals with only CT_{min} or only CT_{max} values. Some families had insufficient replication for certain combinations of sex and rearing temperature (e.g., females at 30 °C), so we subset our dataset to exclude any combinations that had less than 3 tested individuals from a single family. Further, we removed individuals that did not complete thermal tolerance testing within 96 hrs of eclosion. In total, this dataset retained CT_{min} data for 738 individuals from 42 families and CT_{max} data for 723 individuals from 41 families. For each combination of urbanization level, temperature, and sex, we tested a mean 92.25 individuals (range 59–128) across a mean 14.62 families (range 10–20) for CT_{min} and a mean 90.37 individuals (range 56–127) across a mean 14.25 families (range 9–20) for CT_{max} (Table S2).

2.5. Statistical analysis

We performed all analyses in R (version 4.3.0, R Core Team, 2023). We constructed separate linear mixed effects models for CT_{min} and CT_{max} using the *lmer* function in the lme4 package (Bates et al., 2015). In both models, we modeled the response of thermal tolerance as a function of the categorical predictors of population type (urban or rural), rearing temperature (20 °C or 30 °C), sex (male or female), and their three-way interaction. A significant effect of population type, rearing temperature, or their interaction is indicative of urban evolution, plasticity, and evolved plasticity, respectively. We included pre-trial butterfly mass and butterfly age on the day of the trial as covariates. To account for the non-independence of related individuals, we included the family identification code as a random intercept. In preliminary models, we also included capture site (n = 6) as a random intercept, but this explained zero model variance and was excluded from the final model. We ran additional models using either site identification as a factor with six levels or the continuous value of ISA as predictors in the place of population type (urban or rural) to examine the reliance of our results on using ISA values and on binning ISA values into categories respectively.

We additionally constructed separate linear mixed effects models of CT_{min} and CT_{max} for each of the four combinations of population type and rearing temperature to explore the family-level variance in urban and rural populations under each environmental condition. Models were constructed as described above, with body mass and butterfly age as covariates and family as a random intercept, but sex was the only fixed, categorical predictor. We then extracted variance components attributed to the random effect of family and the residual variance using the

summary function in R. We similarly investigated sex-specific differences in the proportion of variance explained by family using eight separate models for each combination of population type, rearing temperature, and sex for both CT_{min} and CT_{max} and report these results in the [supplementary material](#).

We determined parameter significance through analysis of deviance with type III sums of squares using the *Anova* function in the *car* package (Fox and Weisberg, 2019). Model diagnostics were performed using the *simulateResiduals*, *plotResiduals*, and *testDispersion* functions from the DHARMa package (Hartig, 2022). Pairwise comparisons of model estimates were done using the *emmeans* and *contrast* functions in the *emmeans* package (Lenth et al., 2022). Data visualization was done using the *ggplot* package (Wickham, 2016).

3. Results

In our model of CT_{max} , the three-way interaction between population type, rearing temperature, and sex was borderline non-significant for CT_{max} ($\chi^2 = 3.1$, $P = 0.079$). This arises from the pattern that urban butterflies had lower CT_{max} at 20 °C in males but lower CT_{max} at 30 °C in females. However, we did not find strong statistical support for the pairwise comparisons driving this three-way interaction (Table S3). Thus, we interpret the main effects of population type, rearing temperature, and sex below.

We found that CT_{max} responded plastically to rearing temperature, such that butterflies raised at 30 °C had 0.75 ± 0.099 °C (hereafter numbers reported are estimate $\pm$ SE) higher CT_{max} ($\chi^2 = 30$, $P < 0.0001$; Fig. 1a,b). There was no effect of sex ($\chi^2 = 0.12$, $P = 0.73$) or population ($\chi^2 = 0.021$, $P = 0.88$) on CT_{max} (Fig. 1a,b). Body mass was not an important predictor of CT_{max} ($\chi^2 = 0.21$, $P = 0.64$). However, age influenced CT_{max} , such that for each day after eclosion CT_{max} decreased by 0.33 ± 0.10 °C ($\chi^2 = 10$, $P = 0.0013$). Statistical output from the full model is shown in Table S4.

In our model of CT_{min} , the two-way interactions between rearing temperature and population type ($\chi^2 = 2.9$, $P = 0.087$) and rearing temperature and sex ($\chi^2 = 3.4$, $P = 0.066$) were borderline non-significant. However, we did not find strong statistical support for the pairwise comparisons driving either of these interactions. One pattern suggested that urban butterfly cold tolerance might be weakly less plastic in response to rearing temperature (-0.41 ± 0.23 °C, $t_{722} = -1.8$, $P = 0.078$; Fig. 2a,b); however, this too, was borderline non-significant. Thus, as in our model of CT_{max} , we interpret the main effects of population type, rearing temperature, and sex for CT_{min} below.

We found that CT_{min} responded plastically to rearing temperature,

such that CT_{min} was 1.96 ± 0.13 °C lower in butterflies raised at 20 °C ($\chi^2 = 130$, $P < 0.0001$; Fig. 2a,b). Females were more tolerant (-0.41 ± 0.11 °C) of colder temperatures ($\chi^2 = 7.9$, $P = 0.0050$; Fig. 2a,b). We did not detect an effect of urbanization on CT_{min} ($\chi^2 = 0.048$, $P = 0.83$; Fig. 2a,b). Body mass and age were not important predictors of CT_{min} ($P \geq 0.19$). Statistical output from the full model is shown in Table S5.

Our results were largely insensitive to whether sites were binned into urban and rural classifications or if urbanization was treated as a continuous variable (Table S6). Further, our examination of site-level differences did not reveal any notable differences in thermal tolerance between sites (Table S7, Fig. S1, Fig. S2).

Our exploration of family variance showed that family explained more variance in CT_{min} than CT_{max} for each combination of population type and temperature (e.g., family explained 11.2 % of the variance for CT_{min} but 5.5 % of the variance for CT_{max} for rural populations reared at 20 °C; Table 2). Variance explained by family for each population was dependent on temperature, such that family explained less variance in rural populations at 20 °C for both CT_{max} and CT_{min} and less variance in urban populations at 30 °C for CT_{max} (Table 2). There were large sex effects on the percent of variance explained by family for some combinations of population type and rearing temperature (Table S8).

4. Discussion

Uncovering the mechanistic basis of intraspecific trait changes in response to urbanization might aid in understanding patterns of urban persistence (Alberti et al., 2017; Marques et al., 2019). In this study, we examined the phenotypic plasticity and evolutionary divergence of critical thermal limits, CT_{min} and CT_{max} , across an urbanization gradient in the cabbage white butterfly (*Pieris rapae*), a highly successful urban species (Callaghan et al., 2021; Kuussaari et al., 2021; Matteson and Langellotto, 2012; Sobczyk et al., 2017). Although we found evidence of evolutionary potential in both heat and cold tolerance traits, we found no evidence of evolutionary divergence in these traits among urban and rural populations. Heat and cold tolerance traits were plastic in response to developmental acclimation temperature, with warmer temperature enhancing heat tolerance and diminishing cold tolerance. Beneficial acclimation of heat tolerance and retention of baseline cold tolerance could aid in the urban persistence of this species.

4.1. Thermal plasticity but no evolutionary divergence in heat tolerance

The lack of evolutionary divergence in heat tolerance of cabbage white butterflies across the urbanization gradient was surprising given both that we detected evolutionary potential in this trait and

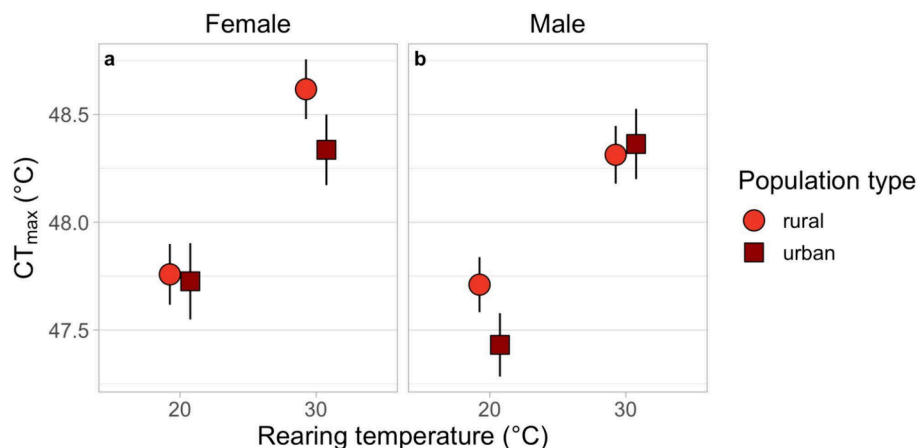


Fig. 1. Plasticity and evolutionary divergence of critical thermal maximum (CT_{max}) in females (a) and males (b) of urban (dark red, squares) and rural (light red, circles) butterflies. Plasticity can be interpreted as the change in value across rearing temperature and evolution as the difference in value between population types within a rearing temperature. Points and error bars are estimated marginal means $\pm$ 1 standard error.

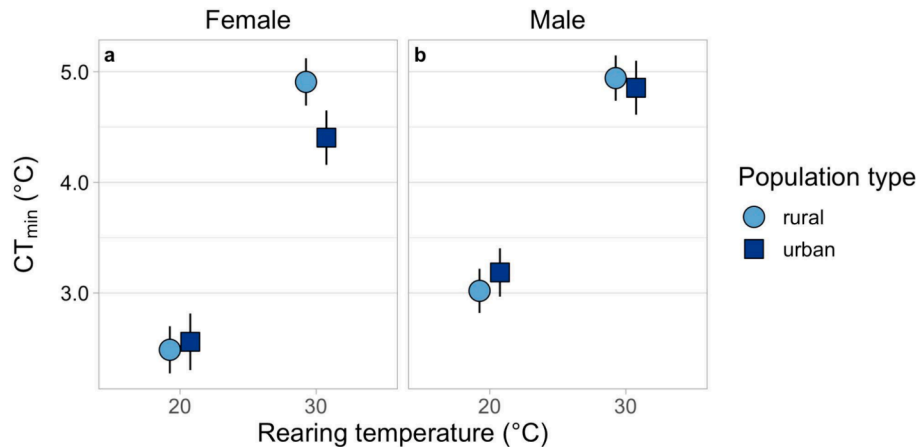


Fig. 2. Plasticity and evolutionary divergence of critical thermal minimum (CT_{min}) in females (a) and males (b) of urban (dark blue, squares) and rural (light blue, circles) butterflies. Plasticity can be interpreted as the change in value across rearing temperature and evolution as the difference in value between population types within a rearing temperature. Points and error bars are estimated marginal means ± 1 standard error.

Table 2

Variance explained by the random intercept ‘family’ in relation to the residual model variance for each thermal tolerance trait (response variable). Variance explained by family and residual model variance is reported from separate models for each combination of population type and rearing temperature. Variance explained by family is also reported as the percent of total variance (family variance + residual variance).

Response variable	Model (Population type / rearing temperature)	Family variance	Residual variance	Percent of total variance explained by family
CT_{min}	Rural / 20 °C	0.246	1.946	11.2 %
	Rural / 30 °C	1.348	1.899	41.5 %
	Urban / 20 °C	0.482	1.451	24.9 %
	Urban / 30 °C	0.586	1.588	27.0 %
CT_{max}	Rural / 20 °C	0.078	1.348	5.5 %
	Rural / 30 °C	0.111	0.786	12.4 %
	Urban / 20 °C	0.316	1.288	19.7 %
	Urban / 30 °C	0.045	0.801	5.3 %

accumulating evidence from other systems showing rapid evolution of physiological traits in response to urban heat islands (Diamond and Martin, 2021). One possibility for this result is that the plasticity in heat tolerance is sufficient to cope with urban warming. Work in acorn ants is suggestive of this pattern, as evolutionary divergence in heat tolerance was only found in cities where the magnitude of the urban heat island effect was high (Diamond et al., 2018). However, in our study on the cabbage white butterfly, we used one of the same gradients (Cleveland, OH) for which evolutionary divergence in heat tolerance had been demonstrated for acorn ants, yet we only found evidence of beneficial thermal acclimation (i.e. higher heat tolerance under warmer conditions), not evolutionary divergence. Importantly, other urban evolution studies have found comparable or greater magnitudes of thermal tolerance plasticity as we found in the cabbage white butterfly, yet plasticity did not preclude evolutionary responses in these other systems (Brans et al., 2017; Diamond et al., 2017; Diamond and Martin, 2021). Furthermore, the magnitude of plasticity in *P. rapae* falls within the typical range for adult insects (Gunderson and Stillman, 2015) and for Lepidoptera (Weaving et al., 2022). Together, these findings suggest that while thermal tolerance plasticity likely benefits cabbage white butterflies in their ability to inhabit urban environments, this plasticity is perhaps on its own insufficient to hinder an evolutionary response.

Another possible explanation for the lack of evolutionary divergence in CT_{max} observed in our study is that species with already-high heat tolerance might be buffered against urban heat island effects. In a

comparison of two species of cicada in urban Seoul, South Korea, Nguyen et al. (2020) showed that heat tolerance was only related to environmental temperature in the less thermally tolerant cicada species. Similarly, Sánchez-Echeverría et al. (2019) suggested that a lack of change in CT_{max} in urban bees was caused by the CT_{max} already being sufficiently high to cope with the maximum temperatures experienced in urban habitats. The estimated CT_{max} of cabbage white butterflies in Cleveland, Ohio was generally high; however, their trait values were still comparable to other very heat tolerant species that have shown evidence of evolutionary divergence across urbanization gradients, such as acorn ants (Diamond et al., 2018).

One possible limitation of our heat tolerance assay is that nearly all individuals (716 of 723) were exposed to low temperatures during CT_{min} testing prior to CT_{max} testing. This approach has been used, albeit infrequently, when testing physiological limits of other insect species (e. g., Bota-Sierra et al., 2022; Dongmo et al., 2021; Gonzalez et al., 2022a). Assessing CT_{min} prior to CT_{max} in the same individual had no effect on the estimation of CT_{max} in a bee species (Gonzalez et al., 2022b); however, similar tests have not been done on butterflies, so we cannot rule out impacts of measuring cold tolerance on subsequent measurement of heat tolerance in our system. We expect any influence of our experimental approach on the interpretation of our results to be minimal as the duration of cold temperature exposure was brief (i.e., time spent between 15 °C and −1 °C did not exceed one hour), and exposure of similar duration had no effect on CT_{max} in another arthropod (Alemu et al., 2017) and on high temperature survival in another Lepidopteran (Chidawanyika and Terblanche, 2011). CT_{min} trials did not have differential effects on survival between urban and rural population, as the few individuals that died after CT_{min} testing (13 out of 799 total individuals tested for CT_{min} from full dataset), were evenly split between urban ($n = 7$) and rural ($n = 6$) populations. While we argue that the effects of prior testing of CT_{min} on the estimation of CT_{max} are likely to be minimal, we cannot rule out the possibility that acute low-temperature exposure might lead to cold injury and reduce subsequent heat tolerance (Rozsypal, 2022) or that urban and rural populations have differential capacities for recovery from low-temperature exposure which masked true population divergence in heat tolerance.

4.2. Thermal plasticity, but no evolutionary divergence in cold tolerance

The evolutionary potential of cold tolerance was often greater compared with that of heat tolerance, which is consistent with the general pattern of cold tolerance being less evolutionary conserved than heat tolerance in ectotherms (Araújo et al., 2013; Hoffmann et al., 2013; Kellermann et al., 2012), yet we found no indication of population

divergence in this trait. The evolutionary retention of baseline cold tolerance might benefit urban cabbage white butterflies as they encounter climatic heterogeneity in cities. The retention of cold tolerance in response to urbanization has been observed in some species, such as the woodlouse *Oniscus asellus* (Yilmaz et al., 2021), though a number of other species show evolutionary losses in cold tolerance (Diamond and Martin, 2021). Further work distinguishing between a lack of selection (either on cold tolerance directly or through its potential correlation with heat tolerance) and adaptive reasons for the maintenance of cold tolerance is needed to understand the retention of cold tolerance across urbanization in the cabbage white butterfly.

Although it is tempting to interpret the role of CT_{min} plasticity in the lack of evolution of baseline trait values, especially since its magnitude was greater in comparison to plasticity in CT_{max} (a finding consistent with other systems; reviewed in Hoffmann et al., 2013), we are cautious to do so. In particular, we did not measure CT_{min} on overwintered butterflies (e.g., on immature developmental stages or early-season emerging adults). As a consequence, it is difficult to interpret how active-season plasticity in CT_{min} might shape the evolution of baseline trait values. It is possible that cold tolerance plasticity would be beneficial by allowing butterflies to be buffered against low temperature challenges, with the consequence of this plasticity hindering trait evolution (Fox et al., 2019; Ghalambor et al., 2007). However, low thermal sensitivity of cold tolerance could likewise be beneficial if it allowed butterflies to maintain low temperature tolerance in highly variable thermal environments (Hallsson and Björklund, 2012; Reed et al., 2010). More work is needed to uncover the causes and consequences of low temperature tolerance plasticity in this and other systems.

4.3. Other factors could constrain tolerance evolution

Several alternative factors in adult butterflies could act in concert with the modest thermal tolerance plasticity in *P. rapae* to constrain evolutionary divergence in thermal tolerance across the urban heat island. Wing melanin, an important trait for capturing solar radiation (Clusella Trullas et al., 2007), shows remarkable seasonal plasticity in *P. rapae* (Stoehr and Goux, 2008). Additional shifts in phenology, growth, and development could mediate temperature exposure and relax selection on thermal limits. For example, phenological shifts observed in urban populations of a widespread moth, *Chiasmia clathrate*, and congeneric *Pieris napi* have been attributed to evolutionary shifts in the critical daylength for direct development and the photoperiod reaction norm for development (Merckx et al., 2021).

Urban organisms, such as *P. rapae*, could also avoid urban heat and regulate body temperature through behavioral plasticity. Butterflies can regulate body temperature through behavioral adjustments including initiating basking or altering basking posture (Kemp and Krockenberger, 2002; Kingsolver, 1985a, 1985b), matching activity time with suitable temperatures (Slamova et al., 2011), and selecting suitable microhabitats (Kleckova et al., 2014). These behaviors can limit exposure to extreme environmental temperatures, therefore shielding phenotypes from selection and hindering evolution of physiological limits, i.e., Bogert effect (Huey et al., 2003; Muñoz and Losos, 2018). It is likely that combined with behavioral modifications in basking and microhabitat choice the existing magnitude of *P. rapae* thermal tolerance and thermal tolerance plasticity is sufficient to cope with urban heat. *Pieris rapae* use open habitats with more variable temperatures, and females select oviposition sites with higher temperatures in comparison to congeneric *P. napi*, which could explain why *P. rapae* larvae are more tolerant of acute extreme high temperature stress (Vives-Inglá et al., 2023). These ecological differences in microhabitat selection might underlie why *P. rapae* is so successful in navigating the variable thermal environment of cities. Although we do not have data on how *P. rapae* use the urban landscape in Cleveland, a previous study on *P. rapae* in New York City showed that butterflies released outside of their garden of capture generally avoided non-natural perches and instead moved back to

available green spaces or remained in flight (Matteson and Langellotto, 2012). Therefore, it is also possible that *P. rapae* is behaviorally avoiding the most extreme urban temperatures.

Because of the potential shielding effect of thermoregulation and other adult traits in urban butterflies, evolutionary divergence in thermal limits might be apparent in other less mobile life stages, such as caterpillars as they are generally thermal conformers (Kingsolver, 2000). In *Bicyclus anynana* butterflies, Klockmann et al. (2017) found partial support for the Bogert effect, as some life stages with less thermoregulatory capacity (e.g., pupae) had higher upper thermal tolerance than others. However, this pattern across ontogeny does not necessarily hold when considering divergence of thermal tolerance between populations (MacLean et al., 2016). It may be especially informative to measure the effect of urbanization on critical thermal limits in *P. rapae* larvae, given that metamorphosis can decouple responses across life stages (Bowler and Terblanche, 2008; Kingsolver and Buckley, 2020). Indeed, in a multi-city study of urban moths, population divergence in heat knock-down time was only found in the adult, not larval, life stage (Merckx et al., 2023). Understanding the role of plasticity in mediating evolved responses across life stages would be especially interesting, as juvenile insects are generally more plastic in their thermal limits than adults (Weaving et al., 2022). Given the recent focus on the importance of characterizing thermal vulnerability across life stages when assessing responses to increased temperature (Medina-Báez et al., 2023; Pottier et al., 2022), assessing thermal tolerance evolution and plasticity across life stages that differ in ability to traverse the urban landscape would be a valuable next step.

5. Conclusion

Urbanization has profound impacts on insect biodiversity. Understanding the mechanisms underlying how insects cope with urbanization is important for informing insect conservation interventions in cities. Lepidoptera are particularly sensitive to urban environments (Fenoglio et al., 2020; Theodorou et al., 2020). Yet, some butterflies have been able to take advantage of urban environments and display strong urban affinity. In studying a butterfly with high urban affinity, we expected high capacity of compensatory responses in thermal tolerance traits, including high capacity for beneficial acclimation of heat tolerance and evidence of rapid evolution in this trait. However, we found no evidence of evolutionary divergence in heat tolerance, and only a modest plastic response to developmental acclimation temperature. Rather, these results suggest that heat tolerance plasticity is likely accompanied by other compensatory mechanisms such as behavioral plasticity and evolution in other traits (e.g., wing melanin, phenology) to facilitate urban persistence of cabbage white butterflies. Further investigation into the use of the urban landscape, the response to urbanization across ontogeny, and the plastic response to acute temperature exposure are necessary to understand the role of plasticity, both physiological and behavioral, in the success and vulnerability of insects inhabiting urban environments.

CRediT authorship contribution statement

Angie Lenard: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sarah E. Diamond:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data are available at Open Science Framework <https://doi.org/10.17605/OSF.IO/KRMP9>.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jinsphys.2024.104648>.

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