

1 Evolution, not transgenerational plasticity, explains the divergence of acorn ant thermal tolerance
2 across an urban-rural temperature cline

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10 Running head: Testing for transgenerational plasticity

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20 **Abstract**

21 Disentangling the mechanisms of phenotypic shifts in response to environmental change is
22 critical, and although studies increasingly disentangle phenotypic plasticity from evolutionary
23 change, few explore the potential role for transgenerational plasticity in this context. Here, we
24 evaluate the potential role that transgenerational plasticity plays in phenotypic divergence of
25 acorn ants in response to urbanization. F2 generation worker ants (offspring of lab-born queens)
26 exhibited similar divergence among urban and rural populations as F1 generation worker ants
27 (offspring of field-born queens) indicating that evolutionary differentiation rather than
28 transgenerational plasticity was responsible for shifts towards higher heat tolerance and
29 diminished cold tolerance in urban acorn ants. Hybrid matings between urban and rural
30 populations provided further insight into the genetic architecture of thermal adaptation. Heat
31 tolerance of hybrids more resembled the urban-urban pure type, whereas cold tolerance of
32 hybrids more resembled the rural-rural pure type. As a consequence, thermal tolerance traits in
33 this system appear to be influenced by dominance rather than being purely additive traits, and
34 heat and cold tolerance might be determined by separate genes. Though transgenerational
35 plasticity does not explain divergence of acorn ant thermal tolerance, its role in divergence of
36 other traits and across other urbanization gradients merits further study.

37

38 *Keywords:* adaptation, maternal effect, thermal physiology, global change, speciation

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40

41 **Introduction**

42 Responses to changing environments can occur either through evolutionary change or through
43 existing phenotypic plasticity (Diamond and Martin 2016). However, the mechanisms underlying
44 observed phenotypic responses to rapid environmental changes, including through anthropogenic
45 effects are largely unknown for most organisms, as distinguishing plastic from evolved responses
46 is often challenging (Merilä and Hendry 2014). Indeed, while the dichotomy between purely
47 plastic and purely evolutionary responses is important and real, the two mechanisms can interact
48 and influence each other (Ghalambor et al. 2007; Diamond and Martin 2016; Kelly 2019). This
49 more complex view posits that along with the concept that plasticity itself evolves (DeWitt and
50 Scheiner 2004), plastic responses to novel environments may often precede and shape the speed,
51 direction, and magnitude of evolutionary change, thereby facilitating adaptation (*i.e.*, *plasticity-*
52 *first hypothesis* or *genetic accommodation*; for theoretical considerations, see Price et al. 2003;
53 West-Eberhard 2003; Levis and Pfennig 2016, and for empirical examples, see Corl et al. 2018;
54 Levis et al. 2018). Transgenerational effects—the influence of parents on the phenotype of their
55 offspring independent from the effects of genetic inheritance (Bernardo 1996; Mousseau and Fox
56 1998; Marshall and Uller 2007)—may be especially effective in facilitating adaptive evolution to
57 novel environments, as parents can alter offspring development in anticipation of the
58 environment they will later experience (Badyaev 2008). Despite this promise, there are few
59 examples of transgenerational plasticity facilitating evolution in response to the altered selective
60 regimes of novel environments (Badyaev et al. 2002a,b, Pfennig and Martin 2009, 2010).

61 Cities are an emerging experimental model system for studying plastic and evolutionary
62 responses to rapid anthropogenic change (Rivkin et al. 2019), and a number of studies find
63 evidence that populations are indeed evolving in response to selective pressures associated with

64 urbanization over contemporary timescales (Johnson and Munshi-South 2017). For example,
65 research suggests that urban populations of the Puerto Rican Crested Anole, (*Anolis cristatellus*)
66 have evolved longer limbs and increased toe pad lamellae in response to selection for locomotor
67 performance on artificial surfaces (Winchell et al. 2016). This and other studies have also found
68 considerable within generational phenotypic plasticity in response to urban environments (e.g.,
69 Diamond et al. 2017, 2018a; Gorton et al. 2018). However, evaluating if transgenerational
70 plasticity contributes to divergence in urban habitats has not yet been explored.

71 A classic approach for disentangling plastic responses occurring both within and across
72 generations from evolved responses is to compare field phenotypic measures between
73 populations with those from individuals of the same populations bred and reared in a controlled,
74 shared environment (i.e., a common garden experiment) across multiple generations (Merilä and
75 Hendry 2014; Donihue and Lambert 2015). By controlling for environmental differences across
76 populations in the common garden, researchers can isolate the genetic component, if any, of the
77 phenotypic divergence measured in the field. Although this approach is conceptually simple,
78 such experiments are often labor intensive and time consuming, and might be challenging or
79 impossible for species that are difficult to rear and breed in the lab (Donelson et al. 2018).
80 Consequently, most urban evolution studies to date have only reared field-caught juveniles or a
81 single generation in the lab and as a result cannot fully disentangle transgenerational phenotypic
82 plasticity from within generation plasticity and purely genetic divergence (but see Brans et al.
83 2017).

84 Here we use a laboratory common garden experiment with a multi-generational breeding
85 design to test for the presence of transgenerational plasticity and also investigate the genetic
86 architecture underlying evolutionary divergence between urban and rural populations of the

87 acorn ant (*Temnothorax curvispinosus*). We focus on thermal tolerance traits (heat and cold
88 tolerance) and their response to urban heat island effects along the urbanization gradient of
89 Cleveland Ohio, USA. F1 offspring (lab-born workers of field-caught queens) of urban acorn ant
90 populations reared under common garden exhibit increased heat tolerance and diminished cold
91 tolerance compared with rural populations; this pattern of differentiation among urban and rural
92 acorn ant populations is apparent for geographically isolated cities, including Cleveland, Ohio
93 and Knoxville, Tennessee (Diamond et al. 2017, 2018a). Moreover, in both cities we found that
94 urban colonies produced more alates (*i.e.*, reproductive offspring) under typical urban rearing
95 temperatures, and rural colonies produced more alates when reared at typical rural rearing
96 temperatures, resulting in fitness tradeoffs across environments for each population (Diamond et
97 al. 2018a). These results strongly suggest that *T. curvispinosus* has adaptively evolved to the
98 warmer environmental temperatures of the urban environment (*i.e.*, the urban heat island effect).
99 However our design could not detect or rule out transgenerational plasticity as a mechanism
100 behind this adaptive divergence.

101 In this study, we tested the heat and cold tolerance of F2 offspring, lab-born workers of
102 lab-born queens. We tested the offspring of pure-type rural population matings, pure-type urban
103 population matings, and hybrids, both where the female was from the rural population and the
104 male from the urban population and vice versa. If transgenerational plasticity were responsible
105 for the increased heat tolerance and diminished cold tolerance we documented previously in
106 urban population acorn ants, then we would expect these phenotypic differences to disappear in
107 the F2 offspring comparisons of pure-type urban and rural populations. If transgenerational
108 plasticity plays only a minor to no role, we would expect the increased heat tolerance and
109 diminished cold tolerance of pure-type urban populations relative to rural populations to remain

110 in the F2 offspring. The hybrid matings further evaluate the role of transgenerational plasticity,
111 where parental effects should differentially affect hybrid offspring depending on the direction of
112 the urban-rural mating. Additionally, comparisons among and against the hybrid tolerance
113 phenotypes allowed us to gain further insight into the genetic architecture underlying heat and
114 cold tolerance. For example, these comparisons allowed us to distinguish a purely additive
115 genetic model of trait inheritance from alternatives, such as dominance or transgressive
116 segregation.

117

118 **Materials and Methods**

119 *Colony collections*

120 We collected queenright (queen present) acorn ant (*Temnothorax curvispinosus*) colonies
121 in early summer 2017 (6 June 2017 – 21 July 2017) from urban and rural sites across Cleveland,
122 Ohio (42°N latitude). To identify urban and rural sites, we used percent developed impervious
123 surface area (ISA) (Imhoff et al. 2010), such that urban sites were categorized as 40-50% ISA,
124 and rural sites were categorized as 0% ISA (see Table S1 for site details).

125

126 *Mating design and common garden rearing*

127 We returned field-caught colonies to the lab and placed them under conditions to
128 facilitate the production of male and female alates (or winged, sexual individuals), including a
129 warm, diurnally fluctuating thermal regime (30 °C daytime, and 26 °C nighttime temperature)
130 synced with a 14:10 short-day photoperiod (following Stuart et al. 1993; Percival 36-VL growth
131 chambers). Each colony was maintained individually in a 120 mL plastic cup. Resource tubes
132 with sugar water (25% solution) and plain tap water were provided to colonies along with a

133 continuous supply of dead mealworms. Once alates were produced within the colonies, we
134 paired males and females in four mating crosses, including two pure-type matings, rural-rural
135 and urban-urban and two hybrid matings, rural-urban (with the maternal source population listed
136 first) and urban-rural. Because acorn ants are suggested to have male-biased dispersal (Stuart et
137 al. 1993), we provided several female alates (typically 5 alates) from the same colony with males
138 from several different colonies (typically 10 alates, all of which were distinct from the female
139 alate source colony). We enclosed alates from each cross in separate glass aquaria (38 L
140 capacity) with a mesh netting top. Alates were provided with the same resources as their source
141 colonies, including water and sugar tubes plus dead mealworms. We provided alates with several
142 uninhabited, hollowed-out acorns that we had sliced horizontally and held together with garden
143 twine. This allowed us to check on the mating status of the paired alates, specifically whether the
144 ants had shed their wings (an indication that they have mated, (Herbers 1990) and whether mated
145 female alates began to lay eggs. All mated females began new egg production by 7 December
146 2017.

147 Once mated female alates began to produce eggs, we placed these newly established
148 colonies individually within 120 mL plastic cups and provided them with water and sugar
149 resource tubes and dead mealworms. We held these colonies at 25 °C (on a 14:10 photoperiod),
150 as this is the optimal temperature for brood development (Diamond et al. 2013). After the newly
151 laid eggs developed into workers, we tested the thermal tolerance of these workers (*i.e.*, the
152 second generation of acorn ant workers reared entirely within the laboratory environment).

153

154 *Thermal tolerance*

155 We used a dynamic temperature ramping protocol to assess the critical thermal maximum
156 and minimum (CT_{\max} and CT_{\min}), each defined as the loss of muscular coordination, which yield
157 ecologically relevant limits on performance and serve as our measures of heat and cold tolerance
158 (Terblanche et al. 2011). We tested workers individually for either CT_{\max} or CT_{\min} , as the
159 assessment of thermal tolerance is a semi-destructive process that precludes assessment of both
160 CT_{\max} and CT_{\min} on the same individual. Ants were placed individually into 1.5 mL Eppendorf
161 tubes with a cotton plug in the lid. Temperatures were manipulated using a dry block incubator
162 (Boekel Scientific Tropicooler), and increased or decreased at a rate of $1\text{ }^{\circ}\text{C min}^{-1}$. Initial
163 temperature for the estimation of CT_{\max} was $34\text{ }^{\circ}\text{C}$, and was $16\text{ }^{\circ}\text{C}$ for CT_{\min} . These starting
164 temperatures lie outside the range needed to induce loss of muscular coordination or death.

165 Thermal tolerances were assessed between 30 August 2018 and 2 September 2018. We
166 tested a total of 487 individuals for thermal tolerance, 251 CT_{\max} , 236 CT_{\min} from 18 different
167 colonies. For the pure mating types, we had four urban-urban colonies and four rural-rural
168 colonies. For the hybrid mating types, we had five urban-rural (with the origin of the maternal
169 population listed first) colonies and five rural-urban colonies. The number of individuals tested
170 per colony (and within each tolerance type, heat or cold tolerance) ranged from 5 to 24 with a
171 mean and SD of 13.6 and 5.17 (see also Table S2).

172

173 *Statistical analyses*

174 We fit linear mixed effects models with either CT_{\max} or CT_{\min} as the response variable
175 and the type of mating (the two pure types, urban-urban and rural-rural, and the two hybrid types
176 with the population origin of the mother listed first, urban-rural and rural-urban) as a predictor
177 variable (treated as a categorical variable with four levels). We used the *lme* function from the

178 *nlme* library in R (Pinheiro et al. 2018; R Core Team 2018) to perform these models. Colony
179 identity was included as a random intercept in each model to account for autocorrelation among
180 individuals from the same colony. The statistical significant of mating type was assessed using
181 likelihood ratio tests. Post-hoc analyses to assess the significant differences between factor levels
182 of the type of mating predictor variable were performed using the *emmeans* function (from the
183 eponymous library) with all pairwise comparisons (Lenth 2018).

184 To model tolerance breadth (the difference between colony-mean CT_{\max} and colony-
185 mean CT_{\min}), we fit a generalized linear model with tolerance breadth as the response and type of
186 mating as a predictor. We modeled tolerance breadth using a Gaussian probability distribution
187 and log link function owing to skew in the untransformed data. Because hybrid tolerance
188 responses were indistinguishable from one another, and because we were more limited in
189 statistical power after computing colony-level means for heat and cold tolerance, we fit a
190 subsequent model wherein each of the pure types (urban-urban and rural-rural) were compared
191 with the hybrid types (considered as a single group). We performed the post-hoc analysis on this
192 model. Although a strict test of maternal effects would involve comparisons of the urban
193 maternal pairings versus rural pairings, our initial analyses showed little evidence of maternal
194 effects (see below), so we used the particular analyses for thermal tolerance breadth to explore
195 the genetic architecture underlying heat versus cold tolerance, and hence justified our combining
196 of offspring results for the hybrid matings.

197

198 **Results**

199 We found no evidence that the evolutionary differentiation in heat and cold tolerance
200 among urban and rural acorn ant populations was driven by transgenerational plasticity.

201 Likelihood ratio tests for the effect of mating type on heat tolerance and cold tolerance were
202 statistically significant (CT_{\max} : $\chi^2 = 44.6$; $P = 1.12E-09$; $df = 3$; CT_{\min} : $\chi^2 = 20$; $P = 0.00017$; $df =$
203 3). Post-hoc tests revealed that F2 offspring of male and female reproductives born, reared, and
204 mated within the lab environment exhibited significantly elevated heat tolerance and
205 significantly diminished cold tolerance in urban-urban crosses as compared with rural-rural
206 crosses (Table 1, Fig. 1).

207 The results of the hybrid pairings (female-male) urban-rural and rural-urban revealed
208 further insights into the contemporary evolution of thermal tolerance traits in response to
209 urbanization. First, the fact that the hybrid matings were successful in producing offspring
210 indicates no evidence of behavioral prezygotic or intrinsic postzygotic reproductive isolation
211 among urban and rural populations. It is possible that F2 offspring may exhibit later signs of
212 reproductive isolation (hybrid inviability or sterility). We also note that male F2 hybrid
213 incompatibility is unclear from our experimental design and study system as males are haploid
214 clones of diploid queens, and thus another round of mating (F3) would be required to test this
215 possibility (Koevoets and Beukeboom 2009). In any case, these are secondary goals of our study.

216 Interestingly, hybrids were more similar to either the rural-rural or urban-urban pure
217 types depending on whether heat tolerance or cold tolerance was assessed. The heat tolerance of
218 hybrids more resembled the urban-urban pure type than the rural-rural pure type (Fig. 1A),
219 whereas the cold tolerance of hybrids more resembled the rural-rural pure type than the urban-
220 urban pure type (Fig. 1B). This provides further evidence that divergence in thermal tolerance
221 does not stem from transgenerational plasticity, as the offspring did not consistently resemble a
222 specific parental population. Moreover, this differential matching of hybrids and pure types

223 across heat and cold tolerance potentially suggests some degree of genetic decoupling of the
224 traits.

225 We further explored the potential ecological consequences of the differential matching of
226 hybrid and pure-type heat and cold tolerance phenotypes by computing their tolerance breadth
227 (difference between colony mean CT_{\max} and CT_{\min}). The likelihood ratio test for the effect of
228 mating type on tolerance breadth was statistically significant ($\chi^2 = 9.22$; $P = 0.0266$; $df = 2$).
229 Post-hoc tests revealed that hybrids exhibited greater tolerance breadth than either of the pure
230 types since the hybrids possessed both the superior heat tolerance value (high CT_{\max}) of urban
231 populations and the superior cold tolerance value (low CT_{\min}) of rural populations (Figure 2;
232 Table 1).

233

234 **Discussion**

235 Populations can adaptively respond to rapidly changing environments through
236 evolutionary change and phenotypic plasticity occurring within or across generations or through
237 their interaction (Merilä and Hendry 2014; Diamond and Martin 2016). Although distinguishing
238 among these mechanisms is often difficult, doing so is necessary to understand and predict future
239 responses to rapid environmental change (Urban et al. 2016). Here we used a multi-generational
240 common-garden study to discriminate between the effects of transgenerational plasticity and the
241 effects of evolutionary change. We explored this question in context of the adaptive divergence
242 of thermal tolerance traits between urban and rural populations of the acorn ant *Temnothorax*
243 *curvispinosus*. We found no evidence that transgenerational plasticity contributed to divergence
244 between urban and rural populations in thermal tolerance, as shifts towards better heat tolerance
245 and worse cold tolerance in urban populations were maintained over two generations in a

246 common-garden laboratory environment. Further, we found that thermal tolerance traits were not
247 inherited either maternally or paternally in the hybrid pairings.

248 Our results align with those of the few other studies that have used multi-generation
249 common garden experiments in the context of thermal tolerance divergence to urban heat islands
250 (Brans et al. 2017). Yet, the overall importance of transgenerational plasticity for evolutionary
251 responses to novel environments is far from known. The thermal environment experienced by
252 parents does appear to adaptively affect the thermal tolerance of their offspring in other contexts
253 and systems, providing strong examples of anticipatory parental effects (Massamba-N'Siala et al.
254 2014; Chirgwin et al. 2018). However, a recent comprehensive synthesis of transgenerational
255 plasticity across taxa and traits found relatively weak effects overall (Uller et al. 2013). Adaptive
256 transgenerational plasticity in thermal tolerance could therefore prove to be relatively
257 uncommon, albeit with important exceptions, such as those mentioned above. Alternatively,
258 previous studies might not have been designed to detect such transgenerational plasticity
259 effectively, or might not have focused on systems where such effects are expected to evolve
260 (Burgess and Marshall 2014). This may occur if researchers are not actually manipulating the
261 aspects of the environment that are most predictive of the conditions offspring will encounter
262 (Uller 2008; Burgess and Marshall 2014).

263 Is there reason to expect adaptive parental effects to evolve in acorn ants as a response to
264 the urban heat islands? Perhaps not. Acorn ant dispersal ability is quite limited (Herbers and
265 Tucker 1986; Prebus 2017), and it is highly unlikely acorn ants could experience urban and rural
266 temperature environments from one generation to the next. Further, within an acorn ant
267 generation, workers experience substantial thermal variation over very rapid spatio-temporal
268 scales, including diurnally within the acorn ant nest environment and spatially as foragers move

269 throughout the landscape. This thermal variability is greater within urban forest patches, and
270 urban population ants have evolved greater within-generation thermal plasticity to rapid
271 temperature increases (Diamond et al. 2018b). Consequently, while both temperature means and
272 variances differ between urban and rural populations, the temporal and spatial scale of this
273 variation is unlikely to select for adaptive transgenerational effects.

274 Although the primary goal of our experiment was to evaluate transgenerational plasticity
275 as an alternative explanation for the adaptive divergence in thermal tolerance between urban and
276 rural *T. curvispinosus* populations, our study is not poised to address the overall presence or
277 absence of parental effects. That is, we cannot rule out *any* role for transgenerational thermal
278 plasticity in this system or others, only that it does not appear to explain the urban-rural
279 divergence in thermal tolerance here. Indeed, because we reared acorn ant colonies under non-
280 stressful temperatures within our multi-generation common garden experiment, it remains
281 unclear whether other manipulations of the thermal environment, such as heat stress (Chirgwin et
282 al. 2018) or seasonal temperature variation (Walsh et al. 2014), could induce transgenerational
283 effects and whether such effects could have played an initial role in facilitating urban-rural
284 divergence (Badyaev et al. 2002a,b; Badyaev 2008; Pfennig and Martin 2009, 2010).

285 While we did not find evidence for transgenerational plasticity as an explanation for the
286 divergence in heat and cold tolerance traits among urban and rural acorn ant populations, the
287 results of the urban-rural hybrid crosses yielded some surprising insights into the genetic
288 architecture of acorn ant thermal tolerance. Specifically, our comparisons of heat and cold
289 tolerance traits between the pure type and hybrid urban-rural crosses revealed that dominant and
290 recessive alleles appear to underlie heat and cold tolerance traits, rather than being determined as
291 purely additive traits. We found that both hybrid crosses resemble urban populations in heat

292 tolerance and achieve greater cold tolerances similar to that of rural populations, rather than
293 exhibiting intermediate phenotypes as would be expected for additive traits (Figure 1, Table 1).
294 Why might both hybrid crosses express the greater heat tolerance of urban populations *and* the
295 greater cold tolerance of rural populations? One explanation is that heat tolerance and cold
296 tolerance are largely determined by different genes, with urban populations contributing a
297 dominant allele(s) for heat tolerance and rural populations a dominant allele(s) for cold tolerance
298 (Chown and Nicolson 2004). Alternatively, heat and cold tolerance could share genes with
299 alternative conditional mutations (Griffiths et al. 2005), resulting in environmentally-dependent
300 dominance. For example, imagine that urban and rural populations are fixed for different alleles
301 of a heat shock protein. At low temperatures, the urban allele suffers loss of function and
302 conversely the rural allele loses function at high temperatures, resulting in temperature-sensitive
303 dominance. The latter scenario would suggest that the losses of cold tolerance in urban
304 population could in part, be a correlated response to selection for increased heat tolerance in
305 urban habitats. However, heat and cold tolerance are not strongly genetically correlated within
306 colonies (Diamond et al. 2018a) suggesting that they may indeed be determined by separate
307 genes. This would also correspond to the general findings from *Drosophila* and other ectotherm
308 systems which show that cold and heat tolerance traits are under independent or semi-
309 independent genetic control (Hoffmann et al. 2003).

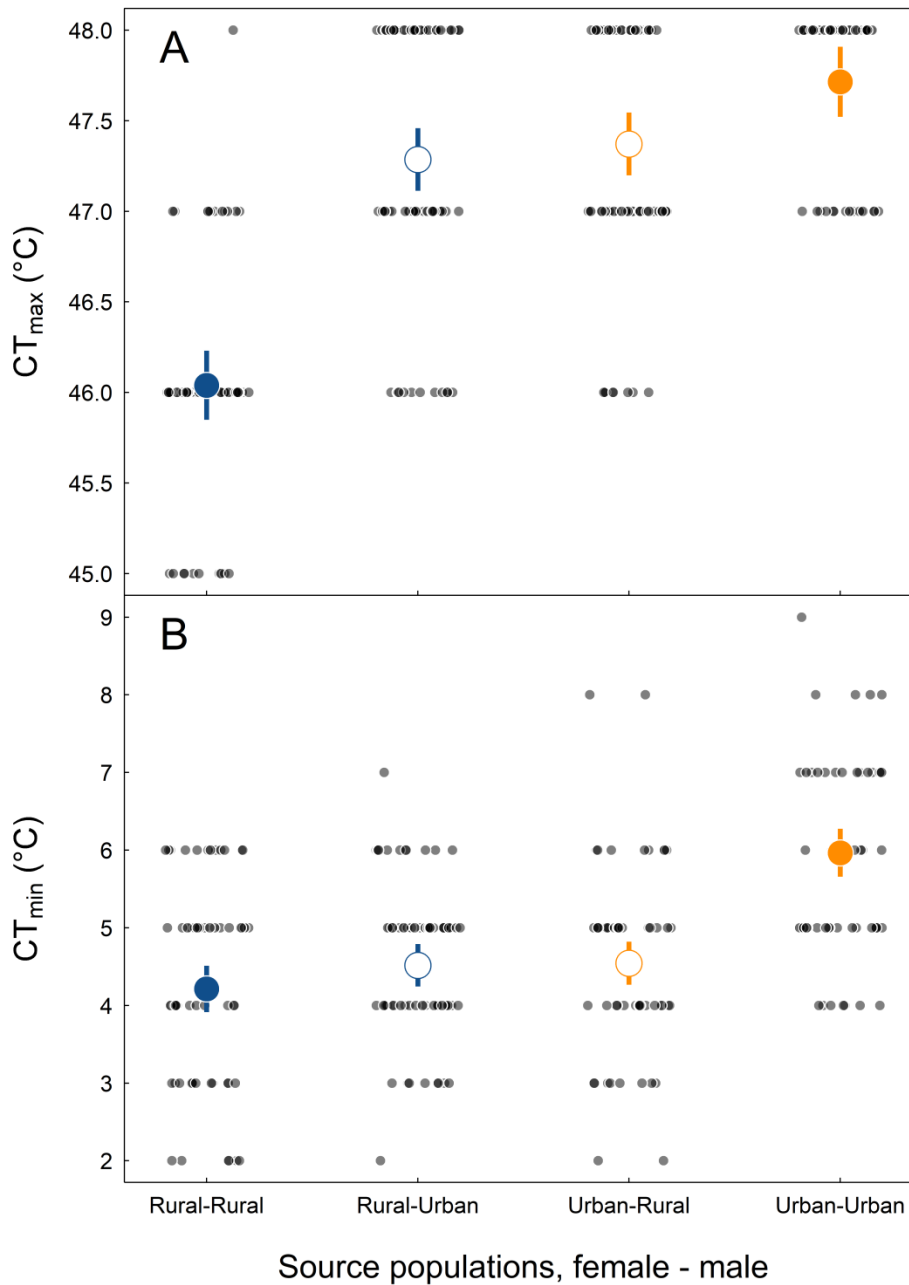
310 Our research provides a case study in how an urban-rural cline in temperature can be
311 used to explore the mechanisms underlying shifts in thermal tolerance trait values, specifically
312 disentangling evolutionary change from transgenerational plasticity, and to understand the
313 genetic architecture underlying these traits. But what else can we gain from such comparisons?
314 With the growing consensus that evolution can occur in the wild on the scale of human lifetimes

315 (Reznick et al. 2018), cities might also allow us to peer into the early stages of speciation as
316 differences accumulate among urban and rural populations (Thompson et al. 2018). Indeed, as
317 *Temnothorax curvispinosus* populations have evolved divergent thermal tolerances in response to
318 urban heat islands, could this ecologically divergent selection promote speciation as well?
319 Ecologically divergent environments can promote reproductive isolation in several ways. For
320 example populations may become reproductively isolated by shifting the timing of reproduction
321 between them (Rundle and Nosil 2005). In the acorn ant system, the date of peak alate
322 production is shifted earlier by as much as 30 days in urban populations compared with rural
323 populations, likely due to the differences in the timing of seasonal temperature cues in rural and
324 urban habitats (Chick et al. 2019). This phenological shift in reproduction is thus likely to reduce
325 possibilities for mating between urban and rural populations. In contrast, our laboratory breeding
326 experiment shows that, given the opportunity, urban and rural alates (*i.e.*, reproductive-caste
327 offspring) will mate and produce fertile offspring, revealing a lack of both behavioral prezygotic
328 and intrinsic genetic postzygotic isolating mechanisms. Although, because male alates are
329 haploid, any male sterility corresponding with Haldane's rule would only be expressed by F2
330 male hybrids who through recombination, carry alleles from both urban and rural populations
331 (Koevoets and Beukeboom 2009), which remains to be tested.

332 Ecologically divergent environments can also impose reproductive isolation by selecting
333 against migrants and/or hybrid offspring (Nosil et al. 2003; Nosil 2004). Local adaptation and
334 divergent selection between urban and rural thermal environments suggest that acorn ant
335 migrants would face negative selection pressures in their non-native habitat. Hybrid acorn ant
336 offspring, in comparison, inherit the combined thermal tolerance of both their parents, with heat
337 and cold tolerances equaling those of urban and rural populations respectively (Figure 1, Table

338 1). This expanded thermal performance (Figure 2, Table 1) breaks the expectation of
339 intermediate hybrid fitness for ecological speciation (Rundle and Nosil 2005) and suggests that
340 hybridization could lead to population collapse rather than population divergence. We then
341 expect that limited dispersal ability, shifted phenologies, or strong selection against migrants has
342 enabled the evolutionary divergence between urban and rural populations in light of the
343 expanded thermal breadth of their hybrid offspring. However, the evidence for any ongoing
344 speciation between urban and rural population is both preliminary and mixed at this time.

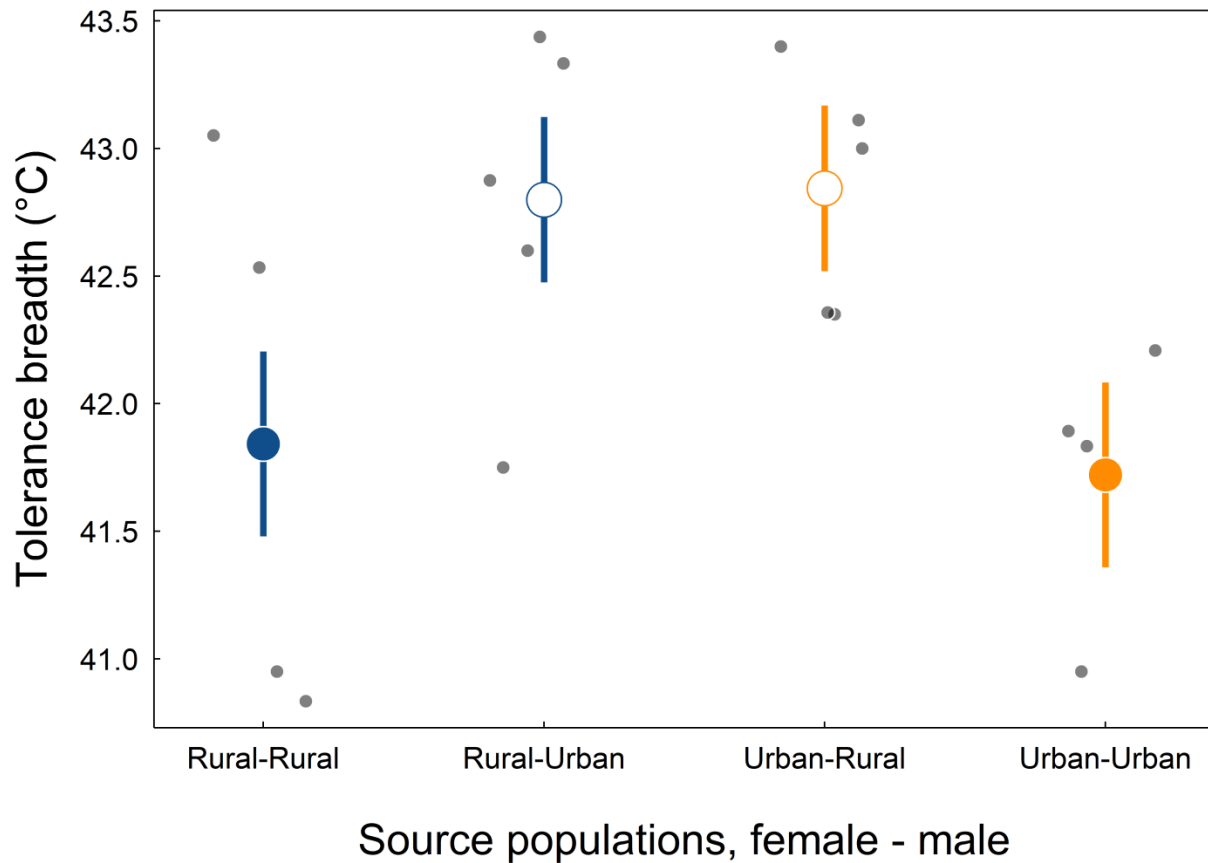
345 In summary, the results from a two-generation common garden rearing experiment
346 support the conclusion that acorn ants have evolved divergent heat and cold thermal tolerances in
347 response to the selective agent of the urban heat island in Cleveland, Ohio. Surprisingly, thermal
348 tolerance appears to be influenced by dominance rather than being a purely additive trait, and
349 heat and cold tolerance might be determined by separate genes. While urban and rural
350 populations have also diverged in their reproductive phenology, there is no evidence for other
351 pre- or postzygotic reproductive isolation between them. *Temnothorax curvispinosus* populations
352 have also diverged in thermal tolerance along at least one other urbanization gradient (Knoxville,
353 Tennessee), and it is an open question remaining to be explored whether the genetic architecture,
354 mating behavior or hybrid success is convergent across this independent selective gradient. As
355 urban environments provide replicated theaters against which the evolutionary play unfolds,
356 future work aimed at comparisons of the mechanisms of trait evolution across multiple urban
357 heat islands could be especially fruitful.



358

359 **Figure 1.** (A) Heat tolerance (CT_{max} , °C) and (B) cold tolerance (CT_{min} , °C) as a function of pure
360 type and hybrid matings across urban and rural acorn ant populations. Small points indicate
361 tolerance values of individual worker ants from each mating type (with the maternal source
362 population listed first): rural-rural, rural-urban, urban-rural, and urban-urban. Predicted mean
363 heat tolerances and standard errors from a linear mixed effects model that accounts for colony-
364 level autocorrelation are presented in large points (means) and line segments (standard errors).
365 Pure types are in filled symbols (blue for rural and orange for urban). Open symbols represent
366 hybrid matings, with the color reflecting the maternal population origin.

367



368

369 **Figure 2.** Tolerance breadth (°C) as a function of pure type and hybrid matings across urban and
370 rural acorn ant populations. Small points indicate tolerance breadth values of whole colonies
371 (colony mean CT_{max} - colony mean CT_{min}) from each mating type (with the maternal source
372 population listed first): rural-rural, rural-urban, urban-rural, and urban-urban. Predicted mean
373 tolerance breadths and standard errors from a generalized linear model are presented in large
374 points (means) and line segments (standard errors). Pure types are in filled symbols (blue for
375 rural and orange for urban). Open symbols represent hybrid matings, with the color reflecting the
376 maternal population origin.

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386 **Table 1.** Post-hoc analyses of the mating type term for models of CT_{\max} , CT_{\min} , and tolerance
 387 breadth are provided including estimates of the contrast, their standard errors and test statistics
 388 and p -values for the pairwise differences between factor levels of the mating type term. Note that
 389 hybrid factor levels are combined for the analysis of tolerance breadth, and that estimates and
 390 test statistics are reported on the natural log scale. Significant p -values at the 0.05 level are
 391 indicated in bold font.

392

Tolerance type	Contrast	Estimate	SE	Test statistic	P
CT_{\max}	rur-rur - rur-urb	-1.25	0.257	-4.84	0.00132
	rur-rur - urb-rur	-1.33	0.257	-5.17	0.000723
	rur-rur - urb-urb	-1.67	0.272	-6.17	0.000128
	rur-urb - urb-rur	-0.0855	0.245	-0.35	0.985
	rur-urb - urb-urb	-0.428	0.259	-1.65	0.384
	urb-rur - urb-urb	-0.343	0.26	-1.32	0.566
CT_{\min}	rur-rur - rur-urb	-0.304	0.405	-0.75	0.875
	rur-rur - urb-rur	-0.331	0.408	-0.813	0.848
	rur-rur - urb-urb	-1.75	0.43	-4.08	0.00548
	rur-urb - urb-rur	-0.0276	0.39	-0.0709	1
	rur-urb - urb-urb	-1.45	0.412	-3.51	0.0161
	urb-rur - urb-urb	-1.42	0.415	-3.42	0.019
Tolerance breadth	hybrid - rur-rur	0.0231	0.00984	2.35	0.0490
	hybrid - urb-urb	0.0260	0.00986	2.64	0.0225
	rur-rur - urb-urb	0.00290	0.0119	0.244	0.968

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562 **Supporting Information**

563 **Table S1.** Geographic coordinates and percent developed impervious surface area (ISA) values
 564 (0% ISA indicates rural sites, 40-50% ISA indicates urban sites) of acorn ant collection sites.

Site name	Longitude	Latitude	Source environment	ISA
University Farm	-81.4245	41.49842	Rural	0
Holden Arboretum	-81.3127	41.6088	Rural	0
Ambler Park	-81.6055	41.4976	Urban	40
Case Western Reserve University				49
Forest Hills	-81.6137	41.50897	Urban	
	-81.5737	41.52751	Urban	44

565

566 **Table S2.** Number of individual worker ants tested for CT_{min} and CT_{max} per each colony. The
 567 type of cross (the two hybrids and two pure types) is also indicated with the maternal source
 568 population listed first.

Cross	Number of individuals tested for CT_{max}	Number of individuals tested for CT_{min}
rur-rur	15	15
rur-rur	12	12
rur-rur	13	12
rur-rur	20	20
rur-urb	16	16
rur-urb	16	16
rur-urb	24	24
rur-urb	6	5
rur-urb	10	10
urb-rur	20	10
urb-rur	11	11
urb-rur	10	10
urb-rur	14	14
urb-rur	9	9
urb-urb	11	9
urb-urb	8	7
urb-urb	24	24
urb-urb	12	12

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