Multivariate phenotypic divergence along an urbanization gradient

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Author Contributions

JSS and KAT planned the study and collected original plant material, and all authors contributed to subsequent study design. JSS wrote the first draft of the paper with sections written by KAT and LRR, and all authors contributed to revisions. CA conducted the common garden experiment and collected data, with assistance from all other authors. JSS oversaw field data collection, with assistance from other authors. JSS analyzed the data with input from KAT and LRR.

Data accessibility

All code and data used throughout this manuscript are presently available for reviewers on the GitHub page for J.S.S (https://github.com/James-S-Santangelo/SIC) and will be permanently archived in a data repository following publication.

Conflicts of interest

The authors declare no conflicts of interest.

Abstract

A growing body of evidence suggests that natural populations can evolve to better tolerate the novel environmental conditions associated with urban areas. Invariably, studies of adaptive divergence in urban areas examine only one or a few traits at a time from populations residing only at the most extreme urban and nonurban habitats. Thus, whether urbanization is driving divergence in many traits simultaneously in a manner that varies with the degree of urbanization remains unclear. To address this gap, we generated seed families of white clover (*Trifolium repens*) collected from 27 populations along an urbanization gradient in Toronto, Canada, and grew them up to measure multiple phenotypic traits in a common garden. Overall, urban populations had later phenology and germination, larger flowers, thinner stolons, reduced cyanogenesis, greater biomass, and were more attractive to pollinators. Pollinator observations revealed near complete turnover between urban and nonurban sites, which may explain some of the observed divergence in floral traits and phenology. Our results suggest that adaptation to urban environments involves multiple organismal traits.

Keywords: Anthropocene; cline; urban evolution; rapid evolution; *Trifolium repens*

Introduction

Urbanization is rapidly changing the face of the planet. As cities develop, natural habitats experience drastic environmental changes, from increased temperatures and pollution to greater impervious surface and habitat fragmentation [1]. Evidence is accumulating to support the hypothesis that the environmental features associated with urbanization drive phenotypic differences between populations in urban and nonurban habitats [2]. For example, urban *Anolis* lizards have evolved longer limbs and more toe lamellae to improve sprint speed on the smooth artificial surfaces common in cities [3,4]. Increased impervious surfaces often lead to warmer air temperatures in cities [5], which has driven increases in thermal tolerance of urban acorn ant [6] and *Daphnia* populations [7]. These studies, and others (see table S1 in [8]) suggest that many urban populations are adapting to the novel environments created by humans.

Despite accumulating evidence of evolution in response to urban-driven environmental change, most studies focusing on phenotypic divergence associated with urbanization have examined just one or a few traits at a time [4,9–12]. However, theoretical and empirical work in other systems suggest that selection can drive the evolution of multivariate phenotypic clines along environmental gradients [13,14]. Multivariate phenotypic analyses incorporate multiple, often correlated, traits to explain overarching shifts in phenotypes across environments. Although we can make informed predictions about how particular traits might respond to urbanization from work in other systems, understanding how cities are driving phenotypic evolution requires quantifying divergence in many traits that simultaneously influence fitness. It is thus presently unclear what suites of traits are most often favored as populations become more urbanized.

Here, we investigate multivariate phenotypic evolution along an urbanization gradient in Toronto, Canada. Traits involved in plant reproduction are particularly likely to show multivariate, genetically-based phenotypic associations with urbanization between urban and nonurban populations due to the direct effect of these traits on fitness. In animal-pollinated plants, urbanization might impact fitness through changes to their pollinator communities. Both the diversity and abundance of pollinators are known to change along urbanization gradients [15,16], with positive [17–19] to negative [20] effects on pollinator visitation. Variation in pollination along urbanization gradients might drive changes in the extent of pollen limitation experienced by outcrossing plants [21,22], which can influence the strength and opportunity for pollinator-mediated selection on plant reproductive traits [23–25]. Consequently, we expect that plants could compensate for the altered pollination environment in cities by evolving altered reproductive trait values relative to non-urban plants.

Methods

Detailed methods can be found in the online supplementary materials.

Common garden experiment

We examined multivariate trait divergence along an urbanization gradient using white clover (*Trifolium repens*) as a model system. We grew 642 F1 generation white clover plants from seed in a common garden. These plants were distributed among 209 plant families' from 27 populations spanning an urban-rural transect in Toronto, Ontario, Canada (Fig. 1a, [10]). The garden was located at the University of Toronto Mississauga in summer 2017. We measured

107 several traits during our experiment, some of which are known to be under selection in this
108 system (Table 1) [26,27].
109
110 *Field observations*
111 We quantified variation in pollination among our study populations to identify possible
112 mechanisms that might underlie phenotypic divergence along the urbanization gradient. We
113 conducted pollinator observations in all study populations using two 1 m × 1 m quadrats (similar
114 to [28]) placed in full sun and counting the number of inflorescences visited by pollinators over
115 20 minutes. We classified pollinators into three morphological groups (termed *morphs* hereafter):
116 honeybees (*Apis mellifera*), bumblebees (*Bombus spp.*) and sweat bees (Halictidae).
117 We additionally collected twenty ripe infructescences (i.e., group of fruit) from the same
118 populations in which we recorded pollinator observations. We counted the number of flowers
119 and seeds in each infructescence and used the number of seeds per flower as a metric of pollen
120 limitation (fewer seeds per flower = more pollen limitation). We generated data for the same
121 metric from plants in the common garden. These data provide information about *in situ* variation
122 in pollen limitation (field-collected inflorescences) and evolved differences in plants' abilities to
123 set seed from pollen (common garden data).
124
125 *Data analysis*
126 We investigated whether urbanization was associated with multivariate phenotypic
127 divergence using canonical redundancy analysis (i.e., RDA, [29]). The RDA regressed a matrix
128 of standardized family mean trait values (209 families × 14 traits) as a response variable against
129 a numeric vector representing the distance (in km) of populations to the urban core as the sole

predictor (see below), which is correlated with % impervious surface cover ($r_{pearson} = -0.63$, Fig. S1a) and human population density ($r_{pearson} = -0.72$, Fig. S1a), has previously been shown to be correlated with cyanogenesis frequency ($r_{pearson} = 0.63$) in the same species and region [10].

The RDA tests whether distance to the urban center explains more phenotypic variation than expected by chance. In principle, we could observe a significant RDA even if just one trait was associated with distance, but our interest lies in understanding whether multiple traits are evolving together along the transect (i.e., including traits that do not vary significantly on their own). Therefore, we used the canonical coefficients from the RDA that describe the individual contribution of phenotypic traits to the first constrained axis of the RDA (RDA1 [i.e., distance]) to calculate a multivariate phenotype score for each individual. This score, referred to as **cline$_{max}$**, is the multivariate quantitative trait that shows the strongest association with distance to the urban center [13]. Regressing **cline$_{max}$** against distance to the urban center enables us to better visualize the shape of the multivariate cline and to quantify how multivariate phenotypes are changing along our urbanization gradient.

To examine variation in pollinator visitation among natural populations, we fit a linear model with the number of visits per inflorescence as a response variable, and distance, pollinator morph, and their interaction as predictors. To assess how the number of seeds per flower varied among field-collected inflorescences and plants in the common garden, we fit a model with population-mean number of seeds per flower as the response and distance, source (field-collected vs. common garden-collected), and their interaction as predictors. All data (see **Data Accessibility**) were analyzed in R version 3.6.0 [31] within the RStudio environment [32].

Results

153 *Common garden*

154 Urbanization was associated with multivariate trait divergence and the evolution of a
155 multivariate phenotypic cline in Toronto, ON (Fig. S2 and Fig. 1b). Distance to the urban core
156 explained 2.7% of the total variation in the multivariate phenotypic composition of populations
157 (RDA, $F_{1, 202} = 5.51$, $P < 0.001$, Fig. S2, Fig. S3). The composite trait showing the strongest
158 association with distance to the urban core (i.e., $\mathbf{cline_{max}}$) showed a positive cline along the
159 urbanization gradient ($\beta = 0.01$, $t_{206} = 9.32$, $P < 0.001$, $r^2 = 0.29$, Fig. 1b) that was at least as
160 strong as any trait when analyzed individually (Fig. S4 and S5, see also standardized beta
161 coefficients in table S1). The six traits that loaded most strongly (|loading| > 0.3, bolded in Fig.
162 S2) onto the first axis of the RDA (RDA1)—germination, phenology, flower size, biomass,
163 HCN, and stolon thickness—and contributed most to the composite trait showing the strongest
164 association with distance all showed significant univariate clines in the direction predicted based
165 on their trait loadings (Fig. S2 and Fig. S6a).

166

167 *Field observations*

168 On average, pollinator visitation per inflorescence was greatest in urban populations (Distance: β
169 $= -0.032$, $F_{1, 75} = 21.96$, $P < 0.001$; thick black line in Fig. 2a). Visitation rate varied with
170 pollinator morphs (Morph: $F_{2, 75} = 11.06$, $P < 0.001$): bumblebees had the highest visitation rate
171 (average = 0.63 ± 1.2 SD visits/inflorescence across all populations), followed by honey bees
172 (average = 0.58 ± 1.05 visits/inflorescence), and sweat bees (average = 0.18 ± 0.41
173 visits/inflorescence). Pollinator morphs varied in their response to urbanization (Distance ×
174 Morph interaction: $F_{2, 75} = 14.11$, $P < 0.001$, Fig. 2a): bumblebee visitation rate was greatest in

urban populations, whereas honeybee visitation was greatest in nonurban populations, and sweat bees showed little change in visitation across the urbanization gradient (Fig. 2a).

While common garden infructescences were on average more thoroughly pollinated than those in natural populations (i.e., more seeds per flower; source $F_{1, 50} = 30.04$, $P < 0.001$, $\text{mean}_{\text{field}} = 1.1$, $\text{mean}_{\text{garden}} = 2.5$, Fig. 2b), the number of seeds per flower was highest in urban populations (Distance effect: $\beta = -0.013$, $F_{1, 50} = 5.48$, $P = 0.03$, Fig. 2b). Urban plants were more pollinated than non-urban plants in both the common garden and in the field (Distance × source interaction: $F_{1,50} = 0.99$, $P = 0.33$, Fig. 2b).

Discussion

Multivariate phenotypic divergence and clinal variation

Most studies of population differentiation and clinal variation along environmental gradients focus on only one or a few traits. We know much less about how suites of traits evolve in concert in response to environmental factors [13,14]. Our study shows clear phenotypic divergence and clinal variation in multivariate phenotypes along an urbanization gradient. After weighting traits based on their loadings onto RDA1 [13], distance explained 29% of the variation in the multivariate phenotype $\mathbf{cline_{max}}$. Urban populations had later germination and flowering (Fig. S4a and S4b), greater vegetative biomass (Fig. S4c), larger banner petals (Fig. S4d), thinner stolons (Fig. S4e), and lower HCN frequencies (Fig. S4f). Many of the traits (three of six) most strongly associated with distance to the urban core were reproductive traits, supporting the prediction that these traits are likely to show evolutionary divergence between urban and

197 nonurban habitats, although some notable traits (e.g. reproductive biomass, number of flowers,
198 and number of inflorescences) showed no association with urbanization.
199
200 *Urban evolution in white clover*
201 Correlational data suggests that lower cyanogenesis in urban populations has evolved due to
202 selection for tolerance to freezing damage [10]. Recent data suggest a cost to producing the two
203 metabolic component of HCN (cyanogenic glucosides and linamarase) under stressful conditions
204 (e.g., frost) [33], which may partially explain the lower HCN frequencies in urban populations.
205 Furthermore, small-leaved plants and those with thinner stolons are more frost-tolerant [34,35],
206 suggesting plants in urban environments should match these phenotypes if they have indeed
207 evolved to better tolerate frost. Our data do not support the hypothesis that urban populations
208 have evolved smaller leaves but do support urban plants having thinner stolons (Fig. S4e, table
209 S1), offering partial support to the hypothesis that urban populations may be more frost tolerant.
210
211 *Plant evolution in urban environments*
212 Our observed differences in flower size corroborate earlier findings of stronger directional
213 selection for larger flowers in urban populations [36,37]. In addition, plants from urban
214 populations were larger than those in nonurban populations, similar to urban and nonurban
215 *Lepidium virginicum* plants grown in a common garden [12]. However, increased vegetative
216 biomass in urban populations is not universal; in ragweed (*Ambrosia artemisiifolia*), there were
217 no differences in plant size between urban and nonurban populations, although urban populations
218 flowered earlier than nonurban populations [11]. This contrasts with our experiment in which
219 urban populations flowered later than nonurban populations. These results suggest that the

effects of urbanization on plant traits vary across species and there currently appears to be no particular combinations of traits consistently favored in cities.

Urban populations of white clover were less pollen limited and were visited primarily by bumblebees whereas nonurban populations were visited primarily by honeybees. A single bumblebee typically visits more white clover flowers per minute than a honeybee [38], potentially explaining the greater seed set of urban plants, and consistent with other work showing higher seed set among bumblebee-pollinated urban white clover plants [28]. The difference in pollinator community between urban and nonurban populations might explain some of the differences in floral size that have evolved if these pollinators have divergent preferences or interactions with the flowers [36,39]. In support of this hypothesis, we found that both urban common garden and field-collected plants set more seed than nonurban plants, suggesting evolved increases in seed set among urban plants which may be due to increased attractiveness to the most abundant urban pollinators. The increased pollen limitation of urban plants might have helped drive the shifts towards—or have resulted from—larger flowers in urban populations.

Conclusion

Our results suggest that natural selection imposed by urbanization is sufficiently strong and multifarious to have driven the rapid evolution of a multivariate phenotypic cline in white clover in a large metropolitan area. The data available demonstrate variable responses of different plant taxa to urbanization, suggesting there is likely no ‘one size fits all’ solution to life in the city. Many of the traits showing divergence between urban and nonurban populations are involved in plant reproduction, and some of this divergence might be due to variation in the pollination

242 environment. Together, our results suggest that natural selection in urban environments is rapidly

243 refining whole-organism phenotypes to facilitate adaptation to cities.

244

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Tables

Table 1: Traits (with units) measured throughout common garden experiment.

Trait	Unit
Flowing phenology	# days to first flower
Banner petal length	mm
Banner petal width	mmm
# of flowers per inflorescence	Count
# of inflorescences	Count
Defensive phenotype	HCN+ (1) or HCN− (0)
Leaf width	mm
Leaf length	mm
Stolon diameter	mm
Petiole length	mm
Peduncle length	mm
Vegetative biomass	g
Reproductive biomass	g
Relative investment in sexual reproduction	Ratio of reproductive biomass and vegetative biomass

Figures

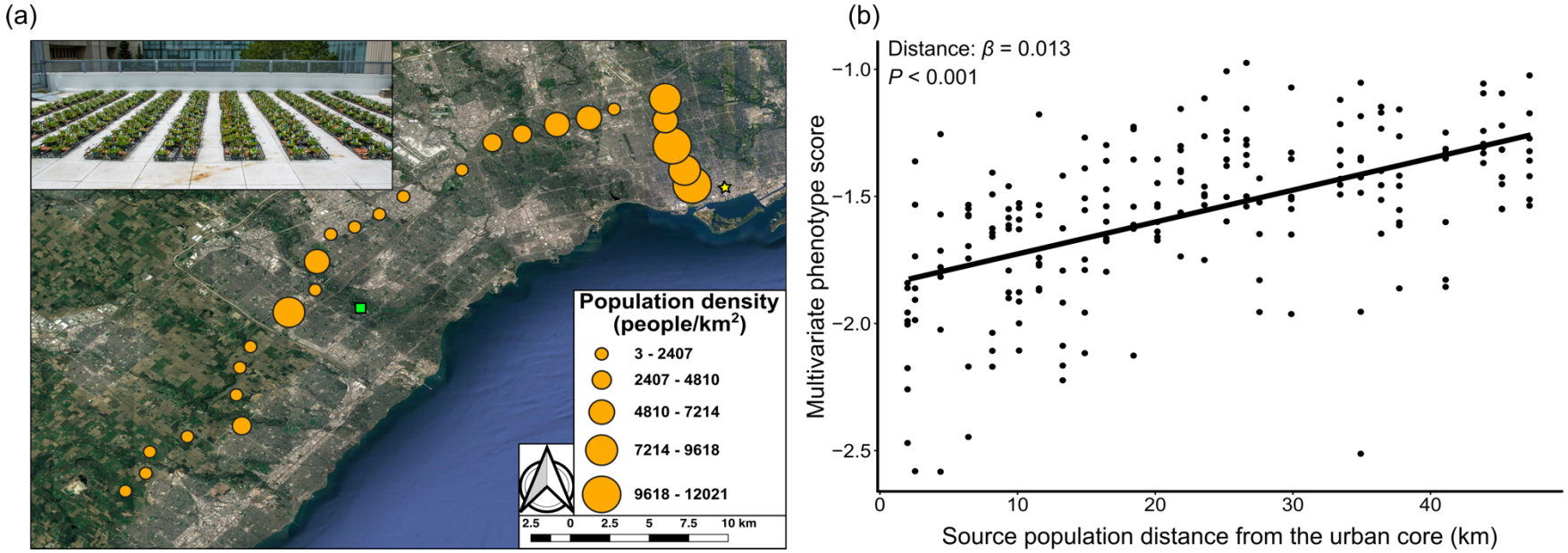

Fig. 1: (a) 27 populations from which we collected plants (orange points). Points are scaled by population density (from [40]) within 1 km^2. The city center (yellow star, Yonge-Dundas Square, lat: 43.6561, long: −79.3803) and common garden location (green square, lat: 43.5494, long: −79.6625) are also shown. Inset: Photograph of the common garden. (b) **cline$_{max}$** scores plotted against distance to the urban core (km).

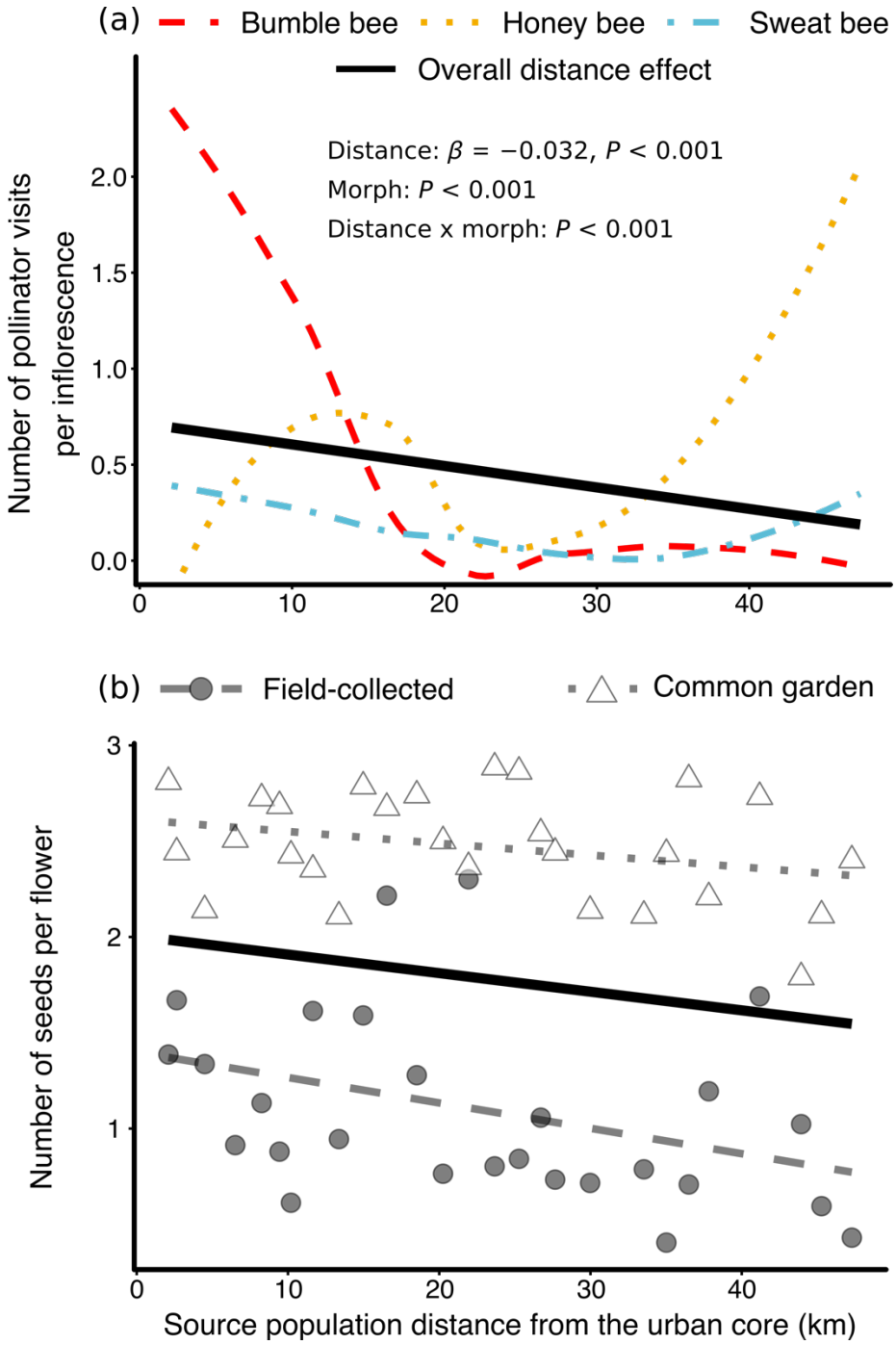

Fig. 2: (a) Mean number of visits per inflorescence against the distance of the population from the urban core (solid black line). We also show a smoothing line (loess line) for bumblebees (red dashed), honeybees (yellow dotted), and sweat bees (blue dots/dashes) separately to illustrate the change in community composition (also see Fig. S7 for point with linear fits). (b) Number of seeds per fruit (a metric of pollen limitation) among field-collected infructescences (grey-filled circles, dashed line) and infructescences from common garden plants (white triangles, dotted line) from these same populations. The thick black line shows the overall decrease in the number of seeds per flower with increasing distance from the urban center.