

Research



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Evolutionary biology

Multivariate phenotypic divergence along an urbanization gradient

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Evidence suggests that natural populations can evolve to better tolerate the novel environmental conditions associated with urban areas. Studies of adaptive divergence in urban areas often examine one or a few traits at a time from populations residing only at the most extreme urban and nonurban habitats. Thus, whether urbanization drives 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, grew them in a common garden, and measured 14 phenotypic traits. Families from urban sites had evolved later phenology and germination, larger flowers, thinner stolons, reduced cyanogenesis, greater biomass and greater seed set. Pollinator observations revealed near-complete turnover of pollinator morphological groups along the urbanization gradient, which may explain some of the observed divergences in floral traits and phenology. Our results suggest that adaptation to urban environments involves multiple traits.

1. 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 supports 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 traits that improve sprint speed on the smooth artificial surfaces common in cities [3,4], and warmer air temperatures in cities [5] have driven increases in thermal tolerance of urban acorn ant [6] and *Daphnia* populations [7]. These studies, among others (see electronic supplementary material, table S1 in [8]), suggest that many urban populations are adapting to urban environments.

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 suggests that selection can drive multivariate phenotypic differentiation along environmental gradients (i.e. multivariate clines) [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

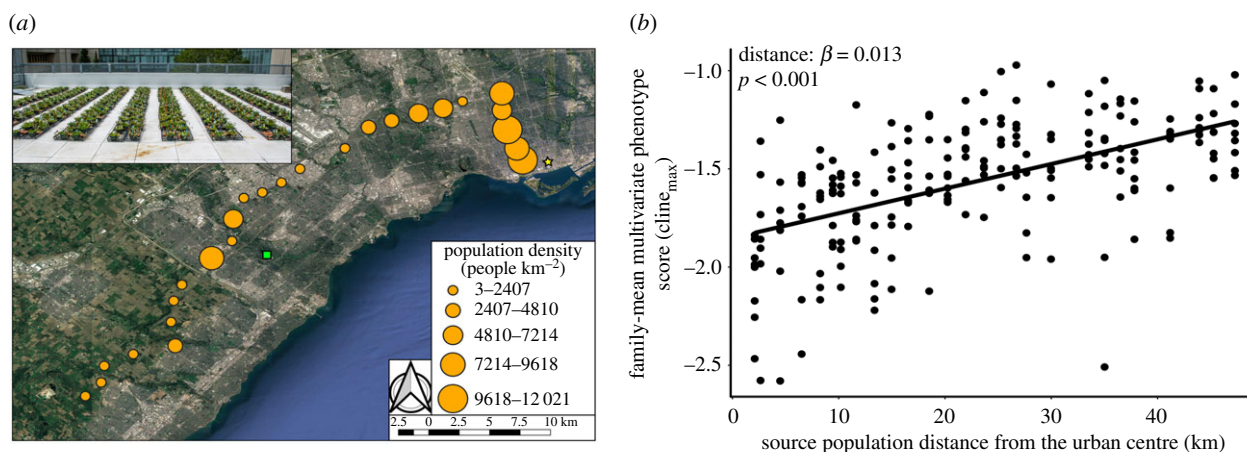


Figure 1. (a) Locations of the 27 populations from which we collected plants (orange points). Points are scaled by human population density (from [26]) within 1 km². The city centre (yellow star, Yonge-Dundas Square, lat.: 43.6561°, long.: -79.3803°) and common garden location (green square, lat.: 43.5494°, long.: -79.6625°) are also shown. Inset: photograph of the common garden. (b) Plot of cline_{max} scores against distance to the urban centre (km).

are driving phenotypic differentiation requires quantifying divergence in many traits that simultaneously influence fitness. It is presently unclear what suites of traits are most often favoured as populations adapt to urban environments.

Here, we investigate multivariate phenotypic divergence along an urbanization gradient in Toronto, Canada. Traits involved in plant reproduction are particularly likely to show phenotypic associations with urbanization between urban and nonurban populations owing 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 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 nonurban plants.

2. Methods

Here, we provide a sufficient but brief overview of our methods—additional details can be found in electronic supplementary material, text S1.1.

(a) Common garden

We examined multivariate trait divergence along an urbanization gradient using white clover (*Trifolium repens*) as a model system (electronic supplementary material, text S1.1). To minimize maternal effects, we grew 642 F₁ generation white clover plants from seed in a common garden at the University of Toronto Mississauga in summer 2017 where plants were naturally pollinated and consumed by herbivores for the duration of the experiment (electronic supplementary material, text S1.2). These plants were distributed among 209 plant families from 27 populations spanning an urban–nonurban transect in Toronto, Ontario, Canada (figure 1a, [10]). We measured 14 traits during our experiment, some of which are known to be under selection in this system (table 1) [27,28] and one of which, cyanogenesis, is known to have evolved along this and nearby urbanization transects [10,29,30].

Table 1. Traits (with units) measured throughout common garden experiment. HCN, hydrogen cyanide.

trait	unit
flowering phenology	no. days from germination to opening of first flower
banner petal length	mm
banner petal width	mm
no. flowers per inflorescence	
no. inflorescences	
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
time to germination	no. days from planting to cotyledon emergence

(b) Field observations

We conducted pollinator observations and quantified seed set in our study populations to identify possible mechanisms that might underlie phenotypic divergence along the urbanization gradient (electronic supplementary material, text S1.3). We assumed floral visitors were pollinators if they successfully peeled back the keel petals and made contact with the anthers (electronic supplementary material, figure S1). We classified pollinators into three morphological groups (termed *morphs* hereafter): honeybees (*Apis mellifera*), bumblebees (*Bombus* spp.) and sweat bees (Halictidae), which are the dominant pollinators of white clover in this region [27].

In each field population, we collected twenty ripe infructescences (i.e. group of fruits) and counted the number of flowers and seeds in each infructescence. We collected the same data from common garden plants. These data inform *in situ* variation in seed set per flower (field-collected infructescences) versus evolved differences in plants' abilities to set seed from pollen

(common garden data). We used seed set per flower as a measure of pollination success to control for the variable number of flowers per inflorescence in white clover; however, results are qualitatively similar when the number of seeds per infructescence is used as a measure of pollination success (electronic supplementary material, text S2).

(c) Data analysis

We investigated whether our urbanization gradient was associated with multivariate phenotypic divergence using principal components analysis (PCA) on the \mathbf{D} matrix, the variance–covariance matrix of standardized population means for the 14 traits (electronic supplementary material, text S1.4.1). We extracted the first principal component of \mathbf{D} , termed d_{\max} , which is the linear combination of traits showing the greatest among-population variance [13]. We used the trait loadings onto d_{\max} to calculate individual-level d_{\max} scores and regressed family mean d_{\max} scores against distance to the urban centre (in km)—our proxy for urbanization (electronic supplementary material, text S1.4.1 and figure S2)—as a global test of multivariate population divergence.

We examined clinal variation in multivariate phenotypes using canonical redundancy analysis (i.e. RDA, [31]). The RDA regressed a matrix of standardized family mean trait values (209 families \times 14 traits) as a response variable against a numeric vector representing the distance of populations to the urban centre as the sole predictor, though we note qualitatively similar results when using percentage impervious surface as a predictor (electronic supplementary material, text S3 and figure S3). 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 composite trait that shows the strongest association with distance to the urban centre [13] and facilitates quantifying how multivariate phenotypes are changing along our urbanization gradient (electronic supplementary material, text S1.4.1).

We estimated variation in pollinator visitation among natural populations by fitting a linear model with the mean number of visits per inflorescence as a response variable, and distance, pollinator morph and their interaction as predictors (electronic supplementary material, text S1.4.2). We assessed variation in the number of seeds per flower among field-collected infructescences and common garden plants by fitting a model with population mean number of seeds per flower as the response, and distance, source (field-collected versus common garden-collected) and their interaction as predictors. All data (see Data accessibility) were analysed in R v. 3.6.3 [32] within the RStudio environment [33].

3. Results

(a) Common garden

Our urbanization gradient was associated with multivariate trait divergence and the evolution of a multivariate phenotypic cline in Toronto, Ontario (figure 1*b*; electronic supplementary material, figures S4 and S5, and table S1). The first principal component of \mathbf{D} , d_{\max} , explained 71.6% of the variation in population means and showed a significant cline along our urbanization gradient ($\beta = 0.005$, $t_{206} = 2.99$, $p = 0.003$; electronic supplementary material, figure S4 d_{\max} loadings are given in electronic supplementary material, table S1). Distance to the urban centre explained 2.7% of the total variation in the multivariate phenotypic composition of populations (RDA, $F_{1,202} = 5.51$, $p < 0.001$; electronic supplementary material, figures S5

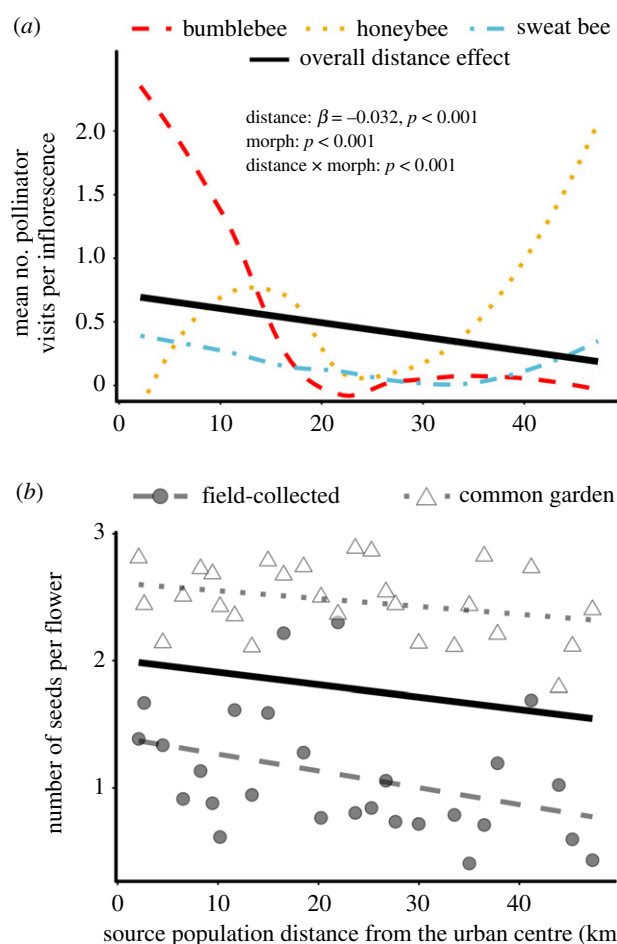


Figure 2. (a) Mean number of visits per inflorescence against the distance of the population from the urban centre (solid black line). We also show a locally estimated scatterplot smoothing (i.e., LOESS) fit for bumblebees (red dashed), honeybees (yellow dotted), and sweat bees (blue dot–dashed) separately to illustrate the change in visitation rate by different pollinator taxa (see electronic supplementary material, figure S10 for plot with linear fits). (b) Number of seeds per flower among field-collected infructescences (grey-filled circles, dashed line) and common garden plants (white triangles, dotted line) from these same populations. The thick black line shows the decrease in the number of seeds per flower with increasing distance from the urban centre.

and S6). The multivariate phenotype, cline_{\max} showed a cline along the urbanization gradient ($\beta = 0.01$, $t_{206} = 9.32$, $p < 0.001$, $r^2 = 0.29$, figure 1*b*; cline_{\max} loading in electronic supplementary material, table S1) that was steeper than any individual trait (electronic supplementary material, figures S7 and S8, and table S2). The six traits that loaded most strongly ($|\text{loading}| > 0.3$; in bold type in electronic supplementary material, figure S5) onto RDA1—germination, phenology, flower size, biomass, hydrogen cyanide (HCN) frequency and stolon thickness—and contributed most to cline_{\max} all shown significant univariate clines in the direction consistent with their trait loadings (electronic supplementary material, figures S5 and S9, and table S1).

(b) Field observations

On average, pollinator visitation rate was greatest in urban populations (distance: $\beta = -0.032$, $F_{1,75} = 21.96$, $p < 0.001$; thick black line in figure 2*a*). Visitation rate varied with pollinator morphs (morph: $F_{2,75} = 11.06$, $p < 0.001$): bumblebees had the highest visitation rate (mean = 0.63 ± 1.2 (s.d.) visits per inflorescence across all populations), followed by honeybees (mean = 0.58 ± 1.05) and sweat bees (mean = 0.18 ± 0.41).

Pollinator morphs varied in abundance along our urbanization gradient (distance \times morph interaction: $F_{2,75} = 14.11$, $p < 0.001$, figure 2a): bumblebee visitation rate increased with urbanization, whereas honeybee visitation decreased with urbanization; sweat bees showed little change in visitation along the transect (figure 2a).

While common garden infructescences contained more seeds per flower than those in natural populations (source $F_{1,50} = 30.04$, $p < 0.001$, $\text{mean}_{\text{field}} = 1.1$, $\text{mean}_{\text{garden}} = 2.5$, figure 2b), the number of seeds per flower was highest in urban populations (distance effect: $\beta = -0.013$, $F_{1,50} = 5.48$, $p = 0.03$, figure 2b), and this was true for both common garden and field-collected plants (distance \times source interaction: $F_{1,50} = 0.99$, $p = 0.33$, figure 2b).

4. Discussion

Most studies of population differentiation and clinal variation along environmental gradients focus on one or a few traits. We know less about how suites of traits evolve in response to environmental factors [13,14]. Our study shows clear phenotypic divergence and clinal variation in multivariate phenotypes along an urbanization gradient. The linear combination of traits showing the greatest among-population variance, d_{max} explained approximately 72% of the variation in population mean trait values and showed a significant cline along our urbanization gradient. Two traits (germination time and cyanogenesis) contributed most strongly to this divergence (electronic supplementary material, table S1), with minor contributions from other traits. After weighting traits based on their loadings onto RDA1 [13], distance explained 29% of the variation in $\text{cline}_{\text{max}}$, the multivariate phenotype that best explains trait variation along our urbanization gradient. Urban populations had later germination and flowering, greater vegetative biomass, larger banner petals, thinner stolons and lower HCN frequencies. Only one or a few of these traits may be direct targets of selection, whereas others may be indirect targets via trait correlations (mean pairwise $r_{\text{Pearson}} = 0.22$). Reciprocal transplant experiments with replicated plant genotypes across urban and nonurban common gardens would facilitate identifying the direct targets of selection, and deeper within-population sampling would enable estimating variation in trait correlations along the cline. These approaches would grant a fuller picture of how urbanization drives differentiation of multiple correlated plant phenotypes, especially when replicated across multiple cities. Finally, three of the six traits most strongly associated with distance to the urban centre were reproductive traits, supporting the prediction that these traits are likely to show phenotypic divergence between urban and nonurban habitats, although some notable traits (e.g. number of flowers) showed no association with our urbanization gradient.

Our observed differences in flower size corroborate earlier findings of stronger directional selection for larger flowers in urban populations [34,35]. In addition, plants from urban populations were larger than those in nonurban populations, similar to urban and nonurban *Lepidium virginicum* plants grown in a common garden [12]. However, increased vegetative biomass in urban populations is not universal; in *Ambrosia artemisiifolia*, urban and nonurban populations did not differ in plant size, although urban populations flowered earlier [11]. This contrasts with our experiment in which urban populations flowered later than nonurban populations.

Earlier flowering in urban populations is typically attributed to the increased temperatures resulting from the urban heat island, although this effect appears to be weaker for later-blooming perennials (e.g. white clover) [36]. Rather than temperature, the later flowering observed here may instead be related to changes in the biotic community (e.g. insects) or other components of the abiotic environment (e.g. soil moisture) [36], all of which require additional study in this system and urban environments more generally. Nonetheless, 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 favoured in cities.

Urban populations of white clover produced more seeds per flower and were visited primarily by bumblebees whereas nonurban populations were visited primarily by honeybees. Greater availability of food resources, reduced parasitism and/or lower pesticide concentrations may lead to greater bumblebee fitness and abundance in cities [37], potentially explaining their higher visitation rates in urban populations. In addition, a single bumblebee typically visits more white clover flowers per minute than a honeybee [38], and the number of seeds per flower is positively correlated with the number of visits in white clover [39,40]. This may explain the greater seed set of urban plants and is consistent with other work showing higher seed set among bumblebee-pollinated urban white clover plants [41]. 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 flower preferences [34,42] or select for different suites of correlated floral traits that improve pollination success and fitness [43]. In support of this hypothesis, we found increasing seed set with increasing urbanization for both common garden and field-collected plants, suggesting evolved increases in seed set among urban plants, which may be due to increased attractiveness to—or better mechanical fit with—urban pollinators. We hypothesize that the increased seed set of urban plants may be due to decreased pollen limitation. However, seed set is limited by both pollen receipt and resource supply [44–46]. While we do not currently have data on the resource environment for field-collected plants, the urban–nonurban difference in seed set persisted among common garden plants where resources were standardized, suggesting urban and nonurban plants are genetically differentiated in their ability to set seed from pollen. Factorial manipulation of the pollination (e.g. through supplemental hand pollination) and the resource environment (e.g. through fertilizer application) on replicate plant genotypes along the cline will help identify how urbanization shapes variation in pollen limitation and its consequences for floral trait evolution.

Data accessibility. All code and data used in this manuscript are available from the Dryad Digital Repository (<https://doi.org/10.5061/dryad.73n5tb2vg>) [47] and on the GitHub page for J.S.S. (github.com/James-S-Santangelo/SIC).

Authors' contributions. J.S.S. and K.A.T. planned the study and collected original plant material, and all authors contributed to subsequent study design. J.S.S. wrote the first draft of the paper, with sections written by K.A.T. and L.R.R., and all authors contributed to revisions. C.A. led the common garden experiment and collected data with assistance from all other authors. J.S.S. oversaw field data collection, with assistance from other authors. J.S.S. analysed the data with input from K.A.T. and L.R.R. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests. The authors declare no conflicts of interest.

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