Multivariate phenotypic divergence along an urbanization gradient

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Abstract

A growing body of evidence suggests that natural populations of dozens of species have undergone adaptive evolution in order to better tolerate the novel environmental conditions in urban areas. Invariably, studies of adaptive divergence in urban areas examine a single or few—often correlated—traits at a time from populations residing only at the most extreme urban and nonurban habitats, and do not control for environment-of-origin (e.g., maternal provisioning) effects. Thus, whether urbanization is driving divergence in many traits simultaneously in a manner that varies with the degree of local urbanization remains unclear. To examine whether urbanization drives clinal multivariate trait divergence, we generated seed families of white clover (*Trifolium repens*) collected from 27 populations along an urbanization gradient in Toronto, Canada, and used them to measure multiple phenotypic traits in a common garden. Our results show that families whose parents were collected from the most urban populations grew larger, had larger flower petals, experienced delayed germination and flowering, had thinner stolons, had reduced cyanogenesis, and were more attractive to pollinators. Each of these traits together exhibited genetically-based changes that varied with the degree of urbanization of the source population. Field observations indicate that the pollinator community exhibits almost complete turnover between urban and nonurban sites, which potentially explains some of the observed divergence in reproductive traits. Our results suggest that urban populations are rapidly tuning their phenotypes to tolerate the local disturbances imposed by humans.

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 (McDonnell, Hahs, & Breuste, 2009). Evidence is accumulating to support the hypothesis that the environmental features associated with urbanization cause the evolution of phenotypic differences between populations in urban and nonurban habitats (Johnson & Munshi-South, 2017). 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 (K M Winchell, Maayan, Fredette, & Revell, 2018; Kristin M Winchell, Reynolds, Prado-Irwin, Puente-Rolón, & Revell, 2016). Increased impervious surfaces often lead to warmer air temperatures in cities (i.e. the "urban heat island effect" Oke 1973), which has driven increases in thermal tolerance of acorn ants (Diamond, Chick, Perez, Strickler, & Martin, 2018) and greater energy storage and stress resistance among urban *Daphnia* populations (Brans, Stoks, & De Meester, 2018). These studies, and others (see table S1 in Rivkin et al. 2019) 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 changes, most studies focusing on phenotypic divergence associated with urbanization have examined just one or a few traits (Cheptou et al. 2008; Thompson et al. 2016; Gorton et al. 2018; Winchell et al. 2016; but see Yakub and Tiffin 2016). However, theoretical and empirical work in other systems suggests that selection can drive the evolution of multivariate phenotypic clines along environmental gradients (Lohman, Berner, & Bolnick, 2017; Stock, Campitelli, & Stinchcombe, 2014). Multivariate phenotypic analyses incorporate multiple, often correlated,

traits to explain overarching shifts in phenotypes across environments. While 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. Studies suggest that natural selection drives genome-wide divergence along urbanization gradients (Harris & Munshi-South, 2017; Theodorou et al., 2018), indicating that the targets of natural selection in urban environments can be multifarious. Nevertheless, it is presently unclear to what extent populations exhibit multivariate phenotypic divergence along urbanization gradients and what suites of traits are most often favored as populations become more urbanized.

Traits involved in plant reproduction are particularly likely to show genetically-based phenotypic associations with urbanization evolutionary responses 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 because both the diversity and abundance of pollinators are known to change along urbanization gradients (A. J. Bates et al., 2011; Larson, Kesheimer, & Potter, 2014), with positive (Theodorou et al. 2016a,b; Desaegher et al. 2017) to negative (Pellissier, Muratet, Verfaillie, & Machon, 2012) effects on pollinator visitation. Variation in pollination along urbanization gradients might drive changes in the extent of pollen limitation experienced by outcrossing plants (Eckert et al., 2009; Johnson, Thompson, & Saini, 2015), which can influence the strength and opportunity for pollinator-mediated selection on plant reproductive traits (Caruso et al., 2017; Sletvold & Ågren, 2016; Trunschke, Sletvold, & Ågren, 2017). For example, urban populations of yellow jasmine (*Gelsemium sempervirens*) experienced stronger directional selection for large floral displays, an effect that was associated with reduced pollen receipt of urban plants (Irwin, Warren, & Adler,

2018). Alternatively, prolonged reductions or inconsistent pollination in cities might lead to greater investment into vegetative propagation over sexual reproduction. Typically, investment into one form of reproduction occurs at the cost of the other (Abrahamson, 1980; Vallejo-Marín, Dorken, & Barrett, 2010) and consequently, we expect that plants could compensate for the altered pollination environment in cities by altering their reproductive strategy.

Here, we investigate multivariate phenotypic evolution along an urbanization gradient in Toronto, Canada. We address the following two specific questions: First, do populations exhibit genetically-based multivariate phenotypic clines along an urbanization gradient? If populations do exhibit multivariate phenotypic clines, we predict that reproductive traits are particularly likely to show divergence in response to urbanization. Second, can multivariate trait divergence or variation in sexual vs. clonal reproduction be explained by variation in the pollination environment along the urbanization gradient? If divergence in reproductive mode is indeed associated with an altered pollination environment, we predict that they will vary in the same direction as variation along an urbanization gradient. Our results demonstrate that natural populations are rapidly refining their phenotypes in response to urbanization.

Methods

Study design

We examined multivariate trait divergence along an urbanization gradient using white clover (*Trifolium repens*) as a model system. White clover is a perennial, herbaceous plant that is native to Eurasia but has been introduced to temperate and tropical areas globally within the past few centuries (Burdon, 1983; Kjærgaard, 2003). White clover is found in many human-altered habitats, including in cities and agricultural lands. Our focal urbanization gradient was located in

Toronto, ON, Canada (population in collection year [2016] approx. 6.5 million humans). White clover is particularly suited for our research questions for several reasons. First, white clover has locally adapted for least one trait—cyanogenesis, an antiherbivore defense—in Toronto (Thompson et al., 2016) and surrounding cities (Johnson, Prashad, Lavoignat, & Saini, 2018), where the frequency of cyanogenesis was positively correlated with the distance from the urban center (Fig. S2A in Thompson et al. 2016; $R^2 = 0.40$, $P < 0.001$). Second, allele frequencies associated with local adaptation of cyanogenesis was identified as a good proxy for local adaptation generally (Wright, Zhou, Kuhle, & Olsen, 2017), suggesting that phenotype-wide local adaptation is likely occurring in Toronto. Third, white clover possesses a considerable amount of genetic variation for many phenotypic traits (Thompson et al. 2016), including investment in sexual vs. clonal reproduction (Griffiths & Bonser, 2013), which can be leveraged to test predictions about the evolution of plant reproduction. Finally, white clover is also an obligate outcrosser that requires pollinators for successful seed set, although occasional self-compatible genotypes have been documented (Burdon, 1983; Michaelson-Yeates, Marshall, Abberton, & Rhodes, 1997).

Common garden experiment

On August 15th, 2016, we collected plant material from 27 white clover populations spanning a 50 km gradient of urbanization intensity in Toronto (Fig. 1). We collected up to 15 stolons (mean = 14.2) —trailing stems that give rise to foliar, floral, and root tissue—from each population for a total of 385 maternal plants, which we propagated in the glasshouse at the University of Toronto Mississauga into four-inch pots filled with Sunshine Mix Potting Soil (Sunshine mix #1;

Sun Gro Horticulture, Agawam, MA, USA). Plants were kept well-watered, with ambient light, 25°C, and 50% humidity.

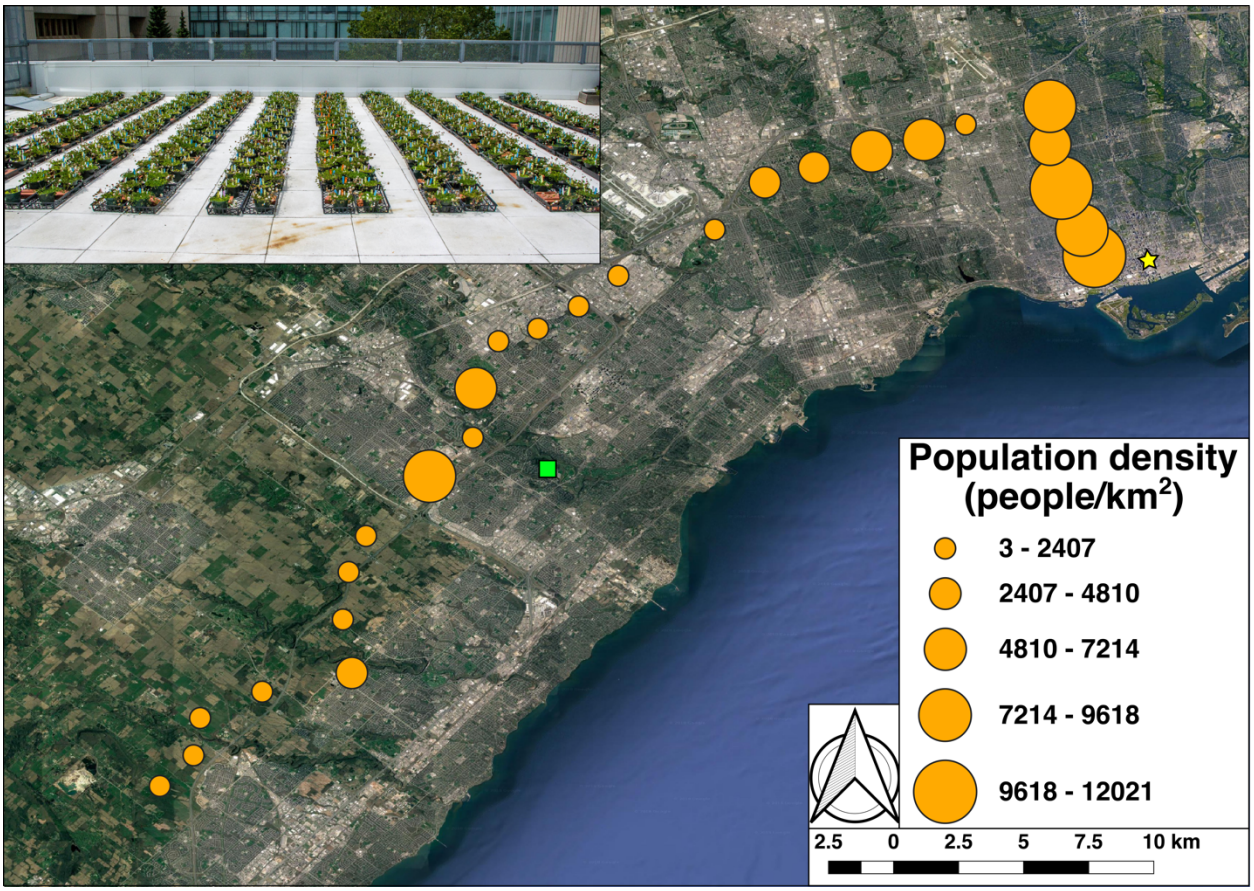

Fig. 1: Map of source populations and photograph of the common garden. Each of the 27 sampling locations from which we collected plants (orange points), with point size scaled by population density within the surrounding square kilometer (population density data from the Center for International Earth Science Information [CIESIN 2016]). The yellow star marks the location used as the city center (Yonge-Dundas Square, lat: 43.6561, long: –79.3803) and the green square marks the location of the common garden at the University of Toronto Mississauga (lat: 43.5494, long: –79.6625). Inset: Photograph of the rooftop common garden on the University of Toronto Mississauga campus.

To minimize maternal effects and ensure that all plants in the experiment were the same age, we started our common garden using glasshouse-generated seeds. To generate these seeds, we hand-pollinated each plant with every other possible donor from its own population and harvested the seeds from these crosses when mature. Although we used individuals collected from the field as parents, this design accounts for environment-of-origin effects because nearly all of the biomass present in the seed parents was generated in the glasshouse. Throughout the article, we refer to seeds produced by an individual maternal plant within a population as a plant 'family'.

To generate our common garden, we selected seeds based on their population and parent. For each of the 27 populations, we sowed five randomly selected seeds from eight randomly selected plant families (*n* seeds sowed = 1080). Due to lack of germination and seedling mortality, we ended up with 642 plants from 209 plant families across the 27 populations (mean families per population = 7.7, mean individuals per family = 3.1). On May 15th, 2017, we transplanted surviving seedlings into 6-inch round pots (~1.8 liter) and randomized their location on the outdoor rooftop patio beside the University of Toronto Mississauga glasshouse (Fig. 1 inset). We allowed the plants to be naturally pollinated and consumed by herbivores throughout the duration of the experiment, which we ended on 30 July 2017 prior to the plants becoming root-bound in the pots.

We measured several reproductive traits during our experiment, some of which are known to be under selection in this system (Santangelo, Thompson, & Johnson, 2018; Thompson & Johnson, 2016). First, we recorded the number of days from germination to the production of the first open flower. Using digital calipers, we then measured the length and width of banner petals on three flowers from two inflorescences per plant. We counted the number of flowers on

the first two inflorescences produced by each plant and recorded the total number of inflorescences produced over the growing season. We determined the cyanotype (i.e., cyanogenic: HCN+ or acyanogenic: HCN–) of each plant using Feigl-Anger assays (Feigl and Anger 1966; methodology from Gleadow et al. 2011).

While harvesting, we measured additional traits that might exhibit clinal variation and have diverged between urban and nonurban population. We measured the length and width of three leaves on each plant, as well as the diameter of two stolons. These traits have previously been associated with increased frost tolerance (Caradus et al. 1989; Svenning et al. 1997), which may be under clinal selection due to elevated frost-exposure in urban clover populations (Thompson 2016). We additionally measured the length of three petioles and three peduncles to quantify plant growth form (shorter peduncles and petioles = more prostrate growth). Leaf size and petiole length were measured, relative to the leading end of a stolon, on the second most recent fully expanded leaf. The diameter of stolons was measured between the node of that leaf and the adjacent interior node. We obtained plant-level estimates of each trait by taking the mean of all measurements per individual. Finally, we harvested the sexual reproductive tissue (peduncles and inflorescences / infructescences) and aboveground vegetative tissue (everything else) separately, dried it for at least 48 h at 60 °C, and weighed it to the nearest 0.001 g. These weights were our measures of sexual and vegetative biomass.

Field observations

We quantified variation in pollination *in situ* among our study populations to identify possible mechanisms that might underlie phenotypic divergence along the urbanization gradient. This was done by conducting pollinator observations in all study populations in early August 2017.

Similar to previously published methods for white clover (Verboven, Aertsen, Brys, & Hermy, 2014), we established two 1 m × 1 m quadrats in full sun in each of the 27 populations along our urbanization gradient and counted all inflorescences with open flowers within each quadrat. We then observed each quadrat for 20 minutes and counted all pollinators and the number of inflorescences they visited during this period. We classified pollinators into three morphological groups (*hereafter*: morphs): honey bees (*Apis mellifera*), bumblebees (*Bombus spp.*) and sweat bees (Halictidae). Recent mowing in one population prevented us from collecting pollinator observations from the exact source location, so we collected data from a population within 100 m.

To complement data from pollinator observations and our field experiment, we collected twenty ripe infructescences (i.e., collection of fruit) from the same populations in which we recorded pollinator observations. Samples were collected at least 1.5 m apart. We counted the number of flowers and seeds produced by each infructescence and used the number of seeds per flower as a metric of pollen limitation (fewer seeds per flower = more pollen limitation). We generated data for the same metric from plants in the common garden by counting flowers and seeds from each of two infructescences per plant and taking the mean of these measurements as our measure of plant-level number of seeds and flowers. These data provide information about evolved differences in plants' abilities to set seed from pollen (common garden data) and *in situ* variation in pollen limitation (field-collected inflorescences).

Data analysis

We analyzed our data in R version 3.6.0 (R Core Team, 2019) within the RStudio environment (RStudio Team, 2016) and used functions within the following packages: *tidyverse* (Wickham,

2017), *vegan* (Oksanen et al., 2018), *car* (Fox & Weisberg, 2011), *lme4* (D. Bates et al., 2015), and *lmerTest* (Kuznetsova, Brockhoff, & Christensen, 2017). See **Data Accessibility** for instructions to access the data and code.

Common garden experiment

We investigated whether urbanization was associated with multivariate phenotypic divergence using canonical redundancy analysis (RDA, Légendre and Légendre 2012). The RDA regressed a matrix of standardized family mean trait values (209 families × 14 traits) as a response variable against a numeric vector representing the distance of populations to the urban core as the sole predictor (see below). RDA is useful here because it allowed us to simultaneously examine divergence in multiple correlated traits (response matrix) and explore combinations of traits most strongly associated with our urbanization gradient (predictor).

Prior to the RDA, we standardized individual trait values by dividing them by their experiment-wide means and, similar to previous work examining multivariate trait divergence across environmental gradients, calculated family-mean trait values (n = 209 families) for use in our RDA (Stock et al., 2014). Since maternal plants are independent and paternal plants were homogenized within populations, we treated families as independent in our analyses. However, results are qualitatively similar when using population means (Fig. S1). Our RDA regressed family-mean trait values (n = 14 traits) against distance to the urban center. We used distance to the urban center as a measure of urbanization because this is correlated with % impervious surface (r = –0.63, Fig. S2A) and human population density (r = –0.72, Fig. S2B), and captures appreciable variation in at least one trait (HCN) along urbanization gradients (Johnson et al., 2018; Thompson et al., 2016). We calculated the distance between population and the urban

center using the haversine formula (Sinnott, 1984), which calculates the distance between a pair of coordinates while accounting for Earth's curvature. We confirmed the statistical significance of distance in explaining variation of multivariate phenotypic trait divergence using 10,000 permutations of the phenotype matrix (*anova.cca* function in 'vegan', see **Results**). This approach tests the extent to which distance predicts the observed distribution of phenotypic traits relative to random rearrangements of the phenotype matrix among families.

The RDA evaluates whether distance to the urban center explains better-than-random variation in family-mean phenotypes. In principle, however, we could observe a significant RDA even if just one trait were strongly associated with distance. Thus, a significant RDA is necessary but is not alone sufficient to test our hypothesis that multivariate phenotypic divergence has occurred along our focal urbanization gradient. To probe the multivariate nature of the cline, 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 $\mathbf{cline_{max}}$, is the multivariate quantitative trait that shows the strongest association with distance to the urban center (Stock et al. 2014) and is calculated as:

$$cline_{max,i} = (r_1 \times Germ_i) + (r_2 \times FF_i) + (r_3 \times NumInf_i) + (r_4 \times RepBio_i)$$
$$+ (r_5 \times VegBio_i) + (r_6 \times BW_i) + (r_7 \times BL_i) + (r_8 \times PetL_i) + (r_9 \times PedL_i)$$
$$+ (r_{10} \times NumFlwr_i) + (r_{11} \times LW_i) + (r_{12} \times LL_i) + (r_{13} \times ST_i) + (r_{14} \times HCN_i)$$

Where $r_1 - r_{14}$ represent the canonical coefficients extracted from the RDA, while the remaining terms (e.g., $Germ_i$, FF_i, etc.) represent the 14 traits for individual *i*. As above, the calculation of $\mathbf{cline_{max}}$ was performed on traits standardized by dividing by the mean across all populations (Stock et al., 2014). Because traits are weighted by their canonical coefficients from the RDA,

traits that are more strongly associated with distance to the urban core contribute more strongly to a population's **cline$_{max}$** score. Regressing **cline$_{max}$** against distance to the urban center thus enables us to better visualize the shape of the multivariate cline and better quantify how multivariate phenotypes are changing along our urbanization gradient.

To complement the multivariate approach taken above, we used univariate models to examine trait change along our urbanization gradient. This allowed us to determine if the traits that contributed most strongly to multivariate trait divergence and clinal variation also showed strong clines when analyzed individually. In these models, we regressed family-mean trait values against distance to urban core as the sole predictor. Second, we used the univariate model to test the specific prediction that urbanization alters relative investment in sexual vs. clonal reproduction. In this model, we used the ratio of sexual biomass to vegetative biomass as our response variable and distance to the urban center as our predictor. Plants with a higher ratio invest more strongly into vegetative biomass and a value of zero means that a plant produced no sexual biomass.

Field observations

We used linear models to assess how pollinator visitation and the number of seeds per flower among field-collected and common garden plants varied along the urbanization gradient. To examine variation in pollinator visitation, we fit a linear model with the number of visits per inflorescence as a response variable, and distance, pollinator morph, and their interaction as fixed effect predictors. We obtained parameter estimates and *P*-values using type III sums of squares. In this model, a significant effect of distance suggests that pollinator visitations vary along our urbanization gradient, a significant effect of morph suggests that pollinator taxa vary in their

overall visitation rates, while a significant distance × morph interaction suggests that visitation rates by at least two pollinator taxa change in different ways along the urbanization gradient.

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 fixed-effect predictors. We obtained parameter estimates and *P*-values using type III sums of squares. In this model, a significant effect of distance suggests that the number of seeds per flowers varies with urbanization, a significant effect of source suggests that field-collected and common garden plants vary in the number of seeds per flower, while a significant distance × source interaction suggests that the effects of population origin on pollen limitation is different in the common garden than at the field sites. This interaction term provides insight into whether variation in pollen limitation was due to extrinsic (reduced pollination) or intrinsic (e.g., increased attractiveness to pollinators) factors.

Results

Common garden

Our analyses indicate that urbanization was associated with multivariate trait divergence and the formation of a multivariate phenotypic cline in Toronto, ON (Fig. 2). The permutation test showed that distance to the urban core explained 2.7% of the variation in the multivariate phenotypic composition of populations ($F_{1, 202} = 5.51$, $P < 0.001$, Fig. 2a, Fig. S3). The composite trait showing the strongest association with distance to the urban core (i.e., $\mathbf{cline_{max}}$) showed a highly significant positive cline along our urbanization gradient ($\beta = 0.01$, $t_{206} = 9.32$,

$P < 0.001$, $R^2 = 0.29$, Fig. 2B) that was as strong or stronger than any of the univariate clines (Fig. S4 and S5, standardized beta coefficients in table S1).

Traits that loaded strongly (|loading| > 0.3) onto the first axis of the RDA (RDA1) and contributed most to the composite trait showing the strongest association with distance—germination time, flowering time, vegetative biomass, banner petal length, stolon thickness, and HCN frequency (bolded in Fig. 2a)—all showed significant univariate clines in the direction predicted based on their trait loadings (Fig. 2a). Specifically, families from urban populations germinated and flowered earlier (Fig. S3a and S3b), had more vegetative biomass (Fig. S3c), produced longer banner petals (Fig. S3d), had thinner stolons (Fig. S3e), and less HCN (Fig. S3f). Relative investment into sexual vs. clonal reproduction did not vary with urbanization ($t_{207} = 1.97$, $P = 0.05$, Fig. S4i).

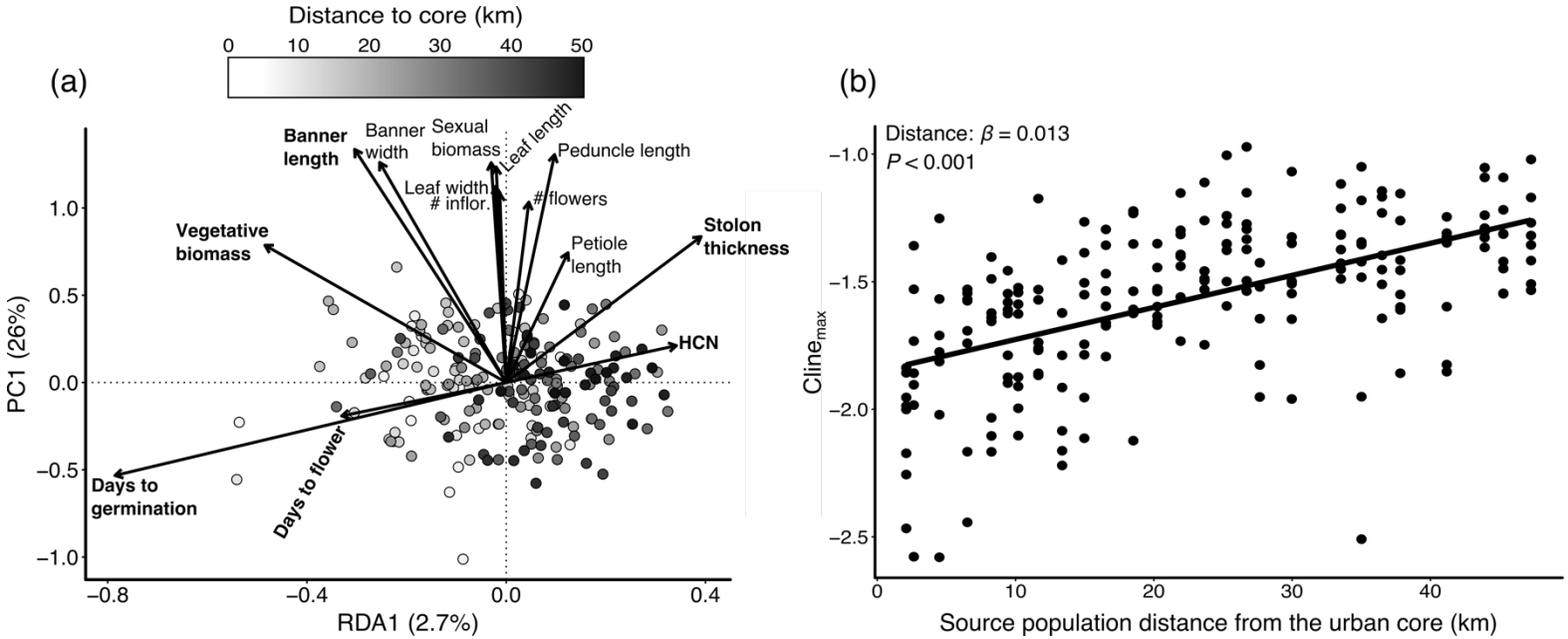

Fig. 2: Multivariate phenotypic divergence along an urbanization gradient. Panel (a) shows an RDA triplot displaying the position of each family along the constrained axis (RDA1) and first unconstrained axis (PC1) of the canonical redundancy analysis. Families are shaded based on their distance to the urban center. Solid arrows represent standardized trait loadings: the direction of arrows relates to the orientation of axes with which they are most strongly correlated while the length of the arrow corresponds to the strength of that association. Bolded traits are significant ($P < 0.05$) when analyzed as univariate clines (Fig. S2). Populations further from the city center (i.e. right along RDA1) had higher HCN frequencies, smaller banner petal widths and lengths, lower vegetative biomass, flowered earlier, and germinated earlier. Other traits were not strongly associated with distance. Note that average seeds per flower (Fig. 3b) was not included in the RDA due to too much missing data at the family level. Panel (b) shows the population **cline$_{max}$** scores plotted against distance to the urban core (km). The distance to the city center was a significant prediction of **cline$_{max}$,** the multivariate trait showing the strongest association with distance based on RDA scores.

Field observations

In field observations of pollinators, both pollinator visitation rate and pollinator composition were influenced by urbanization. On average, pollinator visitation per inflorescence was greatest in urban populations (Distance: $\beta = -0.032$, $F_{1, 75} = 21.96$, $P < 0.001$; thick black line in Fig. 3a). Visitation rate varied with pollinator morphs (Morph: $F_{2, 75} = 11.06$, $P < 0.001$), where bumblebees had the highest visitation rate (average = 0.63 ± 1.2 SD visits/inflorescence across all populations), followed by honey bees (average = 0.58 ± 1.05 visits/inflorescence), and sweat bees (average = 0.18 ± 0.41 visits/inflorescence). However, pollinator morphs varied in their response to urbanization (Distance × Morph interaction: $F_{2, 75} = 14.11$, $P < 0.001$, Fig. 3a): bumblebee visitation rate was greatest in urban populations, whereas honey bee visitation was greatest in nonurban populations, and sweat bees showed little change in visitation across the urbanization gradient (Fig. 3a).

Pollen limitation of flowers also varied with urbanization. While common garden infructescences were on average more thoroughly pollinated than the field trial plants (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. 3b), 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. 3b) independent of inflorescence source (Distance × source interaction: $F_{1,50} = 0.99$, $P = 0.33$, Fig. 3b).

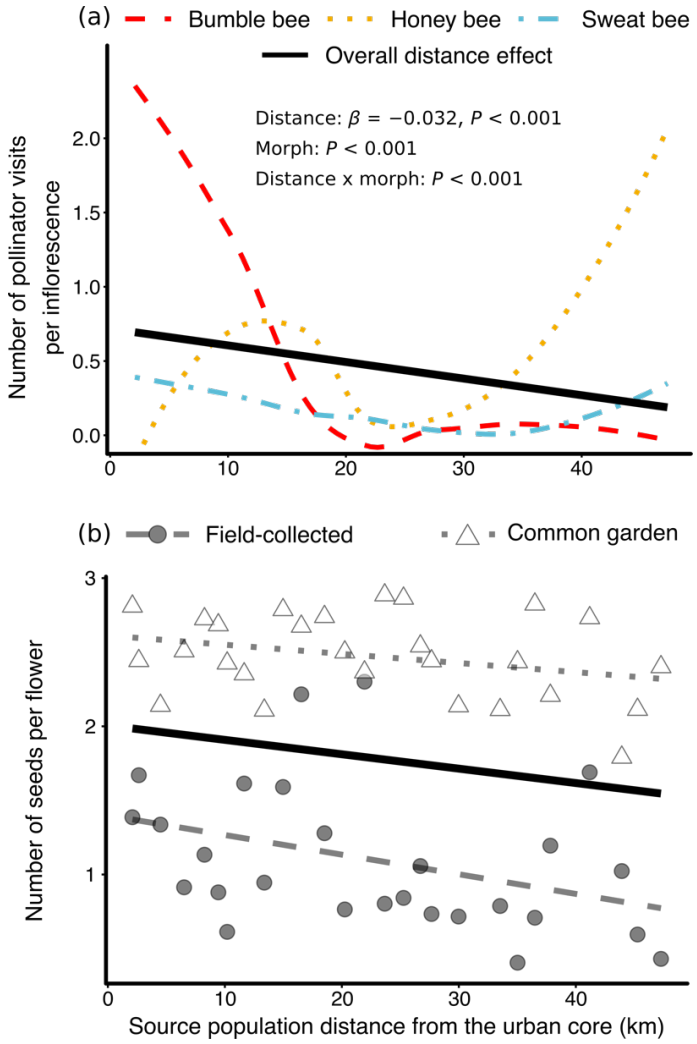

Fig. 3: Changes in pollination, pollinator community and pollen limitation along the urbanization gradient. Panel (a) depicts the relationship between the mean number of visits per inflorescence and the distance of the population from the urban core (solid black line). Because the effects of distance on the number of pollinator visits varied across pollinator taxa, 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. Points and confidence intervals are not shown to avoid clutter (see Fig. S6 for similar figure with points and linear fits). Panel (b) shows the relationship between the number of seeds per senesced flower (a metric of pollen limitation) among field-collected infructescences (grey-filled circles with dashed line) and infructescences from common garden plants (white triangles with 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 among both field-collected and common garden plants ($\beta = -0.01 \pm 0.006$, $P = 0.02$). The effect of distance to the urban core on the number of seeds per flower did not differ between field-collected ($\beta = -0.01 \pm 0.007$) and common garden ($\beta = -0.005 \pm 0.005$) infructescences (Distance × source interaction: $F_{1,50} = 0.99$, $P = 0.33$).

Discussion

We conducted a common garden experiment to investigate whether populations exhibit multivariate phenotypic divergence and clinal variation along an urbanization gradient, and we used field observations of pollination and seed set to provide ecological context to our findings. Our primary result is that populations have undergone multivariate phenotypic divergence along an urbanization gradient in North America's fourth-largest metropolitan area. Contrary to our prediction, we did not find support for the hypothesis that urban populations have evolved altered investment into sexual reproduction, although we did find evidence for evolved increases in flower size and reduced pollen limitation among urban populations. Below, we discuss our findings and place them in the context of other work in white clover and urban environments, and we discuss some of the mechanisms potentially leading to altered evolutionary responses in cities.

Multivariate phenotypic divergence and clinal variation

Most studies of population differentiation and clinal variation along urbanization and other 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 (Lohman et al., 2017; Stock et al., 2014). Our study shows clear phenotypic divergence and clinal variation in multivariate phenotypes along an urbanization gradient. While distance explained only 2.7% of the variation in the multivariate phenotypic composition of plant families, this analysis includes many traits that were not strongly associated with urbanization, thereby lowering the variation explained. Indeed, after weighting traits based on their loadings onto RDA1 (Stock et al., 2014), distance explained 29% of the variation in the multivariate phenotype $\mathbf{cline_{max}}$. In our experiment, urban

populations had lower **cline$_{max}$** scores indicating lower HCN frequencies, later germination and flowering, more vegetative biomass, thinner stolons, and larger flowers (banner petal length). Furthermore, many of the traits (four of seven) 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 nonurban habitats. although some notable traits (e.g. reproductive biomass, number of flowers per inflorescence, and number of inflorescences) showed no association with urbanization.

Relation to other studies of white clover

Our study was able to replicate the cyanogenesis cline described by Thompson et al. (2016) along the western transect of the GTA. Thompson et al. (2016) used correlational data to infer that lower cyanogenesis in urban populations had evolved due to selection for tolerance to freezing damage. HCN results from an epistatic interaction among two loci: *CYP79D15* produces cyanogenic glucosides which are hydrolyzed in the presence of linamarase encoded by the *Li* locus. Recent data suggest a cost to producing glucosides and linamarase under stressful conditions (e.g., frost) (Kooyers, Hartman Bakken, Ungerer, & Olsen, 2018), which may partially explain the lower HCN frequencies in urban populations. Furthermore, small-leaved plants and those with thinner stolons are more frost-tolerant (Caradus et al., 1989; Svenning, Røsnes, & Junttila, 1997), suggesting plants in urban environments should match these phenotypes if they are indeed more prone to frost and have evolved to tolerate these conditions. Our data do not support the hypothesis that urban populations have evolved smaller leaves (width or length) but do support urban plants having thinner stolons (Fig. S2e, table S1). Additional work testing how frost-tolerance traits evolve in cities, particularly cities at northern

latitudes, is needed to better enable us to predict how the phenotypic composition of plant populations will change in response urbanization.

Relation to other work in urban environments

Our data parallels other work examining evolutionary responses of plants to urbanization. Our observed differences in flower size are consistent with studies that found stronger directional selection by pollinators for larger flowers in urban populations (Irwin et al. 2018; Bode and Tong 2018). Similar to work on *Lepidium virginicum*, we found that plants from urban populations were larger than those in nonurban populations when grown in a common garden (Yakub and Tiffin. 2016). However, increased vegetative biomass in urban populations is not universal. In ragweed (*Ambrosia artemisiifolia*), there were no differences in plant size between urban and nonurban populations, although urban populations flowered earlier than nonurban populations (Gorton et al. 2018). This contrasts with our experiment in which urban populations flowered later than nonurban populations. Together, these results suggest that the effects of urbanization on plant traits vary across species and that there appears to be no particular combinations of traits consistently favored in cities.

We observed no evidence of a change in reproductive mode along our urbanization gradient. We tested the hypothesis that divergence in reproductive mode would be associated with variation in the pollination environment due to urbanization. Despite a clear turnover in the pollinator community and evidence of increased pollinator visitation and seed set of urban plants, urbanization did not alter relative investment in sexual vs. asexual reproduction, although vegetative biomass increased with urbanization. rather than altering investment in reproductive

biomass., urban white clover plants may respond to the altered pollination environment by evolving traits that increase pollination efficiency (see paragraph above).

Urban populations of white clover were less pollen limited and were visited primarily by bumblebees whereas nonurban populations were visited primarily by honey bees. This pattern contrasts with other work in this system showing the reverse (Larson et al., 2014), potentially owing to differences in methodology (collection vs. pollinator observations) or time of year (spring vs. late summer). A single bumblebee typically visits more white clover flowers per minute than a honeybee (Howlett, Lankin-Vega, & Jesson, 2018), 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 (Verboven et al., 2014). 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 (Irwin et al., 2018; Irwin, Warren, Carper, & Adler, 2014). 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. Our two primary results regarding the extent of pollination—more observed pollination events and greater seed set per flower—together suggest that urban white clover populations are less pollen limited than nonurban populations, which might have helped drive the shifts towards—or have resulted from—larger flowers in urban populations. Hand pollinations along the urban gradient would help better quantify the extent of pollen limitation due to urbanization and identify pollinators as agents driving divergence of urban and nonurban floral traits.

Conclusion

Our results suggest that natural selection imposed by urbanization is sufficiently strong to have driven the rapid evolution of a multivariate phenotypic cline in white clover in a large metropolitan area. Our results are consistent with the hypothesis that adaptation in response to selection imposed by urbanization is multifarious and exhibits variation in strength continuously over space. Accordingly, 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 environment. Together, our results suggest that natural selection in urban environments is rapidly refining whole-organism phenotypes to facilitate adaptation to cities.

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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.

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