Rural selection drives the evolution of an urban-rural cline in coat color in gray squirrels

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Abstract

Phenotypic differences between urban and rural populations are well-documented, but the evolutionary processes driving trait variation along urbanization gradients are often unclear. We combined spatial data on abundance, trait variation, and measurements of fitness to understand cline structure and test for natural selection on heritable coat color morphs (melanic, gray) of eastern gray squirrels (*Sciurus carolinensis*) along an urbanization gradient. Population surveys using remote cameras and visual counts at 76 sites along the urbanization gradient revealed a significant cline in melanism, decreasing from 48% in the city center to <5% in rural woodlands. Among 76 translocated squirrels to test for phenotypic selection, survival was lower for the melanic than gray morph in rural woodlands, whereas there was no difference in survival between color morphs in the city. These results suggest the urban-rural cline in melanism is explained by natural selection favoring the gray morph in rural woodlands combined with relaxed selection in the city. Our study illustrates how trait variation between urban and rural populations can emerge from selection primarily in rural populations rather than adaptation to novel features of the urban environment.

Keywords: animal color, genetic drift, landscape change, natural selection, relaxed selection, urbanization

1. Introduction

In an era of unprecedented global change, how urbanization affects the evolution of life is a frontier in biology (Donihue and Lambert 2015, Johnson and Munshi-South 2017, Rivkin 2019). Urban areas are the fastest growing ecosystem on Earth (United Nations 2018), transforming land cover, climate, and hydrology, and creating novel interactions between people and wildlife (Grimm et al. 2008, Soulsberry and White 2015). These environmental changes can drive the evolution of novel adaptations within populations (Lambert et al. 2021). Yet, whereas phenotypic differences between urban and rural populations are well-documented (McDonnell and Hahs 2015, Alberti et al. 2017), the evolutionary processes controlling trait variation along urbanization gradients are less clear.

Phenotypic variation between urban and rural areas is not sufficient evidence of adaptive urban evolution. Phenotypic divergence can be due to plasticity rather than genetic variation, such as birds adjusting song pitch to high frequencies in response to low-frequency city noise (e.g., Slabbekoorn and Peet 2003). Demonstrating urban adaptation first requires showing trait variation is at least partially heritable. Genetic divergence between urban and rural populations provides further evidence of adaptation but is not entirely sufficient. Environmental differences between urban and rural areas may cause divergent natural selection on heritable traits, but phenotypic and genetic divergence can also result from genetic drift due to founder events, or low effective population size in combination with limited gene flow along the urbanization gradient (Vasemägi 2006). For example, colonization of cities by European blackbirds (*Turdus merula*) resulted in reduced genetic variation in city populations and divergence in migratory behavior between urban and rural areas (Evans et al. 2009, 2011). Ultimately, urban adaptation requires heritable trait variation that leads to differences in fitness among individuals. Despite a

70 proliferation of case studies of urban-rural trait variation, many including patterns consistent
71 with urban adaptation (Winchell et al. 2022), few studies examine the fitness consequences of
72 trait variation (Lambert et al. 2021).

73 Fitness consequences of heritable trait variation can provide much needed insight into
74 alternative hypotheses on the spatial context of adaptive and non-adaptive mechanisms
75 generating urban-rural clines. Smooth clines along urbanization gradients can result from
76 spatially divergent natural selection in the presence of gene flow (Takahashi 2015). In the case of
77 discrete polymorphisms, divergent natural selection favors different morphs in urban and rural
78 environments, generating a pattern of morph-by-environment interaction for fitness (Fig. 1*a*).
79 Alternatively, urban-rural clines can result from a combination of selection and genetic drift
80 across the urbanization gradient. For example, selection may favor one morph in the city,
81 whereas morph frequencies are controlled primarily by drift in the rural environment (i.e., urban
82 adaptation; Fig. 1*b*). Under this hypothesis, the morph favored by selection in the city should
83 rarely reach high frequency in rural populations due to drift alone, and on average the frequency
84 of the morph favored in the city should be negatively related to distance from the city (Fig. 1*b*).
85 Urban-rural clines can also be generated by the reverse hypothesis of selection operating
86 primarily in the rural environment with drift controlling morph frequencies in the urban
87 environment (i.e., rural adaptation; Fig. 1*c*). Finally, if genetic drift is the salient force in both
88 urban and rural populations, we expect no differences in fitness between morphs in either
89 environment, with no consistent urban-rural clines (Fig. 1*d*).

90 Our goal was to better understand the spatial context of adaptive and non-adaptive
91 mechanisms of urban evolution. We used coat color in eastern gray squirrels (*Sciurus*
92 *carolinensis*) as a model system. *Sciurus carolinensis* has two common color morphs – gray and

melanic – controlled by the melanocortin-1-receptor gene (*Mc1R*; McRobie et al. 2009). Melanism in *S. carolinensis* is caused by a 24-bp deletion allele that is incompletely dominant, with heterozygotes having a brownish-black dorsum and brown underbelly (McRobie et al. 2009). Homozygotes for the deletion allele are black, whereas homozygotes for the recessive wild-type allele are gray. Melanics were the prevailing color morph in the northeastern United States prior to massive deforestation during European colonialism in the 18th and 19th centuries (e.g., Allen 1943, Schorger 1949, Robertson 1973), but today melanics tend to be rare in rural forests and more common in urban areas. Urban-rural clines in melanism occur across multiple cities at varying degrees of strength, and clines are absent in some cities (Cosentino and Gibbs 2022). These urban-rural clines are most evident in regions with relatively cold winter temperatures, where the deletion allele for melanism is likely maintained by selection for greater potential for non-shivering thermogenesis (Ducharme et al. 1989).

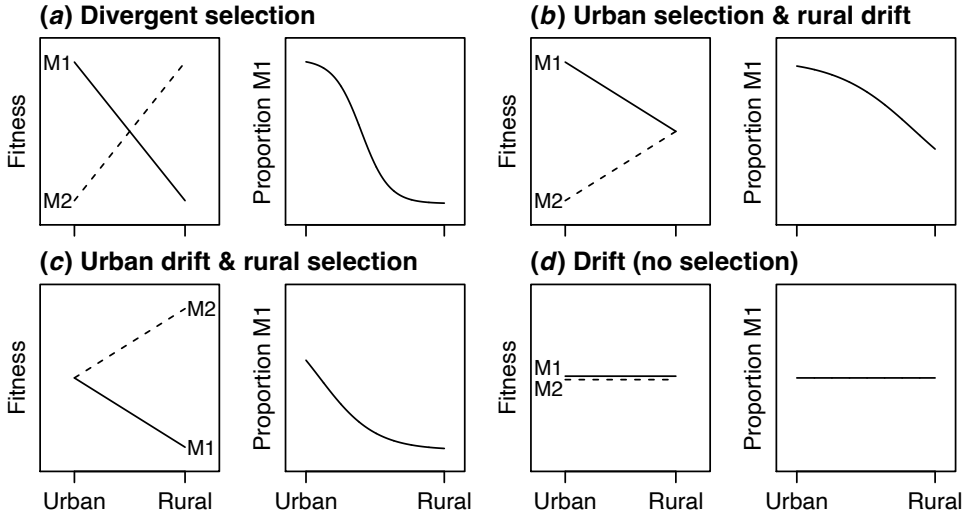

Figure 1. Four hypotheses (*a*-*d*) for spatial variation in the effects of selection and drift on a heritable, discrete polymorphism along urbanization gradients. Predicted relative fitness for two morphs (M1 and M2) in urban and rural environments and mean expectation for clinal variation in the proportion of morph M1 along urbanization gradients are shown for each hypothesis.

We combined two commonly used methods (see Linnen and Hoekstra 2009) to test whether urbanization causes natural selection on *S. carolinensis* coat color in Syracuse, New York, USA and surrounding rural areas. First, we characterized spatial variation in the prevalence of melanism along the urbanization gradient. Previous studies documenting an urban-rural cline in this area were based on observations of squirrels incidentally encountered via community science, which likely have spatial biases in sampling effort and detection of rare color morphs (Cosentino and Gibbs 2022). Here we used standardized surveys via remote cameras and visual counts and a novel hierarchical model that accounts for imperfect detection probability to test for an urban-rural cline. Second, we experimentally translocated squirrels of each color morph from urban to both urban and rural areas and compared survival between morphs in each environment to test for differential fitness. Previous work suggested an urban-rural cline in melanism may be related to two ecological causes of selection (Gibbs et al. 2019, Bryan 2020): 1) selection *against* melanics in rural forests because they are more conspicuous to predators and human hunters than the gray morph, and 2) selection *for* melanics in cities because they are more visible to drivers than the gray morph on roads and less likely to be struck by vehicles. Densities of *S. carolinensis* are significantly greater in urban than rural areas (Merrick et al. 2016), potentially due to relaxed predation pressure in cities relative to rural areas where *S. carolinensis* density is limited by predators (Havera and Nixon 1980, Bowers and Breland 1996). The predominant mortality source for urban tree squirrels is thought to be collisions with vehicles (McCleery et al. 2008). Thus, we predicted survival would be greater for the gray than melanic morph in rural forests where predation is the main driver of mortality, whereas survival would be greater for the melanic than gray morph in the urban environment where vehicular collision is the main driver of mortality.

2. Methods

Study area

Syracuse (43.04068° N, -76.14373° W) is a medium-sized city (ca. 150,000 people) with a land area of 65 km^2 and population density of ca. 2,300 persons/km^2 (U.S. Census Bureau 2022). Mean tree cover is 27%, ranging from 4.5% at the city core to 47% at its periphery (Nowak et al. 2001), with rural areas outside the city consisting of a mosaic of forest and agriculture (Fig. 2).

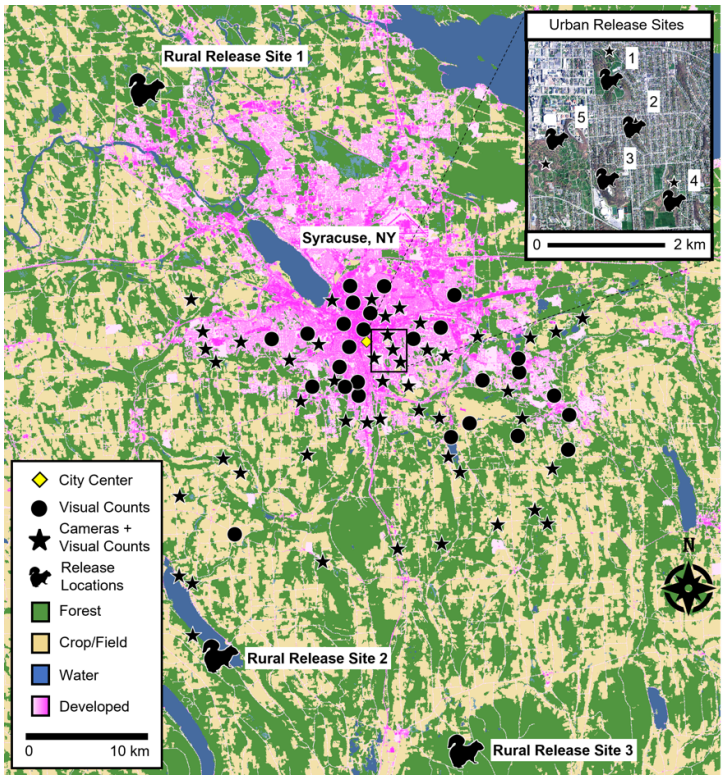

Figure 2. Land cover map of Syracuse, New York and surrounding rural areas. Sites with camera traps and visual counts (stars; n = 49) of eastern gray squirrels (*Sciurus carolinensis*) and sites with visual counts only (circles, n = 27) were distributed across the urbanization gradient. Rural release sites (n = 3) and urban release sites (n = 5; inset) for the translocation experiment are depicted by squirrel silhouettes. Land cover data modified from the National Land Cover Database (Yang et al. 2018). Digital orthoimagery from New York State Office of Information Technology Services GIS Program Office (2022a).

147 *Camera trapping and visual surveys*

148 We estimated the relative abundance of each *S. carolinensis* color morph at 76 sites along a
149 gradient of urbanization (Fig. 2). Squirrel occurrence is limited by tree cover, so we focused
150 surveys on areas within urban greenspaces and rural woodlots with mature deciduous trees
151 (Allen 1982, Parker and Nilon 2008). Sites surveyed were constrained to a sampling area that
152 extended from the city center to rural woodlands 24 km south of the city. Site locations were
153 chosen to represent a gradient in distance from the city center (range = 0.9–23.8 km, median =
154 6.8 km) with sites separated by at least 1 km.

155 We surveyed sites using a combination of remote cameras (i.e., camera trapping; Burton
156 et al. 2015) and visual surveys. Camera trapping was conducted at 49 sites between 16
157 September 2021 and 13 October 2022. A single camera trap (Browning Strikeforce Pro XD) was
158 deployed at each site between September 2021 and March 2022. Each camera was fastened to a
159 tree 30-50 cm off the ground, generally facing north and aimed toward the ground with no
160 obstructions within 2 m. All cameras were removed by October 2022, and cameras were active
161 for 46-379 days (median = 263 days). Images from cameras were viewed by a single observer
162 and classified to generate daily detection histories denoting whether each color morph was
163 detected at each site on a given day.

164 We conducted visual surveys for squirrels at all camera sites and an additional 27 sites
165 without cameras between 12 October 2021 and 18 October 2022 (n = 258 visual surveys). These
166 additional sites were included to fill sampling gaps where permanent camera traps could not be
167 placed. During each survey, a single observer recorded all visible squirrels of each color morph
168 over a 3-min period. Each site was surveyed visually 1-7 times during the study (median = 4).
169 Most sites (n = 57) received >1 visual survey across multiple days to facilitate abundance

estimation with a hierarchical model that included an explicit model for the detection process. Median time between visual surveys was 65 days (range 10–197).

Statistical analysis for cline estimation

We used an integrated hierarchical model (Kéry and Royle 2021) combining camera detection data with visual counts to estimate squirrel abundance and the proportion melanic at each site. The model is hierarchical in that it includes 1) an ecological process model to describe variation in squirrel abundance and the proportion melanic among sites and 2) an observation model to describe variation in detection probability of squirrels during surveys due to measurement error. We used the model to estimate the latent total abundance of squirrels (N_total_i), the latent abundance of each color morph (N_{ik}, where $k = 1$ for the melanic morph and $k = 2$ for the gray morph), the expected proportion of melanic squirrels ($p_melanic_i$), and individual detection probabilities (p_{ijk}) based on repeated surveys j of color morph k at each location i.

We included two submodels to describe the observation process: 1) A binomial distribution describing the counts y_{ijk} of each morph from visual surveys (Royle 2004): $y_{ijk}|N_{ik} \sim Binomial(N_{ik}, p_{ijk})$, and 2) a Bernoulli distribution describing the binary detection observations y_{ijk} of each morph from cameras (Royle and Nichols 2003): $y_{ijk}|N_{ik} \sim Bernoulli(P^*_{ijk})$, where $P^*_{ijk} = 1 - (1 - p_{ijk})^{Nik}$. Because squirrel activity is related to daily ambient temperature (e.g., Wassmer and Refinetti 2019), we modeled individual detection probability as a function of mean daily temperature from a nearby (8.5 km from city center) weather station with a complete record for the study period (Syracuse International Airport, NOAA Climate Data Online, https://www.ncei.noaa.gov/cdo-web/). The detection probability model was $\text{logit}(p_{ijk}) = a0_k + a1_k*\text{temperature}_{ij} + a2_k*\text{temperature}^2_{ij}$, where for each color morph k, $a0_k$ represents the intercept,

193 $a1_k$ represents a linear effect of daily temperature, and $a2_k$ represents a quadratic effect of
194 temperature. We included a quadratic term for temperature because we expected peaks in
195 squirrel activity in fall and spring at intermediate temperatures and lower squirrel activity in
196 winter and summer at temperature extremes (Spritzer 2002; electronic supplementary material,
197 Fig. S1). Regression coefficients for detection probability were estimated with a joint likelihood,
198 with the coefficients assumed to be identical between the count and detection/nondetection
199 datasets. This integrated model formulation allowed us to combine count and
200 detection/nondetection data in a single model, which can improve accuracy and precision of
201 parameter estimates (Kéry and Royle 2021).

202 We included two submodels to describe the ecological state process. First, we used a
203 Poisson distribution to describe the latent total abundance of squirrels: $N_total_i \sim Poisson(\lambda_i)$,
204 where λ_i is the expected total abundance of squirrels at location i. We expected total squirrel
205 abundance to be greatest in the city (Merrick et al. 2016), so we modeled total abundance as a
206 function of distance to city center: $\log(\lambda_i) = \beta 0 + \beta 1 * \text{distance}_i$, where $\beta 0$ represents the intercept
207 and $\beta 1$ represents the effect of distance to city center. We defined the city center as the centroid
208 of the civil boundaries of the city of Syracuse (Fig. 2; New York State Office of Information
209 Technology Services GIS Program Office 2022b). We chose distance to city center as a general
210 spatial index of urbanization because Syracuse has a simple, circular shape, and distance to city
211 center is strongly correlated with multiple metrics commonly used to measure urbanization (Moll
212 et al. 2019; e.g., impervious cover within 1 km: r = -0.70; road length within 1 km: r = -0.72;
213 settlement density within 1 km: r = -0.72), each of which can represent different causal effects on
214 the prevalence of melanism. Second, we used a binomial distribution to describe the latent
215 abundance of the melanic morph: $N_{i1} \sim Binomial(N_total_i, p_melanic_i)$. To estimate the cline in

216 melanism, we modeled the proportion of melanic squirrels as a function of distance to city
217 center: logit($p_melanic_i$) = $\beta 0 + \beta 1$*distance$_i$, where $\beta 0$ represents the intercept and $\beta 1$ represents
218 the effect of distance to city center. The latent abundance of the gray morph was derived as $N_{i2} =$
219 $N_total_i - N_{i1}$.

220 We used JAGS 4.3.0 (Plummer 2017) and the R package *jagsUI* (Kellner and Meredith
221 2021) in R (Version 4.0.2; R Core Team 2020) to fit the integrated model with a Bayesian
222 approach and Markov chain Monte Carlo. Covariates were standardized (mean = 0, SD = 1)
223 before fitting the model. We fit the model with noninformative normal prior distributions for
224 regression coefficients (Gelman and Hill 2007). We ran three chains with 12,000 iterations,
225 discarding the first 2000 iterations as burn-in and thinning the remaining iterations by 10. The
226 resulting 3000 iterations were used to describe the posterior distribution for each parameter. We
227 confirmed convergence of parameter estimates with the Gelman-Rubin statistic (R-hat < 1.1;
228 Gelman and Hill 2007).

229 We used a model of abundance that assumes closure to births, deaths, immigration, and
230 emigration within sites during the survey period because our focus was on spatial variation in
231 abundance of color morphs along the urbanization gradient. The closure assumption is unlikely
232 to be met because *S. carolinensis* has an extended reproductive period, and juveniles can be
233 found year-round (Kirkpatrick and Hoffman 1960; Steele and Koprowski 2003). When closure is
234 not met, the abundance parameter (λ_i) should be interpreted to represent the number of squirrels
235 associated with a given site during the survey period rather than the number of permanent
236 residents at the site (Kéry and Royle 2021). To evaluate whether our results were sensitive to the
237 closure assumption, we fit our model using the same parameterization but with a subset of data
238 during a period when closure was more likely met (16 September 2021 - 15 March 2022).

239 *Translocation experiment*

240 To test for phenotypic selection, we conducted a translocation experiment and compared squirrel
241 survival between color morphs in urban and rural environments. Squirrels of both color morphs
242 were translocated from urban greenspaces in Syracuse to both urban and rural release sites (Fig.
243 2; electronic supplementary material, Table S1). Although we preferred an experimental design
244 with squirrels translocated from both urban and rural environments, the rarity of melanic
245 squirrels in rural areas precluded a full reciprocal design, which ultimately prohibited us from
246 testing for local adaptation (e.g., greater survival of each color morph in its home than away
247 environment; Kawecki and Ebert 2004). However, our design allowed us to compare survival as
248 an index of fitness between morphs within environments as a test of phenotypic selection
249 (Linnen and Hoekstra 2009), which was our focus.

250 We measured survival via radiotelemetry. To do so, we captured and collared 76 squirrels
251 in urban greenspaces between June 2021 and May 2022 using wire cage traps (Tomahawk Live
252 Trap Co., Tomahawk, WI; Model 103) baited with peanut butter. Upon capture we coaxed
253 squirrels into a cloth handling cone for processing and radio-collar attachment (Koprowski
254 2002). Squirrels were anesthetized using isoflurane delivered via a plastic nose cone to minimize
255 squirrel stress and pain response and to maximize human safety (Parker et al. 2008). We
256 recorded squirrel mass, color morph, sex, and reproductive status. We collected a 1x3-mm sliver
257 of ear tissue for future genetic analyses and attached a metal ear tag (National Band Company,
258 Newport KY; Style 1005-3) with a unique numeric code to the other ear. We attached VHF
259 ("very high frequency") radiotransmitters to squirrels via neck collars (Holohil Systems Ltd.
260 Carp, ON; Model RI-2D 14 or 21g; with mortality switch option). We epoxied a metal
261 temperature logger (Maxim Integrated DS1925L-F5, 3.49 g) to the end of collars to record

262 temperature data. Weight of the radiotransmitter, collar, and temperature logger was <5% of the
263 squirrel's mass (mean = 2.7%, range = 2-4%). Pregnant and lactating females were excluded
264 from the sample. Following radio-collar attachment and recovery from isoflurane, we transported
265 each squirrel to a rural or urban release site by vehicle. To control for impacts of travel time, we
266 drove all translocated squirrels for a similar amount of time before release (30 min). Although
267 the process of translocation can be disruptive to an individual by removing it from its home
268 range, any effect of translocation on survival was controlled because all individuals in our study
269 were translocated.

270 Three rural release locations were 22-30 km from trapping locations and composed of
271 deciduous and mixed deciduous forest. Five urban release locations were 0.7-6 km from trapping
272 locations within Syracuse and consisted of a cluster of urban greenspaces with mixed deciduous
273 forest. All urban and rural release locations had known populations of *S. carolinensis*. Release
274 sites for each squirrel were selected systematically to maintain a balanced release of morph types
275 across sites at locations with ample shelter (e.g. tree cavities) and food resources (mast-
276 producing trees). In total, 18-20 squirrels of each morph were translocated to urban and rural
277 environments (Table 1).

278 After release, squirrel survival was monitored weekly (Haughland et al. 2008; Millspaugh
279 et al. 2012) until October 2022. At the end of the study, squirrel status was classified into one of
280 four categories: 1) confirmed and probable mortalities, 2) possible mortalities, 3) missing
281 squirrels that could no longer be found with radiotelemetry, or 4) active (Table 1). When we
282 detected a squirrel's radiotransmitter emitting a mortality signal, we attempted to locate the
283 squirrel as soon as possible. Squirrels with a mortality signal were assigned to the "confirmed
284 and probable" mortality category based on either the presence of a carcass (i.e., confirmed) or

285 significant evidence of a mortality event in the absence of a carcass (e.g. deep, rounded bite
286 marks on the collar assembly, large amounts of squirrel fur, or evidence the collar assembly was
287 removed by a human; Adams et al. 2004, Tebo and Norton 2017, Wilson et al. 2019). When
288 possible, we tried to ascertain the cause of mortality (McCleery et al. 2008). Possible mortalities
289 were based on the presence of a transmitter with a mortality signal but without a carcass or other
290 evidence of a depredation event, including transmitters broadcasting from a consistently
291 immobile position that we were unable to retrieve (e.g., emanating from a treetop).
292
293 **Table 1.** Number of translocated eastern gray squirrels (*Sciurus carolinensis*) and their fates by
294 color morph in each environment to which squirrels were translocated. Fates included confirmed
295 & probable mortalities, possible mortalities (mortalities and collars that were transmitting a
296 mortality signal, but we were unable to be locate), missing squirrels that we could no longer find
297 using radiotelemetry, and squirrels with active collars at the end of the study (October 2022).

		Translocated environment	
Translocations and Fates	Color morph	Urban	Rural
Translocated from urban environment	Melanic	18	18
	Gray	20	20
Confirmed & probable mortalities	Melanic	2	8
	Gray	4	2
Possible mortalities	Melanic	10	8
	Gray	6	12
Missing	Melanic	4	2
	Gray	8	5
Active	Melanic	2	0
	Gray	2	1

298
299

300 *Statistical analysis for survival estimation*

301 We used a Cox proportional hazards model (Cox 1972; Pollock et al. 1989) to relate daily
302 survival of translocated squirrels to color morph (gray, melanic) and translocation environment
303 (urban, rural). Date of translocation was used as the time of entry to maximize the number of
304 squirrels at risk at a given time for estimating daily survival (Karp and Gehr 2020). We used the
305 inferred date of mortality as the exit date in the model for squirrels classified as confirmed and
306 probable mortalities. Date of mortality was estimated as the median date between when the last
307 active signal was received and the first instance a mortality signal was detected. For collars
308 recovered with functioning temperature loggers (n = 21), we determined the date of mortality as
309 the date when collar temperature remained at ambient temperature, no longer rising to core
310 diurnal body temperature for *S. carolinensis* (37.9 °C; Pereira et al. 2002, Wassmer and Refinetti
311 2019). Squirrels not classified as mortalities contributed to the survival analysis via right
312 censoring, as each had a known minimum number of days survived (Murray 2006).

313 We modeled the hazard ratio as a function of morph, environment, and the interaction
314 between morph and environment using the R package *survival* (Therneau 2021). A likelihood
315 ratio test was used to examine the significance of the interaction term, with two-sided Wald tests
316 reported for each term in the most-supported model. We considered a model with additional
317 covariates, including sex, body mass, collar size, and mean daily temperature during the first
318 week after translocation (linear and quadratic effects), but we excluded these variables from the
319 final model because a) none of the variables predicted the hazard ratio (electronic supplementary
320 material, Table S2), b) data on sex was missing for two squirrels, which precluded use of those
321 observations in a model with sex as a covariate, and c) the effects of morph, environment, and
322 their interaction were consistent with or without the additional covariates in the model. No

deviations from the assumption of proportional hazards were evident through analysis of the Schoenfeld residuals for morph ($\chi^2 = 0.24$, d.f. = 1, $P = 0.62$), environment ($\chi^2 = 2.20$, d.f. = 1, $P = 0.14$), the morph-environment interaction ($\chi^2 = 0.01$, d.f. = 1, $P = 0.93$), or the global model ($\chi^2 = 4.06$, d.f. = 3, $P = 0.26$). Kaplan-Meier estimates were used to quantify survival of each color morph over time, which were visualized with the *survminer* package (Kassambara et al. 2021). We also used log rank tests for pairwise comparisons of survival between morphs within each environment. Finally, to test whether the results were sensitive to our classification of mortalities, we refit the Cox proportional hazards model and log rank tests classifying both "confirmed and probable" and "possible" mortality categories as true mortalities.

3. Results

Cline estimation

Across the 76 sites sampled, we detected the melanic morph at 40 sites (53%) and the gray morph at 64 sites (84%) on either point counts or camera imagery. We counted 237 squirrels across the 76 sites during 258 visual surveys, including 69 melanic morphs (0-8 per survey) and 168 gray morphs (range = 0-7 per survey). Among the 49 sites with cameras, the melanic morph was detected at 35 sites (71%), and the gray morph was detected at all sites (100%). The median number of days detected on camera at each site was 23 for the melanic morph (range: 0-321) and 111 for the gray morph (range: 5-351).

The proportion of melanic squirrels was negatively related to distance from the city center, declining from 48% at the city center to <5% in rural woodlands (Fig. 3; electronic supplementary material, Table S3). Total squirrel abundance was also negatively related to distance from city center (Fig. 4; electronic supplementary material, Table S3), but abundance of

346 the melanic morph declined at a rate faster than abundance of the gray morph with increasing
347 distance from the city center (Fig. 4). Detection probability of both morphs peaked at
348 intermediate daily mean temperature, and detection probability was consistently greater for the
349 gray than melanic morph (electronic supplementary material, Fig. S2). All model parameters
350 converged (R-hat < 1.01), with results virtually identical when fitting the model with a subset of
351 data when population closure was more likely met (electronic supplementary material, Table S4,
352 Figs. S3-S5).
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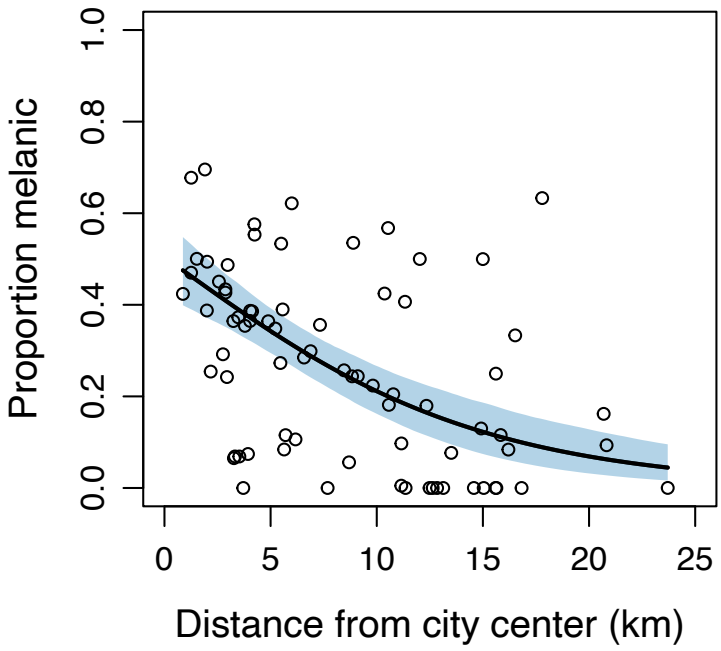

354

355 **Figure 3.** Relationship between the proportion of melanic eastern gray squirrels (*Sciurus*
356 *carolinensis*) and distance to city center for Syracuse, New York based on a hierarchical model
357 integrating camera trap and visual count observations of squirrels across n = 76 sites (Fig. 2).
358 Solid line represents the predicted proportion of melanic squirrels from the model, and shaded
359 area represents a 95% credible interval. Open circles represent estimates of the proportion of
360 squirrels melanic at each site.

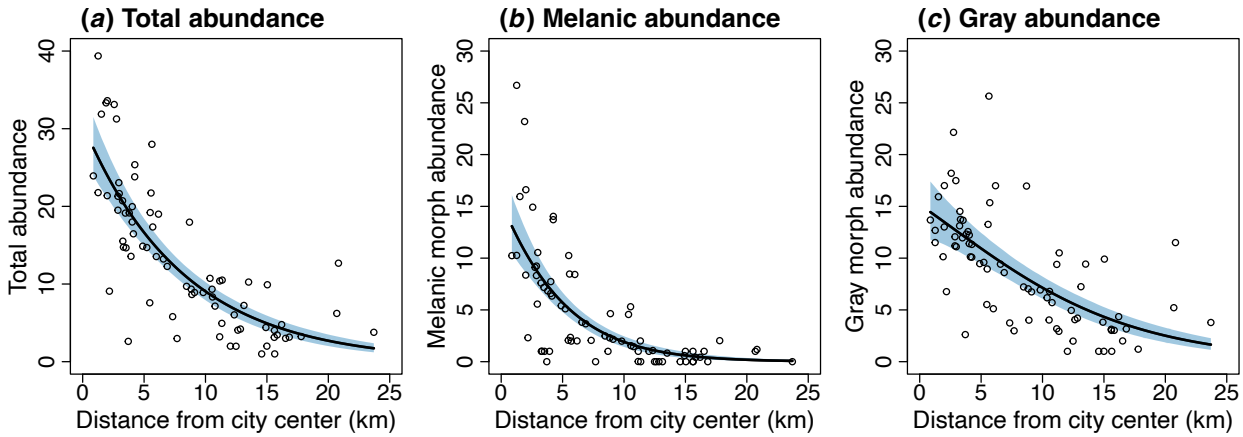

Figure 4. Relationship between the abundance of eastern gray squirrels (*Sciurus carolinensis*) and the distance to city center for Syracuse, New York, in total (*a*) and for each color morph (*b*-*c*), based on a hierarchical model integrating camera trap and visual count observations of squirrels across n = 76 sites (Fig. 2). Solid lines represent predicted abundance from the model, and shaded areas represent 95% credible intervals. Open circles represent estimates of abundance at each site.

Survival estimation

Of the 76 squirrels collared and translocated, we documented 16 confirmed and probable mortalities by the end of the tracking period (Table 1). An additional 36 squirrels with mortality signals were classified as possible mortalities. Nineteen squirrels went missing during the study, and five squirrels were still active at the end of the study. The Cox proportional hazards model revealed an interaction effect between morph and environment on the mortality hazard ratio (Likelihood ratio test: $\chi^2 = 5.70$, d.f. = 1, $P = 0.017$). The model with the interaction term indicated significant hazard ratios for morph, environment, and the morph*environment interaction (Table 2). The melanic morph had eight times the mortality risk as the gray morph in the rural environment (hazard ratio for gray relative melanic in rural areas = 0.12, 95% CI: 0.03 –

379 0.60), whereas there was no difference in the odds of mortality between melanic and gray
380 morphs in the urban environment (electronic supplementary material, Fig. S6). Post-hoc log rank
381 tests showed survival was lower for the melanic than gray morph in the rural environment (Fig.
382 5; $\chi^2 = 7.2$, d.f. = 1, $P = 0.007$), whereas there was no difference in survival between color
383 morphs in the urban environment (Fig. 5; log rank test: $\chi^2 = 0.4$, d.f. = 1, $P = 0.50$).
384 Results of the survival analysis were largely the same when including both the 16
385 confirmed and probable mortalities and 36 possible mortalities as true mortalities in the analysis.
386 The Cox proportional hazard model showed an interaction effect between morph and
387 environment (Likelihood ratio test: $\chi^2 = 3.84$, d.f. = 1, $P = 0.05$), and there was an additional
388 effect of collar size on the mortality hazard ratio (electronic supplementary material, Table S5).
389 Squirrels with the large collar had a greater hazard than squirrels with the small collar (electronic
390 supplementary material, Fig. S7), but the interpretation of the morph*environment interaction
391 remained the same. Survival was lower for the melanic than gray morph in the rural environment
392 (electronic supplementary material, Fig. S8; log rank test: $\chi^2 = 4.2$, d.f. = 1, $P = 0.04$), whereas
393 there was no difference in survival between melanic and gray morphs in the urban environment
394 (log rank test: $\chi^2 = 0.2$, d.f. = 1, $P = 0.60$)
395
396 **Table 2.** Parameter estimates for Cox proportional hazards regression model for effects of color
397 morph (melanic, gray) and environment (rural, urban) on daily survival of translocated eastern
398 gray squirrels (*Sciurus carolinensis*). Reference categories were set as melanic for color morph
399 and rural for environment.

Explanatory variable	Log hazard ratio	SE	*z*	*P*
Color morph	-2.09	0.80	-2.61	0.009
Environment	-2.12	0.80	-2.64	0.008
Color morph * Environment	2.65	1.18	2.24	0.025

400

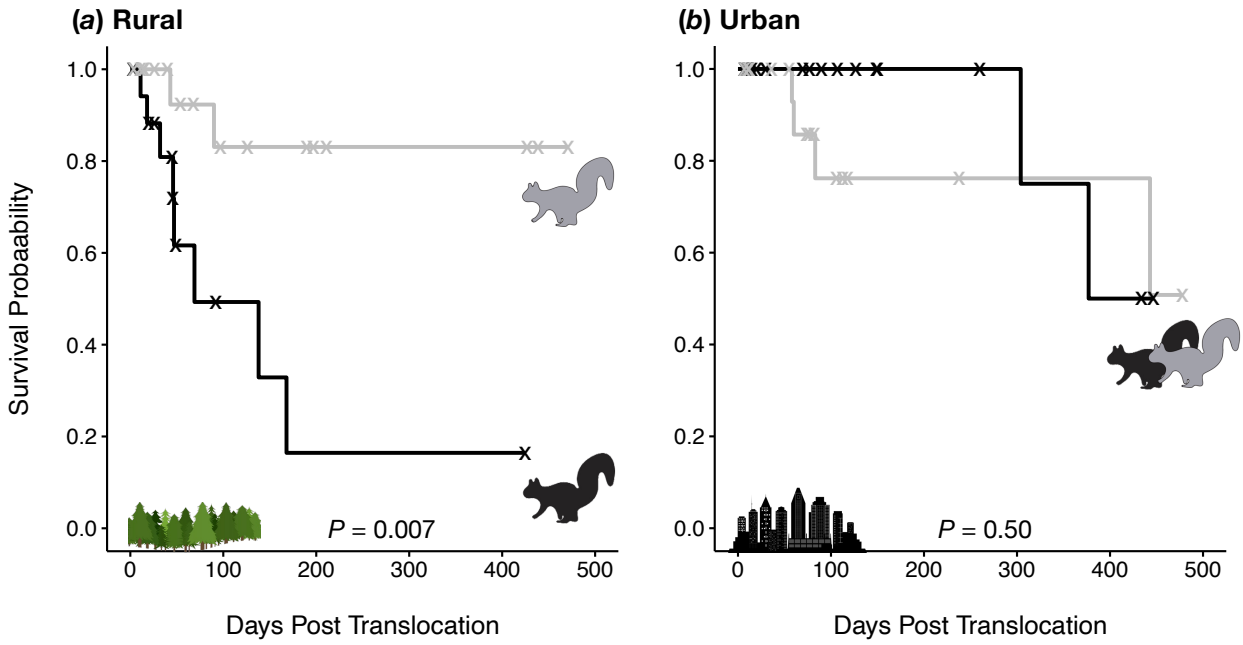

Figure 5. Survival curves for gray (solid gray line) and melanic (solid black line) color morphs of eastern gray squirrels (*Sciurus carolinensis*) translocated to rural (*a*) or urban (*b*) environments (see Table 1 for sample sizes). Each step down represents a confirmed or probable mortality (Table 1), and "X"s represent possible, missing, and active squirrels that were right-censored for the analysis. *P*-values indicate the effect of morph on survival within each environment based on log rank tests.

4. Discussion

Urbanization is hypothesized to foster adaptation to features novel to cities, but empirical studies differentiating between adaptive and non-adaptive drivers of urban evolution have been limited (Lambert et al. 2021). Our work suggests a combination of adaptive and non-adaptive processes can generate urban-rural clines in phenotypic traits. We found evidence of a strong urban-rural cline in melanism in *S. carolinensis* in our study area, with melanism increasing tenfold from the rural areas to the city center. Our translocation experiment provides insight into the evolutionary

416 mechanisms generating this cline. We found evidence of natural selection on coat color in the
417 rural environment, where squirrel survival was significantly greater for the gray than melanic
418 morph. In contrast, there was no evidence of selection on coat color in the urban environment,
419 where squirrel survival was similar between color morphs. These results suggest that adaptive
420 mechanisms of urban evolution do not necessarily result from urban adaptation *per se* (i.e.,
421 adaptation in the city proper), and instead that urban-rural clines in heritable traits can emerge
422 from selection in the rural environment alone.

423 Our finding of an urban-rural cline in melanism is consistent with previous studies on
424 coat color in *S. carolinensis* (Gibbs et al. 2019, Lehtinen et al. 2020, Cosentino and Gibbs 2022)
425 and more broadly with the body of evidence showing phenotypic divergence between urban and
426 rural populations (McDonnell and Hahs 2015, Alberti et al. 2017). The cline unveiled here was
427 significantly stronger than the cline predicted for Syracuse from community science observations
428 (Cosentino and Gibbs 2022), underscoring the importance of conducting standardized surveys
429 (documenting both presence and absence data) and estimating the relative abundance of trait
430 variants with a model that accounts for imperfect detection probability. Notably we found that
431 detection probability during surveys peaked at moderate temperatures typical of spring and fall
432 seasons (electronic supplementary material, Figs. S2), but with detection probability greater for
433 the gray than melanic morph at all but the coldest temperatures (electronic supplementary
434 material, Fig. S2). The melanic morph is generally more visible to human observers than the gray
435 morph (Gibbs et al. 2019, Bryan 2020), so the difference in detection probability between
436 morphs is not likely explained by differences in crypsis. It is possible that melanic *S.*
437 *carolinensis* individuals have lower activity levels than gray individuals, effectively decreasing
438 the availability of melanics to be observed during surveys. Eumelanin production via the

439 melanocortin system is often correlated with a range of physiological and behavioral traits
440 (Roulin and Ducrest 2011), so further evaluation of behavioral differences between color morphs
441 in *S. carolinensis* is needed. Regardless of the mechanisms explaining the difference in detection
442 probability between morphs, our findings highlight the importance of considering intraspecific
443 variation in detection probability when fitting hierarchical models of abundance.

444 Cities are commonly thought to be engines of evolution by creating novel ecological
445 conditions that cause positive selection (Shochat et al. 2006). Indeed, urban evolution studies
446 with strong evidence of adaptive trait variation (*sensu* Lambert et al. 2021) have all found novel
447 selection pressures in the city. For example, Atlantic killifish (*Fundulus heteroclitus*) evolved
448 tolerance to lethal pollutants in urban estuaries (Nacci et al. 2010), and urban water fleas
449 (*Daphnia magna*) evolved a greater critical thermal maximum under urban heat stress (Brans et
450 al. 2017). Fitness tradeoffs have also been documented between urban and rural populations. In
451 the textbook case of peppered moths (*Biston betularia*), selection favored the dark morph in
452 polluted cities with dark tree backgrounds and the light morph in unpolluted rural areas with
453 light tree backgrounds (Cook 2003, Cook et al. 2012). Similarly, urban acorn ants (*Temnothorax*
454 *curvispinosus*) evolved greater heat tolerance than rural ants in the warm urban environment,
455 whereas urban ants had lower cold tolerance than rural ants in the cool rural environment (Martin
456 et al. 2019). In this study, melanic and gray morphs of *S. carolinensis* experienced similar levels
457 of mortality when translocated within the urban environment, suggesting that melanism has not
458 adaptively evolved in response to novel selection pressures in the city. Instead, urban-rural clines
459 in melanism appear to emerge primarily from selection for the gray morph in rural populations,
460 as gray survival was substantially greater than melanic survival among individuals translocated
461 to rural woodlands. Our results suggest that selection in rural areas is relaxed in the city, and the

462 dominant evolutionary process controlling coat color in city populations is likely genetic drift.

463 Our findings can help explain why the prevalence of melanism often varies substantially
464 between nearby cities. Cosentino and Gibbs (2022) found that urban-rural clines in melanism in
465 *S. carolinensis* tend to evolve repeatedly among cities, but they are not universal. Urban-rural
466 clines in melanism are rare in regions with warm winters, where the prevalence of melanism is
467 likely limited due to thermal selection (Ducharme et al. 1989, Cosentino and Gibbs 2022). But
468 even within regions with colder winter temperatures where the melanic morph is regionally
469 common, the prevalence of melanism can vary widely among cities. For example, Lehtinen et al.
470 (2020) found the prevalence of melanism ranged 0-96% among cities in Ohio, and we found
471 melanism ranged 0-50% among cities in upstate New York (Cosentino and Gibbs 2022; B.J.
472 Cosentino, unpublished data). Dramatic variation in the frequency of melanism among nearby
473 cities may be the result of differential introduction history and consequent founder effects, as
474 melanic squirrels have commonly been introduced to cities as novelty (Benson 2013). Limited
475 evidence about temporal variation in melanism in cities also suggests drift is the primary driver
476 of color morph frequencies within the urban core. For example, Lehtinen et al. (2020) found no
477 consistent change in the prevalence of melanism over eight years of monitoring in Wooster,
478 Ohio, consistent with the stochastic effects of drift on morph frequencies (although it is possible
479 for equilibrium conditions to emerge from a balance of selection with gene flow or mutation).
480 More studies are needed to examine the mechanisms driving variation in cline strength among
481 cities. Moreover, although we documented a strong spatial cline in melanism along the
482 urbanization gradient in Syracuse, there is considerable variation in the prevalence of melanism
483 within any segment of the urban-rural gradient (Fig. 3). Landscape heterogeneity within both
484 urban and rural areas could cause microgeographic variation in the strength of selection, drift,

485 and gene flow. Mechanistically linking fine-scale trait variation to landscape heterogeneity
486 should be a priority for future urban evolution research.

487 Relaxed selection can result from environmental change weakening a source of selection
488 on trait variation, resulting in either a new vector of selective costs and benefits or neutral
489 evolution of the trait variation in question (Lahti et al. 2009). Environmental change in cities has
490 been hypothesized to cause relaxed selection in several ways, such as relaxation of food and
491 water limitation (Shochat et al. 2006). For example, Rodewald et al. (2011) described a putative
492 case of relaxed selection in northern cardinals (*Cardinalis cardinalis*), where food subsidies in
493 urban areas break a positive relationship between plumage color and individual condition found
494 in rural areas where food is limited. Although our study does not provide insight into the
495 ecological causes of selection, one hypothesis is that selection via predation and hunting favoring
496 the gray morph in rural woodlands is relaxed in cities. *Sciurus carolinensis* has a variety of avian
497 and mammalian predators and have been hunted by people for centuries (Benson 2013). Our
498 previous work showed the gray morph is more cryptic against tree backgrounds than the melanic
499 morph in regrown, secondary forests that dominate the landscape in rural New York (Gibbs et al.
500 2019, Bryan 2020), potentially leading to lower levels of predation on the gray morph. Selection
501 via predation is likely weaker in cities than rural secondary forests. Hunting is nearly universally
502 banned in cities, and predation rates are often lower in cities than rural areas as food subsidies
503 (e.g., bird seed, pet food, garbage) redirect foraging of predators away from some prey (Fischer
504 et al. 2012). Multiple lines of evidence suggest predation rates on squirrels are lower in urban
505 than rural areas. For example, experiments on giving-up-densities and food supplementation
506 have indicated that rural *S. carolinensis* populations are predator-limited, whereas urban
507 populations are food-limited (Havera and Nixon 1980, Bowers and Breland 1996). Moreover,

508 flight initiation distances from an approaching predator are lowest where human exposure is
509 greatest (Engelhardt and Weladii 2011), and a study of eastern fox squirrel (*S. niger*) mortality
510 showed >60% of fatalities were caused by predation at a rural site compared to <5% at an urban
511 site (McCleery et al. 2008). Ultimately experimental study of attack rates on color morphs of *S.*
512 *carolinensis* in urban and rural areas is needed to clarify whether predation is a cause of selection
513 on coat color in rural populations (i.e., greater attack rates on the melanic than gray morph) and
514 to test whether selection via predation is relaxed in cities. Given the correlation of melanism with
515 other physiological and behavioral traits (Roulin and Ducrest 2011), future studies should also
516 clarify the degree to which selection on melanism is direct (e.g., crypsis) versus indirect via
517 correlations with other traits or early life conditions.

518 Similar survival between color morphs in the urban environment was counter to our
519 prediction. The melanic morph is more visible to humans than the gray morph on roads (Bryan
520 2020), and community science data suggested the melanic morph was underrepresented among
521 road killed squirrels in our study area (Gibbs et al. 2019), so we expected selection for the
522 melanic morph in the city. The gray morph may be more susceptible to vehicular collisions
523 without this source of mortality contributing to the urban-rural cline in melanism. For example,
524 fitness costs of road mortality for the gray morph in urban areas (where traffic volume is
525 greatest) could be balanced by other sources of fitness costs for the melanic morph (e.g., greater
526 visibility to some urban predators than the gray morph), leading to no net difference in fitness
527 between morphs in city populations.

528 Our translocation experiment was limited in multiple ways. First, because all squirrels in
529 the survival analysis were translocated, we were unable to directly test if there was a significant
530 effect of translocation on survival (e.g., greater survival of squirrels in their home range than a

531 translocated environment). However, Koprowski et al. (2016) reports annual survival in *S.*
532 *carolinensis* is often >0.50, which is consistent with annual survival estimates for all translocated
533 squirrels in our study except the melanic morph in rural areas (Fig. 5). Second, we were rarely
534 able to confirm the cause of mortality when retrieving radiotransmitters, and therefore unable to
535 examine variation in the causes of mortality along the urbanization gradient. Nearly half of the
536 translocated squirrels (36 of 76) had ambiguous fates despite an active mortality signal. These 36
537 squirrels were recorded as "possible" mortalities and included 14 cases where the
538 radiotransmitter was unretrievable (e.g., at the top of a tree). Of the 22 possible mortalities where
539 we retrieved the radiotransmitter, we found no evidence of physical damage. Some of these
540 possible mortalities were likely due to slipped collars and did not represent true mortalities,
541 which is why we refrained from including possible mortalities in our main survival analysis.
542 When we conducted the survival analysis with these possible mortalities classified as true
543 mortalities, the hazard of mortality was greater for squirrels with large than small collars,
544 supporting the idea that possible mortalities are sometimes slipped collars (more likely for large
545 collars). Nonetheless, we found a significant morph*environment interaction and the same
546 pattern of differential survival between morphs in each environment regardless of whether
547 possible mortalities were included as true mortalities in the survival analysis. Third, our study
548 focused solely on the vital rate survival as a component of fitness. We lack data on differential
549 reproductive success between morphs, although our focus on adult survival was important given
550 that the best short-term proxies of lifetime fitness are those measured at the recruitment stage
551 (Alif et al. 2022).

552 In conclusion, our study highlights how the effects of urbanization on trait variation can
553 reach beyond the city boundaries, driving adaptive evolution in nonurban areas. The rise of cities

554 was accompanied by conversion of adjacent rural lands – often distant from urban centers – to
555 agriculture and other land uses for resource extraction to support urban populations (Cronon
556 1991). European settlements dramatically reshaped the landscape in the northeastern United
557 States, converting extensive old growth forests to secondary forests that have regrown over the
558 last 200 years (Foster et al. 2010, Thompson et al. 2013). It was this period of time during which
559 melanism in *S. carolinensis* began to decline in rural forests (e.g., Allen 1943, Schorger 1949,
560 Robertson 1973), reinforcing the idea that "urban evolution" not only encompasses the effects of
561 contemporary selective regimes on trait variation in urban centers, but also the historical
562 evolution of trait variation associated with landscape change beyond the city boundaries. Our
563 study underscores the importance of comparing fitness between trait variants in both urban and
564 rural populations to provide insight into the spatial context of adaptive and nonadaptive
565 evolutionary processes along urbanization gradients.
566

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573

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