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 <10% in rural woodlands. Among 76 translocated squirrels, survival was lower for the melanic than gray morph in rural woodlands but equivalent 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. Moreover, our work demonstrates the importance of linking trait variation to fitness in mechanistic studies of urban evolution.

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, while phenotypic differences between urban and rural populations are well-documented for species that persist in cities (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 their 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 indeed 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, demonstrating urban adaptation requires showing that heritable trait variation leads

70 to differences in fitness among individuals. A recent review found that despite a proliferation of
71 case studies of urban-rural trait variation, only six cases conclusively showed evidence of
72 adaptation, mainly because the fitness consequences of trait variation are rarely examined
73 (Lambert et al. 2021).

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

91 Our goal was to better understand the spatial context of adaptive and non-adaptive
92 mechanisms of urban evolution. We used coat color in eastern gray squirrels (*Sciurus*

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 are rare in rural forests and more common in urban areas, with urban-rural clines in melanism apparent across multiple cities (Cosentino and Gibbs 2022). These urban-rural clines are most evident in cities with relatively cold winter temperatures, where the deletion allele for melanism is likely maintained by selection for greater thermogenic capacity at cold temperature (Ducharme et al. 1989).

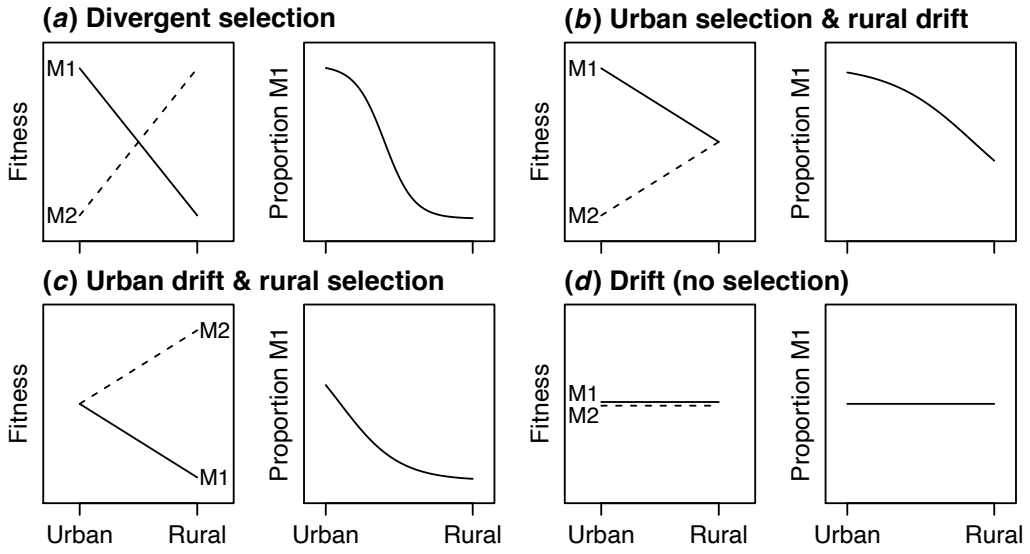


Figure 1. Four hypotheses (*a*-*d*) for spatial variation in the effects of selection and drift on a heritable, discrete polymorphism along urbanization gradients. Predicted relative fitness for two morphs (M1 and M2) in urban and rural environments and mean expectation for clinal variation in the proportion 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 incidentally encountered squirrels 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 other 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, the predominant mortality source for urban squirrels (McCleery et al. 2008). Thus, we predicted survival would be greater for the gray than melanic morph in rural forests, whereas survival would be greater for the melanic than gray morph in the urban environment.

2. Methods

Study area

Syracuse (43.0469° N, -76.1444° 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% and ranges from 4.5% at the city core to 47% at the southern extent (Nowak et al. 2001). The rural areas outside the city consist of a mosaic of forest and agriculture (Fig. 2).

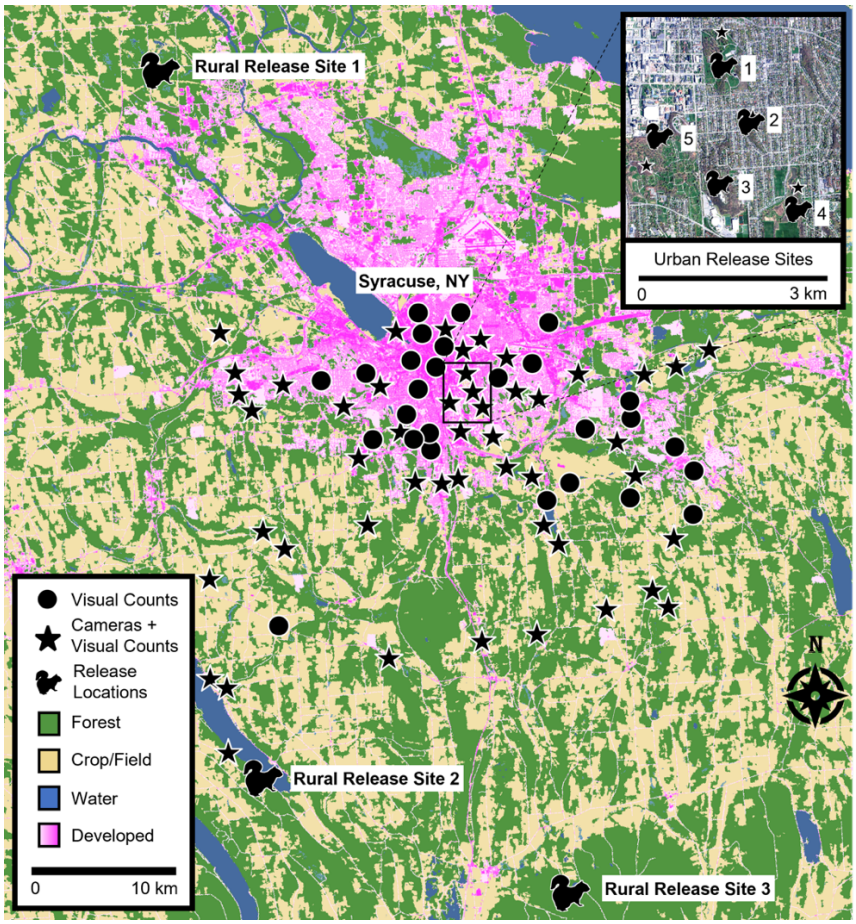


Figure 2. Land cover map of Syracuse, New York and surrounding rural areas. Sites with camera traps and visual counts (stars; n = 49) of eastern gray squirrels (*Sciurus carolinensis*) and sites with visual counts only (circles, n = 27) were distributed across the urbanization gradient. Rural release 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).

Camera trapping and visual surveys

We estimated the relative abundance of each *S. carolinensis* color morph at 76 sites along a gradient of urbanization (Fig. 2). Squirrel occurrence is limited by tree cover, so we focused surveys on areas within urban greenspaces and rural woodlots with mature deciduous trees (Allen 1982, Parker and Nilon 2008). Sites surveyed were constrained to a sampling area that extended from the city center to rural woodlands 26 km south of the city. Site locations were chosen to represent a gradient in distance from the city center (range = 0.9–26.1 km, median = 8.95 km) with sites separated by at least 1 km.

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

We conducted visual surveys for squirrels at all camera sites and an additional 27 sites without cameras between 12 October 2021 and 18 October 2022 (n = 258 visual surveys). These additional sites were included to fill sampling gaps where permanent camera traps could not be placed. During each survey, a single observer recorded all visible squirrels of each color morph over a 3-min period. Each site was surveyed visually 1-7 times during the study (median = 4). 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 $P^*_{ijk} = 1 - (1 - p_{ijk})^{Nik}$. Individual detection probability was modeled as a function of survey day ordered 0-397 (corresponding to the survey period 0 = 16 September 2021 to 397 = 18 October 2022): $\text{logit}(p_{\text{ijk}}) = a0_k + a1_k*\text{date}_{ij} + a2_k*\text{date}^2_{ij} + a3_k*\text{date}^3_{ij} + a4_k*\text{date}^4_{ij}$, where for each color morph k, $a0_k$ represents the intercept, and $a1$-$a4_k$ represent a fourth-order polynomial effect of survey date. We included a fourth-order polynomial function because we expected peaks in squirrel activity in fall and spring (see Spritzer 2002). Regression coefficients for detection probability were estimated with a joint likelihood, with the coefficients assumed to be identical

between the count and detection/nondetection datasets. This integrated model formulation allowed us to combine count and detection/nondetection data in a single model, which can improve accuracy and precision of parameter estimates (Kéry and Royle 2021).

We included two submodels to describe the ecological state process. First, we used a Poisson distribution to describe the latent total abundance of squirrels: $N_total_i \sim Poisson(\lambda_i)$, where λ_i is the expected total abundance of squirrels at location i. We expected total squirrel abundance to be greatest in the city (Merrick et al. 2016), so we modeled total abundance as a function of distance to city center: $\log(\lambda_i) = \beta 0 + \beta 1 * \text{distance}_i$, where $\beta 0$ represents the intercept and $\beta 1$ represents the effect of distance to city center. We defined the city center as the centroid of the civil boundaries of the city of Syracuse (New York State Office of Information Technology Services GIS Program Office 2022b). Second, we used a binomial distribution to describe the latent abundance of the melanic morph: $N_{i1} \sim Binomial(N_total_i, p_melanic_i)$. To estimate the cline in melanism, we modeled the proportion of melanic squirrels as a function of distance to city center: $\text{logit}(p_melanic_i) = \beta 0 + \beta 1 * \text{distance}_i$, where $\beta 0$ represents the intercept and $\beta 1$ represents the effect of distance to city center. The latent abundance of the gray morph was derived as $N_{i2} = N_total_i - N_{i1}$.

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

214 confirmed convergence of parameter estimates with the Gelman-Rubin statistic (R-hat < 1.1;
215 Gelman and Hill 2007).
216 Our model of abundance assumes closure to births, deaths, immigration, and emigration
217 within sites during the survey period. This assumption is unlikely to be met because *S.*
218 *carolinensis* has an extended reproductive period, and juveniles can be found throughout the year
219 (Kirkpatrick and Hoffman 1960; Steele and Koprowski 2003). When closure is not met, the
220 abundance parameter (λ_i) should be interpreted to represent the number of squirrels associated
221 with the site during the survey period rather than the number of permanent residents at the site
222 (Kéry and Royle 2021). To evaluate whether our results were sensitive to the closure assumption,
223 we fit our model with a subset of data during a period when closure was more likely met (16
224 September 2021 - 15 March 2022). We used the same parameterization of the model, except we
225 fit a quadratic effect of survey date on detection probability rather than a fourth-order term. We
226 expected a quadratic effect in the subset dataset due to peaks in squirrel activity in Autumn 2021
227 and Spring 2022.
228
229 *Translocation experiment*
230 To compare survival between color morphs in urban and rural environments, we conducted a
231 translocation experiment and tracked squirrel survival via radiotelemetry. Although a reciprocal
232 transplant is preferred to test for local adaptation (Lambert et al. 2021), the rarity of melanic
233 squirrels in rural areas precluded capture of rural melanics for translocation to urban areas. To
234 test for phenotypic selection, we translocated squirrels of both color morphs from urban
235 greenspaces in Syracuse to both urban and rural release sites (Fig. 2; electronic supplementary
236 material, Table S1). Three rural release locations were 22-30 km from trapping locations and

237 composed of deciduous and mixed deciduous forest. Five urban release locations were 0.7-6 km
238 from trapping locations within Syracuse and consisted of a cluster of urban greenspaces with
239 mixed deciduous forest. All urban and rural release locations had known populations of *S.*
240 *carolinensis*.

241 We captured and collared 76 squirrels in urban greenspaces between June 2021 and May
242 2022 using wire cage traps (Tomahawk Live Trap Co., Tomahawk, WI; Model 103) baited with
243 peanut butter. Upon capture we coaxed squirrels into a cloth handling cone for processing and
244 radio-collar attachment (Koprowski 2002). We anesthetized squirrels using isoflurane delivered
245 via a plastic nose cone to minimize squirrel stress and pain response and to maximize human
246 safety (Parker et al. 2008). We recorded squirrel mass, color morph, sex, and reproductive status.
247 We collected a 1x3-mm sliver of ear tissue for future genetic analyses and attached a metal ear
248 tag (National Band Company, Newport KY; Style 1005-3) with a unique numeric code to the
249 other ear. We attached VHF ("very high frequency") radiotransmitters to squirrels via neck
250 collars (Holohil Systems Ltd. Carp, ON; Model RI-2D 14 or 21g; with mortality switch option).
251 We epoxied a metal temperature logger (Maxim Integrated DS1925L-F5, 3.49 g) to the end of
252 collars to record temperature data. Weight of the radiotransmitter, collar, and temperature logger
253 was <5% of the squirrel's mass (mean = 2.7%, range = 2-4%). Lactating females were excluded
254 from the sample.

255 Following radio-collar attachment and recovery from isoflurane, we transported squirrels
256 to selected release sites by vehicle. To control for impacts of travel time, we drove all
257 translocated squirrels for a similar amount of time before release (approximately 30 min).
258 Squirrels were released at locations with ample shelter (e.g. snags, cavities) and food resources
259 (mast producing trees). Release sites for each squirrel were selected systematically to maintain a

260 balanced release of morph types across sites. In total, 18-20 squirrels of each morph were
261 translocated to urban and rural environments (Table 1).
262
263 **Table 1.** Number of translocated eastern gray squirrels (*Sciurus carolinensis*) and their fates by
264 environment and morph. "Confirmed & probable" = Confirmed and probable mortalities;
265 "Possible" = Possible mortalities and collars that were transmitting a mortality signal, but we
266 were unable to be locate; "Missing" = squirrels that we could no longer find using
267 radiotelemetry; "Active" = squirrels with active collars at the end of the study (October 2022).

Environment	Morph	Translocated	Confirmed & probable	Possible	Missing	Active
Rural	Melanic	18	8	8	2	0
	Gray	20	2	12	5	1
	Rural total	38	10	20	7	1
Urban	Melanic	18	2	10	4	2
	Gray	20	4	6	8	2
	Urban total	38	6	16	12	4
	Grand total	76	16	36	19	5

268
269 After release, squirrel survival was monitored weekly via radiotelemetry (Haughland et
270 al. 2008; Millspaugh et al. 2012) until October 2022. At the end of the study, squirrel status was
271 classified into one of five categories: 1) confirmed mortality, 2) probable mortality, 3) possible
272 mortality, 4) missing, or 5) active (Table 1). When we detected a transmitter emitting a mortality
273 signal, we attempted to locate the squirrel as soon as possible to determine the cause.
274 Transmitters emitting a mortality signal were assigned to the confirmed, probable, or possible
275 mortality categories. Confirmed mortalities were based on the presence of a carcass or significant
276 evidence of a mortality event (e.g. large amounts of squirrel fur, bite marks on collar assembly).
277 When possible, we tried to ascertain the cause of mortality (McCleery et al. 2008). Probable
278 mortalities were based on transmitters without a carcass but noticeable bite marks on the collar

tubing, wire, or transmitter epoxy (Adams et al. 2004). Possible mortalities were based on the presence of a transmitter with a mortality signal but without a carcass or other evidence of a depredation event. Transmitters in this condition could be the result of a squirrel mortality, but more likely represent collar slippage or failure. Squirrels with transmitters broadcasting from a consistently immobile position but that we were unable to retrieve (e.g., emanating from a treetop) were classified as possible mortalities.

Statistical analysis for survival estimation

We used a Cox proportional hazards model (Cox 1972; Pollock et al. 1989) to relate daily survival of translocated squirrels to color morph (gray, melanic) and translocation environment (urban, rural). Date of translocation was used as the time of entry for each squirrel to maximize the number of squirrels at risk during a given time (Karp and Gehr 2020). We used the inferred date of mortality as the exit date in the model for squirrels classified as confirmed or probable mortalities. Date of mortality was estimated as the median date between the last active signal and the first time a mortality signal was detected. For collars recovered with functioning temperature loggers (n = 21), we determined the date of mortality using a graph of the recorded temperature data. Squirrels not classified as mortalities contributed to the survival analysis via right censoring, as each had a known minimum number of days survived (Murray 2006).

We modeled the hazard ratio as a function of morph, environment, and the interaction between morph and environment using the R package *survival* (Therneau 2021). A likelihood ratio test was used to examine the significance of the interaction term, with two-sided Wald tests reported for each term in the most-supported model. We considered a model including sex and collar size as additional covariates, but we excluded these variables from the final model because

302 a) neither variable predicted the hazard ratio (sex: $z = 0.14$, $P = 0.89$; collar size: $z = 0.64$, P
303 $=0.52$), b) data on sex was missing for two squirrels, which precluded use of those observations,
304 and c) the effects of morph, environment, and their interaction were consistent with or without
305 the additional covariates in the model. No deviations from the assumption of proportional
306 hazards were evident through analysis of the Schoenfeld residuals for morph ($\chi^2 = 0.24$, d.f. = 1,
307 $P = 0.62$), environment ($\chi^2 = 2.20$, d.f. = 1, $P = 0.14$), the morph-environment interaction ($\chi^2 =$
308 0.01, d.f. = 1, $P = 0.93$), or the global model ($\chi^2 = 4.06$, d.f. = 3, $P = 0.26$). Kaplan-Meier
309 estimates were used to quantify survival of each color morph over time, which were visualized
310 with the *survminer* package (Kassambara et al. 2021). We also used log rank tests for pairwise
311 comparisons of survival between morphs within each environment. Finally, to test whether the
312 results were sensitive to our classification of mortalities, we refit the Cox proportional hazards
313 model and log rank tests using confirmed, probable, and possible mortalities as true mortalities.
314

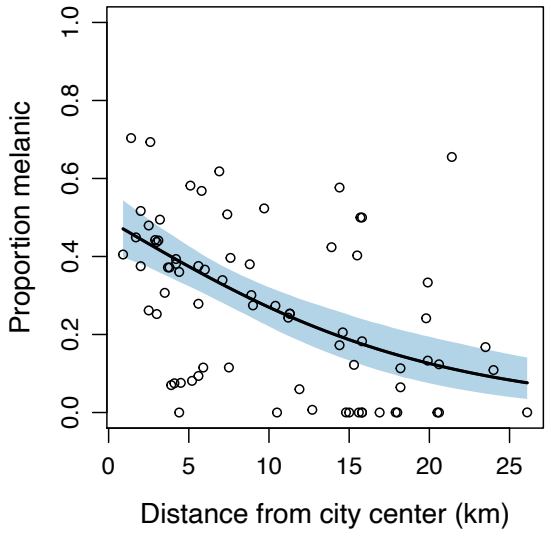
315 **3. Results**

316 *Cline estimation*

317 Across the 76 sites sampled, we detected the melanic morph at 40 sites (53%) and the gray
318 morph at 64 sites (84%) on either point counts or camera imagery. We counted 237 squirrels
319 across the 76 sites during 258 visual surveys, including 69 melanic morphs (0-8 per survey) and
320 168 gray morphs (range = 0-7 per survey). Among the 49 sites with cameras, the melanic morph
321 was detected at 35 sites (71%), and the gray morph was detected at all sites (100%). The median
322 number of days detected on camera at each site was 23 for the melanic morph (range: 0-321) and
323 111 for the gray morph (range: 5-351).

324 The proportion of melanic squirrels was negatively related to distance from the city

325 center, declining from 48% at the city center to <10% in rural woodlands (Fig. 3; electronic
326 supplementary material, Table S2). Total squirrel abundance was also negatively related to
327 distance from city center (Fig. 4; electronic supplementary material, Table S2), but the
328 abundance of the melanic morph declined at a rate faster than the abundance of the gray morph
329 with increasing distance from the city center (Fig. 4). Detection probability of both morphs
330 peaked in September-November 2021 and 2022, and detection probability was consistently
331 greater for the gray than melanic morph (electronic supplementary material, Fig. S1). All model
332 parameters converged (R-hat < 1.03), with results virtually identical when fitting the model with
333 a subset of data when population closure was more likely met (electronic supplementary
334 material, Table S3, Figs. S2-S4).



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

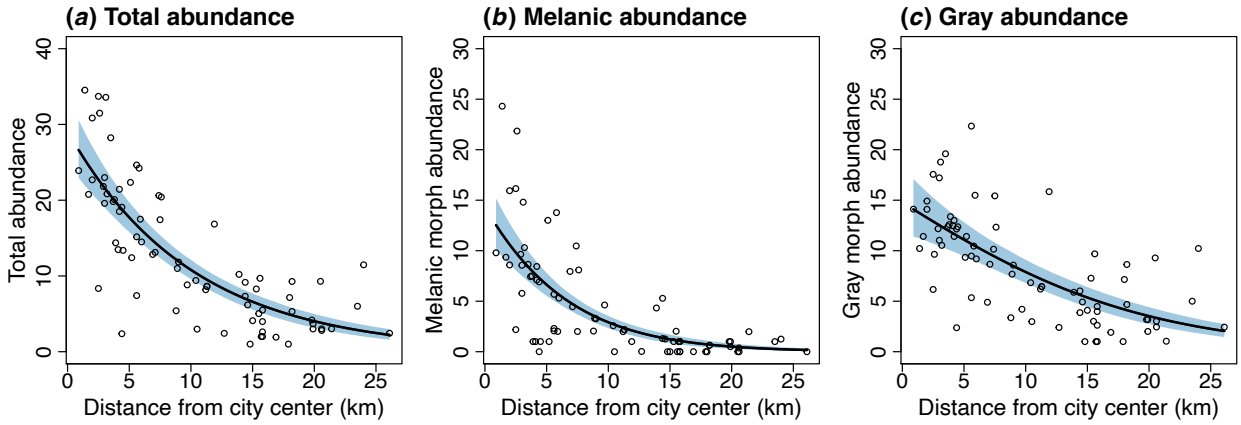

Figure 4. Relationship between the abundance of eastern gray squirrels (*Sciurus carolinensis*) and the distance to city center for Syracuse, New York, in total (*a*) and for each color morph (*b*-*c*), based on a hierarchical model integrating camera trap and visual count observations of squirrels across 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 or probable mortalities by the end of the tracking period, including eight rural melanics, two rural grays, two urban melanics, and four urban grays (Table 1). An additional 36 squirrels with mortality signals were classified as possible mortalities. Nineteen squirrels went missing during the study (unable to locate after the last active signal), 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 (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). Rural melanics exhibited greater hazard than rural grays, and the hazard for

360 rural melanics was greater than both morphs in the urban environment (electronic supplementary
361 material, Fig. S5). Post-hoc log rank tests showed survival was lower for melanics than grays in
362 the rural environment (Fig. 5; $\chi^2 = 7.2$, d.f. = 1, $P = 0.007$), whereas there was no difference in
363 survival between color morphs in the urban environment (Fig. 5; log rank test: $\chi^2 = 0.4$, d.f. = 1,
364 $P = 0.50$). Results of the survival analysis were largely the same when including the 36 possible
365 mortalities as true mortalities in the analysis. The Cox proportional hazard model showed an
366 interaction effect between morph and environment (Likelihood ratio test: $\chi^2 = 3.84$, d.f. = 1, $P =$
367 0.05), and there was an additional effect of collar size on the hazard of mortality (electronic
368 supplementary material, Table S4). Squirrels with the large collar had a greater hazard than
369 squirrels with the small collar (electronic supplementary material, Fig. S6), but the interpretation
370 of the morph*environment interaction remained the same. Survival was lower for the melanic
371 than gray morph in the rural environment (electronic supplementary material, Fig. S7; log rank
372 test: $\chi^2 = 4.2$, d.f. = 1, $P = 0.04$), whereas there was no difference in survival between melanics
373 and grays in the urban environment (log rank test: $\chi^2 = 0.2$, d.f. = 1, $P = 0.60$)
374
375 **Table 2.** Parameter estimates for Cox proportional hazards regression model for effects of color
376 morph (melanic, gray) and environment (rural, urban) on daily survival of translocated eastern
377 gray squirrels (*Sciurus carolinensis*). Reference categories were set as melanic for color morph
378 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

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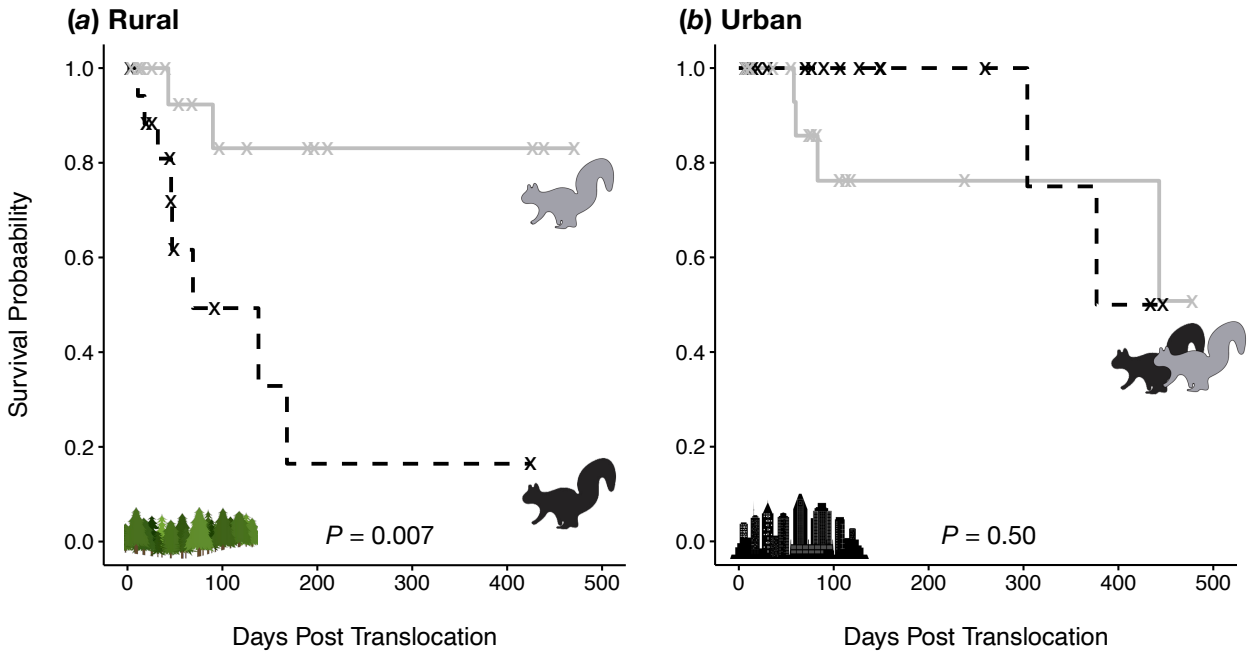

Figure 5. Survival curves for gray (solid gray line) and melanic (dotted black line) color morphs of eastern gray squirrels (*Sciurus carolinensis*) translocated to rural (*a*) or urban (*b*) environments. Steps down represent confirmed or probable mortalities, and "X"s represent possible, missing, and active squirrels that were right-censored for the analysis. *P*-values are shown for the effect of morph 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 fivefold from the rural extremes to the city center. Our translocation experiment provides insight into the evolutionary mechanisms generating this cline. We found evidence of natural selection on coat

397 color in the rural environment, where squirrel survival was significantly greater for the gray than
398 melanic morph. In contrast, there was no evidence of selection on coat color in the urban
399 environment, where squirrel survival was similar between color morphs. These results suggest
400 that adaptive mechanisms of urban evolution do not necessarily result from urban adaptation *per*
401 *se* (i.e., adaptation in the city proper), and instead that urban-rural clines in heritable traits can
402 emerge from selection in the rural environment alone.

403 Our finding of an urban-rural cline in melanism is consistent with previous studies on
404 coat color in *S. carolinensis* (Gibbs et al. 2019, Lehtinen et al. 2020, Cosentino and Gibbs 2022)
405 and more broadly with the body of evidence showing phenotypic divergence between urban and
406 rural populations (McDonnell and Hahs 2015, Alberti et al. 2017). The cline unveiled here was
407 significantly stronger than the cline predicted for Syracuse from community science observations
408 (Cosentino and Gibbs 2022), underscoring the importance of conducting standardized surveys
409 (documenting both presence and absence data) and estimating the relative abundance of trait
410 variants with a model that accounts for imperfect detection probability. Notably we found that
411 detection probability during surveys was significantly greater for the gray than melanic morph
412 throughout the study period (electronic supplementary material, Fig. S1). Melanics are generally
413 more visible to human observers (Gibbs et al. 2019, Bryan 2020), so the difference in detection
414 probability between morphs is not likely explained by differences in crypsis. It is possible that
415 melanic *S. carolinensis* individuals have lower activity levels than gray individuals, effectively
416 decreasing the availability of melanics to be observed during surveys. Eumelanin production via
417 the melanocortin system is often correlated with a range of physiological and behavioral traits
418 (Roulin and Ducrest 2011), so further evaluation of behavioral differences between color morphs
419 in *S. carolinensis* is needed.

420 Cities are commonly thought to be engines of evolution by creating novel ecological
421 conditions that cause positive selection (Shochat et al. 2006). Indeed, urban evolution studies
422 with strong evidence of adaptive trait variation (*sensu* Lambert et al. 2021) have all found novel
423 selection pressures in the city. For example, Atlantic killifish (*Fundulus heteroclitus*) evolved
424 tolerance to lethal pollutants in urban estuaries (Nacci et al. 2010), and urban water fleas
425 (*Daphnia magna*) evolved a greater critical thermal maximum under urban heat stress (Brans et
426 al. 2017). Fitness tradeoffs have also been documented between urban and rural populations. In
427 the textbook case of peppered moths (*Bison betularia*), selection favored the dark morph in
428 polluted cities with dark tree backgrounds and the light morph in unpolluted rural areas with
429 light tree backgrounds (Cook 2003, Cook et al. 2012). Similarly, urban acorn ants (*Temnothorax*
430 *curvispinosus*) evolved greater heat tolerance than rural ants in the warm urban environment,
431 whereas urban ants had lower cold tolerance than rural ants in the cool rural environment (Martin
432 et al. 2019). In this study, melanic and gray morphs of *S. carolinensis* experienced similar levels
433 of mortality when translocated within the urban environment, suggesting that melanism has not
434 adaptively evolved in response to novel selection pressures in the city. Instead, urban-rural clines
435 in melanism appear to emerge primarily from selection for the gray morph in rural populations,
436 as gray survival was substantially greater than melanic survival among individuals translocated
437 to rural woodlands. Our results suggest that selection in rural areas is relaxed in the city, and the
438 dominant evolutionary process controlling coat color in city populations is likely genetic drift.
439 This could help explain why the prevalence of melanism often varies substantially between
440 nearby cities. Lehtinen et al. (2020) found the prevalence of melanism ranged 0-96% among
441 cities in Ohio, and we found melanism ranged 0-50% among cities in upstate New York
442 (Cosentino and Gibbs 2022; B.J. Cosentino, unpublished data). Dramatic variation in the

443 frequency of melanism among nearby cities may be the result of differential introduction history
444 and consequent founder effects, as melanic squirrels have commonly been introduced to cities as
445 novelty (Benson 2013). Limited evidence about temporal variation in melanism within cities also
446 suggests drift is the primary driver of color morph frequencies. For example, Lehtinen et al.
447 (2020) found no consistent change in the prevalence of melanism over eight years of monitoring
448 in Wooster, Ohio, consistent with the stochastic effects of drift on morph frequencies (though it
449 is possible for equilibrium conditions to emerge from a balance of selection with gene flow or
450 mutation).

451 Relaxed selection can result from environmental change weakening a source of selection
452 on trait variation, resulting in either a new vector of selective costs and benefits or neutral
453 evolution of the trait variation in question (Lahti et al. 2009). Environmental change in cities has
454 been hypothesized to cause relaxed selection in several ways, such as relaxation of food and
455 water limitation (Shochat et al. 2006). For example, Rodewald et al. (2011) described a putative
456 case of relaxed selection in northern cardinals (*Cardinalis cardinalis*), where food subsidies in
457 urban areas break a positive relationship between plumage color and individual condition found
458 in rural areas where food is limited. Although our study does not provide insight into the
459 ecological causes of selection, one hypothesis is that selection via predation and hunting favoring
460 the gray morph in rural woodlands is relaxed in cities. *S. carolinensis* has a variety of avian and
461 mammalian predators and have been hunted by people for centuries (Benson 2013). Our previous
462 work showed the gray morph is more cryptic against tree backgrounds than the melanic morph in
463 regrown, secondary forests that dominate the landscape (Gibbs et al. 2019, Bryan 2020),
464 potentially leading to lower levels of predation on the gray morph. Selection via predation is
465 likely weaker in cities than rural secondary forests. Hunting is nearly universally banned in

466 cities, and predation rates are often lower in cities than rural areas as food subsidies (e.g., bird
467 seed, pet food, garbage) redirect foraging of predators away from prey (Fischer et al. 2012).
468 Multiple lines of evidence suggest predation rates on squirrels is lower in urban than rural areas.
469 For example, experiments on giving-up-densities and food supplementation have indicated that
470 rural *S. carolinensis* populations are predator-limited, whereas urban populations are food-
471 limited (Havera and Nixon 1980, Bowers and Breland 1996). Moreover, flight initiation
472 distances from an approaching predator are lowest where human exposure is greatest (Engelhardt
473 and Weladii 2011), and a study of eastern fox squirrel (*S. niger*) mortality showed >60% of
474 fatalities were caused by predation at a rural site compared to <5% at an urban site (McCleery et
475 al. 2008). Ultimately experimental study of attack rates on color morphs of *S. carolinensis* in
476 urban and rural areas is needed to clarify whether predation is a cause of selection on coat color
477 in rural populations (i.e., greater attack rates on the melanic than gray morph) and to test whether
478 selection via predation is relaxed in cities.

479 A lack of difference in survival between color morphs in the urban environment was
480 counter to our prediction. The melanic morph is more visible to humans than the gray morph on
481 roads (Bryan 2020), and community science data suggested the melanic morph was
482 underrepresented among road killed squirrels in Syracuse (Gibbs et al. 2019), so we expected
483 selection for the melanic morph in the city. The gray morph may be more susceptible to
484 vehicular collisions without this source of mortality contributing to the urban-rural cline in
485 melanism. For example, fitness costs of road mortality for the gray morph in urban areas (where
486 traffic volume is greatest) could be balanced by other sources of fitness costs for the melanic
487 morph, leading to no net difference in fitness between morphs in city populations.

488 One limitation of our translocation experiment was that we were rarely able to confirm

489 the cause of mortality when retrieving radiotransmitters, and therefore unable to examine
490 variation in the causes of mortality along the urbanization gradient. Of the 36 squirrels recorded
491 as possible mortalities, 22 were cases where we retrieved the radiotransmitter but found no
492 physical damage. Some of these "possible" mortalities were likely due to slipped collars rather
493 than true mortalities. The hazard of mortality was greater for squirrels with large collars in our
494 analysis including "possible" mortalities as true mortalities, supporting the idea that possible
495 mortalities are sometimes slipped collars (more likely for large collars). Nonetheless, we found
496 the same morph*environment interaction for survival even when including the possible category
497 as true mortalities. An additional limitation of our study is its sole focus on the vital rate survival
498 as a component of fitness. We lack data on differential reproductive success between morphs,
499 although our focus on adult survival was important given that the best short-term proxies of
500 lifetime fitness are those measured at the recruitment stage (Alif et al. 2022).

501 Our study highlights how the effects of urbanization on trait variation can reach beyond
502 the city boundaries, driving adaptive evolution in nonurban areas. The rise of cities was
503 accompanied by conversion of adjacent rural lands – often distant from urban centers – to
504 agriculture and other land uses for resource extraction to support urban populations (Cronon
505 1991). European settlements dramatically reshaped the landscape in the northeastern United
506 States, converting extensive old growth forests to secondary forests that have regrown over the
507 last 200 years (Foster et al. 2010, Thompson et al. 2013). It was this period of time during which
508 melanism in *S. carolinensis* began to decline in rural forests, reinforcing the idea that "urban
509 evolution" not only encompasses the effects of contemporary selective regimes on trait variation
510 in urban centers, but also the historical evolution of trait variation associated with landscape
511 change beyond the city boundaries. Our study underscores the importance of comparing fitness

512 between trait variants in both urban and rural populations to provide insight into the spatial
513 context of selection in urban evolution studies.
514
515 **Data accessibility**
516 Data and R code used for analyses are publicly available at GitHub
517 (https://github.com/bcosentino/squirrel-coatcolor-selection).
518
519 **Ethics**
520 All squirrels were handled with approval of the Institutional Animal Care and Use Committee at
521 Hobart and William Smith Colleges (Protocol 2020-02) and a permit from the New York State
522 Department of Environmental Conservation (License 2823). We followed ethical guidelines of
523 the American Society of Mammologists for the Use of Wild Mammals in Research (Sikes and
524 the Animal Care and Use Committee of the American Society of Mammologists 2016).
525
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532
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