1   **Parallel evolution of urban-rural clines in melanism in a widespread mammal**

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Abstract

Urbanization is the dominant trend of global land use change. The replicated nature of environmental change associated with urbanization should drive parallel evolution, yet insight into the repeatability of evolutionary processes in urban areas has been limited by a lack of multi-city studies. Here we leverage community science data on coat color in >60,000 eastern gray squirrels (*Sciurus carolinensis*) across 43 North American cities to test for parallel clines in melanism, a genetically based trait associated with thermoregulation and crypsis. We show the prevalence of melanism was positively associated with urbanization as measured by impervious cover. Urban-rural clines in melanism were strongest in the largest cities with extensive forest cover and weakest or absent in cities with warmer winter temperatures, where thermal selection likely limits the prevalence of melanism. Our results suggest that novel traits can evolve in a highly repeatable manner among urban areas, modified by factors intrinsic to individual cities, including their size, land cover, and climate.

Introduction

Urban land use is expanding at a rate faster than urban population growth[1], transforming land cover, climate, hydrology, and biogeochemical cycling[2]. The dramatic environmental change associated with urbanization renders cities similar to one another in many abiotic and biotic characteristics[3,4]. For example, cities have extensive impervious cover (e.g., buildings, transportation networks), increased air temperature, and increased air, water, light, and noise pollution relative to their adjacent rural landscapes[2].

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35   The convergence of environmental conditions in cities makes urban ecosystems an ideal

36   focus for addressing longstanding questions in evolutionary biology about the degree to which

37   populations exhibit parallel evolution, that is, when multiple populations occupying similar

38   environments evolve comparable traits[5]. Cities are thought to drive parallel evolution as urban

39   populations adapt to similar selection pressures[6,7,8,9]. Cities are not homogenous, however, and

40   variation in their characteristics likely mediates processes that facilitate or inhibit parallel

41   evolution, generating variation in the shape of genotypic or phenotypic clines along urbanization

42   gradients. Clines may be strongest in large cities where the environmental gradients driving

43   divergent selection between urban and rural areas are steepest. Conversely, clines may be weak

44   or nonexistent in small cities or cities with extensive greenspace connecting urban and rural

45   populations via gene flow. Environmental conditions at large spatial scales – such as regional

46   climate – may also modulate the evolution of urban-rural clines at a smaller scale. For example,

47   directional selection for traits that reduce thermal stress at climate extremes may overwhelm

48   selective pressures associated with local land use, weakening urban-rural clines[10]. Advances in

49   our understanding of how landscape-scale differences among cities facilitate or constrain parallel

50   evolution have been limited by a lack of studies conducted across multiple, replicate cities[7,8,9].

51   Addressing this knowledge gap will help clarify how urbanization, the megatrend of global land

52   use change, will shape biodiversity.

53   Here we test for urban-rural clines as a signal of parallel evolution and examine how

54   landscape features mediate the shape of clines. Our focus is on melanism in eastern gray

55   squirrels (*Sciurus carolinensis*) in 43 cities in North America (Fig. 1, Supplementary Fig. 1).

56   Gray squirrels are among the most ubiquitous of fauna in cities and rural woodlands in eastern

57   North America[11]. Coat color in gray squirrels is typically gray or black (melanic) and inherited in

3

58   a simple Mendelian fashion at the melanocortin 1 receptor gene (*Mc1R*)[12]. Evidence suggests

59   divergent selection associated with crypsis, predation risk, and road mortality favors the melanic

60   morph in cities and the gray morph in rural woodlands[13], which should cause a clinal decrease in

61   melanism from urban to rural areas. However, urban-rural divergence in melanism has been

62   documented in only two cities (Syracuse, NY[13] and Wooster, OH[14]).

63   We used a dataset on coat color from squirrel observations collected via the community

64   science project *SquirrelMapper*[13] to test for parallel clines in melanism along gradients of

65   impervious cover. We included city size, forest cover across each urbanization gradient, and

66   winter temperature in a linear mixed model to test how these landscape-scale characteristics

67   affect the prevalence of melanism in each city (i.e., main effects of each covariate) and drive

68   variation in cline shape among cities (i.e., interaction effects with impervious cover). Gray

69   squirrels can disperse long distances (>10 km)[15], but movements are limited by forest

70   fragmentation[16], so we expected clines to be weakest in small cities and cities with extensive

71   forest cover where gene flow between urban and rural areas should be greatest. We included

72   winter temperature in the model because melanic squirrels are known to have greater

73   thermogenic capacity than the gray morph at cold temperature[17]. Clines may be weak in cities

74   with cold winter temperature if thermal selection overwhelms the selection pressures on

75   melanism associated with urbanization.

76

77   **Results and Discussion**

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79   Our analyses revealed that a positive relationship between the prevalence of melanism and

80   degree of urbanization was replicated across multiple cities in North America (main effect of

81 impervious cover = 0.17, SE = 0.04, $P < 0.001$; Figs. 1-2; Supplementary Table 2). Repeated

82 shifts in phenotype along environmental gradients – such as the general increase in melanism

83 from rural to urban areas documented here – is a hallmark of parallel evolution. Melanism in

84 gray squirrels is a simple Mendelian trait coded by a 24-bp deletion at $Mc1R$[12]. Individuals with

85 at least one copy of the deletion allele are melanic, so clines in melanism very likely occur at

86 both phenotypic and genotypic levels.

87   The presence of parallel clines in heritable traits is often considered evidence of adaptive

88 evolution because gene flow should homogenize populations in the absence of natural

89 selection[18]. Known drivers of parallel adaptive evolution in cities include the urban heat island[19]

90 and pollution[20]. Evidence suggests multiple selective mechanisms involving crypsis help

91 maintain clines in melanism in gray squirrels. Predation – including hunting of squirrels by

92 humans – is often the main driver of squirrel mortality in rural woodlands[21,22,23], and the gray

93 morph is more concealed than the melanic morph in regrown, secondary forests that dominate

94 rural woodlands in our study area[13]. In contrast, predation pressure on tree squirrels tends to be

95 relaxed in cities[21,22], and relaxed selection against melanic squirrels in cities could generate the

96 observed urban-rural clines. On the other hand, novel selection pressures in cities may favor

97 melanics. The primary driver of mortality of tree squirrels in cities tends to be vehicular

98 collisions[22]. The melanic morph is more visible to vehicle drivers than the gray morph on asphalt

99 roads, which may lead to the melanic morph having greater survival in areas with high car

100 traffic[13]. Additional selective mechanisms may play a role in maintaining urban melanism (e.g.,

101 parasite pressure, pollution)[24], including mechanisms involving correlated traits[25]. For example,

102 melanic squirrels have less phylogenetically variable microbiomes than the gray morph in

103 cities[26]. A more complete picture of the selective mechanisms driving urban-rural clines will

104   require linking trait variation to fitness differences between squirrel color morphs in urban and

105   rural areas.

106   An alternative explanation for the urban-rural clines documented here is that melanic

107   squirrels have been introduced by humans to some cities[23]. Strong genetic drift via founder

108   events, combined with restricted gene flow along the urbanization gradient, can generate single-

109   locus clines[27]. Variation in the size of founding populations likely causes some variation in the

110   prevalence of melanism among cities, and thus the strength of urban-rural clines. At an extreme,

111   urban-rural clines are absent in cities where melanism was historically absent and melanic

112   squirrels have never been introduced. However, two factors lead us to believe that urban founder

113   events are not the driving force behind the observed urban-rural clines. First, single-locus clines

114   generated by neutral evolutionary forces require strong isolation-by-distance (IBD)[27]. We

115   suspect IBD between urban and rural areas exists but is limited in strength by high effective

116   population size in cities (e.g., urban squirrel densities 2.5 times greater than rural squirrel

117   densities[28]), high capacity for long-distance dispersal[15], and frequent translocation of squirrels[29].

118   Second, at northern latitudes where we observe most urban-rural clines, melanism was common

119   in rural old growth forests prior to the 1800s[30,31,32], including regions where it is now rare except

120   in cities (e.g., upstate New York[13,31]). Founder events in cities do not explain the paucity of

121   melanics in rural areas relative to the past. We hypothesize that the dramatic shift from old

122   growth to secondary forests following European settlement in North America[33,34,35] changed the

123   degree to which gray and melanic morphs are concealed from visual predators and hunters,

124   favoring the gray morph in regrown forests[13]. Genomic analysis and field experiments are

125   needed to clarify the role of natural selection versus nonadaptive processes in generating the

126   observed urban-rural clines[36].

127　　　　Although clines in melanism tended to take the same form among cities, the strength of

128　　urban-rural clines varied, supporting the idea that repeated evolution is often inconsistent[5]. First,

129　　there was a strong negative relationship between melanism and winter temperature (main effect

130　　of winter temperature = -2.14, SE = 0.33, $P < 0.001$; Figs. 1-2; Supplementary Table 2) that

131　　likely constrains the development of urban-rural clines. Urban-rural clines in melanism were

132　　most common in northern cities with cold winter temperature (Fig. 1b), whereas clines were

133　　absent in cities with warm winters where melanism was rare or nonexistent. The melanic morph

134　　has greater capacity for non-shivering thermogenesis and retains more heat than the gray morph

135　　at cold temperature[17,37,38]. Our results suggest thermal selection at a regional scale plays an

136　　important role in maintaining melanism in the north, allowing other processes operating at a finer

137　　spatial scale to cause the evolution of urban-rural clines. Winter temperature has also been

138　　shown to constrain the evolution of urban-rural clines in cyanogenesis in white clover (*Trifolium*

139　　*repens*)[39]. Importantly, our results do not suggest urban-rural clines in melanism in gray squirrels

140　　are caused by the urban heat island effect, which should cause the opposite clinal pattern than

141　　that observed.

142　　　　　Second, the positive relationship between urbanization and melanism was strongest in

143　　large cities with extensive forest cover (impervious*city size interaction effect = 0.13, SE =

144　　0.03, $P < 0.001$; impervious*forest cover interaction effect = 0.15, SE = 0.03, $P < 0.001$; Fig. 2;

145　　Supplementary Table 2), where melanism increased by nearly 20% from rural to urban areas

146　　(Fig. 2c). In contrast, clines were weak to nonexistent in smaller cities and those with less

147　　extensive forest cover (Fig. 2c). Although gray squirrels have substantial capacity for long-

148　　distance dispersal, gene flow between urban and rural areas should be most limited in large cities

149　　due to isolation-by-distance and mortality caused by extensive road networks during dispersal.

150   Gene flow may be strongest between urban and rural populations in smaller cities, maintaining

151   maladapted morphs across the urbanization gradient[9]. Selection associated with road mortality in

152   urban areas may also be weak in small cities with limited traffic, which could explain the lower

153   prevalence of melanism in smaller cities (main effect of city size = 0.58, SE = 0.28, P = 0.037;

154   Fig. 2). Mechanistic studies are needed to provide insight into how road networks, traffic

155   volume, and road-crossing structures (e.g., aerial utility lines that squirrels use to cross roads[23])

156   contribute to variation in gene flow and selection along urbanization gradients within and among

157   cities.

158   The finding of strong clines in cities with extensive forest cover (Fig. 2) did not support

159   our prediction that forest cover facilitates gene flow and weakens clines along the urbanization

160   gradient. Gray squirrels require trees, and squirrel abundance and distribution along urbanization

161   gradients is limited by tree cover[28], particularly in large cities[40]. It is possible that cities with

162   more extensive forest cover support a greater abundance of gray squirrels, increasing effective

163   population size and thereby making selection more efficient in overcoming the effects of genetic

164   drift on morph frequencies.

165   Community science datasets in ecology and evolution are powerful for conducting

166   studies at large spatial scales and with levels of replication that would otherwise be infeasible.

167   However, one challenge of using community science datasets is the potential for sampling

168   biases[41]. For example, *SquirrelMapper* observers may be more likely to record observations of

169   rare color morphs. Melanics were less common than the gray morph in most cities, so detection

170   bias for rare morphs would inflate the estimated prevalence of melanism. However, we are

171   unaware of a mechanism that would cause sampling bias to vary between urban and rural areas

172   in a way that artificially generates the urban-rural clines documented here (e.g., bias toward

173   recording the melanic morph in urban areas and gray morph in rural areas), so we have no reason

174   to believe the relative change in melanism along urbanization gradients is biased.

175   Our work reinforces the idea that ecological homogenization among cities likely drives

176   parallel evolution[19,20], and it suggests cities can function as refuges for unique phenotypic

177   traits[42]. Our study also provides insight into how landscape-scale differences among cities alter

178   phenotypic clines along urbanization gradients. We show the degree of parallelism in urban-rural

179   clines among cities is imperfect, with environmental heterogeneity among cities contributing to

180   variation in the shape of clines. Greenspace availability in particular has been a focus in the

181   ecological literature for understanding among-city variation in the response of species

182   distributions to urbanization[40] – our results show that greenspace availability in the form of forest

183   cover likely mediates evolutionary responses to urbanization as well. Additional multi-city

184   studies will be essential for understanding how the degree of parallel evolution depends on

185   factors intrinsic to individual cities, including those not examined here (e.g., forest community

186   composition). Finally, our work underscores the value of leveraging community science data to

187   conduct studies of urban evolution across multiple cities.

188

189   **Methods**

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191   **Squirrel datasets.** Squirrel records were sourced from the community science program

192   *SquirrelMapper* (squirrelmapper.org). Starting in 2010, participants used a web platform to

193   submit sightings of *S. carolinensis*, including the date of observation and color morph of each

194   squirrel ("gray", "melanic", or "other"). Spatial coordinates for each observation were secured

195   via Google Maps JavaScript API. A total of 5,899 records were submitted to the original

196   *SquirrelMapper* program from the native range of *S. carolinensis* in North America. Beginning

197   in 2019, *SquirrelMapper* was integrated with *iNaturalist*, enabling users to submit georeferenced

198   photos of each squirrel observation. All images of *S. carolinensis* submitted to *iNaturalist*

199   through 21 December 2020 and classified as "research grade" were included for analysis (n =

200   75,130). Data from the original *SquirrelMapper* program were merged with the *iNaturalist*

201   dataset for analyses.

202   We used the participatory science portal *Zooniverse* to crowdsource the classification of

203   coat color of squirrels in images submitted to *iNaturalist*. Participants were asked to classify the

204   number of squirrels observed in each image ("zero", "one", "two or more") and the coat color of

205   squirrels present in each image ("gray", "melanic", "other", or "unclear"). Each image was

206   classified by a minimum of 10 participants (range: 10-291; median = 10). We retained images

207   with a minimum agreement threshold of 80% for the number of squirrels observed. Of the

208   72,239 images (96%) that met the agreement threshold, 865 were classified as having no squirrel

209   present (e.g., track or other sign), 70,258 had a single squirrel, and 1,116 had two or more

210   squirrels. Of the images classified as having at least one squirrel, we retained 63,506 images that

211   had a minimum agreement threshold of 80% for coat color of at least one squirrel in the image.

212   We removed 5,182 images that did not have location data available and 4,035 images taken

213   outside of North America where *S. carolinensis* is introduced. The final *iNaturalist* dataset

214   consisted of 54,289 images, of which 54,215 were classified as having a single coat color present

215   (gray: 46,630, melanic: 6,728, other: 857) and 74 had two color morphs present (gray and

216   melanic: 68, gray and other: 5, melanic and other: 1). A random sample of 500 images classified

217   by the authors showed that the classification scheme we used with 80% agreement thresholds

218   was 100% accurate for the number of squirrels observed in the image and 99.8% accurate for

219   coat color, the only discrepancy being a single image classified as melanic that the authors

220   classified as unclear. The final *iNaturalist* dataset was merged with squirrel records from the

221   original *SquirrelMapper* project, yielding 60,262 squirrel records in total. Multiple coat colors

222   observed at the same location were treated as separate records in this final dataset. Observations

223   in the final dataset were made between 1980 and 2020 (median = 2019), with 97% of

224   observations being reported since 2010. The 3% of observations prior to 2010 consisted of

225   *iNaturalist* records and records by the authors and colleagues prior to *SquirrelMapper*.

226

227   **City selection**. We selected cities in the native range of *S. carolinensis* that had at least 100

228   squirrel observations in the city and surrounding rural area. We defined the spatial extent of each

229   city's footprint using the Urban Area National Shapefile Record Layout[43] for the United States

230   and the Population Center Boundary File[44] for Canada. We calculated the distance from the

231   centroid of each city footprint to its maximum extent in each of the four cardinal directions. We

232   then buffered each urban area by 25% of the average distance from the city centroid to its extent

233   (Supplementary Fig. 2). This buffer distance was large enough to include a substantial area of

234   rural land surrounding each city but not so large as to produce excessive overlap between

235   polygons of adjacent cities. We determined the number of squirrels in each city polygon

236   (encompassing the city footprint and hinterland) after spatially thinning the squirrel records to a

237   minimum distance of 10 m using the *spThin* package (v. 0.2.0)[45] in R[46], which helps minimize

238   spatial sampling bias. If two cities with ≥100 squirrels had polygons that overlapped, we

239   retained the city with a greater sample size or spatial spread of squirrel observations. The final

240   dataset included 26,924 squirrels among 43 cities (Fig. 1), and the median number of squirrels in

241   each city was 254 (range: 101 – 4,731; Supplementary Table 1; maps of squirrel observations in

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242   each city available for download at https://github.com/bcosentino/squirrelMapper-urban-rural-

243   clines).

245   **Environmental covariates**. We measured percent impervious cover within 1 km around each

246   squirrel observation using the Global Man-made Impervious Surface Dataset (30-m resolution)[47].

247   A 1-km buffer was chosen to encompass the typical home range size of *S. carolinensis* (<5 ha)

248   and long-distance movements typical of males during winter and summer mating seasons[48]. For

249   each city and its surrounding rural area we measured forest cover and winter temperature. Forest

250   cover was measured using the Global 2010 Tree Cover database (30-m resolution)[49], and we

251   used WorldClim (version 2.1) to measure winter temperature as mean temperature of the coldest

252   quarter (BIO11, 30s / 1 km resolution)[50]. We also measured the area of each city footprint as a

253   metric of city size. All spatial analyses were conducted in R using the *raster* (v. 3.3-13)[51] and

254   *exactextractr* packages (v. 0.5.1)[52].

256   **Statistical analyses**. We fit a generalized linear mixed model with a binomial error distribution

257   to test for effects of impervious cover, city size, winter temperature, and forest cover on the

258   distribution of melanism. Melanism was the response variable and represented by a binary

259   indicator, with 1 corresponding to melanic and 0 to other coat colors for each squirrel

260   observation. Impervious cover within 1 km of each observation was included as a fixed effect to

261   represent each city's urbanization gradient. Because the range of impervious cover varied among

262   cities, we rescaled impervious cover within each city to range from 0 (lowest impervious cover)

263   to 1 (greatest impervious cover)[39]. This ensured the slope term for impervious cover has a

264   consistent interpretation among cities, allowing us to test for a general urban-to-rural cline in

265   melanism. City size (log-transformed), forest cover, and winter temperature were included as

266   fixed effects to test whether these variables predict variation in the prevalence of melanism

267   among cities. Two-way interaction terms were included between each predictor and impervious

268   cover to test how city characteristics affect the shape of urban-to-rural clines in melanism. We

269   standardized each predictor so that each variable had mean = 0 and SD = 1, which aided in

270   model convergence and allowed us to compare the effect size of each slope term[53]. We also

271   included city as a random effect to account for the nested observations within each city and to

272   estimate unexplained variance in the prevalence of melanism among cities (Supplementary Table

273   3). The model was fit with the *lme4* package in R (v. 1.1-26)[54]. Two-sided Wald tests were

274   conducted for each parameter in the model. Binned residual plots were generated using the *arm*

275   package (v. 1.11-2)[55] as recommended for models with a binary response variable[56]. Plots of

276   binned residuals on fitted values and each predictor suggested adequate model fit and no

277   substantial deviation from model assumptions.

278   We used the residual autocovariate approach[57] to account for spatial autocorrelation in

279   the residuals of the model. We extracted residuals from the global model and created a spatial

280   autocovariate for the residuals using the *spdep* package (v. 1.1-7)[58]. We set the radius

281   neighborhood to 31 km (the greatest nearest neighbor distance in the thinned dataset) and used

282   an inverse distance weighting scheme with style "B" for symmetric weights[59]. Our global model

283   was then refit with the spatial autocovariate term as an additional predictor, which reduced

284   spatial autocorrelation in the residuals from a maximum of $r = 0.11$ within 1 km to $r < 0.05$

285   (Moran's I; Supplementary Fig. 3).

286   We refit our model using different spatial thinning distances (10 m, 50 m, 100 m) and

287   buffer distances to measure impervious cover (500 m, 1 km, 10 km). These analyses showed the

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288   model results were not sensitive to spatial thinning distances (Supplementary Table 4) or buffer

289   distances to quantify impervious cover around squirrel observations (Supplementary Table 5).

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291   **References**

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427

428   **Author Contributions**

429

430   B.J.C. and J.P.G designed the study. B.J.C. led the data analysis and wrote the first draft of the

431   manuscript. J.P.G. contributed ideas for data analysis and edited the manuscript.
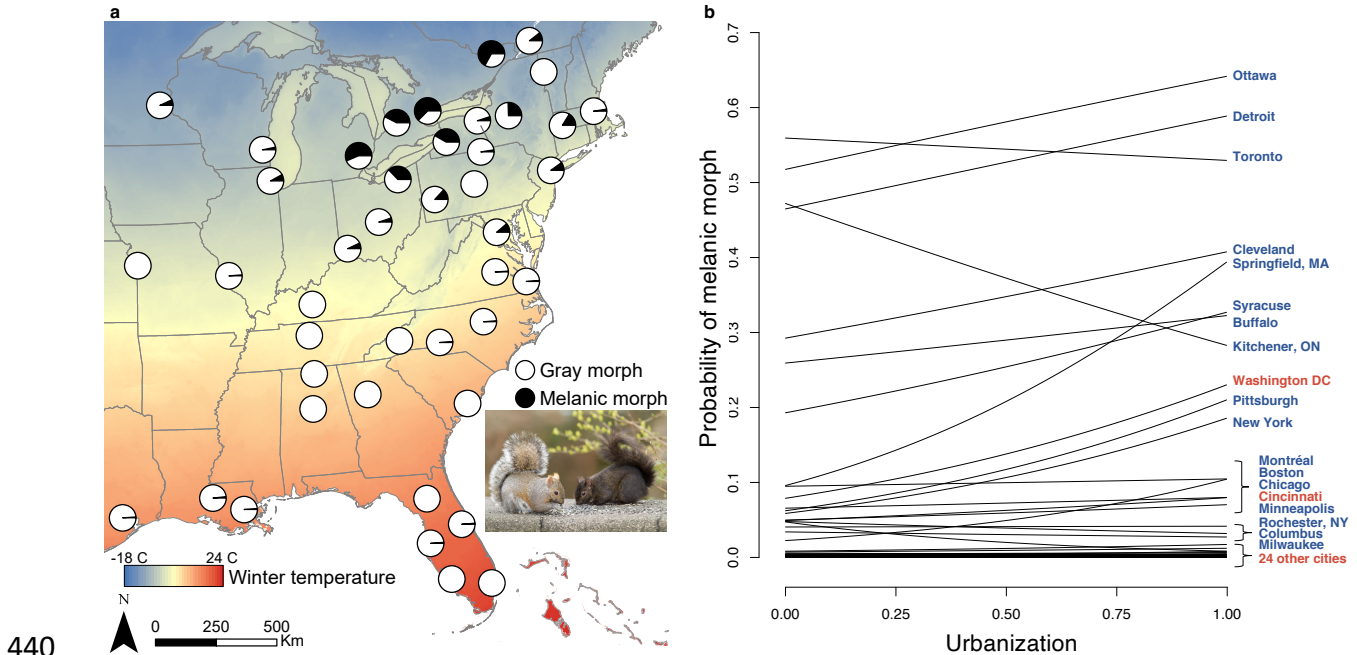
432

433   **Data Availability**

434   The observations of eastern gray squirrels (*Sciurus carolinensis*) generated and analyzed for this

435   project can be found at https://github.com/bcosentino/squirrelMapper-urban-rural-clines.

436

437   **Competing interests**

438

439   The authors declare no competing interests.

Fig. 1. a, Distribution of coat color morphs of 26,924 eastern gray squirrels (*Sciurus carolinensis*) among 43 cities in North America. Pie charts show the proportion of melanic and gray color morphs in each city. Gray morph included rare color morphs that accounted for 2% of observations (Supplementary Table 1). **b,** Clines in melanism along urbanization gradients in each city. Urbanization was measured as standardized impervious cover. Regression lines represent predicted effect of urbanization on melanism in each city based on a linear mixed model including effects of city size, forest cover, and winter temperature (Fig. 2, Supplementary Table 2). City names are color-coded to show winter temperatures above (red) and below (blue) the median winter temperature. Photo 'grey squirrel, black squirrel' (tinyurl.com/pauha835) by Eyesplash Photography is licensed under CC BY-NC-ND 2.0 (https://creativecommons.org/licenses/by-nc-nd/2.0/).

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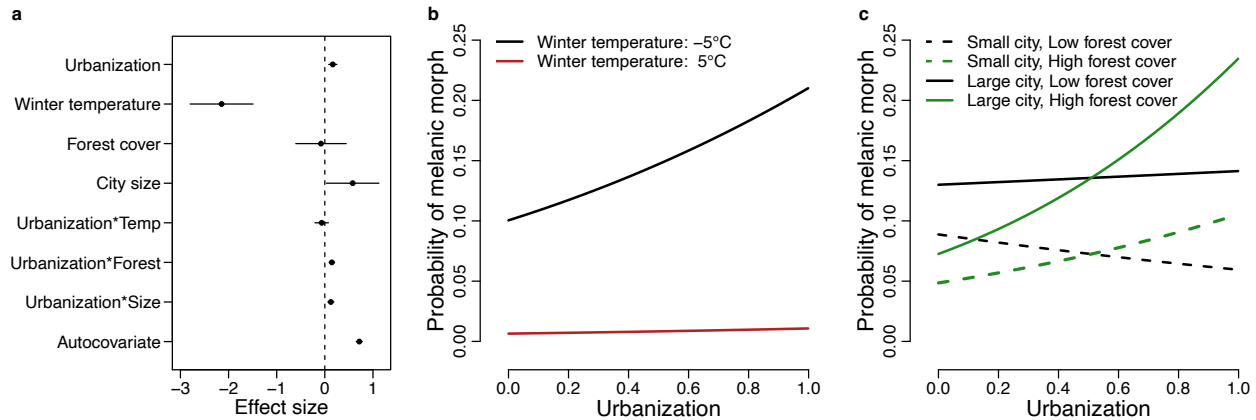
Fig. 2. a, Effect sizes for fixed effects of urbanization, winter temperature, forest cover, city size, interaction terms with urbanization, and a spatial autocovariate from a linear mixed model of melanism in 26,924 eastern gray squirrels *(Sciurus carolinensis)* among 43 cities in North America. Effect sizes represent standardized regression coefficients and include 95% confidence intervals. **b**, Relationship of the probability of melanism to urbanization at low (-5°C) and high (5°C) winter temperature while holding city size and forest cover constant. **c**, Relationship of the probability of melanism to urbanization at varying levels of city size and forest cover. Effects of urbanization on melanism are shown for combinations of small cities (25,000 ha), large cities (350,000 ha), low forest cover (15%), and high forest cover (45%) while holding winter temperature constant at -5°C. Urbanization was measured as standardized impervious cover in all panels.