

1 PLUMAGE BALANCES CAMOUFLAGE AND THERMOREGULATION IN HORNED
2 LARKS (*EREMOPHILA ALPESTRIS*)

3

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23 **Abstract**

24 Animal coloration serves many biological functions and must therefore balance
25 potentially competing selective pressures. For example, many animals have
26 camouflage, in which coloration matches the visual background against which predators
27 scan for prey. However, different colors reflect different amounts of solar radiation and
28 may therefore have thermoregulatory implications as well. In this study, we examined
29 geographic variation in dorsal patterning, color, and solar reflectance among Horned
30 Larks (*Eremophila alpestris*) of the western United States. We found associations
31 between dorsal plumage brightness, hue, and patterning relative to the soil conditions
32 where specimens were collected. Specifically, brighter dorsal plumage corresponded to
33 brighter soil, while redder, more saturated hues in dorsal plumage corresponded to
34 redder soils. Furthermore, backs with more high-contrast patterning were more common
35 among females and also associated with soil that had coarser soil fragments,
36 suggesting that lark plumage has been selected to optimize background matching in
37 different environments. We also found that larks exhibited higher solar reflectance in
38 hotter and more arid environments, which lowers the water requirements for
39 homeothermy. Taken together, these findings suggest that natural selection has
40 balanced camouflage and thermoregulation in Horned Larks across a wide variety of
41 soil types and abiotic conditions.

42 **Introduction**

43 Animal colors and patterns constitute complex phenotypes that are shaped by a
44 wide array of biotic and abiotic processes (Burt 1981; Vo et al. 2011; Cuthill et al.
45 2017). For example, some species have bright colors involved in sexual selection via
46 mate choice (Andersson and Simmons 2006; Shultz and Burns 2017), whereas others
47 have cryptic colors and patterns driven by natural selection to avoid visual detection by
48 predators (Cott 1944; Endler 1978). Furthermore, animal colors have implications for
49 maintaining homeostasis in different environments due to differences in the reflectance
50 of light wavelengths from solar radiation (Walsberg 1983; Wolf and Walsberg 2000).
51 Thus, animal coloration and patterning must balance multiple selective pressures that
52 may conflict or act synergistically to produce multifunctional phenotypes that vary
53 among populations and species (Caro 2017). Among the wide array of biological
54 processes affecting animal colors, camouflage and thermoregulation are thought to
55 have a particularly strong influence on coloration because of their direct impact on
56 survival.

57 Camouflage includes a suite of physical and behavioral attributes that deter
58 visual detection by predators and is prevalent among various animal lineages, including
59 arthropods (Farkas et al. 2013; Stevens et al. 2014) and vertebrates (Rosenblum et al.
60 2009; Isaac and Gregory 2013; Boratyński et al. 2017). Also known as background
61 matching, camouflage favors phenotypes that resemble a random sample of brightness,
62 hue, and patterning of the visual background against which predators actively scan for
63 prey (Endler 1978; Merilaita et al. 1999; Michalis et al. 2017). Thermoregulation is also
64 closely tied to coloration (Walsberg 1983) and sometimes favors phenotypes that either

65 conflict with background matching (Smith et al. 2016) or that simultaneously enable
66 thermoregulation and camouflage (Wacker et al. 2016). Many species conform to
67 ‘Gloger’s Rule,’ a highly prevalent ecogeographic pattern that ascribes lighter colors to
68 more xeric environments and darker colors to more mesic environments (Gloger 1833;
69 Delhey 2019; Marcondes et al. 2020). This widespread trend is thought to be the
70 product of multiple selective pressures, including camouflage and thermoregulation
71 (Burt 2004; Delhey et al. 2019). Plumage reflects and absorbs light that includes
72 wavelengths within the visual range of birds (UV-VIS: 300–700 nm) as well as near-
73 infrared wavelengths (NIR: 700–2,600 nm), both of which serve important roles in light
74 and heat absorptance (Stuart-Fox et al. 2017). Within the visual spectrum, darker
75 feathers tend to absorb more light and heat than lighter feathers (Porter and Gates
76 1969), but the physical properties of feathers and the ability of incident light to travel
77 through or become captured by feather microstructures also play important roles
78 (Walsberg 1988; Wolf and Walsberg 2000). Because NIR wavelengths are not
79 perceived by predators, reflectance at those wavelengths is not related to camouflage
80 but may still play an important thermoregulatory role (Medina et al. 2018).

81 Despite the prevalence of camouflage among animals, the majority of studies to
82 date on background matching have focused on a small number of systems such as
83 peppered moths (Van’t Hof et al. 2011; Cook and Saccheri 2013), pocket mice
84 (Nachman et al. 2003; Linnen et al. 2009), White Sands lizards (Rosenblum et al. 2010;
85 Laurent et al. 2016), and a few ground-nesting birds (Troscianko et al. 2016; Stevens et
86 al. 2017). Most of these systems involve discrete phenotypic variants that occupy
87 visually distinct environments. In comparison, continuous variation in background

88 matching across environmental gradients has received far less attention (Stevens and
89 Merilaita 2009; Caro et al. 2016). Furthermore, studies that simultaneously examine
90 camouflage and thermoregulation remain scarce, especially among endotherms, such
91 as birds. Finally, the role of NIR reflectance in potentially mediating tradeoffs between
92 camouflage and thermoregulation remain largely unexplored (Stuart-Fox et al. 2017;
93 Medina et al. 2018).

94 To address these knowledge gaps, we examined associations between plumage
95 reflectance and patterning, soil color and composition, and thermoregulatory models
96 among geographically variable populations in a widespread songbird, the Horned Lark
97 (*Eremophila alpestris*). Horned Larks occupy a wide variety of open mesic and arid
98 habitats, including deserts, fallow agricultural land, tundra, and grasslands (Beason
99 1995; Mason et al. 2020). Horned larks build nests and glean seeds and insects on the
100 ground (Wiens and Rotenberry 1979; de Zwaan and Martin 2018). Due to their
101 preference for habitats with sparse vegetation, larks are thought to rely on substrate
102 matching to avoid avian predators (Donald et al. 2017). Although camouflage in Horned
103 Larks has been discussed anecdotally (Zink and Remsen 1986; Mason and Unitt 2018),
104 associations between phenotypic and environmental variation have not yet been tested
105 rigorously. Larks also exhibit various physiological adaptations to aridity gradients
106 (Tieleman et al. 2003b, 2003a), making them an excellent system to study interactions
107 between camouflage and thermoregulation. If larks exhibit background matching, we
108 predict that soil conditions will be associated with variation in plumage brightness, color,
109 and patterning. Furthermore, if plumage also plays a thermoregulatory role, we predict

110 that rates of evaporative water loss and solar reflectance will be associated with
111 variation in aridity and temperature.

112 To test these predictions, we combined digital photography, color and plumage
113 analyses, full-spectrum (UV, Visual, NIR) spectroradiometry of museum specimens,
114 remote sensing data, and simulation-based thermoregulatory models of heat flux to
115 examine phenotype-environment associations between plumage coloration and
116 patterning, soil conditions, and climate. This approach allows us to disentangle the
117 effects of camouflage, thermoregulation, and sexual dimorphism in driving the evolution
118 and ecology of lark coloration. More broadly, it illustrates how we can understand limits
119 on the adaptive potential of certain traits such as coloration. For example, warming
120 climates might select for more reflective features, but at a cost to background matching.
121 Likewise, habitat alterations might select for darker feathers that impose greater
122 physiological stress under warming climates. Thus, one selective pressure might have
123 detrimental effects to another in the evolution of phenotypes. Only by integrating both
124 background matching and thermoregulatory performance can we understand
125 evolutionary responses to these different and potentially competing selective pressures.
126

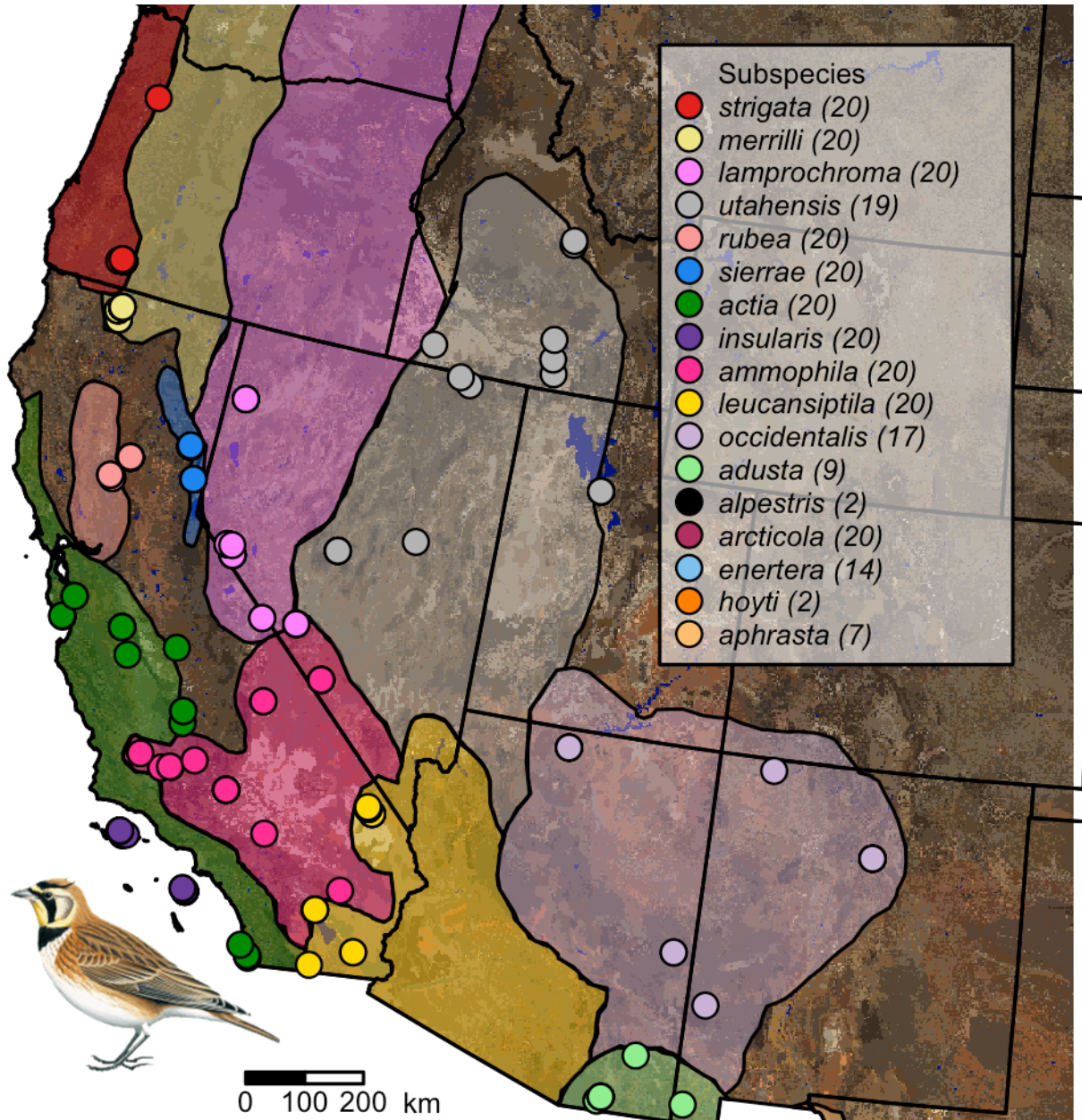
127 **Methods**

128 *Digital Photography and Image Analysis*

129 We photographed the dorsal side of 270 Horned Lark specimens from the
130 Museum of Vertebrate Zoology (MVZ) at the University of California, Berkeley
131 (Supplementary Table S1), using a Nikon D7000 camera modified for full-quartz
132 calibration (Advanced Camera Services, Watton, Norfolk, England). We measured up to

133 10 males and 10 females of 17 different subspecies (Figure 1) in the western United
134 States, preferentially selecting specimens from breeding months (May–August) and with
135 undamaged plumage. We used a Novoflex Noflexar 30mm f/3.5 lens, which does not
136 filter out ultraviolet wavelengths and is therefore suitable for measuring plumage
137 reflectance under an avian visual model. We took two RAW images of each specimen
138 at ISO200: one image used a Baader Venus-U filter, which captures wavelengths
139 between ~320–380 nm, and a second image used a Baader UV/IR cut filter, which
140 captures wavelengths between ~400–680 nm. Each image included a ruler at the height
141 of the specimen’s dorsal plane with 5% and 80% reflectance standards (Labsphere,
142 Hutton, NH, USA). We automatically aligned and linearized images using the Image
143 Calibration Analysis Toolbox (Troschianko and Stevens 2015), which provides a set of
144 plugins for ImageJ (Schneider et al. 2012), and manually drew polygons corresponding
145 to the dorsal region of each specimen in ImageJ (Supplementary Figure S1).

146 After processing each image and delimiting the dorsal region of interest, we
147 converted the channel readings for the UV and visual images to the cone-catch values
148 of a blue tit visual model (Vorobyev and Osorio 1998). We then converted these cone-
149 catch values into tetracolorspace measurements (Vorobyev et al. 1998; *sensu* Stoddard
150 and Prum 2008) of hue, saturation, and chroma using the package pavo v2.2.0 (Maia et
151 al. 2013, 2019) in the R programming environment (R Core Team 2020). We also
152 measured achromatic brightness (i.e., total reflectance or luminosity across all
153 wavelengths) and calculated an index of patterning via a series of Fast Fourier
154 Transform (FFT) bandpass filters at 49 levels (beginning at 2 pixels and increasing
155 exponentially by $\sqrt[8]{2}$ to 128). FFTs are widely applied in digital image analysis



156

157 Figure 1: Sampling map showing localities of vouchered Horned Lark (*Eremophila*
158 *alpestris*) specimens used in this study. Soil color is based on USDA soil surveys, and
159 the approximate range of each subspecies is shown in a different color based on Behle
160 (1942) and new museum records. Some dots may represent more than one individual
161 sampled from the same locality. The number of samples per subspecies is given in
162 parentheses next to the name of the subspecies in the legend in the upper right. Some

163 subspecies (*alpestris*, *arctica*, *enertera*, *hoyti*, *aphrasta*) are not shown because we
164 focused on the geographic area with the most dense sampling.
165
166 (Stoddard and Osorio 2019) and can be used to quantify animal patterns based on
167 neurophysiological processing of spatial patterns (Godfrey et al. 1987; Stoddard and
168 Stevens 2010; Troscianko et al. 2016; Mason and Bowie 2020). In this process, an
169 image is converted into a set of sine waves, each with a different frequency and
170 amplitude. The amplitude or power of each wave indicates how much patterning—or
171 change between light and dark pixels—occurs at a specific spatial scale. Thus, images
172 with high power across FFT bandwidths display more patterning (e.g. the dorsal spots
173 on some larks), whereas images with lower power are more uniformly colored.

174 Finally, we performed a principal component analysis on the three focal plumage
175 characters: brightness, achieved chroma, and back patterning (total power) to
176 summarize plumage variation among the sampled larks. We found that the first principal
177 component axis loaded positively with brightness and dorsal patterning, whereas the
178 second principal component axis represented a tradeoff between brightness and back
179 patterning (Supplementary Table S2).

180

181 *Measuring Full-spectrum Solar Reflectance*

182 We measured solar reflectance of the lark specimens described above using
183 recently published methods for estimating heat stress in birds (Riddell et al. 2019). For
184 each specimen, we measured dorsal and ventral feather reflectance from 350 – 2500
185 nm using an ASD FieldSpec Pro spectroradiometer (ASD, Inc., 1625 S. Fordam Street,

186 Suite 300, Longmont, CO 80503), and standardized each measurement relative to a
187 Spectralon™ white standard before recording our measurements. We used a custom-
188 built tungsten halogen light source to measure feather reflectance 2 cm from the feather
189 surface, with a 45° angle between the light source and fiber optic cable. This light
190 source was built using an AC-to-DC voltage converter to inhibit interference from an
191 alternating source of electrical current. To standardize the angle and distance, we used
192 a RPH-1 reflection probe holder (Ocean Optics, Inc., Largo, FL). We also used the
193 reflection probe to standardize the surface area of each measurement to ensure that the
194 measurements were not influenced by body size. The combination of numerous
195 measurements per specimen (see below) and the diameter of the probe opening (6.35
196 mm) ensured that we captured the average solar reflectance of each side for each
197 specimen.

198 We used ViewSpec software (ASD, Inc.) to measure solar reflectance, recording
199 ten measurements (five dorsal and five ventral) for each of the 270 specimens. On both
200 the dorsal and the ventral sides, we recorded one measurement from the crown or neck
201 and four measurements spread across the breast or mantle for a total of 2,700
202 measurements. We then used a custom script in Python (v. 3.5) to average these
203 values for each individual, and corrected the reflectance curves for solar radiation using
204 the ASTM G-172 standard irradiance spectrum for dry air provided by SMARTs v. 2.9
205 (Gueymard 2001). We calculated the corrected value by multiplying the intensity of solar
206 radiation by the empirical reflectance, integrating across all of the wavelengths, and
207 dividing by the total intensity of solar radiation (Gates 1980).

208

209 *Remote Sensing Data*

210 We compiled two different soil data sets to examine associations between lark
211 dorsal plumage and soil conditions. First, we downloaded a soil color data set based on
212 an extensive series of United States Department of Agriculture soil surveys of the
213 contiguous United States, with values that had been converted from Munsell color
214 charts to RGB color space (Beaudette et al. 2013). Using georeferenced localities of
215 each lark specimen obtained from the MVZ database Arctos
216 (arctos.database.museum), we extracted their respective soil values and performed a
217 principal components analysis to assess soil color. The first principal component axis
218 loaded strongly with all three channels corresponding to soil brightness. The second
219 principal component axis loaded positively with the red channel, but negatively with blue
220 and green (Supplementary Table S3), and therefore corresponded to soil redness. In
221 this manner, we obtained soil color data associated with the site of collection of 224 of
222 our lark specimens. This soil color dataset is limited to the continental United States, so
223 we were unable to include 46 individuals from populations in Alaska, Canada, and
224 Mexico in this part of our analysis.

225 We also downloaded harmonized soil property data for the top five centimeters of
226 soil depth at a 30 arc-second resolution from the WISE30Sec database (Batjes 2016).
227 WISE30Sec data has been used widely to study soil biogeochemistry for quantifying
228 global carbon stocks (Sanderman et al. 2017). Although it has not been applied broadly
229 to organismal biology, this dataset provides ecologically relevant information on clay
230 abundance, proportion of coarse fragments, and other soil properties relevant to
231 terrestrial organisms such as Horned Larks. To generate an index of soil surface

232 granularity, we extracted the volume percentage of coarse fragments (> 2 mm) and the
233 mass percentages of sand, silt, and clay. We then performed a principal component
234 analysis and found that the first principal component axis loaded positively with coarse
235 fragments and sand, and negatively with silt and clay (Supplementary Table S4).

236 To examine associations between climate and plumage, we also downloaded all
237 19 WorldClim bioclimatic variables (worldclim.org; Hijmans et al. 2005) at a resolution of
238 30 arc-seconds to examine associations with dorsal plumage. We conducted a principal
239 component analysis in which the first principal component axis loaded positively with
240 seasonality, the second principal component axis loaded positively with aridity, and the
241 third principal component axis loaded positively with temperature (Supplementary Table
242 S4).

243

244 *Statistical Analyses of Phenotype-Environment Associations*

245 We performed a series of statistical analyses to examine associations between
246 dorsal plumage and the environment. First, we summarized phenotypic variation among
247 subspecies by plotting the first two PCA axes of plumage variation and noted clustering
248 by subspecies and sex. We then compared mean values by first performing an analysis
249 of variance on the output of linear models, with subspecies as the grouping variable and
250 with males and females separately. We subsequently used a Tukey's multiple
251 comparison post-hoc test (Steel et al. 1997) with the HSD test function from the
252 agricolae package v1.3-3 (de Mendiburu 2020) in R (v4.0.1; R Core Team 2020) to
253 assign subspecies to groups within each sex based on their mean values. We then
254 constructed linear models (LMs) to quantify background matching by examining

255 associations between plumage and soil variables with sex included as a main effect.
256 Specifically, we constructed the following LMs: (1) plumage brightness as the response
257 variable with soil brightness (soil color PC1) and sex as main effects; (2) plumage
258 redness as the response variable with soil redness (soil color PC2) and sex as main
259 effects; and (3) plumage patterning as the response variable with soil granularity (soil
260 composition PC1) and sex as main effects. We also calculated Pearson's product-
261 moment correlation coefficients between plumage brightness and soil brightness (soil
262 color PC1), plumage chroma and soil redness (soil color PC2), and plumage patterning
263 (plumage patterning PC1) and soil granularity (soil granularity PC1) using the `cor.test()`
264 function in R (v4.0.1; R Core Team 2020).

265 Finally, we generated additional LMs to simultaneously estimate the influence of
266 soil and bioclimatic conditions on variation in plumage reflectance. Specifically, we
267 constructed a LM with dorsal brightness (UV-VIS) as a response variable and soil color,
268 seasonality, aridity, temperature, and sex as main effects using the `glm()` function in R
269 (R Core Team 2020). We also generated a LM with dorsal solar reflectance (UV-VIS-IR)
270 as a response variable and soil color, seasonality, aridity, temperature, and sex as main
271 effects with the same function and settings.

272

273 *Heat flux simulations*

274 We incorporated the empirical measurements of solar-corrected feather
275 reflectance into a heat flux model to estimate the thermoregulatory differences among
276 subspecies. This model simulates heat balance using the morphological characteristics

277 of the bird in a complex radiative and thermal environment. The simulation output
278 produces estimates of net sensible heat flux (Q), which was calculated using:

279

$$280 \quad Q = M - E - C \frac{dT_b}{dt} = K_e(T_b - T_e) \quad \text{Eq. 1}$$

281

282 where M is the heat generated through metabolic processes, E is the heat lost via
283 evaporative processes, T_b is body temperature, K_e is the effective conductance, and T_e
284 is the operative temperature. The net sensible heat flux equation estimates the heat flux
285 required to maintain a stable body temperature given the morphology of the bird and its
286 interaction with the environment.

287 The heat flux simulation uses biophysical principles to estimate heat flux between
288 the birds and their environment. We used environmental data generated by *NicheMapR*
289 (v1.1.3; Kearney and Porter 2020) to estimate the thermal microclimate for larks. First,
290 we obtained monthly minimum and maximum air temperatures from the *Worldclim*
291 global climate database for the sites at which specimens had been collected. We then
292 corrected these temperatures using *NicheMapR* to reflect conditions relevant to larks,
293 using a reference height of 5 cm above the ground because larks spend most of their
294 time near the ground. We simulated heat flux assuming two types of soil environments:
295 a xeric, desert-like environment (sand, soil reflectance = 0.35) and a more mesic
296 environment (loam, soil reflectance = 0.15; Campbell and Norman 1998). These two
297 environments represent the extremes of the thermal environment that larks inhabit in
298 our study area. Assuming a more reflective soil (i.e., sand) in our simulations did not
299 qualitatively alter our conclusions. We then used these environments to understand the

300 thermal consequences of variation in dorsal and ventral solar-corrected feather
301 reflectance.

302 We incorporated morphological phenotypes that directly influence reflected solar
303 radiation in several ways. Our goal was to isolate the importance of dorsal reflectance
304 for thermoregulation. Thus, we assumed that each phenotype was equivalent among
305 subspecies, with the exception of feather reflectance. In general, variation in body mass
306 among subspecies of western Horned Larks is low (males = $29.7 \text{ g} \pm 2.4$, females =
307 $28.5 \pm 3.5 \text{ g}$; Behle 1942), suggesting that the differences in mass are unlikely to
308 substantially influence thermoregulatory differences among subspecies. Estimates of
309 morphological phenotypes were taken from Riddell et al. (2019) using three specimens
310 that represented the average mass of a lark in our simulations, but here we briefly
311 describe the methods. We estimated plumage depth (sensu Kearney et al. 2016) by
312 measuring the vertical distance from the skin to the outer surface of the feathers using a
313 Fisherbrand™ 150 mm ruler at 10 locations that spanned the dorsal and ventral side of
314 each specimen. We also measured the average length of contour feathers across six
315 feathers per specimen spanning the dorsum and ventrum. To characterize the
316 approximate shape of larks, we used measurements of the height, length, and width of
317 the lark specimens. We measured length from crown to the vent, width from shoulder to
318 shoulder, and height from the back of the dorsal side to the breast at the shoulder
319 (Kearney et al. 2019). We then used these values to estimate the rough dimensions of a
320 lark, assuming a spheroid shape (Porter and Kearney 2009). The dimensions of birds in
321 nature are dependent upon posture and are thus highly variable. By using the same
322 dimensions for each subspecies, our analysis focuses on the thermoregulatory effects

323 of reflectance and avoids possible noise due to specimen preparation and behavioral
324 differences among subspecies. The mean body mass for Horned Larks (29.5 g) was
325 determined by Riddell et al. (2019), which used the Vertnet data aggregator
326 (vertnet.org). Briefly, body mass was based on collection points in western North
327 America ($n = 2,468$). The protocol in Riddell *et al.* (2019) removed data greater or less
328 than two standard deviations from the mean body mass to remove juvenile values
329 erroneously labelled as adult and extreme outliers that were likely a mistake. Estimates
330 of mass agree closely with previously published values for subspecies of larks (Behle
331 1942).

332 The simulation estimates heat flux by integrating morphological phenotypes with
333 environmental biophysics and behavior. Estimating heat flux in endotherms is
334 complicated by properties of the insulation layer. We addressed these issues by
335 integrating a series of equations involved in a two-dimensional heat transfer model to
336 estimate the flux from the dorsal and ventral components of a bird (Bakken 1981). This
337 model calculates the total amount of heat absorbed or lost from the environment, and
338 converts the amount of energy into the physiological response that would be necessary
339 to maintain a stable body temperature (39°C in our simulations). These values represent
340 the amount of heat that needs to be generated via metabolic heat production or lost via
341 evaporative cooling to regulate body temperature. We incorporated sources of heat
342 specifically including air temperature, direct solar radiation, diffuse solar radiation,
343 reflected radiation from the ground and sky, and longwave radiation from the sky and
344 ground. We also calculated standard operative temperature (T_{es}) to equate simulated
345 environments in the field to laboratory conditions as shown in Equation 2:

346

$$T_{es} = T_b - \frac{K_e}{K_{es}}(T_b - T_e) \quad \text{Eq. 2}$$

347 where T_b is body temperature, K_e is effective conductance, K_{es} is standard effective
348 conductance, and T_e is operative temperature. Standard operative temperature equates
349 the heat flux that an organism experiences in a black-body temperature-controlled
350 metabolic chamber under specific convective conditions to that of a complex thermal
351 environment to predict a physiological response. For our simulations, we assumed K_{es}
352 represented the effective conductance at 0.1 m/s for our standard convective
353 conditions. The specific calculations for these simulations can be found in Riddell *et al.*
354 (2019) and the Python script can be found on GitHub (github.com/ecophysiology).

355 We used these simulations to isolate the thermoregulatory consequences of
356 geographic variation in dorsal feather reflectance. We were specifically interested in
357 estimating the water requirements for evaporative cooling (termed ‘cooling costs’)
358 because these costs are highly relevant to the environmental pressures shaping
359 physiological adaptation in larks (Tieleman *et al.* 2003a). We estimated cooling costs
360 across all sites with available climate and elevational data ($n = 66$). For all sites, we
361 generated estimates of cooling costs using two simulations: one with the average dorsal
362 reflectance across all sites (average = 0.65) and the other incorporating the site-specific
363 dorsal reflectance (range = 0.47 – 0.80). Ventral feather reflectance was held constant
364 (average = 0.69) to specifically focus on the role of dorsal feather reflectance in
365 camouflage and thermoregulation. For each site, we subtracted the cooling costs
366 between the two simulations. The difference (termed reduction in cooling costs)

367 provides an index for the site-specific reduction in thermoregulatory pressure driven by
368 geographic variation in dorsal feather reflectance.

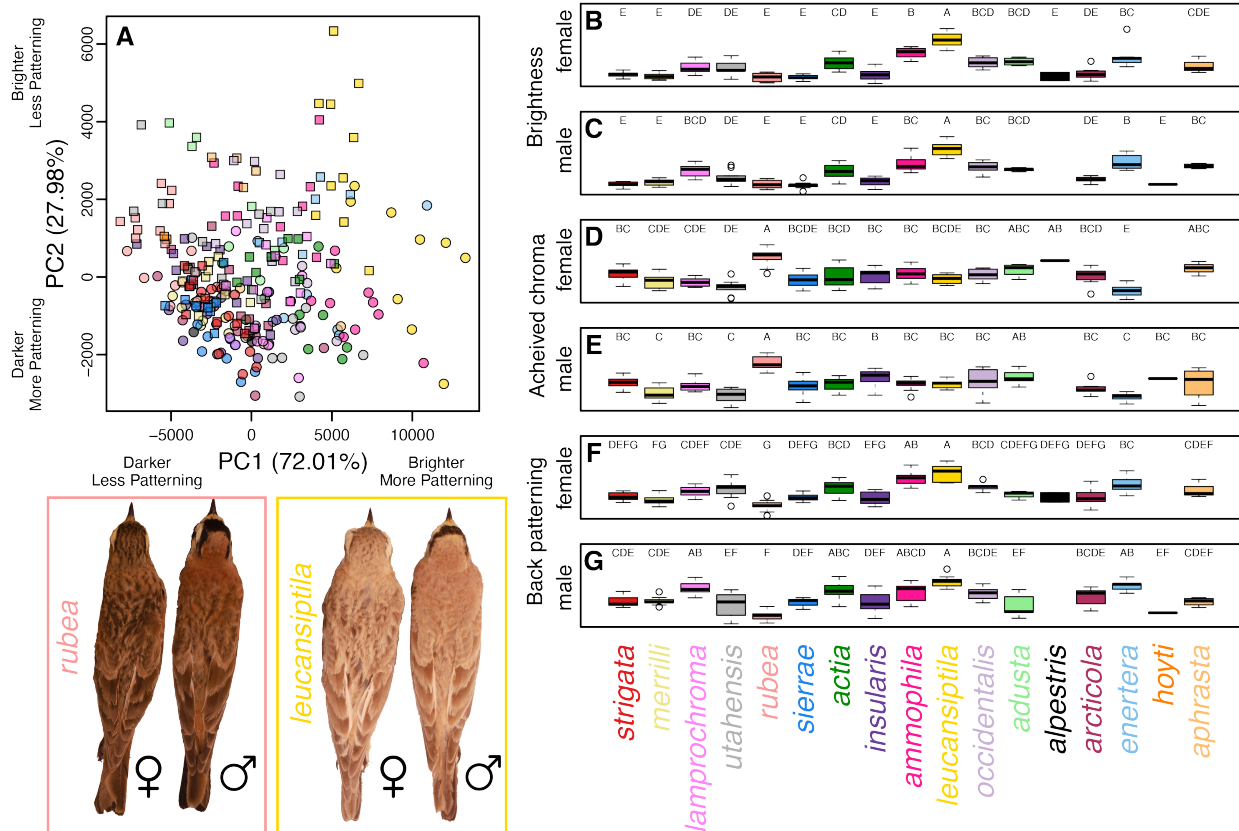
369 We then determined whether the reduction in cooling costs from geographic
370 variation in reflectance was associated with climatic variables. We fit exponential
371 models from the $n/s()$ function in *R* (v4.0.1; R Core Team 2020) to determine the
372 relationship between the reduction in cooling costs and the principal components of
373 climatic variables. For climatic variables, we averaged the principal components that
374 described seasonality (PC1), aridity (PC2), and temperature (PC3; see Table S4 for
375 loadings). We converted the principal components to positive values by adding the
376 lowest value to each principal component, which generated interpretable confidence
377 intervals. Starting values for coefficients were generated using the $n/sLM()$ function. We
378 assessed the significance of the models based on the regression coefficients, standard
379 errors of coefficients, and 95% confidence intervals. We then used an AIC model
380 selection framework to determine whether models with the principal components of
381 seasonality, aridity, or temperature were more likely to explain the variation in the
382 reduction in cooling costs attributed to variation in feather reflectance.

383

384 **Results**

385 *Phenotypic variation*

386 Plumage characters varied among subspecies and sexes, but also exhibited
387 substantial overlap. The PCA of plumage characters revealed general clustering by both
388 subspecies and sex (Figure 2A). For example, *E. a. leucansiptila* tended to have higher
389 PC1 and PC2 scores, indicating that they were lighter and more patterned compared to



390

391 Figure 2: Phenotypic variation in Horned Lark plumage. A principal component analysis

392 (A) reveals clustering by subspecies (colors correspond to labels below boxplots) and

393 sex (males shown with squares, females shown with circles). Panels B–G show box

394 plots of brightness, achieved chroma, and dorsal patterning for males and females.

395 Letters above each boxplot correspond to Tukey's posthoc groupings. Subspecies are

396 ordered to reflect their approximate geographic distributions from northwest to

397 southeast. Inset on the lower left shows is an example of male and female *E. a. rubea*

398 and *E. a. leucansiptila*, which represent opposite ends of the phenotypic distribution of

399 larks included in this study.

400

401 other subspecies. Furthermore, males tended to have lower PC1 scores and higher

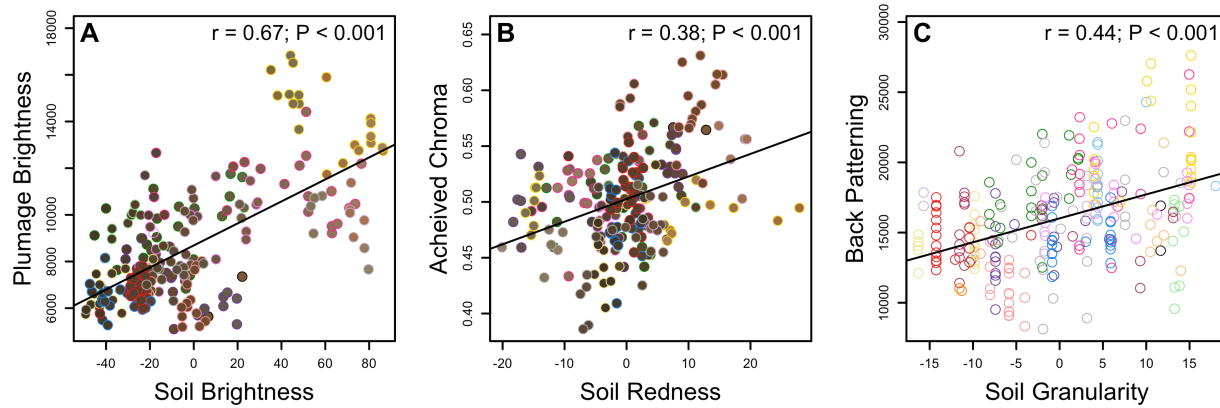
402 PC2 scores than females, indicating that males tended to have less dorsal patterning

403 than females on average. Comparisons of mean values using Tukey's posthoc tests
404 revealed various groupings among both male and female larks, but also indicated
405 substantial overlap or gradations in phenotypes among subspecies of Horned Lark
406 (Figure 2B–G). In parallel with PCA scores and loadings, we found that *E. a.*
407 *leucansiptila* was in its own posthoc grouping for brightness for both males (Figure 2B)
408 and females (Figure 2C). Similarly, *E. a. rubea* tended to differ from other subspecies in
409 mean values for all three plumage variables and was frequently in its own posthoc
410 grouping for both males and females. Box plots also revealed substantial overlap in
411 geographically proximate subspecies. For example, the geographic distribution of *E. a.*
412 *ammophila* overlaps with *E. a. actia* to the west in south-central California, and the two
413 subspecies exhibited substantial overlap in posthoc groupings. Similar overlap was
414 present in other pairs of geographically proximate subspecies, such as *E. a.*
415 *occidentalis* and *E. a. adusta*, which come into contact in southern Arizona. These
416 results suggest ample clinal variation among subspecies of Horned Lark, as has been
417 noted elsewhere (Behle 1942).

418

419 *Phenotype-environment associations*

420 Using linear models, we found multiple associations between dorsal plumage
421 variation and soil conditions underlying background matching in Horned Larks (Figure
422 3). Specifically, we found that dorsal brightness was positively associated with soil
423 brightness ($\beta_{\text{soil brightness}} = 47.17 \pm 3.5$; t-value = 13.49; $P < 0.001$) and differed marginally
424 between sexes ($\beta_{\text{sex_male}} = 485.46 \pm 250.21$; t-value = 1.94; $P = 0.05$). Achieved chroma
425 was positively associated with soil redness ($\beta_{\text{soil redness}} = 2e-3 \pm 3.32e-4$; t-value = 6.02;



426

427 Figure 3: Associations between plumage and soil conditions indicating background
428 matching in Horned Larks. In panels A and B, the fill of each point corresponds to the
429 color of the soil for the locality of the vouchered specimen, which in turn is based on
430 RGB values of USDA soil surveys. The outline of each point corresponds to the
431 subspecies, as seen in Figure 1. Results from Pearson's correlation tests are shown in
432 the upper right hand of each plot. A trend line for the relationship between the two
433 variables is drawn when the Pearson's correlation test is significant ($P < 0.05$).

434

435 $P < 0.001$) and differed marginally between sexes ($\beta_{\text{sex_male}} = -0.01 \pm 5.31e-3$; $t\text{-value} = -$
436 1.9 ; $P = 0.06$). Finally, we found that dorsal patterning was associated with soil
437 granularity ($\beta_{\text{soil granularity}} = 169.23 \pm 19.91$; $t\text{-value} = 8.50$; $P < 0.001$) such that dorsal
438 plumages with higher contrast patterning were associated with more granular soils (i.e.,
439 more coarse fragments, more clay), and that males were less patterned than females
440 ($\beta_{\text{sex_male}} = -2358.47 \pm 354.86$; $t\text{-value} = -6.65$; $P < 0.001$).

441

442 When we expanded our linear models to consider how bioclimatic and soil
443 conditions simultaneously impact dorsal reflectance within the same model, we found a
444 positive association between dorsal plumage brightness (UV-VIS) and soil brightness,

444 seasonality, aridity, and temperature (Table 1). We also found positive associations
445 between dorsal solar reflectance (UV-VIS-IR) and seasonality and aridity, but not
446 between dorsal solar reflectance and soil brightness or temperature (Table 1).

447

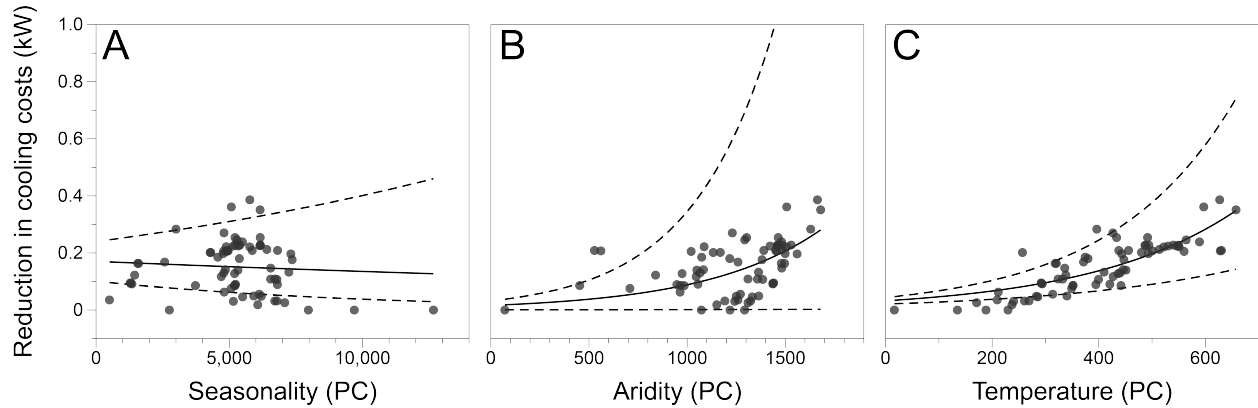
448 *Heat Flux Simulations*

449 The observed variation in feather reflectance contributed to a substantial
450 reduction in cooling costs compared to simulations with average feather reflectance.
451 Incorporation of the observed variation in feather reflectance reduced water
452 requirements for evaporative cooling by 15.1% on average (SD = 9.1%; range = 4.1% to
453 70.2%). The reduction in cooling costs was also positively associated with temperature
454 (PC2) and aridity (PC3) indices but not with seasonality (PC1; Figure 4, Table S5).
455 Although the reduction in cooling costs was associated with aridity, model selection
456 indicated that the model with temperature far outperformed models with aridity and
457 seasonality (Table S6).

458

459 **Discussion**

460 By combining data from digital photography, spectroradiometry, and remote
461 sensing we documented multiple associations between dorsal plumage and
462 environmental conditions of Horned Larks over a broad geographic distribution. These
463 findings emphasize the multifarious role of feathers and animal coloration more
464 generally. Although lark coloration has long been hypothesized to facilitate camouflage
465 (Behle 1942; Donald et al. 2017; Mason and Unitt 2018), our study provides the first
466 empirical evidence of phenotype-environment associations that underlie background



467

468 Figure 4: Variation in feather reflectance reduces thermoregulatory costs in hotter
469 locations. Shown are the relationships between climatic principal components and the
470 reduction in water requirements for evaporative cooling (termed cooling costs) due to
471 variation in dorsal feather reflectance. The reduction in cooling costs was not associated
472 with the principal component for (A) seasonality but was associated with (B) aridity and
473 (C) temperature. The analyses suggest that temperature is a major driver of variation in
474 feather reflectance for thermoregulatory purposes. Exponential models are plotted with
475 95% confidence intervals in the dotted line.

476

477 matching in larks in brightness, hue, and patterning. Furthermore, full-spectrum
478 spectroradiometry combined with simulation-based models of thermoregulation
479 revealed that geographic variation in feather reflectance reduces evaporative cooling
480 costs in hotter environments. While selective pressures imposed on total solar
481 reflectance in feathers have just begun to be explored (Stuart-Fox et al. 2017; Medina et
482 al. 2018), our findings suggest it plays an important role in the thermal ecology of larks.

483 Background matching is one component of a suite of phenotypes that organisms
484 have evolved to avoid visual detection by predators (Merilaita and Lind 2005; Stevens
485 and Merilaita 2009). In order to avoid such detection, natural selection has shaped the

486 appearance of many organisms so that they resemble a random sample of the
487 background against which their predators typically scan for prey (Endler 1978; Michalis
488 et al. 2017). Dorsal brightness and hue in Horned Larks match the background
489 substrate (Fig. 3A, Fig. 3B), as has also been shown in mice (Vignieri et al. 2010),
490 gerbils (Boratyński et al. 2017), moths (Kettlewell 1955), and other taxa (Stevens et al.
491 2014; Troscianko et al. 2016). There is less empirical evidence for background
492 matching in pattern as opposed to brightness or color alone, but it has been reported in
493 cuttlefish (Barbosa et al. 2008), nightjars (Troscianko et al. 2016), and plover eggs
494 (Stevens et al. 2017). In Horned Larks, increased dorsal 'mottling' with more high-
495 contrast spots was associated with increases in sand and other coarse particles rather
496 than with clay and silt (Fig. 3C). Beyond background matching, increased dorsal
497 patterning in larks may also contribute to disruptive patterning that breaks up the visual
498 outline of the bird as seen from above (Cuthill et al. 2005). Disruptive patterning in larks
499 may be associated with molt strategies that promote the retention of worn feathers with
500 lighter edges (Negro et al. 2019).

501 Our study focused on the substrate encountered by Horned Larks during the
502 breeding months. However, migratory populations of larks must avoid detection against
503 multiple, geographically distant substrates that differ in color and composition. Further
504 contributing to the complexity of this challenge, soil color may change over the course of
505 a year as precipitation increases or decreases, especially in more seasonal
506 environments. Horned Larks molt only once per year (Pyle 1997), and thus migratory
507 populations may need to balance competing pressures for background matching
508 against different substrates. Future studies could expand upon our results by examining

509 how natural selection shapes organisms with non-dynamic camouflage against multiple
510 backgrounds.

511 We also uncovered associations between climate and dorsal plumage.
512 Specifically, we found that solar reflectance (i.e., UV-VIS-NIR reflectance) is associated
513 with two climatic variables—seasonality and aridity (Table 1)—but bears no association
514 with soil brightness or temperature (Table 1). Thus, solar reflectance increases as
515 climates become more arid, presumably to prevent the organism from overheating and
516 becoming dehydrated. Interestingly, we found greater reductions in cooling costs
517 associated with temperature than with seasonality or aridity (Fig. 4)—an observation
518 that conflicts with implications from our linear models as we found no association
519 between temperature and dorsal solar reflectance in our linear models (Table 1).
520 However, the modeling approach for cooling costs incorporates additional parameters
521 such as NIR reflectance, feather conductance, direct and diffuse solar radiation, and
522 radiation from the ground (Gates 1980). Thus, these findings may not be in direct
523 conflict with one another, but rather may reflect different methodologies and statistical
524 assumptions. Regardless, NIR reflectance might play a role in allowing larks to increase
525 solar reflectance while maintaining crypsis in the visual spectrum. Future work could
526 further disentangle how the NIR and UV-VIS portions of light co-vary or are
527 independently selected for optimal thermoregulation in different climates (Stuart-Fox et
528 al. 2017).

529 There are many other factors beyond modifications to solar reflectance in UV-VIS
530 and NIR wavelengths that could contribute to the ability of larks to inhabit hot, arid
531 environments (Trost 1972; Dean and Williams 2004). First, many arid-adapted larks

532 have reduced metabolic rates and increased water retention through various
533 physiological adaptations (Tieleman et al. 2002). Second, behavioral adaptations also
534 can reduce heat stress. For example, microhabitat selection such as resting in shade or
535 animal burrows during extreme heat may contribute to thermoregulation in larks
536 (Williams et al. 1999; Hartman and Oring 2003; Walde et al. 2009). While Horned Larks
537 are philopatric and stay close to their breeding territory during the breeding season
538 (Beason 1970), they are nomadic during non-breeding months and may seek out
539 favorable habitat within reach of their individual movements to facilitate
540 thermoregulation. Feathers are one part of a complex suite of phenotypes involved in
541 maintaining homeostasis in thermally challenging environments. The interplay of
542 plumage and other physiological and behavioral adaptations for thermoregulation
543 remains an open avenue of research.

544 In conclusion, our study uncovered empirical evidence for the multifaceted role
545 that plumage plays in mediating both camouflage and thermoregulation in Horned
546 Larks. Dorsal plumage and patterning are associated with soil conditions, whereas
547 feather solar reflectance is associated with abiotic conditions and improved cooling
548 costs in hotter climates. Future studies could leverage these phenotype-environment
549 associations in combination with new genomic resources (Mason et al. 2020) to identify
550 candidate loci driving these local adaptations. Furthermore, Horned Larks are one of
551 approximately 100 species of larks (Alaudidae) globally that vary in habitat affiliations.
552 Phylogenetic comparative studies across the family would shed light on whether the
553 patterns we found here are generalizable across broader taxonomic and evolutionary
554 scales. Interactions between an organisms' body surfaces and light from the sun are

555 complex, and our study illustrates how natural selection has shaped the phenotypic
556 variation across different habitats to meet potentially competing demands.

557

558 **Data and Code Availability**

559 Raw data and code used in the analyses presented here are available via GitHub
560 (<https://github.com/mason-lab/HornedLarkCamoThermo>). Raw data and metadata will
561 be made available via Dryad upon article acceptance.

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777

778 **Tables**

779 Table 1: Linear model output for plumage-environment associations. *P*-values with
 780 asterisks indicate statistically significant terms (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

781

Response Variable	Predictor Variable	Beta + SE	t-value	<i>P</i> -value
Dorsal Brightness	Intercept	8420.53 ± 138.09	60.98	<0.001***
	Sex (male)	418.17 ± 192.97	2.17	0.03*
	Soil brightness	18.59 ± 3.58	5.19	<0.001***
	BioClim PC1 (seasonality)	0.17 ± 0.06	2.56	0.01*
	BioClim PC2 (aridity)	4.53 ± 0.38	11.85	<0.001***
	BioClim PC3 (temperature)	5.17 ± 0.92	5.61	<0.001***
Dorsal Solar Reflectance	Intercept	0.34 ± 6.82e-3	49.78	<0.001***
	Sex (male)	1.15e-3 ± 9.53e-3	0.12	0.90
	Soil brightness	1.48e-4 ± 1.77e-4	0.84	0.40
	BioClim PC1 (seasonality)	2.14e-5 ± 3.20e-6	6.67	<0.001***
	BioClim PC2 (aridity)	8.49e-5 ± 1.88e-5	4.50	<0.001***
	BioClim PC3 (temperature)	4.85e-5 ± 4.55e-5	1.06	0.29

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783 Table 2: Non-linear regression analyses for investigating the relationship between the
784 reduction in cooling costs, temperature, and aridity. Slopes and standard errors are
785 presented and correspond to parameters in the exponential function $f(x) = a^{bx}$.

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Temperature model	<i>a</i>	<i>b</i>
Slope ± SE	0.040 ± 0.005	0.097 ± 0.008
<i>t</i> -value	7.529	12.747
<i>p</i> -value	< 0.001	< 0.001

Aridity model		
Slope ± SE	0.1244 ± 0.0117	0.0017 ± 0.0003
<i>t</i> -value	10.636	4.926
<i>p</i> -value	< 0.001	< 0.001

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