

1 **Tinamou egg color displacement at eco-geographical and song space overlap**
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Abstract

The divergence of reproductive traits frequently underpins the evolution of reproductive isolation. Here we investigated the hypothesis that tinamou (Tinamidae) egg coloration functions as a mating signal and its diversification was driven by reinforcement. For many tinamou species, the male guards the nest that is sequentially visited and laid eggs in by multiple females. The colorations of the existing eggs in the nest could signal mate quality and species identities to both the incubating male and the upcoming females, preventing costly hybridization, thus were selected to diverge among species (Mating Signal Character Displacement Hypothesis). If so, two predictions should follow: (1) egg colors should coevolve with known mating signals as the tinamou lineages diverged; (2) species that partition similar eco-geography should display different egg colors. The tinamou songs are important mating signals and are highly divergent among species. We found that the egg color was significantly associated with the first principal component of the song variables. In addition, tinamou species with similar eco-geography tend to display different egg colors, while controlling for song variation among species. Mating signal evolution could be opportunistic and even exploit post-mating trait as premating signals that undergo character displacement in sympatry.

39

Introduction

40 Reproductive trait divergence is crucial for speciation because these traits frequently
41 underpin barriers to gene flow in the onset of speciation (Koski and Ashman 2016; Pfennig
42 2016). A very puzzling reproductive trait divergence exists in tinamou egg coloration.
43 Tinamiformes (common name: tinamou) is the most species-rich order of Palaeognathae,
44 containing 48 extant species (Figure 1) (Cabot 1992; Davies 2002). Tinamous are mostly dull in
45 plumage, but various species of tinamous lay brightly colored eggs ranging from brilliant
46 magenta, to pink, purple, turquoise, and olive green (Figure 1) (Cabot 1992). Despite its
47 evolutionary uniqueness, tinamou egg color divergence remains a mystery due to the cryptic and
48 elusive plumage and behavior of the birds (Cabot 1992; Davies 2002).

49 Two main hypotheses have been made about evolutionary mechanism of egg color
50 divergence: (1) ‘aposematism hypothesis’ (Swynnerton 1916) states that eggs are brightly
51 colored to warn predators of their distastefulness; (2) ‘mating signal hypothesis’, which predicts
52 that egg color are employed for stimulating reproductive investment of the incubating sex
53 (Weeks 1973; Brennan 2010). However, ‘mating signal hypothesis’ (2) did not explain egg color
54 divergence among tinamou species. We further generalize the ‘mating signal hypothesis’ (2) into
55 “mating signal character displacement hypothesis’ (3), in which the egg colors could signal to
56 both sexes for mate recognition and attraction, that could be divergently selected among closely
57 related species in sympatry. The aposematic function of egg coloration was thought to be
58 unlikely, because egg predators are nocturnal mammals or reptiles that prioritize chemical cues
59 over visual cues (Skutch 1966; Cabot 1992; Brennan 2010). The ‘mating signal character
60 displacement hypothesis’ (3) is plausible considering the ecology and mating systems of tinamou
61 species. Although there is limited understanding of tinamou mating systems, for the species of

62 which mating systems were described, males collect and incubate eggs laid by multiple females
63 (Cabot 1992; Davies 2002). An initial female is attracted by distinctive songs to the nest guarded
64 a male. If mating occurs, the female lays eggs in the nest and leaves before other females come
65 and lay more eggs to be incubated by the same male (Cabot 1992; Davies 2002). The colors of
66 the existing eggs in the nest could signal to both the incubating male and the upcoming females.
67 As predicted by the mating signal hypothesis (2), the salient egg colors could stimulate male
68 incubation and reproductive investment, as well as convey intra-specific mate identity. We
69 further suspect that the egg colors could signal to the upcoming females to the focal nest as mate-
70 copying and species-recognition cues. Such alternative “mating signal channel” might be
71 especially favored when the plumage is adapted for camouflage (Cabot 1992; Davies 2002). If
72 egg colors of different tinamou species is adapted for mate recognition, reinforcement
73 (Dobzhansky 1937; Mayr 1942; Liou and Price 1994; Servedio 2000) could have driven egg
74 color divergence among different tinamou species.

75 Here we investigated the ‘mate-signal character displacement hypothesis’ (3) of tinamou egg
76 color divergence. If tinamou egg colors are employed as a species-specific mate recognition
77 signal, it should coevolve with other mate-recognition signals as the tinamou lineages diverged.
78 Songs of tinamous are known to be important and highly divergent mating signals among species
79 (Cabot 1992; Bertelli and Tubaro 2002; Laverde-R. and Cadena 2014; Boesman et al. 2018). In
80 addition to songs, multimodality of mating signals is selected for efficient mate-recognition in
81 complex environment in a number of taxa (Partan and Marler 1999; Rowe 1999; Secondi et al.
82 2015; Halfwerk et al. 2019). When glamorous plumage is costly due to increased predation risk,
83 alternative signal modality such as egg coloration could be favored. Since the birds themselves
84 (instead of the eggs) tend to attract nest predation (Brennan 2010), egg coloration could be

85 employed to fulfill signal multimodality, bypassing the evolutionary constraints for plumage. If
86 the egg colors provide an additional dimension of mating signal, tinamou species with similar
87 songs and ecogeographical range should have different egg colors. To investigate this possibility,
88 here we ask: (1) whether egg coloration coevolved with songs as tinamou species diverged; and
89 (2) whether species that are more likely to have a sympatric history and similar songs display
90 more divergent egg colors?

91

92 **Methods**

93 To investigate the mating signal character displacement hypothesis in tinamou egg color
94 evolution, we examined the association of song, egg colors, and ecoregion co-partitions among
95 tinamou species.

96 **Tinamou egg color**

97 Tinamou egg coloration data was extracted from existing tinamou egg color analysis in
98 an online article (Schläpfer 2017), which quantified coloration of the eggs in RGB color space
99 from 32 tinamou species based on nest photo citizen science submissions. Since tinamou egg
100 colors are known to decay over the course museum storage, tinamou nest photos are used to best
101 represent the functional egg color among species (Schläpfer 2017). Briefly, for each species, the
102 egg coloration reflected in RGB color space was quantified (Schläpfer 2017). To efficiently infer
103 egg color variation among species, we conducted principal component analysis (PCA) of the
104 RGB color space with RGB axes (values centered around zero and scaled to unit variance).

105 Ideally, egg colors should be quantified in the nest in the tetrahedron color space that is
106 consistent with tinamou vision. However, given the difficulty in nest detection in the clade of
107 elusive avian species, Schläpfer (2017) resorted citizen science nest photos and quantified

108 species-specific egg colors in RGB color space. A caveat of this metric is the lack of detection in
109 color variation in the ultraviolet (UV) axis and nonequal-distance conversion to the tetrahedron
110 space. However, this might not be an problem for tinamou visual system, as an earlier study
111 suggested that tinamous likely do not have UV perception since neither the brushland tinamou
112 (*Nothoprocta cinerascens*) nor the Chilean tinamou (*Nothoprocta perdicaria*) had short
113 wavelength sensitive type1 cones (Mullen and Pohland 2008). Further, RGB color metric has
114 been shown to be highly predictive to avian perception ($R^2 = 0.837$) (Bergeron and Fuller 2018)
115 and thus provides an effective and practical way to detect egg color variation among tinamou
116 species given the challenges in egg-searching, curation, and color preservation.

117 To test the validity of between-species variation in the egg color quantification in RGB
118 space from field nest photos (Schläpfer 2017), we compared the within- versus between-species
119 egg color differences with museum egg specimens of four tinamou species: *Crypturellus*
120 *undulatus*, *Eudromia elegans*, *Nothura boraquira*, *Tinamus major*. Photos of egg collections
121 from the Field Museum of Natural History were taken with a standard color reference chart (by
122 Canon EOS 5D Mark IV; Figure S1 B). All images were set to the RGB 16bit mode in
123 Photoshop. All images were white-normalized to the first white patch of the standard. Central
124 areas 3mm away from the edge of each egg were selected, and all reflection, cracked slots and
125 labels were excluded from further quantification. Colors of the remaining central areas were
126 averaged to generate one RGB reading for each egg. Up to four eggs at the most left and top
127 positions were quantified if more than four eggs from a clutch were photographed in one image.
128 Accounting for the egg color variation over time within species, we tested whether there are
129 species-specific egg color clusters with both field nest photo (Schläpfer 2017) and our museum
130 photo measurements for all the four species.

131 **Tinamou song**

132 Song data was acquired from a previous study (Bertelli and Tubaro 2002), in which four
133 song variables from 40 tinamou species were quantified: maximum frequency (Hz), minimum
134 frequency (Hz), emphasized frequency (frequency of the note with highest amplitude in the song,
135 Hz), and bandwidth (the difference between maximum and minimum frequency, Hz). Because
136 these variables are correlated (Figure S2), we used PCA with scaled values to generate PC1 that
137 captured 83% of the variation in song data. Since previous study showed that song covaries with
138 habitat (Bertelli and Tubaro 2002), we have to consider habitat variation in the current study.

139 **Tinamou ecoregion-partitioning**

140 Species ranges were generated according to their occurrences. Geo-referenced
141 occurrences were downloaded from GBIF (GBIF.org (30 September 2021) GBIF Occurrence
142 Download <https://doi.org/10.15468/dl.fqnhrk>) for all tinamou species. Occurrences with
143 uncertainty in coordinates less than 10km were kept, and were further filtered by removing
144 redundant localities within a 1-km² grid cell via R package *ecospat* (Cola et al. 2017) after
145 Lambert azimuthal equal-area projection (ranging 9 – 9413, median = 469). Ecoregion
146 designation for each species was carried out by overlaying occurrences with the ecoregion map
147 of America. Ecoregion maps were downloaded for North America (<https://www.epa.gov>) and
148 South America (<http://ecologicalregions.info>) separately at level I and then were combined as
149 one to include 11 ecoregions for all occurrences. For each species, an ecoregion within which the
150 proportion of occurrences was greater than 10% (or > 50 if the total number > 1000) was
151 included in the species' ecoregion distribution.

152 With this data, we calculated pairwise probability of ecoregion co-partitioning among
153 tinamou species. For each pair of species, the probability of ecoregion co-partitioning is the sum

154 (across all ecoregions) of the multiplication of the probability of species-specific partitioning
155 within each ecoregion. For each species, the probability of partitioning within an ecoregion is the
156 number of points of occurrence within the ecoregion over the total points of occurrence for the
157 species. If the probability of ecoregion co-partitioning is an effective indicator of sympatry or
158 parapatry in the course of tinamou speciation, closely related species should be more likely to co-
159 partition ecoregions, given the limited dispersibility of tinamous (Davies 2002). We employed
160 Mantel Pearson's correlation test with function *mantel.test* in R to test if the species distance
161 matrix is correlated with the ecoregion co-partitioning probability matrix.

162 **Tinamou phylogeny**

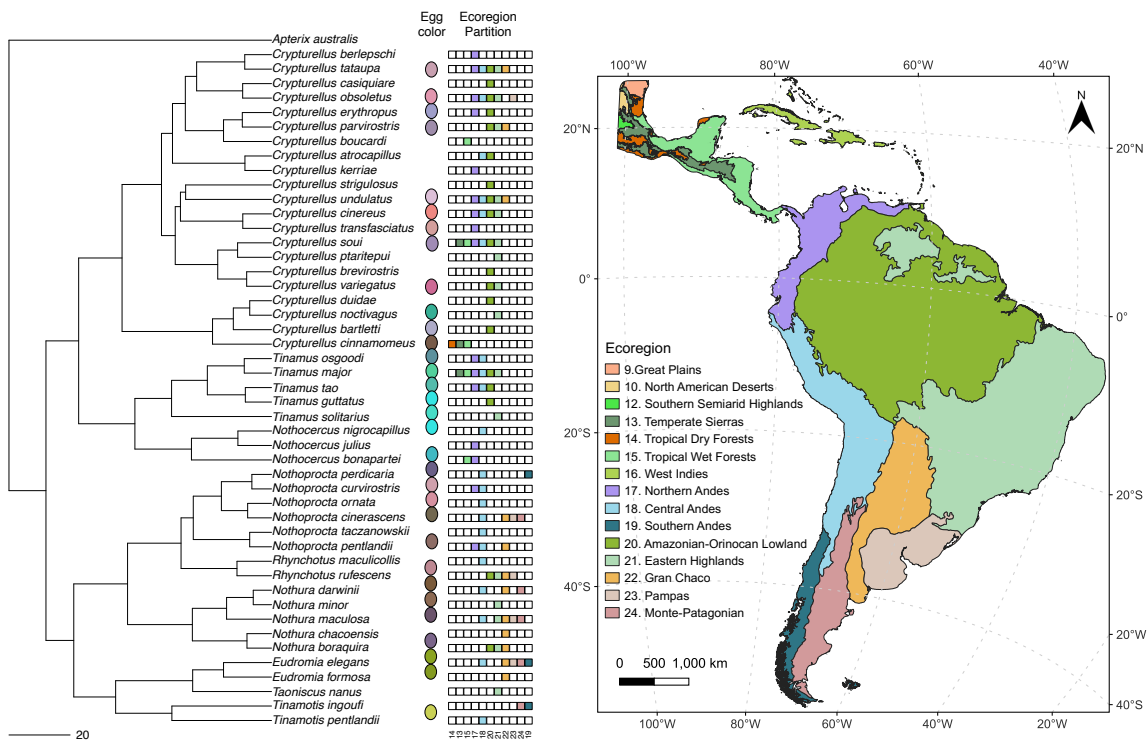
163 To account for phylogenetic uncertainty, we downloaded a phylogeny subset of 500
164 random trees with Hackett backbone for tinamou species from VertLife
165 (<http://vertlife.org/phylosubsets>; Jetz et al. 2012). Additionally, we generated a representative
166 phylogeny based on OneZoom Explorer with branch length as divergence time for visualization
167 (<http://www.onezoom.org>).

168 **Statistical tests**

169 We tested four macroevolutionary associations: (1) egg color and song; (2) song and
170 habitat; (3) egg color and habitat; and (4) egg color displacement at ecoregion and song space
171 (song PC space) co-partitioning among tinamou species. Species-specific habitat type is a
172 variable with “open”, “mixed”, and “close forest” habitat categories acquired from previous
173 study (Bertelli and Tubaro 2002). For (1)-(3), we used *gls* with *corPagel* function in the *ape*
174 package R to account for phylogenetic signals (Paradis and Schliep 2019). For egg color and
175 song variables, we used PC axes that cumulatively explain over 80% of the variation, which
176 resulted in PC1 (54%) and PC2 (38%) for egg color and only PC1 (83%) of song PCA. To

177 account for phylogenetic uncertainty, we used 500 tinamou phylogenies. To correct for multiple
 178 hypotheses testing, we conducted False Discovery Rate correction (Benjamini and Hochberg
 179 1995).

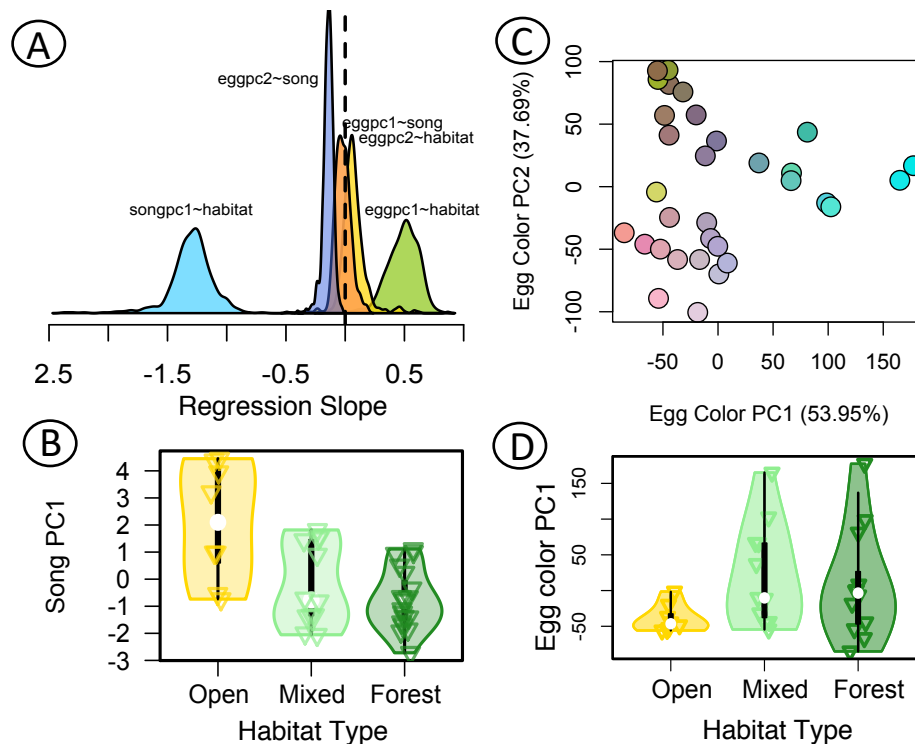
180 Further, we examined whether tinamou egg colors are character displacement trait, by
 181 testing whether there are greater egg color differences between species that are more likely to co-
 182 partition eco-regions. Since song can be a character displacement trait as well, we have to control
 183 for song disparity among species. We first computed the distance matrices of egg color PC1 and
 184 song PC1 respectively with the *dist* function in R. To examine the correlation between the
 185 distance matrix of egg color PC1 and the probability matrix of ecoregion co-partitioning while
 186 controlling for song PC1 distance matrix among species, we used one-tailed Partial Mantel
 187 Pearson's correlation test with 10,000 iterations with the *mantel.partial* function. All the
 188 variables involved in the study is deposited on Dryad (<https://doi.org/10.5061/dryad.rfj6q577g>).



190 **Figure 1. Egg color and ecoregion partition across the Tinamidae phylogeny.** Tinamou
191 ecoregion and song space co-partitioning is significantly associated with egg color difference
192 among species, with greater color divergence among species that partition similar ecoregions
193 after controlling for song variation (partial mantel test, $p < 0.05$). The Tinamidae phylogeny
194 (with *Apterix australis* as the outgroup species) was inferred from OneZoom Explorer
195 (<http://www.onezoom.org>). The Lambert azimuthal equal-area projection was applied for
196 ecoregion map.
197

198 Results

199 Egg color is significantly associated with song and habitat variation among tinamou
200 species (Figure 2 A). The between-species distinction overcomes the within-species variation in
201 egg color variation (Figure S1). Ecogeographic similarity predicts egg color distance among
202 species, after controlling for song variation among tinamou species (Figure 1).



203 **Figure 2. Association of habitat type, song variation, and egg color among tinamou species.**
204 (A) The distribution of regression slope between response variables and predicting variables
205 derived from 500 tinamou phylogenies (Table 1). Three distributions of regression slope were
206 significantly deviated from zero: (1) between song PC1 and habitat type (B), (2) between egg
207 color PC1 (C) and habitat (D), as well as (3) between egg color PC2 and song PC1 (A). (B)
208 Phylogenetic analysis reveals significant association between song PC1 and habitat type among
209 tinamou species, consistent with a previous study (Bertelli and Tubaro 2002). (C) Egg color PC1
210

211 and PC2: the dot color represents the egg RGB color of each species. **(D)** Egg color PC1 is
 212 significantly associated with habitat types among tinamou species.
 213

214 Egg color is associated with song and habitat variation specifically (Figure 2). Among 32
 215 tinamou species, PC1 reflects the warm-to-cold gradient of egg colors, while PC1 reflects the
 216 brightness (Figure 2 C). There was significant association between egg color PC2 and song PC1
 217 (Table 1; Figure 2 A). The relationship is unlikely confounded by habitat types among tinamou
 218 species, as egg color PC1, but not egg color PC2, was significantly associated with habitat types
 219 (Table 2; Figure 2 C-D). The open field tinamou species tend to lay warm-colored eggs, while
 220 there was a wider variation in egg colors (from pink to greenish blue) among the closed forest
 221 tinamou species (Figure 2 D).

222
 223 **Table 1 Phylogenetic regression models investigating the associations among egg color,**
 224 **song, and habitat types across tinamou species.** For each pair of variables, a phylogenetic
 225 regression model was run with each of the 500 tinamou phylogenies to account for phylogenetic
 226 uncertainty. The significant (0 falls out of the 95% confidence interval, CI) intercepts (b_0) and
 227 slopes (b_1) were in bold.

Response variable	Predictor	mean b_1	95% CI b_1	mean b_0	95% CI b_0	R ²
egg color PC1	song PC1	-0.017	(-0.118, 0.128)	-0.007	(-0.274, 0.265)	0.008
	habitat	0.483	(0.151, 0.693)	-1.074	(-1.546, -0.353)	0.119
egg color PC2	song PC1	-0.144	(-0.244, -0.072)	0.157	(0.034, 0.278)	0.069
	habitat	0.071	(-0.090, 0.392)	-0.060	(-0.750, 0.259)	0.010
song PC1	habitat	-1.297	(-1.729, -0.990)	2.929	(2.175, 3.861)	0.383

228
 229
 230 Ecoregion co-occurrence is likely an effective indicator of parapatry or sympatry in
 231 history of tinamou speciation, as closely related species tends to co-occur at ecoregions (Mantel
 232 $Z = 1530.38, p = 0.001$). We further observed greater egg color divergence between species that
 233 co-partition ecoregions after controlling for song variation among tinamou species. The PC1
 234 explains 54% variation of egg colors in the RGB color space. The distance of egg color PC1 is

235 significantly positively associated with the ecoregion co-partitioning among species controlling
236 for song distance among tinamou species (Partial Mantel $r = 0.066$, $p = 0.048$).

237
238

Discussion

239 The macroevolution relationship among egg color disparity, song variation, and habitat
240 and ecoregion partitioning, is consistent with the ‘Mating Signal Character Displacement
241 Hypothesis’ that tinamou egg coloration is an alternative mating signal that is divergently
242 selected among species with similar ecogeographical partition (Weeks 1973; Brennan 2010). Egg
243 colors and songs could be divergently selected as multimodal mate-recognition signals among
244 tinamou species with similar appearance that partition similar ecogeographical range. When the
245 plumage modality is constrained by anti-predation adaptation, egg coloration could be
246 opportunistically adopted to fulfill mating signal multimodality for species recognition in
247 sympatry. This study sheds light on the evolution of multimodal sexual signals that bypasses
248 natural selection for plumage camouflage in the most species-rich order of Palaeognathae.

249 Egg coloration could be both a pre- and post-mating signals in tinamous. In many other
250 bird species, egg color is known to be post-mating signals indicating female quality and can
251 influence male incubation and promiscuity (Soler et al. 2005; English 2009). This is consistent
252 with the Mating Signal Hypothesis in which egg colors could stimulate tinamou male incubation
253 and parental care (Weeks 1973; Brennan 2010). In addition, egg coloration in the nest can be
254 premating signals received by upcoming females. Premating mate-recognition signals among
255 females that mate with the same male could be selected if it enhances the offspring fitness by
256 protecting the reproductive investment of the parental care provider (male tinamous) from costly
257 heterospecific mating interference (Gröning and Hochkirch 2008).

258 Although the mating systems are not well-understood for elusive tinamous, in the species
259 with mating system records, males guard and incubate the eggs laid by multiple females (Cabot
260 1992). The coloration of the existing eggs in the nest could be a mating signal received by
261 upcoming females. As premating signals, egg colors could reflect mate species-identity and
262 quality (Sætre et al. 1997; Servedio and Noor 2003; Secondi et al. 2015), as well as stimulate
263 female mate choice copying (Dugatkin 1992; Gibson and Höglund 1992).

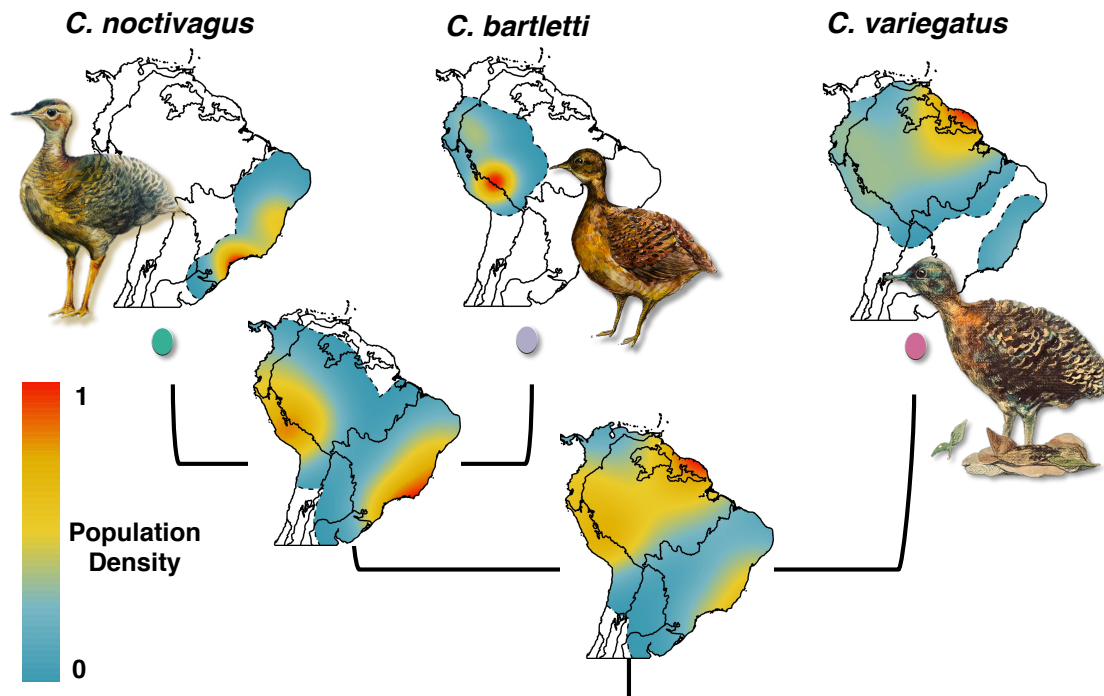
264 Then why do tinamous adopt egg colors as mating signals in addition to songs? Songs
265 that are important mating signals in tinamous (Bertelli and Tubaro 2002) and are involved in
266 duetting between mating partners (Boesman et al. 2018). However, such acoustic modality is
267 usually insufficient for mate communication in complex environments, thus signal redundancy or
268 multimodality is further selected for mate communication to alleviate mate-searching effort
269 and/or hybridization (Partan and Marler 1999; Rowe 1999; Uy et al. 2008; Secondi et al. 2015).
270 Plumage patterning and coloration are frequently adopted as inter-specific and intra-specific
271 mating signals at a finer spatial scale in birds (Uy et al. 2008, 2009; Seddon et al. 2013).
272 However, most tinamou species exhibited cryptic plumage as an adaption for anti-predatory
273 camouflage (Cabot 1992; Davies 2002). Sexual selection for mating signal elaboration would
274 compromise the adaptation for camouflage favored by natural selection (Heinen-Kay et al.
275 2015). Egg coloration can be an alternative modality of mate signaling enrichment towards mate-
276 searching refinement, bypassing the conflict between natural and sexual selection. This idea is
277 supported by the fact that egg color brightness is correlated with song variation among species
278 (Table 1, Figure 2 A-B).

279 Reinforcement may have driven egg color and song divergence among sympatric or
280 parapatric tinamou species. Many closely-related tinamou species demonstrate historical and/or

281 contemporary ecoregion overlap (Cabot 1992) (Figure 1). Ecological and/or intrinsic
282 incompatibility among diverged lineages leads to reduced hybrid fitness, which in turn select for
283 premating signal divergence to avoid costly hybridization (Dobzhansky 1940; Mayr 1942; Liou
284 and Price 1994; Servedio 2000). Mating signal multimodality is needed to ensure mate
285 recognition in complex heterospecific environment (Uy et al. 2008; Secondi et al. 2015). The
286 divergence of tinamou song and egg coloration could jointly reduce heterospecific reproductive
287 efforts among sympatric tinamou species. Closely related tinamou species that are mostly
288 allopatric at present but could still harbor footprints of historical character displacement of egg
289 colors formed at historical sympatry either over secondary contact or sympatry speciation.
290 Notably, the species with greater likelihood of ecoregion co-occurrence tend to display divergent
291 egg colors (Figure 1). For example, the closely related species *Crypturellus noctivagus*, *C.*
292 *bartletti*, *C. variegatus* still partition similar ecoregions and demonstrate distinct egg coloration,
293 which may have been driven by reinforcement at historical sympatry (Figure 3). The association
294 of egg color and habitat types among tinamou species (Table 1, Figure 2 AD) further indicates
295 residual divergent selection on egg colors in the speciation history. Although the speciation event
296 among tinamou species have long passed (> 7 million years), the likelihood of ecoregion co-
297 occurrence is potentially an effective indicator of historical sympatry/parapatry in the history of
298 tinamou speciation because the closely related species tend to share ecoregions.

299 Although we found significant supports for both predictions of the Mating Signal
300 Character Displacement Hypothesis, the significance levels were marginal. This indicates that
301 there are other potential evolutionary forces shaping tinamou egg color divergence. Besides the
302 local tinamou species assembly, other complex aspects of abiotic and biotic interactions such as
303 sexual conflicts, predation, and parasitism, might contribute to the egg color signal diversity.

304



305
306 **Figure 3 Reconstructed ancestral distribution of three *Crypturellus* species.** Black lines
307 depict the boundaries of ecoregions corresponding to Figure 1, and dashed lines depicted
308 boundaries of species' distribution ranges. Ranges of tips were concave hull polygons generated
309 via R package *rangemap* (Cobos et al. 2021) with a hierarchical clustering method, and kernel
310 densities were estimated via R package *spatstat* (Adrian Baddeley, Ege Rubak 2015), to capture
311 the spatial configuration of the occurrences. Distributions of internal nodes were generated by
312 the same method with an intermediate step of rescaling densities (at a 100-km-resolution) of all
313 descendants to offset the effect of variation in abundance. Blue to red colors depict the relative
314 scale of kernel densities from low to high (ranging 0 to 1).
315

316 The genetic underpinning of such song and egg color association is unclear. The simplest
317 genetic mechanism underlying such association is pleiotropy (one gene affecting multiple traits)
318 (Fisher 1930; Williams 1957). For example, the pleiotropic *foraging* gene underpins the
319 association among social behavior and life history traits in natural populations (de Belle et al.
320 1989; Mery et al. 2007; Wang and Sokolowski 2017). There might be an omnipotent pleiotropic
321 gene linking egg coloration and songs, among many other traits. If so, a wide array of traits
322 underpinned by such pleiotropy are expected to coevolve. However, the association between

323 song and egg color of tinamous is specific: only brightness egg color is associated with tinamou
324 songs (Table 1, Figure 2 A-B). Such specificity in song-egg-color association is consistent with
325 mating signal multimodality in which specifically signals were coupled among modalities
326 (Gilliard 1956; Partan and Marler 1999; Hebets and Papaj 2005). Various signal modality can be
327 underpinned by different genes and selected to coevolve as multimodal signals. Future
328 investigation of genetic underpinnings of tinamou song and egg colors would further shed light
329 on this interesting co-divergence. In sum, the results presented herein are concordant with the
330 hypothesis that egg coloration in tinamous serves as mating signals, for mate attraction and/or
331 mate species-recognition and could be divergently selected upon historical sympatry/parapatry.

332

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339

340 **Conflict of interest**

341 There is no conflict of interest in this study.

342

343 **Reference**

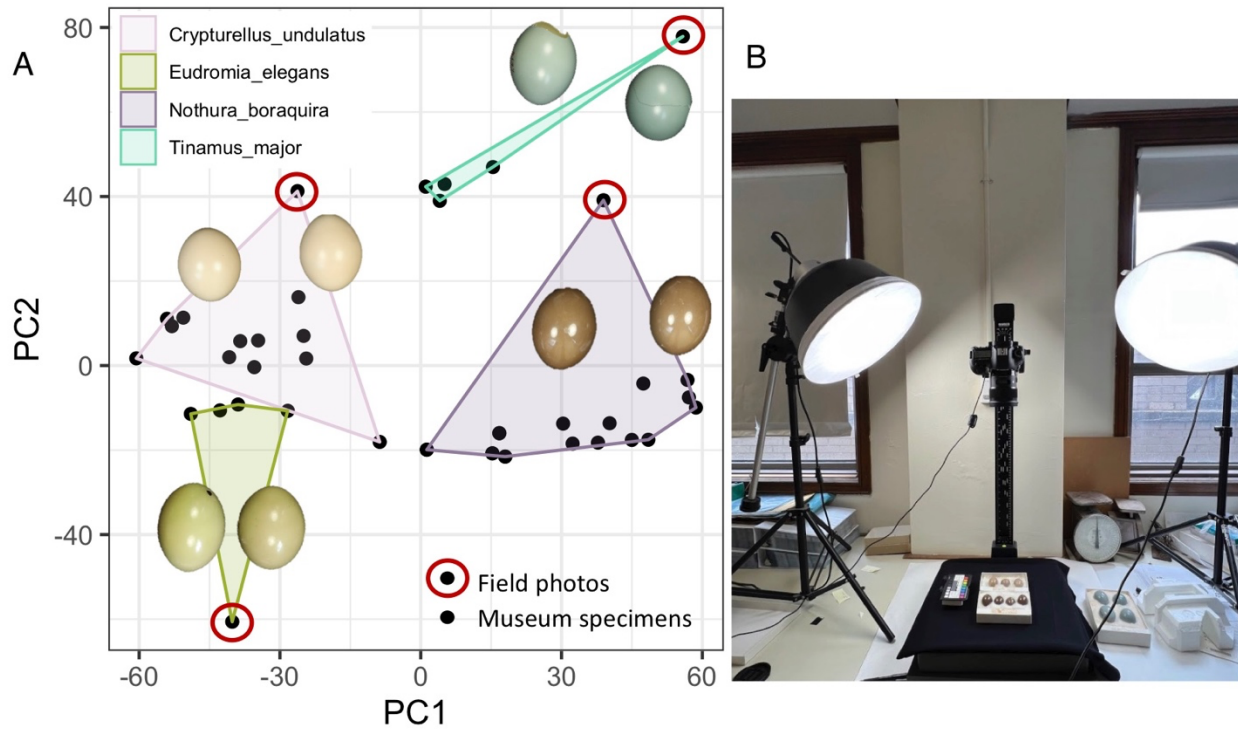
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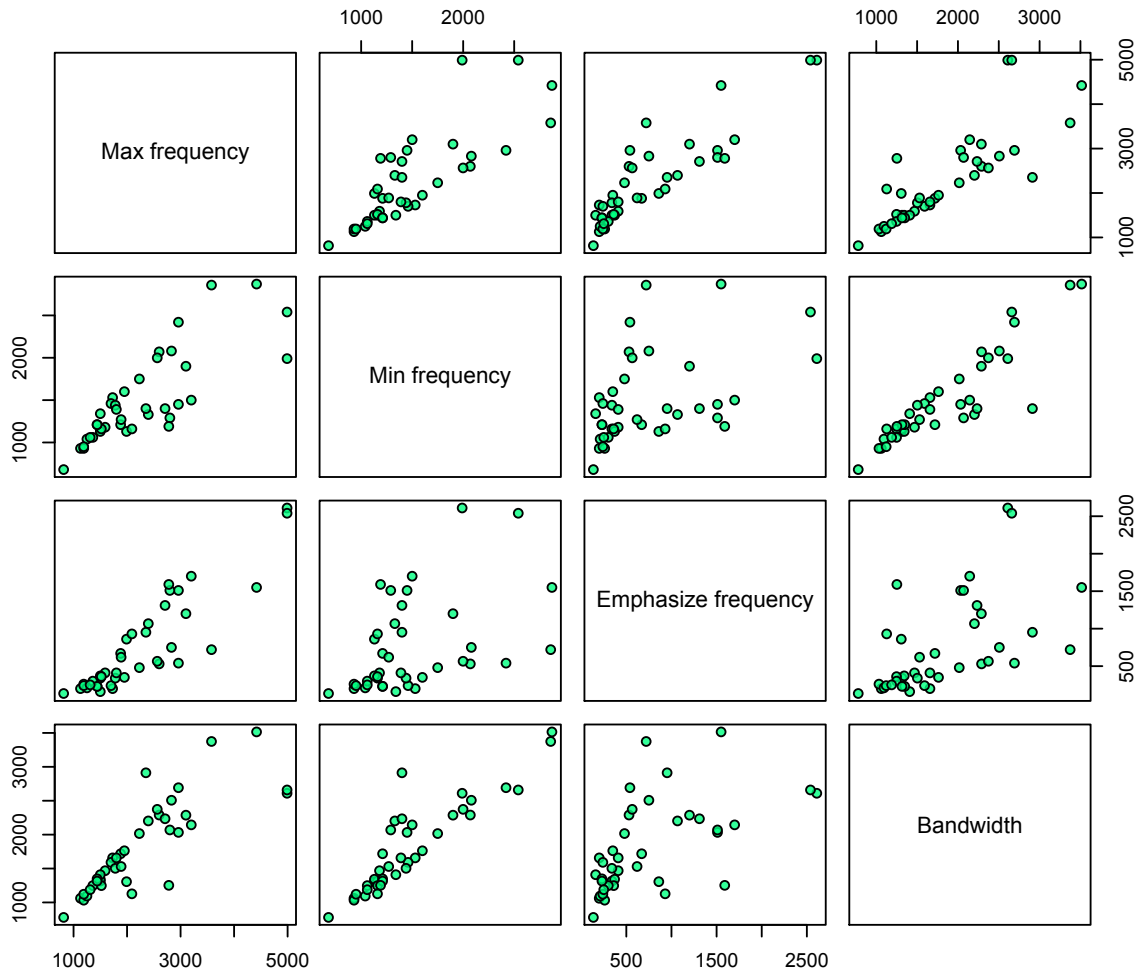
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450 **Supplementary information**



451
452 **Figure S1 Fisher Discriminant Analysis of egg color.** A, Egg colors were quantified in RGB
453 space from field nest photos and museum collection within and between tinamou species.
454 Although there is variation within species and between field and museum RGB quantification,
455 the between-species difference is distinctive. B, Lighting setup at which the museum egg photos
456 were taken.
457



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459

Figure S2 Scatterplots showing correlations among the four song variables.