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

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Abstract

23

24   The divergence of reproductive traits frequently underpins the evolution of reproductive

25 isolation. Here we investigated the hypothesis that tinamou (Tinamidae) egg coloration functions

26 as a mating signal and its diversification was driven by reinforcement. For many tinamou

27 species, the male guards the nest that is sequentially visited and laid eggs in by multiple females.

28 The colorations of the existing eggs in the nest could signal mate quality and species identities to

29 both the incubating male and the upcoming females, preventing costly hybridization, thus were

30 selected to diverge among species (Mating Signal Character Displacement Hypothesis). If so,

31 two predictions should follow: (1) egg colors should coevolve with known mating signals as the

32 tinamou lineages diverged; (2) species that partition similar eco-geography should display

33 different egg colors. The tinamou songs are important mating signals and are highly divergent

34 among species. We found that the egg color was significantly associated with the first principal

35 component of the song variables. In addition, tinamou species with similar eco-geography tend

36 to display different egg colors, while controlling for song variation among species. Mating signal

37 evolution could be opportunistic and even exploit post-mating trait as premating signals that

38 undergo character displacement in sympatry.

Introduction

39

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 citzen science nest photos and quantified

108   species-specific egg colors in RBG color space. A caveat of this metric is the lack of detection in

109   color variation in the ultraviolet (UV) axis and noneuqal-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-km^2 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.

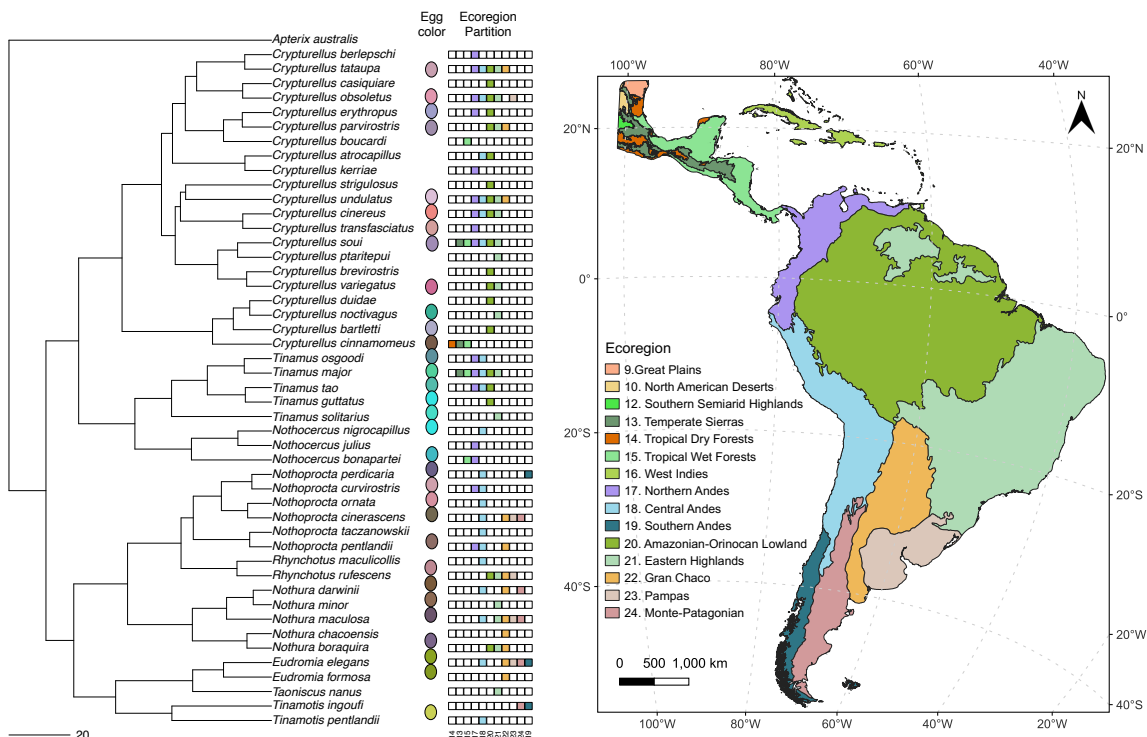162   **Tinamou phylogeny**

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).

189

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

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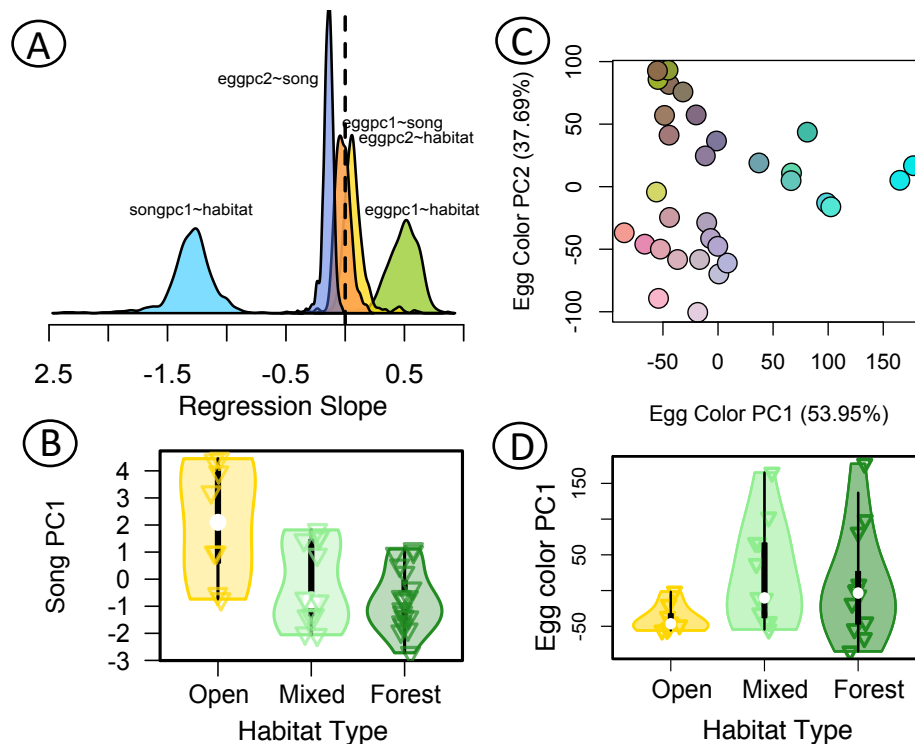
203
204 **Figure 2. Association of habitat type, song variation, and egg color among tinamou species.**
205 (**A**) The distribution of regression slope between response variables and predicting variables
206 derived from 500 tinamou phylogenies (Table 1). Three distributions of regression slope were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

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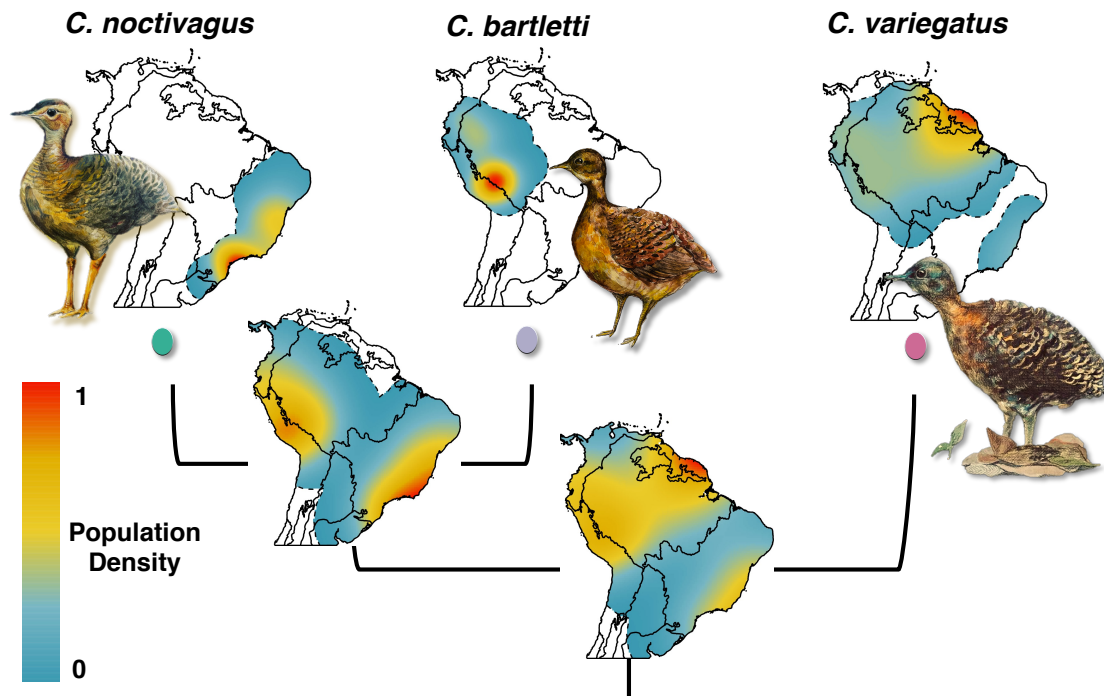
Figure 3 Reconstructed ancestral distribution of three *Crypturellus* species. Black lines depict the boundaries of ecoregions corresponding to Figure 1, and dashed lines depicted boundaries of species' distribution ranges. Ranges of tips were concave hull polygons generated via R package *rangemap*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

The genetic underpinning of such song and egg color association is unclear. The simplest genetic mechanism underlying such association is pleiotropy (one gene affecting multiple traits) (Fisher 1930; Williams 1957). For example, the pleiotropic *foraging* gene underpins the association among social behavior and life history traits in natural populations (de Belle et al. 1989; Mery et al. 2007; Wang and Sokolowski 2017). There might be an omnipotent pleiotropic gene linking egg coloration and songs, among many other traits. If so, a wide array of traits 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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338   comments on the manuscript.

339

340   **Conflict of interest**

341   There is no conflict of interest in this study.

342

343   **Reference**
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450   **Supplementary information**
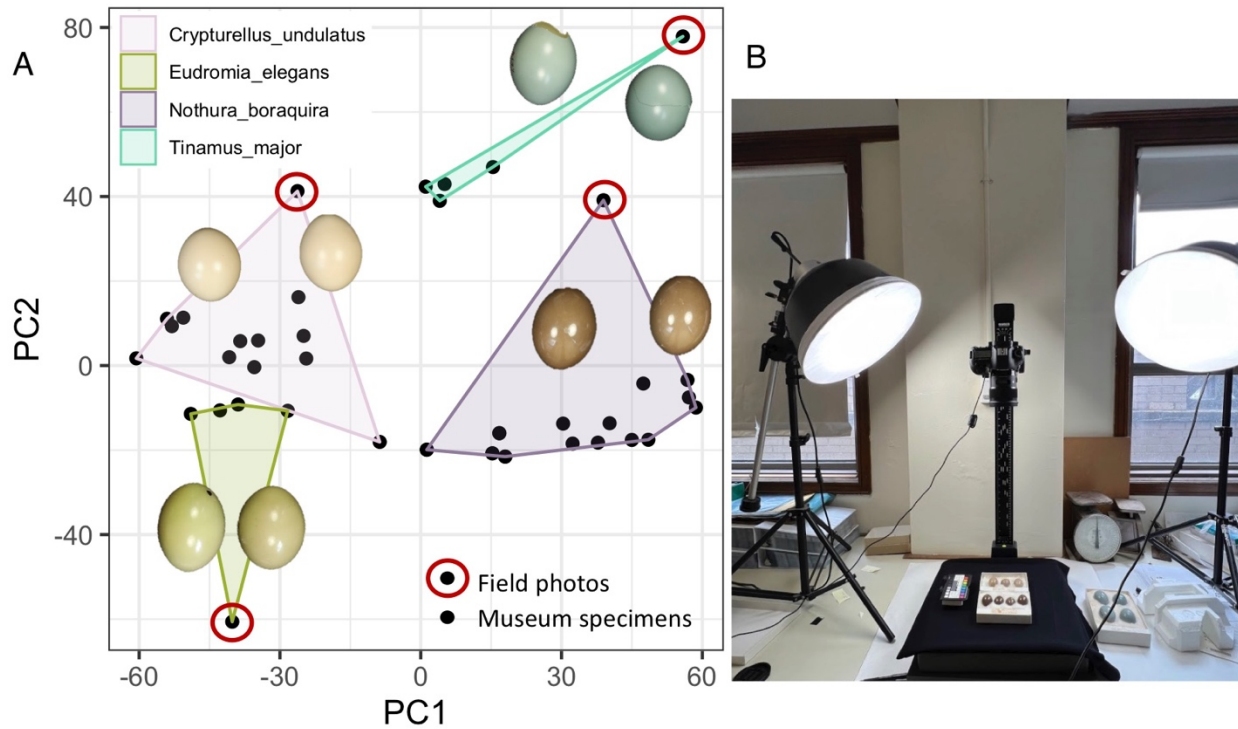
451
452   **Figure S1 Fisher Discriminant Analysis of egg color**. **A**, Egg colors were quantified in RGB
453   space from field nest photos and museum collection within and between tinamou species.
454   Although there is variation within species and between field and museum RGB quantification,
455   the between-species difference is distinctive. **B**, Lighting setup at which the museum egg photos
456   were taken.
457
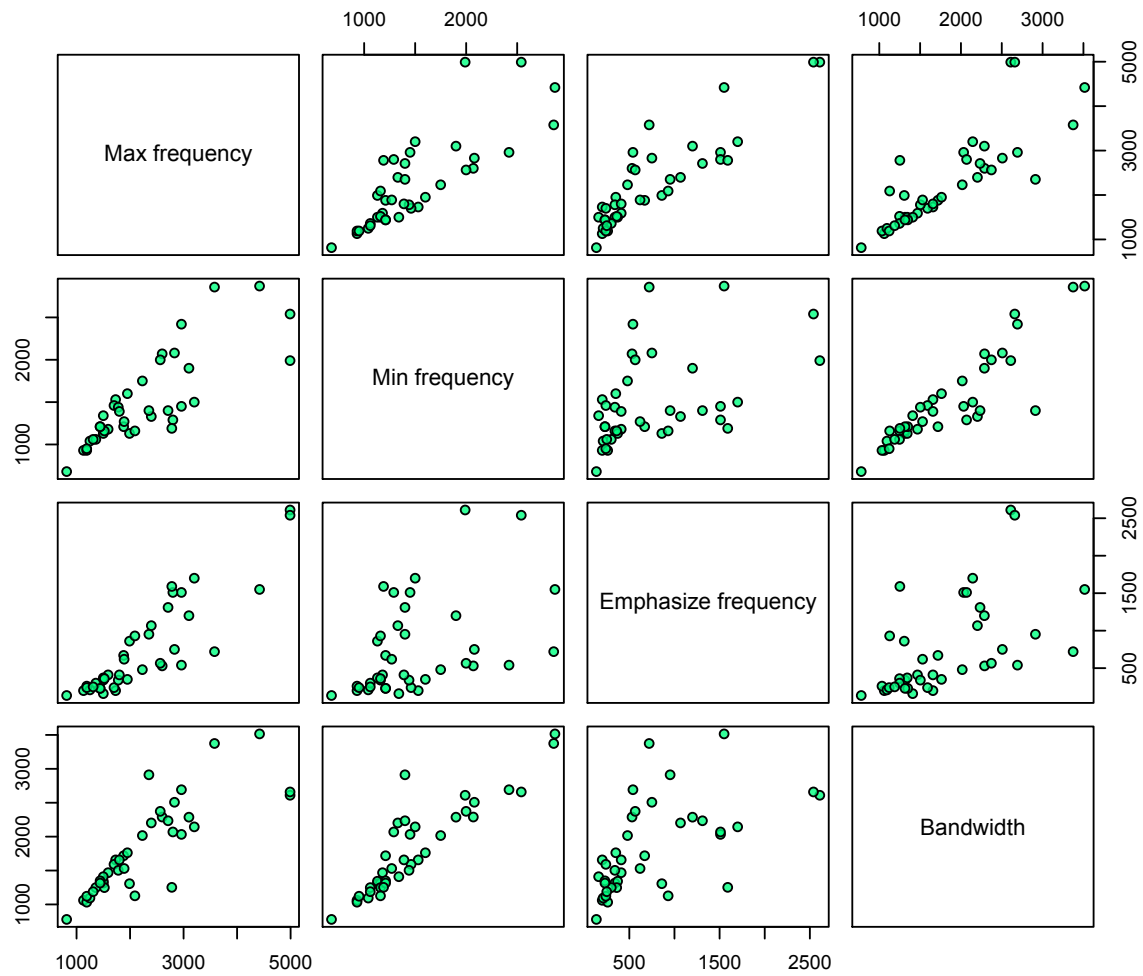
458
459   **Figure S2 Scatterplots showing correlations among the four song variables**.