

1 **Two Genomic Loci Control Three Eye Colors in the Domestic Pigeon (*Columba livia*)**
2

3 **Authors:** Emily T. Maclary¹, Bridget Phillips¹, Ryan Wauer¹, Elena F. Boer¹, Rebecca Bruders¹,
4 Tyler Gilvarry¹, Carson Holt², Mark Yandell², and Michael D. Shapiro¹
5

6 **Affiliations:**

7 ¹School of Biological Sciences, University of Utah, Salt Lake City, UT 84112, USA

8 ² Department of Human Genetics and USTAR Center for Genetic Discovery, University of Utah,
9 Salt Lake City, UT, USA

10

11 *Author for Correspondence:

12 Michael D. Shapiro, School of Biological Sciences, 257 South 1400 East, Salt Lake City, UT
13 84112 USA; phone +1 801 581 5690; email: mike.shapiro@utah.edu

14 **ABSTRACT**

15 The iris of the eye shows striking color variation across vertebrate species, and may play
16 important roles in crypsis and communication. The domestic pigeon (*Columba livia*) has three
17 common iris colors, orange, pearl (white), and bull (dark brown), segregating in a single species,
18 thereby providing a unique opportunity to identify the genetic basis of iris coloration. We used
19 comparative genomics and genetic mapping in laboratory crosses to identify two candidate
20 genes that control variation in iris color in domestic pigeons. We identified a nonsense mutation
21 in the solute carrier *SLC2A11B* that is shared among all pigeons with pearl eye color, and a
22 locus associated with bull eye color that includes *EDNRB2*, a gene involved in neural crest
23 migration and pigment development. However, bull eye is likely controlled by a heterogeneous
24 collection of alleles across pigeon breeds. We also found that the *EDNRB2* region is associated
25 with regionalized plumage depigmentation (piebalding). Our results establish a genetic link
26 between iris and plumage color, two traits that were long known by pigeon breeders to co-occur,
27 and demonstrate the importance of gene duplicates in establishing possibilities and constraints
28 in the evolution of color and color pattern among vertebrates.

29

30 **Keywords:**

31 comparative genomics, QTL mapping, pigeon, iris color, pigment

32 INTRODUCTION

33 A wide variety of genetic and developmental mechanisms influence diversity in pigment
34 type and patterning in the vertebrate epidermis, including epidermal appendages such as hair
35 and feathers (Hoekstra 2006; Kelsh et al. 2008; Hubbard et al. 2010; Kaelin and Barsh 2013;
36 Domyan et al. 2014; Parichy and Spiewak 2014; Bruders et al. 2020; Inaba and Chuong 2020).
37 Pigments are also deposited in non-epidermal tissues in vertebrates, including the iris of the
38 eye. Among vertebrate species, iris color varies widely. Some species have conspicuously
39 colored bright yellow, red, or white irises, while others have dark irises. Iris coloration may be an
40 adaptive trait that, like epidermal coloration, plays roles in crypsis and communication. For
41 example, iris color is correlated with habitat in mantellid frogs, with arboreal species more likely
42 to have bright eyes (Amat et al. 2013). Bright irises probably evolved multiple times in arboreal
43 mantellid species, indicating that this trait might be adaptive. There is evidence that iris color
44 may be adaptive in birds as well. In owls, dark iris color likely coevolved with nocturnal behavior
45 (Passarotto et al. 2018), while the bright white irises of jackdaws communicate that nesting sites
46 are occupied (Davidson et al. 2014; Davidson et al. 2017).

47 The genetic and developmental origins of variation in iris pigmentation are poorly
48 understood. While iris color varies widely among species, variability in iris color is limited within
49 most species (Negro et al. 2017). However, intraspecific variation in iris color is widespread in
50 humans and certain domestic species (Negro et al. 2017). In mammals, iris colors typically
51 include shades of brown, green, and blue. These colors all arise from varying concentrations
52 and deposition patterns of melanin pigments in the iris (Edwards et al. 2016). In contrast, the
53 diversity of eye colors in amphibians and birds also depends on the presence of non-melanin
54 pigments. In birds, brilliant reds, oranges, and yellows arise from multiple non-melanin pigment
55 types, including pteridines, purines, and carotenoids (Oliphant 1987a).

56 The domestic pigeon, *Columba livia*, shows intraspecific iris color variation among its
57 300+ different breeds. This variation, coupled with extensive genetic resources, makes the
58 pigeon an ideal model to understand the genetics of iris pigmentation. Pigeons have three main
59 iris colors: orange, pearl (white), and bull (dark brown; Fig. 1A). Orange iris color is the ancestral
60 state (Bond 1919), and “orange” eyes in actuality range in shades from yellow to red, depending
61 on the density of blood vessels in the eye (Hollander and Owen 1939; Sell 2012). The pearl iris
62 color is white, with tinges of pink and red from blood vessels. Lastly, the bull iris color is named
63 based on the similarity in color to dark bovine eyes, and ranges from dark brown to almost black
64 (Hollander and Owen 1939; Levi 1986). Breeding experiments show that the switch between
65 orange and pearl eye color is controlled by a single autosomal locus, and that orange is

66 dominant to pearl (Bond 1919). Less is known about the inheritance of bull eye color. While
67 orange and pearl irises can be found in a variety of pigeon breeds, the bull iris color is primarily
68 found in birds with white plumage (Hollander, 1939). Breeders have also reported birds with a
69 phenotype known as “odd eyes” (Levi 1986; Sell 2012), where one iris is a dark bull color and
70 the other is either orange or pearl, suggesting that bull eye color may have a stochastic
71 component.

72 Pigeons have two types of non-melanin pigments in the iris, guanidines and pteridines
73 (Oliphant 1987a). Guanidines are whitish opaque pigments, and pteridines are yellow-orange
74 pigments (Oliphant 1987b). In orange-eyed pigeons, both guanidine and pteridine pigments are
75 present in the iris stroma, while in white-eyed pigeons, only guanidine pigment is present
76 (Oliphant 1987b; Oliphant 1987a). In bull-eyed pigeons, both white and orange pigments are
77 absent from the iris stroma, so the underlying dark melanin pigment of the iris pigment
78 epithelium is not obscured (Bond 1919; Oliphant 1987b). The genetic and developmental
79 mechanisms underlying loss of pteridine iris pigment in pearl-eyed birds or both pteridine and
80 guanidine pigment in bull-eyed birds are currently unknown. Loss could arise from defects in
81 pigment production or failure to transport the pigment into the iris stroma, for example.

82 To better understand the genetic mechanisms that control iris color in domestic pigeons,
83 we used a combination of genomic mapping and laboratory crosses to identify two loci that are
84 associated with eye color. We identified a nonsense mutation that segregates with pearl eye
85 color and a second locus associated with bull eye color. We also found a genetic link between
86 iris and feather color in birds with an array of plumage depigmentation phenotypes collectively
87 known as piebalding, thereby establishing a genetic link that explains the anecdotal co-
88 occurrence of iris and plumage color traits.

89

90 RESULTS

91 A single genomic locus is associated with pearl eye color in the domestic pigeon.

92 To determine the genetic basis of pearl eye color, we compared whole-genome
93 sequences from a diverse panel of orange-eyed ($n = 28$ from 17 domestic breeds and feral
94 pigeons) and pearl-eyed pigeons ($n = 33$ from 25 breeds and ferals) using a probabilistic
95 measure of allele frequency differentiation (pF_{ST} ; Domyan et al. 2016; Fig. 1B). We identified a
96 single, 1.5-megabase genomic region on scaffold ScoHet5_1307 that was significantly
97 differentiated between orange-eyed and pearl-eyed birds (ScoHet5_1307:1490703-3019601,
98 genome wide significance threshold $pF_{ST} = 5.4 \times 10^{-10}$).

99 **The pearl eye locus segregates with eye color in F₂ crosses**

100 To confirm the association between the ScoHet5_1307 locus and pearl eye color, we
101 turned to quantitative trait locus (QTL) mapping in an F₂ intercross between an orange-eyed
102 Archangel and a pearl-eyed Old Dutch Capuchin. Within this cross, F₂ birds had either two pearl
103 eyes (n=12), one pearl eye and one bull eye (n=1), two orange eyes (n=40), or one orange eye
104 and one bull eye (n=5). We used a binary QTL model to compare birds with at least one pearl
105 eye to birds with at least one orange eye, and identified a single peak on linkage group 20
106 associated with eye color (Fig. 1C, peak marker ScoHet5_1307 : 1090556; peak LOD score =
107 13.28; candidate region, defined as a 2-LOD interval from the peak marker, spans
108 ScoHet5_149:3706619 - ScoHet5_1307:1911647). The genotype at peak marker
109 ScoHet5_1307:1090556 is perfectly associated with eye color in the cross, with all pearl-eyed F₂
110 birds homozygous for the pearl-eyed Capuchin allele (Fig. 1D). We additionally used targeted
111 genotyping in F₂ individuals from a cross between Racing Homer and Parlor Roller breeds; this
112 cross had four founders, one or more of which was heterozygous for the pearl allele so QTL
113 mapping was impractical. We instead used PCR and Sanger sequencing to genotype a single
114 nucleotide polymorphism (SNP) within the pearl-eye haplotype (ScoHet5_1307:1901234). This
115 SNP again showed perfect association with the pearl eye phenotype (n = 25 F₂ birds; *p* = 2.24 x
116 10⁻⁷, Fisher's exact test). Thus, two independent approaches converged on the same genomic
117 region controlling pearl eye.

118

119 **Pearl-eyed birds harbor a premature stop codon in solute carrier SLC2A11B.**

120 Because pearl eye color is recessive to orange, we searched for SNPs within the
121 overlapping pF_{ST} and QTL peak region to identify polymorphisms where pearl-eyed birds were
122 always homozygous for the reference allele (the Danish tumbler pigeon sequenced for the
123 Cliv_2.1 reference assembly had pearl eyes; (Shapiro et al. 2013; Holt et al. 2018; Fig. S1A).
124 We identified 20 SNPs spanning a 22-kb region (ScoHet5_1307:1895934-1917937) that
125 showed the expected segregation pattern between orange-eyed and pearl-eyed birds, three of
126 which were in protein coding regions, while 17 were intronic or intergenic (Fig. 1E). To evaluate
127 variants in the pearl eye candidate locus, we first assessed the predicted impact of the three
128 coding mutations identified in pearl-eyed birds. Two of these coding mutations are in
129 SLC2A11B, a predicted solute carrier, while the third is in CHEK2, a kinase required for
130 checkpoint-mediated cell-cycle arrest in response to DNA damage (Hirao et al. 2000; Chen et
131 al. 2005). The coding mutation in exon 13 of CHEK2 (position ScoHet5_1307:1917937) results
132 in a synonymous substitution. CHEK2 is not known to play a role in pigmentation or pteridine

133 deposition; thus, this substitution is unlikely to have an effect on protein function or eye color
134 phenotype.

135 The function of the second gene harboring coding mutations, *SLC2A11B*, is not well
136 characterized, but is predicted to be a solute carrier. The first coding mutation in *SLC2A11B*
137 (Position ScoHet5_1307: 1895934) changes a tryptophan (orange allele) to a premature stop
138 codon (pearl allele; Fig. 1F). The second coding mutation (position ScoHet5_1307:1896042)
139 results in a synonymous substitution 36 amino acids downstream of the premature stop codon.
140 Unlike *CHEK2*, *SLC2A11B* is a strong candidate gene for pearl eye color. This gene has
141 orthologs in fish and sauropsids, but not in mammals. Data from fish orthologs suggests that
142 *SLC2A11B* plays a role in pigmentation. In medaka, for example, *SLC2A11B* is involved in the
143 differentiation of pteridine-containing leucophore and xanthophore cells in scales (Kimura et al.
144 2014). The most closely related mammalian gene appears to be *SLC2A11 (GLUT11)*, a glucose
145 transporter (Doege et al. 2001; Kimura et al. 2014).

146 The premature stop codon in pearl-eyed pigeons falls in exon 3 of *SLC2A11B*, which is
147 predicted to severely truncate the resulting protein from 504 to 57 amino acids. Translation
148 initiation at the next in-frame methionine would produce a protein missing the first 95 amino
149 acids, but with the remaining 81% (409 out of 504 amino acids) of the protein intact. To predict if
150 such a truncated protein would be functional, we used InterProScan (Zdobnov and Apweiler
151 2001) and Phobius (Käll et al. 2004) to predict transmembrane domains and conserved
152 functional motifs within the *SLC2A11B* protein, and examined sequence similarity across
153 species (Fig. S1 B-D). The first 94 amino acids of *SLC2A11B* are predicted to code for two
154 transmembrane domains that are highly conserved; removing these domains is predicted to be
155 detrimental to protein function (PROVEAN score of -189.145; Choi et al. 2012; Choi and Chan
156 2015). Therefore, the pearl mutation, which results in a loss of pteridines in the iris, is predicted
157 to truncate a highly-conserved protein that is associated with the differentiation of pteridine-
158 containing pigment cells. The first two transmembrane domains of the *SLC2A11B* protein are
159 highly conserved across species, yet we identified orthologs in two bird species, hooded crow
160 [NCBI accession XP_019140832.1] and wire-tailed manakin [NCBI accession
161 XP_027569903.1], in which the annotated protein sequence is missing the first of these
162 transmembrane domains. While we cannot rule out a misannotation in these genomes, neither
163 species appears to have yellow-orange iris pigment. Hooded crows have dark eyes, while wire-
164 tailed manakins have white irises (Madge 2020; Snow 2020), raising the possibility that neither
165 species is capable of producing pteridine iris pigments due to a hypomorphic or null version of
166 *SLC2A11B*.

167 **Expression of the *SLC2A11B* pearl allele is reduced**

168 Using high-throughput RNA-sequencing (RNA-seq) datasets, we found that *SLC2A11B*
169 shows very low levels of expression in most adult tissues, including the retina, but substantial
170 expression in both Hamburger-Hamilton stage 25 (HH25; Hamburger and Hamilton 1951) whole
171 embryos (n = 2) and embryo heads (n = 12) (Fig. S2 A-B). Based on genotypes at the two
172 coding SNPs in the pearl eye haplotype (nonsense SNP ScoHet5_1307:1895934 and
173 synonymous SNP ScoHet5_1307:1896042), we found that embryo head samples homozygous
174 for the pearl allele show a significant reduction in *SLC2A11B* expression ($p = 3.94 \times 10^{-6}$, two-
175 tailed t-test; Fig. S2C). Analysis of read distribution within the *SLC2A11B* gene shows a
176 decrease in spliced reads specifically within the first three annotated exons, suggesting that
177 alternative splicing or nonsense-mediated decay may be occurring. In summary, the pearl eye
178 phenotype is associated with a nonsense mutation in a known mediator of yellow-orange
179 pigments, which in turn is linked to a significant decrease in *SLC2A11B* expression, possibly
180 due to nonsense-mediated decay of the mutant transcripts.

181

182 **QTL mapping identifies a single genomic locus associated with bull eye color**

183 Variation at *SLC2A11B* appears to act as a switch between two of the major pigeon iris
184 colors, orange and pearl eye, but it does not explain the third major color, bull eye. Bull eyes are
185 dark brown, lacking both orange and white pigment in the iris. However, pigeon breeders
186 observe that bull eye color can occur on either an orange or pearl genetic background (Sell
187 2012), suggesting that the loss of orange pigment in bull eyes likely arises from a mechanism
188 that does not involve *SLC2A11B*.

189 To identify the genetic basis of bull eye color, we used QTL mapping in two independent
190 F_2 intercrosses. In a cross between an orange-eyed Pomeranian Pouter and a bull-eyed
191 Scanderoon, F_2 birds had either two bull eyes (n=41), two orange eyes (n=40), or “odd eyes”,
192 where one eye has a pigmented iris stroma and the other eye is bull (n=12) (Fig. 2A). Using a
193 binary model where odd-eyed birds were included in the “bull eye” group, we identified a QTL
194 on linkage group 15 (Fig. 2C; peak marker ScoHet5_507: 11175287, LOD score = 11.89,
195 genome wide significance threshold = 4.28). The peak region spans 2.0 Mb across two genomic
196 scaffolds, from ScoHet5_507:9736663 to scaffold ScoHet5_683.1: 279252, and includes 42
197 annotated genes. Nearly all (52/53) odd-eyed or bull-eyed F_2 birds have at least one copy of the
198 bull-eyed Scanderoon allele at the peak marker (Fig. 2D), indicating dominant inheritance of bull
199 eye color. However, penetrance is both incomplete and lower in heterozygotes, suggesting a
200 stochastic effect of the bull eye allele. The one odd-eyed bird that is homozygous for the orange

201 Pomeranian Pouter allele may be a recombinant between the peak QTL marker and the
202 causative bull eye variant.

203 In the cross between the orange-eyed Archangel and the pearl-eyed Old Dutch
204 Capuchin that we originally used to map pearl eyes, neither founder had the bull eye phenotype.
205 However, some offspring had either two bull eyes (n = 8) or odd eyes (n = 6) (Fig. 2B). We used
206 a binary model to compare these 14 birds with at least one bull eye to 52 F₂ birds without bull
207 eyes (either two orange or two pearl eyes). Here, too, we identified a single locus associated
208 with bull eye color on linkage group 15 (Fig. 2E; peak marker ScoHet5_1916:103567, peak LOD
209 score = 8.85, genome wide significance threshold = 4.61). The peak region spans 1.5 Mb
210 across eight scaffolds, including the same two scaffolds identified in the Scandaroon x
211 Pomeranian Pouter cross, and captures 44 annotated genes. Although the Old Dutch Capuchin
212 founder does not have bull eyes, nearly all bull-eyed and odd-eyed F₂s carry two copies of the
213 Capuchin allele at the peak marker (Fig. 2F). This suggests that, unlike in the Pomeranian
214 Pouter x Scandaroon cross, inheritance of bull eye color in the Archangel x Capuchin cross is
215 recessive with low penetrance. The lone odd-eyed bird in the latter cross is heterozygous for the
216 Capuchin allele at ScoHet5_1916:103567 and may have a recombination event between the
217 peak QTL marker and the causative bull eye variant.

218

219 **Bull eye color and allelic heterogeneity**

220 QTL mapping identified a single locus associated with bull eye color in two different
221 crosses, but the inheritance pattern of bull eye appears to differ in each case. Furthermore,
222 genome-wide pF_{ST} analysis comparing bull-eyed birds (n = 18) to a background dataset of both
223 orange-eyed and pearl-eyed birds (n = 61) identified a small number of differentiated SNPs
224 across multiple scaffolds, including ScoHet5_507, but did not pinpoint a single well-
225 differentiated region in all bull-eyed breeds (Fig. 2G). Together, these results imply that, while
226 our QTL mapping identified the same genomic region in two separate crosses, the variants that
227 give rise to bull eye color are probably not the same across all pigeon breeds.

228

229 **Bull eye color is associated with plumage depigmentation**

230 Pigeon hobbyists have long noted that bull eye color is most common in birds with white
231 plumage (Sell 2012), including individuals with solid white plumage and those with a range of
232 piebalding phenotypes. Piebalding is characterized by broad regions of white and pigmented
233 feathers, and these regionalized de-pigmentation patterns are often breed-specific. Both the
234 Scandaroon and Pomeranian Pouter cross founders show breed-specific piebald patterning,

235 and the F₂ offspring of this cross show highly variable piebalding across multiple body regions
236 (Fig. 3A-B). We found that plumage color in many body regions is significantly associated with
237 bull eye color in the Pomeranian Pouter x Scandaroon cross. The strength of this relationship
238 varies by region, with areas like the lateral head and dorsal wing (Fig. 3C-D) showing a stronger
239 relationship with eye color than the lateral neck (Fig. 3E; additional body regions are shown in
240 Fig. S3).

241 To further evaluate the genetic relationship between piebalding and bull eye color, we
242 quantified the proportion of white plumage across 15 different body regions in the F₂ progeny of
243 the Pomeranian Pouter x Scandaroon cross. We identified two broad QTL regions associated
244 with white plumage (Fig. 3F-H, Fig. S4). Each locus is associated with white plumage in specific
245 body regions and explains 15-58% of the variance in the cross (Table 1). The QTL on linkage
246 group 1 is associated with white plumage on the neck and dorsal body, and individuals with
247 white plumage carry the Pomeranian Pouter allele at the linkage group 1 candidate locus. The
248 QTL on linkage group 15 is associated with white plumage on the head, wings, and dorsal body;
249 for this locus, the Scandaroon allele is associated with white plumage. The linkage group 15
250 piebalding QTL overlaps with the locus identified for bull eye, suggesting either closely linked
251 variants in the same or different genes, or the same gene controlling both traits. These
252 associations are consistent with breed-specific plumage patterns, as Scandaroon pigeons
253 typically have white plumage on the head, wings, and ventral body, while the Pomeranian
254 Pouter breed is characterized by a white “bib” on the neck (see examples, Fig. 3A). In summary,
255 at least two genetic loci control piebalding in pigeons, one of which overlaps with the bull eye
256 locus, and these loci act in a regionally- and breed-specific manner.

257

258 ***EDNRB2* is a candidate gene for bull eye color and white plumage**

259 We next wanted to identify candidate genes for bull eye color and white plumage within
260 the linkage group 15 region. Of the 60 genes included in at least one of the Pomeranian Pouter
261 x Scandaroon (2.0 Mb, 42 genes) or Archangel x Old Dutch Capuchin (1.5 Mb, 44 genes) bull
262 eye QTL peaks, comparison to gene ontology databases did not identify any genes with GO
263 annotations related to pigmentation. However, we were able to find potential links to pigment
264 patterning for five genes, including the endothelin receptor *EDNRB2* (Table 2). Mutations in
265 *EDNRB2* are associated with depigmentation phenotypes in several domestic bird species,
266 including “Panda” plumage in Japanese Quail, spot patterning in ducks, tyrosinase-independent
267 mottling in chickens, and white plumage with dark eye color in Minohiki chickens (Miwa et al.
268 2007; Kinoshita et al. 2014; Li et al. 2015; Xi et al. 2020). Additionally, changes in the

269 mammalian orthologue *ENDRB* are responsible for piebalding phenotypes in mice and the
270 piebald-like frame overo pattern in horses (Koide et al. 1998; Metallinos et al. 1998). Given the
271 known role of endothelin receptors in piebalding in other vertebrates, *EDNRB2* is a compelling
272 candidate for the linked piebalding and bull eye phenotypes in domestic pigeons. We examined
273 the allele frequencies and genotypes of SNPs within *EDNRB2* coding regions in both the bull-
274 eyed and non-bull-eyed populations used for pF_{ST} analysis and did not identify any coding
275 polymorphisms that were unique to bull-eyed birds, suggesting that noncoding regulatory
276 changes may mediate bull eye color and piebalding in domestic pigeons. Due to the allelic
277 heterogeneity and incomplete penetrance of the bull eye phenotype, however, we cannot rule
278 out coding changes in *EDNRB2*, or other candidate genes within the region, as mediators of the
279 bull eye phenotype.

280

281 **DISCUSSION**

282 ***SLC2A11B* and pearl eyes**

283 Using comparative genomic and classical genetic approaches, we identified two
284 candidate loci that control the three major iris colors of domestic pigeons. A locus on scaffold
285 ScoHet5_1307 is associated with pearl eye color. This region contains a SNP fixed in pearl-
286 eyed birds that changes a tryptophan to a premature stop codon in exon 3 of the solute carrier
287 *SLC2A11B*, and was also recently identified by Andrade et al. and Si et al. as a candidate
288 mutation for pearl eye color in pigeons (Si et al. 2020; Andrade et al. 2021). We found that the
289 nonsense mutation is associated with pearl iris color in individually phenotyped pigeons from a
290 wide array of domestic breeds, consistent with a single mutation arising early in domestication
291 (Si et al. 2020). We also showed that the *SLC2A11B* locus is the one and only genomic region
292 that segregates with pearl eye color in two F_2 crosses. Our results support the trio genotyping of
293 the *SLC2A11B* mutation performed by Andrade et al. (Andrade et al. 2021), and our linkage
294 mapping excludes a role for the remainder of the genome in the switch between orange and
295 pearl eyes. Intriguingly, while all pearl-eyed birds in our sample share a common *SLC2A11B*
296 allele, pigeon breeders have also identified a second locus associated with white iris color that
297 appears to be genetically distinct and is linked to brown plumage color (Levi 1986; Sell 2012).
298 Future analysis of individual birds with this “false pearl” eye color could expand our
299 understanding of the genes affecting pteridine synthesis and localization in the eyes of birds.

300 The *SLC2A11B* gene is not well-characterized, but likely plays an evolutionarily
301 conserved role in the development of pteridine-containing pigment cells. A nonsense mutation in
302 *SLC2A11B* in medaka is associated with loss of mature pteridine-containing leucophores and

303 xanthophores, and the Zebrafish Mutation Project identified differentiation defects in
304 *SLC2A11B*-mutant xanthophores (Kimura et al. 2014). Si et al. (2020) additionally identified a
305 frameshift mutation in *SLC2A11B* in cormorants, which have unique blue eyes and appear to
306 lack pteridine pigments in the iris. Similarly, the missing transmembrane domain in the manakin
307 and crow described here might render *SLC2A11B* incapable of pteridine deposition.

308 *SLC2A11B* does not have a mammalian ortholog, and its presence is restricted to
309 species that have xanthophores or xanthophore-like cells (Kimura et al. 2014). Comparative
310 analysis of solute carriers across species shows that the *SLC2A11B* gene likely originated prior
311 to the teleost fish-specific genome duplication, and was then lost in mammals (Kimura et al.
312 2014). Loss of *SLC2A11B* may have restricted the repertoire of pigments that mammals can
313 synthesize.

314

315 **Allelic heterogeneity at the bull eye locus**

316 Observations by pigeon breeders previously indicated a simple recessive mode of
317 inheritance for pearl eye color (Sell 2012), and this is confirmed by our analyses. The third
318 major iris color in domestic pigeons, bull eye, appears to have a more complicated inheritance
319 pattern. Through QTL mapping in two F_2 crosses, we identified a single genomic locus on
320 linkage group 15 that is associated with bull eye color. As previously noted by breeders, bull eye
321 color is associated with white plumage (Sell 2012), and QTL mapping identified a strong
322 association between the same linkage group 15 locus and piebald plumage patterning on the
323 wing and head.

324 Despite the overlap in QTL for bull eye color in two F_2 crosses and the QTL white
325 plumage, we were unable to pinpoint a single mutation within this locus associated with bull eye
326 color through a comparative genomic approach. This suggests that bull eye may not be caused
327 by a single genetic variant that is shared across breeds. Instead, the linkage group 15 QTL
328 regions may harbor multiple breed-specific mutations. These mutations may affect multiple
329 closely linked genes or may impact a single gene. Future work will examine the genetic
330 underpinnings of regionalized plumage patterning in F_2 crosses and work towards identification
331 of specific genetic variants associated with bull eye color and the piebald plumage that typically
332 accompanies it.

333 ***EDNRB2* and constraints on endothelin receptor evolution**

334 Although the specific mutations that cause bull eye color and white plumage color
335 remain unknown, the linkage group 15 QTL for bull eye color and piebalding contains a strong
336 candidate gene, *EDNRB2*. The endothelin signaling pathway plays critical roles in the
337 development and migration of multiple neural crest cell populations, including pigment cells. In
338 mammals, mutations in the endothelin receptor *ENDRB* are linked to piebalding in mice; lethal
339 white foal syndrome in horses; and Waardenburg Shah syndrome type 4A in humans, which is
340 characterized by changes in hair, skin, and eye pigment, as well as congenital defects in enteric
341 nervous system development (Read and Newton 1997; Koide et al. 1998; Metallinos et al. 1998;
342 Jabeen et al. 2012). In several bird species, coding and regulatory variants of *EDNRB2* are
343 associated with white plumage phenotypes and dark eye color (Miwa et al. 2007; Kinoshita et al.
344 2014; Li et al. 2015; Wu et al. 2017; Xi et al. 2020), but they are not typically linked to other
345 major pathologies. Thus, while endothelin signaling is linked to pigmentation changes across
346 vertebrates, *ENDRB* mutations in mammals are typically associated with deleterious pleiotropic
347 effects, while *EDNRB2* mutations in birds are not.

348 The endothelin signaling pathway in vertebrates evolved through multiple rounds of gene
349 duplication, and most bony vertebrates have three endothelin receptor genes: *EDNRA*,
350 *EDNRB1*, and *EDNRB2* (Braasch, Wolff, et al. 2009). Expression of different combinations of
351 endothelin receptors and ligands characterize unique cell populations. In *Xenopus*, chicken, and
352 quail, for example, *EDNRB2* is expressed specifically in migrating and post-migratory
353 melanophores, while non-pigment neural crest populations, like skeletal and trunk neural crest
354 cells, express *EDNRA* or *EDNRB1* (Square et al. 2016). However, *EDNRB2* was lost in therian
355 mammals, and the sole endothelin B receptor *ENDRB* is expressed in both trunk neural crest
356 populations and melanophores (Braasch, Wolff, et al. 2009; Square et al. 2016). As a result, in
357 therian mammals, changes in endothelin signaling typically affect both pigmentation and
358 neurogenesis. The retention of *EDNRB2* in non-mammalian vertebrates, on the other hand, may
359 permit the evolution and development of novel pigment patterns because the genetic controls of
360 pigment cell migration and neurogenesis are uncoupled.

361

362 **Gene duplication and retention mediate the evolution of pigment diversity**

363 The retention of *EDNRB2* in non-mammalian vertebrates, and the diverse endothelin-
364 mediated pigment patterns identified across bird species, point to a role for gene duplication in
365 mediating or constraining diversity in both pigment type and patterning. In species that retained
366 *EDNRB2*, sub-functionalization mediates the evolution of novel pigment patterns such as

367 piebalding, while in species that lost *EDNRB2*, such changes are severely constrained by the
368 requirement for a functional endothelin receptor B gene. This idea of gene loss restricting
369 pigment phenotypes is also relevant to the retention of our pearl eye candidate gene
370 *SLC2A11B*, which is only present in species with pteridine-containing xanthophore- or
371 leucophore-like cells. Solute carriers in the *SLC2A* family also evolved through multiple rounds
372 of gene duplication, though their evolutionary history is not as clear as that of endothelin ligands
373 and receptors due to multiple segmental duplication events (Kimura et al. 2014; Lorin et al.
374 2018). Gene duplication and retention permitted the striking expansion and evolution of novel
375 pigment types and patterns in teleost fish (Braasch, Brunet, et al. 2009; Lorin et al. 2018). The
376 identification of *SLC2A11B* and *EDNRB2* as candidate genes for pigeon eye color suggests that
377 similar patterns of retention of gene duplicates may mediate the evolution of pigment
378 phenotypes across vertebrate species.

379

380 MATERIALS AND METHODS

381 Animal husbandry and phenotyping of F₂ offspring

382 Pigeons were housed in accordance with protocols approved by the University of Utah
383 Institutional Animal Care and Use Committee (protocols 10-05007, 13-04012, and 19-02011).
384 Two intercrosses were used in these studies. An intercross between a male Pomeranian Pouter
385 and two female Scandaroons was performed to generate 131 F₂ offspring (Domyan et al. 2016).
386 An intercross between a male Archangel and a female Old Dutch Capuchin generated 98 F₂
387 offspring.

388

389 Whole Genome Resequencing

390 DNA was extracted from blood samples collected with breeders' written permission at
391 the annual Utah Premier Pigeon Show or from our lab colony using the Qiagen DNEasy Blood
392 and Tissue Kit (Qiagen, Valencia, CA). Samples were treated with RNase during extraction.
393 Isolated DNA was submitted to the University of Utah High Throughput Genomics Shared
394 Resource for library preparation using the Illumina Tru-Seq PCR-Free library kit. The resulting
395 libraries were sequenced on either the Illumina HiSeq or Illumina NovaSeq platforms. Raw
396 sequence data for 54 previously unpublished samples is available in the NCBI Sequence Read
397 Archive under BioProject accession PRJNA680754. These data sets were combined with
398 previously published data sets (BioProject accessions PRJNA513877, PRJNA428271, and
399 PRJNA167554) for variant calling.

400 **Genomic Analyses**

401 Variant calling was performed with FastQForward, which wraps the BWA short read
402 aligner and Sentieon (sentieon.com) variant calling tools to generate aligned BAM files
403 (fastq2bam) and variant calls in VCF format (bam2gvcf). Sentieon is a commercialized variant
404 calling pipeline that allows users to follow GATK best practices using the Sentieon version of
405 each tool (broadinstitute.org/gatk/guide/best-practices
406 and support.sentieon.com/manual/DNAseq_usage/dnaseq/). FastQForward manages
407 distribution of the workload to these tools on a compute cluster to allow for faster data-
408 processing than when calling these tools directly, resulting in runtimes as low as a few minutes
409 per sample.

410 Raw sequencing reads from the 54 resequenced individuals described above were
411 aligned to the Cliv_2.1 reference assembly (Holt et al. 2018) using fastq2bam. Variant calling
412 was performed for each newly resequenced individual, as well as 132 previously resequenced
413 individuals (Shapiro et al. 2013; Domyan et al. 2016; Vickrey et al. 2018; Bruders et al. 2020),
414 using bam2gvcf and individual genome variant call format (gVCF) files were created. Joint
415 variant calling was performed on a total of 186 individuals using the Sentieon GVCFTyper
416 algorithm. The resulting VCF file was used for all subsequent genomic analyses.

417 The subsequent variant call format (VCF) file was used for pF_{ST} analysis using the
418 GPAT++ toolkit within the VCFLIB software library (<https://github.com/vcflib>). For orange vs.
419 pearl pF_{ST} analysis, the genomes of 28 orange-eyed birds from were compared to the genomes
420 of 33 pearl-eyed birds. For bull eye vs. other color pF_{ST} analysis, the genomes of 18 bull eyed
421 birds were compared to the genomes of 61 non-bull birds (a mix of orange and pearl)

422

423 **Eye color phenotyping**

424 Eye colors of birds in our whole genome resequencing panel were determined from
425 photographs taken at the time of sampling. Each photograph was independently scored by three
426 individuals. In instances where eye color could not confidently be determined from photographs,
427 those individuals were not included in pF_{ST} analysis. Breeds included in the orange-eyed group:
428 American Show Racer, Archangel, Chinese Owl, Damascene, Dragoon, English Carrier, Feral,
429 Granadino Pouter, Hamburg Sticken, Hungarian Giant House Pigeon, Italian Owl, Mindian
430 Fantail, Modena, Pomeranian Pouter, Rafeno Pouter, Saxon Pouter, and Starling. Breeds
431 included in the pearl-eyed group: Australian Tumbler, Bacska Tumbler, Berlin Long Faced
432 Tumbler, Berlin Short Faced Tumbler, Birmingham Roller, Budapest Tumbler, Chinese Owl,
433 Cumulet, Danzig Highflier, English Short Faced Tumbler, English Trumpeter, Feral, Helmet,

434 Indian Fantail, Long Face Clean Leg Tumbler, Naked Neck, Oriental Roller, Polish Lynx,
435 Russian Tumbler, Saint, Temeschburger Schecken, Turkish Tumbler, Uzbek Tumbler, and
436 Vienna Medium Faced Tumbler. Breeds included in the bull-eyed group: African Owl, Canario
437 Cropper, Classic Old Frill, Chinese Nasal Tuft, Fairy Swallow, Ice Pigeon, Komorner Tumbler,
438 Lahore, Mookee, Old German Owl, Oriental Frill, Scandaroon, and Schalkaldener Mohrenkopf.
439 Eye colors of 93 Pomeranian Pouter x Scandaroon and 66 Archangel x Capuchin F₂ birds were
440 recorded based on observation at the time of euthanasia, and live photographs showing eye
441 color were taken for reference.

442

443 **Plumage phenotyping**

444 Following euthanasia, photos were taken of F₂ plumage including dorsal and ventral
445 views with wings and tail spread, and lateral views with wings folded. We divided the body into
446 15 different regions for phenotyping: dorsal head, right lateral head, left lateral head, dorsal
447 neck, ventral neck, right lateral neck, left lateral neck, dorsal body, ventral body, dorsal tail,
448 ventral tail, dorsal right wing, dorsal left wing, ventral right wing, and ventral left wing. To score
449 each region, we imported photos into Photoshop v21.1.0x64 (Adobe, San Jose, CA) and used
450 the magic wand tool to select only the white feathers within the body region. Following this
451 selection, we saved two separate images: one containing the entire region (both pigmented and
452 white feathers) with the color for the white feathers inverted (hereafter, “whole region image”),
453 and one with the selected white feathers removed and only the pigmented feathers remaining
454 (“pigmented region image”). For each body region, we imported these two images into ImageJ
455 (v1.52a; Schneider et al. 2012) and converted them to greyscale, then used the threshold tool to
456 measure the number of pixels in each image. To calculate the proportion of white feathers for
457 each region, we subtracted the number of pixels in the pigmented region image from the
458 number of pixels in the whole region image, then divided by the number of pixels in the whole
459 region image.

460

461 **Genotype by Sequencing**

462 DNA samples from founders of the crosses and their F₂ progeny were extracted using
463 the Qiagen DNeasy Blood and Tissue kit. Our Genotype by Sequencing approach was adapted
464 from a previously published protocol with minor modifications (Elshire et al. 2011; Domyan et al.
465 2016). DNA was digested with ApeKI, and size selected for fragments in the 550-650 bp range.
466 Domyan et al. (2016) performed an initial round of genotyping for the Pomeranian Pouter x
467 Scandaroon cross. These libraries were sequenced using 100- or 125 bp paired-end

468 sequencing on the Illumina HiSeq2000 platform at the University of Utah Genomics Core
469 Facility. Genotype by sequencing for the Archangel x Capuchin founders (n=2) and F₂ offspring
470 (n=98), as well as supplemental sequencing for 20 additional and 17 previously low-coverage
471 Pomeranian Pouter x Scandaroon F₂s, was performed by the University of Minnesota Genomics
472 Center. New GBS libraries were sequenced on a NovaSeq 1x100 SP FlowCell.

473

474 **Linkage Map Construction**

475 Genotype by Sequencing reads were trimmed using CutAdapt (Martin 2011), then
476 mapped to the Cliv_2.1 reference genome reads using Bowtie2 (Langmead and Salzberg
477 2012). Genotypes were called using Stacks2 by running “refmap.pl” (Catchen et al. 2013). In the
478 Pomeranian Pouter x Scandaroon cross, which had three founders, the Pomeranian Pouter and
479 one of the two Scandaroons designated as parents; to account for the three-founder cross
480 structure, all markers where the genotypes of the two Scandaroon founders differed were
481 subsequently removed from the dataset.

482 We constructed genetic maps using R/qtl v1.46-2 (www.rqtl.org; Broman et al. 2003).
483 Autosomal markers showing significant segregation distortion ($p < 0.01$ divided by the total
484 number of markers genotyped, to correct for multiple testing) were eliminated. Sex-linked
485 scaffolds were assembled and ordered separately, due to differences in segregation pattern for
486 the Z chromosome. Z-linked scaffolds were identified by assessing sequence similarity and
487 gene content between pigeon scaffolds and the Z chromosome of the annotated chicken
488 genome (Ensembl Gallus_gallus-5.0).

489 Pairwise recombination frequencies were calculated for all autosomal and Z-linked
490 markers. Markers with identical genotyping information were identified using the
491 “findDupMarkers” command, and all but one marker in each set of duplicates was removed.
492 Within individual Cliv_2.1 scaffolds, markers were filtered by genotyping rate; to retain the
493 maximal number of scaffolds in the final map, an initial round of filtering was performed to
494 remove markers where fewer than 50% of birds were genotyped. Large scaffolds (> 40
495 markers) were subsequently filtered a second time to remove markers where fewer than 66% of
496 birds were genotyped.

497 Within individual scaffolds, R/Qtl functions “dropmarker” and “calc.errorlod” were
498 used to assess genotyping error. Markers were removed if dropping the marker led to an
499 increased LOD score, or if removing a non-terminal marker led to a decrease in length of >10
500 cM that was not supported by physical distance. Individual genotypes were removed if they had
501 error LOD scores >5 (a measure of the probability of genotyping error, see (Lincoln and Lander

502 1992). Linkage groups were assembled from both autosomal markers and Z-linked markers
503 using the parameters (max.rf 0.15, min.lod 6). Scaffolds in the same linkage group were
504 manually ordered based on calculated recombination fractions and LOD scores. Linkage groups
505 in the Pomeranian Pouter x Scanderoon map were numbered by marker number. Linkage
506 groups in the Archangel x Old Dutch Capuchin map were numbered based on scaffold content
507 to correspond with Pomeranian Pouter x Scanderoon linkage groups.

508

509 **Quantitative Trait Locus Mapping**

510 We performed QTL mapping using R/qtl v1.46-2 (Broman et al. 2003). For eye color
511 phenotypes, we used the *scanone* function to perform a single-QTL genome scan using a binary
512 model. In QTL scans for the bull eye phenotype, “odd-eyed” birds with a single bull eye were
513 scored as bull. For piebalding phenotypes, we used the *scanone* function to perform a single-QTL
514 genome scan using Haley-Knott regression. For each phenotype, the 5% genome-wide
515 significance threshold was calculated by running the same *scanone* with 1000 permutation
516 replicates. For each significant QTL peak, we calculated 2-LOD support intervals using the *lodint*
517 function. We calculated percent variance explained (PVE) using the *fitqtl* function.

518

519 **SLC2A11B mutation identification and gene re-annotation**

520 We identified numerous SNPs with maximal pF_{ST} scores, and manually examined
521 genotype calls from the VCF file to identify SNPs that followed the expected recessive
522 inheritance pattern of pearl eye (i.e., all pearl-eyed birds were homozygous for the reference
523 allele and all orange-eyed birds were either heterozygous or homozygous for the alternate
524 allele). We identified SLC2A11B orthologs across species using NCBI blastp
525 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; (Altschul et al. 1990; Johnson et al. 2008). The first 10-
526 20 amino acids of the SLC2A11B protein vary across species, but alignments showed that the
527 annotated pigeon protein was missing >80 amino acids that are well conserved most other
528 species, and was likely incomplete. We then took RNA sequences for the orange and pearl
529 alleles of SLC2A11B and translated each using Expasy Translate
530 (<https://web.expasy.org/translate/>; (Gasteiger et al. 2003). The longest contiguous protein
531 predicted for the pearl allele matched the protein sequence available on NCBI, while the longest
532 contiguous protein for the orange allele was in the same open reading frame, but contained an
533 additional 95 amino acids at the start of the protein sequence. This N-terminal sequence
534 matched the highly conserved SLC2A11B protein sequence annotations across species. The
535 amino acid residue position of the pearl allele mutation is based on this re-annotation.

536 **Expression analysis from RNA-seq data**

537 RNA-sequencing data for whole embryos and adult tissues (retina, liver, olfactory
538 epithelium) were obtained from previously described datasets deposited in the SRA database
539 with sequence accessions SRR5878849-SRR5878856 (Holt et al. 2018). For HH25 Oriental Frill
540 and Racing Homer embryo heads, RNA from whole embryonic heads was isolated using the
541 Qiagen RNEasy Kit, and submitted to the University of Utah High Throughput Genomics Shared
542 Resource for Illumina TruSeq stranded library preparation. Libraries were sequenced on the
543 Illumina HiSeq platform. Data are available in NCBI Sequence Read Archive under BioProject
544 PRJNA680754

545 We mapped reads to the Cliv_2.1 reference genome using STAR (Dobin et al. 2013),
546 and counted reads in features using FeatureCounts (Liao et al. 2014). Reads per million for the
547 *SLC2A11B* gene were calculated based on total number of uniquely mapped reads per sample.
548 For each HH25 embryo head, we looked at reads overlapping two SNPs within the pearl eye
549 haplotype (ScoHet5_1307:1895834 and ScoHet5_1307:1896042) to predict genotypes. We
550 evaluated relative expression level of *SLC2A11B* between orange and pearl alleles using a two-
551 tailed T-test to compare reads per million in each sample set.

552

553 **Protein conservation, structure prediction, and mutation evaluation**

554 We obtained protein sequences for *SLC2A11B* orthologues across species using NCBI
555 blastp and generated multi-species alignments using Clustal Omega (Madeira et al. 2019), and
556 then visualized using Jalview2 (Waterhouse et al. 2009). We assessed the predicted structure
557 of the *SLC2A11B* protein by using Phobius (Käll et al. 2004) to predict cytoplasmic, non-
558 cytoplasmic, transmembrane, and signal peptide domains. As the premature stop codon in
559 *SLC2A11B* occurs very early in the protein sequence, we evaluated the likely impact of the
560 premature stop codon by identifying the next in-frame methionine where translation could initiate
561 to make the longest possible partial protein. We input this truncation into PROVEAN (Choi et al.
562 2012; Choi and Chan 2015) as a deletion of the first 95 amino acids.

563

564 **Gene ontology analysis**

565 We mapped gene ontology annotations to identifiers for genes within the two bull eye
566 candidate regions using DAVID (<https://david.ncifcrf.gov/>; Huang et al. 2009). We used
567 annotations from Biological Process (GOTERM_BP_ALL; GOTERM_BP_DIRECT), Cellular
568 Component (GOTERM_CC_ALL; GOTERM_CC_DIRECT), and Molecular Function

569 (GOTERM_MF_ALL; GOTERM_MF_DIRECT) gene ontology databases, and searched results
570 for GO terms containing the keywords “pigment”, “melanosome” or “melanocyte”.

571

572 **Data Availability**

573 Whole genome sequencing and RNA-sequencing datasets generated for this study have
574 been deposited to the NCBI SRA database under BioProject PRJNA680754. Previously
575 generated whole genome sequencing and RNA-seq data used in this study is available under
576 BioProject accessions PRJNA513877, PRJNA428271, and PRJNA167554.

577

578 **Acknowledgements and Funding Information**

579 We thank current and former members of the Shapiro lab for assistance with sample
580 collection and processing. We thank Eric Domyan, Anna Vickrey, Hannah Van Hollebeke, Alexa
581 Davis, Tennyson George, Marissa Burton, and Lucas Periera for technical assistance and
582 advice. Layne Gardner generously shared the Pomeranian Pouter and Scandaroon
583 photographs featured in Fig. 3A. We thank members of the Utah Pigeon Club for providing
584 samples. We acknowledge a computer time allocation from the University of Utah Center for
585 High Performance Computing. This work was supported by the National Institutes of Health
586 (R35GM131787 to M.D.S. and F32DE028179 to E.B.), the National Science Foundation (GRF
587 1256065 to R.B.), the Jane Coffin Childs Memorial Fund for Medical Research (fellowship to
588 E.M.), and the University of Utah Undergraduate Research Opportunities Program (fellowship
589 support to B.P., R.W., and T.G.).

590

591 **References**

592 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J
593 Mol Biol* 215:403–410.

594 Amat F, Wollenberg KC, Vences M. 2013. Correlates of eye colour and pattern in mantellid
595 frogs. *Salamandra* 49:7–17.

596 Andrade P, Gazda MA, Araújo PM, Afonso S, Rasmussen JacobA, Marques CI, Lopes RJ,
597 Gilbert. MTP, Carneiro M. 2021. Molecular parallelisms between pigmentation in the avian
598 iris and the integument of ectothermic vertebrates. *Plos Genet* 17:e1009404.

599 Bond C. 1919. On certain factors concerned in the production of eye colour in birds. *Journal of
600 Genetics* 9:69–81.

601 Braasch I, Brunet F, Volff J-N, Schartl M. 2009. Pigmentation Pathway Evolution after Whole-
602 Genome Duplication in Fish. *Genome Biol Evol* 1:479–493.

603 Braasch I, Wolff J-N, Schartl M. 2009. The Endothelin System: Evolution of Vertebrate-Specific
604 Ligand-Receptor Interactions by Three Rounds of Genome Duplication. *Mol Biol Evol*
605 26:783–799.

606 Broman KW, Wu H, Sen Š, Churchill GA. 2003. R/qtl: QTL mapping in experimental crosses.
607 *Bioinformatics* 19:889–890.

608 Bruders R, Hollebeke HV, Osborne EJ, Kronenberg Z, Maclary E, Yandell M, Shapiro MD.
609 2020. A copy number variant is associated with a spectrum of pigmentation patterns in the
610 rock pigeon (*Columba livia*). *Plos Genet* 16:e1008274.

611 Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA. 2013. Stacks: an analysis tool
612 set for population genomics. *Mol Ecol* 22:3124–3140.

613 Chen C, Shimizu S, Tsujimoto Y, Motoyama N. 2005. Chk2 regulates transcription-independent
614 p53-mediated apoptosis in response to DNA damage. *Biochem Bioph Res Co* 333:427–431.

615 Choi Y, Chan AP. 2015. PROVEAN web server: a tool to predict the functional effect of amino
616 acid substitutions and indels. *Bioinformatics* 31:2745–2747.

617 Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. 2012. Predicting the Functional Effect of
618 Amino Acid Substitutions and Indels. *Plos One* 7:e46688.

619 Davidson GL, Clayton NS, Thornton A. 2014. Salient eyes deter conspecific nest intruders in
620 wild jackdaws (*Corvus monedula*). *Biol Letters* 10:20131077.

621 Davidson GL, Thornton A, Clayton NS. 2017. Evolution of iris colour in relation to cavity nesting
622 and parental care in passerine birds. *Biol Letters* 13:20160783.

623 Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras
624 TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29:15–21.

625 Doege H, Bocianski A, Scheepers A, Axer H, Eckel J, Joost H-G, Schürmann A. 2001.
626 Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel
627 sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J*
628 359:443–449.

629 Domyan ET, Guernsey MW, Kronenberg Z, Krishnan S, Boissy RE, Vickrey AI, Rodgers C,
630 Cassidy P, Leachman SA, Fondon JW, et al. 2014. Epistatic and combinatorial effects of
631 pigmentary gene mutations in the domestic pigeon. *Curr Biology Cb* 24:459–464.

632 Domyan ET, Kronenberg Z, Infante CR, Vickrey AI, Stringham SA, Bruders R, Guernsey MW,
633 Park S, Payne J, Beckstead RB, et al. 2016. Molecular shifts in limb identity underlie
634 development of feathered feet in two domestic avian species. *Elife* 5:e12115.

635 Edwards M, Cha D, Krithika S, Johnson M, Cook G, Parra EJ. 2016. Iris pigmentation as a
636 quantitative trait: variation in populations of European, East Asian and South Asian ancestry
637 and association with candidate gene polymorphisms. *Pigm Cell Melanoma R* 29:141–162.

638 Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE. 2011. A
639 Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. *Plos*
640 *One* 6:e19379.

641 Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: the
642 proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 31:3784–
643 3788.

644 Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick
645 embryo. *J Morphol* 88:49–92.

646 Harris ML, Hall R, Erickson CA. 2008. Directing pathfinding along the dorsolateral path - the role
647 of EDNRB2 and EphB2 in overcoming inhibition. *Development* 135:4113–4122.

648 Hirao A, Kong Y-Y, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ, Mak TW.
649 2000. DNA Damage-Induced Activation of p53 by the Checkpoint Kinase Chk2. *Science*
650 287:1824–1827.

651 Hoekstra HE. 2006. Genetics, development and evolution of adaptive pigmentation in
652 vertebrates. *Heredity* 97:222–234.

653 Hollander WF, Owen RD. 1939. Iris pigmentation in domestic pigeons. *Genetica* 21:408–419.

654 Holt C, Campbell M, Keays DA, Edelman N, Kapusta A, Maclary E, Domyan ET, Suh A, Warren
655 WC, Yandell M, et al. 2018. Improved Genome Assembly and Annotation for the Rock
656 Pigeon (Columba livia). *G3 Amp 58 Genes Genomes Genetics* 8:1391–1398.

657 Huang DW, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene
658 lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57.

659 Hubbard JK, Uy JAC, Hauber ME, Hoekstra HE, Safran RJ. 2010. Vertebrate pigmentation:
660 from underlying genes to adaptive function. *Trends Genetics* 26:231–239.

661 Inaba M, Chuong C-M. 2020. Avian Pigment Pattern Formation: Developmental Control of
662 Macro- (Across the Body) and Micro- (Within a Feather) Level of Pigment Patterns. *Frontiers*
663 *Cell Dev Biology* 8:620.

664 Jabeen R, Babar ME, Ahmad J, Awan AR. 2012. Novel mutations of endothelin-B receptor gene
665 in Pakistani patients with Waardenburg syndrome. *Mol Biol Rep* 39:785–788.

666 Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. 2008. NCBI
667 BLAST: a better web interface. *Nucleic Acids Res* 36:W5–W9.

668 Kaelin CB, Barsh GS. 2013. Genetics of pigmentation in dogs and cats. *Annu Rev Anim Biosci*
669 1:125–156.

670 Käll L, Krogh A, Sonnhammer ELL. 2004. A Combined Transmembrane Topology and Signal
671 Peptide Prediction Method. *J Mol Biol* 338:1027–1036.

672 Kelsh RN, Harris ML, Colanesi S, Erickson CA. 2008. Stripes and belly-spots -- a review of
673 pigment cell morphogenesis in vertebrates. *Semin Cell Dev Biol* 20:90–104.

674 Kimura T, Nagao Y, Hashimoto H, Yamamoto-Shiraishi Y, Yamamoto S, Yabe T, Takada S,
675 Kinoshita M, Kuroiwa A, Naruse K. 2014. Leucophores are similar to xanthophores in their
676 specification and differentiation processes in medaka. *P Natl Acad Sci Usa* 111:7343–7348.

677 Kinoshita K, Akiyama T, Mizutani M, Shinomiya A, Ishikawa A, Younis HH, Tsudzuki M,
678 Namikawa T, Matsuda Y. 2014. Endothelin receptor B2 (EDNRB2) is responsible for the
679 tyrosinase-independent recessive white (mo(w)) and mottled (mo) plumage phenotypes in
680 the chicken. *Plos One* 9:e86361.

681 Koide T, Moriwaki K, Uchida K, Mita A, Sagai T, Yonekawa H, Katoh H, Miyashita N, Tsuchiya
682 K, Nielsen TJ, et al. 1998. A new inbred strain JF1 established from Japanese fancy mouse
683 carrying the classic piebald allele. *Mamm Genome* 9:15–19.

684 Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods*
685 9:357–359.

686 Levi W. 1986. The Pigeon. ed 2. Revised. Sumter, S.C.: Levi Publishing Co., Inc

687 Li L, Li D, Liu L, Li S, Feng Y, Peng X, Gong Y. 2015. Endothelin Receptor B2 (EDNRB2) Gene
688 Is Associated with Spot Plumage Pattern in Domestic Ducks (*Anas platyrhynchos*). *Plos One*
689 10:e0125883.

690 Liao Y, Smyth GK, Shi W. 2014. featureCounts: an efficient general purpose program for
691 assigning sequence reads to genomic features. *Bioinformatics* 30:923–930.

692 Lincoln SE, Lander ES. 1992. Systematic detection of errors in genetic linkage data. *Genomics*
693 14:604–610.

694 Liu XY, Dangel AW, Kelley RI, Zhao W, Denny P, Botcherby M, Cattanach B, Peters J,
695 Hunsicker PR, Mallon A-M, et al. 1999. The gene mutated in bare patches and striped mice
696 encodes a novel 3 β -hydroxysteroid dehydrogenase. *Nat Genet* 22:182–187.

697 Lorin T, Brunet FG, Laudet V, Volff J-N. 2018. Teleost Fish-Specific Preferential Retention of
698 Pigmentation Gene-Containing Families After Whole Genome Duplications in Vertebrates.
699 *G3 Amp 58 Genes Genomes Genetics* 8:1795–1806.

700 Madeira F, Park Y mi, Lee J, Buso N, Gur T, Madhusoodanan N, Basutkar P, Tivey ARN, Potter
701 SC, Finn RD, et al. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019.
702 *Nucleic Acids Res* 47:W636–W641.

703 Madge S. 2020. Hooded Crow (*Corvus cornix*), version 1.0. In Birds of the World (S. M.
704 Billerman, B. K. Keeney, P. G. Rodewald, and T. S. Schulenberg, Editors). Cornell Lab of
705 Ornithology, Ithaca, NY, USA. <https://doi.org/10.2173/bow.hoocro1.01>

706 Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads.
707 *Emnet J* 17:10–12.

708 Metallinos DL, Bowling AT, Rine J. 1998. A missense mutation in the endothelin-B receptor
709 gene is associated with Lethal White Foal Syndrome: an equine version of Hirschsprung
710 Disease. *Mamm Genome* 9:426–431.

711 Miwa M, Inoue-Murayama M, Aoki H, Kunisada T, Hiragaki T, Mizutani M, Ito S. 2007.
712 Endothelin receptor B2 (EDNRB2) is associated with the panda plumage colour mutation in
713 Japanese quail: Quail plumage colour mutation associated with EDNRB2. *Anim Genet*
714 38:103–108.

715 Negro JJ, Blázquez MC, Galván I. 2017. Intraspecific eye color variability in birds and
716 mammals: a recent evolutionary event exclusive to humans and domestic animals. *Front*
717 *Zool* 14:53.

718 Oliphant LW. 1987a. Pteridines and Purines as Major Pigments of the Avian Iris. *Pigm Cell Res*
719 1:129–131.

720 Oliphant LW. 1987b. Observations on the Pigmentation of the Pigeon Iris. *Pigm Cell Res* 1:202–
721 208.

722 Parichy DM, Spiewak JE. 2014. Origins of adult pigmentation: diversity in pigment stem cell
723 lineages and implications for pattern evolution. *Pigm Cell Melanoma R* 28:31–50.

724 Passarotto A, Parejo D, Cruz-Miralles A, Avilés JM. 2018. The evolution of iris colour in relation
725 to nocturnality in owls. *J Avian Biol* 49.

726 Pla P, Alberti C, Solov'eva O, Pasdar M, Kunisada T, Larue L. 2005. Ednrb2 orients cell
727 migration towards the dorsolateral neural crest pathway and promotes melanocyte
728 differentiation. *Pigm Cell Res* 18:181–187.

729 Read AP, Newton VE. 1997. Waardenburg syndrome. *J Med Genet* 34:656.

730 Sanchez-Ferras O, Bernas G, Farnos O, Touré AM, Souchkova O, Pilon N. 2016. A direct role
731 for murine Cdx proteins in the trunk neural crest gene regulatory network. *Development*
732 143:1363–1374.

733 Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image
734 analysis. *Nat Methods* 9:671–675.

735 Scott GA, Jacobs SE, Pentland AP. 2006. sPLA2-X Stimulates Cutaneous Melanocyte
736 Dendricity and Pigmentation Through a Lysophosphatidylcholine-Dependent Mechanism. *J*
737 *Invest Dermatol* 126:855–861.

738 Sell A. 2012. Pigeon Genetics. Sell Publishing. Verlag Karin und A. Sell

739 Shapiro MD, Kronenberg Z, Li C, Domyan ET, Pan H, Campbell M, Tan H, Huff CD, Hu Haofu,
740 Vickrey AI, et al. 2013. Genomic diversity and evolution of the head crest in the rock pigeon.
741 *Sci New York N Y* 339:1063–1067.

742 Si S, Xu X, Zhuang Y, Luo S-J. 2020. Unpublished Data; The Genetics and Evolution of Eye
743 Color in Domestic Pigeons (*Columba livia*). *Biorxiv*: 2020.10.25.340760. Available from:
744 <https://www.biorxiv.org/content/10.1101/2020.10.25.340760v2>

745 Snow D. 2020. Wire-tailed Manakin (*Pipra filicauda*), version 1.0. In Birds of the World (J. del
746 Hoyo, A. Elliott, J. Sargatal, D. A. Christie, and E. de Juana, Editors). Cornell Lab of
747 Ornithology, Ithaca, NY, USA. <https://doi.org/10.2173/bow.witman2.01>.

748 Square T, Jandzik D, Cattell M, Hansen A, Medeiros DM. 2016. Embryonic expression of
749 endothelins and their receptors in lamprey and frog reveals stem vertebrate origins of
750 complex Endothelin signaling. *Sci Rep-uk* 6:34282.

751 Tamura K, Ohbayashi N, Ishibashi K, Fukuda M. 2011. Structure-Function Analysis of VPS9-
752 Ankyrin-repeat Protein (Varp) in the Trafficking of Tyrosinase-related Protein 1 in
753 Melanocytes*. *J Biol Chem* 286:7507–7521.

754 Vickrey AI, Bruders R, Kronenberg Z, Mackey E, Bohlender RJ, Maclary ET, Maynez R,
755 Osborne EJ, Johnson KP, Huff CD, et al. 2018. Introgression of regulatory alleles and a
756 missense coding mutation drive plumage pattern diversity in the rock pigeon. *Elife* 7:e34803.

757 Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview Version 2—a
758 multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191.

759 Wu N, Qin H, Wang M, Bian Y, Dong B, Sun G, Zhao W, Chang G, Xu Q, Chen G. 2017.
760 Variations in endothelin receptor B subtype 2 (EDNRB2) coding sequences and mRNA
761 expression levels in 4 Muscovy duck plumage colour phenotypes. *Brit Poultry Sci* 58:116–
762 121.

763 Xi Y, Wang L, Liu H, Ma S, Li Y, Li L, Wang J, Chunchun H, Bai L, Mustafa A, et al. 2020. A 14-
764 bp insertion in endothelin receptor B-like (EDNRB2) is associated with white plumage in
765 Chinese geese. *Bmc Genomics* 21:162.

766 Zdobnov EM, Apweiler R. 2001. InterProScan – an integration platform for the signature-
767 recognition methods in InterPro. *Bioinformatics* 17:847–848.

Table 1. Summary of QTL for regional white plumage. PVE, percent variance explained; Pom, Pomeranian Pouter; Scan, Scandaroon.

Body Region	Linkage Group	Peak Marker	Peak LOD score	PVE	Associated Allele
Dorsal Right Wing	15	ScoHet5_507_11304619	19.9	57	Scan
Dorsal Left Wing	15	ScoHet5_507_11304619	15.5	49	Scan
Dorsal Body	1	ScoHet5_80_11511249	9.11	30	Pom
Dorsal Body	15	ScoHet5_683.1_42424	4.45	15	Scan
Dorsal Neck	1	ScoHet5_80_3402497	5.72	22	Pom
Dorsal Head	15	ScoHet5_507_11175287	22.4	63	Scan
Ventral Right Wing	15	ScoHet5_507_11175287	12.2	40	Scan
Ventral Left Wing	15	ScoHet5_507_11227444	9.34	33	Scan
Ventral Body	15	ScoHet5_507_11058018	11.4	38	Scan
Ventral Tail	15	ScoHet5_683.1_42424	17.4	52	Scan
Ventral Neck	1	ScoHet5_80_524713	4.81	21	Pom
Ventral Neck	15	ScoHet5_507_11175287	7.39	30	Scan
Lateral Right Head	15	ScoHet5_507_10468270	20.5	58	Scan
Lateral Right Neck	1	ScoHet5_80_524713	6.56	15	Pom
Lateral Left Head	15	ScoHet5_507_11058018	20.7	58	Scan
Lateral Left Neck	1	ScoHet5_2444_504541	4.37	17	Pom

Table 2. Summary of pigment-associated genes within the LG15 QTL

Gene Name	Scaffold	Role in Pigmentation
<i>CDX1</i>	ScoHet5_507	Involved in neural crest development, Reduction in <i>CDX1</i> is associated with white spotting in mice (Sanchez-Ferraz et al. 2016)
<i>NSDHL</i>	ScoHet5_507	Mice with heterozygous mutations can have striped coats (Liu et al. 1999)
<i>VAMP7</i>	ScoHet5_507	SNARE protein involved in <i>TYRP1</i> trafficking to the melanosome (Tamura et al. 2011)
<i>EDNRB2</i>	ScoHet5_507	Controls migration of neural crest derived pigment cells (Pla et al. 2005; Harris et al. 2008); Linked to plumage pigmentation phenotypes in multiple avian species (Miwa et al. 2007; Kinoshita et al. 2014; Li et al. 2015; Wu et al. 2017; Xi et al. 2020)
<i>GPR119</i>	ScoHet5_1916	G-protein coupled receptor expressed in human melanocytes (Scott et al. 2006)

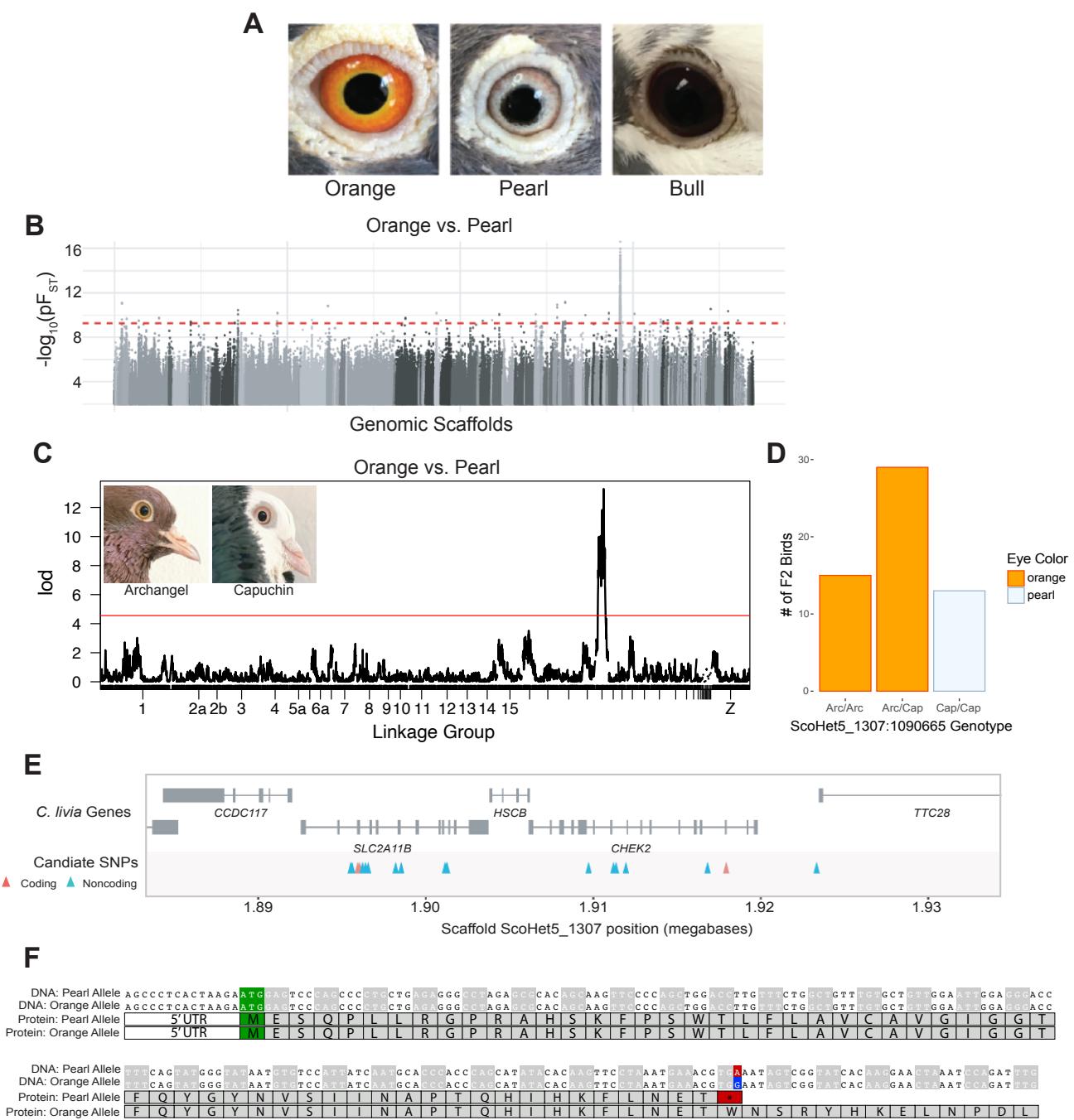


Figure 1. A single genomic locus is associated with pearl iris color in domestic pigeons. (A) Domestic pigeons typically have one of three major iris colors: the wild-type orange, pearl, or bull. (B) Whole-genome pF_{ST} comparisons of orange-eyed and pearl-eyed pigeons. Gray dots represent SNPs, with different shades indicating different genomic scaffolds. Dashed red line indicates genome-wide significance threshold. (C) Genome-wide QTL scan for pearl eye in the Archangel x Old Dutch Capuchin cross. Red line indicates 5% genome wide significance threshold. Insets: Archangel (left) and Capuchin (right) founders. (D) Eye color phenotypes of F_2 progeny with different genotypes at the QTL peak marker. Arc, allele from the Archangel founder. Cap, allele from the Capuchin founder. (E) Genomic context of the pearl eye candidate region. Gene models for the region are shown in gray. SNPs in coding regions are shown in red, SNPs in non-coding regions are shown in blue. (F) Alignment of DNA (top) and predicted protein (bottom) sequences of SLC2A11B for pearl-eyed and orange-eyed pigeons. The start codon is highlighted in green. The DNA polymorphism at position ScoHet5_1307:1895934 is marked in red (pearl allele) or blue (orange allele); the resulting stop codon in the pearl allele is highlighted in red.

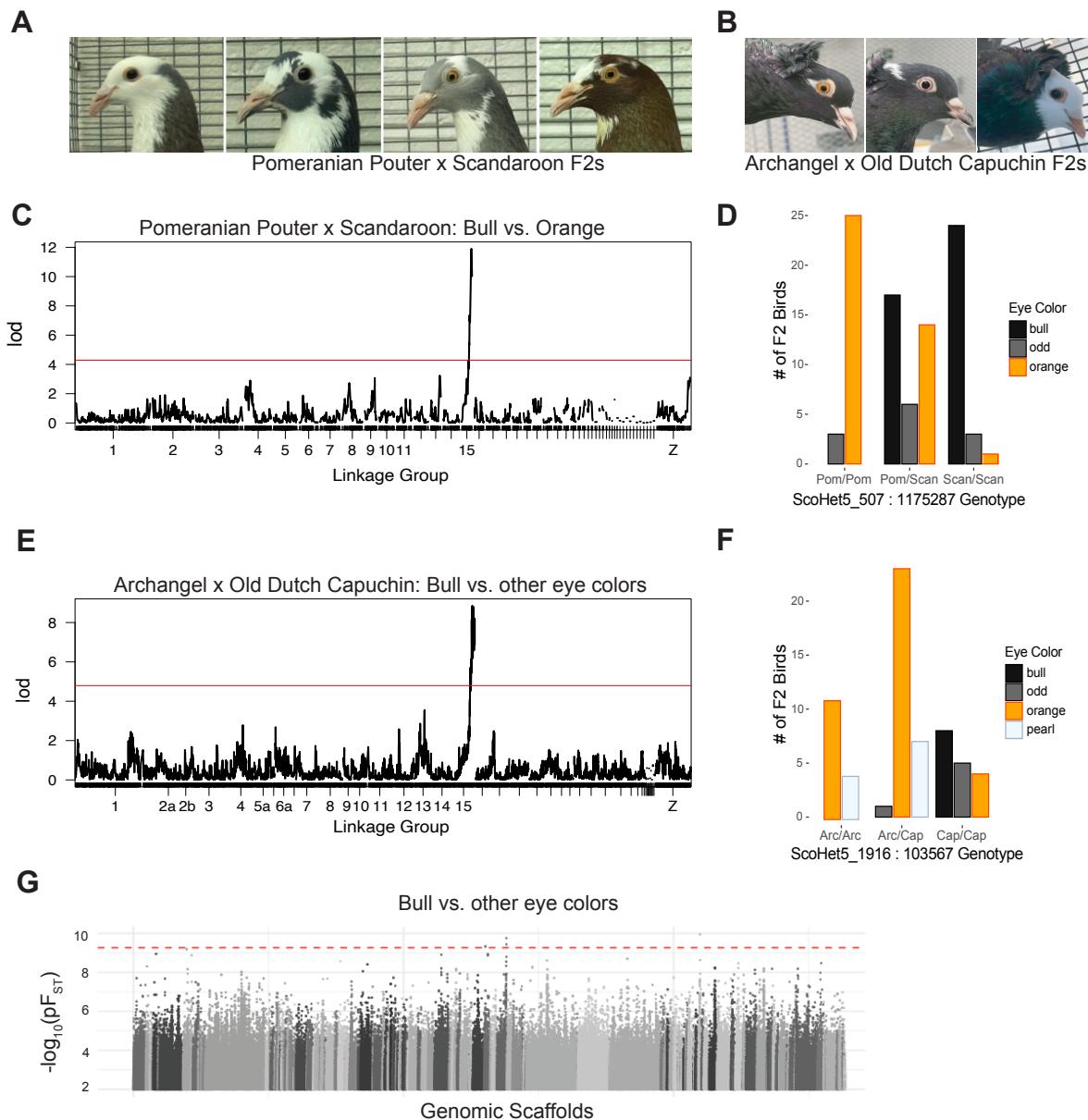


Figure 2. A single genomic locus is associated with bull eye color in two F₂ intercrosses. (A) F₂ offspring from an intercross between a Pomeranian Pouter and a Scandaroon have either bull (left two images) or orange (right two images) eyes. (B) F₂ offspring from an intercross between an Archangel and an Old Dutch Capuchin have orange (left), pearl (center), or bull (right) eyes. (C) Genome-wide QTL scan of the Pomeranian Pouter x Scandaroon cross for bull eye. Red line indicates 5% genome wide significance threshold. (D) Iris color phenotype counts for each genotype at the bull eye peak marker from the Pomeranian Pouter x Scandaroon cross. Pom, allele from Pomeranian Pouter founder. Scan, allele from Scandaroon founder. (E) Genome-wide QTL scan of the Archangel x Old Dutch Capuchin cross for bull eye. Red line indicates 5% genome wide significance threshold. (F) Iris color phenotype counts for each genotype at the bull eye peak marker from the Archangel x Capuchin cross. Arc, allele from the Archangel founder. Cap, allele from the Capuchin founder. (G) Whole-genome pF_{ST} comparisons of bull-eyed birds to birds with non-bull (orange or pearl) eyes. Dashed red line indicates 5% threshold for genome-wide significance.

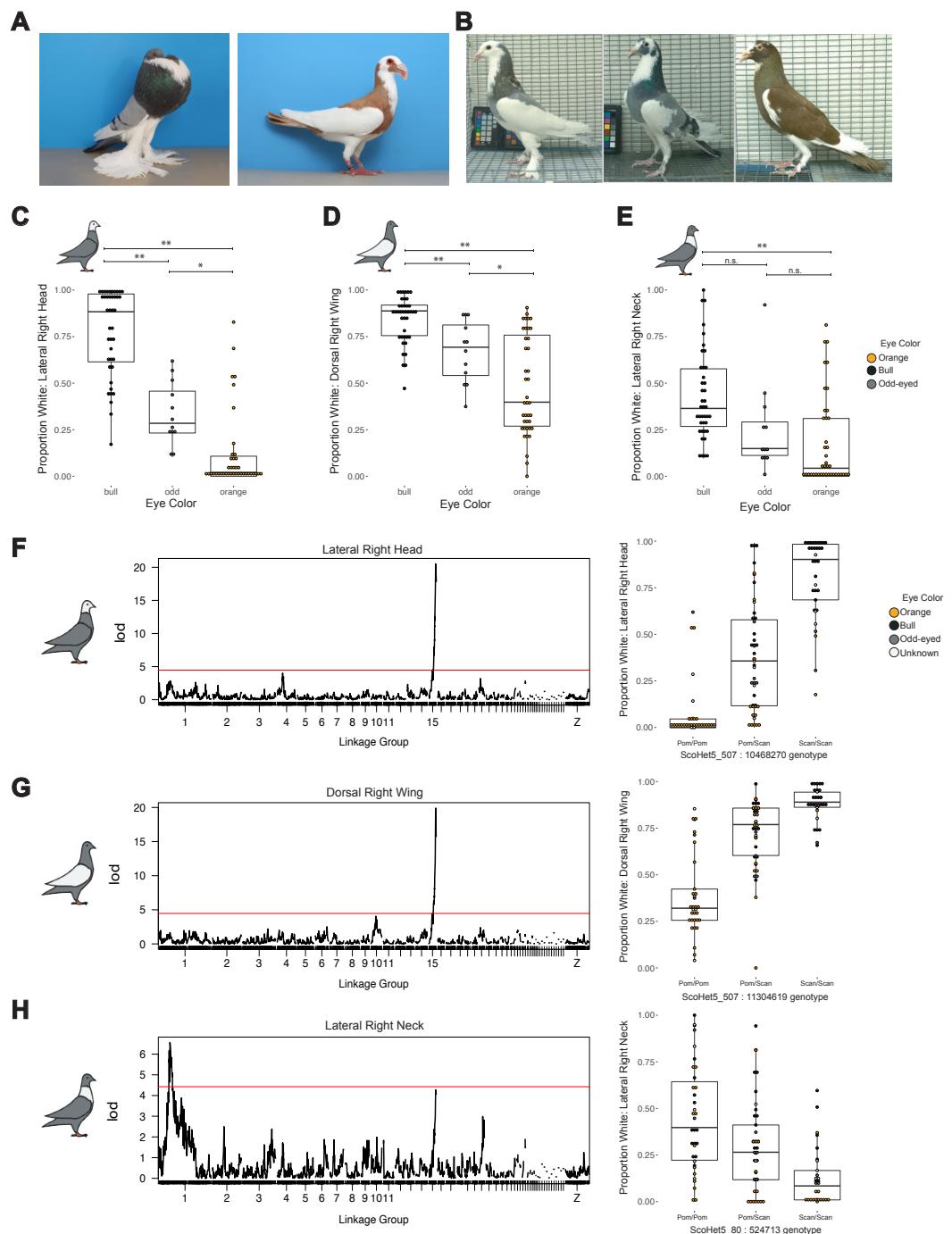


Figure 3. Bull eye color is associated with white plumage in an F₂ intercross. (A) Examples of standard plumage patterning for the Pomeranian Pouter (left) and Scanderoon (right) breeds. Photos by Layne Gardner, used with permission. (B) Examples of variable piebald plumage patterning in Pomeranian Pouter x Scanderoon F₂ offspring. (C-E) Boxplots of association between eye color and proportion of white plumage on the (C) lateral right head, (D) dorsal right wing, and (E) lateral right neck of F₂ birds. **, $p \leq 0.0001$; *, $0.001 < p \leq 0.01$; n.s., $p > 0.01$. Boxes span from the first to third quartile of each data set, with lines indicating the median. Whiskers span up to 1.5x the interquartile range. (F-H) QTL scans for proportion of white plumage (left side of panel) and proportion of white plumage by genotype at the peak marker (right) for (F) lateral right head, (G) dorsal right wing, and (H) lateral right neck. Red line, 5% genome-wide significance threshold.