

1 **Title: A role for differential gene regulation in the rapid diversification of melanic plumage**  
2 **coloration in the dark-eyed junco (*Junco hyemalis*)**

3

4 **Running title: Genetic basis of junco plumage color**

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20

21 **ABSTRACT**

22 Color plays a prominent role in reproductive isolation, therefore understanding the proximal  
23 basis of pigmentation can provide insight into speciation. Subspecies of the dark-eyed junco  
24 (*Junco hyemalis*) have evolved marked differences in plumage coloration since the Last Glacial  
25 Maximum, yet whether color differences are caused by mutations in coding regions of expressed  
26 genes or are instead the result of regulatory differences remains unknown. To address this  
27 question, we studied the pigment composition and the genetic basis of coloration in two  
28 divergent subspecies, the slate-colored and Oregon juncos. We used HPLC and light microscopy  
29 to investigate pigment composition and deposition in feathers from four body areas. We then  
30 used RNAseq to compare the relative roles of differential gene expression in developing feathers  
31 and sequence divergence in transcribed loci under common garden conditions. Junco feathers  
32 differed in eumelanin and pheomelanin content and distribution. Within subspecies, in lighter  
33 feathers melanin synthesis genes were downregulated (including PMEL, TYR, TYRP1, OCA2,  
34 MLANA), ASIP was upregulated. Feathers from different body regions also showed differential  
35 expression of HOX and Wnt genes. Feathers from the same body regions that differed in color  
36 between the two subspecies showed differential expression of ASIP and three other genes  
37 (MFSD12, KCNJ13, HAND2) associated with pigmentation in other taxa. Sequence variation in  
38 the expressed genes was not related to color differences. Our findings support the hypothesis that  
39 differential regulation of a few genes can account for marked differences in coloration, a  
40 mechanism that may underlie the rapid diversification of juncos.

## 41 INTRODUCTION

42 Color traits are among the most rapidly evolving phenotypes in animals and plants  
43 (Hubbard et al., 2010; Protas & Patel, 2008), and they often represent the only phenotypically  
44 diagnosable differences between species (Bourgeois et al., 2017; Campagna et al., 2016). The  
45 rapid evolution of animal color is often attributed to sexual selection (Gray & McKinnon, 2007;  
46 Lande et al., 2001; Naisbit et al., 2001), because rapidly evolving sexually selected color traits  
47 may cause prezygotic reproductive barriers due to differences in mate preference, potentially  
48 leading to reproductive isolation and speciation (Seehausen et al., 2008). Understanding the  
49 mechanisms that underlie color divergence between populations is therefore critical for a better  
50 understanding of the speciation process.

51 This divergence is particularly apparent in birds, where color diversity has three main  
52 components: the diversity of pigments, the patterns of pigment deposition on different parts of a  
53 feather, and the modular organization of feather tracts across the bird's body, which may enable  
54 rapid recombination of color schemes (Badyaev, 2004, 2006). Some of the diversity in feather  
55 color has been shown to evolve as rapidly as within a few thousand years (Milá et al., 2007;  
56 Ödeen & Björklund, 2003; Zink et al., 2003), representing one of the fastest rates of evolutionary  
57 change reported in wild species. In some cases, the main genetic differences between species are  
58 in regions that encode color genes (Campagna et al., 2016; Poelstra et al., 2014), suggesting that  
59 speciation may start from only a few changes in mechanisms underlying color development.  
60 Furthermore, specific patterns of coloration often evolve independently in distantly related  
61 species (Shapiro et al., 2013), suggesting that common mechanisms may underlie major aspects  
62 of bird color diversity by channeling color variation along specific evolutionary trajectories  
63 (Poelstra et al., 2014).

64           Because of the power and promise of genetic studies of color variation, the genetics of  
65 pigment production have been extensively studied in mammals and birds for the better half of the  
66 past century (Hoekstra, 2006; Hofreiter & Schöneberg, 2010; Mundy, 2005; Silvers & Russell,  
67 1955; Yu et al., 2004). This is especially true for melanic color diversity, which has a strong  
68 genetic basis (Roulin & Ducrest, 2013). Melanic color diversity in birds is generated mainly by  
69 two pigments: eumelanin (grey, brown, black colors) and pheomelanin (yellow, red), which are  
70 produced in melanocytes, specialized pigment cells (Galván & Solano, 2016). Color differences  
71 in birds may be due to either differences in the chemical composition of melanin polymers,  
72 differential development of melanocytes, or differential distribution of melanin granules in the  
73 feather. Melanin synthesis has been shown to be regulated via numerous pathways (Hoekstra,  
74 2006), including the melanocortin 1 receptor (MC1R) and its two ligands, the  $\alpha$ -MSH and the  
75 Agouti signaling protein (ASIP)(Gluckman & Mundy, 2017; Yoshihara et al., 2012). Mutations  
76 in the regulatory genes, notably MC1R, have been shown to result in drastic changes in  
77 coloration in domestic and laboratory animals (Hoekstra, 2006; Kijas et al., 1998; Rieder et al.,  
78 2001; Våge et al., 1999) and, to a lesser extent, in wild species (Nachman et al., 2003; Theron et  
79 al., 2001; Uy et al., 2009).

80           While the role of MC1R and ASIP genes in generating color variation in some  
81 domesticated and undomesticated species is appreciated, our understanding of color evolution is  
82 nevertheless incomplete (Hoekstra, 2006). First, although sequence divergence in MC1R has  
83 been associated with melanic coloration in several cases (Theron et al., 2001; Uy et al., 2016), it  
84 often fails to explain polymorphisms (Bourgeois et al., 2016; Cheviron et al., 2006; MacDougall-  
85 Shackleton et al., 2003; Riyahi et al., 2015). Given the number of pathways that have been  
86 shown to regulate melanin production and melanocyte differentiation, this may not be surprising.

87 Indeed, the historical focus on a few candidate genes belies the complexity of the molecular and  
88 genetic networks that underlie melanin-based coloration (San-Jose & Roulin, 2017). Color  
89 variation in the wild can be more subtle and is often continuous, indicating complex interactions  
90 between the mechanisms that regulate local melanin production, polymerization, melanosome  
91 maturation, and deposition in the developing barbs and barbules (Arai et al., 2017; Bourgeois et  
92 al., 2017; Poelstra et al., 2014; Yang et al., 2017). To better understand the genetic basis of the  
93 natural diversity of coloration, we therefore need to expand our scope to identify additional  
94 candidate genes and mechanisms that generate color variation.

95 Second, we know little about the relative roles of gene expression and point mutations in  
96 coding regions in affecting color variation in the wild (Roulin & Ducrest, 2013). Although some  
97 work has been done in domesticated birds (Cooke et al., 2017; San-Jose et al., 2017),  
98 experimental work under controlled conditions aimed at understanding the role of gene  
99 regulation in affecting melanic coloration has been scarce (Ekblom et al., 2012).

100 In this study, we addressed both of these issues by studying gene expression underlying  
101 plumage color divergence in two plumage forms of the dark-eyed junco (*Junco hyemalis*) in a  
102 common garden environment. The dark-eyed junco complex is a quintessential example of rapid  
103 evolution of plumage color (Milá et al., 2007) and consists of at least six distinct, geographically  
104 structured subspecific forms with strikingly different plumage coloration (Nolan et al., 2002).  
105 Recent molecular evidence indicates that the diversification within the dark-eyed junco species  
106 complex has occurred within the last 10,000 years following their post-glacial expansion in  
107 North America (Friis et al., 2016; Milá et al., 2007). Color is the main phenotypic difference  
108 between these taxa, which are otherwise morphologically similar and do not differ in their song  
109 (Nolan et al., 2002). The main color differences between junco subspecies occur on their heads,

110 backs, and flanks. Importantly, variation in plumage traits that delineate subspecies (color of the  
111 head, amount of white on tail feathers), has also been shown to have social significance (Hill et  
112 al., 1999; Holberton et al., 1989). This suggests that the differences in junco feather color are  
113 involved in mate choice and thus may play a role in the development of assortative mating and  
114 prezygotic isolation.

115 We investigated the mechanisms responsible for color divergence in two forms of the  
116 dark-eyed junco: the slate-colored junco and the Oregon junco, which occur in temperate areas  
117 of Eastern and Western North America, respectively (Nolan et al., 2002). Slate-colored juncos  
118 have uniformly slate-gray upper parts, lighter gray flanks, and ventral areas (bellies), whereas  
119 Oregon juncos have black heads, brown backs, light brown flanks, and white ventral areas  
120 (Figure 1). We first asked if the two subspecies differed in the concentration of eumelanin and  
121 pheomelanin in their head, back, flank, and ventral feathers using high performance liquid  
122 chromatography (HPLC). We predicted to find more pheomelanin in the light brown-colored  
123 flanks and brown backs of Oregon juncos, compared to the gray feathers of slate-colored juncos.  
124 We then investigated if the two subspecies differed in the patterns of eumelanin and pheomelanin  
125 deposition in different feather regions (rachis, barbs and barbules) using light microscopy. To  
126 investigate the mechanisms underlying color divergence, we used RNA sequencing to  
127 characterize gene expression differences and sequence variation associated with variation in  
128 feather color between junco subspecies as well as across different body parts (head, flank, back,  
129 belly) within subspecies. Our objectives were to determine (i) whether candidate genes well  
130 known to regulate melanic coloration in domestic and some wild birds were also involved in the  
131 color differences between the two junco subspecies as well as among different feather types  
132 within each subspecies (ii) whether novel genes may be involved in this radiation that have not

133 been known to control color in birds, and (iii) whether color differences among subspecies may  
134 be explained by point mutations in coding regions of the expressed genes or are instead the result  
135 of regulatory differences of these genes.

136

## 137 **MATERIALS AND METHODS**

### 138 *Feather sampling and experimental design*

139 We sampled feathers from two dark-eyed junco (*Junco hyemalis*) subspecies: the Oregon junco  
140 (*J. h. thurberi*, n=4) and the slate-colored junco (*J. h. carolinensis*, n=4). Oregon juncos (*herein*  
141 *abbreviated* ORJUs) were originally captured on University of California San Diego campus and  
142 the Laguna Mountains in the San Diego County, California, USA. Slate-colored juncos (*herein*  
143 *abbreviated* SCJUs) were captured at Mountain Lake Biological Station, Giles County, Virginia,  
144 USA. In order to reduce the influence of external factors on gene expression, birds were kept in a  
145 common-garden environment for at least 7 months (including the fall molt) prior to the collection  
146 of feathers. All birds used in the experiment were placed in cages in a single room, under  
147 identical light and temperature conditions, and were fed the same food. For each individual, we  
148 plucked mature feathers from four distinct body areas: head (coronal region of the capital feather  
149 tract), back (interscapular region of the spinal tract), flank (dorsal side of the sternal region of the  
150 ventral tract), and ventral area (belly; the ventral side of the sternal region of the ventral feather  
151 tract). For each tract, we plucked feathers from an area of 1 cm<sup>2</sup>. Mature feathers were used for  
152 the pigment composition and distribution analysis, while the plucking served to induce feather  
153 development in the plucked area. We monitored feather regrowth every two days following  
154 plucking. We collected developing feathers during the development of the pennaceous vane,  
155 when the first mature barbs started to erupt from the tip of the follicle (8 to 19 days following the  
156 plucking, median 11 days). To collect feather follicles, we applied a topical anesthetic to the

157 skin, and gently plucked individual developing feathers using forceps. Six follicles were  
158 collected from each area. Plucked follicles were immediately placed on pulverized dry ice, and  
159 thereafter frozen at -80 °C until RNA extraction.

160

#### 161 *Quantification of melanins in mature feathers*

162 We examined the patterns of pigment deposition in the feather rachis, barbs, and barbules using a  
163 Leica MZI16A stereomicroscope at a magnification of 100X, and photographed each feather  
164 using a Leica DFC550 camera. We also quantified melanin content using high performance  
165 liquid chromatography (HPLC) to measure degradation products of pheomelanin (4-amino-3-  
166 hydroxyphenylalanine, 4-AHP, and thiazole-2,4,5-tricarboxylic acid, TTCA) and eumelanin  
167 (pyrrole-2,3,5-tricarboxylic acid, PTCA). Feather samples were homogenized with a Ten-Broeck  
168 homogenizer at a concentration of 1 mg/mL H<sub>2</sub>O. 100 µL (0.1 mg) aliquots were subjected to  
169 alkaline hydrogen peroxide oxidation (Ito et al., 2011) and hydroiodic acid hydrolysis  
170 (Wakamatsu et al., 2002).

171

#### 172 *RNA extraction and cDNA library preparation and sequencing*

173 To analyze gene expression, we created 32 separate libraries – one for each of the four body  
174 areas (head, back, flank, ventral) for each of the eight individuals (four per morph). We used 6  
175 developing feathers for each library to ensure sufficient RNA recovery. RNA was extracted in  
176 TRIzol following manufacturer directions (Invitrogen, Carlsbad, CA, USA). cDNA libraries  
177 were prepared for the polyA-enriched fraction of the transcriptome at Macrogen Inc., South  
178 Korea, using Illumina Truseq RNA technology and sequenced in an Illumina HiSeq2000  
179 platform. Only 3 of the ventral region libraries were sequenced per subspecies and one SCJU-

180 Back library failed, so that a total of 29 libraries were successfully sequenced (Oregon junco: 4 x  
181 Back, 4 x Flank, 4 x Head, and 3 x Ventral; slate-colored junco: 3 x Back, 4 x Flank, 4 x Head,  
182 and 3 x Ventral). The sequencing runs produced a total of 129 Gb of cDNA, representing an  
183 average of 22 million high quality read pairs (each about 100 bp long, more than 94% of the  
184 bases with a quality above/below or equal to Q20) per library. The raw read datasets are  
185 available at ArrayExpress database at EMBL-EBI ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) under accession  
186 number E-MTAB-6794.

187

188

### 189 *Mapping of reads*

190 Spliced mapping was performed against the closest and most complete reference genome  
191 available, the zebra finch (*Taeniopygia guttata*, v3.2.4) genome with annotation v3.2.4.89. In  
192 order to map and obtain the read counts per transcript we used the Gemtools RNA-sequencing  
193 pipeline version 1.6, which is based on the GEM mapper (Marco-Sola et al., 2012) and is an  
194 update of the workflow used in (Lappalainen et al., 2013). Because the overall quality of the  
195 reads observed with FastQC was high and because the quality of the reads is taken into account  
196 during the mapping with GEM, no preliminary read cleaning was performed. Mapping statistics  
197 were computed with Gemtools and SAMTOOLS 1.2 *flagstat* (Li et al., 2009).

198

### 199 *Differential regulation analysis*

200 Expression quantification was performed at the gene level, using FeatureCounts (Liao et al.,  
201 2014) and the genome annotation v3.2.4.89. Differential regulation analysis was conducted using  
202 the edgeR package in R (Robinson et al., 2010) with the read counts at the gene level and  
203 comparing expression in body parts within and among subspecies. Normalized read Counts Per

204 Million (CPM) were calculated using the TMM method (Robinson & Oshlack, 2010).  
205 Differential regulation was tested using a generalized linear model approach (FDR <0.05)  
206 comparing each tissue against each other (Table 2). For further analysis of the differentially  
207 expressed genes, we focused on those that were associated with GO term “pigmentation”  
208 (GO:0043473). Genes and GO terms were matched with BioMart filtering API in Ensembl  
209 (Cunningham et al., 2014). This led to a list of 59 genes related to pigmentation to which we  
210 added the MLANA gene which was absent from the GO query results. GO term enrichment  
211 analyses were performed with the R package topGO (Alexa & Rahnenfuhrer, 2016) for  
212 differentially expressed genes in pairwise comparisons between feather types (within and  
213 between subspecies), using Fisher’s test to calculate the significance of gene enrichment. Only  
214 terms that had more than 5 annotated genes and included more than 2 significantly expressed  
215 genes are reported.

216

### 217 *Variant calling*

218 Read mappings from the GEM output were further processed for variant (single nucleotide  
219 polymorphisms (SNPs) and insertions/deletions) calling in the transcribed genes. Read groups  
220 and duplicate markings were added to the bam files using the AddOrReplaceReadGroups and  
221 MarkDuplicates commands from the PICARD package (<http://broadinstitute.github.io/picard>).  
222 We then used GATK (McKenna et al., 2010) to identify putative SNPs and indels. We followed  
223 the guidelines from the GATK best practices for variant calling from RNA-seq data  
224 (<https://www.broadinstitute.org/gatk/guide>) and from (De Wit et al., 2015). Variant calls were  
225 filtered using GATK (filters used: FS > 30.0; QD < 2.0, window 35, cluster 3) and VCFtools  
226 ((Danecek et al., 2011), --max-missing 0.25, --mac 1, --min-alleles 2, --minDP 6, --minGQ 10).

227 Variant locations on the genome were identified using in-house python scripts. Weir and  
228 Cockerham  $F_{ST}$  values between the two forms ORJU and SCJU were computed using VCFtools.  
229 A GO term enrichment analysis was performed on the genes showing variants with  $F_{ST}$  values  
230 equal to one.

231

## 232 **RESULTS**

### 233 *Distribution and quantification of pigments*

234 Inspection of mature feathers from various body parts of SCJU and ORJU individuals  
235 with light microscopy color differences between subspecies are in part to do differential  
236 pigmentation of rachi, barbs and barbules (Figure 1). In the black feathers from ORJU heads,  
237 rachis, barbs, and barbules were uniformly darkly colored, suggesting predominance of  
238 eumelanin pigmentation. In contrast, in ORJU back and flank feathers, only barbules showed  
239 dark coloration consistent with eumelanin, while barbs and rachi showed orange-brown  
240 coloration, consistent with pheomelanin predominance. Feathers from the gray heads, backs, and  
241 flanks of SCJUs had darkly pigmented rachi and barbules, yet barbs contained no apparent  
242 pigment. The pennaceous part of white ventral feathers from both subspecies showed no  
243 apparent pigmentation.

244 Eumelanin and pheomelanin concentrations quantified using HPLC were overall  
245 consistent with the light microscopy observations. Eumelanins were found in feathers from both  
246 subspecies and all body parts, whereas pheomelanins were absent in all SCJU feather samples,  
247 but present in ORJU back, flank feathers, as well as in the black ORJU heads where it may be  
248 masked by eumelanin (Table 1). ORJU back feathers (brown) showed the highest concentration  
249 of pheomelanin. We also found eumelanin in the white ventral feathers. In these feathers, the

250 visible distal vane of the ventral feathers is white, while the more proximal feather plumes,  
251 hidden by other vanes, are gray.

252

253 *Differential gene expression between feather types within subspecies*

254 Using the Gemtools pipeline, an average of 87.1% (min: 83.6%, max: 90.4%) of all reads were  
255 mapped to the zebra finch genome and an average of 61% (min: 59.1%, max: 63.5%) were  
256 properly paired. The relatively low figures are not surprising given the evolutionary distance  
257 between junco and zebra finch, which is the closest species for which a high-quality reference  
258 genome exists. Overall, 346 genes were differentially expressed at a statistically significant level  
259 (FDR threshold of 0.05) between different feather tracts within subspecies (Table 2, Table S1).  
260 Of these, 304 were differentially expressed in Oregon juncos, and 112 were differentially  
261 expressed in slate-colored juncos (overlap of 70 genes).

262       Among the significantly differentially expressed genes between body parts in ORJU were  
263 several members of the canonical melanin synthesis pathway –TYRP1, TYR, OCA2, RAB38,  
264 SLC45A2, SLC24A5, PMEL, and MLANA (Figure 2). Most of these genes were downregulated  
265 in developing white ventral feathers compared to colored feathers (Figure 3). Within SCJU, the  
266 qualitative patterns of expression of these genes were similar, although only SLC45A2 was  
267 significantly downregulated in SCJU ventral feathers compared to other body regions.

268       In ORJU, the white ventral feathers expressed significantly more ASIP, an inhibitor of  
269 eumelanin synthesis, compared to head and back feathers. In both ORJU and SCJU, feathers also  
270 showed differential regulation of Wnt signaling pathway components, including SFRP1 and  
271 DKK3, both Wnt signaling inhibitors (Figure 4). DKK3 expression was lower in the black head  
272 feathers compared to the lighter back, flank, and ventral feathers in both ORJU and SCJU, while

273 SFRP1 expression was lower in the dark ORJU feathers compared to their white ventral feathers.  
274 On the other hand, another Wnt-signaling pathway gene, FRZB, was upregulated in SCJU head  
275 and back feathers compared to the white ventral feathers (Figure 4).

276 Among other significantly differentially expressed genes between the different feather  
277 types were members of the HOX gene group. In ORJU, ten HOX genes were downregulated in  
278 the head feathers compared to back, flank, and ventral feathers. In SCJU, only two HOX genes  
279 were differentially regulated between feather types (Figure 5). Some HOX genes were  
280 differentially expressed with respect to body region rather than melanin type. For example,  
281 HOXA2 and HOXB7 were up and down-regulated, respectively, in the head feathers of both  
282 subspecies, whereas HOXB8 was upregulated only in developing ventral feathers.

283 GO categories that were significantly enriched among the differentially regulated genes  
284 between feather types included categories related to pigmentation (e.g. melanin biosynthetic  
285 process, pigment granule organization, pigment cell differentiation; all significantly enriched  
286 between ventral feathers and colored feathers in ORJUs) as well as morphogenesis (e.g.  
287 developmental process, appendage development, tissue morphogenesis, all between head  
288 feathers and other body feathers in both ORJUs and SCJUs)(Table S2).

289

#### 290 *Differential gene expression between subspecies*

291 Only 10 genes (Table S3) were significantly differentially regulated between the same  
292 feather tracts across the two subspecies (Table 2). Of these, ASIP has been linked to pigment  
293 variation in birds, and three other genes (MFSD12, KCNJ13, and HAND2) have been associated  
294 with pigment production in other vertebrates. ASIP and HAND2 were more highly expressed in  
295 the gray SCJU heads compared to black ORJU heads, while MFSD12 and KCNJ13 were more

296 highly expressed in the light brown ORJU flanks compared to the grey SCJU flanks (Figure 2).  
297 Most of the differential expression (7 out of 10 genes) was between developing ORJU and SCJU  
298 head feathers (black vs gray). Only one gene (FAM172A) was significantly differentially  
299 expressed between all three colored feather tract comparisons.

300

### 301 *Sequence variation between subspecies*

302 A total of 57,214 variant sites were identified between the two morphs. Out of these, only 43  
303 variant sites (located in 20 different genes) were segregating between the two morphs with  
304  $F_{ST}=1$ , but none were located in the pigmentation related gene list obtained from Ensembl. The  
305 highest  $F_{ST}$  value observed for a pigmentation related gene was 0.55 at the FIG4 gene.  
306 Interestingly, the group of 20 genes with a fixed SNP contained two genes associated to the Wnt  
307 signaling pathway, FZD4 and APC.

308

## 309 **DISCUSSION**

310 Color variation between the dark-eyed junco subspecies represents one of the best examples of  
311 rapid plumage color evolution in the wild. To understand the mechanisms that underlie this  
312 diversity, we characterized the pigment composition and deposition patterns in mature feathers  
313 and used RNAseq to ask if differences in coloration between two distinct junco subspecies are  
314 explained by differences in gene expression in developing feathers or by coding differences in  
315 the expressed genes. We show that coloration differences between subspecies are due to the  
316 differential deposition of eumelanin and pheomelanin in different parts of the birds' feathers, and  
317 that variation among body parts within and across subspecies results from the differential  
318 regulation of a potentially small set of genes rather than from point mutations in their coding  
319 regions.

320

321 *Phenotypic difference in melanin deposition*

322           Slate-colored and Oregon junco subspecies differed in the coloration of their flanks,  
323 backs, and heads. As proposed previously (Miller, 1941), the differences in coloration on a  
324 phenotypic level were explained by differences in the type of pigment deposited in the feathers,  
325 as well as the pattern in which this pigment was deposited in the rachis, barbs, and barbules  
326 (Figure 1). Eumelanin was found in both subspecies and all feather types. Pheomelanin was  
327 present in all body parts of black- and brown-colored ORJUs, whereas it was below the detection  
328 limit in the uniformly gray SCJU feathers (Table 1). Pheomelanin values in ORJU were higher  
329 for back, yet values for head, flank, and ventral area were very similar, even though head  
330 feathers are black, flank is light brown, and ventral area is white. Similar patterns of color  
331 differences despite similar pheomelanin levels have also been shown in human hair (Ito et al.,  
332 2011) and human skin (Bino et al., 2015). This can be explained by the casing model, which  
333 proposes that in melanosomes that contain both pigments, pheomelanin is produced first,  
334 followed by synthesis of eumelanin, which surrounds the pheomelanin core (Ito & Wakamatsu,  
335 2008). Interestingly, pigment deposition in feathers was not uniform: the grey SCJU feathers had  
336 pigmented rachis and barbules, whereas barbs appeared unpigmented. In contrast, in ORJU, rachis  
337 and barbs of flank and back feathers showed orange pigmentation, likely due to a presence of  
338 pheomelanin, whereas barbules were much darker, indicating a predominance (or casing) of  
339 eumelanin. In ORJU heads, feather rachis, barbs, and barbules were all dark, suggesting a  
340 predominance of eumelanin pigment. These observations suggest that color differences between  
341 Oregon and slate-colored juncos are due to regulation of both melanin synthesis and the  
342 differential migration of the mature melanosomes in the developing feather matrix. Recent

343 studies have also suggested that color variation in feathers may be a result of the ratios of  
344 different melanin moieties (chemical variants) in the feathers (Galván & Wakamatsu, 2016).

345

346 *Differences in expression between subspecies*

347 We were able to detect only a handful of genes that were differentially regulated at a statistically  
348 significant level between the same body parts of ORJUs and SCJUs (Table 2). The grey SCJU  
349 heads expressed less ASIP and HAND2 compared to the black ORJU heads (Figure 2). ASIP,  
350 which encodes the Agouti-signaling peptide, is an inverse agonist to melanocortin-1 receptor  
351 (MC1R), one of central regulators of melanin synthesis in birds and mammals (Manceau et al.,  
352 2011; Mundy, 2005). Increased expression/signaling by ASIP has been shown to lead to  
353 increased synthesis of pheomelanin (Roulin & Ducrest, 2013) or arrest of melanin synthesis,  
354 leading to absence of pigmentation (Lin et al., 2013). Higher expression of ASIP in grey head  
355 feathers of SCJU suggests that ASIP may be lowering the production of melanin in these  
356 feathers, in contrast to the black ORJU feathers. ASIP has been shown to explain color variation  
357 in a wide variety of taxa (Martin & Orgogozo, 2013). Our study adds to this body of literature  
358 and suggests that the regulation of MC1R by ASIP can lead to rapid changes in the color hue.

359 HAND2 encodes a transcription factor that has been shown to be important in regulating  
360 cell fate during the development of various organs and limbs (Yelon et al., 2000). It is also an  
361 upstream regulator of an important patterning gene, sonic hedgehog (SHH) (Xiong et al., 2009).  
362 HAND2 has been shown to be differentially expressed in cichlid fish fins that differ in color,  
363 indicating that HAND2 may be responsible for regulation of pigment cell development or  
364 function (Santos et al., 2016). Because of this, we hypothesize that HAND2, or genes  
365 downstream in the SHH pathway, may be involved in regulation of melanin production or

366 differential deposition of mature melanosomes in the rachi, barbs, and barbules, which are  
367 distinct tissue types in the developing feather.

368         The light brown pheomelanin-rich ORJU flank feathers expressed more MFSD12 and  
369 KCNJ13 compared to the grey SCJU feathers (Figure 2). MFSD12 was recently identified as one  
370 of the principal genes regulating skin color in humans and coat color in mice (Crawford et al.,  
371 2017). Knockdown studies in mice have shown that MFSD12 inhibits eumelanin synthesis while  
372 being required for pheomelanin synthesis (Crawford et al., 2017). This suggests a conserved role  
373 of this gene across major vertebrate groups and calls for further study of the role of this gene in  
374 generation phenotypic variation. KCNJ12 has been associated with changes in pigmentation  
375 patterns on zebra fishes (Haffter et al., 1996). In zebra fishes, the potassium channel encoded by  
376 this gene regulates the interactions between melanophores and xantophores (Singh & Nüsslein-  
377 Volhard, 2015). KCNJ13 function is unknown in birds, but it may regulate interactions between  
378 pigment cells and the surrounding cellular matrix, perhaps being responsible for the differential  
379 pigment deposition in barbs and barbules.

380

### 381 *Differences in gene expression between feather types within subspecies*

382         Compared to head, back, and flank, the white ventral ORJU feathers had lower  
383 expression of genes that encode four main regulators/catalysts of melanin production in the  
384 melanosome: TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), OCA2 (OCA2  
385 melanosomal transmembrane protein), and SLC45A2 (Solute Carrier Family 45 Member 2,  
386 Figure 2). TYR and TYRP1 catalyze reactions that lead to the conversion of tyrosine to melanin,  
387 while the role of SLC45A2 and OCA2 in bird melanocytes is less well understood (Galván &  
388 Solano, 2016). Mutations in SLC45A2 have been shown to inhibit the synthesis of pheomelanin,

389 suggesting that it may control pheomelanin production (Gunnarsson et al., 2007). White feathers  
390 also showed lower expression of MLANA/MART1 and GPR143/OA1 which regulate  
391 melanosome biogenesis and maturation (Aydin et al., 2012; Cortese et al., 2005; Schiaffino &  
392 Tacchetti, 2005)(Figure 2).

393 Melanin synthesis in birds is regulated by at least three semi-independent pathways:  
394 MC1R, Wnt, and MAPK pathways (Poelstra et al., 2014). White ventral and light brown flank  
395 ORJU feathers expressed more ASIP compared to ORJU heads or backs. ASIP is an inverse  
396 agonist to MC1R, indicating that signaling along this pathway may be responsible for the  
397 suppression of melanin synthesis in the white ventral and light brown flank feathers. We also  
398 found that ventral feathers expressed significantly less SFRP1, a gene encoding frizzled-related  
399 protein that plays an important role in Wnt signaling, and significantly more DKK3, a Wnt-  
400 signaling inhibitor (Figure 4). Genes from the DKK family have been shown to suppress  
401 melanocyte function and proliferation (Yamaguchi et al., 2007). This suggests that white feather  
402 color may also result from inhibition of Wnt-activated melanin synthesis. Surprisingly, another  
403 Wnt-signaling inhibitor that is linked to melanocyte function FRZB (Thomas & Erickson, 2008),  
404 showed the opposite pattern, being expressed at lower levels in the white ventral feathers (Figure  
405 4). This suggests that either the role of FRZB in avian melanocytes may be different compared to  
406 mammalian systems, that FRZB may be responsible for processes other than feather color (see  
407 below), or that FRZB may be involved in arresting melanocyte function following active melanin  
408 synthesis. FRZB has shown to be associated with darker pigmentation in other bird species as  
409 well, suggesting a similar function of FRZB across avian taxa (Poelstra et al., 2015).

410

411 *Differential expression of genes associated with feather type*

412 It is important to note that, instead of regulating color, many of the differentially  
413 expressed genes between different feather types may be regulating feather morphology.  
414 Alternatively, these differences may reflect differences in developmental timing, as we could not  
415 ensure that feathers from different body regions were collected at the exact same developmental  
416 stage. Poelstra et al. (2015) differentiated between the putative functions (color vs. shape) of  
417 differentially expressed genes by asking if expression differences between feather types persist  
418 across taxa, given that at least in one taxon the color is the same between feather types. Because  
419 SCJUs have grey feathers on their heads, backs, and flanks, we applied this logic to ask if genes  
420 differentially expressed in these feathers in SCJU were also differentially expressed in the  
421 equivalent comparisons in ORJU. We found only three genes that were consistently differentially  
422 expressed between feather types across subspecies. Only one of these genes (IL17REL) was  
423 annotated (lower expression in back compared to flank in both subspecies), but it has not been  
424 linked to feather development before.

425

#### 426 *Differential expression of HOX and Wnt genes*

427 We found differential expression in 11 HOX genes between white and darker feathers,  
428 although our experimental design does not allow us to assign precise functions to these genes  
429 (Komiya & Habas, 2008)(Figure 5). HOX genes are transcription factors that regulate  
430 morphogenesis via their time- and location-specific expression (Krumlauf, 1994). All but one  
431 (HOXA2) showed lower expression in the black ORJU head feathers compared to the ORJU  
432 flank, back, and belly feathers. Among these, HOXC8 has been shown to regulate feather  
433 morphology in chickens, and its misexpression can turn head feathers into body-like feathers  
434 (Boer et al., 2017; Wang et al., 2012). SCJU head feathers showed qualitatively similar HOX

435 expression patterns as ORJU head feathers, although only two HOX genes (HOXA2, HOXB8)  
436 were significantly differentially expressed between SCJU head and ventral feathers. These  
437 qualitatively similar patterns suggests that HOX genes may be either regulating head-specific  
438 feather morphology (head feathers are much smaller) or reflect differences in the developmental  
439 stage of feathers across different body regions at the time of tissue collection. On the other hand,  
440 the strong difference in HOX expression between ORJU flank, back, and ventral feathers (which  
441 differ in pigment deposition patterns), and the near absence of such differences in the SCJU  
442 feathers (which have similar pigment deposition patterns), suggests that HOX genes may also be  
443 involved in regulation of feather color, as shown in *Drosophila* (Jeong et al., 2006), perhaps  
444 through their capacity to regulate cell migration (Stoll & Kroll, 2012).

445 In addition to HOX genes, another important signaling pathway for morphogenesis is the  
446 Wnt signaling pathway, which regulates cell fate, migration, and tissue patterning (Komiya &  
447 Habas, 2008). Therefore, differences in expression of Wnt-related genes in white and dark  
448 feathers may reflect the role of these genes in regulating feather growth or differences in feather  
449 shape.

450

#### 451 *Sequence variation and population differentiation*

452 We identified only 43 variant sites segregating SCJU and ORJU forms ( $F_{ST}=1$ ), none of them in  
453 genes closely related to pigmentation, indicating that differential color pigmentation in the two  
454 forms are more likely due to regulatory mechanisms than to sequence variation in the coding  
455 regions of known pigmentation-related genes. Nonetheless, although weakly supported in terms  
456 of number of good quality genotypes called, segregating variants were detected in Wnt-pathway  
457 related genes, providing further support for the potential role of Wnt signaling in feather color

458 regulation. Higher coverage sequencing as well as SNP data in non-coding introns and *cis* and  
459 *trans* regulatory sites could shed light on the implication of sequence variation in the regulation  
460 of pigmentation of the two forms. An additional possibility is that differential expression may be  
461 due to copy number variation of the underlying genes, or due to environment-driven differences  
462 in the epigenetic regulatory mechanisms. However, our common garden approach should have at  
463 least partly eliminated the possibility of environmentally-induced plumage variation.

464

#### 465 *Rapid evolutionary change*

466 We have shown that rapid evolution of feather color in the genus *Junco* can be explained by  
467 changes in pigment composition and pigment distribution. While the role of pigment  
468 composition in the evolution of color is appreciated, few studies have investigated how  
469 differential pigment distribution on rachis, barbs, and barbules, contributes to color divergence  
470 (Galván, 2011). Here we demonstrate striking differences in pigment distribution in feathers  
471 between closely related subspecies. Because developing barbs and barbules occupy distinct  
472 locations in the developing feather (barbules are more peripheral in the cross-section of a  
473 developing follicle (Yu et al., 2004)), these differences could be achieved by relatively few  
474 changes in the regulation of melanosome deposition.

475         Indeed, our data show that feather color differences at the phenotypic level could be  
476 achieved by simple changes in gene expression involving canonical melanocyte signaling  
477 pathways and, possibly, genes that regulate pigment distribution within feathers. These findings  
478 demonstrate that drastic changes in plumage color can evolve rapidly and readily. Furthermore,  
479 although our study might have failed to identify *Junco*-specific pigmentation genes due to the  
480 lack of a complete genomic reference for the species, our findings are consistent with the

481 hypothesis that evolution of coloration in birds and other vertebrates involves the same  
482 molecular pathways and genes (Martin & Orgogozo, 2013). For example, ASIP has been shown  
483 to regulate color in both mammals (Manceau et al., 2011; Rieder et al., 2001; Steiner et al., 2007)  
484 and birds (Campagna et al., 2016; Lin et al., 2013; Nadeau et al., 2008). Many of the studies  
485 investigating the role of ASIP capitalize on color polymorphisms in domesticated or model  
486 organisms. Our study joins the small but growing number of studies showing that ASIP may  
487 regulate evolution of feather color in wild populations. The absence of segregating SNPs in the  
488 coding sequence of ASIP and other genes involved in pigmentation suggests that variation in the  
489 feather color between junco subspecies may be due to variation in the regulatory regions of these  
490 genes, or in the coding or regulatory sequences of upstream transcription factors.

491 In addition to ASIP, we identify three other candidate genes - MFSD12, KCNJ13, and  
492 HAND2 – that have been shown to be important in vertebrate color development and evolution  
493 but have not, to our knowledge, been linked to color differences in birds. Identification of such  
494 genes is important because major candidate genes for feather color, such as ASIP, explain only  
495 part of the phenotypic diversity observed in the wild (San-Jose & Roulin, 2017). Future studies  
496 should further investigate the precise role that these genes may play in regulating melanin  
497 synthesis or melanosome distribution.

498

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502

## 503 **REFERENCES**

504 Arai, E., Hasegawa, M., Makino, T., Hagino, A., Sakai, Y., Ohtsuki, H., ... Kawata, M. (2017).

- 505           Physiological conditions and genetic controls of phaeomelanin pigmentation in nestling  
506           barn swallows. *Behavioral Ecology*, 28, 706–716.
- 507   Aydin, I. T., Hummler, E., Smit, N. P. M., & Beermann, F. (2012). Coat color dilution in mice  
508           because of inactivation of the melanoma antigen MART-1. *Pigment Cell and Melanoma*  
509           *Research*, 25, 37–46.
- 510   Badyaev, A. V. (2004). Integration and Modularity in the Evolution of Sexual Ornaments. In  
511           *Phenotypic Integration: Studying the Ecology and Evolution Complex Phenotypes* (pp.  
512           50–79).
- 513   Badyaev, A. V. (2006). Colorful Phenotypes of Colorless Genotypes: Toward a New  
514           Evolutionary Synthesis of Color Displays. In *Bird Coloration* (pp. 349–379).
- 515   Bino, S. D., Ito, S., Sok, J., Nakanishi, Y., Bastien, P., Wakamatsu, K., & Bernerd, F. (2015).  
516           Chemical analysis of constitutive pigmentation of human epidermis reveals constant  
517           eumelanin to pheomelanin ratio. *Pigment Cell & Melanoma Research*, 28, 707–717.
- 518   Boer, E. F., Van Hollebeke, H. F., & Shapiro, M. D. (2017). Genomic determinants of epidermal  
519           appendage patterning and structure in domestic birds. *Developmental Biology*, 429, 409–  
520           419.
- 521   Bourgeois, Y. X. C., Bertrand, J. A. M., Delahaie, B., Cornuault, J., Duval, T., Milá, B., &  
522           Thébaud, C. (2016). Candidate Gene Analysis Suggests Untapped Genetic Complexity in  
523           Melanin-Based Pigmentation in Birds. *Journal of Heredity*, 107, 327–335.
- 524   Bourgeois, Y. X. C., Milá, B., Thébaud, C., Delahaie, B., Gautier, M., Lhuillier, E., ... Holota,  
525           H. (2017). A novel locus on chromosome 1 underlies the evolution of a melanic plumage  
526           polymorphism in a wild songbird. *Royal Society Open Science*, 4, 1–14.
- 527   Campagna, L., Repenning, M., Silveira, L. F., Suertegaray Fontana, C., Tubaro, P. L., & Lovette,  
528           I. J. (2016). Repeated divergent selection on pigmentation genes in a rapid finch radiation  
529           driven by sexual selection. *Science Advances*, 5, e1602404
- 530   Chevion, Z. A., Hackett, S. J., & Brumfield, R. T. (2006). Sequence variation in the coding  
531           region of the melanocortin-1 receptor gene (MC1R) is not associated with plumage  
532           variation in the blue-crowned manakin (*Lepidothrix coronata*). *Proceedings of the Royal*  
533           *Society of London B: Biological Sciences*, 273, 1613–1618.
- 534   Cooke, T. F., Fischer, C. R., Wu, P., Jiang, T.-X., Xie, K. T., Kuo, J., ... Bustamante, C. D.  
535           (2017). Genetic Mapping and Biochemical Basis of Yellow Feather Pigmentation in  
536           Budgerigars. *Cell*, 171, 427–439.
- 537   Cortese, K., Giordano, F., Surace, E. M., Venturi, C., Ballabio, A., Tacchetti, C., & Marigo, V.  
538           (2005). The Ocular Albinism Type 1 (OA1) Gene Controls Melanosome Maturation and  
539           Size. *Investigative Ophthalmology & Visual Science*, 46, 4358–4364.
- 540   Crawford, N. G., Kelly, D. E., Hansen, M. E. B., Beltrame, M. H., Fan, S., Bowman, S. L., ...  
541           Tishkoff, S. A. (2017). Loci associated with skin pigmentation identified in African  
542           populations. *Science*, 358, eaan8433.
- 543   Cunningham, F., Amode, M. R., Barrell, D., Beal, K., Billis, K., Brent, S., ... others. (2014).  
544           Ensembl 2015. *Nucleic Acids Research*, 43, D662–D669.
- 545   Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., ... others.  
546           (2011). The variant call format and VCFtools. *Bioinformatics*, 27, 2156–2158.
- 547   De Wit, P., Pespeni, M. H., & Palumbi, S. R. (2015). SNP genotyping and population genomics  
548           from expressed sequences--current advances and future possibilities. *Molecular Ecology*,  
549           24, 2310–2323.
- 550   Ekblom, R., Farrell, L. L., Lank, D. B., & Burke, T. (2012). Gene expression divergence and

- 551 nucleotide differentiation between males of different color morphs and mating strategies  
552 in the ruff. *Ecology and Evolution*, 2, 2485–2505.
- 553 Friis, G., Aleixandre, P., Rodríguez-Estrella, R., Navarro-Sigüenza, A. G., & Milá, B. (2016).  
554 Rapid postglacial diversification and long-term stasis within the songbird genus Junco:  
555 phylogeographic and phylogenomic evidence. *Molecular Ecology*, 25, 6175–6195.
- 556 Galván, I. (2011). Feather microstructure predicts size and colour intensity of a melanin-based  
557 plumage signal. *Journal of Avian Biology*, 42, 473–479.
- 558 Galván, I., & Solano, F. (2016). Bird integumentary melanins: Biosynthesis, forms, function and  
559 evolution. *International Journal of Molecular Sciences*, 17. doi:10.3390/ijms17040520
- 560 Galván, I., & Wakamatsu, K. (2016). Color measurement of the animal integument predicts the  
561 content of specific melanin forms. *RSC Advances*, 6, 79135–79142.
- 562 Gluckman, T. L., & Mundy, N. I. (2017). The differential expression of MC1R regulators in  
563 dorsal and ventral quail plumages during embryogenesis: Implications for plumage  
564 pattern formation. *PLoS ONE*, 12, 1–18.
- 565 Gray, S. M., & McKinnon, J. S. (2007). Linking color polymorphism maintenance and  
566 speciation. *Trends in Ecology and Evolution*, 22, 71–79.
- 567 Gunnarsson, U., Hellström, A. R., Tixier-Boichard, M., Minvielle, F., Bed'hom, B., Ito, S., ...  
568 Andersson, L. (2007). Mutations in SLC45A2 cause plumage color variation in chicken  
569 and Japanese quail. *Genetics*, 175, 867–877.
- 570 Haffter, P., Odenthal, J., Mullins, M. C., Lin, S., Farrell, M. J., Vogelsang, E., ... Nüsslein-  
571 Volhard, C. (1996). Mutations affecting pigmentation and shape of the adult zebrafish.  
572 *Development Genes and Evolution*, 206, 260–276.
- 573 Hill, J., Enstrom, D. A., & Ketterson, E. D. (1999). Mate choice based on static versus dynamic  
574 secondary sexual traits in the dark-eyed junco. *Behavioral Ecology*, 10, 91–96.
- 575 Hoekstra, H. E. (2006). Genetics, development and evolution of adaptive pigmentation in  
576 vertebrates. *Heredity*, 97, 222–234.
- 577 Hofreiter, M., & Schöneberg, T. (2010). The genetic and evolutionary basis of colour variation in  
578 vertebrates. *Cellular and Molecular Life Sciences*, 67, 2591–2603.
- 579 Holberton, R. L., Able, K. P., & Wingfield, J. C. (1989). Status signalling in dark-eyed juncos,  
580 Junco hyemalis: plumage manipulations and hormonal correlates of dominance. *Animal*  
581 *Behaviour*, 37, 681–689.
- 582 Hubbard, J. K., Uy, J. A. C., Hauber, M. E., Hoekstra, H. E., & Safran, R. J. (2010). Vertebrate  
583 pigmentation: from underlying genes to adaptive function. *Trends in Genetics*, 26, 231–  
584 239.
- 585 Ito, S., Nakanishi, Y., Valenzuela, R. K., Brilliant, M. H., Kolbe, L., & Wakamatsu, K. (2011).  
586 Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and  
587 pheomelanin in various tissue samples: application to chemical analysis of human hair  
588 melanins. *Pigment Cell & Melanoma Research*, 24, 605–613.
- 589 Ito, S., & Wakamatsu, K. (2008). Chemistry of Mixed Melanogenesis—Pivotal Roles of  
590 Dopaquinone†. *Photochemistry and Photobiology*, 84, 582–592.
- 591 Jeong, S., Rokas, A., & Carroll, S. B. (2006). Regulation of Body Pigmentation by the  
592 Abdominal-B Hox Protein and Its Gain and Loss in Drosophila Evolution. *Cell*, 125,  
593 1387–1399.
- 594 Kijas, J. M. H., Wales, R., Törnsten, A., Chardon, P., Moller, M., & Andersson, L. (1998).  
595 Melanocortin Receptor 1 (MC1R) Mutations and Coat Color in Pigs. *Genetics*, 150,  
596 1177–1185.

- 597 Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, 4, 68–75.
- 598 Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell*, 78, 191–201.
- 599 Lande, R., Seehausen, O., & Alphen, J. J. M. V. (2001). Mechanisms of rapid sympatric  
600 speciation by sex reversal and sexual selection in cichlid fish. *Genetica*, 112–113, 435–  
601 443.
- 602 Lappalainen, T., Sammeth, M., Friedländer, M. R., Hoen, P. A. C., ‘T, Monlong, J., Rivas, M. A.,  
603 ... Dermitzakis, E. T. (2013). Transcriptome and genome sequencing uncovers functional  
604 variation in humans. *Nature*, 501, 506–511.
- 605 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... Durbin, R. (2009). The  
606 sequence alignment/map format and SAMtools. *Bioinformatics*, 25, 2078–2079.
- 607 Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: an efficient general purpose program  
608 for assigning sequence reads to genomic features. *Bioinformatics*, 30, 923–930.
- 609 Lin, S. J., Foley, J., Jiang, T. X., Yeh, C. Y., Wu, P., Foley, A., ... Chuong, C. M. (2013).  
610 Topology of feather melanocyte progenitor niche allows complex pigment patterns to  
611 emerge. *Science*, 340, 1442–5.
- 612 MacDougall-Shackleton, E. A., Blanchard, L., & Gibbs, H. L. (2003). Unmelanized plumage  
613 patterns in Old World leaf warblers do not correspond to sequence variation at the  
614 melanocortin-1 receptor locus (MC1R). *Molecular Biology and Evolution*, 20, 1675–  
615 1681.
- 616 Manceau, M., Domingues, V. S., Mallarino, R., & Hoekstra, H. E. (2011). The developmental  
617 role of Agouti in color pattern evolution. *Science*, 331, 1062–5.
- 618 Marco-Sola, S., Sammeth, M., Guigó, R., & Ribeca, P. (2012). The GEM mapper: fast, accurate  
619 and versatile alignment by filtration. *Nature Methods*, 9, 1185–1188.
- 620 Martin, A., & Orgogozo, V. (2013). The Loci of repeated evolution: a catalog of genetic hotspots  
621 of phenotypic variation. *Evolution*, 67, 1235–50.
- 622 McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernysky, A., ... others.  
623 (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-  
624 generation DNA sequencing data. *Genome Research*, 20, 1297–1303.
- 625 Milá, B., McCormack, J. E., Castaneda, G., Wayne, R. K., & Smith, T. B. (2007). Recent  
626 postglacial range expansion drives the rapid diversification of a songbird lineage in the  
627 genus *Junco*. *Proceedings of the Royal Society B*, 274, 2653–2660.
- 628 Miller, A. H. (1941). *Speciation in the avian genus Junco*. Berkeley: University of California  
629 Press.
- 630 Mundy, N. I. (2005). A window on the genetics of evolution: MC1R and plumage colouration in  
631 birds. *Proceedings of the Royal Society B*, 272, 1633–40.
- 632 Nachman, M. W., Hoekstra, H. E., & D’Agostino, S. L. (2003). The genetic basis of adaptive  
633 melanism in pocket mice. *Proceedings of the National Academy of Sciences*, 100, 5268–  
634 73.
- 635 Nadeau, N. J., Minvielle, F., Ito, S., Inoue-Murayama, M., Gourichon, D., Follett, S. A., ...  
636 Mundy, N. I. (2008). Characterization of Japanese quail yellow as a genomic deletion  
637 upstream of the avian homolog of the mammalian ASIP (agouti) gene. *Genetics*, 178,  
638 777–786.
- 639 Naisbit, R. E., Jiggins, C. D., & Mallet, J. (2001). Disruptive sexual selection against hybrids  
640 contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*.  
641 *Proceedings of the Royal Society B*, 268, 1849–54.
- 642 Nolan, J. V., Ketterson, E. D., Cristol, D. A., Rogers, C. M., Clotfelter, E. D., Titus, R. C., ...

- 643 Snajdr, E. A. (2002). Dark-eyed junco: *Junco hyemalis*. In A. Poole (Ed.), *The Birds of*  
644 *North America*. Ithaca: Cornell Lab of Ornithology.
- 645 Ödeen, A., & Björklund, M. (2003). Dynamics in the evolution of sexual traits: Losses and gains,  
646 radiation and convergence in yellow wagtails (*Motacilla flava*). *Molecular Ecology*, *12*,  
647 2113–2130.
- 648 Poelstra, J. W., Vijay, N., Bossu, C. M., Lantz, H., Ryll, B., Muller, I., ... Wolf, J. B. W. (2014).  
649 The genomic landscape underlying phenotypic integrity in the face of gene flow in crows.  
650 *Science*, *344*, 1410–1414.
- 651 Poelstra, J. W., Vijay, N., Hoepfner, M. P., & Wolf, J. B. W. (2015). Transcriptomics of colour  
652 patterning and coloration shifts in crows. *Molecular Ecology*, *24*, 4617–4628.
- 653 Protas, M. E., & Patel, N. H. (2008). Evolution of coloration patterns. *Annual Review of Cell and*  
654 *Developmental Biology*, *24*, 425–446.
- 655 Rieder, S., Taurit, S., Mariat, D., Langlois, B., & Guérin, G. (2001). Mutations in the agouti  
656 (ASIP), the extension (MC1R), and the brown (TYRP1) loci and their association to coat  
657 color phenotypes in horses (*Equus caballus*). *Mammalian Genome*, *12*, 450–455.
- 658 Riyahi, S., Björklund, M., Ödeen, A., & Senar, J. C. (2015). No association between the  
659 melanocortin-1 receptor (MC1R) and black belly stripe size variation in the Great Tit  
660 *Parus major*. *Bird Study*, *62*, 150–152.
- 661 Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for  
662 differential expression analysis of digital gene expression data. *Bioinformatics*, *26*, 139–  
663 140.
- 664 Robinson, M. D., & Oshlack, A. (2010). A scaling normalization method for differential  
665 expression analysis of RNA-seq data. *Genome Biology*, *11*, R25–R25.
- 666 Roulin, A., & Ducrest, A. L. (2013). Genetics of colouration in birds. *Seminars in Cell and*  
667 *Developmental Biology*, *24*, 594–608.
- 668 San-Jose, L. M., Ducrest, A. L., Ducret, V., Simon, C., Richter, H., Wakamatsu, K., & Roulin,  
669 A. (2017). MC1R variants affect the expression of melanocortin and melanogenic genes  
670 and the association between melanocortin genes and coloration. *Molecular Ecology*, *26*,  
671 259–276.
- 672 San-Jose, L. M., & Roulin, A. (2017). Genomics of coloration in natural animal populations.  
673 *Philosophical Transactions of the Royal Society B*, *372*, 20160337..
- 674 Santos, M. E., Baldo, L., Gu, L., Boileau, N., Musilova, Z., & Salzburger, W. (2016).  
675 Comparative transcriptomics of anal fin pigmentation patterns in cichlid fishes. *BMC*  
676 *Genomics*, *17*, 712–712.
- 677 Schiaffino, M. V., & Tacchetti, C. (2005). The ocular albinism type 1 (OA1) protein and the  
678 evidence for an intracellular signal transduction system involved in melanosome  
679 biogenesis. *Pigment Cell Research*, *18*, 227–233.
- 680 Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D. J., Miyagi, R., ...  
681 Okada, N. (2008). Speciation through sensory drive in cichlid fish. *Nature*, *455*, 620–6.
- 682 Shapiro, M. D., Kronenberg, Z., Li, C., Domyan, E. T., Pan, H., Campbell, M., ... Wang, J.  
683 (2013). Genomic diversity and evolution of the head crest in the rock pigeon. *Science*,  
684 *339*, 1063–1064.
- 685 Silvers, W. K., & Russell, E. S. (1955). An experimental approach to action of genes at the  
686 agouti locus in the mouse. *Journal of Experimental Zoology Part A: Ecological Genetics*  
687 *and Physiology*, *130*, 199–220.
- 688 Singh, A. P., & Nüsslein-Volhard, C. (2015). Zebrafish stripes as a model for vertebrate colour

- 689 pattern formation. *Current Biology*, 25, R81–R92.
- 690 Steiner, C. C., Weber, J. N., & Hoekstra, H. E. (2007). Adaptive variation in beach mice  
691 produced by two interacting pigmentation genes. *PLoS Biology*, 5, 1880–1889.
- 692 Stoll, S. J., & Kroll, J. (2012). HOXC9: A Key Regulator of Endothelial Cell Quiescence and  
693 Vascular Morphogenesis. *Trends in Cardiovascular Medicine*, 22, 7–11.
- 694 Theron, E., Hawkins, K., Bermingham, E., Ricklefs, R. E., & Mundy, N. I. (2001). The  
695 molecular basis of an avian plumage polymorphism in the wild: a melanocortin-1-  
696 receptor point mutation is perfectly associated with the melanic plumage morph of the  
697 bananaquit, *Coereba flaveola*. *Current Biology*, 11, 550–7.
- 698 Thomas, A. J., & Erickson, C. A. (2008). The making of a melanocyte: The specification of  
699 melanoblasts from the neural crest. *Pigment Cell and Melanoma Research*, 21, 598–610.
- 700 Uy, J. A. C., Cooper, E. A., Cutie, S., Concannon, M. R., Poelstra, J. W., Moyle, R. G., &  
701 Filardi, C. E. (2016). Mutations in different pigmentation genes are associated with  
702 parallel melanism in island flycatchers. *Proc. R. Soc. B*, 283, 20160731.
- 703 Uy, J. A. C., Moyle, R. G., Filardi, C. E., & Cheviron, Z. a. (2009). Difference in plumage color  
704 used in species recognition between incipient species is linked to a single amino acid  
705 substitution in the melanocortin-1 receptor. *The American Naturalist*, 174, 244–54.
- 706 Våge, D. I., Klungland, H., Lu, D., & Cone, R. D. (1999). Molecular and pharmacological  
707 characterization of dominant black coat color in sheep. *Mammalian Genome*, 10, 39–43.
- 708 Wakamatsu, K., Ito, S., & Rees, J. L. (2002). The Usefulness of  
709 4-Amino-3-hydroxyphenylalanine as a Specific Marker of Pheomelanin. *Pigment Cell  
710 Research*, 15, 225–232.
- 711 Wang, Y., Gao, Y., Imsland, F., Gu, X., Feng, C., Liu, R., ... Li, N. (2012). The crest phenotype  
712 in chicken is associated with ectopic expression of HOXC8 in cranial skin. *PLoS ONE* 7,  
713 4, e34012.
- 714 Xiong, W., He, F., Morikawa, Y., Yu, X., Zhang, Z., Lan, Y., ... Chen, Y. (2009). Hand2 is  
715 required in the epithelium for palatogenesis in mice. *Developmental Biology*, 330, 131–  
716 141.
- 717 Yamaguchi, Y., Passeron, T., Watabe, H., Yasumoto, K., Rouzaud, F., Hoashi, T., & Hearing, V.  
718 J. (2007). The effects of dickkopf 1 on gene expression and Wnt signaling by  
719 melanocytes: mechanisms underlying its suppression of melanocyte function and  
720 proliferation. *The Journal of Investigative Dermatology*, 127, 1217–25.
- 721 Yang, L., Du, X., Wei, S., Gu, L., Li, N., Gong, Y., & Li, S. (2017). Genome-wide association  
722 analysis identifies potential regulatory genes for eumelanin pigmentation in chicken  
723 plumage. *Animal Genetics*, 1–4.
- 724 Yelon, D., Ticho, B., Halpern, M. E., Ruvinsky, I., Ho, R. K., Silver, L. M., & Stainier, D. Y.  
725 (2000). The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and  
726 pectoral fin development. *Development*, 127, 2573–2582.
- 727 Yoshihara, C., Fukao, A., Ando, K., Tashiro, Y., Taniuchi, S., Takahashi, S., & Takeuchi, S.  
728 (2012). Elaborate color patterns of individual chicken feathers may be formed by the  
729 agouti signaling protein. *General and Comparative Endocrinology*, 175, 495–499.
- 730 Yu, M., Yue, Z., Wu, P., Wu, D. Y., Mayer, J. A., Medina, M., ... Chuong, C. M. (2004). The  
731 developmental biology of feather follicles. *International Journal of Developmental  
732 Biology*, 48, 181–191.
- 733 Zink, R. M., Drovetski, S. V., Questiau, S., Fadeev, I. V., Nesterov, E. V., Westberg, M. C., &  
734 Rohwer, S. (2003). Recent evolutionary history of the bluethroat (*Luscinia svecica*)

735 across Eurasia. *Molecular Ecology*, 12, 3069–3075.  
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738 **DATA ACCESSIBILITY STATEMENT**

739 RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI

740 ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) under accession number E-MTAB-6794.

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742 **AUTHOR CONTRIBUTIONS**

743 B.M. and E.D.K designed the study. M.A.A. and M.P.P. conducted the common-garden

744 experiments. K.W. analyzed the chemical composition of feathers. E.K., P.R. and M.A.A.

745 analyzed the transcriptomic data. M.A.A., E.K., and B.M. wrote the paper with input from all

746 authors. B.M. provided funding for the project.

747

748 **TABLES**

749

750 **Table 1.** Degradation products of eumelanin and pheomelanin pigments measured by HPLC in  
 751 feathers from different body parts of Oregon and slate-colored juncos. Values (n = 4) represent  
 752 sample means and standard deviations (in parentheses).

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	Oregon junco			Slate-colored junco		
	Eumelanin (ng/mg)		Pheomelanin (ng/mg)	Eumelanin (ng/mg)		Pheomelanin (ng/mg)
	PTCA	4-AHP	TTCA	PTCA	4-AHP	TTCA
Head	2468 (332)	46 (8)	207 (117)	1967 (314)	<9	<94
Back	1075 (189)	165 (38)	369 (28)	1796 (163)	<9	<94
Flank	2195 (764)	46 (13)	148 (24)	2707 (323)	<9	<94
Ventral	2063 (535)	36 (7)	132 (100)	2156 (408)	<9	<94

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 757 **Table 2.** Number of differentially regulated genes for each comparison between junco forms and  
 758 body parts (two-fold change, down-regulated/up-regulated). Comparisons of the same tissue  
 759 between sub-species is highlighted in grey. ORJU: Oregon junco; SCJU: Slate-colored junco; B:  
 760 back; F: flank; V: ventral; H: head.

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	ORJU-B	ORJU-F	ORJU-V	SCJU-H	SCJU-B	SCJU-F	SCJU-V
ORJU-H	49/17	65/31	119/79	6/1	67/12	36/8	201/114
ORJU-B		3/3	22/46	10/28	1/0	8/6	38/37
ORJU-F			36/41	13/16	24/11	1/3	67/44
ORJU-V				61/77	71/25	45/57	0/0
SCJU-H					4/0	0/0	42/32
SCJU-B						1/2	8/16
SCJU-F							29/17

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767 **FIGURES**

768

769 **Figure 1.** Light microscopy of junco feathers showing differential distribution of pheomelanin  
770 and eumelanin in barbules, barbs, and rachis (100x magnification). ORJU: Oregon junco; SCJU:  
771 slate-colored junco.

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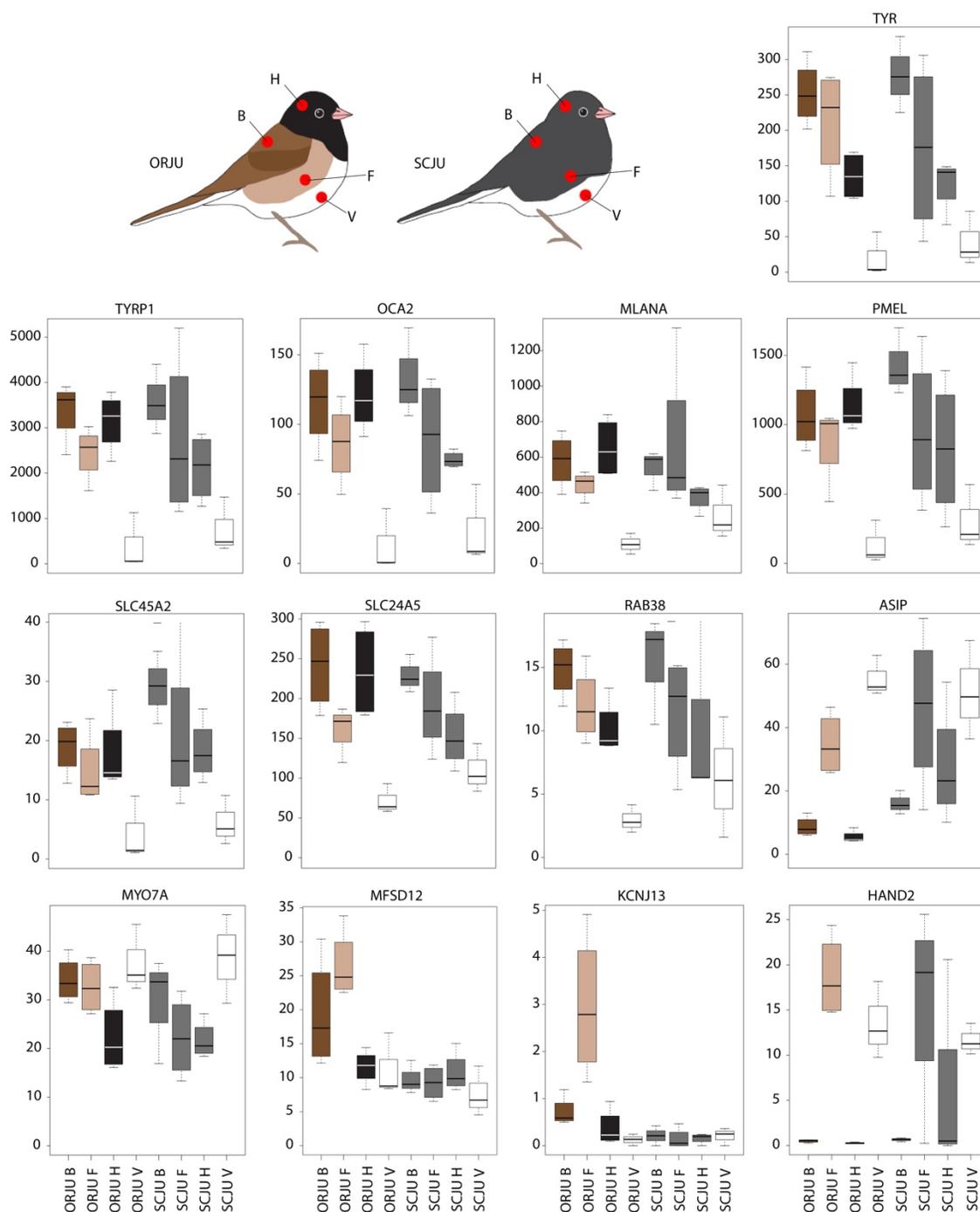


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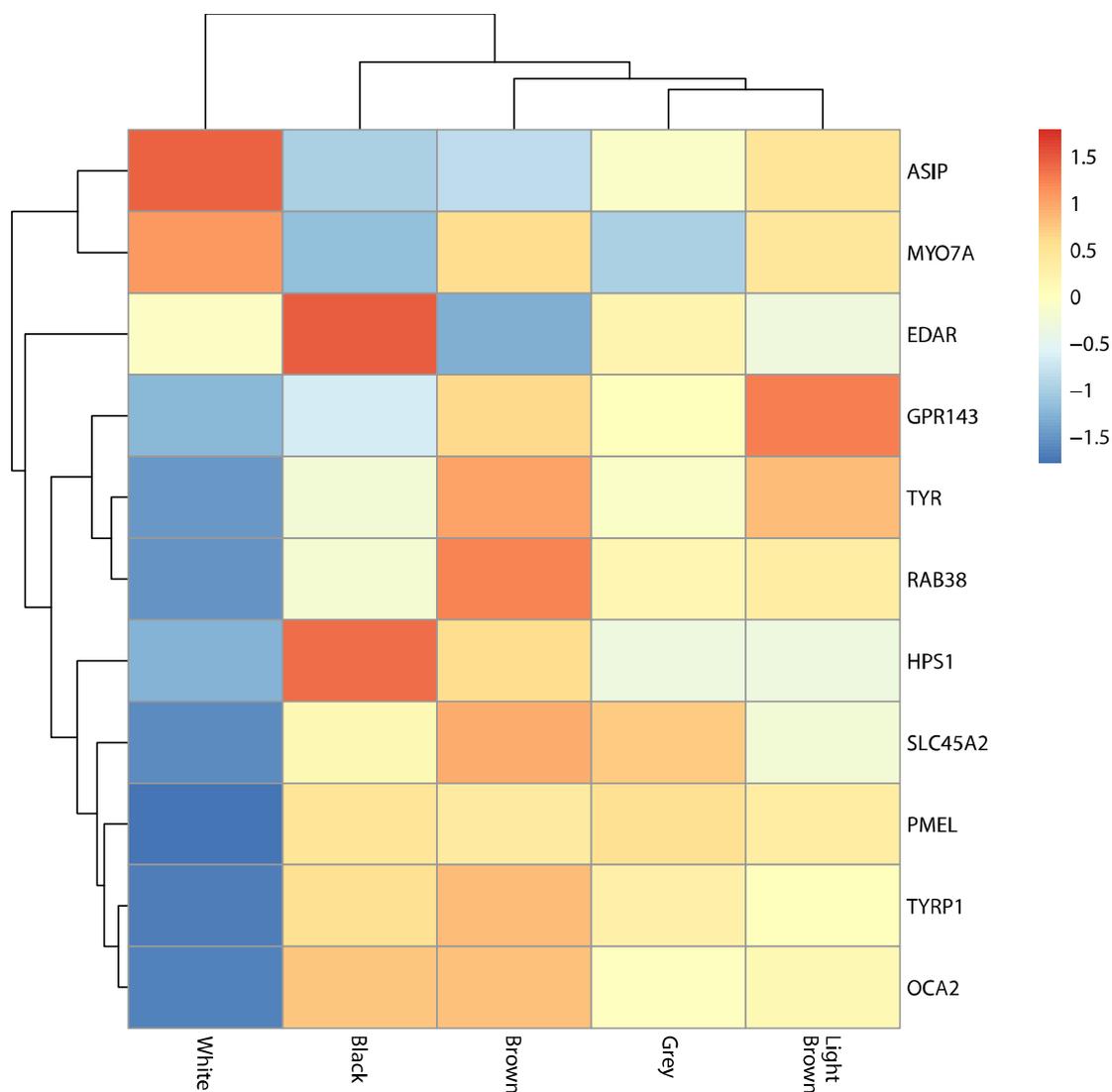
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776 **Figure 2.** Expression (normalized CPMs) of significantly differentially regulated genes  
777 associated with color development between subspecies and body regions. ORJU: Oregon junco;  
778 SCJU: slate-colored junco; H: head; B: back; F: flank; V: ventral.  
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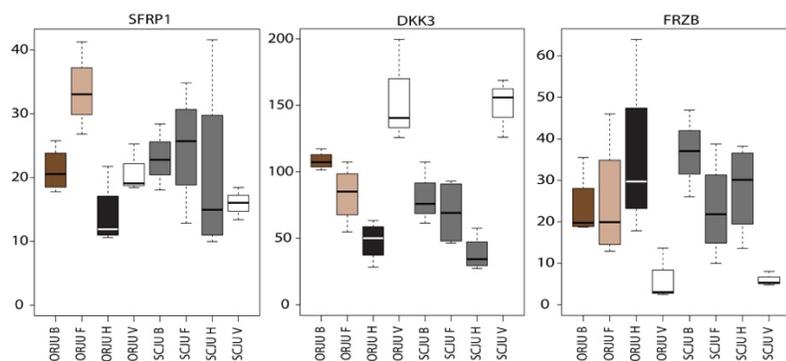
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782 **Figure 3.** Heat map based on the median of counts (normalized CPMs) feather colors for the 11  
783 pigment-associated genes differentially regulated in any comparison between colored feathers  
784 against white (ventral) feathers.  
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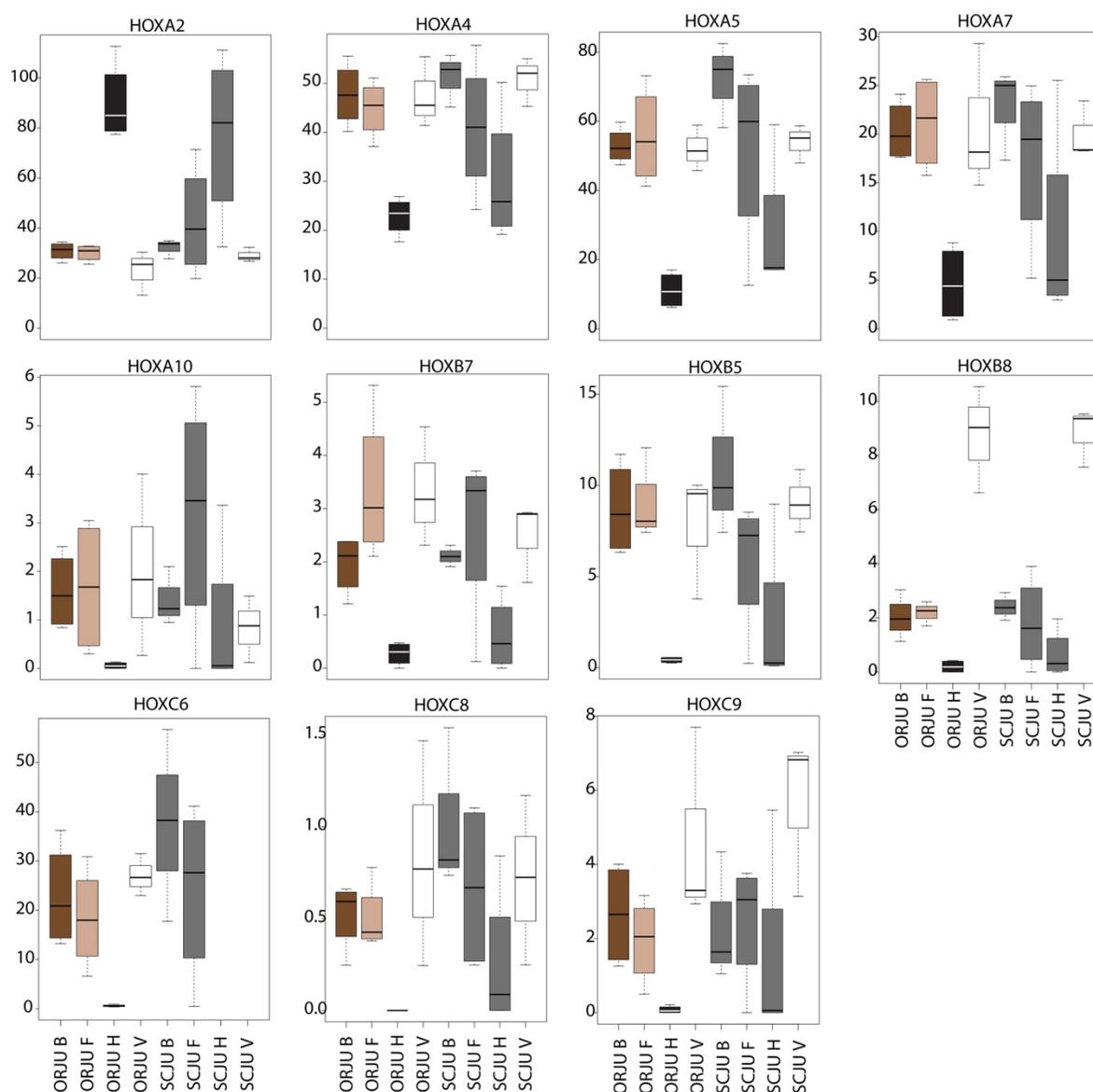
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789 **Figure 4.** Expression (normalized CPMs) of significantly differentially regulated genes  
790 associated with Wnt signaling between subspecies and body regions. ORJU: Oregon junco;  
791 SCJU: slate-colored junco; H: head; B: back; F: flank; V: ventral.  
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797 **Figure 5.** Expression (normalized CPMs) of significantly differentially expressed HOX genes  
 798 between subspecies and body regions. ORJU: Oregon junco; SCJU: slate-colored junco; H: head;  
 799 B: back; F: flank; V: ventral.  
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