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Not All Roads Lead to Red: The Genetics of Sexual Dichromatism in the Northern Cardinal (*Cardinals cardinalis*)

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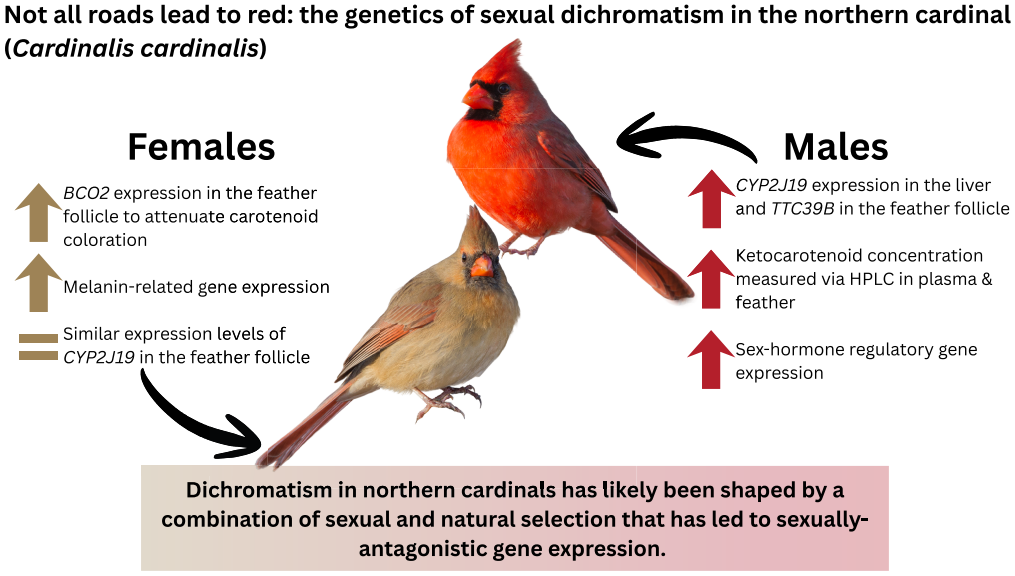
Abstract

Sexual dichromatism, characterized by sex-specific differences in coloration, is widespread among birds and often involves carotenoid-based pigmentation. Despite extensive research on the social and ecological environments favoring sexual dichromatism, the molecular mechanisms underlying its development and evolution remain largely unexplored. In this study, we investigated the genetic and molecular processes giving rise to sexual dichromatism in the red ketocarotenoid-based plumage of northern cardinals (*Cardinalis cardinalis*). We quantified carotenoid concentrations in plasma and feather follicles, confirmed that homologs of *CYP2J19*, *BDH1L*, and *TTC39B* catalyze the production of C-4 ketocarotenoids, and performed gene expression analyses across tissues. Males showed significantly higher plasma and feather ketocarotenoid concentrations, upregulated *CYP2J19* and *TTC39B* expression in liver and feather tissues, and upregulated carotenoid transport gene expression in the gut and feather follicles. Females exhibited a dramatic upregulation of *BCO2* in their feather follicles, facilitating carotenoid degradation and attenuating red pigmentation. Additionally, sex-biased expression of hormonal regulators, such as *HSD17B4* and *ZNF131*, in the feather follicle suggests hormonal modulation influences dichromatism. These findings indicate that sex-specific regulation of carotenoid processing genes underpins the vivid red coloration in males and the drab phenotype in females, likely maintained by a balance between natural and sexual selection, with mechanisms of sexual antagonism affecting divergent gene expression. This work advances understanding of the molecular basis of avian sexual dimorphism, highlighting key genetic pathways involved in carotenoid-based coloration and providing a foundation for further research into the evolution of sexually dichromatic traits.

Graphical Abstract



Key words: avian plumage color, carotenoid-based pigmentation, coloration genetics, feather coloration, sexual conflict, sexual dichromatism.

Significance statement

The molecular mechanisms underlying the development and evolution of sexual dichromatism involving carotenoid-based pigmentation remain largely unexplored. We investigated gene expression during feather growth and find that sexual dichromatism is largely attributed to a few genes in the northern cardinal. In particular, the highly upregulated *BCO2* in developing feathers of females indicates that carotenoid breakdown is the major action leading to a reduction of red plumage color in females, while males have upregulation of *CYP2J19* and *TTC39B* in liver and feather follicles and carotenoid uptake genes in both the gut and feather follicles. These patterns suggest that dichromatism is maintained by both natural and sexual selection, with sexual antagonism driving divergent gene expression. These findings provide new insight into the genetic basis and evolution of avian sexual dichromatism.

Introduction

Sexual dichromatism, where sexes differ in patterning or coloration, is a common form of dimorphism seen across diverse animal taxa. Birds are among the most conspicuous examples and have long been the subject of study regarding the evolution and maintenance of sexual dichromatism (Badyaev and Hill 2000; Shultz and Burns 2017). In birds, sexual dichromatism often involves carotenoid-based pigmentation. Vertebrates cannot synthesize carotenoids, so carotenoid-based pigmentation requires they first obtain carotenoids such as zeaxanthin and lutein from their diet. These dietary carotenoids can either be integrated directly or further modified to produce a wider range of red, orange, and yellow pigments (Hill and McGraw 2006a); however, sexual dichromatism tends to be strongest in species that have red carotenoid-based coloration (Delhey and Peters 2017). Recent progress in discovering the genes involved in avian carotenoid pigmentation has created opportunities to investigate mechanistic hypotheses for the development and evolution of sexual dichromatism.

The expression of gene products mediates carotenoid uptake, binding, metabolism, deposition, and breakdown, shaping plumage coloration and patterning (Hill and McGraw 2006b; Toews et al. 2017). Genes

that regulate the uptake of carotenoids in birds include scavenger receptor B1 (*SCARB1*) and scavenger receptor class F member 2 (*SCARF2*; Brelsford et al. 2017; Toomey et al. 2017). Additionally, 3-hydroxybutyrate dehydrogenase 1-like (*BDH1L*) converts the dietary carotenoids lutein and zeaxanthin to canary xanthophylls (Toomey et al. 2022, 2025), a yellow pigment in the plumage of many birds. In concert with *BDH1L*, cytochrome P450 2J19 encoded by the gene *CYP2J19* catalyzes the production of red C-4 ketocarotenoids (hereafter referred to as "ketocarotenoids") from dietary carotenoids in many birds (Lopes et al. 2016; Mundy et al. 2016; Toomey et al. 2022, 2025). The process of ketocarotenoid production is enhanced by tetratricopeptide repeat protein 39B (*TTC39B*) in birds (Toomey et al. 2022; Hooper et al. 2024). Multiple studies have found that *CYP2J19* (or a homolog) contributes to red coloration in natural populations (Twyman et al. 2018; Hooper et al. 2019; Kirschel et al. 2020; Aguillon et al. 2021; Khalil et al. 2023; Ranasinghe et al. 2025), indicating that the CYP2J19/BDH1L enzymatic pathway is a widespread mechanism of ketocarotenoid synthesis in birds.

In addition to the important roles of uptake and modification on carotenoid pigmentation, the breakdown of carotenoids also contributes to avian coloration. The expression of β-carotene oxygenase 2 (*BCO2*), which catalyzes the cleavage of carotenoid molecules (Toomey et al. 2016), is important for mediating the accumulation of carotenoids in tissues (Eriksson et al. 2008; Amengual et al. 2011, 2013; Ahi et al. 2020) and is linked with the loss of carotenoids in the feathers, skin, and beak of several bird species (Eriksson et al. 2008; Gazda et al. 2020b; Baiz et al. 2021; Enbody et al. 2021). In the "mosaic" breed of the domestic canary (*Serinus canaria*), the mechanism behind dichromatism lies in the selective degradation of carotenoids catalyzed by *BCO2* rather than the upregulation of red carotenoid metabolism via *CYP2J19* expression or any physiological difference in carotenoid uptake or transport (Gazda et al. 2020a). Dichromatism in yellow carotenoid coloration of plumage of European serins (*Serinus serinus*) was similarly attributed to upregulation of *BCO2* in females (Gazda et al. 2020a). However, the mechanisms controlling *BCO2* expression or ketocarotenoid metabolism are not fully understood.

Because reproductively senescent and ovariectomized female canaries develop color patterning similar to that of males (Perez-Beato 2008), *BCO2* expression may be estrogen-dependent (Gazda et al. 2020a). In estrogen-dependent dichromatism, dull plumage develops in the presence of estrogen, while bright coloration develops in its absence (Kimball and Ligon 1999). Prior work has found evidence for localized hormonal regulation between the sexes in birds for the hormone 17β-hydroxysteroid dehydrogenase type 4 (*HSD17B4*) in developing zebra finch (*Taeniopygia guttata*) brains (London et al. 2010; Tomaszycki and Dzubur 2013) and in their melanin-pigmented cheek patch due to sex-biased transcription factor expression (Lin et al. 2025). Steroid hormone-associated genes displaying sex-specific expression patterns have also been implicated as upstream regulators influencing carotenoid-based sexual dimorphism in the toad-headed agamid lizard (*Phrynocephalus putjatai*; Lu et al. 2024). Evidence from feather ketocarotenoid and gene expression analysis in red-backed fairywrens (*Malurus melanocephalus*) suggests that testosterone mediates plumage redness, as testosterone-implanted males had higher feather ketocarotenoid concentrations and a greater magnitude of *CYP2J19* expression compared with control males (Khalil et al. 2023). This work also reported increased expression of *BCO2* in the unornamented compared with the ornamented males. Combined, these studies suggest that divergence in ketocarotenoid metabolism and carotenoid cleavage between the sexes, perhaps due to the influence of sex-biased hormones or other regulatory factors, contributes to the development of carotenoid-based sexual dichromatism in birds.

To provide molecular insights into the development and evolution of sexual dichromatism, we quantified gene expression and carotenoid concentrations in key tissues of male and female northern cardinals (*Cardinalis cardinalis*) during their natural seasonal molt. Adult male northern cardinals possess iconic bright red plumage and bills pigmented with ketocarotenoids (McGraw et al. 2003b; Patton et al. 2026). Contrastingly, adult females largely lack red coloration in their melanin-based body plumage and have orange bills. However, females have some red coloration on their crests, underwing coverts, and flight and tail feathers (Fig. 1). The pigments used by northern cardinals to color both feathers and bills are derived by modifying yellow dietary carotenoids to produce red ketocarotenoids (Patton et al. 2026). Prior analysis shows that the red feathers of male northern cardinals contain the ketocarotenoids α-doradexanthin, astaxanthin, canthaxanthin, and adonirubin (McGraw et al. 2001, 2003b; Toomey et al. 2022). Since both α-doradexanthin and astaxanthin are produced by the action of *CYP2J19*, *BDH1L*, and *TTC39B* in other bird species (Toomey et al. 2022), we hypothesized that homologs of these genes from northern cardinals have the same function in ketocarotenoid production and performed cell-based assays to confirm this mechanism. As the tissue location of ketocarotenoid metabolism in birds seems to vary both by species and mechanism (Hill and McGraw 2006a; Alonso-Alvarez et al. 2022), we investigated

Fig. 1. An adult female (left) and an adult male (right) northern cardinal. The adult female is predominately drab brown in color, whereas the adult male has bright red plumage. Photo © Geoffrey E. Hill.

carotenoid concentrations in plasma and feather follicles in conjunction with gene expression analysis. We hypothesized that carotenoid metabolism occurs in the liver and feather follicle of northern cardinals and that males would have higher plasma and feather follicle concentrations of ketocarotenoids and canary xanthophylls compared with females. Based on the research outlined above, we also hypothesized that male northern cardinals would have an accompanying greater transcription of the genes *CYP2J19*, *TTC39B*, and *BDH1L*, whereas females would have higher transcription of *BCO2*. Thus, we analyzed gene expression in the gut, liver, and feather follicle to investigate if genes related to carotenoid uptake, transport, metabolism, and deposition, along with hormone-related genes, show a sex-biased expression pattern and therefore contribute toward sexual dichromatism.

Results

Male Cardinals Circulate and Deposit More Ketocarotenoids in Their Plasma and Feathers Than Females

The plasma concentrations of lutein and zeaxanthin, canary xanthophylls, and other carotenoids, with spectral characteristics consistent with yellow, non-C4-keto pigments (see Figs. S1 and S2 for representative high-performance liquid chromatography [HPLC] chromatograms and peak UV-Vis spectra), were similar between the male and female northern cardinals (Fig. 2a and c, e and g; Table S1). However, the plasma concentration of ketocarotenoids was significantly higher in males than females ($P < 0.01$), with a mean concentration of 20.01 μg mL^{-1} in the males and 8.65 μg mL^{-1} in the females (Fig. 2a). In the sexually dichromatic breast feather follicles, concentrations of canary xanthophylls, other carotenoids, and ketocarotenoids, but not the dietary carotenoids lutein and zeaxanthin, were significantly different between male and female northern cardinals (Fig. 2b and d, f and h; Table S1). The average concentration of canary xanthophylls present in the males was 10.7 μg mg^{-1}, which was significantly greater than the average of 0.23 μg mg^{-1} in the females ($P < 0.001$, Fig. 2h). The average concentration of other carotenoids was 0.34 and 5.14 μg mg^{-1} in the females and males, respectively ($P < 0.001$, Fig. 2d). For ketocarotenoids, the mean concentration measured in the females was 10.64 μg mg^{-1} whereas that in the males was significantly higher at 125.7 μg mg^{-1} ($P < 0.001$, Fig. 2b). We also found that the concentration of circulating ketocarotenoids correlated significantly (Pearson's product-moment correlation, $r = 0.71$, $P < 0.001$) with the ketocarotenoid concentration of the feather follicles.

A Shared Pathway for Ketocarotenoid Metabolism

Consistent with our hypothesis of conserved biochemical function of carotenoid metabolizing gene products,

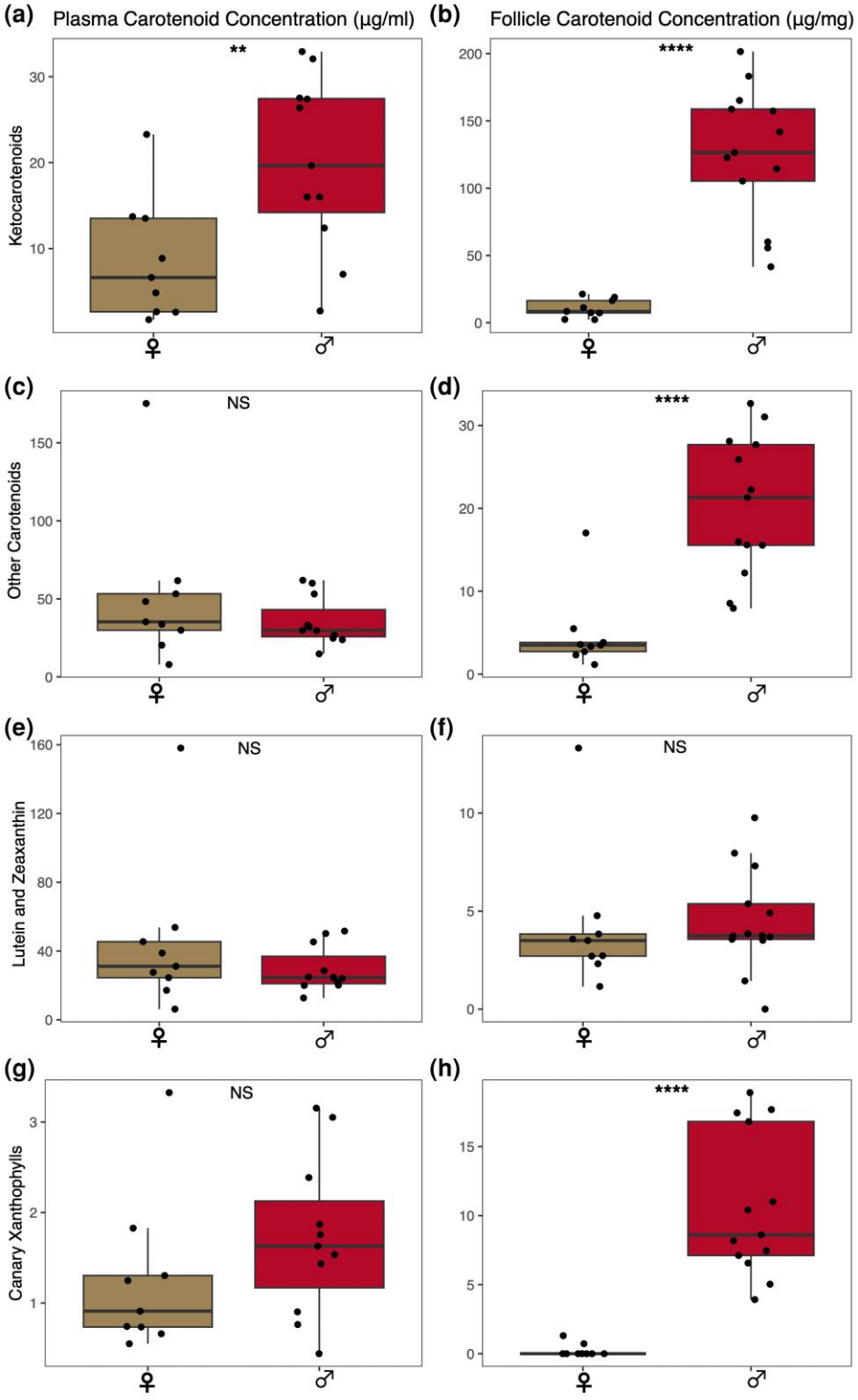


Fig. 2. Boxplots of carotenoid concentrations measured from the plasma (left column) and feather follicle (right column) of female (brown, left side of figures) and male (red, right side of figures) northern cardinals. This includes the ketocarotenoids (a, b), other carotenoids (c, d), combined lutein/zeaxanthin representing dietary carotenoids (e, f), and canary xanthophylls (g, h). Significance was assessed according to Welch's two-sample *t*-test (not significant [NS]: $P > 0.05$; $^{**}P \leq 0.01$; $^{****}P \leq 0.0001$). All values are also indicated with points. The boxes indicate the median, the lower and upper hinges of the boxes correspond to the first and third quartiles, with whiskers extending to the smallest/largest value up to 1.5 times the value of the interquartile range.

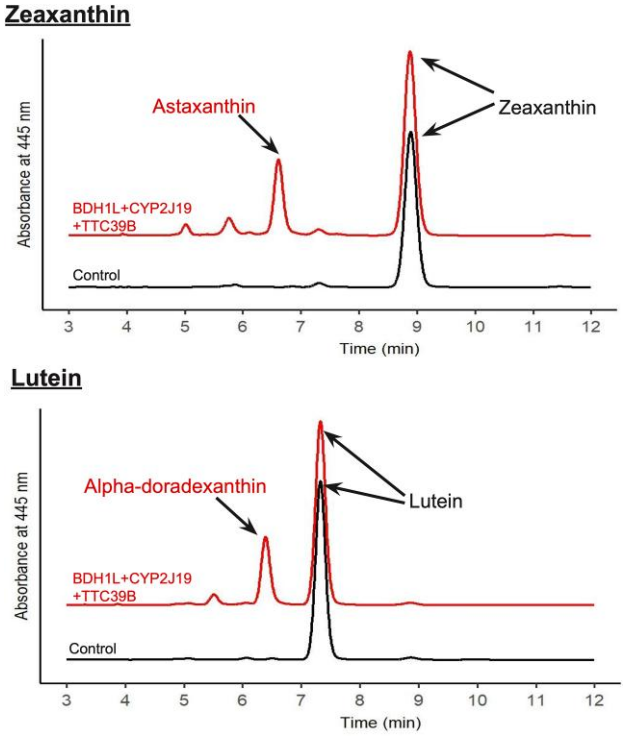


Fig. 3. Representative high-performance liquid chromatography chromatograms from HEK293 cells transfected with cardinal *CYP2J19*, *BDH1L*, and *TTC39B* and provided with a zeaxanthin (a) or lutein (b) substrate.

heterologous expression assays confirm that the co-expression of the northern cardinal homologs of *BDH1L*, *CYP2J19*, and *TTC39B* are sufficient to catalyze the conversion of yellow dietary carotenoids into red ketocarotenoids (Fig. 3). We confirmed the formation of astaxanthin in the cells given zeaxanthin (Fig. 3, top) or of α-doradexanthin in the cells provided lutein (Fig. 3, bottom) as substrate. Thus, the expression of the northern cardinal homologs of *BDH1L*, *CYP2J19*, and *TTC39B* is sufficient to produce the red ketocarotenoid pigments that are enriched in the plasma and feather follicles of male cardinals.

Male Cardinals had Increased Transcription of Genes Involved in Ketocarotenoid Metabolism and Carotenoid Transport Across Tissues

We compared gene expression in the feather follicles, gut, and liver of wild-caught molting male and female cardinals using RNA sequencing. We found 775 differently expressed genes (DEGs) between the male and female cardinals in the breast feather follicle (Fig. 4a, Table S4). In the liver and gut tissue, we found a total of 116 and 298 DEGs between the two sexes, respectively (Fig. 4c and d, Tables S5 and S6). In the feather follicles, 328 genes were upregulated in the males, including *TTC39B* (log fold change [LFC] = -1.36, adj-*P* = 1.26e-07; Figs. 4a and 5b). However, we did not find an accompanying, significant difference in the expression of *CYP2J19* (LFC = -0.47, adj-*P* = 0.2, Figs. 4a and 5a) between the sexes in this tissue. In fact, female and male cardinals had similar expression in *CYP2J19* in their feather follicles quantified as transcripts per million (TPM; Fig. 5a). In contrast, in the liver we found *CYP2J19* was significantly enriched in males (LFC = -2.44, adj-*P* = 0.01, Figs. 4d and 5a) but there were no significant differences in *TTC39B* expression (LFC = -0.59, adj-*P* = 0.35). Interestingly, we found that the third gene playing a key role in converting dietary carotenoids into ketocarotenoids, *BDH1L*, was expressed at similar levels in both males and females (Fig. 5c), suggesting that *BDH1L* is not a gene contributing to sexual dichromatism in northern cardinals. While *BDH1L* is expressed at a higher level in the liver than in feather follicles in both sexes (average expression in the liver = 66.2 TPM, feather follicle average = 34.8 TPM, *P* = 0.003), *CYP2J19* is expressed at a much higher level in feather follicles than in liver tissue (average expression in the liver = 5.3 TPM, feather follicle average = 121.3 TPM, *P* = 1.46e-11). As the average expression levels for *CYP2J19* inclusive of both sexes is 121.26 TPM in the feather follicles and 5.26 TPM in the liver, there is an almost 24-fold difference in transcription (Fig. 5a).

We found expression changes in other genes encoding several lipid transport-related proteins. Interestingly, we did not find evidence of the enrichment for the known carotenoid uptake gene *SCARB1* (Toomey et al. 2017) in the males. However, Solute Carrier Family 27 Member 6 (*SLC27A6*, LFC = -1.48, adj-*P* = 3.91e-08) was upregulated in the feather follicles of males (Fig. 4a). *SLC27A6* is involved in the transport, formation, and secretion of chylomicrons, which are integral in the uptake and transportation of dietary carotenoids (Lee et al. 1999; Hill and Johnson 2012; Nishida et al. 2023). We also found increased transcription of perilipin 2 (*PLIN2*, LFC = -0.79, *P* = 0.01) in male feather follicles. *PLIN2* encodes a structural protein involved in the formation and stabilization of lipids that transport hydrophobic molecules like carotenoids (Liu et al. 2021). Both *SLC27A6* (LFC = -1.02, adj-*P* = 0.0002) and *PLIN2* (LFC = -0.73, adj-*P* = 0.009) were also upregulated in the gut tissues of males (Fig. 4c), suggesting that these genes may increase carotenoid absorption from food contents to the bloodstream. Cluster-determinant 36 (*CD36*), similarly involved in carotenoid uptake and transportation (Liu et al. 2023), was upregulated in male feather follicles (LFC = -1.1, *P* = 0.02; Fig. 4a). *CD36* regulates red skin color in leopard coral grouper (*Plectropomus leopardus*; Jin et al. 2024) and is

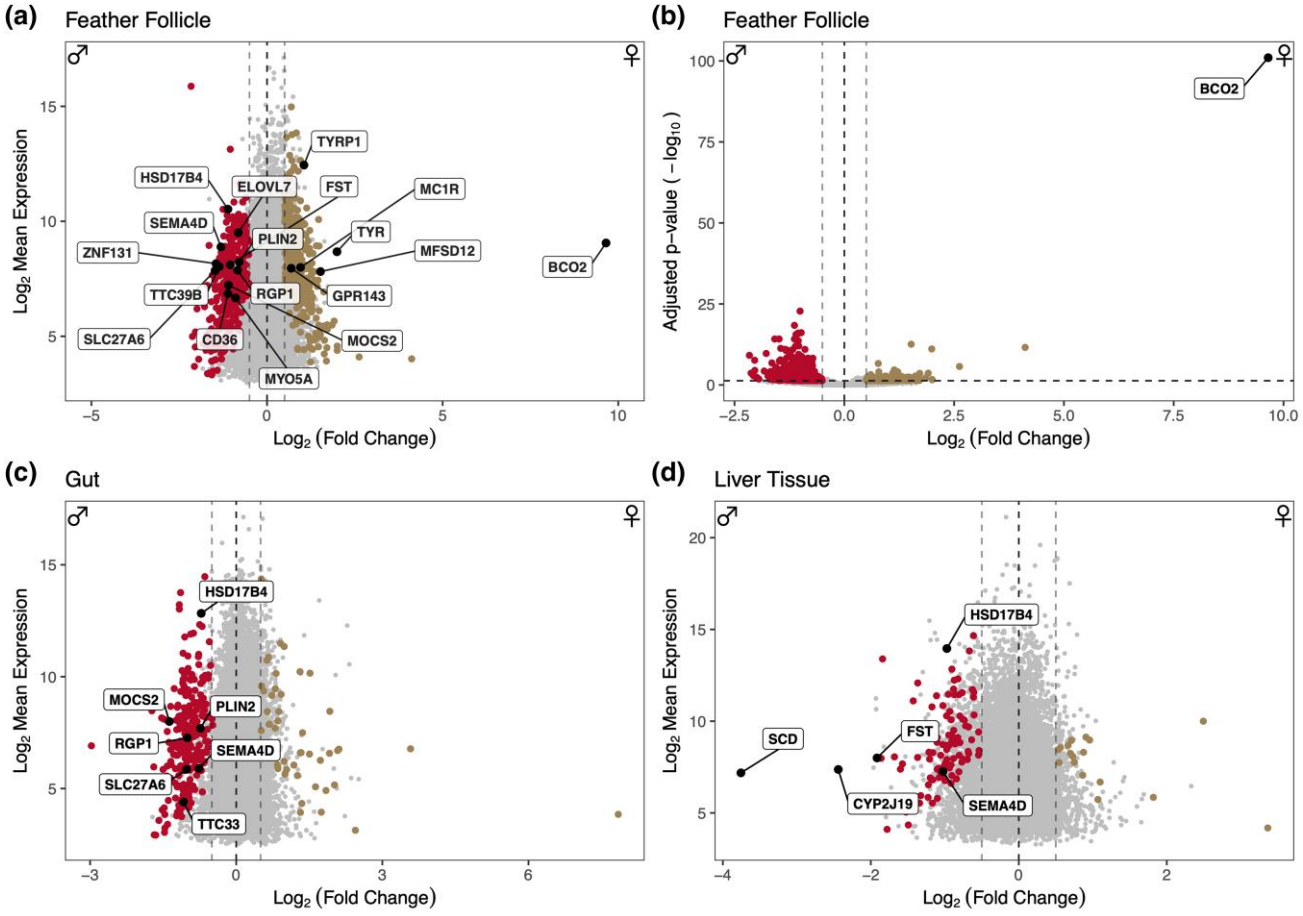


Fig. 4. Differential gene expression between males (left side of plots) and females (right side of plots) in the feather follicle (a, b), gut (c), and liver (d). MAplot (a, c, d) with candidate and putative coloration genes labeled. The dashed line on $x = 0$ indicates a LFC of zero, and the lighter dashed lines on either side of $x = 0$ indicate LFCs of −0.5 and 0.5, respectively. b) A volcano plot of the feather follicle gene expression data demonstrating the high significance of the differential expression in the *BCO2* gene. The dashed line on the y axis indicates the significance cutoff of adjusted $P = 0.05$. LFC > 0 indicates upregulation in females, whereas LFC < 0 indicates upregulation in males. Significantly upregulated genes in males and females are indicated by red and brown dots, respectively.

associated with skin yellowness in chickens (Zhao et al. 2023), suggesting a role in carotenoid transport and coloration in vertebrates.

Females Appear to Moderate Ketocarotenoid Pigmentation by Upregulating *BCO2* Expression

In the liver and gut tissues, we found no carotenoid-related genes in the 17 and 45 genes that were upregulated in females, respectively (Fig. 4c and d, Tables S5 and S6). In the breast feather follicles, we found 447 genes upregulated in the females (Table S4). In particular, *BCO2* was highly upregulated in the females compared with the males (Figs. 4a and 5d). This differential expression of *BCO2* was also highly significant, with over 800-fold greater transcription in females (adj-$P = 8.62e\text{-}102$; LFC = 9.65; Figs. 4b and 5d). Across our analyses, *BCO2* had both the highest LFC and most significant *P*-value for its differential expression between the sexes. *BCO2* codes for an enzyme that plays a key role in carotenoid degradation by catalyzing the 9′,10′ oxidative cleavage of carotenoids to yield shorter apocarotenoids (Dela Seña et al. 2016; Ahi et al. 2020; Gazda et al. 2020a, 2020b).

Differential Melanin Synthesis in Feathers Also Contributes to Sexual Dichromatism

Several genes involved in melanin-based pigmentation were upregulated in females (Fig. 4a). These genes included tyrosinase (*TYR*, LFC = 1.98, adj-$P = 3.7e\text{-}07$), responsible for catalyzing the rate-limiting step of melanogenesis (Hubbard et al. 2010), major facilitator superfamily domain containing 12 (*MFSD12*, LFC = 1.45, adj-$P = 2.3e\text{-}13$), which is essential in importing cysteine into melanosomes (Adelmann et al. 2020),

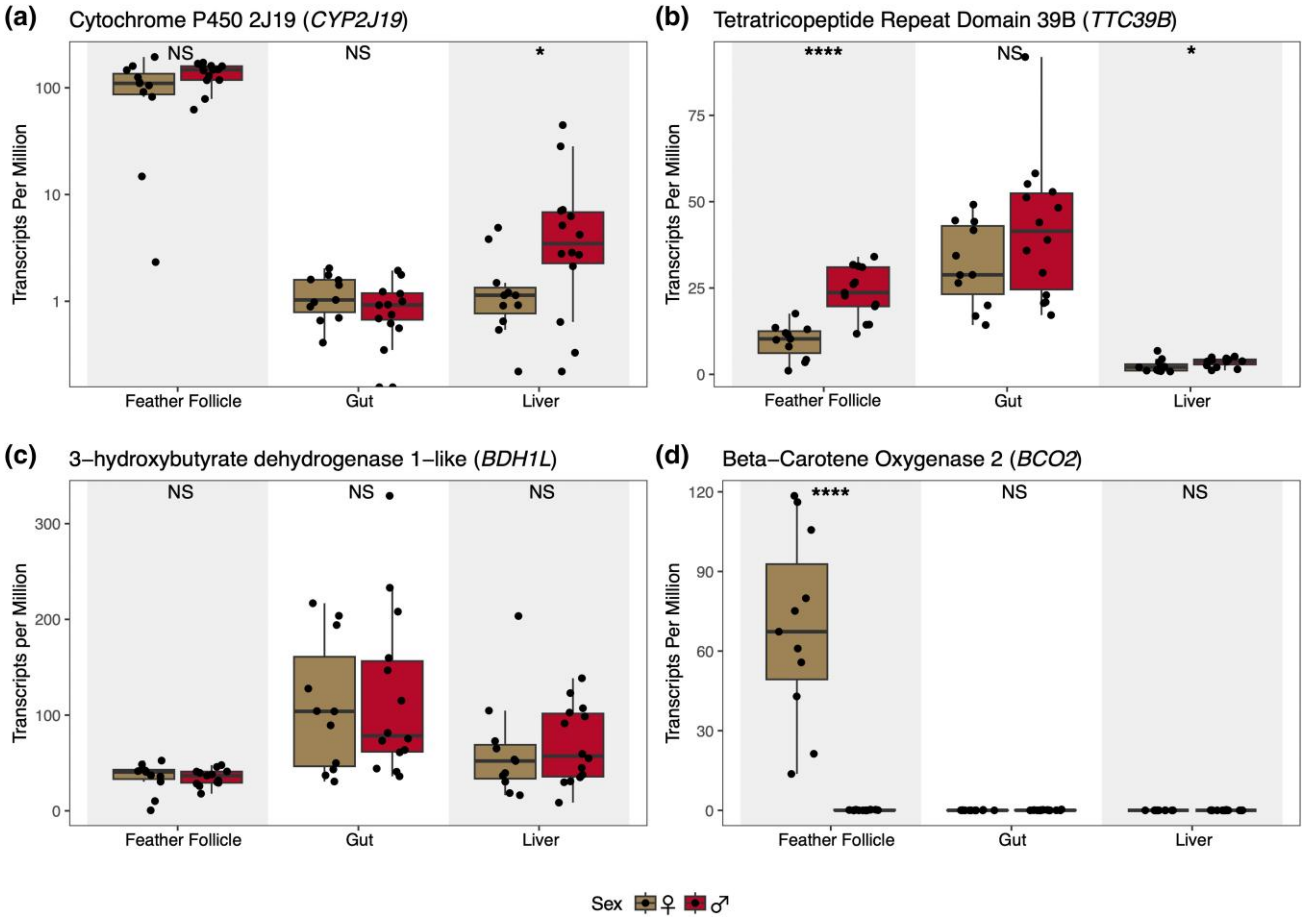


Fig. 5. Gene expression quantification by TPM across the feather follicle (left), gut (middle), and liver (right) for the putative carotenoid coloration genes *CYP2J19* (a), and *TTC39B* (b), *BDH1L* (c), and *BCO2* (d) by sex. For clarity, the *y* axis of (d) has been log-transformed. Significance of the difference in read count assessed using independent hypothesis weighting in DESeq2 and significant differences in read count are indicated in bolded asterisks (not significant [NS]: $P > 0.05$; *$P \leq 0.05$; ****$P \leq 0.0001$). All values are also indicated with points. The boxes indicate the median, the lower and upper hinges of the boxes correspond to the first and third quartiles, with whiskers extending to either the smallest/largest value or up to 1.5 times the value of the interquartile range.

and tyrosinase-related protein 1 (*TYRP1,* LFC = 1.05, adj-*P* = 0.02), which is involved in melanin deposition in many vertebrates (Lyons et al. 2005; Chen et al. 2021; Melo-Rojas et al. 2023). In the female feather follicles, we also found increased transcription in the melanocortin-1 receptor (*MC1R*, LFC = 0.96, adj-*P* = 0.001), which has a diverse role in patterning across species and serves as a regulator of *TYR* (Mundy 2005; Bourgeois et al. 2012; Yan et al. 2022), and ocular albinism type 1 (*OA1/GPR143*, LFC = 0.69, adj-*P* = 0.013), which controls melanosome maturation (Cortese et al. 2005). Altogether, the genes with increased expression in the females were significantly enriched for biological processes (BPs) related to the melanin biosynthetic process (GO:0042438, functional enrichment [FE] = 21.22, false discovery rate [FDR] = 2.54e-04) and pigmentation (GO:0043473, FE = 5.74, FDR = 0.003).

We also found melanin genes with lower expression in females than in males, including a member of the semaphorin (SEMA) gene family with prior associations with melanin-based pigmentation in birds (Poelstra et al. 2015; Aguillon et al. 2021), *SEMA4D* (LFC = -1.31, adj-*P* = 4.37e-09), which promotes melanocyte growth and survival (Soong et al. 2012). Additionally, we found increased expression in the reversibly glycosylated polypeptide homolog, *RAB6A* GEF complex partner (*RGP1*, LFC = -0.84, adj-*P* = 5.8e-07), found in candidate regions of both flickers (*Colaptes auratus auratus/Colaptes auratus cafer*) and warblers (*Setophaga coronata auduboni/Setophaga coronata coronata*) correlating with melanin production (Brelsford et al. 2017; Aguillon et al. 2021) and unconventional myosin-Va (*MYO5A*, LFC = -0.89, adj-*P* = 0.05), which is related to coat color deposition via melanosome transport in other vertebrates

(Christen et al. 2021; Zhang et al. 2021). In addition to the genes known to be involved in carotenoid and melanin-based pigmentation, our weighted gene co-expression analysis (WGCNA) analysis revealed correlated gene modules in the three tissues associated with sexual dichromatism. For details of the WGCNA findings, please refer to Figs. S3 to S8.

Additional Genes May Play a Role in Carotenoid-Based Sexual Dichromatism

Through our transcriptomic analysis, we found additional genes that may play a role in carotenoid-based sexual dichromatism within northern cardinals. Males had upregulated expression of both follistatin (*FST*, LFC = -1.05, adj-*P* = 0.04) and Molybdenum cofactor synthesis protein 2A (*MOCS2*, LFC = -1.1, adj-*P* = 0.001) in the feather follicles (Fig. 4a), which have increased transcription levels in red morphs of Gouldian finches (*Chloebia gouldiae*) compared with the black morphs (Toomey et al. 2018; Kim et al. 2019). *MOCS2*, which was also differentially expressed in the gut, does not appear to be directly linked to pigmentation, but may be associated with the noncoding region upstream of the gene's location. Fatty acid elongase 7 (*ELOVL7*), which regulates red skin color in the leopard coral grouper (Liu et al. 2025), was upregulated in male feather follicles (LFC = -0.82, adj-*P* = 0.02; Fig. 4a). *ELOVL7* may have a role in carotenoid coloration in birds as the *ELOVL* gene family is implicated in lipid deposition in chickens (Wang et al. 2022), and another gene in the same family, *ELOVL6*, was enriched in male red-backed fairywrens with carotenoid-based coloration (Khalil et al. 2023). Gene ontology (GO) analysis of the genes upregulated in the male feather follicles found an overall enrichment for the primary metabolic BP (GO:0044238, FE = 1.45, FDR = 0.01), suggesting metabolic differences in the feather follicle between the sexes.

Outside of the feather follicle, the gene with the greatest magnitude of differential expression in the liver was Stearoyl-CoA desaturase (*SCD*, LFC = -3.76, adj-*P* = 0.004), which was upregulated in the males. *SCD* is a key regulator in unsaturated fatty acid metabolism and biosynthesis (Flowers and Ntambi 2008; Paton and Ntambi 2009). As unsaturated fatty acids can facilitate the uptake of carotenoids into chylomicrons (Guo et al. 2024), there may be a link between increased *SCD* activity and more efficient carotenoid uptake in males. However, we also found the genes *FST* (LFC = -1.92, adj-*P* = 0.01) and *SEMA4D* (LFC = -1.02, adj-*P* = 0.007) upregulated in the livers of males. GO analysis of the DEGs upregulated in the male livers did not reveal any statistically significant FE for the DEGs in this tissue. In the gut, we also found increased transcription in the gene *TTC33* (LFC = -1.08, adj-*P* = 0.004), which does not have a known correlation with carotenoid pigmentation but is located within 5 kb of *TTC39B* in the cardinal genome. This region in the northern cardinal maps to the Z chromosome of the *S. canaria* genome, consistent with other species. While investigating for FE, we found that the genes upregulated in the males were enriched for the BPs of ribosomal small subunit biogenesis (GO:0042274, FE = 8.48, FDR = 6.09e-04), rRNA processing (GO:0006364, FE = 5.65, FDR = 0.002), and DNA repair (GO:0006281, FE = 3.20, FDR = 0.02). We consulted a previously published list of pigmentation-related genes and their associated PANTHER overrepresentation terms (Baxter et al. 2019) and did not find any of these BPs listed. Therefore, it is unclear what role these processes may play in pigmentation.

Potential Hormonal Regulation of Sexual Dichromatism in the Northern Cardinal

Our transcriptomic analysis provides insights into potential candidate genes related to localized hormonal control of coloration in northern cardinals. We found differential expression in two genes, both located on the Z chromosome, that may serve a role related to the hormonal regulation of sexual dichromatism. *HSD17B4* was upregulated in males in all the three tissues, with the strongest effect in feather follicles (LFC = -1.11, adj-*P* = 9e--13; Figs. 4 and S9). *HSD17B4* encodes an alcohol oxidoreductase that plays a role in many processes including fatty acid β-oxidation, bile acid biosynthesis, sterol transport, and the metabolism of sex steroids (Baes et al. 2000; Breitling et al. 2001; Zhang et al. 2017). Specifically, *HSD17B4* inactivates androgens and estrogens by converting their 17β-hydroxysteroid forms into less active 17-keto forms (Adamski et al. 1995). It therefore may play a key role in reducing localized sex steroid receptor signaling activity and hence the associated downstream effects. Another potential regulator is zinc finger protein 131 (*ZNF131*), which was highly expressed in the feather follicles of males but not females (LFC = -1.45, adj-*P* = 4.1e-8; Figs. 4 and S10). *ZNF131* is a repressor of estrogen receptor α (ERα) and negatively regulates ER-mediated signaling by interrupting ERα binding to estrogen response elements (Han et al. 2008; Oh and Chung 2012).

Discussion

Overall, we found many transcriptomic and pigment-concentration differences between male and female cardinals. Male cardinals circulated higher concentrations of ketocarotenoids than females, even though we observed no significant difference between the sexes in the concentrations of dietary carotenoids. This

pattern suggests that at least some sex-specific differences in carotenoid metabolism occurs before pigments reach the feather follicle or bill. Similarly, in zebra finches, which lack carotenoids in their plumage, males have redder bills and circulate higher concentrations of carotenoids in their plasma compared with females (McGraw et al. 2003a). Additionally, in wild-caught white-winged crossbills (*Loxia leucoptera*), which have carotenoid-based plumage dichromatism like cardinals but without ketocarotenoids in their bills, females and males had no significant difference in plasma lutein/zeaxanthin concentrations but the males exhibited significantly higher concentrations of circulating ketocarotenoids (Deviche et al. 2008). Since feather follicles of male cardinals had higher concentrations of both canary xanthophylls and ketocarotenoids than the plasma, it is likely that carotenoid metabolism is occurring primarily within the feather follicles, but it remains to be determined how carotenoid metabolism is coordinated among tissues in the northern cardinal and other birds utilizing carotenoid-based pigmentation.

As we confirmed that the gene products of northern cardinal homologs of *CYP2J19*, *TTC39B*, and *BDH1L* are sufficient to convert lutein/zeaxanthin to red ketocarotenoids, expression of *BDH1L*, *CYP2J19*, and *TTC39B* in both the liver and feather follicles supports our hypothesis that ketocarotenoid metabolism occurs in these two tissues. The almost 24-fold increase in transcription of *CYP2J19* in the feather follicle compared with the liver in both sexes (Fig. 5) suggests that even though some canary xanthophylls and ketocarotenoids are produced in the liver, feather follicles are the major site of ketocarotenoid metabolism. In males, higher *CYP2J19* expression in the liver may contribute to sexual dichromatism by increasing ketocarotenoid production and, therefore, the level of circulating plasma ketocarotenoids. In addition, upregulation of *TTC39B* in male feather follicles likely contributes to the higher concentration of canary xanthophylls and ketocarotenoids in this tissue. However, since *BDH1L*, *CYP2J19*, and *TTC39B* were also expressed in females, and females had a high overall level of *CYP2J19* expression in feather follicles, the differential expression of *CYP2J19* and *TTC39B* cannot entirely explain observations that the breast feathers of females have drab coloration with almost no ketocarotenoids.

Despite the observed differences in genes related to carotenoid uptake, modification, and transport, the large increase in *BCO2* expression in female northern cardinals suggests that this is a gene of major effect in determining the level of carotenoids in feathers. This finding is consistent with prior work concluding *BCO2* is a gene of major effect in modulating carotenoid-based sexual dichromatism in a wild population of European serins (Gazda et al. 2020a). Given that female cardinals expressed *BDH1L*, *CYP2J19*, and *TTC39B* at similar levels to the males in most sampled tissues and, indeed, have red feathers in some plumage patches such as the crest, tail, and flight feathers (Fig. 1), ketocarotenoid production is fully functional and active in in both sexes. Females appear to compensate for ketocarotenoid production by highly upregulating *BCO2* to cleave carotenoids in the feather follicles of unornamented body regions, leading to the loss of ketocarotenoid coloration.

While cleavage of carotenoids in the feather follicles may be the most important mechanism in reducing red coloration in the plumage of female cardinals, it is not the only mechanism. We did not sample feathers in the melanin-dominated black (male) or gray (female) masks of the cardinals, but males and females also differ in melanin-based coloration in the body plumage we sampled. Females retain melanin-based brownish plumage similar to juvenile plumage, whereas males have reduced melanin in their body feathers. The reduction in melanin likely enhances the chroma and brightness of ornamental red coloration of males (Price-Waldman et al. 2025). We found differential expression of many of the genes that are well-known to play a role in melanin-based plumage coloration (McNamara et al. 2021; Wakamatsu and Ito 2021), demonstrating potential molecular mechanisms underlying melanin-based sexual dichromatism. Collectively, the sexual dichromatism of northern cardinals is due to differences in both carotenoid- and melanin-based pigmentation between the sexes.

Our observations of differences in expression of genes that may have roles in carotenoid uptake and transportation likely drive some of the plumage differences observed in sexually dichromatic birds. As carotenoids are hydrophobic molecules transported along with lipids (Granneman et al. 2017; Harrison 2019), the upregulation of lipid transporters in the males compared with the females indicates that sex-specific carotenoid uptake and transport may influence the availability of carotenoid pigments for deposition in the feathers. We observed higher expression of *SLC27A6* and *PLIN2* in the feather follicles and gut tissues of male compared with female cardinals, suggesting enhanced carotenoid absorption from the food to the bloodstream and increased carotenoid transfer from the bloodstream to the feather follicles. Prior work in the red-billed quelea (*Quelea quelea*) established *PLIN* as a candidate carotenoid coloration gene (Walsh et al. 2012) but did not find evidence of sexually dimorphic expression. *PLIN* was later found differentially expressed between red and buff feather patches in male golden pheasants (*Chrysolophus pictus*; Gao et al. 2016),

but this study did not involve female birds for a complete comparison. Collectively, the increased expression of these lipid transport genes suggests that enhanced carotenoid absorption and deposition among male northern cardinals contributes to sexual dichromatism.

The transcriptomic differences between sexes revealed other candidate genes potentially related to sexual dichromatism. One of the most interesting observations of a nontarget gene was the upregulation of *FST* in the males. *FST* has been linked indirectly to coloration through genome-wide association studies in bird species that do not have red carotenoid coloration, including the pied flycatcher (*Ficedula hypoleuca*; Lehtonen et al. 2012), the golden-winged warbler (*Vermivora chrysoptera*) and the blue-winged warbler (*Vermivora cyanoptera*; Toews et al. 2016). *FST* acts as an antagonist on genes in the *TGF-β* superfamily, which may control melanogenesis (Sharov et al. 2005; McDowall et al. 2008; Moustakas 2008). If *FST* contributes to the control of melanogenesis, it may contribute to the reduced expression of most melanin-related genes we observed in male cardinals. This may serve as a mechanism whereby males maximize the intensity of their plumage, as plumage color intensity in northern cardinals correlates with body condition and is positively selected for by females (Jawor 2003; Jawor et al. 2004; Jawor and Breitwisch 2004).

Our investigation into potential hormonal regulators of northern cardinal dichromatism yielded two candidates. As *HSD17B4* was upregulated in males and downregulated in females, upregulation of this gene suggests greater interconversion of estrogens/androgens which may correspond with increased conversion of estradiol into less active estrone in males. If *BCO2* is upregulated by estrogens in northern cardinals, the lower level of estradiol should therefore reduce *BCO2* expression in males. However, while the higher expression of *HSD17B4* was associated with masculinizing male zebra finch brains, the sex differences in *HSD17B4* mRNA levels between the sexes does not translate into a significant difference in the activity of the HSD17B4 enzyme between the sexes (London et al. 2010), so the relationship between *HSD17B4* expression and sex hormone interconversion remains unclear. It is also worth mentioning that our findings of higher expression of *HSD17B4* in all sampled tissues is not congruent with observations of a bilateral gynandromorphic northern cardinal (Peer and Motz 2014), a chimera of male and female that display the typical plumage color of each sex on each half of the body, suggesting the role of localized factors in controlling plumage color. However, as the feather follicle was the only tissue observed with highly differential expressed *ZNF131*, which had higher expression in males, localized inhibition of estrogen signaling may serve as the mechanism whereby males suppress *BCO2* expression. Although the sexual dichromatism in northern cardinals is likely driven by estrogen, androgen-mediated signaling may still contribute. Prior work in northern cardinals reported the presence of transcription factor binding motifs associated with androgen signaling upstream of *CYP2J19* (Sin et al. 2020). An androgen-mediated mechanism may resemble that found in red-backed fairywrens where testosterone implantation led to the upregulation of *CYP2J19* and development of red plumage in otherwise unornamented males (Khalil et al. 2023). However, fully exploring the links between genes that control carotenoid coloration, steroid hormones, and sexual dichromatism is beyond the scope of this work and remains an area ripe for investigation.

Our findings provide further context to and may provide important insight into the debates surrounding the evolutionary origins of sexual dichromatism. Comparative studies in various clades of birds have shown that evolution of sexual dichromatism was often driven by changes in female rather than male coloration (Björklund 1991; Irwin 1994; Burns 1998; Hofmann et al. 2008; Johnson et al. 2013; Dale et al. 2015; Simpson et al. 2015; Maia et al. 2016; Price et al. 2024). Therefore, sexual selection may have a less important role in shaping dichromatism than natural selection that drives the loss of female ornamentation (Martin and Badyaev 1996). However, evidence from Tyrannida birds suggests that sexual selection led to rapid divergence in male carotenoid-based coloration rather than any evolution in female plumage (Cooney et al. 2019). Comparative work across Thraupidae and Cardinalidae has indicated that sexual dimorphism in birds occurs via both sexual and natural selection (Shultz and Burns 2017; Porzio and Mota 2025). Our findings also support both evolutionary mechanisms driving sexual dichromatism in northern cardinals. The high transcription of *BCO2* in the feather follicles of females corresponds with ketocarotenoid breakdown and the loss of red plumage color. This supports natural selection driving the evolution of a molecular mechanism for cryptic coloration in females, as nest site predation correlates with female but not male plumage (Martin and Badyaev 1996). Although females evolved cryptic coloration, they still possess red coloration in their crests, flight feathers, and tails, suggesting a yet-unexplored mechanism whereby *BCO2* expression is modulated in these feather patches. In males, upregulation of *CYP2J19* and *TTC39B* in the liver and feather follicle indicates carotenoid breakdown by *BCO2* in females is not the only mechanism contributing to sexual dichromatism. One possible explanation for the differential expression of these carotenoid metabolism genes is that the red plumage in males

was driven by sexual selection to become redder in color. This led to the elevated expression of *TTC39B*, *CYP2J19*, and genes related to carotenoid transport resulting in higher levels of ketocarotenoids in both plasma and feather follicles. Additionally, the overall reduced transcription of melanogenesis genes in male feather follicles compared with females, accompanied by males having increased transcription of potential melanogenesis-regulating genes like *FST*, may result from males evolving mechanisms to reduce melanin-based coloration to enhance ketocarotenoid plumage coloration instead of females evolving mechanisms to increase melanin production in feathers.

These differing selective pressures experienced between male and female birds could lead to the evolution of mechanisms favoring sexual selection that would be counter to natural selection (Coyne et al. 2007). This may lead to a form of sexual antagonism that would in turn create sex-biased changes in gene expression or other mechanisms of sex-specific regulation (Connallon and Clark 2010). In light of this, the mechanism of *BCO2* upregulation in female birds in several species where males exhibit carotenoid- and ketocarotenoid-based coloration may represent a form of compensation for sexually antagonistic selection. Under sexual conflict driven by sexual antagonism, we would expect to see alleles that favor selection in one sex being detrimental to the other and thus subjected to sex-specific regulation (Coyne et al. 2007). Such a scenario may contribute to the differential expression in *TTC39B,* located on the Z chromosome, in the feather follicle between the sexes (Fig. 5). Sexually antagonistic loci that are detrimental to females are theorized to occur more often on the Z chromosome in birds (Mank and Ellegren 2009; Naurin et al. 2012; Huang and Rabosky 2015). In our findings, the upregulation of *TTC39B* in male feather follicles matches this pattern, as there is incomplete dosage compensation in birds (Ellegren et al. 2007; Arnold et al. 2008), resulting in increased expression of Z chromosome genes in males.

In northern cardinals, sexual selection is promoting mutations that enhance the accumulation of red ketocarotenoids in males. This may account for genomic changes such as the large insertion containing many transcription factor binding sites located upstream of *CYP2J19* (Sin et al. 2020), which is an autosomal gene. As, over time, some of these mutations increased *CYP2J19* expression and thus drove maladaptive, increased ketocarotenoid accumulation in females, females evolved an entirely separate mechanism (*BCO2* upregulation) to modify color. This upregulation of *BCO2* in adult females matches theory on sexual antagonism where female-biased genes are upregulated in mature females (Mank and Ellegren 2009). However, this hypothesis also suggests that female-biased genes would be downregulated in males, but from our experimental design, it is not possible to determine if *BCO2* is actively downregulated in mature males or if little to no transcription of *BCO2* occurs in both immature and mature males. Despite this, since red plumage is sexually selected for in males, it is probable that increased expression of *BCO2* and its resulting enzymatic activity is detrimental to mature males and thus is actively downregulated, perhaps by a localized hormonal mechanism such as repression related to the observed *ZNF131* transcription.

Another possibility contributing to the scenario in northern cardinals not matching classic sexual antagonism theory is that ketocarotenoid coloration may not be completely detrimental to female cardinals. As assortative mating occurs in both sexes of northern cardinals based on the redness of their plumage (Jawor 2003), it is possible that the antagonistic evolution of red coloration generated another selective method whereby females indicate their quality to males despite contrary selection toward cryptic plumage. Prior work has determined that both bill and underwing redness in females correlate with body size and condition (Jawor et al. 2004), further adding to the evidence for a delicate interplay between natural and sexual selection experienced by northern cardinals. Our findings, in this context, provide an intriguing look into the mechanisms promoting, creating, and controlling sexual dichromatism in birds and lead to many exciting avenues of future exploration and investigation from comparative, endocrinological, and genomic standpoints.

Conclusion

By assessing differential expression of genes between male and female northern cardinals in gut, liver, and feather follicle tissues, we determined that sexual dichromatism is largely attributed to the action of a few genes, in particular the breakdown of carotenoids by *BCO2* in the developing feathers of females. In addition to upregulation of *BCO2*, female feather follicles also exhibited a corresponding increase in expression of melanogenesis-related genes. The more saturated and extensive red coloration in males was related to enhanced carotenoid uptake and metabolism. Our results indicate that differences in the absorption, metabolism, and transportation of carotenoids between males and females set the stage for sexual dichromatism in northern cardinals. Importantly, the breakdown of carotenoids at the site of deposition in females results in their much drabber appearance compared with males. Establishing the genetic and molecular basis of the development of sex-specific coloration provides a platform

for further studies into the evolution of sexual dichromatism. In the northern cardinal, a combination of selective forces likely drove and maintains sexual dichromatism. These conflicting, sexually antagonistic evolutionary pressures led to both the high transcription of *CYP2J19* in feather follicles regardless of sex and the resulting compensatory mechanism of *BCO2* cleaving carotenoids in female plumage. Further analysis, including sampling from different feather regions and leveraging epigenomics, could provide insight into the intriguing basis of sexual dichromatism in northern cardinals, especially in determining the genetic control of increased ketocarotenoid metabolism and how and to what degree noncoding regions and localized regulatory factors contribute to sexual dichromatism in this species. This work adds to the rich and rapidly expanding body of literature on coloration genetics as well as opens the floor to new questions and explorations into the mechanisms generating some of the incredible biodiversity of the natural world.

Materials and Methods

Sample Collection and Study Design

Sample collection and study design follows a prior experiment; for full details see Patton et al. (2026). Briefly, we caught 11 female and 14 male cardinals using mist nets and Potter traps in Lee County, Alabama, United States from 2023 August 14 to 30 (see Table S3 for specific dates). This collection period falls after the breeding season of northern cardinals but during definitive prebasic molt (Pyle 1997), which is similar in both timing, duration, and mass of feathers replaced in males and females. Blood samples were taken at capture via puncture of the brachial vein using a 28-gauge sterile needle. Birds were weighed, measured for wing and tail length, and assigned numeric values for molt score following Ginn and Melville (1983), where old feathers were assigned a 0, growing feathers assigned 1 to 4 for increasing stages of development, and fully-grown new feathers given a 5. Average bill hue, average bill saturation, and average bill brightness were assessed using reflectance spectrophotometry in the HSB system. All birds were sampled while in early stages of replacing body plumage estimated as regrowing 4% to 34% of their plumage at the time of sampling, with the remainder of plumage yet to be replaced. Following euthanasia, follicles of similar developmental stage (about 2 d after first emergence) were individually plucked from the breasts of the carcasses with tweezers, transferred to cryotubes (10 to 12 follicles per tube), and frozen at −80 °C until used for either carotenoid or RNA analysis to maintain a consistent growth stage and region for comparison in our analyses. We also dissected and flash-frozen liver and gut tissue from each individual for RNA analysis.

Carotenoid Analysis

To quantify and characterize carotenoids in feather follicles and blood plasma, we used HPLC, also following methods completely outlined in Patton et al. (2026). In brief, depending on the available sample amount, we extracted either 10, 5, or 3 µL of plasma and added it to 250 µL of 100% ethanol. Feather follicles were weighed to the nearest 0.0001 g using an analytical balance and homogenized using 500 µL of 0.9% NaCl in water and 0.1 g of silica beads in a Beadbug Homogenizer (Benchmark Scientific Inc.) at 4 kHz for 60 s. Pilot analysis of feather follicles determined that fatty acid ester removal via saponification was unnecessary. For each tissue type, we then vortexed the solution for 5 s, added 250 µL of 1:1 Hexane:Methyl tert-butyl ether, vortexed again, and then centrifuged for 3 min at 9,400 *g*. The prior steps were repeated until the supernatant became transparent and then we capped the vials under nitrogen gas and stored them in a −80 °C freezer. The samples were dissolved in 120 µL of 44:44:12 mobile phase (acetonitrile:methanol:dichloromethane). One hundred microliters of the sample were injected into an Agilent 1200 Series HPLC with a C30 carotenoid column (5.0 µm, 4.6 × 250 mm, YMC, CT99S05-2546WT) following methods from Koch et al. (2024). Specific carotenoid identities were determined by column retention time and comparison to previously established standards (details in Koch et al. 2025). Carotenoids were quantified by comparison to external standard curves as described in Koch et al. (2025). Carotenoids were measured at 445 nm, while ketocarotenoids were measured at 480 nm wavelength using a UV-Vis photodiode array detector. Representative HPLC chromatograms and peak UV-Vis spectra for carotenoids and ketocarotenoids are included in Figs. S1 and S2. We used detection limits of 0.000203 µg for carotenoids and 0.0003 µg for ketocarotenoids, based on standards of zeaxanthin and astaxanthin, respectively. Tissue concentrations of the specific carotenoids were calculated by dividing the determined carotenoid mass by the mass or volume of the sample. We used R (R Core Team 2023) for all statistical analyses and data plotting. For analysis, we focused on the red pigments previously found in cardinals and the substrates/products involved with the *CY2J19*/*BDH1L*/*TTC39B* pathway. We combined measurements of lutein and zeaxanthin (the dietary carotenoid precursors), canary xanthophylls (the product of BDH1L), all ketocarotenoids, and other carotenoids including β-carotene and

unidentified pigments. We used Wilcoxon rank-sum (also known as Mann–Whitney *U*) nonparametric statistics to compare accumulation between male and female birds.

Functional Testing

We performed functional tests following Koch et al. (2025) and Toomey et al. (2022). We determined the coding sequences of the northern cardinal homologs of *CYP2J19*, *BDH1L*, and *TTC39B* by searching the reference genome (GenBank accession GCA_014549065.1) via BLAST (Sayers et al. 2025) with a query of the known functional house finch homologs (sequences in Koch et al. 2025). We then extracted ~50 kb of the reference sequence centered on the BLAST hit and used AUGUSTUS (Hoff and Stanke 2013) to predict genes within this region. We validated these gene predictions by alignment of raw RNA sequencing reads and comparison to the de novo assembled transcripts (below). We then translated the validated transcript sequences to amino acid sequences (Table S2) and synthesized human codon optimized DNA fragments through a commercial service (Twist Biosciences, San Francisco, California, United States). We cloned these fragments into the first position of the eukaryotic expression vector *pCAG-[first position]-2A-GFP* or *pCAG-[first position]-2A-dsRed*. This vector produces a single transcript containing the candidate gene and the fluorescent tag (GFP or dsRed) that results in the stoichiometric translation of two peptides through a ribosomal skipping event. Thus, the fluorescent protein is indicative of candidate gene expression. We co-transfected the three constructs containing *CYP2J19*, *BDH1L*, or *TTC39*B into HEK293 (CRL-1573, American Type Culture Collection [ATCC]) using polyethylenimine. The HEK293 cells were cultured with the media formulation and protocols recommended by ATCC. As a control, we separately transfected HEK293 cultures with constructs containing fluorescent protein sequences in both positions of the construct (ie *pCAG-dsRed-2A-GFP*). We incubated the cells overnight and confirmed expression by visualizing fluorescence protein with an epifluorescence microscope. We then supplemented the culture media with 1 μg of either lutein (Floraglo, 5011868022; DSM Inc.) or zeaxanthin (Optisharp, 5003563004; DSM Inc.) per 1 mL solubilized with 0.035% (w:v) polysorbate 40 (Acros Organics, AC334142500). For additional details on carotenoid purification and preparation, please refer to Toomey et al. (2025). We then incubated the experimental and control cultures overnight, harvested the cells by scraping the culture plates, washed the cells twice with phosphate-buffered saline, then extracted and analyzed the carotenoid content of the cells by HPLC following the methods described for liver and gut tissue above.

RNA Extraction, Library Preparation, and Sequencing

We extracted RNA from our tissue samples using the Trizol reagent following the manufacturer's suggested protocols and homogenizing on a beadmill (15596026; Invitrogen). Contaminating DNA was removed by treating the samples with Turbo DNase reagent (AM2238; Invitrogen) and the treated RNA recovered by chloroform extraction followed by sodium acetate precipitation. The RNA was then dissolved in 35 μL of RNAse-free water and quantified using a Qubit fluorometer (Thermo Fisher). Library preparation and sequencing was completed by the Oklahoma Medical Research Foundation's Clinical Genomics Center. Briefly, 250 ng of RNA from each tissue type (feather follicle/liver/gut) was prepared for poly-A selected mRNA sequencing using the xGen RNA Library Preparation Kit from Integrated DNA Technologies (Coralville, Iowa, United States). Samples were sequenced on the NovaSeq PE150.

Genome Annotation

For annotation of the existing Northern cardinal genome (Sin et al. 2020), we built a de novo transcriptome using TRINITY v 2.14.0 (Grabherr et al. 2011). We combined RNA-seq data from a cardinal blood sample from the NCBI SRA (SRX7523501), and sequencing data from a single male and female individual feather follicle, liver, and gut tissue. We used the default settings within TRINITY, including the recommended quality trimming using TRIMMOMATIC (Bolger et al. 2014) with the following parameters: "ILLUMINACLIP: TruSeq3-PE.fa:2:30:10 SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25." Following transcript assembly, we used the transcriptome as additional evidence for performing ab initio gene prediction using MAKER v 3.01.03 (Cantarel et al. 2008; Holt and Yandell 2011), the AUGUSTUS chicken database (Stanke et al. 2006), and the RepBase for aves (Bao et al. 2015). We performed annotation finalization and homology prediction using GeMoMa (Keilwagen et al. 2019) and reference annotations from other passerines including the house finch (*Haemorhous mexicanus*; GenBank accession GCA_027477595.1), common canary (*S. canaria*; GenBank accession GCA_022539315.2), great tit (*Parus major*; GenBank accession GCA_001522545.3), and zebra finch (*T. guttata*; GenBank accession GCA_003957565.4). We performed genome re-annotation and functional annotation using eggNOG (Huerta-Cepas et al. 2019).

Transcriptomic Data Analysis

We preprocessed the RNA-seq data using Fastp v0.23.2 (Chen et al. 2018) to remove low-quality bases, adapters, and erroneous polyG tails to correct for two-color sequencing bias in the case of short read inserts, and the first ten bases of read 2, as suggested by the best practices for the xGen RNA library kit. For transcript-based gene expression quantification, we utilized the program RSEM (Li and Dewey 2011). For initial alignment to the reference genome, we used STAR v2.7.9a (Dobin et al. 2013) with modified settings –outFilterType BySJout –outFilterMultimapNmax 20 –alignSJoverhangMin 8 –alignSJDBoverhangMin 1 –outFilterMismatchNmax 999 –outFilterMismatchNoverReadLmax 0.6 –alignIntronMin 20 –alignIntronMax 30000 –alignMatesGapMax 1000000. For secondary alignment, we used the same settings as before but under –quantMode TranscriptomeSAM and using –sjdbFileChrStartEnd with the SJ files generated from the prior alignment. We then passed the output files to rsem-calculate-expression specifying paired-end alignments to calculate expression prior to creating a gene count matrix using rsem-generate-data-matrix.

We read the raw gene counts from all three runs into DESeq2 (Love et al. 2014) using R (R Core Team 2023) and removed genes with no counts across all individuals before checking for library effects using PCA. After confirming the absence of library effects, we collapsed the reads from each library into a single dataset. For tissue-specific analysis, we separated the data by tissue type (feather follicle, gut, and liver) and carried out additional filtration of genes with no counts in the respective tissue. Following that, we used a final filter from edgeR (Robinson et al. 2010) "filterByExpr()" to remove uninformative genes from each dataset. We performed additional independent hypothesis weighting within DESeq2 to adjust for the covariate of the sum of read counts per gene across all samples and control for FDR (Ignatiadis et al. 2016). We then compared expression levels between the males and females to determine differential expression related to sex. When visualizing single genes across tissues, we extracted TPM data from RSEM output files and plotted the data using R.

For WGCNA, we used the WGCNA package in R (Langfelder and Horvath 2008). Following best practices for this analysis, we used a variance stabilizing transformation on our expression data for each tissue type. We removed outlier samples based on visualizing the cluster dendrogram of samples for each analysis. When calculating our soft threshold for adjacency analysis, we utilized the built-in method for power estimation under default settings, which relies on a mean connectivity threshold of 0.85. For the feather follicle, gut, and liver datasets, we used the recommended powers of 6, 9, and 10, respectively. For each tissue type, we set a minimum size per module of 30 genes and used a threshold of 0.25 to merge highly correlated gene modules. For finding correlations between gene modules and phenotypes of interest, we used numerical factors for sex (ie females = 1, males = 2). We also incorporated measurements of wing length, tail length, weight, molt score, average bill hue/saturation/brightness while molting, and plasma/follicle xanthophyll, ketocarotenoid, and other carotenoid measurements from HPLC. As there was missing information in a few individuals for the wing/tail/weight measurements, we imputed these values using the imputePCA() function from the R package missMDA (Josse and Husson 2016) and then calculated a body condition ratio using the wing length and weight. For modules with a significant ($P < 0.05$) correlation with a phenotypic variable, we confirmed that the genes within the module also had a significant correlation between their calculated significance and module membership as well as a significant difference in average eigengene expression between the phenotypes (Welch t-test, $P < 0.05$). We then investigated the GO enrichment of these modules across BPs using the PANTHER database for *Gallus gallus* (Ashburner et al. 2000; Thomas et al. 2022; Aleksander et al. 2023). We used the Fisher test with correction for FDR to assess significance.

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

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Author Contributions

Conceptualization and resources: S.Y.W.S., G.E.H., M.B.T., and Y.Z.; formal analysis and visualization: M.J.W.; funding acquisition: S.Y.W.S., M.B.T., G.E.H., and Y.Z.; investigation: S.P., E.C.S., and R.E.K.; methodology: S.Y.W.S., G.E.H., M.B.T., M.J.W., and Y.Z.; supervision: S.Y.W.S.; writing—original draft: M.J.W. and S.Y.W.S.; writing—review & editing: M.J.W., S.Y.W.S., G.E.H., M.B.T., Y.Z., and R.E.K.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Raw count data for this project are available from the HKU DataHub, DOI: 10.25442/hku.30828212; R scripts for the project can be obtained upon request.

Ethics Statement

All research was conducted under approved IACUC permit #2023-5246 at Auburn University, along with compliance of Alabama state permit #2023124515668680 and United States federal permit #MPBER1562352.

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