SEXUAL DIMORPHISM

A genetic mechanism for sexual dichromatism in birds

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Sexual dichromatism, a difference in coloration between males and females, may be due to sexual selection for ornamentation and mate choice. Here, we show that carotenoid-based dichromatism in *mosaic* canaries, a hybrid phenotype that arises in offspring of the sexually dichromatic red siskin and monochromatic canaries, is controlled by the gene that encodes the carotenoid-cleaving enzyme β-carotene oxygenase 2 (*BCO2*). Dichromatism in *mosaic* canaries is explained by differential carotenoid degradation in the integument, rather than sex-specific variation in physiological functions such as pigment uptake or transport. Transcriptome analyses suggest that carotenoid degradation in the integument might be a common mechanism contributing to sexual dichromatism across finches. These results suggest that differences in ornamental coloration between sexes can evolve through simple molecular mechanisms controlled by genes of major effect.

In sexually dichromatic species, males and females can differ in color or pattern (*1*, *2*). These differences in coloration arise through distinct selective pressures on the two sexes (*3*, *4*), and patterns of dichromatism can shift rapidly in response to changing social environments or predation (*5*, *6*). Whether they arise in response to selection for status signals, honest signals of quality, or simply aesthetic beauty, the genetic and molecular mechanisms that control the differences in male and female coloration remain largely unknown.

Dichromatism in birds can involve pigmentary or structural mechanisms that give rise to feather coloration. However, differences in the coloration of males and females most frequently involve red or yellow carotenoid coloration (*2*, *6*, *7*), which is also the form of ornamental coloration used in sexual signaling that is best understood (*8*, *9*). Carotenoid coloration plays a central role in mate choice and can signal social dominance (*10*). Thus, elucidating the genetic basis of carotenoid-based sexual dichromatism is important to comprehensively understand the evolution of sexual dichromatism and the selective forces that shape ornamentation in animals (*11*, *12*).

In birds, heritable differences in dichromatism are largely fixed between species (*13*). The lack of genetic differences for this trait segregating within populations complicates attempts to link genotype and phenotype. We thus took advantage of the *mosaic* breed of domesticated canaries created by an interspecific cross between the sexually dimorphic red siskin (*Spinus cucullatus*) and common canaries lacking dichromatism (*Serinus canaria*). *Mosaic* canaries are strongly dichromatic, with males accumulating more carotenoid pigment in their feathers than females (Fig. 1 and fig. S1). The *mosaic* phenotype segregates in a Mendelian fashion.

To elucidate the genetic basis of dichromatism, we conducted whole-genome sequencing of two *mosaic* breeds and compared them with four domestic breeds and one wild population of common canary (*14*). After the hybridization of red siskin with canaries, siskin alleles controlling sexual dichromatism were selected through generations of backcrossing to common canaries (Fig. 1). We therefore predict that genome sequences of *mosaic* canaries should be very similar to those of common canaries except in the region mediating dichromatism, which should be derived from the red siskin genome.

We carried out genetic differentiation [fixation index (F_{ST})], association [Cochran-Mantel-Hanzel test (CMH)], and introgression analyses [the fraction of the genome shared through introgression ($f\hat{}_d$) and the relative node depth (RND)] (*14*). These analyses revealed a clear outlier region on scaffold NW_007931177 (Fig. 2, A to D, and figs. S2 and S3), which is homologous to zebra finch chromosome 24. A second weaker signal overlapped *CYP2J19*, a gene associated with red feather coloration in canaries (*15*). Genetic differentiation is inflated at the *CYP2J19* locus by our use of breeds exhibiting both yellow and red coloration, and the signal disappears when the comparison is restricted to breeds exhibiting the same background color (fig. S4). Thus, we do not believe that *CYP2J19* plays a role in dichromatism. By contrast, differentiation, association, and introgression statistics indicate that the outlier locus on scaffold NW_007931177 is a strong candidate for controlling dichromatism in *mosaic* canaries.

Next, we increased mapping resolution at the locus on scaffold NW_007931177 by genotyping 52 variants fixed for alternative alleles between wild canaries and red siskin (Fig. 2E). Because the *mosaic* phenotype follows a recessive inheritance pattern, the expectation is that *mosaic* birds should be homozygous for a haplotype derived from the siskin genome. Consistent with this expectation, we found 12 consecutive variants homozygous for the red siskin allele in all *mosaic* canaries, defining a stretch of ~36 kb (NW_007931177:821,814 to 857,981 base pairs). This interval contained three genes: *PTS* (6-pyruvoyltetrahydropterin synthase), *BCO2* (β-carotene oxygenase 2), and *TEX12* (testis-expressed protein 12).

Given that red siskins and canaries belong to different genera (*16*), it is possible that genomic rearrangements may have occurred between the two species. We thus sequenced a red siskin individual at 6.5× coverage using

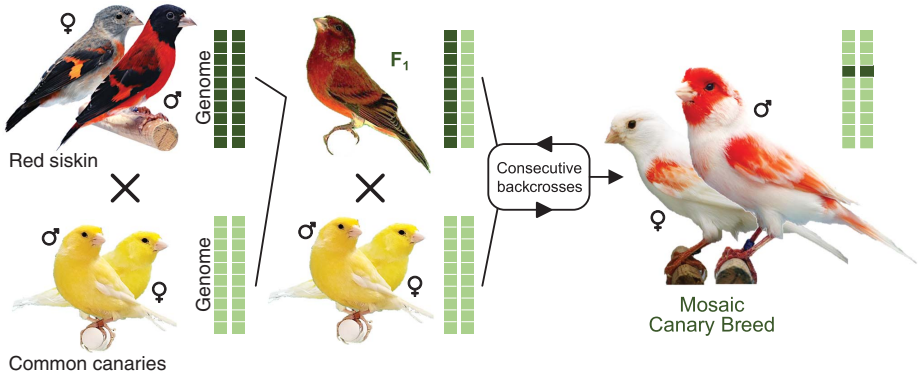

Fig. 1. Mosaic canaries were obtained through an interspecific cross. Diagram of the crosses used by breeders to obtain sexually dimorphic *mosaic* canaries from common canaries and red siskins.

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long-read technology. The synteny in both species was well preserved, and no evidence was observed that genes present in the red siskin dichromatism-associated haplotype were absent from the homologous region in the common canary genome, or vice versa (fig. S5).

Sexually dimorphic phenotypes arise from differences in gene regulation between sexes (*17*, *18*). Accordingly, we measured the expression of *PTS*, *BCO2*, and *TEX12* in regenerating feather follicles from the sexually dichromatic uropygium region of *mosaic* canaries in male and female birds by quantitative polymerase chain reaction (qPCR) (Fig. 3A). We found no significant differences in expression between males and females for *PTS* or *TEX12* [Mann-Whitney *U* rank sum test (MWU), $P > 0.21$]. By contrast, we observed significantly increased expression of *BCO2* in females compared with males (MWU, $P = 0.02$).

BCO2 is a carotene-cleaving enzyme that localizes to mitochondria and catalyzes the 9′,10′ oxidative cleavage of carotenoids, an essential step in carotenoid degradation (*19*, *20*). Missense or knockout mutations in *BCO2* result in increased accumulation of carotenoids in tissues (*19*, *21*). Thus, *BCO2* represents a candidate for mediating the *mosaic* phenotype. The increased expression of *BCO2* in *mosaic* females is predicted to result in enhanced carotenoid degradation and consequent depigmentation of the integument. When we measured *BCO2* expression in the liver, another organ that plays a role in carotenoid metabolism in birds (*22*), expression levels were indistinguishable between males and females (MWU, $P > 0.80$) (Fig. 3A). This indicates that variation at the *mosaic* locus likely alters the expression of *BCO2* between sexes in a tissue-specific manner.

To further characterize *BCO2* expression patterns, we analyzed developing feather follicles of male and female birds by in situ hybridization (Fig. 3B and fig. S6). We observed *BCO2* expression in the barb ridges and barbule cells of developing white feather follicles in both sexes. We did not observe *BCO2* expression in barb ridges of carotenoid-pigmented follicles. This suggests that *BCO2* is selectively expressed in developing white feather follicles and produces the *mosaic* phenotype through the local degradation of carotenoids.

To measure allele-specific expression in regenerating feather follicles, we crossed *mosaic* canaries to common canaries and generated birds heterozygous for the dichromatism-associated siskin allele and for the common canary allele at the *BCO2* locus (Fig. 3C and table S1). In heterozygous birds, the two *BCO2* alleles are influenced by the same trans-acting regulatory elements and other environmental factors; thus, differences in their relative

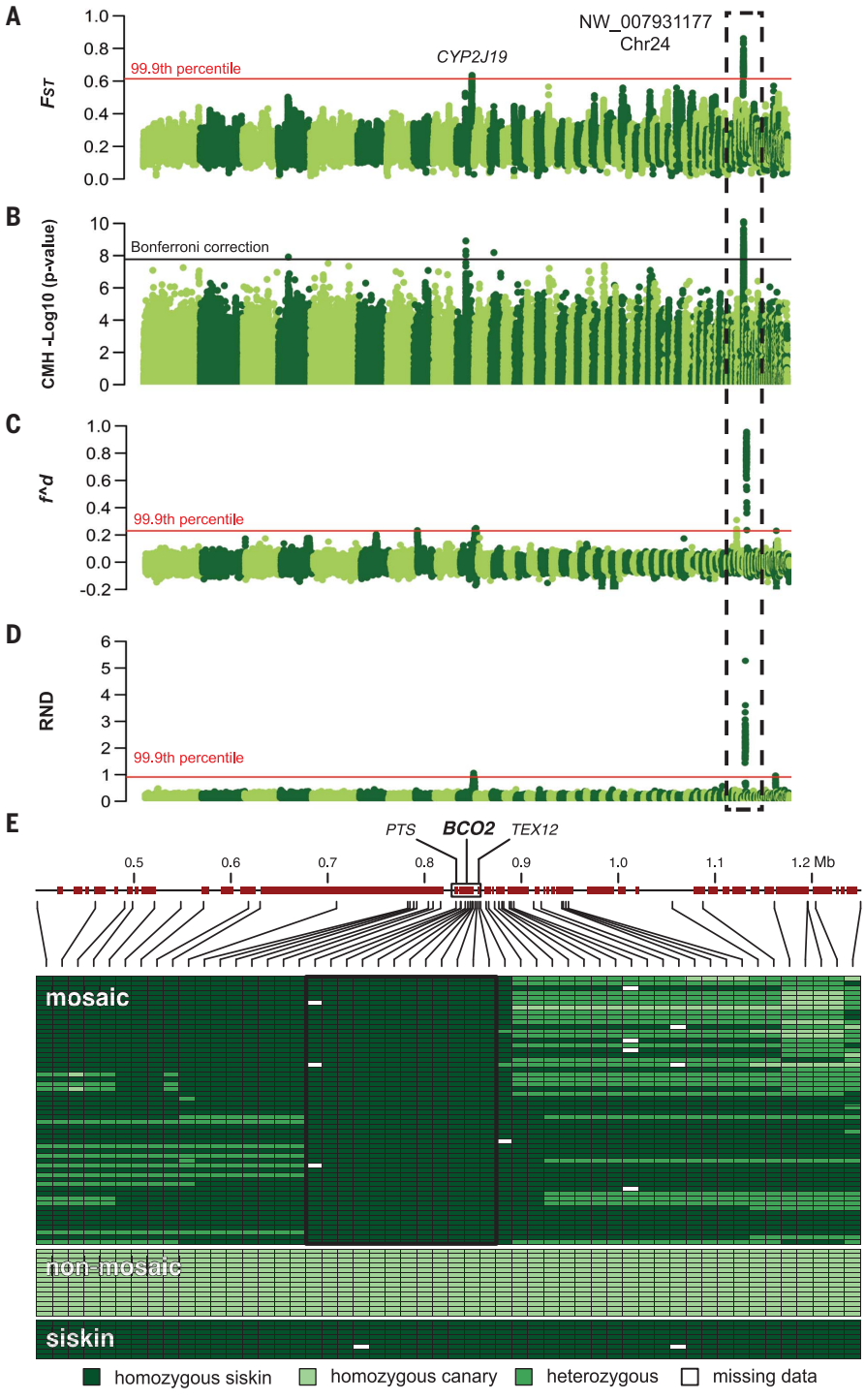

Fig. 2. Genetic mapping using whole-genome sequencing. (**A**) Average F_{ST} values between *mosaic* and non-*mosaic* canaries across the genome (20-kb windows with 5-kb steps) (**B**) −log10 values per variant of the CMH statistic configured to detect consistent differences in allele frequency between *mosaic* and non-*mosaic* canaries. (**C**) The fraction of introgression ($f\hat{}_d$) from red siskin to *mosaic* canaries summarized in nonoverlapping windows of 100 single-nucleotide polymorphisms (SNPs). (**D**) Divergence between *mosaic* and non-*mosaic* canaries summarized across the genome using RND. Dots represent RND values in nonoverlapping windows of 10,000 polymorphic and nonpolymorphic positions passing filters. In (A) to (D), the 99.9th percentile of the empirical distribution and the significance threshold after Bonferroni correction are shown by red and black horizontal lines, respectively. (**E**) Genotyping across the candidate region on scaffold NW_007931177. Each column represents one SNP, and each row represents one individual. Shades of green indicate positions homozygous for the siskin allele, homozygous for the canary allele, and heterozygous. White indicates missing data. Protein-coding genes are indicated by red boxes.

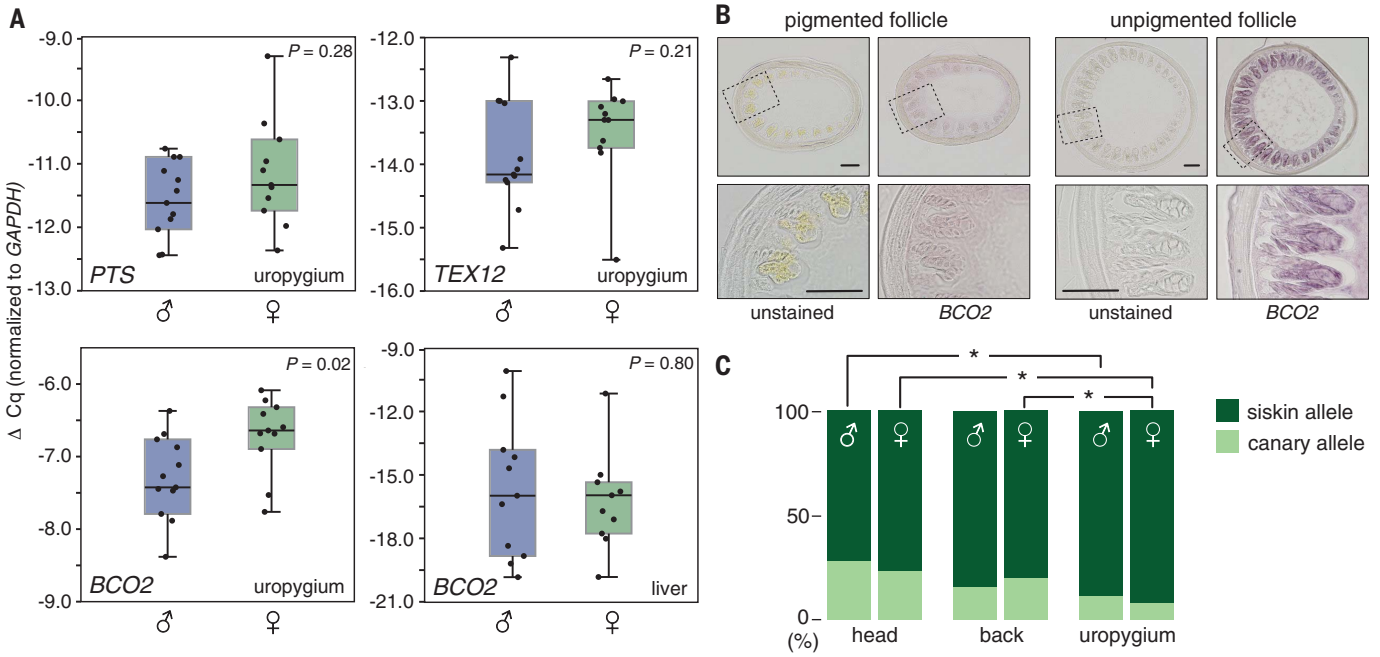

Fig. 3. Gene expression analysis in *mosaic* canaries. (**A**) qPCR measurements of *BCO2*, *PTS*, and *TEX12* normalized to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) in the uropygium skin and liver of *mosaic* canaries. Relative expression (ΔCq) was obtained by averaging quantification cycle (Cq) values of three technical replicates. Boxes represent the 25 and 75 percent quartiles, horizontal lines inside the box mark the median, and the short horizontal lines ("whiskers") indicate the minimal and maximal values. (**B**) Unstained sections and in situ hybridization of *BCO2* in regenerating feather follicles that express or lack carotenoid pigments in the developing barb ridges. The bottom row shows magnified views of the outlined areas in the images in the top row. Scale bars, 50 μm. (**C**) Relative expression (%) of the red siskin and canary alleles in regenerating feather follicles of heterozygous individuals. Values are averages of two amplicons (table S1). Significant comparisons using FET are denoted with an asterisk.

expression should be due to cis-acting regulatory elements (i.e., an enhancer or promoter), which affect gene expression in an allele-specific manner (*23*). We found preferential expression of the siskin allele over the canary allele in both sexes and in the three feather tracts sampled [Fisher's exact test (FET), $P < 10^{-16}$]. This difference is likely due to a more active cis-regulatory element on the siskin haplotype and likely explains why the integument of *mosaic* canaries in both sexes exhibits less carotenoid pigmentation compared with common canaries (Fig. 1).

Our sample size of one male and female is small, and the relative expression of both alleles was not evidently different between sexes (FET, $P > 0.05$). However, we did observe significant differences among the three feather tracts (FET, $P < 0.05$) (Fig. 3C), suggesting that trans-acting regulators in the canary genomic background might regionally modulate *BCO2* expression across the integument. Trans-acting regulators of *BCO2* expression and/or additional genes located elsewhere in the genome, which can alter the rate at which carotenoids are deposited or degraded, could explain why *mosaic* canaries and red siskins exhibit different carotenoid pigmentation patterns (Fig. 1) despite sharing identical DNA sequences at the *BCO2* locus (Fig. 2E). Our results show that dichromatic phenotypes can be produced by genes of large effect; however, they also suggest that additional genetic modifiers might be involved in the fine-tuning of the dichromatism observed in nature.

The canary and red siskin BCO2 proteins also differ at two amino acid positions. However, these substitutions are found in bird species lacking sexual dichromatism or carotenoid pigmentation (fig. S7). Thus, a functional role for these differences is unlikely. Overall, our expression studies suggest that sexual dichromatism in *mosaic* canaries arises because of differences in the activity of *BCO2* throughout the integument. The lack of ornamental coloration in female *mosaic* canaries seems to be estrogen-dependent, because reproductively senescent and ovariectomized females develop a color pattern similar to that of males (*24*). These observations suggest the presence of siskin-derived, hormone-responsive regulatory elements within the introgressed haplotype. These putative regulatory elements have yet to be identified.

To test whether the mechanisms for sexual dichromatism that we uncovered in *mosaic* canaries are present in wild bird species, we examined gene expression in the developing feathers of three species of finches that vary in the extent of carotenoid-based sexual dichromatism (Fig. 4A): common canaries (*S. canaria*) exhibiting wild-type coloration, which exhibit slight sexual dichromatism; the European serin (*Serinus serinus*), the sister species of canaries, which displays more pronounced sexual dichromatism; and the house finch (*Haemorhous mexicanus*), a species in which males display bright red or yellow colors but females are nearly devoid of colorful carotenoids in their plumage. We sampled the same regions of the integument in all three species (chest and belly) and profiled gene expression by RNA sequencing (*14*).

We tested if the degree of sexual dichromatism was correlated with gene expression divergence between males and females. We found that feather patches that differed more strongly in carotenoid pigmentation between sexes (chest and belly in serin and chest in house finch) had a larger number of differentially expressed genes (DEGs) (Fig. 4B), demonstrating that sexual dichromatism correlates with increased sex-biased gene expression.

We also compared gene expression between sexes, sister species, or patches within species showing pronounced differences in levels of carotenoid pigmentation (Fig. 4C). Because the transcriptomes of males and females differ in most tissues (*17*), many of the expression differences observed in our dataset should have no causal relationship with pigmentation differences. We thus reasoned that DEGs shared among the three types of contrasts would be promising candidates mediating dichromatism. Of the DEGs from our total dataset, only 12 genes met this criterion (Fig. 4D and table S2), including *BCO2*.

A closer examination revealed that several aspects of *BCO2* expression varied predictably

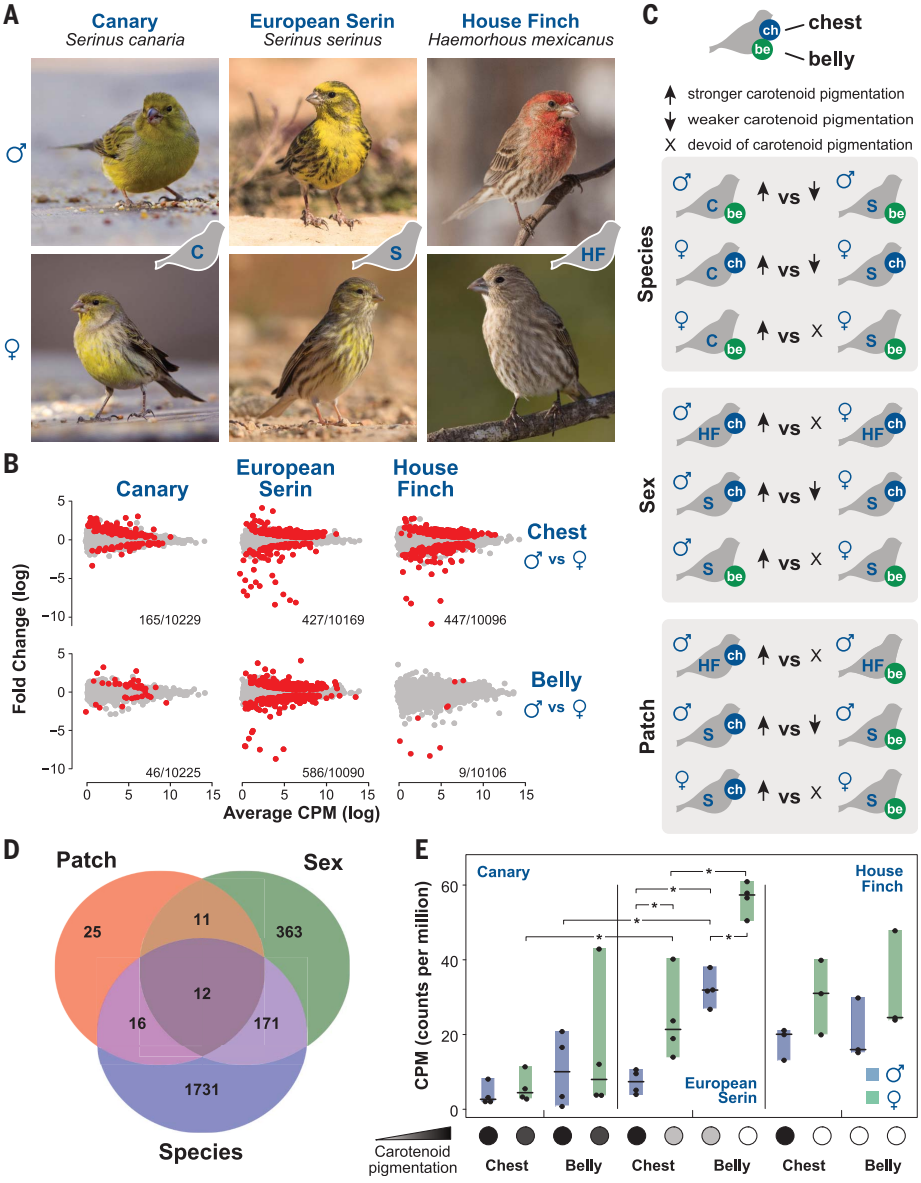

Fig. 4. Transcriptomics along a continuum of sexual dichromatism. (**A**) Representative pictures of male and female canaries (C), European serin (S), and house finch (HF). (**B**) Scatterplots of log-fold change (*y* axis) and log-CPM (counts per million) (*x* axis) in comparisons between males and females. Significant genes are depicted as red dots. For each comparison, the number of DEGs versus the total number of genes in the transcriptome is given at the bottom of the graph. (**C**) Nine different comparisons characterized by pronounced differences in the intensity of carotenoids, which include contrasts between sexes, species, and patches [belly (be) and chest (ch)]. (**D**) DEGs among the types of contrasts defined in (B). (**E**) Patterns of *BCO2* expression in the three finch species. Comparisons where *BCO2* was found significantly differentially expressed by the three methods implemented are denoted with an asterisk (*14*). Carotenoid intensity is indicated by gray scale.

with plumage carotenoid content in comparisons involving the European serin (Fig. 4E). First, serin females exhibit less carotenoid pigmentation than serin males and expressed *BCO2* at higher levels. Second, *BCO2* expression was higher in serin females compared with canary females, whereas the latter exhibit more marked carotenoid pigmentation. Finally, both male and female serins show lower expression of *BCO2* in feather patches exhibiting stronger carotenoid pigmentation. These findings suggest that *BCO2* plays a role in dichromatism in the European serin. *BCO2* expression was largely uncorrelated with the levels of carotenoid pigmentation in comparisons involving the house finch (Fig. 4E), which suggests that finches may use alternative molecular mechanisms to produce sexual dichromatism.

Genetic studies of sexual dichromatism may reveal the molecular mechanisms that enable the expression of differential male and female traits from a single shared genome. Here, we show that differences in carotenoid pigmentation between sexes of *mosaic* canaries are controlled by a single genomic region of red siskin origin. This region contains *BCO2*, a candidate gene for sex-specific pigmentation in birds displaying sexually divergent carotenoid-based coloration. The simplicity of this genetic mechanism may help explain the evolutionary lability of sexual dichromatism and why carotenoid pigmentation is the coloration mechanism most commonly associated with sexual dichromatism in birds. Sexual dichromatism in carotenoid coloration has been proposed to result from sex-specific differences in ingestion, absorption, metabolism, or transportation of carotenoids (*25*). Our observations, however, suggest that selective degradation of carotenoids at different rates in peripheral tissues may be an important mechanism for differences in carotenoid coloration in males and females. These findings add to a growing body of evidence that the *BCO2* locus is a genomic hotspot for the evolution of carotenoid-based pigmentation across multiple tissues and vertebrates (*26*–*28*), which our study extends to sexual dichromatism.

REFERENCES AND NOTES

1. T. Amundsen, *Trends Ecol. Evol.* **15**, 149–155 (2000).
2. A. V. Badyaev, G. E. Hill, *Annu. Rev. Ecol. Evol. Syst.* **34**, 27–49 (2003).
3. J. Dale, C. J. Dey, K. Delhey, B. Kempenaers, M. Valcu, *Nature* **527**, 367–370 (2015).
4. A. E. Johnson, J. Jordan Price, S. Pruett-Jones, *Ecol. Evol.* **3**, 3030–3046 (2013).
5. T. Price, G. L. Birch, *Auk* **113**, 842–848 (1996).
6. A. V. Badyaev, G. E. Hill, *Biol. J. Linn. Soc. London* **69**, 153–172 (2000).
7. D. A. Gray, *Am. Nat.* **148**, 453–480 (1996).
8. R. J. Weaver, E. S. A. Santos, A. M. Tucker, A. E. Wilson, G. E. Hill, *Nat. Commun.* **9**, 73 (2018).
9. R. J. Weaver, R. E. Koch, G. E. Hill, *Philos. Trans. R. Soc. London B Biol. Sci.* **372**, 20160343 (2017).
10. P. A. Svensson, B. B. M. Wong, *Behaviour* **148**, 131–189 (2011).
11. J. A. Coyne, E. H. Kay, S. Pruett-Jones, *Evolution* **62**, 214–219 (2008).
12. E. R. Funk, S. A. Taylor, *Auk* **136**, ukz048 (2019).
13. K. J. Burns, *Evolution* **52**, 1219–1224 (1998).
14. Materials and methods are available as supplementary materials.
15. R. J. Lopes *et al.*, *Curr. Biol.* **26**, 1427–1434 (2016).
16. D. Zuccon, R. Prŷs-Jones, P. C. Rasmussen, P. G. P. Ericson, *Mol. Phylogenet. Evol.* **62**, 581–596 (2012).
17. H. Ellegren, J. Parsch, *Nat. Rev. Genet.* **8**, 689–698 (2007).
18. T. M. Williams, S. B. Carroll, *Nat. Rev. Genet.* **10**, 797–804 (2009).
19. J. Amengual *et al.*, *FASEB J.* **25**, 948–959 (2011).
20. C. Dela Seña *et al.*, *J. Biol. Chem.* **291**, 14609–14619 (2016).
21. M. A. Gazda *et al.*, *Mol. Biol. Evol.* **37**, 1317–1328 (2020).
22. E. del Val *et al.*, *Naturwissenschaften* **96**, 797–801 (2009).
23. P. J. Wittkopp, B. K. Haerum, A. G. Clark, *Nature* **430**, 85–88 (2004).
24. O. Perez-Beato, *Fundamentals of Color Genetics in Canaries: Reproduction and Control* (RoseDog Books, Pittsburgh, 2008).
25. K. J. McGraw, G. E. Hill, R. Stradi, R. S. Parker, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **131**, 261–269 (2002).
26. J. Eriksson *et al.*, *PLOS Genet.* **4**, e1000010 (2008).
27. D. P. L. Toews *et al.*, *Curr. Biol.* **26**, 2313–2318 (2016).
28. P. Andrade *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **116**, 5633–5642 (2019).

29. M. Carneiro *et al*., A genetic mechanism for sexual dichromatism in birds. Dryad (2020); doi:10.5061/dryad.80gb5mknh.
30. P. Pereira, PJADPereira/Dichromatism: Dichromatism. Zenodo (2020); doi:10.5281/zenodo.3648365.

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SUPPLEMENTARY MATERIAL

science.sciencemag.org/content/368/6496/1270/suppl/DC1
Materials and Methods
Figs. S1 to S7
Tables S1 to S6
References (*31*–*61*)
MDAR Reproducibility Checklist

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Canaries changing colors

Many animals are sexually dimorphic, with different phenotypes in males and females. To identify the genetic basis of sexual differences in bird coloration, Gazda *et al.* investigated red coloration in mosaic canaries and related species (see the Perspective by Chen). Using a combination of genetic crosses, genomic mapping, transcriptomics, and comparative analyses, the authors show that trans-regulation of the carotenoid-processing gene *BCO2* is involved in sexual dichromatism. Although such variation in coloration among the sexes is common, particularly in birds, there are few candidate genes known to be involved. This study helps to elucidate the molecular mechanisms that underlie the evolution of dichromatism and may aid in uncovering sexually selected traits.

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