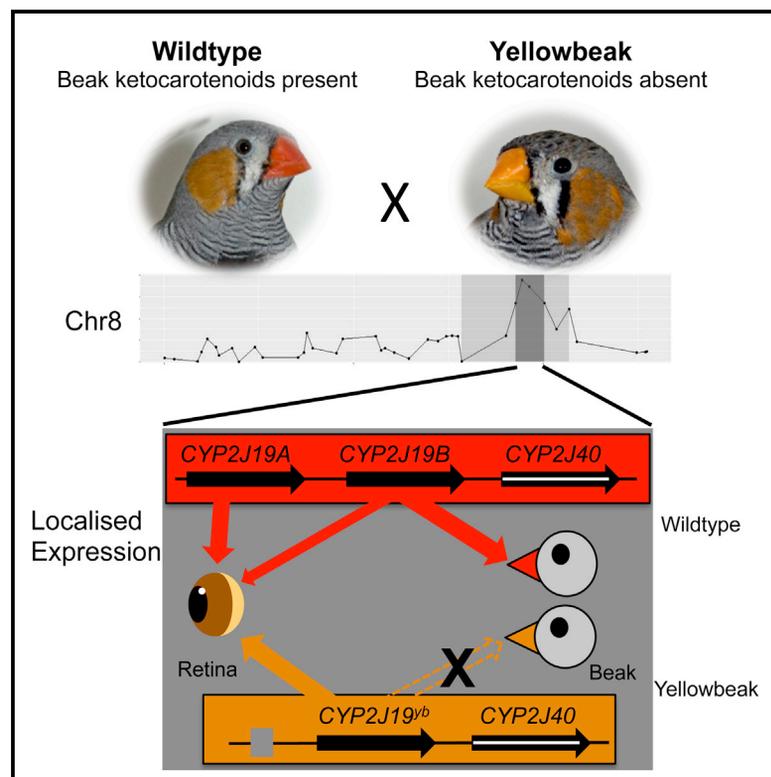


# Current Biology

## Red Carotenoid Coloration in the Zebra Finch Is Controlled by a Cytochrome P450 Gene Cluster

### Graphical Abstract



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### In Brief

Mundy et al. have identified genes required for the bright-red coloration that many birds use for communication, such as attracting mates. They uncover a genetic connection between red coloration and color vision in birds and propose that redness may be an honest signal of mate quality by indicating a bird's ability to detoxify harmful substances.

### Highlights

- The *yellowbeak* mutation maps to a narrow region of chromosome 8 with a CYP cluster
- CYP2J19 loci most likely encode ketolases that generate red ketocarotenoids
- CYP2J19 loci are involved both in red coloration and red retinal oil droplets
- Involvement of cytochrome P450s provides a novel mechanism of signal honesty

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# Red Carotenoid Coloration in the Zebra Finch Is Controlled by a Cytochrome P450 Gene Cluster

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## SUMMARY

Bright-red colors in vertebrates are commonly involved in sexual, social, and interspecific signaling [1–8] and are largely produced by ketocarotenoid pigments. In land birds, ketocarotenoids such as astaxanthin are usually metabolically derived via ketolation of dietary yellow carotenoids [9, 10]. However, the molecular basis of this gene-environment mechanism has remained obscure. Here we use the *yellowbeak* mutation in the zebra finch (*Taeniopygia guttata*) to investigate the genetic basis of red coloration. Wild-type ketocarotenoids were absent in the beak and tarsus of *yellowbeak* birds. The *yellowbeak* mutation mapped to chromosome 8, close to a cluster of cytochrome P450 loci (*CYP2J2*-like) that are candidates for carotenoid ketolases. The wild-type zebra finch genome was found to have three intact genes in this cluster: *CYP2J19A*, *CYP2J19B*, and *CYP2J40*. In *yellowbeak*, there are multiple mutations: loss of a complete *CYP2J19* gene, a modified remaining *CYP2J19* gene (*CYP2J19<sup>yb</sup>*), and a non-synonymous SNP in *CYP2J40*. In wild-type birds, *CYP2J19* loci are expressed in ketocarotenoid-containing tissues: *CYP2J19A* only in the retina and *CYP2J19B* in the beak and tarsus and to a variable extent in the retina. In contrast, expression of *CYP2J19<sup>yb</sup>* is barely detectable in the beak of *yellowbeak* birds. *CYP2J40* has broad tissue expression and shows no differences between wild-type and *yellowbeak*. Our results indicate that *CYP2J19* genes are strong candidates for the carotenoid ketolase and imply that ketolation occurs in the integument in zebra finches. Since cytochrome P450 enzymes include key detoxification enzymes, our results raise the intriguing possibility that red coloration may be an honest signal of detoxification ability.

## RESULTS AND DISCUSSION

Bright-red coloration of skin or plumage is a major component of avian color diversity and depends, with few exceptions, on the

ability to enzymatically convert yellow dietary carotenoids (xanthophylls) to red C4 ketocarotenoid pigments such as astaxanthin or canthaxanthin. Carotenoid ketolation is thus an important key innovation in avian evolution and color diversification [11–13], and many well-known examples of sexually selected visual displays involve bright-red ketocarotenoid coloration in the plumage or beak, functioning to attract partners or to deter rivals (e.g., [5–8]). The anatomical location of ketolation has been contentious. The absence of ketocarotenoids in the blood plasma of some birds suggests peripheral action, close to or in the ketocarotenoid-pigmented tissues, such as the beak or feather follicles [14]. In other species, high concentrations of ketocarotenoids in the plasma imply transport to the periphery from the tissue where ketolation occurs [15, 16], which is likely to be the liver [17].

Ketocarotenoids also occur in avian retinas, where astaxanthin is the pigment in the red oil droplets [18] that are present in longwave-sensitive (LWS) cones and act as cut-off filters to improve color discrimination [19]. The mechanism behind retinal astaxanthin, and its relationship with integumentary ketocarotenoid pigmentation is unknown.

Male zebra finches have ketocarotenoid-based red bill coloration, which is a classical sexually selected trait, with females preferring males with redder bills [6]. Males also have red tarsi, and females have less saturated red bills and tarsi, containing the same ketocarotenoids as the males at lower concentrations [18]. The *yellowbeak* mutation, only described from captive birds, has yellow bills and tarsi in both sexes and is considered an autosomal recessive mutation among cage-bird enthusiasts (Figure 1) [20].

### Ketocarotenoids Are Absent in the Beak of *yellowbeak* Birds

High-performance liquid chromatography (HPLC) analysis of carotenoids extracted from the beak of *yellowbeak* birds revealed the presence of dietary yellow carotenoids and an absence of the four red ketocarotenoids (astaxanthin, canthaxanthin,  $\alpha$ -doradoxanthin, and adonirubin) present in wild-type birds (Figure 2). This is consistent with a role for the *yellowbeak* mutation in the metabolic conversion of dietary carotenoids to ketocarotenoids.

### *Yellowbeak* Maps to a Narrow Region of Chromosome 8 Containing Candidate Ketolase Genes

We first mapped the *yellowbeak* mutation using 352 genome-wide SNPs in a pedigree of 95 birds. A total of 17 (0.28) of the second-generation birds expressed the *yellowbeak* phenotype, almost





**Figure 1. The yellowbeak Phenotype**

Male wild-type (left) and yellowbeak (right) zebra finches. Photo credit: Stuart Dennis.

exactly the 3:1 ratio of wild-type:yellowbeak phenotypes expected for a segregating autosomal Mendelian recessive trait. The yellowbeak trait was genetically linked to a single SNP on chromosome 8 (Tgu8) (two-point linkage of yellowbeak to Tgu\_SNP\_01464; recombination fraction = 0 cM; logarithm of the odds [LOD] = 5.72; position of SNP on assembly = 25.44 Mb) (Figure 3A).

We next performed fine-scale mapping in this region, obtaining data from 39 SNPs between 15.02 and 27.73 Mb on chromosome 8. Six consecutive markers, positioned between 24.01 Mb and 25.35 Mb, produced recombination fractions of zero with yellowbeak (Table S2; Figure 3B). The flanking markers with some recombination to yellowbeak are located at 22.85 Mb and 25.68 Mb, and so yellowbeak must lie within this 2.83 Mb interval. In fact, the fine mapping provides strong support for a much narrower interval. At the central four of the  $r = 0$  markers, the F2 yellowbeak birds and F0 yellowbeak founders had identical genotypes (Figure S1). The first and sixth markers were the least informative and so had lower power to detect recombination events with yellowbeak (Table S2). The two markers with highest LOD scores were rs83092174 (LOD score = 7.53), which is located at 24,442,889 bp on chromosome 8, and rs83092728 (LOD score = 6.92), which is located at 24,628,232 bp. The two flanking markers had weaker support (LOD = 5.42 in both cases), and the interval spanning the flanking markers covers 24.269–25.029 Mb. This interval contains five genes on the zebra finch genome assembly, two CYP2J2-like loci, *NF1A*, *HOOK*, and *FGGY* (Figure 3C). Thus, there is greatest statistical support for yellowbeak to reside within this 0.76 Mb window, but because some F1 parents are not informative at all SNPs, an extended 2.83 Mb cannot be ruled out by the mapping data alone.

The CYPs encode a large family of cytochrome P450 oxidases that are good candidates for carotenoid ketolases. Specifically, a fungal CYP encodes a carotenoid ketolase [21], and vertebrate CYP26s encode retinoic acid oxidases, which perform an oxidation of the terminal carbon ring of retinoic acid to 4-oxo-retinoic acid that is chemically similar to the reaction performed by carotenoid ketolases [22]. The CYP2J2-like cluster on chromosome 8 lies right under the LOD peak in yellowbeak birds at 24.573–24.586 Mb. In contrast, *NF1A* (encoding a nuclear family 1 transcription factor), *HOOK* (encoding a microtubule-binding

protein), and *FGGY* (encoding a carbohydrate kinase) are very unlikely to be involved in carotenoid metabolism. We therefore targeted the CYP2J2-like cluster as a candidate region for yellowbeak.

### Yellowbeak Is Associated with a Deletion of a CYP2J2-like Locus

Preliminary gene expression results indicated an association of expression of a CYP2J19 locus on chromosome 8 and the yellowbeak phenotype (see below). Further investigation led to the discovery

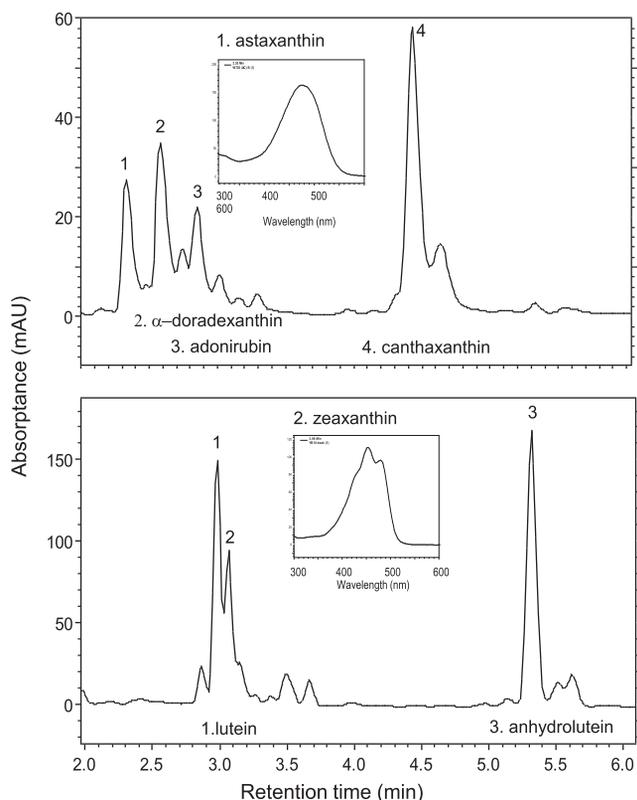
of errors in the current zebra finch genome assembly (Taegut3.2.4) in this region. The existing assembly indicates two adjacent CYP2J2-like loci on chromosome 8, with a further two CYP2J2-like loci on an unassembled chromosome 8 contig. Using a combination of long-range PCR and Illumina MiSeq sequencing, we found direct evidence for three adjacent CYP2J2-like loci on chromosome 8, incorporating some sequence from the previously unanchored region (Figure 3D; Supplemental Experimental Procedures; Figure S1B). Two of these loci, which we term CYP2J19A and CYP2J19B, are close homologs related to chicken CYP2J19, whereas the third, CYP2J40, is more distantly related.

Long-range PCR results indicated the presence of an ~13 kb deletion of a full copy of CYP2J19 in yellowbeak birds (Figure S1D). Using a PCR assay (Figure S1C), we found a perfect association between this deletion and yellowbeak (Fisher's exact test of association between phenotype and absence of the wild-type allele,  $p = 1.16 \times 10^{-8}$ ): all yellowbeak birds were homozygous for the deletion ( $N = 12$ ), whereas wild-type birds were homozygous ( $N = 9$ ) or heterozygous ( $N = 9$ ) for the intact allele.

The single copy of CYP2J19 in yellowbeak birds, CYP2J19<sup>yb</sup>, is divergent from both wild-type CYP2J19 loci and has four unique amino acid substitutions. Phylogenetic reconstruction shows that it is closer to CYP2J19B than CYP2J19A, implying that the mutation may have involved a full deletion of CYP2J19A (Figure S2A). Substantial allelic variation in wild-type alleles of CYP2J19B, but not CYP2J19A, was detected, with two divergent allelic lineages present (Figure S2A). A 76 bp indel polymorphism in CYP2J19<sup>yb</sup> only was found ~600 bp upstream of the start codon. An intact copy of the other CYP2J2-like locus on chromosome 8, CYP2J40, is present in yellowbeak, and this encodes a protein with a single amino acid substitution (Y209N) compared to wild-type CYP2J40.

### CYP2J19 Loci Are Specifically Expressed in Ketocarotenoid-Containing Tissues, and Bill Expression Is Greatly Reduced in yellowbeak

In wild-type birds, we detected expression of CYP2J19 loci in the beak, tarsus, and retina, which are all sites of ketocarotenoid deposition, but not in the heart, or liver and duodenum, which



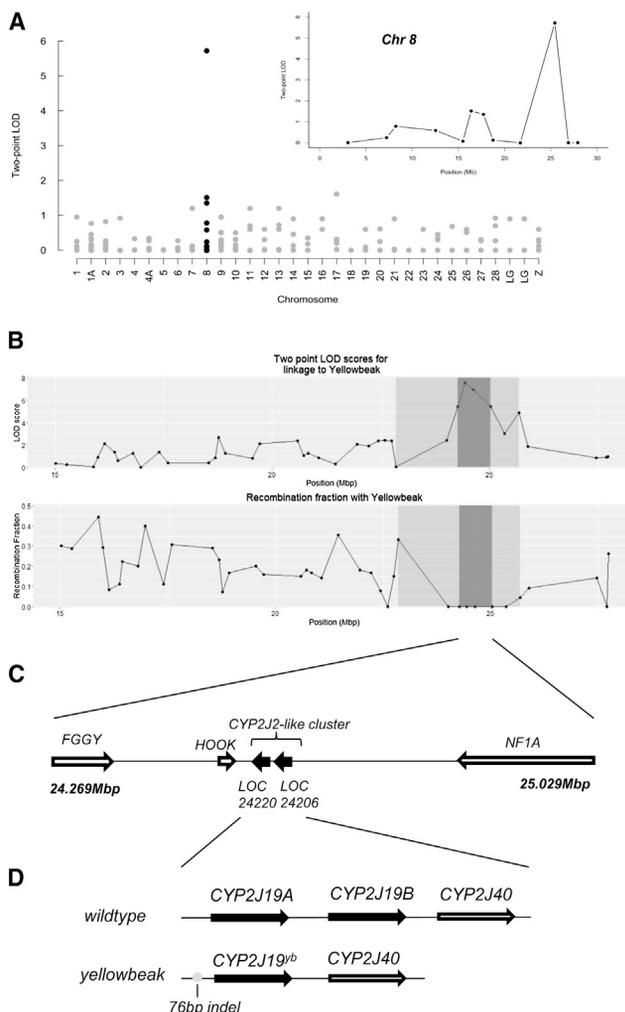
**Figure 2. Representative Chromatograms from HPLC Analyses of Carotenoid Content in Zebra Finch Beaks**

Wild-type (top) and *yellowbeak* (bottom). Insets show absorbance spectra of one of the major dietary precursor carotenoids, zeaxanthin (bottom) and its ketolated derivative, astaxanthin (top). All major, labeled peaks in the wild-type are red ketocarotenoids (peak absorbance 460–475 nm), whereas labeled peaks in the *yellowbeak* are all dietary yellow xanthophylls (peak absorbance <455 nm).

are important sites for carotenoid metabolism and uptake (Figure S2B). *CYP2J19A* expression was detected only in the retina, whereas *CYP2J19B* was strongly expressed in the beak and tarsus, with variable expression in the retina (Figure S2C). In contrast, *CYP2J40* was expressed in all of these tissues (data not shown).

In qRT-PCR experiments, *CYP2J19<sup>vb</sup>* expression was barely detectable in the bill of *yellowbeak* birds (Figure 4A) and was significantly lower than expression of *CYP2J19B* in wild-type birds (two-tailed t test,  $p < 0.05$ ). In contrast, *CYP2J19<sup>vb</sup>* expression was present in *yellowbeak* retinas and similar to that of *CYP2J19A/B* in wild-type retinas (Figure 4B). There were no differences in *CYP2J40* expression between *yellowbeak* and wild-type bills (Figures 4C and 4D). Taking genetic and expression data together, our results suggest that the deletion in *yellowbeak* removed both *CYP2J19A* and *cis*-regulatory sequences driving expression of *CYP2J19B* in the beak, bringing *CYP2J19<sup>vb</sup>* under control of *cis*-regulatory sequences of *CYP2J19A*, thereby driving expression of *CYP2J19<sup>vb</sup>* in the retina.

The bills of female zebra finches have the same ketocarotenoids as males, but at lower concentrations. In an experiment to investigate potential sex-biased expression, we did not find



**Figure 3. Linkage Mapping and Structure of *CYP2J2*-like Cluster**

(A) Low-resolution mapping results. Genome-wide LOD scores are shown in the main panel, and chromosome 8 LOD scores are shown in the inset.

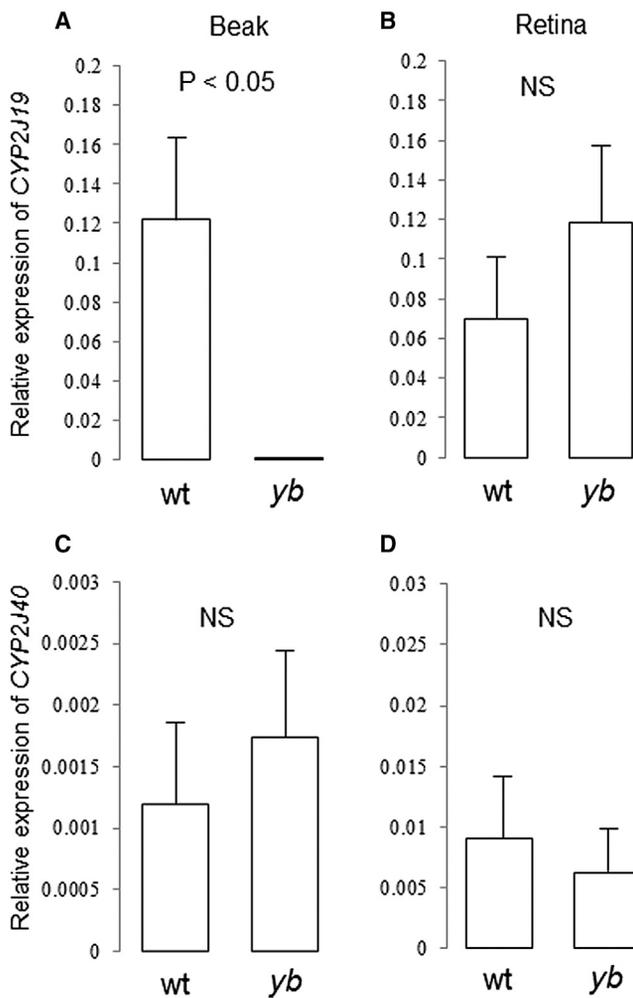
(B) Fine-scale mapping results. The top panel shows the two-point LOD scores, and the lower panel shows the recombination fraction between *yellowbeak* and 39 SNPs. The light-gray shaded rectangle spans the markers flanking the region where the recombination fraction with *yellowbeak* is zero. The dark-gray shaded region (24.269–25.029 Mb) indicates the most likely location of *yellowbeak*.

(C) Genome map from 24.269 to 25.029 Mb.

(D) *CYP2J2*-like cluster in wild-type and *yellowbeak*. A schematic diagram of the experimentally determined gene arrangement on chromosome 8 in wild-type and *yellowbeak* is shown (gene orientation is inverted relative to C). See also Figure S1.

significant differences in *CYP2J19B* expression in the bills of wild-type males and females (Figure S2D).

Taken together, our results strongly suggest that the *CYP2J2*-like cluster is required for red ketocarotenoid coloration. The pattern of expression of *CYP2J19* loci strongly implicates a role of *CYP2J19B* in red beak and tarsus pigmentation and suggests that *CYP2J19A* is probably involved in astaxanthin production in the retina, potentially in combination with *CYP2J19B*. Since *CYP2J40* has a fixed mutational difference in *yellowbeak*, we cannot rule out an additional role of this locus in coloration.



**Figure 4. Quantitative Real-Time RT-PCR Results in Wild-Type and *yellowbeak* Birds**

Expression is normalized relative to  $\beta$ -actin. Error bars indicate the SEM. The top panel shows results for *CYP2J19* loci using primers that amplify all copies (*CYP2J19A*, *CYP2J19B*, and *CYP2J19<sup>nb</sup>*) in the beak (A) and retina (B). The lower panel shows results for *CYP2J40* in the beak (C) and retina (D).  $n = 3$  wild-type and  $n = 3$  *yellowbeak* birds throughout. See also Figure S2.

Overall, the *CYP2J2*-like loci are strong candidates to encode the long-sought ketolase enzyme. These results concur with an independent study that has used introgression of red coloration into the canary to identify *CYP2J19* as a putative ketolase [23]. Direct experiments on the biochemical properties of the enzymes will be required to confirm this. Our results further demonstrate the utility of mutations in domesticated forms for identifying novel loci involved in evolutionarily relevant avian coloration [24, 25].

The specific expression of *CYP2J19* loci in the tissues where ketocarotenoids are deposited (beak, tarsus, and retina) provides strong circumstantial evidence that they play a direct role in ketocarotenoid presence in those tissues. The absence of *CYP2J19* expression in the liver of zebra finches is striking since the majority of *CYPs* are expressed there, where many of them act as detoxification enzymes. Our results therefore provide

clear support for the peripheral model of ketolase conversion in zebra finches, where ketolation occurs in the tissues where ketocarotenoids are deposited. Further, they suggest an intriguing genetic link between ketocarotenoids used for red coloration and those used for color vision. The presence of red oil droplets is thought to be conserved across birds [26], whereas red coloration is patchily distributed [13], so a role of *CYP2J2*-like loci in color vision is likely to have evolved prior to a function in coloration.

#### Ancestry of *yellowbeak* and *CYP2J19* Duplication

The presence of a derived indel polymorphism that only occurs on the *yellowbeak* haplotype and multiple fixed mutations in the *CYP2J2*-like cluster in *yellowbeak* together imply that *yellowbeak* is an old mutation that probably predates domestication. In a preliminary analysis to determine whether the *yellowbeak* allele is still segregating in the wild, we genotyped 50 wild zebra finches from a wild population and did not detect the *yellowbeak* deletion (data not shown). The apparent origin of the *yellowbeak* allele in the wild together with the presence of divergent alleles of *CYP2J19B* segregating in wild-type zebra finches suggests interesting evolutionary dynamics at this locus, perhaps including selection, that warrant further study. The contribution of other genetic variation at *CYP2J19B* to beak color variation is another interesting question. A quantitative trait locus study of beak redness in captive zebra finches did not find evidence for a quantitative trait locus on chromosome 8, although suggestive linkage to loci associated with beak color was detected on four other chromosomes [27].

Blast searches of available avian genomes, published studies on avian *CYPs* [28, 29] and our unpublished data from weaverbirds (Ploceidae) (H.T., unpublished data), which are closely related to estrildids, indicate that most other birds, from chickens to canaries, have a single *CYP2J19* locus. Hence, the duplication of *CYP2J19* in zebra finches into copies with their own tissue-specific expression is relatively recent. It will be interesting to determine when the duplication occurred in the avian phylogeny and whether it was associated with a change in color signaling.

#### Implications for Sexual Selection

A key issue in sexual selection research is the maintenance of honest signaling [30]. For carotenoid-based displays, much of the extensive literature has focused on the resource allocation hypothesis, the notion that it may be costly to irreversibly divert carotenoids to coloration from other physiological roles such as immune defense and antioxidant functions [31–33]. In other hypotheses, such as the “shared-pathway hypothesis,” some or all carotenoids may not themselves be limiting resources, but their uptake and metabolism, in particular ketocarotenoid derivation, may be intricately linked to crucial aspects of phenotypic and/or genetic quality, such as vitamin A homeostasis [34, 35] (but see 36) or respiratory efficiency [37]. Our finding that cytochrome P450s are involved in carotenoid metabolism brings an exciting new perspective to this field. The cytochrome P450 oxidase enzymes, present in all living organisms, are primary metabolizers of foreign substances. In vertebrates, they are best known as detoxification enzymes [38], sharing several basic requirements for their function, including protein cofactors

(cytochrome reductase and/or cytochrome  $b_5$ ) and an optimal cellular redox state. Many cytochrome P450s are expressed in the inner mitochondrial membrane [38], leading to a potential direct association between ketolation and respiration [35]. An alternative and more specific “honest signaling” hypothesis is that *CYP2J2*-based ketocarotenoid coloration is advertising some aspect of cytochrome P450 functionality in general (tissue-specific or systemic), such as detoxification ability.

#### ACCESSION NUMBERS

The new nucleotide sequences reported here have been submitted to GenBank under accession numbers KX024636–KX024638, KX184728, and KX184729.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.04.047>.

#### AUTHOR CONTRIBUTIONS

N.I.M., J. Stapley, J. Slate, S.A., T.B., and T.R.B. designed the experiments; N.I.M., J. Stapley, S.A., C.B., R.T., and H.T. obtained the data; N.I.M., J. Stapley, J. Slate, S.A., and K.-W.K. analyzed the data; N.I.M., S.A., J. Stapley, and J. Slate drafted the manuscript; and all authors verified the final version of the manuscript.

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