BBA - Molecular and Cell Biology of Lipids 1870 (2025) 159654

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

journal homepage: www.elsevier.com/locate/bbalip

Substrates, intermediates, and products of avian ketocarotenoid metabolism☆

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ARTICLE INFO

Keywords:
Carotenoid
Bird
Coloration
Cytochrome P450
Short-chain dehydrogenase/reductase
CYP2J19
BDH1L
Ketocarotenoids
Red

ABSTRACT

Carotenoid-based coloration is an essential feature of avian diversity and has important roles in communication and mate choice. The red feathers of birds from phylogenetically diverse orders and families are pigmented with C4-ketocarotenoids produced via the successive action of Cytochrome P450 2J19 (CYP2J19) and 3-hydroxybutyrate dehydrogenase 1-like (BDH1L) on yellow dietary precursors. Yet, the biochemistry of these enzymes remains incompletely understood. Here we present a series of experiments characterizing the substrates, intermediates, and products of CYP2J19 and BDH1L expressed in heterologous cell culture. We confirm that CYP2J19 preferentially hydroxylates the 4 and 4′ positions of β-ring substrates, but can also hydroxylate the 3 and 3 positions of C4-ketocarotenoids. We confirm that BDH1L catalyzes the conversion of zeaxanthin to canary xanthophyll B (ε,ε'-carotene-3,3′-dione) a major pigment in plumage of many yellow bird species. These results suggest that the actions of CYP2J19 and/or BDH1L can explain the presence of many metabolically transformed carotenoids in avian tissues.

1. Introduction

Elaborate carotenoid-based coloration is an important aspect of avian diversity and members of at least 95 extant families of birds color their feathers with carotenoids [1–3]. In some taxa, much of the genetic differentiation among species is associated with the genes mediating carotenoid-based color expression [4–6]. These colors play a variety of important roles in the lives of birds including crypsis [7], species recognition [8], offspring-parent communication [9], and intraspecific competition [10–12]. However, the function of carotenoid-based colors in avian mate choice and their evolution through sexual selection has received the greatest attention. In many species, carotenoid-based coloration is sexually dimorphic and drab females select mates by assessing their colorful plumes and patches [13–16]. A major unresolved question in avian evolutionary biology is why these preferences for carotenoid-based colors have evolved and how they are maintained over time.

Birds cannot synthesize carotenoids *de novo* and must acquire them through their diet [17]. This observation has led to the hypothesis that the expression of carotenoid-based coloration is limited by, and communicates information about, diet and the allocation of carotenoids to other physiological functions like immune function and antioxidant defense [18–20]. However, recent studies have questioned whether carotenoids are limited in the diet and such trade-offs in signal function are important [21–23]. Hill and colleagues have proposed the shared pathway hypothesis, positing that carotenoid-based coloration communicates reliable information about individual quality and condition because the mechanisms of color expression are inextricably linked to core cellular processes [22,24,25]. A deeper understanding of the biochemistry and biology of carotenoid-based coloration mechanisms is essential to devise definitive tests of these hypotheses.

Although birds cannot synthesize carotenoids *de novo*, they can

☆ This article is part of a Special issue entitled: 'Carotenoid molecular' published in BBA - Molecular and Cell Biology of Lipids.

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https://doi.org/10.1016/j.bbalip.2025.159654
Received 22 April 2025; Received in revised form 8 June 2025; Accepted 24 June 2025
Available online 27 June 2025

metabolize and modify dietary precursors into a variety of forms. Terrestrial birds primarily consume lutein, zeaxanthin and β-carotene, and the direct deposition of these pigments usually produces yellow coloration [17,26]. In many species, red coloration is produced through the addition of ketone groups at the 4 and 4′ positions of the β-ring of these dietary carotenoids to generate C4-ketocarotenoids. Additionally, yellow coloration may include metabolically modified carotenoids like canary xanthophylls [26,27]. Metabolically transformed carotenoids also have an important role in avian vision, as they are deposited in cone oil droplets in the retina where they spectrally filter incoming light to enhance color vision [28–30]. Meta-analysis of multiple studies of color condition-dependence suggests that expression of colors with these metabolically modified carotenoids are especially reliable indicators of individual quality [31]. Thus, these mechanisms of carotenoid transformation may be particularly sensitive to physiological perturbations and there is great interest in understanding the biochemical mechanisms underlying these processes.

For more than four decades biochemists and ornithologists have hypothesized the enzymatic mechanisms of carotenoid transformations in birds. Davies [32] was the first to propose that dietary xanthophylls (zeaxanthin) were the precursors of C4-ketocarotenoids (astaxanthin) in the avian retina and this hypothesis was supported by subsequent tracing studies [33–35]. Similarly, Stradi [36] suggested that canary xanthophylls, first characterized in the yellow feathers of cardueline finches, were derived from dietary xanthophylls (lutein and zeaxanthin). Advances in analytical capabilities have revealed an expanding diversity of carotenoid types in the colorful tissues of birds, leading to the proposal of increasingly complex metabolic mechanisms [2,37,38]. Yet, direct demonstrations of the specific enzymatic pathways that produce this diversity and their precursor-intermediate-product relationships remains incomplete.

In the past decade, comparative genomic analyses of color variant subspecies, morphs, and mutants of domesticated and wild bird species have begun to reveal the enzymatic basis of carotenoid transformations. Studies of yellow and red variants of domesticated canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*) identified the enzyme cytochrome P450 2J19 (CYP2J19) as a key mediator of red C4-ketocarotenoid-based coloration [39,40]. Subsequent studies have implicated CYP2J19 in the red coloration of northern flicker (*Colaptes auratus*) [41], tinkerbird (*Pogoniulus* Sp.) [42], long-tailed finch (*Poephila acuticauda*) [43], diamond firetail (*Stagonopleura guttata*) and the star finch (*Bathilda ruficauda*) [27] suggesting that this mechanism is phylogenetically widespread among birds. However, CYP2J19 alone is not sufficient to catalyze the transformation of yellow dietary carotenoids to C4-ketocarotenoids in heterologous cell culture [27]. Recently, it was discovered that biosynthesis of C4-ketocarotenoids requires a second enzyme, the short-chain dehydrogenase reductase, 3-hydroxybutyrate dehydrogenase 1-like (BDH1L) [27]. Additionally, when BDH1L acts alone, it catalyzes the conversion of yellow dietary carotenoids to pigments consistent with yellow canary xanthophylls found in the feathers of many yellow bird species [27]. Thus, the CYP2J19 and BDH1L—acting individually or in concert—may be a key mechanism for producing many of the metabolically modified carotenoids found in the integument of birds.

Several aspects of CYP2J19/BDH1L-mediated carotenoid metabolism remain unresolved (Fig. 1). Our analyses indicate that the transformation of yellow dietary carotenoid substrates (e.g. zeaxanthin) to C4-ketocarotenoids (e.g. astaxanthin) involves CYP2J19 acting first to produce an intermediate which is then oxidized by BDH1L to produce a ketocarotenoid. The intermediate made by CYP2J19 has not been definitively identified; however, we have observed that this intermediate has increased polarity compared to the substrate carotenoid but an identical UV–Vis spectrum [27]. We hypothesize that CYP2J19 catalyzes the 4,4′-hydroxylation of the substrate molecules, and here we test this hypothesis with expanded functional assays and comparison to 4,4′-hydroxylated standards. We have evidence that BDH1L acting alone on dietary substrates yields products that match the metabolized yellow carotenoids found in the feathers of finches and other bird species [27]. However, questions remain about the precise identity of these products as well. Initial analyses suggested that BDH1L acting on zeaxanthin yields isoastaxanthin (4,4′-dihydroxy-ε,ε-carotene-3,3′-dione) [27]. This compound was first described by Schiedt et al. [34] and was later observed by Stradi et al. [36] in bird feathers and given the name canary xanthophyll D. Yet, canary xanthophyll D is only a minor component of the feather pigment profile and the BDH1L product also has chromatographic and spectral properties consistent with the more abundant canary xanthophyll B (ε,ε-carotene-3,3′-dione) [36]. Here we resolve this ambiguity with expanded analyses and comparison to a validated canary

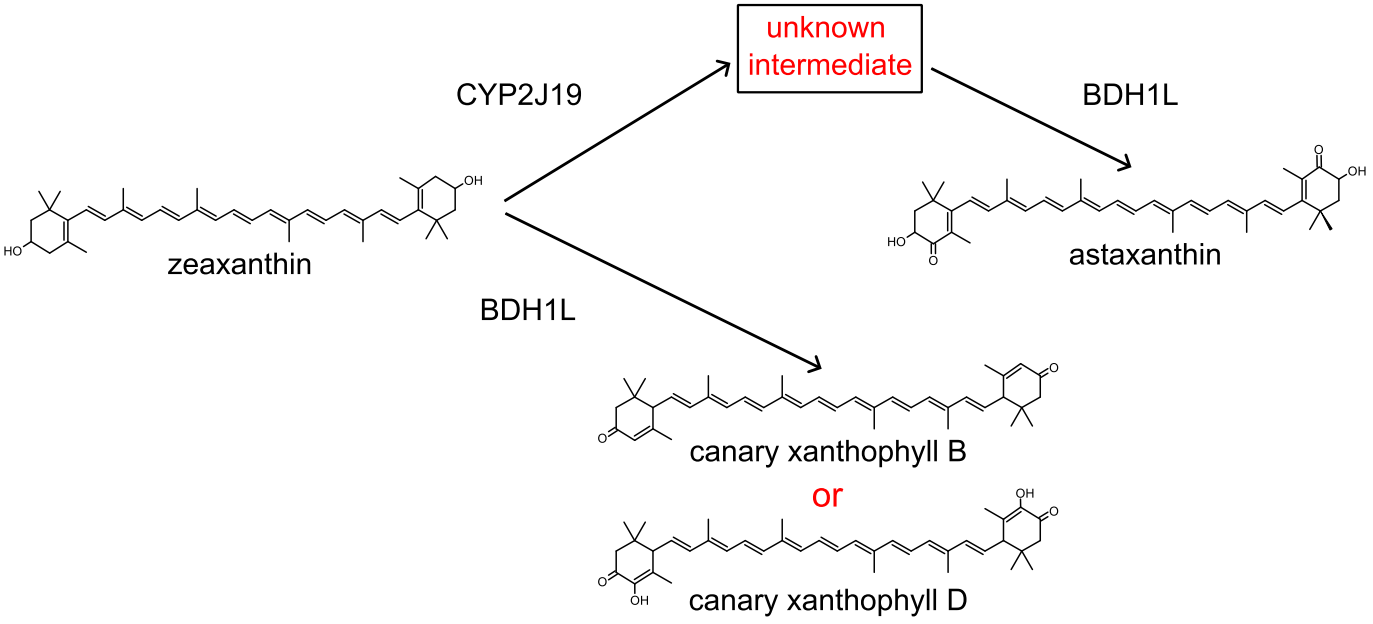

Fig. 1. Two unresolved questions in avian plumage carotenoid metabolism. CYP2J19 and BDH1L together catalyze the sequential oxidation of dietary carotenoid substrates (e.g. zeaxanthin) to C4-ketocarotenoids (e.g. astaxanthin), but the precise identity of the CYP2J19 product has not been determined. BDH1L alone catalyzes the conversion of β-ring substrates (e.g. zeaxanthin) to ε-ring products, but there is uncertainty about the identity of these products.

xanthophyll B (ε,ε-carotene-3,3′-dione) standard. Finally, we tested CYP2J19 on an expanded range of substrates and demonstrate that this enzyme can catalyze the 3,3′-hydroxylation of 4,4′-ketocarotenoids.

2. Materials and methods

2.1. Ethics statement and animal handling procedures

Northern cardinal (*Cardinalis cardinalis*) feathers were sampled from a specimen in the University of Tulsa collection (USGS permit #20948 to C. Brown). Yellow canary (*Serinus canarius*) feathers were collected from captive domesticated birds in an earlier study [39]. Mice were sampled under a protocol approved by Case Western Reserve University's Institutional Animal Care and Use Committee (protocol #2014–0106) and adhered to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The generation of the *Isx*$^{-/-}$; *Bco2*$^{-/-}$ mutant mice, housing conditions, diet formulations, and euthanasia are described in Thomas et al. [44].

2.2. Carotenoid sources and synthesis

Zeaxanthin (Optisharp, 5003563004), lutein (Floraglo, 5011868022), β-carotene (0489999004), and canthaxanthin (5005256004) were gifts from dsm-firmenich (Stroe, Netherlands). We fractioned these carotenoid preparations by HPLC (conditions detailed below), collected, and purified the all-*trans* isomer of each for further experimentation and analysis. We generated crustaxanthin (β,β-carotene 3,4,3′,4′-tetrol), β,ε-carotene 3,4,3′-triol and isozeaxanthin (β,β-carotene 4,4′-tetrol) by reducing astaxanthin, 3,3′-dihydroxy-β,ε-carotene-4-one (α-doradexanthin), and canthaxanthin, respectively. We extracted and fractioned (3*S*,3′*S*)-astaxanthin from a culture of *Haematococcus pluvialis* following the methods described in [45] and purified canthaxanthin as described above. We purified 3,3′-dihydroxy-β,ε-carotene-4-one (putative α-doradexanthin) from the red feathers of northern cardinal following published protocols [26]. The stereochemical designation of 3,3′-dihydroxy-β,ε-carotene-4-one as α-doradexanthin ((3*S*,3′*S*,6′*R*)- 3,3′-dihydroxy-β,ε-carotene-4-one) is based on earlier descriptions of northern cardinal feather composition [46,47]; however we did not directly verify this configuration. We reduced ketocarotenoids by dissolving each in 1 mL of ethanol containing approximately 1 mg of sodium borohydride ($NaBH_4$; Sigma-Aldrich, 480886), capping under nitrogen gas and incubating 30 min at room temperature in the dark [48]. We extracted the reduced products by adding 2 mL of 0.9 % NaCl in water, 1 mL of hexane, mixing, centrifuging 5000 *g*, and collecting and drying the organic fraction under a stream of nitrogen.

2.3. Assays of enzyme function in cell culture

To investigate the substrates and products of avian CYP2J19 and BDH1L, we expressed these enzymes alone or in combination in HEK293 cells (ATTC, CRL-1573) and assayed function by delivering carotenoid substrates via the culture media. We cloned the coding sequences of the house finch (*Haemorhous mexicanus*) homologs of *CYP2J19, BDH1L,* and *TTC39B* into the pCAG expression vector with an in-frame fluorescent tag, cultured cells, and transfected as described in Koch et al. [49]. *TTC39B* has been shown to enhance the activity of CYP2J19 and BDH1L [27,49]; therefore, we co-expressed *TTC39B* along with our enzyme(s) of interest in each of our assays. As a negative control, we transfected cells with the same expression vector containing only the coding sequences of fluorescent proteins (*GFP* and *dsRed*). We incubate transfected cells for 24–48 h and confirmed gene expression by visualizing expression of the fluorescent protein. We then exchanged the culture media for media enriched with one of the carotenoid substrates described above. We solubilized carotenoid substrates in culture media with 0.035 % (w:v) polysorbate 40 (Acros Organics, AC334142500). We incubated the cultures an additional 16–20 h, then collected the experimental cells via centrifugation, washed with phosphate-buffered saline, and stored at −80 °C until further analysis. Additional methodological details are available in Koch et al. [49].

2.4. Carotenoid extraction and analysis

We extracted carotenoids from cultured cells, mouse liver tissue, and feathers as described previously [49]. Briefly, we homogenized cells and liver tissue in 500 μL of 0.9 % NaCl on beadmill (Benchmark Science, Beadbug) at 4 kHz for 30 s, then mixed with 250 μL of ethanol and 500 μL of hexane:tert-butyl methyl ether (1:1, vol:vol; hexane:MTBE) by vortexing. We extracted feather carotenoids by grinding in 1 mL of methanol on the beadmill at 4 kHz for 5 min. For all samples, we centrifuged the homogenates at 10,000 *g* for 3 min, collected the solvent fraction, and dried under a stream of nitrogen.

We compared enzyme products, tissue extracts, and carotenoid standards by high-perfomance liquid chromatography (HPLC) with UV–Vis detection. We dissolved extracts and standards in 120 μL of acetonitrile:methanol:dichloromethane (44:44:12, vol:vol:vol) and injected 100 μL into an Agilent 1200 series HPLC fitted with a C30 YMC carotenoid column (YMC, CT99S05-2546WT). We used a gradient mobile phase beginning with acetonitrile:methanol:dichloromethane (44:44:12) for 11 min, a gradient up to 35:35:30 from 11 to 21 min, and continuing at 35:35:30 until 35 min. We pumped mobile phase solvent at a constant rate of 1.2 mL/min and maintained the column at 30 °C. We monitored the samples with a UV–Vis photodiode array detector at 445 or 480 nm wavelength.

To characterize the stereoisomers of ε,ε-carotene-3,3′-dione we first fractioned the samples by reverse-phase HPLC as described above and collected the putative ε,ε-carotene-3,3′-dione peak from each sample. We then separated and compared these purified peaks with a Daicel Chiralpak IA-3 column (Chiral Technologies) and a mobile phase of hexane:isopropanol (90:10, vol:vol). We set the mobile phase flow rate of 0.5 mL/min, the column temperature to 25 °C, and we monitored the samples at 445 nm wavelength.

3. Results

3.1. CYP2J19 hydroxylates the 4 and 4′ positions of carotenoid β-rings

CYP2J19 catalyzes the first step in the conversion of yellow dietary precursor carotenoids (e.g. zeaxanthin) to red C4-ketocarotenoids (e.g. astaxanthin) generating an oxidized intermediate that is further oxidized to a ketocarotenoid by BDH1L (Fig. 1). We previously hypothesized that these intermediates might be C4-hydroxylated carotenoids [27]. To test this hypothesis, we first generated the C4-hydroxylated carotenoids standards crustaxanthin (β,β-carotene 3,4,3′,4′-tetrol) and β,ε-carotene 3,4,3′-triol, by reducing the C4-ketocarotenoids, astaxanthin and α-doradexanthin with $NaBH_4$, respectively. We then compared these standards with the products of *CYP2J19* expressed in HEK293 cells and provided with zeaxanthin or lutein as substrates (Fig. 2a,d). Reducing (3*S*,3′*S*)-astaxanthin yielded three products that were separable with our chromatography system. Buschor and Eugster [50] report that the reduction of (3*S*,3′*S*)-astaxanthin yields 3,4-*trans*, 3′,4′-*trans*-crustaxanthin, 3,4-*cis*, 3′,4′-*trans*-crustaxanthin, and 3,4-*cis*, 3′,4′-*cis*-crustaxanthin in an ~8:12:5 ratio. Accordingly, based on relative retention times, relative abundance, and UV–Vis spectra we putatively identified our three products as 3,4-*trans*, 3′,4′-*trans*-crustaxanthin, 3,4-*cis*, 3′,4′-*trans*-crustaxanthin, and 3,4-*cis*, 3′,4′-*cis*-crustaxanthin. The retention times and UV–Vis spectrum of presumed 3,4-*cis*, 3′,4′-*cis*-crustaxanthin match the product of CYP2J19 with a zeaxanthin substrate and these two compounds co-elute in our chromatography conditions (Fig. 2b,c). Similarly, the reduction of α-doradexanthin with $NaBH_4$ is reported to yield optical isomers of β,ε-carotene-3,4,3′-triol [51]. Reduction of the α-doradexanthin we

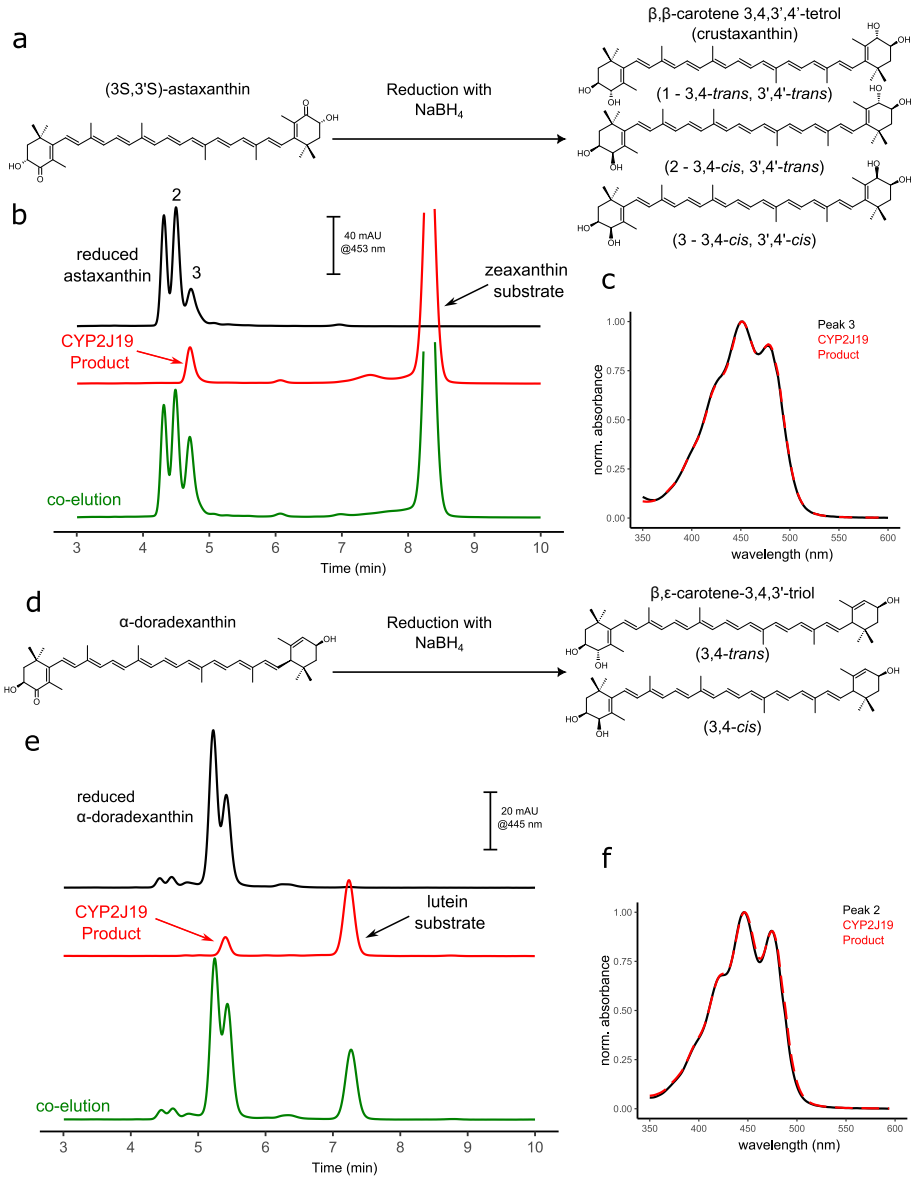

Fig. 2. CYP2J19 catalyzes the oxidation of zeaxanthin to β,β-carotene 3,4,3′,4′-tetrol (crustaxanthin) and lutein to β,ε-carotene 3,4,3′-triol. a) The reduction of astaxanthin with $NaBH_4$ yields three optical isomers of crustaxanthin. b) Top trace - Representative C30 reverse-phase HPLC chromatograms of the crustaxantin isomers. The peaks are numbered with the putative isomer identifications based on retention times and relative abundance, middle trace - the product of CYP2J19-catalyzed oxidation of a zeaxanthin substrate in cell culture, bottom trace - and co-elution of crustaxanthin isomers with the CYP2J19 product. c) The overlaid UV–Vis spectra of *cis,cis*-crustaxanthin (peak 3 - black) and the CYP2J19 product (red). d) The reduction of α-doradexanthin with $NaBH_4$ yields two optical isomers of β,ε-carotene 3,4,3′-triol. e) Top trace - Representative C30 reverse-phase HPLC chromatogram of the β,ε-carotene 3,4,3′-triol isomers. The peaks are numbered with the putative isomer identifications based on retention times and relative abundance, middle trace - the product of CYP2J19-catalyzed oxidation of a lutein substrate in cell culture, bottom trace - and co-elution of crustaxanthin isomers with the CYP2J19 product. f) The overlaid UV–Vis spectra of *cis*- β,ε-carotene 3,4,3′-triol (peak 2 - black) and the CYP2J19 product (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isolated from northern cardinal feathers yields two products with identical UV–Vis spectra (Fig. 2e,f). The retention time and UV–Vis spectrum of one of these β,ε-carotene-3,4,3′-triol isomers match the product of CYP2J19 with a lutein substrate, and both compounds co-elute in our chromatography conditions (Fig. 2e,f).

Finally, we provided HEK293 cells expressing *CYP2J19* with a β-carotene substrate and observed three products that are more polar than β-carotene (Fig. 3b). The earliest eluting peak (peak 1) has a retention time and UV–vis spectrum consistent with β,β-carotene-4,4′-diol (isozeaxanthin) that we produced through the reduction of canthaxanthin (Fig. 3a-c). The second eluting peak (peak 2) has a retention time and UV–vis spectrum consistent with 4′-hydroxy-β,β-caroten-4-one (4-hydroxyechinenone) (Fig. 3a-c). The presence of small amounts of this C4-ketocarotenoid, in the absence of *BDH1L* expression, may be attributable to endogenous short-chain dehydrogenase/reductase enzymes expressed in the HEK293 cells. Peak 3 elutes much later than the other products and we hypothesize that this is β,β-caroten-4-ol (isocryptoxanthin), though we did not have an authentic sample of this compound available for comparison. Taken together, our evidence suggests that CYP2J19 preferentially catalyzes hydroxylation at the 4,4′ position of carotenoid β-rings.

3.2. *CYP2J19 hydroxylates the 3 and 3′ positions of 4,4′-ketocarotenoids*

Co-expressing *CYP2J19* and *BDH1L* in HEK293 cells and providing a β-carotene substrate yields multiple ketocarotenoid products including

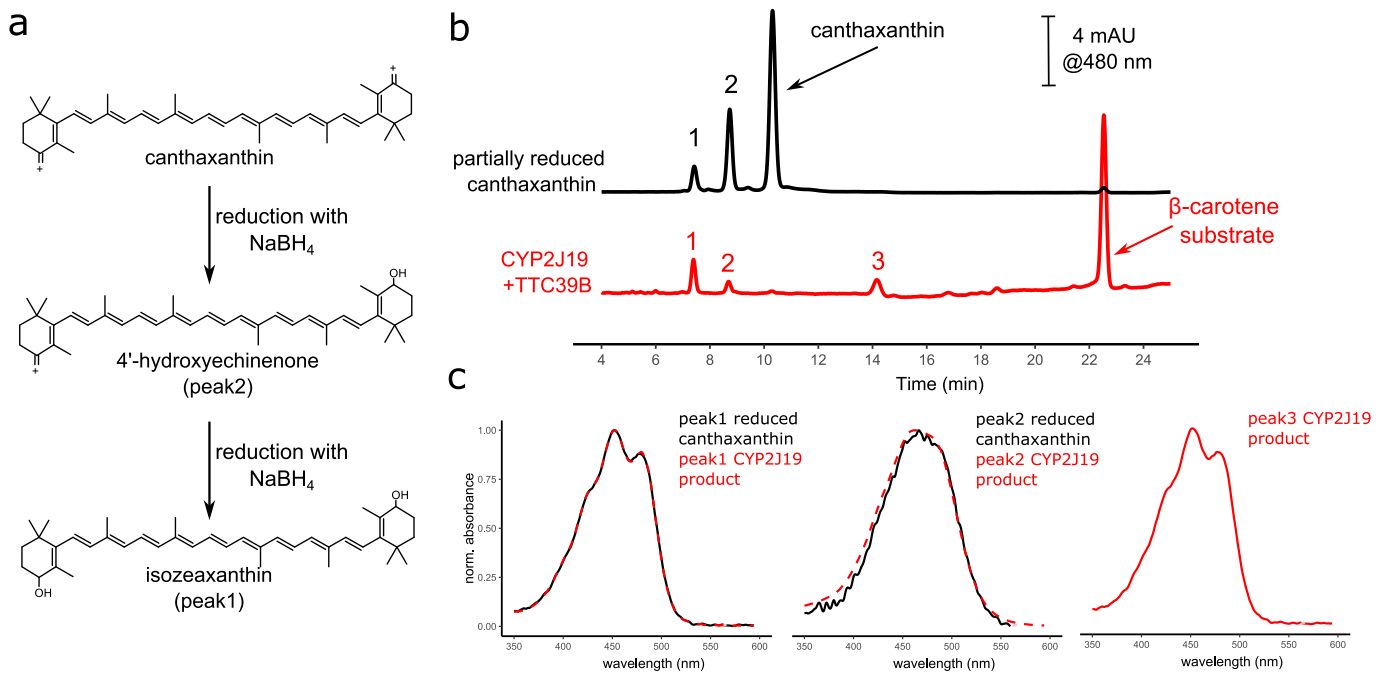

Fig. 3. CYP2J19 catalyzes the addition of hydroxyl groups at the 4 and 4′-positions of β-carotene. a) The partial reduction of canthaxanthin with $NaBH_4$ yields 4′-hydroxyechinenone and complete reduction yields isozeaxanthin. b) Top trace - Representative C30 reverse-phase HPLC chromatograms of the canthaxanthin reduction products. Bottom trace - the product of CYP2J19-catalyzed oxidation of a β-carotene substrate in cell culture. c) The UV–Vis spectra of the three CYP2J19-β-carotene products (red) overlaid on the UV–Vis spectra of the corresponding canthaxanthin reduction products (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

canthaxanthin and astaxanthin (Fig. 4) This suggests that these enzymes not only catalyze C4-keto group addition but may also catalyze C3-hydroxylation of C4-ketocarotenoids. To test this hypothesis, we expressed *CYP2J19* alone and supplied the cells with a canthaxanthin substrate. We observed two products that were more polar than canthaxanthin (Fig. 5). Peak 1 has a retention time and UV–Vis spectra identical to astaxanthin, indicating that CYP2J19 catalyzes the addition of hydroxyl groups at the 3 and 3′ positions (Fig. 5). We hypothesize that peak 2 is most likely the product of a single hydroxylation event, adonirubin (3-Hydroxy-β,β-carotene-4,4′-dione). However, we did not have an authentic standard available to verify this inference (Fig. 5).

3.3. BDH1L catalyzes the conversion of zeaxanthin to ε,ε-carotene 3,3′-dione

The enzyme BDH1L catalyzes the conversion of zeaxanthin a β,β-carotenoid, to an ε,ε-carotenoid that is a major component of the feather carotenoids of a diversity of bird species [17,27,52] (Fig. 1). However, there are conflicting reports about the precise identity of this ε,ε-carotenoid. Toomey et al. [27] suggested that this pigment was canary xanthophyll D (4,4′-dihydroxy-ε,ε-carotene-3,3′-dione), contradicting previous studies of feather pigments that identified this compound as canary xanthophyll B (ε,ε-carotene-3,3′-dione) [27,36]. To resolve this conflict, we harvested liver tissue from zeaxanthin-fed *Isx*$^{-/-}$;*Bco2*$^{-/-}$ mutant mice that are known to accumulate ε,ε-carotene-3,3′-dione [44]. The major carotenoid from liver extracts of these mice matches the retention time and UV–Vis absorbance of the product of BDH1L acting upon zeaxanthin as well as that of one of the two major carotenoids extracted from canary feather (Fig. 6b,c). Next, we fractioned the corresponding peak from each sample, combined them in a single run, and found that all three co-elute (Fig. 6d). Finally, we separated stereoisomers of the fractioned peaks by normal-phase chiral chromatography and observed two peaks in similar proportions for all three samples (Fig. 6e). Taken together, these results confirm that the BDH1L catalyzes the conversion of zeaxanthin to ε,ε-carotene-3,3′-dione (canary xanthophyll B).

4. Discussion

Carotenoid-based ornaments, especially the red coloration of feathers and beaks, play important roles in avian social behavior and have been shaped by powerful forces of sexual and natural selection [53–55]. These colorful traits present an exciting opportunity to integrate molecular and biochemical mechanisms to understand macroevolutionary processes. Here we resolve several outstanding questions regarding the enzymatic mechanisms producing metabolically derived yellow and red carotenoid (Fig. 7). First, we find that CYP2J19 preferentially catalyzes the 4,4′-hydroxylation of the β-rings of common dietary carotenoids, generating intermediates (e.g. crustaxanthin and isozeaxanthin) that can then be further oxidized to C4-ketocarotenoids (e.g. astaxanthin and canthaxanthin) by BDH1L. Second, we show that CYP2J19 catalyzes the 3,3′-hydroxylation of C4-ketocarotneoid substrates. Finally, we find that BDH1L alone is sufficient to catalyze the yellow-to-yellow conversion of zeaxanthin into ε,ε'-carotene-3,3′-dione (canary xanthophyll B) and confirm earlier characterizations of this metabolized yellow carotenoid in avian plumage [53–55].

CYP2J19 catalyzes the hydroxylation of the 4,4′ position of carotenoid β-rings to yield a single optical isomer (e.g. 3,4-*cis*, 3′,4′-*cis*-crustaxanthin) as expected for an enzyme-mediated process. These products are an important intermediate in the production of C4-ketocarotenoids and red color expression, yet they have never been reported in the carotenoid-pigmented tissues of birds [49]. Why are these compounds missing from avian carotenoid profiles? One possibility is that these compounds are rapidly oxidized to ketocarotenoids by BDH1L, which appears to be widely expressed in avian tissues [49]. BDH1L may mediate essential functions other than carotenoid metabolism, as we have never observed a viable BDH1L loss-of-function among numerous red-to-yellow color mutants we have investigated [56]. Alternatively, the increased polarity of 4,4′-hydroxycarotenoids may facilitate their rapid degradation or elimination. For example, tracing studies in rats

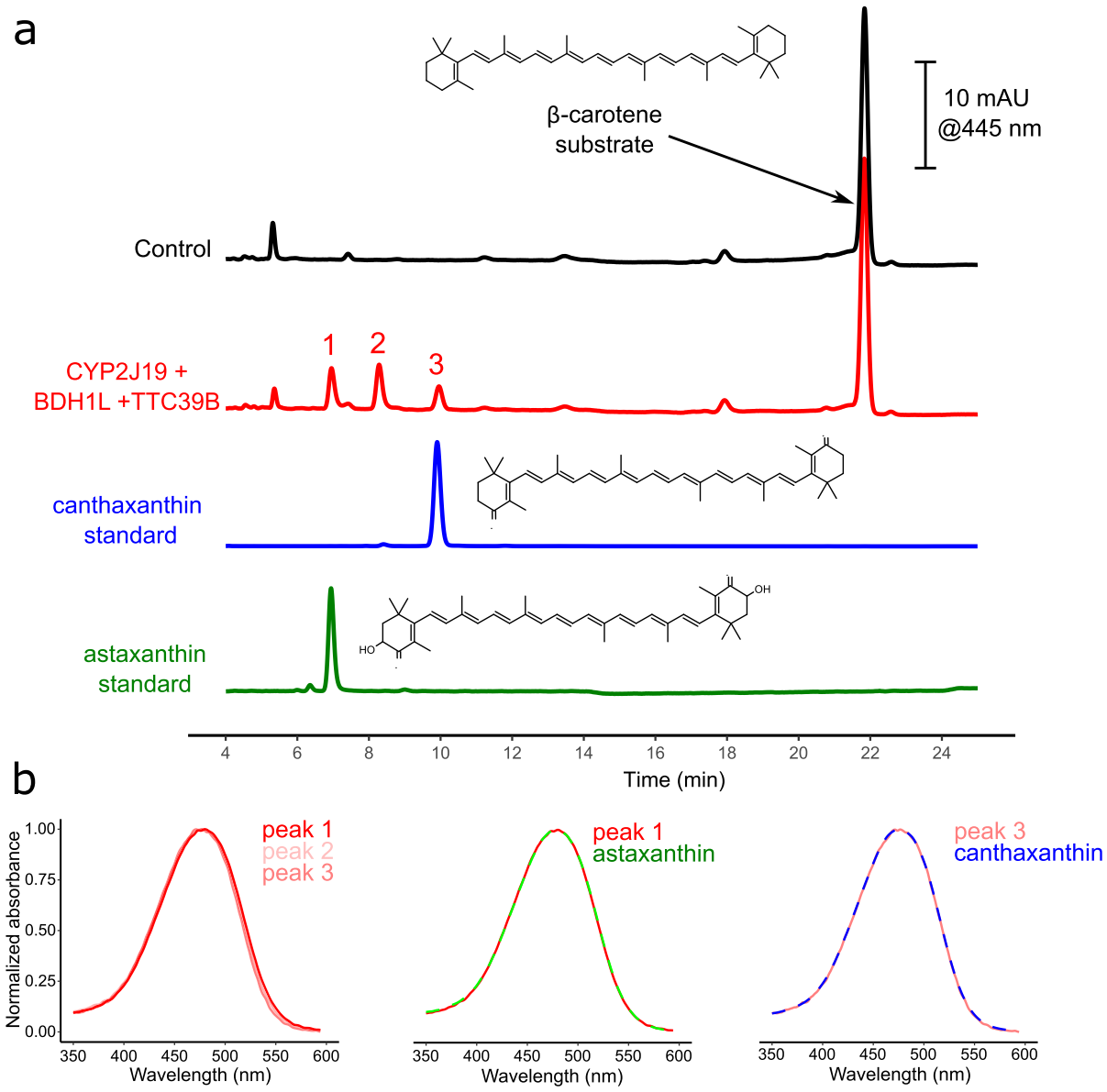

Fig. 4. CYP2J19 and BDH1L together with a β-carotene substrate, yield various products differing in 3 and 3′ hydroxylation. a) Top trace - Representative C30 reverse-phase HPLC chromatograms of the control cell culture supplemented with β-carotene. Top middle trace - the products of CYP2J19-BDH1L catalyzed oxidation of a β-carotene substrate in cell culture, bottom middle trace - the canthaxanthin standard, bottom trace - the astaxanthin standard. b) UV–Vis spectra of the three CYP2J19-BDH1L products (left), the peak 1 overlaid on the astaxanthin spectrum (middle), and the peak 3 spectrum overlaid on the canthaxanthin spectrum (right). We hypothesize that peak 2 is adonirubin (3-Hydroxy-β,β-carotene-4.4′-dione), however we did not have a standard available to confirm this identification.

indicate that exogenous crustaxanthin is rapidly modified and excreted in urine and crustaxanthin is noted to be rapidly destroyed under acidic and alkaline conditions [57]. This may explain the absence of crustaxanthin from avian retinal carotenoid profiles, where sample preparation procedures typically involve alkaline saponification that may lead to selective degradation and loss [57].

Our evidence suggests that CYP2J19 and BDH1L do not act upon ε-rings. When provided with a lutein substrate, CYP2J19 yields β,ε-carotene 3,4,3′-triol (this study), CYP2J19/BDH1L yields 3,3′-dihydroxy-β,ε-carotene-4-one (putatively α-doradexanthin), and BDH1L alone yields a product consistent with canary xanthophyll A (3′-hydroxy-ε,ε-caroten-3-one) [27]. In each case, the ε-ring of the lutein substrate remains unchanged. This may reflect conformational selectivity of the active sites of the enzymes. Modeling of conformational energies indicates that the lowest energy conformation of ε-ring is approximately perpendicular to the conjugated chain, C3-C6-C7 bond angle ~120°, while the β-ring favors a nearly parallel conformation, C3-C6-C7 bond angle ~170° [58,59]. This "'hoe-like' perpendicular conformation of the ε-ring may limit entry to the active sites of CYP2J19 and BDH1L. Thus, the presence of carotenoids like 3-dehydrolutein and papilioerythrinone, which are major components of the plumage of Gouldian finches (*Erythrura gouldiae*), for example, cannot be explained by the actions of CYP2J19 and BDH1L alone (Fig. 7). The presumed direct oxidation of ε-ring groups is likely mediated via a separate, yet-to-be-described mechanism (Fig. 7).

We have observed that CYP2J19 not only catalyzes the hydroxylation of the 4 and 4′ positions of β-rings as previously reported [27,39,52], but also catalyzes the addition of hydroxyl groups at the 3 and 3′ positions of C4 ketocarotenoids (i.e. canthaxanthin). When acting upon a β-carotene substrate, CYP2J19 and BDH1L together produce canthaxanthin, and CYP2J19 can then further oxidize this product to adonirubin and astaxanthin (Fig. 7). The relative abundance of these three ketocarotenoid products vary in the red feathers of different bird species, raising the question of how the balance among these

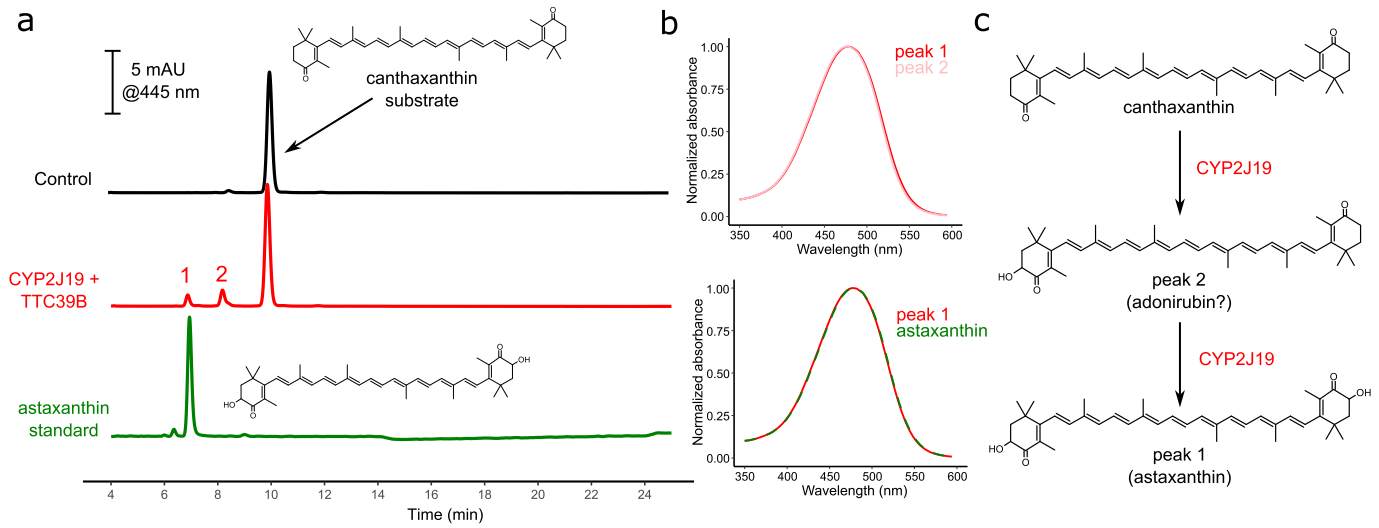

Fig. 5. CYP2J19 catalyzes the addition of hydroxyl groups at the 3 and 3′-positions of canthaxanthin. a) Top trace - Representative C30 reverse-phase HPLC chromatograms of the control cell culture supplemented with canthaxanthin. Middle trace - the products of CYP2J19 catalyzed oxidation of a canthaxanthin substrate in cell culture, bottom trace - astaxanthin standard. b) UV–Vis spectra of the two CYP2J19 products (top) and peak 1 overlaid on the astaxanthin spectrum (bottom). c) We hypothesize that adonirubin is generated as an intermediate of the CYP2J19 catalyzed oxidation of canthaxanthin to astaxanthin.

intermediates and products is determined. For example, red-factor canaries rely on CYP2J19 and BDH1L to pigment their red feathers by primarily accumulating canthaxanthin with only trace amounts of astaxanthin being produced [27–29,60]. Thus, there must be some mechanism that limits the further oxidation of canthaxanthin by CYP2J19. One possibility is that the affinity of CYP2J19 for canthaxanthin is much lower than β-carotene and an abundance of β-carotene during the development of feather pigmentation limits further 3,3′ oxidation. Alternatively, there might be mechanisms of transport and partitioning that rapidly remove canthaxanthin from the site of enzyme action.

In the avian retina, CYP2J19 and BDH1L mediate the production and accumulation of astaxanthin in the oil droplet of the red cone photoreceptor [27–29,60]. Given the broad range of substrates and products of CYP2J19 and BDH1L detailed in this study and elsewhere, the nearly exclusive accumulation of astaxanthin in red cones is surprising. Terrestrial birds typically circulate lutein, zeaxanthin, and β-carotene in their bloodstream [17]. We have demonstrated CYP2J19 and BDH1L-mediated astaxanthin production from both zeaxanthin and β-carotene substrates, but a lutein substrate yields 3,3′-dihydroxy-β,ε-carotene-4-one (putatively α-doradexanthin), a carotenoid that has not been reported in the avian retina. These observations suggest that substrate carotenoid uptake by the red cone photoreceptor may selectively exclude lutein. Consistent with such selectivity, dietary tracing studies indicate that zeaxanthin is the major precursor of astaxanthin in the avian retina [33–35,61]. These mechanisms of selective uptake remain to be determined.

Many bird species accumulate asymmetrically oxidized β,β-carotenoid products, like echinenone and 3-hydroxyechinenone in their red plumage [27,49]. Yet, we have never observed these asymmetric C4-ketocarotenoids among the products of CYP2J19- and/or BDH1L-mediated reactions, regardless of the substrate supplied [49]. We therefore believe it is unlikely that CYP2J19/BDH1L are the primary enzymes producing these ketocarotenoids. Consistent with this notion, we have found that molting house finches (*Haemorhous mexicanus*), a species that primarily accumulates 3-hydroxyechinenone in its red feathers, express little or no *CYP2J19* in their developing feathers or liver [49]. Therefore, it is likely that house finches and other species that accumulate these asymmetric C4-ketocarotenoids rely on a different enzymatic mechanism (Fig. 7). Such asymmetrically acting enzymes have been described in cyanobacteria; though there are no obvious homologs among birds [62,63].

We have confirmed that BDH1L alone converts zeaxanthin into ε,ε-carotene-3,3′-dione (canary xanthophyll B), consistent with earlier studies of avian plumage carotenoid composition [36,53–55]. This contrasts with our previous report [27] that suggested this pigment was 4,4′-dihydroxy-ε,ε-carotene-3,3′-dione (canary xanthophyll D) based on the observation of a 597 *m/z* ion in the mass spectrometry spectrum of this compound. However, the 597 m/z ion was a relatively minor component of the spectrum, and by far the dominant ion was 565 m/z, which is consistent with the ε,ε-carotene-3,3′-dione (canary xanthophyll B) reported by others [36,44]. The higher molecular weight component of our earlier analyses may have resulted from incomplete separation of compounds in our LC system or some unexpected adduct formation during ionization.

Canary xanthophylls have been reported in the plumage of diverse bird species and several lines of evidence suggest that these pigments may have functions distinct from the common dietary yellow carotenoids (e.g. lutein and zeaxanthin). Ancestral reconstruction of the feather carotenoid composition of true finches (Fringillidae) indicates that species with yellow canary xanthophyll-pigmented plumage evolved from a common ancestor with red C4-ketocarotenoid-pigmented feathers [64]. This suggests that the evolution from red to yellow plumage involved selective loss of *CYP2J19* expression with maintenance of *BDH1L* expression. Compared to the typical β,β-carotenoids in the diet, canary xanthophylls have a shorter conjugated system and short-wavelength shifted absorbance spectra. Although the differences in these yellow feathers may not be immediately apparent to the human visual system, objective spectral measures and modeling analyses indicate that the shift from β- to ε-rings can have a significant impact on feather hue [65]. There is growing evidence that feather coloration produced by derived yellow pigments like canary xanthophylls are more reliable indicators of individual quality than plumage colors produced through the direct accumulation of dietary pigments [31]. Thus, sexual selection may favor and maintain canary xanthophyll-based yellow plumage pigmentation because it provides useful and reliable information for mate choice.

Taken together, our results indicate that CYPJ19 and BDH1L catalyzed oxidation of common dietary carotenoids can explain many of the metabolized carotenoid products observed in bird plumage (Fig. 7). These mechanism of pigmentation are likely the targets of natural and sexual selection in many bird species and an important engine of avian

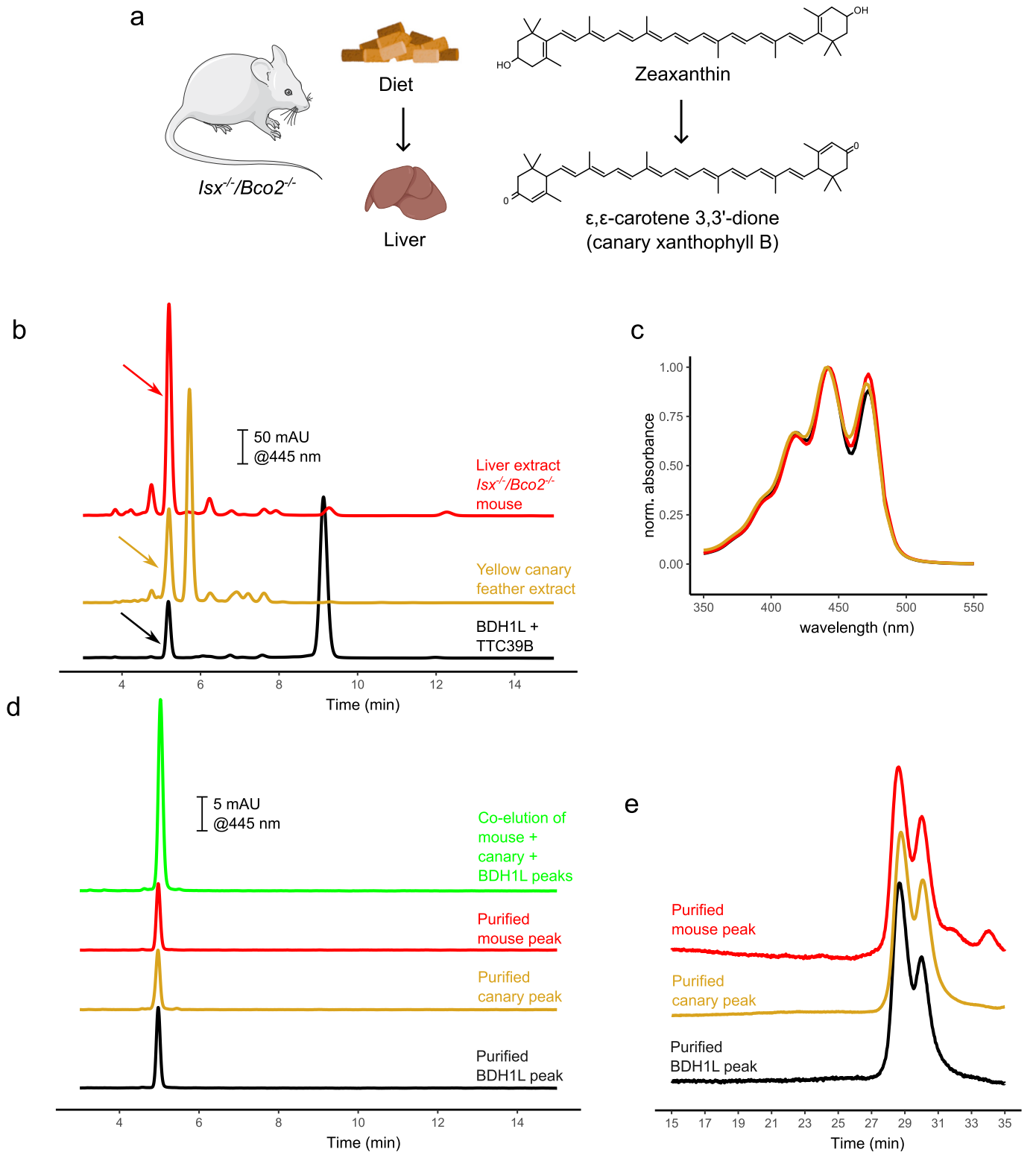

Fig. 6. BDH1L catalyzes the conversion of zeaxanthin to canary xanthophyll B (ε,ε-carotene-3,3′-dione). a) *Isx*$^{-/-}$/*Bco2*$^{-/-}$ mutant mice fed a zeaxanthin diet accumulate ε,ε-carotene-3,3′-dione as a major metabolite in their livers [44]. b) Top trace - Representative C30 reverse-phase HPLC chromatograms of a liver extract from a zeaxanthin fed *Isx*$^{-/-}$/*Bco2*$^{-/-}$ mutant mouse. Middle trace - extract from the yellow feathers of a common canary, bottom trace - the products of BDH1L catalyzed conversion of a zeaxanthin substrate in cell culture. c) The UV–Vis spectra of the ε,ε-carotene-3,3′-dione peak from mouse and the corresponding peaks in the canary feathers and BDH1L product (indicated with arrows in b) d) isolated peaks from each sample, mouse, canary, and BDH1L product, perfectly co-elute. e) Amylose tris(3,5-dimethylphenylcarbamate) Chiral HPLC chromatograms of the purified putative ε,ε-carotene-3,3′-dione peaks from the mouse (top), canary feathers (middle), and BDH1L product (bottom). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

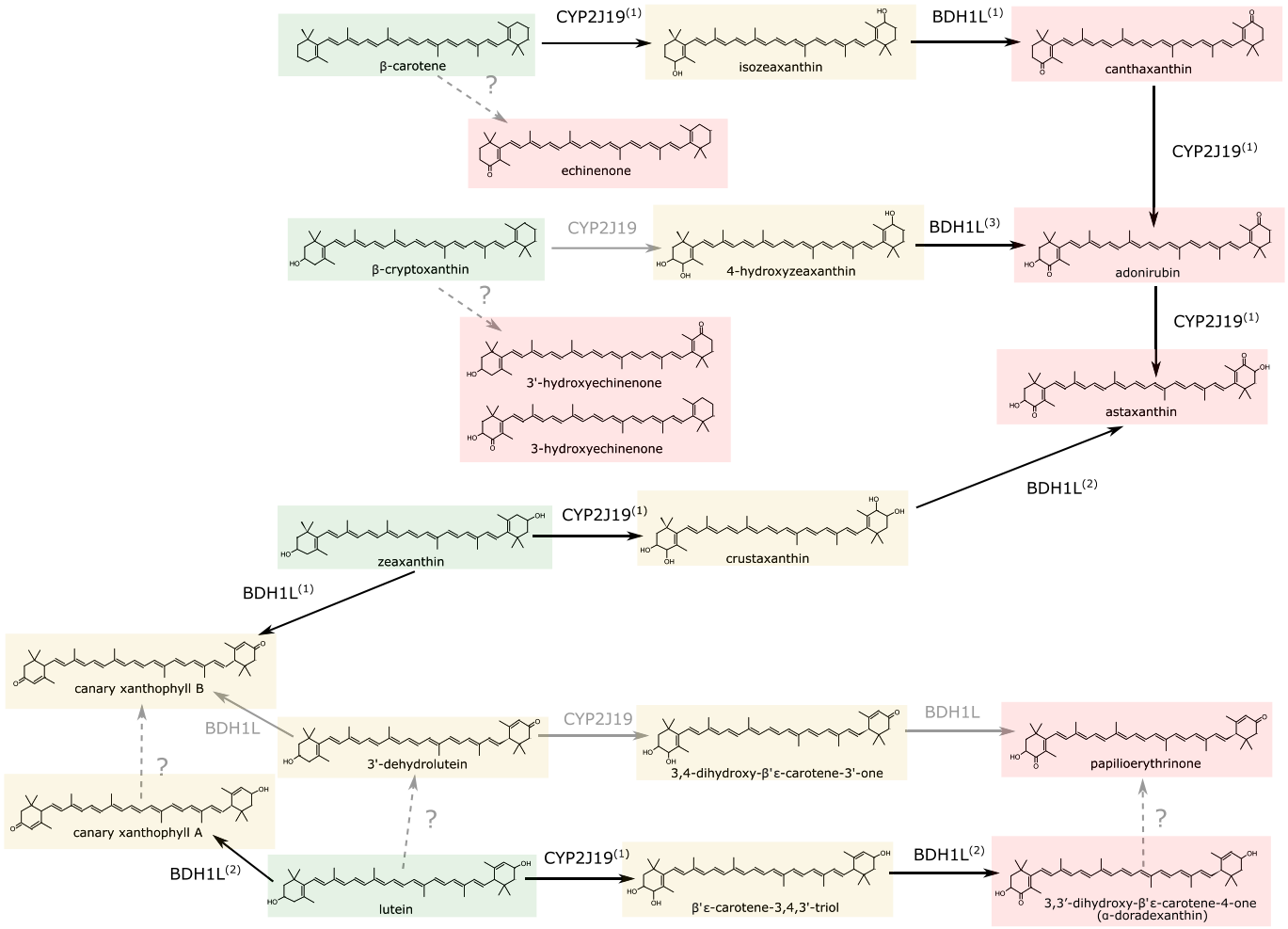

Fig. 7. CYP2J19 and BDH1L-mediated transformations of common dietary carotenoids can generate many, but not all, of the metabolized carotenoids observed in avian tissues. Demonstrated enzyme, precursor, and product relationships are noted with black arrows, text, and superscripts indicate the source of information (1) this study, (2) [27] and (3) [49]. Gray arrows and text indicate likely transformations based on the know activity of the enzymes. Gray dashed lines and "?" indicate transformations that are likely mediated by enzymes other than CYP2J19 and BDH1L. Green, yellow, and red shading indicate unmodified yellow (dietary), modified yellow, and modified red carotenoids, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

diversification [4–6]. It is our hope that better understanding of the enzymes mediating the expression of carotenoid-based colors will open new avenues of investigation and novel insights into the evolution of birds. However, the puzzle of avian carotenoid metabolism is still missing pieces. Specifically, we have yet to identify enzymes that can catalyze the direct transformations of ε-ring groups and the CYP2J19/BDH1L mechanism cannot explain the presence of asymmetrically oxidized β,β-carotenoids. With the growth of genomic resources and molecular tools, we anticipate that these missing pieces may soon be in hand.

CRediT authorship contribution statement

Matthew B. Toomey: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Rebecca E. Koch:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Yu Liu:** Writing – review & editing, Methodology, Investigation. **Johannes von Lintig:** Writing – review & editing, Resources. **Joseph C. Corbo:** Writing – review & editing, Visualization, Supervision, Resources, Investigation. **Geoffrey E. Hill:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Yufeng Zhang:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

Funding

This research was supported by National Science Foundation grant IOS-2037739 and the Oklahoma Center for the Advancement of Science grant PS20-021 to MBT.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matthew B Toomey reports financial support was provided by The University of Tulsa. Matthew B Toomey reports financial support was provided by National Science Foundation. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Elizabeth Scheer, Brian Nguyen, Nathan Reed, Akshaya Pradhananga, Brooke Joski, Hannah Reeb, and Christy Truong for their

assistance in the lab. We thank two anonymous reviewers for their helpful suggestions.

Data availability

All data referenced and analyzed in this manuscript are present in graphical form in the main figures. Raw chromatogram and UV–Vis spectra data are available upon request.

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