Studying ketocarotenoid metabolism in cell culture

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STUDYING KETOCAROTENOID METABOLISM IN CELL CULTURE

by

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ABSTRACT

This study examines the conversion of carotenoids to ketocarotenoids, essential for bright plumage in birds, a trait crucial in sexual selection. We used DF-1 avian cells genetically engineered to express BDH1L, CYP2J19, and TTC39B, key enzymes in this conversion. The genetic modifications and enzyme activity were verified via PCR genotyping and RT-qPCR. These cells were treated with yellow carotenoids (zeaxanthin, β-carotene, lutein) and their conversion to red ketocarotenoids (astaxanthin, canthaxanthin, alpha-doradexanthin) was analyzed using High-Performance Liquid Chromatography (HPLC). The study also assessed the impact of antioxidant treatments and mitochondrial uncouplers on this process. Results indicated that the inclusion of TTC39B enhances ketocarotenoid production, with antioxidants further boosting this conversion, while mitochondrial uncouplers showed minimal effect. This research highlights the pivotal roles of BDH1L, CYP2J19, and TTC39B in the bioconversion process, providing insights into the genetics and environmental factors influencing birds' ornamental traits.
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INTRODUCTION

In the animal kingdom, the evolution of male ornamentation, such as vivid coloration in birds, reptiles, and fish, is primarily influenced by female preference for traits indicating male fitness, a phenomenon grounded in sexual selection theory (1). These traits, often manifested through bright yellow or red hues derived from carotenoid pigments, serve as honest signals of health and vigor (2). Such condition-dependent signals allow individuals to assess potential mates based on phenotypes, playing a pivotal role in mate selection processes (3). This aspect of sexual selection emphasizes the significance of condition-dependent signaling, yet the origins of this dependency continue to spark debate within evolutionary biology circles (3). Individual condition is tied to resource availability, influencing ornamental traits development (1), with sexual selection driving resource allocation to these traits (4). This is encapsulated in the Resource Trade-off Hypothesis, which balances carotenoids between cell function and ornamentation (5). The Shared-Pathway Hypothesis links red ketocarotenoid coloration development to cellular processes via mitochondrial energy metabolism, suggesting ketolation indicates mitochondrial efficiency (6). Despite extensive research, consensus on how red/yellow color intensity links to key traits is elusive. Unlike psittacofulvin or melanin pigmentation, birds must consume dietary yellow carotenoids and then convert them to red ketocarotenoids via ketolation (7). Recent advances in genetic research have shed light on the mechanisms behind carotenoid coloration, identifying bird-specific genes, such as Cytochrome P450 Family 2 Subfamily J Member 19 (CYP2J19) and 3-hydroxybutyrate dehydrogenase 1-like (BDH1L), that are crucial for converting dietary yellow carotenoids into red ketocarotenoids (8).
Additionally, the discovery of the Tetratricopeptide repeat protein 39B (TTC39B) protein's role in amplifying ketocarotenoid production underscores the genetic complexity of these ornamental traits (8). These findings enhance our grasp of coloration's genetic basis and open research paths using transduced cell lines to investigate biochemical mechanisms of carotenoid coloration. (9). Such studies promise to further elucidate the intricate balance between genetic predisposition and environmental factors in the evolution of male ornamentation, contributing to our broader understanding of sexual selection and evolutionary dynamics.

**LITERATURE REVIEW**

**Background Study on Carotenoid**

Carotenoids account for the vivid red, orange, and yellow hues observed in numerous animal species, with such coloration playing a crucial role as a social indicator of an individual's health status (10,11). Carotenoid-based coloration is a widely studied example of a condition-dependent sexual display (5,12,13). In numerous vertebrate species, individuals exhibiting red-shifted coloration have a mating advantage or possess greater resources (2,14). For example, individuals with redder ornaments, as compared to dull individuals, are more proficient in resisting and recovering from parasitic infections, handling oxidative stress, better immune responses, and more efficient cellular respiration (15–18). To exhibit carotenoid-based red coloration, animals must biochemically transform yellow pigments into red ones, or they can ingest red pigments directly (2,19,20). The quality of an individual's color display results from the biochemical processes involved in the absorption, transport, metabolism, and deposition of carotenoids (21). Therefore, these biochemical pathways should play a pivotal role in establishing the links between ornamental coloration and the condition of an individual (16). In vertebrates, there are three primary categories of modified carotenoids: Apocarotenoids, 4-keto-
carotenoids (commonly referred to as 'ketocarotenoids'), and ε,ε-carotenoids (22,23).

Apocarotenoids, including all-trans retinol (vitamin A) and galloxanthin, are produced from dietary yellow carotenoids through the oxidative cleavage of the polyene chain (24,25).

Ketocarotenoids, such as astaxanthin, canthaxanthin, and α-doradexanthin, originate from dietary yellow carotenoids through the addition of ketone groups at the C4 position on one or both β-ionone rings terminal (26–28). The inclusion of these ketone groups extends the conjugated system, consequently granting ketocarotenoids with absorption properties that are shifted toward the red part of the spectrum (26,27). Modified ε,ε-carotenoids, such as canary xanthophyll B, are produced from dietary yellow carotenoids through a shift from β-to-ε in the ring double bond and the oxidation of the 3-hydroxyl group (27). Although the impact of carotenoid-based coloration on individual health and mating success is well understood as honest signals, the process by which honest signals prevent deception is still widely disputed (3). Studies on the signaling of individual conditions through carotenoid coloration have concentrated on the scarcity of resources or the trade-off in utilizing carotenoids to improve physiological functions, an idea broadly referred to as the Resource Trade-off Hypothesis (29,30). The Resource Trade-off Hypothesis suggests that condition-dependent honest signaling is based on a trade-off of resources between ornamental display and bodily maintenance (1). According to this theory, condition dependency could evolve through selection that favors the reallocation of resources towards enhancing ornamentation. On the other hand, index hypothesis posits that mate selection is directed towards carotenoid coloration, which is intrinsically dependent on condition because the creation of this coloration is closely linked to crucial cellular functions (1). A significant form of the index hypothesis is the shared pathway hypothesis, which suggests that traits can be condition-dependent if the production of ornaments is inherently connected to fundamental
cellular pathways necessary for essential life processes (3). A study carried out in Dr. Zhang’s laboratory has shown that (i) red carotenoids are localized within the inner mitochondrial membrane (IMM) and (ii) there is a strong correlation between plumage coloration and mitochondrial function. Therefore, it is concluded that plumage coloration acts as an indicator of mitochondrial function and, consequently, of fundamental cellular activity (6). In the current study, I propose to evaluate these two hypotheses using an in vitro model.

Resource Trade-Off Hypothesis

Condition is traditionally described based on the amount of metabolic resources an individual possesses, with those in high condition having a more substantial pool of resources, thereby granting them a greater capacity for ornament production (30–33). This definition of condition gave rise to the hypothesis that sexual selection favoring increased expression of display traits results in the evolution of mechanisms that divert resources towards the production of ornamental traits (33). Based on this theory, the most efficient distribution of resources is dictated by weighing the comparative advantages and disadvantages of different strategies. It implies that individuals with access to more resources can allocate more towards ornamental traits compared to those with fewer resources (33,34). Many studies have aimed to examine this hypothesis by inducing immune or oxidative stress and subsequently observing an anticipated decline in coloration, as carotenoids are redirected from ornamental purposes to essential maintenance functions (2,30). The persistent challenge for studies aiming to evaluate trade-offs through such experiments lies in the fact that triggering the immune system or inducing oxidative stress can diminish carotenoid coloration not necessarily by reallocating carotenoid molecules themselves but rather by impacting the mechanisms responsible for their absorption, transportation, or metabolism (5,30). In the only experiment where the potential for carotenoid
allocation to ornamentation or bodily maintenance was regulated via genetic manipulation, carotenoid allocation exhibited no discernible impact on bodily function (35). The study analyzed common canaries, focusing on three genetic variations: normal carotenoid processing, inhibited carotenoid uptake, and blocked allocation to feathers. Despite these differences, all groups showed similar immunocompetence and oxidative stress levels, indicating that carotenoid allocation to feather coloration does not affect these health parameters (35). The studies involving canaries represent just one time experiment conducted to directly test the resource trade-off hypothesis, and it's essential to validate the findings of these studies with further data from other systems.

**Shared Pathway Hypothesis**

Unlike the resource trade-off hypothesis, the shared pathway hypothesis proposes that ornament production is fundamentally intertwined with core cellular functions (3). In this framework, certain traits are intrinsically linked to individual conditions as they are associated with crucial cellular processes. Furthermore, the shared pathway hypothesis contradicts the notion that an individual's condition is solely determined by the metabolic resources it possesses. Instead, the condition arises from the functionality of vital cellular processes rather than from resource trade-offs (3). To evaluate the degree of correlation between carotenoid coloration and condition, a study conducted a meta-analysis examining on carotenoid-based plumage ornaments and conditions in birds. Unlike prior reviews and summaries, in this meta-analysis, the authors distinctively evaluated the potency of condition-dependent signaling for the two separate forms of ornamental coloration (5). Their findings revealed that ornaments derived from modified carotenoids, either into yellow ε,ε-carotenoids or red keto-carotenoids, exhibited stronger associations with measures of individual quality compared to ornaments produced using dietary
carotenoids. However, ornaments produced with dietary pigments also tended to serve as signals of individual condition, albeit the associations were not as robust as those observed with modified carotenoids (5). Subsequently, it was suggested that the production of modified carotenoid pigments entails metabolic conversions influenced by mitochondrial function (6).

Carotenoid-modifying enzymes facilitate oxidation-reduction (redox) reactions to modify the functional groups on the carotenoid molecule. Enzymes in this category transfer electrons from an energy-rich donor molecule such as NADH or NADPH to carry out their reactions, resulting in the production of NAD$^+$ or NADP$^+$ as a by-product (36). Once depleted, NAD$^+$ and NADP$^+$ can be replenished with electrons, allowing them to sustain redox reactions in a cyclical manner. This reliance on redox cycles potentially links the process of modifying carotenoids to the mitochondria and mitochondria-associated membranes, which rely on their own set of redox reactions to execute various functions (6,37). The ratios of electron donors such as NADH and NADPH (along with their oxidized forms) or redox products fluctuate within both the mitochondria and the mitochondria-associated membranes of the endoplasmic reticulum (ER). It is conceivable that conditions in one compartment could influence those in another through the transfer of intermediate molecules across membrane barriers (38,39). The variations in the ratios of redox partners and products within the cell, occurring both within the mitochondria and at the mitochondria-ER boundary, create a common redox environment. Enzymes operating within this dynamic electrochemical milieu, which utilize redox reactions, are subject to alterations in their electrochemical potential necessary for carrying out these reactions (10,40). It is hypothesized that carotenoid-modifying enzymes conduct their redox reactions within the shared electrochemical environment of mitochondria or mitochondria-associated membranes of the endoplasmic reticulum (ER) (6,37). Hence, the rate at which an organism can synthesize
ketolated carotenoids might be linked to the metabolically determined redox environment of its pertinent cells.

**Noval genes in carotenoids ketolation pathway**

Recent studies have demonstrated that genes encoding proteins from the cytochrome P450 monooxygenase superfamily are essential for synthesizing red ornamental carotenoids in birds (41–43). An investigation led to identifying an enzymatic pathway that can convert dietary yellow carotenoids into red ketocarotenoids and ε,ε-carotenoids (8). Using yellow dietary carotenoid zeaxanthin as the substrate, CYP2J19 is responsible for the hydroxylation at the end of the zeaxanthin molecule, which adds a hydroxyl group (–OH) to form crustaxanthin (8). Leading to the fact that CYP2J19 alone cannot achieve this chemical transformation to keto-carotenoid, prompting a search for other factors that might collaborate with CYP2J19 to facilitate ketocarotenoid formation. The oxidation of a hydroxyl group (–OH) by BDH1L would result in the formation of a ketone group (C=O), contributing to the conversion of crustaxanthin into astaxanthin. The results indicate that CYP2J19 initially works on zeaxanthin to create an intermediate product, which is subsequently modified by BDH1L, culminating in the formation of astaxanthin, and its efficiency is increased by the presence of TTC39B (8). BDH1L plays a crucial role in the initial phase of transforming dietary carotenoids into red ornamental carotenoids. BDH1L exhibits a significant sequence resemblance to BDH1, an enzyme that facilitates the interconversion between acetoacetate and 3-hydroxybutyrate (8). Gene expression research has associated (TTC39B) with the intensity of red coloration in fish (44,45) and birds (8). TTC39B is prominently recognized in human biomedical research due to its role in the transportation of cholesterol within lipoproteins (45).
Test Shared Pathway Hypothesis and Resource Tradeoff Hypothesis in vitro.

We intended to establish novel stable transduction cell lines in vitro utilizing a lentivirus-mediated gene delivery system (9). Three genes, CYP2J19, BDH1L, and TTC39B, cloned and introduced into chicken embryo fibroblasts (DF-1) cell lines using lentiviral vectors. Subsequently, the genomes of these cells sequenced to validate the integration of the aforementioned three genes. Once confirmed, the cells were treated with yellow dietary carotenoids, and the resulting metabolized products examined and quantified using High-Performance Liquid Chromatography (HPLC). These cellular models hold great potential as valuable instruments for investigating the physiological mechanisms and regulatory pathways involved in the conversion of yellow to red carotenoids. Consequently, we proceeded to titrate these cells with antioxidants (such as N-acetyl cysteine and Mito-tempo) as well as mitochondrial uncouplers (such as 2,4-Dinitrophenol) and examined the effects of these compounds on carotenoid ketolation. N-acetyl cysteine (NAC), a thiol compound, serves as an acetylated precursor to the amino acid L-cysteine and exhibits a direct antioxidant action against specific oxidative agents, including NO$_2$ and hypohalous acids (HOX) at the cellular level (46). Its significance as a powerful antioxidant stems from its capacity to elevate intracellular cysteine levels, leading to an increase in glutathione (GSH). This mechanism positions NAC as a key approach in mitigating oxidative stress damage in scenarios such as xenobiotic intoxication, like with paracetamol, or in conditions associated with GSH depletion, by preserving GSH concentrations across various tissues (47–49). Mito-Tempo (MT) is recognized as an antioxidant specifically designed to target mitochondria. It is a hybrid molecule comprising the antioxidant element piperidine nitroxide (Tempo) and the lipophilic cation triphenylphosphonium (TPP$^+$). Tempo acts as a mimetic of superoxide dismutase (SOD), facilitating the dismutation of superoxide radicals during the catalytic cycle. TPP$^+$, on the other hand, is a cation that can
permeate membranes and is selectively accumulated in the mitochondria in response to the mitochondrial membrane potential, often at concentrations several hundred times higher than in the surrounding environment. This strategic combination yields a compound with potent abilities to neutralize superoxide radicals within the mitochondria (50). 2-4-Dinitrophenol (DNP), introduced in the 1930s as an early form of antiobesity treatment, was found to promote weight loss by disrupting oxidative phosphorylation. This disruption accelerates metabolic rate and enhances fat burning. The mechanism behind its effect involves altering the proton electrochemical gradient across mitochondrial membranes, causing potential energy to be released as heat rather than being transformed into ATP (51). By utilizing these innovative cell models, we aimed to investigate both the Resource Trade-off and Shared-Pathway hypotheses, thus gaining insights into the biochemical and cellular mechanisms that regulate carotenoid coloration.

**Hypothesis**

We hypothesized that it is possible to establish an *in vitro* model for the continuous expression of genes involved in the conversion of carotenoids into ketocarotenoids and that this will result in increased ketocarotenoid levels. Furthermore, we hypothesized that antioxidant treatments will increase ketocarotenoid production as suggested by the Resource Trade-off Hypothesis, while mitochondrial uncouplers are expected to decrease production aligning with the Shared-Pathway Hypothesis. We acknowledge that both hypotheses may intersect, indicating that elements of each could simultaneously influence ketocarotenoid production.
METHOD

Materials, Methods, and Procedures

This study used a lentiviral mediated gene delivery system to express specific genes in DF-1 cells. After establishment of the stable cell line, the cells were treated with yellow carotenoids (β-carotene, lutein, and zeaxanthin). Following treatment, the cells were processed to isolate the metabolites and measured the carotenoids using sonication, solvent extraction, and High-Performance Liquid Chromatography (HPLC). The methodology included the insertion of genes into vectors, the production of viruses, the infection and selection of DF-1 cells, the treatment with carotenoids, and their analytical quantification to explore the effects of carotenoids on genetically modified cells. We then used a pharmacological method to examine if there's a link between the ketolation of carotenoids and mitochondrial respiratory function or the antioxidant capabilities of cells. This involves adjusting mitochondrial respiratory function by administering varying doses of 2,4-Dinitrophenol (DNP), which dissociated mitochondria in cells. Concurrently, to alter the cells' antioxidant abilities, we introduced a range of antioxidants, such as the broad-spectrum antioxidant N-Acetyl Cysteine and mitochondria-specific antioxidants like mitoQ, aiming to enhance the cells' antioxidant defenses. The impact of these interventions on carotenoid bio-conversion was assessed.

Cell Culture

The avian (DF-1) cell line was purchased from ATCC. Cells were cultured according to ATCC guidelines.
Experiment 1

Development and production of lentivirus-based vectors

To generate lentiviral vectors for the expression of BDH1L, CYP2J19, and TTC39B, we designed specific primers for each gene: BDH1L (forward: 5'-CACCATGTGGGCTGCCGCACC 3'; reverse: 5'-CTAAGCCAGTTTCACTTTAGACAGGGCG 3'), CYP2J19 (forward: 5'-CACCATGGATTTTCGCTTTGGCCCATTTCCCAATTG -3'; reverse: 5'-TCAGCAGCGAGGCAGGGCG -3'), and TTC39B (forward: 5'-CACCATGGCGAGCGTCGGAAAC 3'; reverse: 5'-TCAAGGTGTGAAGCAGGTGACACTTTC 3') to amplify their corresponding DNA fragments. These fragments were initially sub-cloned into the pENTR/D-TOPO vector, and then the expression cassette transferred into the pLV7 destination vector using Gateway cloning. The destination vector had mammalian selection markers such as neomycin, puromycin, and blasticidin resistance genes. This cloning process created pLV7-CAG-gene of interest (GOI) expression vectors. The lentiviral particles were produced in 293T cells using the GOI expression vector and viral packaging vectors (GAG-POL, VSV-G and REV) using PEI-mediated transient transfection protocols. After viral particles production, the viral particles were concentrated through ultracentrifugation and used to infect DF-1 cells, aiming to create stable cell lines expressing the genes of interest. Selection of infected cells will occur in stages: neomycin selection for BDH1L expression, followed by puromycin for CYP2J19, and blasticidin for TTC39B. Eight different cell lines were established: one without any genes but empty vector as a control, and others expressing single genes (BDH1L, CYP2J19, TTC39B), pairs of genes (BDH1L and CYP2J19, BDH1L and TTC39B, CYP2J19 and TTC39B), and one expressing all three genes (BDH1L, CYP2J19, and TTC39B). Although the focus of subsequent experiments
was on the cell lines with all three genes, the remaining cell lines, including the control, were valuable for future studies. After we generated a stable cell line, PCR genotyping confirmed the chromosomal integration of all three genes. These stable cell lines, capable of expressing all three target genes, were subsequently utilized in assays to assess their ability to convert ketocarotenoids.

**RNA Isolation and qPCR Confirmation**

To verify the expression of the introduced genes (CYP2J19, BDH1L, and TTC39B) in DF-1 cells, total RNA was isolated. The quality and concentration of the purified RNA was determined spectrophotometrically by measuring absorbance ratios at 260/280 nm, ensuring the RNA is of sufficient purity and quantity for reliable qPCR analysis. Following RNA isolation, reverse transcription (RT) was carried out to synthesize cDNA from the mRNA, providing a template for quantitative PCR (qPCR). Specific primers for CYP2J19, BDH1L, and TTC39B were used to selectively amplify these target genes. For the quantitative analysis of CYP2J19, BDH1L, and TTC39B gene expression, qPCR was conducted using SYBR Green, a fluorescent dye that binds to double-stranded DNA, allowing for the real-time monitoring of the amplification process. As the qPCR product accumulates, the fluorescence signal increased proportionally, allowing for the quantification of gene expression levels. The expressions of CYP2J19, BDH1L, and TTC39B were normalized to GAPDH gene expression, providing a relative measure of gene expression to confirm the presence and initial expression levels of the genes of interest in the DF-1 cells.

**Carotenoids Treatment**

DF-1 cells containing CYP2J19, BDH1L, and TTC39B were exposed to yellow carotenoids (β-carotene, lutein, and zeaxanthin) dissolved in 0.035% Tween 40 to achieve a
concentration of 1 μg/ml for 48 hours. Tween 40 was chosen for its ability to effectively dissolve hydrophobic compounds such as carotenoids offering a simple, rapid, and non-toxic method for delivering carotenoid mixtures to cells in culture (52). Fresh media and carotenoids were replaced every 24 hours during the treatment period. After 48 hours, the cells were trypsinized, and the resulting cell pellets were stored at -80°C for further experiments.

**Carotenoids Isolation and Measurements**

The cell pellets were resuspended in 500 μl of 0.9% NaCl. Following this, the cells underwent disruption using a sonicator for 30 seconds at 4 kHz. Subsequently, 250 μl of 100% ethanol was added to the solution, followed by thorough mixing via vortexing. To this solution, 500 μl of hexane:tert-butyl methyl ether (1:1, vol:vol) was added, and the mixture underwent an additional round of sonication for 30 seconds at 4 kHz. The resulting cell homogenate was then centrifuged at 10,000 g for 3 minutes, and the upper solvent fraction containing carotenoids was carefully collected in a 2 mL glass vial. This hexane:tert-butyl methyl ether extraction process was repeated two more times, and subsequently, the solvent was completely evaporated under a stream of nitrogen.

**High-Performance Liquid Chromatography**

We prepared carotenoids for analysis by dissolving them in the solvent used for high-performance liquid chromatography (HPLC) according to (8) and then introduced them into an Agilent 1200 series HPLC system, which was equipped with a specialized YMC carotenoid column (5.0 μm, 4.6 mm × 250 mm, YMC, CT99S05-2546WT). The procedure involved a dynamic elution process starting with a mixture of acetonitrile, methanol, and dichloromethane
in the ratio of 44:44:12 for the first 11 minutes, adjusting the mixture to 35:35:30 for the next 10 minutes, and then holding these conditions stable until the 35-minute mark. The flow rate of the solvent was kept steady at 1.2 mL per minute, while the column's temperature was controlled at 30°C. Detection of the carotenoids as they elute was carried out with a UV-Visible photodiode array detector, tuned to specific wavelengths, and identification was achieved by comparing the results to known standards of carotenoids available on the market.

**Experiment 2**

To explore the link between carotenoid conversion and cellular mitochondrial health and antioxidant capabilities, we implemented a pharmacological approach, adjusting mitochondrial activity within DF-1 cells. Our strategy involved the application of 2,4-Dinitrophenol (DNP), a mitochondrial uncoupler, along with N-Acetyl Cysteine (NAC), a general antioxidant, and Mito-tempo, a mitochondria-targeted antioxidant. We conducted titration experiments, and the titration ranged from 0.1 μM to 1 mM for DNP, 1 mM to 10 mM for NAC, and 10 nM to 100 μM for Mito-tempo. The cells were treated these chemicals for 24-hour to establish the most effective function. Following treatment, cells were trypsinized, and the cell pellet was stored at -80°C for subsequent carotenoid isolation and High-Performance Liquid Chromatography (HPLC) analysis. This method ensures precise dosage determination for each compound, which is essential for evaluating their effects on mitochondrial function and antioxidant defenses accurately.
RESULTS

Experiment 1

Prior to confirming gene expression through qPCR, we successfully developed and characterized lentiviral mediated gene delivery system in our in vitro cellular model. These vectors were designed to express BDH1L, CYP2J19, and TTC39B, either individually or in combination. The genotype results confirm that all three genes were integrated into the chromosome of the DF-1 cell line. Subsequently, quantitative PCR (qPCR) was utilized to establish and confirm the presence and expression of BDH1L, CYP2J19, and TTC39B genes in the avian cell line. Specifically, we established the cell line that were engineered to express the individual genes (BDH1L, CYP2J19, TTC39B), two gene combinations (BDH1L+CYP2J19), and the three gene combination (BDH1L+CYP2J19+TTC39B). The successful establishment of these cell lines was ascertained through the relative quantification of gene expression, confirming the transcriptional activity of the inserted genes compared to a control group with an empty vector. This strategy provided a diverse array of cell lines for subsequent analyses of carotenoid conversion efficiency, thereby facilitating a comprehensive evaluation of the genetic factors involved in the ketolation pathway (Figure 1).
Figure 1. Relative Gene Expression in DF-1 Cell Lines. (A) to (C) display DF-1 cells with an empty vector, and cells expressing BDH1L and CYP2J19 genes, respectively. (D) to (F) show DF-1 cells expressing TTC39B, BDH1L+CYP2J19 combination, and all three genes (BDH1L, CYP2J19, TTC39B), demonstrating the successful establishment and expression levels of each genetic modification in comparison to the control.

Cell lines expressing the three genes of interest—BDH1L, CYP2J19, and TTC39B—were subjected to treatments with zeaxanthin, β-carotene, and lutein, each at a concentration of 1µg/ml, dissolved in 0.035% Tween 40 for a duration of 48 hours. Post-treatment, these cell lines exhibited production of red ketocarotenoids, astaxanthin, canthaxanthin and alpha-doradexanthin which were successfully detected and quantified using High-Performance Liquid Chromatography (HPLC) (Figure 2). Notably, the cell lines expressing only two genes (BDH1L+CYP2J19) and treated with β-carotene also demonstrated conversion to the
corresponding red ketocarotenoid. These observations provide empirical support for the functionality of the inserted genes in the bioconversion of yellow carotenoids to their red ketocarotenoid counterparts within the cellular environment (Figure 2).

Figure 2. Carotenoid uptake and ketocarotenoid production in DF-1 cells. Carotenoid levels following treatment with dietary carotenoids zeaxanthin, lutein, and beta-carotene in DF-1 cells without gene modification (empty vector) and cells modified to express BDH1L, CYP2J19, and TTC39B (A). Resultant ketocarotenoid production, shows astaxanthin, alpha-doradexanthin, and canthaxanthin levels (B).

Experiment 2

Treatment with both antioxidants and a mitochondrial uncoupler resulted in a decreased uptake of yellow carotenoids by the cells. However, while antioxidant treatment led to an increase in the ketolation ratio, indicating an enhancement of ketocarotenoid production, the treatment with mitochondrial uncouplers did not alter this ratio. These findings suggest that
bolstering the cellular antioxidant defenses can potentially elevate the efficiency of ketocarotenoid biosynthesis. This observation is consistent with the idea that antioxidants may play a role in promoting the conversion of yellow carotenoids to red ketocarotenoids, whereas mitochondrial uncoupling does not have a significant impact on this process. These results provide insight into the complex interplay between cellular redox states and carotenoid metabolism, highlighting the potential for antioxidants to influence the pathway of carotenoid ketolation within the cells (Figure 3).

Figure 3. Impact of Antioxidants and a Mitochondrial Uncoupler on Carotenoid Metabolism. DF-1 cells expressing BDH1L, CYP2J19, and TTC39B were treated with beta-carotene and various modifiers: N-Acetyl Cysteine (NAC), 2,4-Dinitrophenol (DNP), and MitoTEMPO. (A) presents beta-carotene uptake; (B) quantifies canthaxanthin production; and (C) illustrates the beta-carotene to canthaxanthin to conversion ratio across treatments. The treatments demonstrate differing impacts on carotenoid processing, with the ratio graph highlighting the relative efficiency of conversion in the presence of each modifier.
DISCUSSION

This study established DF-1 cell line as an *in vitro* model for investigating the carotenoid metabolism. We genetically modified DF-1 cells to overexpress the genes BDH1L, CYP2J19, and TTC39B, either individually or in combination. The recombined cell line containing all three genes were then treated with dietary yellow carotenoids (zeaxanthin, β-carotene, and lutein), and cells containing BDH1L and CYP2J19 were treated with β-carotene. Using HPLC analysis, we observed the red product of these yellow carotenoids into their corresponding red ketocarotenoids (astaxanthin, canthaxanthin, and alpha-doradexanthin). Additionally, cells expressing TTC39B with BDH1L + CYP2J19 resulted in higher ketocarotenoid products compared to cells expressing only BDH1L + CYP2J19. To investigate the influence of antioxidant and mitochondrial uncoupling treatments on the ketolation process, cells were treated with antioxidants (N-Acetyl cysteine and Mito-Tempo) and a mitochondrial uncoupler (DNP) together with yellow carotenoids. We observed an increase in ketocarotenoid production following antioxidant treatment. However, treatment with the mitochondrial uncoupler did not significantly alter ketocarotenoid production.

The establishment of stable transduced DF-1 cell lines for carotenoid metabolism was achieved using lentiviral mediated gene delivery system. (9). To drive robust and consistent expression of the target genes, we selected the CAG promoter. This hybrid promoter which is a composite of the cytomegalovirus (CMV) immediate-early promoter/enhancer, chicken β-actin promoter, and rabbit β-globin splice acceptor, is known for its ability to drive high levels of gene expression (53). We opted to use the CAG promoter, primarily influenced by its successful application in a related study detailed in (8). Utilizing the same promoter in the present study
allows for more direct comparability of results, facilitating a clearer understanding of how our findings align with or differ from previous research. Furthermore, incorporating selection markers alongside our target genes enabled us to select and propagate the transduced cells for downstream experiments.

Overexpression of BDH1, which shows structural similarity to BDH1L, significantly decreases mitochondrial ROS production, stabilizes mitochondrial membrane potential and reduces oxidative stress. This reduction in ROS not only prevents cellular damage but also decreases inflammation and apoptosis, particularly in liver cells under stress from conditions like fatty liver disease. The protective effects of BDH1 are mediated through its regulation of metabolic flux involving β-Hydroxybutyrate (β-OHB), acetoacetate (AcAc), succinate, and fumarate. The latter activates the nuclear factor red 2-related factor 2 (Nrf2) pathway, enhancing the antioxidant defense mechanism essential for mitigating cellular inflammation and damage. Additionally, the possible localization of BDH1 to mitochondria, where it modulates the redox potential, suggests that BDH1L may also target mitochondria, thereby linking its activity, including effects on feather coloration, to mitochondrial respiration (54–56). Enhanced activity of CYP2J19 could lead to greater red pigmentation, potentially enhancing the signaling traits used in reproductive success. However, this might come at a cost, affecting mitochondrial respiration, and potentially altering the balance of reactive oxygen species (ROS), which are crucial for cellular health (57). Specifically, the presence of CYP2J19 was crucial for the hydroxylation steps necessary for converting zeaxanthin to astaxanthin, while BDH1L was essential for introducing keto groups into the carotenoid structure, a critical step for red coloration development. Moreover, TTC39B's role in enhancing ketocarotenoid coloration in birds may relate to its involvement in lipid metabolism, similar to its function in mammals. In
mice, TTC39B influences LXR, a regulator of lipid transporter genes like ABCA1, which is crucial for HDL formation and carries lipid-soluble carotenoids (58). Since HDL transports carotenoids to feathers in birds (59), TTC39B could enhance carotenoid deposition and feather coloration by modulating LXR and increasing lipid transporter activity. However, findings on red-throated parrotfinch suggest a different perspective (8). The mutation in TTC39B results in a clear decrease in ketocarotenoid levels in the feathers and retinas of the birds, which corresponds to a change in feather color from red to yellowish-orange. This effect does not appear to be mediated through changes in HDL levels or total circulating carotenoid levels, which remain unchanged. Instead, TTC39B might influence ketocarotenoid biosynthesis directly at the local sites, such as in the feather follicles and the red cone photoreceptors. This suggests that while TTC39B might influence lipid transport pathways, its primary impact on feather coloration may involve localized metabolic processes, highlighting a more complex interaction within carotenoid metabolism and deposition than previously understood. This direct action on the sites of carotenoid conversion is further supported by experimental evidence showing TTC39B’s enhancement of the conversion of zeaxanthin and β-carotene into ketocarotenoids in cultured cells, suggesting a direct regulatory role at the enzymatic level. The expression and functional demonstration of these genes in DF-1 cells confirm their pivotal role in the biosynthesis of red ketocarotenoids, aligning with previous suggestions made by avian and comparative genomic studies (8,42,60).

Furthermore, we investigated two central hypotheses within evolutionary biology—the Resource Trade-off Hypothesis and the Shared-Pathway Hypothesis—using our established in vitro system. DNP, as a mitochondrial uncoupler, facilitates an insight into the mitochondrial function’s role in carotenoid metabolism and sexual signaling. In our experiment, no change was
observed in the ratio of canthaxanthin to β-carotene when treated with DNP compared to controls. This finding is significant as the DNP dosage was chosen to alter mitochondrial membrane potential without affecting ATP production. Specifically, it increased the rate of respiration during oxidative phosphorylation (OXPHOS), accelerating redox reactions to compensate for the lack of ATP produced, yet it did not increase reactive oxygen species (ROS) production (61–66). This uncoupling mechanism suggests that the carotenoids were not consumed in oxidative stress responses since there was no increase in ROS, which aligns with the resource trade-off hypothesis. The observation that DNP treatment did not alter the canthaxanthin/β-carotene ratio in our study may pose a challenge to the Shared Pathway Hypothesis (SPH), as it suggests that not all carotenoid metabolic pathways are directly affected by changes in mitochondrial function induced by DNP. However, this result does not completely refute the SPH. Furthermore, a study on the metabolism of red carotenoids in marine copepods showed that DNP treatment increased ketocarotenoid accumulation in red male copepods after 7 days, along with an increase in respiration rate (67). However, similar results were not observed within a shorter 3-day period. This suggests that the 24-hour treatment duration in our experiment may not have been sufficient to induce observable changes in ketocarotenoid levels. This temporal factor might explain the stability in the canthaxanthin/β-carotene ratio under our experimental conditions. Recent findings indicate that while CYP2J19 is not localized within the mitochondria, enzymes like BDH1L may interact with mitochondrial functions by potentially being located at mitochondria-associated membranes (MAMs) between the ER and mitochondria (68,69). This positioning allows BDH1L to access substrates and maintain efficient function despite mitochondrial uncoupling, thereby supporting the stability of carotenoid-based coloration as a reliable indicator of individual quality (37).
Coenzyme Q10 primarily facilitates electron transfer from complex I to III within the mitochondrial electron transport chain. Its reduced form, QH2, serves a dual purpose: it not only shuttles electrons but also acts as an antioxidant, safeguarding the inner mitochondrial membrane (IMM) against oxidative damage to lipids (70). MitoQ, a mitochondria-targeted derivative of ubiquinone, penetrates the IMM and stimulates the production of QH2 at complex II, which is then transferred to complex III. This process is thought to stabilize the IMM and enhance the function of ketolase enzymes, crucial for ketocarotenoid synthesis (71).

Experimental results with male zebra finches have shown that MitoQ can intensify the red coloration of bills in individuals that were initially red, indicating its effectiveness in enhancing ketocarotenoid presence (72). Conversely, studies involving crossbills have shown that while MitoQ reduces xanthophyll levels, it does not increase ketocarotenoids, possibly due to its longer ten-carbon alkyl chain affecting ROS production. On the other hand, MitoTEMPO has been observed to boost total ketocarotenoid levels, including canthaxanthin, especially in birds initially exhibiting high-quality red coloration (73). This aligns with our findings where MitoTEMPO enhanced canthaxanthin production from beta-carotene, reflecting its role in recycling ubiquinol back to ubiquinone (74). These outcomes underscore the influence of mitochondria-targeted molecules on ketocarotenoid synthesis, supporting the Shared Pathway Hypothesis, which posits a role for the ubiquinone biosynthesis pathway in these processes. Moreover, the use of N-acetylcysteine (NAC), a precursor to glutathione, also led to increased ketocarotenoid levels in our study, suggesting a potential link with the Resource Trade-Off Hypothesis (RTOH). This hypothesis considers the allocation of antioxidants like glutathione in managing oxidative stress, which may also influence carotenoid metabolism. Additionally, a study conducted on great tits (75) investigated the relationship between environmental stressors,
oxidative stress, and plumage coloration by examining carotenoid-based yellow plumage and glutathione levels in urban and rural populations. This research revealed that urban birds, exposed to higher pollution levels, exhibited paler plumage, and altered glutathione dynamics, suggesting that environmental stress might limit the availability of carotenoids for coloration due to their diversion to combat oxidative stress. In our study, the administration of N-acetylcysteine (NAC), a precursor to glutathione, potentially bolstered the antioxidant defense system, which could allow more carotenoids to be allocated towards plumage coloration rather than being used to combat oxidative damage. This aligns with the Resource Trade-off Hypothesis, supporting the idea that enhancing internal antioxidant resources allows for greater external allocation of carotenoids to sexual signaling traits like plumage coloration, thereby providing a mechanistic link between antioxidant supplementation and enhanced ornamental trait expression.

Nevertheless, it is critical to consider the limitations inherent to in vitro studies. While cell culture models like ours are invaluable for studying specific biochemical pathways in a controlled environment, they do not fully represent the intricate physiological and ecological contexts in which these mechanisms operate in vivo.

CONCLUSION AND FUTURE DIRECTIONS

Our study successfully established an in vitro model that demonstrates the genetic basis of carotenoid to ketocarotenoid conversion in avian cells. We confirmed the functionality of the genes BDH1L, CYP2J19, and TTC39B in this process. Antioxidant treatments were shown to increase ketolation, suggesting a potential for enhanced ornamental pigment production, while
mitochondrial uncoupler treatments did not affect the ketolation ratio. These findings advance our understanding of carotenoid bioconversion and provide a platform for future research into the genetic and environmental factors influencing male ornamentation in birds. This study lays the groundwork for numerous avenues of future research. To understand the dynamic regulation of carotenoid-based coloration throughout a bird's life cycle, we need to elucidate how hormones, transcription factors, and environmental stimuli modulate the expression of BDH1L, CYP2J19, and TTC39B. Further investigations into redox dynamics, employing targeted redox probes, manipulations of specific redox couples (e.g., NAD+/NADH, NADP+/NADPH), and examining subcellular localization, are crucial for illuminating the precise redox environments that influence the ketolation pathway. Finally, to grasp the evolutionary forces shaping these mechanisms, comparative studies are needed. Utilizing cell lines derived from bird species with contrasting diets, lifestyles, and coloration patterns will reveal how ecological and evolutionary factors have driven the diversification of carotenoid metabolic pathways across avian lineages.
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