Understanding Ornamental Carotenoid Metabolism in the House Finch
(Haemorhous mexicanus)

by

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Abstract

Carotenoids are the most common source of yellow, orange and red coloration in birds. However, like other vertebrates, birds cannot synthesize carotenoids de novo. To produce carotenoid-based coloration they must ingest carotenoids from their diet. Most songbirds that deposit red carotenoids in their feathers, skin or beaks ingest only yellow or orange dietary carotenoids, which they further oxidized to red ketocarotenoids via a ketolation reaction. Despite of the extensive effort to understand carotenoid-based coloration in birds, fundamental questions regarding ornamental carotenoid metabolism remain unanswered. In this dissertation, I used house finches (*Haemorhous mexicanus*) as a model animal to help us better understand this carotenoid metabolism in birds. I collected tissues and extracted subcellular fractions from wild house finches and surveyed the ornamental ketoarotenoid content from both tissue and subcellular fractions to locate the potential site of carotenoid ketolation. I then compared the carotenoid content across sexes and seasons to get a bigger picture of how carotenoid metabolism might be like in the house finch. Last but not least, I collected other bird species with red carotenoid coloration but different display strategies to try to understand the general pattern of carotenoid metabolism in the avian world. We found the highest concentration of ketocarotenoid inside the hepatic mitochondria, or more specifically, in the inner mitochondria membrane in the house finch, suggesting liver as the primary site of carotenoid ketolation. Ketocarotenoids were detected in both males and females during the molting season, but no ketocarotenoid was detected out of the molting window. And the patterns of ketocarotenoid

distribution varied among birds with different red carotenoid color displays, indicating the possibility of multiple strategies of ornamental carotenoid metabolism in birds.

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List of Abbreviations

ANT	Adenine nucleotide translocator protein
COX	Cytochrome c oxidase
CPT	Carnitine palmitoyltransferase
CS	Citrate synthase
ETS	Electron transport system
HNE	Hydroxynonenal
HPLC	High-Performance Liquid Chromatography
IMM	Inner Mitochondrial Membrane
MMP	Mitochondrial Membrane Potential
OMM	Outer Mitochondrial Membrane
PGC	Peroxisome proliferator-activated receptor Gamma Coactivator
PBS	Phosphate-buffered Saline
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel Electrophoresis

Chapter 1

High concentrations of ketocarotenoids in hepatic mitochondria of *Haemorhous mexicanus*

Introduction

Many species of fish, reptiles, and birds have carotenoid-based integumentary coloration. No vertebrate species can synthesize carotenoids de novo; they must ingest carotenoid pigments to use them as colorants (Fox and Vevers, 1960). Once ingested, however, carotenoid pigments can be modified via enzyme-supported redox reactions. Such redox reactions can change the fundamental hue of carotenoids, including transformations of yellow dietary hydroxycarotenoids (e.g. lutein, zeaxanthin) to red ketocarotenoids (e.g. astaxanthin, canthaxanthin) (Brush, 1990).

Oxidation of dietary carotenoids is a particularly widespread and important source of coloration in songbirds (Order Passeriformes). The diets of most songbirds contain four primary carotenoid pigments, all of which are yellow or orange: β-carotene, β-cryptoxanthin, zeaxanthin, and lutein (McGraw, 2006). Some songbirds use these dietary pigments directly (Partali et al., 1987). Others oxidize lutein or zeaxanthin to produce the yellow pigments canary xanthophyll a and b (Stradi et al., 1995). Still other songbirds ketolate dietary pigments by introducing ketone groups to produce red ketocarotenoids. Ketolation of yellow dietary pigments is the primary source of red coloration in songbirds (Inouye et al., 2001; Stradi et al., 1997; Stradi et al., 1996) (Fig 1.1).

Extensive research has been conducted on both the function (Hill, 2002, 2006) and production (García-de Blas et al., 2015; Hill and Johnson 2012) of red carotenoid pigmentation in birds. Types and concentrations of carotenoid pigments have been characterized in internal tissues, including blood, fat, liver, and retina (Toomey and McGraw, 2010), as well as in external structures with ornamental coloration, including feathers, bills, and skin (McGraw et al., 2003;

McGraw et al., 2006; McGraw and Toomey, 2010). Among bird species in which carotenoids have been identified from colorful feathers and bare parts, about half display red coloration (McGraw 2006). Despite this interest in red carotenoid coloration in songbirds, the site of carotenoid metabolism remains uncertain and contentious. The liver has been proposed as the likely site of metabolism (del Val et al., 2009a, b), but that hypothesis has been disputed (McGraw 2009) as universally applicable based on the observations that integumentary pigments are absent from liver and plasma in some species (García-de Blas et al., 2015; McGraw, 2004). More recently it was proposed that hepatic carotenoid metabolism should occur within or in close association with mitochondria, because (1) of evolutionary relationships between carotenoids and quinones (Johnson and Hill, 2013), (2) oxidation potential is adequate at the inner mitochondrial member (Johnson and Hill, 2013), (3) the mitochondria is closely linked to mechanisms for transport and packaging of lipids and lipid-like molecules including carotenoids (Hill and Johnson, 2012), (4) there are plausible oxidation sites such as Complex III that could serve as a location for carotenoid oxidation (Johnson and Hill, 2013), and (5) carotenoids are known to be transported to the mitochondria in some bird species (Mayne and Parker, 1986).

Here, we studied pigment accumulation in the livers of male house finches (*Haemorhous mexicanus*) that were undergoing molt and hence synthesizing ketocarotenoids from dietary carotenoids. Our goal was to describe the specific subcellular locations of carotenoids in a putative site for production of ornamental red pigments. Male house finches have extensive carotenoid plumage coloration that varies from red to yellow and that is developed in a relatively short window of time in the late summer during prebasic molt (Hill, 2002). Based on likely precursor-product relationships, it has been argued that house finches derive their red coloration by oxidizing primarily dietary β-cryptoxanthin into primarily the red ketocarotenoid 3-hydroxy-

echinenone (Inouye et al., 2001; McGraw et al., 2006). We collected wild molting male house finches and analyzed the carotenoid content of cellular fractions of homogenized liver to test a key assumption of the hypothesis that carotenoid pigments used for ornamental feather coloration are oxidized in hepatic mitochondria. Within this context, high concentration of ketocarotenoids would be expected inside the mitochondria, while the level of precursor β-cryptoxanthin should be much lower.

Materials and Methods

Collection of liver samples

In August 2014 four hatching-year molting House Finches were captured at a feeding station in Lee County, Alabama (32° 35' 46.480" N Latitude, -85° 29' 29.270" W Longitude). Males were killed by asphyxiation in a CO2 chamber following protocols approved by the Institutional Animal Care and Use Committee at Auburn University. The whole liver of each male was removed within 1 min of euthanasia, and within 20 s chilled on ice. Livers and extracts were kept on ice throughout the process of mitochondria isolation.

Mitochondrial preparation

Mitochondria were prepared by differential centrifugation, using the protocol of Trounce et al (1996) modified by Ingraham and Pinkert (2003). Within 20 s of dissection from carcasses (n=4), livers were rinsed and minced in mitochondria isolation buffer. During the entire procedure, each bird's liver was processed separately and all liver mitochondria preparations were kept at 4℃. Minced livers were then homogenized with a 2 ml glass Teflon homogenizer at 300 mg tissue per ml of buffer. The crude homogenate was centrifuged twice at 750G for 10 min. Remaining

pellets were collected and re-suspended in 1 ml SMEE as Fraction 1. The 750G supernatant was centrifuged for 15 min at 9800G and the resulting supernatant was collected carefully as Fraction 2. The pellet was re-suspended slowly by drop-wise addition of SMEE. This suspension was then centrifuged for 10 min at 9800G again, and supernatant for this spin was collected as Fraction 3. The final purified mitochondria pellets were re-suspended in 1ml SMEE (Fraction 4) and stored at -80℃.

Immunocytochemistry - Western Blot Analysis

We used marker proteins to identify fractions isolated by centrifugation containing mitochondria. Total protein concentration for the various fractions was determined using Bradford assay (Bradford, 1976), with fat-free BSA as standard. A Shimadzu UV-2450 Spectrophotometer (Chiyoda-ku, Tokyo) was used to measure absorbance of the Bradford reagent at 595 nm. We made a standard curve of protein concentration that we used to estimate the protein content of isolated fractions.

Immunoblots of putative mitochondria pellets were performed using rabbit polyclonal antibody adenine nucleotide translocator proteins 1, 2, 3 and 4 (ANT1/2/3/4, Santa Cruz Biotechnology), which is a multi-pass membrane protein of the mitochondrial inner membrane that can be used to quantify mitochondria (Olvera-Sanchez, S. et al. 2011). Isolated mitochondria extracts were suspended in 1× phosphate buffered saline (PBS) solution and incubated in boiling water for 5 min to break the mitochondria membrane and denature the proteins released. Proteins (28.5µg/well) were separated by SDS-PAGE in 10% gels at 200 V for 40 min and transferred to polyvinylidene fluoride (PVDF) membranes via a semidry electrophoretic transfer cell at 20 V for 30 min. Membranes were washed 3 times for 5 min each with PBS containing 0.05%

Tween-20 (wash buffer), followed by blocking with 5% non-fat powdered milk in washing buffer for 90 min. Primary polyclonal antibody for ANT1/2/3/4 (Santa Cruz Biotechnology. Inc, USA) was diluted by 1/1000 in wash buffer respectively. Membranes were incubated with primary antibody at room temperature overnight (12hrs), followed by two 5-min washes. The secondary antibody (Goat anti-Rabbit IgG (Heavy & Light Chain) antibody (HRP)) was diluted to 1/5000 in wash buffer before being added to membranes for 40 min incubation. Membranes were then washed 3 times before visualization onto photographic x-ray film with Amersham™ ECL select™ (GE Healthcare, Pittsburgh, PA, USA) according to manufacturers' protocols.

Carotenoid Analysis

Methods for carotenoid extraction and high-performance liquid chromatographic (HPLC) analyses generally follow those in McGraw and Toomey (2010). We sequentially extracted carotenoids from 10 mL of each thawed liver fraction using ethanol and then 1:1 (v/v) hexane:methyl tert butyl ether, vortexing for 5 s after adding each solvent. We centrifuged the tubes, transferred the colored supernatant to a fresh tube, and evaporated the solution to dryness under a stream of nitrogen. We re-suspended residue in 200 μL HPLC mobile phase (42:42:16, methanol:acetonitrile:dichloromethane, v/v/v) and injected 50 μL into a Waters Alliance HPLC instrument (Waters Corp., Milford, MA) equipped with a reverse-phase C-30 YMC carotenoid column (YMC Co. Ltd., Kyoto, Japan) and a column heater set to 30° C. The column was pre-treated with 1% ortho-phosphoric acid to permit recovery of ketocarotenoids (Toomey and McGraw, 2007). Runs consisted of isocratic elution in the mobile phase for 11 min, followed by a linear gradient that finished at 42:23:35 methanol:acetonitrile:dichloromethane at 21 min; these conditions were held until 25 min, at which point the run returned to initial conditions until 29.5

min. Carotenoids were identified by comparison to standards and quantified using external standard curves. We detected lutein, β-carotene, and 3-hydroxy-echinenone in several forms (cis isomers plus in pure and esterified form), and all data are presented in 'carotenoids/10mg protein' (Palczewski et al., 2014).

Results

Identification of isolated liver fractions

Based on validations in previous studies of the standard protocols that we employed (Johnson et al., 1967; Prudent et al., 2013), we classified Fraction 1 that was deep brownish red, the same color as homogenized whole liver to contain mostly tissue debris and blood. The soluble fraction of the second centrifugation step, Fraction 2 should contain microsomes, fat, soluble debris, broken mitochondria membrane and other organelle debris. Fraction 2 had a slight orange color. Fraction 3, a second supernatant is proposed to be the suspension of similar cellular fraction residues from Fraction 2, was pale in color with the lowest protein concentration among all fractions, suggesting that most microsomal and cytosolic components were removed after the first high-speed spin. Finally, we took Fraction 4 to be the crude mitochondria pellet. It was orange-red in color. Support for Fraction 4 being a mitochondrial pellet came from direct observation of the fraction using transmission electron microscopy (TEM). The TEM image shows dense and unbroken mitochondria in Fraction 4 (Fig 1.2). We also used polyclonal antibody for ANT1/2/3/4 which is a mitochondrial marker to test fractions for mitochondrial content. ANT1/2/3/4 increased in concentration through the extraction steps with the highest

concentration in Fraction 4, the mitochondria pellet (Fig 1.3). Few total proteins were detected in Fraction 3, so it was not included in western blot.

Carotenoid Concentration

Carotenoids were detected in three fractions, but the highest concentration was in Fraction 4, the crude mitochondrial pellet (Fig 1.4). Approximately 85% of the total carotenoids, as well as 67% of the total ketocarotenoids, were found in the crude mitochondrial pellet. Ketocarotenoids were nearly twice as abundant in the crude mitochondrial fraction as were other carotenoids, suggesting that ketocarotenoids were located either within hepatic mitochondria or closely associated with them.

Discussion

Red pigmentation of the feathers and bare parts of birds has been the focus of intensive research by behavioral and evolutionary biologists interested in understanding the function and evolution of ornamentation (Hill, 2002; Hill and McGraw, 2006). The physiological mechanisms that underlie the production of ornamental coloration are central to hypotheses related to the signal content of red coloration (Hill, 2011; Hill and Johnson, 2012; García-de Blas et al., 2015). Yet, key aspects of the production of red coloration remain unstudied. Here we made the first attempt to determine the carotenoid content of subcellular fractions of hepatic tissue and determine the site of accumulation of metabolically produced ketocarotenoids in a songbird that uses these pigments for integumentary coloration. We detected high concentrations of carotenoids in the mitochondria of hepatocytes, which turned out to be 15 times higher (carotenoids/10mg protein) than in the mitochondria of spleen and brain of these same birds, and 10 times higher than in the mitochondria of heart (Ge and Hill unpublished data). These observations suggest that the liver is

a major site of ketolation or storage of the red carotenoid pigments used by songbirds to color feathers. In other orders of birds and especially for carotenoid coloration of bare parts, ketolation may occur at sites other than the liver, such as the integument (García-de Blas et al., 2015). More comparative data are certainly needed.

Our data suggest that the subcellular location of carotenoid ketolation is either within mitochondria, on the outer membrane of mitochondria, or in close association with Golgi apparatus-linked mitochondrial associated ER membranes (MAMs). As we proceeded toward more purified hepatic mitochondria through rounds of centrifugation, ketocarotenoid concentrations in the mitochondrial layer increased, such that the enriched mitochondrial fraction had the highest levels of ketocarotenoids. Microscopic examination of this fraction also showed a high density of unbroken mitochondria. Because of the limits of the mitochondria isolation technique, there was undoubtedly loss and damage of intact mitochondria in each round of centrifugation, leading to carotenoids leaking from ruptured mitochondrial membranes. The first high speed supernatant (Fraction 2) would be expected to have the highest concentration of carotenoids released from the breakage of mitochondria due to the attraction of carotenoids to the high fat content of debris, and that is what we observed.

High concentration of ketocarotenoids in mitochondria of hepatocytes is consistent with a key prediction of the hypothesis that oxidation of dietary carotenoids occurs in mitochondria of liver cells (Hill and Johnson 2012, Johnson and Hill 2012) and that the liver is a primary site of red carotenoid ketolation in red cardueline finches (del Val et al. 2009a,b). This conclusion, however, rests on the assumption that a high concentration of ketolated carotenoids is indicative of the site where the ketolation occurred. In our study, low levels of β-cryptoxanthin were detected in crude mitochondria pellets suggesting the presence of the substrate for ketolation;

almost no β-cryptoxanthin found in other fractions (data not presented). Low levels of β-cryptoxanthin have also been detected in plasma in a recent analysis of plasma of molting House finches from Arizona (Giraudeau and McGraw 2014), suggesting the possibility of rapid conversion from β-cryptoxanthin to 3-hydroxy-echinenone. More data are needed to demonstrate that hepatic mitochondria are the primary site of metabolism of the red pigments used to color the feathers of songbirds. Even if technically difficult, an informative approach would be to use radiolabeled carotenoids or stable carotenoid tracers to track production pathways for carotenoids (Scheidts et al. 1999).

Our observations of high concentrations of carotenoids within or in intimate association with the mitochondria of the hepatic cells of molting hatching-year male House Finches have important implications for the cellular mechanisms that regulate the production of ornamental plumage coloration. Ornament elaboration is a function of the redness of plumage display in the house finch and other birds (Hill, 1996) and plumage redness is a function of the ratio of ketocarotenoids versus xanthophylls deposited in feathers (Inouye et al., 2001; McGraw et al., 2006). If carotenoid ketolation occurs within or on the outer surface of mitochondria, then carotenoid oxidation will likely to be linked to core electron transporting events including cytochrome b5 and vitamin C reduction on the outer mitochondrial membrane and oxidative phosphorylation on inner mitochondria membrane. The efficiency of the ketolation process is likely to reflect core electron transport efficiency and this should be reflected in plumage coloration (Hill, 2011; Johnson and Hill, 2013). Females can potentially assess the efficiency of cellular respiration of prospective mates by assessing plumage redness (Hill 2014). This hypothesis could be tested directly by relating the redness of growing feathers to respiratory efficiency.

In future studies, it would be informative to obtain more highly purified hepatic mitochondria from density centrifugation to better address the direct correlation between carotenoids ketolation and functional status of mitochondria of avian hepatic cells.

Figures and Legends

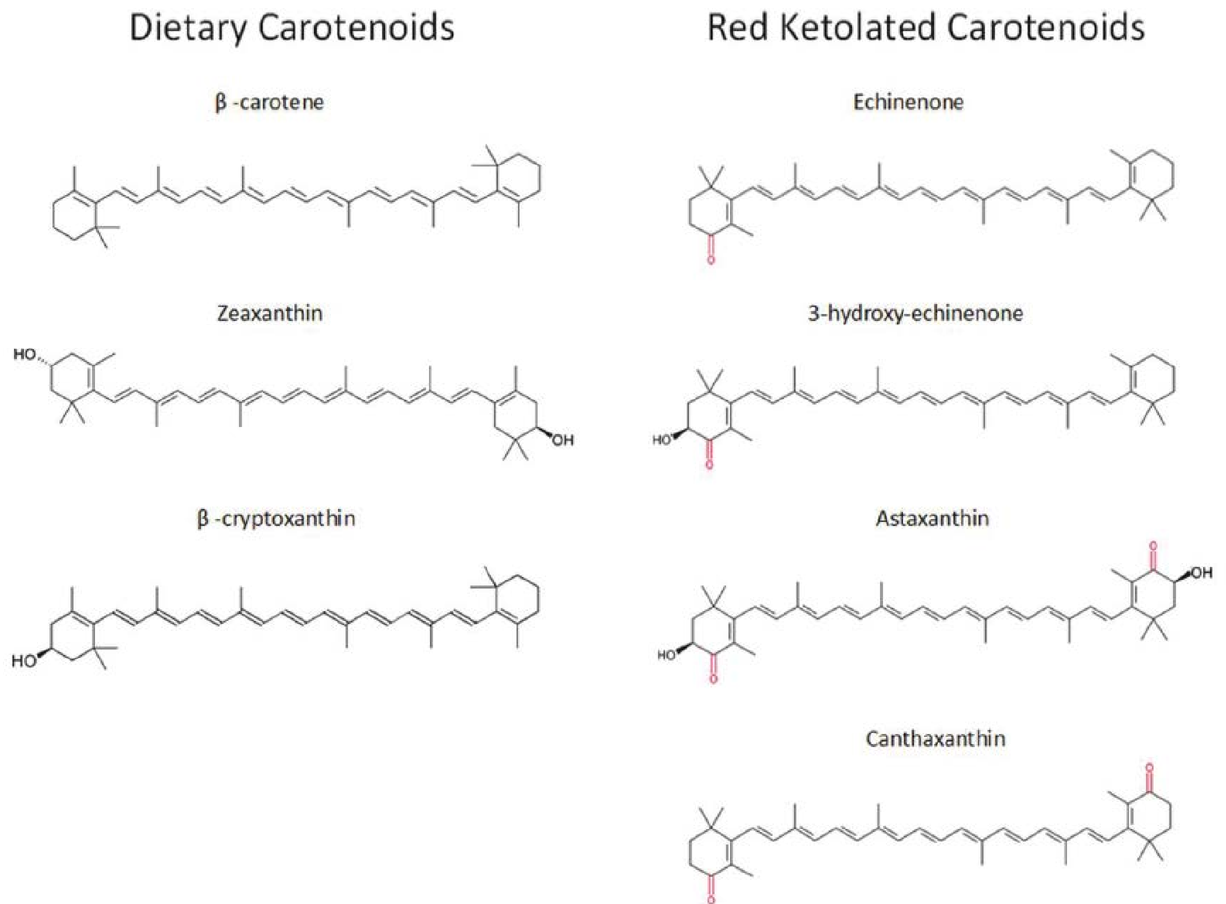

Figure 1.1 The chemical structure of the primary carotenoids that are in the diets of songbirds and that are metabolically derived through ketolation reactions. The red portions of the figure indicate the positions of ketone groups that are the product of biochemical modification within birds.

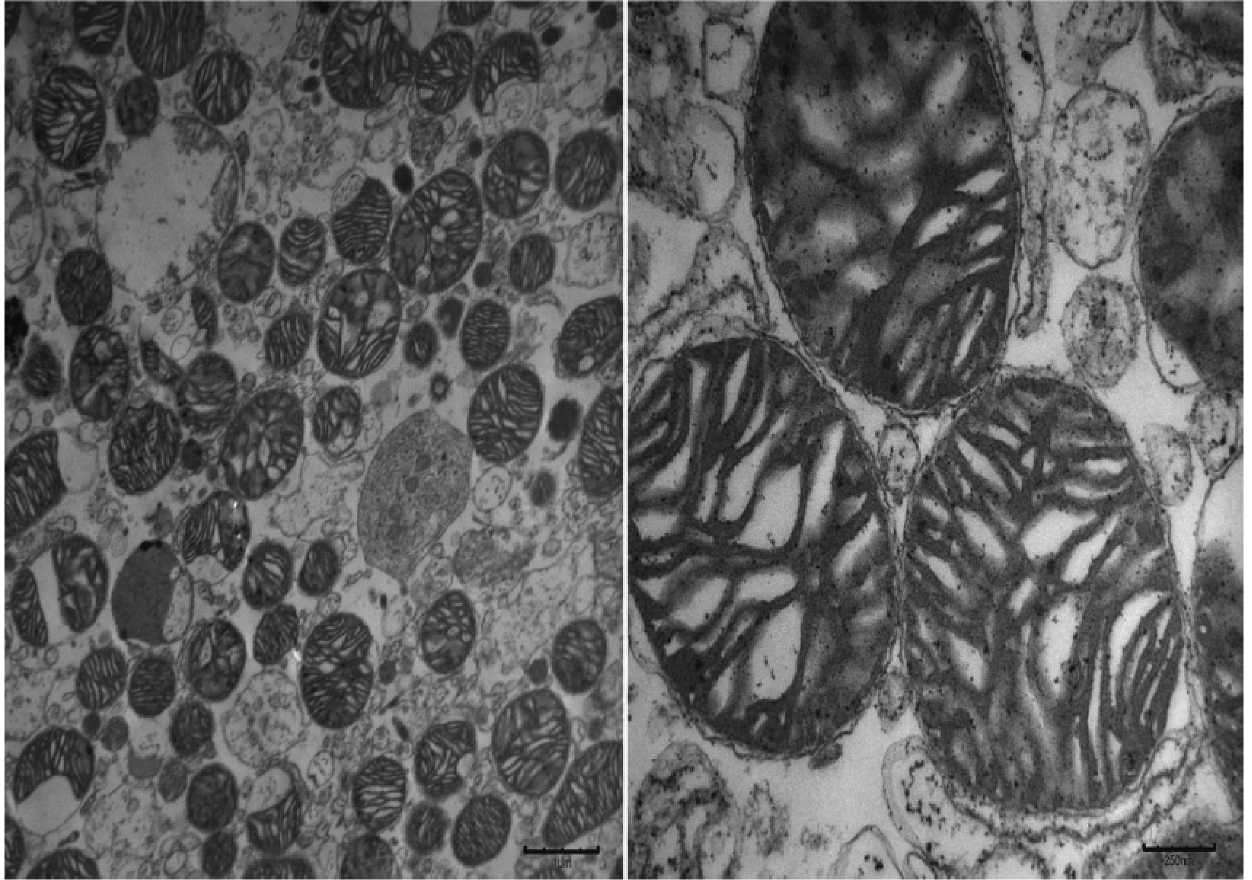

Figure 1.2 Images of Fraction 4 in the mitochondrial isolation series used in this study, made with a transmission electron microscope at a magnification of ×12,500 (left) and ×50,000 (right). Mitochondria appear unbroken and are present in high density.

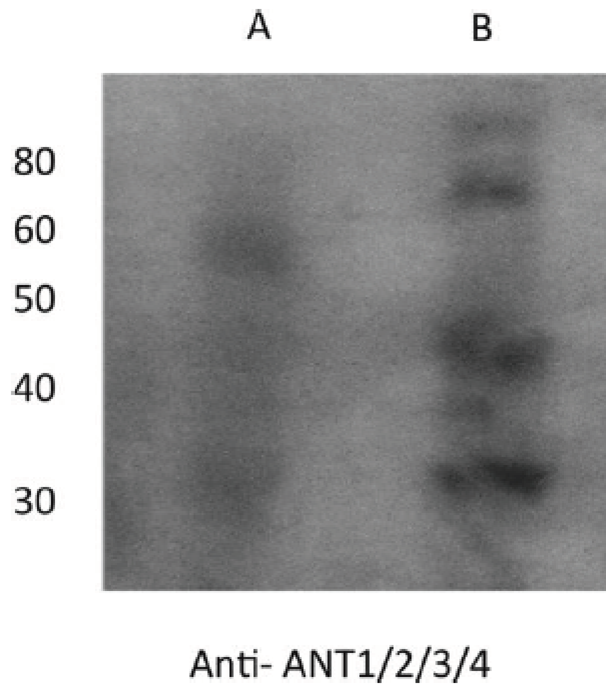

Figure 1.3 Immunoblot analysis of the mitochondrial protein ANT1/2/3/4 in fractions from mitochondrial isolation protocol. Lane A is the supernatant from the initial high speed spin (Fraction 2) and Lane B is the enriched mitochondrial pellet (Fraction 4). Equal protein loading as determined by Bradford assay. The darker staining in Lane B indicates a higher concentration of mitochondria in Fraction 4.

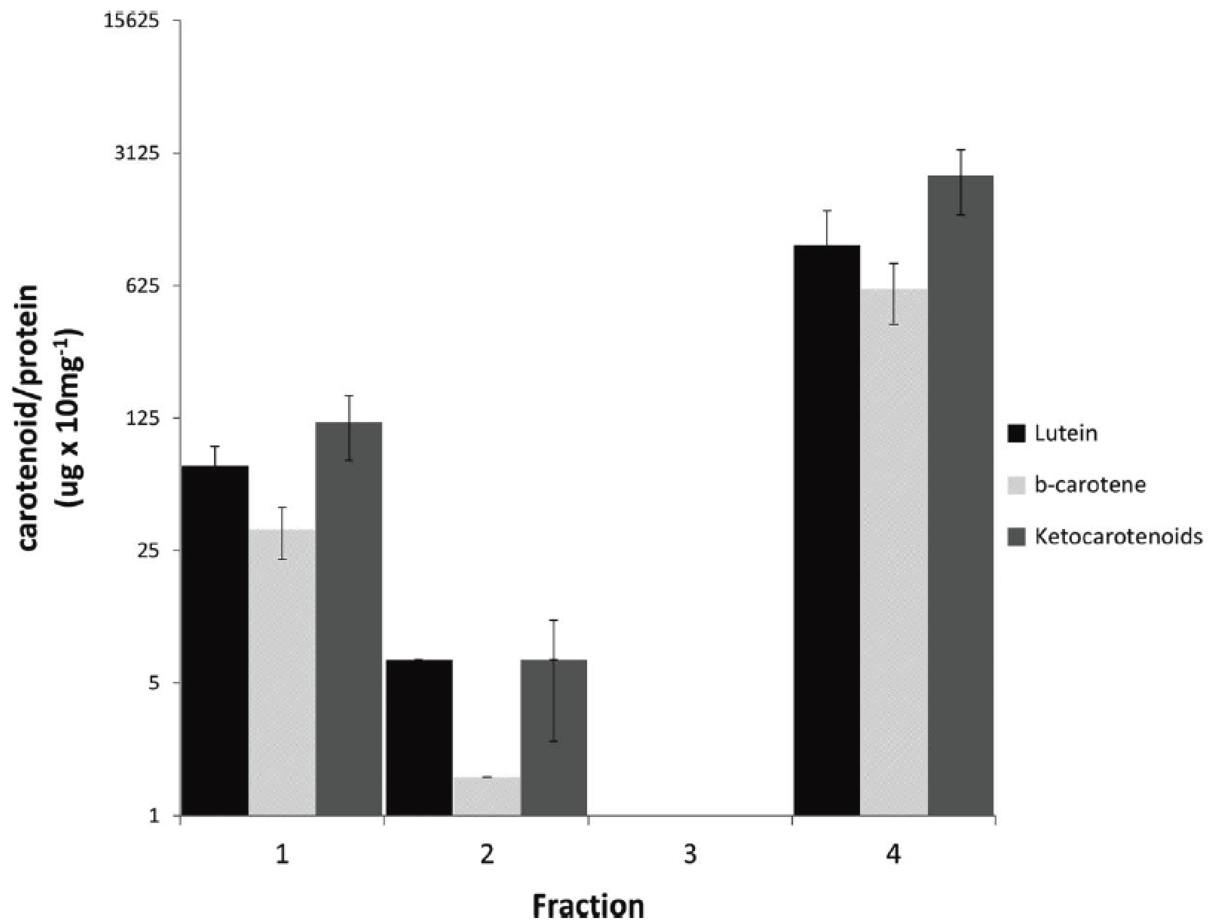

Figure 1.4 The concentration of carotenoids in four fractions from liver tissue of molting male house finches. Carotenoids were found in three fractions. The crude mitochondria pellets (Fraction 4) had the highest concentration of ketocarotenoids. Data represents means ± SD of 4 birds/data point.

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Chapter 2

Plumage redness signals mitochondrial function in the House Finch

Main Text

Red carotenoid coloration is an important social signal in many species of animals. Individuals that display color that is shifted toward longer wavelengths gain a mating advantage or hold more resources (Svensson and Wong, 2011). In addition, carotenoid coloration is among the most commonly cited examples of a condition-dependent signal of individual quality (Hill, 2006; Blount and McGraw, 2008). Compared to animals with less red ornamentation, individuals with redder ornaments are better at resisting and recovering from parasites, managing oxidative stress, and providing resources to mates and offspring, among other measures of individual performance (Svensson and Wong, 2011; Blount and McGraw, 2008; Hill, 2014).

Most animals that display red carotenoid coloration ingest only yellow carotenoids (McGraw, 2006), which they must oxidize using a ketolase enzyme to produce red pigments (Lopes et al., 2016; Mundy et al., 2016) (Fig 2.1). Hypotheses for how carotenoid coloration serves as signals of individual condition have traditionally focused on resource limitations, invoking either the challenge of accruing scarce pigments (Hill, 1991) or the need to trade off use of pigments for physiological processes over ornamentation (Alvarez et al., 2008) More recently, it has been proposed that ketolation of yellow dietary pigments takes place in mitochondria and that inexorable link between mitochondrial function and carotenoid ketolation makes red carotenoid coloration an uncheatable signal of condition (Johnson and Hill, 2013; Hill, 2014).

The discovery of CYP2J19 as the gene that codes for the ketolase enzyme that catalyzes the oxidation of yellow dietary carotenoids to red ornamental carotenoids in birds (Lopes et al., 2016; Mundy et al., 2016) provided support for the hypothesis that oxidation of yellow dietary carotenoids might be linked to cellular respiration in mitochondria. CYP2J19 is a cytochrome P450 enzyme that belongs to a subfamily of enzymes, CYP2s, that are frequently bimodally targeted to endoplasmic reticulum and mitochondria (Avadhani et al., 2011). Locating the production of red pigments in mitochondria in general and the inner mitochondrial membrane in particular could make the production of red pigments responsive to the redox state and mitochondrial membrane potential and hence make red carotenoid coloration a reliable signal of cellular respiration (Johnson and Hill, 2013) and overall individual condition.

To explore the location of carotenoid metabolism in songbirds, we undertook a study of molting house finches, a species in which carotenoid-based red feather coloration has been well documented to be a condition-dependent signal of male condition (Hill, 2002). These molting birds were actively growing and pigmenting feathers. In a previous study, we documented that high levels of red keto-carotenoids are associated with mitochondria in the livers of molting house finches (Ge et al., 2015) implicating mitochondria as a site of carotenoid metabolism. In this previous study, however, we could not rule out the possibility that carotenoid metabolism occurred in mitochondrial associated membranes external to mitochondria. To test whether keto-carotenoids are localized inside mitochondria, we used high-speed centrifugation to separate inner mitochondrial membrane and mitochondrial matrix from outer membrane and intermembrane space. Protein markers confirmed separation of mitochondrial components (Fig 2.2A-C). Our marker of mitochondrial matrix, citrate synthase, can also bind to the inner mitochondrial membrane as indicated by its equal abundance in both inner membrane and matrix.

The red keto-carotenoid 3-hydroxy-echinenone, which is the main pigment creating the red coloration of house finches (Inouye et al., 2001), was present in all fractions, and it was at greatest concentration in the inner membrane fraction compared to either the outer membrane or matrix (Fig 2.2D). There are no hypothesized functions for carotenoids in mitochondria of vertebrates; indeed, they are actively prevented from entering the mitochondria of mammals (Lobo et al., 2012). Concentrations of ketolated carotenoids in the inner mitochondrial membrane and matrix of house finches implicate the inner membrane as the site of the oxidation of yellow carotenoids into red pigments used in sexual displays.

Carotenoid ketolation is predicted to be dependent on local redox condition (Johnson and Hill, 2013; Hill and Johnson, 2012). In the mitochondria of liver cells, redox conditions arise in large part from the relative performance of the electron transport system (ETS) in the inner mitochondrial membrane (Brand and Nicolls, in press). Thus, we predicted that the redness of the feathers of an individual house finch, which is determined by the ketolation of yellow dietary pigments to red carotenoids (Hill and Johnson, 2012), would depend on the performance of the electron transport system (ETS) in the inner mitochondrial membrane. Specifically, we predicted that there would be a positive association between the hue of an individual's feathers and both the respiratory control ratio (RCR) and mitochondrial membrane potential (MMP) of the ETS in its liver mitochondria. RCR is a measure of mitochondria's relative coupling ability when oxygen, ADP, and substrate are abundant (Brand and Nicholls, in press). Low RCR values are associated not only with reduced energetic performance but also with disease and senescence (Porter et al., 2015).

We tested these predictions by capturing from a wild population and sacrificing molting male house finches, quantifying the coloration of ornamental carotenoid pigmentation on

growing feathers, and isolating intact and functional mitochondria from liver. Thus, we compared the quality of carotenoid ornamentation currently under production to key measures of cellular respiration (Lopes et al., 2016). As predicted if red carotenoid coloration is associated with mitochondrial function, we found significant positive associations between the hue of growing feathers and both RCR and MMP (Fig 2.3A, B). Redder birds had demonstrably better mitochondrial function. Variables that significantly contributed to a backwards regression model to predict male plumage hue included the RCR (Fig 2.3A), state II respiration (Fig 1S) and MMP when provided complex I substrates (Fig 2.3B), state IV respiration when provided complex II substrate, PGC-1a (Fig 2.3D), a transcriptional coactivator that regulates mitochondrial biogenesis, and 4-HNE adducts (Fig 2.3C), which is a marker of lipid peroxidation. Taken together, these observations indicate the birds that are capable of generating redder feathers have higher performing mitochondria but also greater lipid oxidative damage.

The observations that redder birds display higher mitochondrial membrane potential, have greater proton leak when respiring the carbohydrate substrate, and have higher state IV respiration while using lipid substrate suggest that a higher MMP is favored during ketolation (Johnson and Hill, 2013). Control of proton leakage and higher state IV respiration could also play a role in maintaining the appropriate redox conditions for ketolation (Hill and Johnson, 2012). Proton leak plays an important role in regulating the production of reactive oxygen species. By the uncoupling-to-survive hypothesis (Brand, 2000), individuals with greater leak may experience lower oxidative damage, and consequently, live longer. Previous research showed that male house finches with redder plumage have higher survival than drab males (Hill, 1991).

While higher oxidative damage in redder animals seems counterintuitive, increasing evidence suggests that both ROS and 4-HNE adducts (a by-product of lipid peroxidation) serve as important signaling molecules that can benefit mitochondrial performance (Leonarduzzi et al., 2004). Given the highly reactive nature of ROS, it is not surprising that differences in lipid peroxidation but not hydrogen peroxide (a measure of ROS emission) where observed in this study. When ROS occur at modest levels, they can stimulate increased production of antioxidants that prevent oxidative damage, upregulate repair mechanisms that remove damage, and induce mitochondrial biogenesis that compensates for damage (Zhang and Hood, 2016). Because ROS can upregulate repair mechanisms that render modest levels of damage ephemeral (Zhang and Hood, 2016; Zhang et al., 2017), modest oxidative damage is not necessarily tied to the reduced condition of the mitochondria or the cell. The positive association between PGC-1a and plumage redness that we observed supports this idea. Birds with higher oxidative damage had higher RCR and lower PGC-1a, indicating that the mitochondria of red birds not only had higher respiratory function, but the mitochondria weren't being signaled for replacement (Lee and Wei, 2005). One interpretation of these data is that the poor performance of the mitochondria of dull birds induced signaling for generation of more mitochondria.

Linking ornamental feather coloration with mitochondrial function suggests a possible solution to a long-standing puzzle in evolutionary and behavioral biology: what maintains the honesty of signals of individual condition? (Weaver et al., 2017). Carotenoid coloration has been documented to signal a wide range of measures of individual performance such as foraging ability, overwinter survival, immune system function, predator avoidance, and cognition (Svensson and Wong, 2011; Hill, 2006). Mitochondria produce 90% of the energy available to birds and other animals as well as 90% of the free radicals that contribute to oxidative stress

(Lane, 2014). Mitochondrial function is intimately linked to immune system function (Koch et al., 2017), development of complex organ systems including the nervous system (28), and reproductive performance (Martikainen et al., 2017). Numerous inherited diseases are caused by mutations in both mitochondrial and nuclear genes that affect the ETS and mitochondrial function (Gorman et al., 2015). Linking the ornamentation used in mate choice to function of core respiratory processes provides a novel mechanistic explanation for why carotenoid coloration relates to a range of aspects of individual performance and why females use plumage redness as a key criterion in choosing mates. While the implications of our results are exciting, at this point we have strong correlations between mitochondrial function and red coloration. The next step in testing the shared pathway hypothesis will be experimental manipulation of mitochondrial function, such as uncoupling the inner mitochondrial membrane or impairing the function of specific OXPHOS complexes, to more directly test the hypothesis that production of red carotenoid coloration is linked to aerobic respiration.

Materials and Methods:

Field collection

We captured wild house finches at feeding stations using a walk-in basket trap as described in (Hill, 2002). All birds included in this study were males in the hatching year that were in active molt. These birds were transitioning from juvenal to first basic plumage via prebasic molt. In the juvenal plumage, house finches have no feathers with carotenoid pigmentation, so any red, orange, or yellow feathers in the breast plumage of birds had recently been grown. In this way, we could match the current physiological state of birds to ornamentation that they had just produced. We recorded the stage of molt of each bird on a five-point scale.

We captured birds between 0700 and 1000 on eleven mornings at 7 locations in Lee County Alabama between July 20 and August 20, 2017. Our protocol was to rapidly approach a trap holding birds—finches are undisturbed in a trap until approached— and then to quickly move the birds from trap to brown paper bags. Paper bags enable birds to stand in a non-stressful position and block all threatening visual and most auditory stimulation (Hill, 2002). Thus, birds remained relatively calm between the time of capture and a maximum of three hours later when we removed them from the bag. Birds were taken from the bags and immediately anesthetized with isoflurane vapors and then sacrificed to collect tissues for physiological analyses. All procedures in this study were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2016-2922).

Color analysis

The coloration of growing breast feathers was quantified from digital images of the ventral plumage of carcasses that each included a color standard. We used Adobe Photoshop color sampler tool (Adobe Photoshop CS3 extended, v. 10.0, Adobe Systems, San Jose, CA, USA) to quantify the hue and saturation of feathers with carotenoid pigmentation at three points in each photo. We first measured hue of the color standard and found minimal variation between photos. We then quantified carotenoid coloration of each male. Molting birds have uneven coloration and we focused color quantification on the largest unbroken patches of red/orange/yellow coloration where feathers were positioned to create a plane of coloration relative to the camera angle. Color patches were preselected before moving the selection wand over them and then the wand was moved to the center of the chosen patch and hue and saturation recorded. The PI who made these measurements was blind to the physiological measures for the birds. We averaged

the three-color measurements to arrive at a single hue and saturation value for each bird. Our hypotheses focused on the ketolation of carotenoids, which affects the hue of coloration so a priori we focused our analysis on the hue of feathers.

Tissue collection

Upon sacrifice, the liver was quickly removed from each bird and weighed. The livers of seven males were pooled for fractionation of mitochondria. Livers and extracts were kept on ice throughout the process of mitochondria isolation. For the other 37 males used in this study, a small cube of the right lobe of the liver was removed, flash frozen in liquid nitrogen, and then stored at -80ºC for later analyses. The left and remaining right lobe of the liver was used for mitochondrial isolation and immediate testing of mitochondrial function.

Fractionation of mitochondria

Within 20 s of dissection from carcasses (n = 7), livers were rinsed and minced together in mitochondria isolation buffer. During the entire procedure, all liver mitochondria preparations were kept at 4°C. Minced livers were then homogenized with a 2-mL glass Teflon homogenizer at 300 mg tissue per milliliter of buffer. Following the protocol in (Trunce et al., 1996) as modified by (Ingraham and Pinkert, 2003), the homogenate was centrifuged at 750 g for 10 min. The supernatant was then centrifuged at 9,800 g for 15 min. The resulting supernatant was discarded. The pellet was resuspended slowly by drop-wise addition of SMEE. This suspension was then centrifuged again at 9,800 g for 10 min. The pellet was resuspended in SMEE buffer and layered on top of 30% Percoll SMEE solution and subjected to ultracentrifugation at

150,000g for 60 min. The mitochondrial-rich layer was carefully harvested and washed 3 times with centrifugation at 7000g for 10 min in SMEE at 4°C.

For submitochondrial fractionation, we followed a method previously described by (Palczewski et al., 2014), with slight modification. Isolated mitochondria were diluted with SMEE to a concentration of 50 mg mitochondrial protein /ml. Digitonin was added to a final concentration of 0.12mg/mg protein. The solution was stirred on ice for 2 min and then diluted with 1.5 volume of SMEE. The solution was then centrifuged at 12,000g for 10 min. The supernatant, which contained the outer mitochondrial membrane was saved. The pellet, which contained mitoplast fraction was resuspended in SMEE and then sonicated in an ice bath for 30s (4s on, 10s off cycles). The sonicated material, as well as the previously saved supernatant, were then ultracentrifuged at 150,000g for 60 min at 4°C.

We predicted that the ultracentrifuged pellet from the mitoplast fraction would be inner mitochondrial membrane, while the supernatant from the same fraction would be matrix. With this conservative method, however, instead of having just inner mitochondrial membrane, the pellet should be considered more a mixture of mostly inner mitochondrial membrane and some matrix content. And pellet from digitonin treated supernatant contained outer mitochondrial membrane. Fractions were then verified by immunoblot against carnitine palmitoyltransferase 1A (CPT1A), citrate synthase (CS) and COX IV.

Mitochondria activity

Mitochondria were isolated following procedures outlined previously (Zhang et al., 2017; Kuzmiak et al., 2012). The fresh liver was minced and then homogenized in a Potter-Elvhjem PTFE pestle and glass tube. The resulting homogenate was centrifuged and the supernatant was

then decanted through cheesecloth and centrifuged again. The resulting supernatant was discarded, and the mitochondria pellet was washed in liver isolation solution. This solution was again centrifuged, and the final mitochondria pellet was suspended in a mannitol-sucrose solution.

Mitochondrial respiration was determined polarigraphically (Oxytherm, Hansatech Instruments, UK) following procedures outlined previously (Zhang et al., 2017; Kuzmiak et al., 2012). In one chamber, respiration was measured using 2 mM pyruvate, 2 mM malate, and 2 mM glutamate as a substrate. In the second chamber, respiration was measured using 5 mM succinate as a substrate. State II respiration was defined as the respiration rate in the presence of substrates, maximal respiration (state III) was defined as the rate of respiration following the addition of 0.25 mM ADP to the chamber containing buffered mitochondria and respiratory substrates, and state IV respiration was defined as the respiration rate measured after the phosphorylation of added ADP was complete. State II, III, and IV respirations were measured at 40℃ and were normalized to mitochondrial protein content. Respiratory control ratio (RCR) was calculated by dividing state III respiration by state IV respiration.

Mitochondrial membrane potential was measured as described by (Lambert et al., 2008). Briefly, mitochondrial membrane potential was followed using the potential-sensitive dye safranin O. Isolated mitochondria were incubated in standard buffer containing 3 mM HEPES, 1 mM EGTA, 0.3% (W/V) BSA, 1 μg/ml oligomycin, and 120 mM potassium chloride (pH = 7.2 and 40°C). Mitochondria were incubated at a concentration of 0.35 mg/ml mitochondrial protein in standard buffer with 5 μM safranin O. The change in fluorescence were measured in cuvette by Spectramax M (Molecular Devices, Sunnyvale, CA) at an excitation of 533 nm and an emission of 576 nm. In the end of each run, membrane potential was dissipated by addition of 2

μM FCCP. The relative decrease in fluorescent signal on energization of the mitochondria is used to represent the membrane potential. Results are reported as the absolute magnitude of this change in fluorescence, with larger changes in relative fluorescence units (RFU) indicating higher membrane potentials.

The measurement of H2O2 emission in isolated mitochondria was conducted using Amplex Red (Thermofisher, Waltham, MA)(Miwa et al., 2016). Formation of resorufin (Amplex Red oxidation) by H2O2 was measured at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a Synergy H1 Hybrid plate reader (BioTek; Winooski, VT, USA), at 40ºC in a 96-well plate using succinate. To eliminate carboxylesterase interference, 100 μM of phenylmethyl sulfonyl fluoride were added into experimental medium immediately prior measurement according to (Miwa et al., 2016). Readings of resorufin formation were recorded every 5 minutes for 15 minutes, and a slope (rate of formation) was produced from these. The obtained slope was then converted into the rate of H2O2 production using a standard curve and were normalized to mitochondrial protein levels.

Western blots were conducted on liver samples to analyze a marker of lipid peroxidation (4-Hydroxynonenal; 4-HNE; ab46545; Abcam, Cambridge, MA), and a marker of protein oxidation (protein carbonyls; OxyBlot; s7150; EMD Millipore, Billerica, MA and marker of mitochondrial biogenesis (PGC-1a, GTX37356; Genetex, Irvine, CA). Each membrane was stained by Ponceau S, and was used as the loading and transfer control. A chemiluminescent system was used to visualize marked proteins (GE Healthcare Life Sciences, Pittsburgh, PA). Images were taken and analyzed with the ChemiDocIt Imaging System (UVP, LLC, Upland, CA). Citrate synthase activities was measured in liver homogenate as a function of the increase in absorbance from 5,5′-dithiobis-2-nitrobenzoic acid reduction. We used backward stepwise

selection (SigmaStat 3.5, Systat Software, Inc., Point Richmond, CA, USA) procedure for multiple regression to evaluate the relationship between hue and physiological markers. Significance was established at α=.05.

Analyses of tissue carotenoid content

We used high performance liquid chromatography (HPLC) to examine the carotenoid content of mitochondrial fractions proposed to be IMM, OMM, matrix. The pelleted fractions were resuspended in 0.5 ml mitochondrial extraction buffer, and we subsampled 50 µl of the resuspension and extracted with 250 µl of ethanol, 500 µl water, and 1.5 ml of hexane:tert-Butyl ethyl ether 1:1 vol:vol. We collected the organic phase from the samples, dried this under a stream of nitrogen gas, then resuspended in 200 µl of mobile phase. We injected 50 µl of the resuspended extract in to an Agilent 1100 series HPLC equipped with a YMC carotenoid 5.0 µm column (4.6 mm × 250 mm, YMC). We eluted the samples with a gradient mobile phase consisting of acetonitrile:methanol:dichloromethane (44:44:12) (vol:vol:vol) through 11 minutes then a ramp up to acetonitrile:methanol:dichloromethane (35:35:30) from 11-21 minutes followed by isocratic conditions through 35 minutes. The column was held at 30°C, and the flow rate was 1.2 ml/min throughout the run. We monitored the samples with a photodiode array detector at 400, 445, and 480 nm, and carotenoids were identified by comparison to authentic standards or published accounts. Carotenoid concentrations were determined based on standard curves established with astaxanthin (for ketocarotenoids) and zeaxanthin (for xanthophylls) standards.

Figures and Legends

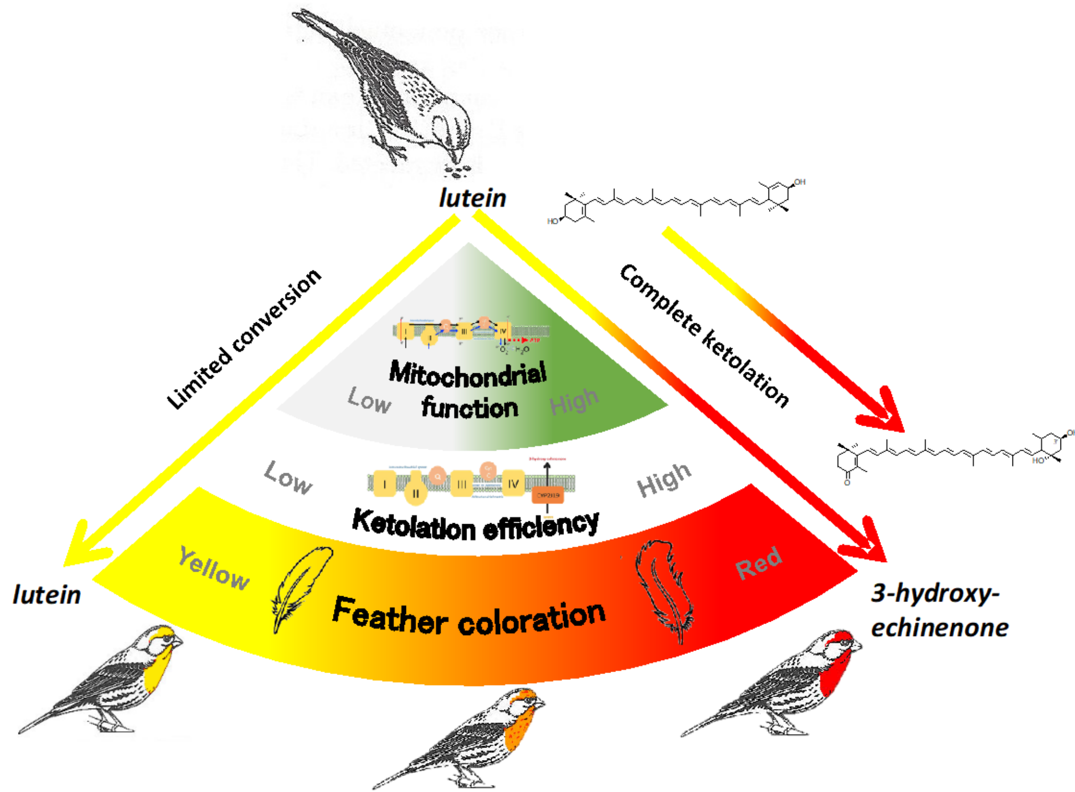

Figure 2.1 A schematic summary of the hypothesized links between red feather coloration and mitochondrial function. To produce red feathers, songbirds ingest yellow dietary carotenoids such as lutein and oxidize the yellow pigments via a ketolase enzyme. We hypothesized that ketolation efficiency is linked to the function of the electron transport system and the inner mitochondrial membrane potential. By this hypothesis, birds with poor mitochondrial function also have poor ketolation capacity and produce yellow feathers. Birds with high mitochondrial function have excellent capacity to ketolate carotenoids and produce red feathers.

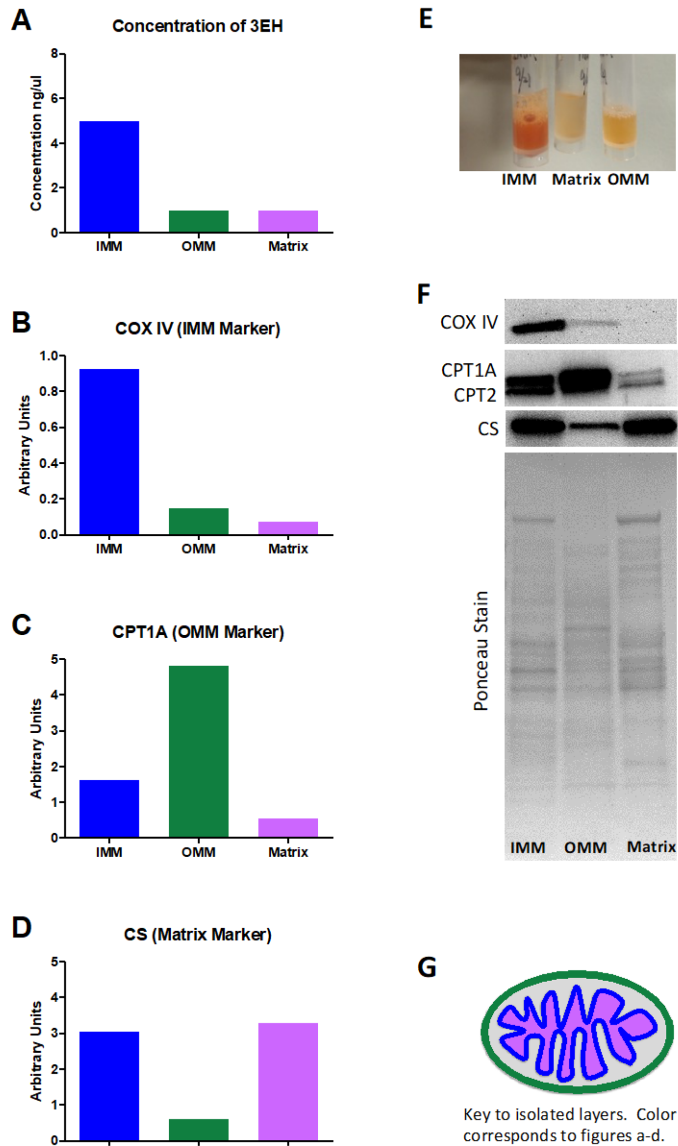

Figure 2.2 Carotenoid content of inner and outer mitochondrial membranes and the mitochondrial matrix with fraction identities confirmed by region-specific proteins. (A) The concentration of the red carotenoid 3-hydroxy-echinenone (3HE) in each of three fractions of mitochondria isolated from house finch livers. The relative 3HE content of each fraction was also apparent visually (E). (B) The relative levels of COX IV, a protein of the electron transport system in the inner mitochondrial membrane (IMM), (C) carnitine palmitoyltranferase 1 (CPT1), a marker of the outer mitochondrial membrane (OMM), and (D) citrate synthase (CS), a citric

acid enzyme located primarily in the matrix that also binds to the IMM. (F) The Western blots with Ponceau staining from which protein concentrations were estimated. The stylized illustration of a mitochondrion (G) provides a color key to organelle locations.

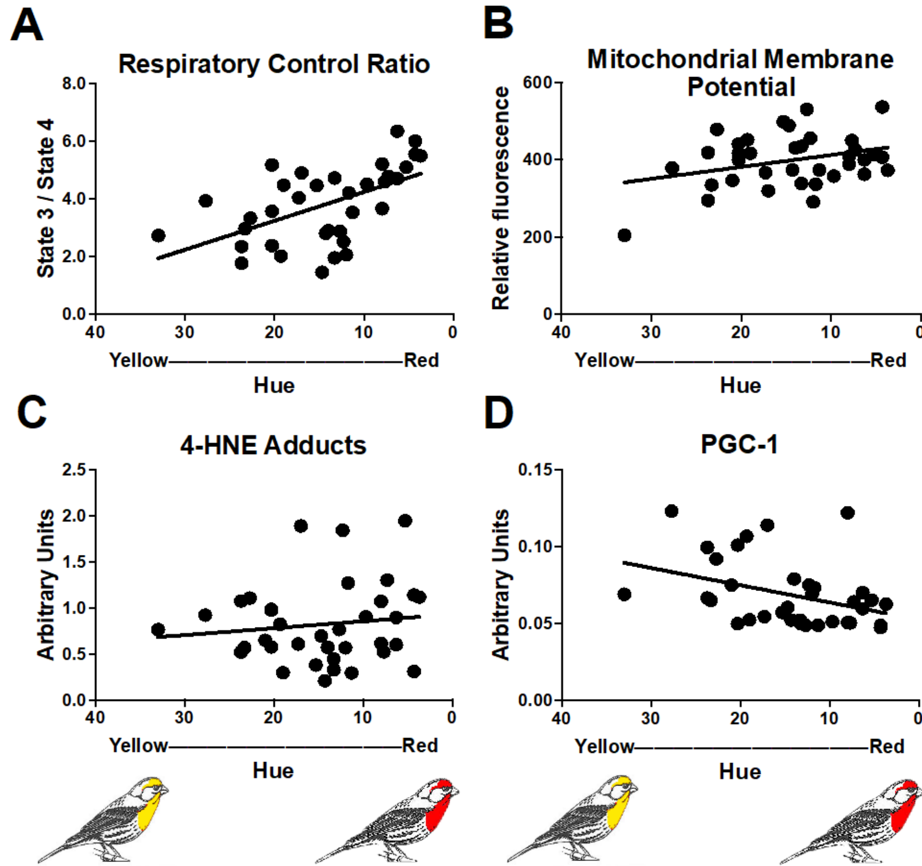

Figure 2.3 The relationship between feather redness and mitochondrial respiratory capacity, membrane potential, lipid peroxidation, and PGC-1a. The relative contribution of liver mitochondrial variables to the plumage coloration of wild caught house finches were evaluated using backward stepwise regression. Variables included in the model are described in he methods section. The final model (F=9.32, df=6.25, P < 0.001) included (A) potential (7,19) (P=0.028), (C) relative level of lipid peroxidation (4-HNE adducts; P=0.031)(22), and (D) PGC-

1a protein level (P=0.002)(25). State II respiration with complex I substrate and state IV respiration with complex II substrate were also significant predictors of carotenoid coloration.

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Chapter 3

Variation in carotenoid metabolism by season and sex in the house finch (*Haemorhous mexicanus*)

Introduction

Carotenoids are the most common source of yellow, orange and red coloration in the feathers and bare parts of birds (Fox, & Vevers, 1962;Hill & McGraw, 2006). Carotenoids cannot be synthesized by animals *de novo*, thus birds have to acquire these pigments from their diet (Volker, 1938;Yokoyama, 1982;Goodwin,1984). Birds cannot synthesize carotenoids, but they can modify the pigments that they ingest (Brush, 1978). Most birds that use red carotenoids in color displays acquire the red pigments by metabolizing yellow dietary pigments into red (Schiedt, 1998;Brush, 1990). Although these carotenoid pathways have been studied for decades via analyses of dietary precursors and integumentary products (Hudon, 1991;Stradi, 1998;McGraw et al., 2001), only recently did researchers identify the potential subcellular location of carotenoid ketolation in birds when high concentration of ketocarotenoids were isolated from the hepatic mitochondria of house fines (*Haemorhous mexcianus*) (Ge et al., 2015). Finding high concentrations of ketolated carotenoids in the hepatic mitochondria of male house finches during molting season was a breakthrough in avian color research, but it also raised further questions about the process of carotenoid ketolation. First, initial observations were made exclusively on males that were in the process of molting feathers, which is the period when carotenoid pigmentation occurs. However, the period of molt occupies less than 10% of the annual cycle of House Finches and other birds (Pimm, 1976;Hill, 2002). For a temperate bird species like the house finch, which breeds seasonally, physiology can change drastically and rather rapidly as individuals transition into and out of breeding condition. For example,

testosterone levels drop quickly after a breeding season, and a drop in testosterone can cause seasonal changes in aggressiveness and song rate (Nolan et al., 1992;Kurvers et al., 2008). Components of an animal's body can also be redistributed between seasons; for instance, bone is demineralized and fat is mobilized during lactation in small mammals (Kovacs, 2001; Prentice, 2000;Hood et al., 2006). Carotenoids have been recognized as having multiple important biological functions in living organisms such as antioxidants, immune booster and photoprotective agents (Mezzomo & Ferreira, 2016; Schroeder & Johnson, 1993; Young, 1991;McGraw et al., 2011), so re-allocation of carotenoids across seasons seems likely. Thus, an obvious and important question with regard to carotenoid metabolism is whether house finches maintain high concentratin of ketocarotenoids in their hepatic mitochondria throughout the year.

Second, in earlier research we focused exclusively on liver tissue because theoretical work suggested that the liver would be a primary site of ketolation (Hill & Johnson, 2012). However, other research groups have published data that suggests tissues other than liver can be the site of carotenoid ketolation (McGraw, 2004, 2009). In McGraw's work, liver and blood samples from 11 colorful bird species with carotenoid-based coloration were examined and no ketocarotenoids were detected in either liver or plasma, suggesting integumentary tissues like skin might be the site for ketolation. Therefore, it was important to also survey for ketocarotenoids in tissue other than liver in house finches to better understand the pigmentation strategies of these birds.

And finally, because the house finch is sexually dichromatic songbird in which males have bright red coloration while females are drab, previous studies on the coloration of house finches targeted colorful males (Brush & Power, 1976; Mcgraw et al., 2006). However, female coloration is also of interest. There are similar seasonal patterns for changing hues of plasma in

both male and female house finches (Hill, 1995) suggesting changes in the levels of circulating carotenoids. Researchers have studied a variety of factors that might affect female coloration, including age, availability of dietary carotenoids, parasites, and body conditions in both controlled experiments and wild populations (Potti & Merino, 1996; Hill, 1993a, 1993b,2000). Although female house finches lack the intensive red coloration of males, some female house finches show a wash of red coloration in their feathers (Hill, 1993a, 1993b, 2010). The degree to which female House Finches concentrate red ketocarotenoids in the mitochondria of their liver cells, however, is unknown.

To address these basic questions regarding carotenoid metabolism, we conducted a longitudinal survey in male house finch by including individuals from both molting and non-molting season, and we examined a variety of tissues that might be the alternative sites for ketolation in these males. We also collected molting females from the field to compare the ketocarotenoid status in both males and females so that we can deduce whether carotenoid ketolation is a sex-specific event. Our goal was to better understand the physiology that underlies carotenoid ornamentation in house finches and improve our overall knowledge of carotenoid ketolation in wild birds.

Materials and Methods

Bird collection

We captured wild house finches at feeding stations using a walk-in basket trap described in previous studies (Geoffrey E. Hill, 2010).All molting birds included in this study were in their hatching year that were actively replacing their feathers in their first prebasic molt, and the sex of the females were confirmed using PCR (Dubiec & Zagalska-Neubauer, 2006).

In total 31 molting males, 11 molting females and 14 non-molting male house finches were collected for this project between 0700 and 1000 am at 7 locations in Lee County Alabama between summer 2014 and winter 2017. After blood collection, birds were immediately anesthetized with isoflurane vapors and then sacrificed to collect tissues, all these were done within 3 hours upon capture. All procedures in this study were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2016-2922)

Plasma and tissue collection

Approximately 100 ul of blood was collected from the brachial vein of each bird, and the plasma was separated by centrifuging at 3000 rpm for 10 mins. Plasma was then stored at -80C until carotenoid analysis. Upon sacrifice, tissues were quickly removed from each bird and weighed. Livers and extracts were kept on ice through the process of mitochondria isolation. Other tissues were kept on ice through the process of homogenization and were stored in -80C upon carotenoid extraction.

Mitochondria extraction

Mitochondria were isolated following procedures outlined previously (Mowry et al., 2016). The fresh liver was minced and then homogenized in a Potter-Elvhjem PTFE pestle and glass tube. The resulting homogenate was centrifuged, and the supernatant was then decanted through cheesecloth and centrifuged again. The resulting supernatant was discarded, and the mitochondria pellet was washed in liver isolation solution. This solution was again centrifuged, and the final mitochondria pellet was suspended in a mannitol-sucrose solution.

Carotenoid extraction

Carotenoids were extracted following previously described protocols with slight modification (Sassani et al., 2016). For plasma, 10ul of plasma was combined with 150ul of acetone and

centrifuged at 3000 rpm for 10 min. The supernatant was collected and dried off in nitrogen. For homogenized tissues, we subsampled 50 ul of the homogenates and extracted with 250 ul ethanol, 500ul H2O, and 500ul of hexane:tert ethyl ether 1:1 (v:v). We then centrifuged it at 3000 rpm for 10min, collected the organic phase from the samples and the dried it off in nitrogen. All samples were stored at -80C until analysis.

Carotenoid analysis

Target ketocarotenoids were separated using a Shimadzu HPLC system from a 50 µL injection on to a Sonoma C18 column (10 µm, 250 × 4.6 mm, ES Technologies) fitted with a C18 guard cartridge. We used mobile phases A 80:20, methanol: 0.5 M ammonium acetate, B 90:10, acetonitrile:H2O and C ethyl acetate in a tertiary linear gradient as follows: 100% A to 100% B over 4 min, then to 80% C: 20% B over 14 min, back to 100% B over 3 min and returning to 100% A over 5 min and held for 6 min (Wright et al., 1991). Total run time was 32 min at a flow rate of 1 mL min −1. Absorbance was measured at 450 nm using an ultraviolet (UV)/visible detector. Ketocarotenoids were identified and quantified by comparison to authentic standards. And all tissue sample data were presented in carotenoids/mg protein.

Results

As in previous studies (Brush & Power, 1976; Inouye et al., 2001),we were able to identify 3-hydroxy-echinenone as the dominant ornamental ketocarotenoids in house finch. The highest concentration of 3-hydroxy-echinenone was found in liver of molting male house finches. Modest level of 3-hydroxy-echinenone was also detected in the skin of molting males. Other organs of molting male house finches had either trace levels or not detectable levels of ketocarotenoids (Fig 3.1).

In contrast to the high levels of 3-hydroxy-echinenone in both the liver and blood of molting male house finches, we detected no ketocarotenoids in either plasma or liver in non-molting males. When not molting, house finches were found to be completely devoid of ketocarotenoids (Fig 3.2). And similar patterns were also observed for dietary carotenoids. The level of dietary carotenoids in the liver and plasma dropped precipitously from molting to non-molting seasons. Dietary carotenoids were present in modest levels in hepatic mitochondria during molt but at barely detectable levels in non-molting individuals (Fig 3.3).

The pattern of distribution of both dietary and keto-carotenoids among tissues were very similar between male and female house finches during molting season. The highest concentration of ketocarotenoids were all detected in liver, or more specifically in liver mitochondria (Fig 3.4). However, the concentration of ketocarotenoids detected in males was almost 13 folds higher than in females. However, the levels of dietary carotenoids detected in the tissues of males and females were not significantly different (Fig 3.5).

Discussion

In previous studies, we detected high concentrations of ketocarotenoids in liver mitochondria in molting male house finches, implicating liver mitochondria as a primary site of carotenoid ketolation in house finches (Ge et al., 2015). Here we confirmed that observation. The levels of ketolated carotenoids in liver tissue was more than four times higher than the levels that we measured in integumentary tissue, the only other tissue with appreciable quantities of carotenoids. These observations further implicate the liver as the primary site of carotenoid ketolation. Given the relatively high levels of ketocarotenoids that we also observed in integumentary tissue, however, we cannot rule out feather follicles as a secondary site of

carotenoid metabolism. Alternatively, the integument at the site of feather growth may be a site of concentration of ketolated carotenoids that were metabolized in the liver, with no ketolation at the integument in House Finches. These alternative hypotheses can be tested tracking isotope- or raido-labelled precursor carotenoids in future work.

By comparing ketocarotenoids data from molting and non-molting male house finches, we showed that this carotenoid ketolation was completely shut down during non-molting seasons, with no ketocarotenoids detected anywhere in those non-molting males including plasma. This was consistent with the visual assessment of hue of plasma throughout the year (Hill, 1995) in which the hue of male plasma was observed to increase sharply during molting season and then decline to a pale yellow, like the plasma of mammalian blood that has no ketolated carotenoid, after molting ended. However, reasons for such pattern can be more than just a simple demand and supply relationship. Other factors may also play a role in this seasonal regulation of carotenoid ketolation. Melanin, another common pigment in bird's world, is known to be affected by endocrine factors like testosterone (Bókony et al., 2008;Fargallo et al., 2007).Similarly, the effect of testosterone on coloration was also seen in experiments conducted in house finch (Stoehr & Hill, 2001) in which testosterone implantation disrupted the carotenoid pigmentation of feathers.

Observations from fish and mammals suggest that carotenoids can become reactive and cause harm within mitochondria (Lietz et al., 2012). In mice, reactive oxygen species (ROS) was induced when a key gatekeeping gene (*bco2*) that prevents carotenoids from accumulating in mitochondria got knocked out. In zebrafish, when carotenoid accumulated in mitochondria blood cell apoptosis was induced, leading to the cessation of embryonic development (Amengual et al., 2011; Lobo et al., 2012). And in captive experiments, high level of carotenoid accumulation was

linked to impaired skeletal muscle function and to muscle breakdown in American Goldfinch (*Spinus tristis*) (Huggins et al., 2010). Therefore, even though no obvious negative effect was observed from carotenoid accumulation inside mitochondria during molt, we cannot rule out the possibility of potential tissue or cellular damage if such high carotenoids levels are maintained beyond molting season.

Levels of dietary carotenoids levels both in circulation and in hepatic mitochondria were significantly lower in non-molting birds compared to molting male finches. This drop in circulating dietary pigments could reflect a change in diet between molting and non-molting season. It could also reflect a change in the way in which male house finches used dietary carotenoids. Carotenoid uptake is controlled by proteins including SR-B1 and CD36 (Toomey et al., 2017; Walsh et al., 2012). These uptake proteins could be regulated between seasons to reduce the levels of circulating carotenoids when they are not needed for ornamentation. Further experiments would be required to distinguish between these alternative explanations for changes in circulating dietary carotenoids between seasons.

We detected 3-hyroxy-echinenone in the tissue of both molting male and female house finches, indicating that both sexes were capable of ketolating carotenoids. The dichromatism of the sexes that is obvious in feather coloration was also evident in the levels of carotenoids that we observed. Males had much higher concentration of ketocarotenoids than females in their liver and liver mitochondria. Interestingly, dietary carotenoids were found to be present at similar levels between males and females during molting season. These observations suggest that differences in diets and access to precursor pigments is not the basis for the differences in color expression between male and female finches. It is still unclear why some females pigment feathers with ketolated carotenoid while most do not (Hill, 1993b). However, it seems that most

female house finches that grow feathers with ketocarotenoids are in their first breeding (Hill, 1993b). Recently, the efficiency of carotenoid ketolation has been predicted to be dependent on local redox condition, which again linked it to mitochondria condition (Hill, 2014; Johnson & Hill, 2013). And mitochondrial research done in mice have shown that life history events such as reproduction can alter mitochondria conditions like cellular respiration and oxidative damage load, with carryover effects months or even years after the event (Hyatt et al., 2017; Zhang & Hood, 2016). In house finches, molting season comes right after breeding season, so in their second and subsequent breeding cycles, females will have just completed breeding when they grow feathers (Hill, 2002). Effects of breeding may in some way inhibit ketolation of carotenoids in these birds. Alternatively, yearling and older females may pursue different strategies in social signaling (Hill, 2002).

In summary, carotenoid ketolation inside mitochondria can be carried out by both male and female house finches in liver mitochondria during molting season. However, ketolation is completely shut down outside the molting window. These results provided some firsthand data on the physiology underlies ornamental pigment production in house finches and could be really important for the future research in carotenoid-based coloration in other wild birds.

Figures and Legends

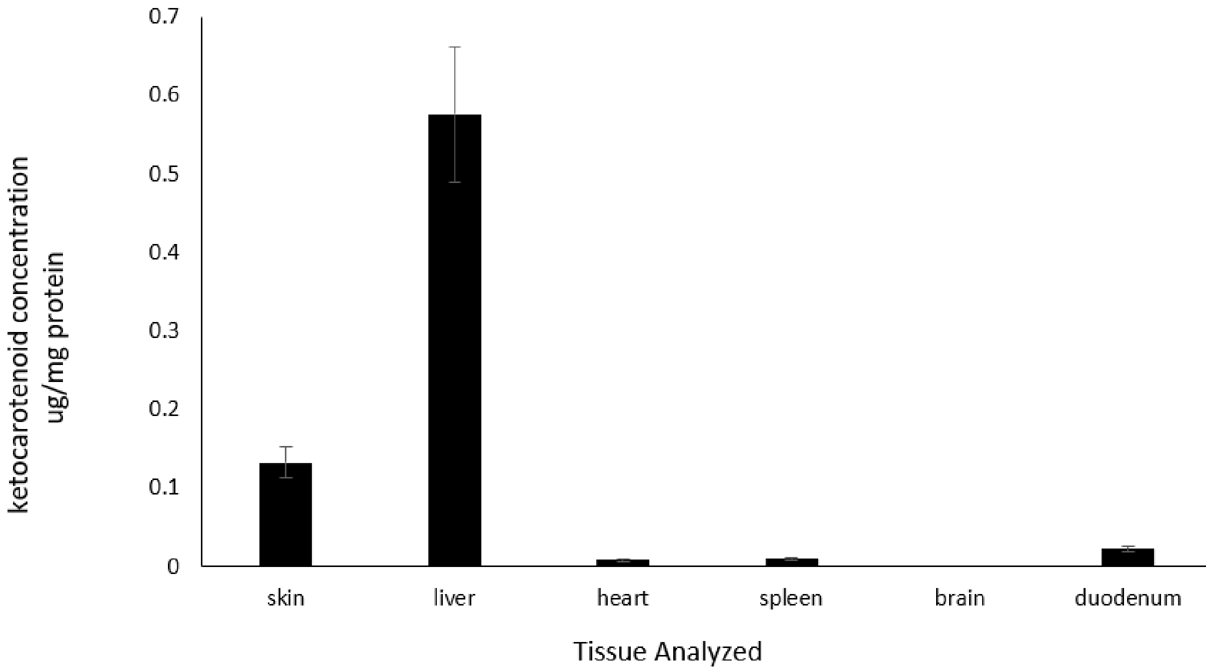

Figure 3.1 Distribution of 3-hydroxy-echinenone in molting male house finch. Liver was found to have the highest concentration of 3-hydroxy-echinenone.

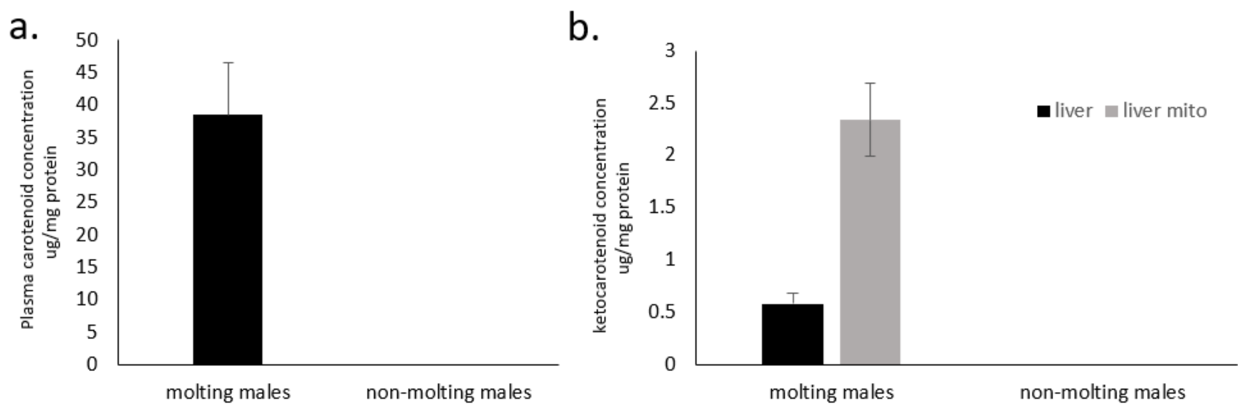

Figure 3.2 Comparison of the distribution of 3-hydroxy-echinenone between molting and non-molting male house finches. High levels of 3-hydroxy-echinenone were detected in

molting male house finches in both plasma(a.) and liver(b.). No 3-hydroxy-echinenone was detected in non-molting male house finches.

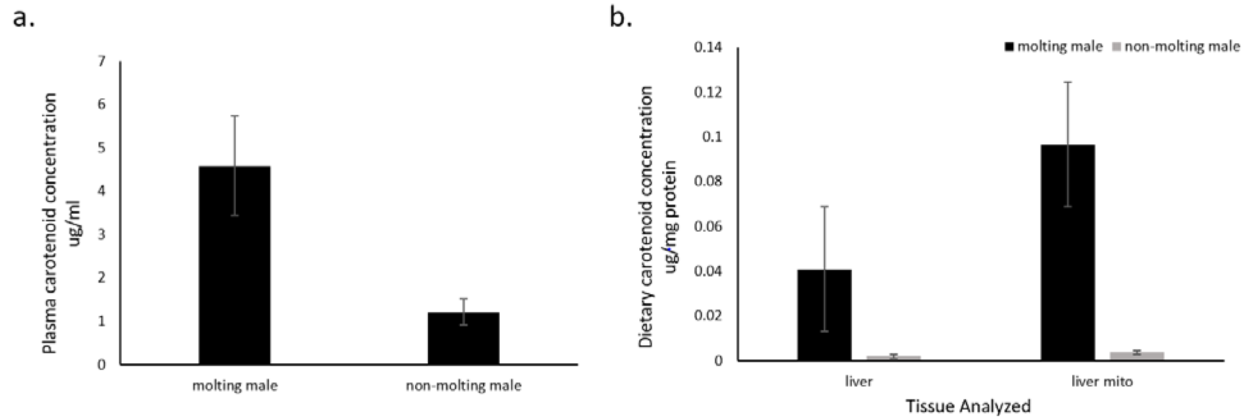

Figure 3.3 Comparison of the distribution of dietary carotenoids between molting and non-molting male house finches. Dietary carotenoids were detected in both molting and non-molting male house finches in both their plasma(a.) and liver(b.). While the concentration of dietary carotenoids in molting birds were significantly higher than those in non-molting birds.

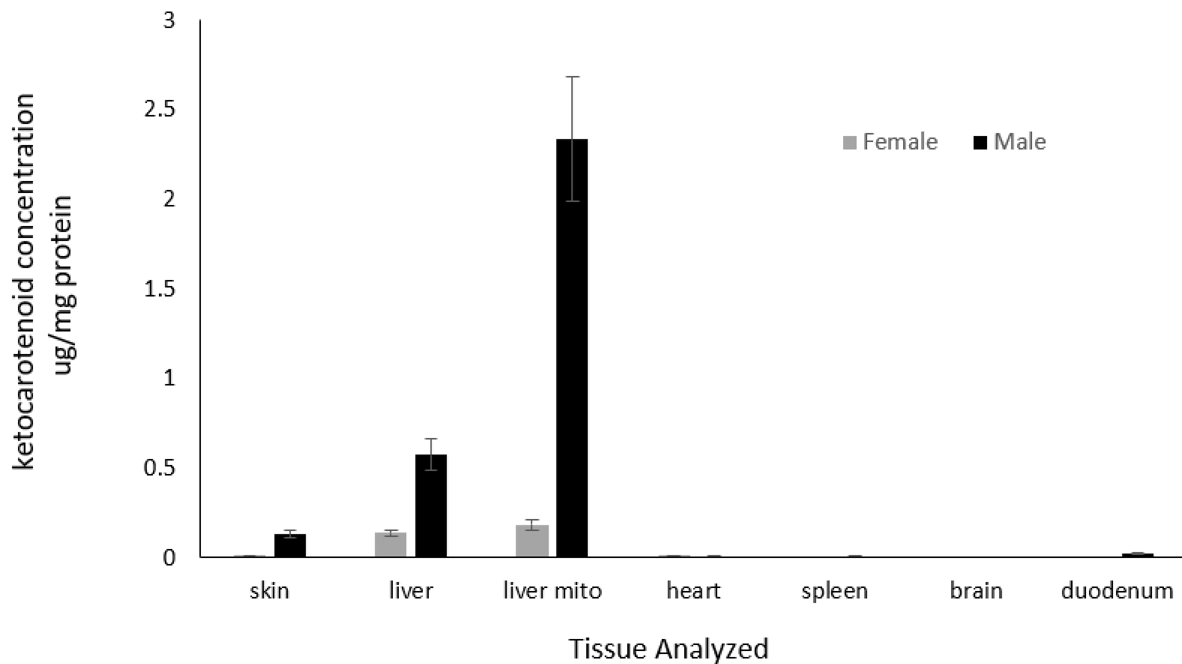

Figure 3.4 Comparison of the distribution of 3-hydroxy-echinenone in male and female house finches. Both male and female house finch showed similar pattern of ketocarotenoid distribution, with the highest concentration of ketocarotenoids were detected in liver mitochondria. But the absolute concentration of 3-hydroxy-echinenone in males (2.33±0.55ug/mg protein) were much higher than in females (0.18±0.06ug/mg protein).

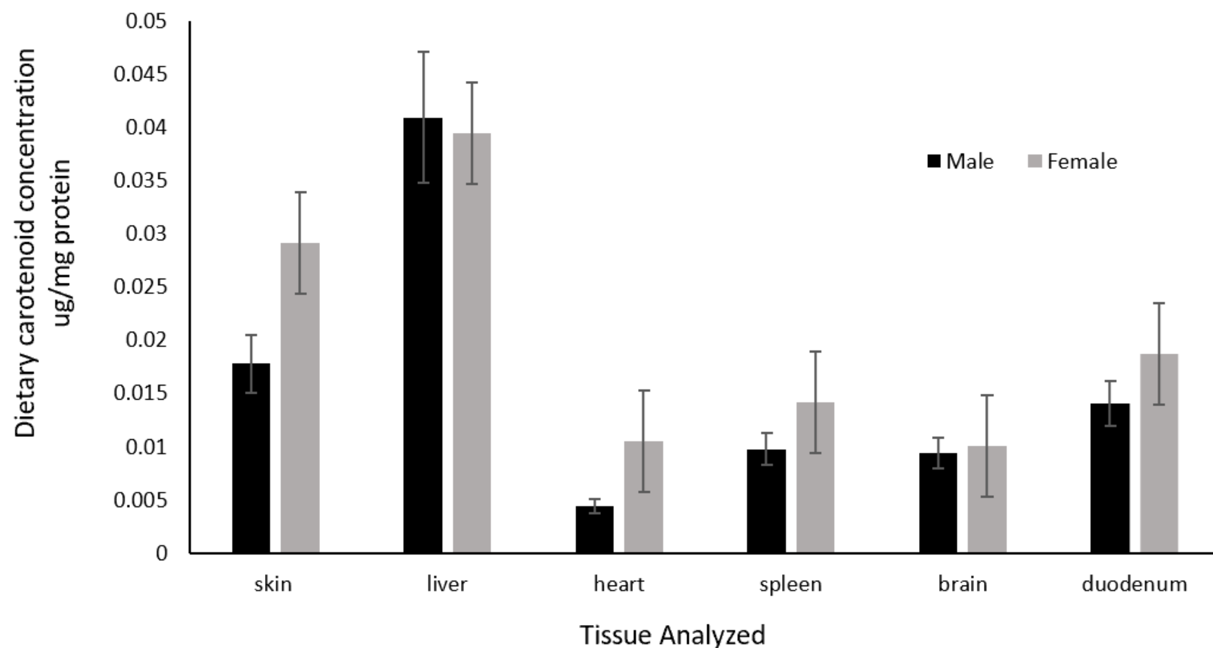

Figure 3.5 Comparison of the distribution of dietary carotenoids in male and female house finches. Both male and female house finch showed similar pattern of dietary carotenoids distribution as well as the levels of dietary carotenoids found in both sexes.

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Chapter 4

An investigation of tissues that support metabolism of red ketocarotenoids in wild birds with red feathers or skin

Introduction

Carotenoids are a group of naturally occurred organic pigments, which can be found in almost all living organisms and which are the primary source of red, yellow, and orange coloration in the feathers and bare parts of birds (Goodwin, 1980 ;Hill & McGraw, 2006). Vertebrates cannot synthesize carotenoids *de novo*, so birds with carotenoid-based color displays must obtain these pigments from their diet (Fox & Vevers, 1962; Milne & Milne, 1961). Birds ingest abundant carotenoids from their diets and the great majority of carotenoids in the diets of most birds are yellow pigments like lutein, zeaxanthin, beta-cryptoxanthin and beta-carotene (Stradi, 1998; McGraw et al., 2001).

Birds utilize two primary strategies in pigmenting integumentary structures with carotenoids: use dietary pigments unaltered or metabolic conversion of dietary pigments into modified carotenoid pigments before deposition (Brush, 1990; Stradi, 1998). For instance, many yellow songbirds like the Yellow-breasted Chat (*Icteria virens*) utilizes exclusively dietary lutein for feather coloration (Mays Jr. et al., 2004). On the other hand, red songbirds like House Finch (*Haemohorus mexicanus*) uses mostly red ketocarotenoids for their coloration, which are rare to non-existent in their diets (McGraw et al., 2001).These species of birds with red carotenoid displays ingest yellow dietary carotenoids as metabolic substrates that are converted them into more oxidized carotenoids before being deposited into their feathers (Schiedt, 1998;Brush, 1990). Despite of an extensive effort to understand carotenoid metabolism in birds (A H Brush,

1990; Brush & Power, 1976; Davies, 1985; Schiedt, 1989;Hill et al., 2002), fundamental questions remain unanswered.

The site of ketolation within the bodies of birds remains an open question. Liver has long been recognized as the most active organ in carotenoid metabolism in other vertebrates (Deming & Erdman, 1999; Eroglu & Harrison, 2013). Thus, it was also hypothesized as the most logical site of ketolation in birds (Brush, 1990). By this hypothesis, dietary carotenoids are enzymatically modified in the liver and with final products transported via bloodstream to integumentary tissues. Similar pathways have been proposed in multiples studies for explaining red coloration in red finches (Hill et al., 1994;Hill, 2000) and this hypothesis was supported by a carotenoid survey done in Common Crossbill (*Loxia curvirostra*) (del Val et al., 2009) in which researchers successfully detected ornamental ketocarotenoid in both plasma and liver. And in strong support of the hypothesis that the liver is a site of carotenoid ketolation, high concentrations of ketocarotenoids were also found inside the hepatic mitochondria in molting male house finch (Ge et al., 2015).

It remains uncertain whether the liver is the exclusive site of ketolation in the birds and whether all birds follow this pattern. The fact that multiple studies reported lack of metabolically derived carotenoid pigments in liver and plasma (Stradi, 1998; Inouye, 1999; McGraw, 2004) led to an alternative hypothesis for carotenoid metabolism in birds. Instead of liver, integumentary tissues like skin were proposed as the likely site for ketolation in some birds. The strongest support for this local ketolation hypothesis came from a study in which the carotenoid content of various tissues was analyzed for 11 songbird species with carotenoid-based coloration. Derived ornamental carotenoids were detected in the integumentary tissue, but none were detected in liver or plasma. Unfortunately, this survey relied on analytical methods that may have lacked the

sensitivity to detect small quantities of ketocarotenoids when they conducted this carotenoid survey. Using more sensitive analytical techniques, the same lab group reported the presence of circulating 3-hydroxy-echinenone in their later work (Toomey & McGraw, 2007).

Despite of the mixed results from various studies, rarely considered in discussions of the site of carotenoid metabolism is the type of color displays used by different species of birds. Many species of birds deposit carotenoids in what are often referred to as “bare parts”—the bare skin on legs or eyerings, the outer sheath of bills, or eyes. Some deposit carotenoids both in bare parts and feathers. While relatively fewer species deposit carotenoids in only feathers. Previous studies reporting ketocarotenoids in liver included exclusively birds with only feather ketocarotenoid coloration, while no ketocarotenoids were ever reported in the liver of birds with bare part red ketocarotenoid coloration.

To test whether the type of carotenoid display determines the site of carotenoid metabolism, we examined the carotenoid content of three species of wild birds with the latest HPLC protocol. We included in our study 1) Mourning Doves (*Zenaida macroura*), which have red ketocarotenoids in the skin of their legs and feet but no feather carotenoids coloration, 2) Northern Cardinals (*Cardinalis cardinalis*), which have red ketocarotenoids in both their bills and their feathers, and 3) House Finches (*Haemorhous mexicanus*), which have red carotenoids in their feathers but not in any bare parts. Our goal was to test the hypothesis that the site of carotenoid deposition determines the site of carotenoid metabolism.

Materials and Methods

Bird collection

We captured wild house finches, northern cardinals and mourning doves at feeding stations using either a walk-in basket trap or a modified rodent trap described in previous studies (Hill, 2002). All birds included in this study were males in the hatching year that were in active molt and thus actively metabolizing carotenoids and pigmenting structures. Our analyses included 31 molting house finches, 15 northern cardinals and 10 mourning doves that were collected for this project between 0700 and 1000 am at 2 locations in Lee County Alabama between summer 2014 and early spring 2018. All birds were kept in brown paper bags after being collected from the trap, and this also helped to keep them relatively calm through transportation. After blood collection, birds were immediately anesthetized with isoflurane vapors and then sacrificed to collect tissues, all these were done within 3 hours upon capture. All procedures in this study were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2016-2922)

Plasma and tissue collection

Approximately 100 ul of blood was collected from the brachial vein of each bird, and the plasma was separated by centrifuging at 3000 rpm for 10 mins. Plasma was then stored at -80C until carotenoid analysis. Upon sacrifice, tissues were quickly removed from each bird and weighed. Tissues were kept on ice through the process of homogenization and were stored in -80C upon carotenoid extraction.

Carotenoid extraction

Carotenoids were extracted following previously described protocols with slight modification (Sassani et al., 2016). For plasma, 10ul of plasma was combined with 150ul of acetone and centrifuged at 3000 rpm for 10 min. The supernatant was collected and dried in nitrogen. For homogenized tissues, we subsampled 50 ul of the homogenates and extracted with 250 ul ethanol, 500ul H_2O, and 500ul of hexane:tert ethyl ether 1:1 (v:v).Due to the difficulty of

homogenizing leg skin from the doves, carotenoids were extracted with 600 ul acetone ,600ul 0.02M KOH (dissolved in methanol) (4 hrs saponification), 250 ul H2O, and 500 500ul of hexane:tert ethyl ether 1:1 (v:v). We then centrifuged it at 3000 rpm for 10min, collected the organic phase from the samples and the dried it off in nitrogen. All samples were stored at -80C until analysis.

Carotenoid analysis

Target ketocarotenoids were separated using a Shimadzu HPLC system from a 50 µL injection on to a Sonoma C18 column (10 µm, 250 × 4.6 mm, ES Technologies) fitted with a C18 guard cartridge. We used mobile phases A 80:20, methanol: 0.5 M ammonium acetate, B 90:10, acetonitrile:H2O and C ethyl acetate in a tertiary linear gradient as follows: 100% A to 100% B over 4 min, then to 80% C: 20% B over 14 min, back to 100% B over 3 min and returning to 100% A over 5 min and held for 6 min (Wright *et al.*, 1991). Total run time was 32 min at a flow rate of 1 mL min^{-1}. Absorbance was measured at 450 nm using an ultraviolet (UV)/visible detector. Ketocarotenoids were identified and quantified by comparison to authentic standards. And all tissue sample data from house finch and northern cardinals were presented in carotenoids/mg protein. Data from mourning dove was presented in carotenoids/g tissue.

Results

With current HPLC protocol, we were able to detect and quantify most of the ketocarotenoids in our selected bird species. We confirmed 3-hydroxy-echinenone to be the dominant ketocarotenoids in house finch, while alpha-doradexanthin and astaxanthin were responsible for red coloration in cardinal. Carotenoids in mourning dove were examined for the first time, and

astaxanthin was found to be the major ketocarotenoids in the red pigmented skin of the leg and feet, along with an unidentified ketocarotenoid.

In house finch, the highest concentration of ketocarotenoids was detected in liver but with significant quantities also detected at the site of deposition in the skin (Fig 4.1).

In northern cardinal, the dominant ketocarotenoid is alpha-doradexanthin, followed by astaxanthin. These ketocarotenoids can be detected in multiple tissues, with slightly different distribution pattern (Fig 4.2). The highest concentration of astaxanthin was detected in skin, while concentration of alpha-doradexanthin were found similar in both liver and skin.

In mourning doves, ornamental ketocarotenoids were detected only in skin of the leg and feet (Fig 4.3).

Discussion

Consistent with previous studies, we found multiple likely sites of carotenoid metabolism within the bodies of the birds that we studied, with some interesting differences among species. Different red birds seemed to have different strategies for where to modify their dietary carotenoids. We found high concentration of ketocarotenoids in the liver of molting male house finches, which was consistent with what we found in our previous studies (Ge et al., 2015). These observations implicate the liver as the primary site for carotenoid ketolation in House Finches. However, we also detected significant quantities of ketolated carotenoids at the feather follicle, suggesting the carotenoid ketolation may also occur at the site of deposition.

In the mourning dove, ornamental ketocarotenoids were detected exclusively in the red skin of the legs and feet. No ketocarotenoids were detected in any other tissues that we tested including plasma, suggesting no transportation of ketocarotenoids. Taken together, these observations indicate that ketocarotenoids are produced and utilized locally in the integumentary

tissue in the Mourning Dove. This was consistent with an independent study done in red-legged partridge, a bird also grows red skin on their legs and feet, astaxanthin was only detected in bare parts but not in liver or plasma (García-de Blas, Mateo, & Alonso-Alvarez, 2015).

Northern cardinals have both red feathers and red bare parts in the form of red bills. Perhaps not surprisingly, Northern Cardinals appear to utilize multiple sites for carotenoid metabolism. We observed substantial quantities of ketocarotenoids in multiple tissues, with the highest concentration detected in both liver and skin. These observations suggest that cardinals may ketolate carotenoids in multiple tissues, including at the site of deposition and in the liver. This finding further supported a result from previous study of captive northern cardinals (McGraw et al., 2001b) that showed cardinals were capable of producing feathers pigmented with red ketocarotenoids from plain seed diet without extra carotenoid supplementation.

Somewhat surprisingly, we also detected ornamental ketocarotenoids in the gut content from these cardinals, suggesting the possibility of cardinals ingested some of their red pigments that pigment their bills and feathers. Red ketocarotenoids are generally considered rare in plant-based diet (Stradi, 1998; McGraw et al., 2001), but we cannot exclude the possibility of birds foraging on food source that contains certain ketocarotenoids during certain period of time like molting season to supplement their own red pigment production. The potential role of red dietary pigments in the production of coloration has been reported in multiple field and captive studies before (Hill, 1992; Kodric-Brown & Brown, 1984; Poyser et al., 1997;Grether et al., 2001). An alternative explanation for this finding might be the microbial source of carotenoids from the gut microbiota, however, little was known about the microbial environment in the gut of these birds and this may need further investigation in future research.

Recently a significant advance has been made in understanding the genetic control of ketolation of red pigments in birds. A gene called CYP2J19 was discovered in domestic birds (Lopes et al., 2016; Mundy et al., 2016). This gene allows birds to produce and display the color red by converting yellow dietary carotenoids into red ketocarotenoids. Our observations support and extend recent observations associated with the discovery of Cyp2J19 as the ketolase enzyme in birds. This gene was found to be highly expressed in birds with red feather and bill coloration, but the sites of this gene expression varied among bird species. In red factor canaries, which have red feathers, this gene was found to up-regulated in both liver and skin. In zebra finch, which has red carotenoids only in the bill, this gene was only up-regulated in beak tissue. Further genetic tests done on African weaver birds, which have red carotenoid pigments only in feathers, also reported that Cyp2J19 was found to be highly expressed in liver but not skin (Twyman et al., 2018). And unlike feather coloration, which can only be replaced during molt, changes in coloration of bare parts like beak can occur more rapidly and frequently throughout the year (Anderson, 1975). Some researchers believe that rapid changes in bare part coloration may reflect more dynamic changes than feathers (Ardia et al., 2010; Rosenthal et al., 2012; Velando et al., 2006) . Thus, producing and depositing pigments locally could be a more efficient means to achieve bare part coloration. For birds like cardinal, which has both bare part and feather coloration, both remote and local carotenoid ketolation systems may be active during molting. This can be further tested by including non-molting birds with red bare part carotenoid coloration, when tissues supporting feather coloration should be shut down.

Our work suggests that different bird species utilize different strategies for metabolizing dietary carotenoid. Birds were capable of producing red ketocarotenoids through different strategies at different sites. Future works can be done on more wild bird species with red

ketocarotenoid coloration to present a clearer pattern of red ketocarotenoid production and the potential implications of these different site choice.

Figures and Legends

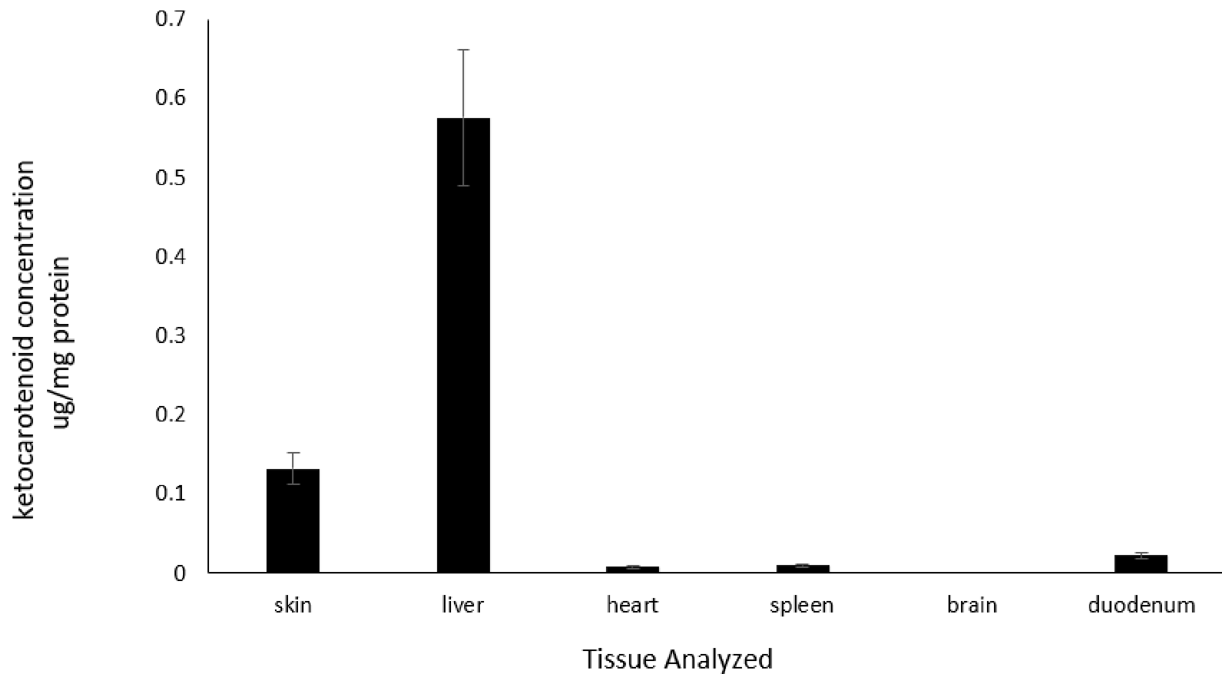

Figure 4.1 The concentration of 3-hydroxy-echinenone in various tissues in molting male house finch. Liver was found to have the highest concentration of 3-hydroxy-echinenone.

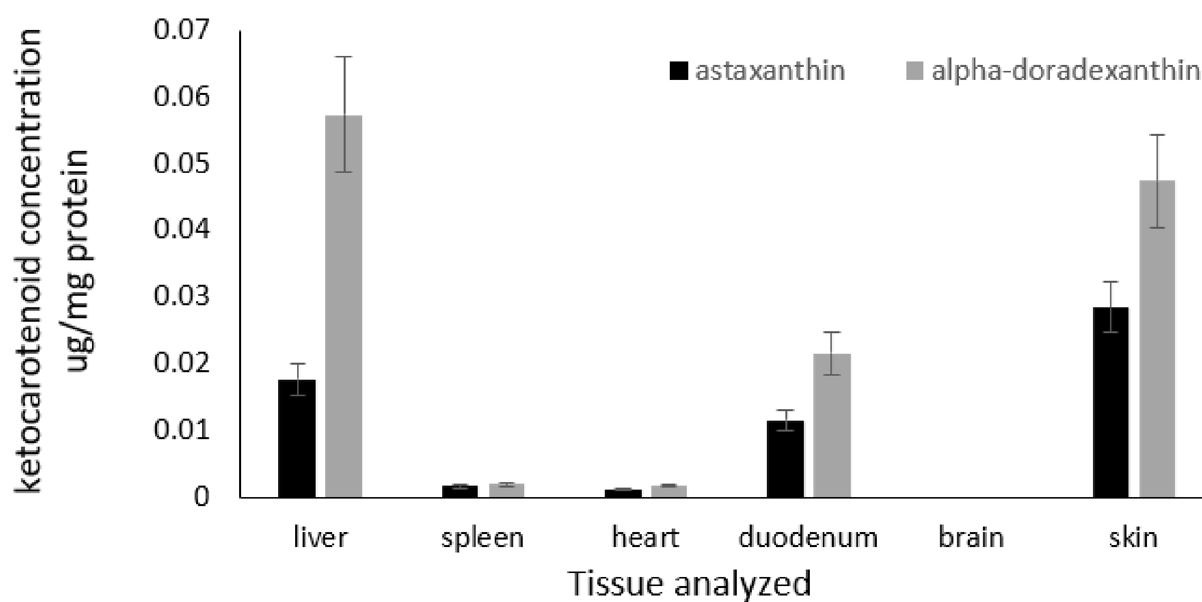

Figure 4.2 The concentration of red ketocarotenoids in molting male northern cardinal (NOCA). High concentration of alpha-doradexanthin was detected in both liver (0.06±0.01ug/mg protein) and skin (0.05±0.01ug/mg protein), while high concentration of astaxanthin was also detected in skin (0.03±0.01ug/mg protein) and liver (0.02±0.02ug/mg protein).

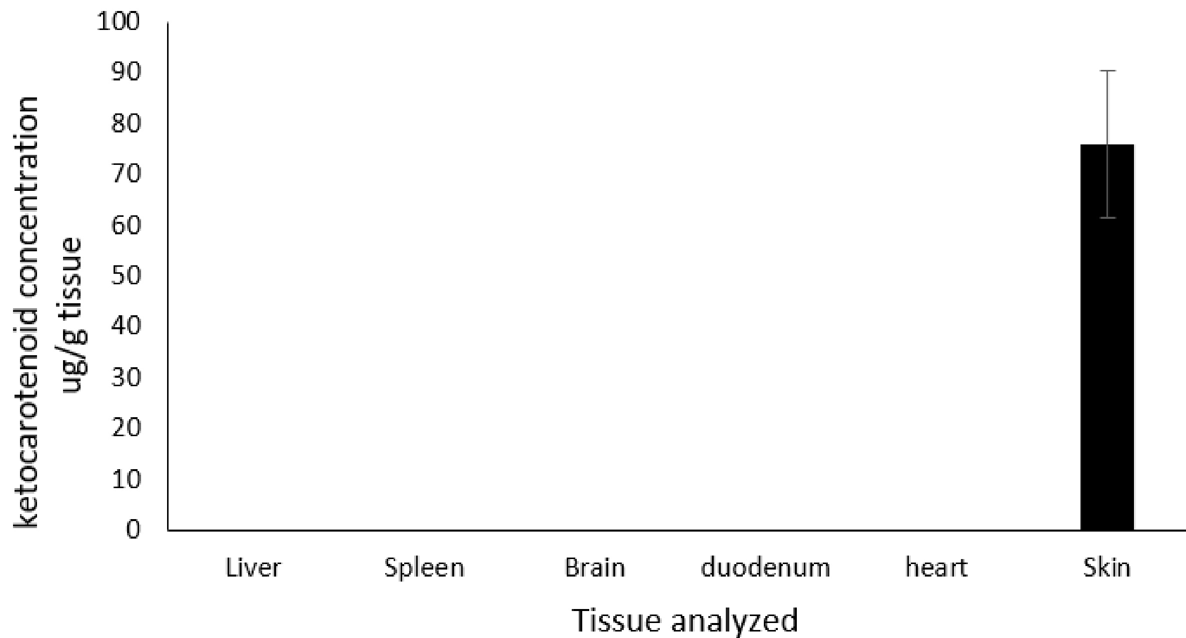

Figure 4.3 The concentration of ketocarotenoids in molting male mourning doves.

Ketocarotenoids were only detected in the leg skin of mourning doves.

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Supplemental Material

Dietary carotenoids were also measured in this study, lutein was found to be the most dominant dietary carotenoids in all of our samples, with modest amount of β-carotene also found in the leg skin of mourning dove. In mourning dove, just like ketocarotenoids, dietary carotenoids were found to be most abundant in the skin of the feet. While in cardinals, similar high concentrations of dietary carotenoids were detected in duodenum, liver and skin.

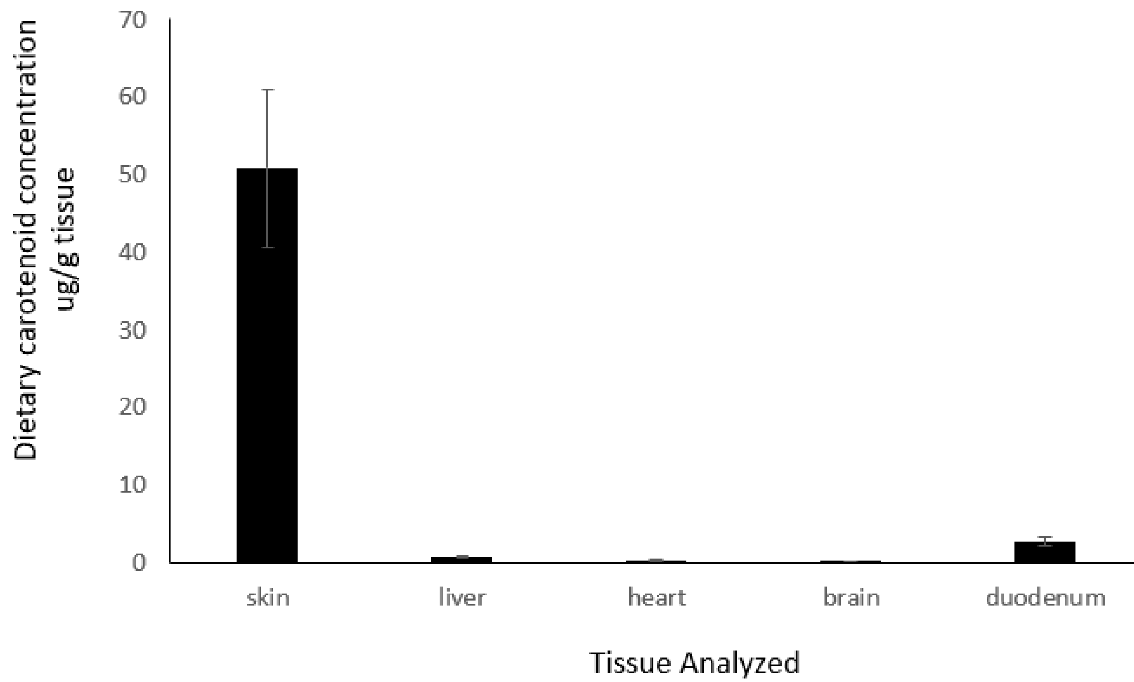

Figure S4-1. The concentration of dietary carotenoids in molting male mourning doves (MD). Dietary carotenoids were found to be most abundant in the leg skin of mourning doves.

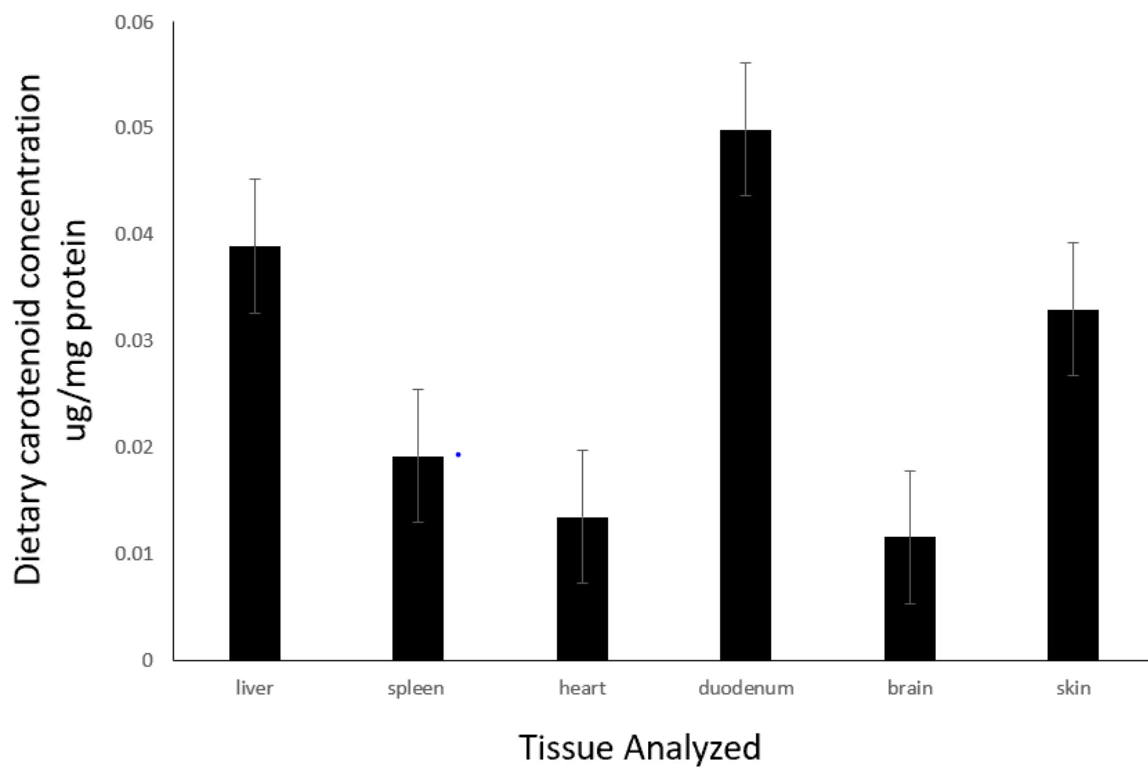

Figure S4-2. The concentration of dietary carotenoids in molting male northern cardinal (NOCA). High concentration of dietary carotenoids was detected in duodenum (0.05±0.01ug/mg protein), liver (0.04±0.01ug/mg protein) and skin (0.03±0.01ug/mg protein).