Where Have the Carotenoids Gone?

Physiology of Carotenoid Absorption And Distribution in Birds

by

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ABSTRACT

Organisms regularly face the challenge of having to accumulate and allocate limited resources toward life-history traits. However, direct quantification of how resources are accumulated and allocated is rare. Carotenoids are among the best systems for investigating resource allocation, because they are diet-derived and multi-functional. Birds have been studied extensively with regard to carotenoid allocation towards life-history traits, but direct quantification of variation in carotenoid distribution on a whole-organism scale has yet to be done. Additionally, while we know that scavenger receptor B1 (SCARB1) is important for carotenoid absorption in birds, little is known about the factors that predict how SCARB1 is expressed in wild populations. For my dissertation, I first reviewed challenges associated with statistically analyzing tissue distributions of nutrients (nutrient profiles) and tested how tissue carotenoid distributions (carotenoid profiles) varied by sex, season, health state, and coloration in two bird species, house finches (*Haemorhous mexicanus*) and zebra finches (*Taeniopygia guttata*). Then, I investigated the relationship between dietary carotenoid availability, relative expression of SCARB1, and extent of carotenoid-based coloration in a comparative study of wood-warblers (Parulidae). In my review of studies analyzing nutrient profiles, I found that multivariate analyses were the most common, but studies rarely reported intercorrelations among nutrient types. In house finches, all tissue carotenoid profiles varied by sex, season, and coloration. For example, males during autumn (molt) had higher concentrations of 3-hydroxyechinenone (the major red carotenoid in sexually attractive male feathers) in most but not all tissues compared to other season and sex combinations. However, the relationship between color and carotenoid profiles depended on the color

metric. In zebra finches, only muscle and spleen carotenoid profiles varied between immune-challenged and control birds. In wood-warblers, I found that capacity to absorb carotenoids was positively correlated with the evolution of carotenoid-based coloration but negatively associated with liver carotenoid accumulation. Altogether, my dissertation illustrates (a) the context-dependence of tissue carotenoid profile variation, (b) that carotenoid-based integumentary coloration is a reflection of tissue carotenoid profiles, and (c) that digestive physiology (e.g., carotenoid absorption) is an important consideration in the study of diet and coloration in wild birds.

DEDICATION

For my partner and best friend, Joe. I could not have done this without his love and support.

For my parents, whose unwavering support and unconditional love allowed me to pursue my dream of becoming a biologist.

For Dr. Jed Burtt, who inspired me to become the teacher and scientist I am today.

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PREFACE

No organism has infinite resources that they can maximally apportion to all investments (e.g., growth, reproduction, somatic maintenance) throughout their life history. Because resources are limited, there are inherent trade-offs that must be made between investing in different life-history traits and stages (Antonovics 1980; Boggs 1992, 2009; Saeki et al. 2014). This suggests that patterns of resource distribution or allocation have the potential to reveal information about different life-history strategies (e.g., indeterminate vs. determinate growth, growth vs. reproduction, semelparity vs. iteroparity, fast vs. slow pace-of-life; Stearns 1989). Condition-dependent signals can also reveal an organism's ability to accumulate and allocate resources, such that an individual in better condition is one who can either accumulate more resources or allocate resources more appropriately and produce or maintain a stronger signal (i.e., more elaborate song, brighter color) than an individual in worse condition (Lorch et al. 2003; Tomkins et al. 2004). However, despite intense research efforts to understand the control, function, and evolution of different life-history strategies (e.g., Lemaître et al. 2015; Healy et al. 2019) and condition-dependent signals (e.g., Hill 2011; Giery and Layman 2015), we have a surprisingly limited understanding of the specific resource-allocation patterns underlying these, including how a resource may be distributed among body tissues in space and time (Saeki et al. 2014).

Among the several resources that could be specially tracked and quantified in these contexts, including energy (King et al. 2011), biomass (Saeki et al. 2009), time (Angelier et al. 2007), and metabolites (Gleibs and Mebs 1999), nutrients are among the most useful for studying allocation mechanisms (Boggs 1992; Raubenheimer et al. 2009).

Nutrients are ideal because they have quantifiable inputs (e.g., diet) and often are more explicit and functionally linked than other resources. There are also multiple sources of environmental (e.g., nutrient location, selection, and intake) and physiological (e.g., nutrient assimilation, transport, and use) variation that could help explain dynamic nutrient-allocation patterns within individuals (Zera and Harshman 2001; Birnie-Gauvin et al. 2017). Relatively few studies have investigated this full complement of factors governing nutrient allocation (e.g., direct biochemical quantification, stable isotope tracking; Medford and Mackay 1978; Min et al. 2006; Judd et al. 2011; McCue 2011), and those that have typically examine nutrients in only a handful of tissues (e.g., gonad, liver). Even fewer studies have measured nutrient accumulation and distribution as they relate to condition-dependent signals (Scheuber et al. 2003).

For my dissertation, I aimed to help fill this knowledge gap and understand how environmental and physiological factors shape variation in multi-tissue nutrient distributions (hereafter nutrient profiles) and to what extent condition-dependent signals are a reflection of such nutrient profiles. Just as there are several types of resources that could be quantified, there are also many nutrients that could be selected to study allocation patterns. Morehouse (2014) proposed that the best approach for measuring allocatable nutrients is to choose one that is limited to the focal organism and required for multiple key traits. Both macro- and micro-nutrients, including proteins and salt, can be limited and important for producing and maintaining multiple traits (White 1993; Elser et al. 2000; Kaspari et al. 2008). Carotenoids are also an ideal system for investigating this, because they can be limiting nutrients, have many health benefits (Britton et al. 2008), and serve as pigments that produce red, orange, and yellow condition-dependent color

signals in plants and animals (Maoka 2020). Among the most well-studied nutritional functions of carotenoids are their roles as a vitamin A precursors (von Lintig 2010), immune-system modulators (Chew and Park 2004), antioxidants (Krinsky 2001), yolk nutrients (McGraw et al. 2005), and eye photoprotectants (Demmig-Adams and Adams 2013). Despite the extensive history of carotenoid research, there are still many "black boxes" in the field, where we do not fully understand mechanisms underlying carotenoid accumulation and allocation toward tissues and life-history traits in animals.

I investigated how several sources of environmental and physiological variation—including season or life-history stage, sex, health state, and carotenoid-based coloration—contribute to multi-tissue carotenoid distributions (hereafter carotenoid profiles) of birds, particularly house finches (*Haemorhous mexicanus*) and zebra finches (*Taeniopygia guttata*). In addition to examining carotenoid distributions, I also investigated carotenoid absorption and accumulation as possible mechanisms explaining interspecific variation in carotenoid-based coloration in wood-warblers (Parulidae). Carotenoids have been studied extensively in birds from both morphological and physiological perspectives, because carotenoid-based coloration is widespread in this taxon (Thomas et al. 2014a), and because the health functions of carotenoids have inspired a large body of recent research on the links between pigment accumulation and condition-dependent carotenoid-based color signaling (Møller et al. 2000; Peters, Denk, et al. 2004; Remeš et al. 2011; Garratt and Brooks 2012; Simons, Cohen, et al. 2012; Merrill et al. 2016). Carotenoids are also important to study in birds because of their relevance to health, both as it applies to human consumption of poultry (Akdemir et al. 2012; Rajput et al. 2014) and to wild bird conservation (Ewen et al. 2006; Kennedy 2019). Still, in nearly all of these novel and

influential studies on avian carotenoids, we lack a basic understanding of how carotenoid pigments are directly acquired, distributed, and allocated.

House finches and zebra finches were chosen as study species because there is a robust base of information on their carotenoid physiology (Hill 1995; McGraw and Ardia 2004; McGraw et al. 2006; McGraw and Toomey 2010; Rowe et al. 2012; George et al. 2017), and they both have carotenoid-based coloration that has been established as sexually selected, condition-dependent signals (Hill 1990; Simons and Verhulst 2011). I chose house finches for an observational study specifically, because I wanted to investigate variation in carotenoid profiles of a wild population of birds, and they are a locally abundant native songbird with carotenoid-based ornamental plumage coloration. I chose zebra finches for an experimental study specifically, because they have bare-part (e.g., bill, legs) carotenoid coloration that may respond rapidly to current conditions (e.g., color change observed as soon as 6.5 hours post-capture in goldfinch bills; Rosenthal et al. 2012). I also had access to a large breeding colony of zebra finches through a collaborator in Williamsburg, Virginia, USA. For my comparative study (see more below), I also chose wood-warblers (Parulidae) because there is considerable variation in both the extent of carotenoid-based plumage coloration and in dietary availability of carotenoids (e.g., relative contributions of fruit and variably carotenoid-rich insects to the diet) in this relatively large family of birds (Olson 2006; Winkler et al. 2020). I was also able to collect relatively large numbers of species within this family within a short period of time (i.e., two weeks) by harvesting tissues post-mortem from individuals that died via window-strike during migration.

Multi-tissue carotenoid profiles inherently have a certain level of complexity, because (a) there are over a thousand carotenoid types that could be measured (although a dozen or so may be found in a given organism; Yabuzaki 2017), (b) carotenoid types are not independent from one another since they can co-occur in foods (Yang et al. 2020) or share a metabolic network (Morrison and Badyaev 2016a), (c) different carotenoid types can be present in different tissues (e.g., retina vs. liver; Toomey and McGraw 2009), and (d) tissue carotenoids are not independent of the carotenoids found elsewhere in the body (e.g., due to mobilization from storage, prioritized body-wide accumulation of particularly valuable or abundant pigments; Metzger and Bairlein 2011). There is potential here for both carotenoid and nutrient profiles more broadly to be challenging to statistically analyze. No review exists to date that addresses data treatment and statistical analyses of nutrient profiles, so I reviewed the nutritional-physiology and -ecology literatures from the last decade by identifying studies containing nutrient profile data relevant to the physiology or ecology of wild animals and summarized the statistical methods currently used. Specifically I collected information from each nutrient profile study about intercorrelations among nutrient types, number of nutrient types, nutrient metrics used (e.g., amount, concentration, percent), and number of hierarchical levels (e.g., different nutrient types within a tissue, nutrients across tissues within an organism, amounts of nutrients in an organism within a population). To get a more practical sense for how different statistical approaches impact results and conclusions, I also evaluated the effect of these different, published statistical analyses on the results of nutrient profile comparisons using house finch tissue carotenoid profiles as an example dataset (Chapter 1).

For my empirical studies of carotenoid profiles (which comprise Chapters 2-4), I first evaluated tissue carotenoid profile variation in a wild population of house finches in relation to season, sex, and coloration, where season was associated with life-history stages during which carotenoids may serve different functions (e.g., feather coloration during fall molt, egg yolk production during spring breeding; Chapter 2). Next, I examined the effect of innate immune system activation on carotenoid profiles of male zebra finches as well as how differences in carotenoid profiles co-varied with bill color (Chapter 3). Finally, in addition to studies of carotenoid profiles, which are key for understanding carotenoid allocation physiology, I also investigated another important aspect of carotenoid physiology—absorption. Carotenoid absorption and its relationship with tissue carotenoid accumulation has been studied to some extent *in vitro* and in artificially bred species (Widjaja-Adhi et al. 2015; Toomey et al. 2017), but expression of genes associated with carotenoid absorption such as scavenger receptor B1 (SCARB1) has not yet been studied in a wild population of animals. I addressed this knowledge gap through an analysis of the relationships between dietary carotenoid availability, carotenoid absorption (i.e., SCARB1 expression), carotenoid accumulation (i.e., lutein concentration in the liver), and extent of carotenoid-based plumage coloration in wood-warblers (Parulidae). Across these studies, I hoped to contribute toward a better understanding of the mechanisms underlying resource allocation to life-history traits generally and those underlying carotenoid distribution patterns in animals specifically. I expect that the results of my initial descriptive, experimental, and comparative studies will serve as a rich resource for generating hypotheses that can be examined and tested more explicitly in the future.

CHAPTER 1

AN ECOPHYSIOLOGIST'S GUIDE TO ANALYZING NUTRIENT PROFILES

Abstract

The assessment of nutrient profiles, or distributions of nutrients (e.g., macronutrients, fatty acids, antioxidants) in tissues and foods, of wild animals is widespread in nutritional ecology and physiology. However, finding and interpreting biologically meaningful effects and mechanisms can be difficult when there are multiple challenges associated with statistical analyses of nutrient profiles, which may affect decisions regarding which method to use in a given context. I identified four such challenges based on the math-statistics literature including intercorrelations among nutrient types, number of nutrient types, nutrient metrics (e.g., concentration, proportion), and hierarchical levels of nutrient profiles (i.e., different nutrient types within tissues within an organism, the whole-organism nutrient profile across all tissues). Here I reviewed and summarized the ways in which and the extent to which authors of nutrient-profile studies in the field of nutritional physiology and ecology have addressed these challenges. Last, to give detailed and consistent empirical treatment to these approaches, I also tested an assumption or recommendation from each of these challenges using tissue nutrient (carotenoid) profile data from a free-living bird species, the house finch (*Haemorhous mexicanus*). Despite heavy emphasis in the math-statistics literature on the importance of considering intercorrelations, intercorrelations among nutrient types were rarely examined or reported in nutrient profile studies. Most nutrient profiles had high numbers of nutrient types that were also represented as proportions; multiple nutrients were generally statistically

examined via greater use of non- or semi-parametric analyses (e.g., permutational multivariate analysis of variance). Very few studies on more than one level of nutrient profiles (e.g., multiple nutrient types within multiple tissues) addressed the challenge of multiple hierarchical levels in any way. My tests of these challenges supported the importance of accounting for intercorrelations among nutrient types as well as of transforming proportion data or using non-parametric analyses, but I did not find support for an optimum number of nutrient types needed to run multivariate analysis of variance (as opposed to running multiple univariate analyses) or use of tissue type in models to account for two hierarchical levels of nutrient profiles. Where possible, I recommend that nutritional physiologists and ecologists test these assumptions with their own data before deciding which statistical analyses to run.

Introduction

Many physiologists and ecologists have interest in describing the nutrients available to animals as well as those ingested, absorbed, circulated, and stored internally. These so-called "nutrient profiles" have been used to evaluate differences within and among animal species in nutrient composition among diets (Willig and Lacher 1991; Ramírez et al. 1996; Izhaki 1998; Schairer et al. 1998; Arnold et al. 2010; Geiger et al. 2013) and body tissues (e.g., fat, muscle, liver; Mos and Ross 2002; Craven et al. 2008; Leiber et al. 2008; McGraw and Toomey 2010; Ben-Hamo et al. 2011; Budge et al. 2011; Ruiz et al. 2017). Nutrients in these studies range from macro- (i.e., protein, carbohydrates, lipids) to micro-nutrients (e.g., calcium, magnesium) including antioxidants (e.g., carotenoids). Nutrient profiles also vary in complexity from a simple comparison of one nutrient type

(e.g., vitamin A) between two diets to more complex comparisons of several nutrient types (e.g., fatty acids, amino acids, and micronutrients) including their subtypes (e.g., linoleic acid) in multiple tissues (e.g., liver, muscle) between two or more study groups (e.g., different sexes, species, supplemented vs. control groups in an experiment). These ecophysiological complexities of nutrient profiles are critical to explore, because nutrient profile research in animals helps to elucidate physiological mechanisms of nutrient absorption, transport, metabolism, turnover, and excretion, all of which have implications for human, animal, and ecosystem health (for examples, see Havel et al. 1962; Rumsey and Levine 1998; Jankowska et al. 2010; Filipiak and Weiner 2017).

However, with complexity comes potential challenges to the statistical analyses of nutrient profiles. Based on the complexity of nutrient profiles and math-statistics literature recommendations surrounding these features, I identified four such challenges that might affect outcomes of statistical analysis. Here I describe each of the challenges in more detail, and, in the sections below, I will elaborate on why these challenges are important to consider. The first challenge is intercorrelations among nutrient types; some nutrient types may be more likely to be intercorrelated with each other, due to shared solubilities, occurrences in foods, physiological processes, and/or functional uses (e.g., fatty acids, Han 2016; carotenoids and other lipid-soluble nutrients, Morrison and Badyaev 2016). The second challenge is number of nutrient types; nutrient profiles can have variable numbers of nutrient types, and number of response variables can be a limitation of some statistical methods. This could be complicated further when multiple tissue types are examined, hence adding another analytical level (i.e., the nutrient profile of a tissue containing different nutrient types, in addition to the nutrient profile of the

whole organism containing multiple tissue-nutrient profiles; Figure 1), which represents the third challenge of hierarchical levels. Nutrient profile data also come in many different quantitative forms, including amounts, concentrations, and percentages/proportions data, not all of which often meet assumptions required for parametric analyses (Aitchison 1986; Reyment 1989; Nikolaidis and Mougios 2004), which represents the fourth and final challenge of nutrient metrics.

To date, there is no systematic literature review of the general statistical approaches to analyzing nutrient profile data, so these challenges continue to hamper new studies and thus general progress in the field. Here, I reviewed the literature in nutritional ecology and physiology to (a) assemble the methods used by research for quantifying and analyzing different nutrient profiles in animals, (b) showcase how these challenges are being addressed across nutrients, data metrics, tissues, and species, and (c) to deploy an example dataset—tissue carotenoids in house finches (*Haemorhous mexicanus*)—to compare different statistical methods and illustrate the effect of different data and analytical approaches and challenges on results, outcomes, and interpretations. I also weave in commentaries on best statistical practices in hopes of advancing robust multivariate approaches. This article as a whole is intended to be a practical guide for nutritional ecologists and physiologists to select the most appropriate analyses for their nutrient profile data.

Methods

I searched for peer-reviewed articles published in the last ten years (2009-2019), first using Google Scholar and the search terms “nutri* (physiology OR ecology)” to

capture a wide range of contemporary studies of nutritional physiology and ecology, and then using a more inclusive search engine (Arizona State University library's OneSearch) and more search terms ("nutri* (physiology OR ecology)" OR (protein OR carbohydrate OR lipid OR micronutrient OR macronutrient OR antioxidant OR "fatty acid") AND profile AND animal NOT (human OR domestic* OR agriculture OR aquaculture OR "food chemistry" OR "meat science" OR "journal of food composition and analysis" OR pet OR "food research international" OR medi*)). I searched within the last ten years to ensure that only recently used statistical methods were included in the review. Then articles were included in the study if they contained ecologically relevant nutrient profile data. Nutrient profile data included any data with either (a) one nutrient type that was measured in more than one tissue, (b) multiple nutrient types measured in one tissue or diet item, or (c) multiple nutrient types measured in more than one tissue or food. Lab and domesticated animals were excluded to ensure that the results would reflect the intersection between physiology and ecology, which was the intended audience for this review. In total, my review yielded 2,521 article hits and 56 studies that matched the criteria.

Once all articles meeting these criteria had been collected, I extracted the statistical methods used for each study as text and then used conventional qualitative content analysis, which is a widely used qualitative research technique for interpreting meaning from the content of text data (Hsieh and Shannon 2005), to identify themes or categories of statistical methods used. I also collected data related to the challenges associated with analyzing nutrient profiles, including whether or not intercorrelations among nutrient types were examined, number of nutrient types, nutrient profile data

metric (e.g., percent, concentration), and number of hierarchical levels of nutrient profiles (e.g., multiple nutrient types within multiple tissue types).

I also tested some of the assumptions and recommendations of these nutrient-profile statistics (see more below) using an example nutrient-profile dataset on tissue carotenoid profiles of house finches (*Haemorhous mexicanus*) (Chapter 2).

Review of Statistical Analyses

In total, I collected data from 56 nutrient profile studies. With qualitative content analysis, two categories of statistical analysis were identified in these studies: multiple univariate (e.g., ANOVA; n = 21) and multivariate (e.g., MANOVA, PERMANOVA, PCA; n = 33) tests. Some studies used both methods. Some nutrient profiles were not analyzed statistically at all but rather qualitatively compared in more descriptive studies looking to characterize the nutrient content of food items of relevance to human and animal health or ecology (n = 6). I should also note here that, in the vast majority of cases, nutrient profiles were treated as response variables, so that is the context within which I focused this review, although I recognize that they can and have been used previously as predictors in some cases. Below I provide a brief summary, with literature examples, of the two categories.

Multiple univariate analyses

Some recent nutrient-profile studies have employed univariate analyses (i.e., one response variable) to examine predictors of both categorical (e.g., using t-test, ANOVA) and continuous (e.g., using linear regression) nutrient variables. This is straightforward

for analyses of only one nutrient and tissue type (e.g., biotin in liver), but it has also been used to analyze multiple nutrient types. For example, Caputo et al. (2009) used multiple t-tests to determine if there was an effect of surgically implanted telemetry tags on the plasma nutrient profiles (Ca^{2+}, Mg^{+}, phosphorus, total protein, triglycerides, and cholesterol) of largemouth bass (*Micropterus salmoides*). Additionally, they used multiple linear regressions (one per nutrient type) to examine the relationship between body mass and plasma nutrient concentrations among the tagged fish. A few, but not the majority of, studies that used multiple univariate analyses also used some form of multiple testing correction (e.g., Bonferroni).

Multivariate analyses

Other published studies used multivariate analyses to examine predictors of or effects on multiple nutrients and tissues; however, the multivariate method that was used varied widely. The most frequently used multivariate methods were (a) permutational multivariate analysis of variance (PERMANOVA) or analysis of similarities (ANISOM; n = 11) and (b) principle components analysis (PCA) or non-metric dimensional scaling (NMDS; n = 11). The next most frequently used methods were (c) multivariate analysis of variance (MANOVA) or multivariate analysis of covariance (MANCOVA; n = 5), and (d) correspondence analysis (CA; n = 4). Miscellaneous multivariate statistical methods were each only used once (n = 4), including canonical analysis of principle components, redundancy analysis, multivariate partial least squares, and multivariate pathway analysis. An example of a nutrient-profile study using PERMANOVA was by Cárdenas-Palomo et al. (2018), who examined variation in 32 subdermal tissue fatty acids of whale sharks

(*Rhincodon typus*) by location, year, sex, and age class. PERMANOVA/ANISOM were also frequently used in combination with PCA or NMDS. For example, Ronconi et al. (2010) explored species differences and interannual variation in plasma fatty acid profiles (including 47 different fatty acids) in two birds (greater shearwater, *Puffinus gravis*; sooty shearwater, *P. griseus*) using multi-dimensional scaling (MDS) and an analysis of similarities (ANOSIM). Some studies, such as by Maruyama et al. (2019), just used PCA or NMDS. An example of a nutrient-profile study using MANOVA was by Deans et al. (2016), who examined differences in the protein and carbohydrate content of four tissue types (young leaves, mature leaves, squares, and bolls) in cotton (*Gossypium hirsutum* and *G. barbadense*) as a nutritional resource for insect herbivores.

Statistical Challenges of Analyzing Nutrient Profiles

I will address four challenges associated with statistically analyzing nutrient profiles that have the potential to impact the results and interpretations of the nutritional data: (A) intercorrelations among nutrient types, (B) number of nutrient types, (C) nutrient profile data metric, and (D) hierarchical levels of nutrient profiles. In the sections below, I summarize the importance of the challenge in deciding which statistical analysis method to use (e.g., multiple univariate vs. multivariate analysis of variance) as well as review how the challenge has been addressed in recent ecological or physiological studies of nutrient profiles in animals and plants.

(A) Intercorrelations Among Nutrient Types

Intercorrelations among nutrient types are important to consider, because of the statistical assumption of independence. Specifically, for univariate analyses, if the assumption of independence is violated (i.e., high intercorrelation or multicollinearity), then you run the risk of redundant analyses, spurious results, and reduced statistical power (Fish 1988; Thompson 1999; Thompson et al. 2005). In contrast, for multivariate analyses where nutrients are response variables, if nutrient types are relatively weakly correlated with one another (i.e., more independent), then the power of MANOVA is also reduced (Cole et al. 1994). As such, correlations among nutrient types should be explicitly examined for evidence of non-independence before using univariate or multivariate approaches (Huberty and Morris 1989). However, there is no clear precedent for how to handle mixed response variables (i.e., nutrient types) where some are strongly correlated and others are weakly correlated.

Typically, independence should be considered at the conceptual level (i.e., if the nutrients are mechanistically or functionally unrelated) before examining intercorrelations, and then intercorrelations should be used to help to support (or not) *a priori* determinations of conceptual independence. This is particularly relevant for nutrient profiles, because some nutrient types are not conceptually independent because of known co-occurrence in foods and/or shared metabolic processes (e.g., fatty acids, Han 2016; carotenoids, Morrison and Badyaev 2016). An example of a nutrient profile with conceptually independent nutrient types is a macronutrient profile (i.e., total carbohydrates, proteins, and lipids), because typically nutritional physiologists are interested in, for example, the effect of a treatment on each of these particular nutrient

types separately. However, even this could be considered as conceptually dependent if the research question centers on the idea of whole-body nutrient composition.

From my literature review of nutrient-profile studies, only 4 out of 56 sets of authors reported that they conducted correlation analyses to determine the degree to which their nutrient types were intercorrelated. It is difficult to say with only four studies if there is a pattern of when or which intercorrelations are considered and how that information informs the decision regarding statistical methods chosen. McIntyre and Flecker (2010) found weak negative correlations between nitrogen and phosphorous ($r = -0.31$, $p = 0.002$) in whole bodies of fish and then used multivariate analysis of variance to examine effects of fish family and dietary strategy on these elements. In contrast, Larson et al. (2017) examined fatty acid profiles of filter-feeding aquatic organisms as they related to ecological variables (e.g., water quality, and land cover) and found that alpha linolenic acid was highly positively correlated with 10 of the other 14 fatty acids (10 out of 15) in mussel tissues. They used a multiple univariate Bayesian approach to analyze their data. Then there were two studies with a mixture of weak and strong correlations among their nutrient types. Velurtas et al. (2011) found weak (~0.3) to very strong (>0.95) positive correlations between glucose, total protein, and cholesterol depending on tissue type (e.g., hemolymph, midgut gland) in two shrimp species (*Artemesia longinaris* and *Pleoticus muelleri*) and then used multiple univariate analyses of variance to examine differences by species and diet. Morris et al. (2019) examined differences in metabolite profiles (e.g., sugars, amino acids, amines, bile acids, fatty acids, phospholipids, acylcarnitines, myelins; 148 total) of polar bears (*Ursus maritimus*) from different parts of the Hudson Bay. They measured intercorrelations among 29 metabolites of interest,

which were mostly phospholipids, as identified by variable importance in projection after partial least squares discriminant analysis and found that the metabolites were mostly positively correlated with each other, but there was a mix of strong and weak relationships. Three out of four of these studies used multiple univariate analyses, although two of these were less conventional univariate analyses (i.e., not ANOVA or t-test), and the pattern of decisions made about the statistical analysis method (univariate vs multivariate) in the few studies that did look at intercorrelations between nutrient types did not match expectations based on previous literature (i.e., weak relationships = univariate, strong relationships = multivariate; Cole et al. 1994). However, one clear finding from this review is that intercorrelations among nutrient types are either underanalyzed or underreported; in either case, this should be addressed in future studies of nutrient profiles.

(B) Number of Nutrient Types

In the studies I reviewed for this paper, I found a wide range of nutrient numbers used, from two (Finkler et al. 2014; Deans et al. 2016) up to as many as 148 (Morris et al. 2019). Number of response variables or nutrient types used in a statistical analysis of nutrient profiles is important to consider for different reasons depending on whether multiple univariate or multivariate analyses are used. For univariate analyses of multiple nutrient types, when more nutrient and tissue types are included in the analyses, we are less certain that we are detecting actual biological significance rather than false significance due to random chance. However, there is a lot of debate in the statistics and ecology literatures about how to deal with this issue. Some statisticians suggest that it is

important to control for type I error from multiple testing by using adjusted p-values or other metrics to prevent falsely rejecting null hypotheses (Moyé 1998; Ottenbacher 1998). However, others suggest that it is better to falsely determine that something is significant and then find out later that it is not actually significant than to overlook something biologically significant in favor of avoiding type I error (Rothman 1990; Perneger 1998, 1999; Cabin and Mitchell 2000; Feise 2002; Moran 2003). This is not dissimilar to recent arguments in the literature about using p-values to determine significance more broadly (Yoccoz 1991; Sterne and Smith 2001; Martínez-Abraín 2008; Smith 2020).

Statistical analyses of nutrient profile data in the nutritional physiology and ecology literature appears to align more with the latter philosophy, because out of 21 studies using multiple univariate analysis methods, only two used some form of multiple testing correction. Caputo et al. (2009) used Bonferroni corrections for multiple tests when using paired t-tests to evaluate differences in nutrient profiles with 11 nutrient types. Meier et al. (2016) used Benjamini and Hochberg multiple comparison procedure (Benjamini and Hochberg 1995) when using a univariate distribution-free permutation test to evaluate differences in nutrient profiles among 42 fatty acids. In both cases, the number of nutrient types was relatively high (> 10), but there were also 8 studies that did not use multiple testing corrections with more than ten nutrient types (Zhukova and Eliseikina 2012; Bártů et al. 2013; Ghazali et al. 2013; Ying et al. 2014; Larson et al. 2017; Zhang et al. 2017; Shi et al. 2018; Eglite et al. 2019).

For multivariate analyses, classic parametric tests (e.g., MANOVA, MANCOVA) are limited by the number of response variables (i.e., nutrient types) that can be included.

Specifically, the number of response variables cannot exceed the group sample size and the math-statistics literature suggests that there is an optimum range of response variables (~2-5) for maximizing power and minimizing type I error rate (Young 2006; Al-Abdullatif 2020). There are two possible solutions if analyzing nutrient profiles with more than five nutrient types: 1) collapse variation by biologically meaningful or quantitatively similar categories *a priori* before using MANOVA/MANCOVA, or 2) use non- or semi-parametric tests (e.g., PERMANOVA).

Nutrient-profile studies largely follow the recommendation that nutrient profiles with 2-5 response variables can use classic parametric tests (e.g., MANOVA, MANCOVA) and that nutrient profiles with >5 response variables must use an alternative method. It is worth noting that the number of studies with multivariate analyses of 2-5 nutrient types is fairly low (5 out of 33 studies). Three out of the five studies analyzing nutrient profiles with 2-5 nutrient types used MANOVA/MANCOVA (McIntyre and Flecker 2010; Finkler et al. 2014; Deans et al. 2016), and the other two used PCA/NMDS (Madgett et al. 2019; Maruyama et al. 2019); however, there were a few exceptions where a MANOVA was used to analyze nutrient profiles with more than five nutrient types (McMeans et al. 2013; Chen et al. 2017). Although McMeans (2013) took an interesting approach where they used PCA on all 16 fatty acids to identify 9 fatty acid indicators that were then used in MANOVAs (one per tissue). The number of response variables was still greater than five, but the approach to reducing the number of response variables was unique. For studies with greater than five nutrient types, the authors seemed to primarily use the strategy of choosing non- or semi-parametric tests. There were a few studies that collapsed variation into biologically meaningful categories, but

this was done as a separate univariate analysis in addition to the non- or semi-parametric multivariate analysis of all nutrient types. For example, Cashman et al. (2016), in a study of fatty-acid profiles in tissues throughout an ecosystem (e.g., mussel, wood, leaves, grass, algae), included all fatty acids in a multivariate analysis using ANOSIM, but then fatty acids were also subdivided into the four major fatty-acid classifications (saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, and highly unsaturated fatty acids) for multiple univariate comparisons.

In sum, the consensus among nutritional physiologists and ecologists for multiple univariate analysis of variance appears to be that multiple testing corrections are not used, even with particularly high numbers of nutrient types. For multivariate analysis of variance, non- or semi-parametric statistical techniques tend to be used with higher (>5) numbers of nutrient types, and the two most common methods are PERMANOVA or ANOSIM and PCA or NMDS.

(C) Nutrient Metrics

The type of quantitative metric used is another challenge to analyzing nutrient profile data, largely due to the fact that different nutrient metrics (e.g., amount, concentration, proportion) may not all meet the assumptions (i.e., normality) of parametric analyses (e.g., ANOVA, MANOVA). Proportional data (i.e., percentage/compositional) and zero-inflated data (i.e., when not all nutrients are found in every sample) are the two biggest culprits for violating normal distributions. The latter is probably less of an issue for studies of essential nutrients (e.g., carbohydrates, protein), but it could pertain when studying any nutrient with variants that may or may not be

present in a given group or tissue type (e.g., carotenoids, fatty acids). Consequences to violating parametric assumptions include an inflated type I error rate and reduced statistical power (Lix et al. 1996; Delacre et al. 2019). When proportional, zero-inflated, or other nutrient metrics do not meet assumptions of normality and heterogeneity, a common statistical approach is to transform the data. If the data cannot achieve normality with transformation, then non-parametric tests must be used.

Since some nutrient-profile studies used more than one metric, I found that the most common metric by far was nutrient proportion (n = 45) followed by concentration (n = 13), ratio (n = 3), and then amount (n = 1). The prevalence of proportion as a nutrient-profile metric was likely driven by overrepresentation of fatty acids in my review of nutrient profiles more broadly (38 out of 56 total studies). Many nutritional physiologists and ecologists dealt with proportions by either transforming prior to doing parametric analysis (e.g., arcsine; Riley et al. 2013) or using non- or semi-parametric methods (e.g., ANISOM; Ronconi et al. 2010). Because proportion data were largely dealt with as needed when they were used, I encourage the continued use of literature-recommended practices.

(D) Hierarchical Levels of Nutrient Profiles

Some nutritional physiologists and ecologists are interested in a single measurement of nutrient composition in a tissue or the body (e.g., Botić et al. 2015; Zhang et al. 2017), whereas others measure multiple nutrients within or across tissues (e.g., liver, muscle; Giraldo et al. 2015). The latter is the situation where there is more than one hierarchical level of nutrient profiles. Theoretically, this is a problem of individualistic fallacy, where

inferences are being drawn at the higher level based on lower-level data, which could lead to incorrect conclusions (Diez-Roux 1998). However, this might not become a practical problem for statistical analysis unless more than one nutrient profile is measured per level, because then the data are less likely to meet the assumption of independence. For example, liver and muscle carotenoid profiles might not be independent from each other because they occupy the same individual's body, much like different sites within the same region in an ecological study might not be independent from each other.

When I explored recent studies of nutrient profiles for number of hierarchical levels and whether or not the authors accounted for these levels in any way statistically, I found that there was a relatively even mix of studies with one (n = 32) or two (n = 24) nutrient-profile levels. An example of a study with one nutrient-profile level was by Ghazali et al. (2013), who examined fatty-acid profiles of European eels (*Anguilla anguilla*) in muscle tissue. An example of a study with two levels was by DesRochers et al. (2009), who examined macronutrient profiles of several wetland plant species consumed by the Hawaiian moorhen (*Gallinula chloropus sandvicensis*), including the profiles of multiple plant tissues such as leaves, seed stalks, stems, flowers, seeds, and young shoots. Most studies analyzed tissues separately and did not account for the potential non-independence of tissue nutrient profiles within individuals. Some studies accounted for this partially by including tissue as a covariate rather than analyzing each tissue separately (e.g., Deans et al. 2016; Díaz de Vivar et al. 2019).

Tests of Statistical Challenges with Analyzing Nutrient Profiles Using an Example Dataset

In association with the challenges described in the previous section, there are several important considerations and recommendations from the math-statistics literature that, in theory, affects interpretation of results from statistical analysis. In the sections below, I tested one of these considerations or recommendations for each challenge using a subset of data from house finch carotenoid profiles generated in Chapter 2. I also discuss implications of these tests for nutrient-profile statistical analyses more broadly.

(A) Intercorrelations Among Nutrient Types

To evaluate the importance of considering intercorrelations among nutrient types with a case study, I considered the relationships among carotenoid concentrations in two tissues—brain and liver—from the house finch, where I expect to find season and/or sex differences (Hill 1995; Mcgraw et al. 2006a; Toomey and McGraw 2009). I chose brain, because it is a tissue in which there is no correlation between the two dominant carotenoid types (lutein and zeaxanthin; $r = 0.21$), and I chose liver because it is a tissue with a strong positive correlation between the same two carotenoid types ($r = 0.93$). In this case, I compared the results of multiple univariate analyses of variance (ANOVA) and multivariate analysis of variance (MANOVA) to test the assumption that correlation structure affects the ability of ANOVA to detect differences between groups (e.g., season, sex). I found that both ANOVA and MANOVA detected significant differences by season and sex in brain carotenoid profiles (i.e., lutein and zeaxanthin concentrations), meaning that they were equally able to detect effects in the tissue with weak correlations.

In contrast, I also found different results between the ANOVA and MANOVA for liver carotenoid profiles. Specifically, there was a significant effect of season detected by the MANOVA, but this effect was not significant with the ANOVA, which means that only the MANOVA detected differences in the tissue with strong positive correlations (Table 1). This example illustrates the importance of examining intercorrelations among nutrient types when deciding which type of test to use, since only the MANOVA was able to detect a significant effect of season in the tissue with strong intercorrelations (i.e., liver). The impact of correlation structure on MANOVA results is still complex, so this should still be considered beyond the basic guideline that stronger correlations dictate running multivariate analyses (see Cole et al. 1994).

(B) Number of Nutrient Types

To evaluate the importance of choosing the right statistical test for the number of response variables in a given study, in my example dataset I chose to use eye carotenoid profiles with MANOVA and PERMANOVA to test for season and sex differences with variable numbers of response variables. Eye tissue in house finches contains carotenoid types that could be easily collapsed or reduced *a priori* (e.g., multiple isomers of the same carotenoid type, astaxanthin; Toomey and McGraw 2009), so that I could compare results with more than five response variables (i.e., lutein, lutein isomer, zeaxanthin, epsilon-carotene, astaxanthin, astaxanthin isomer 1, astaxanthin isomer 2, astaxanthin isomer 3, astaxanthin isomer 4, astaxanthin isomer 5, astaxanthin isomer 6, galloxanthin), five response variables (i.e., lutein, zeaxanthin, epsilon-carotene, astaxanthin, galloxanthin), and two response variables (i.e., astaxanthin, galloxanthin) using the same

data. I chose to compare the outcomes (i.e., significant effects by season and sex) of MANOVA and PERMANOVA, because MANOVA is more constrained by number of nutrient types than PERMANOVA, which does not require that a specific number of response variables be used to maximize effectiveness. Also, PERMANOVA is similar to ANOSIM, but is more robust (i.e., less sensitive to dispersion effects) and should be preferred (Anderson 2001; Jari Oksanen et al. 2020). I found that there was no consequence for results (in terms of which factors were significant, including season, sex, and their interaction) due to number of nutrient types (Table 2), meaning that the results were the same qualitatively no matter how many nutrient types were used. However, there was a difference in results between MANOVA and PERMANOVA that was not associated with number of carotenoid types. MANOVA was consistently able to detect a significant effect of sex, where PERMANOVA did not detect this effect in all three sets of tests. This effect was possibly due to the greater power of MANOVA to detect small effects in a nutrient profile like house finch eye carotenoids that has strong intercorrelations among nutrient types.

(C) Nutrient Metrics

To examine effect of the type of nutrient metric used on nutrient profile statistical analysis, I considered brain carotenoid data as concentrations, untransformed proportions, and transformed proportions and tested for season and sex differences in house finch brain carotenoid profiles again using MANOVA and PERMANOVA. I found differences in results according to which nutrient metric was used (Table 3). Specifically, I found both season and sex differences in brain carotenoid concentrations and transformed

proportions using MANOVA, but only sex differences for untransformed proportions. In other words, MANOVA results were consistent for carotenoid concentrations and proportions as long as the proportion data were transformed. In contrast to the MANOVA findings, PERMANOVA results were robust to data format (i.e., same results no matter which nutrient metric was used). However, I again observed that there were different results between MANOVA and PERMANOVA, but this time the carotenoid profile used had weak instead of strong intercorrelations, so the difference is not just that MANOVA has more power to detect small effects in nutrient profiles with strong intercorrelations.

(D) Hierarchical Levels of Nutrient Profiles

I aimed to test for season and sex differences across multiple tissues (i.e., two hierarchical levels rather than one) by comparing results from analyses of carotenoid profiles within individual tissues with those across tissues using both MANOVA and PERMANOVA. Qualitatively, there were only subtle differences in the results between individual-tissue analyses and multi-tissue analyses, since all effects including tissue were significant in slightly different ways (Table 4). The main difference between these two approaches (i.e., individual vs. multi) of course was being able to directly examine the effect of tissue and its interactions (i.e., season*tissue, sex*tissue) as opposed to determining tissue-specificity of effect by qualitatively comparing individual tissue results. However, then it is not possible to tell which tissues have which effects unless a follow-up analysis is conducted. There are recommendations in the math-statistics literature for how to follow up from multivariate analyses to consider; for example, descriptive discriminant analysis (DDA) is preferred over multiple ANOVA (Huberty

1984; Huberty and Morris 1989; Thompson et al. 2005), because ANOVA requires independence, which has already been determined not to be the case for a given nutrient-profile dataset if multivariate analyses are being conducted.

Future directions

The statistical methods reviewed here were primarily been restricted to classic analyses typically used in the field of nutritional ecophysiology. However, there are other methods for analyzing nutrient-profile-like data that could be borrowed and adapted from other fields, such as psychology, education, landscape ecology, and molecular biology. Below I provide a summary of some of these additional methods. It would also be helpful if these novel methods could be validated in collaboration with statisticians through simulation studies to determine assumptions and limitations as they pertain to the data structure of nutrient profiles.

Currently, for multi-level nutrient data, there are no clear recommendations for how to properly account for non-independence of tissue nutrient profiles within whole organisms. One possibility involves using principles of multi-level modeling previously developed in fields such as education (Paterson and Goldstein 1991) to capture the nested nature of student data within schools and districts in combination with a compositional technique to capture differences among nutrient profiles that account for the fact that tissues exist non-independently within individual organisms. However, this would be another example of statistical innovation that would require validation.

Additionally, nutritional physiologists and ecologists may seek other creative ways of analyzing nutrient profiles besides comparing groups or exploring correlations

among variables and nutrient profiles. For example, although no study in this review used it, there is a Mantel test (Mantel 1967) that is similar to a PERMANOVA but it allows you to test the correlation between two matrices rather than two variables, so you could directly compare the nutrient profiles of tissue types to better understand similarity of tissue carotenoid profiles within groups (e.g., similarity between plasma and liver profiles within a treatment group). Another application of interest for nutritional ecologists and physiologists would be to calculate the ratio between acquisition and allocation of nutrients for the purposes of evaluating potential trade-offs across tissues both within and between groups (King et al. 2011; Morehouse 2014). Other possibilities include using network analysis or source-sink models (Gravel et al. 2010) to gain a better understanding of nutrient allocation among tissues.

Conclusions/Summary

In this study, I have reviewed the recent statistical methods used by nutritional physiologists and ecologists to analyze nutrient profiles, and I have tested a few of the assumptions surrounding common challenges associated with statistically analyzing nutrient profiles including intercorrelations between nutrient types, number of nutrient types, nutrient metrics, and the hierarchical nature of nutrient profiles. There was an even mix of multiple univariate and multivariate methods used to statistically analyze nutrient profile data, with a majority of univariate analyses being classics such as analysis of variance (ANOVA) and with a majority of multivariate analyses being non-parametric (e.g., PERMANOVA, ANOSIM, PCA, NMDS). I found that intercorrelations among nutrient types were reported infrequently, which may be concerning given the impact of

intercorrelations on the results of house finch carotenoid profile analysis. Most nutrient profiles containing high numbers of nutrient types tended to not use multiple testing corrections (if univariate) or use non-parametric analyses (if multivariate). Most nutrient profiles contained proportions (rather than concentrations or ratios), whose standardly non-normal distributions were addressed either through the use of transformation or non-parametric analyses. There was also an even mix of one or two hierarchical levels of tissue nutrient analysis, although very few studies addressed this challenge.

Based on the tests of my own house finch carotenoid profile data, I confirmed the importance of accounting for intercorrelations among nutrient types as well as transforming proportion data or using non-parametric analyses, but I was not able to find sufficient evidence in support of the caution surrounding other challenges. However, it is possible that the cases that I used were just not able to reveal the weaknesses associated with those challenges. For example, even if you are not limited in number of nutrient types by your sample size, it might be prudent to only use MANOVA (as opposed to non-parametric methods like PERMANOVA) if you have relatively few nutrient types (e.g., 2-5). Regardless of how the challenges were addressed, the tests also revealed that there were differences in results depending on whether a MANOVA or PERMANOVA was used. This could just be highlighting that parametric analyses (e.g., MANOVA) are more powerful, if your data meet their assumptions, than semi- or non-parametric analyses (e.g., PERMANOVA; see Vickers 2005). The challenge of dealing with hierarchical levels of nutrient profiles should be addressed through validation of statistical techniques borrowed from other disciplines.

Figures

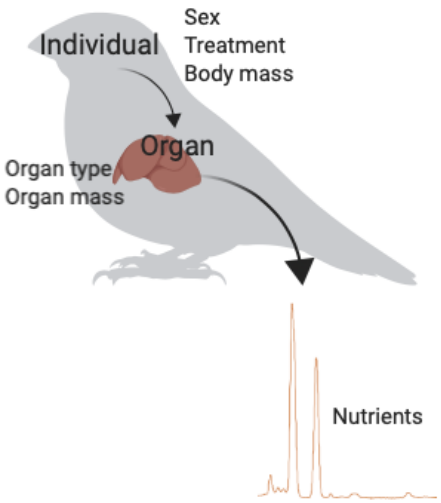

Figure 1. Conceptual diagram of organizational levels of tissue nutrients in an animal. There can be multiple nutrients per tissue, multiple tissues per individual, and multiple ways to consider data of individual animals (e.g., in relation to sex, treatment, body/tissue mass).

Tables

Table 1. Univariate analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) examining season and sex differences in a tissue in house finches with weak intercorrelations among carotenoid (lutein and zeaxanthin) concentrations (brain) and a tissue with strong intercorrelations (liver) between lutein and zeaxanthin concentrations.

Predictor	F-value	P
Brain (weak intercorrelations)		
ANOVA		
Lutein		
Season	4.14	0.005
Sex	16.94	0.0001
Season*Sex	0.91	0.46
Zeaxanthin		
Season	1.09	0.37
Sex	7.83	0.007
Season*Sex	0.52	0.73
MANOVA		
Season	2.29	0.02
Sex	11.87	0.00004
Season*Sex	0.71	0.68
Liver (strong intercorrelations)		
ANOVA		
Lutein		
Season	1.46	0.23
Sex	1.03	0.32
Season*Sex	0.35	0.84
Zeaxanthin		
Season	1.66	0.17
Sex	1.17	0.28
Season*Sex	0.16	0.96
MANOVA		
Season	2.17	0.03

Sex	0.58	0.56
Season*Sex	0.57	0.80

Table 2. Multivariate analysis of variance (MANOVA) and permutational multivariate analysis of variance (PERMANOVA) examining season and sex differences in house finch eye carotenoid profiles with variable numbers of response variables achieved through consolidation of the twelve original carotenoid types to five carotenoid types by identity and then reduction to only the two dominant carotenoid types.

Predictors	**F-value**	**P**
All carotenoid types (12)		
MANOVA		
Season	5.49	<0.0001
Sex	2.10	0.03
Season*Sex	1.17	0.22
PERMANOVA		
Season	5.59	0.001
Sex	0.73	0.45
Season*Sex	1.66	0.13
Consolidated carotenoid types (5)		
MANOVA		
Season	4.33	<0.0001
Sex	3.07	0.02
Season*Sex	1.04	0.41
PERMANOVA		
Season	4.32	0.007
Sex	0.40	0.58
Season*Sex	1.77	0.13
Dominant carotenoid types (2)		
MANOVA		
Season	7.82	<0.0001
Sex	5.11	0.009

Season*Sex	1.17	0.32
PERMANOVA		
Season	4.84	0.001
Sex	0.81	0.37
Season*Sex	1.71	0.14

Table 3. Multivariate analysis of variance (MANOVA) and permutational multivariate analysis of variance (PERMANOVA) examining season and sex differences in house finch brain carotenoid profiles with different nutrient metrics: concentration, proportion without arcsine transformation, and proportion with arcsine transformation.

Predictors	**F-value**	**P**
Concentration		
MANOVA		
Season	3.11	0.0004
Sex	3.42	0.02
Season*Sex	0.84	0.61
PERMANOVA		
Season	1.71	0.11
Sex	4.30	0.02
Season*Sex	0.60	0.77
Proportion without transformation		
MANOVA		
Season	1.75	0.06
Sex	4.32	0.008
Season*Sex	0.70	0.75
PERMANOVA		
Season	1.42	0.21
Sex	3.97	0.04
Season*Sex	0.72	0.66
Proportion with transformation		
MANOVA		
Season	3.13	0.0004

Sex	3.03	0.04
Season*Sex	0.73	0.72
PERMANOVA		
Season	1.24	0.29
Sex	4.08	0.04
Season*Sex	0.72	0.63

Table 4. Multivariate analyses of variance (MANOVA) and permutational analyses of variance (PERMANOVA) exploring season and sex effects in tissue carotenoid profiles of house finches. Individual analyses of brain, eye, and liver carotenoid profiles were compared with single analyses of all three tissues simultaneously with tissue as a factor in the model.

Predictors	**F-value**	**P**
Individual tissue analyses		
Brain		
MANOVA		
Season	2.59	0.01
Sex	3.87	0.03
Season*Sex	1.06	0.40
PERMANOVA		
Season	2.31	0.03
Sex	5.58	0.01
Season*Sex	0.66	0.69
Eye		
MANOVA		
Season	4.28	0.0001
Sex	0.60	0.55
Season*Sex	0.96	0.47
PERMANOVA		
Season	3.65	0.005
Sex	-0.01	0.93

Season*Sex	1.13	0.35
Liver		
MANOVA		
Season	2.48	0.02
Sex	1.19	0.31
Season*Sex	2.35	0.02
PERMANOVA		
Season	2.60	0.02
Sex	4.21	0.01
Season*Sex	1.62	0.13
Multi-tissue analyses		
MANOVA		
Season	3.37	0.0009
Sex	2.41	0.09
Tissue	260.09	<0.0001
Season*Sex	1.52	0.15
Season*Tissue	2.19	0.005
Sex*Tissue	0.99	0.41
Season*Sex*Tissue	2.15	0.006
PERMANOVA		
Season	2.29	0.04
Sex	5.21	0.02
Tissue	690.40	0.001
Season*Sex	1.00	0.40
Season*Tissue	2.65	0.002
Sex*Tissue	4.27	0.01
Season*Sex*Tissue	1.32	0.21

CHAPTER 2

SEASON-, SEX-, AND PLUMAGE COLOR-BASED VARIATION IN TISSUE CAROTENOID PROFILES OF HOUSE FINCHES (*HAEMORHOUS MEXICANUS*)

Abstract

Animals have limited resources that they allocate toward life-history traits such as reproduction and self-maintenance. Trade-offs can occur when resources are allocated toward one trait at the expense of another. However, resources are not frequently directly quantified as they are allocated to multiple traits. One method for directly quantifying the allocation of resources would be to measure distributions of resources in tissues associated with different life-history traits (e.g., spleen with self-maintenance, gonad with reproduction). Carotenoids are ideal for measuring the distribution of resources in tissues, because they can only be obtained from the diet and they have functions (e.g., antioxidant capacity, immune modulation, photoprotection, color production) that likely lead to tissue- and context-specific distributions of carotenoids in the body. I investigated natural variation in tissue carotenoid distributions (hereafter carotenoid profiles) as a function of season, sex, and ornamental coloration in house finches (*Haemorhous mexicanus*), a popular model for studies of carotenoid physiology and coloration. I found tissue- and carotenoid-type-specific differences in carotenoid profiles among seasons, sexes, and in relation to ornamental plumage coloration. Specifically, some tissues had carotenoid

profiles that varied only by season (adipose, eye, plasma, lung), only by sex (brain, gonad), or both (heart, kidney, liver, lung, muscle, spleen), and many of these seasonal and sex differences could be attributed to the proportional contribution of particular carotenoid types, especially 3-hydroxyechinenone and beta-carotene. Male plumage redness (hue) only reflected heart carotenoid profiles during molt, where less red males had more astaxanthin in heart tissue. Male plumage saturation reflected carotenoid profiles in nearly all tissues regardless of season, where, generally, males with more saturated plumage had greater lutein concentrations in tissues but lower concentrations of ketocarotenoids (e.g., astaxanthin, 3-hydroxyechinenone) were negatively associated with saturation. These findings show that carotenoids are distributed in tissue-specific and carotenoid-type-specific ways in different contexts that are consistent with the idea that carotenoids are being allocated to and traded-off among life-history traits, although there should be a follow-up with more explicit tracing of the movement of carotenoids, not just their location at one point in time. These results also indicate that different measures of carotenoid-based coloration reveal different information about internal carotenoid profiles.

Introduction

Animals routinely face behavioral and physiological challenges of finding resources in their environment (i.e., foraging), accumulating them in their bodies (i.e., assimilating nutrients), and apportioning those resources toward life-history traits such as reproduction and self-maintenance (i.e., health, growth; Stearns 1989). The resulting pattern of resource allocation is itself an adaptive trait that can change dynamically with

the environment (e.g., seasonal weather, availability of resources) and individual physiological state (e.g., life stage; Antonovics, 1980). For example, an individual in search of a mate will have different investments, and therefore different allocation patterns, in self-maintenance and reproduction than one attempting to survive harsh seasonal weather (Stearns 1989).

Although there are several fitness-critical resources that could be quantified to test principles of allocation theory, including energy (King et al. 2011), biomass (Saeki et al. 2009), time (Angelier et al. 2007), and metabolites (Gleibs and Mebs 1999), among the most useful resources for studying allocation patterns are nutrients (Boggs 1992; Raubenheimer et al. 2009). Even among nutrients, there are many that could be quantified, including various macro- (e.g., protein; Elser et al., 2000; White, 1993) and micro-nutrients (e.g., salt; Kaspari et al., 2008). Nutrients in general are rarely quantified in more than a handful of tissues to explain allocation of resources to different life-history traits. However, carotenoids, a group of lipid-soluble pigment-nutrient molecules, are ideal for studying dynamic allocation of nutrients to life-history traits, because they serve a multitude of life-history functions in animals including growth, reproduction, sexual attractiveness, and health. Specifically, in addition to carotenoids being used as integumentary coloration to signal quality in the context of sexual selection (Blount & McGraw, 2008; Koch & Hill, 2018; Simons et al., 2012; Svensson & Wong, 2011; Weaver et al., 2017), carotenoids provide many health benefits such as antioxidant activity (Krinsky 2001), photoprotection in the eye (Demmig-Adams and Adams 2013; Balić and Mokos 2019), and modulation of immune responses (Chew and Park 2004).

Despite all of the attention paid to diverse carotenoid mechanisms and functions in wild animals over the years, surprisingly little has been paid to where in the body carotenoids may be exerting their functions. The vast majority of studies on carotenoids in animals use plasma and/or a few tissues (e.g., liver, fat) as an indicator of carotenoid accumulation in the body, even though carotenoids have been found in many different tissues at varying concentrations and proportions (Black et al., 2014; McGraw et al., 2006; Negro et al., 2001; Rowe et al., 2012; Surai et al., 2000; Zhang et al., 2019), and there are known on-site unique functions for tissues like eye (visual tuning) and integument (visual signaling; e.g., Blount & McGraw, 2008; Demmig-Adams & Adams, 2013). One means by which to investigate how wild animals allocate nutrients like carotenoids throughout the body is to track tissue-specific carotenoid distributions on a whole-organism scale (hereafter carotenoid profiles) under a variety of environmental and physiological conditions.

Only one study to my knowledge has previously described the carotenoid profile of more than a few (2-4) tissues at a time in a wild population of animals. Surai et al. (2000) characterized the carotenoid profiles of 12 breeding female lesser black-backed gulls (*Larus fucus*) in seven tissues. However, there is still a need to understand biological predictors of variation (e.g., females vs. males, breeding vs. non-breeding) in tissue carotenoid profiles of free-living animals. It is possible that one of the reasons there are so few tissue-carotenoid-profile studies is because there is little precedent in the ecophysiology literature for how to statistically analyze complex differences in nutrient profiles (e.g., by carotenoid and tissue type, as a function of body/tissue mass, sex, age, population, species, etc.) (see Chapter 1). It can become unwieldy very quickly to

interpret correlations in carotenoid levels among carotenoid and tissue types as well as differences in carotenoid concentrations between groups of animals for more than a few tissues. However, there are statistical techniques that could handle the analysis of carotenoid profiles across many tissues, many carotenoid types, and many groups (e.g., populations, experimental treatment vs. control animals; Qian et al. 2010; Maus et al. 2011; Nyman et al. 2011; Anderson 2017).

I studied tissue carotenoid profiles and how they vary as a function of season, sex, and carotenoid-based plumage coloration in wild house finches (*Haemorhous mexicanus*), a popular model for studies of carotenoid nutrition and physiology (Hill et al., 2006; see more below). Specifically, I tested the following hypotheses: 1) tissue carotenoid profiles are season- and sex-specific, 2) tissue carotenoid profiles are correlated with expression of plumage coloration, and 3) if there are differences in carotenoid profiles across seasons, sexes, or in relation to coloration, then those differences are tissue-specific rather than systemic. Previous studies show that plasma, liver, and retinal carotenoid levels vary with sex and season in house finches (Giraudeau & McGraw, 2014; Hill, 1995; McGraw et al., 2006; Toomey & McGraw, 2009), suggesting that there may be sex- or season-specific carotenoid allocation strategies (i.e., optimizing distribution of carotenoid types and amounts in specific tissue locations for localized functions; McGraw et al., 2011). I expected to see seasonal differences in tissue carotenoid profiles because of the different life-history stages and carotenoid uses of house finches across seasons, such as fall molt (i.e., development of ketocarotenoid-derived ornamental coloration; McGraw et al., 2013) and spring breeding (i.e., allocation of carotenoids to egg yolk by females; Navara et al., 2006). Male house finches also vary

widely in extent and redness of carotenoid-based plumage coloration, which is produced through the metabolism of dietary carotenoids into ketocarotenoids (e.g., 3-hydroxyechinenone; Inouye et al., 2001), and females prefer males with redder plumage patches as mates (Hill 1990). As an honest signal of condition (Hill 1991), this coloration is expected to reflect internal carotenoid levels (e.g., resource trade-off hypothesis; Koch & Hill, 2018; Weaver et al., 2017), so I expected plumage coloration to covary with tissue carotenoid profiles (as McGraw et al., 2006 found for liver and plasma in molting male house finches), potentially in a context-dependent (i.e., season-dependent) manner among males. In addition to describing variation in tissue carotenoid profiles of wild house finches across seasons, between sexes, and in relation to plumage coloration, I intended for this study to also be used as an empirical example for how to analyze multi-tissue carotenoid profiles (Chapter 1).

Methods

I used sunflower-seed-baited basket traps to capture 80 house finches on the Arizona State University-Tempe campus (Tempe, Arizona, USA) over five seasons (Table 1). I sampled more males than females in order to examine tissue carotenoid profiles in relation to variation in male ornamental carotenoid-based plumage coloration. Birds with obvious signs of avian pox virus infection (i.e.,, lesions on bare parts) or with low body mass (< 15 g) were not included in the study. At time of capture, I processed each individual by recording sex (based on plumage characteristics initially, then confirmed with gonads when possible) as well as measuring tarsus length (to the nearest 0.01 mm with digital calipers) and body mass (to the nearest 0.01 g with an electronic balance).

Then I took digital photographs of three carotenoid-colored plumage regions (crown, breast, and rump) in males and measured plumage hue, saturation, and brightness (*sensu* Giraudeau et al., 2015; see more below); note that lower hue scores are associated with redder plumage. One person (E.A.W.), who was not aware of tissue carotenoid levels for individual birds at the time, measured the three color variables for each body region and all individuals in the study. I intend to validate the precision and repeatability of these measurements with independent raters who are unaware of the hypotheses before publication. After taking photographs, I humanely euthanized all birds by approved methods (IACUC protocol #18-1665R) and drew blood from the jugular vein immediately post-mortem into heparinized capillary tubes. Plasma was obtained by centrifuging to separate plasma from blood cells. I then dissected, collected, and weighed all major tissues whole: muscle (pectoralis major), heart, liver, spleen, lung, gonad, kidney, adipose (furcular), eye, and brain. For all paired organs (e.g., lung, kidney, eye), both left and right ones were taken. Gonad and adipose were not collected for every individual, because gonads are regressed in fall and winter and because some birds did not have fat deposits. All tissues were stored immediately at -80°C until carotenoid extraction.

Carotenoid Extraction and Saponification

I extracted carotenoids from all tissue types using a standard organic-solvent protocol (Butler & McGraw, 2010; McGraw et al., 2008). I extracted carotenoids from 5-20uL of plasma by mixing it with 200uL ethanol followed by 200uL of 1:1 hexane:methyl tert-butyl ether (MTBE). This mixture was centrifuged, and the resulting carotenoid-

containing supernatant was evaporated to dryness under a stream of nitrogen gas. I extracted carotenoids from all other tissues by homogenization via ball grinder (Retsch MM200) with 1:1 hexane:MTBE (500 - 2000 μL, depending on tissue) and glass beads. Prior to homogenization, muscle and heart tissues were cut into smaller pieces to facilitate carotenoid extraction. Similar to plasma, the homogenate was centrifuged, and the resulting carotenoid-containing supernatant was evaporated. After extraction, carotenoids from fat and eye samples were saponified according to previously described procedures (Negro et al. 2001; Butler and McGraw 2010; Toomey and McGraw 2010), because these sample types are known to contain carotenoid esters, which make quantification of individual carotenoid types more challenging (Oliver and Palou 2000; Negro et al. 2001; Toomey and McGraw 2007). Fat carotenoid extracts were capped with nitrogen gas and incubated in 1 mL of 0.5 M methanolic NaOH for 4 hours at room temperature in the dark. Eye carotenoid extracts were split in half and incubated similarly in 0.2 M or 0.02 M methanolic NaOH for optimal saponification of xanthophylls and ketocarotenoids, respectively. After incubation, all saponification reactions were stopped with 1 mL of saturated NaCl followed by 2 mL of doubly-distilled water, and then carotenoids were re-extracted from the organic-solvent fraction by vortexing with 2 mL of 1:1 hexane:MTBE, centrifuging, and evaporating the supernatant.

High-Performance Liquid Chromatography

Carotenoid extracts were prepared for high-performance liquid chromatography (HPLC) by resuspending in 200 uL of mobile phase (42:42:16 methanol:acetonitrile:dichloromethane). Then 50 uL of sample was injected into an

Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) fitted with a YMC Carotenoid C30 column (IDxL = 4.6x250 mm; silica; particle size = 5 um) and a built-in column heater set at 30°C. I used a three-step gradient solvent system and identified carotenoid types *sensu* McGraw et al. (2006). For eye carotenoid extracts, the first step of the HPLC run used a solvent ratio of 44:44:12 methanol:acetonitrile:dichloromethane instead of 42:42:16, to separate the polar carotenoid types more effectively (Toomey and McGraw 2009).

Data Collection and Preparation for Analysis

Areas collected from HPLC chromatograph peaks were converted into carotenoid concentration using a standard curve for lutein and custom Python code stored on GitHub (https://github.com/TheEmilyAves/avian-nutrient-distributions). In cases where a carotenoid type was known to be present in a particular tissue type but at a concentration too low for integration (i.e., a peak was detectable but area was < 5), then the threshold concentration at an area of 5 was calculated for that carotenoid type to avoid underestimating total carotenoid concentration due to threshold-detection issues. Similarly, because a few tissue types (e.g., spleen, non-breeding testes) were particularly small (mass < 0.005 g), concentrations were high at the threshold level even for barely detectable peaks (i.e., areas just above 0), so minimum detectable carotenoids were calculated and included for major carotenoid types in these tissues, again to avoid skewing results due to threshold-detection issues. For example, a spleen with a mass of 0.005 g and an area of 5 (which is the minimum detectable area) has a minimum detectable carotenoid concentration of 26 µg/g, whereas pectoralis major (muscle) with a

mass of 5.00 g and an area of 5 has a minimum detectable carotenoid concentration of 0.03 ug/g. Low tissue carotenoid concentrations, therefore, have a problem with zero inflation when carotenoids are not detected. Using a lower threshold of detectability reduces, but it does not eliminate, this issue.

Statistical Analyses

All statistical tests were performed in the R environment (R Core Team 2017) with RStudio (RStudio Team 2015). The three main predictors (i.e., season, sex, and color) could not be included in one model, because I collected color data for only male house finches, so separate tests were run for season*sex and season*color. The three color metrics that I measured—hue, saturation, and brightness averaged across all three body regions (crown, breast, rump)—were tested for intercorrelations, and I found that saturation and brightness were highly correlated with each other (cor = -0.56, $t = -4.77$, $df = 49$, $p < 0.0001$), whereas hue was not correlated with either saturation ($t = -0.42$, $df = 49$, $p = 0.67$) or brightness ($t = 1.00$, $df = 49$, $p = 0.32$). As a result, I only used two color metrics, hue and saturation, in the models that included color. Saturation was selected over brightness, because saturation is typically more closely associated with carotenoid content of ornaments including house finch feathers (Butler et al. 2011). To consider the role of tissue size (mass) in explaining variation in carotenoid profiles, I ran all statistical models with and without whole tissue mass as a covariate, but the results were not qualitatively different (data not shown), so tissue mass was excluded from final models.

Three different levels of tests were conducted to thoroughly explore differences in carotenoid profiles as a function of season, sex, and plumage coloration: 1) tissue

carotenoid profiles (i.e., concentrations of each carotenoid type by tissue; Figure 1A), 2) total tissue-carotenoid concentrations (Figure 1B), and 3) total body-carotenoid concentration (Figure 1C). I chose these three levels, because I wanted to be able to distinguish between carotenoid profile differences that were due to the relative proportions of carotenoid types within a given tissue (Figure 1A), total carotenoid concentrations within a given tissue relative to other tissues (Figure 1B), and total carotenoid accumulation in the body independent of tissue and carotenoid type differences (Figure 1C). For tissue carotenoid profile analyses, only carotenoid types found consistently (i.e., in more than 10% of individuals) within a tissue type were used. For calculations of total tissue-carotenoid concentrations, all compounds that were identified as carotenoids by characteristic absorbance spectra (e.g., three-peaked with a maximum absorbance between 400 nm and 480 nm for xanthophylls/carotenes or single-peaked with a maximum absorbance of 475nm for some ketocarotenoids; Britton et al., 2004) were included, even if a particular carotenoid type was infrequently identified among individuals, unlike with tissue carotenoid profile analyses where these were excluded. For total body carotenoid concentration, I added the total tissue-carotenoid concentrations together to get a proxy for total body-carotenoid concentration. Although this figure is likely an underestimate since it is missing carotenoids from the carcass (i.e., skin and bones) and gastrointestinal tract (including pancreas), it still represents a proxy of total carotenoid accumulation in the body that is closer to the total than if only a few tissues had been measured.

To test for statistical effects of season, sex, and plumage color, and their interactions and tissue carotenoid profiles, I used permutational multivariate analysis of

variance (PERMANOVA) with Bray-Curtis dissimilarities and 999 random permutations (Anderson 2001). I chose this semi-parametric test based on my findings from Chapter 1, since these data had variable intercorrelation structure (i.e., some weak and some strong relationships depending on tissue and carotenoid type), which I did not want to influence tissue specificity of results. Additionally, although number of nutrient types was within the acceptable range for a MANOVA given my sample sizes (three to six per tissue) and the data metric (i.e., concentration) did not necessarily require the use of a PERMANOVA, there were multiple tissue carotenoid concentrations that were not normally distributed (e.g., zero inflated) in a way that was difficult to correct with transformation alone. It is worth noting that, because PERMANOVAs are multivariate analyses, they intentionally only determine whether carotenoid profiles are different between groups and do not provide statistical information about directionality or which carotenoid types contribute to differences between groups. Any time when directionality is implied, this comes from a qualitative evaluation of group differences after statistical significance has been confirmed. PERMANOVAs were carried out using the "adonis" function in R with package *vegan* (Oksanen et al., 2020). Seasonal differences in tissue carotenoid profiles were examined further using pairwise post-hoc tests (with package *pairwiseAdonis*; Arbizu, 2021).

To test for statistical effects of season, sex, plumage color, and their interactions on total tissue-carotenoid concentrations (Figure 1B), I conducted PERMANOVAs where the dependent variables were concentrations of each of the carotenoid types added together to get total tissue carotenoid concentrations. This allowed me to detect differences between groups that were driven by total carotenoid concentrations in tissues

rather than contributions of particular carotenoid types. Last, I used a generalized linear model and a univariate analysis of variance (ANOVA) to test for differences in total body carotenoid concentration (Figure 1C) by season*color and season*sex, respectively. This was necessary to check for differences in total carotenoid accumulation in the body regardless of tissue or carotenoid type. Adipose and gonad were excluded from analyses of total body carotenoid concentrations because I did not have adipose and gonad samples from each individual in the study (as above).

Results

General aspects of tissue carotenoid profiles

All carotenoid types detected were identified for 7 out of 11 house finch body tissues. For the remaining 4 tissue types (adipose, heart, liver, and muscle), at least 90% of carotenoids by concentration were identified (90%, 98%, 93%, and 90%, respectively). When I ran tissue carotenoid-profile models with and without unidentified carotenoid types, the results were qualitatively the same. Across all tissues, I detected 9 different carotenoid types that have previously been identified in house finches: lutein, zeaxanthin, beta-carotene, beta-cryptoxanthin, 3-hydroxyechinenone, astaxanthin, echinenone, epsilon-carotene, and galloxanthin.

All tissues contained lutein and zeaxanthin. Most tissues (adipose, brain, gonad, heart, kidney, liver, lung, muscle, plasma, spleen) had a similar composition of primarily lutein followed by zeaxanthin and some combination of beta-carotene, beta-cryptoxanthin, 3-hydroxyechinenone, and/or astaxanthin (though the latter two were seasonally restricted to around the summer-autumn molt period). Echinenone was only

found in liver. Eye was distinct from other tissues in the sense that it contained two unique carotenoid types (epsilon-carotene, galloxanthin) as well as those found in other tissues (e.g., astaxanthin, lutein). Among tissues not including plasma, the eye was the most carotenoid concentrated tissue followed by adipose, spleen, lung, liver, kidney, gonad, heart, muscle, and then brain. This order is not necessarily directly associated with tissue mass. For example, eye is much more carotenoid concentrated than expected for its mass. Most other tissues fall within the expected range of carotenoid concentrations by tissue mass, with some (e.g., brain) being a little lower than expected based on its mass while others (e.g., spleen) were higher than expected.

Sexual and seasonal differences in carotenoid profiles

I found significant differences in carotenoid profiles among seasons and sexes, and these differences were tissue-specific (Table 2, Figure 2). Carotenoid profiles of some tissues varied only by season (adipose, eye, lung, and plasma) or sex (brain, gonad), while others varied either by season and sex (heart and spleen) or by the season*sex interaction (kidney, liver, and muscle). Since gonad profiles were examined only by sex due to small gonad size in late summer-winter, seasonal differences in gonad carotenoid profiles could not be examined.

When examining pairwise differences among seasons for tissues where season was significant (Table 3), carotenoid profiles (for all tissues except adipose) in fall 2018 were distinct from those in other seasons, which is associated with the higher concentrations of ketocarotenoids (i.e., 3-hydroxyechinenone, astaxanthin, echinenone), especially 3-hydroxyechinenone, in tissues during the molt period. Similarly, for lung and

spleen, carotenoid profiles were most different between fall and spring; heart carotenoid profiles differed most between fall and winter. However, although ketocarotenoids (i.e., 3-hydroxyechinenone, astaxanthin) varied seasonally in all three of these tissues (lung, spleen, heart), beta-carotene also seasonally varied in the spleen. Additionally, there were tissues, such as kidney, liver, and muscle, where carotenoid profiles were distinct in nearly every season.

I also found significant differences in total tissue-carotenoid concentrations by season ($F = 2.34$, $R^2 = 0.11$, $P = 0.004$) and sex ($F = 3.70$, $R^2 = 0.04$, $P = 0.013$), but not the season*sex interaction ($F = 1.49$, $R^2 = 0.07$, $P = 0.13$; Figure 3). Post-hoc tests of seasonal differences (Table 4) indicated that liver carotenoid concentrations were higher and spleen carotenoid concentrations were lower in fall 2018 compared to spring. There were also season-specific sex differences in liver, spleen, and eye carotenoid concentrations. Males had higher liver carotenoid concentrations in the fall and spleen carotenoid concentrations in the winter compared to fall females and to males in other seasons. Females had higher eye carotenoid concentrations than males in every season except fall 2018 (peak molt). Since gonad and adipose were not included in the overall tissue-carotenoid-profile analyses (as above), their total carotenoid concentrations were analyzed separately, and I found that female gonads were more carotenoid concentrated than male gonads ($F_{1,13} = 30.91$, $P<0.0001$), but that there were no effects of season, sex, or the season*sex interaction on total adipose carotenoid concentrations (season: $F_{4,52} = 1.84$, $p = 0.14$; sex: $F_{1,52} = 1.98$, $p = 0.17$; season*sex: $F_{4,52} = 0.57$, $p = 0.69$). There were also only sex differences in total body carotenoid concentrations (sex: $F_{1,70} = 5.57$, $p =$

0.021; season: $F_{4,70} = 1.705$, $p = 0.16$; season*sex: $F_{4,70} = 1.62$, $p = 0.18$), where males had more than females.

Plumage coloration as a predictor of carotenoid profiles

Male plumage hue was not a significant predictor of tissue carotenoid profiles for all tissues. However, there was a season*hue interaction for two tissue types, heart and lung (Table 5, Figure 4, see more below). There were also no relationships between male plumage hue and total tissue carotenoid concentrations (hue: $F_{1,50} = 0.34$, $p = 0.85$; hue*season: $F_{1,50} = 1.57$, $p = 0.13$) or total body carotenoid concentration ($t = -0.93$, $p = 0.36$). Additional analyses for gonad (t-value = 1.33, $p = 0.22$) and adipose (t-value = 0.14, $p = 0.89$) also revealed no association with plumage hue.

Pairwise comparisons of heart carotenoid profiles among seasons (Table 6) revealed that winter 2017 was distinct from all other seasons. Regarding individual heart carotenoid types, there was no association between plumage hue and lutein concentration in all seasons, except during winter 2017 and spring 2018, when there was a positive association between hue and lutein concentration, such that (due to the fact that lower hue scores represent redder birds0 finches with redder plumage had lower heart lutein levels. There were positive associations between zeaxanthin concentration and hue in all seasons except winter 2018, when there was a negative relationship. There were no associations at all between beta-cryptoxanthin concentrations and hue. There was a positive association between astaxanthin concentration and hue but only during fall 2018. There

was a negative association between 3-hydroxyechinenone concentration and hue but only during fall 2017 and 2018.

Plumage hue was also associated with differences in lung carotenoid profiles in fall 2017, but not the other seasons. Specifically, there were positive associations between lutein concentration and hue in winter 2017 and spring 2018, no association in fall 2018, and negative associations in winter 2018 and fall 2017. There was a positive association between zeaxanthin concentration and hue in spring 2018 but no association in any other season. There were negative associations between 3-hydroxyechinenone concentration and hue during fall (both 2017 and 2018) but no other season.

In contrast, male plumage saturation was a significant predictor of tissue carotenoid profiles for nearly all tissues (Table 7). I found a similar pattern of carotenoid type-specific differences in the association between saturation and concentration of a particular carotenoid type in all tissues except adipose, brain, and gonad, where, generally, plumage saturation was positively associated with dietary carotenoid (e.g., lutein) concentrations, but negatively associated with metabolized ketocarotenoid (e.g., 3-hydroxyechinenone, astaxanthin) concentrations (Figure 5). There was also a significant relationship between total tissue carotenoid concentrations and male plumage saturation ($F_{1,50} = 51.65$, $p = 0.001$) but not the interaction between saturation and season ($F_{1,50} = 0.79$, $p = 0.58$). Specifically, I found positive associations between plumage saturation and total carotenoid concentrations in eye, kidney, lung, plasma, and spleen, but no association in brain; and negative associations in heart, liver, and muscle. However, I did not find an association between total body carotenoid concentrations and male plumage saturation ($t = 1.05$, $p = 0.30$), nor was plumage saturation correlated with total gonad

carotenoid concentration (t-value = 1.95, p = 0.09) or total adipose carotenoid concentration (t-value = -0.14, p = 0.89).

Discussion

I investigated tissue carotenoid profiles of wild-caught house finches in a suite of eleven internal body tissues and how they varied with season, sex, and expression of male ornamental plumage coloration. Previous studies that characterized the carotenoid profiles of more than a few (2-4) tissues either used artificially bred or captive populations (Fox 1962; McGraw and Toomey 2010; Phelan et al. 2018), or described carotenoid profiles without evaluating ecological or life-history sources of variation (e.g., seasonal or sex differences; Surai et al., 2000). In house finches, carotenoid profile variation by season, sex, and plumage coloration was previously examined in a few tissues (e.g., retina, liver, plasma; McGraw et al., 2006; Toomey & McGraw, 2009). My aim with this study was to evaluate the extent of carotenoid profile variation in a wild population of animals by more thoroughly characterizing carotenoid profiles of house finches across seasons, sexes, and plumage color variation simultaneously and in many tissue types.

Tissue carotenoid profiles varied with both season and sex, and this variation was mostly consistent with prior work in house finches. Toomey and McGraw (2009) found similar seasonal differences in retinal carotenoid levels (i.e., higher eye carotenoid concentrations during winter/pre-breeding) and no sex differences, although it is worth

noting that, while I found no sex differences in eye carotenoid profiles (i.e., distribution pattern of carotenoid types), I did find sex differences in total eye carotenoid concentration. Also McGraw et al. (2006) found that, during molt, males generally had higher carotenoid concentrations (in plasma and liver) than females. However, in their study, this was driven by males having greater concentrations of both beta-carotene and beta-cryptoxanthin, whereas I found that, of those two carotenoid types, only beta-carotene in the liver differed between sexes, and I also found that males had more ketocarotenoids such as 3-hydroxyechinenone. I did not find sex differences in plasma carotenoid profiles independent of total carotenoid concentrations, which means that males did not have higher relative concentrations of ketocarotenoids (e.g., 3-hydroxyechinenone), but instead total concentrations of ketocarotenoids were higher, consistent with McGraw et al. (2013). Giraudeau and McGraw (2014) examined seasonal differences in plasma carotenoid profiles of males and found a spike in 3-hydroxyechinenone with a concurrent drop in lutein during molt, and I observed a similar pattern. Hill (1995) found season and sex differences in plasma hue (which is associated with carotenoid concentrations), such that female plasma had lower overall hue than that of males, but a similar seasonal pattern, which is supported by my findings with total plasma carotenoid concentration.

At least for the tissues most frequently analyzed for carotenoid content in previous research (e.g., plasma, liver, eye/retina), my findings both strengthen and clarify existing knowledge about carotenoids in these tissues as they relate to the life history of birds. First, despite the sex difference in eye carotenoid concentration that I uncovered, this study provides support for the lack of sex differences in eye or retinal carotenoid

profiles, which highlights their importance in both males and females, likely for some combination of mutual mate selection (Hill, 1993; Toomey et al., 2015), foraging (Toomey and McGraw 2011), and photoprotection (Demmig-Adams and Adams 2013). Additional support for the importance of mutual mate selection is that winter or pre-breeding was associated greater concentrations of eye and retinal carotenoids. Second, this study joins many others in support of the link between the molt period and the presence of 3-hydroxyechinenone in house finches (Hill, 1995; Hutton et al., 2021.; Inouye et al., 2001; McGraw et al., 2006). However, my research has also revealed that 3-hydroxyechinenone is much more widespread in tissues than just liver, plasma, and feathers, which suggests that distribution of this carotenoid either is not tightly regulated (exclusively for coloration) mechanistically or functionally may play more diverse roles in the body (e.g., antioxidant, immune) than previously thought. Third, I believe that my results for tissue carotenoid profiles and total tissue carotenoid concentrations clarify that, in plasma, sex differences in carotenoids are due more to differences in total carotenoid concentrations than to proportional differences in specific carotenoid types (e.g., ketocarotenoids). This could be due to sex differences in the ingestion, absorption, or transport of carotenoids, as opposed to differences in specific metabolism of ketocarotenoids, all of which have been suggested previously as possible mechanisms (McGraw et al. 2002), although this would have to be tested more explicitly to confirm. Finally, the discrepancy in drivers of sex differences in liver carotenoids between previous literature and my study could be a result of interannual variation in dietary availability of relatively minor carotenoids in house finches (e.g., beta-carotene, beta-cryptoxanthin) compared to major carotenoids such as lutein and zeaxanthin. This is

supported by my finding that differences in concentrations of these carotenoids did not vary consistently by season, suggesting that interannual variation in the availability or circulation of these minor carotenoids could be large.

Although there are no studies in house finches that have examined season and sex differences in carotenoid profiles of the remaining tissues (i.e., adipose, brain, gonad, heart, kidney, lung, muscle, and spleen), one of these tissues (adipose) has at least been examined in wild birds along at least one axis of variation, and all of these tissues have been analyzed for carotenoid content in birds (Fox 1962; Surai et al. 2000; Phelan et al. 2018), although not in wild populations or along one of these axes of variation. Sex differences in adipose carotenoid concentration or proxy associated with carotenoid concentration (i.e., saturation) have been examined previously in wild-caught graylag geese (*Anser anser*) and garden warblers (*Sylvia borin*). I found that there were no sex differences in adipose carotenoid profiles or total concentrations, which was consistent with Metzger and Bairlein (2011) in warblers but not Negro et al. (2001) in geese (studied during winter), who found that males had higher adipose carotenoid concentrations than females. One possible explanation for why there are not consistent sex differences in adipose carotenoid concentration among species is life history variation. Since female geese had lower adipose carotenoid concentrations than males in the winter (post-breeding), then this could be because females used up their carotenoid stores during their breeding attempts. The lack of sex difference in house finches and garden warblers compared to the sex difference in geese could then be attributed to the extent to which these species are income versus capital breeders (Stephens et al. 2009), where geese would be capital breeders that save up their carotenoids stored in adipose

and use them up during breeding, and house finches and garden warblers would be income breeders that use carotenoids as they are acquired.

It is not surprising to find tissue-specific differences by season and sex in house finch carotenoid profiles, based on previous work showing differences in carotenoid profiles for a smaller subset of tissues (Giraudeau & McGraw, 2014; McGraw et al., 2006; Toomey & McGraw, 2009). These results, however, allow us to explore the implications of tissue-specific carotenoid distributions on carotenoid functions and mechanisms in ways that have not been previously examined in birds. I found sex or sex*season differences in carotenoid profiles for most tissues (brain, gonad, heart, kidney, liver, lung, muscle, and spleen; but not eye, adipose, or plasma) and this resulted in sex differences in total body carotenoid concentrations. These observations suggest that there may be sex differences in the physiological mechanisms underlying the accumulation (e.g., absorption, metabolism, deposition, transport) of total, but not specific, carotenoids. Sex differences in expression of genes associated with these elements of carotenoid physiology (e.g., micellar uptake in the gut, lipoprotein transport, tissue carotenoid-binding proteins, metabolism, SCARB1; Bhosale & Bernstein, 2007; Lopes et al., 2016; Toomey et al., 2017) are rarely studied in any animal (Zhang et al. 2003; Gazda et al. 2020). Additional studies are needed to test if the differences I observed are reflected in these genetic mechanisms and their potential regulation by sex steroids. For example, testosterone may regulate the expression of CYP2J19, a member of the cytochrome P450 family of monooxygenases, which leads to sex differences in production of red ornaments through metabolism of dietary carotenoids into red ketocarotenoids (Khalil et al. 2020). There are also likely differences in carotenoid

metabolism between males and female house finches, because I observed a season*sex interaction effect in liver, where molting males (fall 2018) had higher concentrations of 3-hydroxyechinenone (the primary pigment in red feathers of male house finches; Inouye et al., 2001) than other season and sex combinations; however, this should be confirmed with an analysis of CYP2J19 (i.e., carotenoid ketolation enzyme; Hill et al., 2019) and BCO2 (i.e., carotenoid cleaving enzyme; Gazda et al., 2020) expression in males and females during molt.

I found season or season*sex differences in carotenoid profiles among all tissues except brain. Much of this seasonal variation could be explained by greater contributions of 3-hydroxyechinenone to the carotenoid profile by molting individuals, particularly males. This would explain why birds from the fall 2018 (peak molt) study period have a distinct carotenoid profile in so many of the tissue types. It appears as though there is mostly systemic, rather than tissue-specific, accumulation of 3-hydroxyechinenone during molt. At present, it is unclear if 3-hydroxyechinenone is important only for feather color development and only distributed to (or mobilized from) other body tissues, or whether this pigment conveys additional, systemic benefits (e.g., antioxidant activity) to finches during the molt period in which it is produced. One example in support of the latter is a study linking higher circulating 3-hydroxyechininenone levels with lower likelihood of being infected with a common bacterium (*Mycoplasma gallisepticum*) in male house finches (McGraw et al., 2013). No experimental studies have directly examined the potential health benefits, or lackthereof, of 3-hydroxyechinenone (e.g., via repletion/depletion). However, another ketocarotenoid commonly found in birds (astaxanthin) is a particularly potent antioxidant (Negro and Garrido-Fernández 2000).

Thus, 3-hydroxyechinenone may be similarly beneficial, which might explain why females also convert dietary carotenoids into this compound, in addition to the fact that females sometimes also produce carotenoid-based ornaments (Hill 1993). In my study, very few females had carotenoid-based ornaments, likely because I collected my samples from individuals in an urban environment, where female house finches are less ornamented (Sykes et al. 2021).

Besides 3-hydroxyechinenone, the other carotenoid types appear to vary seasonally in a tissue-specific manner. For example, beta-carotene is only present in adipose, gonad, kidney, liver, muscle, and spleen, and patterns of accumulation by season and sex do not align with differences in circulating levels. This is particularly evident in the spleen, where females in most seasons, as well as males during spring, have higher levels of beta-carotene relative to other season-sex combinations. The spleen is an immune organ and the only immune tissue that is not tightly integrated with other tissues in adult birds (as opposed to the mucosal immune tissues of the respiratory, digestive, and reproductive tracts Kaiser & Balic, 2015). These results suggest particular importance of beta-carotene for supporting the immune response in a season- and sex-specific manner in house finches. While there are no studies evaluating the effect of beta-carotene on spleen function in birds, there is evidence of antioxidant benefits for higher levels of beta-carotene levels in the spleens of rats (Bendich and Shapiro 1986).

Perhaps the most surprising result was a lack of strong relationship between male plumage hue and carotenoid profiles in house finches, given all previous evidence that male plumage hue reflects internal carotenoid levels (Hill 1995; McGraw et al. 2006b; Toomey and McGraw 2009). These studies in molting house finches found that redder

birds have higher total concentrations of plasma, liver, and eye carotenoids, but I did not find this relationship in my sample of molting males (n = 21) when I analyzed it directly (plasma: estimate = -0.28, t = -0.26, p = 0.80; liver: estimate = -0.61, t = -1.26, p = 0.22; eye: estimate = -0.31, t = 0.28, p = 0.28). However, my results are not entirely comparable to those of prior studies, because of different color-metrics used. For example, Hill's (1995) metric of plumage coloration included saturation and brightness. Rowe et al. (2012) also found no relationship during the breeding season between male plumage hue and carotenoid concentrations in the plasma, liver, and testes. This is more consistent with my results, which show that hue is not associated with carotenoid concentrations in these and other tissues (e.g., liver, testes) during the breeding season. However, it seems likely that this lack of relationship during molt is due to plumage hue insufficiently capturing all aspects of carotenoid investment in house finch tissues, because we did find significant relationships between male plumage saturation and tissue carotenoid profiles.

In contrast to my results for plumage hue, in nearly all tissues and regardless of season, I found the same general trends of a positive associations between plumage saturation and concentrations of the most abundant dietary carotenoid (e.g., lutein) and a negative association between plumage saturation and metabolized ketocarotenoids (e.g., 3-hydroxyechinenone, astaxanthin) regardless of season. Since higher plumage saturation is often associated with greater concentrations of total carotenoids in the plumage (Hill, 1995; McGraw et al., 2006; Toomey & McGraw, 2009), it makes sense that there would be a positive association for the most abundant carotenoid. The negative association between plumage saturation and internal tissue ketocarotenoid levels could be explained

by greater deposition of ketocarotenoids into feathers by birds with more saturated (i.e., more carotenoid concentrated) plumage, since ketocarotenoids are the primary component of house finch feathers (Inouye et al. 2001). This finding suggests that there could be a trade-off between internal tissue levels of ketocarotenoids and ketocarotenoids that are used to produce more saturated plumage coloration.

Despite the lack of overall strong relationships between male plumage hue and tissue carotenoid profiles, there was one tissue (heart) that emerged whose carotenoid profile was linked to the hue*season interaction, such that the heart carotenoid profile covaried with hue in every season except winter 2017. In fall 2017 and winter 2018, redder males tended to have a higher concentration of 3-hydroxyechinenone in heart, which is notable, because redder males did not have higher concentrations of 3-hydroxyechinenone in plasma, so this suggests specific accumulation of 3-hydroxyechinenone in heart tissue. However, in fall 2018 (peak molt), less red males appeared to have higher heart astaxanthin concentrations than redder males. Since astaxanthin was only detected in three tissues (heart, kidney, and muscle), this suggests that astaxanthin was accumulated in a tissue-specific manner. One of the known functions of astaxanthin is to promote cardiovascular health, in part via antioxidant defense in a tissue that produces reactive oxygen species in excess relative to other tissues (Negro and Garrido-Fernández 2000). Concentration of astaxanthin along with other feather ketocarotenoids are also positively correlated with plumage hue in male house finches (Inouye et al. 2001). Although it might seem counterintuitive at first that *less*-red males had higher heart concentrations of astaxanthin, this result fits with an alternative prediction of the resource trade-off hypothesis (Koch and Hill 2018), which

states that beneficial carotenoids are found in higher concentrations in tissues of lower-quality males, because they lack the good genes that would give them inherently good cardiovascular health and thus redirect astaxanthin away from feathers to meet this heart-specific need.

While I did not find support for the classic prediction of the resource trade-off hypothesis that plumage coloration is an indicator of total internal carotenoid accumulation (i.e., redder birds have more carotenoids; Koch & Hill, 2018), I did find support for the honesty of male plumage coloration (i.e., both hue and saturation) in house finches as a reflection of tissue carotenoid profiles (e.g., relative contributions of specific carotenoid types to the overall profile). However, I found that different aspects of male plumage coloration reflected different aspects of tissue carotenoid profiles, which highlights the importance for future work to analyze elements of integumentary coloration separately rather than as a single composite metric. Plumage redness (i.e., hue) did not predict carotenoid profiles in most contexts with the exception of heart carotenoid profiles of males during molt, whereas plumage saturation predicted investment of total carotenoids in different tissues (e.g., negative relationship in heart, positive relationship in eye) and a potential trade-off between ketocarotenoids in internal tissues and color production. Although I have observed evidence of potential trade-offs in this observational study of carotenoid profiles along natural gradients like season and sex, it would still be useful to further explore carotenoid profile variation and its links to color in naturally challenged (i.e., sick/stressed) birds and/or via an experimental manipulation (e.g., immune challenge), because physiological limitation of carotenoids by such a challenge is more likely to elicit an explicit trade-off.

Figures

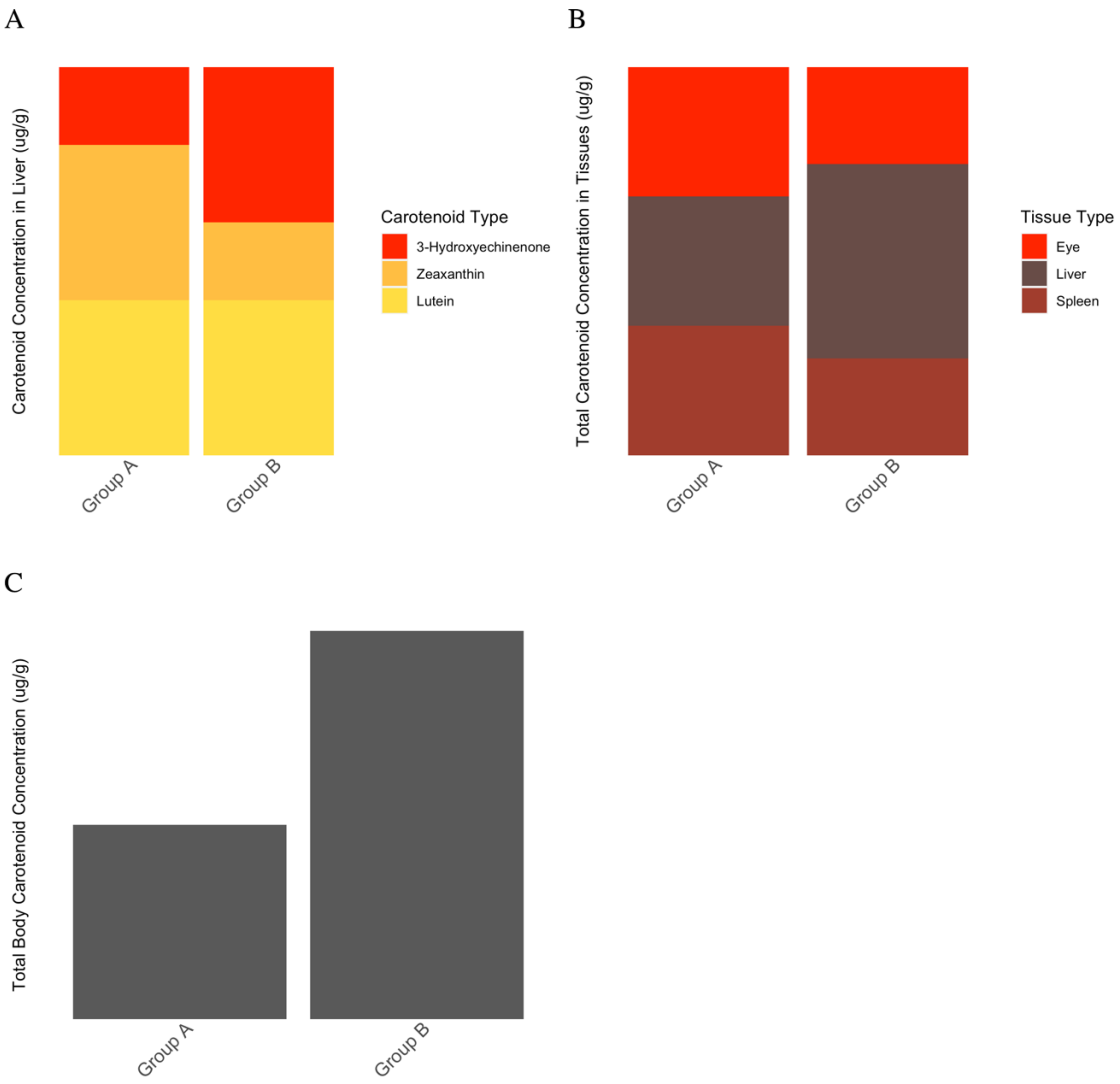

Figure 1. Theoretical diagram to illustrate the differences between my three metrics of carotenoid distribution in this study: (A) tissue carotenoid profiles, (B) total tissue carotenoid concentrations, and (C) total body carotenoid concentration. In (C), groups A

and B represent theoretical groups that could differ in carotenoid profiles (e.g., treatment vs. control, male vs. female, fall vs. spring).

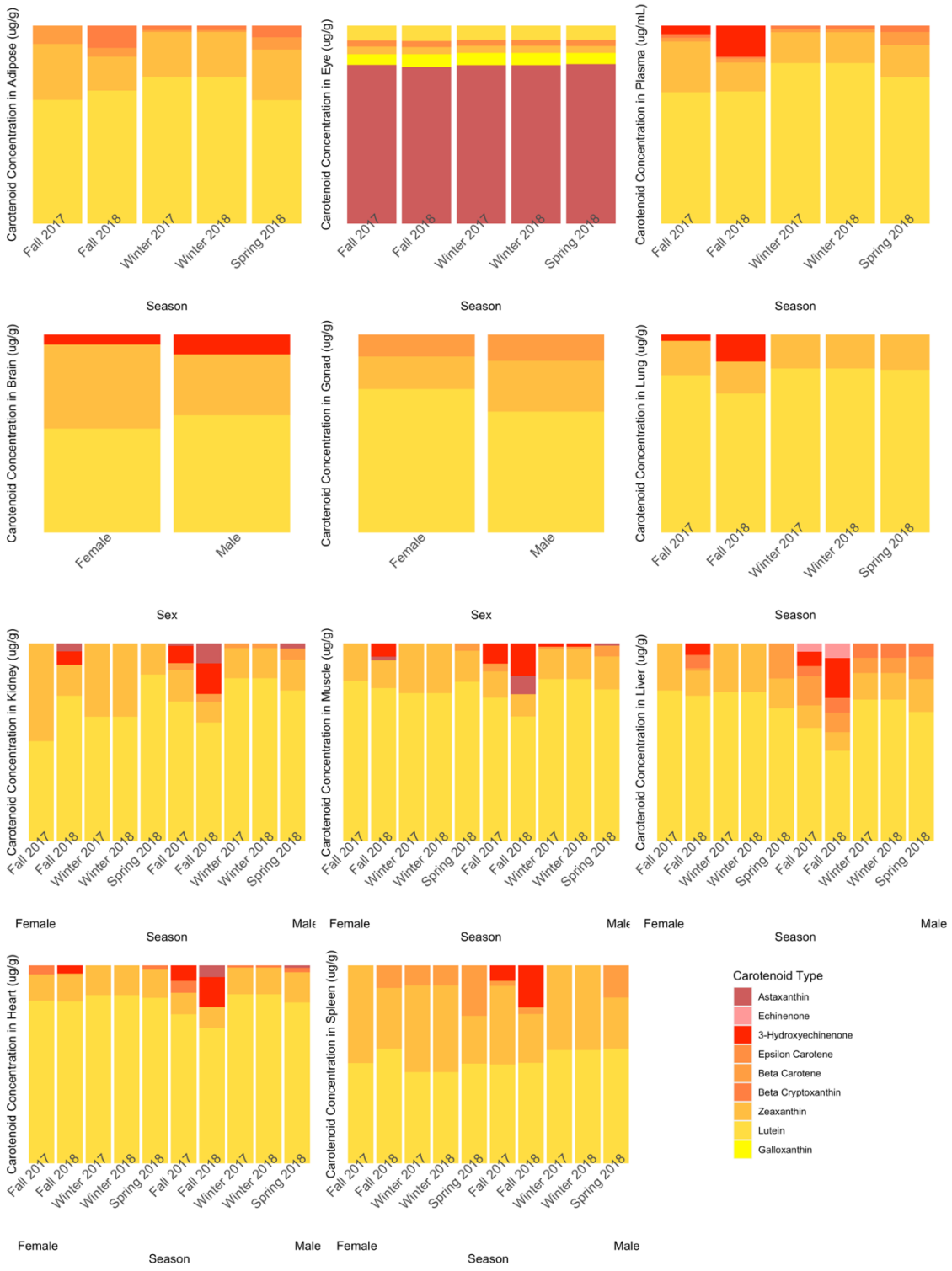

Figure 2. Seasonal and sexual variation in carotenoid profiles. The proportion of each carotenoid type by carotenoid concentration is represented for each season-sex combination and tissue type.

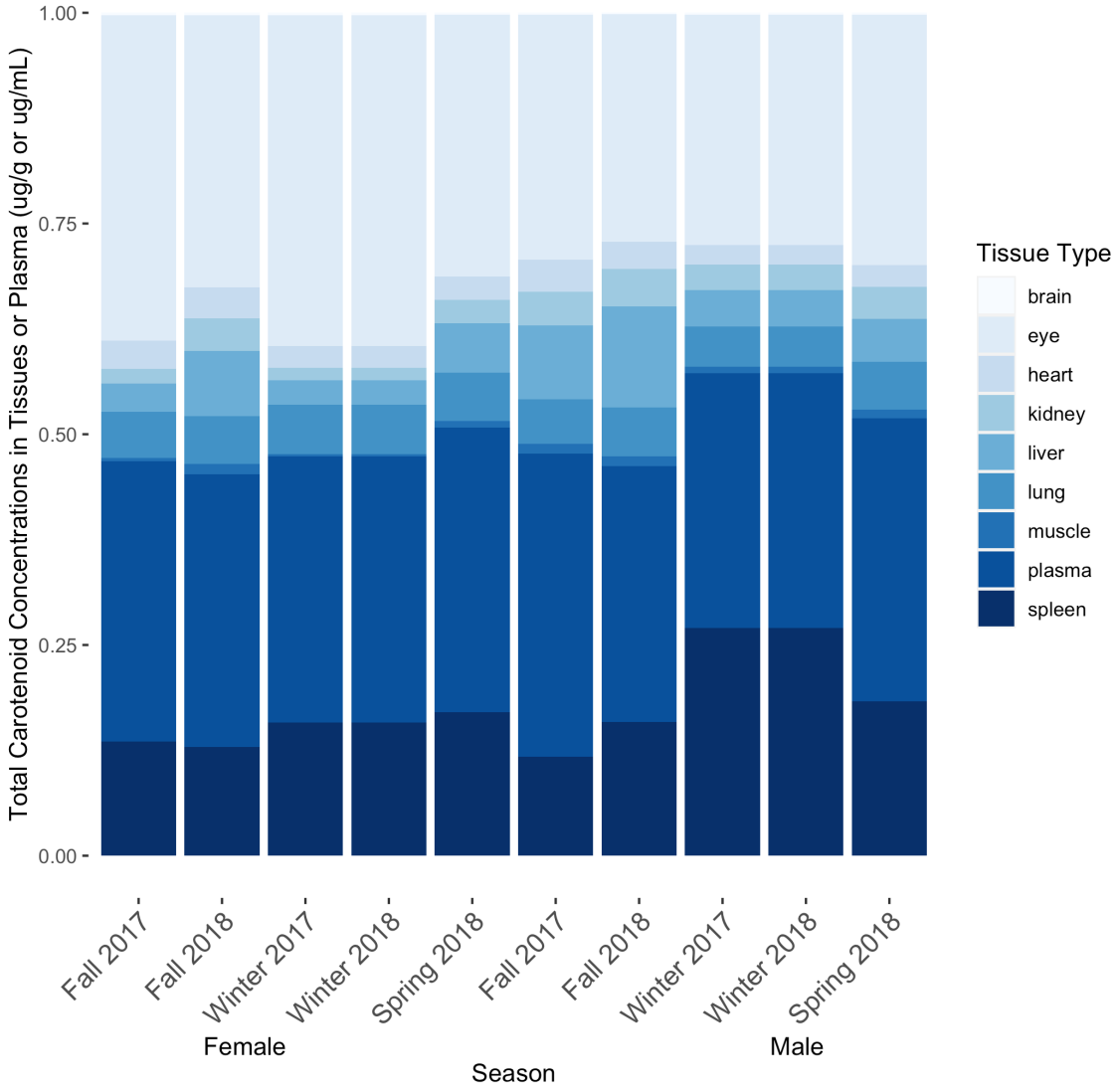

Figure 3. Seasonal and sex differences in tissue carotenoid concentrations, here shown in proportions to highlight relative differences in tissue concentrations independently of total body carotenoid concentration differences.

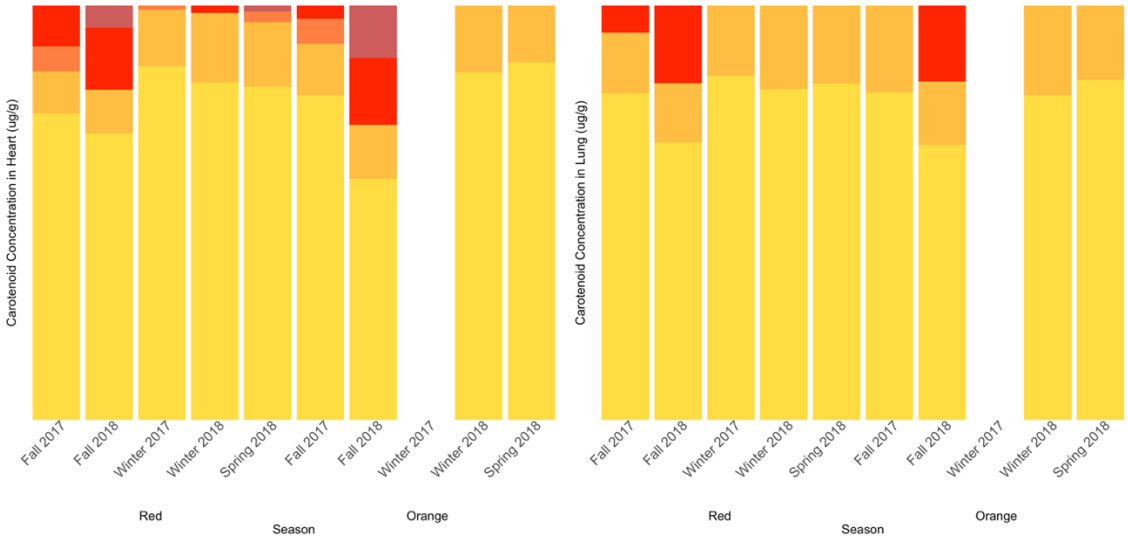

Figure 4. Carotenoid profiles for heart and lung as a function of season and plumage coloration, where there was a significant hue*season interaction. The proportion of total carotenoid concentration by tissue is represented for each hue-season combination, where hue has been reduced to red (mean hue < 15) and orange (mean hue > 15) for the purposes of visualization.

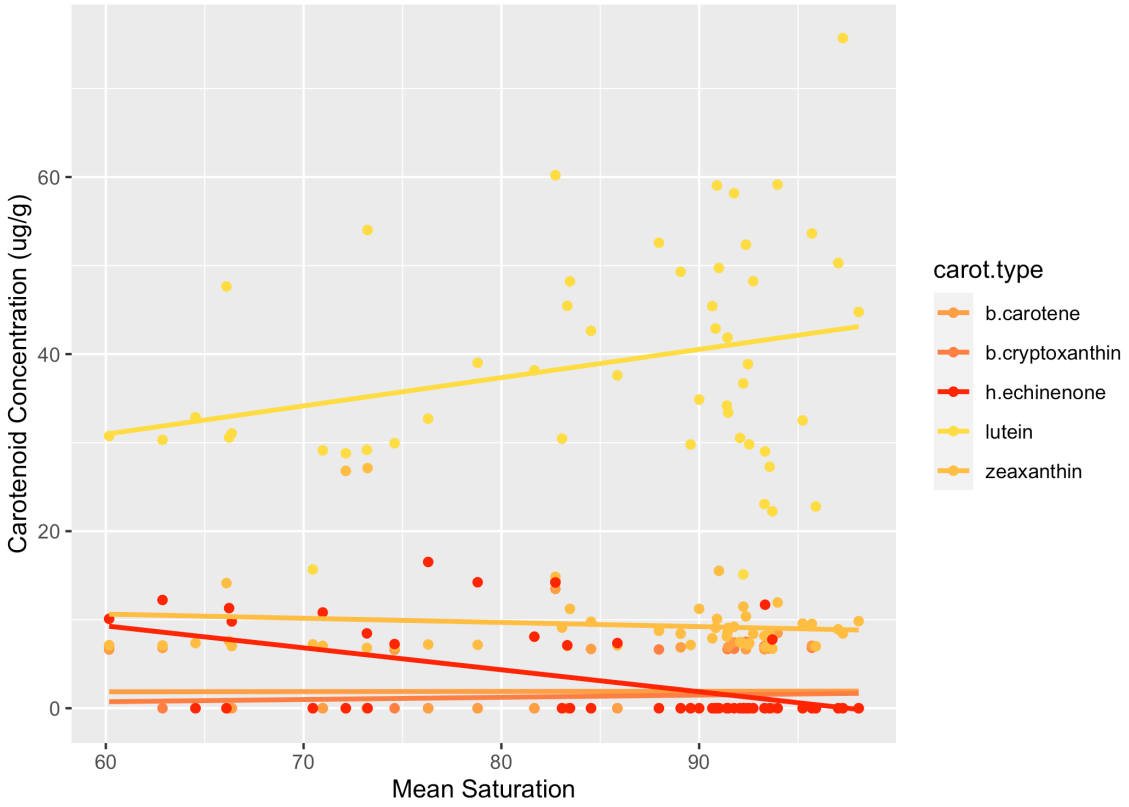

Figure 5. Carotenoid profile of plasma where there was a significant association between tissue carotenoid profiles and mean male plumage saturation. Concentrations (µg/g) of individual carotenoid types (i.e., beta-carotene, beta-cryptoxanthin, 3-hydroxyechinenone, lutein, and zeaxanthin) are presented.

Tables

Table 1. Sample sizes of wild-caught house finches by season and sex.

Date Ranges (Season Year, Life History Stage)	Female	Male
8 - 15 October (fall 2017, post-molt)	5	9
24 August - 6 September (fall 2018, peak molt)	7	12
15 January - 3 February (winter 2017, pre-breeding)	5	10
5 December - 30 January (winter 2018, pre-breeding)	7	10
21 April - 1 May (spring 2018, breeding)	5	10

Table 2. Permutational multivariate analysis of variance (adonis) for tissue carotenoid profile variation as a function of season, sex, and their interaction, using Bray-Curtis dissimilarity matrices. Terms were added sequentially (first to last) to the model. Significant predictors are denoted by bold text ($\alpha = 0.05$); underlined text shows Bonferroni-corrected alpha of 0.005.

Predictor	df	F	R^2	p
Adipose				
Season	4	3.61	0.20	**0.001**
Sex	1	1.23	0.02	0.27
Season*Sex	4	1.05	0.06	0.42
Residuals	52		0.72	
Total	61		1.00	
Brain				
Season	4	1.74	0.08	0.12
Sex	1	4.19	0.05	**0.02**

Season*Sex	4	0.60	0.03	0.79
Residuals	70		0.84	
Total	79		1.00	
Eye				
Season	4	4.32	0.18	**0.003**
Sex	1	0.40	0.004	0.58
Season*Sex	4	1.77	0.07	0.13
Residuals	70		0.74	
Total	79		1.00	
Gonad				
Sex	1	10.36	0.44	**0.003**
Residuals	13		0.56	
Total	14		1.00	
Heart				
Season	4	5.59	0.22	**0.001**
Sex	1	3.48	0.03	**0.03**
Season*Sex	4	1.42	0.06	0.17
Residuals	70		0.69	
Total	79		1.00	
Kidney				
Season	4	8.78	0.21	**0.001**
Sex	1	37.57	0.22	**0.001**
Season*Sex	4	6.95	0.16	**0.001**
Residuals	70		0.41	
Total	79		1.00	
Liver				
Season	4	5.85	0.20	**0.001**
Sex	1	9.85	0.09	**0.001**
Season*Sex	4	3.02	0.10	**0.002**
Residuals	70		0.61	
Total	79		1.00	
Lung				
Season	4	3.41	0.15	**0.003**
Sex	1	1.72	0.02	0.20
Season*Sex	4	1.39	0.06	0.21

Residuals	70		0.77	
Total	79		1.00	
Muscle				
Season	4	8.18	0.24	**0.001**
Sex	1	17.87	0.13	**0.001**
Season*Sex	4	4.54	0.13	**0.001**
Residuals	70		0.50	
Total	79		1.00	
Plasma				
Season	4	5.32	0.22	**0.001**
Sex	1	1.44	0.01	0.23
Season*Sex	4	1.31	0.05	0.23
Residuals	70		0.71	
Total	79		1.00	
Spleen				
Season	4	3.34	0.15	**0.001**
Sex	1	2.84	0.03	**0.04**
Season*Sex	4	1.28	0.06	0.22
Residuals	70		0.77	
Total	79		1.00	

Table 3. Pairwise permutational multivariate analysis of variance (pairwiseAdonis) for tissue carotenoid profile variation as a function of season, using Bray-Curtis dissimilarity matrices. Significant predictors are denoted by bold text ($\alpha = 0.05$); underlined text shows Bonferroni-corrected alpha of 0.005. If the model had a significant season*sex interaction from the initial permutational multivariate analysis of variance, then significant ($p<0.05$) sex or season*sex effects were denoted by an asterisk (*).

Pairs	df	F	R^2	p
Adipose				
Fall 2017 vs Winter 2017	16	1.13	0.07	0.32
Fall 2017 vs Spring 2018	13	4.33	0.26	**0.01**

Fall 2017 vs Fall 2018	20	1.35	0.07	0.26
Fall 2017 vs Winter 2018	18	0.35	0.02	0.76
Winter 2017 vs Spring	24	3.18	0.12	**0.02**
Winter 2017 vs Fall 2018	31	0.82	0.03	0.49
Winter 2017 vs Winter	29	3.84	0.12	**0.01**
Spring 2018 vs Fall 2018	28	4.24	0.13	**0.009**
Spring 2018 vs Winter	26	11.81	0.30	**0.001**
Fall 2018 vs Winter 2018	33	5.26	0.14	**0.007**
Eye				
Fall 2017 vs Winter 2017	28	3.89	0.13	0.06
Fall 2017 vs Spring 2018	28	4.85	0.15	**0.03**
Fall 2017 vs Fall 2018	32	1.19	0.03	0.29
Fall 2017 vs Winter 2018	30	3.69	0.11	0.06
Winter 2017 vs Spring	29	0.28	0.01	0.64
Winter 2017 vs Fall 2018	33	9.58	0.22	**0.004**
Winter 2017 vs Winter	31	0.57	0.02	0.47
Spring 2018 vs Fall 2018	33	11.66	0.26	**0.003**
Spring 2018 vs Winter	31	1.78	0.05	0.20
Fall 2018 vs Winter 2018	35	7.85	0.18	**0.004**
Heart				
Fall 2017 vs Winter 2017	28	6.72	0.19	**0.004**
Fall 2017 vs Spring 2018	28	3.63	0.11	**0.04**
Fall 2017 vs Fall 2018	32	4.53	0.11	**0.008**
Fall 2017 vs Winter 2018	30	9.98	0.25	**0.001**
Winter 2017 vs Spring	29	2.15	0.07	0.16
Winter 2017 vs Fall 2018	33	7.90	0.18	**0.002**
Winter 2017 vs Winter	31	0.83	0.03	0.41
Spring 2018 vs Fall 2018	33	4.82	0.12	**0.01**
Spring 2018 vs Winter	31	4.33	0.13	**0.01**
Fall 2018 vs Winter 2018	35	10.22	0.21	**0.001**
Kidney				
Fall 2017 vs Winter 2017	28	2.69	0.03	**0.09***
Fall 2017 vs Spring 2018	28	8.05	0.13	**0.003***
Fall 2017 vs Fall 2018	32	13.97	0.17	**0.001***
Fall 2017 vs Winter 2018	30	8.59	0.12	**0.001***

Winter 2017 vs Spring	29	7.90	0.13	**0.009***
Winter 2017 vs Fall 2018	33	17.91	0.23	**0.001***
Winter 2017 vs Winter	31	8.28	0.13	**0.005***
Spring 2018 vs Fall 2018	33	7.77	0.17	**0.001***
Spring 2018 vs Winter	31	1.66	0.05	0.20
Fall 2018 vs Winter 2018	35	9.27	0.18	**0.001***
Liver				
Fall 2017 vs Winter 2017	28	2.87	0.06	0.05*
Fall 2017 vs Spring 2018	28	0.86	0.02	0.400*
Fall 2017 vs Fall 2018	32	3.59	0.08	**0.022***
Fall 2017 vs Winter 2018	30	5.82	0.12	**0.006***
Winter 2017 vs Spring	29	2.42	0.07	0.096*
Winter 2017 vs Fall 2018	33	8.94	0.19	**0.001***
Winter 2017 vs Winter	31	3.97	0.10	**0.018***
Spring 2018 vs Fall 2018	33	6.51	0.15	**0.001***
Spring 2018 vs Winter	31	5.05	0.15	**0.009**
Fall 2018 vs Winter 2018	35	11.65	0.23	**0.001***
Lung				
Fall 2017 vs Winter 2017	28	1.61	0.06	0.20
Fall 2017 vs Spring 2018	28	6.37	0.19	**0.009**
Fall 2017 vs Fall 2018	32	2.99	0.08	0.05
Fall 2017 vs Winter 2018	30	3.72	0.12	**0.04**
Winter 2017 vs Spring	29	1.04	0.04	0.33
Winter 2017 vs Fall 2018	33	4.21	0.11	**0.01**
Winter 2017 vs Winter	31	0.69	0.02	0.49
Spring 2018 vs Fall 2018	33	7.53	0.17	**0.002**
Spring 2018 vs Winter	31	0.47	0.02	0.59
Fall 2018 vs Winter 2018	35	5.58	0.13	**0.006**
Muscle				
Fall 2017 vs Winter 2017	28	1.68	0.03	0.17*
Fall 2017 vs Spring 2018	28	5.65	0.13	**0.005***
Fall 2017 vs Fall 2018	32	11.05	0.19	**0.001***
Fall 2017 vs Winter 2018	30	9.56	0.19	**0.002***
Winter 2017 vs Spring	29	7.63	0.15	**0.002***
Winter 2017 vs Fall 2018	33	16.40	0.25	**0.001***

Winter 2017 vs Winter	31	11.53	0.20	**0.001***
Spring 2018 vs Fall 2018	33	8.04	0.19	**0.002***
Spring 2018 vs Winter	31	2.70	0.08	0.07
Fall 2018 vs Winter 2018	35	6.53	0.15	**0.001***
Plasma				
Fall 2017 vs Winter 2017	28	2.94	0.10	0.06
Fall 2017 vs Spring 2018	28	4.82	0.15	**0.006**
Fall 2017 vs Fall 2018	32	5.86	0.14	**0.002**
Fall 2017 vs Winter 2018	30	1.51	0.05	0.20
Winter 2017 vs Spring	29	1.97	0.07	0.15
Winter 2017 vs Fall 2018	33	12.95	0.27	**0.001**
Winter 2017 vs Winter	31	0.79	0.03	0.44
Spring 2018 vs Fall 2018	33	19.28	0.35	**0.001**
Spring 2018 vs Winter	31	2.08	0.07	0.11
Fall 2018 vs Winter 2018	35	8.11	0.18	**0.001**
Spleen				
Fall 2017 vs Winter 2017	28	2.06	0.07	0.09
Fall 2017 vs Spring 2018	28	8.05	0.23	**0.004**
Fall 2017 vs Fall 2018	32	2.07	0.06	0.10
Fall 2017 vs Winter 2018	30	1.41	0.05	0.22
Winter 2017 vs Spring	29	3.24	0.11	**0.02**
Winter 2017 vs Fall 2018	33	3.46	0.09	**0.02**
Winter 2017 vs Winter	31	0.81	0.03	0.49
Spring 2018 vs Fall 2018	33	8.15	0.19	**0.001**
Spring 2018 vs Winter	31	3.05	0.09	**0.04**
Fall 2018 vs Winter 2018	35	2.60	0.06	0.08

Table 4. Pairwise permutational multivariate analysis of variance (pairwiseAdonis) for tissue carotenoid concentrations by season using Bray-Curtis dissimilarity matrices. Significant predictors are denoted by bold text for an alpha of 0.05 and underlined text for a Bonferroni-corrected alpha of 0.005. If the model had a significant season*sex

interaction from the initial permutational multivariate analysis of variance, then significant ($p<0.05$) sex or season*sex effects were denoted by an asterisk (*).

Pairs	df	F	R^2	p
Fall 2017 vs Winter 2017	28	1.43	0.05	0.18*
Fall 2017 vs Spring 2018	28	4.23	0.13	**0.006**
Fall 2017 vs Fall 2018	32	1.69	0.04	0.15*
Fall 2017 vs Winter 2018	30	0.92	0.03	0.40
Winter 2017 vs Spring	29	0.91	0.03	0.47
Winter 2017 vs Fall 2018	33	3.21	0.08	0.007*
Winter 2017 vs Winter	31	0.76	0.02	0.57
Spring 2018 vs Fall 2018	33	5.29	0.13	**0.002**
Spring 2018 vs Winter	31	3.21	0.10	0.05
Fall 2018 vs Winter 2018	35	3.48	0.09	**0.03**

Table 5. Permutational multivariate analysis of variance (adonis) for tissue carotenoid profile variation by mean hue and season using Bray-Curtis dissimilarity matrices. Terms were added sequentially (first to last) to the model. Significant predictors are denoted by bold text for an alpha of 0.05.

Tissue	df	F	R^2	p
Adipose				
Mean Hue	1	1.40	0.03	0.24
Season	4	2.95	0.26	**0.001**
Mean Hue*Season	4	0.34	0.03	0.99
Residuals	31		0.68	
Total	40		1.00	
Brain				
Mean Hue	1	0.17	0.004	0.82
Season	4	1.37	0.11	0.21
Mean Hue*Season	4	0.53	0.04	0.81
Residuals	41		0.84	
Total	50		1.00	

Eye				
Mean Hue	1	0.23	0.005	0.71
Season	4	1.94	0.15	0.11
Mean Hue*Season	4	0.54	0.04	0.71
Residuals	41		0.80	
Total	50		1.00	
Gonad				
Mean Hue	1	0.10	0.21	0.14
Residuals	8	0.05	0.79	
Total	9		1.00	
Heart				
Mean Hue	1	0.41	0.005	0.68
Season	4	7.60	0.37	**0.001**
Mean Hue*Season	4	2.36	0.12	**0.03**
Residuals	41		0.50	
Total	50		1.00	
Kidney				
Mean Hue	1	1.56	0.02	0.20
Season	4	5.79	0.33	**0.001**
Mean Hue*Season	4	1.05	0.06	0.40
Residuals	41		0.59	
Total	50		1.00	
Liver				
Mean Hue	1	0.76	0.01	0.52
Season	4	5.33	0.32	**0.001**
Mean Hue*Season	4	0.86	0.05	0.59
Residuals	41		0.62	
Total	50		1.00	
Lung				
Mean Hue	1	1.50	0.02	0.25
Season	4	4.15	0.23	**0.003**
Mean Hue*Season	4	3.01	0.17	**0.008**
Residuals	41		0.58	
Total	50		1.00	
Muscle				

Mean Hue	1	0.84	0.01	0.46
Season	4	4.09	0.26	**0.002**
Mean Hue*Season	4	1.00	0.06	0.44
Residuals	41		0.66	
Total	50		1.00	
Plasma				
Mean Hue	1	1.89	0.03	0.16
Season	4	4.90	0.29	**0.001**
Mean Hue*Season	4	1.30	0.08	0.25
Residuals	41		0.61	
Total	50		1.00	
Spleen				
Mean Hue	1	0.41	0.007	0.77
Season	4	3.33	0.22	**0.003**
Mean Hue*Season	4	1.21	0.08	0.25
Residuals	41		0.69	
Total	50		1.00	

Table 6. Pairwise permutational multivariate analysis of variance (pairwiseAdonis) for tissue carotenoid profile variation by mean hue and season using Bray-Curtis dissimilarity matrices. Degrees of freedom (df), F value (F), R^2, and p-values (p) in this table are associated with effect of hue, not season. Values in parentheses represent p-values of the hue*season interaction term. Significant effects of hue or hue*season are denoted by bold text for an alpha of 0.05 and underlined text for a Bonferroni-corrected alpha of 0.005.

Tissue	Df	F	R2	p
Heart				
Fall 2017 vs Winter 2017	18	0.50	0.02	0.58 (**0.02**)
Fall 2017 vs Spring 2018	18	0.38	0.02	0.68 (0.06)
Fall 2017 vs Fall 2018	20	0.90	0.04	0.46 (0.63)

Fall 2017 vs Winter 2018	18	2.23	0.07	0.12 (0.88)
Winter 2017 vs Spring	19	5.38	0.24	**0.02** (0.53)
Winter 2017 vs Fall 2018	21	1.03	0.03	0.34 (**0.03**)
Winter 2017 vs Winter	19	0.60	0.03	0.49 (**0.04**)
Spring 2018 vs Fall 2018	21	0.52	0.02	0.64 (0.09)
Spring 2018 vs Winter	19	0.71	0.03	0.43 (0.10)
Fall 2018 vs Winter 2018	21	0.59	0.02	0.57 (0.45)
Lung				
Fall 2017 vs Winter 2017	18	1.10	0.05	0.32 (**0.005**)
Fall 2017 vs Spring 2018	18	2.37	0.09	0.12 (**0.02**)
Fall 2017 vs Fall 2018	20	2.63	1.00	0.10 (0.16)
Fall 2017 vs Winter 2018	18	5.50	0.21	**0.007** (0.20)
Winter 2017 vs Spring	19	6.10	0.27	**0.03** (0.48)
Winter 2017 vs Fall 2018	21	1.22	0.04	0.31 (0.08)
Winter 2017 vs Winter	19	0.71	0.03	0.50 (**0.02**)
Spring 2018 vs Fall 2018	21	1.58	0.05	0.22 (0.22)
Spring 2018 vs Winter	19	0.50	0.02	0.51 (**0.05**)
Fall 2018 vs Winter 2018	21	-0.01	-0.0003	0.93 (0.50)

Table 7. Permutational multivariate analysis of variance (adonis) for tissue carotenoid profile variation by mean saturation and season using Bray-Curtis dissimilarity matrices. Terms were added sequentially (first to last) to the model. Significant predictors are denoted by bold text for an alpha of 0.05.

Tissue	df	F	R^2	p
Adipose				
Mean Saturation	1	2.15	0.04	0.10
Season	4	3.18	0.25	**0.002**
Mean Saturation	4	1.35	0.11	0.16
Residuals	31		0.68	
Total	40		1.00	
Brain				
Mean Saturation	1	2.87	0.05	0.07

Season	4	0.87	0.07	0.54
Mean Saturation	4	1.37	0.10	0.22
Residuals	41		0.78	
Total	50		1.00	
Eye				
Mean Saturation	1	4.63	0.09	**0.03**
Season	4	0.81	0.06	0.51
Mean Saturation	4	0.63	0.05	0.66
Residuals	41		0.80	
Total	50		1.00	
Gonad				
Mean Saturation	1	2.54	0.24	0.11
Residuals	8	0.05	0.79	
Total	9		1.00	
Heart				
Mean Saturation	1	12.95	0.18	**0.001**
Season	4	3.56	0.20	**0.002**
Mean Saturation	4	0.89	0.05	0.54
Residuals	41		0.57	
Total	50		1.00	
Kidney				
Mean Saturation	1	8.51	0.13	**0.001**
Season	4	3.74	0.22	**0.001**
Mean Saturation	4	0.74	0.04	0.63
Residuals	41		0.61	
Total	50		1.00	
Liver				
Mean Saturation	1	7.46	0.12	**0.001**
Season	4	3.44	0.22	**0.002**
Mean Saturation	4	0.60	0.04	0.83
Residuals	41		0.63	
Total	50		1.00	
Lung				
Mean Saturation	1	5.31	0.09	**0.01**
Season	4	2.32	0.15	**0.04**

Mean Saturation	4	1.17	0.08	0.34
Residuals	41		0.68	
Total	50		1.00	
Muscle				
Mean Saturation	1	5.4	0.09	**0.003**
Season	4	3.09	0.20	**0.003**
Mean Saturation	4	1.14	0.07	0.35
Residuals	41		0.65	
Total	50		1.00	
Plasma				
Mean Saturation	1	5.99	0.10	**0.002**
Season	4	3.33	0.21	**0.001**
Mean Saturation	4	0.60	0.04	0.82
Residuals	41		0.65	
Total	50		1.00	
Spleen				
Mean Saturation	1	5.25	0.09	**0.004**
Season	4	2.23	0.15	**0.01**
Mean Saturation	4	0.84	0.06	0.62
Residuals	41		0.70	
Total	50		1.00	

CHAPTER 3

BILL COLORATION MODIFIES THE EFFECT OF IMMUNE ACTIVATION ON TISSUE CAROTENOID PROFILES IN ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

Abstract

Both immune function and condition-dependent signals are important for the survival and reproductive success of animals, but investment in one often comes at the cost of the other. To date, however, the investment of resources allocated toward each has rarely been directly quantified. Carotenoids are nutrient resources that can modulate the immune response and produce condition-dependent signals (i.e., carotenoid-based coloration), and their distributions throughout tissues in the body (i.e., carotenoid profiles) can be quantified to represent relative investment in these two somatic functions. To understand the potential for a trade-off, a perturbation to the system is needed, such as experimental manipulation of a function (e.g., immune), as well as tracking of carotenoid profiles to determine where carotenoids are being apportioned. I investigated this by measuring the effect of innate immune activation (via bacterial

lipopolysaccharides, LPS) on carotenoid profiles of male zebra finches (*Taeniopygia guttata*). I hypothesized that there are tissue-specific and/or carotenoid-type-specific differences in carotenoid profiles between LPS-injected and control birds. I also hypothesized that differences in carotenoid profiles would co-vary with bill coloration, which would have implications for the trade-off between condition-dependent signals and immune function. While there were many possible outcomes, one prediction was that carotenoid profiles of LPS-injected birds would reflect a greater investment in immune organs (e.g., spleen) relative to control birds and that this investment would scale with variation in bill-color change during the experiment (e.g., decreased bill color could be associated with greater investment in the spleen). I found that there were tissue-specific (e.g., spleen, muscle), but not carotenoid-type-specific, differences in carotenoid profiles of LPS-injected birds relative to control birds. In the case of spleen carotenoid profiles, this effect was bill-color-dependent, such that, among redder birds (but not for less-red birds), those that were immune-challenged had greater spleen carotenoid concentrations than redder control birds. Treatment effects on breast-muscle carotenoid profiles, however, were color-independent; treatment birds had lower concentrations of carotenoids in breast-muscle (the site of LPS injection) than control birds. Interestingly, there were also treatment-independent relationships between bill color and carotenoid profiles of the heart and adipose tissue. Neither LPS treatment nor bill color predicted carotenoid profiles of the remaining tissues: eye, kidney, liver, lung, plasma, and testes. These results support the role of carotenoids regardless of type in modulation of the immune response at the spleen and at the site of injection (muscle). Moreover, although there was no evidence of an explicit trade-off in carotenoid investment between signal

and immune function (i.e., bill-color change did not predict differences in immune-organ carotenoid profiles between treatment and control groups), there was some evidence of a trade-off independent of the immune challenge, as demonstrated by the negative association between bill-color change and tissue carotenoid concentrations for heart and adipose in both treatment groups.

Introduction

A robust and effective immune response is critical for an animal's survival, but carries notable resource costs. Investment in constitutive and inducible immune functions can trade-off with other life-history traits, including growth and reproduction, so allocation of specific resources to immune activation must be precisely routed and timed (Rauw 2012). While there are several fitness-critical resources that could be quantified to assess the potential for immune trade-offs, including energy (King et al. 2011), biomass (Saeki et al. 2009), time (Angelier et al. 2007), and metabolites (Gleibs and Mebs 1999), among the most useful resources for studying allocation patterns are nutrients (Boggs 1992; Raubenheimer et al. 2009). However, direct investment of nutrients in relation to immune system activity has rarely been quantified.

Expression of condition-dependent signals (e.g., coloration, courtship displays) can also carry resource costs and potentially be linked to a trade-off with an immune response. Prior work has shown that immune activation can decrease investment in a condition-dependent signal (Alonso-Alvarez et al. 2004; Jacot et al. 2004; Clotfelter et al. 2007; López et al. 2009; Ibáñez et al. 2014; Desprat et al. 2015). Ideal nutrients for quantifying potential trade-offs between condition-dependent signals and immunity

would be those that directly contribute to both to production of the signal and the immune response. Carotenoid pigments produce red, orange, and yellow condition-dependent ornaments (e.g., feathers, bare parts in birds; Hill et al. 2006) as well as modulate the immune response. There are many mechanistic hypotheses for the role of carotenoids in the immune response, including stimulating the growth of immune organs (e.g., thymus in mammals), stimulating lymphocyte blastogenesis, increasing the strength of immune cell activity (e.g., enhanced NK cell cytotoxicity, higher bacterial killing ability of neutrophils, increased peroxidase activity in macrophages), quelling reactive oxygen species (ROS) which stimulate production of cytokines (i.e., modulating inflammation), and facilitating cell-cell communication via membrane fluidity (Chew and Park 2004).

The effect of immune activation on avian carotenoid-based ornamental coloration has been studied extensively (Faivre et al. 2003; Peters, Delhey, et al. 2004; Aguilera and Amat 2007; Fitze et al. 2007; Casagrande and Groothuis 2011; Rosenthal et al. 2012), and in these studies a decrease in carotenoid-based color expression following immune-system activation is often described as evidence of a trade-off between investment of carotenoid pigments in the immune response versus the color signal. However, there is still a need to examine how carotenoids are actually distributed throughout the body, including coloration, after immune-system activation, because, without measuring carotenoids in tissues where they could influence the immune response, it is difficult to determine if a change in carotenoid-based coloration is actually associated with a redirection of resources toward immunity. Carotenoids have already been measured in a handful of tissues as a response to immune challenges, although not explicitly linked to how they trade-off with sexual signal expression. For example, Toomey et al. (2010)

found that carotenoids were depleted from the retina after immune activation in house finches (*Haemorhous mexicanus*), and Koutsos et al. (2003) found that liver carotenoids were depleted and immune-tissue (e.g., bursa, thymus) carotenoids increased after immune activation in juvenile chickens (*Gallus gallus domesticus*). Also Rosenthal et al. (2012) showed that immune activation decreased the expression of a carotenoid-based signal in male American goldfinches (*Spinus tristis*), but males that were originally more colorful (i.e., higher hue, saturation, and brightness) prior to immune activation were still more colorful than males that were initially less colorful. Since more colorful birds typically have stronger immune responses (McGraw and Ardia 2003) and remain comparatively more colorful in the face of an immune challenge (Rosenthal et al. 2012), this suggests that less colorful birds could also have different internal patterns of carotenoid distribution (hereafter carotenoid profiles) relative to more colorful birds. For example, if redder birds are redder because they are better at accumulating carotenoids in their bodies overall, then they may also be better at maintaining their color and directing more carotenoids toward the immune response (e.g., in spleen) compared to less red birds, which may not maintain their color but have either the same or lower levels of spleen carotenoids.

In this study, I investigated the effect of immune-system activation on tissue carotenoid profiles of male zebra finches (*Taeniopygia guttata*) as well as how the carotenoid profile co-varies with both pre-experimental bill coloration and bill color change. Zebra finches are a popular model for studies of carotenoid-based coloration (Blount et al. 2003; McGraw, Gregory, et al. 2003; Eraud et al. 2007; Ardia et al. 2010; McGraw et al. 2011; Spickler et al. 2020), and studies of avian bill color generally are

key for improving our understanding of how morphological traits can respond to change in rapid and current environmental or physiological conditions (Faivre et al. 2003; Ardia et al. 2010; Rosenthal et al. 2012; George et al. 2017b), as opposed to feather color change which is restricted to a brief (molting) time period during the year. Specifically, I compared tissue carotenoid profiles of control birds with those of lipopolysaccharide (LPS)-injected birds to test the following hypotheses: 1) not all tissue carotenoid profiles respond the same way to immune activation, 2) pre-treatment bill coloration predicts tissue carotenoid profile after immune activation, and 3) the ability to maintain bill coloration during the experiment (i.e., stay red) is associated with the tissue carotenoid profile after immune activation. I hypothesized that some tissues, especially those associated with immunity (e.g., spleen), would differ between control and LPS-injected birds, whereas other tissues would not differ. I also hypothesized that, relative to control males, LPS-injected males that either started the experiment with redder bills or were able to maintain bill color after immune-system activation would have different tissue carotenoid profiles than individuals with less red bills or that did not maintain bill color, because bill color is a sexually selected, condition-dependent signal in zebra finches (Simons and Verhulst 2011; Simons, Briga, et al. 2012). This could manifest in many different ways. For example, there could be no tissue-carotenoid differences between treatment and control birds, except that redder males are able to maintain higher carotenoid concentrations in an immune tissue (like the spleen). Less red or LPS-injected birds could also redistribute carotenoids from those tissues not associated with the immune response to those that are involved. Evidence of the trade-off between a

condition-dependent signal and the immune response would be if I observed that greater decreases in bill color correlate with higher levels of immune-organ carotenoids.

Methods

From November - December 2017, male zebra finches from a large, outbred domesticated colony were housed in an indoor room at the College of William and Mary (Williamsburg, VA, USA) in group cages (approximately 0.76 x 0.46 x 0.46 m) of five individuals under constant environmental conditions (14:10 light:dark photoperiod, at approximately 22ºC) and with *ad libitum* access to food and drinking water. Thirty birds were randomly assigned to two groups (immune-challenged = 18; control = 12) that did not differ in pre-treatment bill color ($W = 351$, $P = 0.18$) or body mass ($t = -0.41$, $P = 0.68$). Innate immune responses were activated with three intramuscular (pectoralis major) injections (0.15mL) of LPS (*E. coli* serotype O55:B5 diluted in phosphate-buffered saline, concentration = 0.1 mg/mL, dose = 1 mg/kg) on days 0, 14, and 26. Control birds received three injections of vehicle solution (i.e.,, 0.15 mL of PBS) on the same days. I included more birds in the immune-challenged group, because I expected more variation in carotenoid profiles after LPS injection than injection of vehicle solution. I used this sequential injection regime to simulate an ongoing bacterial infection, as conducted previously by Toomey et al. (2010), rather than just the acute phase response after a single injection. I chose this time course because long-term (i.e., weeks to months) infections are common in wild birds (McClure 1989; Kollias et al. 2004). All birds were humanely euthanized 48 hours after the last injection (day 28); I chose this time instead of 24 hours because I wanted to capture long-term effects of the

injections that would not be confounded with the acute phase effects of the third injection (Xie et al. 2000).

For sample processing immediately after euthanasia, I bled birds and dissected the major body tissues (eye, furcular adipose, heart, kidney, liver, lung, pectoralis major muscle, spleen, and testes). I did not measure carotenoids in bill and leg tissue because these tissues were considered to be carotenoid sinks, meaning, once carotenoids are deposited there, they cannot be retrieved to rejoin the internal pool, even though ongoing deposition into these tissues can change (resulting in bare part color change) during the experiment. I only measured carotenoids in internal tissues, part of the mobilizable pool of carotenoids, to determine how carotenoids were distributed after an immune challenge as well as what bill color signals about carotenoid profiles in this context. I put heparinized whole blood on ice until it could be centrifuged for extracting plasma. I stored all plasma and tissues at -80°C until further analyses.

I followed general methods described in Chapter 2 for extracting zebra finch tissue carotenoids, and identified them based on retention time, absorption spectra, and comparisons to chromatographs from zebra finch tissue carotenoids in McGraw and Toomey (2010). Bill color was measured in three standardized locations from each individual before and after the experiment but before euthanasia (days -7 and 27, respectively) with a USB2000 UV-VIS portable reflectance spectrometer with a PX-2 pulsed xenon lamp (Ocean Optics Inc., Dunedin, FL). All reflectance curves were processed using a Java-based CLR program (Montgomerie 2008) to generate a metric of bill coloration, S1R, which represents saturation in the red part of the spectrum (625-700nm) and has been shown previously to respond to an immune challenge in zebra

finches (Merrill et al. 2016). I calculated both pre-experiment S1R (pS1R) and the difference between pre- and post-S1R (dS1R, or bill-color change). During the experiment, dS1R was mostly below zero or zero, meaning that zebra finch bill color mostly either did not change or became less red.

I performed all statistical tests in the R environment (R Core Team 2017) with RStudio (RStudio Team 2015). I conducted three different levels of tests (as categorized in Chapter 2) to identify differences in carotenoids between experimental groups: 1) tissue carotenoid profiles, 2) total carotenoid concentration within each tissue, and 3) total body carotenoid concentration (i.e., sum of values in 2 across all tissues). I entered bill color (pS1R or dS1R), treatment group, and their interaction as predictors in all three levels of testing; separate models were run for the two color variables, including bill color at the start of the experiment (pS1R), and bill color change during the experiment (dS1R). I did not include body mass in these models, because there was no difference between LPS-injected and control groups in the mass change between pre- and post-experiment ($t = 1.29$, $P = 0.21$). I statistically tested differences by group and color using the same method as for Chapter 2, with permutational multivariate analysis of variance (PERMANOVA). As a reminder, note that PERMANOVAs are multivariate analyses, which means that they intentionally only determine whether carotenoid profiles are different between groups and do not provide statistical information about directionality or which carotenoid types contribute to differences between groups. Any time when directionality is implied, this comes from a qualitative evaluation of group differences after statistical significance has been confirmed.

Results

General carotenoid profiles

All detected carotenoid types were identified in 9 out of 10 zebra finch body tissues. For the remaining tissue type (eye), at least 90% of carotenoids by average concentration were identified. Across all tissues, I detected 8 different carotenoid types that have been previously identified in zebra finches: lutein, zeaxanthin, anhydrolutein, dehydrolutein, beta-cryptoxanthin, galloxanthin, astaxanthin, and epilutein. All tissues contained lutein and zeaxanthin. Almost all tissues (adipose, heart, kidney, liver, lung, muscle, plasma, spleen, and testes) had a similar composition of primarily lutein, zeaxanthin, and anhydrolutein followed by dehydrolutein and beta-cryptoxanthin to a lesser extent. Eye was distinct from other carotenoid profiles in that it contained three unique carotenoid types (galloxanthin, astaxanthin, and epilutein) as well as those found in other tissues (lutein and zeaxanthin).

Effects of immune activation and pre-experiment bill color on carotenoid profiles

When I used pre-experiment bill color (pS1R) in the model for tissue carotenoid profiles, I found no effects of treatment, bill color, or their interaction on carotenoid levels in adipose, eye, kidney, heart, liver, lung, muscle, plasma, and testes. However, I found a significant effect of treatment*color interaction on spleen carotenoid profiles (Table 1; Figure 1). Among control birds, individuals with redder bills had lower concentrations of all carotenoid types in spleen, whereas, among treatment birds, there was no link between pre-treatment bill color and concentrations of the major spleen carotenoids (zeaxanthin, lutein, anhydrolutein), although redder birds were more likely to have detectable levels of

less abundant carotenoid types (dehydrolutein, beta-cryptoxanthin). Even though there were no effects of treatment, bill color, or their interaction on total tissue carotenoid concentrations (Table 2), I found an effect of the treatment*color interaction on total body carotenoid concentration (Table 3), where, among redder birds, those in the treatment group had greater concentrations of total body carotenoids than those in the control group, but among less-red birds there was no difference in total body carotenoid concentration between treatment and control birds (Figure 2).

Effects of immune activation and bill color change on carotenoid profiles

When using bill color change (dS1R) in the models, I found no effects of treatment, bill color, or their interaction on tissue carotenoid profiles of eye, kidney, liver, lung, plasma, spleen, and testes. However, I found a significant effect of treatment on muscle carotenoid profiles (Table 1). This effect was not driven by any one particular carotenoid type (Figure 3), but generally treatment birds (3.9±1.5 ug/g) had lower concentrations of carotenoids in breast muscle than control birds (4.7±1.2 ug/g). There were also two tissues (adipose, heart) for which I found effects of bill color change, but not treatment, on carotenoid profiles (adipose: $F = 5.99$, $p = 0.007$; heart: $F = 10.77$, $p = 0.002$). Specifically, birds with a greater reduction in bill redness, regardless of treatment, had higher concentrations of carotenoids in both fat and heart tissue. Additionally, bill color (not treatment or treatment*color interaction) was the only significant predictor of total tissue carotenoid concentrations (Table 2) as well as total body carotenoid concentrations (Table 3). In both cases, higher concentrations of carotenoids were associated with greater losses in bill color.

Discussion

I investigated the effect of an immune challenge on tissue carotenoid profiles in male zebra finches, and the extent to which these effects depended upon bill color before the experiment and bill color change during the immune challenges. I found tissue-specific effects of LPS-injection on carotenoid profiles that co-varied with carotenoid-based bill coloration. Of the ten tissues, I found that muscle and spleen carotenoid profiles differed significantly between treatment and control groups, where the treatment effect on muscle carotenoid profiles was independent of color and the treatment effect on spleen carotenoid profiles was dependent on pre-experiment bill color. No other tissue carotenoid profiles differed by treatment or the treatment*color interaction. By contrast, there were no carotenoid-type-specific effects of treatment or the treatment*color interaction on tissue carotenoid profiles, meaning that, where carotenoid concentrations differed, it was due to changes in concentrations of all carotenoid types, not one or a few types in particular. One exception was less abundant carotenoid types (dehydrolutein, beta-cryptoxanthin) in spleen carotenoid profiles, because these carotenoid types were found in amounts that were low enough such that sometimes they were measurable and other times they were not. The former meant that the minimum concentration was much higher than zero, creating a positive association where there likely is not one, as this effect was likely largely driven by differences in total carotenoids, which would indicate that it is not truly carotenoid-type specific.

Although carotenoid profiles of many animal or avian tissues had not been measured in response to an immune challenge before this study, the tissue carotenoid

profiles that have been measured previously (e.g., plasma, eye) responded fairly consistently between my study and past experiments, with some explainable exceptions. First, the effect of the immune challenge on zebra finch spleen carotenoids was consistent with results of Koutsos et al. (2003), who previously found that immune tissues (e.g., bursa, thymus) in juvenile chickens had higher carotenoid concentrations in immune-challenged individuals relative to controls. It is worth noting that the effect of LPS injection on spleen carotenoids in zebra finches was bill-color-dependent, but at least among zebra finches with redder bills at the start of the experiment, I found that immune-challenged birds generally had higher tissue carotenoid concentrations. Second, while much previous work has shown that immune challenges deplete plasma carotenoids (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Rosenthal et al. 2012; Simons et al. 2012; George et al. 2017), I did not find that post-treatment plasma carotenoid concentrations differed between treatment and control birds. However, many of the previous studies used acute (short-term) immune challenges, and my results in this study are consistent with studies that used multiple injections to simulate prolonged bacterial infection and also found no effect of immune challenge on plasma carotenoids (Koutsos et al. 2003; Toomey et al. 2010). This lack of effect on plasma carotenoid concentrations may also be because I only examined post-treatment differences in plasma carotenoid concentrations (I did not gather pre-treatment blood samples) rather than the change in plasma carotenoid concentration between the start and end of the experiment. My finding that liver carotenoid concentrations were not influenced by the immune challenge is consistent with a study in house finches (Toomey et al. 2010), but contrary to this study's results I did not find that eye carotenoids differed. This is possibly due to the difference

in length of study, since the previous study was for 8 weeks and this study was 4 weeks, which suggests that 4 weeks was not sufficient to allow changes in eye carotenoid profiles to be observed.

In breast muscle, I observed that, regardless of bill color, carotenoid concentrations were lower in the treatment group compared with control birds. This has interesting implications for the role of carotenoids in the immune response, because breast muscle was the tissue site of LPS injection (i.e., simulated bacterial infection). Lower carotenoid concentrations in LPS-injected muscle tissue suggests that carotenoids may be consumed at sites of infection, perhaps due to their free-radical scavenging role (e.g., high reactive oxygen species production during immune response; Siems et al. 2002, 2005; Sommerburg et al. 2003), as opposed to being recycled (Edge et al. 1998; Young and Lowe 2001). Sommerburg et al. (2003) found that white blood cells that are key in the innate immune response break down beta-carotene *in vitro*, which supports the idea that carotenoids are consumed on-site. To my knowledge, the present study is the first to observe evidence of this *in vivo*. A follow-up study where LPS is always injected on the same side of the body (left or right breast muscle) would help to determine if there were differences in muscle carotenoid concentrations specific to the site of infection as opposed to muscle tissue more broadly.

The finding that there was a significant treatment*color interaction in both spleen carotenoid profiles and total body carotenoid concentrations has important implications for the hypothesis that there is a trade-off between bill color and immune function, as previously suggested (McGraw and Ardia 2003; Alonso-Alvarez et al. 2004; Rosenthal et al. 2012; Simons et al. 2012; George et al. 2017). The only difference in spleen

carotenoid profiles between immune-challenged and control groups was among birds that were redder at the start of the experiment (pS1R), which suggests that redder birds may be apportioning their carotenoids more appropriately (i.e., less when healthy, more when immune-challenged) than less red birds. However, because I observed the same directionality in total body carotenoid concentrations as well, it could also be that redder birds are eating or absorbing more carotenoids during an immune challenge. This result supports the idea that bill color reflects an interaction between internal carotenoid levels and health state rather than either of these factors alone (Koch and Hill 2018). There was also a notable absence of the treatment*color interaction effect, where color was measured as the change between the start and end of the experiment (dS1R), which is not consistent with the classic trade-off idea that a reduction in bill color leads to an increase in internal carotenoid accumulation. However, for some concentrations of tissue carotenoids, I did observe an effect of bill color change independent of treatment effects, where birds whose bill became less red during the experiment had greater carotenoid concentrations in adipose and heart. It is difficult to say whether or not this explicit trade-off exists without doing additional studies to trace carotenoids (e.g., radio- or stable-isotope labeling; Schiedt 1989; Hardy et al. 1990), which would permit tracking of carotenoid movement across tissues rather than just acquiring a snapshot of tissue carotenoid profiles at the end of the experiment.

Although I did not have *a priori* predictions for the relationship between bill color and carotenoid profiles in this study independent of the immune challenge, I did find that adipose and heart carotenoid concentrations were lower for males that maintained their bill color (i.e., no change) and higher for males that were not able to maintain their bill

color (i.e., bill redness decreased). I observed a similar pattern in the relationship between dS1R and total body carotenoid concentration, meaning that males that maintained their bill color had lower total body carotenoid concentration and males that were not able to maintain their bill color had higher total body carotenoid concentration. It is difficult to draw causal inferences here, without knowing how tissue carotenoid levels actually changed from the beginning through the end of the experiment, but this finding is consistent with the idea of a trade-off between bill color and internal carotenoid levels, independent of an immune challenge. Specifically, it suggests that bill color maintenance comes at the cost of internal carotenoid pools, both overall and especially in adipose and heart tissue. It is possible that the birds in both treatment and control groups experienced generalized systemic stress associated with a needle injection (including handling and the injection itself, which occurred three times over four weeks) and this affected both bill color change (i.e., dS1R) and internal carotenoid levels. Without measuring carotenoids before and after in muscle tissue, it is difficult to say whether the changes were seen in the control group were due to stress at the site of injection. Future research should attempt to identify other treatment or treatment*color interaction effects on carotenoid profiles where treatment is an experimental manipulation of other stressors associated with carotenoid functions, particularly in ecologically relevant contexts. For example, it would be interesting to examine if bill color change covaries with heart and muscle carotenoid profiles during a physiological oxidative stress challenge.

Overall, I found that there were tissue-specific, but not carotenoid-type-specific, differences in carotenoid profiles between LPS-injected and control birds. This result supports the hypothesis of a general role of carotenoids in the avian immune response but

not a unique function for any particular type of carotenoid. However, as this study focused on tissues, it is still possible that there are carotenoid-type-specific allocations to support immune function at a cellular or subcellular level (e.g., immune cells in the blood, mitochondria). I found mixed results with regard to implications of my findings for trade-offs between condition-dependent signals and immune function during an innate immune challenge, since there were effects of bill color change and carotenoid profiles, but these effects were independent of immune activation treatment. Since carotenoids have multiple hypothesized functions for modulating the immune response, not just as part of the innate immune response, additional studies should evaluate the effect of different immune challenges (e.g., adaptive immune response) on carotenoid profiles to better understand how carotenoids are mobilized as part of the immune response and, therefore, how lipid-soluble nutrients more broadly may be invested in different life history traits including condition-dependent signals and health (i.e., immune function).

Figures

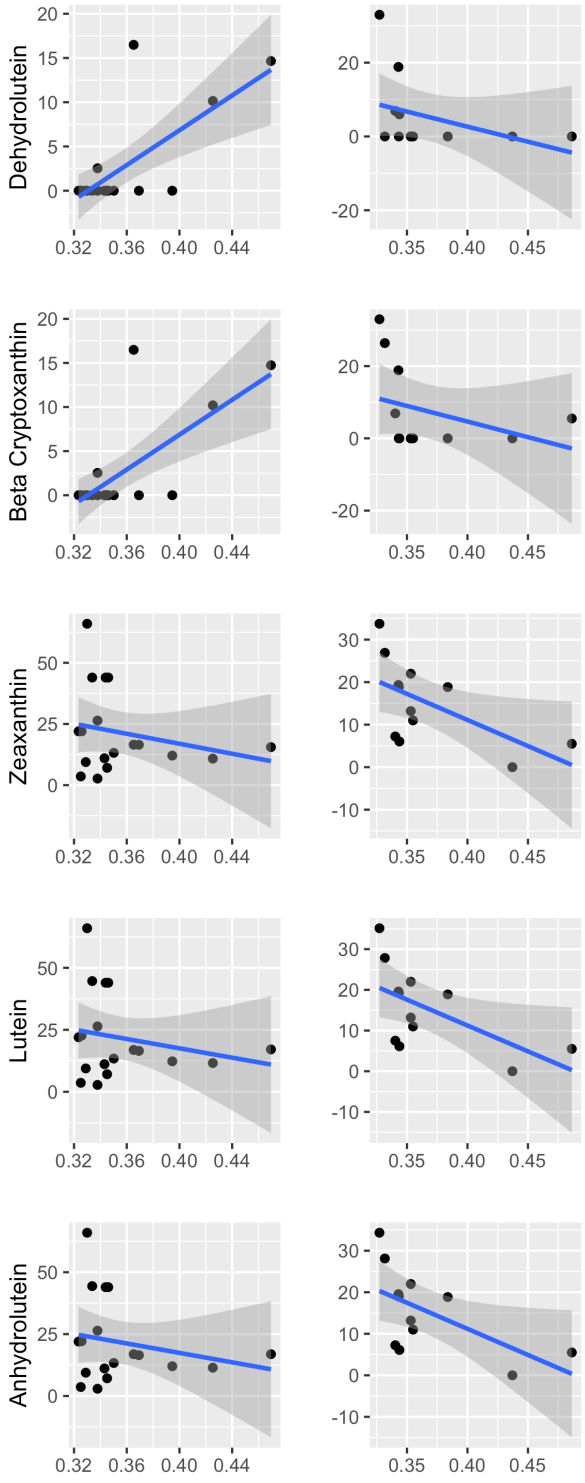
Dehydrolutein
Beta Cryptoxanthin
Zeaxanthin
Lutein
Anhydrolutein
0.32 0.36 0.40 0.44
0.35 0.40 0.45

Figure 1. Spleen carotenoid profile by experimental group and pre-experiment S1R (pS1R), where the y-axis for each row of graphs represents carotenoid concentration (ug/g) by carotenoid type, and the x-axis for all graphs represents pS1R. LPS-injected birds are in the left column and control group birds are in the right column.

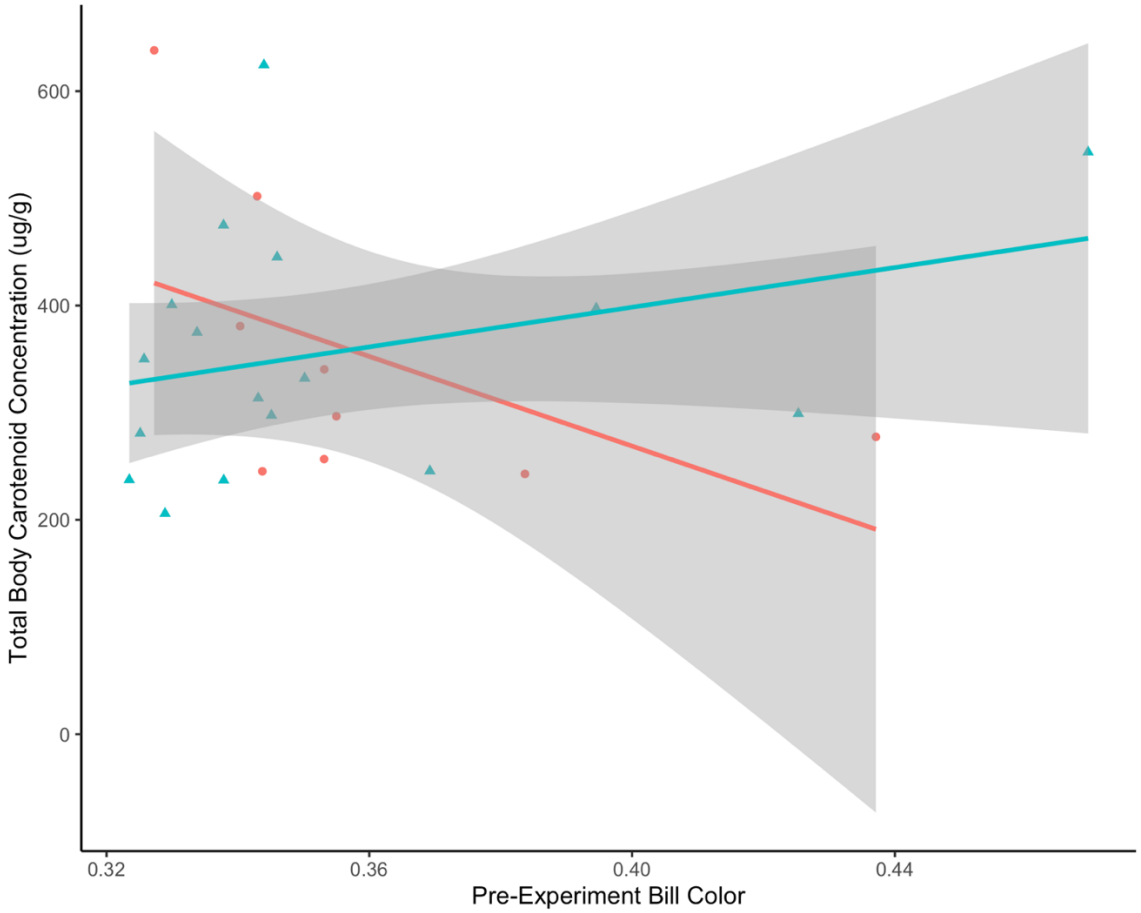

Figure 2. Total body carotenoid concentration by pre-experiment bill color (pS1R), where the red-orange line with circle markers represents the control group, and the blue-green line with triangle markers represents the treatment group. The shaded gray areas represent 95% confidence intervals.

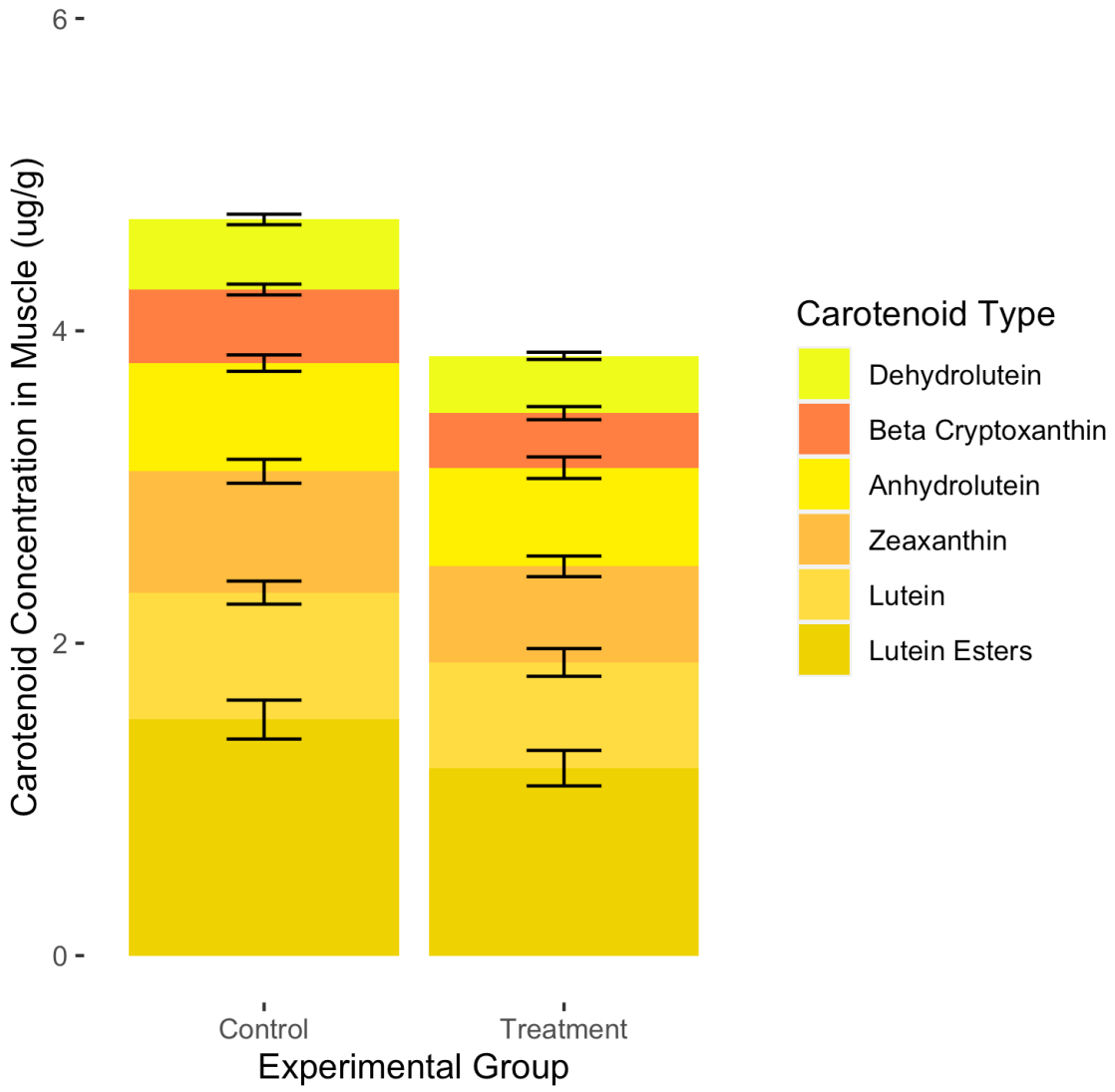

Figure 3. Muscle carotenoid profile by experimental group. Error bars represent standard error.

Tables

Table 1. Permutational multivariate analysis of variance (adonis) for tissue carotenoid profile variation as a function of experimental group, pre-experiment bill color (pS1R), and difference between pre- and post-experiment bill color (dS1R), as well as the interaction between group and bill color, using Bray-Curtis dissimilarity matrices. Terms were added sequentially (first to last) to the model. Significant predictors are denoted by bold text ($\alpha = 0.05$).

Predictor	F-value	R^2	p
Eye			
pS1R			
Group	0.50	0.02	0.55
Color	0.64	0.02	0.45
Group*Color	0.03	0.001	0.96
dS1R			
Group	0.21	0.01	0.76
Color	0.87	0.04	0.40
Group*Color	0.10	0.004	0.90
Fat			
pS1R			
Group	0.16	0.01	0.87
Color	0.59	0.02	0.54
Group*Color	0.17	0.01	0.86
dS1R			
Group	0.27	0.01	0.77
Color	5.99	0.19	**0.007**
Group*Color	1.55	0.05	0.21
Heart			
pS1R			
Group	1.58	0.05	0.20

Color	0.78	0.03	0.43
Group*Color	0.83	0.03	0.40
dS1R			
Group	3.05	0.08	0.08
Color	10.77	0.28	**0.002**
Group*Color	0.25	0.01	0.74
Kidney			
pS1R			
Group	2.21	0.08	0.09
Color	0.09	0.003	0.96
Group*Color	0.26	0.01	0.87
dS1R			
Group	2.66	0.09	0.06
Color	0.83	0.03	0.43
Group*Color	1.76	0.06	0.18
Liver			
pS1R			
Group	0.16	0.01	0.87
Color	0.56	0.02	0.57
Group*Color	1.55	0.06	0.22
dS1R			
Group	0.16	0.01	0.86
Color	1.18	0.05	0.32
Group*Color	0.95	0.04	0.37
Lung			
pS1R			
Group	0.45	0.02	0.71
Color	0.40	0.01	0.68
Group*Color	0.22	0.01	0.84
dS1R			
Group	0.30	0.01	0.73
Color	2.16	0.08	0.12
Group*Color	1.02	0.04	0.37
Muscle			
pS1R			

Group	2.80	0.09	0.08
Color	0.59	0.02	0.51
Group*Color	0.35	0.01	0.70
dS1R			
Group	3.59	0.12	**0.04**
Color	1.04	0.03	0.32
Group*Color	2.40	0.08	0.11
Plasma			
pS1R			
Group	0.29	0.01	0.66
Color	0.37	0.01	0.59
Group*Color	1.01	0.02	0.33
dS1R			
Group	0.17	0.004	0.75
Color	2.70	0.06	0.12
Group*Color	1.57	0.03	0.20
Spleen			
pS1R			
Group	0.60	0.02	0.68
Color	1.78	0.06	0.15
Group*Color	3.96	0.12	**0.005**
dS1R			
Group	0.89	0.03	0.47
Color	1.45	0.05	0.22
Group*Color	0.82	0.03	0.49
Testes			
pS1R			
Group	0.06	0.002	0.98
Color	1.73	0.06	0.23
Group*Color	3.47	0.11	0.06
dS1R			
Group	0.22	0.01	0.73
Color	1.12	0.04	0.31
Group*Color	1.14	0.04	0.31

Table 2. Permutational multivariate analysis of variance (adonis) for total tissue carotenoid concentration variation as a function of experimental group, pre-experiment bill color (pS1R), and difference between pre- and post-experiment bill color (dS1R), as well as the interaction between group and bill color, using Bray-Curtis dissimilarity matrices. Terms were added sequentially (first to last) to the model. Significant predictors are denoted by bold text ($\alpha = 0.05$).

Predictor	F-value	R^2	p
pS1R			
Group	0.46	0.02	0.79
Color	0.84	0.03	0.52
Group*Color	2.21	0.09	0.09
dS1R			
Group	0.49	0.02	0.76
Color	4.26	0.16	**0.01**
Group*Color	1.14	0.04	0.32

Table 3. Generalized linear model for total body carotenoid concentration variation as a function of experimental group, pre-experiment bill color (pS1R), and difference between pre- and post-experiment bill color (dS1R), as well as the interaction between group and bill color. Significant predictors are denoted by bold text ($\alpha = 0.05$).

Predictor	Estimate (SE)	t-value	p
pS1R			
Group	538.7 (260.7)	2.07	0.051
Color	-583.1 (724.5)	-0.81	0.43
Group*Color	-1508.6 (724.5)	-2.08	**0.049**
dS1R			
Group	-8.9 (21.0)	-0.42	0.68
Color	-1083.8 (397.4)	-2.73	**0.01**
Group*Color	-162.9 (397.4)	-0.41	0.69

CHAPTER 4

SCARB1 EXPRESSION AND DIETARY CAROTENOID AVAILABILITY PREDICT EXTENT OF CAROTENOID-BASED PLUMAGE COLORATION IN WOOD-WARBLERS

Abstract

Researchers have commonly investigated the effects of dietary variation on digestive physiology and on the expression of condition-dependent traits, but rarely have the interaction of all three variables (diet, digestive physiology, and condition-dependent traits) been studied in wild animals. Carotenoids are a great system for studying associations between diet, digestive physiology, and condition-dependent traits in animals because they can only be obtained from the diet and are frequently used by animals such as birds to produce red, orange, and yellow condition-dependent coloration. Scavenger receptor B1 (SCARB1) is a transporter that facilitates the absorption of carotenoids in the intestines of birds, so SCARB1 expression may be a key mechanism underlying the association between dietary availability of carotenoids and extent of carotenoid-based coloration. Using comparative phylogenetic analyses, I investigated these relationships in wood-warblers (Parulidae), which exhibit interspecific variation in both diet and extent of carotenoid-based plumage coloration. I first conducted a broad-scale analysis in 56 wood-warbler species to evaluate the relationship between dietary carotenoid availability and carotenoid-based coloration. Then, I conducted a fine-scale analysis of 11 species examining links between dietary variation in carotenoids, SCARB1

expression, and plumage coloration. Additionally, I tested the association in these species between SCARB1 expression and carotenoid accumulation in the liver, which is a carotenoid storage site, to gain insight into how SCARB1 could be regulated in these free-ranging species. As predicted, I found that dietary carotenoid availability and SCARB1 expression together significantly explained variation in extent of carotenoid-based coloration in wood-warblers. There was no relationship between dietary carotenoid availability and extent of carotenoid-based plumage coloration in the broad- or fine-scale analyses when only diet and color were part of the models, but, once SCARB1 expression was included, in the fine-scale models there was a positive relationship between both diet and color as well as SCARB1 expression and color. In the fine-scale analyses, I also found a negative association between SCARB1 expression and carotenoid accumulation in the liver. These findings indicate that digestive physiology can be an important mediator between diet and carotenoid coloration in birds and suggest that carotenoid uptake mechanisms, particularly via SCARB1, at the species level are carefully regulated.

Introduction

Animals can vary tremendously in their diets and types of nutrients ingested, and such variation carries significant mechanistic and functional consequences. Mechanistically, physiological systems for digesting and absorbing nutrients are affected by the types and forms of nutrients that are present in the diet. This includes short-term changes in the expression or activities of digestive enzymes and transporters (Leigh et al. 2018; Trotta et al. 2020) as well as long-term seasonal (Naya et al. 2009) or developmental (Jackson and Diamond 1995) changes in gut structure and function. Functionally, nutrients can shape

the general performance and condition of animals (Maklakov et al. 2008), which can lead to selection for longer-term changes in digestive physiology at the level of populations or species (Chen and Zhao 2019; Heras et al. 2020; Wang et al. 2020). However, because the relationship between diet and digestive physiology itself varies, it is important to consider both diet and digestive physiology, not just one or the other, when trying to understand how they impact animal phenotypes and fitness. For example, the availability of some nutrients, such as carbohydrates, frequently positively correlate with digestive enzyme production (i.e., carbohydrases, amylases; Trotta et al. 2020), whereas for other nutrients such as essential fatty acids more limited dietary availability prompts greater digestive enzyme expression (i.e., carboxyl ester lipase; Leigh et al. 2018). Yet, especially in wild animals, surprisingly few studies have carefully integrated dietary and digestive-physiological variation when trying to understand the function or evolution of various phenotypic traits (Clauss et al. 2013; Charron et al. 2015; Borzoui et al. 2018).

Variation in animal diets has also been linked to expression of condition-dependent ornamental traits. Typically, within species, reduced nutrient availability leads to lower expression of a condition-dependent signal (e.g., smaller, duller ornament) and inversely, supplementation leads to enhanced expression of a condition-dependent signal (e.g., larger, brighter ornament; McGlothlin et al. 2007; Kopena et al. 2011; Taylor et al. 2011; Katsuki et al. 2012; Rahman et al. 2013; Koch et al. 2016). However, across species, there are mixed results in regards to the relationship between diet and condition-dependent signals, and there are far fewer interspecific studies on this subject than intraspecific studies (Mahler et al. 2003; Tella et al. 2004; Olson and Owens 2005). Since dietary availability of nutrients is only one possible mechanism underlying variation in

investment of those nutrients in the development of condition-dependent signals, consideration should also be given to nutrient intake and processing mechanisms to explain the evolution of condition-dependent signals. No study to date has examined relationships among diet, digestive physiology, and condition-dependent signals simultaneously, either within or across species.

Carotenoids in birds are an excellent system for studying the associations between diet and condition-dependent signals, because carotenoids cannot be synthesized *de novo* and so they must be consumed, and birds exhibit wide variation in production of carotenoid-based coloration as well as dietary availability of carotenoids (Olson and Owens 2005). Additionally, the only studies so far that have examined diet and condition-dependent signals across species are of carotenoid-based coloration in birds. Mahler et al. (2003) found that the evolution of carotenoid-based coloration in dove plumage was associated, although not exclusively, with fruit-eating behavior as opposed to seed-eating. Using general categorizations of diet (e.g.,, herbivore v. carnivore), Tella et al. (2004) found that diet and presence of carotenoid-based coloration explained some of the interspecific variation in plasma carotenoids, but there were strong effects of phylogeny with broad categorizations of diet. Olson and Owens (2005) conducted a comparative study of birds at the family level and found that the relationship between dietary carotenoid availability (scored based on food item type; see Olson, 2006) and carotenoid-based coloration was dependent on the type of coloration; specifically, carotenoid availability only significantly explained variation in plumage, but not bare-part, coloration, and red plumage in particular tended to be restricted to birds with the most carotenoid-rich diets. Because of these mixed results linking dietary carotenoid

availability to the evolution of carotenoid-based coloration, the next logical step for investigation is to incorporate a measure of digestive physiology, such as carotenoid uptake in the gut.

A few prior studies have suggested an important role for digestive physiology in shaping the expression and evolution of carotenoid-based colors in birds. First, a small comparative study showed that carotenoid extraction efficiency, measured as the ratio of plasma-carotenoid concentration to dietary-carotenoid concentration, was higher in passerines than in galliforms (McGraw 2005); since passerines more frequently express integumentary carotenoid coloration than galliforms (Thomas et al. 2014b), this suggests that carotenoid absorption may be important for explaining some of the variation in carotenoid-based coloration. Second, genomic investigations of artificially bred birds revealed that genes associated with the absorption and transport of lipids were responsible for the absence of carotenoid-based coloration (Attie et al. 2002; Toomey et al. 2017), including scavenger receptor B1 (SCARB1). SCARB1 has previously been identified as a high-density lipoprotein receptor that mediates the uptake of lipids in the intestine (Acton et al. 1996) and has been suggested to mediate carotenoid uptake as well in fruit flies, silkworms, fish, rats, and humans (Kiefer et al. 2002; Sundvold et al. 2011; Borel et al. 2013; Tsuchida and Sakudoh 2015; Shivananju| 2019; Ahi et al. 2020), suggesting that it is conserved in animals. However, no study has yet examined the expression of SCARB1 in wild populations.

I conducted a comparative study investigating if and how digestive physiology, measured as relative SCARB1 expression, may interact with dietary carotenoid availability to explain variation in the extent of carotenoid-based color in birds. I focused

on wood-warblers (Parulidae), because they vary in both dietary availability of carotenoids (e.g., degree of frugivory and insectivory) and extent of carotenoid-based integumentary coloration (Winkler et al. 2020), and because I had access to tissue samples from multiple species within a relatively narrow time window. I first used previously published information about diet and coloration for a large subset of parulid species to conduct a broad-scale analysis of the relationship between dietary carotenoid availability and extent of carotenoid-based color. Then, using a subset of wood-warbler species for which I could directly assess SCARB1 expression, I conducted a fine-scale analysis to determine if dietary carotenoid availability and/or SCARB1 expression together might explain plumage color variation. Finally, since intraspecific variation in SCARB1 expression has not been measured previously in wild birds, I also evaluated the relationship between SCARB1 expression and carotenoid accumulation, measured as carotenoid (i.e., lutein) concentration in the liver.

In the broad-scale analysis, similar to previous studies examining diet and color in birds more broadly (Tella et al. 2004; Olson and Owens 2005), I predicted that I would find a weak association between dietary carotenoid availability alone and extent of carotenoid-based color. In the fine-scale analysis, I expected SCARB1 expression to mediate the relationship between diet and color in wood-warblers, meaning that dietary availability of carotenoids and relative expression of SCARB1 should together explain variation in carotenoid-based coloration better than diet alone. For the relationship between relative expression of SCARB1 and carotenoid accumulation, I could find either a positive or negative association, which would have different implications for the regulation and role of SCARB1 at the species level. If a positive relationship is observed,

then it suggests that SCARB1 is upregulated to facilitate greater absorption of carotenoids when there is more dietary availability of carotenoids, similar to the way that carbohydrase activity increases when more carbohydrates are introduced into the diet (Trotta et al. 2020). If a negative relationship is observed, then it suggests that SCARB1 is downregulated when carotenoids are abundant and upregulated when carotenoids are scarce, similar to the way that carboxyl ester lipase activity is carefully regulated relative to intake of essential fatty acids (Leigh et al. 2018).

Methods

Sample Collection

All individuals in this study died as a result of building collisions in downtown Cleveland (Ohio, USA) and were collected during fall migration (mid- to late-September) in 2019. Trained volunteers with Lights Out Cleveland checked transects at least twice per morning. The first check started at 0400 hours and each subsequent check was completed within one hour of the previous check. After the first check, any individual that was not detected during the previous check was included in this study and dissected within one hour (i.e., within two hours of presumed death of that individual) to ensure that RNA degradation would be minimized. I dissected the first loop of small intestine (from end of gizzard to where the pancreas ends) and whole liver samples from males and females of 11 species of wood-warblers belonging to 4 different genera (Table 1). This sample represents 10% (10/110) of all warbler species and 29% (4/14) of warbler genera. Although differential expression of SCARB1 in different sections of intestine has not yet been studied in birds, I chose the first loop of the small intestine as the site for measuring

SCARB1 expression, because SCARB1 expression was highest in the duodenum of mice relative to other sections of the intestine (Mapelli-Brahm et al. 2019). I only measured SCARB1 expression in the intestine, not other parts of the body, because, in this study, I was interested in measuring carotenoid uptake or absorption independently of deposition or distribution within the body. However, it is worth noting that SCARB1 expression has been measured in other songbird tissues (including the liver; Toomey et al. 2017), and the expression of liver SCARB1 in particular might also play a role in the absorption and accumulation of carotenoids; unfortunately I could not measure SCARB1 in liver here due to logistical challenges with separate preparations of the tissue for each analysis. It is also worth noting that SCARB1 expression was measured in intestines of migrating individuals, which may have different expression patterns than molting individuals who are developing their carotenoid-based coloration; however, I assume for the purposes of this study that expression during migration is still representative of species-level differences in SCARB1 expression.

Liver was chosen for measuring carotenoid accumulation, because it is where carotenoids are first delivered immediately post-absorption (Surai et al. 2001), and it is a rapidly mobilizable pool as demonstrated by its flexible response to dietary carotenoids (Koutsos et al. 2003). I chose to measure SCARB1 expression, as opposed to the genotype of SCARB1, because of the possibility that SCARB1 could have a difference in regulation between species that did not depend on the genotype of SCARB1, such as mediated regulation by other genes associated with the metabolism and transport of carotenoids. I stored small intestine samples immediately in RNAlater and incubated them at ambient temperatures, which ranged from 11-30°C, for at least 1 hour but no

more than 4 hours before freezing on dry ice in the field. Liver samples were also stored on dry ice in the field. I moved samples to a -20ºC freezer at the Cleveland Museum of Natural History for up to two weeks and then transported on dry ice to a -80ºC freezer at Arizona State University until RNA and carotenoids could be extracted from small intestine and liver samples, respectively.

Gene Expression via Quantitative Real Time Polymerase Chain Reaction

I isolated total mRNA from the small intestines of 33 individuals and reverse transcribed it into cDNA using separate kits. I performed RT-qPCR for SCARB1 and the endogenous control, TAB, in parallel with three technical replicates per sample. TAB was selected as a reference gene, because of previous literature providing evidence of the stability of this gene in studies of gene expression in chickens (Simon et al. 2018; Hassanpour et al. 2019). I specifically designed both the SCARB1 and TAB primers to amplify short fragments (75-150bp), avoid genomic DNA amplification by amplifying fragments along an exon-exon boundary, and maximize the possibility that these fragments would be amplified in all species by only amplifying regions that were conserved between the common yellowthroat (*Geothlypis trichas*) and Kirtland's warbler (*Setophaga kirtlandii*) genomes, which were publicly available on NCBI GenBank. I measured florescence of the green dye, SYBR green using ROX as a passive reference to normalize the fluorescence signal, in a 7900HT Real-Time PCR system with SDS 2.4 software (10uL reactions, cycling parameters: 95ºC for 10 minutes, then 40 cycles of 95ºC for 30s, 49ºC for 60s, and 72ºC for 30s). A dissociation phase was also added to check that only the desired product was amplified. If more than one amplification product was detected, then

those data were discarded. This filtering process left 25 viable individual samples belonging to 12 of the original 13 species collected. Raw data were exported as fluorescence by cycle for each well and converted into initial fluorescence measurements (R_0) using the amplification plot method *sensu* Peirson et al. (2003). Relative expression was calculated as a ratio of SCARB1 to TAB R_0 for all samples. At this point, one extreme outlier was removed, leaving 24 individuals in 11 species across 4 genera for the final analyses. Generally, across species, with a few exceptions, SCARB1 expression was at or below the same level of expression as the reference gene (1.02 ± 0.31).

Liver Carotenoid Analyses

High-performance liquid chromatography methods for measuring carotenoids in liver samples follow those in Chapters 2 and 3. However, only lutein concentration was used in analyses for this chapter, because it explains the majority of variation in carotenoid concentrations across species. For example, the median percent of lutein out of total carotenoids across four randomly selected individuals was 79%. Lutein is also likely the primary carotenoid deposited in feathers for most wood-warbler species, since common yellowthroat (*Geothlylpis trichas*) feathers are 100% lutein (McGraw, Beebee, et al. 2003). However, I intend to analyze the full liver carotenoid profile prior to publication.

Estimated Dietary Carotenoid Availabilities

I estimated dietary carotenoid availability of wood-warbler species (Parulidae) using the fine-scale method *sensu* Olson (2006) and based on diet descriptions in Birds of the World (Billerman et al. 2020). I only included species that had sufficient data quality to

resolve variation in dietary carotenoid availability among wood-warblers. For instance, since nearly all warblers are primarily insectivores, I only used diet data if the description specified which invertebrate orders (e.g., Hymenoptera, Coleoptera) rather than just describing the diet as primarily insects with or without some fruit. Insect identification is important, because there is an order of magnitude difference in the estimated carotenoid concentration of some groups (e.g., Lepidoptera) compared to others (e.g., Isoptera; Olson 2006). Out of 110 parulid species worldwide, 57 met this data-quality criterion. However, it is worth noting that this restriction resulted in the loss of many warblers native to Central and South America due to lack of access to detailed diet data in these regions. While some species had only one summary of the major and minor components of the diet, other species had multiple entries. For example, the American redstart (*Setophaga ruticilla*) had seven quantitative descriptions of their diet summarized from different studies in different locations (e.g., New Hampshire, Venezuela, Jamaica, Louisiana) and at different points in their life history (e.g., breeding, wintering, migration). For these species, I separated the entries, estimated dietary carotenoid availability for each, and then averaged all entries to get a single diet score per species. One person (E.A.W.) scored dietary carotenoid availability for each species. Because bird diets are known to vary seasonally, I calculated the average repeatability of diet scores by species for those that had more than one diet description. For the broad-scale analysis, 22 out of 56 species had more than one diet description, and, for the fine-scale analysis, there were 7 out of 11 species. I calculated standard error as a repeatability metric for species in the broad-scale analysis (22) as well as the fine-scale subset (7), and found that repeatability was 0.90 and 0.77, respectively. These repeatability scores suggest that

variation within species is less than variation between species, since species-level diet scores varied from 9 to 24. I intend to validate the precision and repeatability of these scores with independent raters who are unaware of the hypotheses and species color scores before publication.

Extent of Carotenoid-based Coloration

I used descriptions and photos in Birds of the World (Billerman et al. 2020) to identify the presence or absence of carotenoid-based coloration in each of 10 plumage regions (eyebrow, crown, cheek, throat, nape, breast, belly, back, tail, and wings) from each considered wood-warbler species. I focused on plumage over bare part coloration, even though parulids have both, because there was not sufficient variation in extent of bare part coloration for the fine-scale analysis. Although warbler plumage regions are not each equal in area (e.g., eyebrow vs. belly), this method of scoring does still provide an approximate measure of extent of carotenoid plumage pigmentation. Measuring total coverage area of carotenoid-based coloration more directly may provide different results, and I aim to adjust measures as such prior to publication. Red, orange, and yellow feathered body regions were assumed to be carotenoid-based (Gray 1996; Tella et al. 2004; Olson and Owens 2005), as well as olive plumage, which typically contains carotenoids in addition to melanin (Delhey et al. 2010). Reddish-brown and orangish-brown patches (e.g., ovenbird crown, Cape May warbler cheek) were scored as carotenoid-absent, because they were assumed to be produced by pheomelanin (e.g., Toral et al. 2008). I chose extent of carotenoid-based coloration rather than other metrics of carotenoid-based coloration (e.g., hue, saturation, brightness), because there are known

genetic mechanisms underlying the presence or absence of multiple carotenoid-based plumages in warbler species (Brelsford et al. 2017; Baiz et al. 2020), whereas other metrics of carotenoid-based coloration have much weaker genetic underpinnings (e.g., heritability; Hadfield et al. 2006; Drobniak et al. 2013). I focused on plumage over bare part coloration, even though parulids have both, because there was not sufficient variation in extent of bare part coloration for the fine-scale analysis.

I coded plumage coloration in terms of presence/absence, where 0 indicated absence of carotenoid-based coloration and 1 indicated presence within a plumage region, and then I added up the presence/absence scores for each body region to get the color score (0-10) for each species, so that the color score captured both presence/absence (0 vs 1) as well as extent (1-10) of carotenoid-based coloration. For this study, I restricted the color data to just male breeding plumage coloration. It is worth noting that, while male and female individuals for this study were sampled during fall migration (i.e., not in breeding plumage), this score was meant to be a species-level representation of how ornate coloration, which is typically breeding plumage in males, has evolved in warblers. Moreover, male breeding plumage was highly correlated with female breeding coloration in 56 species of parulids (Pearson's product-moment correlation: 0.83, $t = 11.19$, $df = 55$, $p < 0.001$), so it would be redundant to also analyze female breeding coloration here. One person (E.A.W.) scored extent of carotenoid-based plumage coloration for each species. I intend to validate the precision and repeatability of these scores with independent raters who are unaware of the hypotheses and species diet scores before publication.

Statistical Analyses

For this and all subsequent tests, I used the R environment (R Core Team 2017) with RStudio (RStudio Team 2015). In the broad-scale analysis, where I used data from 56 wood-warbler species, I evaluated the relationship between species-level scores of dietary carotenoid availability and extent of carotenoid-based plumage coloration by estimating parameters for the phylogenetic variance-covariance matrix and fitting a linear model using generalized least squares using R packages *Rphylopars* (Goolsby et al. 2017) and *nlme* (Pinheiro et al. 2021), respectively. I estimated parameters with a previously published phylogenetic tree of wood-warblers (Lovette et al. 2010) that was pruned to contain these 56 species. This was one fewer species than I had diet data for, because the Lovette phylogeny does not include the blue-winged warbler (*Vermivora cyanoptera*). However, before fitting the linear model, I estimated parameters using different assumptions (e.g., Brownian motion, Ornstein-Uhlenbeck) and I used Bayesian information criterion (BIC) to select the best model.

In the fine-scale analysis, where I used data from 11 warbler species for which I had relative expression of SCARB1 data, I tested the hypothesis that dietary carotenoid availability and SCARB1 expression together explain more variation in extent of carotenoid-based coloration than diet alone. Here I also used the *Rphylopars* package but this time for both estimation of parameters (phylopars function) for model selection and fitting a linear model using generalized least squares (phylopars.lm function). This choice was because the phylopars.lm function accounts for within-species variation in relative expression of SCARB1. However, to include both individual-specific variables like

SCARB1 expression and species-specific variables like diet and color scores in the same analysis, I had to generate individual-level variation in the species-specific variables. For example, if the color score of a species was 3, and there were 3 individuals, then their color scores were 2.99, 3.00, and 3.01. The idea is similar to adding an inconsequential amount (e.g., 0.01) to raw data before log transforming to prevent errors due to the presence of zeros. I used the same warbler phylogeny for this analysis, but I pruned the tree to contain these 11 species. I tested for multicollinearity between dietary availability of carotenoids and SCARB1 expression by calculating their variance inflation factor (VIF) scores and found that they were within acceptable levels of multicollinearity (< 5; diet = 1.51, scarb1 = 4.19; García et al. 2015). I used model selection with Akaike information criterion (AIC) to determine which predictors (diet = species score of dietary carotenoid availability, scarb1 = individual measurement of relative SCARB1 expression, and conc = individual measurement of liver lutein concentration) best explained extent of carotenoid-based plumage coloration (color). The best model with the lowest AIC (22.14) included diet and scarb1 as predictors. All other models (diet, scarb1, conc, diet + conc, scarb1 + conc) had an AIC that was at least 9.27 higher. Then I ran two models, one with just dietary carotenoid availability and extent of carotenoid-based plumage coloration (formula: color ~ diet), and another with relative expression of SCARB1 included (formula: color ~ diet + scarb1). Additionally, I tested the hypothesis that there is an association between SCARB1 expression and liver lutein accumulation, measured as lutein concentration in liver, using the same method as described for the previous hypothesis, but there was only one model that included liver lutein concentration and relative expression of SCARB1 (formula: conc ~ scarb1).

Results

In the broad-scale analysis, I found that there was no significant correlation (i.e., standard error of coefficient overlaps with zero) between dietary carotenoid availability and plumage color extent in the full dataset of warblers (coefficient = -0.07±0.26, t-value = -0.28, p-value = 0.78; Figures 1 and 2). In the fine-scale analysis (containing the 11 species for which SCARB1 expression and liver lutein concentration data were available), I found significant effects of dietary carotenoid availability (coefficient = 0.41±0.06, z-value = 7.39, p-value < 0.0001) and SCARB1 expression (coefficient = 8.45±0.55, z-value = 15.49, p-value < 0.000001; Figures 3 and 4) on plumage color extent. In this same set of 11 parulid species, I also found a significant negative association between SCARB1 expression levels and liver lutein concentration (coefficient = -5.91±1.10, z-value = -5.37, p-value < 0.001; Figures 5 and 6).

Discussion

I investigated the relationships between dietary carotenoid availability, tissue expression of a gene related to carotenoid uptake in the gut, and carotenoid-based plumage color variation in a comparative study of wood-warblers. To my knowledge, no previous study has directly evaluated the evolutionary relationship between dietary availability of carotenoids and extent, rather than presence or absence, of carotenoid-based coloration in birds at the interspecific level or evaluated how carotenoid absorption modulates this relationship. The best model explaining extent of carotenoid-based coloration included both dietary carotenoid availability and relative expression of SCARB1. When only

dietary carotenoid availability was included as a predictor in both the broad-scale analysis (incorporating 56 of the 110 wood-warbler species) and the fine-scale analysis (which included 11 species, for which I also had SCARB1 and liver carotenoid data), I found no relationship. Diet was only weakly positively associated with color after SCARB1 expression was included in the model.

I found what I expected to find with regard to the relationship between dietary carotenoid availability and extent of carotenoid-based coloration, which was a weak association between the two. This finding is consistent with the idea that either dietary availability as defined here is an unreliable proxy for plumage coloration or male wood-warbler coloration at the family level may not be limited by dietary availability of carotenoids. In other words, wood-warblers, for the most part, have sufficient carotenoids in their diet to produce up to full-body carotenoid-based plumage coloration. Additional evidence that wood-warblers may not be carotenoid-limited in their diet are recent findings that genetic differences in beta-carotene oxygenase 2 (BCO2; Toews et al. 2016; Baiz et al. 2020), an enzyme that cleaves carotenoids, is responsible for differences in extent of carotenoid-based coloration. If dietary carotenoids were limited, then it is more plausible that carotenoid-based coloration would be produced through selective deposition in particular plumage body regions (e.g., via scavenger receptors) rather than through indiscriminate deposition into all feather tracts and then selective degradation (e.g., by BCO2) in a particular pattern. While this finding does not rule out the importance of dietary carotenoid availability for the production of carotenoid-based coloration, it does provide support for the idea that dietary carotenoid availability is not as important as other factors such as carotenoid uptake in the intestine or selective

degradation of carotenoids. In addition to these possible mechanistic explanations associated with carotenoid availability in the diet, there are other factors at a functional level that potentially constrain the evolution of carotenoid-based coloration, such as conspicuousness (Simpson et al. 2020) and sympatry (Simpson et al. 2021).

Despite these other factors, relative expression of SCARB1 and dietary availability of carotenoids combined best explained the variation in extent of carotenoid-based coloration in the fine-scale subset of 11 species. This finding supports my hypothesis that greater capacity to absorb carotenoids has facilitated the evolution of greater extent of carotenoid-based coloration in male wood-warblers. However, it is also possible that factors at a functional level (e.g., natural selection on conspicuousness) are directly constraining the evolution of carotenoid-based coloration, and the physiological mechanism (i.e., SCARB1 expression) is only an indirect effect of that shift in demand for carotenoids. This is not unlikely for a family in which BCO2, but not SCARB1, genotypes were associated with differences in extent of carotenoid-based coloration for many species pairs (Toews et al. 2016; Baiz et al. 2020) with one notable exception, the two subspecies of yellow-rumped warbler (*Setophaga coronata*; Brelsford et al. 2017), which was not included in the fine-scale analysis. It is also possible that, while SCARB1 was not genetically different between species (Toews et al. 2016; Baiz et al. 2020), other genes that were different might still be affecting SCARB1 regulation and expression. For example, when SCARB1 gene expression is measured in BCO2 knockout mice, SCARB1 expression is greater compared to wild-type mice, and this relationship is at least partially mediated through the transcription factor ISX (Widjaja-Adhi et al. 2015). These alternative hypotheses, one where carotenoid absorption facilitates the evolution of

more carotenoid-based coloration and the other where carotenoid absorption is modulated in response to changes in carotenoid-based coloration, should be parsed out in future studies.

While I did find a positive association between relative expression of SCARB1 and extent of carotenoid-based coloration, the idea that carotenoid absorption facilitates carotenoid accumulation in internal tissues and therefore production of carotenoid-based coloration is based on the assumption that carotenoid absorption is related to carotenoid accumulation. This is why I also tested the association between relative SCARB1 expression and lutein concentration in the liver as a proxy for carotenoid accumulation in the body, given that we studied these birds in autumn and could not examine this directly in colorful nuptial feathers. At first, the finding that there is a negative association between SCARB1 expression and liver carotenoid concentration seems counterintuitive, given that SCARB1 function is associated with carotenoid accumulation in the case of the SCARB1-knockdown canary model (Toomey et al. 2017). However, because I examined variation in SCARB1 expression among free-ranging species, these results have implications for SCARB1 regulation beyond presence versus virtual absence of SCARB1 expression that we see in artificially selected species. At the species level, this negative association could represent the relationship between capacity to absorb carotenoids from food and need for carotenoid storage. For example, if a species has higher capacity for carotenoid absorption, then it might not need to store carotenoids in the liver or other tissues (Negro et al. 2001; Koutsos et al. 2003), because it can absorb carotenoids from the diet and use them as needed. Alternatively, this observed relationship could be an artifact of SCARB1 regulatory mechanisms that are not

necessarily associated with species differences in SCARB1 expression. Specifically, if SCARB1 exhibits negative feedback on carotenoid accumulation in tissues like the liver, where more carotenoid accumulation leads to lower expression of SCARB1, then we would see this pattern of low SCARB1 expression when carotenoid concentrations are higher and high SCARB1 expression when carotenoid concentrations are lower. Although results from *in vitro* studies show a positive association between SCARB1 and carotenoid absorption (Borel et al. 2013), there are no studies taking into account the effect of other tissue carotenoid supplies on SCARB1 regulation. However, there are hypothesized mechanisms of negative feedback based on the regulatory relationship between BCO2 and SCARB1 (Widjaja-Adhi et al. 2015).

I have shown in this study that a measure of digestive physiology, specifically the expression of a lipid (including carotenoid) transporter (i.e., SCARB1) in the small intestine, can be an important mediator in the relationship between diet and color in warblers, so it is worth exploring other possible cases where digestive physiology could be mediating relationships between diet and condition-dependent signals in animals more broadly. Future analyses could also be expanded to include expression of a suite of genes or transcriptomes associated with the uptake and metabolism of nutrients in the intestine rather than just a single gene. The finding that there was a negative association between SCARB1 expression and lutein concentration in the liver provides some support for the regulatory behavior of genes associated with carotenoid uptake being more like that of carefully balanced micronutrient (e.g., essential fatty acid) uptake rather than more-is-better uptake of macronutrients (e.g., carbohydrates), which is also consistent with the idea that carotenoid accumulation must be regulated such that costs of too much

carotenoids are not incurred (Huggins et al. 2010). This should be validated with more explicit experimental tests of the relationship between SCARB1 expression and carotenoid accumulation to better understand mechanisms of lipid-soluble nutrient absorption and transport in animals.

Figures

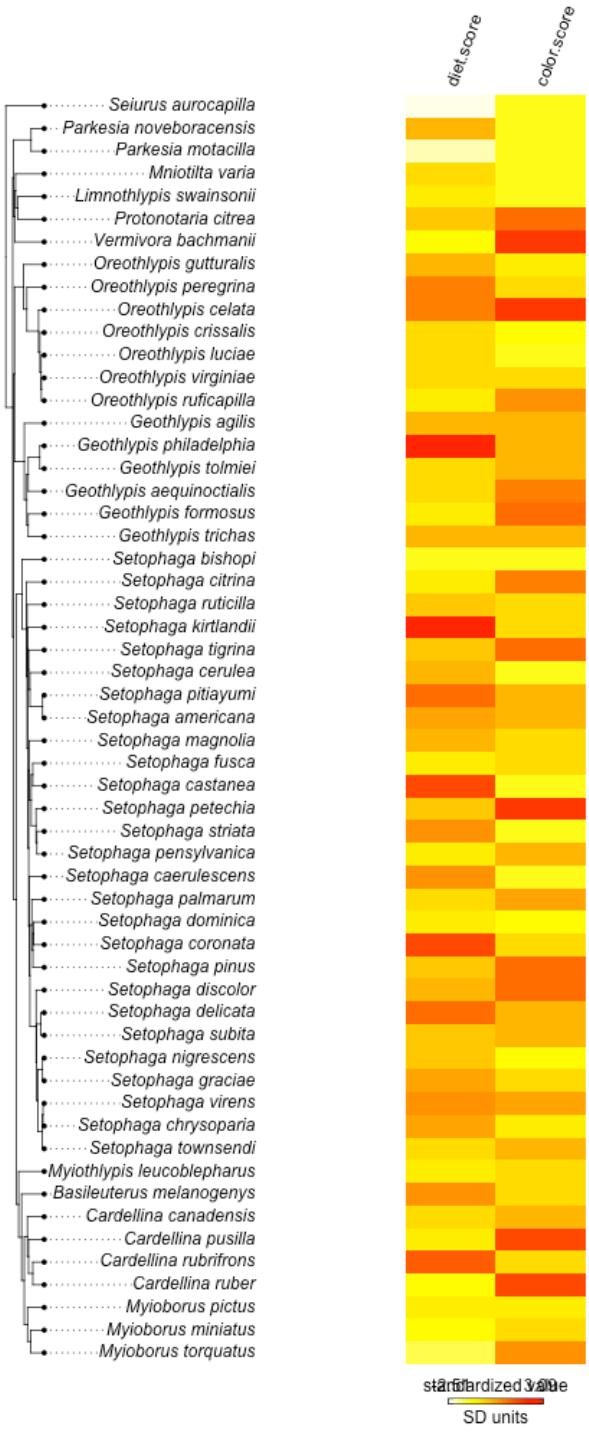
diet.score
color.score
Seiurus aurocapilla
Parkesia noveboracensis
Parkesia motacilla
Mniotilta varia
Limnothlypis swainsonii
Protonotaria citrea
Vermivora bachmanii
Oreothlypis gutturalis
Oreothlypis peregrina
Oreothlypis celata
Oreothlypis crissalis
Oreothlypis luciae
Oreothlypis virginiae
Oreothlypis ruficapilla
Geothlypis agilis
Geothlypis philadelphia
Geothlypis tolmiei
Geothlypis aequinoctialis
Geothlypis formosus
Geothlypis trichas
Setophaga bishopi
Setophaga citrina
Setophaga ruticilla
Setophaga kirtlandii
Setophaga tigrina
Setophaga cerulea
Setophaga pitiayumi
Setophaga americana
Setophaga magnolia
Setophaga fusca
Setophaga castanea
Setophaga petechia
Setophaga striata
Setophaga pensylvanica
Setophaga caerulescens
Setophaga palmarum
Setophaga dominica
Setophaga coronata
Setophaga pinus
Setophaga discolor
Setophaga delicata
Setophaga subita
Setophaga nigrescens
Setophaga graciae
Setophaga virens
Setophaga chrysoparia
Setophaga townsendi
Myiothlypis leucoblepharus
Basileuterus melanogenys
Cardellina canadensis
Cardellina pusilla
Cardellina rubrifrons
Cardellina ruber
Myioborus pictus
Myioborus miniatus
Myioborus torquatus
standardized value
SD units

Figure 1. Phylogenetic distribution of dietary availability of carotenoids (diet.score) and extent of carotenoid-based coloration (color.score) in standardized units for 56 species of New World warblers. Standardized units (where mean and variance are set to zero and one, respectively) are used to make changes in diet scores and color scores more easily comparable, since they have different scales.

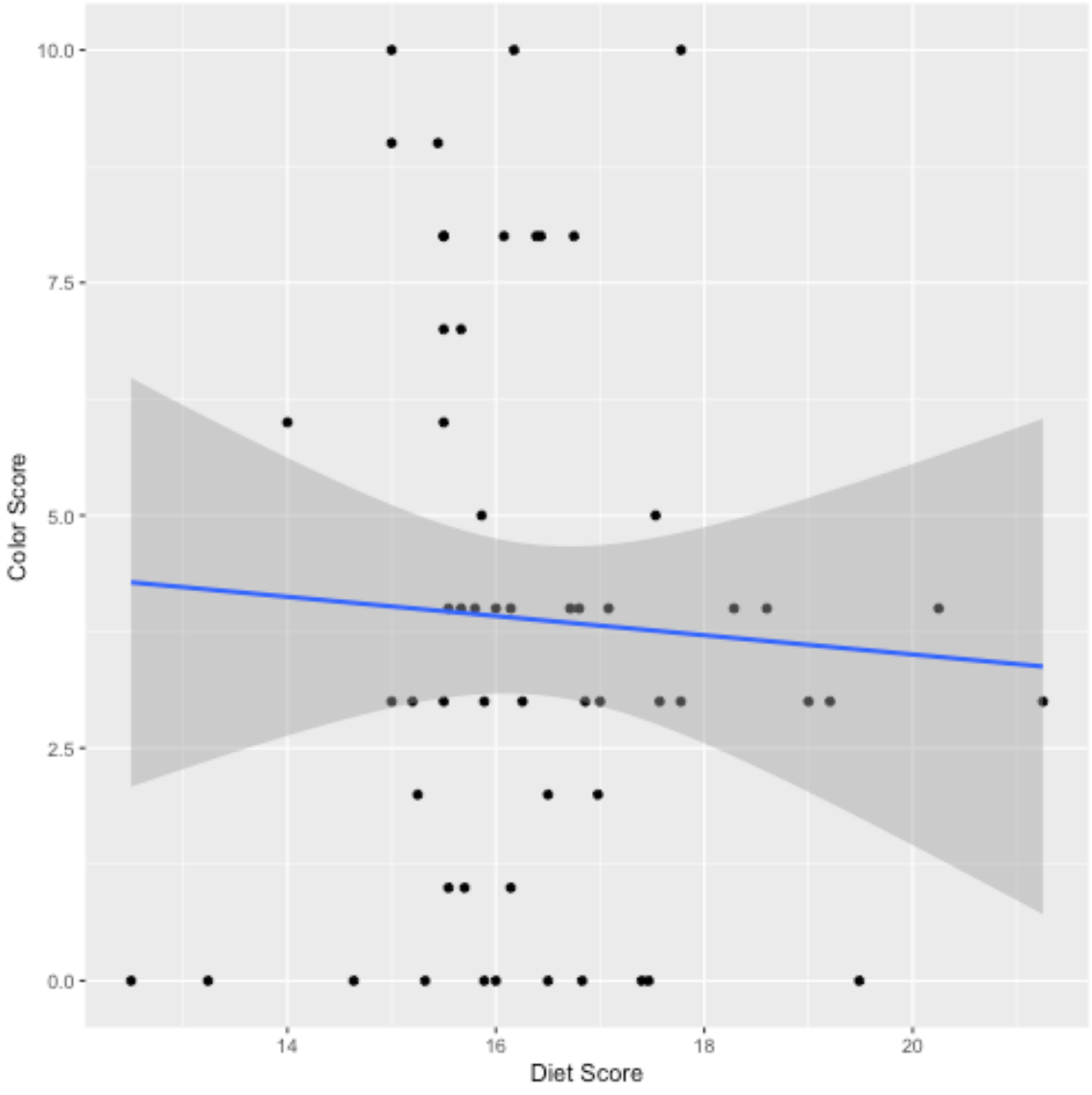

Figure 2. Scatterplot showing the relationship between dietary availability of carotenoids (diet score) and extent of carotenoid-based coloration (color score) in the 56 wood-warbler species included in the broad-scale analyses.

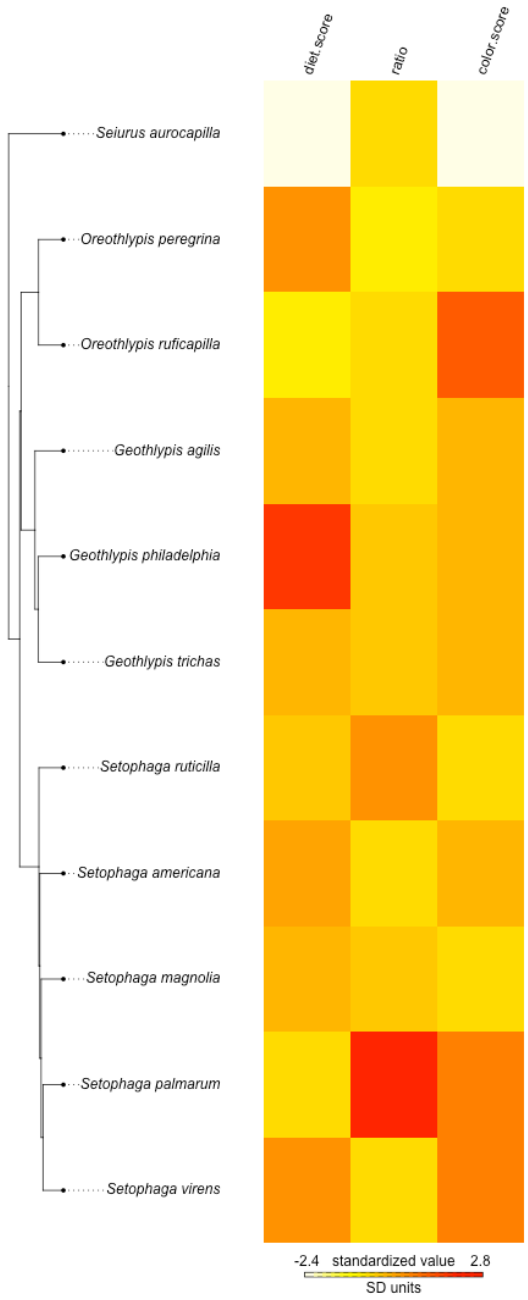

Figure 3. Phylogenetic distribution of dietary availability of carotenoids (diet.score), relative expression of SCARB1 (ratio), and extent of carotenoid-based coloration

(color.score) in standardized units for 11 species of New World warblers. Standardized units (where mean and variance are set to zero and one, respectively) are used to make changes in diet scores, SCARB1 expression, and color scores more easily comparable, since they have different scales.

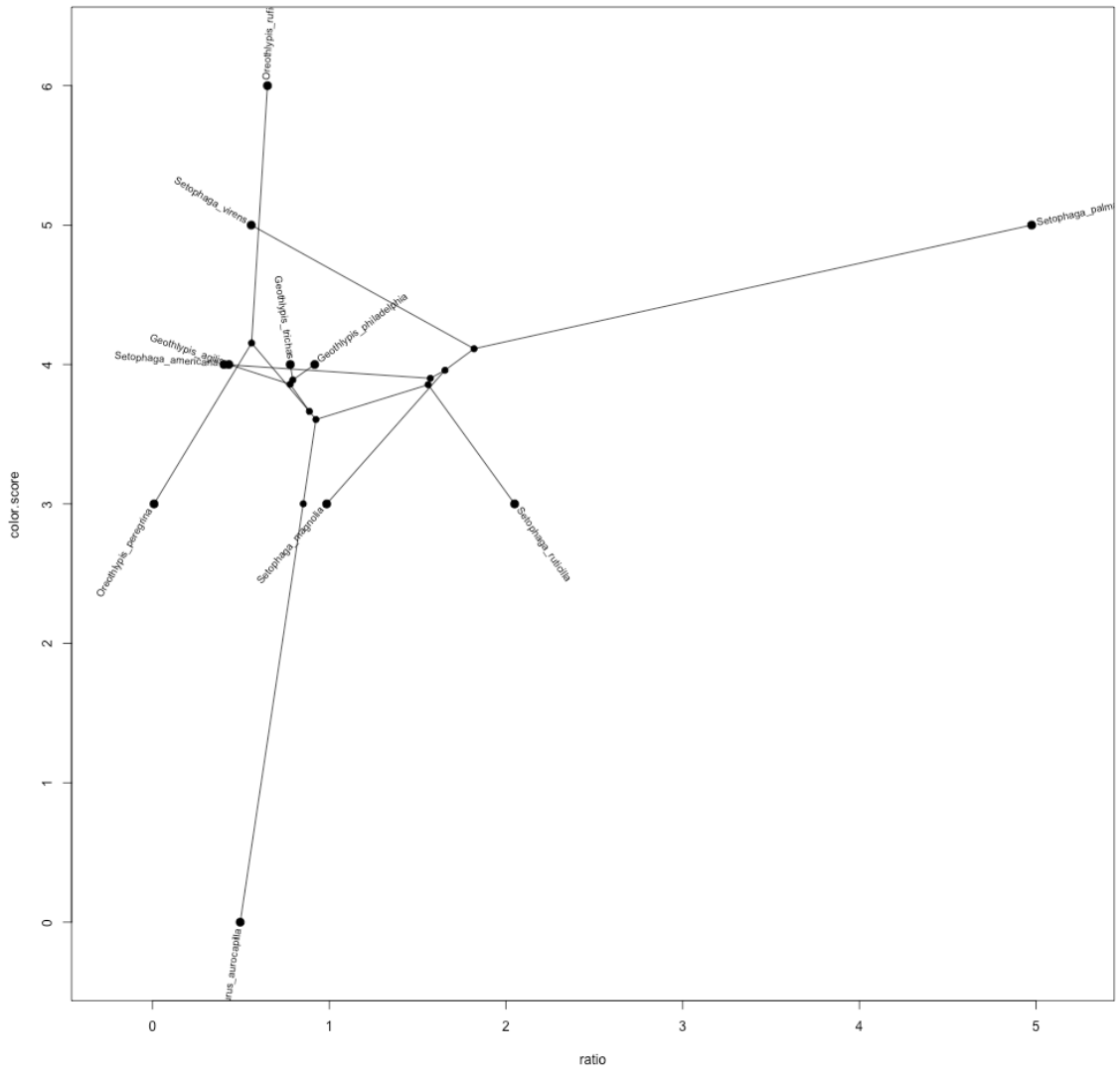

Figure 4. Graph of phylomorphospace for relative expression of SCARB1 (ratio) and extent of carotenoid-based coloration (color.score) in the 11 wood-warbler species included in the fine-scale analyses.

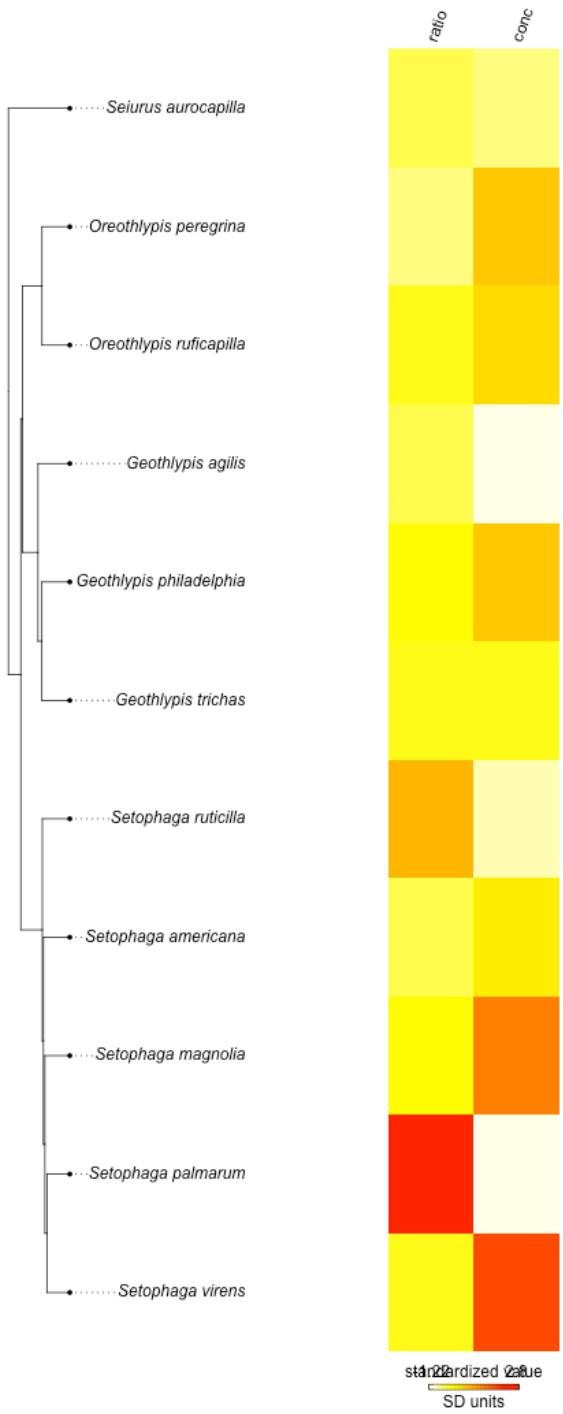

Figure 5. Phylogenetic distribution of relative expression of SCARB1 (ratio) and liver lutein concentration (conc) in standardized units for 11 species of wood-warblers. Standardized units (where mean and variance are set to zero and one, respectively) are

used to make changes in SCARB1 expression and liver lutein concentration more easily comparable, since they have different scales.

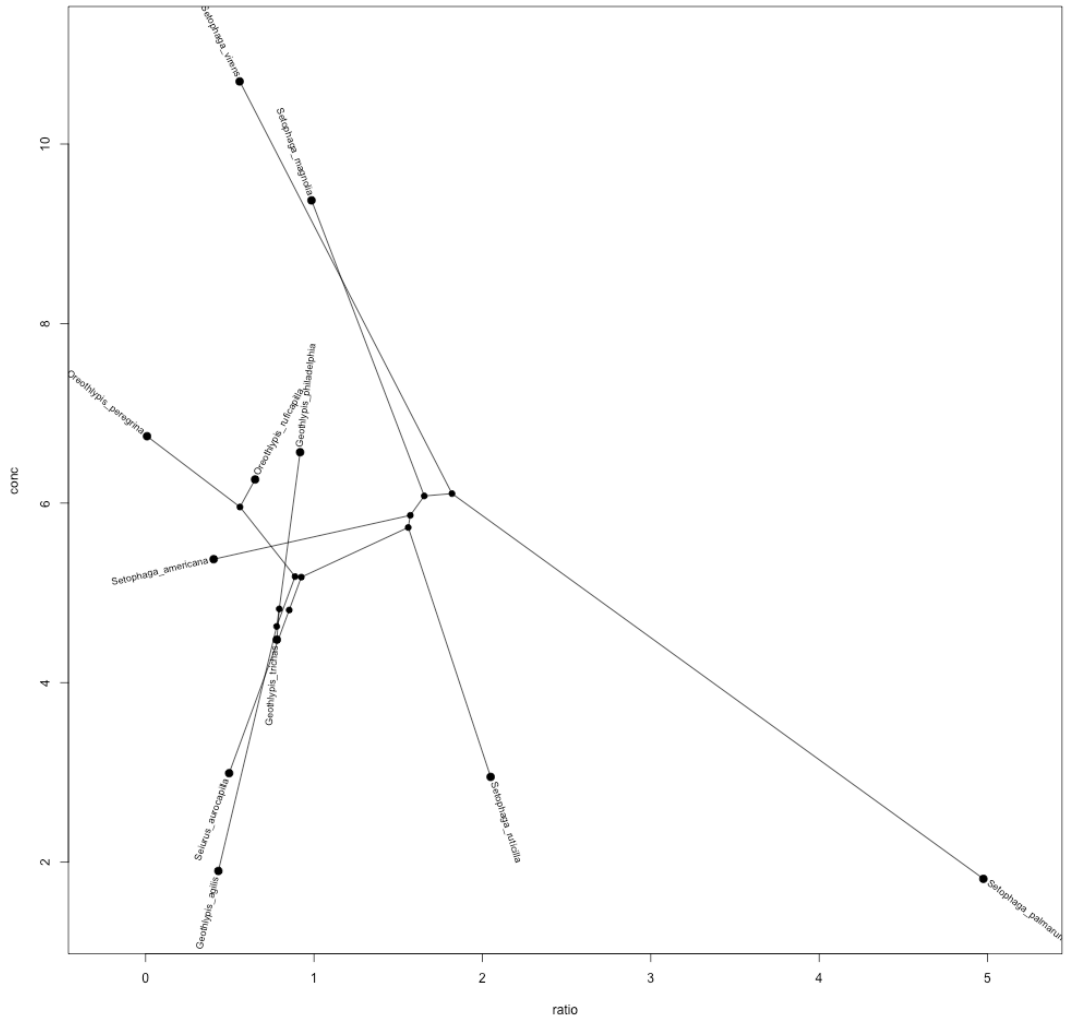

Figure 6. Graph of phylomorphospace for relative expression of SCARB1 (ratio) and liver lutein concentration (conc).

Tables

Table 1. Species and sample sizes by sex (female, male) and age (hatch year, after hatch year) for small subset of wood-warblers with SCARB1 expression measurements.

Species	Sex	Age	Total
Seiurus aurocapilla	2, 1	1, 2	3
Oreothlypis peregrina	0, 1	0, 1	1
O. ruficapilla	1, 2	3, 0	3
Geothlypis agilis	1, 0	1, 0	1
G. philadelphia	0, 1	1, 0	1
G. trichas	4, 2	2, 4	6
Setophaga ruticilla	2, 1	2, 1	3
S. americana	1, 0	0, 1	1
S. magnolia	2, 1	2, 1	3
S. palmarum	0, 1	1, 0	1
S. virens	1, 0	0, 1	1

CONCLUDING REMARKS

The overarching aims of my dissertation were to understand how certain environmental and physiological factors shape variation in tissue carotenoid profiles in birds and to what extent condition-dependent signals reflect those carotenoid profiles as well as of the ability to absorb carotenoids. Although several studies have investigated variation in carotenoid profiles of a few tissues, and in some cases in relation to expression of condition-dependent signals (Negro and Garrido-Fernández 2000; Koutsos et al. 2003; McGraw et al. 2006; Butler and McGraw 2010; McGraw and Toomey 2010; Rowe et al. 2012), in very few studies have avian carotenoid profiles been described in more than a handful of tissues (Fox et al. 1967; Surai et al. 2000). Moreover, to my knowledge, never before had sources of variation in such whole-organism-scale carotenoid profiles been examined, and no studies have simultaneously addressed the relationships between diet (e.g., dietary carotenoid availability, nutrient availability), digestive physiology (e.g., nutrient absorption, expression of a carotenoid transporter), and condition-dependent signals (e.g., carotenoid-based coloration). Because of these knowledge gaps, I asked the following specific questions in my dissertation: (1) How are carotenoid and other nutrient profiles statistically analyzed? (2) How do carotenoid profiles vary along natural gradients (e.g., season, sex, coloration) in a wild population of birds? (3) How do carotenoid profiles differ between immune-challenged and control individuals? (4) Do dietary carotenoid availability and carotenoid absorption (i.e., SCARB1 expression) explain variation in carotenoid-based coloration across species better than dietary carotenoid availability alone?

In my first chapter (Chapter 1), I reviewed the statistical approaches used to analyze nutrient profiles in recent nutritional-physiology and -ecology literature, and I tested assumptions about recommendations for analyzing nutrient profiles. With tests of an example nutrient profile dataset from house finches, I found that intercorrelations among nutrient types, but not number of nutrient types, type of nutrient metric used, or hierarchical levels of analysis, qualitatively affected the outcome of statistical analyses, yet intercorrelations among nutrient types were rarely reported in analyses of nutrient profiles. I also discovered that MANOVA had more power to detect statistically significant differences in nutrient profiles than PERMANOVA in multiple contexts, but many more nutrient profile studies used non- or semi-parametric multivariate tests instead of MANOVA. This observation was likely a reflection of the difference in power between parametric and non- or semi-parametric analyses (Vickers 2005). Overall, these results emphasize the importance of choosing suitable and well-justified statistical test in future studies of nutrient profiles, because there are consequences measured both in this study and in other studies examining the impact of intercorrelations, number of dependent variables, and data metric (Fish 1988; Cole et al. 1994; Lix et al. 1996; Thompson 1999; Thompson et al. 2005; Young 2006; Delacre et al. 2019; Al-Abdullatif 2020). Nutritional physiologists and ecologists in particular should conduct and report intercorrelations between nutrient types more frequently as well as carefully consider whether parametric multivariate analyses (e.g., MANOVA) could be applied before deciding whether or not to use non- or semi-parametric analyses (e.g., PERMANOVA) instead.

In my second (Chapter 2) and third (Chapter 3) chapters, I focused on understanding variation in carotenoid profiles of two songbird species with carotenoid-based coloration. In both species, I found differences in carotenoid profiles along all axes of variation (e.g., season, sex, health state, color), and these differences exhibited a mix of tissue- and carotenoid-type-specificity. Although these concepts of tissue- and carotenoid-specificity have been studied previously in a handful of tissues (e.g., immune organ but not liver and canthaxanthin but not lutein response to immune challenge; Koutsos et al. 2003), measuring whole-organism carotenoid profiles provides a rich new source of descriptive data from which hypotheses for the tissue- and type-specific functions and mechanisms of carotenoids can be generated or refined.

For example, in Chapter 2, I observed an interesting pattern of carotenoid type specificity in the spleen, where spring individuals (i.e., male and female) and females during any time of year had greater concentrations of beta-carotene in the spleen. This suggests that there could be a role for beta-carotene in the immune response, particularly during breeding. There is some support for this role for beta-carotene in the literature, including in birds (Bendich and Shapiro 1986; Cucco et al. 2007; Fitze et al. 2007), but I was unable to test this in my experiment with zebra finches (Chapter 3) because there were no detectable levels of beta-carotene in zebra finch tissues. Valdebenito et al. (2021) recently conducted a meta-analysis evaluating season and sex differences in immunity of wild birds, and found that females had a reduced immune response by certain metrics (e.g., macrophage counts, phytohaemagglutinin, and haemolysis) compared to males during the breeding season but not during the non-breeding season. They also found trends of higher heterophil counts and lower bacterial killing ability in females during the

non-breeding season. Because this mirrors the season- and sex-specific differences in spleen carotenoid profiles, particularly with respect to beta-carotene concentrations, it suggests that these may be possible mechanisms by which beta-carotene modulates the immune response in birds. There is some support for a relationship between white blood cell production and beta-carotene in general, although not in birds (Chew and Park 2004). Although previous research does not support strong links between bacteria-killing ability and carotenoids in general (Koch et al. 2018, 2019; but see Rowe et al. 2011; Merrill et al. 2014), this may be due to the fact that the specific link between beta-carotene concentration in spleen and bacteria-killing ability has not yet been tested directly. The best way to test whether the differences in concentrations of specific carotenoid types (e.g., beta-carotene) are functional or a byproduct would be to directly manipulate the movement of carotenoids in and out of specific tissues (e.g., with RNAi; Waters et al. 2009) and measure functionality (e.g., lymphocyte production). However, a much deeper understanding of carotenoid physiology is needed to be able to conduct such experiments.

Another example of the value of whole-organism carotenoid profiles for generating and refining hypotheses about the mechanisms of carotenoid distribution was that three tissues not typically associated with the immune response (e.g., muscle, heart, adipose) were identified as having significant variation in carotenoid profiles along one or more axes of variation (e.g., innate immune activation versus control, bill coloration) in Chapter 3. The fact that I found differences in muscle carotenoid concentrations between treatment and control groups suggests a potential immunomodulatory role for carotenoids at the site of infection, since bacterial lipopolysaccharide (LPS) was injected into the muscle of treatment birds. In fact, white blood cells are known to break down

carotenoids *in vitro* during the innate immune response (Sommerburg et al. 2003), although this has not yet been tested *in vivo* or in birds. The relationship between bill color change and heart carotenoid profiles was particularly interesting, given that plumage coloration was also related to heart carotenoid profiles in house finches (Chapter 2). Specifically, in Chapter 3, I found that greater reductions in zebra finch bill redness were associated with higher heart carotenoid concentrations, regardless of immune treatment (i.e., LPS vs. control) and carotenoid type. In Chapter 2, I found mixed associations (positive and negative) between concentrations of different carotenoid types and plumage hue in male house finches that was dependent on season. While these results are difficult to compare directly due to the use of different species, study approaches (correlational vs. experimental), color metrics, and integument types (i.e., bare part vs. feather), the common significance of heart carotenoid profiles in association with color does suggest that heart may be an important tissue for carotenoid functions in birds. The benefits of carotenoids for cardiovascular health has been studied extensively in humans (see reviews Ciccone et al. 2013; Maria et al. 2015), but cardioprotective effects of carotenoids and their link to signaling in wild animals has not yet been studied.

One hypothesis that was refined rather than generated from my whole-organism carotenoid profile studies (Chapters 2 and 3) was the resource trade-off hypothesis for honest signaling, which states that carotenoid coloration is honest because high-quality individuals can better afford to allocate carotenoids both toward production of color ornaments and internal carotenoid health functions (e.g., immune response, antioxidant defense; see review Koch and Hill 2018). This hypothesis is based on an assumption that high-quality individuals are less challenged by the trade-off between pigment and health

functions of carotenoids, because they are healthier (i.e., color reflects current health state), have larger internal carotenoid pools (i.e., color reflects ability to absorb and accumulate more carotenoids), and/or are intrinsically higher performing (i.e., "good genes"). The data I generated from my dissertation were best equipped to evaluate the second of these three assumptions, because I measured carotenoid concentrations in all major internal tissues. Overall, I found little support for the assumption that high-quality individuals were more colorful because they had larger internal carotenoid pools. In house finches, I did not find an association between male plumage color (hue or saturation) and total body carotenoid concentrations, and, in zebra finches, there was an immune treatment-dependent relationship between bill color and total body carotenoid concentration, but redder birds in the control group had lower concentrations of total body carotenoids than redder birds in the treatment group or less red birds in either group. However, I did find associations in both species between carotenoid-based integumentary coloration and patterns of tissue carotenoid distribution independent of total accumulation. Thus, I contend that, based on my studies, it is unlikely that carotenoid coloration is an honest signal of the overall concentrations/amounts of body carotenoids, but carotenoid coloration is instead an honest signal of internal resource allocation or distribution among tissues.

In my fourth chapter (Chapter 4), I shifted my focus away from studying tissue distributions of carotenoids and toward the physiology of carotenoid absorption. I examined the relationships between dietary carotenoid availability, carotenoid absorption (i.e., expression of SCARB1), carotenoid accumulation (i.e., liver lutein concentration), and extent of carotenoid-based plumage coloration in wood-warblers (Parulidae). I found

that dietary carotenoid availability and SCARB1 expression together explained variation in carotenoid-based coloration better than dietary carotenoid availability alone; specifically greater access to dietary carotenoids as well as more SCARB1 expression was associated with greater extent of carotenoid-based plumage coloration. However, although carotenoid accumulation did not help explain more variation in color, there was a negative relationship between SCARB1 expression and liver lutein concentration, which suggests that carotenoid absorption and accumulation are carefully regulated (i.e., not too much and not too little, as opposed to a more-is-better regulatory pattern). This regulatory behavior would be consistent with other aspects of carotenoid physiology that have been studied previously. Specifically, carotenoids have been shown to have pro- rather than anti-oxidant effects under certain conditions including particularly high concentrations (Huggins et al. 2010), which would make careful regulation of absorption and accumulation important. Expression of SCARB1 has also been shown to exhibit negative feedback in the same co-regulatory system as a gene for carotenoid metabolism (beta-carotene oxygenase 2; Widjaja-Adhi et al. 2015). Since this study focused only on the role of intestinal SCARB1 expression in carotenoid accumulation and carotenoid-based plumage coloration, future studies should also incorporate SCARB1 expression in other tissues, particularly liver SCARB1 expression, as it is likely to play a role in the absorption of carotenoids in concert with intestinal expression of SCARB1.

Through my dissertation, I have demonstrated that variation in carotenoid profiles is quite context-dependent, and carotenoid-based integumentary coloration reflects tissue carotenoid profiles, although this relationship is also context-dependent. I have shown that an element of digestive physiology, carotenoid absorption, is important to consider

when studying relationships between diet (e.g., dietary carotenoid availability, nutrient availability) and color (e.g., extent of carotenoid-based plumage coloration). By studying whole-organism carotenoid profiles rather than just a handful of tissues, we can gain a much better understanding of potential mechanisms underlying the distribution of carotenoids and of other lipid-soluble nutrients more broadly (Morehouse 2014; Koch and Hill 2018). Unfortunately, studying whole-organism carotenoid profiles comes at the cost of sacrificing birds, but this impact could be mitigated somewhat by continuing to study whole-organism carotenoid profiles primarily in a system similar to the one I used in Chapter 4 (i.e., harvesting tissues from window-strike birds), which is something that I plan to do in the future. Additionally, studying relationships between SCARB1 expression and other metrics associated with carotenoid physiology in wild birds allows us to gain a deeper understanding of the regulation of carotenoid absorption and accumulation (Toews et al. 2017). Overall, I hope that my dissertation highlights the value of whole-organism nutrient profile studies as well as validates the need to incorporate measures of digestive physiology in studies attempting to understand the relationship between diet and color in animals.

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