Exploring the role of carotenoid pigments in physiological function and color signals

by

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Abstract

The study of carotenoid-based coloration, particularly in bird species, is a large and growing literature that is increasingly defined by the framework of a single overarching concept, the resource tradeoff hypothesis. Contrary to widespread acceptance of resource tradeoffs in maintaining the condition-dependence of carotenoid-colored signals, methodological and theoretical challenges alike have complicated my ability to conclusively test the hypothesis. Here, I first review the existing empirical literature that has attempted to test the resource tradeoff hypothesis in bird species, and I highlight outstanding uncertainty in several key components of the hypothesis as well as important directions for future research. Given that one of the primary complications in testing for differential resource allocation of carotenoid resources is the ability to experimentally manipulate dietary intake, I next present a meta-analysis that quantitatively describes the relationships between dietary carotenoid supplementation and changes to circulating carotenoid levels or carotenoid-based coloration. A second main complication in testing the resource tradeoff hypothesis is the ability to use experimental challenges to physiological systems to assess whether carotenoid pigments play a role in the response. While immune challenges, such as pathogen infection or parasite administration, have been better studied, experimental techniques that induce increased oxidative stress remain new and relatively unexplored; I therefore present the first comprehensive review of a variety of methods that have been or could potentially be used to induce increased oxidative stress in animal bodies without confounding side-effects. Before applying one of these

techniques to my own study system, I first describe that system—the domestic canary (*Serinus canaria*)—in a short comparative study that uses feather samples and museum specimens to establish the evolution of sexual monochromatism through domestication. Finally, I perform my own empirical test of the core assumption of the resource tradeoff hypothesis, that carotenoid pigments provide direct physiological benefits, by examining whether carotenoid-free mutant canaries are unable to perform as well as carotenoid-rich wild-type canaries on immune and oxidative tests. The results of my analyses are a conclusive demonstration that birds do not need internal carotenoid pigments to defend against pathogens or prevent oxidative stress, at least when the confounding effects of retinol are removed. Collectively, my dissertation presents a critical examination of all angles of the resource tradeoff hypothesis, from reviewing existing evidence to raising methodological concerns to finally challenging the hypothesis with my own empirical study. My research offers an important advancement to understanding the role of carotenoid pigments in physiological processes in animals and to how future researchers study carotenoids, those physiological processes, and coloration itself.

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corgis Daisy and Colby keep me grounded in the unpredictable, maddening, and sometimes terrifying world of research; my parents, too, are always just a phone call away, no matter the hour and no matter the topic. Finally, I send a big *thank you* to my canaries.

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CHAPTER 1

Do carotenoid-based ornaments entail resource tradeoffs? An evaluation of theory and data

Manuscript in preparation with co-author Geoffrey Hill

Introduction

Carotenoid-based displays are textbook examples of honest signals of individual quality that are assessed during mate choice (Dugatkin 2013). Although some studies have failed to find consistent evidence that carotenoid coloration is related to measured aspects of condition in their particular systems (e.g. Dale 2000; Navara & Hill 2003; Dowling & Mulder 2006; Smith *et al.* 2007), behavioral ecologists have generally embraced that the idea that carotenoid-based coloration often serves as condition-dependent signals of quality. Accordingly, recent research on carotenoid-based ornaments has focused on seeking an explanation for how carotenoid coloration can be a reliable signal of fundamental aspect of performance like immunocompetence or oxidative balance.

Recent discussions of the mechanisms that might create a link between carotenoid coloration and performance have been dominated by the resource tradeoff hypothesis. The specific tradeoffs involved vary by the system in which they are discussed, but in all manifestations of the hypothesis, carotenoids are considered limited resources that must be traded-off between production of visual displays and key functions in physiological systems (Møller *et al.* 2000; Faivre *et al.* 2003; Blount 2004). Versions of the resource tradeoff hypothesis have invoked differential allocation of carotenoid pigments between offspring versus self (e.g. McGraw 2006; Morales, Velando & Torres 2009; Remes & Matysiokova 2013), between ornamentation versus self-maintenance (e.g. Faivre *et al.* 2003; Fitze *et al.* 2007; Baeta

et al. 2008; McGraw, Nolan & Crino 2011), or between different internal processes (e.g. Mougeot *et al.* 2009a; Toomey, Butler & McGraw 2010). Importantly, the fundamental framework of the resource tradeoff hypothesis is founded on a few key assumptions: 1) carotenoid pigments play important roles in physiological processes, particularly in boosting immune response and/or antioxidant defenses; 2) carotenoid pigments are limiting within animal systems such that individuals do not possess sufficient carotenoids to maximize performance in all avenues simultaneously; and hence 3) carotenoid-based ornaments reflect quality because only high quality individuals can best withstand tradeoffs associated with carotenoid pigments.

The assumptions that underlie the resource tradeoff hypothesis, while addressed in numerous studies, remain contentious. For example, in avian species—perhaps the best-studied taxa for carotenoid-based ornaments—there remains considerable debate regarding whether carotenoid pigments function as beneficial antioxidant molecules *in vivo* (Hartley & Kennedy 2004; Costantini & Møller 2008). The large and growing number of studies that have addressed the resource tradeoff hypothesis in relation to carotenoid-based coloration present a daunting challenge to researchers who attempt to assess the validity of the key assumptions that underlie the hypothesis. Existing reviews consider several of the key tenets of carotenoid tradeoffs listed above from various perspectives (Møller *et al.* 2000; Chew & Park 2004), or they provide broader overviews of the structure and function of carotenoid color signals (Svensson & Wong 2011). To date, there is no published review that explicitly outlines and discusses the carotenoid resource tradeoff hypothesis with respect to theory, physiology, and existing case studies.

Here, I present an assessment of the resource tradeoff paradigm for explaining honesty in carotenoid-based coloration. I limit the scope of my discussion to studies of birds because they are, by far, the most extensively studied group of animals with regard to carotenoid signaling.

Comparisons among taxa can provide important insights, but differences in the basic physiology of different animal groups can also lead to confusion when direct comparisons are attempted. In order to present a clear analysis of the state of my understanding of the focal taxon studied with respect to carotenoid coloration, I focus on birds. I anticipate that the patterns revealed in birds will have important implications for other taxa.

To establish the current state of understanding of the three main assumptions of the carotenoid resource tradeoff hypothesis, I performed a qualitative assessment of the methods, results, and author conclusions presented in more than 60 empirical studies of carotenoid-based traits in birds published between 1996 and 2016. From the patterns that emerge in these studies, I assess tradeoff mechanisms proposed to explain condition-dependence in carotenoid-colored ornaments while also considering evidence for the direct physiological benefits of carotenoids to the individual. My goal is to provide an overview of current issues related to carotenoid resource tradeoffs and the honesty of carotenoid-based colored signals in birds to highlight questions that remain to be fully resolved and to clarify needs for further study.

The Carotenoid Tradeoff Hypothesis

As described above, the resource tradeoff hypothesis for signal honesty in carotenoid-based traits proposes that only high quality individuals can "afford" to allocate valuable and limited carotenoid pigments away from internal processes and toward coloring ornamentation (Figure 1). The three key assumptions of this overarching hypothesis can be further broken down into three testable underlying hypotheses: 1) carotenoids boost physiological function, specifically immunocompetence and/or antioxidant capacity; 2) finite carotenoid resources are sequestered or used up during physiological function such that they are no longer available for ornamentation;

and, 3) individuals of different underlying quality will have different relationships between internal carotenoid levels, immune/oxidative performance, and coloration.

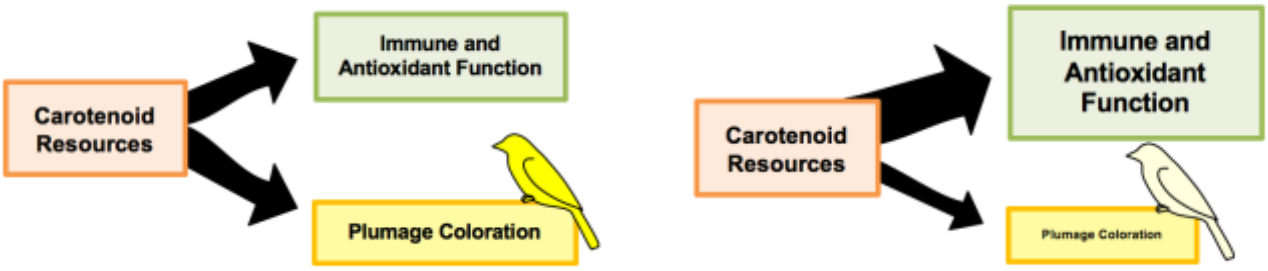

Figure 1. Carotenoid resource tradeoffs. Diagrams illustrating the hypothesized tradeoff that may explain the signal honesty of carotenoid-based ornamental traits, like plumage color. The left image depicts how carotenoid resources may be allocated between maintenance functions (like standing immune and antioxidant support) and boosting plumage coloration in a healthy, high quality bird. The right image depicts a hypothetical shift in resources toward boosting immune and antioxidant function in a sick or otherwise challenged bird, leaving few resources to color ornamental plumage.

Within the following sections, I discuss these key components of the resource tradeoff hypothesis and consider specific experimental studies that investigate them in bird species. While several important questions regarding carotenoid use in immune or antioxidant function or carotenoid limitation could potentially be discussed for the length of an entire review paper, I aim to provide sufficient detail to clarify a more explicit framework for forming specific experimental hypotheses and predictions related to carotenoid tradeoffs.

Are carotenoids limited?

Without carotenoid limitation, carotenoid resource tradeoffs cannot be the basis for honest signaling. Thus, the question of whether carotenoids are limiting for wild birds is the central question related to the resource tradeoff hypothesis in avian species. However, the quantities of carotenoid resources available to wild birds and whether individuals might be sufficiently limited in carotenoid access for allocation tradeoffs to be relevant has long been a matter of debate (Hill

1994; Hudon 1994; Olson & Owens 1998; Hadfield & Owens 2006). The restriction that birds must acquire carotenoids through their diet is sometimes itself seen as clear evidence that carotenoids are a limited resource, but it is also possible that essentially all wild birds ingest sufficient carotenoids to meet all physiological needs. Unfortunately, questions related to carotenoid limitation are difficult to answer under natural conditions when a population of birds may consume a wide variety of food items, individuals may differ in which and how much of those various items they consume, and the internal processes of carotenoid absorption (which may differ among individuals, species, seasons, and even specific carotenoid pigments; Tella *et al.* 2004; McGraw 2005) are largely unknown in any given study system. Evidence suggesting internal carotenoid limitation in wild populations includes correlations between gut carotenoid content and ornamental coloration (Hill, Inouye & Montgomerie 2002) and field studies that find increases in coloration or other parameters when individuals are provided with supplemental carotenoid sources (e.g. Ewen *et al.* 2008; Sternalski *et al.* 2012). However, other field studies have detected little to no effect of carotenoid supplementation on coloration or focal parameters in wild individuals (e.g. Marri & Richner 2014a). As a result, studies on captive species comprise the majority of investigations of carotenoid limitation and allocation.

In captive systems, finding a positive effect of carotenoid supplementation on coloration or other physiological measurements—and particularly, finding evidence that supplementation reduces or eliminates one or more negative effects associated with a physiological challenge—is often interpreted as evidence that unsupplemented individuals are carotenoid-limited (Hill 1992; McGraw & Ardia 2003; Eraud *et al.* 2007; Aguilera & Amat 2007; O'Brien & Dawson 2008; Sternalski *et al.* 2012). Such studies are complicated by dose and type of carotenoid supplemented (Koch, Wilson & Hill 2015); the potential effects of supplementing ornamented

birds with the carotenoid pigments used directly as colorants will differ from the effects of supplementing with dietary carotenoid precursors and will also vary with the physiological properties of those carotenoids, including whether or not they may be converted into vitamin A (Hill & Johnson 2012). Unfortunately, we currently lack a detailed understanding of the quantities of carotenoids absorbed by an individual, the internal locations to which those carotenoids are transported, where they are stored, how and when stored carotenoids may be used, the quantities and locations of carotenoids needed to achieve full coloration of an ornament, and the effects of experimental supplementation or physiological challenge on processes like carotenoid absorption and distribution. Without such information, the question of whether or not carotenoids are limiting resources remains an open question.

To provide more definitive and quantitative evidence for how carotenoids are allocated through the avian body under varying conditions and in various species, there is a critical need for future studies to directly measure the carotenoid content of the diets of wild birds and to use carefully controlled experiments to manipulate carotenoid access within natural ranges. Perhaps even more critically needed are studies that track carotenoids in the body, using radiolabeling or other experimental techniques. Performing such analyses may require sacrificing individuals under different treatments (e.g. ingesting different dietary carotenoid levels and/or undergoing different experimental challenges) and measuring precisely where, in what forms, and in what quantities carotenoid pigments are found throughout the body. It may be logistically impossible to perform these studies in natural populations where dietary intake cannot be fully controlled or quantified, but captive studies can potentially provide foundational information regarding the plausibility of tradeoffs underlying the signal honesty of carotenoid coloration.

Moreover, the mechanisms responsible for carotenoid pigmentation remain remarkably poorly known. There is little information, particularly outside of poultry, regarding where, when, and how carotenoids are absorbed, transported, metabolically transformed from dietary to ornamental pigments, and deposited in the integument, and how these processes vary among individuals and among species (McGraw 2005; McGraw, Nolan & Crino 2006; Ge *et al.* 2015; García-de Blas, Mateo & Alonso-Alvarez 2016). Continued advances in understanding the genetic bases for variation in coloration (Lopes *et al.* 2016; Mundy *et al.* 2016; Toews, Hofmeister & Taylor 2017) combined with the aforementioned controlled experiments should be a top priority for elucidating the nature of carotenoid limitation among tissues and physiological functions and how these patterns may differ among individuals, sexes, and species.

Do carotenoids play important roles in immune and antioxidant function?

It is widely believed and stated that carotenoids play important an role in immune system function in birds—perhaps through antioxidant effects—though their direct involvement as antioxidants is considered more equivocal (Hartley & Kennedy 2004; Costantini & Møller 2008; Perez-Rodriguez 2009; Simons, Cohen & Verhulst 2012). In this section, I assess the evidence that carotenoids play key roles in immune defense and maintaining oxidative balance. Studies aimed at addressing these questions have been conducted with such diverse approaches that a quantitative meta-analysis, while useful to take into consideration when looking for general patterns (e.g. Simons *et al.* 2012), misses important relationships between particular experimental designs, study systems, and results. Thus, my assessment will be qualitative in order to fully describe the complexity of results and discussion pertaining to carotenoid physiological function. Moreover, I aim to avoid over-generalization of studies by "vote-

counting" positive or negative relationships. Instead, I focus on a handful of representative studies to illustrate several important points and outstanding questions within the avian carotenoid literature. The question of whether or not carotenoids play important physiological roles besides serving as pigments for coloration is fundamental to determining whether carotenoids are valuable resources that must be differentially allocated among functions at all.

Carotenoids as immune boosters. *Rationale for the hypothesis.* An important role for carotenoids in enhancing immune function is widely accepted in studies examining carotenoid-based coloration in birds such that some recent studies state the role of carotenoids as beneficial to immunocompetence as a well-established truth (e.g. Benito, Gonzalez-Solis & Becker 2011; Giraudeau *et al.* 2015; Merrill, Naylor & Grindstaff 2016). Originally founded on a small literature examining the potential benefits of a carotenoid-rich diet in humans, studies have since examined correlations between carotenoid-based coloration, circulating carotenoid levels, carotenoid consumption, and measurements of immune response to test whether carotenoids enhance immunocompetence.

The specific means by which carotenoids may improve immune system performance, particularly in non-mammalian species, are rarely articulated and remain largely unresolved. Studies investigating carotenoids and immune benefits often cite reviews largely on mammalian species (Bendich 1989; Chew & Park 2004). Briefly, carotenoids have been implicated in lymphocyte proliferation and activity, though their exact biochemical participation in such processes remains uncertain; it is possible that carotenoids may be localized to critical subcellular locations, such as the mitochondria, where they may prevent oxidative damage and therefore facilitate proper cellular function in immune cells (Chew & Park 2004; Hill 2014;

Koch, Josefson & Hill 2016)—but this remains to be tested, particularly in the avian species that are often studied with respect to coloration (and in fact, the antioxidant properties of carotenoids *in vivo* are debated; see below). Carotenoids have also been cited as important to preventing damage to self during the innate immune process of respiratory burst in which immune cells target pathogens for oxidative damage by rapidly releasing pro-oxidants (Chew and Park 2004), though the process functions differently in mammalian versus avian cells (Harmon 1998) and thus far there is little evidence to suggest a role of carotenoids in respiratory burst in birds (Sild *et al.* 2011).

Within behavioral ecology and evolutionary biology, researchers are generally limited in their means of immune system stimulation and response quantification, so they often rely on one of several common established methods to estimate immunocompetence. While the emerging field of eco-immunology is rapidly expanding the breadth and interpretation of techniques used by non-biomedical researchers to assess the health and immune performance of animal subjects, the carotenoid tradeoff literature is still dominated by results of either parasite load assessment or of phytohaemagluttanin (PHA), sheep red blood cell (SRBC), and/or bacterial lipopolysaccharide (LPS) injection, combined with swelling, hemagluttination, or direct antibody response measurements. Unfortunately, the specific roles that carotenoids may play in responses to parasites or to any of these foreign substances remain a matter of conjecture, though it has been proposed that carotenoids may be important by generally boosting immune processes through their hypothesized roles in lymphocyte development or as antioxidants to combat reactive oxygen species produced by immune cells. In addition, immunocompetence quantification techniques that are based on measuring standing variation in parameters like natural antibody levels may be poorly suited for examining potential carotenoid resource tradeoffs because it is

difficult to assess the role of carotenoids in processes that are ongoing or may have been determined in past time points when individuals may have had different levels of internal carotenoids. I encourage researchers to perform a critical examination of how a potential immune measurement technique may specifically involve carotenoids—and how to explicitly test if it does—before carrying out a new experiment, rather than relying on techniques used in previously published studies.

Case studies. Questions of specific immune measurement techniques aside, existing studies of the interactions between internal carotenoids, ornamental coloration, and immune performance are testament to the complexity of the predictions of the resource tradeoff hypothesis and to how the quality of individuals is interpreted. For example, McGraw *et al.* (2011) studied house finches (*Haemorhous mexicanus*) in captivity both over winter and during the following summer's molt, the period where the finches actively deposited carotenoid pigments in growing feathers. They manipulated carotenoid access by supplementing experimental groups of birds with the dietary pigments lutein and zeaxanthin in both seasons, and they assessed immunocompetence by quantifying swelling response to PHA injection and the ability of isolated whole blood samples to reduce *E. coli* growth relative to controls (in a bacterial killing assay; BKA). Interestingly, they discovered that while supplemented birds always had increased concentrations of plasma carotenoids relative to control birds, they had greater PHA-induced swelling and blood bacterial killing relative to controls only during the molt season. While the scope of conclusions that can be drawn from isolated measures of immune system function—and, in fact, the meaning (or lack thereof) of particular measures itself—is a matter of debate (Adamo 2004; Kennedy & Nager 2006), that the effects of carotenoid supplementation on immune parameters are only clear during molt are notable. If

internal carotenoid pigments are universal immune boosters, we might expect supplementation to *always* increase immune performance.

The authors discuss their results as potential evidence that supplementation relieved the need for a tradeoff between allocating limited carotenoids to the immune system versus ornamentation. Interestingly, supplemented molting birds also possessed greater feather color saturation (but not hue), indicating increased carotenoid pigment concentration (but no changes in pigment composition). This result draws further questions: if carotenoids are limited resources during molt and the supplementation quantity was within natural bounds, and if the immune response required the use of carotenoids for a beneficial function, then we might expect large immune responses to be associated with depleted (or at least unchanged from controls) coloration, plasma carotenoids, or both; instead, the opposite was detected. I suggest that the observed positive correlations between supplementation-boosted plasma carotenoid levels, immune performance, and coloration during molt are indicative that the color signal may be honest, but not necessarily that the honesty is maintained by a resource tradeoff. As the authors suggest, it is unknown how other aspects of the internal physiology of songbirds may be altered during molt to cause such patterns in their results.

While McGraw *et al.* (2011) illustrates that birds circulating higher levels of carotenoids *during molt* may be considered higher quality birds with superior immune performance, a study by Mougeot *et al.* (2009b) in red-legged partridges (*Alectoris rufa*) demonstrates a potential case in which high quality individuals differ not just in resource quantities, but in ability to use those resources both for immune benefits and for bolder ornamental coloration. In the absence of experimental carotenoid supplementation, Mougeot *et al*. (2009) examined standing variation in PHA swelling response, coccidian gut parasite load, circulating carotenoid levels, and three

measurements of ornamental coloration, among other variables. In sum, the redness and extent of eye ring pigmentation—but not beak redness—positively correlated with magnitude of PHA-induced swelling, and though eye ring pigmentation alone reflected circulating carotenoid concentration, and levels of circulating carotenoids positively correlated with PHA response. These interactions were complicated by coccidian load, which was related to lower circulating carotenoids and trait redness, but not extent of eye ring pigmentation. These complex results may indicate that "high quality" birds were relatively resistant to coccidia and were able to produce large red ornaments while also maintaining high levels of circulating carotenoids *and* mounting a large response to PHA injection—essentially, some birds appeared capable of maximizing many parameters of coloration and immune defense simultaneously. While the mechanisms maintaining greater expression of ornamentation, circulating carotenoids, and these measures of immune responsiveness are not clear, the results point toward a more inclusive interpretation of quality where carotenoid levels and colors may be *indicative* of rather than the *determinant* of performance. As the authors note, the decrease in plasma carotenoid levels in the presence of high levels of coccidia might have be due to inhibition of carotenoid absorption mechanisms rather than the use of carotenoids in defenses. Moreover, it is interesting that a *negative* relationship between coccidia and carotenoid levels was said to indicate the direct involvement of carotenoids in immune defenses, while a *positive* relationship between PHA-induced swelling and carotenoids was also said to indicate the same pattern; this apparent contradiction is testament to the general lack of information into how carotenoids are involved in immune defenses: are they "used up" to boost response (causing a negative relationship), or is their presence in greater concentrations beneficial in itself (positive relationship; see below)? Though the authors invoke a resource tradeoff between carotenoid use for coccidian defense versus for

PHA swelling response and coloration in their discussion of results, a clear mechanistic understanding of how carotenoids may be involved in either anti-parasitic defenses or in PHA-induced swelling is necessary to form a conclusive determination of the factors determining variation in health and coloration.

Both of the studies discussed thus far have utilized PHA as an immune measurement, and indeed, PHA injection followed by quantification of swelling response is a particularly common method in studies of carotenoids in birds; more than half of the individual studies I reviewed that incorporated at least one measure of immunocompetence included a measurement of PHA-induced swelling. While a recent meta-analysis found small but significant overall effect sizes linking PHA responses and ornament redness or plasma carotenoid levels (Simons *et al.* 2012), I caution against drawing the general conclusion that coloration reflects immunocompetence because circulating carotenoids mediate PHA response—or even that PHA response is dependent on carotenoids. Correlations between response to PHA and carotenoid supplementation, plasma carotenoid levels, and/or expression of carotenoid-based coloration have rarely been straightforward (e.g. McGraw & Ardia 2003; Blount *et al.* 2003), are often context-dependent and vary with interactions among variables (e.g. McGraw & Ardia 2007; Biard *et al.* 2009; Alonso-Alvarez *et al.* 2009), and sometimes lack a positive relationship at all (e.g. Navara & Hill 2003; Hõrak *et al.* 2006; Smith *et al.* 2007; Benito *et al.* 2011). The example of PHA emphasizes both the heterogeneity in the literature testing for relationships between carotenoids and measures of immunocompetence and the complexity in making predictions based on the resource tradeoff hypothesis when results appear highly variable based on study system and experimental design.

Conclusions. Despite general acceptance that carotenoids are essential to proper immune function, I conclude that empirical evidence for carotenoids as immune boosters that must be differentially allocated either to immune function or to ornamentation is limited and not sufficiently conclusive to justify such widespread acceptance without further testing. There is persistent and notable variation in the results of studies testing for relationships between carotenoids and immunocompetence such that a straightforward, universally beneficial effect of carotenoids seems unlikely. I argue that it is important for future studies to elucidate the mechanisms driving variation in the measured immune function parameters, and how carotenoid pigments may or may not be involved.

Carotenoids as antioxidants. *Rationale for the hypothesis.* Whether carotenoids function as important antioxidants in the bodies of birds is contentious and the subject of current research. Two meta-analyses have examined relationships between circulating carotenoids and/or carotenoid-based coloration and oxidative stress parameters and both reported small and generally nonsignificant effect sizes (Costantini & Møller 2008; Simons *et al.* 2012). Others have suggested that carotenoids may indicate oxidative state without directly participating as antioxidants, such as by becoming "bleached" during oxidative stress (Hartley & Kennedy 2004) or because only particular oxidative states (García-de Blas *et al.* 2016) or high levels of cellular functionality (Johnson & Hill 2013) facilitate the conversion of dietary carotenoids into ornamental carotenoids. While carotenoids are, by their chemical nature, effective electron receivers under most conditions and hence potential antioxidants (Krinsky & Yeum 2003), it is uncertain whether the physiological systems of birds are adapted to deploy carotenoids as important antioxidants to maintain redox balance. The key question is whether the right forms of

carotenoids are present in sufficient quantities in the necessary organ-level, cellular, or even subcellular locations to have a functional effect on an individual's ability to maintain healthy oxidative balance.

A challenge central to any studies examining oxidative stress parameters is that the constant interplay between pro-oxidants and antioxidants causes complex and variable fluctuation in levels of antioxidants, pro-oxidants, and oxidative damage (Monaghan, Metcalfe & Torres 2009; Perez-Rodriguez 2009), and such levels may vary throughout an individual's body. Many studies on live animals are restricted to assessing oxidative stress from blood samples to avoid terminal experimentation, and levels of oxidative damage markers or antioxidant capacity in the blood may have limited relevance to oxidative stress in more metabolically active organs. While studies of blood parameters may be useful for providing at least a peripheral indicator of oxidative stress within an animal, it is important to note that experiments would ideally examine the relationships between antioxidants, pro-oxidants, and damage in the specific tissues most pertinent to the research objectives.

Case studies. Many studies examining interactions between carotenoid levels and measurements of antioxidant capacity in birds have yielded nonsignificant or inconsistent results (Costantini *et al.* 2006; Hõrak *et al.* 2006; Morales *et al.* 2009; Perez-Rodriguez, Mougeot & Alonso-Alvarez 2010; Marri & Richner 2014a; Leclaire *et al.* 2015). Other studies have found difficult-to-interpret correlations between carotenoids and/or coloration and some, but not other, measures of oxidative stress (e.g. Hõrak *et al.* 2007; Alonso-Alvarez *et al.* 2008). However, still more studies have found some indication that carotenoids may play a beneficial role in antioxidant defenses: for example, Alonso-Alvarez *et al.* (2004) found that zebra finches (*Taeniopygia guttata*) that increased circulating carotenoid levels the most also had the highest

performance on a test of the ability of red blood cells to resist oxidative challenge, although there was no clear direct effect of dietary carotenoid supplementation on this measurement of antioxidant defense; in contrast, Bertrand *et al.* (2006) did find a positive direct effect of carotenoid supplementation on the same measurement of red blood cell defense in zebra finches laying large clutches. Additionally, several recent studies have found a relationship between physiological oxidative challenge and ornamental coloration, either indicating increased (Romero-Haro & Alonso-Alvarez 2015; García-de Blas *et al.* 2016) or decreased (Alonso-Alvarez & Galván 2011; Tomášek *et al.* 2016) color expression after the challenge, though other studies have also found no effect (Isaksson & Andersson 2008; Hõrak *et al.* 2010). These results indicate the potential for carotenoid-based colors to be sensitive to oxidative stress whether or not carotenoids serve a direct antioxidant function themselves—though the direction and breadth of that relationship across study systems is uncertain.

Giving ongoing ambiguity in the antioxidant function of carotenoids in the avian body, I encourage researchers to avoid making potentially problematic conclusions that invoke carotenoid antioxidant activity to explain results. For example, several studies—even those not measuring oxidative stress parameters directly, or finding nonsignificant results in oxidative stress measures—have conjectured that carotenoid antioxidant function may the explain complex patterns they observed. The potential antioxidant function of carotenoids has been invoked in this manner to explain a wide variety of results: increased hatch success in lutein-injected eggs (Marri & Richner 2014b), depressed mass gain after LPS injection in unsupplemented (but not supplemented) adults (Hõrak *et al.* 2006), decreased PHA response in cross-fostered chicks with unsupplemented (but not supplemented) parents (Berthouly, Helfenstein & Richner 2007), a lack of immune responsiveness during supplementation (because of a surplus of antioxidant activity

reducing pro-oxidant-mediated signals; Bedecarrats & Leeson 2006), relationships between UV chroma and plasma carotenoid levels in mallard (*Anas platyrhynchos*) bills (Peters *et al.* 2004), and benefits of carotenoids on birds with increased activity levels (Simons *et al.* 2014). While it is important to discuss a variety of interpretations for the results of any study—particularly when a clear answer is not evident—I argue that calling upon the possible antioxidant function of carotenoids when it was not tested in any direct manner (or when it was tested and found to be statistically nonsignificant) may only increase misunderstandings in the literature.

A recent study by Tomášek *et al.* (2016) has proposed that an important explanation for inconsistency in relationships between carotenoids and oxidative stress is that the current commonly used methods for measuring antioxidant defenses fail to capture the effects of carotenoids by focusing on hydrophilic rather than lipophilic parameters (given that carotenoids themselves are lipophilic). In support, they examined the effects of carotenoid supplementation and/or oxidative challenge administration on zebra finches and found that supplementation had no effect on a traditional hydrophilic measure of antioxidant capacity, but *decreased* a new measurement that estimated some types of lipophilic antioxidant defenses; both measures of antioxidant defense increased in response to oxidative challenge, but the lipophilic antioxidant measure increased less when the challenged birds were also supplemented with carotenoids. The authors concluded that increased carotenoid levels in supplemented birds served as important lipophilic antioxidants such that other, non-carotenoid lipophilic antioxidants—the ones estimated by their measurement—were not required. Further, because challenged birds decreased their expression of carotenoid-based bill redness, the authors suggest that carotenoids were traded-off between serving as colorants or as antioxidants. However, even supplemented birds decreased coloration during challenge, which may suggest mechanisms besides differential

resource allocation of limited carotenoid resources may have caused decreased coloration during the experiment (Tomášek *et al.* 2016). A more direct measurement of the potential lipophilic antioxidant activity of carotenoids themselves is needed to provide further explanation for these results.

The results of Tomášek *et al.* (2016) present an important consideration for future studies, which may consider whether measuring estimates of lipophilic antioxidant capacity may more accurately capture the activity of carotenoids. Indeed, García-de Blas *et al.* (2016) similarly suggest that their measure of hydrophilic antioxidant capacity in red-legged partridges could have been more representative of the effects of other antioxidants than of carotenoid activity in their study system. The implication that the most commonly used methods for estimating antioxidant defenses in studies of carotenoid-based coloration in birds are unable to directly detect the activity of carotenoids themselves is intriguing and may provide some explanation for the inconsistency of previous studies, though measurement of antioxidant capacity is only one aspect of oxidative stress that has been tested. It is also important to consider one reason that a wide variety of studies may have be using largely inappropriate methods to estimate carotenoid antioxidant potential: we currently know very little about the mechanisms for how carotenoids may serve as antioxidants in the avian body.

Conclusions. Considering estimates of lipo- rather than hydrophilic antioxidant capacity and testing alternative hypotheses for how carotenoids may relate to oxidative stress may yield important new answers to longstanding questions about whether carotenoids directly benefit individuals through their antioxidant activity. However, as with tests of the immune benefits of carotenoids, I believe that a critical next step is performing detailed experiments to try to isolate where and in what quantities carotenoids are present in the avian body and how, specifically,

these carotenoids may or may not interact with pro-oxidants. A better understanding of the subcellular locations of carotenoids and the role that they play in oxidative stress states is essential to drawing conclusions about whether the antioxidant potential of carotenoids *in vitro* does in fact translate to an important and significant role of carotenoids as antioxidants *in vivo*—and how the deposition of carotenoids in colored ornaments may or may not alter the ability of an individual to maintain a healthy oxidative balance.

What makes a high quality individual?

Even if we accept the hypothesis that carotenoids serve a beneficial physiological function, predicted patterns of carotenoid storage and use in high and low quality individuals are complicated, depend on assumptions of physiological mechanisms, and are rarely discussed in detail in empirical tests of the tradeoff hypothesis. Defining the concept of individual quality or condition is a theoretical challenge in its own right that is beyond the scope of this current review (see Hill 2011); however, it is important to address how researchers who have conducted studies looking for carotenoid tradeoffs have interpreted the results of their experiments based on one or more different underlying frameworks for understanding individual quality. Given that carotenoid resource tradeoff hypotheses ultimately link back to mate choice for higher quality individuals, a consideration of how one recognized quality becomes fundamental. I argue that there is a general lack of consistency within studies that test carotenoid resource tradeoffs in how individual quality is defined, resulting in added confusion in the literature. Here, I break down the component parts of a variety of common frameworks for individual quality within the context of carotenoid-based traits.

Studies of the honesty of carotenoid-based ornaments tend to invoke one or more of the following three aspects of individual condition: 1) oxidative or immune health state, 2) magnitude of internal carotenoid resource pools, or 3) some aspect of inherent, underlying functionality (Table 1). Studies focused on health state often use immune or oxidative challenges to assess predictions of honest signaling hypotheses; studies focused on resource pools typically manipulate access to dietary carotenoids; and, studies focused on general underlying functionality may consider variation in the performance of individuals held under the same conditions. The differences in how these disparate approaches affect the way in which carotenoid signaling is assessed are best illustrated in examples.

In studies of carotenoid signaling that are focused on health state, it is commonly observed that birds presented with an immune challenge, such as a pathogen or parasite infection, develop paler carotenoid coloration than control individuals (e.g. Brawner, Hill & Sundermann 2000; Faivre *et al.* 2003; Rosenthal *et al.* 2012). In these cases, carotenoid-based traits may be interpreted as honest signals of quality because only individuals that are *not* currently undergoing a physiological challenge exhibit full color expression. In parallel experiments, sometimes by the same lab group on the same population of birds, studies also report positive relationships between supplementation of dietary carotenoids, amount of circulating carotenoid pigments (a proxy for the size of internal carotenoid resource pools), and the expression of colorful traits. Such observations suggest that individuals with more pigments have higher quality ornaments and that coloration can be altered by altering access to dietary pigments. When these two general patterns are considered together with the hypothesis that carotenoids may have direct involvement in physiological function, predictions regarding the

relationships between internal carotenoid pigments, internal physiological performance, and external carotenoid coloration become extremely challenging to formulate.

The key problem in trying to articulate a hypothesis that incorporates the negative effects of physiological challenges on carotenoids, positive effects of dietary carotenoids on coloration, and positive effects of carotenoids on physiological function is that we do not know how carotenoids are used by immune or antioxidant systems (if at all)—where, when, how, and in what quantities. Thus, it is not possible to make realistic predictions in experiments that investigate the interactions among these variables. For example, one might propose that internal carotenoid pigments are "used up" while boosting physiological function, in which case the prediction would be a *negative* relationship between strength of physiological response and carotenoid levels. Alternatively, carotenoids might boost immune function without being consumed, in which case the prediction would be a *positive* relationship between physiological response and carotenoid levels. These two predictions both assume that carotenoids serve a direct beneficial role in individual performance, but they yield opposite predictions regarding the direction of the relationship between internal carotenoid levels and measures of that performance. Further, introducing a supplemental carotenoid diet to challenged birds imposes additional uncertainty; for example, if carotenoids are not "used up" during beneficial function, then supplementation may have little to no effect whether or not carotenoids are important to physiological performance.

There is also a fundamental difference between studies that use categorical analyses to assess differences in performance among discrete treatment groups and studies that consider continuous variation among individuals within one group. Many studies examine the patterns of their results to test not only for the discrete effects of treatment differences, such as the effect of

immune challenge on circulating carotenoid levels, but also finer interactions among continuous variables, such as how the circulating carotenoid levels of immune-challenged birds relate to the strength of response to the challenge. For example, the above-mentioned study by Alonso-Alvarez *et al.* (2004) found no direct, categorical effect of either LPS stimulation or carotenoid supplementation on the ability of finches' red blood cells to resist oxidative attack; however, they did find that individuals with the greatest *increase* in plasma carotenoid content had the strongest performance on the oxidative attack test. This latter result could be seen as supportive of the resource tradeoff hypothesis: perhaps individuals that were able to allocate the greatest quantity of carotenoids to internal tissues gained the largest benefit in terms of resistance to oxidative damage, assuming that carotenoids were serving as antioxidants and were not consumed in the process (despite no detected benefit of supplementation itself). However, without knowing how and where carotenoids may perform such antioxidant function, we cannot separate this resource tradeoff interpretation from alternatives hypotheses. It is also possible, for example, that some individuals (i.e. high quality individuals) had fundamentally superior performance in physiological arenas such that they inherently had both stronger defense against free radical attack and a greater capacity to absorb carotenoids from the diet (Hill 2011; index citation?). This latter hypothesis predicts the same pattern of association between increase in plasma carotenoid levels and increase in resistance to oxidative stress, and it also explains a lack of direct effect of supplementation on oxidative damage resistance. It is important to consider the complexity of the system under study and to be explicit regarding the assumptions that lead to a conclusion that carotenoid tradeoffs are involved. Without such understanding of specific mechanisms and careful testing of predictions, we cannot perform tightly controlled, definitive tests of resource tradeoff hypotheses for carotenoid-based tradeoffs.

To summarize, widespread uncertainty exists in the carotenoid literature about whether I define high quality individuals as those that are 1) currently healthy, 2) currently possessing large quantities of carotenoids, some combination or both, or 3) inherently superior in one or more physiological metrics. The result of this uncertainty is a wide range of observations can be used to support resource tradeoff hypothesis even if alternative explanations exist, such as index hypotheses that propose trait quality as an indicator of internal conditions rather than as a direct product of costly trade-offs (Hill 2011). While there is nothing inherently erroneous in discussing how results fit particular frameworks, the danger is in suggesting most every observation to be supportive of a favored hypothesis. This hinders the ability to draw accurate conclusions about support for or against the resource tradeoff hypothesis, and discourages consideration of alternative hypotheses as stated evidence for the current tradeoff paradigm continues to build.

Conclusions

A common thread throughout discussions of carotenoid limitation, beneficial physiological functions of carotenoids, and metrics of considering individual quality is that there is a critical need for biologists to address the fundamental science of the biochemical activity of carotenoids in the animal body, the quantities and types of carotenoids present in the body and differences among organs and cellular locations, and how this ultimately affects carotenoid function and the sensitivity of coloration to individual health status or underlying quality. I encourage new studies of old questions, using improvements in genetic and biomedical techniques to perform carefully controlled and detailed analyses that may answer how carotenoid absorption, modification, transportation, ornamental deposition, and/or biochemical function change under varying conditions, and whether the quantities and dynamics of carotenoid movement patterns necessitate

resource allocation tradeoffs. Until we have established more clear answers to these longstanding questions, I urge researchers of carotenoid coloration to maintain an open perspective with regard to whether or not carotenoids are indeed beneficial physiological molecules and whether resource tradeoffs are the sole basis for honest carotenoid coloration. Correlations between carotenoid levels, coloration, and physiological performance can only yield so much information without a better understanding of underlying mechanisms.

Questions of carotenoid function and allocation clearly have complicated answers, as they have defied the efforts of innumerable scientists that have worked to find a clear and comprehensive explanation for the patterns of color variation observed in nature. The result of decades of research is a large and sprawling literature of studies that vary widely across the phylogeny of birds (and beyond) and that address questions ranging from the influence of yolk carotenoids fledging success to the evolution of multiple ornaments to the basics of interindividual variation, each using a broad suite of techniques and experimental approaches. The central goal of this review is to attempt to clarify variations of the resource tradeoff hypothesis for signal honesty in carotenoid-based ornaments by breaking down the hypothesis into component parts and discussing the issues that arise within each. I argue that there is as yet no definitive answer to the question of whether carotenoids are directly involved in immune function or antioxidant response, and that the ability to interpret resource tradeoffs from correlative experiments is limited by complex interactions among measured variables and different predictions that may be related to tradeoffs. Increasing clarity into the specific mechanisms driving variation in carotenoid-based traits is important as the literature investigating the condition dependence in carotenoid-colored ornaments continues to grow as an important component of studies into sexual selection and physiology.

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Table 1. Axes of quality. The table outlines several different perspectives on how to approach testing relationships between carotenoid levels, coloration, and individual quality.

Axis of quality	Description	What distinguishes higher quality individuals?	How is this axis tested?	What are the predicted results?
Health state	Carotenoid coloration reflects current disease state, or a similar environmentally mediated physiological state	Higher quality individuals have been subjected to fewer physiological challenges	Experimental alterations of health state (e.g. administering an oxidative or pathogen challenge)	Challenging an individual will decrease his carotenoid coloration
Internal carotenoid levels	Carotenoid coloration reflects internal carotenoid resource availability	Higher quality individuals have greater pools of internal carotenoid resources	Internal carotenoid levels can be manipulated with dietary carotenoid supplementation or deprivation	Individuals with larger carotenoid resource pools will have increased carotenoid coloration
Interaction of health state and carotenoid levels	Carotenoid coloration reflects the combined effects of health state and internal carotenoid resource availability	Uncertain	Factorial-type manipulations of health state and dietary carotenoid access	Uncertain
Underlying functionality	Carotenoid coloration reflects some aspect of intrinsic physiological performance	Higher quality individuals are those that perform best within one treatment group (e.g. the individuals with best coloration despite an immune challenge)	Comparisons between variables in resting individuals, or individuals within one treatment group	Under identical conditions, individuals will vary in response variables (immune response, oxidative stress measures, carotenoid coloration); some individuals will exhibit superior performance across multiple metrics compared to others

CHAPTER 2

The importance of carotenoid dose in supplementation studies with songbirds

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Introduction

Carotenoid-based ornaments in birds have drawn substantial attention as indicator traits because numerous studies have reported correlations between the expression of carotenoid coloration and aspects of male quality, including fat reserves, basal metabolic rate, effectiveness of immune response (immunocompetence), and oxidative state (reviewed in Hill 2002, Hill 2006, Svensson and Wong 2011). Carotenoid pigments are responsible for most of the vibrant red, orange, and yellow coloration of the feathers and soft parts of birds (McGraw 2006), and they may also play important physiological roles as vitamin A precursors, boosters of the immune system, and antioxidants (Mougeot *et al.* 2010; Pérez-Rodríguez, Mougeot & Alonso-Alvarez 2010; Hill & Johnson 2012). Because these pigments cannot be synthesized in the bodies of animals and must be acquired from the diet (Goodwin 1984), carotenoids are often considered limited resources such that only birds in the best condition can "afford" to allocate carotenoid pigments toward colored ornaments rather than retain them for potential internal benefit; thus, carotenoid resource trade-offs have been hypothesized to maintain signal honesty in these traits (Møller *et al.* 2000; Alonso-Alvarez *et al.* 2004).

Numerous studies of carotenoid ornamentation aim to establish and clarify whether this hypothesized carotenoid resource trade-off may explain the condition-dependence of carotenoid

coloration in birds by validating that: 1) higher levels of circulating carotenoids improve immune function and/or oxidative stress maintenance, 2) restricted dietary intake limits the quantity of circulating carotenoids, and 3) generation of a high quality ornament sequesters circulating carotenoids such that colored traits impose a cost on other processes that utilize carotenoids (von Schantz *et al.* 1999; Møller *et al.* 2000; Alonso-Alvarez *et al.* 2008). Fundamental to testing these predictions of the trade-off hypothesis are experiments that manipulate carotenoid availability and measure the effect of carotenoid dose on both ornamentation and physiology. In laboratory settings, researchers commonly supplement or restrict dietary carotenoid levels and evaluate the resulting effects on various measures of ornamentation and internal condition (Hill 2006). However, the results of these studies are often inconclusive, and the importance of allocation trade-offs to carotenoid-based signal honesty as well as the physiological functions of carotenoid themselves remain debated (Hill 1994; Hudon 1994; Hill 1999; Hartley & Kennedy 2004; Hadfield & Owens 2006; Costantini & Møller 2008; Hill 2011; Hill 2014).

One critical but often overlooked complication of carotenoid-manipulation studies is the biological relevance of the quantities of carotenoids that are administered to test animals. Commonly, supplemental carotenoids are provided *ad libitum* in food or water without an assessment of the amounts of pigments that are actually ingested and without proper consideration for how levels of supplemental carotenoids compare to quantities ingested by birds under natural conditions. Moreover, the quantitative relationship between the amount of carotenoids ingested and quantities of circulating carotenoids is usually not measured in either lab or field systems, so it is difficult to judge the results of carotenoid supplementation. For example, the quantity of ingested carotenoids may greatly exceed that which is present in the plasma if birds rapidly transport consumed carotenoids to storage in fat, ornamentation, or other

tissues; therefore, birds with vastly different carotenoid access may have the same levels of plasma carotenoids if the bird with greater consumption allocates his excess carotenoids outside of circulation. For this reason, comparing plasma carotenoid levels of captive birds to wild conspecifics is insufficient to justify that the captive supplementation dose mimics the levels of carotenoids available to wild birds. Because the differential allocation of limited carotenoids is key to the resource trade-off hypothesis, it is essential to better track carotenoid usage through quantifying the relationships among amounts ingested, circulated, and deposited in ornaments.

Several studies have addressed this issue by using dosage trials to compare supplementation levels to levels of circulating carotenoids in order to identify doses that do not saturate their subjects' systems (e.g. Alonso-Alvarez *et al.* 2004, Aguilera and Amat 2007). Too often, however, the carotenoid supplementation regimens used in avian studies are based on methods developed for other species or from studies of different dietary carotenoids (e.g. Navara and Hill 2003, Baeta *et al.* 2008); carotenoid consumption and absorption varies markedly across species with different masses and life histories (Tella *et al.* 2004; McGraw 2005), so extrapolating carotenoid doses among species with no validation could lead to experiments that provide carotenoid doses that are too high or too low to yield meaningful results.

Because the focus of most studies utilizing carotenoid supplementation is testing for tradeoffs in the use of limited carotenoid resources for ornamentation versus body maintenance, poorly controlled dosing undermines the goals of the research. For a study of resource limitation or tradeoff to be meaningful, then the resource must be provided at a level below saturation. If the lowest supplementation level provides sufficient carotenoids for both body maintenance and ornament production, then studying the effects of dose become meaningless. As fundamental as

these ideas appear to be, many studies proceed on the unstated assumption that supplementation levels are below saturation.

To better quantify the effects of supplementation on circulating carotenoid availability and carotenoid-based ornamentation, I performed a meta-analysis of 15 published studies that include groups of both carotenoid supplemented and unsupplemented birds and that report the resulting plasma carotenoid levels and/or ornamental color of each group. A previous meta-analysis investigated correlations among these variables, but it grouped studies as either "supplemented" or "unsupplemented" without including supplementation dose as a co-factor (Simons, Cohen & Verhulst 2012), missing a critical source of variation among studies. In my analysis, I built on the existing literature by first using published levels of carotenoid supplementation and allometric scaling equations to estimate individual consumption of carotenoids. I then modeled how variation in intake between supplemented and control groups affected the relationships between circulating carotenoids and allocation to ornamentation in songbirds. By quantifying the physiological responses to varying levels of carotenoid ingestion in different studies and seven different songbird species, I provide a foundational model for predicting the biological relevance of particular carotenoid supplementation regimens and can assess the variables that modulate response to carotenoid intake.

Methods

Literature search

I surveyed the existing carotenoid literature using the Web of Science database on 23 March 2014, using the keywords "carotenoid*" AND "supp*" AND "bird" OR "avian." I included only studies: a) reporting the level of carotenoid supplementation as well as the food source provided;

b) including data on both carotenoid-supplemented and control groups of individuals; c) reporting the values of at least one target physiological variable (plasma carotenoid level or coloration); d) not repeating measures on the same group of birds that were reported in a study already incorporated into the meta-analysis (a potential source of pseudoreplication); e) testing adult male birds rather than nestlings (in which both carotenoid physiology and ornamental function differ greatly from sexually reproducing adult birds, and the quantity of carotenoids acquired from egg yolk or parental provisioning is often unknown; Hill and McGraw 2006); and, f) supplementing with only the carotenoids lutein and/or zeaxanthin, the most prevalent carotenoid pigments in the avian diet (McGraw 2006). With the exception of one study supplementing only with lutein (Stirnemann *et al.* 2009), all studies included in my meta-analysis supplemented primarily with lutein and trace amounts of zeaxanthin (e.g. 20:1 lutein:zeaxanthin; Blount *et al.* 2003, Hõrak *et al.* 2007, Karu *et al.* 2007, Baeta *et al.* 2008, Sild *et al.* 2011, Sepp *et al.* 2011).

This latter point is important because most terrestrial birds consume diets containing primarily these two yellow and structurally similar carotenoid pigments, which many species must then metabolize into red pigments (in species with red coloration) or ornamental yellow pigments (e.g. canary xanthophylls). Critically, chemical properties and therefore potential physiological functions vary across these dietary and ornamental pigments, and the costs of converting dietary to ornamental pigments may itself play a key role in the honesty of carotenoid-based coloration (Hill 1996; Hill & Johnson 2012; Johnson & Hill 2013). Studies supplementing with other pigments, particularly the red carotenoids at the end points of these carotenoid conversion pathways (such as canthaxanthin; e.g. McGraw *et al.* 2002, Smith *et al.*

2007) bypass some of the mechanisms relating coloration to physiology that may be important to carotenoid signal honesty, so such studies are not appropriate for this analysis.

Despite the extensive literature on carotenoid ornamentation (more than 300 results to my initial keyword search), only 19 studies met my criteria of providing measurable carotenoid supplementation quantities to adult birds. Because 16/19 studies investigated songbird species (order Passeriformes), I excluded one study of red junglefowl (*Gallus gallus*; McGraw and Klasing 2006), one study of mallards (*Anas platyrhynchos*; Butler and McGraw 2013), and one of kestrels (*Falco tinnunculus*; Costantini *et al.* 2007) to capture the majority of available data while avoiding comparing data from phylogenetically distant taxa with different physiologies. I also excluded one study on society finches (*Lonchura striata domestica*; McGraw *et al.* 2006) because this species lacks carotenoid-based ornamentation and so is not subject to the potential costs of allocating carotenoids as colorants. I performed my analysis on the remaining 15 studies of seven songbird species with carotenoid-based ornaments: the American goldfinch (*Carduelis tristis*) with yellow plumage and pink-red bill ornamentation; the house finch (*Haemorrhous mexicanus*) with red plumage ornamentation; the zebra finch (*Taeniopygia guttata*) with red bill ornamentation; the diamond firetail (*Stagonopleura guttata*) with red bill ornamentation; the great tit (*Parus major*) with yellow plumage ornamentation; the Eurasian blackbird (*Turdus merula*) with red-orange bill ornamentation; and the European greenfinch (*Carduelis chloris*) with yellow plumage ornamentation.

Carotenoid supplementation calculations

Most experiments supplemented carotenoids to the main food or water supply and reported doses as the concentration of carotenoids added per unit food or water. One study, Peters *et al.* (2011),

quantified daily carotenoid intake of individuals during the experiment, so these values were used in my analysis. For all other studies, I estimated the quantity of carotenoids consumed by each bird by first calculating the average daily food or water intake of an individual of the focal species, using allometric scaling equations to account for the non-linear relationship between species size and consumption. When carotenoids were supplemented in the water supply, I estimated daily water intake using the mass of the study species and the scaling equation for passerines reported in Calder and Braun (1983). When a study supplemented carotenoids in the food supply, I estimated the daily food intake of the study's focal species by using the energy content of the food provided (often, millet or sunflower seeds; Caraco *et al.* 1980, Hõrak *et al.* 2003) and a scaling equation for passerines that predicts the consumption needed to meet daily energetic requirements (Nagy, Girard & Brown 1999). When the exact mass of individuals included in the study was not reported, I estimated the average mass of the species from the *Handbook of the Birds of the World* (del Hoyo *et al.* 2010). From my estimates of daily food or water intake, I then used each study's published details on the concentration of carotenoids supplemented to calculate the quantity of carotenoids ingested along with food or water. I also calculated the carotenoid content of the basic diet provided to both control and supplemented birds using reported carotenoid content values or published measurements of the content of the seeds supplied (McGraw *et al.* 2001; Peters *et al.* 2008) to account for dietary carotenoids acquired independently of supplementation.

To standardize levels of supplemental carotenoids ingested in species of varying body sizes, I divided daily carotenoid consumption amount by species mass in grams. I then calculated the difference in carotenoid intake between supplemented and control groups for each study ("carotenoid intake difference"). Most often, this measure of intake difference was nearly

identical to the actual intake of the supplemented group, since most control groups acquired negligible levels of carotenoids.

Effect size calculations

I calculated the natural log response ratio and its variance from reported means and standard deviations of control and supplemented groups according to the formulae outlined in Koricheva *et al.* (2013); the response ratio allows for the standardization of measurements across studies by converting each measured effect into a unitless ratio of the mean response of the supplemented group to the mean response of the control group. When other experimental manipulations were present in a study, I used data from the otherwise unaltered control groups that varied only in carotenoid supplementation. I calculated two types of effect sizes per study, when possible, to measure the effects of supplementation on plasma carotenoid levels and ornamental coloration. When necessary, I extracted means and errors from figures using either ImageJ (Rasband 1997-2014) or WebPlotDigitizer v. 2.6 (Rohatgi 2013). When mean values were not published in text or figures, I contacted authors to retrieve the raw data and calculate mean values. Along with effect size, I recorded each study's focal species and the number of days that supplementation was provided. If a study reported multiple response values over time, I recorded only values from before supplementation and at the end of supplementation for consistency among studies. I included multiple effect sizes for one study only if each differed in a key variable, such as a different carotenoid supplementation dose or ornament measured. In addition, because the color of feathers is determined only during molt when carotenoids are actively deposited in growing feathers (Hill 2002), I extracted plumage color effect sizes only from studies of molting individuals; I calculated effect sizes from non-molting birds with plumage ornaments only for

the relationship between carotenoid intake and plasma carotenoid concentration. The color of a "soft part," such as the bill, can change rapidly during any season (Rosenthal *et al.* 2012), so I could extract effect sizes of both types from studies of these ornaments regardless of molt status.

The means of assessing ornamental coloration is important to consider in my analysis because color is generally quantified along one or more of three main axes: hue, or the shade of the color (e.g. red, orange, yellow); chroma, or the intensity of the color (also called "saturation"); and brightness, or the lightness/darkness of the color. In addition, principle component analysis (PCA) can be used to create a composite metric directly from the reflectance spectrum of a color (Montgomerie 2006). Each of these axes of color tends to relate to different properties of the colored ornament itself. For example, chroma may be a good generalization of pigment density, while hue may be more representative of the proportion of red to yellow pigments in a carotenoid-colored ornament (Inouye *et al.* 2001; Hill & McGraw 2006). The choice of color parameter used in a particular study is therefore important to include in my analysis because it may affect study conclusions by representing different properties of the ornament measured.

Statistical analyses

I performed all analyses using the "metafor" package (version 1.9-7; Viechtbauer 2010) in R version 3.2.1 (R Core Team 2015). I ran two separate overall meta-analyses, one for plasma carotenoid levels and a second for ornamental coloration. Both analyses used meta-regression to estimate the dose-dependent effect of carotenoid intake difference (between supplemented and unsupplemented groups) on response, also including species, supplementation duration, and measure type (for color measurements) as moderators, and including a random effect of study (to

control for multiple effect sizes from one experiment). Each model took within-study variation, or the error around each effect size, into account when estimating overall effects.

After initial investigation, I discovered that one study in my plasma carotenoid content analysis (Peters, Magdeburg & Delhey 2011) had a modest effect size but an orders-of-magnitude larger daily carotenoid consumption per individual than any other study, so I ran a separate meta-regression omitting this outlying data point to better model the patterns in the remaining studies. I also performed two subgroup analyses for studies of the plasma content of greenfinches and zebra finches, which had the greatest number of individual effect sizes (9 and 7, respectively) and allowed an opportunity to specifically assess the relationships among parameters in these species. I also performed a separate analysis of zebra finches for the relationship of supplementation to coloration. I ran several further subgroup analyses to better parse the effects of particular model variables when significant sources of heterogeneity were held constant (e.g. on data with only hue or only chroma color parameters). Lastly, to examine whether the effects of supplementation on coloration depend on plasma carotenoid content, I performed an additional meta-regression to investigate the effects of species, carotenoid intake difference, color parameter measured, duration of supplementation, and plasma effect size on coloration effect size; this analysis was performed on the subset of studies that measured both plasma carotenoid content and the color of ornaments.

I investigated the extent of publication bias in the main plasma carotenoid content and coloration data sets using funnel plots of effect size versus standard error, a measure of study precision, according to the guidelines of Koricheva *et al.* (2013). Along with a visual examination of plots, I statistically tested for funnel plot asymmetry using a regression test (Viechtbauer 2010). To estimate the impact of study heterogeneity on meta-regression results, I

calculated Q values, which test whether there was significant residual heterogeneity in effect sizes that could not be attributed to variation in carotenoid consumption level and other moderators (Viechtbauer 2010; Koricheva, Gurevitch & Mengersen 2013).

Results

Overall, I calculated 40 effect sizes from 15 studies of seven species: the diamond firetail, European greenfinch, house finch, European blackbird, great tit, American goldfinch, and zebra finch. Among the studies I assessed, carotenoid intake between supplemented and control groups of birds differed by an average of 19.9 (±15.1) μg/day/g body mass, and ranged from 0.01 (Stirnemann *et al.* 2009) to 432.2 μg/day/g body mass (Peters, Magdeburg & Delhey 2011). Duration of supplementation was similarly variable, with an average of 39.0 (± 4.70) days, and a range from 7 (Karu, Saks & Horak 2007) to 84 days (Navara and Hill 2003).

Effect of carotenoid supplementation on plasma carotenoid levels

I calculated 21 effect sizes for plasma carotenoid content from 13 studies of six species: the diamond firetail, European greenfinch, house finch, European blackbird, great tit, and zebra finch (Figure 2). When I included all data, no variable significantly predicted the effect of supplementation on plasma carotenoid response (all $p > 0.12$; Table 2); however, when I omitted one effect size from a study featuring an exceptionally large daily carotenoid intake (Peters, Magdeburg & Delhey 2011), I found that carotenoid intake difference between supplemented and unsupplemented groups had a significant effect on plasma carotenoid content response ratio (Table 2). Subgroup models where only greenfinches or zebra finches were included also revealed either a trend (greenfinch) or a significant effect (zebra finch) of

carotenoid intake on the response of plasma carotenoid levels to supplementation, though the slope of this relationship differed between the two species: greenfinches exhibited a larger increase in coloration with increasing carotenoid intake, on average, than zebra finches (Table 2; Figure 3).

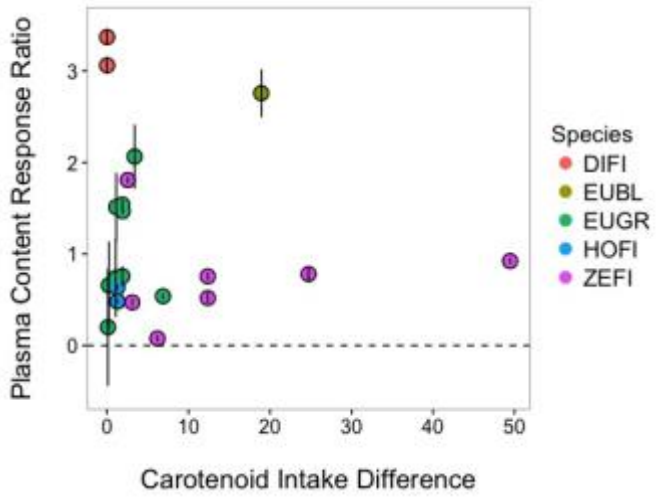

Figure 2. Plasma vs. intake. Plasma carotenoid content response ratio (±SE) relative to the difference in carotenoid intake between supplemented and unsupplemented groups. The dashed line represents an effect size of zero, or no difference in plasma carotenoid content between supplemented and control groups. Not pictured is the effect size from Peters *et al.* (2011), an outlier excluded from main analyses.

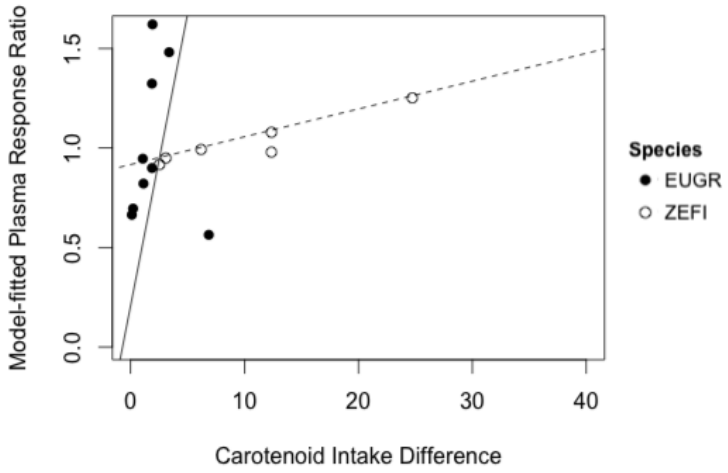

Figure 3. Plasma vs. intake for EUGR and ZEFI. The model-fitted plasma carotenoid response ratios relative to the difference in carotenoid intake between supplemented and unsupplemented groups for two subgroup models comprising data from only zebra finches (open circles) or greenfinches (closed circles). Lines indicate the model-predicted slope of the response of zebra finches (dashed line) or greenfinches (solid line) to increasing carotenoid intake, assuming a constant supplementation duration of 25 days.

In addition, I found that duration of supplementation had a significant negative effect on response in the greenfinch subgroup, indicating that increasing the number of days of supplementation tended to *decrease* the effect of supplementation on the response of plasma carotenoid levels in this species (Table 2). This relationship appeared driven by a single study with a 60-day duration (Peters *et al.* 2008) and a comparatively low effect size relative to carotenoid intake difference, so I ran an additional meta-regression on a dataset excluding this data point and found that the negative effect of supplementation duration was no longer significant, while the difference in daily carotenoid intake continued to trend toward significance ($p = 0.054$; Table 2). Interestingly, the study of Peters *et al.* (2008) was exceptional not only in its long duration, but also in that it was the single study of greenfinch plasma carotenoid levels performed while the birds were undergoing molt; if the process of depositing carotenoids in the growing feathers significantly altered plasma carotenoid levels, then molt (rather than supplement duration) could be responsible for the lower effect size relative to carotenoid intake observed in this study.

Significant residual heterogeneity remained in the full dataset model, the full model excluding the Peters *et al.* (2011) outlier (described above), and the model including only the zebra finch data, but not in the models containing only greenfinch data (both with and without Peters *et al.* 2008; Table 2).

Effect of carotenoid supplementation on coloration

I extracted 19 coloration effect sizes from eight studies of five species: the American goldfinch, diamond firetail, European greenfinch, Eurasian blackbird, and zebra finch (Figure 4). Meta-regression indicated that only the type of measurement used to quantify coloration (i.e. hue,

chroma, PCA) was significant in predicting the magnitude of the effect of supplementation on coloration. While the presence of supplementation increased coloration in most studies (Figure 4), neither increasing the difference in carotenoid intake between supplemented and unsupplemented birds nor increasing the duration of supplementation affected the difference in color between experimental and control groups of birds (Table 2). Carotenoid intake continued to have no significant relationship with effect size even in subgroup models isolating studies only measuring the parameters of hue or chroma ($p > 0.4$), indicating that variation in color measurement was not obscuring effects of variation in carotenoid intake in the overall model (Table 2).

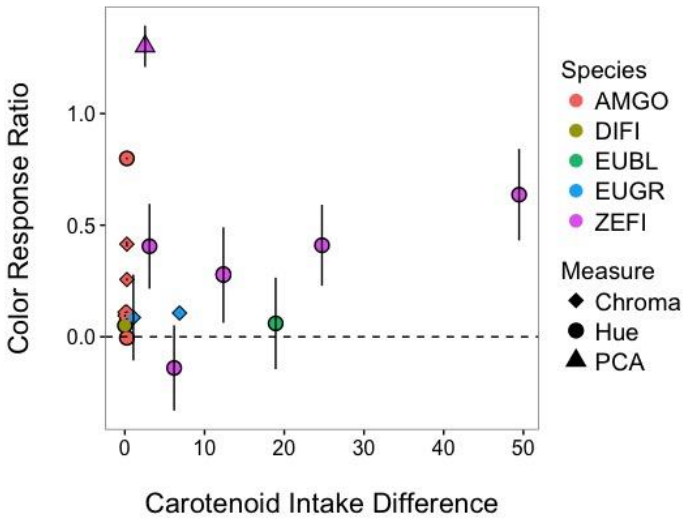

Figure 4. Intake vs. color. Ornamental coloration response ratio (±SE) relative to carotenoid intake. Points with no visible error bars represent errors less than the diameter of the point. Shapes of points indicate the aspect of color that was measured.

The separate analysis of zebra finch data also revealed a significant effect of only measurement type on the response of coloration to supplementation (Table 2). Performing an additional analysis of the zebra finch data set comprising only effect sizes measured with hue (excluding one effect size of PCA; McGraw and Ardia 2003) did not alter the significance of other model variables; neither days of supplementation nor the magnitude of carotenoid intake

had a significant effect on the color difference between experimental and control groups of zebra finches (Table 2).

When I examined the relationship between the responses of coloration and plasma carotenoid content, I found no significant effect of any model parameter. Incorporating the plasma carotenoid content effect size in the coloration model did reduce the effects of residual heterogeneity from highly significant in the overall model to nonsignificant (Table 2), indicating that variation in plasma carotenoid content likely caused some of the variation in effect sizes present in the overall coloration data set. Visual inspection of the plotted relationship between plasma content and coloration effect sizes revealed that most points fell below the 1:1 line (Figure 5), so the effect of supplementation on coloration tended to be smaller than the effect of the same supplementation regimen on plasma carotenoid content.

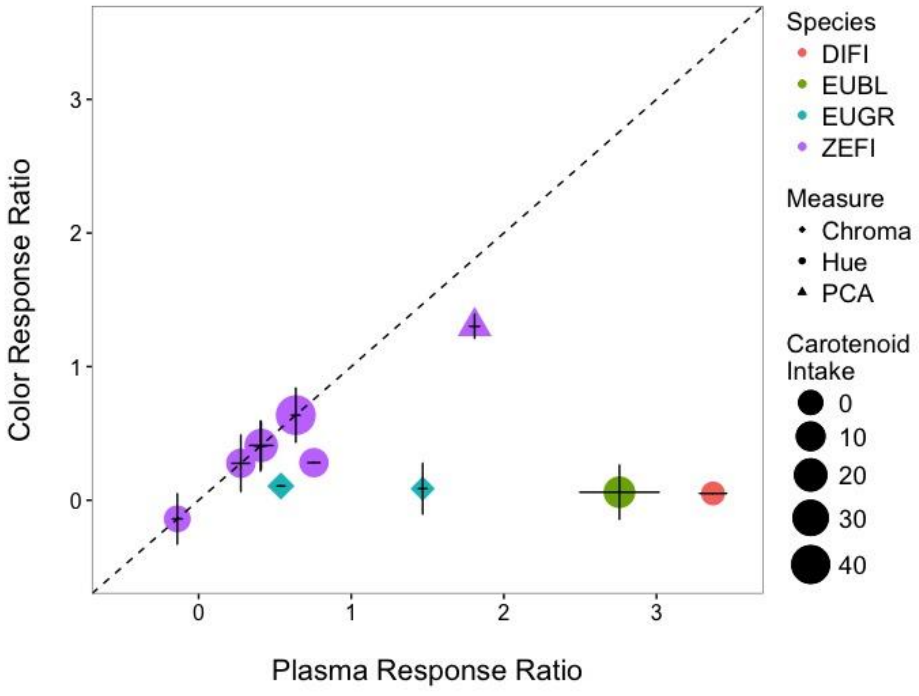

Figure 5. Color vs. plasma. Ornamental coloration response ratio (±SE) relative to plasma carotenoid content response ratio (±SE). Points with no visible error bars represent errors less than the diameter of the point. The dashed line represents a 1:1 relationship between the two effect sizes. The size of each point represents the magnitude of the carotenoid intake difference between supplemented and unsupplemented birds for the given effect size.

Significant residual heterogeneity remained in the overall model and the models of only hue or chroma, but not in the zebra finch or plasma carotenoid content models (Table 1).

Publication bias

Visual inspection of funnel plots indicates little bias in the studies examined in my analyses, though many effect sizes were positive; this is not unexpected, given the predicted physiological relationships between carotenoid intake, plasma carotenoid content, and coloration. Regression analyses of funnel plot asymmetry indicated no significant bias in either of the two sets of data (plasma: $z = -0.83$, $p = 0.40$; color: $z = 1.03$, $p = 0.30$).

Discussion

For studies to meaningfully test differential allocation of a limited pool of carotenoid resources acquired from the diet, they must provide experimental subjects with biologically relevant carotenoid doses. Saturating the diets of birds with carotenoids will obscure physiological trade-offs that may occur between carotenoid absorption, circulation, and use for ornamentation. However, little justification is generally given for the dosage and experimental design used in studies that aim to test for carotenoid trade-offs. To assess the effect that carotenoid dose has on the physiological responses of birds, I performed meta-regressions on data extracted from 15 published studies of seven songbird species. Not surprisingly, and as demonstrated in a previous meta-analysis (Simons *et al.* 2012), the presence of carotenoid supplementation tended to increase plasma carotenoid levels and the expression of carotenoid-based coloration. However, I found that supplementing an experimental group of birds for a longer period of time or with a

larger dose of carotenoids did not increase the difference in color between control and supplemented birds.

Because all carotenoids in the system of an adult bird are derived from the diet, both plasma carotenoid levels and the expression of carotenoid-based coloration are often assumed to directly reflect carotenoid intake (Hill, Inouye & Montgomerie 2002; McGraw 2005). The results of my meta-regression of plasma carotenoid levels indicates that this assumption is correct across the range of supplementation doses provided in studies of captive birds, although the number of days of supplementation did not affect the response. A dose- but not time-dependent effect of supplementation on plasma carotenoid response suggests that the presence of supplementary carotenoids causes an increase in plasma carotenoid levels to a stable level that varies according to the dose offered, but which does not continue to increase over the duration of the experiment; supplementation appears to cause the same pattern of increase followed by stabilization in the expression of ornamental coloration, though this effect is not dose-dependent. While it is always important to validate whether these trends hold true in a particular study system before applying them to other experimental designs, my results indicate that future studies need not supplement birds for long periods of time in order to collect meaningful data on either plasma carotenoid levels or coloration.

Interestingly, I found a more strongly positive relationship between increasing supplementation dose and increasing plasma carotenoid content in greenfinches than in zebra finches. A fundamental difference between these two species is that greenfinches have yellow feathers that are colored only during the annual molt, while zebra finches have red bills that can be rapidly colored or re-colored at any time of the year (Hill & McGraw 2006; Rosenthal *et al.* 2012). The different patterns of carotenoid absorption and circulation in these two species may

reflect the different physiological requirements for pigmenting feathers versus bare parts. Specifically, most greenfinches were not undergoing molt at the time of plasma carotenoid content measurement in the studies I examined, so it is possible that they retained higher levels of ingested carotenoids than zebra finches, which may have been actively depositing carotenoids in their bill ornaments at the time of measurement. The single data point from molting greenfinches (Peters *et al.* 2008) showed the lowest plasma content effect size for its given supplementation regimen, which may have been a consequence of the active deposition of carotenoids into feathers. Unfortunately, the range of studies available in the literature for my analysis did not have the breadth required for separate investigations of whether carotenoid metabolism to produce ornamental pigments from dietary pigments (e.g. to produce red vs. yellow coloration) also affected the relationship between carotenoid intake and plasma content (Hill & Johnson 2012).

One study of great tits, Peters *et al.* (2011), had a supplementation dose that was orders-of-magnitude larger than that of the other studies included in this analysis; however, the plasma carotenoid levels measured in this experiment were within the range of those of other studies. One explanation for this finding is that the great tits in this study may have been at the point of maximal carotenoid absorption from their diet such that even their exceptionally large consumption did not cause a corresponding increase in circulating carotenoids (the point of physiological carotenoid saturation). It is also possible that the insect-rich diet of great tits, as opposed to the seed-based diet of many of the finch species in my analysis, necessitates corresponding differences both in carotenoid access and metabolism; however, both the supplemental carotenoid dose and plasma effect size of another insect-eating species, the Eurasian blackbird, was more similar to the finch species in my study than to these

measurements of the great tit. Further examination of the dose-dependent responses of adult great tits to carotenoid supplementation as well as measurement of the quantity of these carotenoids that are allocated to ornamentation will be essential to extricating how this species makes use of dietary carotenoids, and how it may differ from the cardueline finches commonly studied in analyses of carotenoid-based ornamentation.

In contrast to the strong positive relationship between levels of carotenoid supplementation and levels of circulating carotenoids, I found that, while the presence of supplementation tended to enhance ornamental coloration, increasing the dose used in supplementation did not cause a corresponding increase in the response of ornamental coloration. Moreover, the only significant predictor of how strongly color responded to supplementation was the parameter used to quantify coloration. These results call into question the general and perhaps overly simplistic assumption that greater carotenoid intake should inexorably lead to showier coloration. The complexity of physiological systems involved in carotenoid coloration (Hill & Johnson 2012) and the links between carotenoid coloration and metabolism (Johnson & Hill 2013; Hill 2014) make simple associations between intake and coloration unlikely, as the expression of coloration is dependent on a variety of physiological variables beyond carotenoid availability alone. In fact, my observation that the response of plasma carotenoid content to supplementation tended to exceed that of coloration indicates that the levels of carotenoids present in circulation were more than adequate for expressing colorful ornaments in the species examined, so factors other than carotenoid limitation appear responsible for the variation in coloration responses observed.

Even after accounting for the effects of moderators, many of my models contained significant residual heterogeneity that could not always be eliminated in subgroup analyses by

species or by measurement. The persistent variation in effect sizes within each model emphasizes the unpredictability of response to supplementation among studies, even within one species and controlling for variation in carotenoid dose, supplementation duration, and measurement type. My results substantiate the importance of validating that a particular supplementation regimen is appropriate for a particular experimental design, perhaps through dosage trials, which are currently used in only a minority of studies (Alonso-Alvarez *et al.* 2004; Aguilera & Amat 2007).

An additional source of variation in my meta-analysis may be my estimates of carotenoid intake, which are calculated from predicted food or water intake based on the diet and mass of each focal passerine species. In fact, while my analyses were limited to an average measure of consumption for a particular species, an important consideration for future supplementation experiments is how food or water intake may vary among individuals or among treatment groups. The possibility that birds may use behavioral changes to alter physiological carotenoid access remains largely unexplored (but see Hill 1995, Peters *et al.* 2011), and poses a challenge to detecting internal resource trade-offs. Incorporating measures of water or food intake with analyses of circulating carotenoids and ornamental coloration is a simple but highly valuable step to understand the true magnitude—and, consequently, biological relevance—of supplementation.

Despite the large number of studies that have tested the physiological effects of carotenoids on ornamentation, only a small sample of studies performed controlled supplementation of adult birds with carotenoid-based coloration. Although this small sample size necessarily limits the breadth of the inferences that can be drawn from my study, I found some intriguing patterns that are not necessarily intuitive. My ultimate goal is to emphasize important methodological and theoretical considerations for future studies using carotenoid

supplementation to assess the condition-dependence of carotenoid-based ornaments. Improving the clarity of the relationships between carotenoid intake, circulation, and deposition in ornamentation in a variety of species will be an important step to better understanding the size and function of the pool of dietary carotenoids available to songbirds, and may reduce ambiguity in the results of studies searching for carotenoid allocation trade-offs.

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Table 2. Meta-regression model and test of residual heterogeneity results. Cells containing "N/A" designate moderators that were not included in the given model.

			Model effect estimates ± SE						
Model	Subgroup	Number of effect sizes	Intercept	Carotenoid Intake Difference	Supplemen-tation Duration	Species	Measure-ment Type	Plasma Effect Size	Cochran's Q
Plasma Carotenoid Content	Overall	21	2.47 ± 0.65**	0.0001 ± 0.002	-0.0065 ± 0.17	-0.28 ± 0.18	N/A	N/A	112.3**
	Overall, Without Outlier	20	2.59 ± 0.58**	0.014 ± 0.005**	-0.015 ± 0.016	-0.24 ± 0.16	N/A	N/A	117.1**
	ZEFI	7	1.01 ± 2.27	0.014 ± 0.005**	-0.004 ± 0.05	N/A	N/A	N/A	46.0**
	EUGR	9	1.61 ± 0.18**	0.29 ± 0.16	-0.047 ± 0.018**	N/A	N/A	N/A	4.36
	EUGR, Without Outlier	8	0.90 ± 0.55	0.44 ± 0.23	-0.03 ± 0.02	N/A	N/A	N/A	3.33
Coloration	Overall	19	0.12 ± 0.45	0.0050 ± 0.011	-0.0004 ± 0.0057	0.11 ± 0.09	-0.14 ± 0.050**	N/A	197.0**
	ZEFI	7	-2.22 ± 1.14	0.011 ± 0.012	0.001 ± 0.008	N/A	1.15 ± 0.39**	N/A	0.9
	ZEFI, Hue Only	6	0.098 ± 0.52	0.011 ± 0.012	0.0013 ± 0.0089	N/A	N/A	N/A	0.9
	Hue	10	-0.038 ± 0.084	0.0081 ± 0.010	-0.0002 ± 0.0008	0.046 ± 0.038	N/A	N/A	149.9**
	Chroma	8	0.33 ± 0.44	0.036 ± 0.081	-0.0013 ± 0.0044	-0.097 ± 0.19	N/A	N/A	11.3*
	Color vs. Plasma	11	-2.80 ± 1.81	0.0016 ± 0.011	0.0036 ± 0.011	0.55 ± 0.44	0.005 ± 0.48	0.50± 0.47	9.35

* $0.01 < p < 0.05$

** $p < 0.01$

CHAPTER 3
An assessment of techniques to manipulate oxidative stress in animals

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Introduction

Free radical biology was once the exclusive domain of biochemists and biomedical researchers, but beginning with the publication of an influential paper by von Schantz (1999), physiological ecologists began to consider the role of free radicals in a host of biological processes, including life history evolution, condition-dependent signaling, and senescence (Costantini 2008; Monaghan, Metcalfe & Torres 2009; McGraw *et al.* 2010; Hill & Johnson 2012; Costantini 2014). High concentrations of pro-oxidants, most prominently including reactive oxygen species (ROS), have negative effects on fundamental cellular processes because they compromise genomic stability and disrupt protein or lipid structures. The capacity to resist and recover from states of increased oxidative stress can be a central component of an animal's fitness.

Pro-oxidant molecules, like ROS produced during oxidative phosphorylation (OXPHOS) and the activity of redox enzymes, are more than damaging byproducts: they serve as essential signaling molecules that regulate mitochondrial activity and other processes (Seifried *et al.* 2007; Valko *et al.* 2007; Sena & Chandel 2012). In a healthy organism, oxidative damage from pro-oxidants is largely held in check by the activity of antioxidants. Under states of increased oxidative stress, however, pro-oxidant molecules are generated at a rate that exceeds the capacity for antioxidant molecules to mitigate their effects (Scherz-Shouval & Elazar 2007). Importantly, there is individual variation in susceptibility to oxidative stress such that some individuals appear better able to avoid oxidative damage and its accompanying harmful effects, even when faced

with stress-inducing environmental challenges (Monaghan, Metcalfe & Torres 2009). Discovering the causes and consequences of this variability is a main focus of oxidative stress ecology.

To disentangle the effects of oxidative stress from the confounding effects of other physiological processes, researchers have looked for methods to experimentally manipulate levels of oxidative stress by performing oxidative challenges. The ideal oxidative challenge is a relatively simple and cost-effective treatment that is accessible to and safe for use by organismal biologists outside of the biomedical sciences. Perhaps most critically, effective challenges must induce a measurable change in oxidative stress without causing significant side effects. As the field of oxidative stress ecology continues to expand, there is a need for a critical assessment of techniques for manipulation of oxidative stress.

In this review, I assess the pros and cons of different methods that have been shown or that show promise to be used to manipulate oxidative stress in live animals, particularly non-model vertebrate species. As I assess techniques, I emphasize the mechanism of action of each potential oxidative challenge with the purpose of describing the effects that may make a treatment more or less suitable for particular experimental designs, and I describe potential confounding side-effects of the challenge that may occur independently of oxidative stress pathways and interfere with results interpretation. The primary goal of this paper is to provide a resource for organismal biologists and ecologists that highlights the complex effects of oxidative challenges used to experimentally study oxidative stress in animals.

Pro-oxidant Generators

Because oxidative stress depends on both the rate of pro-oxidant generation and the counteracting effects of antioxidants, experimental oxidative challenges can target either production of ROS or suppression of antioxidants. Treatments that increase ROS production are particularly useful in studies that aim to quantify individual ability to defend against pro-oxidants while possessing their full suite of antioxidant defenses. However, by their nature, substances that increase ROS levels tend also to increase the risk of non-targeted tissue damage or death of experimental subjects, and may pose additional risks to researchers performing the administration. It is therefore critical to consider the specific biochemical means of effect, the clinical symptoms of exposure, and the potential side-effects of methods that increases pro-oxidant generation.

Paraquat

One of the most common means to induce oxidative stress via increased ROS production in vertebrate species is ingestion or injection of paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), an herbicide that is well studied because of its toxic effects on humans following accidental exposure (Suntres 2002; Dinis-Oliveira *et al.* 2008). Once inside the body, paraquat spreads rapidly and causes effects largely through ROS generation via redox cycling and subsequent disruption of redox pathways (Dinis-Oliveira *et al.* 2008; Table 3). Because it rapidly increases ROS generation and oxidative damage in the same redox systems involved in naturally induced states of increased oxidative stress, paraquat is a promising option for an oxidative challenge; however, researchers should be mindful of the potential for selective impacts on organs, particularly the lungs and brain. As a result of harmful clinical effects to exposed wildlife

and agricultural workers, the use of paraquat in agriculture has been banned or discouraged in a number of European countries (InfoCuria 2007; Kervégant *et al.* 2013). However, paraquat may yet be an effective tool within oxidative stress ecology when paired with careful use of protective equipment for personal safety and thorough use of pilot studies to determine doses that induce increased ROS production without causing organ failure or fatality.

Surprisingly little is known about the effects of low doses of paraquat administered in an experimental setting because most biomedical studies have examined model systems receiving high doses to mimic acute paraquat poisoning (e.g. Fukushima *et al.* 1994; Tawara *et al.* 1996) or to induce the symptoms of diseases associated with exceptionally high oxidative stress (e.g. Andreyev, Kushnareva & Starkov 2005; Cicchetti *et al.* 2005; McCormack *et al.* 2005; Gomez, Bandez & Navarro 2006) . Because of interest in determining the ecological effects of agricultural paraquat use, the effects of low doses of paraquat on non-mammalian and non-model systems are perhaps best understood for wild gamebirds (reviewed in Hoffman *et al.* 1987; Table 4) and fish (Gabryelak & Klekot 1985; Parvez & Raisuddin 2006). Importantly, these studies reveal wide variation in susceptibility to paraquat exposure depending on dose, species, and duration of exposure. It is therefore important that researchers perform pilot studies on their own species to identify experimental designs that are effective—and ethical—in inducing a controlled increase in ROS generation.

As of February 2016, paraquat had been used in five ecologically oriented experimental studies, all on bird species (Galvani *et al.* 2000; Isaksson & Andersson 2008; Meitern *et al.* 2013; Lucas, Morales & Velando 2014; Giraudeau *et al.* 2015; Table 4), which varied widely in both their experimental approaches and their findings. Two of these studies used dosage trials to find a concentration of paraquat that caused no apparent clinical symptoms in their focal species

(Isaksson & Andersson 2008; Lucas, Morales & Velando 2014); one study chose an arbitrary low dosage well below the species' LD-50 (Galvani *et al.* 2000); and, two based their doses on that of Isaksson and Andersson (2008; Meitern *et al.* 2013; Giraudeau *et al.* 2015). Giraudeau *et al*. (2015) also tested the dose on three individuals of their study species prior to the start of the main experiment. Unfortunately, it is difficult to draw conclusions from these studies about the effectiveness of paraquat as an oxidative challenge because the findings are generally mixed (Table 4). It is often unclear whether a lack of significant treatment effect is due to an ineffective oxidative challenge (too low or too high a dose) or due to a true lack of relationship between oxidative stress and the focal variables.

For instance, Isaksson & Andersson (2008) were the first to introduce the use of paraquat as an oxidative challenge to the field of behavioral ecology. They exposed juvenile great tits (*Parus major*) to paraquat in the drinking water and found no relationship between paraquat exposure and concentration of carotenoid pigments in plasma or feathers. They concluded that their experiment provided evidence against the widespread hypothesis that oxidative stress depletes circulating carotenoids and hence reduces the coloration of carotenoid-based ornaments (Møller *et al.* 2000; Alonso-Alvarez *et al.* 2008; Perez-Rodriguez 2009; Perez-Rodriguez, Mougeot & Alonso-Alvarez 2010; Alonso-Alvarez & Galvan 2011; but see Costantini and Møller 2008; Hartley and Kennedy 2004). While this study was a pivotal first step in the use of chemical challenges within oxidative stress ecology, it did not incorporate measures of oxidative stress into the experiment, so it is difficult to parse how antioxidant capacity or oxidative damage levels may have been affected in the tissues important to carotenoid metabolism and coloration.

Several years later, Meitern *et al.* (2013) exposed European greenfinches (*Carduelis chloris*) to either the same or twice the dose of paraquat previously used by Isaksson &

Andersson (2008), and they measured multiple aspects of oxidative damage and antioxidant capacity. However, 50% of the finches on the higher of the two doses died during the experiment. Those birds surviving the high dose had increased levels of DNA damage and the antioxidant glutathione in erythrocytes, but no significant change in any of six other measurements related to oxidative stress; none of the birds on the low dose had any measurable change in oxidative damage or antioxidant measurements (Table 4). While it appears that the lower of the two doses (identical to that used on great tits in Isaksson & Andersson 2008) was insufficient to cause a measurable response in greenfinches, the higher dose was evidently too high for this species, causing the death of birds that were unable to withstand the paraquat challenge. It is uncertain whether the surviving birds on the higher dose had generally nonsignificant measurements of oxidative stress because they recovered from the challenge before blood was collected, because they underwent a hormetic response to the increased ROS exposure, or because these measures never were elevated due to treatment. While the study of Meitern *et al.* (2013) is a valuable addition to the literature on oxidative stress ecology, performing a pilot study to isolate a suitable dose for this species prior to experimentation may have both prevented unintended bird mortality and increased the quality and quantity of data.

The primary challenges with using paraquat to induce oxidative stress in birds and other non-model species are 1) finding an appropriate dose and 2) accounting for non-target effects, such as accumulation in lungs or toxicity at low doses (Table 4). Paraquat may be a useful tool for inducing increased oxidative stress, but researchers should carefully consider the effects of dose, means of dosage, and exposure duration of treatment on their study species—as well as proper use of protective equipment to minimize risk of exposure to the researchers themselves—before using the technique.

Diquat

Diquat (1,1'-ethyline-2,2'-bipyridylium; Table 3) is similar in both structure and function to paraquat and has also been used to induce increased ROS generation in animals (Sewalk, Brewer & Hoffman 2000; Xu *et al.* 2007; Alonso-Alvarez & Galvan 2011). Because diquat is not commonly used in agriculture, exposure to humans, wildlife, and livestock is infrequent and its toxic effects are less well studied, though they generally have been found to be comparable to the effects of paraquat (Gage 1968; Rawlings, Wyatt & Heylings 1994; Jones & Vale 2000; Drechsel & Patel 2009). Clinically, the main difference between acute paraquat and diquat poisoning is that diquat does not accumulate in the lungs, and instead primarily causes damage to the kidneys and liver (Rose, Smith & Wyatt 1974; Jones & Vale 2000). For this reason, diquat may be a useful alternative if experimental subjects are found to be particularly prone to paraquat-caused respiratory damage.

At the cellular level, diquat induces ROS generation by the same redox cycling reactions that characterize paraquat (Wong & Stevens 1986; Rawlings, Wyatt & Heylings 1994; Drechsel & Patel 2009). However, diquat has a lower activation energy than paraquat, so redox cycling is more readily triggered in diquat than in paraquat. As a consequence, diquat can cause stronger effects more quickly and at lower doses (Baldwin *et al.* 1975; Witschi *et al.* 1977; Suleiman & Stevens 1986; Drechsel & Patel 2009). Thus, if diquat is used as an agent to induce oxidative stress in live animals, it is especially important to determine an appropriate dosing treatment. Accordingly, Galván and Alonso-Alvarez (2009) and Alonso-Alvarez and Galvan (2011) performed thorough dosage trials prior to experiments with diquat, and they were able to minimize negative side-effects while still inducing an increase in oxidative stress in their animal subjects. In their pilot studies of red-legged partridges (*Alectoris rufa*), they divided 36 birds into

groups receiving one of four diquat doses in their drinking water, and measured consumption as well as mass loss and a blood-based measure of oxidative damage (lipid peroxidation). Based on this dose assessment, they were able to increase oxidative damage in their main experiment without causing the birds to lose weight or show other signs of distress (Galván & Alonso-Alvarez 2009; Alonso-Alvarez & Galvan 2011). These experiments demonstrate how a potentially toxic chemical such as diquat can be used with minimal harm to the animals while manipulating the target variable. To better characterize the mechanisms by which diquat induced an increase in the measure of blood lipid peroxidation, however, it would be valuable to assess how other measures of oxidative damage, ROS production, and/or antioxidant capacity varied after diquat exposure.

Though the studies of Galván and Alonso-Alvarez (2009) and Alonso-Alvarez and Galvan (2011) successfully used diquat in their experiments after careful pilot study, paraquat may generally be a better choice than diquat as an oxidative challenge because diquat is both less well studied and more reactive—and hence potentially more toxic—than paraquat.

Heavy metals

Another means by which ROS production can be increased in an organism is through ingestion or injection of heavy metals (Table 3). The effects of transition metals on biological systems are well studied because of the harmful effects of overdose on organisms from plants to fish to humans (Sorensen 1991; Stohs & Bagchi 1995; Jezierska & Witeska 2001; Clemens 2006; Jomova & Valko 2011). Although metal ions are essential for basic cellular processes and form the catalytic centers of many critical enzymes, high concentrations of metals can induce ROS production and can also interfere with antioxidants (Ercal, Gurer-Orhan & Aykin-Burns 2001).

However, metal ions can also interact directly with cellular components to impair other physiological pathways independently of oxidative stress, which may confound the results of an experimental challenge (Bal & Kasprzak 2002; Kasprzak 2002; Leonard, Harris & Shi 2004). Exposing experimental subjects to heavy metals through ingestion or injection is therefore not an effective means to manipulate system-wide ROS levels in a controlled fashion because of the direct interactions of metal ions with non-redox cellular pathways.

Radiation

Another potential means for inducing systemic increase in free radical levels in animals is controlled exposure to ionizing radiation using medical machinery (Table 3). The connections between ionizing radiation and ROS generation are intimate and direct (Lane 2002), and because total-body radiation potentially affects all parts of an organism uniformly, it avoids the issue of unintended localization of pro-oxidants and oxidative damage that may occur in other treatments.

Radiation is well studied for its effects on human health (Weiss & Landauer 2003; Hendry *et al.* 2009; Morgan & Sowa 2009; Goodhead 2010). As a result, many of the molecular processes triggered by radiation, the pathological effects of high doses, and the cellular pathways responsible for mediating radiation exposure are well characterized (Riley 1994; Weiss & Landauer 2003; Spitz *et al.* 2004; Kim, Chandrasekaran & Morgan 2006; Dauer *et al.* 2010; Szumiel 2012). Low-energy radiation, such as from UV and visible light, is not adequate as an experimental inducer of ROS because such radiation does not pass beyond the skin of animals and does not directly generate ROS from water contained in the body (Riley 1994; Ichihashi *et al.* 2003). High-energy ionizing radiation (such as gamma rays; Riley 1994), on the other hand,

is better suited as a generalized oxidative challenge due to its ability to stimulate system-wide increased ROS.

Ionizing radiation induces damage at the cellular level both directly (through primary effects of ionization itself) and indirectly (through ROS generation). Primary radiation effects occur when high-energy radiation ionizes molecules by causing the ejection of an outer shell electron. This ionization can cause cellular damage directly, such as by breaking DNA strands. However, because animal cells predominately contain water, radiation is most likely to ionize water rather than critical cellular molecules (Riley 1994; Lane 2002). Once ionized, water rapidly reacts with other water molecules to form the superoxide radical and subsequently other ROS, including hydrogen peroxide and hydroxyl radicals (Riley 1994; Lane 2002). Thus, radiation generates pro-oxidants through the ionization of water molecules present everywhere in the body.

Within the ecological literature, most studies investigating the effects of ionizing radiation have focused on changes in the molecular and morphological phenotypes, as well as survival, of species at sites of the nuclear power plant accidents at Chernobyl in 1986 (Møller & Mousseau 2006) and Fukushima in 2011 (Beresford & Copplestone 2011; Mousseau & Møller 2012; Strand *et al.* 2014). A recent meta-analysis assessing the effects of chronic low-dose radiation on wildlife found that exposure to radioactive contaminants tended to cause small-to-moderate increases in oxidative damage and decreases in antioxidant capacity, though responses varied significantly among species (Einor *et al.* 2016).

The caveat to using ionizing radiation as a pro-oxidant stimulant is the potential for ROS generation in locations and rates not typically found in a wild animal's body, which may induce physiological responses that are not standard oxidative stress responses (Szumiel 2012). The

recovery from and prevention of radiation damage is generally mediated by signaling pathways that cause up-regulation of antioxidants and DNA repair mechanisms, or activation of autophagy or apoptosis of damaged structures (Spitz *et al.* 2004; Dauer *et al.* 2010; Pazhanisamy *et al.* 2011; Szumiel 2012). These responses are characteristic of a defensive response during most states of high oxidative stress (Sies 1997; Valko *et al.* 2007). However, high dose radiation can also trigger the release of inflammatory cytokines, which in turn activate ROS production and innate immune activity, which is not a typical response to increased oxidative stress (Iyer & Lehnert 2000; Kim, Chandrasekaran & Morgan 2006; Jang *et al.* 2013). The effects of low doses of ionizing radiation, as might be utilized in oxidative challenges within ecological experiments, are less well understood than the pathological effects of high doses, but may be more comparable to naturally stimulated states of increased oxidative stress than are the effects of high doses.

Low doses of ionizing radiation may have a hormetic (or "radioadaptive") effect, causing the down-regulation of cellular processes that produce ROS and up-regulation of antioxidant production to boost resistance to future oxidative challenge (reviewed in Szumiel 2012; Spitz *et al.* 2004), which has been observed in bird species occupying the Chernobyl area (Galván *et al.* 2014). Much as in other instances of increased pro-oxidant burden, increased antioxidant levels correlate with a decrease in the detrimental effects of radiation (Spitz *et al.* 2004; Pazhanisamy *et al.* 2011; Jang *et al.* 2013). In fact, many of the reactions to low-dose radiation exposure are representative of response to oxidative damage and increased ROS abundance, rather than radiation-specific effects, though empirical data in non-model species is lacking. One of the few studies to examine the response of wild animals to a controlled exposure of low-dose, whole-body ionizing radiation found that gamma radiation treatment consistently increased DNA oxidative damage, though there were significant differences in how two different songbird

species responded to varying levels of radiation over varying time periods (Luloff *et al.* 2011). These results provide important baseline data for how response may vary in a dose- and duration-dependent manner, at least in songbirds, and again emphasize the importance of assessing how any particular study species may respond to radiation. Further experimentation is needed to determine how other aspects of oxidative stress may vary during low-dose treatment, and whether any non-target damage incurred from the primary effects of radiation is observed.

With careful study of potential side effects, low doses of ionizing radiation may offer a useful method for increasing system-wide ROS levels. Furthermore, and important to the logistical feasibility of performing such trials, the machinery required to expose animals to ionizing radiation is often available in major research institutions for medical use. However, additional research is needed to gauge whether even low doses of ionizing radiation cause too much direct damage to biological molecules to isolate the effects of oxidative stress alone, or whether the body's response to system-wide pro-oxidant stimulation differs significantly from the typical pathways of response to increased oxidative stress. Until these questions can be more definitively answered with respect to the non-model systems used within ecology, radiation may be best suited for captive experiments aimed at inducing especially strong oxidative challenges without the immediate toxic side-effects associated with many chemical treatments.

*t*BHP

A fourth potential means to elevate system-wide ROS in animals is exposure to *tert*-butyl-hydroperoxide (*t*BHP), a membrane-permeable, stable analog of hydrogen peroxide that is metabolized within the cell into unstable forms that can induce oxidative damage (Table 3; Piret *et al.* 2004; Oh *et al.* 2012; Slamenova *et al.* 2013). The cellular effects of *t*BHP have been found

to be comparable to those of H_2O_2 exposure, though *t*BHP has the logistical advantage of remaining chemically stable until administered to cells or tissue (Spector *et al.* 2002; Alia *et al.* 2005; Slamenova *et al.* 2013). Cellular exposure to *t*BHP not only induces oxidative damage, but also stimulates mitochondrial membrane depolarization, cytochrome *c* release, and apoptotic signaling through direct interaction with the mitochondrial permeability transition pore in the outer mitochondrial membrane (Haidara *et al.* 2002; Piret *et al.* 2004). These latter effects of *t*BHP occur independently of ROS generation, but the apoptotic signals generated are typical of a mitochondrial response to high rates of oxidative damage (Hamanaka & Chandel 2010). High concentrations of *t*BHP, however, can induce cell necrosis rather than apoptosis (Haidara *et al.* 2002; Oh *et al.* 2012), underscoring the importance of careful dose determination to avoid harmful effects beyond increased oxidative stress.

*t*BHP is primarily known from biomedical research on isolated cell lines in which cell cultures are bathed in a solution containing *t*BHP to test the efficacy of various antioxidants and to determine the specifics of how cells respond to oxidative damage (e.g. Liu *et al.* 2002; Lazze *et al.* 2003). However, only a limited number of studies in vertebrates—specifically, in rats—have administered *t*BHP *in vivo*, finding increased oxidative damage and apoptosis in liver tissue after intraperitoneal injection of *t*BHP (Liu *et al.* 2002; Oh *et al.* 2012). While these studies indicate the potential for *t*BHP as an inducer of oxidative damage, further research is needed to investigate other potential tissue-specific effects of the chemical in vertebrates and how effects vary among species and methods of administration.

Interestingly, *t*BHP has recently gained popularity as an oxidative stress inducer outside of the biomedical literature in studies of the remarkable longevity of bivalve mollusks, such as ocean quahog clams (*Arctica islandica*; Ungvari *et al.* 2011) and freshwater pearl mussels

(*Margaritifera margaritifera*; Ridgway *et al.* 2014). These studies indicate that *t*BHP may be particularly useful in aquatic systems where subjects can be exposed to the chemical by adding *t*BHP to the water surrounding the animal. In the published studies on bivalves, oxidative damage and apoptosis were increased in the gill tissue exposed directly to the dissolved *t*BHP (Ungvari *et al.* 2011; Ungvari *et al.* 2013; Ridgway *et al.* 2014). Though only tested in invertebrates to date, these methods may prove to be broadly applicable to a variety of aquatic species.

In summary, *t*BHP represents a promising avenue of future research and may prove a valuable and safe inducer of widespread oxidative damage, particularly given easy administration to aquatic organisms, though the specific effects of the chemical in most systems and the potential for the chemical to induce widespread oxidative stress after injection remain to be examined.

Oxidized dietary lipids

Another means to induce increased oxidative stress has emerged through the study of animals raised for human consumption, particularly poultry and fish. Because of the effects of increased oxidative damage on a variety of aspects of both animal health and meat quality, researchers have manipulated the oxidation state of lipids in captive animal diets both to examine the effect of diet quality on the animal's oxidative stress levels and to gauge the efficacy of various dietary antioxidants in preventing increased oxidative stress (Table 3). Dietary lipids with high concentrations of polyunsaturated fatty acids (PUFA) are prone to oxidation (Frankel 1980; Kubow 1992; Sargent *et al.* 1999). While PUFA are essential nutrients, the byproducts of their peroxidation via heat or chemical catalysis can be damaging both through the formation of

reactive radicals and through the direct covalent interactions between some peroxidation products, such as 4-hydroxynonenal, and biological molecules (Kubow 1992; Bacot *et al.* 2007; Awada *et al.* 2012). Adding oxidized (often, heat-treated) lipids to the diet has been shown to slow growth rate and increase oxidative damage in many fish species (Nakano *et al.* 1999; Peng *et al.* 2009; Yuan *et al.* 2014) and poultry (Zhang *et al.* 2010; Açıkgöz *et al.* 2011; Delles *et al.* 2014). Similar experiments have also been performed in mammal species, including the domestic pig (Shi-bin *et al.* 2007) and rat (Izaki, Yoshikawa & Uchiyama 1984; Keller, Brandsch & Eder 2004), and consumption of oxidized lipids and their byproducts is considered a human health risk (Kanner 2007).

Providing experimental subjects with dietary oxidized lipids to induce oxidative challenge has the advantage of being a simple experimental design, requiring no injections and posing no safety risks to researchers, that has been widely performed in several vertebrate species. However, this technique may not be suitable for broad application within studies of oxidative stress ecology because of the large variation in assimilation and responses observed among subjects (e.g. Tocher *et al.* 2003). This variation may be due at least in part to differences in the consumption and absorption of dietary oxidized lipids (Tocher *et al.* 2003; Dong *et al.* 2011), but it is also important to consider that the conditions in which the lipids oxidize may cause variation in the products consumed by the animals, some of which may have toxic properties that can potentially damage the digestive tract membrane (Kaneda & Miyazawa 1987; Engberg *et al.* 1996). Moreover, the antioxidant and other properties of the diet beyond its oxidized lipid content can influence the effects of treatment on oxidative stress (Kubow 1992; Kanner 2007; Awada *et al.* 2012).

Administration of dietary oxidized lipids can be an effective means of inducing increased oxidative stress, and is an important method for determining the effects of diet properties on the health and quality of animals; it may also be particularly useful if a study is interested in examining the antioxidant properties of the gut, or interactions between dietary oxidants and antioxidants. If used as an oxidative challenge, it is important to account for the fact that some oxidized lipids and their products may be assimilated directly into the body, inflating quantitative measurements intending to estimate lipid oxidative damage to the body (Awada *et al.* 2012). However, the effects of dietary oxidized lipids are subject to variation from a variety of sources that may be difficult to control, and use of this technique outside of agricultural and model species is largely unstudied. Thus, I do not recommend this technique for use as a general oxidative stress challenge.

Antioxidant Knock-Downs

As an alternative to increasing ROS production directly, decreasing the levels of antioxidants may induce a controlled oxidative challenge by shifting the oxidative balance toward increased levels of pro-oxidants. Such a manipulation has rarely been explored within ecological studies because, until recently, accessible methods did not exist or were not commonly known. However, a chemical manipulation—buthionine sulfoximine (BSO)—and the rapidly advancing field of RNAi research both offer useful methods to eliminate antioxidants within the animal body without additional side-effects. Importantly, neither of these techniques poses any risk of harm to the researcher administering the oxidative challenge.

BSO

Injection of BSO is an effective means to systemically lower levels of glutathione (γ-glutamylcysteinylglycine), one of the most important antioxidants in the bodies of animals (Table 3; Anderson 1998). BSO is unusual among chemical agents because of its specificity: once injected, it suppresses glutathione production throughout the body by irreversibly binding to γ-glutamylcysteine synthetase, an enzyme involved in the initial catalytic steps of glutathione synthesis (Griffith & Meister 1979; Griffith 1982).

Because BSO is rapidly cleared from an animal's system, chronic effects of BSO on glutathione synthesis are not expected, but repeated injections may continuously deplete glutathione levels. A study investigating the effects of chronic BSO injection in rats found that intraperitoneal injection twice daily for 30 days caused 85% reduction in γ-glutamylcysteine synthetase activity in the lung tissue, but only a 36% decrease in glutathione levels (Thanislass, Raveendran & Devaraj 1995). However, BSO treatment in this study increased lipid peroxidation and decreased the levels of a multitude of other cellular antioxidants. These effects were likely due to both a decrease in the antioxidant activity of glutathione and a handicap to other antioxidants, particularly ascorbic acid, which rely on glutathione reactions for proper function (Meister 1992; Thanislass, Raveendran & Devaraj 1995). This study illustrates the cascading effects of BSO-driven glutathione depletion, and how even a modest decrease in glutathione appears to shift the balance of pro- and antioxidants to induce a measurable increase in oxidative stress.

Within the ecological literature, Galván & Alonso-Alvarez (2008) and Romero-Haro & Alonso-Alvarez (2015) have performed controlled experiments in which they injected nestling great tits or zebra finches (*Taeniopygia guttata*), respectively, with four doses of BSO over six

days and measured the effects of treatment on adult phenotype. Importantly, BSO exposure did not affect adult body condition or survival in either species, though both studies found complex interactions between glutathione depletion, ornamentation, and oxidative state. In particular—and in contrast to the decreased antioxidant levels found in the study of rats (Thanislass *et al.* 1995)—treated nestlings but not always adults showed *increased* total antioxidant capacity, despite lower glutathione levels. Two additional studies on adult greenfinches (Hõrak *et al.* 2010) and domestic canaries (*Serinus canaria*; Costantini *et al.* 2015) followed similar designs of repeated BSO injections and found that BSO successfully increased a measure of plasma oxidative stress and decreased erythrocyte glutathione levels, though the former study found no effect of treatment on plasma total antioxidant capacity. These studies exemplify the variation in how different species (and different sexes, ages) may respond to depletion of a key antioxidant, but all three show clear evidence that BSO exposure did indeed decrease glutathione levels within the animal body and cause measurable changes in oxidative damage and/or the activity of other antioxidants.

The advantage of BSO is that it targets one antioxidant, causing limited effects on other cellular components. As with all techniques, however, the usefulness of BSO as an oxidative challenge in a particular experimental design will depend on the nature of the research questions being addressed. While BSO may be an effective means to increase pro-oxidant burden on the cellular antioxidants that remain after glutathione depletion, the challenge may not induce overall increased oxidative stress if those other antioxidants are able to successfully compensate for the inhibition of glutathione (Galván & Alonso-Alvarez 2008; Hõrak *et al.* 2010). In addition, BSO may not be appropriate for studies interested in the response of an animal's total antioxidant defenses to oxidative challenge because suppressing glutathione handicaps one major pathway of

pro-oxidant resistance. On the other hand, a BSO challenge may be particularly well suited to answering questions about the specific function of glutathione in antioxidant response, or to examining the ability of other cellular antioxidants to up-regulate in response to increased pro-oxidant burden.

RNAi

A promising but largely untested option for a targeted manipulation of antioxidants is RNA interference (RNAi; Table 3). This technique causes post-transcriptional degredation of specific mRNAs, reducing expression of the corresponding proteins. RNAi techniques are rapidly being developed for vertebrate models and even non-model systems (reviewed in Sifuentes-Romero *et al.* 2011; Perrimon *et al.* 2010), though the technique remains most commonly employed in invertebrate species. mRNA knock-downs via RNAi are invaluable tools for isolating the function of particular gene sequences, and have been fundamental to advancing the fields of functional genomics and developmental biology. A recent study on copepods (*Tigriopus californicus*) exemplifies how RNAi methodology may have application to evolutionary ecology: Barreto *et al.* (2014) developed a method of using double-stranded RNA to suppress the expression of five target genes by at least 50%; they then used this technique to determine that inhibiting a heat shock protein gene caused a significant increase in mortality of copepods during thermal stress, which is relevant to the survival of this species to high temperatures in tidal pools. RNAi techniques have also been used successfully to knock-down the genes for antioxidants, including mitochondrial superoxide dismutase in *Drosophila* (Kirby *et al.* 2002; Martin, Jones & Grotewiel 2009; Martin *et al.* 2009).

While the methodology for using RNAi in the non-model vertebrate systems often important to ecological studies remains largely in development, RNAi techniques represent an important future avenue for reducing the function of antioxidants and other cellular proteins in order to induce increase oxidative stress. As with BSO, the specificity of RNAi represents a valuable opportunity to manipulate the activity of target enzymes without risking the broad spectrum of clinical effects that can be caused by harmful compounds like paraquat.

Conclusions

Researchers currently have at least six methods for increasing ROS production—paraquat, diquat, heavy metals, radiation, oxidized dietary lipids, and *t*BHP—and at least two methods for decreasing production of antioxidants—BSO and RNAi—as means to manipulate oxidative stress. Selecting the most suitable oxidative challenge method for a particular experiment depends largely on balancing two primary concerns: 1) the biochemical and physiological effects of the challenge suit the research goals of the study with minimal non-target effects, and 2) the risk of accidental overdose to animal subjects and the safety concerns to researchers administering the challenge are minimized. Given these parameters, paraquat is the best option for inducing increased ROS production within animals, but its toxic chemical properties necessitate particular care during dosage trials and diligent use of personal protective equipment. Conversely, BSO and RNAi offer non-toxic alternatives to paraquat, but knocking-down the activity of specific antioxidants may not be appropriate for particular experimental questions. I therefore recommend that researchers carefully evaluate the type of oxidative stress challenge that will best address their research goals and select the corresponding method that minimizes the risk of accidental harm.

Regardless of the oxidative challenge used, I encourage researchers performing oxidative challenges to measure at least some aspects of oxidative damage, antioxidant capacity, and/or ROS production to ascertain that the treatment caused the desired response. Such measurements not only reveal whether or not the oxidative challenge had an effect, but also the nature of the effect (Monaghan, Metcalfe & Torres 2009). Many established methods exist for measuring different components of oxidative stress (Mateos & Bravo 2007), and *in vivo* methods for measuring the specific rate of ROS production from subcellular sites, such as the mitochondria, may become increasingly useful to pinpoint the effect of oxidative challenge (Stier *et al.* 2013). With conscientious application of pilot study, choice of appropriate treatment method, and validation of effects, oxidative challenges can yield invaluable information about the interactions among oxidative stress and other physiological pathways—a core component of oxidative stress ecology.

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Table 3. Oxidative challenges. Summary of the potential oxidative challenges discussed within this review, including their desired effects and some potential problems to consider before their use in ecological experiments.

Challenge	Target effect	Method(s) of dosing	Main mechanism for increased oxidative stress	Other effects	Potential problems
Paraquat	ROS generation	Ingestion, injection, dermal application, environmental water exposure (aquatic organisms)	Redox cycling: Accepts electron from strong reducing enzyme, passes electron to oxygen to form superoxide anion and regenerate oxidized form	Disrupts redox signaling through oxidative damage and by accepting electrons meant for other functions	Causes localized damage to lungs; interacts with microglia to induce increased neural damage, particularly to dopaminergic neurons; researchers must take precautions to prevent exposure
Diquat	ROS generation	Same as paraquat	Same as paraquat	Same as paraquat	More reactive—and potentially more damaging—than paraquat; researchers must take precautions to prevent exposure
Heavy metals	ROS generation	Ingestion, injection, environmental water exposure (aquatic organisms)	Can generate ROS through redox reactions, or interact with antioxidant structures to inhibit function	Reacts with and compromises cellular pathways independently of oxidative stress	Organ-specific toxicity; oxidant-independent effects
Ionizing radiation	ROS generation	Exposure in biomedical setting	Forms ROS from ionizing water found in the body	Also damages molecular structures	Oxidant-independent effects; body-wide induction of ROS may not represent an oxidative stress response experienced in natural conditions
***t*BHP**	ROS generation	Environmental water exposure (aquatic organisms), injection	Acts as a pro-oxidant similarly to H_2O_2	Interacts with mitochondrial permeability transition pore	The specific locations of *t*BHP activity remain largely unexplored
Oxidized dietary lipids	ROS generation	Ingestion	Ingested oxidized lipids cause oxidative damage through further reactions	May compromise lipid assimilation, interfere with structures independently of ox. stress	Response varies greatly among species with changing consumption and absorption; non-target effects on lipid absorption and cellular structures may confound results
BSO	Reduced glutathione levels	Injection	Binds to glutathione synthetase to	Affects activity of other antioxidants that rely on	Not appropriate for studies requiring activity of all

			inhibit glutathione production	glutathione pathways	naturally occurring antioxidants
RNAi	Knocked-down antioxidant gene expression	Injection	Degrades mRNA related to expression of a specific antioxidant	None	Requires development of specific RNAi, not currently available for most vertebrate species; not appropriate for studies requiring activity of all naturally occurring antioxidants

Table 4. Paraquat in birds. Summary of the experimental parameters and results of studies involving controlled paraquat exposure in bird species, the taxa most commonly involved in paraquat experiments within ecology. Doses are expressed in g paraquat per L drinking water (g/L), mg paraquat per kg animal body weight (mg/kg), or ppm (paraquat parts per million parts substrate).

Species	Dose(s)	Adminis-tration	Duration	Symptoms	Source(s)
Mallard (*Anas platyrhynchos*)	1048 ppm	Diet	5 days	50% mortality	Heath *et al*. 1972, Hill & Camardese 1986
American kestrel (*Falco sparverius*)	10, 25, or 60 mg/kg	Oral dose	10 days	Reduced skeletal growth in all doses; 44% dead within 4 days on highest dose	Hoffman *et al.* 1987
Northern bobwhite (*Colinus virginianus*)	981 ppm	Diet	5 days	50% mortality	Heath *et al.* 1972; Hill & Camardese 1986
	25 or 100 ppm	Drinking water	60 days	No clinical symptoms in either dose	Bunck *et al.* 1985
Japanese quail (*Coturnix japonica*)	970 ppm	Diet	5 days	50% mortality	Heath *et al*. 1972, Hill & Camardese 1986
	100 ppm	Drinking water	7 days	100% mortality	Paulov 1977
	10 mg/kg	Intra-peritoneal injection	7 days	Increased glutathione peroxidase activity and decreased glutathione levels in blood; increased lipid peroxidation (MDA[1]) in lung but not blood	Galvani *et al.* 2001
Ring-necked pheasant (*Phasianus colchicus*)	1468 ppm	Diet	5 days	50% mortality	Heath *et al.* 1972, Hill & Camardese 1986
Domestic turkey (*Meleagris gallopavo*)	100 mg/kg	Intra-peritoneal injection	Single dose	50% mortality; anorexia, diarrhea, inactivity	Smalley 1973
	20 mg/kg	Intravenous injection	Single dose		
	290 mg/kg	Oral dose	Single dose		
	500 mg/kg	Dermal application	Single dose		
Domestic chicken (*Gallus gallus*)	262 mg/kg	Diet	Single dose	50% mortality	Clark, McElligott & Hurst 1966
	40 ppm	Drinking water	14 days	Increased numbers of eggs did not hatch or produced abnormal chicks	Fletcher 1967
Yellow-legged gull (*Larus michahellis*)	2.3 mg/kg	Oral dose	Single dose	Higher total antioxidant capacity, but no change in lipid peroxidation (MDA), protein carbonyl levels, total reactive oxygen metabolites, or mass; all oxidative stress assays were blood-based	Lucas, Morales & Velando 2014

Great tit (*Parus major*)	1.5, 0.75, 0.38, 0.19, or 0.09 g/L	Drinking water	6 weeks	All but lowest dose were lethal or caused clinical symptoms of distress (mass loss, decreased activity); lowest dose had no effect on plasma carotenoid concentrations or ornamental plumage coloration	Isaksson & Andersson 2008
European greenfinch (*Carduelis chloris*)	0.1 or 0.2 g/L	Drinking water	7 days	Significant mass loss in both doses; 50% mortality in higher dose; in higher dose: higher levels of DNA damage (comet assay) and glutathione, but no change in levels of protein carbonyls, lipid peroxidation (MDA), overall antioxidant protection (TAC[2] and OXY[3]), uric acid, or carotenoids; no oxidative stress effects on low dose; all oxidative stress assays were blood-based.	Meitern *et al.* 2013
House finch (*Haemorhous mexicanus*)	0.1 g/L	Drinking water	28 days	Significant mass loss in all treated birds; increase in oxidative damage (TBARS[4]) and trend toward decrease in activity levels in birds supplemented with carotenoids, but not unsupplemented birds; no effect on plumage hue or plasma carotenoid levels.	Giraudeau *et al.* 2015

[1] MDA = malondialdehyde; a measure of oxidative damage to lipids
[2] TAC = total antioxidant capacity
[3] OXY = oxygen radical absorbance; a measure of antioxidant function
[4] TBARS = thiobarbituric acid-reactive substances; a measure of oxidative damage

CHAPTER 4
Rapid evolution of bright monochromatism in the domestic Atlantic canary

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Introduction

In many species of songbirds, males and females look alike, and the plumage brightness of such monochromatic species ranges from drab and cryptic to brilliant and conspicuous. Other species are dichromatic, in which females are duller and more camouflaged than males. In no species of passerine—and in few species of other avian taxa—are females more colorful than males (Hoyo 2010). Interestingly, studies that have deduced change in male and female plumage patterns across passerine lineages have found that changes along the monochromatic drab/dichromatic/monochromatic bright continuum are relatively frequent (Price and Birch 1996, Burns 1998, Hofmann *et al.* 2008, Friedman *et al.* 2009, Johnson *et al.* 2013). Monochromatism evolves from a dichromatic ancestral state either by males losing their sex-specific coloration (causing dull monochromatism) or by females gaining colorful plumage (bright monochromatism; Irwin 1994, Omland 1997, Gluckman 2014). Contrary to the expectation that conspicuous ornaments are distinctly male traits, gain of bright and conspicuous coloration by females appears to occur more commonly than loss of coloration in females (Irwin 1994, Peterson 1996, Badyaev and Hill 2003, Price and Eaton 2014). A study of more than 5000 passerine species indicated that shifts between monochromatism and dichromatism and vice versa are not uncommon compared to other evolutionary events, occurring at rates of about 0.1 to 0.4 per species per million years (Price and Birch 1996).

Color is among the most mutable characteristics in domesticated bird species (Price 2002), but it has not been examined with respect to sex-specific changes in captivity. Captive species have long been critical to understanding selection and trait evolution. The range of phenotypes in domestic strains compared to what is observed in wild progeners provides a framework for anticipating what sorts of changes are physiologically possible in a particular species (Darwin 1868, Bartley 1992, Zann and Bamford 1996, Price 2002, Sol 2008). Studies of domesticated species that have been under known selection for finite periods of time allow investigators to directly observe the effects of selection on phenotype (Conner 2003).

The Atlantic canary (*Serinus canaria*) presents an opportunity to study the evolution of bright monochromatic plumage from dichromatic plumage. Wild canaries are native to the Canary Islands and have sexually dichromatic plumage coloration with males more conspicuous yellow than females (Hoyo 2010). Modern domestic canaries (hereafter "domestic canaries") are descended from Atlantic canaries that were brought into captivity by Europeans approximately 500 years ago because of their elaborate song (Birkhead *et al.* 2004). Lineages of domestic canaries have subsequently also been bred selectively for carotenoid-based coloration. Artificial selection has produced lines of "color-bred" canaries, including the solid bright yellow of the stereotypical pet domestic canary (Birkhead 2003, Birkhead *et al.* 2004). Breeders used traditional methods of line-breeding to create canaries of such exaggerated color, pairing their most colorful males with their most colorful females to produce exceptionally colorful offspring (Walker 1993). Artificial selection through line-breeding color-bred canaries has therefore imposed strong selection for a monochromatic bright population by favoring increased coloration in *both* sexes. Studying a domestic species like the canary presents a rare opportunity to study

trait evolution because there is a known starting point (wild-type plumage) and a known duration of selection (at most 500 years).

Moreover, historical canary breeding manuals reveal that dichromatism has existed in domestic canaries as late as the end of the 19th century. A 1848 guide to caged songbirds states that both sexes of canaries are similar, but males generally have "deeper and brighter" colors than females (Bechstein 1848:271); 40 years later, a different manual states firmly that "in no class of Canaries do the females exhibit the depth of color which males possess, therefore the colors and never so brilliant" (Holden 1888:30). Several decades later, however, biologist Alexander Wetmore notes in his 1923 manual that the sexes cannot be distinguished except by the presence of song and by vent appearance during the breeding season (Wetmore 1923), indicating that a shift from dichromatism to monochromatism occurred early in the 1900's. The records kept by these and other canary fanciers present a detailed time window for loss of dichromatism. Each of these authorities examined a variety of canary lines from the United States and Europe, so it is unlikely that the differences noted are unique to one particular strain of canaries (Bechstein 1848, Holden 1888, Page 1898, Galloway 1909, Plath 1922, Wetmore 1923).

In addition, in the late 1930's canaries were crossed with a heterogeneric species, the black-hooded red siskin (*Carduelis cucullata*), which has bright red, sexually dichromatic feather coloration (Hoyo 2010). This cross was affected specifically to transfer the red coloration of the siskin to canaries (Birkhead 2003). Because the goal was a red canary and not a new hybrid taxon, breeders subsequently selected against all siskin traits except redness (Birkhead 2003). The result is a bird that is phenotypically indistinguishable from a non-hybrid color-bred canary except for red feather coloration. Some early siskin/canary hybrids inherited genes responsible

for the strong dichromatism present in the red siskins because female hybrids resembled dull female siskins instead of bright red males (Birkhead 2003). Red canaries, therefore, present a second episode of strong selection to remove dichromatism, in this case by selecting against all siskin traits in the hybrid progeny; moreover, it is likely that current red canaries are products of even more recent hybridization, as modern canary breeders have continued to generate their own lines of red canaries from the original canary-siskin cross.

In this study, I quantified change in sexual dichromatism in domestic color-bred yellow and red canaries, wild Atlantic canaries, and wild black-hooded red siskins. Because human vision may not detect color differences visible to birds (Eaton 2005), I analyzed the spectral characteristics of feathers using quantum catch values and I assessed the ability of the avian eye to discriminate differences in male and female coloration in each of these groups. My goal was to assess the degree to which sexual monochromatism can evolve over a brief period under strong selection for male-like coloration in females.

Methods

To obtain samples for color measurements, I acquired study specimens of wild canaries and wild black-hooded red siskins and breast feather samples from domestic red and yellow canaries. From the American Museum of Natural History, I obtained 41 specimens of wild canaries (21 male, 20 female) and 22 specimens of black-hooded red siskins (15 male, 7 female) that were captured in their native ranges. I received breast feather samples (approximately 8-12 feathers each) from domestic canaries of both red (62 male, 37 female) and yellow (13 male, 21 female) color types from canary breeders in the United States.

I measured color using an Ocean Optics USB4000 spectrophotometer and the OOIBase32 program according to standard color measurement protocols (Saks *et al.* 2003). The spectrophotometer was standardized in a dim room against a 100% white reflectance standard (Labsphere, North Sutton, NH, USA) before each use. Wild canary and red siskin breast feather coloration was measured on intact specimens; all birds were positioned with their ventral side face-up with feathers smoothed into natural positions, and the probe was lightly pressed at a 90° angle to the same point of the breast on every bird. I measured only the breast region on these birds because yellow coloration was localized and was least confounded by additional melanin coloration in this location in both species. To measure color of the feather samples from domestic canaries, I stacked 6-10 feathers from each sample to concentrate the color against a black, non-reflective cardstock background. The probe was then pressed lightly to the surface of the feather stack such that the region evaluated by the probe consisted entirely of the colored feathers of interest. I took six repeated measures of both feather samples and intact birds, removing the probe from the surface between measurements, and I calculated average values for each bird.

To interpret the raw reflectance data from the spectrophotometer, I modified and updated Dr. Jarrod Hadfield's "SPEC" code (2004) written for the program R (version 3.0.2; R Core Team 2014) to calculate the photon catches of each avian cone type for each sample. Photon catches predict the relative stimulation of each of the four avian cone types to a particular color by incorporating the effects of the irradiance spectrum of the environment, light transmission through avian ocular media, and the reflectance spectrum from the measured surface (the feather sample or plumage patch) across all wavelengths detected by each cone (Vorobyev *et al.* 1998, Hadfield 2004, Montgomerie 2006). Because detailed vision data exists for very few avian

species, I used data of the cone sensitivities and transmission properties of the ocular media of another passerine species, the blue tit (*Parus caeruleus*). The blue tit is commonly used as a model species for the study of avian visual perception (Eaton 2005, Stoddard and Stevens 2011), and the few visual parameters of the canary that have been measured closely resemble those of the blue tit (Das *et al.* 1999, Hart and Vorobyev 2005). I used MANOVAs within each group to evaluate whether there were statistical differences between the sexes in the four quantum catch values. I did not assess differences in values among groups (i.e. between wild and domestic yellow canaries) because my analyses focused on changes in levels of sexual dichromatism, not the effects of domestication on coloration.

Lastly, to gauge the biological significance of statistical color differences detected by MANOVA, I used SPEC code (Hadfield 2004) in R to evaluate the discriminability of color differences between the sexes according to the Photoreceptor Noise Model (Vorobyev and Osorio 1998). My model estimated receptor noise under the assumption of a Weber fraction of 0.05 for all cone types, and was adjusted according to the relative abundances of cone types in the blue tit eye (Vorobyev *et al.* 1998, Hadfield 2004). Discriminability is reported in units of JND's, or "Just Noticeable Differences," where JND = 1 is the lowest threshold for successful color discrimination (i.e. JND > 1 indicates a pair of colors that are predicted to be distinguishable by the avian eye). I calculated the average visual discriminability of all pairs of specimens of the same sex ("within-sex") or of the opposite sex ("between-sex") within the red siskin, wild canary, and red and yellow domestic canary groups. I performed one-sample T-tests to compare whether the average JND differed significantly from the threshold value of 1 for both within- and between- sex comparisons in each group. I also used two-sample T-tests to evaluate whether the average JND differed between within-sex and between-sex comparisons within each

group. All statistical analyses were performed in R version 3.0.2 (R Core Team, 2014), and all graphs were created using the R package ggplot2, version 0.9.3.1 (Wickham 2009).

Results

Wild canaries and red siskins but not domestic canaries of either color differed between the sexes in plumage quantum catch values: MANOVAs revealed that wild canaries and red siskins, but not domestic red or yellow canaries, had statistically significant differences in quantum catch values between the sexes (wild canary: $F_{4,36} = 27.45$, $p < 0.001$; red siskin: $F_{4,17} = 14.06$, $p < 0.001$; red canary: $F_{4,94} = 1.34$, $p = 0.26$; yellow canary: $F_{4,28} = 1.49$, $p = 0.23$). I therefore found evidence for sexual monochromatism in the quantum catches of domestic red and yellow canaries, in contrast to the dichromatism I detected in the measurements of wild canaries and red siskins.

My visual discrimination estimates based on the Photoreceptor Noise Model indicated that these color differences (or lack thereof) were biologically relevant. In wild canaries, I observed that the avian eye could distinguish between the coloration of individuals of different sexes (average JND > 1 for between-sex comparisons; $t = 27.30$, $df = 579$, $p < 0.001$; null hypothesis that JND = 1 is rejected), but not among different individuals of the same sex (average JND < 1 for within-sex comparisons; Fig. 1; $t = -6.11$, $df = 799$, $p < 0.001$). The difference in ability to discriminate between individuals of the same sex versus between individuals of the opposite sex was also statistically significant ($t = -25.63$, $df = 1132.4$, $p < 0.001$). In red siskins as well as domestic red and yellow canaries, the average JND values for both within- and between-sex comparisons were greater than 1 (Figure 6; all $p < 0.001$); this result

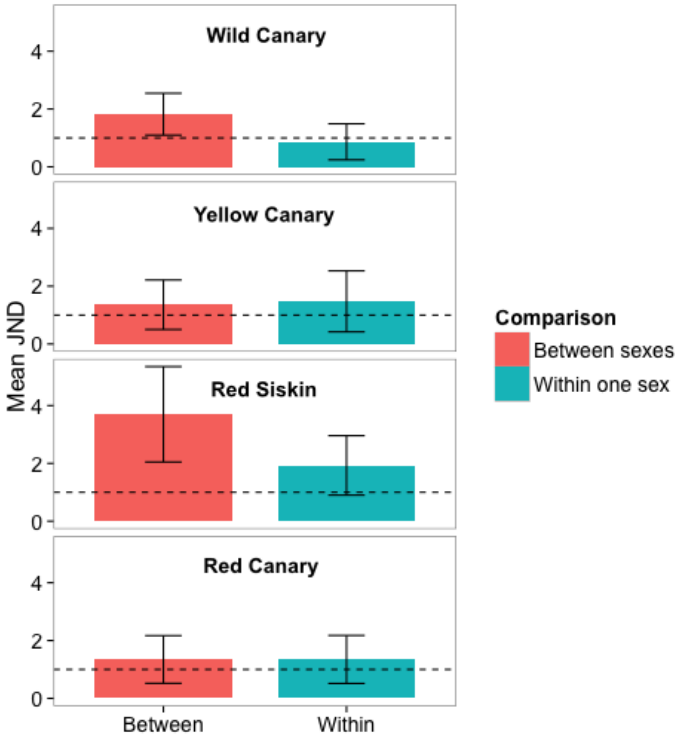

Figure 6. Sexual dichromatism in canaries and the red siskin. Bar graphs show the mean (± standard deviation) Just Noticeable Differences of comparisons between sexes (male vs. female) or within one sex (female vs. female or male vs. male). The dashed line indicates the threshold of JND = 1, where values exceeding this line indicate that groups are distinguishable by the avian eye.

indicates that these groups had high variability in color such that many individuals, even within one sex, could be distinguished based on color. However, there was no significant difference in the ability of a bird to discriminate among domestic canaries of the same sex compared to those of the opposite sex (red: $t = -0.01$, $df = 2952$, $p = 0.99$; yellow: $t = -1.15$, $df = 280$, $p = 0.25$), yet red siskins had far higher average discriminability between individuals of different sexes than within individuals of the same sex ($t = 9.54$, $df = 168$, $p < 0.001$; Figure 6). Given that my model assumed optimal conditions of lighting, these results may indicate that red siskin sexes—but not domestic canaries—may still be distinguishable by color in less ideal conditions.

I therefore found evidence that the sex-based differences in quantum catch I measured in wild canaries and red siskins are both statistically significant and biologically relevant, but I found no evidence that birds could visually distinguish between sexes in domestic canaries based on color. My results therefore indicate that canaries have evolved sexual monochromatism from dichromatism during the process of domestication.

Discussion

The Atlantic canary has been under strong artificial selection for enhanced coloration in both sexes since first brought into captivity more than 500 years ago (Birkhead *et al.* 2004). To determine the effect that this strong selection has had on dichromatism in this species, I evaluated the color differences between sexes in wild canaries, wild black-hooded red siskins, and domestic canaries of both red and yellow color types. I observed dichromatism in wild canaries and red siskins, as has been previously described in field guides (Hoyo 2010) but not previously quantified. In contrast, I found no differences in the plumage coloration of males and females in either red or yellow domestic canaries; both color types of domestic canary have monochromatic bright plumage coloration. My results provide empirical evidence that bright sexual monochromatism can evolve from a dichromatic ancestor in a short time interval under selection for male-like coloration in females.

Given evidence in historical breeding manuals, it is likely that the monochromatism I detected evolved within the time frame of several decades at the start of the 20th century. Such rapid evolution is expected under strong artificial selection; for example, lines of male guppies selected for increased or decreased expression of an orange color patch differed in patch size by two-fold after only three generations of selection (Houde 1994), and *Bicylus* butterflies selected

for increased structural coloration shifted from UV brown to violet scale color in six generations (Wasik *et al.* 2014).

However, traditional means of artificial selection involve modifying a trait already expressed; sexual patterns are rarely altered. My study is the first account of a complete loss of dimorphism in a domesticated species. Although many domestic chicken breeds have been under strong selection for increased mass in egg- or meat-producing hens for thousands of years, a recent study of more than 100 domestic breeds revealed that the extent of male-biased sexual size dimorphism has remained largely unchanged from the ancestral condition in chickens (Remes and Szekely 2010). The speed with which canaries evolved monochromatism contrasts markedly with the persistence of size dimorphism in chickens. The only other species known to have altered patterns of sexual dimorphism through domestication is the rock pigeon (*Columbia livia*), in which wild birds are monochromatic but in which some domestic varieties are dimorphic (Blechman 2007, Baptista *et al.* 2009).

The domestic canary presents a rare opportunity to compare the ancestral and derived forms of a species—before and after the evolution of monochromatism from dichromatism in response to artificial selection. Phylogenetic studies indicate that selection for female coloration is the most common mechanism that causes the evolution of dichromatism from monochromatism, or vice versa, in wild birds (Bjorklund 1991, Peterson 1996, Amundsen 2000, Badyaev and Hill 2003, Cardoso and Mota 2010, Wyman *et al.* 2013, Gluckman 2014, Price and Eaton 2014). My study is the first to directly demonstrate such a change in sexual dichromatism after strong selection for coloration in females. It is possible that increased female coloration could instead be a natural result of releasing females from predation pressure or a product of genetic carryover from strong selection for color in males; however, given the methods of line-

breeding and conclusions from the phylogenetic analyses of wild species (Amundsen 2000), it is most likely that females were strongly selected to be colorful in order to produce offspring of unusually bold color.

Although the Atlantic canary is a popular system for studies of song expression and development (Nottebohm *et al.* 1986, Suthers *et al.* 2012, Garcia-Fernandez *et al.* 2013, Müller *et al.* 2013, Mundinger and Lahti 2014), color in canaries has rarely been investigated under an evolutionary context (Heindl and Winkler 2003). I suggest that the canary is an ideal species in which to pursue questions of the evolution of ornamental coloration. Future research may provide novel insight into the hormonal and developmental mechanisms that evolved to change dichromatism in this passerine species.

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CHAPTER 5

Immune and antioxidant functionality of carotenoid-free canaries questions the value of internal carotenoid resources

Manuscript in preparation for submission as a *short communication* with co-authors Andreas Kavazis, Dennis Hasselquist, Wendy Hood, Yufeng Zhang, Matt Toomey, and Geoffrey Hill

Carotenoid pigments are commonly cited as beneficial physiological molecules that boost immune system and/or antioxidant function in a wide range of animal species, including humans, domestic dogs and cats (Chew & Park 2004), and birds (Møller *et al.* 2000; Svensson & Wong 2011). However, the detailed mechanisms by which carotenoids may serve as antioxidants or otherwise improve immune system functionality remain uncertain and difficult to separate from the beneficial functions of retinol produced from many dietary carotenoids (Hill & Johnson 2012), and it is a matter of contention whether carotenoids play a significant and direct role in antioxidant or immune defenses in vertebrates (Olson & Owens 1998; Hartley & Kennedy 2004; Costantini & Møller 2008). Here, I demonstrate that internal carotenoids are not critically important to oxidative stress or immune response because a carotenoid-free strain of domestic canary (*Serinus canaria*) performs identically to a carotenoid-rich strain on a suite of physiological measures. These results definitively challenge the conventional wisdom that carotenoid pigments themselves are necessary for high functioning internal systems, with important implications both for understanding the role of dietary pigments in animal health and interpreting the potential costs of using such pigments as colorants in signaling theory. While many carotenoid pigments may provide indirect benefits as dietary precursors to retinol, I argue

that the pigments themselves play little to no role in an animal's performance even during states of oxidative or immune stress.

In this study, I test the immune and antioxidant benefits of carotenoid pigments in a novel system comprising both carotenoid-rich yellow and carotenoid-free domestic canaries. White recessive (WR) canaries are the only known vertebrate with a knock-out mutation that specifically affects carotenoid absorption from the diet, essentially eliminating internal carotenoid stores from tissue; these birds must be supplemented with retinol because they lack the dietary carotenoid resources needed to produce their own. I compared these carotenoid-free WR canaries with wild-type yellow (Y) canaries, both of which are allelic variants of the domestic canary that differ only in the form of a transmembrane transport protein responsible for carotenoid transport into the body from the intestinal lumen (Toomey *et al.* submitted). By testing metrics of antioxidant function and immune system performance in both carotenoid-free WR canaries and carotenoid-rich Y canaries (Figures 78), I present the first direct challenge to the benefit of possessing internal carotenoid pigments while controlling for the effects of retinol.

Figure 7. Y and WR canaries. Y canaries (left) feature carotenoid-rich plumage and plasma (left inset), while WR canaries (right) feature pure white plumage and clear or white plasma (right inset).

I performed all physiological tests on male and female canaries of both color types held in a long-term research colony at Auburn University; I confirmed the observed differences in tissue carotenoid content using high performance liquid chromatography (Table 5). To evaluate physiological performance, I assessed one metric of adaptive immune response (antibody production), two of innate immune response (oxidative burst response and bacterial killing ability), one general indicator of immune activation (heterophil to lymphocyte ratio), and two related to antioxidant defenses (total antioxidant capacity and total glutathione). Notably, oxidative damage levels in canary plasma samples were too low for accurate detection by any of three measures attempted: d-ROMs (Diacron International), 4-HNE (Cell BioLabs), or protein carbonyls (Cell BioLabs). I analyzed all results in R (v. 3.2.3; R Core Team 2017) using ANOVAs to test the effects of color, sex, and their interaction on response, except where otherwise noted (see Tables 6-9). Further details for husbandry, laboratory methods, results, and analyses are described in Appendix 1.

First, in July-August 2016, I activated the immune systems of experimental canaries with an intra-abdominal injection of bacterial lipopolysaccharide (LPS) from *Escherichia coli* (O55:B5; 1 mg/mL dissolved in phosphate-buffered saline; List Biological Laboratories, Inc.), commonly used in songbirds and other species to initiate a transient acute phase immune response without causing lasting sickness (e.g. Owen-Ashley & Wingfield 2006). I measured a slight fever response (increase in body temperature; one-way t-test: H_0=0 temperature change; $t=5.01$, $df=25$, $p<0.001$) and decrease in mass (one-way t-test: H_0=0 mass change; $t=8.44$, $df=50$, $p<0.001$), but not food consumption (one-way t-test: H_0=0 food consumption change; $t=0.074$, $df=46$, $p=0.94$); importantly, none of these responses differed between WR and Y birds, although the experimental WR canaries had slightly larger average mass (Tables 5-6). 8 hours after

injection, I extracted a small blood sample from birds to test total antioxidant capacity (TAC), oxidative burst response (both peak response and average response for one minute post-stimulation), and heterophil to lymphocyte ratio (a broad measure related to immune stress in songbirds). LPS injection has been shown to pose an oxidative stress challenge in birds (Costantini & Møller 2009), so my measure of post-LPS total antioxidant capacity is related to antioxidant defenses present during immune activation. I found no significant differences between carotenoid-free WR and carotenoid-rich Y canaries in any of the parameters examined (all $p > 0.15$; Figure 8; Tables 5-6). While not statistically different, I did observe a trend toward increased oxidative burst response in WR birds compared to Y birds (Tables 5-6, Figure 8), indicating that a lack of carotenoid pigments was not inhibiting the ability of WR canaries to mount the response.

In August 2016, at least two weeks after individuals completed the LPS challenge and associated measurements, I extracted another small blood sample and then injected the birds intramuscularly in the breast muscle with human diphtheria-tetanus vaccine; this vaccine has been used successfully to induce antibody production in songbirds without causing lasting sickness. 10 days after injection, at the predicted peak of antibody response for songbirds (Westneat, Hasselquist & Wingfield 2003; Poston *et al.* 2005), I took a blood sample for use in quantifying each bird's antibody response to vaccination. In collaboration with Dr. Dennis Hasselquist (Lund University, Sweden), I used ELISA to estimate antibody production response by comparing antibody levels in the plasma of birds before vaccination to the levels 10 days post-vaccination. All experimental canaries had previously been dosed with the same vaccine in December 2015 (using identical methods), so I was able to compare the results of this secondary response to those of the primary response a year prior for a subset of individuals. Interestingly,

both Y and WR birds appeared to mount a strong primary but not secondary response to diphtheria (Tables 5 and 7); it is possible that the canaries were exposed to a pre-existing bacterial pathogen in summer 2016 that caused them to have high levels of circulating antibodies responsive to diphtheria during both the pre- and post-vaccination measurements, resulting in no noticeable antibody production response. However, both Y and WR birds mounted both a primary response and a stronger secondary response to the *tetanus* injection, and I focus on those results here. I found no significant difference in anti-tetanus antibody responses between Y and WR birds (Tables 5 and 7; Figure 8).

In the same blood sample extracted immediately before vaccination, I isolated a subset of plasma for use in a bacterial killing assay (BKA). I challenged each plasma sample with *E. coli* (ATCC 8739; Microbiologics) in triplicate, using a modified protocol based on that of French and Neumann-Lee (2012). Interestingly, I detected substantial inter-individual variation in bacterial killing capacity such that individuals within one color type tended to kill either >90% or <10% of challenged bacteria relative to controls; I found no significant difference in the likelihood of either WR or Y individuals to fully-kill their bacterial challenge (binomial regression; Tables 5 and 8).

Lastly, in December 2016, I exposed the birds to an oxidative challenge in the form of low-dose (50 rads) X-irradiation to induce increased pro-oxidant production *in vivo*. Low-dose ionizing radiation is a relatively novel method of oxidative challenge that has the advantage of inducing system-wide increases in pro-oxidants through the excitement of electrons in water molecules, without causing clinical disease symptoms or organ-specific dysfunction (as may be observed in chemical oxidative challenges; see Chapter 3; Koch & Hill 2017). 24 hours after irradiation, I extracted a blood sample for oxidative stress analysis. In addition to repeating the

same TAC measurement I quantified after LPS injection, I assessed the levels of an endogenous antioxidant (total glutathione; Cell BioLabs) in red blood cells as an indicator of whether WR birds may compensate for a lack of carotenoids by upregulating the production of other antioxidants. While the radiation challenge did induce a physiological response—TAC values were markedly higher for individuals post-radiation than they were post-LPS-injection (Tables 6 and 9)—I found no significant differences between Y and WR birds in TAC or total glutathione (Table 9; Figure 8). Interestingly, when I added total glutathione levels as a covariate in a model for TAC, there was a trend toward a significant contribution of glutathione to the variation I observed in TAC (Table 9); this suggests that glutathione played a role in overall antioxidant defenses but other factors were also important, which is appropriate for the goals of my study.

Collectively, my results show a clear lack of benefit to possessing internal carotenoid pigments across a variety of immune and oxidative stress parameters and under two different experimental physiological challenges (Figure 8). These data are the first to show unequivocal and definitive evidence against a direct role of internal carotenoids in boosting physiological function, in absence of a potential confounding effect of retinol. I argue that while the role of some carotenoids as retinol precursors may provide indirect benefits to physiological processes, carotenoids themselves pose little value to vertebrate immune or antioxidant function.

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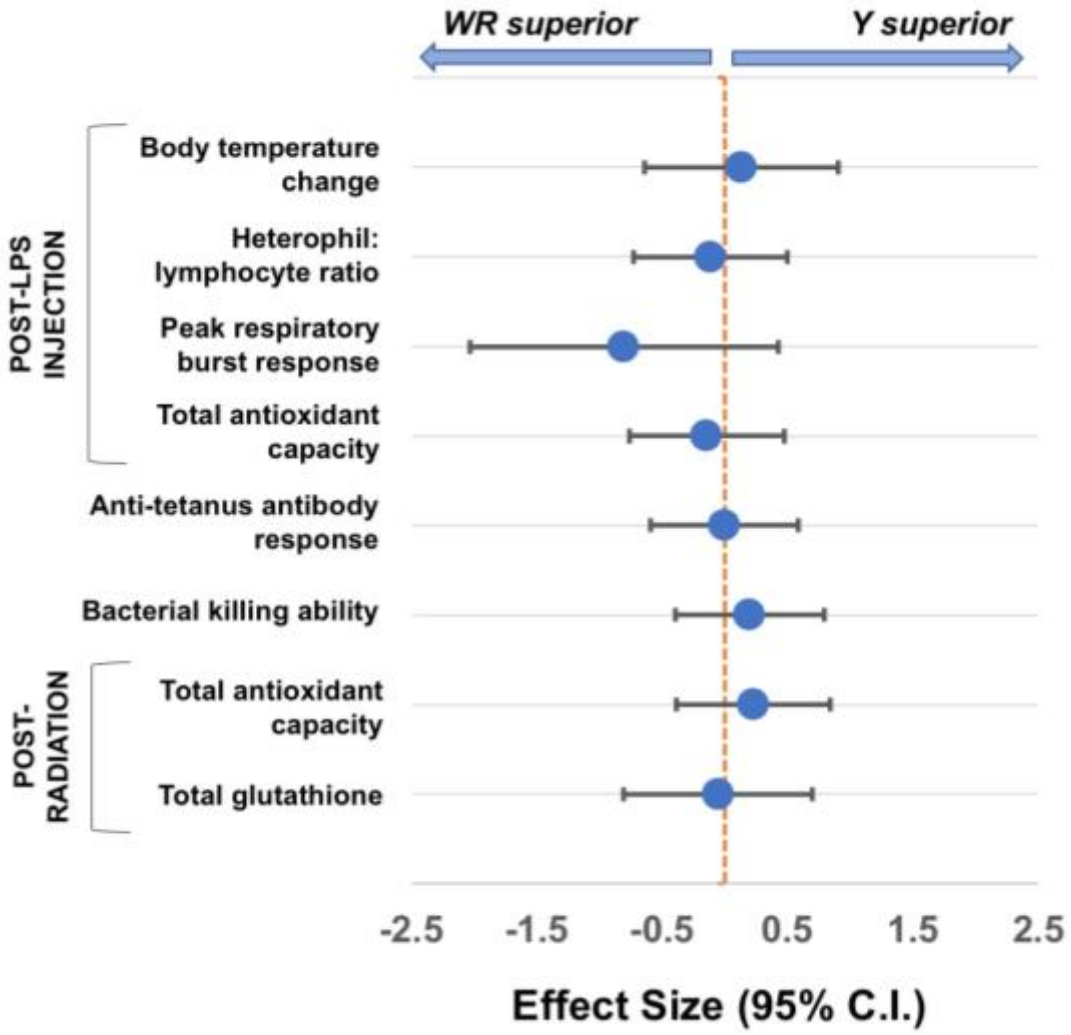

Figure 8. Summary of major WR vs. Y results. Standardized effect sizes (Hedges' *g*; Nakagawa & Cuthill 2007) with 95% confidence intervals to enable simple visual comparison of a representative subset of major measurement results for Y and WR canaries; points to the right of the midline (dashed orange line) indicate a superior response by Y canaries, while points to the left represent superior response by WR canaries.

Table 5. Immune and antioxidant parameters of white and yellow canaries. Average (± standard deviation) results for measurements of WR and Y canaries. See Tables 6-9 for statistical analysis results, and Appendix 1 for further detail.

		WHITE RECESSIVE	YELLOW
DESCRIPTION	**Plumage color**	White	Yellow
	Fat color	White	Yellow
	Plasma color	Clear	Yellow
	Average tissue lutein (μg lutein/g tissue)[1]	9.3 ± 18	83 ± 150
POST-LPS CHALLENGE	**Temperature change (ºC)**	0.47 ± 0.47	0.41 ± 0.44
	Mass change (g) (% change relative to baseline)	-0.58 ± 0.40 (-2.2 ± 1.4)	-0.48 ± 0.52 (-1.97 ± 2.08)
	Total antioxidant capacity (CRE)[2]	0.70 ± 0.34	0.66 ± 0.23
	Respiratory burst peak luminescence (nm)	44960 ± 30810	26094 ± 7420
	Respiratory burst average luminescence (nm)	20400 ± 12450	11790 ± 3672
	Heterophil:lymphocyte ratio	0.37 ± 0.19	0.34 ± 0.26
POST-VACCINATION	**Anti-tetanus primary antibody response[3]**	1.02 ± 0.57	0.67 ± 0.27
	Anti-tetanus secondary antibody response	1.60 ± 0.92	1.60 ± 0.81
	Anti-diphtheria primary antibody response	0.87 ± 0.81	1.01 ± 1.10
	Anti-diphtheria secondary antibody response	0.30 ± 0.81[4]	0.25 ± 0.75[4]
BKA	**% individuals fully killing bacterial challenge (average % killed[5])**	67% (64.6% ± 45.4%)	53% (66.6% ± 44.0%)
POST-RADIATON CHALLENGE	**Total antioxidant capacity (CRE)[2]**	1.05 ± 0.36	0.97 ± 0.39
	Total glutathione (μM)	38.17 ± 5.24	38.46 ± 5.51

[1] Tissue lutein levels were determined using high performance liquid chromatography on breast muscle, fat, and skin tissue, and are representative of differences in total carotenoid levels.
[2] Total antioxidant capacity is reported in units of copper reduction equivalent (CRE).
[3] Antibodies produced in response to secondary tetanus vaccine injection, quantified in relative optical density via ELISA in the lab of Dr. Dennis Hasselquist in Lund University, Sweden.
[4] The canaries did not appear to upregulate anti-diphtheria antibodies in response to secondary exposure.

[5] Percent *E. coli* killed in plasma samples relative to controls.

Table 6. Post-LPS measurement statistical analysis results. Results of ANOVAs investigating the effect of sex, color, and their interaction on response variables. See Table 5 for average values for WR and Y birds. Further details are presented in Appendix 1.

Focal measurement	Sample sizes	Variable	Sum of squares	F	p
Baseline mass	26 WR (16 M, 10 F); 25 Y (17 M, 8 F)	Color	93.9	8.92	0.004
		Sex	10.7	1.02	0.32
		Color*Sex	2.5	0.24	0.63
Change in mass		Color	0.65	0.20	0.66
		Sex	0.03	0.01	0.92
		Color*Sex	1.63	0.50	0.48
Baseline food consumption	26 WR (16 M, 10 F); 22 Y (15 M, 6 F)	Color	0.60	0.44	0.60
		Sex	3.27	0.08	3.27
		Color*Sex	1.23	0.27	1.23
Change in food consumption		Color	3.43	2.27	0.14
		Sex	2.77	1.84	0.18
		Color*Sex	3.86	2.56	0.12
Baseline body temperature	13 WR (8 M, 5 F); 13 Y (6 F, 7 M)	Color	0.49	2.52	0.13
		Sex	0.01	0.06	0.81
		Color*Sex	0.03	0.17	0.68
Change in body temperature		Color	0.03	0.14	0.72
		Sex	0.48	2.46	0.13
		Color*Sex	0.12	0.63	0.44
Total antioxidant capacity	23 WR (14 M, 9 F); 18 Y (13 M, 5 F)	Color	0.02	0.20	0.66
		Sex	0.07	0.78	0.38
		Color*Sex	0.01	0.07	0.79
Respiratory burst peak luminescence	6 WR (3 M, 3 F); 5 Y (3 M, 2 F)	Color	9.70×10^8	1.45	0.27
		Sex	2.25×10^8	0.34	0.58
		Color*Sex	6.07×10^7	0.09	0.77

Respiratory burst average luminescence		Color	2.02×10^8	2.10	0.19
		Sex	1.09×10^8	1.13	0.32
		Color*Sex	4.34×10^7	0.45	0.52
Heterophil:lymphocyte ratio	18 WR (11 M, 7 F); 16 Y (11 M, 5 F)	Color	0.0006	0.01	0.92
		Sex	0.0004	0.007	0.94
		Color*Sex	0.053	1.01	0.32

Table 7. Post-vaccination measurement statistical analysis results. Statistical results for anti-diphtheria secondary response are not reported because neither WR nor Y birds mounted a meaningful response (i.e. no difference in pre- vs. post-vaccination values). All tests reported here are for fixed effects linear models examining the effects of color (WR vs. Y) on antibody response, including sex as a covariate. Appendix 1 contains further detail.

Focal measurement	Sample sizes	Variable	Sum of squares	F	p
Anti-tetanus primary antibody response	23 WR (9 M, 14 F); 5 Y (3 F, 2 M)	Color	0.93	0.14	<0.001
		Sex	-0.35	0.26	0.19
		Color*Sex	0.23	0.2	0.27
Anti-tetanus secondary antibody response	23 WR (13 M, 10 F); 21 Y (14 M, 7 F)	Color	<0.001	<0.001	0.99
		Sex	4.00	5.89	0.020
		Color*Sex	0.44	0.65	0.43
Anti-diphtheria primary antibody response	23 WR (9 M, 14 F); 5 Y (3 F, 2 M)	Color	0.50	1.83	0.19
		Sex	0.35	1.27	0.27
		Color*Sex	0.37	1.35	0.26

Table 8. Bacterial killing ability analysis results. Statistical results of a generalized linear model (binomial) testing for a relationship between color (WR or Y) and tendency to fully-kill an *in vitro* bacterial challenge, including sex as a covariate. Appendix 1 contains further detail.

Focal measurement	Sample sizes	Variable	Estimate	SE	z	p
Bacterial killing	18 WR (9 F, 9 M); 22 Y (15 M, 7 F)	Intercept	1.02	0.65	1.58	0.11
		Color	0.18	0.7	0.26	0.79
		Sex	-0.62	0.73	-0.86	0.39

Table 9. Post-radiation challenge antioxidant analysis results. Statistical results of ANOVAs testing for relationships between color (WR vs. Y) and total antioxidant capacity or total glutathione, including sex and the interaction between sex and color as covariates, using measurements from plasma taken 24 hours after X-irradiation treatment. In the third analysis, total glutathione was added as a covariate in the total antioxidant capacity model to assess whether glutathione levels contributed to total antioxidant capacity measurements. For further detail, see Appendix 1.

Focal measurement	Sample sizes	Variable	Sum of Squares	F	p
Total antioxidant capacity	19 WR (11 M, 8 F); 26 Y (15 M, 11 F)	Color	0.08	0.569	0.455
		Sex	0.041	0.292	0.592
		Color*Sex	0.23	1.641	0.207
Total glutathione	13 WR (8 M, 5 F); 14 Y (7 M, 7 F)	Color	0.6	0.024	0.8773
		Sex	132.8	5.514	0.0278
		Color*Sex	38.6	1.604	0.218
Total antioxidant capacity (with glutathione)		Total Glutathione	0.467	2.731	0.113
		Color	0.002	0.009	0.925
		Sex	0.127	0.746	0.397

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APPENDIX 1
Detailed methods to accompany Chapter 5

Canary Husbandry

I performed the experiments described in Chapter 5 on a long-term research colony of after-hatch-year color-bred canaries held at the Auburn University Avian Research Laboratory 1 in Auburn, AL. Briefly, I held the canaries on a carotenoid-controlled diet of mixed canary seed (predominantly canary grass seed, mixed with rapeseed and thistle; All Natural Canary Blend, Jones Seed Company) coated with a carotenoid-free vitamin powder (AviVita Plus, Avitec Bird Supplies); the vitamin powder includes retinol (Vitamin A), which is critical to supply to white recessive birds that lack the necessary retinol precursor carotenoids. This diet contains a low quantity of carotenoids present in seeds, which should enable me to detect any trade-offs occurring; my primary goal was to avoid supplementing the birds with large amounts of carotenoids that might obscure any trade-off that could occur at lower carotenoid levels (see Chapter 2). Birds were sexed by vent morphology and behavior (singing, egg-laying) in the previous breeding season.

Statistics

For the data sets I describe below and in Chapter 5, I performed ANOVAs to test for significant differences in response measurements based on color type (Y vs. WR), sex (male vs. female), or the interaction of sex and color type. Measurements of internal performance have often been found to differ between the two sexes (e.g. Laaksonen *et al.* 2008; Love *et al.* 2008; McGraw *et al.* 2011). For LPS results analysis, I additionally performed two one-sample t-tests to assess whether the differences in mass or temperature before versus after LPS injection were significantly different than zero. All statistical analyses described were performed in R (version

3.2.3; R Core Team 2017). Detailed statistical results and average values for most measurements can also be found in Tables 5-9 of Chapter 5. When measurements were statistically significant, I also report least-square mean values and their standard errors in this Appendix.

LPS Challenge Experimental Methods

I monitored the canary colony for molt onset and progression during summer 2016. In July-August 2016, I ran a bacterial lipopolysaccharide (LPS; see below) challenge on birds that had fully initiated molt (new feathers emerging from pins on breast and belly, pins growing on back, rump, wings, tail, and face). Briefly, working with 2-6 individuals per day, I moved birds from their long-term colony cages to individual 12 x 16 x 16 inch cages. 24 hours prior to injection, I provided each bird with a known quantity of canary seed mix; I then collected all remaining seeds and husks from each bird's cage immediately prior to LPS injection (after 24 hours) to calculate resting food consumption for each bird (adjusting for each individual's baseline mass and the exact hours elapsed between measurements, resulting in units of mg seed consumed per hour per g body mass). I repeated this process for birds for the 24 hours immediately after LPS injection to test for any changes in food consumption following the challenge. I also measured the mass and temperature (using a Leaton Digital Thermocouple Thermometer inserted ~1 cm into the vent) of each bird 24 hours before, immediately before, and 8 hours after LPS injection. LPS injection has been found to decrease mass, body temperature, and food consumption in many species (Owen-Ashley & Wingfield 2006). For calculation of mass loss due to LPS injection, I subtracted final mass from initial mass, then divided the difference by initial mass and multiplied by 100 to estimate the percent of initial mass that was lost during the challenge.

On the morning of the LPS challenge, I injected each experimental bird intra-abdominally with 1 mg/mL lipopolysaccharide from *Escherichia coli* (O55:B5; List Biological Laboratories, Inc.) dissolved in PBS. 8 hours post-injection, I collected blood samples from each experimental bird by puncturing the wing vein with a 26 gauge needle. I dispensed approximately 30 μL of blood into a heparin-coated 1 mL capped tube for use in respiratory burst assays; I spread an additional small drop of blood evenly on a microscope slide for cell count analyses; and, I collected a final 150 μL of blood in two heparinized capillary tubes, which I immediately centrifuged to extract plasma and red blood cell samples for storage at -80C until further analysis. It is important to note that I intended to perform bacterial killing assays on plasma samples extracted 8 hours after LPS injection, but freezing for 1-2 weeks appeared to eliminate all bacterial killing ability in these canary samples, so bacterial killing assays were performed on fresh plasma samples on a later date.

Description of symptoms of LPS injection. On average, birds lost mass within 8 hours post-injection (see Chapter 5), and there was no difference in mass loss between WR and Y birds, although WR birds tended to have a slightly higher baseline average mass (least-square means ± standard error: 25.94 ± 0.65g (WR); 23.42 ± 0 0.70g (Y); Figure 9; Table 6, Chapter 5). There was no average difference in mass between the sexes (Table 6, Chapter 5). Birds also tended to show a slight increase in body temperature after 8 hours post-injection, which again did not differ (in either baseline or change) between WR and Y birds or between the sexes (Figure 9; Table 6, Chapter 5). Finally, I found that LPS injection did not affect food consumption (when comparing consumption over the 24 hours prior to injection to the 24 hours after injection, adjusted relative to baseline body mass), and that baseline food consumption did not differ

between WR and Y canaries, though individuals varied widely in their consumption. There was a trend toward females consuming slightly more food, on average, than males (Figure 10; Table 6, Chapter 5).

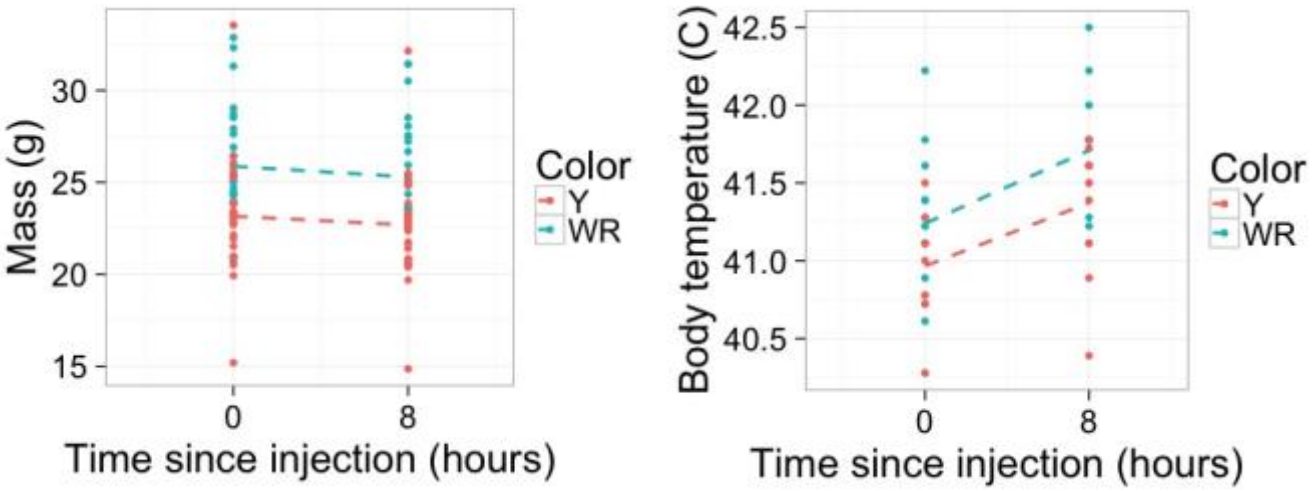

Figure 9. Effect of LPS on mass and body temperature. Scatterplots illustrating the baseline (0 hours) and post-injection (8 hours) mass (left plot) or body temperature (right plot) measurements for experimental Y and WR birds. Dashed lines indicate the change in average measurements between baseline and 8 hours after injection. The change in mass or temperature over time

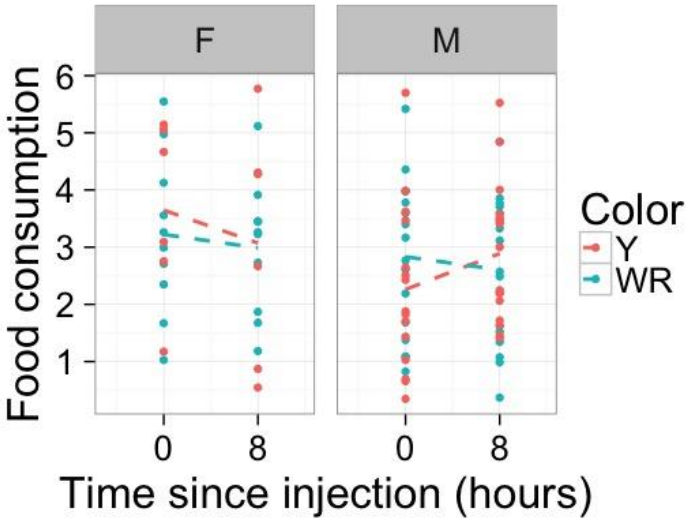

Figure 10. Effect of LPS on food consumption. Scatterplot illustrating the baseline (0 hours) and post-injection (8 hours) food consumpsion measurements for experimental Y and WR birds. Dashed lines indicate the change in average food consumption between baseline and 8 hours after injection. The left facet shows data for female canaries, the right facet shows data for males. Food consumption is in units of mg seed consumed per hour per g initial body mass.

Respiratory burst assay. I assessed the respiratory burst potential of immune cells contained in the fresh whole blood sample taken 8 hours post-LPS injection. Respiratory burst is the process by which immune cells produce large amounts of reactive oxygen species (ROS) to disrupt potential pathogens (Harmon 1998); however, because ROS cannot be "aimed" at specific targets, there is the potential for respiratory burst to also damage host tissue. As a consequence, one of the main roles for carotenoid pigments as antioxidants and immune boosters has been proposed to be defense against damage to self during respiratory burst (potentially by boosting immune cell development, or by quenching ROS as they are produced). Respiratory burst of songbird whole blood samples was previously analyzed by Sild & Hõrak (2010) and Sild *et al.* (2011), and they found no effect of dietary carotenoid supplementation on respiratory burst parameters. Here, I use nearly identical methods to test whether a complete absence of carotenoids affected the ability of immune cells contained within whole blood to mount a strong respiratory burst response.

I followed the kit protocol of the Analysis By Emitted Light (ABEL) Cell Activation Kit with Pholasin (Knight Scientific), modified for use without reagent injectors; this protocol uses a chemiluminscent reagent (derived from mollusks) that releases light upon reaction with ROS or other free radicals. I performed the respiratory burst assay in duplicate for each individual within 2 hours of post-LPS blood draw. First, I diluted 20 μL of each individual's whole blood in 2 mL of dilution buffer. Then, working with a single well at a time, I mixed 85 μL of assay buffer with 20 μL of adjuvant-K, 50 μL of pholasin (the chemiluminescent reagent), and 20 μL of diluted whole blood. I incubated the well at 37C for 5 mins, then read the luminescence in a luminometer every second for 60 seconds for baseline measurements. I then used a pipette to manually inject 25 μL of 1 mg/mL LPS (same solution as above), mixed once, then immediately

began reading luminescence every second for 60 seconds. Manual LPS stimulant injection with the pipette resulted in a 2 second delay between the addition of LPS and the beginning of the plate reading, but the delay was uniform among all samples and therefore was unlikely to affect relative differences between WR and Y birds.

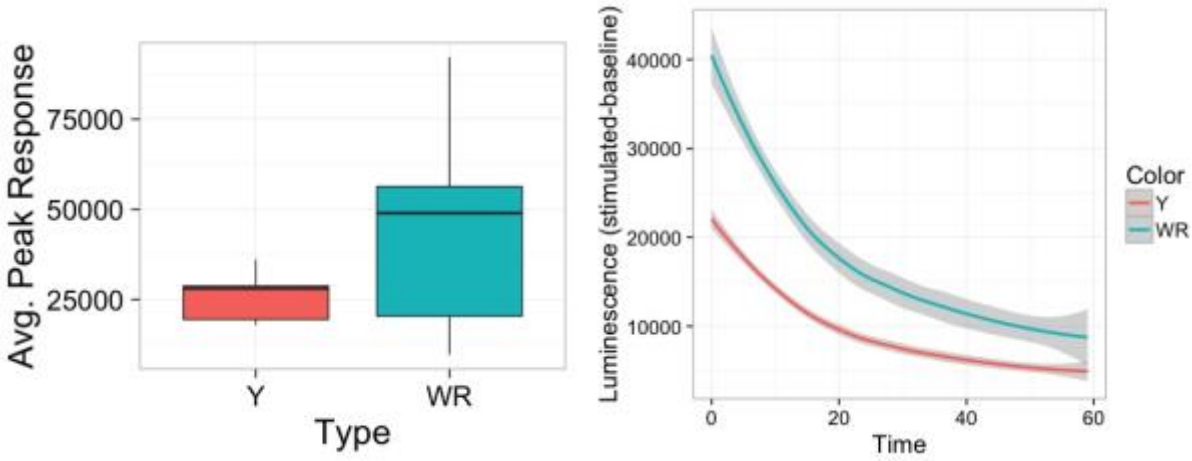

Figure 11. Post-LPS oxidative burst results. *Left:* Box plots illustrating differences in the peak luminescence (maximum oxidative burst response) of Y and WR individuals, averaged within each individual's two replicates. *Right:* Curves illustrating the average change in net luminescence (luminescence after LPS stimulation minus baseline luminescence) over time. Luminescence is reported in units of RLU (relative luminescence unit).

Due to unexpected loss of efficacy of reagents after one week of assays, I measured respiratory burst in only a subset of birds (6 WR and 5 Y). For each sample, I subtracted the baseline values from the experimental values to calculate the net increase in luminescence due to LPS-stimulated respiratory burst. I examined peak response (maximum luminescence) as well as average luminescence over the 60 second post-injection interval to estimate duration of response (Figure 11). Interestingly, there was a trend toward a stronger response in WR birds compared to Y birds (see Table 6), which indicates full immune cell functionality in carotenoid-free birds; in this *in vitro* test, it is also possible that carotenoids in the plasma of Y birds indeed served to dampen ROS reactivity with the luminescent reagent by quenching the ROS rapidly upon production. However, I also observed considerable variation among individuals—particularly in

the peak response of WR canaries (Figure 11)—which questions the presence of a consistent difference in carotenoid-free vs. carotenoid-rich birds.

Heterophil:lymphocyte ratio. The ratio of heterophils (the avian analog to mammalian neutrophils) to lymphocytes, while not a definitive reflection of any one immune parameter, has previously been cited as a general indicator of overall immune activation in birds (Gross & Siegel 1983; Al-Murrani *et al.* 2006), and has specifically been related to health (e.g. Davis, Cook & Altizer 2004) and carotenoid-based coloration (e.g. Maney *et al.* 2008) in songbirds. To assess H:L ratio in WR and Y canaries, I first fixed and stained (Fisher HealthCare PROTOCOL Hema 3 Fixative and Solutions) slides of whole blood smears (collected 8 hours post-LPS injection; see above) to visualize cell types. Using standard techniques for avian blood cell counting (e.g. Maney *et al.* 2008) and type identification, I counted at least 10,000 total cells per individual (estimated based on total slides viewed and average cell counts per 3 representative slide views); I examined multiple regions of each blood smear to gain an accurate reading of blood cell parameters across the slide, and I only measured views of cells evenly spread in a single layer. I counted total cells, number of heterophils, number of lymphocytes, and number of thrombocytes; no other cell types were present across all individuals or were consistently identified. I divided total number of heterophils by total number of lymphocytes to calculate the H:L ratio for each individual (Figure 12).

Post-LPS total antioxidant capacity. I measured total antioxidant capacity in plasma samples using the TAC kit (Cell BioLabs) according to the provided protocol. After preliminary testing, I established that a 1:4 dilution of canary plasma yielded best results within the provided standard

curve. I diluted 5 μL of plasma in 15 μL of PBS in duplicate for each individual. Results are reported in units of μM Copper Reduction Equivalents (CREs), which relates to the relative capacity of the measured samples to reduce copper (II) to copper (I).

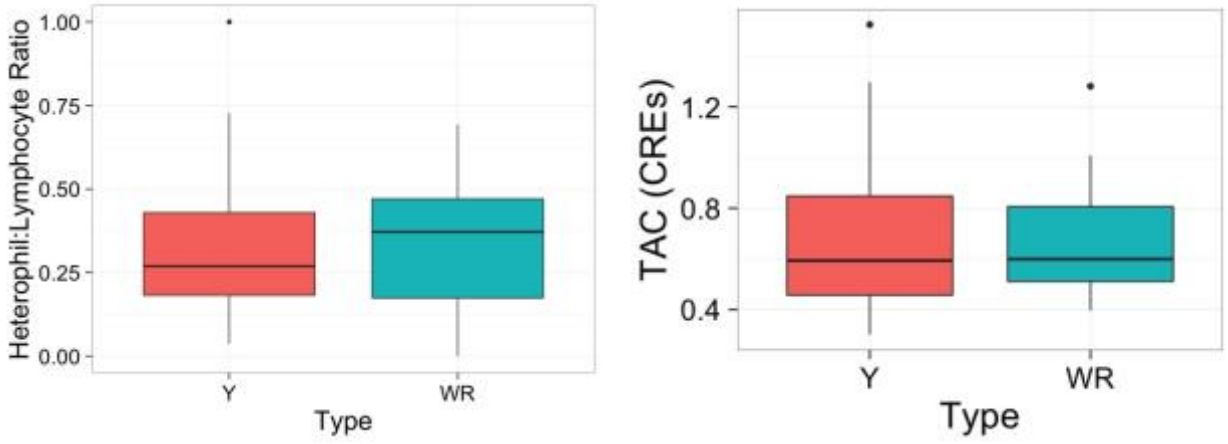

Figure 12. Post-LPS H:L ratio and total antioxidant capacity results. Box plots of average H:L ratios (left plot) or TAC values (right plot) detected 8 hours post-LPS injection.

Vaccination Experimental Methods

Primary vaccination. In December 2015, I extracted 75 μL of blood (pre-vaccination sample) from each experimental canary and then immediately injected each bird intramuscularly with 100 μL of pharmaceutical-grade diphtheria-tetanus vaccine (2.7 lf of diphtheria toxoid and 2 lf of tetanus toxoid), dispensing 50 μL into each breast muscle. 10 days later, I extracted a second 75 μL blood sample (the post-vaccination sample). All blood samples were kept on ice and centrifuged to extract plasma from red blood cells for storage at -80C until analysis.

Secondary vaccination. In August 2016, I repeated these methods (pre-vaccination blood sampling, injection, and 10-day-post-vaccination blood sampling); a subset of plasma from the

pre-vaccination sample was used immediately in bacterial killing assays (20 μL; see below), while the remainder of plasma was frozen at -80C until further analysis.

Analysis. In September 2016, frozen pre- and post-vaccination plasma samples (~15 μL each) were transported to Lund University (Lund, Sweden) for antibody analysis in the lab of Dr. Dennis Hasselquist. Antibody responses were assessed using ELISA methods developed in several songbird species for use quantifying anti-diphtheria and anti-tetanus antibodies in avian plasma. Briefly, plasma samples were aliquoted in 3 μL replicates and diluted 1:500, 1:1000, or 1:2000 (the former two dilutions were used in anti-diphtheria analyses, and the latter two were used for anti-tetanus analyses). Preliminary tests indicated that these dilutions produced measurable responses falling within the bounds of measurements from a positive control used on all plates (serial dilutions of plasma from great tits (*Parus major*) with known strong responses to both diphtheria and tetanus). Working with one toxoid and one sample dilution per plate, diluted samples and the great tit controls were added in duplicate to 96-well microplates previously coated in either tetanus or diphtheria toxoids and incubated overnight. After washing the plates, leaving the layer of canary antibodies conjugated to the diphtheria or tetanus toxoids coating the plates, a dilution of rabbit anti-songbird antibodies was then added to conjugate with the canary antibodies. After incubating and further washing, an anti-rabbit antibody was added, the plate was incubated and washed one more time, and finally reagents were added to allow for the measurement of density of conjugated antibodies in each well spectrographically. Color change was assessed in a 10-minute kinetic assay, and the slopes of color-change for each sample were analyzed for strength of response (i.e. steeper slope indicated stronger response). Slope measurements are in the units of milli-optical-density (milliOD) per minute. Each

measurement was adjusted according to among-plate variation (using variation in the measurements of the great tit control samples), duplicate measurements of each individual were averaged, (for duplicates with less than 10% variation), and the results were log-transformed the results for linearity. Finally, the results were z-transformed into unitless values around a mean of zero to allow for the two different dilutions within one antigen for each individual to be averaged for a final, composite score for each bird for both pre-vaccination and post-vaccination samples. The net "antibody response" for each individual was calculated as the difference between pre- and post-vaccination composite scores.

See previous publications from the Hasselquist lab for more detailed methodological descriptions (e.g. Hasselquist *et al.* 1999; Ilmonen, Taarna & Hasselquist 2000).

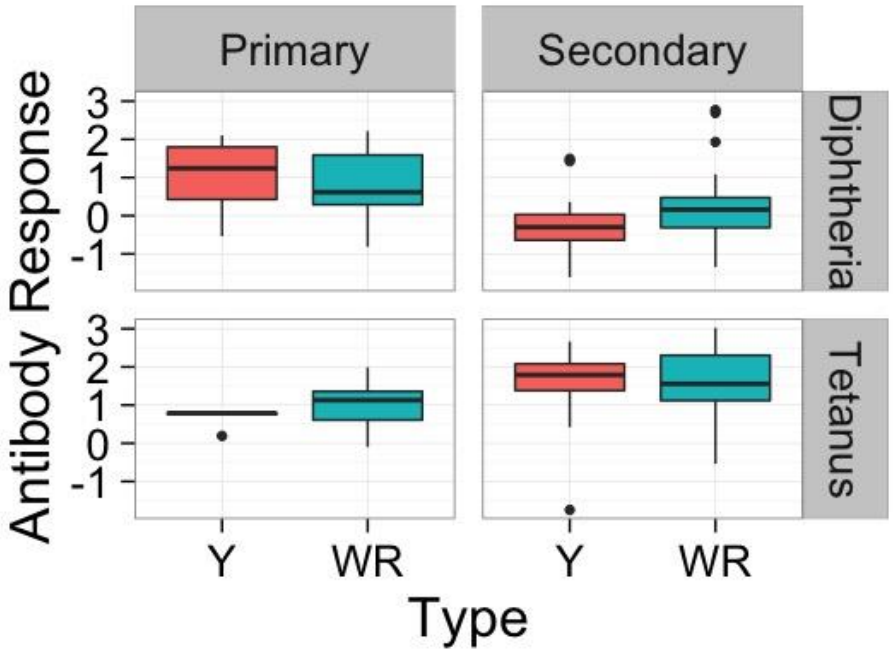

Figure 13. Antibody responses to vaccination. Boxplots illustrating the relative antibody responses (the difference in measured response after injection relative to before injection) of canaries to primary (left) and secondary (right) injections and diphtheria (top) or tetanus (bottom) antigens. Note that secondary responses to diphtheria were centered at or below 0, indicating no functional response.

Bacterial Killing Assay

On a subset of plasma extracted pre-vaccination in August 2016, I performed a microplate-based plasma bacterial killing assay modified from the methods described in French and Neumann-Lee (2012). Briefly, the day prior to assays, I reconstituted a bacterial pellet (*E. coli*, ATCC 8739; Microbiologics Epower) in 40 mL of warm, sterile PBS and assessed colony forming units (CFUs) using standard agar plating protocols under sterile conditions in a laminar flow hood. Based on calculated CFUs, I diluted the stock bacterial solution to obtain a working solution of ~1 x 10^5 CFUs for assays.

From blood samples collected 8 hours post-LPS-injection (see above), I extracted plasma from each capillary tube under sterile conditions and retained 20 μL of each individual's plasma in a single, sterile 1.5 mL pop-cap tube for BKA use (the remaining plasma and packed red blood cells were frozen at -80C). I diluted plasma with 80 μL of sterile PBS, vortexed the mixture thoroughly, then plated 20 μL of each diluted sample to a "negative plasma control" well on a 96-well, round-bottomed microplate. These negative plasma controls served as a test for blood sample contamination—no bacterial growth should be evident in these samples unless the plasma was contaminated. I repeated these methods for one sterile fetal bovine serum (FBS) control, which served as a point of comparison for bacterial growth in the presence of plasma *without* bacterial killing components. This FBS control was used as my primary base of comparison for later analysis because I found that the bacteria used in my assays tended to grow more vigorously when supplied with plasma (in the absence of significant killing), so the PBS-only positive control growth values were uninformative as a comparison to the plasma sample growth values.

I added 8 μL of my prepared bacterial working solution to each individual's remaining 80 μL of diluted plasma, vortexed the mixture, then incubated each tube at 37.4C for 30 minutes. 80 μL of diluted FBS and 80 μL of sterile PBS were each also mixed with 8 μL of bacterial working solution for positive controls. After incubation, I vortexed samples again and plated 20 μL of each sample (canary, FBS, or PBS) to additional wells on the microplate; I tested each control and each canary's plasma in triplicate, with one additional negative control well (with no bacteria added) for every individual. Lastly, I plated three wells of sterile PBS as negative controls. I added 125 μL of sterile tryptic soy broth to each well, mixed gently using a multichannel pipette, and read the plate at a wavelength of 600 nm (for baseline absorbance). I then covered the plate and incubated on a rocker for 12 hours at 37.4C before performing a second reading at 600 nm.

These methods were determined after several pilot analyses involving different concentrations of plasma, bacteria, or whole blood samples, and different incubation lengths. I selected the above dilutions because I found them to best isolate a range of results among individuals; other dilutions tended to result in either no bacterial growth or no evidence of killing in experimental wells. However, the results were unusual in that individuals tended to consistently either completely kill (<10% bacterial growth compared to FBS positive controls) or completely fail to kill (>90% bacterial growth compared to FBS positive controls) their bacterial challenge. As such, I performed a binomial regression analysis to assess statistical patterns in my data. First, I calculated an average net absorbance for each individual and control by subtracting baseline (time 0) values from 12-hour values to remove any underlying differences in absorbance between samples. I then eliminated any outlying values in each individual's triplicate of absorbance readings that differed more than 10% from the other two values. Finally, I averaged

the remaining net absorbance values for each individual and control. I divided this final net absorbance for each individual by the final net absorbance of the FBS positive control on the same microplate to obtain a value for percent difference in absorbance—or, more specifically, percent difference in bacterial growth—between samples and control. This percent-bacteria-killed value for each individual was used in further analyses.

I excluded five data points with percent-bacteria-killed values between 10 and 90% (i.e. those with partial killing) so that all remaining individuals could be categorized as having fully killed or fully failed to kill their bacterial challenge (Figure 14). After the exclusions, I assessed bacterial killing using a binomial regression testing for differences among colors and sexes (Table 8, Chapter 5).

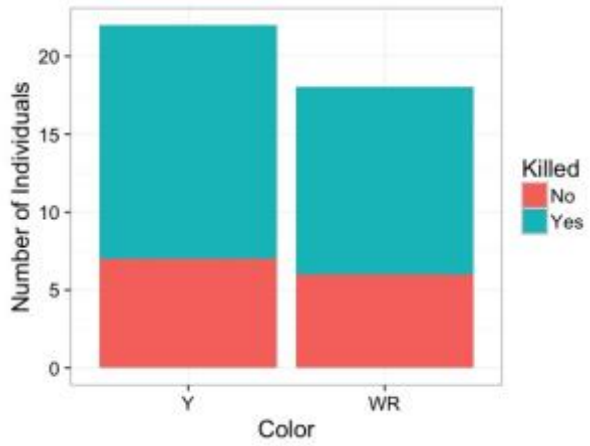

Figure 13. Bacterial killing results. Plot illustrates the proportion of individuals that either completely killed (top of bar, turquoise) or completely failed to kill (bottom of bar, coral) their bacterial challenge.

Radiation Challenge Experimental Methods

In December 2016, I dosed experimental canaries with 50 rads of X-irradiation using a PRIMUS (Siemens) linear acceleration at the Radiology Department of Clinical Science in the Auburn University College of Veterinary Medicine. Each bird was secured in a brown paper bag during exposure. The dose was based on a low dose previously reported to induce an oxidative damage

increase in two other songbird species (Luloff *et al.* 2011). I observed no change in mass or clinical signs of distress after the procedure. 24 hours post-irradiation, I extracted a 150 μL blood sample and centrifuged to isolate plasma from red blood cells; I froze both for further analysis.

Post-radiation total antioxidant capacity. I performed total antioxidant capacity measures on the post-radiation plasma samples, using identical methods as described above (Figure 15).

Total glutathione. I assessed the total glutathione concentration of post-radiation red blood cell samples using the Total Glutathione Assay Kit (Cell BioLabs). Briefly, I mixed 10 μL of red blood cell pellet from each individual with 40 μL of a 5% metaphosphoric acid solution; after centrifuging the mixture, I extracted the supernatant and diluted it 1:250 (optimized according to pilot assay results) in an assay buffer containing 0.5% metaphosphoric acid. I then followed the kit protocol as listed; the procedure converts any reduced glutathione to oxidized glutathione, which is then measured through a chromogenic reaction, in order to assess *total* glutathione levels without regard to specific oxidation state. My goal was to gain an indicator of an endogenous antioxidant level in both WR and Y canaries in order to gauge whether carotenoid-free birds may have upregulated other antioxidants to compensate for the absence of carotenoids. I assessed each individual's total glutathione levels in duplicate and compared them to a standard curve of known total glutathione concentration (Figure 15).

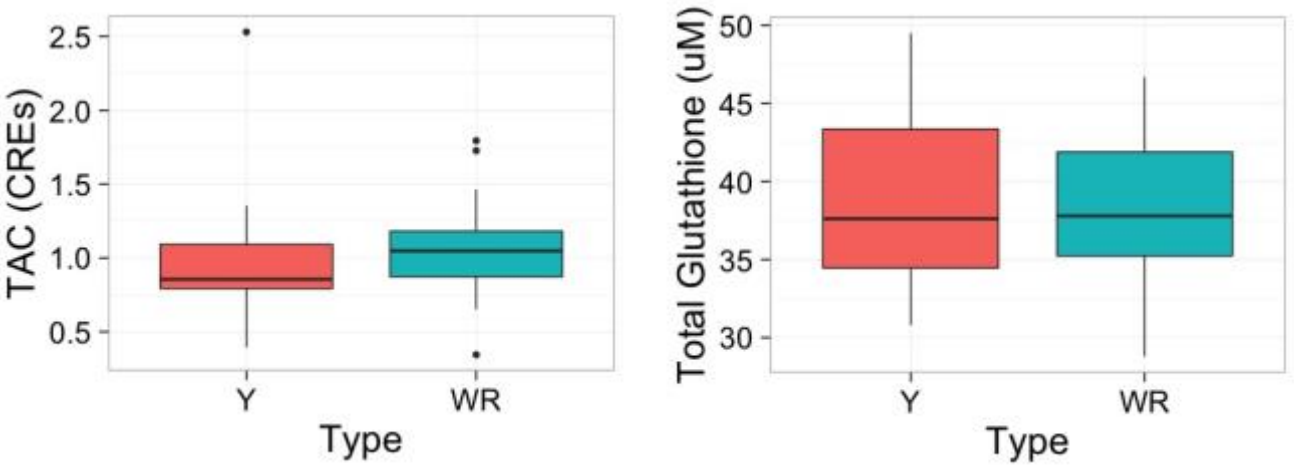

Figure 15. Post-radiation total antioxidant capacity and glutathione results. Box plots of average total antioxidant capacity (in CREs; left) and total glutathione values (right, in μM) detected 24 hours post-irradiation.

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