Evolutionary and physiological processes involved in carotenoid coloration in animals

by

Ryan Jeffrey Weaver

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 4, 2018

Keywords: honest signaling, carotenoids, *Tigriopus*,
sexual selection, mitonuclear

Approved by

Geoffrey Hill, Chair, Professor, Department of Biology
Paul Cobine, Associate Professor, Department of Biology
Jennifer Newbrey, Associate Professor, Department of Biology, Columbus State University
Alan Wilson, Associate Professor, School of Fisheries, Aquaculture, and Aquatic Science

Abstract

Colorful animal ornaments have been a core research focus of the field of sexual selection in evolutionary biology, and carotenoid-based coloration has been particularly well-studied. Animals as diverse as fish, reptiles, and birds, have co-opted carotenoids from their potential physiological roles for use as colorants of conspicuous yellow, orange, and red sexual ornaments. Nearly 40 years of research has revealed a striking pattern in which animals sometimes prefer to mate with individuals with the most richly colored carotenoid-based ornament because they tend to be of higher quality than duller individuals of the same species. What is less clear however, is how variation in coloration between individuals is created. Here, I first discuss two hypotheses about the mechanisms that underlie variation in coloration of sexual ornaments within a species. I compare and contrast the costly signaling hypothesis, which posits that differential costs among high and low-quality individuals maintains honesty from colorful ornaments and the index signaling hypothesis, which states that color signals can be cost-free and honest when their production is intimately tied to core cellular process. Next, I present a test of the predictions of those hypotheses by examining patterns of condition-dependent signaling from carotenoids in the bird coloration literature using meta-analysis. Most coloration from carotenoids comes from two distinct pathways: animals either deposit carotenoids unmodified from their diet to the integument, or they metabolize dietary carotenoids though oxidation reactions to produce ketolated carotenoids used for coloration. I show that coloration derived

from converted, but not dietary, carotenoids is tightly linked to measures of individual quality, suggesting that carotenoid conversion is an important factor in maintaining honesty from carotenoid-based ornaments. Despite the long history of carotenoid research, a vertebrate model system that is amenable to study the mechanistic basis for variation in carotenoid coloration and metabolism has not been identified. The biochemical processes of converting yellow dietary carotenoids to red carotenoids are conserved across taxa; from crustaceans to birds. Considering this conservation, I then propose a novel system for carotenoid physiology research, the red marine copepod *Tigriopus californicus*, and demonstrate that they convert dietary yellow carotenoids to the red carotenoid, astaxanthin. Next, using this copepod system, I test predictions of the hypothesis that mitochondrial function mediates the conversion of yellow carotenoids to red. I employ two methods to manipulate mitochondrial function: exogenous heavy metal exposure and hybridization. I demonstrate that modulation of aspects of mitochondria are associated with reduced accumulation of astaxanthin, which may be due to impairment of bioconverting yellow dietary carotenoids. Overall, my dissertation highlights key details about carotenoids used for coloration of sexual ornaments that have not been incorporated in hypotheses for the maintenance of honesty from colorful ornaments. Additionally, it describes a system that is amenable to experimental manipulations and is poised to answer decade's old questions about the evolution of carotenoid coloration in animal ornaments.

Acknowledgements

Nearly 10 years ago, I quit my career as a chef to pursue a degree in science at Virginia Commonwealth University where I eventually met amazing and inspiring people who would have profound impacts on both my personal and academic pursuits. While there are many who deserve recognition, two people in particular have my greatest gratitude and respect: Dr. Lesley Bulluck and Anna Tucker. The mentorship of Dr. Bulluck and the opportunities she has provided for me will remain among the greatest gifts that I've received, without which, any successes I may achieve in science would not be possible. Anna Tucker, Dr. Bulluck's Master's student at the time, has also played a major role in my life. Starting from being classmates, to her field assistant, to friends, and now as her husband and partner as power-couple scientists, I am deeply indebted to my wonderful Anna for her support, encouragement, and help with gglplot, tidyverse, and writing R functions that help figure out what time to leave for the Atlanta airport to not be rushed or miss our flight. That time change is tricky! My advisor Dr. Geoff Hill has been instrumental in my development as a creative and critical thinker and I am grateful for his excellent mentorship, which I will benefit from for the rest of my career. My labmates Becca and Roy have also been a great source of inspiration and friendship that certainly made my time as a graduate student better and my time outside of the lab much more enjoyable. Lastly, I'll thank my dog Cedar for being a good boy (mostly) and giving me a reason to get out of bed (to feed him) and to enjoy time off (by playing fetch).

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AB – Abalone Cove, CA

ANOVA – Analysis of variance

ASW – Artificial seawater

ATP – Adenosine tri-phosphate

CI – Confidence interval

COI – Cytochrome c oxidase, subunit I

CRI – Credible interval

DNA – Deoxy ribonucleic acid

GAPDH – Glycerol somthinge phosphatase dehydrogenase

GR – Glutathione reductase

HL – Heterophil:lymphocyte ratio

HPLC – High performance liquid chromatography

HSD – Honestly significant difference

MCMC – Monte Carlo Markov Chain

PCA – Principal component analysis

PCR – Polymerase chain reaction

PHA – Phaeghaemagluttination response assay

PO – Phenol oxidase

PRISMA – Preferred reporting is systematic reviews and meta-analysis

qPCR – Quantitative polymerase chain reaction

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SD – San Diego, CA

SE – Standard error

UPLC – Ultra-high performance liquid chromatography

UV – Ultra-violet

WBC – White blood cell count

Chapter 1. What maintains honesty from colorful ornaments used in mate choice?

Manuscript published with co-authors Rebecca Koch, and Geoffrey Hill in 2017 in *Philosophical Transactions of the Royal Society, B.* **372**, 20160343.

Introduction

Female mate choice is hypothesized to be an important form of selection on bold and brilliant color displays of male animals (Darwin, 1871; Andersson, 1994; Hill, 2006b). In particular, it is widely proposed that choosing females assess prospective males by their colorful ornaments because color displays can be honest signals of individual quality (Hamilton and Zuk, 1982; Hill, 1991; von Schantz *et al.*, 1999). For signals of quality to be effective and evolutionarily stable, they must be reliable such that there is a consistent correlation between ornament expression and some unobservable male quality that is of interest to the female (Zahavi, 1975, 1977; Grafen, 1990; Maynard Smith and Harper, 2003). Thus, it has been proposed that ornaments should evolve and be the object of female assessment only if they remain honest signals of the true condition or quality of a male (i.e. condition-dependent ornaments, Grafen, 1990).

The logical challenge to the concept of honest signaling is cheating. During mate choice, the interests of a choosing female and a displaying male are fundamentally different (Parker, 2006; Arnqvist and Rowe, 2013). It is to each male's advantage to present a signal that maximizes his chances of mating—even if it is a dishonest reflection of his quality—while it is to each female's advantage to sort prospective mates by aspects of quality that will affect her reproductive success (Hill, 1994b; Gavrilets *et al.*, 2001). A wealth of empirical data from

diverse taxa shows that coloration can be an honest signal of male quality (Hill, 2006a; Svensson and Wong, 2011; Garratt and Brooks, 2012), so the fundamental question is: what maintains the honesty of colorful ornaments in mate choice systems (Maynard Smith and Harper, 2003; Searcy and Nowicki, 2005; Higham, 2013)?

In this essay, I review two hypothesized mechanisms for the enforcement of the honesty of color signals assessed by females during mate choice: 1) the Costly Signaling Hypothesis (Zahavi, 1975) and 2) the Index Hypothesis (Maynard Smith and Harper, 1995; Biernaskie *et al.*, 2014). The former is the foundation for the most widely accepted and cited hypotheses for how color signals remain honest, proposing that costs entailed in the production or maintenance of color displays ensure the honesty of the signals. On the other hand, the Index Hypothesis, which proposes that signal honesty is maintained by cost-free mechanisms that arise from the inexorable links between male condition and signal expression, is an often-overlooked but important alternative as the basis for honest signaling from coloration. I first briefly review the framework of the Costly Signaling and Index Hypotheses and consider their application to color signals. I assess how including the perspective from the Index Hypothesis provides important alternative explanations for experimental results from two empirical studies of colored ornaments. Finally, I discuss how future studies can better tease apart whether honest color signals used in mate choice are shaped by costly or cost-free mechanisms—or a combination of both.

Mechanisms of honest signaling

The Costly Signaling Hypothesis

The Costly Signaling Hypothesis requires that signals be costly to be reliable (Spence, 1973; Zahavi, 1975; Johnstone, 1995). In the realm of animal coloration, costs come in a variety

of forms such as increased predation risk (1a, Table 1-1) or resource allocation towards ornamentation instead of body maintenance (1b, Table 1-1). However, for signaling to be evolutionarily stable with little cheating, these costs associated with ornamentation must ultimately result in a decrease in fitness (Zahavi, 1975; Grafen, 1990; Maynard Smith and Harper, 1995, 2003; Hasson, 1997). Moreover, a key premise of costly signaling theory is that ornamentation will impose a higher cost for low quality males than high quality males (Pomiankowski, 1987; Grafen, 1990; Hurd, 1997; Getty, 1998a). Consequently, signals remain honest because males of low quality simply cannot recover sufficient fitness from dishonest signaling to compensate for the high cost of ornamentation. For example, bold and conspicuous coloration may slightly increase the predation risk for a high quality male with good stamina and agility, but the same coloration may *greatly* increase the predation risk for a low quality male that is less able to evade predators (Candolin, 1999). Similarly, high and low quality males may suffer differential effects of allocating resources to ornament production and away from physiological functions that contribute to fitness (Halliday, 1987; Zera and Harshman, 2001; 1b, Table 1-1). As with predation risk, allocating critical resources to ornamentation rather than body maintenance may cause low quality males to suffer higher fitness costs than high quality males (Kodric-Brown and Brown, 1984; Møller *et al.*, 2000; McGraw *et al.*, 2010). The unifying theme of costly signaling is that the fitness costs of ornamentation maintain signal honesty because only high quality males gain a net fitness benefit from producing the color displays most preferred by females (Grafen, 1990).

Zahavi (Zahavi, 1975) proposed the first conceptualization of the Costly Signaling Hypothesis in biology with his handicap principle, which states that ornaments pose a survival cost on the signaler such that only high quality males can withstand the handicap of being fully

ornamented. Bearing a large ornament is a strategic cost –a cost in excess of what is needed to unambiguously convey information (Maynard Smith and Harper, 2003)– and thus this form of honest signaling has been termed wasteful signaling (Zahavi and Zahavi, 1997). Zahavi's hypothesis proposed survival costs as an intuitive mechanism for maintaining signal reliability, but his verbal model was met with much early criticism (reviewed in Maynard Smith and Harper, 2003). The plausibility of the handicap principle was not validated until the 1980s through mathematical modeling by Enquist (Enquist, 1985), Pomiankowski (Pomiankowski, 1987), and especially Grafen (Grafen, 1990). Grafen's game-theoretic approach showed that strategic costs could maintain honesty if lower quality individuals paid more than high quality individuals to achieve full ornamentation (Grafen, 1990). This validation bolstered Zahavi's handicap principle as the paradigm for the evolution and persistence of honest animal signals in the wild.

Many recent critiques of the handicap principle argue that the *realized* costs of signaling—the actual decrease in fitness incurred by giving the signal—required by the handicap principle are not wholly sufficient, or required, to prevent cheating. (Lachmann *et al.*, 2001; Bergstrom *et al.*, 2002; Grose, 2011; Számadó, 2011; Higham, 2013; Kane and Zollman, 2015). The key points of these critiques are that a high signal cost is not the only mechanism that maintains honesty, and that cheap or cost-free signals (i.e., signals imposing little or no reduction in fitness) can be honest. For example, when the *potential* costs of cheating are sufficiently high—such as risk of punishment through harassment of cheaters—the realized costs of honest signals could be zero (Számadó, 1999, 2011; Lachmann *et al.*, 2001); in other words, a signal that is not difficult to produce or maintain can still be honest if cheaters run the risk of paying high costs through social mediation or other means. As long as high-quality individuals and not low-quality individuals gain a net fitness benefit from giving a high-quality signal, costs paid for

giving the signal are arbitrary in maintaining signal honesty (Számadó, 1999; Lachmann *et al.*, 2001; Bergstrom *et al.*, 2002). Despite the mounting evidence that handicap-like costs are perhaps unlikely to maintain honesty in most signaling systems (Bergstrom *et al.*, 2002; Getty, 2006; Számadó, 2011; Zollman, 2013; Huttegger *et al.*, 2015), research on animal coloration frequently cites costly mechanisms, like those of the handicap principle, for maintaining signal honesty (Pryke *et al.*, 2001; Alonso-Alvarez *et al.*, 2008; Freeman-Gallant *et al.*, 2011; Roulin, 2016).

While Zahavi's handicap principle of honest signaling originally focused on maintenance costs and increased mortality as the means to ensure signal honesty (Zahavi, 1975), research on honest signaling via color displays in recent decades has largely extended the concept of costly signals to include production costs and especially resource tradeoffs (discussed in detail below) as the potential mechanisms for maintaining honesty in color signals (Verhulst *et al.*, 1999; Roulin, 2016). The Costly Signaling Hypothesis is an overarching concept that extends the Handicap Principle (Table 1-1) to propose that the costs associated with ornament production or maintenance are key to signal honesty. However, others have shown that alternative cheap or cost-free mechanisms can also maintain signal honesty (Hamilton and Zuk, 1982; Getty, 1998b; Számadó, 1999, 2011; Lachmann *et al.*, 2001; Biernaskie *et al.*, 2014; Kane and Zollman, 2015) but have largely been ignored in the animal coloration field.

The Index Hypothesis

The second major explanation for honest signaling in the context of mate choice is the Index Hypothesis, which proposes that coloration can serve as an uncheatable signal of individual quality if it is inexorably linked to individual condition (Maynard Smith and Harper, 1995; Hill, 2011; Biernaskie *et al.*, 2014). By this idea, signal honesty is not maintained by costs but is instead ensured because signal production relies directly on the function of internal

processes that cannot be faked. Proponents of the Index Hypothesis recognize that all behaviors and physiological processes inevitably require energy, resources, and/or time that could potentially have been allocated toward other functions. However, the Index Hypothesis proposes that the requirements of producing or maintaining the signal are not sufficient to impose handicaps or other costly constraints described by the Costly Signaling Hypothesis. Under the Index Hypothesis, signals are honest because their expression is fundamentally linked to core physiological processes—not because of costs associated with that signal.

The concept of index traits has long been accepted as a cost-free mechanism to ensure honesty of vocal and behavioral displays (Maynard Smith, 1956; Riechert, 1978; Enquist, 1985; Maynard Smith and Harper, 1995). For example, in Red Deer, the pitch of roaring vocalizations is causally linked to body size (larger males have a longer vocal apparatus that produces a deeper pitch) and thus fighting ability (larger males are also more successful in intrasexual competition) (Clutton-Brock and Albon, 1979). In this example, roaring is an honest signal of fighting ability because it is an index of body size that cannot be faked (Maynard Smith and Harper, 2003). However, the concept of index traits has rarely been invoked to explain the signal honesty of animal coloration (Searcy and Nowicki, 2005; Irschick *et al.*, 2015).

Hamilton and Zuk's essay on plumage color as an honest signal of parasite resistance was the first time a cost-free mechanism was proposed to maintain honesty in animal color signals (2a, Table 1-1; Hamilton and Zuk, 1982). They concluded that bright feather coloration acts as an honest signal of heritable parasite resistance. While the mechanisms of honestly signaling genetic parasite resistance through feather coloration was not explicitly discussed, Hamilton and Zuk rejected the idea that females would select for a handicap. Instead, they proposed that females would select for a 'demonstration of health that cannot be bluffed' (note 20 in Hamilton

and Zuk, 1982), which is precisely what I and others (Maynard Smith and Harper, 1995) argue to be an index signal.

More recently, Hill (Hill, 2011) revived the idea that a cost-free mechanism could ensure honesty from animal color signals by proposing that condition-dependent expression of colorful ornaments can arise when the underlying physiological pathways that produce coloration are dependent on the same core cellular processes that are the key determinants of individual condition (2b, Table 1-1). By this 'shared pathway' concept, color displays can be fully executed only if the biochemical and cellular processes of an individual are functioning well. System functionality, in turn, depends on the combination of genetic, environmental, and gene by environment interactions that determine an individual's condition (Hill, 2011, 2014). Ornament expression matches the condition of the male because maximum expression is trivial if the individual is functioning well, but impossible if the individual is functioning poorly. Mitochondrial function in particular has been proposed to be a key physiological process that links ornamentation to overall condition (2c, Table 1-1) (Koch *et al.*, 2016).

For example, several recent investigations have shown that colorful ornament expression can be honest signals of condition because the pigments themselves are sensitive to oxidative stress from free radicals, such high oxidative stress levels would reduce the colorfulness of the pigments (Blount *et al.*, 2003; Pérez-Rodríguez *et al.*, 2010). Oxidative stress arises when production of free radicals exceeds the capacity of antioxidant systems (Monaghan *et al.*, 2009; Costantini, 2014). Because core system processes, including the immune response and mitochondrial respiration, can be major contributors to oxidative stress when they are dysfunctional (von Schantz *et al.*, 1999), the Index Hypothesis predicts that colorful ornaments

can honestly signal condition because of these inherent connections between oxidative stress, system functionality, and ornamentation (Hill, 2014; Koch *et al.*, 2016).

Assessing studies of honest signaling from coloration ornaments

While the Costly Signaling and Index Hypotheses are both theoretically viable and not mutually exclusive, research on honest signaling via coloration has been dominated by an assumption of the necessity of costs. In this section, I briefly review how signaling costs have been specifically proposed to maintain signal honesty in male coloration, focusing on the prominent concept of resource tradeoffs (1b, Table 1-1). I then introduce how a consideration of cost-free index mechanisms can both refocus interpretation of experimental and correlational studies and offer new opportunities for future study.

The resource tradeoff hypothesis (1b, Table 1-1) posits that honest signaling from coloration is due to differential allocation of the substrates used to produce ornamental coloration, and it is among the most commonly invoked mechanisms to maintain the honesty of color signals under the Costly Signaling Hypothesis (Folstad and Karter, 1992; Baeta *et al.*, 2008; Blount and McGraw, 2008; McGraw, 2008). Pigments, the substrates used to synthesize pigments, or the components needed for integumentary microstructures potentially have uses within the body other than color production, so individuals might face a direct allocation tradeoff (Zera and Harshman, 2001). By this idea, only high quality males can afford the cost of allocating resources to produce a good color signal and still meet the demands of body maintenance (Morehouse, 2014). Thus, this resource tradeoff conceptualization of the Costly Signaling Hypothesis has two key assumptions: 1) the resources needed for coloration are scarce

or difficult to obtain, and 2) the same resources needed to produce coloration also serve important physiological functions.

Costly signaling through differential resource allocation is perhaps most often applied to carotenoid-based ornaments because carotenoid pigments cannot be synthesized *de novo* within the animal body and must be acquired from the diet (Goodwin, 1986). Whether or not carotenoids are a limited resource in the diets of most animals has been debated for 25 years (Hill, 1994a; Hudon, 1994; Olson and Owens, 1998), with still surprisingly few direct measurements of the carotenoid contents of diets of wild animals and carotenoid requirements of ornaments (Grether *et al.*, 1999; Hill *et al.*, 2002; McGraw, 2006). Moreover, carotenoids are thought to be relevant, powerful antioxidants and important boosters of immune response such that allocating carotenoids towards coloration comes at the cost of those pigments not being available for these other functions (Chew and Park, 2004; Aguilera and Amat, 2007; Baeta *et al.*, 2008). Carotenoid coloration may therefore be an honest signal of oxidative stress or immune system function because allocating potentially scarce and important carotenoid resources to coloration is thought to be too costly for males of poor quality. Related hypotheses propose that testosterone may be a critical mediator of the relationship between ornament color and individual quality by affecting both carotenoid allocation and immune function (1c, Table 1-1) or oxidative stress (1d, Table 1-1); in this case, only high quality individuals could afford to pay potential costs of high testosterone—costs that could potentially be offset by the beneficial function of carotenoids. Although the concept of the resource tradeoff is widely cited as the mechanism that maintains honesty from carotenoid signals, whether carotenoids are limiting resources for most animals with carotenoid-colored signals and whether they are relevant to animal antioxidant and

immune systems remains largely unresolved (Olson and Owens, 1998; Young and Lowe, 2001; Costantini and Møller, 2008; Isaksson and Andersson, 2008).

This resource tradeoff hypothesis has been applied less extensively to melanin pigmentation, but the substrates needed for production of melanic ornaments are also ultimately sourced from an animal's diet and therefore may be limited resources requiring differential allocation among functions. For example, the melanin pigments that produce black, brown, and rufous coloration are synthesized from an essential amino acid that is also used for myriad physiological functions, including hormonal cascades and immune responses (Kanehisa *et al.*, 2006). An attempt to manipulate the access to key precursor amino acids in the diets of House Sparrows (*Passer domesticus*), however, failed to affect black melanin coloration (Poston *et al.*, 2005). In addition, melanin synthesis requires trace metals that have been proposed to be limited in the diet, which could maintain honesty if there is a cost to allocating those metals to melanin synthesis instead of to internal maintenance functions (McGraw, 2008). However, the role of these potential resource limitations and physiological tradeoffs in production of melanin ornamentation remains contentious (Jawor and Breitwisch, 2003; Griffith *et al.*, 2006).

In contrast to pigmentary coloration, much less attention has been focused on honesty-maintaining mechanisms for structural coloration, though these ornaments may also function as honest signals of quality. Structural coloration requires the precise arrangement of integumentary microstructures, and the integrity of microstructures has been shown to be an honest signal of male body condition in some systems (Keyser and Hill, 2000; Shawkey *et al.*, 2003; Siitari *et al.*, 2007). The Costly Signaling Hypothesis has also been adopted to explain honesty from structurally colored ornaments with proposed costs related to production and precise organization of the components that produce coloration (Fitzpatrick, 1998).

While the importance of resource tradeoffs to signal honesty is widely accepted, definitive testing of how differential resource allocation shapes condition-dependent coloration is challenging. It is typically extremely difficult to know the quantity of pigments or other resources available to the individual producing the ornament, how pigment resources are allocated within the body, and whether patterns of allocation change in the manners predicted by tradeoff hypotheses. For example, an individual may vary the amount of pigments it has available for ornamental use at any of the various stages of in the pigmentation process, including absorption from the diet (in the case of strictly dietary pigments, like carotenoids), transportation to target tissues, metabolic transformation (if necessary), as well as deposition in the integument (Hill *et al.*, 1994). The complexities of allocation make the interpretations of whole-animal experimentation problematic because non-terminal investigations generally rely on the quantities of pigments in circulation as a proxy for the internal pigment stores. Such methods, however, do not allow researchers to distinguish between re-allocation versus disruption of uptake, transport, or metabolism. This problem of "blackboxing" the physiological and biochemical processes that determine pigment usage within the body of an animal presents a major challenge to understanding the mechanism that give rise to honest signaling.

For example, a foundational experiment performed by Faivre et al. (Faivre *et al.*, 2003) is often cited as compelling evidence for carotenoid pigment allocation away from ornamental coloration and toward immune and antioxidant function during immune activation. In this experiment, male European Blackbirds (*Turdus merula*) were injected with a foreign substance (sheep red blood cells) to induce an innate immune response that was quantified via a blood haemagluttination test; the coloration of each male's characteristically orange bill was measured before and after injection. Faivre et al. (Faivre *et al.*, 2003) reported that bill coloration became

duller after injection in all males, and that dullness of bill coloration correlated positively with strength of haemagluttination response. These results were interpreted as being supportive of the Costly Signaling Hypothesis, with carotenoids being allocated away from coloration and toward an immune response. However, without understanding the specific allocation processes occurring within the blackbirds, the results of Faivre et al. (Faivre *et al.*, 2003) are also consistent with the Index Hypothesis. Challenged birds may have exhibited duller ornaments because of a change in underlying system functionality independent of reallocation of carotenoids for immune defense. For instance, challenged blackbirds may have absorbed fewer carotenoid pigments from the diet to avoid potential pro-oxidative effects of carotenoids (Huggins *et al.*, 2010). The relationship between dullness of bill and strength of haemagluttination response is also potentially consistent with the Index Hypothesis because a decreased haemagluttination reaction may indicate a bird that was less challenged by an antigen, and therefore may have better functioning core cellular processes that also enable it to produce a bright orange bill. By the Index Hypothesis, bill coloration is a signal of the core system functionality that underlies processes mediating both immunocompetence and ornament production (Hill, 2011).

Experiments with melanin pigmentation are also inconclusive with regard to the mechanisms that link ornamentation to condition. For example, the dark melanin patches on the wings of male damselflies are sexually selected traits that have been shown to honestly signal parasite resistance (Siva-Jothy, 2000). Importantly, melanin production in this and other insect species is dependent on a shared pathway with immune defense, the phenoloxidase (PO) pathway. Using an experimental parasite challenge, Siva-Jothy (Siva-Jothy, 2000) found that males with higher parasite burdens after inoculation also had elevated PO levels but lighter, more heterogeneous wing patches. The author concluded that melanic wing pigmentation is an honest

signal of male parasite resistance because the cost of diverting resources to melanin production was too great for poor condition males. However, alternative conclusions are plausible under the Index Hypothesis. If individual condition is considered to comprise the functionality of core physiological processes—in this case, the PO immune response pathway—then it might be expected that an individual in poor condition to also exhibit poor functionality of melanin production pathways. Melanin ornaments can thus serve as honest signals of condition through cost-free mechanisms because melanin production is an index of immunocompetence through a shared pathway between melanin pigment production and immune function.

Discerning costly tradeoffs from cost-free indices

I have used two empirical examples to demonstrate that the acceptance of the Costly Signaling Hypothesis framework in interpretation of experimental results is not always warranted because experimental designs often cannot rule out alternative explanations. However, just as it is challenging to clearly demonstrate that resource allocation is a cost to ornamentation, it can be equally difficult to definitively demonstrate links to biochemical pathways that enforce honesty in signaling systems. I propose that future studies work to clarify the mechanisms of honest signaling by performing targeted experimental manipulations that can better isolate the importance of costs versus physiological function in determining ornament expression. Indeed, I argue that better awareness of the Index Hypothesis and its potential application to male coloration used in mate choice displays will lead to more decisive experimental design and effective interpretation of results.

Specifically, recent and ongoing advancements in biomedical, molecular, and genetic technologies present new opportunities for testing the roles of resources, energy, cellular

processes, and other key factors in maintaining honesty in colored ornaments. For example, the primary roadblock to definitively demonstrating the role of differential resource allocation in condition-dependent carotenoid color expression is the inability to clarify where, when, and in what quantities carotenoids are moved through the bodies of animals. Relatively unexplored techniques, such as designing radiolabels for carotenoid pigments, may be key to isolating the actual size of the pool of carotenoid resources to which an individual has access and how those resources are allocated under changing conditions. Studies of pigment resource abundance and movement can be paired with the use of new genetic discoveries—such as key genes in the biosynthesis of melanin (Mundy, 2005) and the metabolism of red carotenoids (Lopes *et al.*, 2016; Mundy *et al.*, 2016)—to consider how the genetic regulation of pigment uptake and/or transformation may interact with resource use in defining both signal expression and individual performance. To test the role of allocation or resource tradeoffs in maintaining honesty in color signaling, we must isolate specific tradeoffs, their fitness costs to males of differing quality, and their role in color signaling systems.

Testing the Index Hypothesis is particularly challenging because the physiological processes and potential shared pathways that define individual quality are difficult to isolate. Perhaps the most effective means to test a potential index signal is to focus on the mechanisms generating that signal and to consider how such mechanisms may overlap with other physiological pathways involved in individual performance (such as the immune system; Koch *et al.*, 2016). By performing a targeted experimental manipulation that alters the functionality of the hypothesized controlling mechanism and then measuring the resulting effects on ornament expression, we may be able to elucidate whether central pathways drive variation in ornament production.

For example, it was recently proposed that mitochondrial cellular respiration is the core pathway that links ornamentation to aspects of condition (2c, Table 1-1; Hill, 2011). Many aspects of individual condition that are usually invoked with respect to condition-dependent ornaments, such as immune function and reproductive success, can be traced through pathways that intersect at the level of the mitochondrion (Hill and Johnson, 2012; Johnson and Hill, 2013; Hill, 2014; Koch *et al.*, 2016). This is important because the mitochondrion is both the core energy producer of the animal body and also a critical integrator organelle that interprets, transduces, and responds to a multitude of internal signals. As such, functionality of an individual's mitochondria may be a primary mechanistic basis for the Index Hypothesis; mitochondrial processes could be considered the fundamental physiological processes that determine both ornament and individual quality, thereby maintaining uncheatable signals (Hill, 2014). Basic understanding of mitochondrial function is progressing rapidly and leading to novel means to manipulate mitochondrial function (Huang *et al.*, 2005; Desquiret *et al.*, 2006; Salin *et al.*, 2012), providing new means to test the idea that colorful ornaments act as index signals because mitochondrial function potentially mediates both individual condition and ornamentation. Future research into how ornament quality varies with mitochondrial function as well as with the performance of traditional measures of individual condition, such as immunocompetence or oxidative stress balance, will be important to test ideas related to honest signaling. Laboratory techniques, such as quantifying mitochondrial ATP production efficiency or production of free radicals (Gerencser *et al.*, 2009; Brand and Nicholls, 2011; Stier *et al.*, 2013) or performing biochemical manipulations that inhibit various aspects of mitochondrial function with or without corresponding changes to free radical production (Huang *et al.*, 2005; Desquiret *et al.*, 2006; Salin *et al.*, 2012), will be critical to advancing these ideas. For example,

detecting a change in carotenoid coloration after experimental inhibition of mitochondrial function in the absence of increased free radical production would indicate that color expression is dependent on internal functionality rather than on costs of using the pigments as antioxidants. Determining a direct mechanistic link between mitochondrial function and ornament production will be important to determine whether colored signals are true indices of cellular function.

Conclusion

"*The diversity of …mechanisms … producing cost-free, minimal-cost signals, and handicaps at the equilibrium, will reveal itself in its full shape and beauty when researchers are primed to look for it"* (Számadó, 2011).

The entrenchment of the paradigm that signals must be costly to be honest has resulted in a widespread acceptance that allocation tradeoffs define color signals, while experimental data is often equivocal and correlational evidence does not rule out alternative explanations. To advance understanding of the mechanisms that maintain signal honesty, I encourage scientists to design experiments and collect data that can disentangle competing hypothesis and to consider whether alternative hypotheses may provide valid alternative explanations to observations. The Costly Signaling and the Index Hypotheses are not mutually exclusive; it may prove that both the functionality of core pathways and costs resulting from resource limitations contribute to color expression and establish the observed links between condition and ornamentation. It is time, however, to open the black box that has been erected around many physiological systems so as to better understand the mechanisms that underlie honest signaling in animal coloration.

References

Aguilera, E. and Amat, J.A. (2007) Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (Carduelis chloris). *Naturwissenschaften*, 94, 895–902.

Alonso-Alvarez, C., Pérez-Rodríguez, L., Mateo, R., Chastel, O. and Viñuela, J. (2008) The oxidation handicap hypothesis and the carotenoid allocation trade-off. *J. Evol. Biol.*, 21, 1789–1797.

Andersson, M. (1994) *Sexual Selection*, Princeton University Press, Princeton.

Arnqvist, G. and Rowe, L. (2013) *Sexual conflict*, Princeton University Press.

Baeta, R., Faivre, B., Motreuil, S., Gaillard, M. and Moreau, J. (2008) Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (Turdus merula). *Proc. Biol. Sci.*, 275, 427–34.

Bergstrom, C.T., Számadó, S. and Lachmann, M. (2002) Separating equilibria in continuous signalling games. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 357, 1595–1606.

Biernaskie, J.M., Grafen, A. and Perry, J.C. (2014) The evolution of index signals to avoid the cost of dishonesty. *Proc. R. Soc. B Biol. Sci.*, 281, 20140876–20140876.

Blount, J. and McGraw, K. (2008) *Signal functions of carotenoid colouration*. *Carotenoids* (ed. by G. Britton), S.H.C. Liaaen-Jensen), and H. Pfander), pp. 213–236. Birkhäuser Basel.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R. and Surai, P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science*, 300, 125–127.

Brand, M.D. and Nicholls, D.G. (2011) Assessing mitochondrial dysfunction in cells. *Biochem. J.*, 435, 297–312.

Candolin, U. (1999) The relationship between signal quality and physical condition: is sexual signalling honest in the three-spined stickleback? *Anim. Behav.*, 58, 1261–1267.

Chew, B.P. and Park, J.S. (2004) Carotenoid action on the immune response. *J. Nutr.*, 134, 257S–261S.

Clutton-Brock, T.H. and Albon, S.D. (1979) The Roaring of Red Deer and the Evolution of Honest Advertisement. *Behaviour*, 69, 145–170.

Costantini, D. (2014) *Oxidative Stress and Hormesis in Evolutionary Ecology and Physiology: A Marriage Between Mechanistic and Evolutionary Approaches*, Springer Berlin Heidelberg.

Costantini, D. and Møller, A.P. (2008) Carotenoids are minor antioxidants for birds. *Funct. Ecol.*, 22, 367–370.

Darwin, C.R. (1871) *The descent of man, and selection in relation to sex.*, Appleton, New York.

Desquiret, V., Loiseau, D., Jacques, C., Douay, O., Malthièry, Y., Ritz, P. and Roussel, D. (2006) Dinitrophenol-induced mitochondrial uncoupling in vivo triggers respiratory adaptation in HepG2 cells. *Biochim. Biophys. Acta*, 1757, 21–30.

Enquist, M. (1985) Communication during aggressive interactions with particular reference to variation in choice of behaviour. *Anim. Behav.*, 33, 1152–1161.

Faivre, B., Grégoire, A., Préault, M., Cézilly, F. and Sorci, G. (2003) Immune Activation Rapidly Mirrored in a Secondary Sexual Trait. *Science (80-.).*, 300, 103–103.

Fitzpatrick, S. (1998) Colour schemes for birds: structural coloration and signals of quality in feathers. *Ann. Zool. Fennici*, 35, 67–77.

Folstad, I. and Karter, A.J. (1992) Bright Males, and the Immunocompetence Handicap. *Am. Nat.*, 139, 603–622.

Freeman-Gallant, C.R., Amidon, J., Berdy, B., Wein, S., Taff, C.C. and Haussmann, M.F. (2011) Oxidative damage to DNA related to survivorship and carotenoid-based sexual ornamentation in the common yellowthroat. *Biol. Lett.*, 7, 429–432.

Garratt, M. and Brooks, R.C. (2012) Oxidative stress and condition-dependent sexual signals: more than just seeing red. *Proc. Biol. Sci.*, 279, 3121–3130.

Gavrilets, S., Arnqvist, G. and Friberg, U. (2001) The evolution of female mate choice by sexual conflict. *Proc. Biol. Sci.*, 268, 531–9.

Gerencser, A. a., Neilson, A., Choi, S.W., Edman, U., Yadava, N., Oh, R.J., Ferrick, D. a., Nicholls, D.G. and Brand, M.D. (2009) Quantitative microplate-based respirometry with correction for oxygen diffusion. *Anal. Chem.*, 81, 6868–6878.

Getty, T. (1998a) Handicap signalling: when fecundity and viability do not add up. *Anim. Behav.*, 56, 127–30.

Getty, T. (1998b) Reliable signalling need not be a handicap. *Anim. Behav.*, 56, 253–255.

Getty, T. (2006) Sexually selected signals are not similar to sports handicaps. *Trends Ecol. Evol.*, 21, 83–88.

Goodwin, T. (1986) Metabolism, nutrition, and function of carotenoids. *Annu. Rev. Nutr.*, 6, 237–97.

Grafen, A. (1990) Biological signasl as handicaps. *J. Theor. Biol.*, 144, 517–546.

Grether, G.F., Hudon, J. and Millie, D.F. (1999) Carotenoid limitation of sexual coloration along an environmental gradient in guppies. *Proc. R. Soc. B Biol. Sci.*, 266, 1317.

Griffith, S.C., Parker, T.H. and Olson, V.A. (2006) Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Anim. Behav.*, 71, 749–763.

Grose, J. (2011) Modelling and the fall and rise of the handicap principle. *Biol. Philos.*, 26, 677–696.

Halliday, T.R. (1987) *Physiological constraints on sexual selection. Sexual Selection: Testing the Alternatives* (ed. by J.W. Bradbury) and M. Andersson), John Wiley and Sons Inc., New York.

Hamilton, W.D. and Zuk, M. (1982) Heritable true fitness and bright birds: a role for parasites? *Science (80-.).*, 218, 384–387.

Hasson, O. (1997) Towards a general theory of biological signaling. *J. Theor. Biol.*, 185, 139–156.

Higham, J.P. (2013) How does honest costly signaling work? *Behav. Ecol.*, 25, 8–11.

Hill, G.E. (2014) Cellular respiration: The nexus of stress, condition, and ornamentation. *Integr. Comp. Biol.*, 54, 1–13.

Hill, G.E. (2011) Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol. Lett.*, 14, 625–634.

Hill, G.E. (2006a) *Environmental regulation of ornamental coloration*. *Bird Coloration, Volume 1: Meachanisms and Measurements* (ed. by G.E. Hill) and K.J. McGraw), Harvard University Press, Cambridge MA.

Hill, G.E. (2006b) *Female choice for ornamental coloration*. *Bird Coloration, Volume 2: Function and Evolution* (ed. by G.E. Hill) and K.J. McGraw), Harvard University Press, Cambridge, MA.

Hill, G.E. (1994a) House finches are what they eat: a reply to Hudon. *Auk*, 111, 221–225.

Hill, G.E. (1991) Plumage coloration is a sexually selected indicator of male quality. *Nature*, 350, 337–339.

Hill, G.E. (1994b) Trait elaboration via adaptive mate choice: sexual conflict in the evolution of signals of male quality. *Ethol. Ecol. Evol.*, 6, 351–370.

Hill, G.E., Inouye, C.Y. and Montgomerie, R. (2002) Dietary carotenoids predict plumage coloration in wild house finches. *Proc. R. Soc. Biol. Sci. Ser. B*, 269, 1119–1124.

Hill, G.E. and Johnson, J.D. (2012) The vitamin A-redox hypothesis: A biochemical basis for honest signaling via carotenoid pigmentation. *Am. Nat.*, 180, E127-150.

Hill, G.E., Montgomerie, R., Inouye, C.Y. and Dale, J. (1994) Influence of dietary carotenoids on plasma and plumage colour in the house finch: intra- and intersexual variation. *Funct. Ecol.*, 8, 343–350.

Huang, L.-S., Cobessi, D., Tung, E.Y. and Berry, E.A. (2005) Binding of the respiratory chain inhibitor antimycin to the mitochondrial bc1 complex: a new crystal structure reveals an altered intramolecular hydrogen-bonding pattern. *J. Mol. Biol.*, 351, 573–97.

Hudon, J. (1994) Showiness, carotenoids, and captivity: A comment on Hill (1992). *Auk*, 111, 218–221.

Huggins, K. a, Navara, K.J., Mendonça, M.T. and Hill, G.E. (2010) Detrimental effects of carotenoid pigments: the dark side of bright coloration. *Naturwissenschaften*, 97, 637–44.

Hurd, P.L. (1997) Is signalling of fighting ability costlier for weaker individuals? *J. Theor. Biol.*, 184, 83–88.

Huttegger, S.M., Bruner, J.P. and Zollman, K.J.S. (2015) The handicap principle is an artifact. *Philos. Sci.*, 82, 997–1009.

Irschick, D.J., Briffa, M. and Podos, J. eds. (2015) *Animal Signaling and Function: An Integrative Approach*, John Wiley and Sons Inc.

Isaksson, C. and Andersson, S. (2008) Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. *Proc. R. Soc. B-Biological Sci.*, 275, 309–314.

Jawor, J.M. and Breitwisch, R. (2003) Melanin ornaments, honesty, and sexual selection. *Auk*, 120, 249–265.

Johnson, J.D. and Hill, G.E. (2013) Is carotenoid ornamentation linked to the inner mitochondria membrane potential? A hypothesis for the maintenance of signal honesty. *Biochimie*, 95, 436–444.

Johnstone, R.A. (1995) Sexual selection, honest advertisement and the handicap principle: Reviewing the evidence. *Biol. Rev.*, 70, 1–65.

Kane, P. and Zollman, K.J.S. (2015) An evolutionary comparison of the handicap principle and hybrid equilibrium theories of signaling. *PLoS One*, 10, 1–14.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K., Itoh, M., Kawashima, S., Katayama, T., Araki, M. and Hirakawa, M. (2006) From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res.*, 34, D354-7.

Keyser, A.J. and Hill, G.E. (2000) Structurally based plumage coloration is an honest signal of quality in male blue grosbeaks. *Behav. Ecol.*, 11, 202–209.

Koch, R.E., Josefson, C.C. and Hill, G.E. (2016) Mitochondrial function, ornamentation, and immunocompetence. *Biol. Rev.*, 92, 1459–1474.

Kodric-Brown, A. and Brown, J.H.J. (1984) Truth in advertising: the kinds of traits favored by sexual selection. *Am. Nat.*, 124, 309–323.

Lachmann, M., Számadó, S., Bergstrom, C.T., Lachmann, M., Szamadt, S. and Bergstrom, C.T. (2001) Cost and conflict in animal signals and human language. *Proc. Natl. Acad. Sci.*, 98, 13189–13194.

Lopes, R.J., Johnson, J.D., Toomey, M.B., Ferreira, M.S., Araujo, P.M., Melo-Ferreira, J., Andersson, L., Hill, G.E., Corbo, J.C. and Carneiro, M. (2016) Genetic basis for red coloration in birds. *Curr. Biol.*, 26, 1427–1434.

Maynard Smith, J. (1956) Fertility, mating behaviour and sexual selection in Drosophila subobscura. *J. Genet.*, 54, 261–271.

Maynard Smith, J. and Harper, D.G.C. (2003) *Animal Signals*, Oxford University Press, London.

Maynard Smith, J. and Harper, D.G.C. (1995) Animal signals: Models and terminology. *J. Theor. Biol.*, 177, 305–311.

McGraw, K.J. (2008) An update on the honesty of melanin-based color signals in birds. *Pigment Cell Melanoma Res.*, 21, 133–138.

McGraw, K.J. (2006) *Mechanics of carotenoid coloration*. *Bird Coloration, Volume 1: Measurements and Mechanisms* (ed. by G.E. Hill) and K.J. McGraw), Harvard University Press, Cambridge, Massachusetts.

McGraw, K.J., Cohen, A. a., Costantini, D. and Hõrak, P. (2010) The ecological significance of antioxidants and oxidative stress: A marriage between mechanistic and functional perspectives. *Funct. Ecol.*, 24, 947–949.

Møller, A.P., Biard, C., Blount, J.D., Houston, D.C., Ninni, P., Saino, N. and Surai, P.F. (2000) Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian Poult. Biol. Rev.*, 11, 137–159.

Monaghan, P., Metcalfe, N.B. and Torres, R. (2009) Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecol. Lett.*, 12, 75–92.

Morehouse, N.I. (2014) Condition-dependent ornaments, life histories, and the evolving architecture of resource-use. *Integr. Comp. Biol.*, 54, 591–600.

Mundy, N. (2005) A window on the genetics of evolution : MC1R and plumage colouration in birds A window on the genetics of evolution : MC1R and plumage colouration in birds. *Proc. Biol. Sci.*, 272, 1633–1640.

Mundy, N.I., Stapley, J., Bennison, C., Tucker, R., Twyman, H., Kim, K.-W., Burke, T., Birkhead, T.R., Andersson, S. and Slate, J. (2016) Red Carotenoid Coloration in the Zebra Finch Is Controlled by a Cytochrome P450 Gene Cluster. *Curr. Biol.*, 26, 1435–1440.

Olson, V.A. and Owens, I.P.F. (1998) Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol. Evol.*, 13, 510–514.

Parker, G.A. (2006) Sexual conflict over mating and fertilization: an overview. *Philos. Trans. R. Soc. B Biol. Sci.*, 361, 235–259.

Pérez-Rodríguez, L., Mougeot, F. and Alonso-Alvarez, C. (2010) Carotenoid-based coloration predicts resistance to oxidative damage during immune challenge. *J. Exp. Biol.*, 213, 1685–9160.

Pomiankowski, A. (1987) The costs of choice in sexual selection. *J. Theor. Biol.*, 128, 195–218.

Poston, J.P., Hasselquist, D., Stewart, I.R.K. and Westneat, D.F. (2005) Dietary amino acids influence plumage traits and immune responses of male house sparrows, Passer domesticus, but not as expected. *Anim. Behav.*, 70, 1171–1181.

Pryke, S.R., Andersson, S. and Lawes, M.J. (2001) Sexual selection of multiple handicaps in the red-collared widowbird: female choice of tail length but not carotenoid display. *Evolution*, 55, 1452–63.

Riechert, S.E. (1978) Games spiders play: Behavioral variability in territorial disputes. *Behav. Ecol. Sociobiol.*, 3, 135–162.

Roulin, A. (2016) Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. *Biol. Rev.*, 91, 328–348.

Salin, K., Luquet, E., Rey, B., Roussel, D. and Voituron, Y. (2012) Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (Rana temporaria) tadpoles. *J. Exp. Biol.*, 215, 863–9.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. and Wittzell, H. (1999) Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. London B*, 266, 1–12.

Searcy, W.A. and Nowicki, S. (2005) *The evolution of animal communication: reliability and deception in signaling systems*, Princeton University Press.

Shawkey, M.D., Estes, A.M., Siefferman, L.M. and Hill, G.E. (2003) Nanostructure predicts intraspecific variation in ultraviolet-blue plumage colours. *Proc. R. Soc. London Ser. B-Biological Sci.*, 270, 1455–1460.

Siitari, H., Alatalo, R. V, Halme, P., Buchanan, K.L. and Kilpimaa, J. (2007) Color Signals in the Black Grouse (Tetrao tetrix): Signal Properties and Their Condition Dependency. *Am. Nat.*, 169, Suple, S81–S92.

Siva-Jothy, M.T. (2000) A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proc. Biol. Sci.*, 267, 2523–2527.

Spence, M. (1973) Job Market Signaling. *Q. J. Econ.*, 87, 355–374.

Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., Gros, F., Royer, C., Massemin, S. and Criscuolo, F. (2013) Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. *Front. Zool.*, 10, 33.

Svensson, P.A. and Wong, B.B.M. (2011) Carotenoid-based signals in behavioural ecology: a review. *Behaviour*, 148, 131–189.

Számadó, S. (2011) The cost of honesty and the fallacy of the handicap principle. *Anim. Behav.*, 81, 3–10.

Számadó, S. (1999) The Validity of the Handicap Principle in Discrete Action + Response Games. *J. Theor. Biol.*, 198, 593–602.

Verhulst, S., Dieleman, S.J. and Parmentier, H.K. (1999) A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. *Proc. Natl. Acad. Sci. U. S. A.*, 96, 4478–4481.

Young, A.J. and Lowe, G.M. (2001) Antioxidant and Prooxidant Properties of Carotenoids. *Arch. Biochem. Biophys.*, 385, 20–27.

Zahavi, A. (1975) Mate selection - A selection for a handicap. *J. Theor. Biol.*, 53, 205–14.

Zahavi, A. (1977) The cost of honesty. Further Remarks on the Handicap Principle. *J. Theor. Biol.*, 67, 603–605.

Zahavi, A. and Zahavi, A. (1997) *The Handicap Principle*, Oxford University Press.

Zera, A.J. and Harshman, L.G. (2001) The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.*, 32, 95–126.

Zollman, K.J.S. (2013) Finding alternatives to handicap theory. *Biol. Theory*, 8, 127–132.

Table 1-1. Key hypotheses proposed to maintain honesty from animal color signals.

Overarching principle	Underlying concepts	Description	Key reference(s)
1. Costly Signaling Principle	a) Zahavi handicap principle	Signals are honest because they act as handicaps in contexts other than mating that are too costly for low-quality males.	(Zahavi, 1975, 1977)
	b) Resource Tradeoff Hypothesis	The production of ornaments requires the irreversible allocation of resources that could otherwise serve a beneficial function; as a result, only individuals in good health and otherwise good condition can "afford" to produce high quality ornaments.	(Kodric-Brown and Brown, 1984)
	c) Immunocompetence Handicap Hypothesis	Testosterone both suppresses immune function and enhances ornament production, so only healthy individuals can withstand the high testosterone required for good ornamentation.	(Folstad and Karter, 1992)
	d) Oxidative Handicap Hypothesis	Testosterone mediates a trade-off between ornament expression and internal system function by posing an oxidative challenge, such that only good condition individuals can withstand the increased oxidative burden of high testosterone and still produce high quality ornaments.	(Alonso-Alvarez *et al.*, 2008)
2. Index Hypothesis	a) Good genes (the Hamilton-Zuk Hypothesis)	Full expression of colorful ornaments is an 'unbluffable display' dependent on male condition that signals genetic parasite resistance.	(Hamilton and Zuk, 1982)

	b) Shared Pathway Hypothesis	Honest signaling of male condition from colorful ornaments arises because of physiological links between vital cellular processes and coloration.	(Hill, 2011)
	c) Mitochondrial Function Hypothesis	Mitochondrial function is the key shared pathway that integrates immunocompetence, oxidative stress maintenance, and other aspects of individual condition with ornament expression.	(Hill, 2014; Koch *et al.*, 2016)

Chapter 2. Carotenoid metabolism strengthens the link between feather coloration and individual quality.

Manuscript published with co-authors Eduardo Santos, Anna Tucker, Alan Wilson, and Geoffrey Hill in 2018 in *Nature Communications* **9**, 73.

Introduction

The red, yellow, and orange coloration produced by carotenoids has been studied extensively by behavioral ecologists and evolutionary biologists interested in understanding the signal content of animal ornaments (Hill, 2007; Blount and McGraw, 2008; Garratt and Brooks, 2012; Simons *et al.*, 2012). Under the indicator model, carotenoid coloration serves as an honest signal of individual quality (Kodric-Brown and Brown, 1984; Hill, 1991), and this hypothesis has been supported in many studies of fish, reptiles, lepidopterans, and birds that showed associations between carotenoid coloration and various measures of individual quality (Svensson and Wong, 2011). Despite decades of research, however, the mechanisms that maintain honesty and link carotenoid coloration to individual quality remains contentious (Hill, 2011; Weaver *et al.*, 2017).

Understanding the connections between individual quality and carotenoid ornamentation demands an understanding of the biochemistry of the pigments involved (Box 1; Hill and Johnson, 2012; García-de Blas *et al.*, 2015). Carotenoids are perhaps best known for the attractive coloration that they produce in animals, but carotenoids are ubiquitous cellular components with many functions. Carotenoids serve as vitamin A precursors in vertebrates (Stahl and Sies, 2003), and they may act as antioxidants or play a role in bolstering immune defenses (Blount *et al.*, 2003; Chew and Park, 2004). Despite their important physiological functions, carotenoids cannot be synthesized by animals *de novo;* whether they are used as

external colorants or serve functions in cellular pathways, ultimately all carotenoids must be obtained from the diet (Goodwin, 1992).

Studies on the function and evolution of carotenoid coloration have frequently focused on birds, particularly bird plumage, and sufficient studies have now been published to identify patterns among studies for associations between individual quality and feather coloration resulting from different categories of carotenoid pigments. In this quantitative synthesis, I categorize carotenoids used for feather coloration as "dietary" if the pigments are present in food and deposited in feathers without further modifications (Fig. 2-1). The two most common dietary pigments used as colorants in birds are lutein and zeaxanthin. If dietary carotenoids are metabolically oxidized internally by the bird to form ketocarotenoids before deposition to feathers (Fig. 2-1; Box 1) I refer to them as "converted", e.g., echinenone, canthaxanthin, and canary xanthophylls (McGraw, 2006). Yellow carotenoid-based coloration can result from the deposition of either non-pro-vitamin A dietary carotenoids (*i.e.*, lutein and zeaxanthin) or their metabolized products (*i.e.* canary xanthophylls; Box 1; Fig. 2-1). In contrast, red carotenoid pigments are generally absent from the diets of songbirds, so red feather coloration results from deposition of converted carotenoids that are metabolic products of pro-vitamin A carotenoids (*i.e.*, β-carotene and β-cryptoxanthin) or of dietary hydroxy-carotenoids (Fig. 2-1; Brush, 1990). Many empirical studies have demonstrated associations between the hue, chroma, or multivariate principal components of reflectance spectra from carotenoid-pigmented feathers (hereafter collectively referred to as "richness of coloration") and various proxies of individual condition including size-adjusted body mass, immune function, parasite resistance, and aspects of reproductive and parental performance (Table 2-1; Hill, 1991; Hill and Montgomerie, 1994; von Schantz *et al.*, 1999; McGraw and Hill, 2000a; Cotton *et al.*, 2006; Ferns and Hinsley, 2007;

Murphy *et al.*, 2009; del Cerro *et al.*, 2010; Edler and Friedl, 2010). The result is a perplexing collection of studies with inconsistencies in the direction and magnitude of associations between carotenoid coloration and individual (Isaksson *et al.*, 2007; Karu *et al.*, 2007; Fitze *et al.*, 2009; Pérez-Rodríguez *et al.*, 2010; Garratt and Brooks, 2012).

Previous reviews of condition-dependence of carotenoid pigmentation in birds have not considered whether the ornamentation involved dietary or converted carotenoid pigmentation as a potential moderator to explain heterogeneity of their results (Griffith *et al.*, 2006; Simons *et al.*, 2012, 2014). I hypothesized that a lack of consideration of the biochemical pathways involved in production of carotenoid displays of different species might account for some apparent inconsistencies in the literature between condition and carotenoid coloration. Specifically, I hypothesized that the strength of the relationship between coloration signal and individual quality is dependent on whether or not the color display involved metabolic conversion of dietary carotenoids. I predicted that because the mechanisms of carotenoid metabolism might be linked to basic cellular function (Weaver *et al.*, 2016, 2017), feather coloration requiring carotenoid metabolism would have a stronger positive relationship with measures of individual quality than feather coloration derived from deposition of unaltered dietary carotenoids (Hill, 1999; Hill and Johnson, 2012). Additionally, metabolism of carotenoids requires the maintenance of enzyme systems (Box 1; Lopes *et al.*, 2016) and perhaps additional transport of carotenoids (García-de Blas *et al.*, 2015) that may create stronger links between coloration and system performance for ornaments produced from converted pigments versus unaltered dietary pigments. I tested our hypothesis using meta-analysis techniques to quantitatively synthesize published results on the relationships between individual quality and plumage coloration that was produced from dietary versus converted carotenoids.

Methods

Literature search

A literature search of the *Web of Science* and *Google Scholar* databases using combinations of the keywords "carotenoid", "color", "condition", "signal", "feather" and "quality" returned over 1,600 potentially eligible published articles. This search was based on the Preferred Reporting Items for Systematic reviews and Meta-Analyses statement (PRISMA; Moher *et al.*, 2009). I also considered as potentially eligible articles data from studies included in published meta-analyses of bird coloration (Griffith *et al.*, 2006; Simons *et al.*, 2012) and references therein. The online database search was last performed on 11 April 2017 on titles, abstracts and keywords in both databases. I did not request unpublished data sets from colleagues, because of the risk of biasing the estimates of effect size (see Jennions *et al.*, 2013). Because I was interested in the signal content of carotenoid-based plumage, I focused on studies that quantified feather color using standardized color metrics (see *Color Metrics* below) of natural adult color levels. Therefore, I excluded studies from our meta-analysis for any of the following reasons: only plasma concentrations of carotenoids were measured; only coloration of non-feathered structures was measured (*e.g.* wattles, legs, beaks); or only nestling or juvenile coloration was studied. I did not include measures of feather brightness as it is sensitive to factors unrelated to pigmentation (see below). Additionally, to be included in our meta-analysis, studies must have investigated one of the following proxies of individual quality: (1) nutritional condition, (2) immune response, (3) parasite resistance, or (4) reproductive or parental quality (Table 2-1).

Data extraction and coding

I used the correlation coefficient, Pearson's *r*, as the effect size metric to describe the association between measurements of feather coloration and aspects of individual quality. The effect size metric was extracted directly from each study when available. For cases in which studies did not provide Pearson's *r* (*n*=44), the reported test statistics (*F, ρ,* χ^2, τ, t, and means and standard deviations or standard errors) were used to estimate *r* (Lipsey and Wilson, 2001). Pearson's *r* was Fisher's *Z*-transformed to for statistical analyses to meet normality assumptions of linear models. Researchers commonly refer to *r* = 0.1, 0.3 and 0.5 as small, medium and large effect sizes (Cohen, 1988; these benchmarks are equivalent for *Zr* values), respectively. The sign of the correlation coefficient was changed in some cases to facilitate comparisons across metrics of individual quality; for example, some measures —such as parasite load— were often negatively correlated with richness of coloration but indicated a positive relationship with quality (e.g., McGraw and Hill, 2000b).

Original studies often include multiple effect size estimates because of the measurement of several life history traits associated with individual condition or performance (*e.g*., immune function or fledging success) and/or from different color metrics (see below). When more than one effect size is reported these data points cannot be assumed to be independent. To deal with this issue, I ran multi-level, random-effects meta-analytic models that allowed me to include multiple, non-independent effect sizes per study. This was accomplished by including the study identity and species identity as random effects (Nakagawa and Santos, 2012).

Color Metrics

Standing variation in richness of color between individuals is requisite for assessing quality from carotenoid-based ornaments. Common metrics used to quantify this variation in

feather reflectance include comparisons to standard color charts (*e.g.* Munsel), calculations of hue, chroma and brightness or composite metrics such as principal components (PCA) from spectrophotometer data or digital photographs. Hue describes the unique spectral color (*e.g.* "red", "orange", "yellow") and chroma describes the saturation or spectral purity of the color display relative to brightness, (*i.e.* total reflectance; Montgomerie, 2006). I did not include measures of brightness in the analyses because it is strongly influenced by the physical structure of the feather which may be altered by abrasion and wear and is difficult to interpret for carotenoid content (Shawkey and Hill, 2005).

Meta-analysis Technique

To evaluate the strength of correlation between all measures of individual quality and overall feather color richness, I first used a Bayesian mixed-effects meta-analytic model without distinguishing between carotenoid types (*i.e.* "Combined"; intercept only model). To further partition heterogeneity, I conducted a Bayesian meta-regression analysis with carotenoid type included as a categorical moderator to test the effect of the source of ornamental coloration (either dietary or converted carotenoids) on the overall relationship between color and quality. I then performed a second meta-regression analysis to test the strength of association of each quality category with both dietary and converted carotenoid-based plumage. Lastly, I used a third meta-regression model to quantify the relationship between coloration and quality for each bird species examined in the data set. I used the *MCMCglmm* package (Hadfield, 2010; Hadfield and Nakagawa, 2010) in program R version 3.3.0 (R Core Team, 2017) to conduct the Bayesian mixed-effects meta-analyses.

To adequately correct for non-independence, it is necessary to model the correlations among effect sizes. For instance, effect sizes for immune response and fledging success are

likely to be correlated when the traits were measured from the same group of individuals. Unfortunately, such correlations are almost never reported in the original studies, and are seldom accounted for in meta-analytic models (but see Booksmythe *et al.*, 2017), potentially biasing general finding. Thus, to conservatively account for such correlations, I report multi-level models that assume all correlations to be 0.5. Additionally, in the supplementary material, I report the results of the same models assuming correlations of zero. I note that qualitatively the results are very similar among these meta-analytic models.

I also accounted for the evolutionary history of the bird species used in the study by including a phylogenetic random effect to the models. These meta-analytic models used avian phylogenetic trees with the Ericson backbone from (Jetz *et al.*, 2012). To account for uncertainty in the phylogenetic reconstruction, I used a sample of 1,000 trees, so that each tree was sampled at iteration, *t*. I calculated phylogenetic heritability H^2 as an index of phylogenetic signal. H^2 can be defined as the proportion of phylogenetic variance in relation to the sum of all other variance components, with the exception of sample error variance. When the unit of analysis is at the species level, H^2 is equivalent to Pagel's λ (Housworth *et al.*, 2004).

For all multi-level models, I used parameter expanded priors (V=1, nu=1, alpha.mu = 0, alpha.V = 1000) for all random effects. I used a combination of total iterations and burn-in period so that the posterior distributions consisted of 1,000 samples for the model parameters. I report point estimates from the models based on the posterior means, and considered fixed factor estimates to be statistically significant if the 95% credible interval (95% CI) did not overlap zero. To quantify heterogeneity in multi-level meta-analytic models, I calculated a modified version of the I^2 statistics (following Nakagawa and Santos, 2012). This procedure partitions the proportion of variance not attributable to sampling variance into the contribution of the random factors of

the model. In these models, these are the variance in effect sizes due to phylogenetic relatedness, differences among species, differences among studies, and differences in within-study variation (residual variation). The sum of the percentages of total variation due to these sources yields the original I^2 proposed by (Higgins *et al.*, 2003).

To test for potential publication bias, I visually evaluated funnel plot asymmetry of effect size as a function of sample size. Then, I conducted an Egger's regression (Egger *et al.*, 1997) to test statistically for publication bias. The analyses of bias for the multi-level models was conducted on the meta-analytic residuals (see Nakagawa and Santos, 2012), as this ensures that I meet the assumption of independence.

Results

The final dataset included 197 effect size estimates from 52 studies of 21 bird species published from 1994 to 2013. Detailed results from each model are listed in Table 2-2. Mean effect size estimates and 95% CI are reported unless otherwise noted. Mean estimates with credible intervals that do not include zero are a statistically significant effect at $\alpha = 0.05$.

Carotenoid-based Plumage Coloration as an Honest Signal of Individual Quality

Consistent with previous meta-analyses and reviews that did not account for carotenoid type, I found a small positive correlation between richness of coloration and individual quality overall, but note that the 95% CI slightly overlaps zero ($Zr = 0.195$, 95% CI: -0.012 to 0.416; $I^2 = 98.12\%$). However, when I parsed the data between species with plumage coloration resulting from converted versus dietary carotenoids, I found that the relationship between richness of coloration and individual quality held only for species that used converted pigments (Converted: $Zr = 0.276$, 95% CI: 0.066 to 0.478; Dietary: $Zr = 0.129$, 95 % CI: -0.061 to 0.360; Fig. 2-2a). On average, I found that dietary carotenoid coloration is a weaker predictor of overall quality

than converted carotenoid coloration ($\beta_{(Dietary)}$ = -0.156, 95% CI: - 0.351. to 0.056), though the credible intervals from this comparison slightly overlap zero.

Body Condition

Measures of body condition were not reliably positively correlated with carotenoid-based feather coloration (*Zr* = 0.11, 95% CI: -0.104 to 0.329, Fig. 2-2b), and I found no clear difference in the relationship between carotenoid types (Converted: *Zr* = 0.123, 95% CI: -0.118 to 0.359; Dietary: *Zr* = 0.144, 95% CI: -0.104 to 0.399).

Immune Function

On average across all measures of immune function, I found that feather coloration was not a reliable predictor (*Zr* = 0.146, 95% CI: -0.069 to 0.407; Fig. 2-2c) with no clear difference in the relationship between carotenoid types (Converted: *Zr* = 0.150, 95% CI: -0.086 to 0.421; Dietary: *Zr* = 0.162, 95% CI: -0.083 to 0.384).

Parasite Resistance

When no distinction was made between carotenoid types, richness of coloration was positively correlated with parasite resistance (*Zr* = 0.285, 95% CI: 0.076 to 0.535). When I added carotenoid type as a moderator to the model I found that coloration from dietary carotenoids was weakly correlated with parasite resistance (*Zr* = 0.055, 95% CI: -0.168 to 0.320; Fig. 2-2d). In contrast, coloration from converted carotenoid-based plumage was strongly positively associated with parasite resistance (*Zr* = 0.455, 95% CI: 0.240 to 0.707; Fig. 2-2d) and was a more reliable signal than coloration from dietary carotenoid-pigmented feathers ($\beta_{(Dietary)}$ = -0.412, 95% CI: -0.652 to -0.167).

Reproductive and Parental Quality

I found that overall, carotenoid coloration positively correlated with aspects of reproductive and parental quality (*Zr* = 0.259, 95% CI: 0.0435 to 0.478; Fig. 2-2e), but that the pattern was driven by converted carotenoid feather coloration (Converted: *Zr* = 0.353, 95% CI: 0.087 to 0.621; Dietary: *Zr* = 0.142, 95% CI: -0.074 to 0.387). However, converted feather color was only a marginally better predictor of quality than dietary feather color ($\beta_{(Dietary)}$ = -0.206, 95% CI: -0465. to 0.065).

Species-level analysis

The strength of the relationship between feather coloration and combined measures of individual quality was dependent on the individual species (Fig. 2-3). However, I found only a weak phylogenetic signal among carotenoid types (I^2*= 2.12%)* in the overall analysis (Table 2-2).

Residual Heterogeneity

As mentioned above, heterogeneity in the overall meta-analytic model was high (I^2 = 98.12%; see Higgins *et al.*, 2003 for used benchmarks). Including carotenoid type as a moderator in the overall model reduced heterogeneity (I^2 = 71.97; Table 2-2). More variance was partitioned by the addition of quality category as a model moderator further decreasing heterogeneity (Table 2-2). High amount of heterogeneity remained after I included carotenoid type and quality category as moderators in the model indicating that other variables not included in this analysis may be influencing the relationship between feather coloration and aspects of individual quality (Table 2-2; Viechtbauer, 2010).

Publication Bias

Using sampling variance as a predictor, results from the Egger's regression did not indicate any funnel plot asymmetry based on meta-analytic residuals from the overall model (t_{195} = 1.974, p = 0.05).

Discussion

Carotenoid coloration is among the most frequently cited examples of a condition-dependent signal of quality assessed by females during mate choice across many animal taxa (Cotton *et al.*, 2006; Svensson and Wong, 2011), but there is only mixed empirical support for the assertion that carotenoid coloration is a reliable signal of individual quality (Hõrak *et al.*, 2001; Isaksson *et al.*, 2007; Isaksson and Andersson, 2008). I hypothesized that a key confounding variable that has not been adequately considered in previous comparative studies of the signal function of carotenoid coloration is whether the color display is derived from dietary versus converted carotenoids. Displays derived from converted carotenoids might be better signals of quality if carotenoid metabolism creates stronger connections between color displays and the cellular processes that give rise to the state of condition in the animal (Hill, 2011; Hill and Johnson, 2012).

The patterns that I observed in this meta-analysis of studies testing the condition-dependency of carotenoid coloration indicate that whether the carotenoid coloration under study is dietary or converted is an important predictor of the likelihood that a color display will be correlated to a measure of individual quality. Importantly, I did not observe a clear phylogenetic pattern in the strength of the relationship between coloration and quality (Fig 2-3.) When I assessed all studies of carotenoid feather coloration, without regard for whether the pigments were dietary of converted, there was some evidence that carotenoid coloration was condition-dependent (but note that the 95% CI slightly overlapped zero; Fig. 2-2a). This pattern was

driven by significant effect of converted carotenoids. Dietary carotenoids showed much weaker condition dependency that was not significantly different than no effect. Studies of converted carotenoids showed consistent correlations between parasite resistance and aspects of parental and reproductive quality. These results have important implications for understanding the mechanisms that link carotenoid coloration and individual condition.

Endler (Endler, 1980) first proposed that carotenoid coloration in animals could serve as an honest signal of quality because carotenoids are scarce resources that are challenging to accrue. Experiments with guppies (Kodric-Brown, 1989) and house finches (Hill, 1992) showed that depriving individuals of carotenoids during ornament production caused loss of color, but whether resource limitation was the basis for signal honesty in natural environments remained contentious (Hill, 1994; Hudon, 1994; Thompson *et al.*, 1997).

The dual role of carotenoids—either ornamental or physiological—is the foundation of the resource allocation trade-off hypothesis for why carotenoid coloration is a reliable signal of individual quality in many animals (Weaver *et al.*, 2017). Under this hypothesis, carotenoids are needed both for body maintenance and for ornamentation and thus only individuals with large stores of carotenoids or with low demands for body maintenance due to superior health can afford to allocate sufficient carotenoids for production of full ornamentation. This hypothesis makes two key predictions: 1) carotenoids are a limiting resource and 2) there is direct competition between ornament production and body maintenance for the same pool of carotenoids. Therefore, by this hypothesis carotenoid coloration links to foraging abilities by invoking a tradeoff in use of carotenoids between physiological demands and production of ornamentation (Lozano, 1994; von Schantz *et al.*, 1999; Møller *et al.*, 2000).

Alternatively, carotenoid coloration may serve as a reliable signal of individual quality because the mechanisms involved in the metabolic conversion of carotenoid pigments (Fig. 2-1; Box 1) are intimately linked to vital cellular pathways (Hill, 2011; Hill and Johnson, 2012; Weaver *et al.*, 2017). This shared pathway hypothesis predicts that regardless of the carotenoid resources that are available, disruption of core cellular processes, and particularly cellular respiration, will reduce production of carotenoid ornamentation (Johnson and Hill, 2013; Hill, 2014). Moreover, under the shared pathway hypothesis, metabolism of dietary carotenoids for ornamentation should create stronger connections between cellular processes and carotenoid-based signals because the pathways required for carotenoid metabolism are sensitive to the cellular environment (Hill, 1999; Hill and Johnson, 2012). The process of transforming dietary carotenoids to ketocarotenoids (Box 1) could be a key mechanism responsible for maintaining honesty from converted yellow and red carotenoid-based feather coloration and explain why yellow dietary carotenoid coloration is a not reliable signal of individual quality on average (Fig. 2-2).

If the links between carotenoid coloration and condition arise simply because allocation of carotenoids to ornaments signals the ability to efficiently acquire scarce resources, then under the resource tradeoff hypothesis, all forms of carotenoid coloration should serve as robust signals of body condition. Alternatively, the shared pathway hypothesis predicts that carotenoid coloration will be weakly associated with body condition and no difference should exist between carotenoid types. I show that on average, across multiple species and a range of condition proxies, the signal of body condition from plumage coloration was not robust for either dietary or converted carotenoids (Fig. 2-2b). These observations could reflect the seasonal changes in

variation in body condition that are not consistently correlated with feather coloration, which is dependent on the condition of the bird at the time of molt.

Perhaps one of the most ubiquitous statements made of carotenoids is their powerful antioxidant capacity and immune boosting properties demonstrated by *in vitro* studies (Krinsky, 1989; Chew and Park, 2004; Hix *et al.*, 2004). However, the specific roles that carotenoids might play *in vivo* in free radical scavenging or immune system function are uncertain and contentious (Hartley and Kennedy, 2004; Costantini, 2008; Costantini and Møller, 2008; Pérez-Rodríguez, 2009). A previous meta-analysis by Simons *et al.* (Simons *et al.*, 2012) examined the relationship between carotenoid content and multiple measures of immunocompetence in birds to test the idea that carotenoid content or coloration is an honest signal of this quality. They found that circulating carotenoid levels in the plasma related to only two out of seven measures; PHA response — a commonly used skin swelling assay of immunocompetence — and antioxidant capacity. Additionally, carotenoid coloration of feathers or skin was not a reliable signal for six out of seven proxies of immune function or oxidative stress (Simons *et al.*, 2012). These results are generally consistent with those of Simons *et al.* in that feather coloration was weakly correlated with measures of immune function, and was not a robust signal on average (Fig. 2-2c). Furthermore, whether coloration was derived from dietary or converted carotenoids had no effect on the honesty of coloration as a signal of immune function. The complex pathways involved in innate and acquired immunity may obscure meaningful interpretation of immunocompetence from only measuring the relative abundances of different types of white blood cells in circulation.

Unlike measures of immune function, studies on internal or surface parasites in birds benefit from a simple methodology that is straightforward to interpret. One of the earliest and

widely cited examples of honest signaling of parasite resistance from feather coloration was Hamilton and Zuk's (Hamilton and Zuk, 1982) study that proposed that "bright" feather coloration evolved to signal heritable parasite resistance. This seminal study served as the cornerstone for new ideas about why females might pay attention to male ornaments. I found that birds with converted carotenoid feather coloration had a strong relationship with parasite resistance. In contrast, birds that directly deposited carotenoids from their diet showed a weak and unreliable correlation with parasite resistance (Fig. 2-2d). The mechanisms involved in the transformation of dietary carotenoids might act as a signal of the underlying genetic or physiological mechanisms that provide parasite resistance.

The stronger associations between reproduction and plumage color derived from converted versus dietary pigments could be a consequence of stronger associations between male condition and ornamentation produced by converted versus dietary carotenoids. The proxies for reproductive and parental quality that were used in this study are a composite of the strategies and performances of both the ornamented male bird and its mate (Burley, 1986; Harris and Uller, 2009; Ratikainen and Kokko, 2010). Female mate choice for male coloration should be stronger when the signal of condition is stronger, so the stronger association between reproduction and converted carotenoids could arise through stronger female choice for such signals. Moreover, if the stronger preference for coloration resulting from converted carotenoids means that such males attract higher quality mates then color based on converted carotenoids would be more strongly linked to reproductive success. In many species of perching birds, males directly participate in nesting so there should also be direct effects of male condition on reproductive success. Any of these factors could have contributed to the stronger associations of color produced by converted carotenoids and reproduction.

The proxies for quality used in nearly every study of carotenoid coloration are rooted in the ability to efficiently produce ATP via oxidative phosphorylation at a level necessary to meet physiological demand. Thus far, the mechanism by which carotenoid coloration links to quality has remained speculative. Direct measures of both the allocation of specific pools of carotenoids to physiological function versus ornament production and the effects of direct manipulation of mitochondrial performance on carotenoid production are needed to definitively test the resource allocation and shared pathway hypotheses.

Conclusions and Future Research Directions

Overall, in this meta-analysis I found support for the hypothesis that carotenoid-based plumage coloration serves as an honest indicator of individual quality in birds (Hill, 1991). The strength of this signal, however, is greater for species that convert dietary carotenoids to ketolated carotenoids before utilizing them for feather coloration. These results are not consistent with the resource allocation hypothesis because dietary carotenoids are the pigments primarily implicated in immune enhancement and free radical scavenging, and yet they were not consistently associated to proxies for individual quality. These results are consistent, however, with the hypothesis that conversion of dietary carotenoids acts to strengthen the relationship between feather coloration and measures of quality and may be an important mechanism in maintaining honesty from carotenoid-based ornaments.

References

Blount, J. and McGraw, K. (2008) *Signal functions of carotenoid colouration*. *Carotenoids* (ed. by G. Britton, S.H.C. Liaaen-Jensen, and H. Pfander), pp. 213–236. Birkhäuser Basel.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R. and Surai, P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science*, 300, 125–127.

Booksmythe, I., Mautz, B., Davis, J., Nakagawa, S. and Jennions, M.D. (2017) Facultative adjustment of the offspring sex ratio and male attractiveness: a systematic review and meta-analysis. *Biol. Rev.*, 92, 108–134.

Brush, A.H. (1990) Metabolism of carotenoid pigments in birds. *FASEB J.*, 4, 2969–2977.

Burley, N. (1986) Sexual selection for aesthetic traits in species with biparental care. *Am. Nat.*, 127, 415–445.

del Cerro, S., Merino, S., Martínez-de la Puente, J., Lobato, E., Ruiz-de-Castañeda, R., Rivero-de Aguilar, J., Martínez, J., Morales, J., Tomás, G. and Moreno, J. (2010) Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (Cyanistes caeruleus). *Oecologia*, 162, 825–835.

Chew, B.P. and Park, J.S. (2004) Carotenoid action on the immune response. *J. Nutr.*, 134, 257S–261S.

Cohen, J. (1988) *Statistical Power Analysis for the Behavioral Sciences*, Second. Lawrence Erlbaum Associates, Hillsdale.

Costantini, D. (2008) Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol. Lett.*, 11, 1238–1251.

Costantini, D. and Møller, A.P. (2008) Carotenoids are minor antioxidants for birds. *Funct. Ecol.*, 22, 367–370.

Cotton, S., Small, J. and Pomiankowski, A. (2006) Sexual selection and condition-dependent mate preferences. *Curr. Biol.*, 16, R755–R765.

Edler, A.U. and Friedl, T.W.P. (2010) Individual quality and carotenoid-based plumage ornaments in male red bishops (Euplectes orix): plumage is not all that counts. *Biol. J. Linn. Soc.*, 99, 384–397.

Egger, M., Smith, G.D., Schneider, M. and Minder, C. (1997) Bias in meta-analysis detected by a simple, graphical test. *Brittish Med. J.*, 315, 629–634.

Endler, J.A. (1980) Natural selection on color patterns in Poecilia reticulata. *Evolution (N. Y).*, 34, 76–91.

Ferns, P.N. and Hinsley, S.A. (2007) Carotenoid plumage hue and chroma signal different aspects of individual and habitat quality in tits. *Ibis (Lond. 1859).*, 150, 152–159.

Fitze, P.S., Cote, J., San-Jose, L.M., Meylan, S., Isaksson, C., Andersson, S., Rossi, J.M. and Clobert, J. (2009) Carotenoid-based colours reflect the stress response in the common lizard. *PLoS One*, 4, e5111.

García-de Blas, E., Mateo, R. and Alonso-Alvarez, C. (2015) Accumulation of dietary carotenoids, retinoids and tocopherol in the internal tissues of a bird: a hypothesis for the cost of producing colored ornaments. *Oecologia*, 177, 259–271.

Garratt, M. and Brooks, R.C. (2012) Oxidative stress and condition-dependent sexual signals: more than just seeing red. *Proc. Biol. Sci.*, 279, 3121–3130.

Goodwin, T. (1992) Distribution of Carotenoids. *Methods Enzymol.*, 213, 167–172.

Griffith, S.C., Parker, T.H. and Olson, V.A. (2006) Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Anim. Behav.*, 71, 749–763.

Hadfield, J.D. (2010) MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *J. Stat. Softw.*, 33, 1–22.

Hadfield, J.D. and Nakagawa, S. (2010) General quantitative genetic methods for comparative biology: Phylogenies, taxonomies and multi-trait models for continuous and categorical characters. *J. Evol. Biol.*, 23, 494–508.

Hamilton, W.D. and Zuk, M. (1982) Heritable true fitness and bright birds: a role for parasites? *Science*, 218, 384–387.

Harris, W.E. and Uller, T. (2009) Reproductive investment when mate quality varies: differential allocation versus reproductive compensation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 364, 1039–1048.

Hartley, R.C. and Kennedy, M.W. (2004) Are carotenoids a red herring in sexual display? *Trends Ecol. Evol.*, 19, 353–354.

Higgins, J.P.T., Thompson, S.G., Deeks, J.J. and Altman, D.G. (2003) Measuring inconsistency in knowledgebases. *Brittish Med. J.*, 327, 557–560.

Hill, G. (1991) Plumage coloration is a sexually selected indicator of male quality. *Nature*, 350, 337–338.

Hill, G. and Montgomerie, R. (1994) Plumage colour signals nutritional condition in the house finch. *Proc. R. Soc. Lond. B*, 258, 47–52.

Hill, G.E. (2014) Cellular respiration: The nexus of stress, condition, and ornamentation. *Integr. Comp. Biol.*, 54, 1–13.

Hill, G.E. (2011) Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol. Lett.*, 14, 625–634.

Hill, G.E. (1994) House finches are what they eat: a reply to Hudon. *Auk*, 111, 221–225.

Hill, G.E. (1999) Is there an immunological cost to carotenoid-based ornamental coloration? *Am. Nat.*, 154, 589–595.

Hill, G.E. (2007) *Melanins and carotenoids as feather colorants and signals*. *Reproduction Biology and Phylogeny of Birds* (ed. by B.G.M. Jamieson), pp. 41–74. Science Publishers, Endfield, New Hampshire.

Hill, G.E. (1992) Proximate basis of variation in carotenoid pigmentation in male house finches. *Auk*, 109, 1–12.

Hill, G.E. and Johnson, J.D. (2012) The vitamin A-redox hypothesis: A biochemical basis for honest signaling via carotenoid pigmentation. *Am. Nat.*, 180, E127-150.

Hix, L.M., Lockwood, S.F. and Bertram, J.S. (2004) Bioactive carotenoids: potent antioxidants and regulators of gene expression. *Redox Rep.*, 9, 181–191.

Hõrak, P., Ots, I., Vellau, H., Spottiswoode, C. and Møller, A.P. (2001) Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. *Oecologia*, 126, 166–173.

Housworth, E.A., Martins, E.P. and Lynch, M. (2004) The phylogenetic mixed model. *Am. Nat.*, 163, 84–96.

Hudon, J. (1994) Showiness, carotenoids, and captivity: A comment on Hill (1992). *Auk*, 111, 218–221.

Isaksson, C. and Andersson, S. (2008) Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. *Proc. R. Soc. B-Biological Sci.*, 275, 309–314.

Isaksson, C., McLaughlin, P., Monaghan, P. and Andersson, S. (2007) Carotenoid pigmentation does not reflect total non-enzymatic antioxidant activity in plasma of adult and nestling great tits, Parus major. *Funct. Ecol.*, 21, 1123–1129.

Jennions, M.D., Lortie, C.J., Rosenberg, M.S. and Rothstein, H.R. (2013) *Publication and related biases*. *Handbook of Meta-Analysis in Ecology and Evolution* (ed. by J. Koricheva, J. Gurevitch, and K. Mengersen), pp. 207–236. Princeton University Press, Princeton.

Jetz, W., Thomas, G.H., Joy, J.B., Hartmann, K. and Mooers, A.O. (2012) The global diversity of birds in space and time. *Nature*, 491, 444–448.

Johnson, J.D. and Hill, G.E. (2013) Is carotenoid ornamentation linked to the inner mitochondria membrane potential? A hypothesis for the maintenance of signal honesty. *Biochimie*, 95, 436–444.

Karu, U., Saks, L. and Hõrak, P. (2007) Carotenoid coloration in greenfinches is individually consistent irrespective of foraging ability. *Physiol. Biochem. Zool.*, 80, 663–70.

Kodric-Brown, A. (1989) Dietary carotenoids and male mating success in the guppy: an environmental component to female choice. *Behav. Ecol. Sociobiol.*, 25, 393–401.

Kodric-Brown, A. and Brown, J.H.J. (1984) Truth in advertising: the kinds of traits favored by sexual selection. *Am. Nat.*, 124, 309–323.

Krinsky, N.I. (1989) Antioxidant functions of carotenoids. *Free Radic. Biol. Med.*, 7, 617–635.

Lipsey, M.W. and Wilson, D.B. (2001) *Practical Meta-analysis. (Applied Social Research Methods Series, Vol. 49)*, (ed. by L. Bickman and D.. Rog) Sage, Thousand Oaks, CA.

Lopes, R.J., Johnson, J.D., Toomey, M.B., Ferreira, M.S., Araujo, P.M., Melo-Ferreira, J., Andersson, L., Hill, G.E., Corbo, J.C. and Carneiro, M. (2016) Genetic basis for red coloration in birds. *Curr. Biol.*, 26, 1427–1434.

Lozano, G. (1994) Carotenoids, parasites, and sexual selection. *Oikos*, 70, 309–312.

McGraw, K. and Hill, G. (2000a) Carotenoid-based ornamentation and status signaling in the house finch. *Behav. Ecol.*, 11, 520–527.

McGraw, K.J. (2006) *Mechanics of carotenoid coloration*. *Bird Coloration, Volume 1: Measurements and Mechanisms* (ed. by G.E. Hill) and K.J. McGraw), Harvard University Press, Cambridge, Massachusetts.

McGraw, K.J. and Hill, G.E. (2000b) Differential effects of endoparasitism on the expression of carotenoid- and melanin-based ornamental coloration. *Proc. R. Soc. Biol. Sci. Ser. B*, 267, 1525–1531.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G. and Group, T.P. (2009) Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *PloS Med.*, 6, e1000097.

Møller, A.P., Biard, C., Blount, J.D., Houston, D.C., Ninni, P., Saino, N. and Surai, P.F. (2000) Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian Poult. Biol. Rev.*, 11, 137–159.

Montgomerie, R. (2006) *Analyzing Colors*. *Bird Coloration, Volume 1: Measurements and Mechanisms* (ed. by G.E. Hill and K.J. McGraw), Harvard University Press, Cambridge, Massachusetts.

Murphy, T.G., Hernandez-Mucino, D., Osorio-Beristain, M., Montgomerie, R. and Omland, K.E. (2009) Carotenoid-based status signaling by females in the tropical streak-backed oriole. *Behav. Ecol.*, 20, 1000–1006.

Nakagawa, S. and Santos, E.S.A. (2012) Methodological issues and advances in biological meta-analysis. *Evol. Ecol.*, 26, 1253–1274.

Pérez-Rodríguez, L. (2009) Carotenoids in evolutionary ecology: Re-evaluating the antioxidant role. *BioEssays*, 31, 1116–1126.

Pérez-Rodríguez, L., Mougeot, F. and Alonso-Alvarez, C. (2010) Carotenoid-based coloration predicts resistance to oxidative damage during immune challenge. *J. Exp. Biol.*, 213, 1685–9160.

R Core Team (2017) *R: A language and environment for statistical computing*, R Foundation for Statistical Computing, Vienna, Austria.

Ratikainen, I.I. and Kokko, H. (2010) Differential allocation and compensation: Who deserves the silver spoon? *Behav. Ecol.*, 21, 195–200.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. and Wittzell, H. (1999) Good genes, oxidative stress and condition–dependent sexual signals. *Proc. R. Soc. B Biol. Sci.*, 266, 1–12.

Shawkey, M.D. and Hill, G.E. (2005) Carotenoids need structural colours to shine. *Biol. Lett.*, 1, 121–124.

Simons, M.J.P., Cohen, A.A. and Verhulst, S. (2012) What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds-A meta-analysis. *PLoS One*, 7, e43088.

Simons, M.J.P., Maia, R., Leenknegt, B. and Verhulst, S. (2014) Carotenoid-dependent signals and the evolution of plasma carotenoid levels in birds. *Am. Nat.*, 184, 741–751.

Stahl, W. and Sies, H. (2003) Antioxidant activity of carotenoids. *Mol. Aspects Med.*, 24, 345–351.

Stradi, R., Celentano, G., Boles, M. and Mercato, F. (1997) Carotenoids in Bird Plumage: The Pattern in a Series of Red-Pigmented Carduelinae. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.*, 117, 85–91.

Stradi, R., Hudon, J., Celentano, G. and Pini, E. (1998) Carotenoids in bird plumage: the complement of yellow and red pigments in true woodpeckers (Picinae). *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.*, 120, 223–230.

Svensson, P.A. and Wong, B.B.M. (2011) Carotenoid-based signals in behavioural ecology: a review. *Behaviour*, 148, 131–189.

Thompson, C., Hillgarth, N., Leu, M. and McClure, H. (1997) High parasite load in house finches (Carpodacus mexicanus) is correlated with reduced expression of a sexually selected trait. *Am. Nat.*, 149, 270–294.

Viechtbauer, W. (2010) Conducting meta-analyses in R with the metafor package. *J. Stat. Softw.*, 36, 1–48.

Weaver, R.J., Hill, G.E., Kuan, P.-L. and Tseng, Y.-C. (2016) Copper exposure reduces production of red carotenoids in a marine copepod. *Ecol. Indic.*, 70, 393–400.

Weaver, R.J., Koch, R.E. and Hill, G.E. (2017) What maintains signal honesty in animal colour displays used in mate choice ? *Philos. Trans. R. Soc. B Biol. Sci.*, 372, 20160343.

Boxes

Box 1: Carotenoid structure, nomenclature, and metabolic transformations.

Carotenoids used for coloration in most animals are C_{40} tetraterpenoids. They consist of a central polyene chain — a system of conjugated carbon bonds that comprises most of the *chromophore* (*i.e.* part of the molecule that reflects light) — with ionone rings at either end (Fig 1). These hydrocarbon carotenoids are called *carotenes* whose specific names are derived from the types of ionone end rings present. β-carotene (β, β-carotene) contains two β-ionone rings, while α-carotene (β, ε -carotene) contains one β-ionone and one ε-ionone ring (Britton & Goodwin 1982). Carotenoids containing at least one unmodified β-ionone ring can be cleaved by most animals to yield retinal and thus have pro-vitamin A potential. Modifications to the end rings though oxidation reactions determine the function and color of the carotenoid by increasing its polarity and/or chromophore length. The addition of conjugated double bonds lengthens the chromophore and increases peak light absorption from shorter to longer wavelengths, causing a shift from yellow towards red color (*a bathochromic shift*).

Carotenes can be modified by the addition of oxygen (as hydroxyl or ketone functional groups) to carbons 3 or 4 of the ionone end rings through oxygenation or dehydrogenation reactions. These oxygenated carotenoids are broadly known as *xanthophylls.* Specific xanthophyll names are determined by the presence of either one or more hydroxyl groups (*hydroxy-carotenoids*) and/or ketone groups (*keto-carotenoids*) to the ionone rings. Zeaxanthin (3,3'-dihydroxy-β-carotene) and lutein (3,3'-dihydroxy- α -carotene) are common hydroxy-carotenoids that are abundant in the diet of many herbivorous and insectivorous animals. In contrast, keto-carotenoids such as echinenone (4-keto-β-carotene), canthaxanthin (4,4'-diketo-β-carotene), and astaxanthin (3,3'-dihydroxy-4,4'diketo -β-carotene) are mostly absent from animal diets. Instead, keto-carotenoids are produced either through ketolation of hydroxy-carotenoids or through hydroxylation and ketolation of carotenes and are responsible for most of the vibrant red hues of animal integuments. However, not all keto-carotenoids yield red coloration; *canary xanthophylls*—ketolated products of lutein and zeaxanthin that are derived from dehydrogenation of the existing hydroxyl groups —produce a rich yellow color used by some songbirds as feather pigments. The mechanism by which the ketone is formed includes a change from β-ionone rings to ε-ionone rings which shortens the conjugated system (shortens the chromophore) causing canary xanthophylls appear yellow and not red.

Despite the prevalence and importance of carotenoids in animals, the genetic architecture and physiological mechanisms involved in carotenoid metabolism have only recently been identified. In 2016, two independent lab groups characterized the genetic basis for red bill and red feather coloration, dubbed the redness gene. This gene encodes a cytochrome P450 oxidoreductase CYP2J19 that catalyzes the oxidative transformation of dietary carotenoids to hydroxy- or keto-carotenoids Identification of the particular mechanisms and cellular locations involved in hydroxylation and ketolation of carotenoids in animals is currently underway.

Tables

Table 2-1. Commonly used proxies for quality to evaluate the relationship between carotenoid-based ornaments and individual quality.

Category	Metric	Relationship with Quality
Condition	Body Mass	+
	Size Adjusted Body Mass	+
	Ptilochronology	+
Immune	Anti-Oxidant Capacity	+
	Antibody	+
	Disease Survival	+
	Environmental Pollutant	-
	HL Ratio	-
	Pathogen Challenge	-
	Oxidative Damage	-
	PHA response	+
	WBC count	+
Parasite	Load	-
	Resistance	+
Reproduction	Clutch Size	+
	Feeding Rate	+
	Fledgling number	+
	Fledgling Success	+
	Lay Date	-
	Nest Attentiveness	+

Table 2-2. Results from mixed-effects multilevel phylogenetic meta-analyses that assume effect sizes within studies are correlated (*r=0.5) k = number of effect sizes, m = number of species.* Category (combined) represents estimates from a meta-analytic model with life history trait category as a predictor, while Category (Converted) and Category (Dietary) represent estimates from a meta-analytic model with an interaction between life history trait category and type of carotenoid as predictor variables. Effect sizes in bold are considered to be statistically significantly different from 0, as the 95% credible interval did not overlap 0.

Analysis	*k*	*m*	Mean (*Zr*)	Lower CI (2.5%)	Upper CI (97.5%)	I^2 (%)	Heterogeneity (Var_{study}) %	Heterogeneity ($Var_{species}$) %	Heterogeneity (phylogeny) %
Overall	197	21	0.195	-0.012	0.416	98.12	0.581	1.06	2.12
Carotenoid Type:						71.97			
Converted	95	13	0.276	0.066	0.478				
Dietary	102	8	0.129	-0.061	0.360				
Category (combined)						72.63			
Condition	37	14	0.111	-0.104	0.329				
Immune Function	42	10	0.146	-0.069	0.407				
Parasite Resistance	49	11	0.285	0.076	0.535				
Reproductive and Parental Quality	69	11	0.259	0.035	0.478				
Category (Converted)						95.37			
Condition	19	9	0.123	-0.118	0.359				
Immune Function	21	6	0.150	-0.086	0.421				

Parasite Resistance	30	8	0.455	0.240	0.707
Reproductive and Parental Quality	25	4	0.353	0.087	0.621
Category (Dietary)					
Condition	18	5	0.144	-0.104	0.399
Immune Function	21	4	0.162	-0.083	0.384
Parasite Resistance	19	3	0.055	-0.168	0.320
Reproductive and Parental Quality	44	7	0.142	-0.074	0.387

Figures

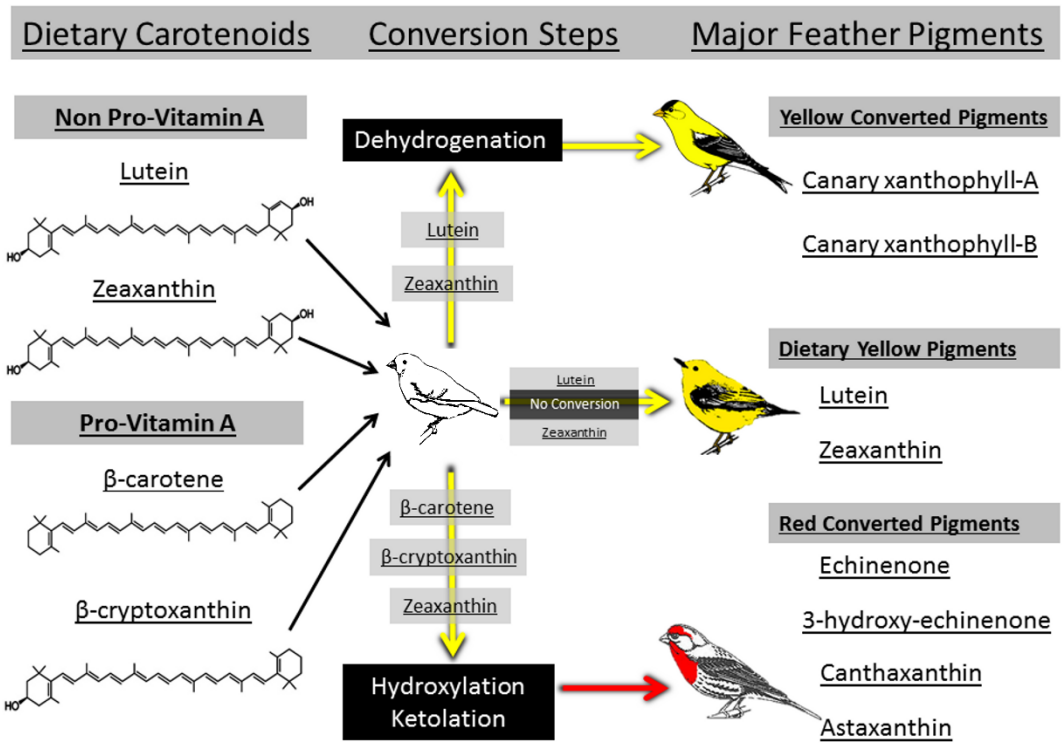

Figure 2-1. Bioconversion pathways of carotenoids in birds. To produce feather coloration, passerine birds either use dietary carotenoids unaltered or use carotenoid pigments that are metabolically derived from dietary pigments. Depicted are the proposed metabolic pathways by which the common dietary carotenoids in the diets of passerine birds can be converted into the red and yellow ketolated carotenoids found in feathers (Stradi *et al.*, 1997, 1998; McGraw, 2006)

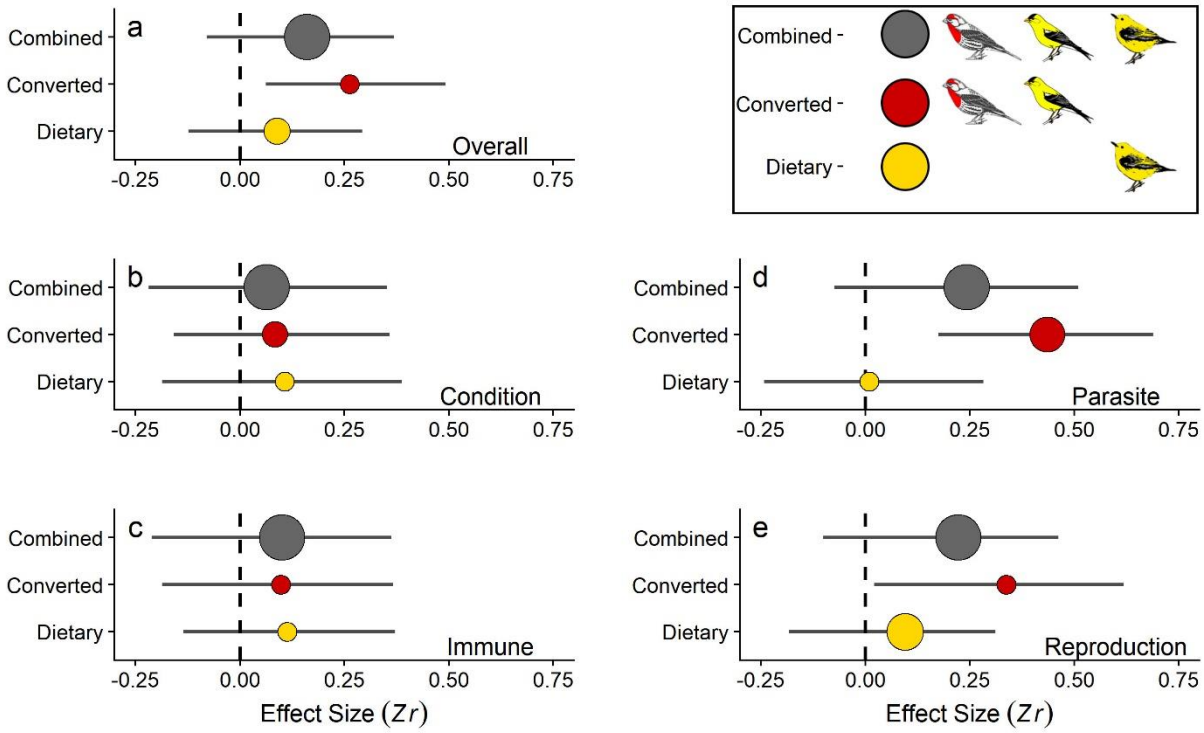

Figure 2-2. The weighted mean correlation (Zr) of feather color richness in relation to individual quality. The strength of the association was calculated for all published studies without consideration of the carotenoid type in the feathers of the study bird (Combined, black squares), for only studies of bird species with plumage coloration derived from converted carotenoids (red squares), and for only studies of bird species with plumage coloration derived from dietary carotenoids (yellow squares). Proxies of quality were divided into b) body condition, c) immune function, d) parasite resistance, and e) aspects of parental and reproductive quality. Square size is inversely proportional to the variance of the mean effect size. Horizontal lines represent 95% credible intervals. Effect size estimates with credible intervals that do not include zero are statistically significant effects (α =0.05).

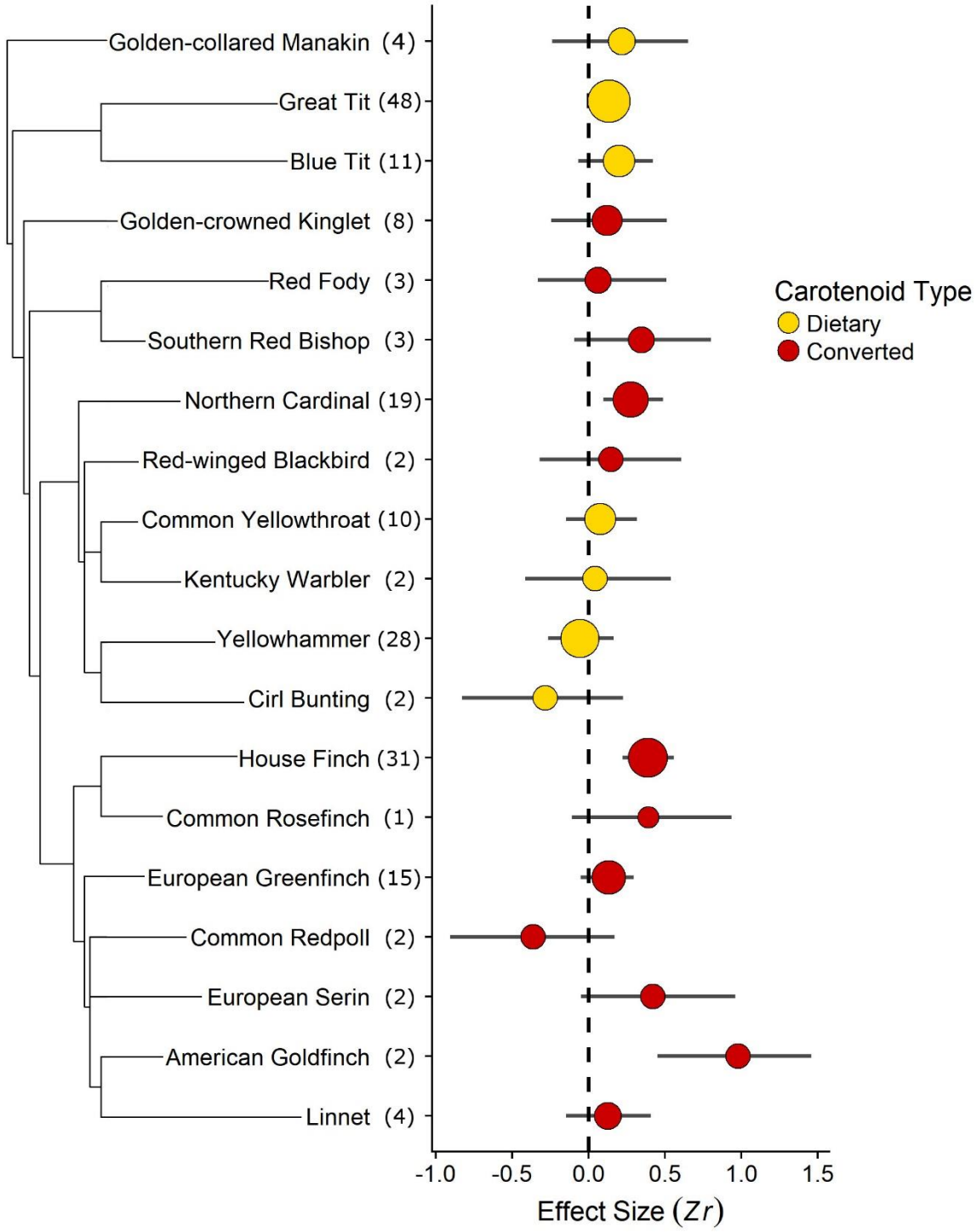

Figure 2-3. The contribution of each species in the overall relationship between feather color richness and measures of individual quality. Boxes represent weighted mean correlation (*Zr*) effect sizes for species with plumage coloration from converted (red) and dietary carotenoids (yellow). Box size is inversely proportional to the variance of the mean effect size. Horizontal lines represent 95% credible intervals. Effect size estimates with credible intervals that do not include zero are statistically significant effects ($\alpha = 0.05$).

Chapter 3. On the bioconversion and distribution of astaxanthin in the marine copepod, *Tigriopus californicus*

Manuscript published with co-authors Geoffrey Hill and Paul Cobine in 2018 in *Journal of Plankton Research* **40**, 142-150.

Introduction

With few exceptions, animals cannot synthesize carotenoids *de novo* from basic biological precursors (Britton and Goodwin 1982). To use carotenoids as external colorants, animals as diverse as flamingos and lobsters must obtain carotenoids from their diet (Fox and Hopkins 1966; Cianci et al. 2002). Animals with red coloration can derive red carotenoid pigments via two distinct pathways: they can ingest yellow pigments and oxidize them to produce red keto-carotenoids (Fig. 3-1) or they can ingest red pigments directly. For most metazoans, yellow carotenoids are much more common components of diets and thus most metazoans derive red coloration through the conversion of yellow dietary pigments (Goodwin 1984; Brush 1990). The processes involved in carotenoid bioconversion have far reaching implications for research across fields of study ranging from ecotoxicology (Weaver et al. 2016) to sexual selection for colorful ornaments (Hill 1991; Weaver et al. 2017). Yet, despite the long history of research on the evolution and distribution of carotenoid coloration across diverse taxa such as birds, fish, and crustaceans, an ideal model system for studying the genetic and physiological mechanisms involved in carotenoid metabolism in animals does not exist.

Recently, I have begun investigating the potential of the red marine copepod *Tigriopus californicus* (Baker 1912) to serve as a model system for the study of carotenoid physiology. Over the past four decades, *Tigriopus* copepods have become model organisms for studies on

ecotoxicology (Raisuddin et al. 2007), phylogeography (Edmands 2001), local adaptation (Pereira et al. 2016), and mitochondrial-nuclear interactions (Ellison and Burton 2008). As a result, a wealth of physiological and genetic data exist that will facilitate detailed investigations in the molecular mechanisms involved in carotenoid coloration. However, fundamental aspects of their pigment physiology are relatively unexplored.

In the wild, *Tigriopus californicus* and other species within the genus *Tigriopus* typically have bright orange-red coloration (Fig. 3-2 a,c) that is produced via accumulation of the red keto-carotenoid astaxanthin (Goodwin and Srisukh 1949; Davenport et al. 2004; Weaver et al. 2016). The orange-red coloration is visible to the naked eye, but upon closer inspection under a dissecting microscope the pigment appears to be in the highest concentration along the gut lining and in lesser amounts throughout the exoskeleton. Adults retain a single naupliar eye spot located on the dorsal anterior cephalosome that is a vibrant red color (Fig 3-2). Previous studies have examined the microstructure of the naupliar eye and speculated that the red color was the result of carotenoids, but no formal analysis was undertaken (Martin et al. 2000). Carotenoids may also be deposited into eggs (Goodwin and Srisukh 1949). Wild gravid females and gravid females fed microalgae in the lab carry a single median egg sac of 15-40 developing embryos (Burton 1985) that transitions from a dark gray to rich red color as development progresses (Fig 3-2c).

The dietary source of carotenoid coloration of *Tigriopus* copepods is supported by the observation of Davenport et al. (1997) that when they are reared on a diet lacking dietary carotenoids, they lose their characteristic red color and appear white. However, to date, the dietary requirements and the bioconversion of dietary carotenoids to astaxanthin by *Tigriopus californicus* has not been the subject of well-controlled studies.

In this study, I first characterized the molecular source of pigmentation in *T. californicus*. I compared the body coloration and identified the carotenoid content of animals fed a live microalgae diet and carotenoid-free yeast diet. Next, I tested whether the red coloration of the naupliar eye spot (Fig. 3-2) and the coloration of the egg sacs of gravid females (Fig. 3-2c) was due to the presence of astaxanthin. Finally, I performed carefully controlled precursor-product carotenoid-supplement feeding experiments to study the bioconversion of dietary precursor carotenoids to astaxanthin by *T. californicus*.

Methods

Copepod culturing

Tigriopus californicus copepods collected from the wild in the vicinity of San Diego, California have been raised in my laboratory since 2014 in 10 L aquaria in filtered artificial seawater (ASW) at salinity of 32 at 24 °C on a 12 h light: 12 h dark light cycle and fed live microalgae *Tetraselmis chuii* and *Synechococcus spp.* This diet contains algal-derived carotenoids α- and β-carotene, lutein, and zeaxanthin (Guillard et al. 1985; Brown and Jeffrey 1992), which are hypothesized to be substrates for bioconversion to astaxanthin (Fig. 3-1). I refer to copepods raised under these conditions as the 'stock population'.

Testing the dietary origin of red coloration in T. californicus

To test the hypothesis that the characteristic orange-red coloration of *T. californicus* depends on the presence of carotenoids in their diet, in 2015 I switched a sample of approximately 1,000 stock population copepods to a carotenoid-free diet of nutritional yeast (Bragg, Santa Barbara, CA). The nutritional yeast diet contains inactive dry yeast and a mix of B-complex vitamins but lacks carotenoids. I cultured these copepods in 15 L containers with

opaque sides and lids to deter algal growth under gentle aeration; changing the water and mixing copepods from separate containers approximately once every three months. I refer to copepods raised under these conditions as 'yeast-fed copepods'. I sampled 10 adult copepods from the stock population in triplicate and 10 yeast-fed copepods in triplicate then visually assessed coloration and tested for the presence of carotenoids in their bodies as described below.

Qualitative carotenoid analysis of red eyespots

To determine whether the red coloration of the naupliar eyespot was carotenoid-based, I dissected the portion of the cephalosome containing the eyespot from 10 adult yeast-fed copepods. All 10 dissected eyespots were pooled in one tube and the corresponding 10 bodies were pooled in a separate tube then processed and analyzed for carotenoids as described below.

Maternal deposition of astaxanthin to developing embryos

In a separate experiment, I sought to determine whether the red coloration of mature egg sacs attached to stock population gravid females was carotenoid-based. I carefully removed red egg sacs from three gravid females using a fine needle under a dissecting scope and processed each clutch individually for carotenoid analysis as described below.

Bioconversion to astaxanthin from carotenoid supplementation of yeast-fed copepods

To definitively test for the bioconversion of dietary carotenoids to astaxanthin by *T. californicus,* yeast-fed copepods were supplemented with either β-carotene, lutein, zeaxanthin, and canthaxanthin. I chose these four precursor carotenoids for the feeding experiments because they occupy various positions in the putative pathway used by copepods to produce astaxanthin (Fig. 3-1). Stock solutions of β-carotene, lutein, zeaxanthin, and canthaxanthin were made from water-soluble carotenoid beadlettes (DSM, Basel, Switzerland) in ASW and diluted to a working carotenoid concentration of 2 μg mL^{-1}. Each carotenoid-supplement contained only the

carotenoid listed, except the lutein supplement. Of the total carotenoid content, 90.6% was pure lutein, but zeaxanthin comprised 7.8%. Therefore, the "lutein" supplement contained 1.984 µg mL^{-1} lutein, and 0.016 µg mL^{-1} zeaxanthin. For each carotenoid supplement group, 10 adult copepods were placed in 5 mL carotenoid solution in each well of a six-well plate (n = 6 for each supplement group) with 0.75 mg nutritional yeast as food for 48 h, then processed for carotenoid analysis (see below).

Carotenoid analysis

After each experiment, copepods were placed in fresh ASW to clear gut contents, then rinsed with de-ionized water, dried and –for the bioconversion experiment – weighed to the nearest 0.01 mg. The mass from one sample from the β-carotene supplement group was not recorded and one sample from the zeaxanthin supplement group was destroyed before carotenoid analysis.

Carotenoids were extracted from copepods by sonicating in 500 µL HPLC-grade acetone in a 1.7 mL microcentrifuge tube for 10s at 10W; I then capped tubes with nitrogen gas and incubated them overnight at 4 °C in the dark. Samples were centrifuged at 3,000g for 5 min, the supernatant was removed to a new tube and evaporated to dryness at 40 °C under vacuum, then resuspended in 50 µL acetone. Carotenoids were separated using a Shimadzu HPLC system from a 40 µL injection on to a Sonoma C18 column (10 µm, 250 x 4.6 mm, ES Technologies) fitted with a C18 guard cartridge. I used mobile phases A) 80:20, methanol: 0.5 M ammonium acetate, B) 90:10, acetonitrile:H_2O, and C) ethyl acetate in a tertiary linear gradient as follows: 100% A to 100% B over 4 min, then to 80% C: 20% B over 14 min, back to 100% B over 3 min and returning to 100% A over 5 min and held for 6 minutes (Wright et al. 1991). Total run time was 32 min at a flow rate of 1 mL min^{-1}. Absorbance was measured at 450 nm using a UV/VIS

detector. Carotenoids were identified and quantified by comparison to authentic standards. Astaxanthin concentration was normalized to copepod dry weight.

Statistical analysis

I tested for a difference in the amount of astaxanthin produced by copepods from each group using ANOVA and evaluated pairwise comparisons between groups using Tukey HSD post-hoc test.

Results

Dietary origin of the red coloration of T. californicus

The major carotenoid found in stock population copepods was free astaxanthin (mean ± SE; 49.38 ± 2.19 ng copepod $^{-1}$, n = 3). Minor amounts of mono and di-esterified astaxanthin were detected comprising 3.22% and 8.83% of the total carotenoid content, respectively. I found that when stock population copepods were switched to a carotenoid-free yeast diet they lost their characteristic orange-red coloration and appeared clear (Fig. 3-2b). Biochemical analysis revealed that a small but measurable amount of astaxanthin was detected in the yeast-fed copepods (mean ± SE; 0.54 ± 0.016 ng copepod $^{-1}$, n = 3).

Astaxanthin analysis of red eyespots

I detected no carotenoids in the red eyespot-enriched fraction of yeast-fed *Tigriopus californicus.* Analysis of the corresponding body fraction returned a similar concentration of astaxanthin as from the yeast-fed copepods in the previous experiment (0.3 ng copepod $^{-1}$).

Astaxanthin analysis of red egg sacs

I found that the red egg sacs of stock population gravid females contained astaxanthin (mean ± SE; 10.53 ± 3.31 ng egg sac $^{-1}$, n = 3).

Bioconversion of dietary supplemented carotenoids

I found that *T. californicus* copepods from each carotenoid supplement group accumulated astaxanthin after 48 h when none was present in their diet (Fig. 3-3). The amount of astaxanthin converted depended on the specific carotenoid supplemented (mean astaxanthin in µg mg $^{-1}$ dry mass of copepod ± SE); zeaxanthin (0.99 ± 0.11 µg mg $^{-1}$, n =5) > canthaxanthin (0.90 ± 0.12 µg mg $^{-1}$, n =6) > β-carotene (0.38 ± 0.06 µg mg $^{-1}$, n =5) > lutein (0.21 ± 0.02 µg mg $^{-1}$, n =6). I detected significant differences in astaxanthin production among supplement groups (ANOVA, F (4,23) = 29.83, P < 0.0001). Post-hoc pairwise comparisons revealed that the amount of astaxanthin produced was not significantly different between zeaxanthin and canthaxanthin groups (Tukey HSD, difference, (95% CI): -0.09, (-0.41 to 0.23) µg mg $^{-1}$, P=0.91) or between β-carotene and lutein groups (Tukey HSD, difference, (95% CI) : -0.17, (-0.5 to 0.14) µg mg $^{-1}$, P=0.49). However, I found that copepods supplemented with zeaxanthin and canthaxanthin produced significantly more astaxanthin than copepods fed either β-carotene or lutein ($P < 0.001$). In each supplement group, only free astaxanthin and the supplemented carotenoid were detected. It is possible that exiguous amounts of intermediate carotenoids were present in samples, but were not detected on the HPLC system.

Discussion

In this study, I conducted carefully controlled precursor/product tests to document the bioconversion of dietary carotenoids to the red ketocarotenoid astaxanthin in the marine copepod *T. californicus*. When they were maintained on a yeast diet that provided no carotenoids, copepods became clear with no hint of red or yellow. Biochemical analysis confirmed that these animals essentially lacked carotenoids in their tissues. When I supplemented yeast-fed carotenoid-free copepods with β-carotene, lutein, zeaxanthin, or canthaxanthin for 48 h, I

observed significant production of astaxanthin (Fig. 3-3). These experiments confirmed that *T. californicus* requires carotenoid-rich foods to obtain their characteristic orange-red color and that they bioconvert precursor carotenoids from their diet to astaxanthin.

The amount of astaxanthin produced depended on which carotenoid was the precursor for bioconversion (Fig 3-3). Copepods fed zeaxanthin and canthaxanthin produced more astaxanthin in 48h than copepods fed β-carotene or lutein. This pattern suggests that the number of oxidation reactions required to convert the supplemented precursor carotenoid to astaxanthin may mediate the rate of astaxanthin production in this species. Canthaxanthin and zeaxanthin require two hydroxylation or two ketolation reactions respectively to form astaxanthin; whereas, β-carotene requires 4-7 reactions, and lutein requires 3 reactions and a conversion from α-doradexanthin to β-doradexanthin (Fig 2-1). Interestingly, a similar effect of supplementation with zeaxanthin versus lutein was observed in a study of American Goldfinches, which transform dietary carotenoid to Canary Xanthophyll A and B, and Northern Cardinals which transform dietary pigments to astaxanthin. Both goldfinches and cardinals produced more oxidized pigments and more colorful integumentary structures when they were fed zeaxanthin compared to when they were fed lutein (McGraw et al. 2014).

In the wild, copepods that feed on micro and macro algae ingest relatively large quantities of β-carotene, zeaxanthin, and lutein (Brown and Jeffrey 1992; Buffan-dubau et al. 1996; Sigaud-Kutner et al. 2005; Takaichi 2011; Wang et al. 2015). The bioconversion of dietary carotenoids to astaxanthin has been documented in other copepod species (Rhodes 2007; Caramujo et al. 2012), crustaceans (Hsu et al. 1970; Tanaka et al. 1976) and fish (Hsu et al. 1972) and these authors have concluded that the pathway begins with β-carotene. However, in addition to being used as a pigment, β-carotene is also the main precursor for vitamin A

synthesis in animals (Parker 1996), which may cause an allocation tradeoff between vitamin A production and coloration (Hill and Johnson 2012). Alternatively, these results suggest that *T. californicus* may utilize multiple carotenoids as substrates for bioconversion to astaxanthin depending on which carotenoids are available in their diet, and/or the body's need for vitamin A. Zeaxanthin has no vitamin A capacity and I found that copepods fed this precursor produced significantly more astaxanthin than β-carotene supplemented copepods. While I did not detect any intermediates along the proposed bioconversion pathways, these results demonstrate that *T. californicus* use zeaxanthin as a substrate for astaxanthin production. It is possible that zeaxanthin is the start of a more efficient bioconversion pathway for astaxanthin production by *T. californicus.* Future experiments that analyze larger amounts of copepods within shorter sampling intervals may identify intermediate carotenoids and help resolve which astaxanthin bioconversion pathway(s) is used by *T. californicus*.

It is unclear from this study whether *T. californicus* uses lutein as a substrate for astaxanthin production because the lutein supplement also contained trace amounts of zeaxanthin. Lutein is common in marine phytoplankton as well, and some marine animals are thought to preferentially use lutein as the substrate for bioconversion to astaxanthin and other ketocarotenoids. Hsu (Hsu et al. 1972) and Katayama (Katayama et al. 1973) have shown that goldfish (*Carassius auratus*) potentially use lutein as precursor to astaxanthin. However, this bioconversion pathway requires the isomerization of α-doradexanthin to β-doradexanthin, a transformation that others have shown to be unlikely in fungus, plants, and other marine animals (Matsuno et al. 1999; Ohkubo et al. 1999).

The red coloration of the eyespot is not dependent on diet; *Tigriopus* copepods fed either algae or yeast both have red eye coloration (Fig 3-2). I found that the bright red eyespot

coloration of *T. californicus* is not from astaxanthin, or any other carotenoid that I was able to detect, and that the trace astaxanthin content of yeast-fed copepods is not located in the eye. These results clarify that the red eye coloration is not from astaxanthin, but may be from the visual pigment rhodopsin that may utilize 3-hydroxyretinal as the chromophore (Cronin 1986).

I found that females deposit astaxanthin to developing embryos, supporting previous reports of this ketocarotenoid occurring in the egg sacs of other *Tigriopus* species (Goodwin and Srisukh 1949). It has been proposed that deposition of astaxanthin to developing eggs provides embryos photoprotection from solar UV radiation (Dethier 1980). Won et al. (2014) have shown that experimental UV exposure reduced hatching success of *Paracyclopina nana* nauplii, although the specific roles that carotenoids may play in survival following UV exposure remain unclear.

Investigations into the genetic architecture and physiological mechanisms involved in carotenoid metabolism in animals has only recently begun. The gene responsible for ketolation of yellow dietary carotenoids in birds - dubbed the redness gene - was independently discovered by Lopes et al (2016) and Mundy et al (2016). This gene encodes a cytochrome P450 oxidoreductase enzyme, CYP2J19, that has sequence motifs that implicate subcellular localization to mitochondria. The enzyme that enables conversion of yellow to red pigments in arthropods in general and *Tigriopus* copepods in particular has yet to determined, but *Tigriopus* are poised to be the model for identification of the physiological mechanisms and cellular locations involved in hydroxylation and ketolation of carotenoids in animals.

Conclusion

I have demonstrated that the orange-red coloration of *Tigriopus californicus* is a result of bioconverting dietary yellow carotenoids to the red keto carotenoid astaxanthin. The

experimental design of producing carotenoid-deficient copepods then supplementing with a single precursor carotenoid could serve as a powerful system for elucidating the genetic underpinnings and physiological constraints of carotenoid metabolism in this species, with broad implications applicable to understanding the evolution of carotenoid-based ornaments in honest signaling systems.

References

Baker CF (1912) Notes on the Crustacea of Laguna Beach. Annu Rep Laguna Mar Lab 1:100–117.

Britton G, Goodwin T (1982) Carotenoid chemistry and biochemistry. Pergamon Press Ltd, New York

Brown MR, Jeffrey SW (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. J Exp Mar Bio Ecol 161:91–113. doi: 10.1016/0022-0981(92)90192-D

Brush AH (1990) Metabolism of carotenoid pigments in birds. FASEB J 4:2969–2977.

Buffan-dubau E, Wit R De, Castel J (1996) Feeding selectivity of the harpacticoid copepod Canuella perplexa in benthic muddy environments demonstrated by HPLC analyses of chlorin and carotenoid pigments. 137:71–82.

Burton RS (1985) Mating system of the intertidal copepod Tigriopus californicus. Mar Biol 86:247–252. doi: 10.1007/BF00397511

Caramujo M-J, de Carvalho CCCR, Silva SJ, Carman KR (2012) Dietary carotenoids regulate astaxanthin content of copepods and modulate their susceptibility to UV light and copper toxicity. Mar Drugs 10:998–1018. doi: 10.3390/md10050998

Cianci M, Rizkallah PJ, Olczak A, Raftery J, Chayen NE, Zagalsky PF, Helliwell JR (2002) The molecular basis of the coloration mechanism in lobster shell: beta-crustacyanin at 3.2-A resolution. Proc Natl Acad Sci U S A 99:9795–9800. doi: 10.1073/pnas.152088999

Cronin TW (1986) Photoreception in Marine Invertebrates. Am Zool 415:403–415.

Davenport J, Barnett PR., McAllen RJ (1997) Environmental tolerances of three species of the harpacticoid copepod genus Tigriopus. J Mar Biol Assoc United Kingdom 77:3–16.

Davenport J, Healy A, Casey N, Heffron J (2004) Diet-dependent UVAR and UVBR resistance in the high shore harpacticoid copepod Tigriopus brevicornis. Mar Ecol Prog . 276:299–303.

Dethier MN (1980) Tidepools as refuges: Predation and the limits of the harpacticoid copepod Tigriopus californicus (Baker). J Exp Mar Bio Ecol 42:99–111. doi: 10.1016/0022-0981(80)90169-0

Edmands S (2001) Phylogeography of the intertidal copepod Tigriopus californicus reveals substantially reduced population differentiation at northern latitudes. Mol Ecol 10:1743–1750. doi: 10.1046/j.0962-1083.2001.01306.x

Ellison CK, Burton RS (2008) Interpopulation hybrid breakdown maps to the mitochondrial genome. Evolution 62:631–8. doi: 10.1111/j.1558-5646.2007.00305.x

Fox DL, Hopkins TS (1966) Comparative metabolic fractionation of carotenoids in three flamingo species. Comp Biochem Physiol 17:841–56. doi: 10.1016/0010-406X(66)90125-3

Goodwin T (1984) The Biochemistry of the Carotenoids: Volume II Animals. Chapman and Hall, New York, New York

Goodwin T, Srisukh S (1949) Some observations on astaxanthin distribution in marine Crustacea. Biochem J 45:268–270.

Guillard RR., Murphy LS, Foss P, Liaaen-Jensen S (1985) Synechococcus spp. as zeaxanthin-dominant ultraphytoplankton in the North Atlantic. Limnol Oceanogr 30:412–414. doi: 10.4319/lo.1985.30.2.0412

Hill G (1991) Plumage coloration is a sexually selected indicator of male quality. Nature 350:337–338.

Hill GE, Johnson JD (2012) The Vitamin A-Redox Hypothesis: A Biochemical Basis for Honest Signaling via Carotenoid Pigmentation. Am Nat 180:E127-50. doi: 10.1086/667861

Hsu WJ, Chichester CO, Davies BH (1970) The metabolism of beta-carotene and other carotenoids in the brine shrimp, Artemia Salina L. (Crustacea: Branchiopoda). Comp Biochem Physiol 32:69–79. doi: 10.1016/0010-406X(70)90156-8

Hsu WJ, Rodriguez DB, O. CC (1972) The biosynthesis of astaxathin VI. The conversion of [14C] lutein and [14C] beta-carotene in goldfish. Int J Biochem 3:333–338.

Katayama T, Kunisaki Y, Shimaya M, Sompson KL, Chichester CO (1973) The biosynthesis of astaxanthin XIV. The conversion of labelled beta-carotene 15,15'-3H2 into astaxanthin in the crab, portunus trituberculatus. Comp Biochem Physiol 46B:269–272.

Lopes RJ, Johnson JD, Toomey MB, Ferreira MS, Araujo PM, Melo-Ferreira J, Andersson L, Hill GE, Corbo JC, Carneiro M (2016) Genetic Basis for Red Coloration in Birds. Curr Biol 1–8. doi: 10.1016/j.cub.2016.03.076

Martin GG, Speekmann C, Beidler S (2000) Photobehavior of the harpacticoid copepod Tigriopus californicus and the fine structure of its nauplius eye. Invertebr Biol 119:110–124. doi: 10.1111/j.1744-7410.2000.tb00179.x

Matsuno T, Ohkubo M, Toriiminami Y, Tsushima M, Sakaguchi S, Minami T, Maoka T (1999) Carotenoids in food chain between freshwater fish and aquatic insects. Comp Biochem Physiol Part B Biochem Mol Biol 124:341–345. doi: 10.1016/S0305-0491(99)00125-X

McGraw KJ, Hill GE, Navara KJ, Parker RS (2014) Differential accumulation and pigmenting ability of dietary carotenoids in colorful finches. Physiol Biochem Zool 77:484–91. doi: 10.1086/383506

Mundy NI, Stapley J, Bennison C, Tucker R, Twyman H, Kim K-W, Burke T, Birkhead TR, Andersson S, Slate J (2016) Red Carotenoid Coloration in the Zebra Finch Is Controlled by a Cytochrome P450 Gene Cluster. Curr Biol 26:1435–1440. doi: 10.1016/j.cub.2016.04.047

Ohkubo M, Tsushima M, Maoka T, Matsuno T (1999) Carotenoids and their metabolism in the goldfish Carassius auratus (Hibuna). Comp Biochem Physiol Part B Biochem Mol Biol 124:333–340. doi: 10.1016/S0305-0491(99)00124-8

Parker RS (1996) Absorption, metabolism, and transport of carotenoids. Faseb J 10:542–551.

Pereira RJ, Sasaki MC, Burton RS (2016) Adaptation to a latitudinal thermal gradient within a widespread copepod species: the contributions of genetic divergence and developmental plasticity.

Raisuddin S, Kwok KWH, Leung KMY, Schlenk D, Lee J-S (2007) The copepod Tigriopus: a promising marine model organism for ecotoxicology and environmental genomics. Aquat Toxicol 83:161–73. doi: 10.1016/j.aquatox.2007.04.005

Rhodes ACE (2007) Dietary effects on carotenoid composition in the marine harpacticoid copepod Nitokra lacustris. J Plankton Res 29:i73–i83. doi: 10.1093/plankt/fbl068

Sigaud-Kutner TCS, Neto AMP, Pinto E, Colepicolo P (2005) Diel activities of antioxidant enzymes, photosynthetic pigments and malondialdehyde content in stationary-phase cells of Tetraselmis gracilis (Prasinophyceae). Aquat Bot 82:239–249. doi: 10.1016/j.aquabot.2005.02.011

Takaichi S (2011) Carotenoids in algae: distributions, biosyntheses and functions. Mar Drugs 9:1101–18. doi: 10.3390/md9061101

Tanaka Y, Matsuguchi H, Katayama T, Simpson KL, Chichester CO (1976) The biosynthesis of astaxanthin- XVI. The carotenoids in Crustacea. Comp Biochem Physiol B 54:391–393.

Wang G, Xu J, Zeng C, Jia Q, Wu L, Li S (2015) Pelagic microalgae as suitable diets for the benthic harpacticoid copepod Tigriopus japonicus. Hydrobiologia. doi: 10.1007/s10750-015-2339-5

Weaver RJ, Hill GE, Kuan P-L, Tseng Y-C (2016) Copper exposure reduces production of red carotenoids in a marine copepod. Ecol Indic 70:393–400. doi: 10.1016/j.ecolind.2016.06.040

Weaver RJ, Koch RE, Hill GE (2017) What maintains signal honesty in animal colour displays used in mate choice ? Philos Trans R Soc B Biol Sci 372:20160343. doi: http://dx.doi.org/10.1098/rstb.2016.0343

Won EJ, Lee Y, Han J, Hwang UK, Shin KH, Park HG, Lee JS (2014) Effects of UV radiation on hatching, lipid peroxidation, and fatty acid composition in the copepod Paracyclopina nana. Comp Biochem Physiol Part - C Toxicol Pharmacol 165:60–66. doi: 10.1016/j.cbpc.2014.06.001

Wright S, Jeffrey S, Mantoura RFC, Llewellyn CA, Bjornland T, Repeta D, Welschmeyer N (1991) Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. Mar Ecol Prog Ser 77:183–196.

Figures

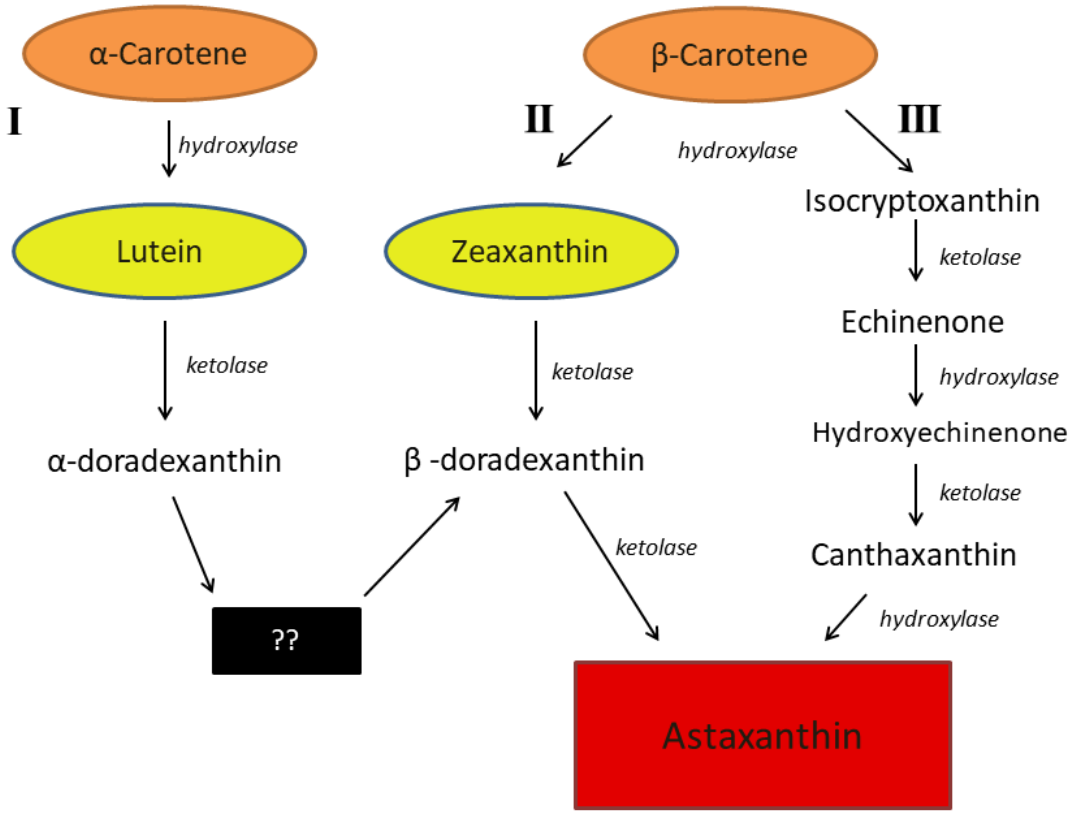

Figure 3-1. Proposed bioconversion pathways for astaxanthin production in animals (modified from Rhodes, 2007). Pathway I is used by some fishes, including goldfish, that utilizes lutein as a substrate. ?? represents the putative transformation of α to β-doradexanthin. Pathway II utilizes β-carotene or zeaxanthin as a substrate to astaxanthin. Pathway III begins with β-carotene and includes canthaxanthin as an intermediate to astaxanthin. The class of enzyme responsible for each transformation is italicized.

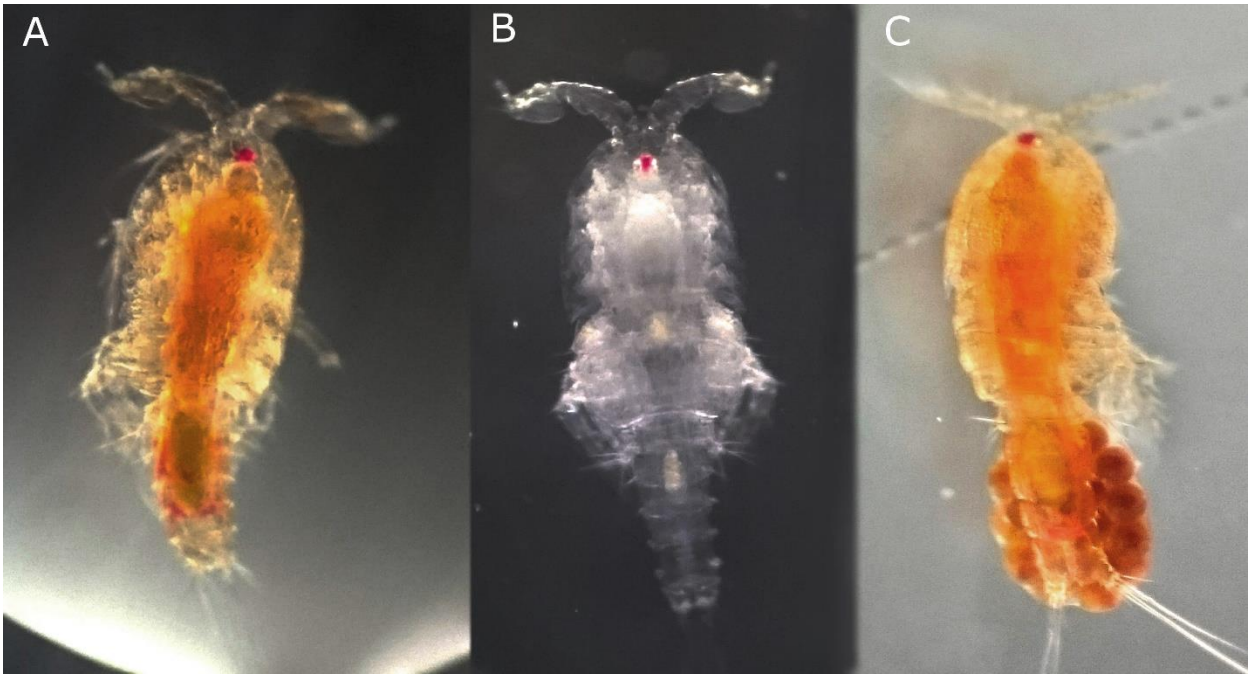

Figure 3-2. Coloration of T. californicus fed different diets. Typical coloration of *Tigriopus californicus* fed microalgae (A,C) and nutritional yeast (B). The red naupliar eye spot is present in copepods fed both carotenoid-rich and carotenoid-deficient diets suggesting that production of that eye pigment is not diet-dependent. (C) Females carry egg sacs that transition from dark gray to red as embryos develop.

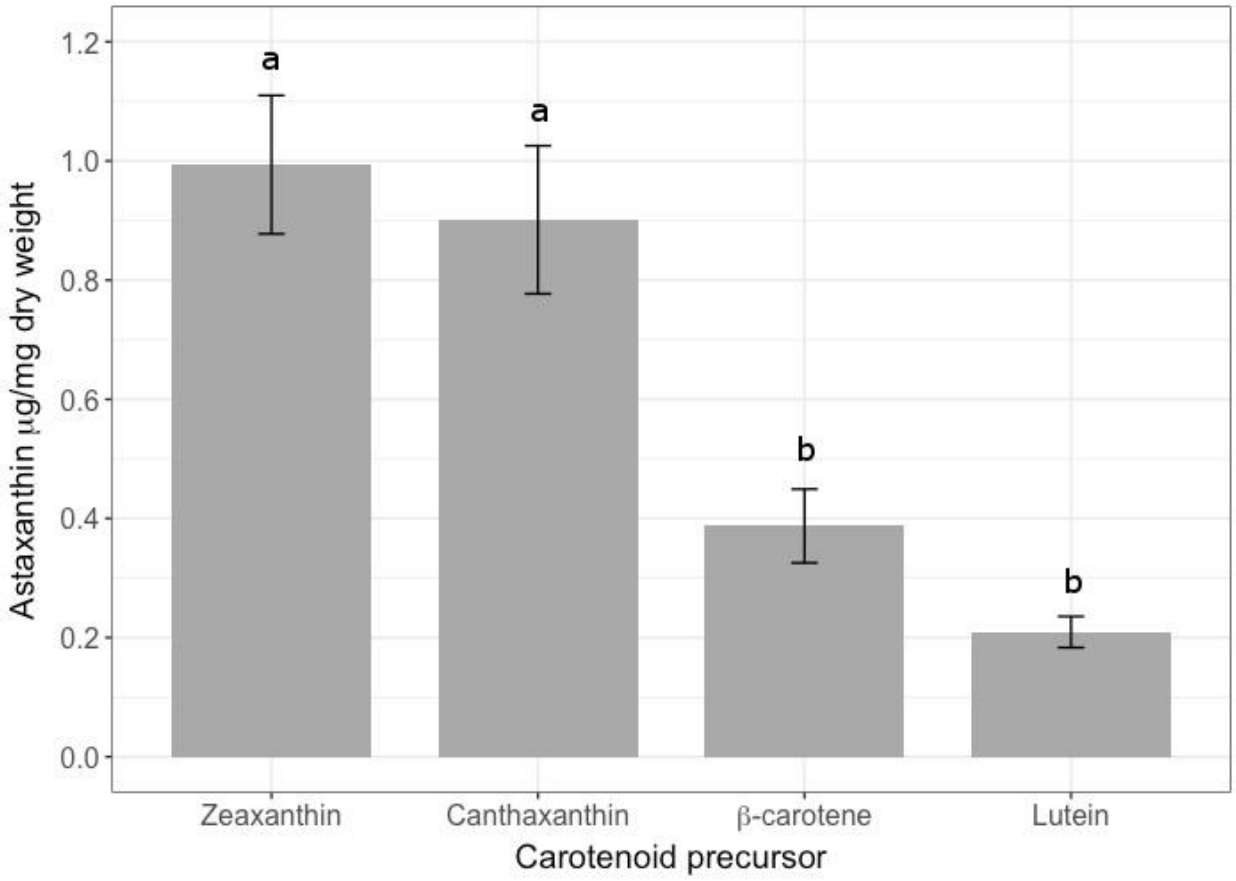

Figure 3-3. Bioconversion of dietary carotenoids. Astaxanthin content of *T. californicus* copepods after supplementation with a precursor carotenoid upstream of astaxanthin for 48h. Least-squared means and standard error bars are shown and significantly different means are indicated by separate letters.

Chapter 4. Copper exposure reduced production of red carotenoids by the marine copepod, *Tigriopus japonicus*

Manuscript published with co-authors Geoffrey Hill, Pou-Long Kuan, and Yung-Che Tseng in 2016 in *Ecological Indicators* **70**, 393-400.

Introduction

Copper is a widespread and damaging marine pollutant. It is an essential micronutrient for the synthesis of cofactors needed for basic cellular functions in animals, including aerobic respiration, but at elevated levels, copper has wide-ranging deleterious effects both from acute exposure and from the bioaccumulation and transfer to higher trophic levels (Rainbow, 2007). Copper enters marine environments via leaching from antifouling paints used on ships throughout the world (Matthiessen *et al.*, 1999; Valkirs *et al.*, 2003), from mining and smelting operations (Castilla and Nealler, 1978), and from natural sources including hydrothermal vents and atmospheric deposition (Lewis, 1995). Many marine waterways have high concentrations of copper with serious negative effects on humans and wildlife (Georgopoulos *et al.*, 2001; Marsden and Rainbow, 2004; Rainbow, 2007).

Copper toxicity compromises the health of marine animals by catalyzing the formation of reactive oxygen species (ROS) that damage cellular components (Valko *et al.*, 2006; Jomova and Valko, 2011) and by depleting antioxidants including glutathione (Speisky *et al.*, 2009). The damaging effects from copper-induced ROS include reduced energy metabolism and growth (Bancroft et al., 2007; Sabatini et al., 2009), decreased fecundity and longevity (Munkittrick and Dixon, 1988; Bielmyer *et al.*, 2006), and ultimately disruptions to population and food web

dynamics (Real *et al.*, 2003; Hamilton, 2004). Free copper ions require existing ROS (*e.g.* superoxide or hydrogen peroxide; H_2O_2) or other reducing equivalents as redox partners to induce oxidative damage (Jomova and Valko, 2011), and mitochondria are the main site of ROS generation (Brookes, 2005; Kowaltowski *et al.*, 2009). As a result, mitochondrial membranes in particular take the brunt of heavy-metal-induced oxidative damage through lipid peroxidation of the inner mitochondrial membrane (Gaetke and Chow, 2003), and oxidative stress has been shown to reduce the rates of oxidative phosphorylation and activity of complex IV (cytochrome c oxidase) of the electron transport system (Sokol *et al.*, 1993; Krumschnabel *et al.*, 2005; Belyaeva *et al.*, 2008). Disruption of core mitochondrial processes such as oxidative phosphorylation has systemic deleterious effects. For example, copper-exposed copepods show decreased growth and development rates (Lee *et al.*, 2008a) and modulated expression of key genes involved in mitochondrial respiration and antioxidant defense (Lee *et al.*, 2008b; Ki *et al.*, 2009).

Aerobically respiring organisms have evolved a complex network of enzymatic and non-enzymatic antioxidants as an innate defense system to maintain ROS homeostasis and minimize oxidative damage. Isoforms of superoxide dismutase are enzymatic antioxidants that reduce the primary ROS generated, superoxide, to the less reactive secondary ROS, hydrogen peroxide (H_2O_2). Glutathione can reduce H_2O_2 to water, thus eliminating one of the substrates by which copper ions catalyze hydroxyl radical production. Through the loss of an electron to H_2O_2, glutathione becomes oxidized thus losing antioxidant potential. However, radical scavenging by glutathione is not a consumptive process; glutathione reductase (GR) regenerates glutathione to its reduced state, restoring its antioxidant capacity. Glutathione also directly binds with free copper ions, thus directly diminishing glutathione availability, and creating a more oxidized

cellular environment which favors the production of ROS (Freedman *et al.*, 1989). Gene expression profiling of antioxidant defense response to heavy metal exposure has frequently included GR to understand physiological consequences of environmental stressors in organisms at the base of the marine food web (Kwok and Leung, 2005; Raisuddin *et al.*, 2007; Ki *et al.*, 2009; Rhee *et al.*, 2013).

Because of the targeted effects of copper on mitochondrial function, I was intrigued by the potential of monitoring copper pollution via phenotypic traits that are sensitive to mitochondrial function (Hill, 2014). The development of a bioassay using a model species that reliably reflects copper contamination or other environmental pollutants would facilitate monitoring of marine food webs. The marine copepod *Tigriopus japonicus* is widely used in ecotoxicology (Raisuddin *et al.*, 2007), and this species has three key attributes that make it an ideal system for studying the impact of copper pollution on marine ecosystem health. First, *T. japonicus* is easy to collect in large quantities along the rocky shores of the East Asian Pacific coast and the species does well in laboratory settings. Second, the toxicities of many environmental stressors have been studied using *T. japonicus* (Raisuddin *et al.*, 2007). Third, the entire mitochondrial genome and many nuclear genes have been annotated from recent transcriptome analyses (Jung *et al.*, 2006). Here, I propose that the orange-red color of *T. japonicus,* which results from metabolic conversion of dietary yellow algal carotenoids to the red ketocarotenoid, astaxanthin (Goodwin, 1986), holds potential to be an excellent bioassay of copper pollution. The red carotenoid astaxanthin comprises more than 95% of the carotenoids detected in *Tigriopus* copepods (Weaver *et al*., 2018) and is almost exclusively responsible for their characteristic orange-red coloration. T. *japonicus* has been well studied in ecotoxicology,

but the use of its red carotenoid production has not been investigated as a biomarker for environmental stress.

Carotenoids have great potential as a sensitive indicator of marine ecosystem health because of the dual role they play in animal physiology and coloration (Goodwin, 1986), and because they are sensitive to environmental stressors (Hill, 1995, 2006). Although most animals cannot synthesize carotenoids *de novo*, herbivorous consumers such as zooplankton, can metabolically convert yellow dietary carotenoids into red ketocarotenoids (Goodwin, 1986). Astaxanthin is a particularly important ketocarotenoid in marine systems; in addition to its use as a colorant and for protection from UV radiation, it also potentially provides antioxidant defense against ROS (Goodwin, 1986; Davenport *et al.*, 2004; Caramujo *et al.*, 2012). Although the extent of antioxidant activity by carotenoids *in vivo* is uncertain, there is a large body of literature suggesting that carotenoids are biologically relevant antioxidants based on theoretical and *in vitro* evidence (Mortensen and Skibsted, 1997; Kobayashi and Sakamoto, 1999; Stahl and Sies, 2003; Chew and Park, 2004; Higuera-Ciapara *et al.*, 2006; Freeman-Gallant *et al.*, 2011). Recently, new ideas that link carotenoid metabolism to the redox reactions involved in cellular respiration implicate proper mitochondrial function as a requisite for efficient conversion of dietary yellow carotenoids into their red ketolated forms (Hill and Johnson, 2013). It follows that disruption of mitochondrial function from oxidative damage will inhibit production of astaxanthin, thus serving as a biomarker of environmental stress. Regardless of the mechanism by which carotenoids are sensitive to ROS, carotenoids hold the potential to act as sensitive indicators of environmental stress.

In this study, I tested the hypothesis that production of red ketocarotenoids is sensitive to environmental stressors by exposing *T. japonicus* copepods to copper (Cu^{2+} from $CuSO_4$) and

measuring changes in expression of mitochondrial and antioxidant genes and in carotenoid content. I predicted that if environmental stressors induced mitochondrial dysfunction and increased ROS production then exposure to copper would decrease the production of red carotenoid pigments by *T. japonicus* copepods.

Materials and methods

Animal collection and culturing conditions

T. japonicus were collected from Hoping Dao Island, Keelung City, Taiwan (25°09'47.2"N, 121°45'48.2"E) and reared in the lab in 0.22µm filtered artificial seawater (ASW, 35 psu salinity, pH 8.03) at 26 C (± 0.5), on a 14h light: 10h dark cycle. Copepods were fed live microalga *Tetraselmis chui* (Butcher), daily. This species of microalgae provides complete nutrition for *T. japonicus* as well as the yellow precursor carotenoids— β-carotene and zeaxanthin— that are necessary for the production of astaxanthin (Goodwin and Srisukh, 1949; Brown and Jeffrey, 1992). Importantly, *Tetraselmis chui* contains no red ketolated carotenoids. Therefore, any astaxanthin detected in *T. japonicus* will have come from oxidation of dietary yellow carotenoids by the copepods. Copepods were acclimated to lab conditions for 2 weeks prior to exposure experiments.

Copper exposure

To test for the effect of environmental stressors on copepod carotenoid content I exposed *T. japonicus* to sub-lethal levels of copper in an acute 24h exposure experiment. Copper test solutions were made from 1M stock solution of $CuSO_4$ (Wako Pure Chemicals Osaka, Japan) and diluted to a concentration of 2 mg/L using filtered ASW, pH 8.03. This copper concentration has been shown to have low mortality, but still modulate antioxidant gene transcript levels in *T.*

japonicus (Lee *et al.*, 2007, 2008b; Rhee *et al.*, 2013), which suggests that there are physiological consequences at this exposure level. All stock solutions were prepared the same day as the beginning of the exposure experiment.

Experiments were carried out in 200 mL beakers containing 100 mL of test solution (ASW or Cu). Copepods were contained in test chambers consisting of a 5.7 x 3.8cm section of polycarbonate tube fitted with 100uM synthetic nylon mesh (Nitex) bottom and suspended in test beakers (Ziegenfuss and Hall, 1998). Approximately 200 adult copepods —males and non-gravid females — were randomly distributed to each treatment test beaker in replicates of five (copper n=5, control n=5). Each beaker was gently aerated to prevent particulate settling and to maintain high air saturation.

To measure how carotenoid content and expression of antioxidant (glutathione reductase; GR) and mitochondrial (cytochrome c oxidase I; COI) genes of copepods responds to Cu exposure 50 individuals were sampled from each replicate at each 0, 6, 12, and 24h time point. Ten adults were processed for carotenoid analysis and the remaining copepods (~40) were processed for gene expression analysis. The number of copepods per sample was determined from a pilot study that found the minimum number necessary for detection of carotenoids and extraction of high quality RNA. Test solution temperature, pH, and salinity were recorded at each sampling point after harvesting copepods followed by the addition of 1mL aliquot of *T. chui* alga.

Carotenoid analysis using ultra high performance liquid chromatography (UPLC)

At the time of sampling, copepods were homogenized in 500 µL HPLC-grade acetone and incubated in the dark at 4C for 24h. After incubation, supernatant containing carotenoids was filtered through 0.2 µm hydrophilic polypropylene discs (Pall Corp), evaporated to dryness under

vacuum at 40C then re-suspended in 100 µL acetone and kept at -80C until all samples were collected. All samples were run at the same time under the following conditions: 10 µL of sample was injected to a Waters Acquity H-Class BEH C18 (1.7 µm, 2.1 mm X 50 mm) UPLC column kept at 7C. The sample was delivered to the column using a linear gradient mobile phase consisting of A: 100 % dH2O, B: Acetonitrile:Methanol, 70:30 v/v at flow rate of 0.2 mL min^{-1}. Initial ratio of 15% A: 85% B for 10 seconds, 100% B for 5.86 min, then returned to initial ratio over 4 min. Relative amounts of carotenoids were quantified by an ultraviolet/visible detector set to 450nm by calculating the area under the curve of each peak. Free astaxanthin was identified by comparison of peak retention time to an analytical standard obtained under identical UPLC conditions (Sigma). Carotenoid extraction and processing were done under low light conditions to prevent carotenoid artefacts from photo-oxidation.

mRNA preparation and RT-qPCR

Whole adult copepods were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) on ice immediately after sampling. Total RNA was extracted and purified following manufacture's protocol and DNA contaminants were removed using DNase I (Promega, Madison, WI, USA). Total RNA quality and quantity was assessed by measuring absorbance at 260 and 280nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from 0.5 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA); total reaction volume was 20 µL. mRNA transcript abundance of GR and COI genes were measured using real time RT-PCR along with reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Table 4-1). SYBR Green (Invitrogen) was used as a fluorescent dye for the detection of target and reference gene PCR products. Amplification and detection was carried out on a Roche

LightCycler 480 using 1µL of cDNA with 0.2µL of each primer set of target and reference genes were reacted under the following conditions: 1 cycle for 5 min at 95°C followed by 45 cycles of 95°C, 58°C, and 72°C for 10 seconds each. To confirm amplification of target genes melting curve analysis was conducted with 1 cycle of 95 °C for 5s at a rate of 4.4 °C/s, 65 °C for 1min at a rate of 2.2 °C/s, then increased to a hold temperature of 97 °C at a rate of 0.11 °C/s with 5 readings/°C. qPCR reactions for each of the five biological replicates per treatment were analyzed in triplicate in a 384-well format with no-template and no-reverse-transcription controls.

Statistical Analyses

Modeling RT-qPCR data

Reference gene (GAPDH) and target gene (GR and COI) raw threshold cycle (Ct) values were exported from Roche LightCycler 480 instrument software to LinRegPCR (v. 2015.3) to calculate PCR efficiencies using a window-of-linearity approach. Statistical analyses of gene expression were performed using the MCMC.qpcR package (Matz et al. 2013) in R-Studio (version 0.99.878). Raw cycle data were log-transformed and modeled using a generalized linear mixed model with a Poisson distribution, to estimate the fold change in target gene mRNA in response to fixed effects of treatment and time point. Beaker ID was set as a random factor to incorporate variation from grouping experimental units into beakers that were repeatedly sampled. Parameter estimates were obtained using a Bayesian Markov Chain Monte Carlo (MCMC) process, using expression of the reference gene as an informative prior for expression of the target genes. Parameter estimates of fold-change in gene expression are reported as posterior means and 95% credible intervals. Pairwise differences between exposure treatment and controls at each time point were calculated, controlling for multiple testing.

Real-time quantitative PCR was used to measure changes in mRNA abundance of GR and COI of *T. japonicus* copepods during exposure to Cu relative to unexposed copepods. Target gene transcript abundance was normalized to GAPDH mRNA levels and reported as the mean fold change in transcript abundance relative to control with 95% credible intervals (CRI). Credible intervals are Bayesian analogs to confidence intervals and can be interpreted as a 95% probability that the true fold change in mRNA abundance occurs in this range

Astaxanthin Analysis

To evaluate how copper exposure affected the production of carotenoids I report carotenoid amounts as the mean relative change in carotenoids (with 95% confidence intervals) from time zero. Preliminary results found that free astaxanthin (*i.e.* not esterified) to be the dominant carotenoid of this species (95%) with mono- and di-esters of astaxanthin and unidentified minor carotenoids comprising approximately 5% of the total carotenoid content. Because relative amounts of total carotenoids and free astaxanthin were highly correlated (R^2 = 0.987), I used only relative amount of free astaxanthin from time 0h as the response variable in the models. I compared relative astaxanthin amount between treatment groups over time using a linear mixed effects model, including fixed effects of treatment and time point as well as an interaction between treatment and time. I also included a random effect of beaker ID to account for repeated sampling from the same beaker.

Results

Changes in target gene mRNA abundance during 24h Cu exposure

I found that COI transcript abundance of Cu-exposed copepods was significantly higher than control copepods at each time point (Fig. 4-1). GR mRNA was more abundant in Cu-

exposed copepods at each time point but these increases were only significantly different from control copepods at 12h (Table 4-2).

Changes in astaxanthin during 24h Cu exposure

I found that copepods in both groups increased the amount of astaxanthin after 24h relative time 0h (Fig. 4-2). After six hours, both control and copper exposed copepods increased the amount of astaxanthin in their tissues (Table 4-3). At both 12h and 24h control copepods continued to increase the amount of astaxanthin, while copper exposed copepods had significantly less astaxanthin (Table 4-3).

Discussion

I investigated the effects of copper exposure on mitochondrial and antioxidant gene expression and the production of red carotenoid pigments in the marine copepod *Tigriopus japonicus* and observed that copper-exposed copepods had a relatively higher abundance of COI and GR transcripts and accumulated less astaxanthin than copepods that were not exposed to environmental copper. These observations are consistent with the prediction that production of ketolated carotenoids is sensitive to mitochondrial function and oxidative state. These findings suggest that red ketocarotenoid content in copepods could serve as a sensitive indicator of marine copper pollution.

Classical ecotoxicology endpoints for exposure to sub-lethal concentrations of toxicants focus mainly on population ecology dynamics such as growth rates and individual endpoints including larval development and mortality (Cairns Jr *et al.*, 1981; Bechmann, 1994). More recently, transcriptomic analyses have been used to examine the effects of stressors on the up and down regulation of components of stress responses (Raisuddin *et al.*, 2007; Ki *et al.*, 2009).

Behavioral endpoints such as swimming speed also link exposure and physiological responses to a functional consequence (Rice *et al.*, 1997; Cohen *et al.*, 2007; Garaventa *et al.*, 2010). Carotenoid pigmentation is unique in that it reveals immediate functional consequences of pollutant exposure at a molecular level. Moreover, carotenoids can be quantified either through simple HPLC methods or by measurement of the coloration of tissues (Saks *et al.*, 2003; McKay, 2013).

The chemical properties of animal carotenoids lie at the core of their sensitivity as indicators of environmental stress. Production of red carotenoids from primary consumers requires enzymatic oxidation of precursor yellow carotenoids obtained through the diet (Britton and Goodwin, 1982; Goodwin, 1986; Lopes *et al.*, 2016). These oxidation reactions have been proposed to be dependent on cellular redox homeostasis and functionality of mitochondria (Hill and Johnson, 2012; Johnson and Hill, 2013). The observation that copper exposure not only upregulated mitochondrial and antioxidant genes, likely in response to increased copper-induced ROS levels, but also decreased production of astaxanthin is consistent with the former hypothesis. Cytotoxicity of copper has been shown to directly modulate both redox homeostasis and mitochondrial function (Stohs and Bagchi, 1995; Craig *et al.*, 2007; Belyaeva *et al.*, 2008; Speisky *et al.*, 2009).

The specific mechanism by which environmental copper is proposed to disrupt carotenoid pigmentation arises from the redox capacity of free intracellular copper ions. Physiological levels of copper are essential to cell function. Cellular copper ions are redox active in both oxidation states (Cu^{2+} or Cu^{1+}) and serve critical functional roles including facilitating electron transfer through cytochrome *c* oxidase in mitochondria during cellular respiration and cytosolic free radical defense, (*e.g.* Cu/Zn superoxide dismutase). Because of its high reactivity,

copper is transported and bound to these proteins in tightly controlled processes through chaperones. Therefore, intracellular copper is almost completely absent in its free form (Rae *et al.*, 1999). However, when copper levels exceed biological demands or storage capacity, free copper ions accumulate in cells and react with H_2O_2 to form hydroxyl free radicals through Fenton-like reactions (Valavanidis *et al.*, 2006; Macomber and Imlay, 2009; Jomova and Valko, 2011). Hydroxyl radicals preferentially damage lipid-bound proteins like those in the inner mitochondrial membrane which are integral to cellular respiration (Sokol *et al.*, 1993; Macomber and Imlay, 2009).

In this study I found that copper exposure modulated COI transcript levels in a time-dependent manner suggesting that copper-induced ROS likely damaged these mitochondrial proteins causing an upregulation of COI gene transcription. The most pronounced increase in COI mRNA was after 12h of copper exposure with intermediate increases at 6 and 24h (Fig. 4-2). This pattern of gene expression suggests that as antioxidant systems were upregulated (GR; Fig. 4-2), copper-induced ROS decreased, leading to less damage to mitochondrial membranes, which in turn, decreased the expression of mitochondrial proteins that were upregulated to replace damaged mitochondria. The North American sister species, *T. californicus*, had similar physiological responses to increased ROS production and mitochondrial dysfunction following interpopulation hybridization (Barreto and Burton, 2013; Barreto *et al.*, 2015). Previous studies on copper toxicity have shown modulation of mitochondrial and antioxidant genes following copper exposure (Scott and Major, 1972; Bremner, 1998; Craig *et al.*, 2007; Ki *et al.*, 2009; Rhee *et al.*, 2013). Although I did not measure ROS levels or mitochondrial function directly, the observation that COI and GR transcript levels were increased during Cu exposure suggests that mitochondria of *T. japonicus* copepods were damaged through lipid peroxidation from copper-

induced hydroxyl radicals and that there was a physiological response to Cu exposure. At the same time, I observed more than a 50% reduction in the amount of astaxanthin found in Cu exposed copepods relative to control copepods (Fig. 3) suggesting that a drastic phenotypic change over a relatively short period of time is acutely signaling physiological responses to an environmental stressor.

The mechanisms that link red carotenoid production to physiological stress through ROS or mitochondrial function have not fully been elucidated. The most widely purported explanation is that because carotenoids have the potential to act as antioxidants, oxidative stress causes carotenoids to be used as free radical scavengers instead of being oxidized into red pigments (von Schantz *et al.*, 1999; Pérez-Rodríguez, 2009). To date there is conflicting empirical support for the role of carotenoids to act as antioxidants *in vivo* (Olson and Owens, 1998; Costantini and Møller, 2008). For example, susceptibility to pro-oxidant stressors of the meiobenthic copepod *Amphiascoides atopus,* was dependent on astaxanthin content of the copepods at the beginning of the experiment (Caramujo *et al.*, 2012), which suggests a possible protective role for astaxanthin. However, several studies have found evidence for the pro-oxidant effects of carotenoids (Britton, 1995; Paiva and Russell, 1999; Huggins *et al.*, 2010; Lee *et al.*, 2012). Carotenoid supplementation of the cyclopoid copepod, *Paracyclopina nana* under high density culture stress not only had no effect on naupliar production, but also was associated with upregulation of GR gene expression (Lee *et al.*, 2012), suggesting that carotenoids do not mediate, and may actually exacerbate, the effects of oxidative stress on antioxidant systems. In this study and many others on *T. japonicus* heavy metal and other pro-oxidant stressors were also associated with upregulation of GR and other antioxidant genes (Kwok and Leung, 2005; Seo *et al.*, 2006; Lee *et al.*, 2008b; Kim *et al.*, 2011).

Copper exposure has been shown to decrease activity levels, including feeding rates, in other studies on marine animals and could explain some of the patterns observed in this study (De Boeck *et al.*, 1997; Chen and Lin, 2001; Correia *et al.*, 2001). Lee et al. (2008b) and Seo et al., (2006), among others, examined the physiological consequences of Cu exposure in *T. japonicus* during starvation and found similar modulation of key antioxidant genes but did not measure how carotenoid levels changed in response to exposure. Kwok and Leung (2005) noted that *T. japonicus* copepods exposed to copper subjectively appeared less colorful than unexposed copepods (*i.e.* had less astaxanthin) when both groups were unfed, but did not quantify this apparent change in coloration. Caramujo et al (2012) also conducted a copper-exposure experiment using unfed *A. atopus* copepods, and found a 20 – 47% decrease in astaxanthin compared to un-exposed copepods, and a 70% decrease when copper exposure was combined with UV-light stress. However, Pérez-Rodríguez and Viñuela (2008) found that food restriction had a positive effect on eye ring redness of red-legged partridges, *(Alectoris rufa*), where food restricted birds had redder eye rings, but fewer circulating carotenoids. In the present study, whether astaxanthin content decreased during copper exposure because toxicant exposure affected feeding and therefore access to the precursors need for its formation, or because damage to mitochondrial-associated proteins that may be involved in the bioconversion of dietary precursors to astaxanthin prevented astaxanthin accumulation cannot be resolved.

The ultimate goal of this study was to determine if red carotenoid production was sensitive to environmental stressors. Carotenoid content and color in other taxa have been used as bio-indicators of environmental stress (Isaksson *et al.*, 2005; Lopez-Antia *et al.*, 2015; Vallverdú-Coll *et al.*, 2016). In this study, I used copper exposure as a source of pro-oxidant stress, but these findings are consistent with those from studies that examined the effect of other

pro-oxidants on carotenoids including lead (Vallverdú-Coll *et al.*, 2015, 2016), testosterone (Alonso-Alvarez *et al.*, 2008), and other pollutants (Isaksson *et al.*, 2005; Pérez-Rodríguez *et al.*, 2010). Isaksson and others found that great tits (*Parus major*) from an urban environment, likely with more pollution, had fewer carotenoids in their feathers, and had a relatively larger pool of oxidized glutathione than birds from rural sites (Isaksson *et al.*, 2005). In a follow-up study in which oxidative stress was experimentally induced with the pro-oxidant paraquat, however, carotenoids levels of great tits were not affected by increased levels of free radicals (Isaksson and Andersson, 2008). It is interesting to note that this bird species has yellow feathers and does not modify dietary yellow carotenoids; instead they directly deposit them to feathers (McGraw, 2006). Alonso-Alvarez et al (2008) found that testosterone-supplemented red-legged partridges *(Alectoris rufa*) had higher levels of yellow circulating carotenoids and decreased red coloration of their red carotenoid-pigmented facial mask (De Blas *et al.*, 2014). Conversely a recent study on the same species found a positive relationship between red coloration and pesticide-induced oxidative stress (Lopez-Antia *et al.*, 2015). Thiram-exposed red-legged partridges had higher levels of circulating yellow carotenoids and redder beak coloration than un-exposed partridges (Lopez-Antia *et al.*, 2015). Clearly, more research is needed to deduce the complex relationships of oxidative stress, antioxidant systems, carotenoid metabolism, and carotenoid coloration.

Objectively measured carotenoid color values have been strongly linked to internal concentrations of carotenoids (Saks *et al.*, 2003). The observation that copper-exposed copepods had a physiological response that correlated with decreased red carotenoid production is encouraging for further tests on the efficacy of carotenoid color as a biomarker for environmental stress. Indeed, carotenoid coloration has been shown to be sensitive to other environmental stressors such as UV-radiation (Caramujo *et al.*, 2012; Brüsin *et al.*, 2016), other heavy metals

(Isaksson *et al.*, 2005; Eeva *et al.*, 2008), and parasites (Hill, 1995; McGraw and Hill, 2000). Future studies should address the functional role of carotenoids as antioxidants *in vivo* through carefully planned experiments to further elucidate the mechanisms by which carotenoid metabolism links to oxidative stress in animals.

References

Alonso-Alvarez, C., Pérez-Rodríguez, L., Mateo, R., Chastel, O. and Viñuela, J. (2008) The oxidation handicap hypothesis and the carotenoid allocation trade-off. *J. Evol. Biol.*, 21, 1789–1797.

Bancroft, B.A., Baker, N.J. and Blaustein, A.R. (2007) Effects of UVB radiation on marine and freshwater organisms: a synthesis through meta-analysis. *Ecol. Lett.*, 10, 332–45.

Barreto, F.S. and Burton, R.S. (2013) Elevated oxidative damage is correlated with reduced fitness in interpopulation hybrids of a marine copepod. *Proc. Biol. Sci.*, 280, 20131521.

Barreto, F.S., Pereira, R.J. and Burton, R.S. (2015) Hybrid Dysfunction and Physiological Compensation in Gene Expression. *Mol. Biol. Evol.*, 32, 613–622.

Bechmann, R.K. (1994) Use of life tables and LC50 tests to evaluate chronic and acute toxicity effects of copper on the marine copepod *Tisbe furcata* (Baird). *Environ. Toxicol. Chem.*, 13, 1509–1517.

Belyaeva, E.A., Dymkowska, D., Wieckowski, M.R. and Wojtczak, L. (2008) Mitochondria as an important target in heavy metal toxicity in rat hepatoma AS-30D cells. *Toxicol. Appl. Pharmacol.*, 231, 34–42.

Bielmyer, G.K., Grosell, M. and Brix, K. V (2006) Toxicity of silver, zinc, copper, and nickel to the copepod Acartia tonsa exposed via a phytoplankton diet. *Environ. Sci. Technol.*, 40, 2063–2068.

De Blas, E.G., Mateo, R., Guzman Bernardo, F.J., Rodríguez Martín-Doimeadios, R.C. and Alonso-Alvarez, C. (2014) Astaxanthin and papilioerythrinone in the skin of birds: A chromatic convergence of two metabolic routes with different precursors? *Naturwissenschaften*, 101, 407–416.

De Boeck, G., Vlaeminck, A. and Blust, R. (1997) Effects of sublethal copper exposure on copper accumulation food consumption, growth, energy stores, and nuclein acid content in Common Carp. *Arch. Environ. Contam. Toxicol.*, 33, 415–422.

Bremner, I. (1998) Manifestations of copper excess. *Am. J. Clin. Nutr.*, 67.

Britton, G. (1995) Structure and properties of carotenoids in relation to function. *FASEB J.*, 9, 1551–1558.

Britton, G. and Goodwin, T. (1982) *Carotenoid chemistry and biochemistry*, (ed. by G. Britton) and T. Goodwin) Pergamon Press Ltd, New York.

Brookes, P.S. (2005) Mitochondrial H + leak and ROS generation: An odd couple. *Free Radic. Biol. Med.*, 38, 12–23.

Brown, M.R. and Jeffrey, S.W. (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. *J. Exp. Mar. Bio. Ecol.*, 161, 91–113.

Brüsin, M., Svensson, P.A. and Hylander, S. (2016) Individual changes in zooplankton pigmentation in relation to ultraviolet radiation and predator cues. *Limnol. Oceanogr.*

Cairns Jr, J., Alexander, M., Cummins, K.W., Edmondson, W.., Goldman, R., Harte, J., Insensee, A.R., Levin, R., McCormick, J.F., Peterle, T.J. and Zar, J.H. (1981) *Testing for effects of chemicals on ecosystems*. National Academy Press, Washington, DC.

Caramujo, M.-J., de Carvalho, C.C.C.R., Silva, S.J. and Carman, K.R. (2012) Dietary carotenoids regulate astaxanthin content of copepods and modulate their susceptibility to UV light and copper toxicity. *Mar. Drugs*, 10, 998–1018.

Castilla, J.C. and Nealler, E. (1978) Marine environmental impact due to mining activities of El Salvador copper mine, Chile. *Mar. Pollut. Bull.*, 9, 67–70.

Chen, J.-C. and Lin, C.-H. (2001) Toxicity of copper sulfate for survival, growth, molting and feeding of juveniles of the tiger shrimp, Penaeus monodon. *Aquaculture*, 192, 55–65.

Chew, B.P. and Park, J.S. (2004) Carotenoid action on the immune response. *J. Nutr.*, 134, 257S–261S.

Cohen, J.H., Tester, P.A. and Forward, R.B. (2007) Sublethal effects of the toxic dinoflagellate Karenia brevis on marine copepod behavior. *J. Plankton Res.*, 29, 301–315.

Correia, A.D., Costa, F.O., Neuparth, T., Diniz, M.E. and Costa, M.H. (2001) Sub-lethal Effects of Copper-Spiked Sediments on the Marine Amphipod Gammarus locusta : Evidence of Hormesis ? *Ecotoxicol. Environ. Restor.*, 4, 32–38.

Costantini, D. and Møller, A.P. (2008) Carotenoids are minor antioxidants for birds. *Funct. Ecol.*, 22, 367–370.

Craig, P.M., Wood, C.M. and McClelland, G.B. (2007) Oxidative stress response and gene expression with acute copper exposure in zebrafish (Danio rerio). *Am. J. Physiol. Integr. Comp. Physiol.*, 293, R1882-1892.

Davenport, J., Healy, A., Casey, N. and Heffron, J. (2004) Diet-dependent UVAR and UVBR resistance in the high shore harpacticoid copepod Tigriopus brevicornis. *Mar. Ecol. Prog. Ser.*, 276, 299–303.

Eeva, T., Sillanpää, S., Salminen, J.P., Nikkinen, L., Tuominen, A., Toivonen, E., Pihlaja, K. and Lehikoinen, E. (2008) Environmental pollution affects the plumage color of great tit nestlings through Carotenoid Availability. *Ecohealth*, 5, 328–337.

Freedman, J.H., Ciriolo, M.R. and Peisach, J. (1989) The role of glutathione in copper metabolism and toxicity. *J. Biol. Chem.*, 264, 5598–5605.

Freeman-Gallant, C.R., Amidon, J., Berdy, B., Wein, S., Taff, C.C. and Haussmann, M.F. (2011) Oxidative damage to DNA related to survivorship and carotenoid-based sexual ornamentation in the common yellowthroat. *Biol. Lett.*, 7, 429–432.

Gaetke, L. and Chow, C. (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology*, 189, 147–163.

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M. and Faimali, M. (2010) Swimming speed alteration of Artemia sp. and Brachionus plicatilis as a sub-lethal behavioural end-point for ecotoxicological surveys. *Ecotoxicology*, 19, 512–519.

Georgopoulos, P.G., Roy, A., Yonone-Lioy, M.J., Opiekun, R.E. and Lioy, P.J. (2001) *Copper: Environmental Dynamics and Human Exposure Issues*, Piscataway, NJ.

Goodwin, T. (1986) Metabolism, nutrition, and function of carotenoids. *Annu. Rev. Nutr.*, 6, 237–97.

Goodwin, T. and Srisukh, S. (1949) Some observations on astaxanthin distribution in marine Crustacea. *Biochem. J.*, 45, 268–270.

Hamilton, S.J. (2004) Review of selenium toxicity in the aquatic food chain. *Sci. Total Environ.*, 326, 1–31.

Higuera-Ciapara, I., Félix-Valenzuela, L. and Goycoolea, F.M. (2006) Astaxanthin: a review of its chemistry and applications. *Crit. Rev. Food Sci. Nutr.*, 46, 185–96.

Hill, G.E. (2014) Cellular respiration: The nexus of stress, condition, and ornamentation. *Integr. Comp. Biol.*, 54, 1–13.

Hill, G.E. (2006) *Environmental regulation of ornamental coloration*. *Bird Coloration. vol. 1.* (ed. by G.E. Hill) and K.J. Mcgraw), Harvard University Press, Cambridge, Massachusetts.

Hill, G.E. (1995) Ornamental Traits as Indicators of Environmental Health. *Bioscience*, 45, 25–31.

Hill, G.E. and Johnson, J.D. (2013) The mitonuclear compatibility hypothesis of sexual selection. *Proc R Soc B*, 280.

Hill, G.E. and Johnson, J.D. (2012) The vitamin A-redox hypothesis: A biochemical basis for honest signaling via carotenoid pigmentation. *Am. Nat.*, 180, E127-150.

Huggins, K. a, Navara, K.J., Mendonça, M.T. and Hill, G.E. (2010) Detrimental effects of carotenoid pigments: the dark side of bright coloration. *Naturwissenschaften*, 97, 637–44.

Isaksson, C. and Andersson, S. (2008) Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. *Proc. R. Soc. B-Biological Sci.*, 275, 309–314.

Isaksson, C., Örnborg, J., Stephensen, E. and Andersson, S. (2005) Plasma Glutathione and Carotenoid Coloration as Potential Biomarkers of Environmental Stress in Great Tits. *Ecohealth*, 2, 138–146.

Johnson, J.D. and Hill, G.E. (2013) Is carotenoid ornamentation linked to the inner mitochondria membrane potential? A hypothesis for the maintenance of signal honesty. *Biochimie*, 95, 436–444.

Jomova, K. and Valko, M. (2011) Advances in metal-induced oxidative stress and human disease. *Toxicology*, 283, 65–87.

Jung, S.-O., Lee, Y.-M., Park, T.-J., Park, H.G., Hagiwara, A., Leung, K.M.Y., Dahms, H.-U., Lee, W. and Lee, J.-S. (2006) The complete mitochondrial genome of the intertidal copepod Tigriopus sp. (Copepoda, Harpactidae) from Korea and phylogenetic considerations. *J. Exp. Mar. Bio. Ecol.*, 333, 251–262.

Ki, J.-S., Raisuddin, S., Lee, K.-W., Hwang, D.-S., Han, J., Rhee, J.-S., Kim, I.-C., Park, H.G., Ryu, J.-C. and Lee, J.-S. (2009) Gene expression profiling of copper-induced responses in the intertidal copepod Tigriopus japonicus using a 6K oligochip microarray. *Aquat. Toxicol.*, 93, 177–87.

Kim, B.M., Rhee, J.S., Park, G.S., Lee, J., Lee, Y.M. and Lee, J.S. (2011) Cu/Zn- and Mn-superoxide dismutase (SOD) from the copepod Tigriopus japonicus: Molecular cloning and expression in response to environmental pollutants. *Chemosphere*, 84, 1467–1475.

Kobayashi, M. and Sakamoto, Y. (1999) Singlet oxygen quenching ability of astaxanthin esters from the green alga Haematococcus pluvialis. *Biotechnol. Lett.*, 21, 265–269.

Kowaltowski, A.J., de Souza-Pinto, N.C., Castilho, R.F. and Vercesi, A.E. (2009) Mitochondria and reactive oxygen species. *Free Radic. Biol. Med.*, 47, 333–43.

Krumschnabel, G., Manzl, C., Berger, C. and Hofer, B. (2005) Oxidative stress, mitochondrial permeability transition, and cell death in Cu-exposed trout hepatocytes. *Toxicol. Appl. Pharmacol.*, 209, 62–73.

Kwok, K.W.H. and Leung, K.M.Y. (2005) Toxicity of antifouling biocides to the intertidal harpacticoid copepod Tigriopus japonicus (Crustacea, Copepoda): Effects of temperature and salinity. *Mar. Pollut. Bull.*, 51, 830–837.

Lee, K.-W., Raisuddin, S., Hwang, D.-S., Park, H.G., Dahms, H.-U., Ahn, I.-Y. and Lee, J.-S. (2008a) Two-generation toxicity study on the copepod model species Tigriopus japonicus. *Chemosphere*, 72, 1359–65.

Lee, K.-W., Raisuddin, S., Rhee, J.-S., Hwang, D.-S., Yu, I.T., Lee, Y.-M., Park, H.G. and Lee, J.-S. (2008b) Expression of glutathione S-transferase (GST) genes in the marine copepod Tigriopus japonicus exposed to trace metals. *Aquat. Toxicol.*, 89, 158–66.

Lee, K., Raisuddin, S., Hwang, D.-S., Park, H.G. and Lee, J.-S. (2007) Acute toxicities of trace metals and common xenobiotics to the marine copepod Tigriopus japonicus: evaluation of its use as a benchmark species for routine exotoxicity tests in Wester Pacific coastal regions. *Environ. Toxicol.*, 22, 532–538.

Lee, K.W., Rhee, J.S., Han, J., Park, H.G. and Lee, J.S. (2012) Effect of culture density and antioxidants on naupliar production and gene expression of the cyclopoid copepod, Paracyclopina nana. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.*, 161, 145–152.

Lewis, A. (1995) Copper in water and aquatic environments. *Int. Copp. Assoc. Reports*, 71.

Lopes, R.J., Johnson, J.D., Toomey, M.B., Ferreira, M.S., Araujo, P.M., Melo-Ferreira, J., Andersson, L., Hill, G.E., Corbo, J.C. and Carneiro, M. (2016) Genetic basis for red coloration in birds. *Curr. Biol.*, 26, 1427–1434.

Lopez-Antia, A., Ortiz-Santaliestra, M.E., Blas, E.G. De, Camarero, P.R., Mougeot, F. and Mateo, R. (2015) Adverse effects of thiram-treated seed ingestion on the reproductive performance and the offspring immune function of the red-legged partridge. *Environ. Toxicol. Chem.*, 34, 1320–1329.

Macomber, L. and Imlay, J. a (2009) The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc. Natl. Acad. Sci. U. S. A.*, 106, 8344–8349.

Marsden, I.D. and Rainbow, P.S. (2004) Does the accumulation of trace metals in crustaceans affect their ecology—the amphipod example? *J. Exp. Mar. Bio. Ecol.*, 300, 373–408.

Matthiessen, P., Reed, J. and Johnson, M. (1999) Sources and potential effects of copper and zinc concentrations in the estuarine waters of Essex and Suffolk, United Kingdom. *Mar. Pollut. Bull.*, 38, 908–920.

McGraw, K.J. (2006) *Mechanics of carotenoid coloration*. *Bird Coloration, Volume 1: Measurements and Mechanisms* (ed. by G.E. Hill) and K.J. McGraw), Harvard University Press, Cambridge, Massachusetts.

McGraw, K.J. and Hill, G.E. (2000) Differential effects of endoparasitism on the expression of carotenoid- and melanin-based ornamental coloration. *Proc. R. Soc. Biol. Sci. Ser. B*, 267, 1525–1531.

McKay, B.D. (2013) The use of digital photography in systematics. *Biol. J. Linn. Soc.*, 110, 1–13.

Mortensen, A. and Skibsted, L.H. (1997) Importance of Carotenoid Structure in Radical-Scavenging Reactions. *J. Agric. Food Chem.*, 45, 2970–2977.

Munkittrick, K. and Dixon, D. (1988) Growth, Fecundity, and Energy Stores of White Sucker (Catostomus commersoni) from Lakes Containing Elevated Levels of Copper and Zinc. *Can. J. Fish. Aquat. Sci.*, 45, 1355–1365.

Olson, V.A. and Owens, I.P.F. (1998) Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol. Evol.*, 13, 510–514.

Paiva, S. a R. and Russell, R.M. (1999) Review Series : Antioxidants and their Clinical Applications Beta-Carotene and Other Carotenoids as Antioxidants. *J. Am. Coll. Nutr.*, 18, 426–433.

Pérez-Rodríguez, L. (2009) Carotenoids in evolutionary ecology: Re-evaluating the antioxidant role. *BioEssays*, 31, 1116–1126.

Pérez-Rodríguez, L., Mougeot, F. and Alonso-Alvarez, C. (2010) Carotenoid-based coloration predicts resistance to oxidative damage during immune challenge. *J. Exp. Biol.*, 213, 1685–9160.

Pérez-Rodríguez, L. and Viñuela, J. (2008) Carotenoid-based bill and eye ring coloration as honest signals of condition: An experimental test in the red-legged partridge (Alectoris rufa). *Naturwissenschaften*, 95, 821–830.

Rae, T.D., Schmidt, P.J., Pufahl, R.A., Culotta, V.C. and O'Halloran, T. V (1999) Undetectable Intracellular Free Copper : The Requirement of a Copper Chaperone for Superoxide Dismutase. *Science (80-.).*, 284, 805–808.

Rainbow, P.S. (2007) Trace metal bioaccumulation: models, metabolic availability and toxicity. *Environ. Int.*, 33, 576–82.

Raisuddin, S., Kwok, K.W.H., Leung, K.M.Y., Schlenk, D. and Lee, J.-S. (2007) The copepod Tigriopus: a promising marine model organism for ecotoxicology and environmental genomics. *Aquat. Toxicol.*, 83, 161–73.

Real, M., Munoz, I., Guasch, H., Navarro, E. and Sabater, S. (2003) The effect of copper exposure on a simple aquatic food chain. *Aquat. Toxicol.*, 63, 283–291.

Rhee, J.-S., Yu, I.T., Kim, B.-M., Jeong, C.-B., Lee, K.-W., Kim, M.-J., Lee, S.-J., Park, G.S. and Lee, J.-S. (2013) Copper induces apoptotic cell death through reactive oxygen species-triggered oxidative stress in the intertidal copepod Tigriopus japonicus. *Aquat. Toxicol.*, 132–133, 182–189.

Rice, P.J., Drewes, C.D., Klubertanz, T.M., Bradbury, S.P. and Coats, J.R. (1997) Acute toxicity and behavioral effects of chlorpyrifos, permethrin, phenol, strychnine, and 2,4-dinitrophenol to 30-day-old Japanese medaka (Oryzias latipes). *Environ. Toxicol. Chem.*, 16, 696–704.

Sabatini, S.E., Juárez, A.B., Eppis, M.R., Bianchi, L., Luquet, C.M. and Ríos de Molina, M.D.C. (2009) Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. *Ecotoxicol. Environ. Saf.*, 72, 1200–6.

Saks, L., McGraw, K. and Hõrak, P. (2003) How feather colour reflects its carotenoid content. *Funct. Ecol.*, 17, 555–561.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. and Wittzell, H. (1999) Good genes, oxidative stress and condition–dependent sexual signals. *Proc. R. Soc. B Biol. Sci.*, 266, 1–12.

Scott, D.M. and Major, C.W. (1972) The effect of copper (II) on survival, respiration, and heart rate in the common blue mussel, Mytilus edulis. *Biol. Bull.*, 143, 679–688.

Seo, J.S., Lee, K.-W., Rhee, J.-S., Hwang, D.-S., Lee, Y.-M., Park, H.G., Ahn, I.-Y. and Lee, J.-S. (2006) Environmental stressors (salinity, heavy metals, H2O2) modulate expression of glutathione reductase (GR) gene from the intertidal copepod Tigriopus japonicus. *Aquat. Toxicol.*, 80, 281–9.

Sokol, R., Devereaux, M., Khandwala, R. and Loehr, J. (1993) Abnormal hepatic mitochondrial respiration and cytochrome C oxidase activity in rats with long-term copper overload. *Gastroenterology*, 105, 178–187.

Speisky, H., Gómez, M., Burgos-Bravo, F., López-Alarcón, C., Jullian, C., Olea-Azar, C. and Aliaga, M.E. (2009) Generation of superoxide radicals by copper-glutathione complexes: Redox-consequences associated with their interaction with reduced glutathione. *Bioorganic Med. Chem.*, 17, 1803–1810.

Stahl, W. and Sies, H. (2003) Antioxidant activity of carotenoids. *Mol. Aspects Med.*, 24, 345–351.

Stohs, S.J. and Bagchi, D. (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.*, 18, 321–336.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.*, 64, 178–189.

Valkirs, A.O., Seligman, P.F., Haslbeck, E. and Caso, J.S. (2003) Measurement of copper release rates from antifouling paint under laboratory and in situ conditions: implications for loading estimation to marine water bodies. *Mar. Pollut. Bull.*, 46, 763–79.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M. (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Interact.*, 160, 1–40.

Vallverdú-Coll, N., Mougeot, F., Ortiz-Santaliestra, M.E., Rodriguez-Estival, J., López-Antia, A. and Mateo, R. (2016) Lead exposure reduces carotenoid-based coloration and constitutive immunity in wild mallards. *Environ. Toxicol. Chem.*, 1–10.

Vallverdú-Coll, N., Ortiz-Santaliestra, M.E., Mougeot, F., Vidal, D. and Mateo, R. (2015) Sublethal Pb exposure produces season-dependent effects on immune response, oxidative balance and investment in carotenoid-based coloration in red-legged partridges. *Environ. Sci. Technol.*, 150212120745006.

Ziegenfuss, M. and Hall, L. (1998) *Standard operating procedures for conducting acute and chronic aquatic toxicity tests with Eurytemora affinis, a calanoid copepod*, Report for U.S. EPA, Chesapeake Bay Program Office, Annapolis, Maryland.

Tables

Table 4-1. Primers used for RT-qPCR

Protein name	Abbreviation	Primer sequence	Amplicon size (bp)
Glutathione Reductase	GR	F 5'-CCATGACGGACAGAAAGCAGATGAC-3'	234
		R 5'- CTCCCATCTTGATGGCAACTCC-3'	310
Cytochrome C Oxidase subunit I	COI	F 5'-GGTCAACAAATCATAAAGATATTGG-3'	300
		R 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	339
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	F 5'-GAGCTGGACAGAACATCATC-3'	299
		R 5'-GAATACCCCAAGTATCCCTTC-3'	303

F, forward primer; R, reverse primer

Table 4-2. Bayesian MCMC mean fold change in mRNA transcripts of target genes in response to copper exposure over 24h. 95% CRI are Bayesian analogs to frequentist credible intervals.

Timepoint	Gene	Fold Change	Lower 95% CRI	Upper 95% CRI	MCMC p-value
6h	COI	1.58	1.08	2.52	0.029
	GR	1.23	0.84	1.73	0.266
12h	COI	2.66	1.84	3.97	0.000001
	GR	1.58	1.11	2.19	0.010
24h	COI	1.56	1.07	2.29	0.023
	GR	1.32	0.97	1.84	0.108

Table 4-3. Restricted maximum likelihood estimated mean relative change in astaxanthin over 24h from a linear mixed effects model.

Time	Exposure	Relative Change	Lower 95%	Upper 95%	t-value	p-value
6h	Control	1.73	1.12	2.33	-0.373	0.712
	Copper	1.56	0.64	2.47		
12h	Control	2.60	1.99	3.20	-2.82	0.0095
	Copper	1.31	0.39	2.22		
24h	Control	3.18	2.57	3.78	-3.92	0.0007
	Copper	1.32	0.37	2.26		

Figures

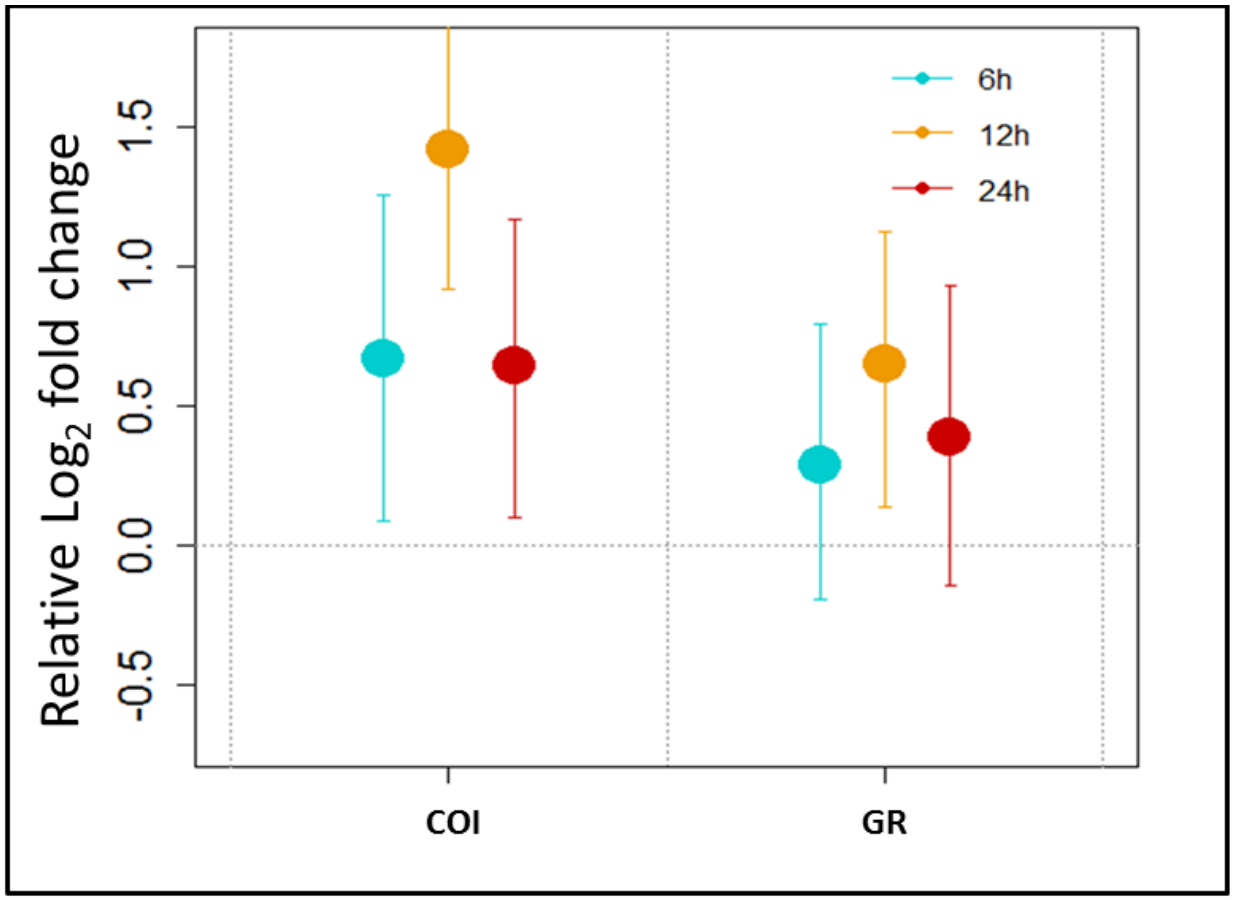

Fig 4-1. Log_2 fold change in transcript abundance of cytochrome c oxidase 1 (COI) and glutathione reductase (GR) during 24h copper exposure relative to control copepods. Abundance data were normalized to reference gene (GAPDH) transcript abundance. Mean ±95% credible intervals. Credible intervals that do not overlap zero indicate a significant difference from control exposure.

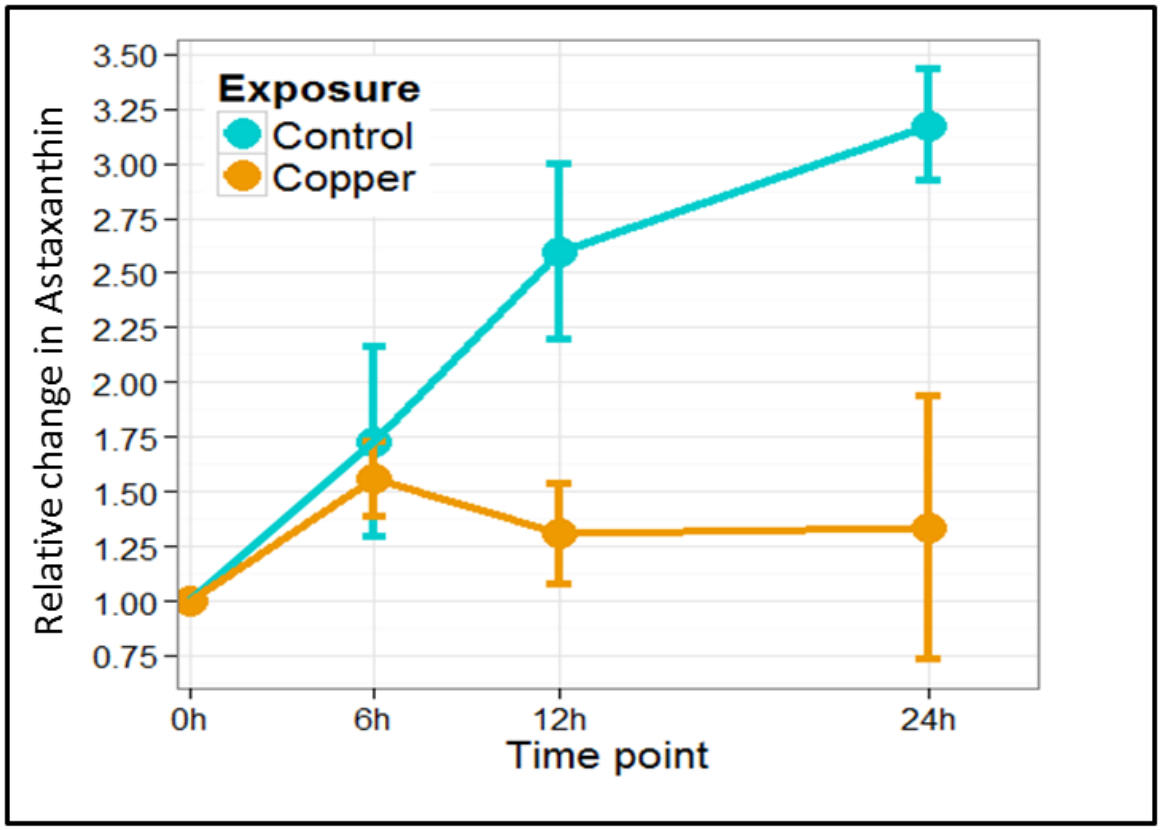

Fig 4-2. Relative change in astaxanthin levels in copepods relative to time 0h. Mean (± SE). Values greater than 1 indicate an increase in astaxanthin while a value less than 1 indicates decreased astaxanthin relative to time 0h.

Chapter 5. Hybrid breakdown in red carotenoid production from interpopulation crosses of *Tigriopus californicus*

Introduction

Cellular respiration requires extensive interaction between proteins encoded by both the nuclear and mitochondrial genomes. Consequently, coevolution of the two genomes within populations results in mitonuclear coadaptation that is fundamental in shaping the patterns and processes of hybridization, speciation, and adaptation in eukaryotes by enabling cellular respiration (Hill, 2015). Hybridization between populations can lead to the pairing of mitochondrial and nuclear genes that are not fully coadapted, resulting in reduced ATP production, increased release of reactive oxygen species, and loss of fitness in a portion of recombinant individuals (Arnqvist *et al.*, 2010; Lane, 2012; Jovanović *et al.*, 2014; Immonen *et al.*, 2015).

Among the best studied animal systems with respect to hybrid breakdown resulting from mitonuclear incompatibilities is the marine copepod, *Tigriopus californicus* (Burton, 1990; Burton *et al.*, 2006; Harrison and Burton, 2006). The F_1 offspring of crosses between divergent populations of *T. californicus* show no loss of fitness because all individuals receive a complete set of maternal nuclear genes that are coadapted with the maternally inherited mitochondrial genes (Burton *et al.*, 2006; Ellison and Burton, 2008a). In F_2 and subsequent crosses, however, recombination shuffles nuclear genes such that two non-adapted paternal alleles can be expressed at some loci, resulting in mitonuclear incompatibilities (Ellison and Burton, 2008b; Barreto and Burton, 2013). The result is variable performance among F_2 hybrids depending on the specific combinations of nuclear genes that they carry, but on average F_2 hybrid offspring have lower fitness than parental individuals due to mitonuclear incompatibilities (Ellison and Burton, 2008a).

Previously unstudied in *T. californicus* is the physiological constraints on their orange-red coloration that comes from the red ketocarotenoid astaxanthin. *Tigriopus californicus* and other crustaceans produce astaxanthin by bioconverting yellow carotenoids obtained from their microalgae diet (Weaver *et al.*, 2018). The pathways for production of red carotenoid pigments are the same in copepods as in vertebrate animals, although the specific enzymes used by arthropods and vertebrates are likely different (Mojib *et al.*, 2014). Numerous studies on birds, fish, and reptiles have demonstrated that the hue and saturation of red carotenoid-based color displays is associated with the condition and quality of individuals, but a major question remains: what mechanism gives rise to an association between carotenoid coloration and individual quality (Weaver *et al.*, 2017)?

It was recently proposed that carotenoid-based ornaments are an index signal of mitochondrial performance because the conversion of yellow dietary carotenoids to red carotenoids shares a pathway with redox reactions of mitochondrial respiration (Johnson and Hill, 2013; Hill, 2014). Here I tested the hypothesis that red carotenoid production is sensitive to mitonuclear incompatibility by performing interpopulation crosses of *T. californicus* and comparing the amount of astaxanthin produced in parental and hybrid copepods. I predicted that if production of red carotenoids is tied to mitochondrial function, then hybrids from populations that have previously shown consistent breakdown in mitochondrial function from mitonuclear incompatibilities would also show breakdown in production of the red carotenoid, astaxanthin.

Methods

Copepod collection and rearing

I collected *Tigriopus californicus* from Abalone Cove, Los Angeles, California, USA (AB: 33 44’ N, 118 22’ W) and San Diego, California, USA (SD: 32 45’ N, 117 15’ W). These two populations have over 7% mitochondrial cytochrome *b* sequence divergence (Barreto and Burton, 2013), but laboratory hybrids are viable and fecund. I kept copepods in 200 mL beakers in artificial seawater (32 psu) at 24C on a 12h light/dark cycle and fed them the live microalga, *Tetraselmis chuii*. To test for the effects of hybridization on carotenoid bioconversion without the confounding effect of stored carotenoids, prior to the experiment I switched both populations of copepods to a carotenoid-deficient diet of nutritional yeast (Bragg, Santa Barbara, CA), which produced copepods that were essentially carotenoid-free (Weaver *et al.*, 2018).

Generation of inbred hybrid and parental lines

I produced hybrid and parental inbred lines of copepods following Ellison and Burton (Ellison and Burton, 2008b). I experimentally crossed virgin females from one population with adult males from the other population (e.g., AB x SD) to produce F_3 hybrids. A reciprocal hybrid cross (SD x AB) and parental control cross (e.g., AB x AB) was performed as well. To generate inbred recombinant and parental lines, F_3 females with egg sacs from hybrid and parental lineages were placed in six-well plates. After the first clutch of eggs hatched, the female was removed and F_4 nauplii were reared as discrete family units and forced to inbreed. Lineages were inbred for one generation to produce F_5 hybrid and parental inbred individuals for carotenoid analysis.

Bioconversion of dietary carotenoids

To measure the effect of hybridization on astaxanthin production, F_5 copepods were switched to a diet of live microalga, *Tetraselmis chuii*, which contains the carotenoid precursors

required for the bioconversion to astaxanthin (Brown and Jeffrey, 1992; Weaver *et al.*, 2018) *ad libitum* for 7 days.

Carotenoid analysis

I pooled all individuals from each inbred clutch per line then extracted carotenoids from copepods using acetone. Carotenoids were separated using reversed-phase HPLC following (Weaver *et al.*, 2018). Astaxanthin was identified and quantified by comparison to an authentic standard. Astaxanthin concentration was normalized to copepod dry weight.

Statistical analysis

The values of astaxanthin concentration were log-transformed for analysis to meet the assumption of normally-distributed residuals. The data were fit to a mixed effects model with generation as a fixed effect and line as a random effect using maximum likelihood by the *nmle* package (Pinheiro *et al.*, 2017) in R (R Core Team, 2014). I report parameter estimates from a model that assumed unequal variances between parental and hybrid lineages because it performed better than when equal variance was assumed (variance ratio: 2.35; ΔAIC = -2.44, p = 0.03).

Results

Before the hybridization experiment, I found that parental SD copepods contained more astaxanthin than parental AB copepods (t =-7.08, df= 2.98 p= 0.005). After four hybrid generations and one generation of inbreeding, nine AB parental lines and eight AB x SD hybrid lines were viable, while SD parental lines and SD x AB hybrid lines did not survive to carotenoid analysis. Previously, *T. californicus* has been shown to be sensitive to inbreeding (Brown, 1991), which is likely the cause of the mortality that I observed in SD parental and SD x AB hybrid

lines. Based on the viable lines, I found that, overall, hybrids converted less astaxanthin than parental lines (mean log astaxanthin (ng mg $^{-1}$) ± s.e. ; hybrid: 2.01 ± 0.064, parental: 2.18 ± 0.04 ; t = -2.19, df =15, p = 0.04, Fig. 5-1a).

Discussion

I tested the hypothesis that hybridization between populations with known mitonuclear incompatibilities would decrease bioconversion of dietary carotenoids to the red carotenoid astaxanthin in *T. californicus*. I found that, on average, hybrid lineages produced less astaxanthin than parental lineages (Fig. 5-1a). As has been demonstrated in previous studies of *T. californicus* (Ellison and Burton, 2010), hybrid lineages were more variable in phenotype than were parental lineages (Fig 5-1b). Links between mitochondrial function and astaxanthin production have been found in the sister species, *T. japonicus*, in response to an environmental stressor (Weaver *et al.*, 2016), but to my knowledge, I present the first report on the effects of hybridization on red carotenoid coloration from populations that are known to have mitonuclear incompatibilities.

Previous research demonstrated that the negative fitness consequences resulting from crosses of divergent populations of *T. californicus* can be traced, at least in part, to mitonuclear incompatibilities leading to poor function of the mitochondrial electron transport system (Ellison and Burton, 2008b) as well as reduced function of transcription of mitochondrial genes (Ellison and Burton, 2008a, 2010). The physiological outcome of mitonuclear incompatibilities in copepod hybrids includes release of more free radicals (Barreto and Burton, 2013), production of less ATP (Ellison and Burton, 2006), and presumably a low inner mitochondrial membrane potential.

The mechanism that gives rise to associations between carotenoid coloration and measures of individual quality and that prevents cheating strategies from evolving remains a major unresolved issue in physiological and evolutionary ecology (Weaver *et al.,* 2017). The observed reduction in astaxanthin production that was associated with hybrid breakdown in this study supports the hypothesis that red carotenoid coloration is a signal of mitochondrial function and thus, mitonuclear compatibility (Hill and Johnson, 2013; Hill, 2014). Whether loss of red pigmentation in hybrid copepods is a result of shared pathways related to redox processes within mitochondria (Johnson and Hill, 2013) or of more indirect effects via increased free radicals or reduced ATP (Woodall *et al.*, 1997) will require additional experiments to resolve.

Numerous experiments have been performed on animals with carotenoid coloration in which environmental conditions are manipulated and the effects on coloration measured (Griffith *et al.*, 2006; Simons *et al.*, 2012). While such studies have partially supported the idea that carotenoid coloration is condition dependent, the data that such experiments provide cannot distinguish between resource allocation and shared pathway hypotheses (Weaver *et al.*, 2017). Directly manipulating core cellular processes—whether through hybridization, chemical disruptors, or genetic knockdown or knockout methods— provides a new avenue for investigating the mechanisms that make carotenoids a reliable signal of quality.

References

Arnqvist, G., Dowling, D.K., Eady, P., Gay, L., Tregenza, T., Tuda, M. and Hosken, D.J. (2010) Genetic architecture of metabolic rate: environment specific epistasis between mitochondrial and nuclear genes in an insect. *Evolution*, 64, 3354–63.

Barreto, F.S. and Burton, R.S. (2013) Elevated oxidative damage is correlated with reduced fitness in interpopulation hybrids of a marine copepod. *Proc. Biol. Sci.*, 280, 20131521.

Brown, A.F. (1991) Outbreeding depression as a cost of dispersal in the harpacticoid copepod, Tigriopus californicus. *Biol. Bull.*, 181, 123–126.

Brown, M.R. and Jeffrey, S.W. (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. *J. Exp. Mar. Bio. Ecol.*, 161, 91–113.

Burton, R.S. (1990) Hybrid Breakdown in Developmental Time in the Copepod Tigriopus californicus. *Evolution (N. Y).*, 44, 1814–1822.

Burton, R.S., Ellison, C.K. and Harrison, J.S. (2006) The sorry state of F-2 hybrids: Consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am. Nat.*, 168, S14–S24.

Ellison, C.K. and Burton, R.S. (2010) Cytonuclear conflict in interpopulation hybrids : the role of RNA polymerase in mtDNA transcription and replication. *J. Evol. Biol.*, 23, 528–538.

Ellison, C.K. and Burton, R.S. (2006) Disruption of mitochondrial function in interpopulation hybrids of Tigriopus californicus. *Evolution*, 60, 1382–91.

Ellison, C.K. and Burton, R.S. (2008a) Genotype-dependent variation of mitochondrial transcriptional profiles in interpopulation hybrids. *Proc. Natl. Acad. Sci. U. S. A.*, 105, 15831–6.

Ellison, C.K. and Burton, R.S. (2008b) Interpopulation hybrid breakdown maps to the mitochondrial genome. *Evolution*, 62, 631–8.

Griffith, S.C., Parker, T.H. and Olson, V.A. (2006) Melanin- versus carotenoid-based sexual signals: is the difference really so black and red? *Anim. Behav.*, 71, 749–763.

Harrison, J. and Burton, R. (2006) Tracing hybrid incompatibilities to single amino acid substitutions. *Mol. Biol. Evol.*, 23, 559–564.

Hill, G.E. (2014) Cellular respiration: The nexus of stress, condition, and ornamentation. *Integr. Comp. Biol.*, 54, 1–13.

Hill, G.E. (2015) Mitonuclear Ecology. *Mol. Biol. Evol.*, 32, 1917–1927.

Hill, G.E. and Johnson, J.D. (2013) The mitonuclear compatibility hypothesis of sexual selection. *Proc R Soc B*, 280.

Immonen, E., Liljestrand-Rönn, J., Watson, C., Berger, D. and Arnqvist, G. (2015) Complex mitonuclear interactions and metabolic costs of mating in male seed beetles. *J. Evol. Biol.*, 29, 360–370.

Johnson, J.D. and Hill, G.E. (2013) Is carotenoid ornamentation linked to the inner mitochondria membrane potential? A hypothesis for the maintenance of signal honesty. *Biochimie*, 95, 436–444.

Jovanović, D.Š., Đorđević, M., Savković, U. and Lazarević, J. (2014) The effect of mitochondrial complex I inhibitor on longevity of short-lived and long-lived seed beetles and its mitonuclear hybrids. *Biogerontology*, 15, 487–501.

Lane, N. (2012) The problem with mixing mitochondria. *Cell*, 151, 246–8.

Mojib, N., Amad, M., Thimma, M., Aldanondo, N., Kumaran, M. and Irigoien, X. (2014) Carotenoid metabolic profiling and transcriptome-genome mining reveal functional equivalence among blue-pigmented copepods and appendicularia. *Mol. Ecol.*, 23, 2740–2756.

Pinheiro, J., Bates, D., DebRoy, S., Deepayan, S. and Team, R.C. (2017) nmle: Linear and nonlinear mixed effects models.

R Core Team (2014) *R: A language and environment for statistical computing*, R Foundation for Statistical Computing, Vienna, Austria.

Simons, M.J.P., Cohen, A.A. and Verhulst, S. (2012) What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds-A meta-analysis. *PLoS One*, 7, e43088.

Weaver, R.J., Cobine, P.A. and Hill, G.E. (2018) On the bioconversion of dietary carotenoids to astaxanthin in the marine copepod, Tigriopus californicus. *J. Plankton Res.*, 40, 142–150.

Weaver, R.J., Hill, G.E., Kuan, P.-L. and Tseng, Y.-C. (2016) Copper exposure reduces production of red carotenoids in a marine copepod. *Ecol. Indic.*, 70, 393–400.

Weaver, R.J., Koch, R.E. and Hill, G.E. (2017) What maintains signal honesty in animal colour dispays used in mate choice? *Philos. Trans. R. Soc. B Biol. Sci.*, 372, 20160343.

Woodall, A.A., Lee, S.W.M., Weesie, R.J., Jackson, M.J. and Britton, G. (1997) Oxidation of carotenoids by free radicals: Relationship between structure and reactivity. *Biochim. Biophys. Acta - Gen. Subj.*, 1336, 33–42.

Figures

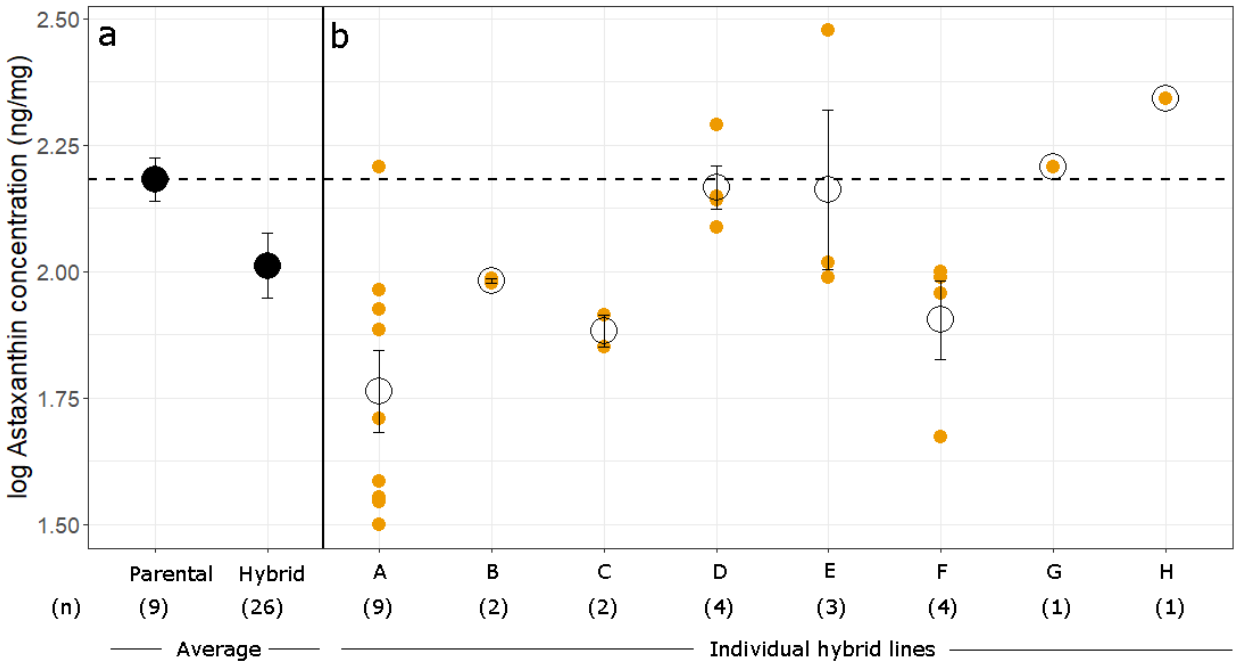

Figure 5-1. Log-transformed astaxanthin concentrations of average (a) parental and hybrid lineages and (b) individual isofemale hybrid lineages. Filled black circles (a) are maximum likelihood estimated means, open circles (b) are raw means with standard error bars. The horizontal dashed line marks the parental mean. Small yellow points show the individual measurements of astaxanthin concentrations within each lineage. (n) = number of inbred clutches per line.