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A Review and Assessment of the Shared-Pathway Hypothesis for the Maintenance of Signal Honesty in Red Ketocarotenoid-Based Coloration

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Synopsis For decades, scientists have noted connections between individual condition and carotenoid-based coloration in terrestrial and aquatic animals. Organisms that produce more vibrant carotenoid-based coloration tend to have better physiological performance and behavioral displays compared with less colorful members of the same species. Traditional explanations for this association between ornamental coloration and performance invoked the need for color displays to be costly, but evidence for such hypothesized costs is equivocal. An alternative explanation for the condition-dependence of carotenoid-based coloration, the Shared-Pathway Hypothesis (SPH), was developed in response. This hypothesis proposes that red ketocarotenoid-based coloration is tied to core cellular processes involving a shared pathway with mitochondrial energy metabolism, making the concentration of carotenoids an index of mitochondrial function. Since the presentation of this hypothesis, empirical tests of the mechanisms proposed therein have been conducted in several species. In this manuscript, we review the SPH and the growing number of studies that have investigated a connection between carotenoid-based coloration and mitochondrial function. We also discuss future strategies for assessing the SPH to more effectively disentangle evidence that may simultaneously support evidence of carotenoid-resource tradeoffs.

The integrative nature of carotenoids

Carotenoids are wonderfully diverse pigments that are synthesized primarily by plants, algae, and bacteria, and then consumed and metabolized by animals. These pigments, which typically produce yellow, orange, or red coloration, are pervasive across plants and animals and in different organismal tissues where they perform a surprising variety of functions (Miki 1991; Britton 2008; Landrum 2009). The coloration produced by carotenoids deposited in integumentary structures is used by some vertebrate and invertebrate species as ornamentation that conveys information either to the opposite sex (i.e., in mating situations) or to the same sex (e.g., as in contests between individuals) (Andersson 1994; Hill 2002; Svensson and Wong 2011). Alternatively, the coloration produced by carotenoids may not be used for inter-individual signaling, but nevertheless the pigments may play critical physiological roles in homeostatic processes (Maoka 2011; Walter and Strack 2011; Barros et al. 2018) or as components of camouflage (Matsuno 2001; Maoka 2011). Researchers noted that carotenoid pigmentation covaries with other measurable traits that are important components of survival and reproduction in many different vertebrate and invertebrate species (Blount and McGraw 2008). Thus, even in taxa that do not visually assess carotenoid-based coloration of members of their own species, there can be tight associations between carotenoid-based coloration and system function that human observers can use to extract information about the individual under observation. These characteristics of carotenoids make carotenoid-based coloration a useful tool with which researchers can approximate individual condition. Despite a growing consensus that carotenoid-based coloration is a reliable signal of condition in a host of animal species, the mechanism that link carotenoids to condition remains the focus of research and debate.

In this review, we first provide an overview of a traditional explanation for the condition-dependency of carotenoid-based coloration that involves a resource-based tradeoff between coloration and body

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maintenance. We then detail an alternative hypothesis for explaining carotenoid condition-dependency that implicates a shared pathway between carotenoid ketolation and core energetic processes in the mitochondria. We evaluate past and recently published evidence from studies that tested the link between carotenoid-based coloration and condition under the specific assumption of a shared metabolic pathway between the mitochondria and carotenoid bioconversion. We then present a small set of unpublished data that suggest a relationship between mitochondrial energetics and carotenoid bioconversion, using this to illustrate that there is much work to be done to understand the mechanism linking carotenoids and condition. Finally, we detail approaches that may be useful to better isolate the mechanisms that could underlie a potential shared pathway between carotenoid bioconversion and mitochondrial energetics.

Carotenoids as honest signals of individual condition

A discussion of the mechanisms that might link carotenoid-based coloration to individual condition requires an unambiguous definition of condition. One key point of confusion in the literature on honest signaling is the distinction between the terms "condition" and "quality"; in this essay we will use the terms interchangeably as there is no agreed upon distinction in the literature (Johnstone et al. 2009). For a definition of condition, we follow the definition given by Hill (2011) that "condition is the relative capacity to maintain optimal functionality of essential cellular processes." We will take this definition as our starting point and explore the specific cellular processes that might link carotenoid-based coloration to individual performance and current evidence for and against various mechanisms.

There is an expansive literature documenting associations between carotenoid-based coloration and various measures of individual performance or direct fitness. We provide but a subset of key examples to illustrate this point. Carotenoid-based coloration is negatively correlated with parasite load in avian and aquatic species, such that individuals more resistant to parasite infections also possess more colorful carotenoid pigmentation (Milinski and Bakker 1990; Houde and Torio 1992; Thompson et al. 1997; Brawner et al. 2000). Related to the ability to fight parasitic infections, carotenoid pigmentation has been positively correlated with mounting an effective immune response in house finches (*Haemorhous mexicanus*) (Hill and Farmer 2005), greenfinches (*Carduelis chloris*) (Lindström and Lundström 2000), and kestrels (*Falco sparverius*) (Dawson and Bortolotti 2006). Carotenoid pigmentation may also indicate the oxidative state of an individual (Lozano 1994; von Schantz et al. 1999; Moller et al. 2000; Alonso-Alvarez et al. 2004; McGraw et al. 2010), or alternatively, carotenoids themselves may act as antioxidants or stimulators of other antioxidant molecules thereby linking carotenoid-based coloration to oxidative state (Moller et al. 2000; Svensson and Wong 2011; Simons et al. 2012). In house finches, increased fecundity and increased parental care has been positively associated with redder carotenoid-based coloration in male birds (McGraw et al. 2001; Badyaev and Hill 2002; Hill 2002).

These pervasive connections between aspects of individual condition and carotenoid-based coloration give rise to the hypothesis that the condition-dependency of carotenoid-based coloration evolved under sexual selection (Rowe and Houle 1996). Alternatively, however, there may be inherent links between carotenoid pigmentation and individual performance that are simply exploited by signaling systems (Hill 2014; Weaver et al. 2017). The best evidence for inherent connections between carotenoid-based coloration and individual performance is found in systems in which sexual selection functions of carotenoid-based coloration have been tested and falsified. For example, copepods are small marine crustaceans that likely do not visually assess carotenoid pigments (Powers et al. 2020a, 2020b). Nevertheless, carotenoid-based coloration in copepods still reflects individual condition upon exposure to pro-oxidants (Weaver et al. 2018c), UV-stress (Davenport et al. 2004), acute mitochondrial toxicity (Caramujo et al. 2012; Weaver et al. 2016), and immune challenges (Babin et al. 2010). The capacity to accurately signal condition may be an inherent property of carotenoid pigmentation independent of sexual selection—one that reflects an integration with all the biological systems described above. Thus, describing a mechanism that explains the integrative nature of carotenoids is critical for understanding how it is a condition-dependent trait.

Traditional hypotheses of carotenoid signaling lack a clear mechanism

For carotenoid pigmentation to honestly signal information about other traits, it must be uncheatable. In other words, carotenoids would not be good signals of individual condition in systems where any individual could artificially increase its carotenoid pigmentation. This observation has led scientists to propose hypotheses in which a cost is imparted on carotenoid-based coloration to maintain the honesty of the information it can convey.

Such costs are proposed to arise because animals cannot synthesize carotenoids *de novo* (with very few exceptions, e.g., Altincicek et al. 2012). Animals must ingest dietary carotenoids from plants, algae, or other animals before using them for displays or in other roles; thus, the earliest ideas regarding the maintenance of honesty in carotenoid-based coloration focused on challenges to accruing limited pigment resources with finite time and energy (Endler 1980; Hill 1990). This idea of limited pigment resources underlying honest signaling via carotenoid-based coloration was subsequently expanded to invoke tradeoffs between use of carotenoid for body maintenance versus use of the pigments for ornamentation (Hill 1999; Moller et al. 2000). Under this assumption, carotenoids used to create a color display are a resource that cannot be devoted to body maintenance (Lozano 1994). This would suggest a carotenoid-based resource tradeoff between using carotenoids for ornamental coloration versus using them in other processes in the body (Alonso-Alvarez et al. 2008) as it relates to the theory of an immunocompetence handicap on ornamentation (Folstad and Karter 1992).

These hypotheses describe a carotenoid resource trade-off at the organismal level, proposing interactions between pools of carotenoids and testosterone (Peters 2007) or antioxidants (Alonso-Alvarez et al. 2008). A potential cost may be imparted on the production of carotenoid-based coloration by testosterone, either via suppression of the immune system or the generation of oxidative stress (Alonso-Alvarez et al. 2008), although the role of testosterone in a potential resource tradeoff is not clear, with effects of the hormone differing across studies (Blas et al. 2006; Cantarero et al. 2019). Moreover, it is unclear where in the cell these interactions might take place or by what biochemical mechanism such an interaction is mediated. Empirical observations interpreted in favor of these hypotheses often simply describe a loss in carotenoid-based coloration upon exposure to an external or internal stressor, which may indicate that carotenoids are somehow being shunted away from coloration such that only the fittest individuals can afford to maintain bright coloration. However, some have challenged these observations and the idea of a carotenoid resource trade-off, pointing out biases in carotenoid supplementation to captive animals (Koch et al. 2016), describing inconsistencies in the usefulness of carotenoids as immune-stimulants or pro-oxidants (Koch and Hill 2018; Koch et al. 2018, 2019), and questioning whether carotenoids are truly limited in the wild (Hudon 1994; Moller et al. 2000; Hadfield and Owens 2006). Moreover, hypotheses concerning a carotenoid resource tradeoff may downplay the importance of a particular step in the production of carotenoid-based coloration: the metabolism of dietary carotenoids into new forms.

The shared-pathway hypothesis and a mechanism for ketocarotenoid signaling

Some species produce their carotenoid-based coloration via metabolic processes that bioconvert dietary carotenoid pigments into other carotenoids (explained in more detail in the "Proposing a mechanism and location of bioconversion enzymes" section). Many dietary carotenoids are yellow in color; but bioconverted carotenoids are often orange or red (Hill and McGraw 2006; LaFountain et al. 2015). Importantly, the metabolism of carotenoids strengthens the connection between coloration and individual quality (Weaver et al. 2018b) compared with organisms that directly deposit dietary carotenoids into their tissues to produce coloration. Many organisms described in the studies listed in the "Carotenoids as honest signals of individual condition" section above utilize red ketolated carotenoids (called ketocarotenoids) to produce coloration. For example, one of the best case-studies concerning the condition-dependency of ketocarotenoid-based coloration involves the house finch. Males of this species bioconvert dietary carotenoids into red ketocarotenoids; however, only the highest quality males in the best condition produce the reddest feathers while males unable to do so produce drab, yellow feathers (Hill 2002).

Alternative explanations for the condition-dependence of carotenoids have been developed to overcome inconsistencies or gaps in resource-tradeoff hypotheses and some researchers have sought to focus on metabolized carotenoids specifically. It has been suggested that bioconverted carotenoids pigments are honest not because they are costly, but rather, because carotenoid metabolism is an index of uncheatable internal processes in the body (Hill and Johnson 2012; Biernaskie et al. 2014; Weaver et al. 2017). This hypothesis may explain the stronger connection between red ketocarotenoid bioconversion and individual quality if the process of metabolizing carotenoids is tied to core cellular processes (Fig. 1). This idea forms the basis for the Shared-Pathway Hypothesis (SPH) of carotenoid condition-dependence (Hill 2011). This hypothesis asserts that ornamentation shares a biochemical pathway with energy production in the mitochondria (Hill 2011, 2014). Thus, the SPH places the efficiency of mitochondrial function at the core of what

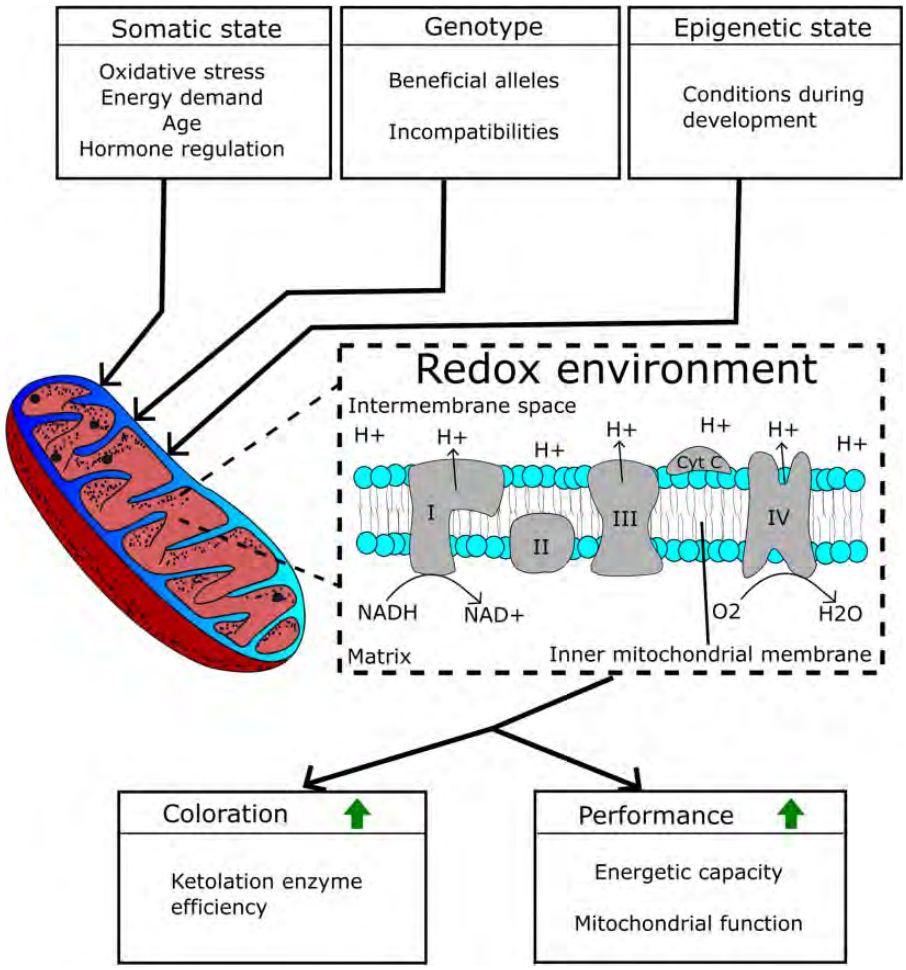

Fig. 1. The relationship between carotenoid coloration and mitochondrial function underlying the integrative nature of carotenoid signaling proposed by the SPH. Adapted from Hill (2011, 2014).

ketocarotenoids signal about individual condition and places less emphasis on tradeoffs created by a finite pool of carotenoid resources (Fig. 2). It should be noted here that, more broadly, tradeoffs may not always be resource dependent, and that direct costs from somatic damage may also influence the expression of condition-dependent traits (Tatar and Carey 1995). However, the SPH may excel at addressing this possibility, as it could explain mechanistically how a direct cost from damage to the mitochondria could turn ketocarotenoid-based coloration into an index of mitochondrial function.

Currently, researchers are working in both vertebrate and invertebrate systems to evaluate the SPH, using observational and experimental techniques. However, just as a clear concept of condition is necessary for a meaningful discussion of honest signaling, an unambiguous understanding of mitochondrial function and dysfunction is essential for a discussion of how mitochondrial processes might underlie honest signaling via ketocarotenoid-based coloration. Mitochondria are primarily known as producers of energy in the form of ATP, but they also play critical roles in regulating hormone responses, the immune system, and broad responses to external or internal stress (Picard and Sandi 2020). Therefore, changes to mitochondrial function in any of these arenas may affect carotenoid bioconversion if the two processes share a cellular environment (Fig. 1; Koch et al. 2017). The challenge for researchers is establishing causal relationships between mitochondrial function and carotenoid bioconversion. Importantly, such relationships do not necessarily invoke the use of ketocarotenoid-based coloration in visual signaling. The central premise of the SPH is the inherent link between carotenoid processing and cellular function.

Measuring mitochondrial function under the framework of the SPH

The way in which mitochondrial function is defined and measured is important to the design of tests of the SPH and to the interpretation of experimental

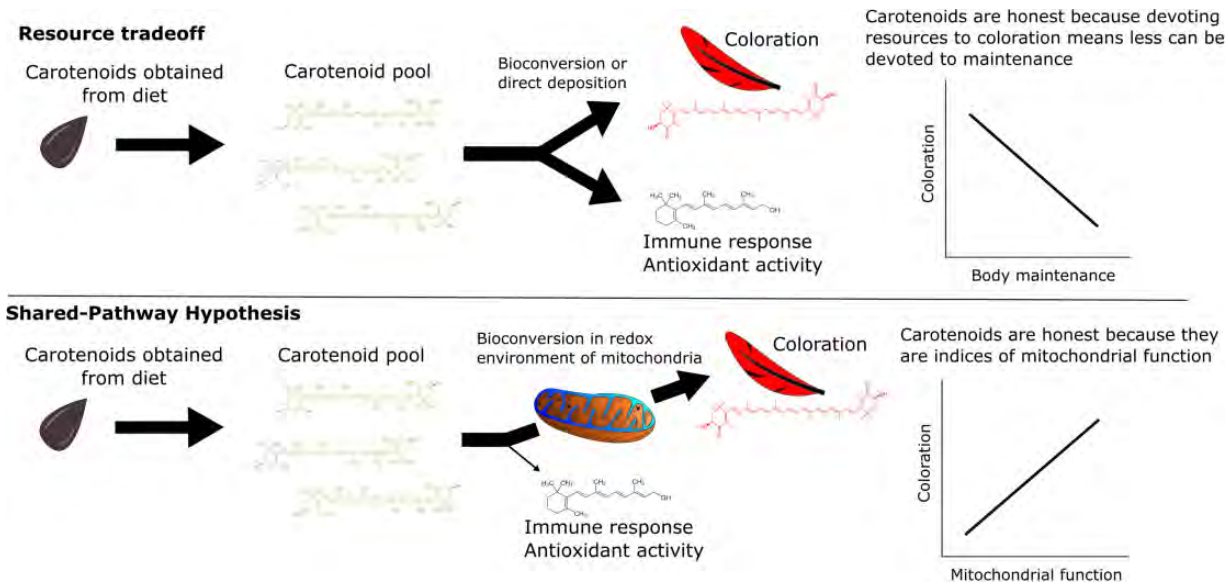

Fig. 2. A diagram showing a simplified overview of the differences between the SPH and carotenoid resource tradeoff hypotheses. Both sets of hypotheses involve many of the same components, but the mechanism for maintaining the honesty of carotenoid coloration is different. The SPH does not ignore that some carotenoids may be devoted to maintaining homeostasis but, instead, places the primary focus on a shared redox environment between mitochondrial metabolism and carotenoid bioconversion.

results. Mitochondrial function (or dysfunction) is no longer best defined by mean energy production; ATP production often is meaningless without further context (Brand and Nicholls 2011; Salin et al. 2015). Even though total energy production may be generally related to numerous processes throughout the body, including carotenoid ketolation, the mechanism linking carotenoid bioconversion and mitochondrial function may be more intimate (Hill and Johnson 2012). For example, the efficiency at which redox reactions occur in the electron transport system (ETS) (as determined by the intracellular redox environment) might underlie positive or negative associations between ATP and ketocarotenoid production observed at the whole-animal level (Figs. 1 and 2).

Mitochondrial efficiency can be measured in numerous ways thanks to advancements made in the field of mitochondrial physiology (Koch et al. 2021). For example, the respiratory control ratio (RCR), a measure of energetic capacity, has been heralded as the most appropriate tool for quantifying mitochondrial dysfunction in isolated mitochondria and in intact cells (Brand and Nicholls 2011), and this may allow researchers to test for associations between carotenoid bioconversion and the ability of the mitochondria to alter their rate of redox reactions. ATP production can be contextualized by measuring oxygen consumption, which is a classic method for estimating metabolic rate (Salin et al. 2015; Petrick and Holloway 2021), and this may also help shed light on how redox activity affect carotenoid bioconversion enzymes. The activity of ETS complexes can be measured individually or in conjunction with one another, allowing researchers to isolate inefficiencies in oxidative phosphorylation to specific ETS proteins (Barrientos et al. 2009), with the prediction under the SPH that inefficiencies in ETS complexes may be reflected in inefficiencies in carotenoid oxidizing enzymes. Measures of oxidative damage to either DNA or lipid membranes can help researchers estimate the degree of oxidative stress suffered by mitochondria, and by extension their redox efficiency (Metcalfe and Alonso-Alvarez 2010; Barreto and Burton 2013). In contrast, measures of mitochondrial turnover rate or mitochondrial volume can inform researchers of the degree to which mitochondria can withstand stress or can allude to the ability of mitochondria to dynamically adapt to oxidative stress (Larsen et al. 2012; Hill et al. 2019b). Measures of oxidative damage and responses to that damage can help test the prediction under the SPH that carotenoid bioconversion enzymes are influenced by redox conditions of the mitochondria. Mitochondrial redox potential and electron donor/acceptor ratios can also be quantified to test specific

predictions concerning the redox environment required by ketolase enzymes (Hanson et al. 2004; Canelas et al. 2008).

Identifying carotenoid bioconversion enzymes

So far, researchers have implicated several different enzymes that are thought to bioconvert dietary carotenoids into ketocarotenoids. Perhaps most prominent in relation to recent tests of the SPH is the enzyme CYP2J19 (Fig. 3), a member of the cytochrome P450 superfamily of oxygenases (Omura 1999). CYP2J19 has been implicated as the carotenoid ketolase in birds and turtles (Lopes et al. 2016; Mundy et al. 2016; Twyman et al. 2018a, 2018b, 2016; Khalil et al. 2020; Kirschel et al. 2020). Recently, another cytochrome P450, labeled CYP3A80, has been identified to control the presence or absence of ketocarotenoid-based coloration in poison dart frogs (*Ranitomeya sirensis*) (Twomey et al. 2020).

As mentioned in the "Carotenoids as honest signals of individual condition" section, crustaceans are another taxonomic group frequently studied in conjunction with ketocarotenoid-based coloration. So far, no direct homolog to the avian and turtle CYP2J19 enzyme has been found in crustaceans. However, other candidate cytochrome P450 oxygenases (CrtS-like beta-hydroxylases) have been putatively identified in both copepods and shrimp that produce coloration using bioconverted ketocarotenoids (Mojib et al. 2014; Weaver et al. 2020). Prado-Cabrero et al. (2020) suggest that carotenoid enzymes in *Tigriopus californicus* copepods and other crustaceans should be able to perform both ketolation and hydroxylation reactions to produce a variety of ketocarotenoids. They also suggest that if avian species display a similar flexibility in their carotenoid metabolism, it would only strengthen the honesty of ketocarotenoid-based coloration in signaling the efficiency of core cellular processes in the mitochondria, as is suggested by the SPH (Prado-Cabrero et al. 2020).

Proposing a mechanism and location of bioconversion enzymes

The biochemistry of carotenoid conversion is relatively straightforward. Carotenoids exist as molecules with a carbon backbone with alternating double bonds with an ionone ring at each end. The backbone of alternating double bonds makes carotenoids photoactive, while the ionone rings, or more specifically, the functional groups attached to the rings, give each carotenoid molecules their properties and colors (Hill and Johnson 2012).

The oxidation reactions performed by carotenoid ketolase enzymes effectively swap out or add functional groups to dietary carotenoids to produce new carotenoid forms, such as red ketocarotenoids. For example, the yellow dietary carotenoid β-cryptoxanthin has a single hydroxyl group on one of its rings. In avian species, this carotenoid is converted into the red ketocarotenoid 3-hydroxy-echinone through the addition of a ketone group on its hydroxylated ring (McGraw et al. 2006; LaFountain et al. 2015). The ketolation of the one ring causes a shift in the carotenoid's absorption properties, causing different wavelengths of light to be reflected (in this case, a change from yellow to red). Enzymes that perform hydroxylation reactions to produce new ketocarotenoids work via a similar process, instead adding a

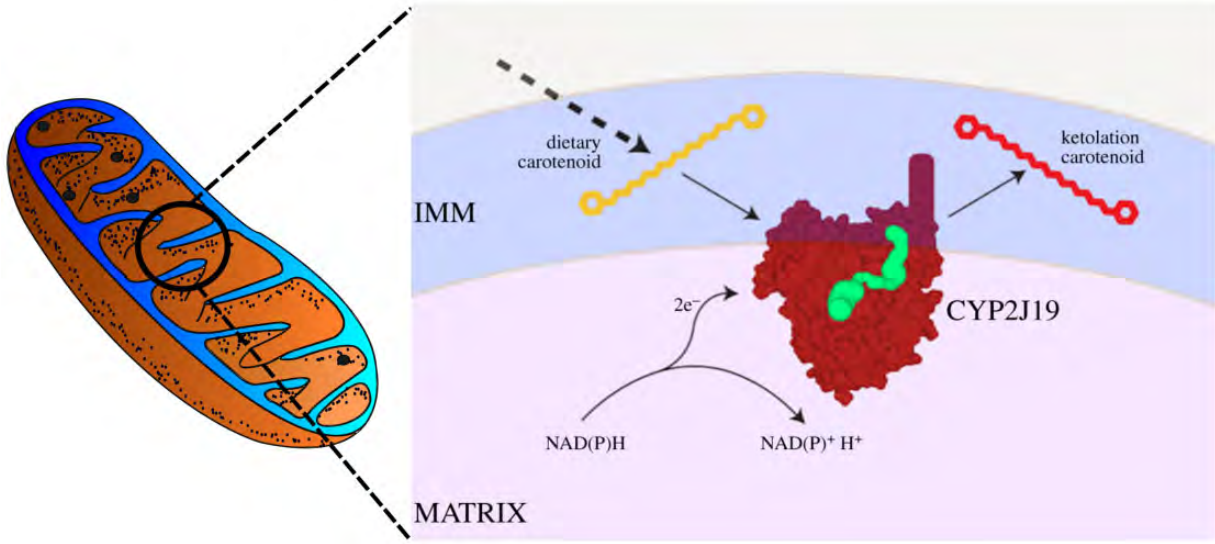

Fig. 3. The predicted structure, mechanism, and location of the avian carotenoid ketolase enzyme, CYP2J19. The right panel showing the hypothesized structure of CYP2J19 is reproduced from Hill et al. (2019b).

hydroxyl functional group. Importantly, there are different pathways to reach the same ketocarotenoid. For example, the marine copepod *T. californicus* can produce its primary ketocarotenoid astaxanthin via hydroxylation of canthaxanthin or ketolation of zeaxanthin (Weaver et al. 2018a).

The process of adding functional groups via ketolation or hydroxylation reactions requires donation and acceptance of electrons during the oxidation–reduction (redox) reaction. Cytochrome P450 oxidases work in conjunction with Cytochrome P450 reductases (CPRs) to perform redox reactions (Strohmaier et al. 2019). The preferred electron donor for CPRs is NADPH, but NADH is also a possible electron donor (Csernetics et al. 2015; Strohmaier et al. 2019). These biochemical details become vital information when we reconsider the process of how carotenoid bioconversion might relate to mitochondrial function according to the SPH. Importantly, energy production in the mitochondria is also accomplished via redox reactions. The process of oxidative phosphorylation by the ETS is regulated by the same types of electron donors as those proposed to be used by carotenoid ketolation enzymes (Karpac and Jasper 2013; Hill 2014; Hill et al. 2019b). The SPH suggests that the redox environment of the mitochondria, maintained via the ratios of electron donors and acceptors (like NADH/NAD+ or NADPH/NADP+) and/or electron shuttlers (like ubiquinone), may influence the effectiveness of carotenoid ketolation enzymes (Hill and Johnson 2012; Johnson and Hill 2013; Cantarero et al. 2020b). If carotenoid bioconversion and energy production do share a redox environment, it suggests an intimate level of integration between carotenoid conversion and any other energy-requiring system in the body.

Evaluating the SPH

Researchers have approached an evaluation of the SPH from diverse perspectives. Some focus on observations of wild-caught populations. Others have opted for controlled experimentation in captive organisms. The question about the integration of ketocarotenoids with the mitochondria has spurred truly integrative collaborations between experts in unique and complex fields. Studies testing for links between carotenoid bioconversion and mitochondria have united mitochondrial and stress physiologists, evolutionary biologists, animal behaviorists, mitochondrial and nuclear geneticists, biostatisticians, and applied and theoretical biochemists. Herein, we discuss some of their findings, as well as their conclusions.

Observing and experimenting using natural systems

The SPH as it has been applied to red ketocarotenoid-based coloration assumes a tight association between the functions of carotenoid ketolase enzymes and mitochondria. Thus, establishing that ketocarotenoids and carotenoid enzymes are located in or near the mitochondria in the cell is paramount. Researchers have proposed that the site of carotenoid bioconversion is either at the integument near where they will ultimately be deposited (McGraw 2004) or in the metabolically active liver (del Val et al. 2009a, 2009b; Hill et al. 2019b). High levels of ketocarotenoids have been found not only in the liver of house finches, but specifically inside the inner membranes of their mitochondria, right alongside the redox enzymes of the ETS (Ge et al. 2015). Moreover, transcript of CYP2J19 has been found at elevated levels in both the integument and liver of songbirds (Lopes et al. 2016; Mundy et al. 2016). Molecular modeling of the avian ketolase suggests it may be associated with the inner mitochondrial membrane (IMM; Fig. 3), or in the mitochondrial associated membranes (MAMs) of the endoplasmic reticulum (ER) (Johnson and Hill 2013; Hill et al. 2019b). Studies in mammals have found that carotenoids are prohibited from reaching cytotoxic levels within mitochondria through the operation of carotenoid cleaving enzymes (Amengual et al. 2011; Lobo et al. 2012), suggesting that careful regulation of carotenoids in mitochondria is important.

Following the observation that concentrations of ketolated carotenoids are found in the mitochondria of molting songbirds (Ge et al. 2015), Hill et al. (2019b) performed a study on wild populations of house finches. This study focused on natural variation in the coloration of molting male house finches that were captured during feather molt, when male finches are actively bioconverting dietary carotenoids to produce the red ketocarotenoids in their feathers. These researchers found that the redness of the male house finches' feathers correlated with several measures of mitochondrial capacity. The male birds with the reddest feathers had the highest RCR, which is a measure of the capacity of mitochondria to respond to increased demand for ATP (Hill et al. 2019b). The reddest males also had lower turnover of new mitochondria (measured via PGC-1α, a marker of biogenesis) despite being able to sustain higher levels of lipid degradation (measured via 4-HNE adducts)

(Hill et al. 2019b). These observations supported the hypothesis that red ketocarotenoids in house finches signal the capacity of their mitochondria to withstand stress and effectively respond to increased demand of energy.

Studies involving CYP2J19 expression

Hybridization is well documented to cause mitochondrial dysfunction (reviewed by Hill 2019), so ketocarotenoid production in hybrid individuals provides an interesting test of the SPH. Hudon et al. (2021) observed that some hybrid offspring from crosses between two genetically divergent populations of northern flickers (*Colaptes auratus cafer* × *Colaptes auratus auratus*) had reduced ketolation capacity compared with flickers from parental populations, as indicated by the carotenoid composition of feathers. They suggested that this loss of red coloration could be consistent with the SPH if hybrid flickers also display reduced redox capacity in their mitochondria. However, this remains to be tested empirically in this system. Similarly, asymmetric color introgression in hybridizing red-fronted (*Pogoniulus pusillus*) and yellow-fronted (*Pogoniulus chrysoconus*) tinkerbirds may be explained by altered expression of CYP2J19 resulting from genetic incompatibilities that affect mitochondrial function (Fig. 1; Kirschel et al. 2020). In northern flickers there is no documented evidence for mate choice based on ketocarotenoid-based coloration (Hudon et al. 2021), but in tinkerbirds asymmetric introgression of red coloration may be due to strong sexual preference for red feathers (Kirschel et al. 2020). The implication of these studies is that hybrids with compromised mitochondrial function may not ketolate carotenoids as effectively, resulting in females selecting against hybrid males with yellower feathers.

As discussed in the "Traditional hypotheses of carotenoid signaling lack a clear mechanism" section, researchers have long suspected a connection between carotenoid-based coloration and testosterone (Stoehr and Hill 2001; Blas et al. 2006; Peters 2007). Mitochondria play integral roles in the synthesis and regulation of testosterone and other sex hormones (Velarde 2014). Recently, a possible association between CYP2J19 ketolase and testosterone was observed in wild populations of red-backed fairywrens (*Malurus melanocephalus*) where testosterone levels and CYP2J19 expression were positively correlated (Khalil et al. 2020). The authors speculated that male fairywrens capable of maintaining higher testosterone levels could be more capable of quickly producing ketocarotenoid-based coloration (Khalil et al. 2020). However, this study offers no definitive support of this speculation in part because the conversion site of carotenoids in this species is unknown, and because this result contrasts with previous hypotheses about a potential tradeoff between testosterone levels and carotenoid-based coloration (von Schantz et al. 1999; Alonso-Alvarez et al. 2007; Peters 2007). Rather, we suggest that mitochondrial function may be a tether between testosterone signaling and carotenoid ketolation, although the relationship between testosterone signaling and the redox environment of the mitochondrion remains unclear (Fig. 1). A hormone-mediated connection between condition and ketocarotenoid-based coloration may be an alternative mechanism by which ketocarotenoids signal somatic state, and this alternative mechanism would not necessarily be mutually exclusive to the mechanism proposed by the SPH (Cantarero et al. 2020a). Alternatively, it may be that testosterone levels are simply a mediator of the redox state of the ETS and carotenoid bioconversion enzymes.

The effects of thyroid hormone signaling and altering the redox state of the mitochondria via mitochondrially targeted compounds have been demonstrated to affect CYP2J19 levels at the integument in zebra finches (*Taeniopygia guttata*) without changing the levels of circulating carotenoids in the birds (Cantarero et al. 2020a), thus demonstrating a link between carotenoid ketolation and the mitochondria. It was hypothesized that lower levels of superoxide in the Zebra Finches may have disrupted redox signaling, altering CYP2J19 expression (Cantarero et al. 2020a). Increasing levels of thyroid hormone may have induced oxidative stress through generation of excessive reactive oxygen species, which then inhibited the expression or function of CYP2J19. Interestingly, they found that the combined effects of increasing thyroid hormone and altering superoxide levels erased changes to CYP2J19 expression and, at moderate thyroid levels, even increased expression of CYP2J19. It should be noted that the authors also posited that thyroid hormone regulation may complicate the ability of CYP2J19 ketolation to act as a reliable index trait, and that this may reflect confounding effects of phenotypic plasticity on the condition-dependence of ketocarotenoid-based coloration (Cantarero et al. 2020a).

Targeted mitochondrial manipulations using avian species

The SPH is best tested with well-designed and carefully controlled experiments. To this end, some researchers are treating animals with mitochondrial-

targeted chemicals that were developed as therapies for human maladies arising from mitochondrial dysfunction. In the first study to use mitochondria-targeted molecules to test the SPH, Cantarero and Alonso-Alvarez (2017) began with the assumption that the ratios of ubiquinol and ubiquinone (redox cyclers involved in the shuttling of electrons across the ETS) might affect the efficiency of carotenoid ketolation in zebra finches. To test this hypothesis, they targeted the IMM with specific molecules designed to either stabilize (using coQ10—mitoQ) or disrupt (using dTPP) the redox environment of the IMM. They found that mitoQ improved bill redness while dTPP caused bills to become less colorful. The researchers interpreted this result as evidence that carotenoid ketolases are indeed localized to the IMM (Cantarero and Alonso-Alvarez 2017).

As a follow-up study, these same researchers again tested the effects of mitoQ, this time in conjunction with mitoTEMPO, a superoxide dismutase mimetic targeted specifically to the IMM thought to help recycle ubiquinone to help maintain effective redox conditions in the mitochondria (Cantarero et al. 2020b). They performed this experiment in red crossbills (*Loxia curvirostra*), which bioconvert carotenoids in the liver like house finches (Hill et al. 2019b). They found that mitoQ reduced circulating carotenoids in the blood but that the treatment did not change the redness of the crossbills' feathers. In contrast, they observed a strong effect of individual condition on the outcome of mitoTEMPO treatment. In low quality birds with already poor coloration, mitoTEMPO increased blood ketocarotenoids, but not feather ketocarotenoids. In high quality birds with already vibrant coloration, mitoTEMPO did not increase circulating carotenoids but were still able to increase feather redness. One interpretation of these results is that poor quality birds revealed their inefficiencies in carotenoid ketolation due to a reduced mitochondrial response to mitoTEMPO, while high quality bird mitochondria and ketolase enzymes responded much more efficiently (Cantarero et al. 2020b).

In a study utilizing thyroid hormones in zebra finches (also discussed in the "Studies involving CYP2J19 expression" section), these authors observed a significant effect of altering hormone levels on ketocarotenoid-based coloration (Cantarero et al. 2020a). At the highest levels of artificially increased thyroid hormone, zebra finches displayed redder bills. However, they also discovered an interaction with the mitochondria-targeted molecule mitoTEMPO, where this molecule counteracted the effect of thyroid hormone at its two highest administered doses (Cantarero et al. 2020a). The authors concluded that these counteractive effects between mitoTEMPO (a reducer of oxidative stress) and thyroid hormone (a stimulator of oxidative stress at high levels) could underlie both an acute and delayed response by the mitochondria (Cantarero et al. 2020a). Thus, it may be that hormone levels are one of many somatic influences on the redox state of the mitochondria, and this may in turn influence redox reactions involving carotenoid bioconversion (Fig. 1).

Lastly, in an experimental manipulation of birds naturally infected by coccidia and *Trichomonia*, Lind et al. (2021) demonstrated that certain antibiotic compounds may interact with carotenoid bioconversion and the production of ketocarotenoid-based coloration in feathers. Greenfinches administered the anticoccidial toltrazuril showed no change in their carotenoid-based coloration, but greenfinches given metronidazole grew significantly more colorful feathers (Lind et al. 2021). The authors suggested that naturally low levels of coccidial infections reduced the effect of the anticoccidial, but that the antibiotic metronidazole improved mitochondrial function. They noted that this antibiotic may improve gut microbiota that produce higher levels of short-chain fatty acids and that these short-chain fatty acids can act as sources of energy and influence mitochondrial function (Lind et al. 2021). At this point, the connection between gut microbiota, mitochondrial function, and ornamentation is purely speculative. Alternatively, changes to gut microbiota may simply affect changes to carotenoid absorption (Brawner et al. 2000).

Using a crustacean to study the relationship between energy and carotenoid metabolism

The majority of empirical studies concerning the SPH have been conducted with birds, in part because red ketocarotenoid-based coloration in birds has been shown to be a criterion in mate choice (Svensson and Wong 2011). Indeed, the SPH has rarely been studied in non-vertebrate taxa. However, our laboratory group has recently begun work to test the SPH in an invertebrate model system. Marine copepods belonging to the *Tigriopus* genus have bright red coloration from the accumulation of primarily a single ketocarotenoid, astaxanthin, in their tissues (Weaver et al. 2018a). In carefully controlled mate choice studies, we found no evidence that these copepods visually assessed ketocarotenoid pigmentation despite producing a bright red ornament (Powers et al. 2020a). Regardless, astaxanthin coloration in *Tigriopus* copepods is still highly condition-dependent (Davenport

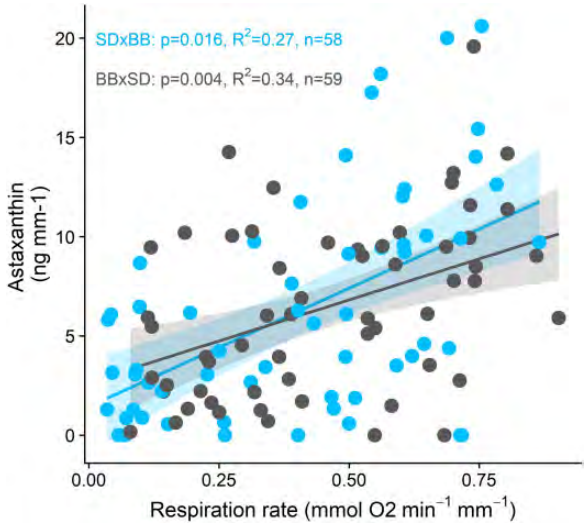

Fig. 4. Blue and black dots represent individual copepods. Lines are the estimated effect of increasing respiration on astaxanthin. The shading behind the line is the 95% confidence interval around this estimate effect. The results of a mixed effects linear model with oxygen plate as a random effect are shown in the top left. R^2 values are adjusted for terms in the model.

et al. 2004; Weaver et al. 2018c), making it useful for testing the integration of carotenoid bioconversion with mitochondrial performance.

To test the connection between a disruption of mitochondrial function and carotenoid bioconversion, Weaver et al. (2016) exposed *Tigriopus japonicus* copepods to copper, increasing oxidative damage in the mitochondria and depleting antioxidants. They measured the effect on ketocarotenoid-based coloration, noting that copepods exposed to copper did not convert as much dietary carotenoids to astaxanthin as unexposed copepods (Weaver et al. 2016). They noted that copper ions likely disrupt the redox environment of the mitochondria, thereby possibly affecting the redox reactions performed by carotenoid bioconversion enzymes. It is possible that astaxanthin was consumed in defense of oxidative stress in copper treated copepods (Weaver et al. 2018c), and this would support the idea of a carotenoid resource tradeoff. However, *Tigriopus* copepods must continuously deposit ketocarotenoids in their tissues, and the algae Weaver et al. (2016) provided the copepods as food contained no astaxanthin for the copepods to directly consume (Brown and Jeffrey 1992). This means that even if the copepods were shunting carotenoids away from coloration to use for antioxidant defense, copper-induced disruptions to the intracellular redox environment of the mitochondria still decreased the rate of carotenoid bioconversion.

Current work being done in the copepod model system

The latest methods for testing the SPH in copepods involve inducing dysfunction in the mitochondria via specific mitonuclear incompatibilities in interpopulation hybrids of *T. californicus* (Burton et al. 2006; Pereira et al. 2016; Hill et al. 2019a). This dysfunction results in an increase in mitochondrial oxidative damage, and a decrease in complex activity and energy production of the mitochondria of hybrids (Ellison and Burton 2006; Ellison and Burton 2008, 2010; Barreto and Burton 2013). The induction of dysfunction that is localized specifically to the mitochondria allows researchers to test the effects of mitochondrial dysfunction on carotenoid bioconversion.

As an illustration of how associations between mitochondrial function and color production might be studied in invertebrates, we present the results of a study on the production of ketolated carotenoids by hybrid copepods from a cross between *T. californicus* copepods from San Diego (SD), in southern California and another population from Bodega Bay (BB), CA in the north of the state. These populations are highly diverged (21.7% nucleotide divergence across mt genome [Barreto et al. 2018]) and show signs of specific adaptive evolution in proteins involving mitochondrial metabolism (Pereira et al. 2016). Thus, we predicted that hybridizing these populations in the laboratory should result in hybrid copepods with altered mitochondria function, as shown by previous crosses between SD and BB copepods (Pereira et al. 2016), and changes to mitochondrial function should correlate with variation in carotenoid bioconversion. To test this prediction, we measure whole-animal respiration and astaxanthin production in hybrid individuals (detailed methods in Supplementary Documents).

Speculating on results from this experiment in the context of other work with *T. californicus*

With this experiment, parental cultures were contaminated with algae during the experiment, so we could not assess astaxanthin bioconversion on these copepods. Thus, we only present results on hybrids from both cross directions (SD♀ × BB♂ and BB♀ × SD♂). We found a statistically significant positive relationship between oxygen consumption and astaxanthin bioconversion in hybrid copepods (Fig. 4). This result was observed in hybrid copepods from both cross directions. Unfortunately, because there were no parental copepods to which we could compare the rate of astaxanthin bioconversion, it is

unclear whether hybrids from this particular cross had less efficient carotenoid ketolation.

The positive relationship between oxygen consumption and astaxanthin production indicated that carotenoid metabolism is correlated with the rate at which oxidative phosphorylation occurs in the mitochondria of *T. californicus*. This could indicate that astaxanthin production is indicative of energetic capacity in the mitochondria of this species. Prior work demonstrated that *T. californicus* hybrids from SD and BB crosses had high levels of oxidative damage (Barreto and Burton 2013). Under the assumption that copepods in our cross also suffered from an increase in oxidative damage, it is possible that they were compensating for this effect through mitochondrial uncoupling (Brand 2000; Speakman et al. 2004), increasing mitochondria volume (Heine and Hood 2020; Heine et al. 2020), or upregulating the activity of alternative oxidases (AOX) (Weaver 2019). These compensatory changes may stabilize the redox environment of the mitochondria against an increase in oxidative stress, thereby increasing respiration. Under these circumstances, astaxanthin bioconversion could predict the ability of hybrid copepods to withstand and respond to oxidative stress (Hill et al. 2019b). The capacity to respond to changes in the need for energy may be a key trait signaled (Hill et al. 2019b), in contrast to mean ATP production alone, which can be deceptive (Brand and Nicholls 2011; Salin et al. 2015; Petrick and Holloway 2021). Indeed, Hill et al. (2019b) show a positive correlation between respiratory capacity and coloration in house finches that is similar to the positive relationship we show between whole animal respiration and astaxanthin in copepods (Fig. 4).

In copepods facing oxidative stress, electron donors such as NADH and NADPH may be in higher demand in the ETS as the rate of oxidative phosphorylation increases in complex 1 (Willis et al. 2016). If complex 1 of the ETS consumes these electron donors at a higher rate, they may not be readily available for use by carotenoid ketolation enzymes, thus creating the potential for a trade-off in energy, but not necessarily carotenoid resources. Under this assumption, carotenoid bioconversion may even negatively correlate with energy produced. In the absence of a stressor, when the demand for energy is lower, less electron donors may be consumed and the relationship between energy and carotenoid ketolation might then be positive. A negative relationship between energy production and carotenoid bioconversion in the presence of an acute stressor may also suggest a carotenoid resource tradeoff because this is the expected association if carotenoids must be diverted away from bioconversion and in support of homeostatic processes. However, it is unclear how a resource tradeoff might facilitate a positive relationship between energy production and carotenoid bioconversion within an individual. Under nonstressful conditions, the presence of a correlation between energy production and carotenoid bioconversion might diminish rather than becoming positive. However, if we expand our view to among-individual variation, it may then be possible to observe a positive relationship between two traits involved in a tradeoff (in this case, coloration and body maintenance), particularly if there is greater variation in resource acquisition than in resource allocation among individuals (van Noordwijk and de Jong 1986; Zera and Harshman 2001). Regardless, a correlation between bioconversion and oxygen consumption represents a single example of a connection between mitochondrial function and carotenoid metabolism and extrapolating these results to evaluate the SPH will take further work.

The next steps for evaluating the SPH

The studies and results presented in the "Evaluating the shared-pathway hypothesis" section offer an overview of the current approaches being employed by researchers who are testing the SPH. However, while patterns observed to date are consistent with the SPH, and in certain instances seem inconsistent with a carotenoid-based resource tradeoff, this field of investigation is far from settled. Indeed, a shared pathway between carotenoid bioconversion and mitochondrial metabolism may not be mutually exclusive to a tradeoff of carotenoid resources in certain cases. To move this field forward, tightly controlled experiments are needed.

Targeted experiments that isolate the effects of the experimental treatment to the cellular systems hypothesized to be integrated with carotenoid bioconversion hold the potential to provide much cleaner tests of the SPH. The results from the few pioneering experimental studies detailed in the "Evaluating the shared-pathway hypothesis" section cannot fully disentangle effects on coloration resulting from changes in the function of mitochondria versus effects arising from the allocation of ketocarotenoids to respond to stress. Evidence that carotenoids play critical roles in maintaining homeostasis is at best contentious in some systems (Koch et al. 2019) yet seems ubiquitous in others (Maoka 2011). Thus, the key to future studies will be designing experiments that can isolate

the specific mechanisms hypothesized by SPH to underlie ornament production.

Chemical manipulation of the mitochondria

One method that may continue to help test the proposed mechanism of the SPH is to observe the effects on ketocarotenoid-based coloration of targeted chemical manipulation of the mitochondria. Tests in zebra finches and red crossbills have employed such methodology (see the "Studies involving CYP2J19 expression" section). However, experiments like these may still suffer from the challenge to distinguish changes in red ketocarotenoid-based coloration due to poor ketolase efficiency versus consumption of ketocarotenoids for antioxidant function (but see Cantarero et al. [2020a] for a good example of how these effects may be disentangled). One strategy that may help clarify interpretations of these types of experiments is to use animals devoid of ketocarotenoids in their system. If these animals are provided dietary carotenoids for a set time period during exposure to chemical manipulations of the mitochondrial redox environment, it is possible to test their efficiency of carotenoid bioconversion under a time-constraint. We have begun using this methodology in *T. californicus* copepods, exposing individuals to mitochondrial uncouplers and chemical inhibitors of specific ETS complexes to disrupt the redox environment in the mitochondria. Employing these methods in avian species could be more challenging, but also very informative.

Genetic knockdown of mitochondrial components

Cause and effect can be difficult to establish in when dealing with complex physiological pathways. Correlations between measured traits may arise through the effects of unmeasured traits. An approach that might allow investigators to make more concrete deductions is to manipulate the expression of specific key genes in pathways of interest. To this end, genetic knockdown of specific enzymes in the mitochondria to manipulate the redox environment of carotenoid ketolase may be particularly informative. This is where model systems like *T. californicus* copepods might excel, considering the molecular resources now available and large body of literature on the genetics for this species on the population level (Pereira et al. 2016; Barreto et al. 2018; Lima et al. 2019). Genetic knockdown of core stress response genes has already been performed on this species (Barreto et al. 2015), but has yet to be conducted under a framework of carotenoid signaling.

In vitro methods for testing the SPH

Cell culture might be one of the best tools for evaluating the proposed mechanism linking carotenoid ketolation and core cellular processes in the mitochondria. Separating individual cell lines from the noise associated with *in vivo* hormone signaling, gene expression and gene regulation may offer more powerful tests of the SPH if combined with actual fitness measures *in vivo*. The use of avian liver hepatocytes may offer a model system in which this is possible since the liver is the main site of carotenoid bioconversion for many songbird species (del Val et al. 2009a, 2009b).

In vitro methods may also help researchers more accurately determine the localization of carotenoid ketolase enzymes. The relationship between mitochondrial function and carotenoid bioconversion becomes much more complicated if carotenoid ketolase enzymes are not localized to the IMM nor any MAMs. The use of protein labeling techniques to track the transport and localization of ketolase enzymes would be informative, but no studies using this methodology have been completed to date. So far, researchers have to rely on predictive modeling for the location and function of ketolase enzymes (Hill et al. 2019b).

Summary

The biochemical activities of mitochondria are among the most complex processes under study by biologists (e.g., Anderson et al. 2019; Bykov et al. 2020; Lechuga-Vieco et al. 2021), so it is not surprising that studying the relationships between carotenoid bioconversion and mitochondrial function presents an enormous challenge to behavioral and physiological ecologists (Koch et al. 2021). However, evidence thus far concerning the integration of ketocarotenoids and core cellular processes is promising and is expanding rapidly with studies of diverse animal systems. A growing sophistication in the application of cell biology to studies of animal signaling is enabling increasingly better tests of the SPH. These advances have been largely due to the collaboration among experts from diverse fields of research. This integration of disciplines is the path to a better understanding of ketocarotenoid-based coloration and all forms of sexual signaling.

Data availability

Presented data and R code for analysis can be found in the Online Supplementary Material for this article.

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Supplementary data

Supplementary data are available at *ICB* online.

Conflict of interest

The authors declare no competing interest.

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