Testing the Shared-Pathway Hypothesis through experimentation on the mating behavior, carotenoid biosynthesis, and mitochondrial function of *Tigriopus californicus*

by

Matthew J. Powers

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Approved by

Geoffrey E. Hill, Chair, Professor of Biological Sciences
Andreas N. Kavazis, Professor of Kinesiology
Tonia S. Schwartz, Associate Professor of Biological Sciences
Matthew E. Wolak, Assistant Professor of Biological Sciences
Todd D. Steury, Associate Professor of Wildlife Sciences

Abstract

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 to 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, 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 many species. In this dissertation, I review the Shared-Pathway Hypothesis and the growing number of studies that have investigated a connection between carotenoid-based coloration and mitochondrial function. I then present experimental tests of the Shared-Pathway Hypothesis using the model organism, *Tigriopus californicus*. I also discuss future strategies for assessing the Shared-Pathway Hypothesis to more effectively disentangle evidence that may simultaneously support evidence of carotenoid-resource tradeoffs.

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Chapter 1

A review and assessment of the Shared-Pathway Hypothesis for the maintenance of signal honesty in red ketocarotenoid-based coloration

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1.1 Abstract

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 (Britton, 2008; Landrum, 2009; Miki, 1991). The coloration produced by carotenoids deposited in integumentary structures are 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 (for example, 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 (Barros et al., 2018; Maoka, 2011; Walter and Strack, 2011) or as components of camouflage (Maoka, 2011; Matsuno, 2001). 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 remain 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 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 suggests 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.

1.2 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 (Brawner III et al., 2000; Houde and Torio, 1992; Milinski and Bakker, 1990; Thompson et al., 1997). 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 (Alonso-Alvarez et al., 2004; Lozano, 1994; McGraw et al., 2010; Moller et al., 2000; von Schantz et al., 1999), 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; Simons et al., 2012; Svensson and Wong, 2011). In house finches, increased fecundity and increased parental care has been positively associated with redder carotenoid-based coloration in male birds (Badyaev and Hill, 2002; Hill, 2002; McGraw et al., 2001).

These pervasive connections between aspects of individual condition and carotenoid-based coloration gives 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 are 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; Powers et al., 2020c). 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.

1.2.1 Traditional hypotheses of carotenoid signaling lack a clear mechanism

For carotenoid pigmentation to honestly signal information about other traits, it must be un-cheatable. In other words, carotenoids would not be good signals of individual condition in systems where any individual could artificially increase its carotenoid concentration. 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; Koch et al., 2019), and questioning whether carotenoids are truly limited in the wild (Hadfield and Owens, 2006; Hudon, 1994; Moller et al., 2000). 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.

1.2.2 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 section 2.2). Many dietary carotenoids are yellow in color; but bioconverted carotenoids are often orange or red (Hill and McGraw, 2006b; LaFountain et al., 2015). Importantly, the metabolism of carotenoids strengthens the connection between coloration and individual quality (Weaver et al., 2018b) compared to organisms that directly deposit dietary carotenoids into their tissues to produce coloration. Many organisms described in the studies listed in section 1.1 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 (Biernaskie et al., 2014; Hill and Johnson, 2012a; 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 (Figure 1.1). 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; Hill, 2014). Thus, the SPH places the efficiency of mitochondrial function at the core of what ketocarotenoids signal about individual condition and places less emphasis on tradeoffs created by a finite pool of carotenoid resources (Figure 1.2). 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).

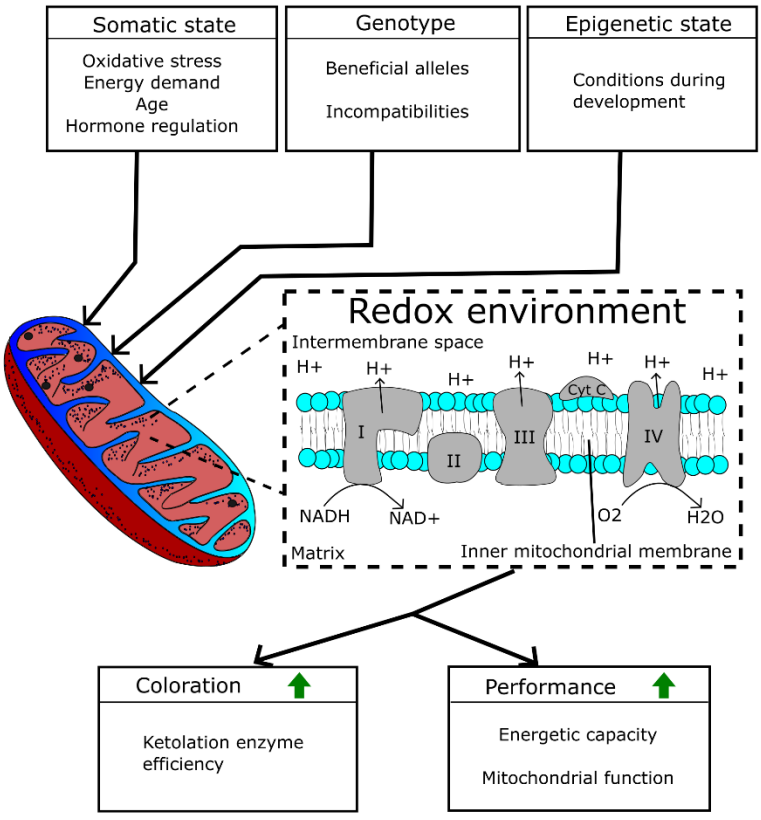

Figure 1.1 The relationship between carotenoid coloration and mitochondrial function underlying the integrative nature of carotenoid signaling proposed by the SPH. Adapted from Hill (2011); Hill (2014)

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 (Figure 1.1) (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.

1.2.3 Measuring mitochondrial function under the framework of the Shared-Pathway Hypothesis

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 results. Mitochondrial function (or dysfunction) is no longer best defined by mean energy production; ATP production

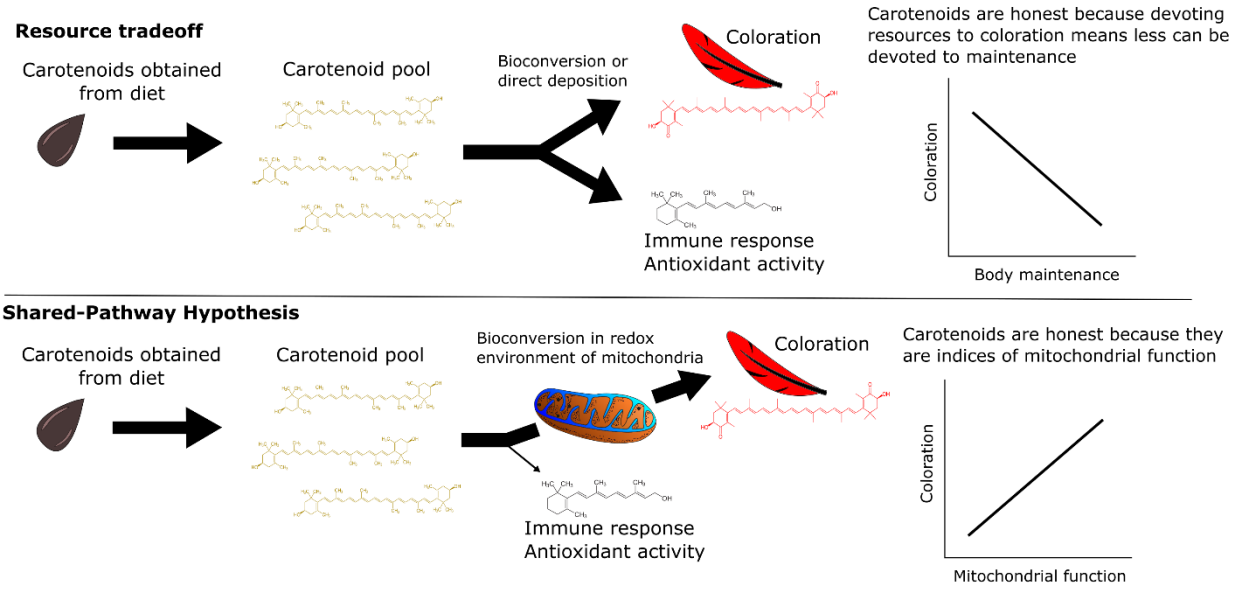

Figure 1.2 A diagram showing a simplified overview of the differences between the SPH and carotenoid resource tradeoff hypotheses. Both sets of hypotheses involve many of the same components, but the mechanism for maintaining the honesty of carotenoid coloration is different. The SPH does not ignore 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.

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, 2012a). 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 (Figures 1.1, 1.2).

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 (Petrick and Holloway, 2021; Salin et al., 2015), 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 (Barreto and Burton, 2013; Metcalfe and Alonso-Alvarez, 2010). 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 (Hill et al., 2019b; Larsen et al., 2012). 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 (Canelas et al., 2008; Hanson et al., 2004).

1.2.4 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 (Figure 1.3), a member of the cytochrome P450

superfamily of oxygenases (Omura, 1999). CYP2J19 has been implicated as the carotenoid ketolase in birds and turtles (Khalil et al., 2020; Kirschel et al., 2020; Lopes et al., 2016; Mundy et al., 2016; Twyman et al., 2018a; Twyman et al., 2018b; Twyman et al., 2016). 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 section 1.1, 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

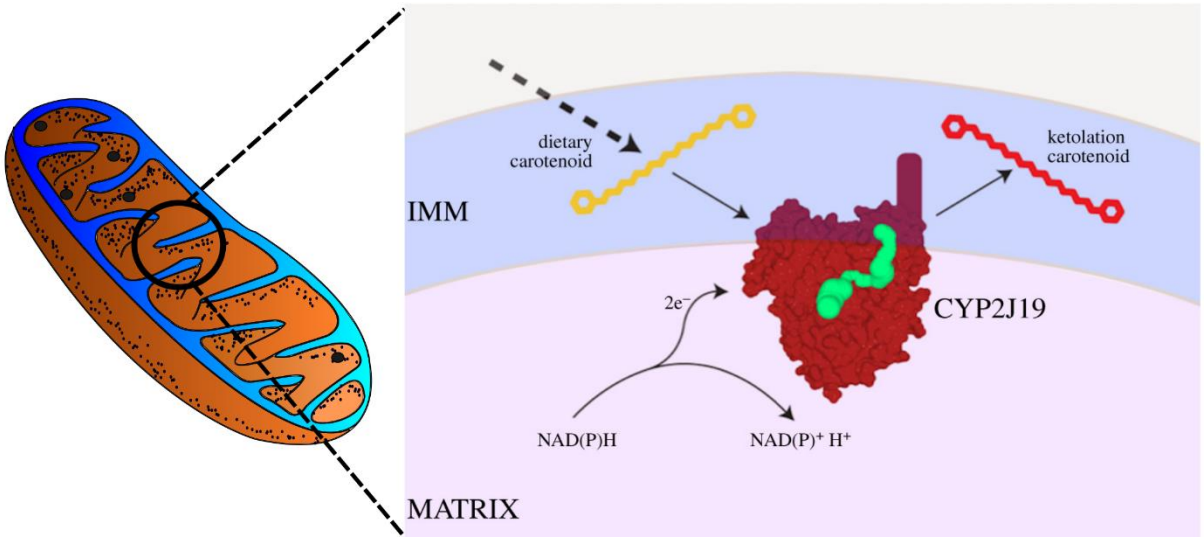

Figure 1.3 The predicted structure, mechanism, and location of the avian carotenoid ketolase enzyme, CYP2J19. The right panel showing the hypothesized structure of CYP2J19 is reproduced from Hill et al. (2019b)

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).

1.2.5 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, 2012a).

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 (LaFountain et al., 2015; McGraw et al., 2006). 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 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 electron transport system (ETS) is regulated by the same types of electron donors as those proposed to be used by carotenoid ketolation enzymes (Hill, 2014; Hill et al., 2019b; Karpac and Jasper, 2013). 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 (Cantarero et al., 2020b; Hill and Johnson, 2012a; Johnson and Hill, 2013). 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.

1.3 Evaluating the Shared-Pathway Hypothesis

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.

1.3.1 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; del Val et al., 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; Figure 1.3), or in the mitochondrial associated membranes (MAM) of the endoplasmic reticulum (ER) (Hill et al., 2019b; Johnson and Hill, 2013). 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 respiratory control ratio, 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 signals the capacity of their mitochondria to withstand stress and effectively respond to increased demand of energy.

1.3.2 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* x *C. a. 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 section 1.2, researchers have long suspected a connection between carotenoid-based coloration and testosterone (Blas et al., 2006; Peters, 2007; Stoehr and Hill, 2001). 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 in unknown, and because this result contrasts with previous hypotheses about a potential tradeoff between testosterone levels and carotenoid-based coloration (Alonso-Alvarez et al., 2007; Peters, 2007; von Schantz et al., 1999). 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 has 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).

1.3.3 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 section 3.2), 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 III et al., 2000).

1.4 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 taxa non-vertebrate taxa. However, our lab 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 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.

1.4.1 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; Hill et al., 2019a; Pereira et al., 2016). This dysfunction results in an increase in mitochondrial oxidative damage, and a decrease in complex activity and energy production of the mitochondria of hybrids (Barreto and Burton, 2013; Ellison and Burton, 2006; Ellison and Burton, 2008; Ellison and Burton, 2010). 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 lab 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 supplemental documents).

1.4.2 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♀ x BB♂ and BB♀ x SD♂). We found a statistically significant positive relationship between oxygen consumption and astaxanthin bioconversion in hybrid copepods (Figure 1.4). This result was observed in hybrid copepods from both cross directions.

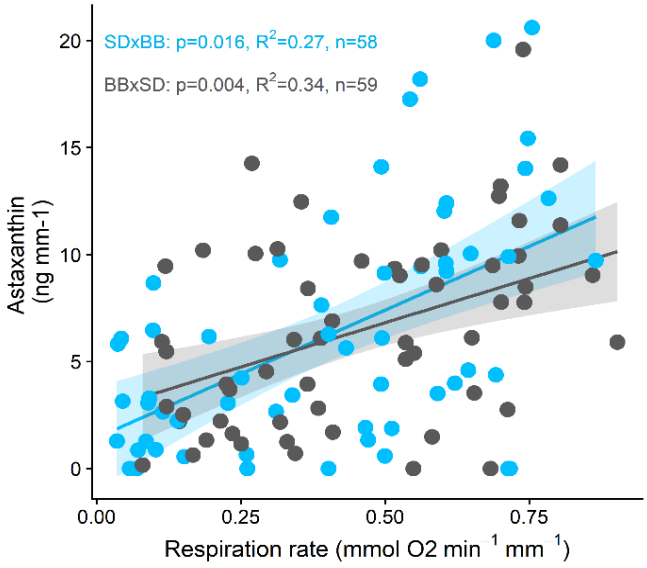

Figure 1.4 The relationship between respiration rate and astaxanthin in hybrids. Blue and black dots represent individual copepods. Lines are the estimated effect of increasing respiration on astaxanthin. The shading behind the line is the 95% confidence interval around this estimate effect. The results of a mixed effects linear model with oxygen plate as a random effect are shown in the top left. R2 values are adjusted for terms in the model.

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; Petrick and Holloway, 2021; Salin et al., 2015). 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 (Figure 1.4).

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 non-stressful 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.

1.4.3 Methods for the above experiment

Copepods were collected from San Diego (SD; 32°44'49.67"N, 117°15'13.34"W) and Bodega Bay (BB; 38°19'59.70"N, 123° 2'53.01"W) and cultured in a laboratory setting. Cultures were fed ground nutritional yeast (Bragg, Santa Barba, CA). All copepod cultures were maintained in 35 psu artificial saltwater in a climate controlled incubator set to 20°C with a 12h day:night cycle. The reciprocal cross between SD and BB was made by taking 15 males from SD and 15 virgin females from BB (and vice versa); these were paired together in a petri dish in triplicate. F1 males and females were mated to individuals from a different replicate dish to avoid sibling matings. F2 hybrid copepods were then subjected to carotenoid and metabolic rate measurements (see below).

Astaxanthin biosynthesis was assayed using a single dietary carotenoid, zeaxanthin. Carotenoid-deficient copepods were isolated in a 24-well plate and provided zeaxanthin (20 µg mL^{-1}) for a period of 48 hours. After the 48-hour period, copepods were isolated in clean artificial saltwater for 2 hours so they could empty their gut contents of remaining dietary carotenoids before being added to a microcentrifuge tube for storage at -80° C until carotenoid extraction. We chose to use zeaxanthin as the solitary precursor in order to measure the ketolation rate of a single dietary carotenoid in conjunction with metabolic rate. Zeaxanthin is a hydroxylated precursor to astaxanthin; copepods fed this dietary carotenoid produced high levels of the ketocarotenoid astaxanthin compared to individuals fed lutein, canthaxanthin and *β*-carotene (Weaver et al., 2018a). However, another study reported the ketolase enzymes in *T. californicus* preferentially metabolize non-hydroxylated carotenoids, such as *β*-carotene (Prado-Cabrero et al., 2020). It is possible that copepod ketolase enzymes exhibit a high degree of flexibility in the type of carotenoid precursor they utilize (Prado-Cabrero et al., 2020).

Carotenoids were extracted from copepod tissues using acetone with sonication and centrifugation to remove cellular debris. Final carotenoid extract was resuspended in 40 µL acetone for HPLC analysis. We injected 10 µL of suspended carotenoid extract on to a Sonoma C18 column (10 µm, 250 x 4.6 mm, ES Technologies, New Jersey, USA) fitted with a C18 guard cartridge using a Shimadzu Prominence HPLC system. We used a flow rate of 1mL/min using mobile phases adapted from (Wright et al., 1991) described briefly here. The mobile phases were: A) 80:20 methanol: 0.5M ammonium acetate; B) 90:10 acetonitrile: H2O; and C) ethyl acetate. We used a tertiary linear gradient that consisted of 100% A to 100% B over 4 min, then 80% C: 20% B over 14 minutes, then 100% B over 3 minutes, ending with 100% A over 11 minutes. We visualized and detected carotenoid absorbance using a Prominence UV/Vis detector set to 450 nm. We identified and quantified carotenoids by comparison to calibration curves of authentic standards that included: astaxanthin, zeaxanthin, β-carotene, lutein, and canthaxanthin. We normalized carotenoid concentration by the size of each copepod (reported as µg carotenoid per mm copepod length).

We measured oxygen consumption using a calibrated Microplate sensor system (Loligo Systems, Copenhagen, Denmark). Prior to adding copepods to the wells of the microplate, we soaked the microplate sensors in artificial saltwater for 30 minutes in order to equilibrate them. After this period, we added individual copepods to 80 µL of 35 psu artificial saltwater in a single well of the microplate and sealed the wells using a gel seal and weighted block. Copepods were left undisturbed in the wells for 30 minutes prior to recording oxygen consumption. We recorded oxygen consumption as mmol O_2 consumed min^{-1} per mm of body length over 30 minutes after an initial burn-in period of 5 minutes. During measurement, temperature was maintained at precisely 23°C. Final oxygen consumption was calculated as mmol oxygen consumed min^{-1} per

mm of copepod body length. Copepod body length was measured before oxygen consumption was measured by photographing copepods on a scaled glass calibration slide.

To analyze the relationship between astaxanthin production and oxygen consumption in individual copepods, we used a linear model that included the oxygen microplate as a random effect. Even though the sensor plate was calibrated between each run, this random effect was still included in the statistical analyses to account for variation in the microsensor values among data collection runs. All statistical analyses were performed in R (R_Core_Team, 2019) using the 'lme4' package (Bates et al., 2014). Figures were produced using the 'ggpubr' package (Kassambara, 2018).

1.4.4 Data Availability

Presented data and R code for analysis can be found at Integrative & Comparative Biology online (https://doi.org/10.1093/icb/icab056).

1.5 The next steps for evaluating the Shared-Pathway Hypothesis

The studies and results presented in section 3 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 section 3 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.

1.5.1 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 section 3.2). 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.

1.5.2 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 (Barreto et al., 2018; Lima et al., 2019; Pereira et al., 2016). 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.

1.5.3 In vitro methods for testing the Shared-Pathway Hypothesis

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; del Val et al., 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 inner mitochondrial membrane nor any mitochondrial associated membranes. 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).

1.6 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. (2020)), 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.

Chapter 2

An experimental test of mate choice for red carotenoid coloration in the marine copepod *Tigriopus californicus*

Manuscript published in *Ethology (Powers et al., 2020a)*

2.1 Abstract

Colorful ornaments are hypothesized to have evolved in response to sexual selection for honest signals of individual quality that provide information about potential mates. Red carotenoid coloration is common in diverse groups, and in some vertebrate taxa red coloration is a sexually selected trait whereby mates with the reddest ornaments are preferred**.** Despite being widespread among invertebrates, whether red carotenoid coloration is assessed during mate choice in these taxa is unclear. The marine copepod *Tigriopus californicus* displays red coloration from the accumulation of the carotenoid astaxanthin. Previous research on copepods has shown that astaxanthin provides protection from UV-radiation and xenobiotic exposure and that carotenoid production is sensitive to external stressors. Because of the condition-dependency of the red coloration, we hypothesized that *Tigriopus* would use it as a criterion during mate choice. To test this hypothesis, we conducted trials in which males chose between females that were wild-type red (carotenoid-rich algae diet) or white (carotenoid-deficient yeast diet). To control for dietary differences and to isolate the effect of carotenoid coloration, we also presented males with restored-red females fed a carotenoid-supplemented yeast diet. We found that wild-type red females were weakly preferred over white females. After controlling for diet, however, we found that restored-red females were avoided. Our observations do not support the hypothesis that male copepods prefer the carotenoid coloration of females during mate choice. We hypothesize that

algal-derived compounds other than carotenoids play a role in mate choice. Red coloration in copepods appears to be a condition dependent trait that is not assessed during mating.

2.2 Introduction

The indicator model of sexual selection proposes that ornamental traits function as reliable signals of the quality or condition of potential mates (Andersson and Iwasa, 1996; Weaver et al., 2017). One of the best studied condition-dependent ornaments is the vibrant yellow to red coloration produced through the deposition of carotenoid pigments in body tissues. It is hypothesized that animals in good condition tend to display more richly-colored ornaments than animals in poor condition (Svensson and Wong, 2011). One possible explanation for the link between carotenoid coloration and quality is that animals cannot synthesize carotenoids de novo and must rely on ingested precursors for color displays (Hill, 2002). In taxa that consume photosynthetic organisms directly or that consume herbivorous prey, the majority of dietary carotenoids are yellow in color (Hill and McGraw, 2006a). Once ingested, carotenoids can either be deposited to tissues unmodified, or they can be oxidatively metabolized to produce red ketocarotenoids (K. J. McGraw, 2006; Weaver, Santos, Tucker, Wilson, & Hill, 2018). The association between individual condition and red carotenoid coloration is hypothesized to arise either through internal tradeoffs between the production of red color and the use of carotenoids to aid homeostasis (Alonso-Alvarez et al., 2004; Koch & Hill, 2018) or through pathways shared by pigmentation and vital cellular processes related to energy production (Hill et al., 2019).

Evidence for mate choice for carotenoid-ornamented traits has been demonstrated in a variety of vertebrate animals. For example, male two-spotted gobies prefer females with more red carotenoids (Amundsen and Forsgren, 2001), male spotted pipefish prefer females with more blue

carotenoids (Berglund et al., 1986; Czeczuga, 1980), female guppies (Houde, 2019; Kodric-Brown, 1989) and sticklebacks (Milinski and Bakker, 1990) prefer males with more red carotenoids, female chuckwalla lizards prefer males with redder carotenoid coloration (Kwiatkowski and Sullivan, 2002), and female house finches prefer males with more red carotenoids in their feathers (Hill, 1991; Hill, 2006). In these species, mating preference for red coloration may have evolved because carotenoid coloration serves as a reliable signal of condition (Endler, 1980; Hill, 1991; Svensson and Wong, 2011) such that preference for redder mates selects for increased reproductive fitness (Candolin, 2000; McGraw and Ardia, 2003). In these cases, individuals select for mates with a greater concentration of ornamental carotenoids. Examples of sexual selection for carotenoid-based coloration in invertebrates are rare (but see: Baldwin and Johnsen (2009a); Detto (2007)) despite the wide taxonomic distribution of this trait, particularly in crustaceans (Maoka, 2011).

The marine copepod *Tigriopus californicus* (Figure 2.1) has striking coloration that comes from the bioconversion of yellow dietary carotenoids to the red ketocarotenoid astaxanthin (Weaver et al., 2018a). In the wild, *Tigriopus* copepods acquire dietary carotenoids, such as zeaxanthin, needed to make astaxanthin from a grazing diet that is rich in photosynthetic micro-algae (Powlik et al., 1997). In copepods, astaxanthin accumulation has been shown to protect against damage from UV-radiation (Caramujo et al., 2012; Davenport et al., 2004; Weaver et al., 2018c). In these studies, copepods with more astaxanthin had improved survival when exposed to short wavelength UV-radiation. Moreover, copepods exposed to UV-radiation lost between 77% to 92% of their astaxanthin coloration during treatment, in contrast to unexposed controls (Caramujo et al., 2012). Copepods, including *T. californicus*, also lose red astaxanthin coloration when exposed to toxic concentrations of metals, such as copper (Caramujo et al., 2012; Weaver et

al., 2016), suggesting that carotenoid accumulation and coloration is correlated with the health or condition of the animal. In vertebrates, the benefits of information about the condition of prospective mates is hypothesized to maintain mating preferences for carotenoid coloration which in turn maintain the color displays (Hill, 2006; Svensson and Wong, 2011). However, evidence that copepods assess mates based on carotenoid coloration is limited.

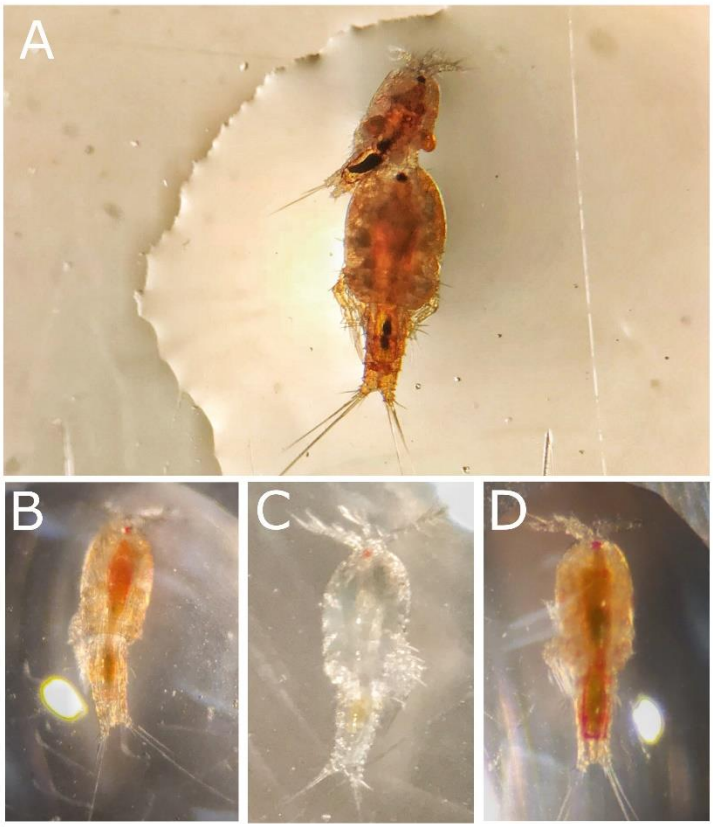

Figure 2.1 *Tigriopus californicus* under a dissecting scope. A) Adult male *T. californicus* clasping and guarding a stage IV female copepodite, B) wild-type red female fed a diet of mixed-algae, C) white female fed a diet of nutritional yeast deficient of carotenoid precursors, and D) restored-red female fed a diet of yeast and supplemented with 20 µg mL^{-1} zeaxanthin.

In the single empirical test for preference related to carotenoid coloration in *T. californicus* copepods, females were observed to have a slight but non-significant preference for red males (Palmer and Edmands, 2000). There was no control for chemical cues in these female preference trials and the authors also suggested this result may have been complicated by differences in mate body size. Palmer and Edmands (2000) also documented female avoidance of inbreeding. However, since the completion of this study, female choice in *T. californicus* has been documented only once more: (Tsuboko-Ishii and Burton, 2017) observed that already-mated females attempt to escape male clasping though increased physical effort.

In many animals where males mate multiple times and females only once, females are the more selective sex. In *T. californicus,* however, males appear to engage in more active mate choice than females because they coerce and clasp the individual that they choose. Although there is evidence that females try to escape unwanted male clasping attempts (Tsuboko-Ishii and Burton, 2017), in most cases females appear to have little choice but to mate with the male that successfully clasps them. Active choice by *Tigriopus* males makes sense because male investment in a single fertilization is large: they engage in precopulatory mate guarding by physically clasping immature females (Figure 2.1A) for up to two weeks or until females reach maturity. This period can last up to approximately 25% of the male's reproductive lifespan (Alexander et al., 2014; Burton, 1985). Indeed, the large investment for males in a single mating event is cited as a major driver for the evolution of selective behavior in response to a variety of mating criteria (Burton, 1985; Frey et al., 1998; Ting and Snell, 2003). Because *Tigriopus* males exhibit a variety of non-random mating behavior, and because male mate choice for astaxanthin coloration has been demonstrated in other marine taxa such as gobies (Amundsen and Forsgren, 2001), crabs (Baldwin and Johnsen, 2009a) and pipefish (Berglund et al., 1986; Czeczuga, 1980), we hypothesized that male *T. californicus* may also assess carotenoid content in females. To date, no test for male preference for red coloration in this species has been completed.

While there have been functional tests of vision in *Tigriopus* copepods, they have been mostly framed in the context of measuring responses to UV or polarized light (Martin et al., 2000; Porter et al., 2017). The *T. californicus* eyespot contains a layer of red pigment located between two sensory cells and next to a retroreflective layer used to concentrate low levels of light (Martin et al., 2000). The function of this pigment has not yet been determined; however, the pigmented layer in the eye of marine animals may serve to enhance discrimination of UV and red light and

may aid pooling of vitamin A for vision (Chiou et al., 2012; Price et al., 2008). Despite the physiology mentioned above, whether *T. californicus* can or cannot detect differences between colors has not been tested, even though other copepod species have been documented to rely on visual cues during mating (Land, 1988). Because *T. californicus* vision may be limited compared to higher taxa, they may only be able to detect large differences in coloration between potential mates.

In this study, we tested whether carotenoid-based coloration is a criterion for mating in *Tigriopus californicus* by conducting male mate-choice experiments using females fed either a wild-type algal diet, a carotenoid-deficient nutritional yeast diet, or a carotenoid-restored nutritional yeast diet. These experimental diets produced a red carotenoid-rich coloration (wild type algal and carotenoid-restored) and a white, carotenoid-deficient coloration (nutritional yeast). We used white copepods in comparison to copepods with red coloration as a coarse-level test to see if color appeared to be a criterion in copepod mate choice. Furthermore, we fed yeast copepods a carotenoid-restored diet to isolate the effect of carotenoid color from other dietary differences in the yeast and algae diets. A similar all-or-none experimental manipulation of ornamentation was used in the initial experiments to test whether birds respond behaviorally to red feather coloration (Peek, 1972; Smith, 1972) or to ultraviolet coloration (Bennett et al., 1996), and these experiments formed the foundations for numerous subsequent studies. As was argued in the foundational bird studies, if *Tigriopus* copepods do not discriminate between two very different color phenotypes, then we would not predict that they would discriminate based on finer color differences as is observed in vertebrates (Svensson and Wong, 2011). We predicted that if red carotenoid coloration is used during mate choice, males would clasp red females more than white females in both wild type-red versus white and restored-red versus white mating trials.

2.3 Materials and Methods

2.3.1 Animal husbandry and generation of color groups

Our lab has maintained large panmictic populations of *Tigriopus californicus* since 2014 under the following conditions: 35 psu artificial seawater at approximately 23 ºC on a 12:12h light:dark cycle and fed live microalgae (*Isochrysis galbana* and *Tetraselmis chuii*). We refer to copepods raised under these conditions as 'wild-type red' (Figure 2.1B).

Because carotenoids must be acquired through the diet, feeding *Tigriopus* copepods a carotenoid-free diet of nutritional yeast (Bragg, Santa Barbara, CA, USA) results in copepods that are colorless and contain only minute amounts of astaxanthin in their bodies (Figure 2.1C) (Weaver et al., 2018a). To produce white copepods, we switched a subset of the wild-type red population to a nutritional yeast-only diet for several generations prior to the experiments to ensure cultures were clear of carotenoid precursors (Weaver et al., 2018c). Nutritional yeast lacks the precursors necessary to produce astaxanthin. We refer to these copepods as 'white' copepods (Figure 2.1C). To isolate the effect of carotenoid coloration and control for dietary differences between algae and yeast-fed copepods, we fed a subset of copepods a nutritional yeast diet supplemented with zeaxanthin - an astaxanthin precursor (Weaver et al., 2018a), at a concentration of 20 µg mL^{-1}. We refer to these copepods as 'restored-red copepods' (Figure 2.1D). In summary, our experimental design generated three phenotypes: wild-type red, white, and restored-red (Figure 2.1).

2.3.2 Obtaining virgin females

Adult male *Tigriopus* perform precopulatory mate-guarding by clasping immature females with their first antennae, forming a 'clasped pair' until she molts into the adult stage. To obtain virgin

females, we first isolated clasped pairs from the populations, then carefully separated the virgin female from the male using fine needles under a dissecting scope. We kept separated females in isolation for 1 day before trials to allow for recovery and only free-swimming males were used in trials. We took pictures of each female under a dissecting scope on a calibrated slide (Figure S2.1) and measured body length and width from the images using Fiji (Schindelin et al., 2012). We used these measurements to size-match red and white females to control for preferences due to developmental stage.

2.3.3 Mitigation of chemosensory signals via trypsin treatment

Previous work has shown that male *Tigriopus* copepods clasp the largest, most developmentally advanced virgin females over smaller, less developed females (Burton, 1985). Male copepods detect differences in female development through chemical cues that are likely amino-acid based pheromones (Kelly et al., 1998), or surface-bound glycoproteins (Ting et al., 2000; Ting and Snell, 2003). Treatment with the protease trypsin to remove these glycoproteins results in highly reduced male discrimination for developmental stage (Ting et al., 2000; Ting & Snell, 2003). To control for the known preference for developmental stage/size, we treated subsets of female copepods in our red versus white choice trials with trypsin (625 U in artificial seawater) (Figure 2.2). After 2h of trypsin exposure we rinsed individuals three times with artificial seawater and conducted the mate choice trials.

To confirm that trypsin could remove chemical signals responsible for male preference for developmentally advanced females, we conducted separate choice trials ($n = 80$) in which a male was presented with a small, early development wild-type red virgin female or a large, late-development wild-type red virgin female (Figure 2.2). In approximately half of the trials ($n = 41$)

both females were treated with trypsin, and in the remainder of the trials (n = 39) both females were untreated.

2.3.4 Color choice trials procedure

In every choice trial (Figure 2.2), one male was placed in single 3.4 mL well of a 24-well plate in 600 µL artificial seawater with one red female and one white female for 2 hours. Sample sizes for each experiment group are listed in Figure 2.2. We determined male choice by recording, at regular intervals over 2 hours, which female was clasped. We did not record full copulations. The guarding behavior associated with clasping has frequently been used as an acceptable correlate of mate choice in copepods (Snell, 2010; Titelman et al., 2007). Our trials only lasted for 2 hours, and males can clasp females for up to 2 weeks before copulation occurs (Burton, 1985) making sperm transfer a difficult measure to quantify choice in this experiment. Each 2-hour trial consisted a male choosing between one white female and either a wild-type red or a restored red female (Figure 2.2). Females were kept individually in labelled wells of a 24-well plate for approximately two hours before the start of mating trials.

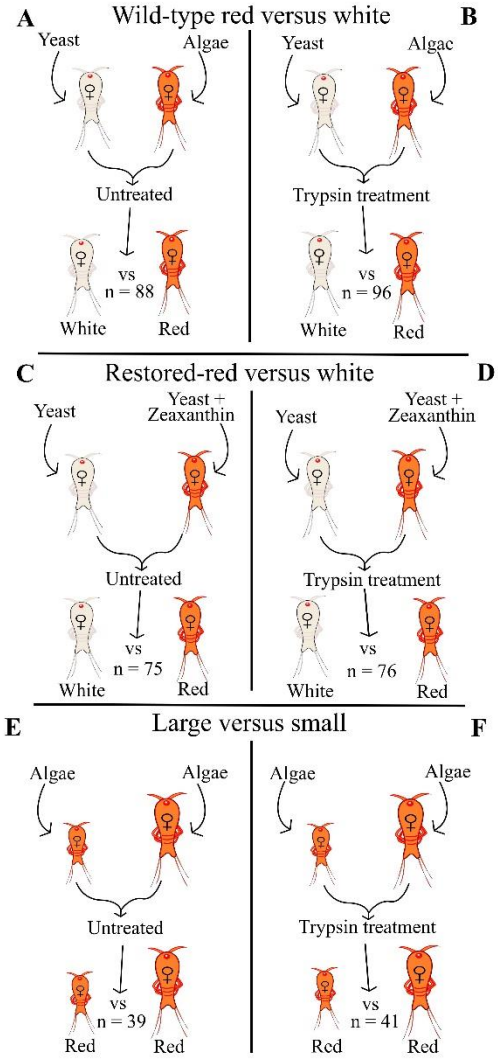

Figure 2.2 A summary of the experimental manipulations, feeding regimes, and comparisons made in this study. A) Wild-type red vs. white females untreated with trypsin, B) wild-type red vs. white females treated with trypsin, C) restored-red vs. white females untreated with trypsin, D) restored-red vs. white females treated with trypsin, E) large vs. small females untreated with trypsin, and F) large vs. small females treated with trypsin. In E and F, both large and small females were colored red from a diet of natural mixed algae.

To begin each trial, we first placed red and white females in a single well of a clean 24-well plate filled with 600 µL of fresh artificial seawater. Once both females were in the well, the male was added, and the trial was started. We recorded which female, if any, the male had clasped every 5 minutes until the 30th minute, after which we recorded two more observations at the 1-hour and 2-hour marks. We recorded which female the male clasped first during this 2-hour time period. If a male still had not clasped a female by the end of the 2-hour observation window, we recorded this as "no choice". The majority of clasping events occurred within the first 10 minutes (Figure S2.2) as previously observed in *Tigriopus* species during mate choice experiments (Kelly et al., 1998). At the end of each mating trial, both the male and two females were placed in stock laboratory cultures not used for further mating trials.

2.3.5 Statistical analyses and presentation of results

We tested for male preference for female coloration and size using a logistic regression model using the 'multinom' function from the 'nnet' package v7.3.12 (Venables and Ripley, 2013). This model estimates the likelihood of a male clasping a red female, a white female, or neither. We report the results as the mean probability and 95% confidence interval of a male clasping one female over another, called the relative risk (Andrade, 2015). A relative risk with confidence intervals that do not include 1 are equivalent to the p-value being < 0.05. All statistical analyses were performed in R v3.6.1 (R_Core_Team, 2019) and graphed using the package 'ggpubr' v0.2.1 (Kassambara, 2018) and 'cowplot' (Wilke, 2016).

2.3.6 Data Availability

Presented data and R code for analysis can be found at Ethology online (**https://doi.org/10.1111/eth.12976**).

2.4 Results

2.4.1 The influence of trypsin on male preference for large or small females

We tested male preference for large or small females that had or had not been treated with trypsin (Figure 2.2). When females were untreated (n = 39 trials), males were 2.89 (95% CI: 1.35 – 6.17) times as likely to clasp large females than small females and this was statistically significant (Figure 2.3). When females were treated with trypsin (n = 41 trials), males were only 1.14 (95% CI: 0.56 – 2.34) times as likely to choose large over small females, but this was not statistically significant (Figure 2.3).

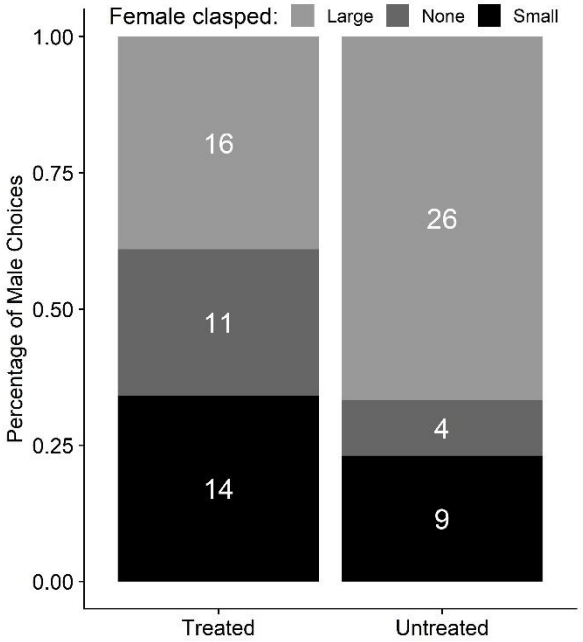

Figure 2.3 Results from size choice trials. Bars add up to 100% and individual segment sizes correspond with the percent of males that clasped each type of female. Numbers inside each bar are raw count values for the number of males that made each choice. Males significantly prefer larger, developmentally mature females over females of earlier stages in normal mating situations (untreated group). When treated with 625U trypsin, stage-specific glycoproteins are removed from the carapace of large and small females. Therefore, males cannot distinguish between developmental stages and have no significant preference for large or small females (trypsin-treated group).

2.4.2 The effect of color on male choice for wild-type red or white females

When females were untreated (n = 88 trials) (Figure 2.2A), males were 1.48 (95% CI: 0.93 – 2.38) times as likely to clasp wild-type red females over white females, but this result was not significant (Figure 4). When males were presented with trypsin-treated females (n = 96 trials)

(Figure 2.2B), males were 1.64 (95% CI: 0.99 – 2.69) times as likely to clasp wild-type red over white females, but this result was also not significant (Figure 4).

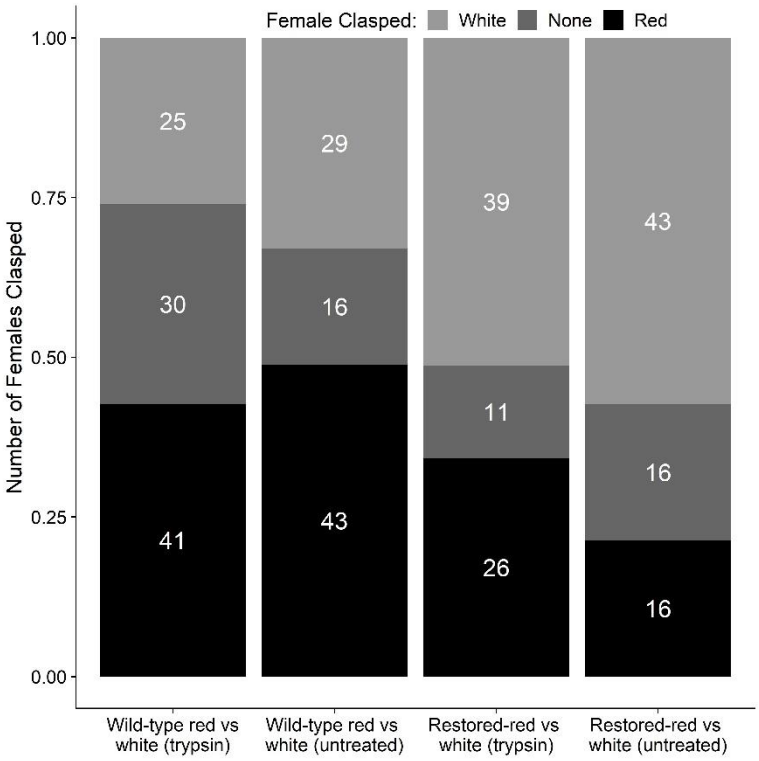

Figure 2.4 Results from mating trials testing male preference for wild-type red versus white females, and male preference for restored-red females versus white females. Both experiments were completed with untreated females and females washed in trypsin to remove surface-bound glycoproteins. Bars add up to 100% and individual segment sizes correspond with the percent of males that clasped each type of female. Numbers inside each bar are raw count values for the number of males that made each choice. Significantly fewer males clasped restored-red females over white females when females were not treated with trypsin.

2.4.3 The effect of color on male choice for restored-red or white females

When females were untreated (n = 75 trials) (Figure 2.2C), males were only 0.37 (95% CI: 0.21 – 0.66) times as likely (63% less likely) to clasp restored-red females over white females and this result was statistically significant (Figure 4). When females were treated with trypsin (n = 76 trials) (Figure 2.2D), males were 0.67 (95% CI: 0.41 – 1.10) times as likely (33% less likely) to clasp restored-red females over white females, but this result was not significant (Figure 4).

2.5 Discussion

Through a series of experiments in which males were given a choice of females that were either red or white, we found little to no support for the hypothesis that male *T. californicus* use

redness as a criterion in mate choice. We found that male copepods clasped wild-type red females more frequently than white females, but not at a rate that differed significantly from a random pattern. The strength of preference observed in these trials was similar to that observed by Palmer and Edmands (2000) when they tested female *Tigriopus* preference for red and white males. In our study, the result was the same whether females were treated with trypsin or not, but the trend toward preference for wild-type red females was stronger in the trypsin-treated group. We may have observed this increased attraction to red females because without chemical cues, wild-type red females stood out more since they were darker. However, an increased reliance on sight would not explain why trypsin treatment removed male preference for larger females in our size trials, since it would be predicted that males would still be able to visually distinguish between large and small mates. Based on these results, *T. californicus* eyespots may be better at detecting differences between light and dark, than resolving shape and size. This is certainly supported by the fact that *T. californicus* can distinguish between water in shade and bright light (Martin et al., 2000), and that other copepods species must use mechanical sensation to determine mate size in the absence of chemical cues (Ceballos and Kiorboe, 2010).

We isolated the effect of red coloration by supplementing carotenoid-deficient females with a single precursor carotenoid used to make the red carotenoid astaxanthin. In these red-restored trials, males not only failed to show a preference for red over white females, they showed a significant preference for white females. This strong pattern of choice is difficult to reconcile with the hypothesis that male copepods use red coloration as a positive criterion in mate choice. Moreover, this lack of choice for red when we controlled for the gross dietary differences that were present in the yeast versus algae-fed trials suggests that the apparent preference for red in those trials was an artefact of preference for algae-related compounds. Compounds derived from an algal

diet may produce a more desirable chemical signal during mate choice in *Tigriopus* copepods than carotenoid coloration.

Conversely, aqueous supplementation of powdered zeaxanthin to produce restored-red individuals may have masked or changed the chemical signal of restored-red females so that males preferred to clasp white females instead (Figure 4). It is possible for dietary carotenoids to associate with glycoproteins (Teng et al., 2016; Zsila et al., 2005; Zsila et al., 2006). This hypothesis is speculative, and we have no data to support the idea that zeaxanthin may interrupt *T. californicus* chemical cues. It is very rare to see a white *T. californicus* copepod in the wild. In other species, such as the freshwater copepod *Acanthodiaptomus denticornis*, certain populations exposed to high predation levels have lost red astaxanthin coloration and have become white (Sereda et al., 2016). In these populations, white individuals may be preferred over red if they are better able to avoid detection by predators when forming clasped pairs. This situation is unlikely to be the case in *T. californicus*, who limit their exposure to predation by inhabiting elevated tide pools that are more difficult for predators to reach (Dethier, 1980). Moreover, high UV-exposure in these tidepools would likely select against any white phenotype.

Copepods produce red integumentary coloration using the same carotenoid precursors and following the same conversion pathways as many vertebrates (Hill and McGraw, 2006b; Weaver et al., 2018a). Also, as in vertebrates, red carotenoid coloration in copepods is sensitive to environmental perturbations. Copepods lose their red coloration after exposure to UV-radiation (Davenport et al., 2004; Weaver et al., 2018c), predation (Brüsin et al., 2016), and toxic metal exposure (Caramujo et al., 2012; Weaver et al., 2016). It is widely stated and experimentally confirmed that red coloration in copepods plays an important role in protecting them from photo-

oxidation (Caramujo et al., 2012; Hairston, 1976; Moeller et al., 2005; Weaver et al., 2018c), and this is in agreement with other marine invertebrates (Babin et al., 2010; Maoka, 2011).

Unlike in vertebrates, experimental evidence of sexual selection for carotenoid color in crustaceans is rare. This could be due to a lack of empirical studies on the use of visual cues during mating events (Elofsson, 2006). The role of carotenoids in crustacean mate choice and the ability of crustaceans to perceive carotenoid-based signals has been identified as an area of research that needs more attention (Baldwin and Johnsen, 2012). Moreover, current evidence for the role of carotenoid-based visual cues in crustacean mating is inconsistent. As a primary example, Baldwin and Johnsen (2009a) showed that blue crabs (*Callinectes sapidus*) prefer mates with claws displaying redder coloration; however, (Bushmann, 1999) showed no decrease in the tendency for blue crabs to mate when blindfolded. This could indicate that visual signals are only part of the information assessed by crustaceans and other signals, such as chemical cues**,** may be more important.

In contrast to the paucity of research on mate choice in crustaceans, numerous studies have investigated carotenoid pigmentation in relation to physiological functions. The dominant red carotenoid in *Tigriopus* copepods and other crustaceans, astaxanthin, has mostly been studied in the context of its roles as an antioxidant and in pigmentation, photoprotection, and production of provitamin A (Britton, 2008; Linan-Cabello et al., 2002). The association of carotenoids with improved growth, immune response, stress response, and disease resistance in crustaceans is well-established (Niu et al., 2009; Niu et al., 2014; Paibulkichakul et al., 2008; Supamattaya et al., 2005). There is also evidence that astaxanthin is important during crustacean reproductive events and gonadal development (reviewed by Linan-Cabello et al. (2002)). Astaxanthin accumulation has been shown to correlate with additive genetic effects and is likely heritable, as shown by a

single trait mixed model used to estimate heritability of shrimp astaxanthin coloration across a reconstructed pedigree (Nguyen et al., 2014). Genetic indicator models of sexually selected traits assume that there needs to be sufficient heritability of a trait for it become preferred (Andersson and Iwasa, 1996).

The apparent associations between astaxanthin, body condition, and improved physiological responses in crustaceans, coupled with the possibility that astaxanthin accumulation is a heritable genetic trait, begs further investigation into the role of astaxanthin coloration in crustacean behavior. Our results with *Tigriopus* copepods show that although astaxanthin accumulation is related to body condition, it has not been co-opted as a sexually selected trait as seen in birds and other vertebrates. However, our results are gathered from laboratory populations of *T. californicus* raised under optimal conditions exclusively.

Red coloration in *T. californicus* appears to be a striking, condition-dependent visual display that according to our study and results shown by Palmer and Edmands (2000) did not evolve as a signal used in mate assessment. It is interesting to speculate that condition dependency is an inherent property of carotenoids (Weaver et al., 2017) whether or not they are assessed in mate choice, and that the coevolution of condition dependency and sexual selection (Rowe and Houle, 1996) may be unnecessary in crustaceans. In order to evaluate this hypothesis, more empirical work in crustaceans testing the role of carotenoid coloration in sexual signaling is needed. Coupling knowledge on the physiological functions of carotenoids with inquiries on their potential roles in modifying behavior may prove valuable to understanding the evolution of condition-dependency of carotenoid-based ornaments.

2.6 Supplemental Figures

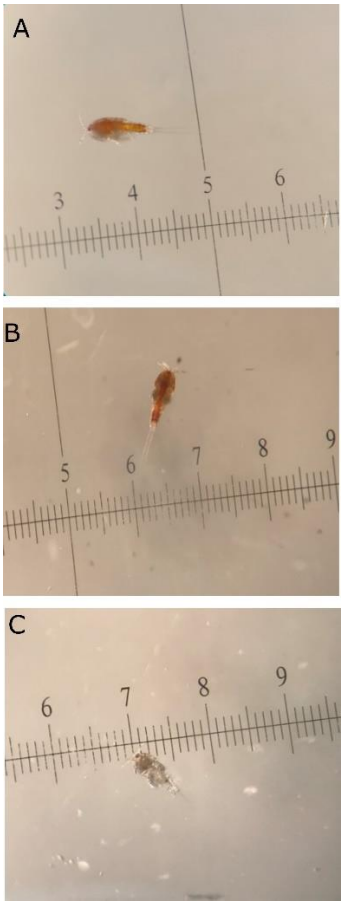

Figure S2.1 Female copepods on a glass calibration slide used to measure their body size when matching females by developmental stage. A) Wild-type red female, B) restored-red female, and C) white female. All measurements were taken using Fiji software (Schindelin et al., 2012). Briefly, we standardized each individual measurement to the millimeter increment scale seen in each picture. We measured copepod body size as a function of the length from eyespot to caudal ramus, and the lateral width from one side of the prosome to the other.

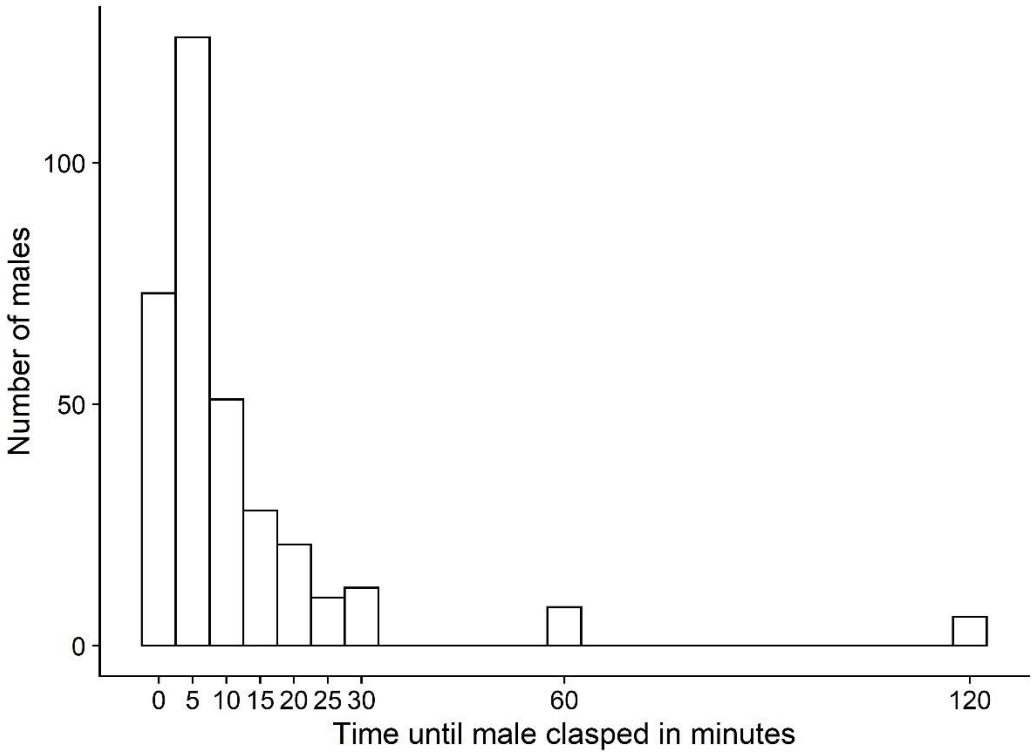

Figure S2.2 A histogram of the number of males that were observed to have clasped a female at a given time point. The majority of males clasped a female within the first 10 minutes of a mating trial. Males that did not clasp either female in the 2-hour trial were recorded as making "no choice".

Chapter 3

Evidence for hybrid breakdown in production of red carotenoids in the marine invertebrate *Tigriopus californicus*

Manuscript published in *PLOS ONE (Powers et al., 2021)*

3.1 Abstract

The marine copepod, *Tigriopus californicus*, produces the red carotenoid pigment astaxanthin from yellow dietary precursors. This 'bioconversion' of yellow carotenoids to red is hypothesized to be linked to individual condition, possibly through shared metabolic pathways with mitochondrial oxidative phosphorylation. Experimental inter-population crosses of lab-reared *T. californicus* typically produces low-fitness hybrids is due in large part to the disruption of coadapted sets nuclear and mitochondrial genes within the parental populations. These hybrid incompatibilities can increase variability in life history traits and energy production among hybrid lines. Here, we tested if production of astaxanthin was compromised in hybrid copepods and if it was linked to mitochondrial metabolism and offspring development. We observed no clear mitonuclear dysfunction in hybrids fed a limited, carotenoid-deficient diet of nutritional yeast. However, when yellow carotenoids were restored to their diet, hybrid lines produced less astaxanthin than parental lines. We observed that lines fed a yeast diet produced less ATP and had slower offspring development compared to lines fed a more complete diet of algae, suggesting the yeast-only diet may have obscured effects of mitonuclear dysfunction. Astaxanthin production was not significantly associated with development among lines fed a yeast diet but was negatively related to development in early generation hybrids fed an algal diet. In lines fed yeast, astaxanthin was negatively related to ATP synthesis, but in lines fed algae, the relationship was reversed. Although the effects of the yeast diet may have obscured evidence of

hybrid dysfunction, these results suggest that astaxanthin bioconversion may still be related to mitochondrial performance and reproductive success.

3.2 Introduction

Carotenoids pigments are widely distributed across diverse groups of organisms where they serve critical roles in physiological processes. Despite their utility, carotenoids are produced only by plants, fungi, algae, and bacteria. With a few notable exceptions(Oliver et al., 2010), animals lack the biochemical pathway necessary to synthesize carotenoids and therefore must obtain carotenoids from their diet to support key functions within their bodies and to produce colorful external displays (Svensson and Wong, 2011). Once ingested and absorbed, carotenoids may then be metabolized into new forms that perform distinct functions such as vitamin-A synthesis, pro-oxidant defense, and others (Hairston, 1976; Koch et al., 2018; Koch et al., 2016; Schneider et al., 2016; Stephensen, 2013; Von Lintig, 2010). Some animal taxa have co-opted carotenoids for their capacity to reflect yellow to red wavelengths of light and use them in colorful external displays (Svensson and Wong, 2011). The identity of carotenoids used by many animals for coloration have been identified using chromatography and spectrophotometric techniques. This foundational research has identified carotenoid pigments in the integuments of some animals that are not typically found in their diet, suggesting that they are metabolizing or 'bioconverting' carotenoids from their diet. For example, yellow dietary carotenoids, such as zeaxanthin or β-carotene, can be hydroxylated or ketolated into the red ketocarotenoid astaxanthin (LaFountain et al., 2015; Weaver et al., 2018a), and astaxanthin, in turn, may perform dual roles as a vibrant colorant and potent protector against oxidative stress, particularly in marine animals (Caramujo et al., 2012; Davenport et al., 2004; Maoka, 2011).

The observation that animals bioconvert dietary carotenoids to new forms used for coloration begs the question as to what genes are involved and what is the intracellular site of the metabolic pathways? Recently, *CYP2J19* was identified as the gene that encodes a putative β-carotene ketolase responsible for the bioconversion of yellow carotenoids to astaxanthin is birds and turtles (Mundy et al., 2016; Twyman et al., 2018a). Other taxa including frogs (Twomey et al., 2020), mites (Wybouw et al., 2019), and crustaceans (Weaver et al., 2020) have provided some candidate genes for the yellow to red carotenoid bioconversion, but more work is required. The exact intracellular arena for carotenoid bioconversion within tissues, however, has yet to be determined (del Val et al., 2009a; del Val et al., 2009b; Hill, 2014; McGraw, 2004; von Lintig et al., 2019). Recent hypotheses and molecular modeling place the cellular site of carotenoid bioconversion within the mitochondria or in association with mitochondrial associated membranes, implicating a shared metabolic pathway between ketocarotenoid conversion and cellular respiration (Hill, 2011; Hill, 2014; Hill et al., 2019b; Johnson and Hill, 2013).

The idea that carotenoid metabolism is linked to cellular respiration has been supported by observations in diverse species of birds. Ketocarotenoids were found in high concentrations in the liver mitochondria of house finches (*Haemorhous mexicanus*) (Ge et al., 2015). Within the mitochondria of this bird, ketocarotenoids were localized in the highest concentrations at the inner mitochondrial membrane (Hill et al., 2019b). Preliminary models predict CYP2J19 should localize either in or around the mitochondria (Cojocaru et al., 2007; Hill et al., 2019b; Šrejber et al., 2018). Moreover, the concentration of ketocarotenoids found in the feathers of house finches correlated strongly with the ability of their mitochondria to respond to changes in respiration and to withstand greater cumulative levels of mitochondrial stress (Hill et al., 2019b). Correlations between hormonal signaling, longevity, and pigmentation have also been observed in red-legged

partridges (*Alectoris rufa*), with mitochondrial function implicated as the underlying causative factor (Cantarero et al., 2019). Along with positive associations between mitochondrial function and production of red pigments, experiments with zebra finches (Cantarero and Alonso-Alvarez, 2017) and red crossbills (Cantarero et al., 2020b) revealed an effect on ketocarotenoid bioconversion by redox-active compounds targeted to the inner mitochondrial membrane. However, to better understand the link between mitochondrial respiration and carotenoid ketolation, observations in taxa outside of Aves are needed.

Bioconversion and accumulation of ketocarotenoids is a prominent feature of many crustaceans. In this often-colorful group, red and blue pigmentation is frequently produced by the accumulation of the ketocarotenoid astaxanthin, along with other ketocarotenoids (Maoka, 2011). Notably, the precursors and products in ketocarotenoid pathways in many crustaceans are the same as those in birds, and the oxidizing enzymes involved in these crustacean pathways are predicted to be functionally similar to the enzymes employed by avian species (Mojib et al., 2014; Prado-Cabrero et al., 2020; Weaver et al., 2020). One particular crustacean group, oceanic and lake-dwelling copepods, have frequently been utilized in carotenoid pigmentation studies due to their critical link in ecological food webs and their amenability to experimental manipulation (Raisuddin et al., 2007; Schneider et al., 2016; Sommaruga, 2010). Marine copepods from the genus *Tigriopus* have been particularly useful to investigate the condition-dependency of carotenoid pigmentation (Davenport et al., 2004; Weaver et al., 2016) and the dynamics or pathways of carotenoid bioconversion (Prado-Cabrero et al., 2020; Weaver et al., 2018a). Previous work has established that the major carotenoid accumulated in the tissues of *T. californicus* is free astaxanthin, compared to much lower concentrations of esterified astaxanthin and dietary carotenoid precursors (Prado-Cabrero et al., 2020; Weaver et al., 2018a). However,

Tigriopus californicus copepods are perhaps best known as a model for studies of mitonuclear coadaptation (Barreto et al., 2018; Burton et al., 2013a).

Interpopulation crosses of *T. californicus* show highly variable and often reduced mitochondrial function and reproductive fitness (Barreto and Burton, 2013; Burton et al., 2006; Ellison and Burton, 2006). These negative effects of hybridization have been traced to incompatibilities between the encoded products of one population's nuclear genome, which consists of 12 chromosomes (Barreto et al., 2018), and the products of the non-coadapted mitochondrial genome of the other population (Burton et al., 2013a; Ellison and Burton, 2008). Nuclear and mitochondrial genes within populations co-evolve to maintain key interactions between proteins that make up Complexes I, III, IV and V of the electron transport system (ETS) (Burton and Barreto, 2012; Willett and Burton, 2004). In *T. californicus*, hybridization between genetically divergent populations breaks up co-evolved combinations of nuclear and mitochondrial genes thanks to maternal inheritance of mt-DNA and sexual recombination of nuclear DNA. The end result in terms of mitochondrial performance and organismal fitness is variable in the second generation of hybrids and beyond (once hybrid offspring no longer retain at least one full copy of maternal genes) (Burton et al., 2006). Some hybrid lines show decreased function in all ETS protein complexes (with the exception of Complex II that is entirely nuclear encoded), increased oxidative stress, and reduced fecundity (Barreto and Burton, 2013; Burton, 1990; Ellison and Burton, 2006; Ellison and Burton, 2008; Harrison and Burton, 2006).

In this study, we utilized interpopulation hybrids of *Tigriopus californicus* that have previously shown mitochondrial dysfunction from mitonuclear incompatibilities (Barreto and Burton, 2013; Burton et al., 2013a; Ellison and Burton, 2006; Ellison and Burton, 2008; Pereira et al., 2016). We tested the hypothesis that the efficiency of carotenoid ketolation is correlated

with mitochondrial function and predicted that mitonuclear mismatched hybrid lines would produce less astaxanthin than corresponding non-hybrid, parental lines. We also assessed the relationship between astaxanthin production and measures of mitochondrial performance and fitness among all lines. Establishing links between carotenoid metabolism, mitochondrial function, and fitness measures have outstanding potential to better understanding the evolution of this conspicuous coloration in animals.

3.3 Materials and Methods

3.3.1 Copepod sampling and culturing

Tigriopus californicus copepods were collected along the west coast of California and Baja California (Figure 3.1). Copepods were collected under collection permit #SCP-339 from California Fish and Wildlife. We used copepods sampled from the following locales listed geographically from most southern to most northern: La Bufadora (BUF), San Diego (SD), Bird Rock (BR), Catalina Island (CAT), Abalone Cove (AB), Santa Cruz (SCN) and Pescadero (PES). We reared copepods from each population separately in large beakers containing 35 psu seawater maintained at 20 C on 12h light: dark cycles. We fed copepods used in carotenoid bioconversion assays a diet of ground nutritional yeast (Bragg, Santa Barbara, CA) from birth to produce individuals deficient of carotenoids and clear/white in color. This ground yeast is inactive yeast powder, supplemented with B vitamins, and contains no fats.

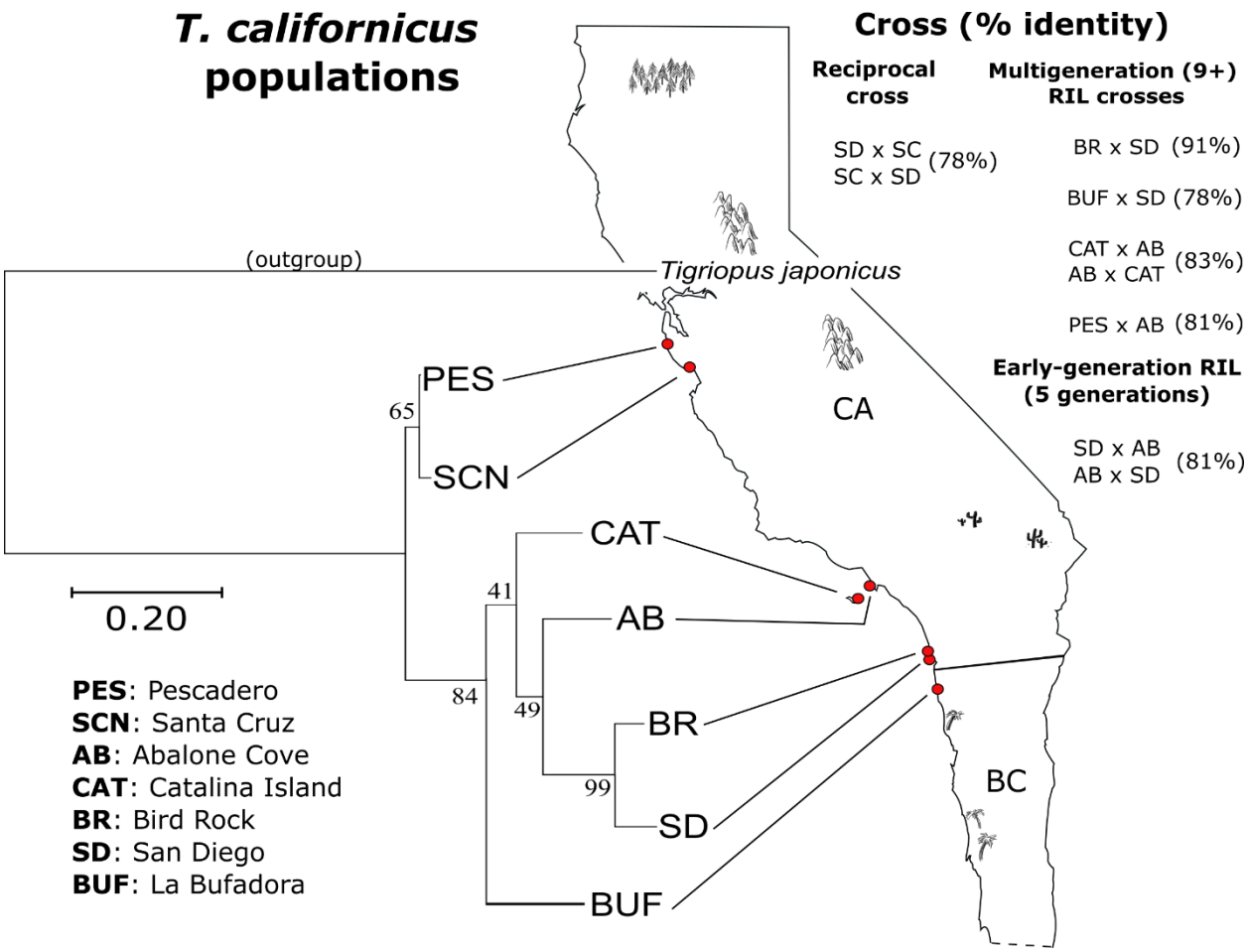

Figure 3.1 Sampling locations along the Pacific Ocean stretching from La Bufadora (BUF) in Baja California (BC) to Pescadero (PES) in the north of California (CA). Percent identity of mitochondrial CO1 gene sequences are shown in parentheses. Populations sampled in this study are shown on the left organized into a phylogenetic tree in Mega X based on 611 bp CO1 sequences, using *Tigriopus japonicus* as a rooted outgroup. For the full methods used to create the tree, see Supplemental Information section 1.6. Key: PES=Pescadero, SCN=Santa Cruz, AB=Abalone Cove, CAT=Catalina, BR=Bird Rock, SD=San Diego, BUF= La Bufadora

3.3.2 Experimental crosses and creation of recombinant inbred lines (RILs)

The methods used to create hybrid copepod lines (see Figure 3.1 for summary) are described here briefly. For a more detailed description of hybrid line generation, see Table S1 for a summary of all crosses and the supplemental information for expanded methodological details.

Multigeneration RILs were formed by crossing the SD population with either BUF or BR populations (Southern RILs), and by crossing the AB population with the CAT or PES populations (Northern RILs). Southern crosses involved pairing SD males with either BR or BUF females (SD nuclear genes tested against BR or BUF mitochondrial genes), and the Northern crosses involved the pairing of AB males with either CAT or PES females (AB nuclear genes tested against CAT or PES mitochondrial genes) and also one pairing of AB females with CAT males (CAT nuclear genes tested against AB mitochondrial genes). Hybrid matings were performed in duplicate and offspring from one replicate were paired with offspring from another to avoid inbreeding. This was repeated until the F3 generation, at which point iso-female, inbred lines were established from a single F3 female's clutch. Beginning in the F4, each generation was initiated with a single pair of full sibs. Discrete generations were maintained until the seventh generation, at which point offspring were allowed to mate continuously and were maintained with overlapping generations. Discrete generations were maintained through the seventh generation in order to allow inbreeding among siblings and purge variation in alleles so that each line more closely represented a unique mitonuclear genotype after the effects of recombination in the first three non-inbred generations. This entire process was repeated using males and females from the same population to make PILs (parental inbred lines) as controls for comparison (Table S1). Similarly, we generated short-term RILs and PILs from a cross of the southern SD and northern AB populations using 20 males and females each in triplicate; however, with this cross we assayed astaxanthin bioconversion at the 5^{th} generation only (Table S1).

After analyzing mitochondrial data collected from our multigeneration RILs (see results below), we became concerned that the yeast diet fed to those copepods created a confounding

effect of dietary stress on mitochondrial and fitness measurements. Copepods from the BUF and BUFSD19 lines fed only ground yeast produced less ATP than copepods fed a complete algal diet (Figure S3.1A-D). Among all parental lines (controlling for non-independence of data within lines), copepods fed yeast had offspring that developed significantly slower compared to parental lines fed algae (Figure S3.1E; mean days to copepodid stage ± 95% CI; yeast-only: 7.21 ± 0.41, algae-fed: 6.29 ± 0.73, $t = 2.16$, $p = 0.037$). Thus, we performed a fresh reciprocal cross between SD and SCN populations, this time feeding the copepods *Tetraselmis* algae for one week prior to measuring mitochondrial function and during offspring development. The reciprocal cross between SD and SCN populations was formed by pairing 40 males from SD with 40 virgin females from SCN and vice versa in two petri dishes. F1 mating pairs were transferred to a new dish to produce F2 offspring. This process was repeated to produce F3 offspring which were then subjected to fitness and carotenoid measurements (described below).

3.3.3 Carotenoid bioconversion assays

Prior to the start of the experiment, RIL copepods were switched to a diet of ground nutritional yeast until they became clear in color and deficient of both carotenoid precursors and the primary red carotenoid, astaxanthin (Powers et al., 2020a; Weaver et al., 2018a). To test carotenoid bioconversion rate, carotenoid-deficient copepods were provided *Tetraselmis chuii* algae *ad libitum* for 7 days to mimic the copepod's natural diet of photosynthetic algae rich with carotenoid precursors (Vittor, 1971). *T. chuii* algae produces multiple dietary carotenoid precursors that can be converted into astaxanthin (Ahmed et al., 2014; Prado-Cabrero et al., 2020; Weaver et al., 2018a). On the seventh day, copepods were moved to clean artificial saltwater for a minimum of 2 hours to clear any algae remaining in their gut. Copepods were

then dried and weighed (±0.001 mg) before being stored in a microcentrifuge tube at -80°C until HPLC analysis (see below).

3.3.4 Carotenoid extraction and HPLC analysis

Carotenoids were extracted from dried copepod tissues using acetone with sonication and centrifugation to remove cellular debris. Final carotenoid extract was resuspended in 50 µL acetone for HPLC analysis. We separated and quantified copepod carotenoids using HPLC following Weaver et al (2018). Briefly, we injected 10 µL of suspended carotenoid extract on to a Sonoma C18 column (10 µm, 250 x 4.6 mm, ES Technologies, New Jersey, USA) fitted with a C18 guard cartridge. Carotenoids were separated using a Shimadzu Prominence HPLC system with mobile phases A 80:20 methanol: 0.5M ammonium acetate, B 90:10 acetonitrile: water, and C ethyl acetate in a tertiary gradient of 100%A to 100%B over 4 min, then to 80% C: 20% B over 14 min, back to 100% B over 3 min, and returning to 100% A over 5 min and held for 6 min (Weaver et al., 2018a; Wright et al., 1991). We visualized and detected carotenoid absorbance using a Prominence UV/Vis detector set to 450 nm. We identified and quantified carotenoids by comparison to calibration curves of authentic standards that included: astaxanthin, zeaxanthin, β-carotene, lutein, hydroxyechinenone, and canthaxanthin. We normalized carotenoid concentration by the dry weight of each copepod sample (reported as µg carotenoid per mg copepod tissue).

Due to restrictions related to the SARS-CoV-2 pandemic, we used a different method to measure astaxanthin content of copepods from the reciprocal cross between SD and SCN populations (analyzed mid-year 2020; see Supplemental Information 1.3). Briefly, astaxanthin content of copepods from these crosses (~10 ind. per replicate) was quantified using an Agilent

HPLC 1260 Infinity II LC system with the following mobile phases: A) 50:25:25 Methanol: Acetonitrile: 0.25M Aqueous Pyridine and B) 20:60:20 Methanol: Acetonitrile. For full descriptions of this mobile phase linear gradient, as well as the carotenoid extraction methods for this analysis, see Supplemental Information section 1.3.

3.3.5 Offspring development rate assay

Gravid females (sample sizes in Table S1) were removed from yeast-fed cultures and placed into filtered seawater in a 6-well plate. These copepods were supplemented with powdered Spirulina (Jade Spirulina, Salt Creek Inc., Salt Lake City, USA) instead of powdered yeast because mortality of gravid females in 6-well plates was high when fed yeast. The Spirulina powder is not the same *Tetraselmis* algae used to resupply copepods with a diet rich in carotenoids. The main pigment in Spirulina are anthocyanins. The Spirulina diet provides enough nutrition to consistently allow reproduction; *T. californicus* females will delay reproduction or cannibalize offspring if they cannot acquire enough quality food (Powlik et al., 1997). Plates were monitored daily, and whenever an egg sac hatched, the date was recorded, and the female and any unhatched eggs were removed. The freshly hatched offspring were then monitored daily, and the number of individuals that metamorphosed to the copepodid I form (sixth molt, halfway point of development to full maturity) was recorded for each well on each day (Powers et al., 2020b).

For the F2 and F3 generations of the reciprocal SD and SCN crosses, development rate was assayed slightly differently due to time constraints. 50 gravid females were placed in a petri dish with filtered seawater and Spirulina. The following day, all non-gravid females and unhatched egg sacs were moved to a new dish, leaving behind any nauplii that hatched. This was

repeated for 5 days, and the hatch date of each dish was recorded. Dishes with freshly hatched offspring were monitored daily, and any animals that metamorphosed to the first copepodid stage were counted and removed so they were not confused with newly molted copepodids the following day.

3.3.6 ATP synthesis and citrate synthase assays

We measured *in vitro* ATP production from isolated mitochondria following methods from (Ellison and Burton, 2006; Harada et al., 2019). For each sample, ten males and ten females were homogenized in 800 µL isolation buffer (400 mM sucrose, 100 mM KCl, 6 mM EGTA, 3 mM EDTA, 70 mM HEPES, 1% w/v BSA, pH 7.6), and their mitochondria were isolated by sequential centrifugation. The isolated mitochondrial pellet was resuspended in ~55µL assay buffer (560 mM sucrose, 100mM KCL, 10mM KH_2PO_4, and 70mM HEPES). Suspended mitochondria (25µL) from each sample were then added to 5µL of either Complex I (CI) substrate (1 mM ADP, 2 mM malate, 10 mM glutamate, and assay buffer) or Complex II (CII) substrate [1 mM ADP, 10 mM succinate, 0.5 µM rotenone (Complex I inhibitor)]. Samples were incubated for 10 minutes at 20°C to allow for ATP synthesis. After the incubation period, 25µL of each sample was added to a 96-well assay plate with 25µL of CellTitre-Glo (Promega)—which halts ATP synthesis. Luminescence of the samples and ATP standards was measured on the same plate using a Fluoroskan Ascent FL plate reader (Thermo Labysystems).

A citrate synthase (CS) activity assay was performed on the remaining mitochondrial suspension to estimate mitochondrial volume and standardize ATP production per sample following the methods of (Spinazzi et al., 2012b). Mitochondrial volume (or mitochondrial content) represents the fraction area of cell volume taken up by the mitochondria (Larsen et al.,

2012; Parry et al., 2020). CS activity correlates strongly with the fraction of mitochondrial surface area to total cell surface area in the cell (Larsen et al., 2012), thus we use it as a proxy for mitochondrial content or mitochondrial volume (Parry et al., 2020) when standardizing ATP production (Spinazzi et al., 2012a). CS activity was determined as follows: a 5µL aliquot of the mitochondrial suspension was added to 50µL of 200mM Tris buffer with 0.2% Triton-X (Sigma), 24µL DI water, 10µL 1mM DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)), and 6µL acetyl coenzyme A (0.3mM). After estimating background activity, 5µL of 10mM oxaloacetic acid was added to each sample and absorbance read at 412nm. The change in absorbance measured over 5 minutes was used to calculate CS activity.

3.3.7 Statistical analyses

Samples sizes for our measurements on each line are shown in Table S1. We used mixed-effects linear models and pairwise contrasts corrected for multiple comparisons (using 'emmeans') to compare astaxanthin concentration among hybrid and non-hybrid copepod lines. These models included a random effect of line ID to account for non-independence of data within each line. We repeated this same analysis on dietary carotenoid concentrations and the ratio of astaxanthin to dietary carotenoid concentrations among lines. We also used linear models and paired contrasts corrected for multiple comparisons to analyze differences in ATP production, and offspring development rate among hybrid and non-hybrid copepods lines.

Because we lacked data from female copepods in several crosses, we used male data to analyze the relationships between astaxanthin production and ATP production and offspring development rate among all of the lines using mixed effects models. We included cross ID (i.e., "BR x SD", "SD x SD", etc.) as a random effect to account for non-independence of data from

the same hybrid cross or parental control line. Before fitting the models, ATP production and development rate data were log transformed, scaled, and centered to achieve normality of model residuals. We averaged replicates from each line to avoid pseudoreplication and because the same individuals could not be used for all measurements (i.e., the same copepods could not be sacrificed for both astaxanthin extraction and mitochondrial measurements).

All statistical analyses were performed in R. For the full list of packages used, see supplemental info section 1.5.

3.3.8 Data availability

Presented data and R code for analysis can be found at PLOS ONE online (https://doi.org/10.1371/journal.pone.0259371).

3.4 Results

3.4.1 Recombinant inbred lines

Among the multigeneration inbred lines, we found no clear pattern between non-hybrids and hybrids in ATP production (Figure S3.2) or offspring development rate (Figure S3.3). Two lines were significantly higher than others in ATP production: the CAT parental line (Complex I, Figure S3.2B) and the BRSD56 hybrid line (Complex II, Figure S3.2C). Thus, in the multigeneration RILs fed yeast only, we detected no evidence of mitochondrial dysfunction, even in RILs from crosses of highly diverged populations.

However, we did observe differences in astaxanthin production among male copepods from hybrid and non-hybrid lines (Figure 3.2), but no difference in astaxanthin bioconversion among female copepods from different lines (Figure S3.4). Additionally, males produced

significantly more astaxanthin than females on average, even while controlling for line ID (mean μg mg^{-1} astaxanthin ± SE; male = 0.27 ± 0.03; female = 0.21 ± 0.03; n = 124; df = 7; p = 0.001). Because of this sex effect and the lack of female data in the northern lines (Figure S3.4B), we analyzed male data only for the rest of the analyses involving the RILs and PILs below.

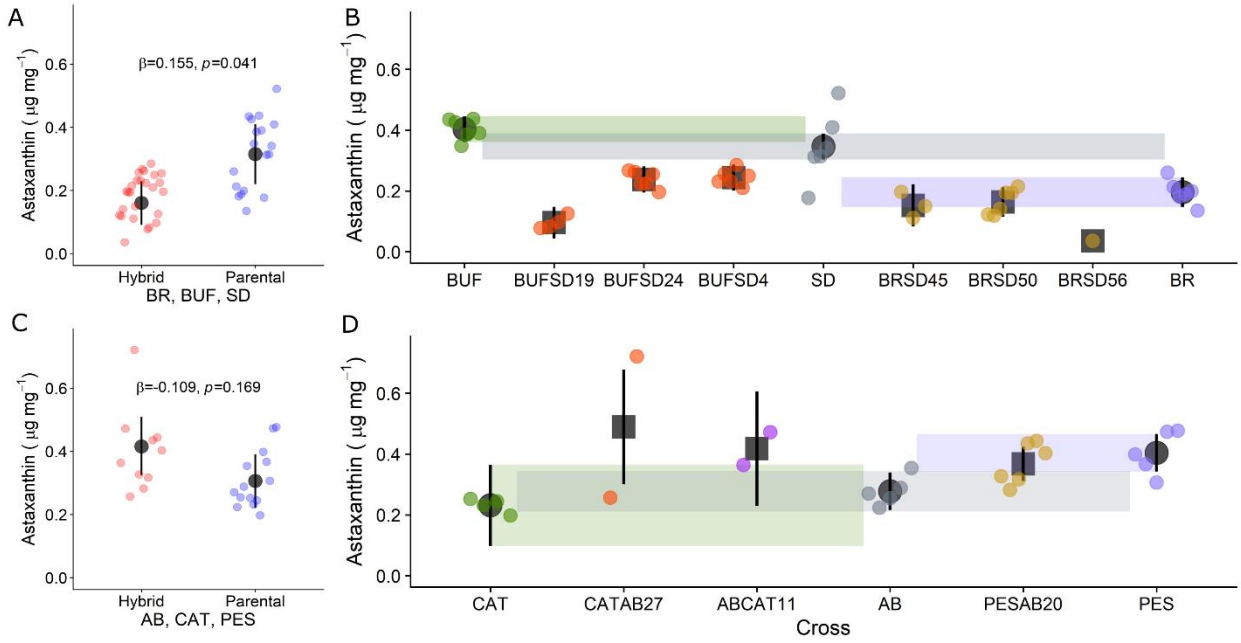

Figure 3.2 Differences among hybrid RILs and non-hybrid PILs from multigeneration crosses. Colored dots represent replicates within each line that are the average astaxanthin concentration of approximately 10 copepods. Dark grey circles (parental lines) and dark grey squares (hybrid lines) are the line average. Black lines and colored shading are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines (Table S2).

The difference in astaxanthin production among male copepods across all of the multigenerational RIL and PIL lines was not consistent, nor did it closely match with pairwise genetic divergence (Figs 1, 2). Hybrid males from crosses of southern populations produced less astaxanthin than parental lines, on average (mean μg mg^{-1} astaxanthin ± SE; hybrid = $1.6e^{-3}$ ± $3.6e^{-4}$; parental = $3.2e^{-3}$ ± $4.9e^{-4}$; n = 44; df = 7; p = 0.035; Figure 3.2A). Moreover, this difference in astaxanthin production was driven by RILs of BUF and SD hybrids (Figure 3.2B). RILs from the hybrid crossing of female BUF copepods with male SD copepods (BUFSD19,

BUFSD24, BUFSD4) accumulated significantly less astaxanthin compared to both SD and BUF parental lines (Figure 3.2, Table S2). RILs from the crossing of female BR copepods with male SD copepods (BRSD45, BRSD50, BRSD56) accumulated significantly less astaxanthin than the SD parental line but only slightly less than the BR parental line (Figure 3.2, Table S2). Individual RIL lines from the Northern crosses (ABCAT11, CATAB27, and PESAB20) did not produce a significantly different amount of astaxanthin that any of their corresponding parental lines (Figure 3.2, Table S2). The pattern observed in males from crosses of southern populations was similar to the pattern of astaxanthin production observed in lines from the cross between southern SD and northern AB populations. In this cross, some RILs produced significantly less astaxanthin compared to non-hybrid controls, while other RILs did not (Figure S3.5, Table S3); however, overall, hybrid lines from this cross converted less astaxanthin than parental lines (mean log astaxanthin ± SE; hybrid = 0.88 ± 0.04 ug mg; parental = 0.71 ± 0.06; n = 35; df = 15; $p = 0.044$; Figure S3.5, Table S3).

The patterns in astaxanthin concentrations across RILs described above was mirrored when we repeated the analysis using the ratio of astaxanthin: dietary carotenoids (Figure S3.6, Table S4). We consistently detected two dietary carotenoids in our yeast-fed inbred line samples: β-carotene and hydroxyechinenone (Figure S3.7). Both are precursors to astaxanthin found in *T. californicus* fed *Tetraselmis* algae (Prado-Cabrero et al., 2020). Hydroxyechinenone is an intermediate between β-carotene and astaxanthin (Weaver et al., 2018a). Hydroxyechinenone was the most abundant dietary carotenoid found in our yeast-fed inbred lines ($p < 0.001$; Figure S3.8). Dietary carotenoid concentrations did not significantly vary among yeast-fed inbred line samples (Figure S3.9, Table S5).

Although the data from yeast-fed lines shows no clear breakdown of mitonuclear function in RILs compared to PILs, we found significant relationships between astaxanthin bioconversion and energy production across all lines. We found a statistically significant, negative relationship between astaxanthin production and Complex I ATP production (β_1 = -0.08 µg astaxanthin mg^{-1}; p = 0.020; R^2_{adj} = 0.44; n = 13) (Figure 3.3A), but no clear relationship between astaxanthin production and Complex II ATP production (β_1 = -0.03 µg astaxanthin mg^{-1}; p = 0.610; R^2_{adj} = 0.52; n = 12) (Figure 3.3B). We did not find a statistically significant relationship between astaxanthin production and offspring development rate (β_1 = 0.05 µg astaxanthin mg^{-1}; p = 0.200; R^2_{adj} = 0.72; n = 11) (Figure 3.3C). When we repeated these analyses using the ratio of astaxanthin to dietary carotenoids, we found that the relationship between astaxanthin: dietary carotenoids and Complex I ATP production was still negative but fell short of statistical significance (Figure S3.10). However, the relationship between astaxanthin: dietary carotenoids and offspring development rate was significantly positive (Figure S3.10).

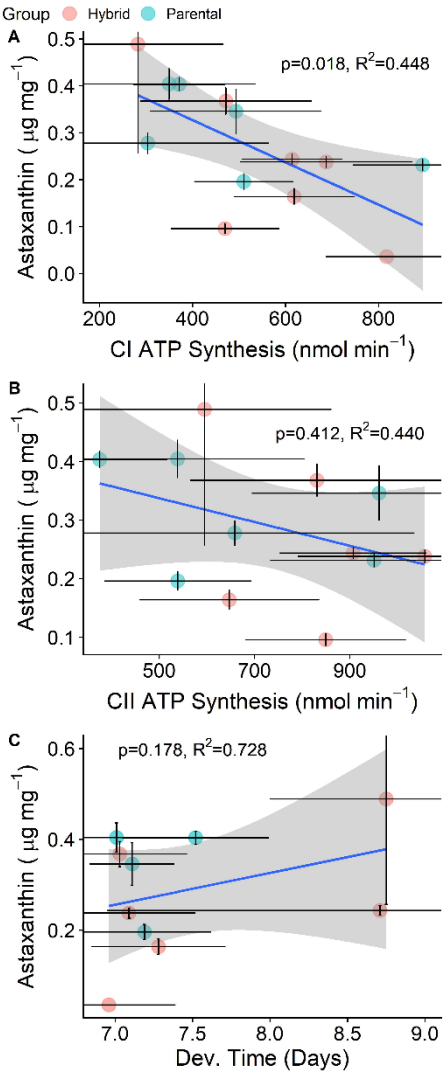

Figure 3.3 Relationships between astaxanthin concentration and either ATP production or offspring development rate among all hybrid and non-hybrid lines. Colored dots represent line averages and the vertical and horizontal black bars extending from the colored dots represent the standard error. The grey shading is the 95% confidence interval around the model estimated slope. Adjusted R^2 values are shown.

Among all lines, the relationship between ATP production and offspring development was not statistically significant (Complex I: β_1 = -0.40 log nmol ATP min^{-1}; p = 0.118; R^2_{adj} =

0.323; n = 10, and Complex II: β_1 = -0.24 log nmol ATP min^{-1}; p = 0.445; R^2_{adj} = 0.21; n = 9) (Figure S3.11A, S3.11B). Unexpectedly, the relationship between ATP production and mitochondrial volume was not significant either (Complex I: β_1 = -0.01 log nmol ATP min^{-1}; p = 0.963; R^2_{adj} = 0.30; n = 13, and Complex II: β_1 = 6.2e^{-3} log nmol ATP min^{-1}; p = 0.979; R^2_{adj} = 0.46, n = 13) (Figure S3.11C, S3.11D).

3.4.2 The reciprocal cross between SD and SCN populations

We repeated our experiment using a fresh cross between SD and SCN populations, this time feeding copepods *Tetraselmis* algae one week prior to measuring ATP production and offspring development rate to mitigate potential confounding effects of the yeast-only diet.

We found no significant difference in astaxanthin production between parental SD and SCN copepods and copepods from the F1 and F2 generations (mean µg mg^{-1} astaxanthin ± SE; F1 generation: parental = 0.14 ± 0.01; F1 hybrid = 0.16 ± 0.01; n = 52, df = 61, p = 0.552; F2 generation: parental = 0.14 ± 0.01; F2 hybrid = 0.16 ± 0.02, n = 36, df = 60, p = 0.870) (Figure 3.4). However, by

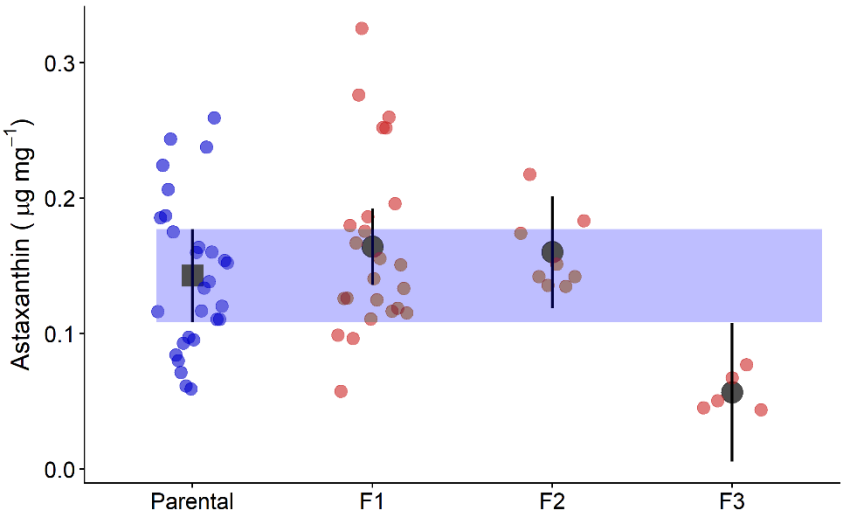

Figure 3.4 Astaxanthin produced by copepods in each generation of a reciprocal cross between SD and SCN populations. The parental group includes both SD and SCN copepods and each hybrid generation includes copepods from both cross directions (i.e., SD x SCN and SCN x SD. For the crosses split, see Figure S5. Colored dots represent replicates within each line that are the average astaxanthin level of approximately 10 copepods. Dark grey circles show the generation average. Black lines and colored shading are 95% confidence intervals around the group average. Where confidence intervals do not overlap, there is a statistically significant difference between groups.

the third generation, F3 hybrid copepods showed a statistically significant decrease in astaxanthin production when compared to parental copepods (mean µg mg^{-1} astaxanthin ± SE; parental = 0.14 ± 0.01; F3 hybrid = 0.06 ± 0.03, n = 33, df = 60, p = 0.014) (Figure 3.4). This decrease in astaxanthin by generation three was observed in both cross directions (SD x SCN and SCN x SD) (Figure S3.12). Dietary carotenoids were observed in only a small subset of samples from this cross. We observed lutein, which is not a precursor to astaxanthin, and a peak putatively identified as echinenone/hydroxyechinenone (see supplemental data file for raw concentration values).

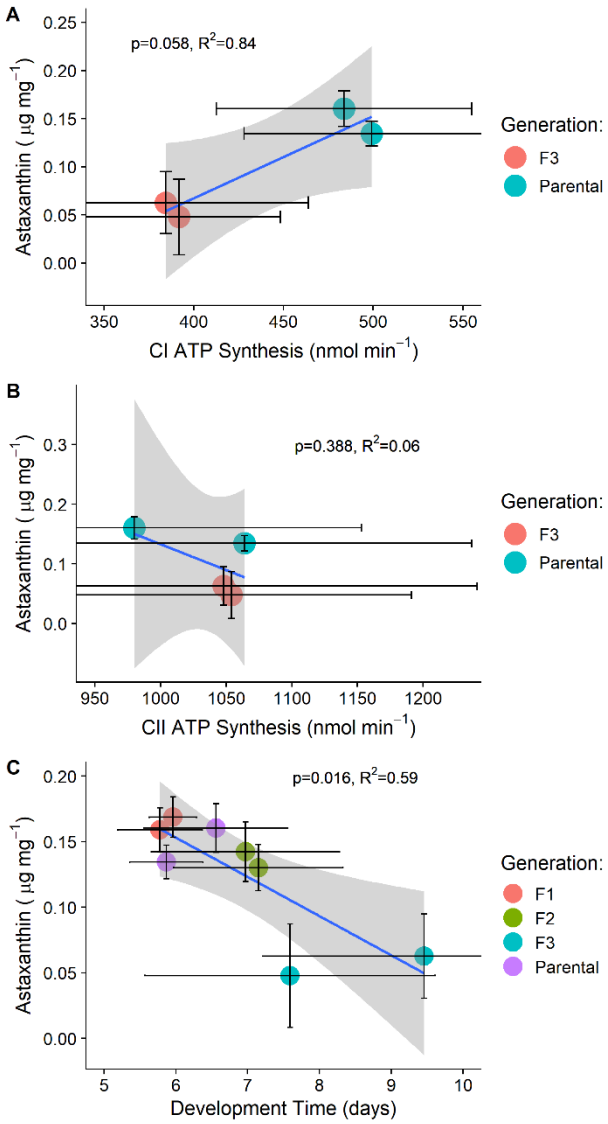

Figure 3.5 Relationships between astaxanthin production and either ATP production (panels A and B) and offspring development rate (panel C) among hybrid and non-hybrid lines from the cross between SD and SCN. Colored dots represent line averages and black bars extending from the colored dots represent the standard error of each trait on the x- and y-axis. The grey shading is the 95% confidence interval around the model estimated slope. Adjusted R^2 values are shown.

We found that some relationships between astaxanthin production and fitness-based traits measured using individuals fed *Tetraselmis* algae did not match the relationships observed with the yeast-only inbred lines. The relationship between astaxanthin production and Complex I ATP production was positive this time, trending towards statistical significance (β_1 = 0.05 µg astaxanthin mg^{-1}; p = 0.056; R^2_{adj} = 0.84; n = 4; Figure 3.5A), but

there was no significant relationship between astaxanthin production and Complex II ATP production (β_1 = -0.03 μg astaxanthin mg^{-1}; p = 0.388; R^2_{adj} = 0.06; n = 4; Figure 3.5B). The relationship between astaxanthin production and offspring development rate was negative and statistically significant (β_1 = -0.04 μg astaxanthin mg^{-1}; p = 0.016; R^2_{adj} = 0.59; n = 8; Figure 3.5C).

Unlike with the multigeneration inbred lines fed yeast during fitness measurements, copepods from the SD x SCN crosses fed algae showed a positive, statistically significant relationship between Complex I ATP production and mitochondrial volume (β_1 = 0.98 log nmol CS min^{-1}; p = 0.023; R^2_{adj} = 0.93; n = 4; Figure S3.13A), but there was no significant relationship between Complex II ATP production and mitochondrial volume (β_1 = 0.63 log nmol CS min^{-1}; p = 0.371; R^2_{adj} = 0.09; n = 4; Figure S3.13B). The relationship between Complex I ATP and offspring development rate was negative, but not statistically significant (β_1 = -0.73 log days; p = 0.087; R^2_{adj} = 0.75; n = 4; Figure S3.13C). There was no significant relationship between Complex II ATP production and offspring development rate (β_1 = 0.13 log days; p = 0.837; R^2_{adj} = -0.46; n = 4; Figure S3.13D).

Comparing F3 hybrid lines and parental lines, there were no statistically significant differences in Complex I ATP production or Complex II ATP production. However, F3 hybrids did consistently make less Complex I ATP on average with reduced variation compared to parental lines (Figure S3.14A, S3.14C). This decrease was not observed in Complex II ATP production (Figure S3.14B). Offspring development rate increased progressively from the parental generation to the F3 hybrid generation. F3 hybrids had a statistically significant longer offspring development rate than parental copepods (mean log days ± SE; parental = 6.3 ± 0.56; F3 hybrid = 8.4 ± 0.49, n = 21, df = 43, p = 0.036) (Figure S3.14C) and F1 hybrid copepods

(mean log days ± SE; F1 hybrid = 6.6 ± 0.0.42; F3 hybrid = 0.06 ± 0.03, n = 28, df = 43, p = 0.045) (Figure S3.14C).

3.5 Discussion

Previous research demonstrated that interpopulation hybrid lines of *T. californicus* showed high variability but a consistent pattern of reduction in ATP production (Ellison and Burton, 2008), ETS complex activity (Ellison and Burton, 2006), oxidative homeostasis (Barreto and Burton, 2013), fecundity (Barreto and Burton, 2013; Burton et al., 2006), offspring survivorship (Ellison and Burton, 2008), and offspring development (Ellison and Burton, 2008). It was demonstrated that the observed decline of performance in hybrid copepods was likely due to mitochondrial dysfunction caused by mitonuclear incompatibilities brought on through the shuffling of nuclear alleles through sexual recombination (Burton et al., 2013a; Ellison and Burton, 2010). Thus, we predicted that if ketolation of yellow dietary carotenoids to red carotenoids is tied to mitochondrial function, then we would observe reduced astaxanthin content in hybrid copepods that also showed evidence of reduced mitochondrial performance and fitness. However, we observed no clear reduction in mitochondrial performance or offspring development in our hybrid RIL copepods compared to parental lines (Figs S2, S3). Yet, some RILs bioconverted significantly less astaxanthin compared to corresponding PILs, while others did not (Figs 2, S5, S6). This variation is to be expected since each RIL captures a unique set of parental alleles; on average, the expectation is that mitonuclear coadaptation will be disrupted. However, those lines with the most severe disruption will die (and not appear in our data set) while some RILs will obtain favorable mitonuclear combinations and continue to propagate. It is unclear if there is any way to rescue RILs with severely incompatible mitonuclear combinations

that are in danger of extinction. Experiments have shown that backcrossing to the maternal population can reintroduce compatible nuclear alleles and may rescue viability (reviewed in (Burton et al., 2013b)). However, there have also been experiments that show that mitonuclear incompatibilities differ among crosses (Healy and Burton, 2020; Lima et al., 2019; Pereira et al., 2020). Therefore, "fixes" for dying lines may not work equally in all cases. Moreover, fixes that alter the environment (for example, via diet or temperature changes) to relieve the stress of mitonuclear incompatibilities may only make it more difficult to compare fitness measures among lines.

We hypothesize that a key confounding factor in this study was dietary stress resulting from the poor yeast diet we used to produce copepods deficient of carotenoid pigments. Malnourishment has been observed to negatively impact oxidative state and mitochondrial function (Akinola et al., 2010; Feoli et al., 2006). We found that lines fed the carotenoid-devoid yeast diet produced less ATP (via Complex I substrates) than the same lines fed a complete, lipid and antioxidant rich diet of photosynthetic algae (Figure S3.1). We also observed that offspring from yeast-fed parental lines developed significantly slower than offspring from parental lines fed algae (Figs S1). Moreover, among lines fed yeast only, we found no significant relationship between ATP production and mitochondrial volume (Figure S3.11C, S3.11D). This is an important result because typically, we expect a positive relationship between ATP production and CS activity (Barrientos et al., 2009; Brand and Nicholls, 2011). Indeed, CS activity is used to standardize measurements of ATP production to account for variation in mitochondrial volume when sampling (Barrientos et al., 2009; Spinazzi et al., 2012a). Thus, it is possible that dietary restriction obscured the relationship between ATP production and CS activity. Whether or not this means a link between astaxanthin bioconversion and mitonuclear incompatibilities was

artificially obscured is unclear, especially considering that we observed little to no variation in female copepods from the yeast-fed multigeneration lines (Figure S3.4). However, the difference that we observed between male and female copepods may agree with other evidence that female *T. californicus* are more resistant to stressful conditions (Foley et al., 2019; Healy et al., 2019; Willett, 2010; Willett and Son, 2018). It is also possible that males are less resistant to changes in oxidative stress (Li et al., 2020). Female *T. californicus* must sequester enough astaxanthin not only for themselves, but also for their developing offspring. Carotenoids must be transferred to the developing eggs in order to provide freshly hatched offspring with the UV-protection they need to survive their first two naupliar instars (Weaver et al., 2018a). During this early naupliar period, offspring (which are the same bright red as adults) cannot eat (Powlik et al., 1997) and, therefore, cannot acquire their own carotenoid precursors. Indeed, it is common for mothers to deposit carotenoids into developing eggs in oviparous taxa (Green, 1965; Koutsos et al., 2003; Torrissen and Christiansen, 1995).

Switching diets appeared to alleviate problems with the initial experiment and yielded results that were more suggestive of hybrid breakdown (Figure S3.13, S3.14). When we fed hybrid and parental copepod lines algae one week prior to making mitochondrial measurements, the relationship between Complex I ATP production and mitochondrial volume was significant and positive (Figure S3.13A) across lines as expected from the perspective of mitochondrial physiology (Barrientos et al., 2009; Brand and Nicholls, 2011). , We also found a more consistent pattern between hybrid and parental lines: hybrid animals made slightly less Complex I ATP (Figure S3.14A), and had offspring with significantly longer development rates by the third generation (Figure S3.14C). These results represent only one reciprocal cross, however, and it is unclear if these results would also be seen in crosses between other populations.

Despite a lack of observable mitonuclear dysfunction in RILs fed yeast, we found an interesting relationship between astaxanthin bioconversion and ATP production among these lines (Figure 3.3). After statistically controlling for the non-independence of data within hybrid vs. parental lines, we found a significant, negative relationship between Complex I ATP production and astaxanthin bioconversion (Figure 3.3A). We found no clear relationship between astaxanthin bioconversion and Complex II ATP production (Figure 3.3B). This negative relationship was also seen when ATP production was modeled against the ratio of astaxanthin: dietary carotenoids, although it fell short of statistical significance (Figure S3.10). This difference is interesting for two reasons. First, unlike Complex I, Complex II is encoded solely by the nuclear genome (Hill, 2019; Willett and Burton, 2004). This suggests astaxanthin pigmentation may better predict Complex I function than Complex II function within a given line. In previous studies, Complex II was found to be the only ETS Complex not impaired by hybridization (Ellison and Burton, 2006). Likewise, we found a slight decrease in Complex I ATP production, but not in Complex II ATP production, in the SD x SCN reciprocal cross fed algae (Figure S3.9A, S3.9B). Second, Complex II utilizes a different electron donor and acceptor system than Complex I (Hatefi, 1985). Complex I uses NADH as its primary electron donor, producing NAD+ as a result, while Complex II uses FADH as an electron donor, producing FAD+. It is possible that carotenoid ketolase enzymes, probably members of the Cytochrome P450 family or CrtS-like enzymes (Lopes et al., 2016; Weaver et al., 2020), also operate using NADH/NAD+ or NADPH/NADP+ systems (Blacker and Duchen, 2016; Csernetics et al., 2015; Hill, 2014; Hill et al., 2019b). Perhaps the stronger relationship between astaxanthin bioconversion and Complex I ATP production could be explained by a shared pool of electron donors (Blacker and Duchen, 2016).

A shared pool of electron donors between Complex I and carotenoid ketolase enzymes may also explain why we observed a negative relationship between Complex I ATP production and astaxanthin bioconversion. It is possible that under stressful conditions, when demand for energy is high relative to available substrates for oxidative phosphorylation, NADH or NADPH used to catalyze reactions in Complex I will no longer be available to catalyze carotenoid ketolation, thus invoking trade-off between production of ATP or production of astaxanthin. However, under benign conditions when the demand for ATP is lower, the relationship could then be positive, as we observed with the SD x SCN reciprocal cross fed a complete algae diet during fitness measurements. In molting house finches, males that bioconverted the most red ketocarotenoids also showed signs of withstanding higher levels of cellular stress in their mitochondria, as indicated by lower rates of mitochondrial replacement despite higher levels of oxidative damage to their cellular membranes (Hill et al., 2019b). This may help explain why we found no significant relationship between ATP production and mitochondrial volume in multigeneration inbred lines fed yeast only.

Alternatively, some invertebrates, including *T. californicus,* can also make use of a nuclear-encoded alternative oxidase (AOX) in their ETS to combat oxidative stress (Fernandez-Ayala et al., 2009; Tward et al., 2019; Weaver, 2019). An increase in AOX expression would continue to consume oxygen for respiration, but would decrease ATP production due to reduced electron flow to Complex IV (Weaver, 2019). Similarly, the use of uncoupling proteins to reduce reactive oxygen species and maximize survival in the face of oxidative stress may also have resulted in a decrease in ATP (Brand, 2000; Caldeira da Silva et al., 2008; Speakman et al., 2004). If copepods in our study were using AOX or uncoupling enzymes to effectively combat oxidative stress due to hybridization and the poor yeast diet, it may explain why our most

colorful lines also tended to produce less ATP (Figure 3.3). Certainly, ATP production can be informative in many situations, but it can also be an ambiguous metric when measured without the added context of metabolic rate (Salin et al., 2015). Indeed, a deficiency in ATP can be compensated for through many different changes in mitochondrial dynamics (Anderson et al., 2019; Celotto et al., 2011; Heine and Hood, 2020; Heine et al., 2020; Picard and Sandi, 2020). Even though these ideas are largely speculative, a positive relationship between the metabolic rate and astaxanthin bioconversion has been observed in hybrid *T. californicus* (Powers and Hill, 2021). In wild male house finches, individuals with greater energetic capacity are better able to bioconvert carotenoids (Hill et al., 2019b). Thus, carotenoid bioconversion may predict the capacity to meet changes in energy demand or withstand oxidative stress, rather than signal raw energy production.

The unexpected relationship between astaxanthin bioconversion and offspring development rate in multigeneration inbred lines (Figure S3.10) may also have been influenced by the limited yeast-only diet. Indeed, we observed that in the yeast-only lines, offspring development time was longer in lines with more efficient astaxanthin bioconversion (Figure S3.10C). Thus, paradoxically, in copepods fed yeast-only, lines with more astaxanthin produced less ATP and had slower developing offspring. However, this relationship was reversed in the reciprocal cross between SD and SCN when the dietary stress was relieved (Figure 3.5). In the yeast-only inbred lines, we found that offspring from all lines developed at similar rates (Figure S3.3); however, in the reciprocal cross between SD and SCN fed algae, we found that development rate was significantly longer in hybrids by the third generation (Figure S3.14C). We have frequently observed longer development rates in laboratory cultures fed yeast-only diets, and in the data in this study we also observed that parental copepods fed yeast only

produced offspring with longer development rates on average compared to parental copepods fed algae (Figure S3.1E). It could be that the malnourishing yeast diet limited variation in offspring development, slowing all lines and changing the relationship between offspring development and astaxanthin bioconversion. These results could support the idea that astaxanthin pigmentation is fundamentally tied to individual condition (Hill, 2011; Svensson and Wong, 2011), even in systems that do not experience sexual selection on carotenoid coloration (Powers et al., 2020a).

Across diverse vertebrate (Hill et al., 2019b; Hill and Montgomerie, 1994; Kodric-Brown, 1985; Simons et al., 2012) and invertebrate species (Baldwin and Johnsen, 2009b; Baldwin and Johnsen, 2012; Weaver et al., 2016), coloration derived from the deposition of carotenoids is recognized as an honest signal of individual condition (Endler, 1980; Hill, 2007; Nguyen et al., 2014; Peneaux et al., 2021; Svensson and Wong, 2011; Weaver et al., 2017). For many years, it was assumed that carotenoid pigments were a limiting resource for many animals (Endler, 1980; Hill, 1990). It has been asserted that carotenoids perform physiological roles in body maintenance in many species, making them costly to devote toward pigmentation alone (Alonso-Alvarez et al., 2008; Lozano, 1994; Moller et al., 2000). Carotenoids may be important signaling molecules in immune responses and may help combat oxidative stress through free radical scavenging or the stimulation of antioxidant activity (Alonso-Alvarez et al., 2008; von Schantz et al., 1999). Previous hypotheses suggest there is a carotenoid-based resource trade-off between pigmentation and body maintenance (Koch and Hill, 2018; Verhulst et al., 1999). However, the hypothesis that carotenoid coloration entails a costly trade-off has been called into question (Hadfield and Owens, 2006; Hill, 2011; Koch et al., 2019; Simons et al., 2012; Simons et al., 2014). An alternative to the resource tradeoff hypothesis is the Shared-Pathway Hypothesis, which proposes that the bioconversion and accumulation of red carotenoids for

coloration shares a biochemical pathway with mitochondrial metabolism in the cell (Hill, 2011; Hill, 2014; Hill and Johnson, 2012b; Johnson and Hill, 2013; Koch et al., 2017). The general prediction derived from this hypothesis is that ketocarotenoid bioconversion is an index that signals mitochondrial efficiency, flexibility, and capacity. Our results in *T. californicus*, may indicate that the Shared-Pathway Hypothesis is applicable in taxa beyond avian species, if indeed carotenoid bioconversion is an indicator of mitochondrial capacity to withstand stress. However, our results do not refute the idea that there is a tradeoff between body maintenance and carotenoid bioconversion in *T. californicus*. To better evaluate the hypotheses concerning a shared pathway between carotenoid bioconversion and mitochondrial function, specific measures of oxidative phosphorylation in the mitochondria, such as oxygen consumption, electron donor/acceptor ratios, and redox potential, may be important to consider in the future in order to contextualize energy production.

3.6 Methods supplement (as published in Marine Biology)

3.6.1 Experimental crosses and creation of recombinant inbred lines (RILs) (further details)

We sampled from the following locations: La Bufadora (BUF; 31°72'37.25"N, 116° 72'18.15"W), San Diego (SD; 32°44'49.67"N, 117°15'13.34"W), Bird Rock (BR; 32°48'41.63"N, 117°15'58.11"W), Catalina Island (CAT; 33°23'57.45"N, 118°23'26.33"W), Abalone Cove (AB; 33°44'29.43"N, 118°22'45.59"W), Santa Cruz (SCN; 36°57'4.35"N, 122° 1'52.65"W), and Pescadero (PES; 37°15'43.79"N, 122°24'47.90"W). The experimental crosses used in this study were performed over several years at two different institutions. The creation of RILs and the reciprocal cross between SD and SCN were performed at the Scripps Institute of Oceanography

at UC San Diego. The RILs from crossing SD and AB populations were created at Auburn University.

To create the early generation RILs from crossing SD and AB populations (completed in 2016), 20 males from SD and 20 virgin females from AB were paired together in petri dishes in triplicate in order to start with sufficient genetic variation to produce offspring with a variety of nuclear allelic combinations working against the single maternal mitochondrial type. When clasped pairs formed, they were moved to a well of a 6-well plate to produce F1 offspring. When F1 offspring began to form clasped pairs, these were separated and males from one of the three replicates were paired in a new well of another 6-well plate with females from one of the other cross replicates to avoid sibling pairings. When F2 offspring were formed, this process was repeated to produce the F3 generation. At the F3 generation we formed iso-female recombinant inbred lines by taking gravid F3 females and isolating them each in a well of a 6-well plate. When a female's eggs hatched, the female was removed the offspring were allowed to inbreed to produce an F5 generation used for carotenoid analyses (see section below). We stopped hybrid generation at the 5^{th} generation as a precautionary measure to ensure enough lines could be sampled before dying out due to the effects of hybridization. This entire process was repeated by crossing males and females from the same population to make parental inbred lines (PILs).

Briefly, the multi-generational RILs from the crosses between (SDxBUF, SDxBR, ABxCAT, and ABxPES) were generated using the following methods. Males from one population were mated to virgin females of the second population in two replicates for the same reasons described above for the SDxAB cross. When these replicate pairings bore F1 offspring, males from one replicate were mated with females from the second replicate to avoid inbreeding. This process was repeated until the F3 generation. When F3 matings produced gravid females,

the egg-bearing mothers were isolated in the well of a 6-well plate until their eggs hatched; once the eggs hatched, the females were removed. At this point, F4 siblings were allowed to inbreed, and mated pairs were again isolated to produce the F5 generation. This process was repeated until F7 offspring were produced, at which point F7 individuals were allowed to mate continuously to form recombinant inbred lines (originally formed from a single F3 mother) with overlapping generations. Discrete generations were maintained through the seventh generation in order to allow inbreeding among siblings and purge variation in alleles so that each line more closely represented a unique mitonuclear genotype after the effects of recombination in the first three non-inbred generations. These lines were maintained as continuous cultures prior to our study once the recombinatory genotypes were established so that logistically we could sample the lines as needed.

The reciprocal cross between SD and SCN populations was formed by pairing 40 males from SD with 40 virgin females from SCN and vice versa in two petri dishes. We started with 40 individuals per replicate instead of the 20 (described in the previous crosses) in order to minimize the chance of sibling pairings. Once every female in a dish formed eggs, males were removed, and the eggs allowed to hatch. When the F1 offspring began to form mating pairs, they were transferred to a new generation to produce F2 offspring. With this cross, we made no attempt to avoid sibling pairings in any generation. This process was repeated once more to produce F3 offspring which were then subjected to fitness and carotenoid measurements (described below). We did not form recombinant inbred lines with this cross due to time limitations imparted by the timing of the work amidst other projects and by the eventual onset of the SARS-CoV-2 pandemic in early 2020.

3.6.2 Carotenoid bioconversion assays (further details)

Prior to the start of the experiment, RIL copepods were switched to a diet of ground nutritional yeast until they became clear in color and deficient of both carotenoid precursors and the primary red carotenoid, astaxanthin (Powers et al., 2020a; Weaver et al., 2018a). To test carotenoid bioconversion rate, carotenoid-deficient copepods were provided *Tetraselmis chuii* algae ad libitum for 7 days to mimic the copepods natural diet of photosynthetic algae rich with carotenoid precursors (Vittor, 1971). *Tetraselmis chuii* algae produces multiple dietary carotenoid precursors that can be converted into astaxanthin, most notably violaxanthin, lutein, and β-carotene (Ahmed et al., 2014). Recent work shows that while *Tigriopus californicus* can convert violaxanthin and lutein into astaxanthin, the species does so only to a small degree and instead favors β-carotene as the substrate of choice to produce astaxanthin (Prado-Cabrero et al., 2020). Thus, we assumed that the majority of free astaxanthin produced by copepods came primarily from the hydroxylation of β-carotene (Prado-Cabrero et al., 2020). On the seventh day, copepods were moved to clean artificial saltwater for a minimum of 2 hours to clear any algae remaining in their gut. Copepods were then dried and weighed (±0.001 mg) before being stored in a microcentrifuge tube at -80°C until HPLC analysis (see below).

3.6.3 Carotenoid extraction and HPLC analysis: SD and SCN cross (further details)

Approximately 280mg of .5mm zirconia-silicate ruptor beads and 500uL of HPLC grade acetone was added to the microtubes containing dried and weighed copepods. Tubes were placed in a Mini-Beadbeater machine (Biospec Products) and homogenized at top speed for 30 seconds to break apart the copepod tissue and suspend the pigments in acetone. The acetone was then transferred into a 1.7mL microcentrifuge tube and spun down at 5000g for 5 minutes. The

supernatant was then passed through a disposable 13mm .45um polypropylene filter (Whatman) with an additional 500uL of HPLC grade acetone into a fresh 1.7mL microcentrifuge tube. Tubes were evaporated to dryness in a centrivap concentrator (Labconco). The concentrated contents were resuspended in 50uL HPLC grade acetone, capped with nitrogen gas, and stored at -80°C until ready for HPLC pigment analysis.

3.6.4 HPLC pigment analysis (further details)

Samples were removed from the -80°C freezer. 40uL of HPLC grade water was added to each sample, and samples were vortexed and then spun down. The entire sample (90uL) was loaded into a 200uL flat bottom glass insert within 2mL autosampler vial (Agilent). Pigments were separated using an Agilent HPLC 1260 Infinity II LC system from a 75ul injection into a Waters Symmetry C8 column with a guard cartridge. We used mobile phases A) 50:25:25 Methanol: Acetonitrile: 0.25M Aqueous Pyridine and B) 20:60:20 Methanol: Acetonitrile: Acetone in a linear gradient as follows: starting at 100% A: 0% B to 60% A: 40% B over 18 min, then to 0% A: 100% B over 4 min, hold at 0% A: 100% B for 16 minutes, then back to 100% A: 0% B over 2 min and held for 8 minutes (Zapata, Rodríguez, & Garrido, 2000). Absorbance was measured at 450nm. Carotenoid peaks were identified by comparison to authentic standards (DHI) run through the same method. A final measurement of astaxanthin concentration was normalized to dry weight, resulting in a final measurement of micrograms of astaxanthin per milligram of copepod tissue.

3.6.5 ATP synthesis and mitochondrial volume assays (further details)

We measured ATP synthesis following an assay derived from Ellison & Burton, 2006. Ten males and ten females were homogenized with Dounce homogenizers. Their mitochondria were isolated by placing homogenized samples in a 4°C centrifuge at 1000g for 5 minutes. The supernatant was transferred into a new tube and placed in the centrifuge at 11000g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in assay buffer (560mM sucrose, 100mM KCL, 10mM KH2PO4, and 70mM HEPES). Suspended mitochondria (25uL) from each sample were then added to 5uL of either Complex I (CI) substrate (100mM ADP, 800mM malate, 2M glutamate, and assay buffer) or Complex II (CII) substrate (100mM ADP, 1M succinate, 15uM rotenone, and assay buffer). Samples incubated for 10 minutes at 20°C on a thermo cycler. During incubation, ATP standards of 10,000nM, 5,000nM, 1,000nM, 500nM, 100nM, 100nM, 10nM, 5nM, and 0nM concentrations were added into an opaque white 96-well assay plate preloaded with 25uL of CellTitre-Glo (Promega). A time=0 control group was created, where 25uL of each mitochondrial resuspension was quickly added to 5uL of CI substrate, mixed briefly, and then immediately added into the assay plate preloaded with CellTitre-Glo. After incubation, 25uL of each sample was loaded into the plate and run immediately on a Fluoroskan Ascent FL plate reader (Thermo Labysystems). Using the generated standard curve and subtracting the control group, a final measurement of nanomoles of ATP produced per minute was reported.

The remainder (30uL) of each sample used in the ATP synthesis assay was saved for the citrate synthase assay (Spinazzi et al., 2012b). Five microliters of each sample were added to 50uL of 200mM Tris buffer with 0.2% Triton-X (Sigma), 24uL DI water, 10uL 1mM DTNB (5, 5’-dithiobis(2-nitrobenzoic acid)), and 6uL acetyl coenzyme A (0.3mM). Absorbance was measured at 412nm for 5 minutes to check for background activity. Immediately after the

background run, 5uL of 10mM oxaloacetic acid was added to each sample and read again at 412nm. The slope measured over 5 minutes was used to calculate the concentration of mitochondria in the original sample. ATP synthesis results were divided by this concentration to normalize the data.

3.6.6 Statistical analyses (further details)

All statistical analyses were performed in R (R_Core_Team, 2019) using the 'lme4' (Bates et al., 2014), 'nlme' (Pinheiro et al., 2013), 'lmerTest' (Kuznetsova et al., 2017), 'emmeans' (Lenth et al., 2018), 'MASS' (Venables and Ripley, 2013), 'agricolae' (De Mendiburu, 2014), 'tidyverse' (Wickham, 2017), and 'MuMIn' (Barton, 2009) packages. See R code in the supplement for notation about the role of each package. Figures were built in R using the 'ggpubr' (Kassambara, 2018) and 'cowplot' (Wilke, 2016) packages.

We used mixed-effects linear models and pairwise contrasts corrected for multiple comparisons (using 'emmeans') to see if there were significant differences in astaxanthin accumulation among hybrid and non-hybrid copepod lines. When looking for differences in astaxanthin content between hybrid and non-hybrid copepods on average, we fit models that included a random effect of line ID to account for non-independence of data within each line. We also used linear models and paired contrasts corrected for multiple comparisons to look for statistically significant differences in ATP production and offspring development rate among hybrid and non-hybrid copepods lines.

Because we lacked data from female copepods with many of the crosses, we used male data to analyze the relationships between astaxanthin production, ATP, mitochondrial volume, and offspring development time among all of the lines. We used linear models to assess the

relationship between astaxanthin, ATP production, mitochondrial volume, and offspring development rate. We included mitochondrial type (i.e., the mito type of the maternal population in hybrid crosses) as a random effect. Before fitting the models, ATP, mitochondrial volume, and development time data were log transformed, scaled, and centered to achieve normality (assessed by inspecting model residuals). We averaged replicates from each line to avoid pseudoreplication.

When assessing if there were significant differences in astaxanthin production, ATP production and offspring development rate between generations from the reciprocal cross between SD and SCN populations, we used a linear model and pairwise contrasts corrected for multiple comparisons including a random effect of maternal mito type to account for non-independence of data from each line. To examine the relationship between astaxanthin production, ATP, mitochondrial volume, and offspring development rate with the reciprocal cross, we averaged replicates from each line to avoid pseudoreplication and used linear models. However, because we had a large number of replicates per generation (compared to ATP and mitochondrial volume measurements) when assessing offspring development rate, we also analyzed the relationship between offspring development rate and astaxanthin production within each generation using separate linear models.

3.6.7 Phylogenetic analysis

The evolutionary history of the *Tigriopus californicus* populations used in this study was inferred by using the Maximum Likelihood method and Tamura-Nei model, using *Tigriopus japonicus* as a rooted outgroup (Tamura and Nei, 1993). The tree with the highest log likelihood (-3168.00) is shown. The percentage of trees in which the associated taxa clustered together is

shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 8 nucleotide sequences of the CO1 gene. There were a total of 613 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). Gene sequences were generously shared with us by Reggie Blackwell.

3.7 Supplemental Tables

Table S3.1 A list of the lines from the experimental crosses used in this study. The number of the replicates per line for each measurement and the approximate number of individual copepods per replicate. Shown as: # of line replicates (approx. # of individuals per replicate)

Line (Female pop. x Male pop.)	Astaxanthin	Offspring development rate	ATP	Citrate synthase
SD x AB early generation RILs				
AB x AB	9 (10)	-	-	-
AB x SD	8 (10)	-	-	-
SD-centric and AB-centric multigeneration RILs				
SD x SD	6 (14)	15 (15)	2 (20)	2 (20)
BUF x BUF	7 (14)	5 (6)	7 (20)	7 (20)
BR x BR	7 (16)	6 (15)	6 (20)	6 (20)
AB x AB	5 (16)	-	1 (20)	1 (20)
CAT x CAT	4 (15)	-	3 (20)	3 (20)
PES x PES	6 (15)	6 (13)	2 (20)	2 (20)
BR x SD 45	3 (12)	-	-	-
BR x SD 50	7 (14)	6 (21)	4 (50)	4 (50)
BR x SD 56	1 (10)	6 (8)	4 (56)	4 (56)
BUF x SD 4	7 (14)	6 (16)	6 (20)	6 (20)
BUF x SD 19	4 (15)	-	5 (20)	5 (20)
BUF x SD 24	6 (13)	6 (12)	2 (20)	2 (20)
AB x CAT 11	2 (8)	1 (6)	-	-
CAT x AB 27	2 (9)	2 (12)	2 (20)	2 (20)
PES x AB 20	7 (14)	6 (10)	2 (20)	2 (20)
Reciprocal cross between SD and SCN				
SD parental	9 (10)	7 (20)	5 (20)	4 (17)
SCN parental	19 (10)	8 (20)	5 (20)	5 (11)
F1 generation	24 (10)	-	-	16 (10)
F2 generation	8 (10)	-	-	10 (5)
F3 generation	5 (10)	12 (20)	12 (20)	12 (6)

Table S3.2 Results from statistical models of paired contrasts of astaxanthin production among male copepods from multigeneration RILs and PILs. The model beta estimate represents the difference in the means per group. The confidence limits in the right two columns represent the confidence boundaries around the model estimate. **Bold** lines denote a significant difference between the contrast and reference groups.

Reference group	Contrast	Model estimate (β_1)	SE	*P*-value	Lower 95% CL	Upper 95% CL
Southern RILs and PILs						
BUF	**BUFSD19**	**0.308**	**0.033**	**<0.0001**	**0.212**	**0.404**
	BUFSD24	**0.166**	**0.030**	**<0.0001**	**0.080**	**0.251**
	BUFSD4	**0.160**	**0.030**	**<0.0001**	**0.074**	**0.246**
SD	**BUFSD19**	**0.250**	**0.033**	**<0.0001**	**0.154**	**0.346**
	BUFSD24	**0.108**	**0.030**	**0.0076**	**0.022**	**0.194**
	BUFSD4	**0.102**	**0.030**	**0.013**	**0.016**	**0.188**
	BRSD45	**0.193**	**0.042**	**0.0005**	**0.073**	**0.313**
	BRSD50	**0.182**	**0.034**	**0.0001**	**0.084**	**0.280**
	BRSD56	**0.310**	**0.063**	**0.0003**	**0.127**	**0.494**
BR	BRSD45	0.043	0.042	0.8330	-0.077	0.164
	BRSD50	0.032	0.034	0.8777	-0.066	0.130
	BRSD56	0.160	0.063	0.1106	-0.023	0.344
Northern RILs and PILs						
CAT	CATAB27	-0.257	0.102	0.1222	-0.576	0.061
	ABCAT11	-0.186	0.102	0.3225	-0.505	0.132
AB	CATAB27	-0.211	0.099	0.2118	-0.518	0.097
	ABCAT11	-0.1397	0.097	0.5199	-0.447	0.168
	PESAB20	-0.090	0.034	0.0398	-0.176	-0.004
PES	PESAB20	0.036	0.034	0.5468	-0.050	0.122

Table S3.3 Results from statistical models of paired contrasts of astaxanthin production among copepods from preliminary RILs from the cross between SD and AB populations. The model beta estimate represents the difference in the means per group. The confidence limits in the right two columns represent the confidence boundaries around the model estimate.

Reference group	Contrast	Model estimate (β_1)	SE	*P*-value	Lower 95% CL	Upper 95% CL
Hybrid vs non-hybrid averages						
Non-hybrids	Hybrids	-0.169	0.077	0.0444	-0.320	-0.018
Parental lines vs individual RILs (letters match figure S…)						
AB x AB	A	-0.417	0.085	<0.0001	-0.591	-0.243
	B	-0.199	0.140	0.1675	-0.487	0.089
	C	-0.298	0.140	0.0434	-0.586	-0.009
	D	-0.014	0.108	0.8994	-0.235	0.208
	E	-0.020	0.120	0.8717	-0.265	0.226
	F	-0.276	0.108	0.0167	-0.498	-0.054
	G	0.026	0.189	0.8910	-0.363	0.415
	H	0.162	0.189	0.3996	-0.227	0.551

Table S3.4 Results from statistical models of paired contrasts of astaxanthin: dietary carotenoids among male copepods from multigeneration RILs and PILs. The model beta estimate represents the difference in the means per group. The confidence limits in the right two columns represent the confidence boundaries around the model estimate. **Bold** lines denote a significant difference between the contrast and reference groups.

Reference group	Contrast	Model estimate (β_1)	SE	*P*-value	Lower 95% CL	Upper 95% CL
Southern RILs and PILs						
BUF	**BUFSD19**	**4.375**	**0.859**	**0.0001**	**1.910**	**6.841**
	BUFSD24	**3.582**	**0.768**	**0.0004**	**1.377**	**5.788**
	BUFSD4	1.741	0.768	0.1796	-0.465	3.946
SD	**BUFSD19**	**7.066**	**0.859**	**<0.0001**	**4.600**	**9.531**
	BUFSD24	**6.273**	**0.768**	**<0.0001**	**4.068**	**8.478**
	BUFSD4	**4.431**	**0.768**	**<0.0001**	**2.226**	**6.636**
	BRSD45	**4.536**	**0.957**	**0.0004**	**1.764**	**7.308**
	BRSD50	**6.535**	**0.782**	**<0.0001**	**4.271**	**8.798**
	BRSD56	**7.495**	**1.463**	**0.0001**	**3.262**	**11.729**
BR	BRSD45	-1.247	0.957	0.6916	-4.019	1.524
	BRSD50	0.751	0.782	0.8702	-1.512	3.015
	BRSD56	1.712	1.463	0.7676	-2.522	5.946
Northern RILs and PILs						
CAT	CATAB27	-2.35	3.73	0.9202	-13.995	9.30
	ABCAT11	-10.37	3.73	0.0837	-22.02	1.28
AB	CATAB27	-2.83	3.61	0.8600	-14.08	8.429
	ABCAT11	-10.85	3.61	0.0592	-22.10	0.404
	PESAB20	-1.270	0.658	0.1550	-2.928	0.388
PES	PESAB20	0.637	0.658	0.6040	-1.021	2.295

Table S3.5 Results from statistical models of paired contrasts of dietary carotenoid concentration among male copepods from multigeneration RILs and PILs. The model beta estimate represents the difference in the means per group. The confidence limits in the right two columns represent the confidence boundaries around the model estimate. **Bold** lines denote a significant difference between the contrast and reference groups.

Reference group	Contrast	Model estimate (β_1)	SE	*P*-value	Lower 95% CL	Upper 95% CL
Southern RILs and PILs						
BUF	BUFSD19	-0.0030	0.0142	0.9995	-0.0438	0.0377
	BUFSD24	**-0.0567**	**0.0127**	**0.0007**	**-0.0931**	**-0.0202**
	BUFSD4	0.0021	0.0127	0.9998	-0.0344	0.0386
SD	BUFSD19	-0.0342	0.0142	0.1373	-0.0749	0.0066
	BUFSD24	**-0.08778**	**0.0127**	**<0.0001**	**-0.1243**	**-0.0513**
	BUFSD4	-0.0290	0.0127	0.1730	-0.0655	0.0074
	BRSD45	0.0017	0.0122	0.9999	-0.0337	0.0372
	BRSD50	**-0.0507**	**0.0187**	**0.0001**	**-0.0801**	**-0.0222**
	BRSD56	-0.0004	0.0187	1.000	-0.0545	0.0537
BR	**BRSD45**	**0.0383**	**0.0122**	**0.0287**	**-0.0029**	**0.0738**
	BRSD50	-0.0145	0.0010	0.5986	-0.0435	0.0144
	BRSD56	0.0366	0.0010	0.3204	-0.0179	0.0903
Northern RILs and PILs						
CAT	CATAB27	-0.0105	0.0188	0.9420	-0.0690	0.0481
	ABCAT11	0.0327	0.0188	0.3576	-0.0259	0.0913
AB	CATAB27	0.0332	0.0181	0.3192	-0.0233	0.0898
	ABCAT11	**0.0764**	**0.0181**	**0.099**	**-0.1330**	**0.0198**
	PESAB20	0.0225	0.0133	0.2318	-0.0110	0.0560
PES	PESAB20	-0.0049	0.0133	0.9276	-0.0384	0.0286

3.8 Supplemental Figures

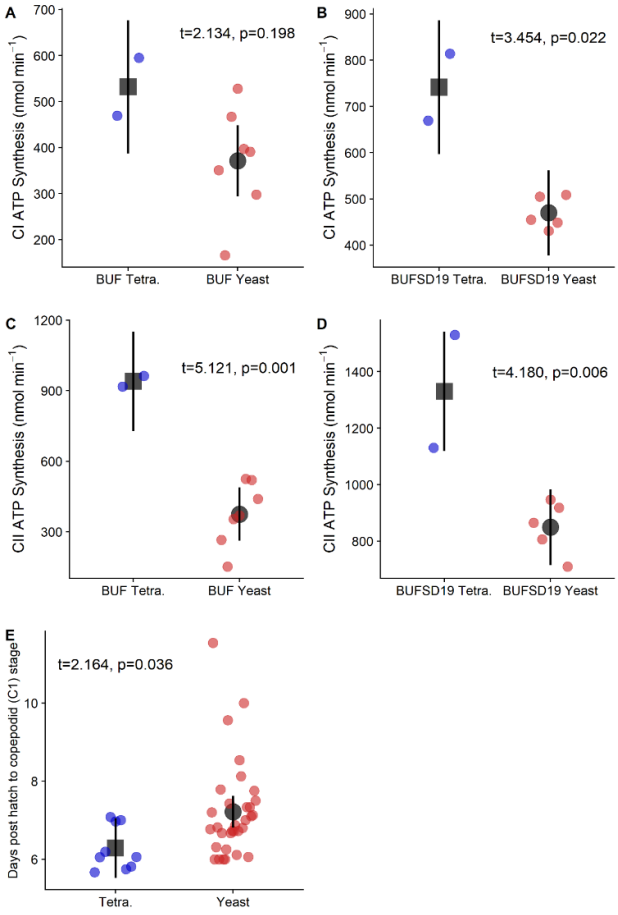

Figure S3.1 ATP production and offspring development between algae-fed (Tetra.) and yeast-fed copepods. Individual replicates from yeast-fed copepods are shown in red and data from algae-fed copepods are in blue. A) Difference in Complex I ATP production between the La Bufadora parental line on each diet, B) difference in Complex I ATP production between hybrid copepods from the BUF x SD 19 line on each diet, C) difference in Complex II ATP production from the La Bufadora parental line on each diet, D) difference in Complex II ATP production between the hybrid copepods from the BUF x SD 19 line on each diet, and E) difference in offspring development time between all parental copepod lines fed either algae or yeast. Black dots are group averages and black bars are 95% confidence intervals around the mean.

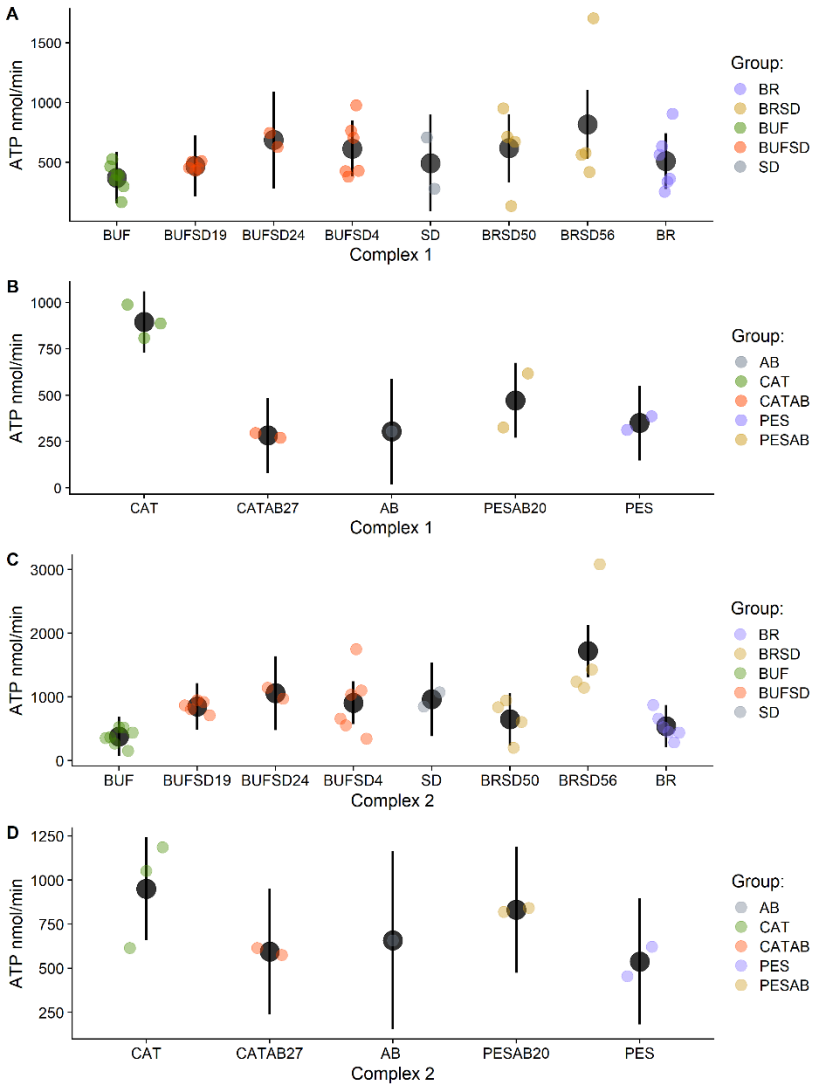

Figure S3.2 Differences among hybrid RILs and non-hybrid PILs from multigeneration crosses fed yeast only in A-B) Complex I ATP production and C-D) Complex II ATP production. Colored dots represent replicates within each line that are the average ATP produced by isolated mitochondria from 20 pooled copepods. Dark grey circles are the line average. Black lines and colored shading are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines.

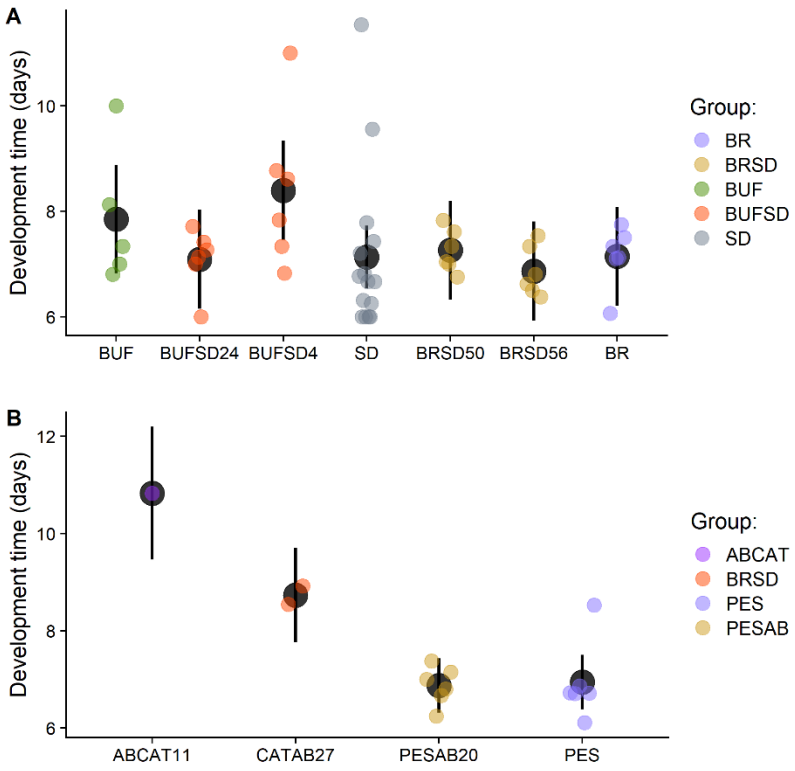

Figure S3.3 Differences among hybrid RILs and non-hybrid PILs from multigeneration crosses fed yeast only in offspring development time (days post hatch to first copepodid stage). Colored dots represent replicates within each line that are the average values produced by individual females. Dark grey circles are the line average. Black lines and colored shading are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines.

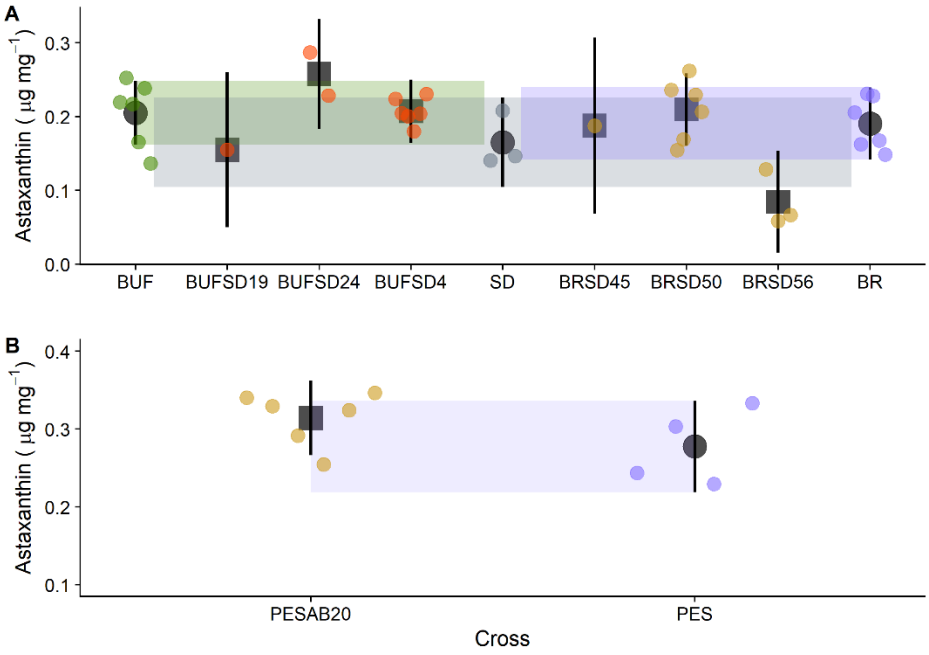

Figure S3.4 Astaxanthin production among female hybrid RILs and non-hybrid PILs from multigeneration crosses. Colored dots represent replicates within each line that are the average astaxanthin level of approximately 10 copepods. Dark grey circles (parental lines) and dark grey squares (hybrid lines) are the line average. Black lines and colored shading are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines.

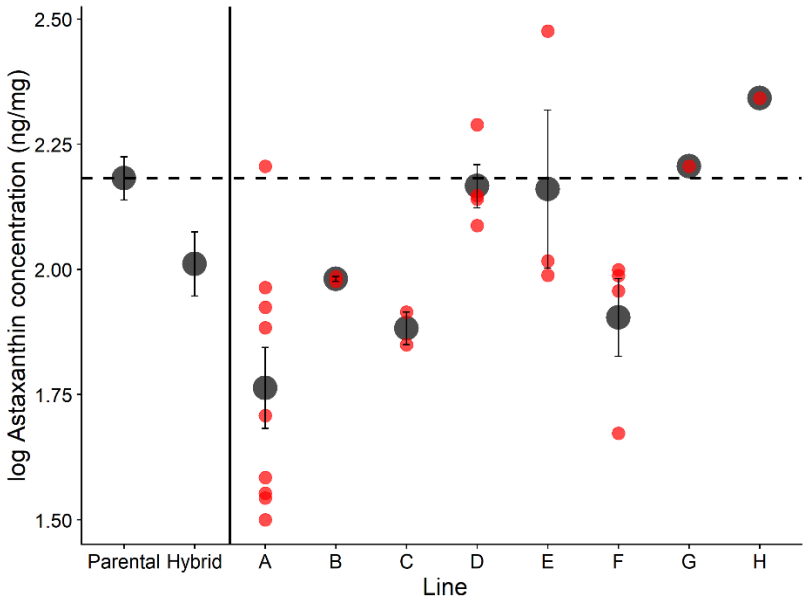

Figure S3.5 Line averages (black dots) and individual replicates (red dots) in astaxanthin production among AB x SD hybrid lines (A-H; 'Hybrid' represents average among all lines) and in parental (AB) copepods. Black bars show standard error. After one generation of inbreeding, 9 AB parental lines and 8 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 highly sensitive to inbreeding (Brown, 1991), which is likely the cause of the mortality that we observed in SD parental and SD x AB hybrid lines.

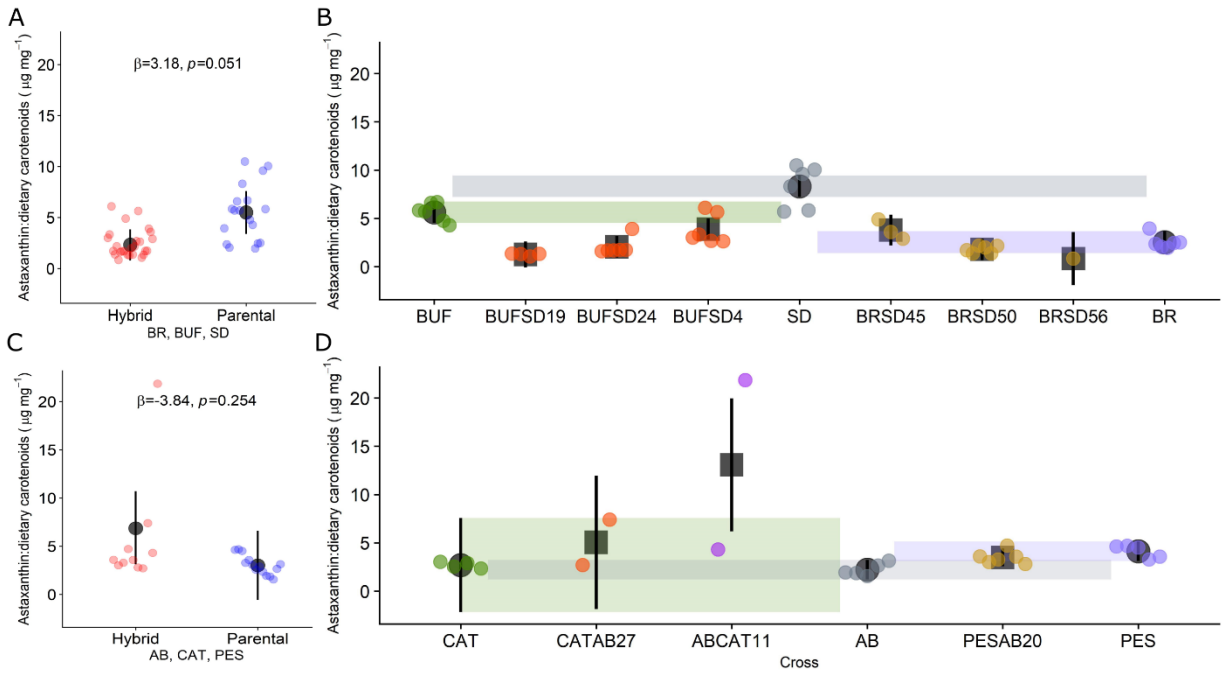

Figure S3.6 Differences in astaxanthin: dietary carotenoid concentration ratio among hybrid RILs and non-hybrid PILs from multigeneration crosses. Colored dots represent replicates within each line that are the average carotenoid ratio of approximately 10 copepods. Dark grey circles (parental lines) and dark grey squares (hybrid lines) are the line average. Black lines and colored shading are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines (Table S4).

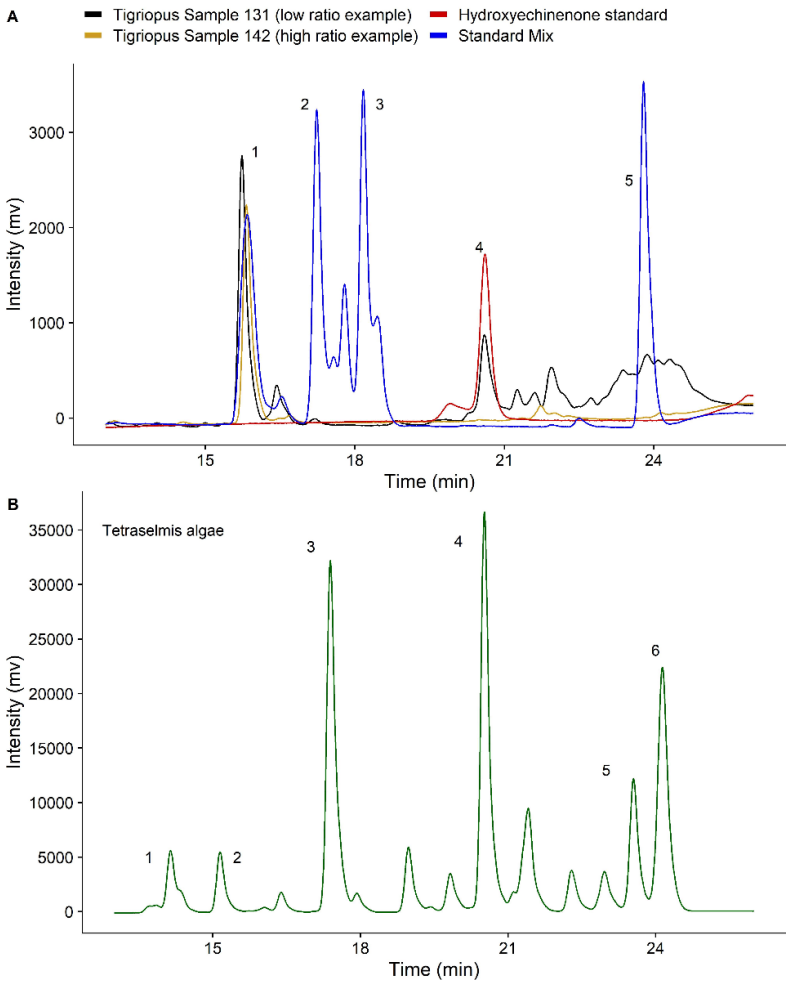

Figure S3.7 A) HPLC chromatograms of a mix of carotenoid standards (blue), hydroxyechinenone standard (red), a copepod sample with a low ratio of astaxanthin to dietary carotenoids (black), and a copepod samples with a high ratio of astaxanthin to dietary carotenoids (yellow). Numbered peaks are as follows: 1) astaxanthin, 2) lutein, 3) canthaxanthin, 4) hydroxyechinenone, 5) β-carotene. B) HPLC chroma profile of the Tetraselmis algae we fed the copepods during the experiment. Numbered peaks are as follows: 1) violaxanthin isomer, 2) trans-violaxanthin, 3) lutein, 4) hydroxyechinenone, 5) α-carotene, 6) β-carotene. Of these carotenoids, only trans-violaxanthin, hydroxyechinenone and β-carotene are precursors to astaxanthin [9, 32].

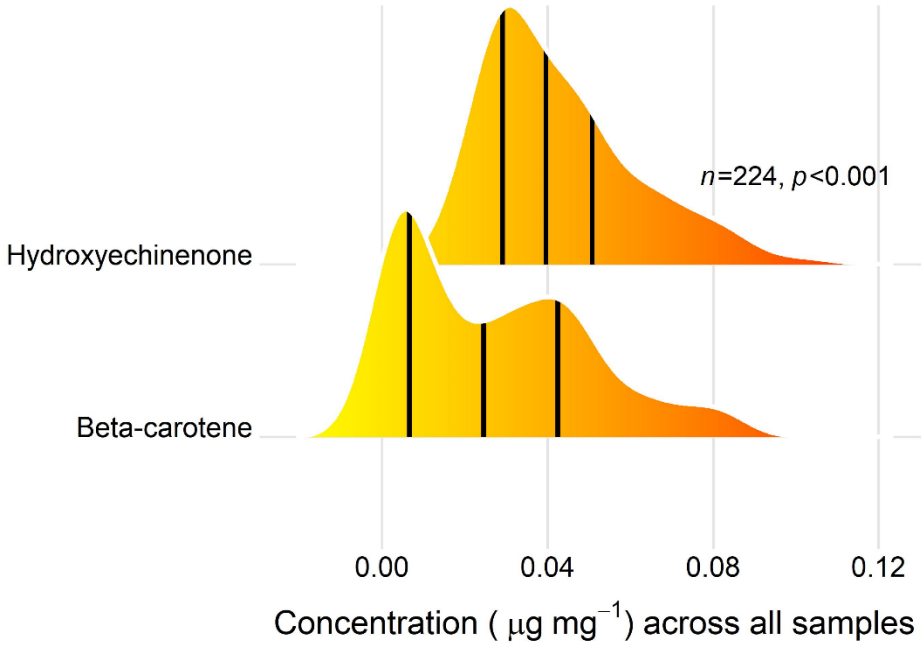

Figure S3.8 Ridgeline plot showing the distribution of hydroxyechinenone and β-carotene concentrations across all copepod samples from the yeast-only RILs and PILs. The black lines represent the median and upper/lower quartiles of each group. The p-value was derived from a linear mixed effects model with inbred line ID encoded as a random effect.

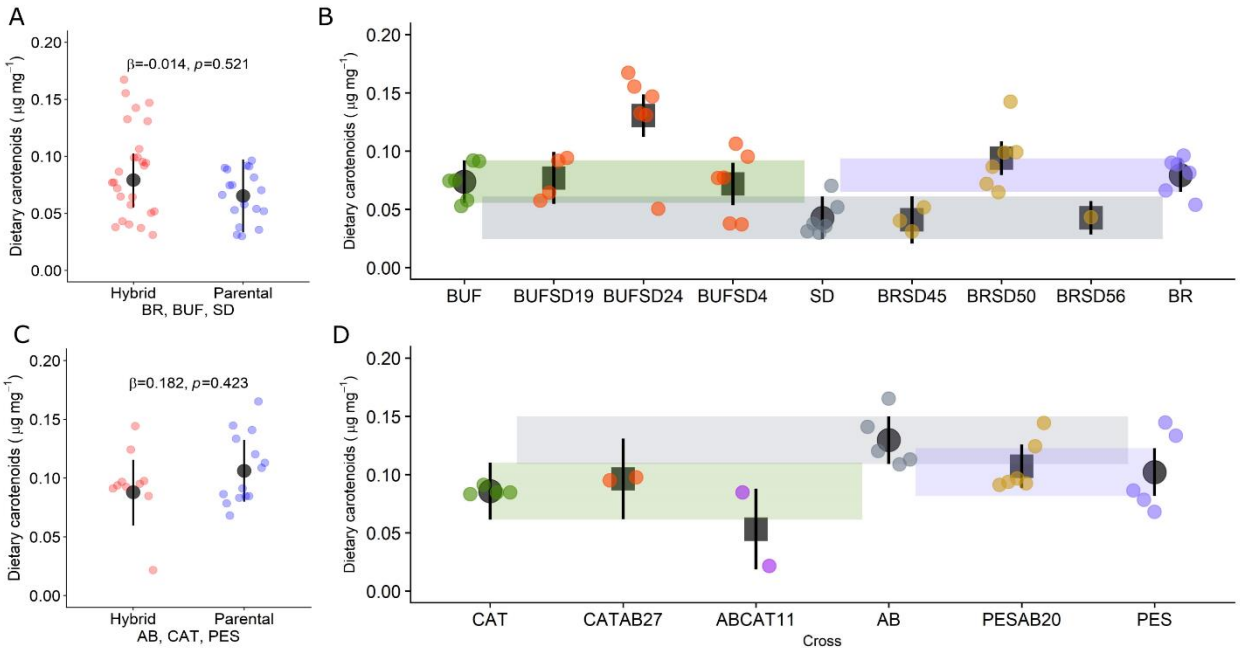

Figure S3.9 Differences in dietary carotenoid concentration among hybrid RILs and non-hybrid PILs from multigeneration crosses. Colored dots represent replicates within each line that are the average of approximately 10 copepods. Dark grey circles (parental lines) and dark grey squares (hybrid lines) are the line average. Black lines and colored shading are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines (Table S5).

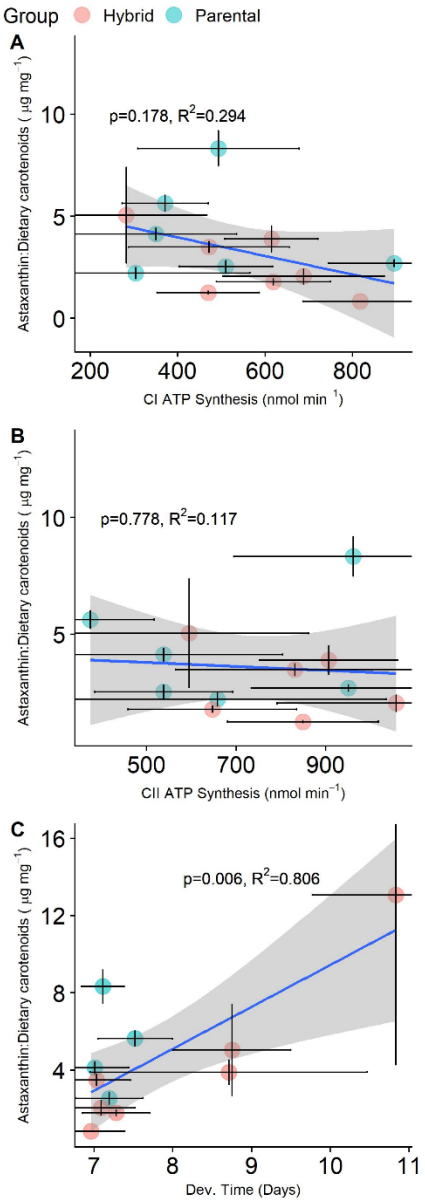

Figure S3.10 Relationships between astaxanthin: dietary carotenoids and either ATP production or offspring development rate among all hybrid and non-hybrid lines. Colored dots represent line averages and the vertical and horizontal black bars extending from the colored dots represent the standard error of each trait. The grey shading is the 95% confidence interval around the model estimated slope. Adjusted R^2 values are shown.

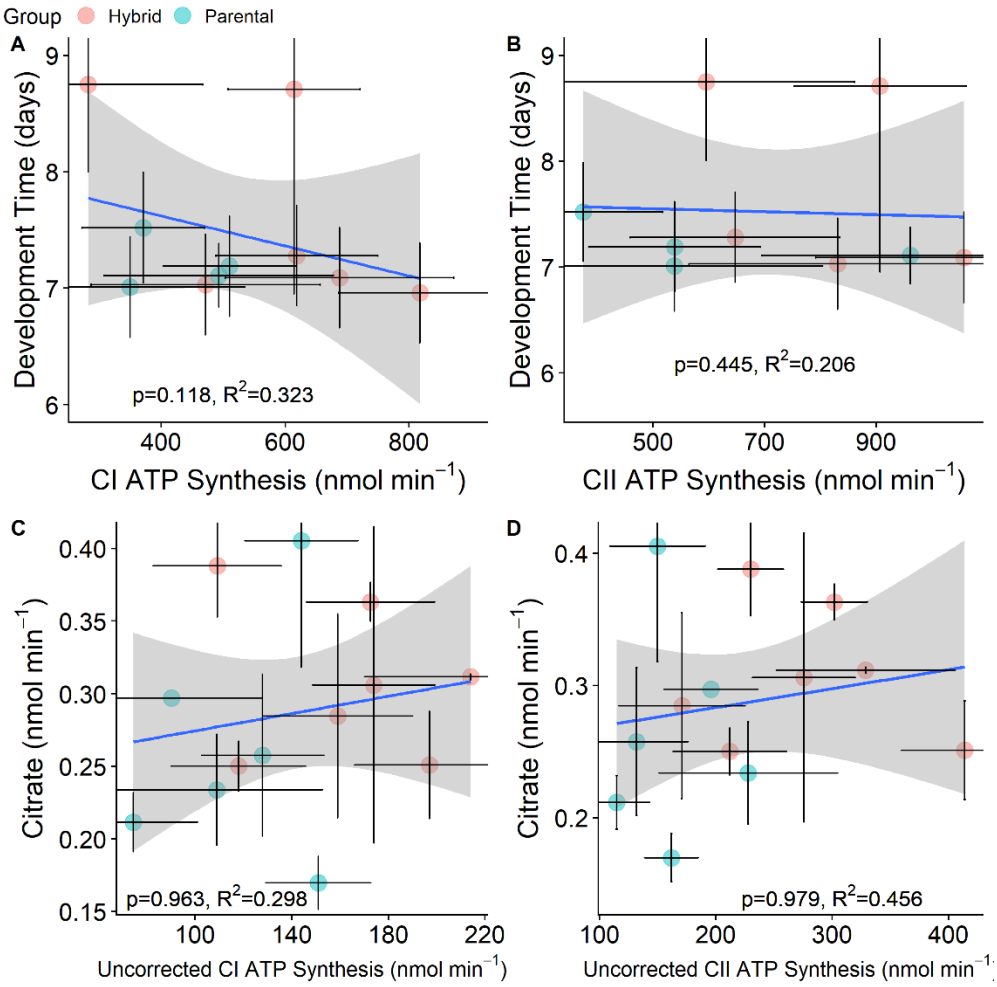

Figure S3.11 Relationships between ATP production and either offspring development time (days post hatch to first copepodid stage; A and B) or mitochondrial volume (C and D) among hybrid and non-hybrid lines. Colored dots represent line averages and the vertical and horizontal black bars extending from the colored dots represent the standard error. The grey shading is the 95% confidence interval around the model estimated slope. Adjusted R^2 values are shown.

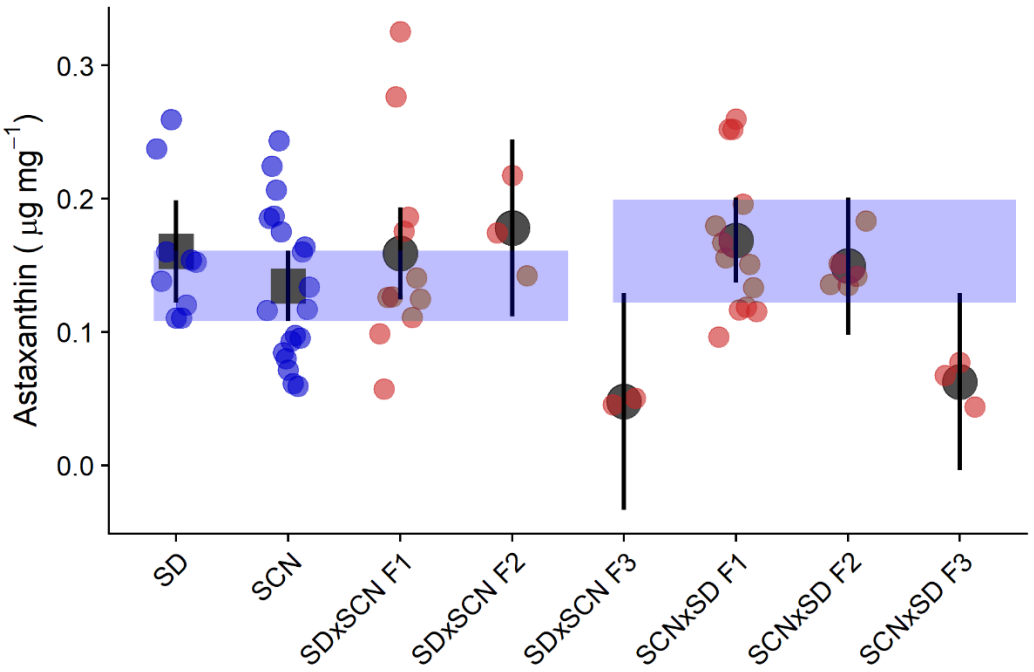

Figure S3.12 Astaxanthin produced by copepods each generation of a reciprocal cross between SD and SCN populations. Groups are split by the direction of the cross (SCN female x SD male on the left, and SD female x SCN male on the right). Colored dots represent replicates within each group that are the average astaxanthin level of approximately 10 copepods per replicate. Dark grey circles are the group average. Black lines and colored shading are 95% confidence intervals around the group average. Where confidence intervals do not overlap, there is a statistically significant difference between groups.

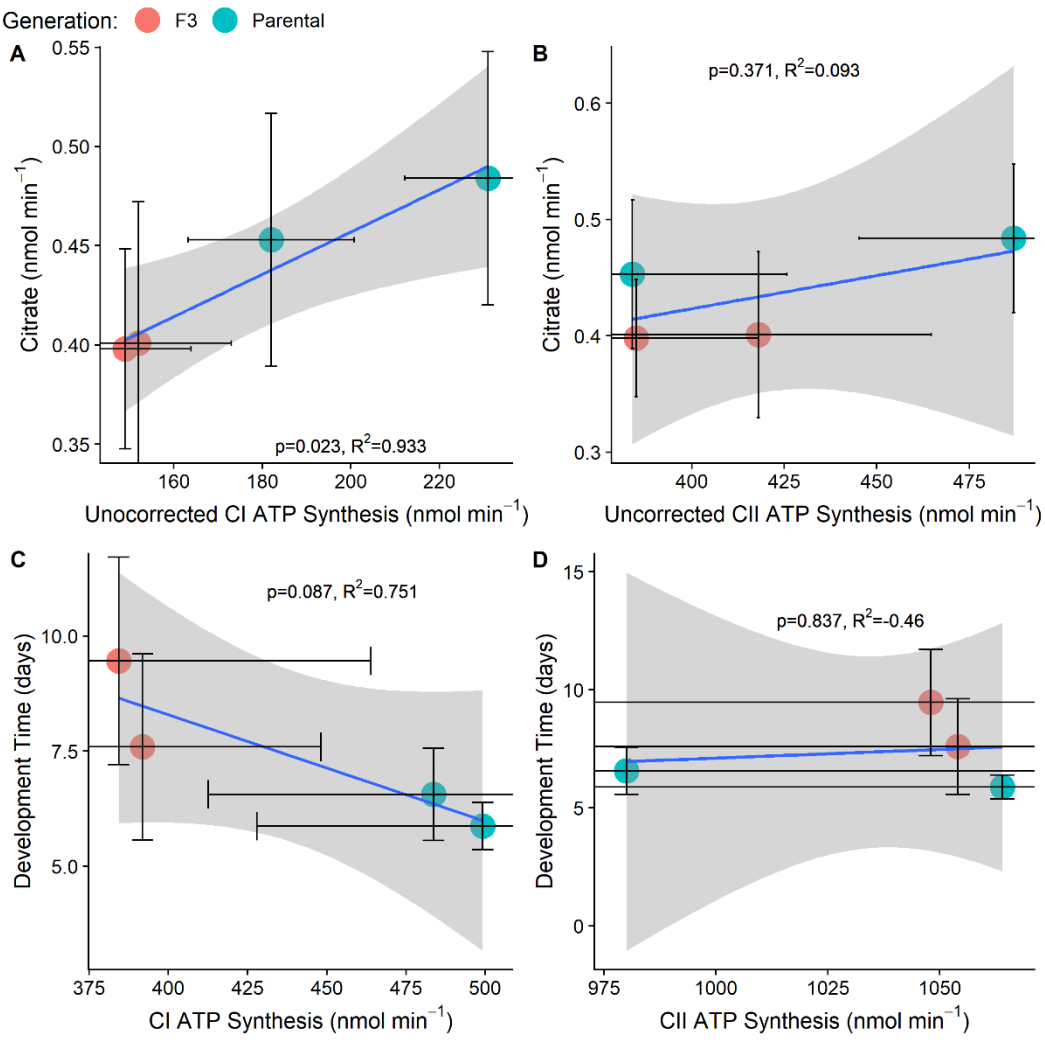

Figure S3.13 Relationships between ATP production and mitochondrial volume (A and B) or offspring development time (C and D) among hybrid and non-hybrid lines from the reciprocal cross between SD and SCN populations. Colored dots represent line averages and the vertical and horizontal black bars extending from the colored dots represent the standard error. The grey shading is the 95% confidence interval around the model estimated slope. Adjusted R^2 values are shown.

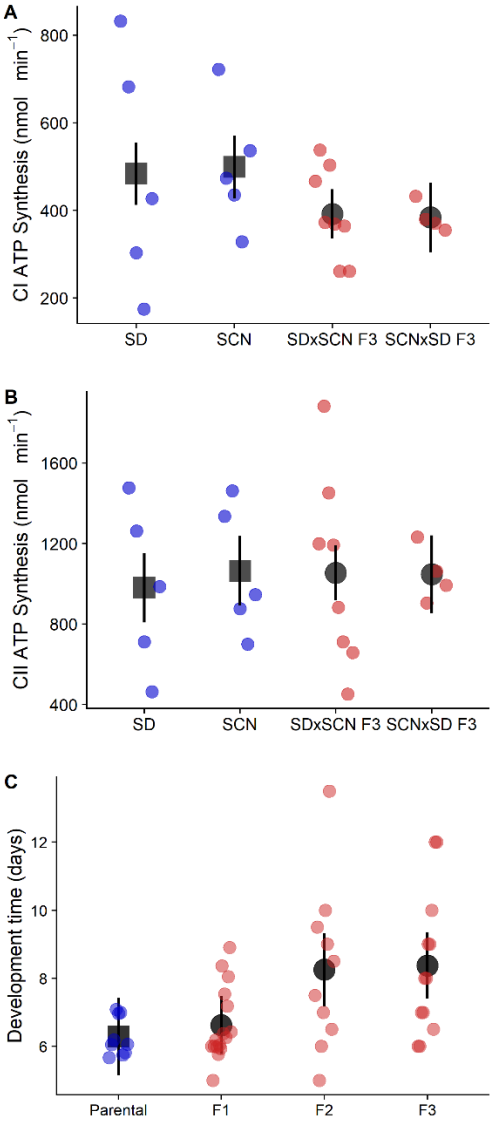

Figure S3.14 Differences among hybrid RILs and non-hybrid PILs from the reciprocal cross in A) Complex I ATP production, B) Complex II ATP production, and C) offspring development rate. Colored dots represent replicates within each line that are either the average ATP produced by isolated mitochondria from 20 pooled copepods (A and B) or clutch averages from individual females (C). Large, dark grey circles and squares are the line average. Black lines are 95% confidence intervals around the line average. Where 95% confidence intervals do not overlap, there is a statistically significant difference between lines.

Chapter 4

Chemical manipulation of mitochondrial function affects metabolism of red carotenoids in a marine copepod *(Tigriopus californicus)*

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4.1 Abstract

The Shared-Pathway Hypothesis offers a cellular explanation for the connection between ketocarotenoid pigmentation and individual quality. Under this hypothesis, ketocarotenoid metabolism shares cellular pathways with mitochondrial oxidative phosphorylation such that red carotenoid-based coloration is inextricably linked mitochondrial function. To test this hypothesis, we exposed *Tigriopus californicus* copepods to a mitochondrially-targeted protonophore, 2-4-dinitrophenol (DNP), to induce proton leak in the inner mitochondrial membranes. We then measured whole-animal metabolic rate and ketocarotenoid accumulation. As observed in prior studies of vertebrates, we observed that DNP treatment of copepods significantly increased respiration and that DNP-treated copepods accumulated more ketocarotenoid than control animals. Moreover, we observed a relationship between ketocarotenoid concentration and metabolic rate, and this association was strongest in DNP-treated copepods. These data support the hypothesis that ketocarotenoid and mitochondrial metabolism are biochemically intertwined. Moreover, these results corroborate observations in vertebrates, suggesting a fundamental connection between ketocarotenoid pigmentation and mitochondrial function.

4.2 Introduction

Ornamental traits can provide reliable information about the condition or quality of an organism (Bonduriansky, 2007; Hill, 2011; Rowe and Houle, 1996). The mechanism by which such honest signaling is achieved remains a focus of debate (Higham, 2014; Searcy and Nowicki, 2010; Smith and Harper, 2003). The Shared-Pathway Hypothesis proposes that some ornaments act as condition-dependent signals of quality because their production is intimately tied to the same vital cellular processes that give rise to individual performance, regardless of the influence of sexual selection (Hill, 2011; Hill, 2014a; Hill, 2014b; Powers and Hill, 2021; Weaver et al., 2017). The hypothesized consequence of such a link is that condition-dependent traits serve as indices of individual quality, such that expression of the ornamental trait reflects the function of internal processes required for the maintenance of life (Biernaskie et al., 2014; Hill, 2011). Specifically, the dynamics of the mitochondrial organelle, a critical, cellular hub of organismal function and condition (Havird et al., 2019; Heine and Hood, 2020; Picard and Sandi, 2020), has been identified as a candidate regulator in the expression of condition-dependent traits (Hill, 2014a; Hill, 2014b; Koch et al., 2017).

A primary and well-studied example of a condition-dependent ornamental trait is bright red coloration that arises from the accumulation of carotenoid pigments in animal tissues (Blount and McGraw, 2008; Hill, 2002; Svensson and Wong, 2011). With few exceptions, animals cannot synthesize carotenoid pigments *de novo*; they must acquire carotenoids through their diet in a trophic manner (Svensson and Wong, 2011). Further, once ingested, many animals metabolize dietary carotenoids into other carotenoid forms in order to produce pigments with new functional roles or new colors (Maoka, 2011; Von Lintig, 2010). This process can occur through a series of oxidation reactions, such as hydroxylations and/or ketolations, that may cause

a color change from the yellow hues of dietary carotenoids to red hues of metabolized carotenoids (Meléndez-Martínez et al., 2007). For example, many crustaceans convert yellow dietary xanthophylls found in algae to red ketolated carotenoids (ketocarotenoids) like astaxanthin. Many animals display astaxanthin-based coloration including birds (Hill and McGraw, 2006), insects (Kayser, 1982), reptiles (Czeczuga, 1980), amphibians (Baruah et al., 2012), fish (Seabra and Pedrosa, 2010), and crustaceans (Maoka, 2011). Astaxanthin is also often recognized as a potent antioxidant and potential immune system regulator (Dose et al., 2016; Naguib, 2000; Weaver et al., 2018b).

The Shared-Pathway Hypothesis, as it applies to ketocarotenoid-based coloration, posits that ketocarotenoid accumulation is intrinsically linked to individual condition through metabolic pathways shared with oxidative phosphorylation (OXPHOS) in the mitochondria (Hill, 2014a; Hill, 2014b; Koch et al., 2017; Powers and Hill, 2021). Under this hypothesis, the bioconversion of red ketocarotenoid pigments is hypothesized to share a biochemical environment with the enzyme complexes in the electron transport system (Hill et al., 2019; Powers and Hill, 2021). These complexes are positioned within the inner mitochondria membrane (IMM) and produce a proton gradient across the membrane needed for the synthesis of ATP. This activity requires a series of reduction-oxidation (redox) reactions driven by coupled electron donors and acceptors, such as NADH/NAD+. The enzymes responsible for the production of ketocarotenoids also metabolize dietary carotenoids using coupled redox reactions, likely utilizing similar electron donor/acceptor systems (Hill and Johnson, 2012; Lopes et al., 2016; Munro et al., 2007; Twyman et al., 2018). Indeed, ketocarotenoids have been observed at the inner mitochondrial membrane in red birds and molecular modeling of the avian carotenoid bioconversion enzyme, CYP2J19, suggests it may localize either in the IMM or mitochondria-associated membranes (MAM) (Ge

et al., 2015; Hill et al., 2019). Moreover, positive relationships between resting metabolic rate, mitochondrial damage, lower rates of mitochondrial replacement, and ketocarotenoid-based coloration have been observed in red birds, suggesting a potential for ketocarotenoid coloration to reflect metabolic capacity in the face of ongoing cellular stress (Hill et al., 2019). Observations in arthropod systems corroborate these observations in vertebrate systems. In the marine copepod *Tigriopus californicus* (Baker, 1912), interpopulation hybrids show a significant positive relationship between oxygen consumption and ketocarotenoid accumulation (Powers and Hill, 2021) and a significant negative relationship between ATP production and ketocarotenoid coloration (Powers et al., 2021), thus indicating that ketocarotenoid accumulation may reliably indicate the capacity to withstand stress and exert energy (Völker, 1957) in invertebrate species as well.

To test the Shared-Pathway Hypothesis, we experimentally exposed *T. californicus* copepods to a mitochondrially targeted compound, 2-4-dinitrophenol (DNP) and measured their accumulation of ketocarotenoid pigmentation. This copepod, native to splash-pools along the west coast of North America, accumulates astaxanthin to produce its vibrant, red coloration, using many of the same carotenoid substrates preferred by red birds and other terrestrial organisms (Prado-Cabrero et al., 2020; Weaver et al., 2018a). Moreover, *T. californicus* ketocarotenoid-based coloration has also been shown to be dependent on individual condition (Powers et al., 2021; Weaver et al., 2016; Weaver et al., 2018b). In birds, mitochondria-targeted compounds, such as synthetic ubiquinones and superoxide dismutase mimetics, have been observed to alter ketocarotenoid metabolism (Cantarero and Alonso-Alvarez, 2017; Cantarero et al., 2020a; Cantarero et al., 2020b). However, these compounds may also increase ROS levels as they alter oxidative state in the mitochondria, and this may, in turn, influence ketocarotenoid

levels given their possible role in ROS scavenging (Dose et al., 2016; Naguib, 2000). In contrast, the ionophore DNP does not incur this complication (Goldgof et al., 2014; Korde et al., 2005; Leverve et al., 1998; Lozinsky et al., 2013; Rognstad and Katz, 1969; Stier et al., 2014). This compound functions as a mitochondrial "uncoupler", acting as a protonophore in the IMM of living mitochondria to dissipate the proton gradient required to make ATP (Fig. 1A) (Pinchot,

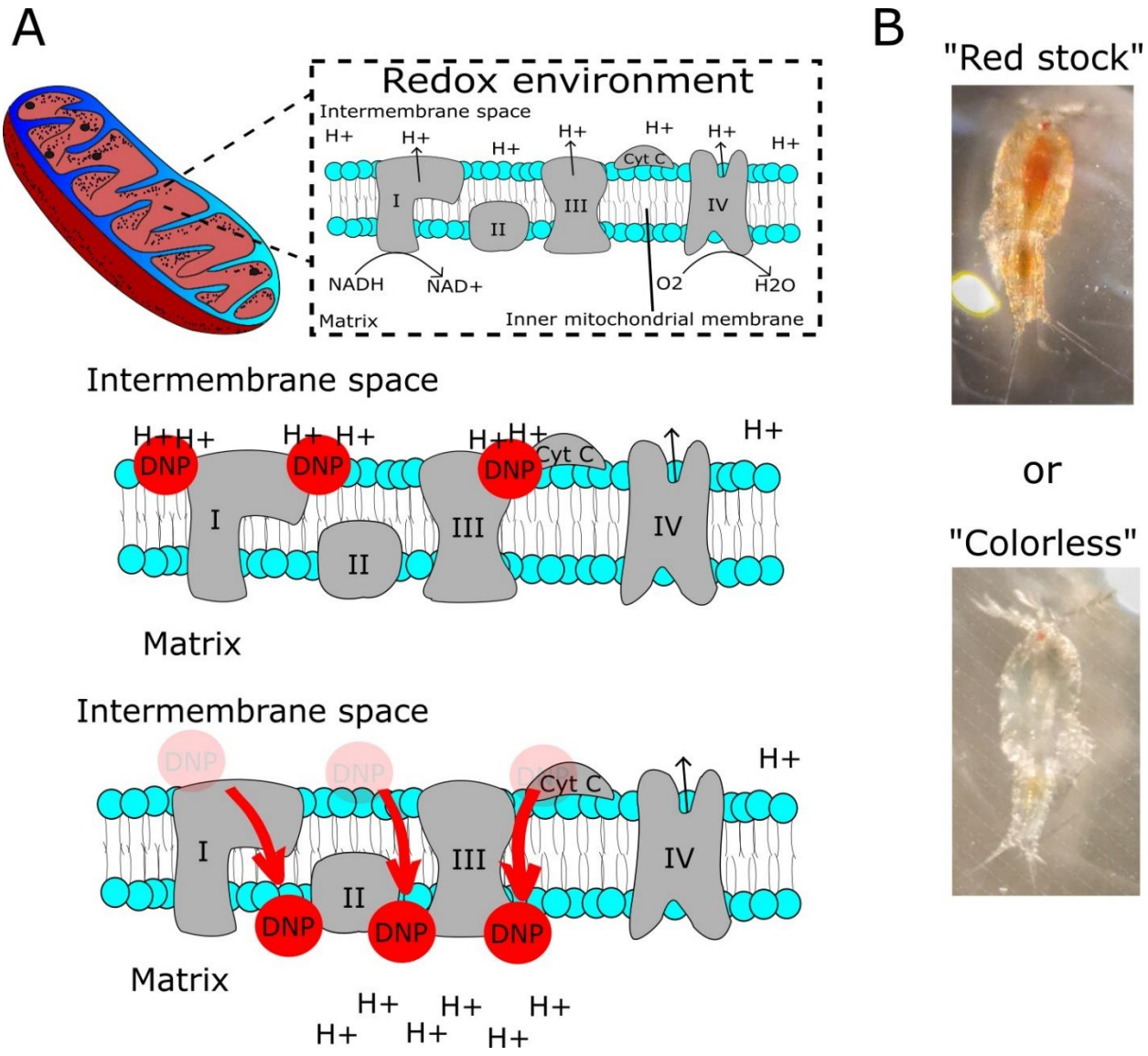

Figure 4.1 A simple representation of the mechanism by which DNP acts as a protonophore in the mitochondria. A) DNP binds to hydrogen protons that have been pumped into the intermembrane space during the process of oxidative phosphorylation in the electron transport system. DNP moves across the inner mitochondrial membrane and releases the hydrogen protons back into the mitochondrial matrix, thereby dissipating the proton gradient required to produce ATP via ATP synthase (not pictured). B) Photographs of the red stock copepods and "colorless" copepods cultured in our laboratory.

1967). At sublethal, acute doses, DNP, therefore, increases the rate of respiration in animals, including crustaceans (Caldeira da Silva et al., 2008; Cantelmo et al., 1978; Geisler et al., 2017). We predicted that, if carotenoid metabolizing enzymes share an oxidative environment with mitochondrial OXPHOS enzymes, DNP should simultaneously increase respiration rate and ketocarotenoid metabolism in *T. californicus* copepods. We tested this prediction using copepods with natural levels of astaxanthin and copepods deficient of carotenoids to test their ability to restore pigmentation from a colorless state (Figure 4.1).

4.3 Materials and Methods

4.3.1 Copepod Culture Conditions

Tigriopus californicus cultures were maintained in a climate-controlled incubator under the following conditions: 32 psu artificial seawater (ASW) at 20 ºC on a 12:12h light: dark cycle and fed live microalgae (*Tetraselmis chuii*). The cultures used in this experiment were maintained for approximately three years as continuous cultures. Cultures were a mix of commercially obtained copepods (Reef Nutrition, Campbell, CA, USA) and admixed wild populations collected from San Diego, Bird Rock, Abalone Cove, Santa Cruz, and Bodega Bay, California. The cultures used in this experiment were admixed once a month (approx. once per generation) via transfer of water and individuals amongst all culture vessels in attempt to maintain one, large panmictic culture. Lab-reared copepods fed *Tetraselmis* retain their red coloration similar to wild populations (Davenport et al., 2004; Weaver et al., 2018a) and the *Tetraselmis* diet approximates the algae-based diet found in natural splash-pools (Powlik et al., 1997; Vittor, 1971). *Tetraselmis* algae provides dietary carotenoid precursors which *T. californicus* metabolize to synthesize astaxanthin, specifically β-carotene and

hydroxyechinenone (Ahmed et al., 2014; Powers et al., 2021; Prado-Cabrero et al., 2020; Weaver et al., 2018a). We refer to copepods cultured under these conditions as “red stock copepods”.

“Colorless” *T. californicus* cultures were established by isolating gravid female copepods from the stock conditions described above and gently removing their eggs. Eggs were placed in a separate container containing only clean artificial saltwater and after hatch, were reared on ground nutritional yeast powder, but no algae (and thus, no dietary carotenoids). These nearly clear copepod cultures were kept in the dark 24 hours of the day to minimize the chance of algae growth in the culture containers. As these copepods did not have access to dietary carotenoids, they remained almost entirely clear in color throughout their entire development (Powers et al., 2020; Powers et al., 2021; Weaver et al., 2018a).

4.3.2 2,4-Dinitrophenol Dosages and Lethality Assay

Before experimentation, we performed survival assays to identify a maximum concentration of DNP (TCI, Portland, OR, USA) that did not produce lethality. DNP was dissolved in 32 psu ASW to make the following stock concentrations: 100µM, 50µM, 25µM, 10µM, 2µM, 1µM, 0.5µM, and 0µM (control). To assay survival at each of these concentrations, we placed five copepods in a single well of a 24-well plate with 50µL of concentrated *Tetraselmis* algae and monitored for individual death every day for four days. This was repeated five times each for each concentration, for a total of 25 copepods per DNP concentration. The highest concentration of DNP tested, 100uM, was more than 80% lethal after just 24 hours (Figure S4.1). The next highest concentration, 50µM, reached 50% lethality after 48 hours (Figure S4.1). 25µM DNP neared 65% lethality after four days exposure. 10µM DNP saw less than 15% lethality after four days, was comparable to the death observed in the control group

(0µM, Figure S4.1). The lowest concentrations of DNP tested, 2µM, 1µM and 0.5µM, all showed minimal or no lethality after four days (Figure S4.1). This was less than the death observed in the control group (0µM DNP) after four days (Figure S4.1). Based on these results, 10µM and 2µM DNP were chosen as the maximum acute dosages for the experiment. These two concentrations were chosen to maximize the effect of DNP on respiration over a short period of time, without reaching 50% lethality during experimental exposure. Thus, the lowest concentrations, 1uM and 0.5uM, and highest concentrations, 25µM and higher, were not tested in the subsequent respiration and carotenoid metabolism assays to minimize the risk of observing no effect on respiration and to minimize the risk of cytotoxicity, respectively.

4.3.3 2,4-Dinitrophenol Exposure and Carotenoid Metabolism Assay

We exposed adult copepods (three individuals per replicate) to either 0 (control), 2, or 10 µM DNP by placing them in 5 mL test solutions of ASW in six-well plates. Each replicate of three copepods was maintained through respiration and HPLC analyses (i.e., the three individuals were isolated as one unit). We separated adult copepods by sex (using morphology of forward antennules) due to observed differences in carotenoid ketolation in previous experiments (Powers et al., 2021) and maintained them under the standard rearing conditions described above for three or seven days (Table 1). At the beginning of the experiments, we added 50 µL of concentrated, live *Tetraselmis* algae as food and a source of dietary carotenoids (Table 1). This serving of algae was *ad libitum*: at the end of the DNP exposure time, the water was still green with algal cells. We exposed red stock copepods to DNP under the following conditions: 10uM DNP for three days, 10uM DNP for seven days, and 2uM DNP for seven days. We first started with only 3-day exposure to the higher dose of DNP, 10µM, in order to minimize the risk of death. When this period of time did not result in elevated death compared to controls, we

extended the exposure time to seven days to test whether oxygen consumption remained elevated at a slightly longer exposure period. We then exposed red stock copepods to 2μM DNP for the same week-long period in order to facilitate a comparison to colorless copepods exposed to DNP under the same regime (see further rationale below).

Table 1: A summary of experimental trials and treatments used in this study.

Treatment	**Copepod type**	**Days Exposure**	**Culture food source**	**Measurements**
10μM DNP	Red stock	3	*Tetraselmis chuii*	Respiration + HPLC
10μM DNP	Red stock	7	*Tetraselmis chuii*	Respiration*
2μM DNP	Red stock	7	*Tetraselmis chuii*	Respiration + HPLC
2μM DNP	Colorless	7	Yeast powder	Respiration + HPLC

*These tissues were not stored at -80°C following respiration. Thus, we could not perform HPLC analysis.

We exposed colorless copepods to DNP in a single concentration and exposure time, 2μM DNP for seven days, in order to test carotenoid metabolism from a colorless state during acute exposure to DNP. 2μM DNP was chosen over 10μM for the experiment with colorless copepods due to previous observations that yeast-fed, colorless copepods may be more susceptible to oxidative stress than red stock, algae fed copepods (Powers et al., 2021). The exposure time of seven days was chosen to ensure copepods had time to 1) respond to mitochondrial uncoupling and 2) metabolize enough astaxanthin to produce a strong signal during HPLC analyses of copepod body tissue. At a minimum, *T. californicus* needs 48 hours to produce detectible amounts of astaxanthin from a colorless state (Weaver et al., 2018a), and takes about two weeks to fully restore astaxanthin concentrations to that of red stock cultures when feeding on *Tetraselmis* algae (Davenport et al., 2004). At the end of DNP exposure, algae feeding, and carotenoid restoration, the colorless copepods regained visible red coloration; thus,

we refer to these as “color-restored” in Figures displaying the carotenoid content of these copepods.

4.3.4 Whole Animal Respiration Rate

We measured whole animal oxygen consumption rates of control and DNP-exposed copepods using a 24-well microplate respirometer (Loligo Systems, Copenhagen, Denmark). Prior to respiration measurement, copepods were moved to a 6-well plate with clean ASW and allowed to clear their gut contents for a minimum of one hour. After this period, the three copepods from each replicate of the DNP-exposure experiment were added to separate 80 μL wells of the microrespirometer filled with 32 psu ASW. Copepods were left undisturbed in the wells for 30 minutes prior to recording oxygen consumption. We then sealed the wells using a gel seal and weighted block. Oxygen consumption was recorded over 30 minutes after an initial burn-in period of 5 minutes. Throughout the measurement process, the temperature was 22°C. Final oxygen consumption was calculated as mmol oxygen consumed min^{-1} per mg of copepod body weight. This was calculated by calculating the slope of the raw oxygen values over time in minutes and subtracting the average slope of empty blank wells (minimum 3 per plate). Copepod body weight was measured using a precision microscale (accurate to 0.001 mg) following oxygen consumption. Prior to weighing, the copepods were dried under air flow in a fume hood for 30 minutes to obtain dry body weight. The copepods were then placed in microtubes and frozen in a -80C freezer until HPLC analysis.

4.3.5 HPLC Analysis of Carotenoids

Carotenoids were extracted from dried copepod tissues using sonication in acetone. We extracted from the three copepods per replicate as a single pooled sample. Centrifugation was used to pellet and remove cellular debris, saving the carotenoid extract suspended in acetone.

The carotenoid extract was evaporated to dryness and resuspended in 50 µL acetone for HPLC analysis. We separated and quantified copepod carotenoids using HPLC following the methods of Weaver et al. (2018a) and Wright et al. (1991). We injected 10 µL of carotenoid extract in acetone on to a Sonoma C18 column (10 µm, 250 x 4.6 mm, ES Technologies, New Jersey, USA) fitted with a C18 guard cartridge. Carotenoids were separated using a Shimadzu Prominence HPLC system with mobile phases A 80:20 methanol: 0.5M ammonium acetate, B 90:10 acetonitrile: water, and C ethyl acetate in a tertiary gradient of 100%A to 100%B over 4 min, then to 80% C: 20% B over 14 min, back to 100% B over 3 min, and returning to 100% A over 5 min and held for 6 min [9, 52]. We visualized and detected carotenoid absorbance using a Prominence UV/Vis detector set to a wavelength of 450 nm. We identified and quantified carotenoids by comparison to authentic standards that included: astaxanthin, zeaxanthin, β-carotene, lutein, hydroxyechinenone, and canthaxanthin (Figure S4.2). We normalized carotenoid concentration of each carotenoid detected by the dry weight of each copepod sample (reported as µg carotenoid per mg copepod tissue).

4.3.6 Statistical Analyses

All statistical analyses were performed in R (R_Core_Team 2019) using the following packages: ‘lme4’ (Bates et al., 2014), ‘Hmisc’ (Harrell Jr and Dupont, 2021), ‘lmerTest’ (Kuznetsova et al., 2017), ‘agricolae’ (De Mendiburu, 2014), ‘MASS’ (Venables and Ripley, 2013), ‘emmeans’ (Lenth et al., 2018), ‘car’ (Fox and Weisberg, 2018), ‘sf’ (Pebesma, 2018), ‘MuMIn’ (Barton, 2009), and ‘bestNormalize’ (Peterson, 2021). Data wrangling was performed in R with the help of the follow packages: ‘tidyverse’ (Wickham, 2017), ‘dplyr’ (Wickham et al., 2015), ‘reshape2’ (Wickham, 2007), ‘plotrix’ (Lemon, 2006), and ‘HH’ (Heiberger et al., 2015). Figures were produced using the following packages: ‘ggpubr’ (Kassambara, 2018), ‘cowplot’

(Wilke, 2016), 'RColorBrewer' (Neuwirth, 2014), and 'ggjoy' (Wilke, 2017). For a full review of our statistical analyses, please refer to the annotated R-code included in the supplemental material; we provide below a brief description of each analysis and the type of modeling used.

To test for statistically significant differences in respiration rate between DNP-treated and control copepods, we encoded a linear model with treatment (DNP vs control) as a fixed effect, an interaction with sex, and copepod mass (dry body weight) as a fixed effect. We inspected pairwise comparisons between DNP-treated and control copepods, blocked by sex, using a Tukey correction for multiple pairwise comparisons. In these models, mass-independent respiration rate was used as the dependent variable instead of mass-corrected respiration rate. As noted above, the effect of mass was controlled by including this variable as a fixed effect (Lighton, 2018; Niitepõld, 2019; Schmidt-Nielsen and Knut, 1984). We included mass a covariate for all models with respiration rate as the dependent variable. For models where respiration rate was included as a fixed effect (i.e., independent variable), we use mass-corrected respiration rate as a covariate (see below).

To test for differences in carotenoid concentration, we fit a linear model with treatment, sex, and their interaction as fixed effects. We then inspected pairwise comparisons between DNP-treated and control copepods, blocked by sex, using a Tukey correction for multiple pairwise comparisons. To model the effect of increasing respiration on carotenoid concentrations across all samples, we specified a linear mixed effects model with mass-corrected respiration rate and sex as fixed-effect covariates and diet (red stock vs colorless) as a random effect. We subset our data by "control" and "DNP-treated" copepods and used the same model for each subset to inspect the relationship between respiration rate and carotenoid concentration. Prior to analyzing the effect of respiration rate on dietary carotenoid concentration, we transformed dietary

carotenoid concentrations using an ordered quantile normalization (chosen using the 'bestNormalize' package) due to observed non-normality in the data frequency distribution. To test for any statistical relationship between dietary carotenoid and astaxanthin concentrations in our control and DNP-treated samples, we fit a mixed effects model with astaxanthin concentration as a dependent variable, transformed dietary carotenoid concentration and sex as fixed effects, and diet as a random effect.

To test whether DNP treatment altered the body mass of copepods compared to controls, we fit a linear mixed effects model with treatment and sex encoded as fixed effects and a random effect of "diet" (i.e., red stock or colorless).

4.3.7 Data Availability

All relevant data are presented within this manuscript or in the online supplementary materials at the Journal of Experimental Biology.

4.4 Results

We found no significant difference in the mass of copepods exposed to DNP and control copepods across all treatments, after controlling for differences due to sex and diet (Figure S4.3; mean tissue mass in mg±SE; DNP: 0.0453±0.0031; Control: 0.0451±0.0031).

We detected three primary carotenoids across all copepod samples. These were identified as astaxanthin, β-carotene, and hydroxyechinenone (Figure S4.2). Astaxanthin was the primary, most abundant carotenoid present in the *T. californicus* tissues, making up 97.6% of the quantifiable carotenoid content in stock red copepods on average and 95.3% in color-restored copepods on average (Figure S4.4; mean μg astaxanthin mg^{-1} tissue±SD; red stock: 1.29±0.45;

color-restored: 0.51±0.14). The second-most abundant carotenoid in both stock red and color-restored copepods was hydroxyechinenone (Figure S4.4; mean μg hydroxyechinenone mg^{-1} tissue±SD; red stock: 0.020±0.023; color-restored: 0.024±0.011). Finally, β-carotene was present and quantifiable in only some, but not all samples and was the least abundant precursor to astaxanthin (Figure S4.4; mean μg β-carotene mg^{-1} tissue±SD; red stock: 0.012±0.018; color-restored: 0.0095±0.0038). In both red stock and color-restored copepods, the concentration of hydroxyechinenone was statistically greater than the concentration of β-carotene (β_1 = model estimate in μg mg^{-1} tissue, *p*-value; stock red: β_1=0.0076, *p*=0.0184; color-restored: β_1=0.023, *p*<0.001).

4.4.1 Red stock copepods

After three days exposure to 10μM DNP, the respiration of red stock copepods was significantly elevated in both sexes (Figure 4.2A; mean mmol O_2 min^{-1}±SE; DNP Male: 2.14±0.20; Control Male: 1.17±0.19; DNP Female: 2.06±0.17; Control Female: 1.54±0.18). Males treated with DNP consumed on average 0.53 mmol O_2 per minute more than control males (CI = confidence interval; CI = 0.036-1.01), and females treated with DNP consumed on average 0.97 more mmol O_2 per min than control females (CI = confidence interval; CI = 0.40-1.53).

When 10uM DNP exposure is extended to seven days, respiration was again elevated in male copepods given DNP, but similar to control levels in females (Figure 4.2B; mean mmol O_2 min^{-1}±SE; DNP Male: 1.30±0.17; Control Male: 0.89±0.21; DNP Female: 1.29±0.20; Control Female: 1.30±0.18). However, the difference was not statistically significant in either sex (Figure 4.2B). When the exposure time was kept at seven days, but the concentration of DNP was lowered to 2uM, female copepod respiration dropped significantly below that of control females (Figure 4.2C; mean mmol O_2 min^{-1}±SE; DNP Female: 0.81±0.09; Control Female: 1.12±0.11; β_1 = model estimate in mmol O_2 min^{-1}, CI = confidence interval; β_1= -0.311, CI = (-0.60)-(-0.023)). At this concentration and exposure time, male respiration was significantly elevated (Figure 4.2C; mean mmol O_2 min^{-1}±SE; DNP Male: 0.98±0.11;

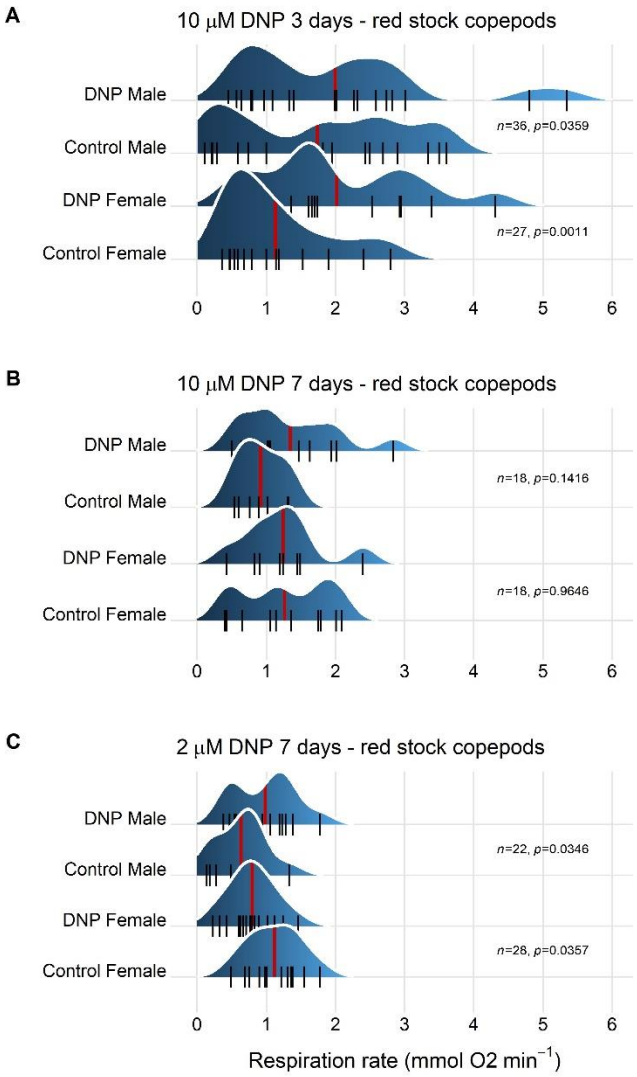

Figure 4.2 Ridgeline plot showing the distribution of oxygen consumption measurements for DNP-treated and control red stock copepods, separated by sex. The label at the top of each panel indicates the concentration of DNP tested and the exposure time. The red line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex and mass as a fixed effect. The samples sizes indicated by n represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

Control Male: 0.63±0.11; β_1 = model estimate in mmol O_2 min^{-1}, CI = confidence interval; β_1=0.35, CI=0.026-0.67).

After three days exposure to 10µM DNP, the astaxanthin concentration in male and female copepod tissues was not significantly different from that in control copepod tissues (Fig. 3A; mean µg astaxanthin mg^{-1} tissue±SE; DNP Male: 1.50±0.12; Control Male: 1.52±0.12; DNP Female: 1.24±0.13; Control Female: 1.35±0.12). However, after seven days exposure to 2µM DNP, astaxanthin concentrations were increased in both sexes, significantly in males (Fig. 3B; mean µg astaxanthin mg^{-1} tissue±SE; DNP Male: 1.64±0.16; Control Male: 0.97±0.15; DNP Female: 1.13±0.13; Control Female: 0.97±0.14). Male copepods exposed to DNP contained 0.67 more µg astaxanthin per mg of body tissue than control copepods (CI = confidence interval; CI = 0.23-1.10).

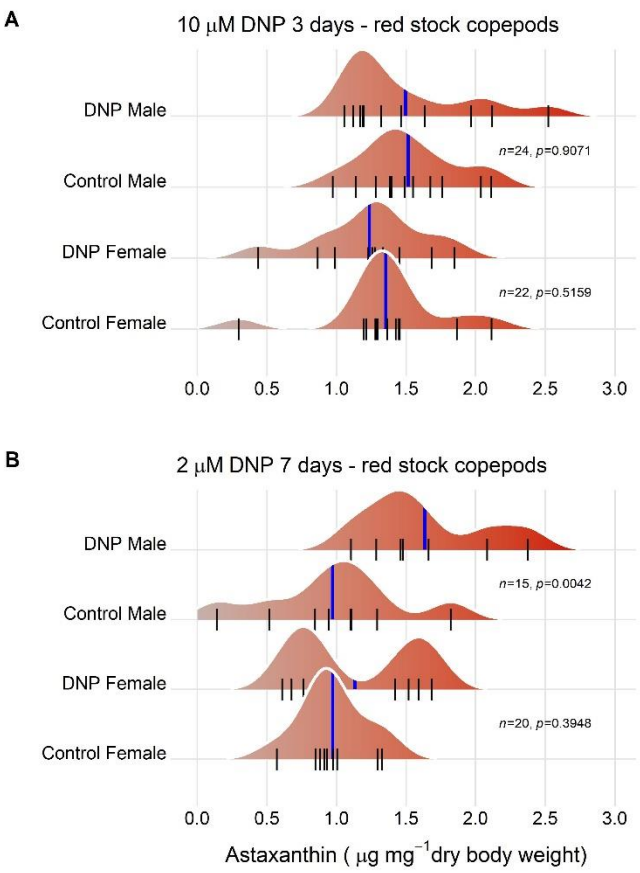

Figure 4.3 Ridgeline plot showing the distribution of astaxanthin concentration measurements for DNP-treated and control red stock copepods, separated by sex. The label at the top of each panel indicates the concentration of DNP tested and the exposure time. The blue line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex. The samples sizes indicated by n represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

After three days exposure to 10µM DNP, we observed no clear or significant difference between the concentration of dietary carotenoids in the tissues of DNP-treated copepods compared to tissues of control copepods (Figure S4.5A; mean µg dietary carotenoids mg^{-1}

tissue±SE; DNP Male: 0.050±0.012; Control Male: 0.040±0.12; DNP Female: 0.047±0.013; Control Female: 0.046±0.012). Similarly, we found no significant difference between the concentration of dietary carotenoids in copepods treated with 2µM DNP for three days compared to control copepods (Figure S4.5B; mean µg dietary carotenoids mg^{-1} tissue±SE; DNP Male: 0.028±0.0067; Control Male: 0.018±0.0063; DNP Female: 0.0081±0.0054; Control Female: 0.0095±0.0059).

4.4.2 Color-restored copepods

In colorless copepods, DNP increased the rate of respiration after seven days in both males and females (Figure 4.4A; mean mmol O_2 min^{-1}±SE; DNP Male: 1.02±0.062; Control Male: 0.82±0.073; DNP Female: 0.84±0.076; Control Female:

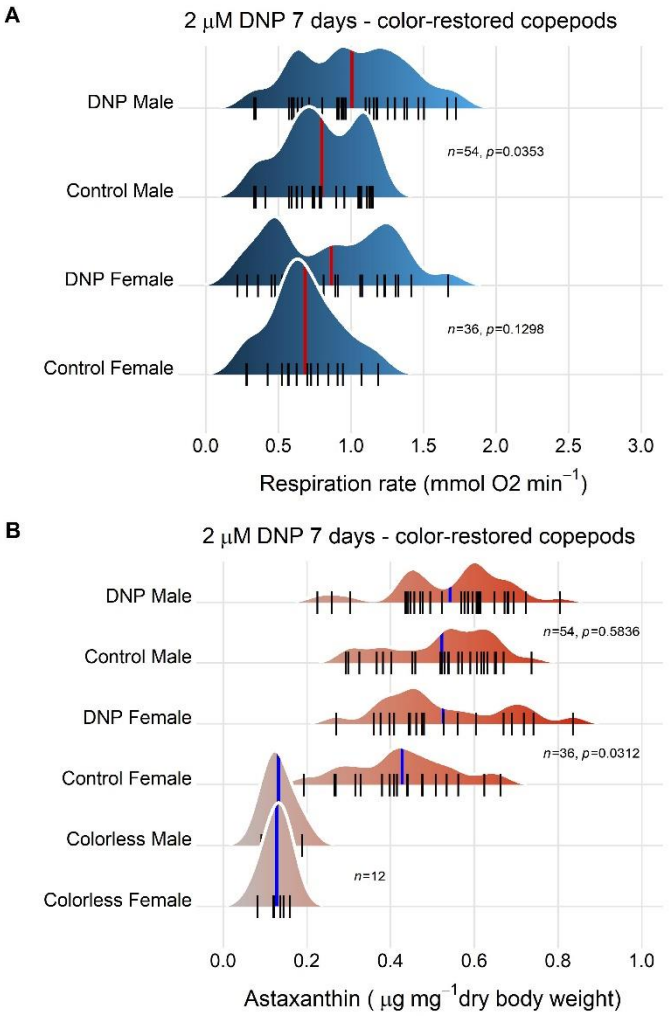

Figure 4.4: Ridgeline plot showing the distribution of oxygen consumption measurements for DNP-treated and control color-restored copepods, separated by sex (A) and the distribution of astaxanthin concentration measurements for DNP-treated and control color-restored copepods, separated by sex (B). The astaxanthin concentration of colorless copepods are included in panel B for visual comparison. The label at the top of each graph indicates the concentration of DNP tested and the exposure time. The blue or red line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex and, in the case of analyzing the respiration data, mass as a fixed effect. The models analyzing astaxanthin concentration did not include mass because astaxanthin concentrations were already standardized by mass. The samples sizes indicated by n represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

0.68±0.080). This was statistically significant in males (β_1 = model estimate in mmol O_2 min^{-1}; CI = confidence interval; β_1=0.20, CI=0.014-0.39).

When allowed to restore red coloration through supplementation with *Tetraselmis* algae, male copepods treated with DNP produced only slightly more astaxanthin than controls (Figure 4.4B; mean µg astaxanthin mg^{-1} tissue±SE; DNP Male: 0.54±0.025; Control Male: 0.52±0.028). However, female copepods treated with DNP accumulated significantly more astaxanthin compared to controls (Figure 4.4B; mean µg astaxanthin mg^{-1} tissue±SE; DNP Female: 0.53±0.032; Control Female: 0.43±0.032; β_1 = model estimate in µg astaxanthin mg^{-1} tissue, CI = confidence interval; β_1=0.098, CI=0.0091-0.19). There were no significant differences between the average concentration of dietary carotenoids in copepods treated with DNP and in control copepods (Figure S4.6; mean µg dietary carotenoids mg^{-1} tissue±SE; DNP Male: 0.021±0.0020;

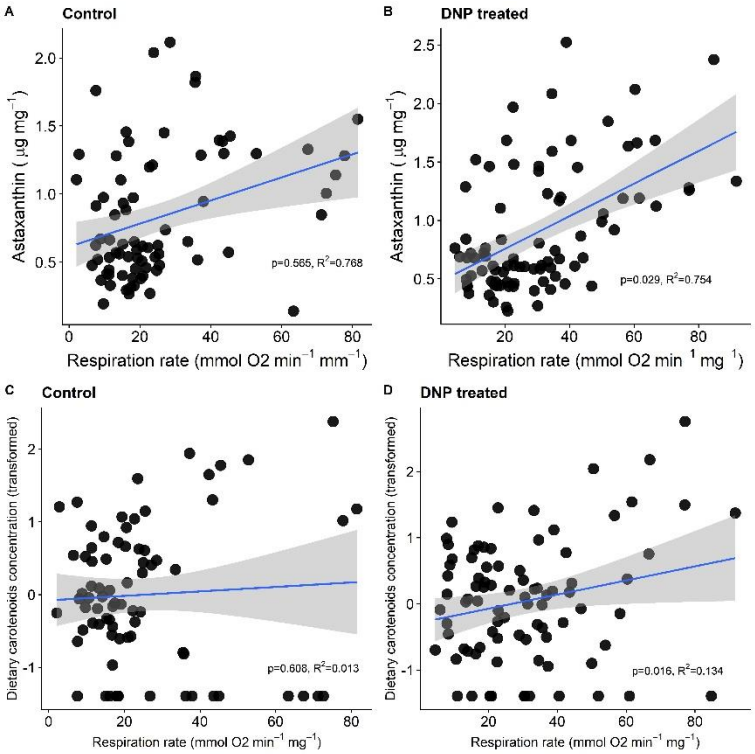

Figure 4.5 Scatterplots showing the relationship between respiration rate (x-axis, all panels) and either A) astaxanthin in control copepods, B) astaxanthin in DNP-treated copepods, C) dietary carotenoids in control copepods, and D) dietary carotenoids in DNP-treated copepods. P-values are reported from linear mixed effects models with sex included as a fixed effect and diet (i.e., red stock or color-restored) coded as a random effect. Using raw dietary carotenoids concentration value, model residuals were initially skewed and non-normal, so we transformed dietary carotenoid values using an ordered quantile transformation. R2 values are adjusted values, accounting for the additional terms encoded in the model. The blue line is a smoothed line of best fit and the grey shading represents the 95% confidence interval around this line. Each black dot represents a single experimental trial, with 3 copepods per trial.

Control Male: 0.020±0.0022; DNP Female: 0.033±0.0025; Control Female: 0.030±0.0025). Both DNP-treated and control copepods of both sexes contained significantly more astaxanthin than colorless males and females never given access to algae to restore their coloration ($p<0.0001$ for each comparison; Figure 4.4B; mean μg astaxanthin mg^{-1} tissue±SE; colorless males: 0.133±0.052; colorless females; 0.128±0.052).

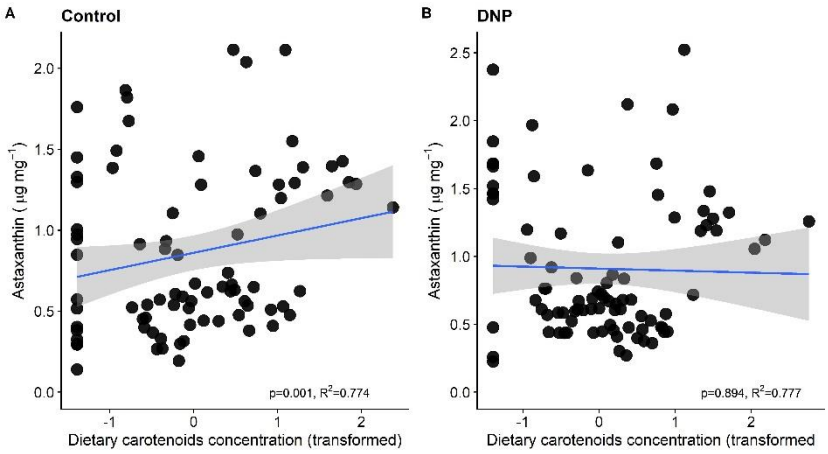

Figure 4.6 Scatterplots showing the relationship between dietary carotenoid data (x-axis, all panels) and either A) astaxanthin in control copepods, or B) astaxanthin in DNP-treated copepods. P-values are reported from linear mixed effects models with sex included as a fixed effect and diet (i.e., red stock or color-restored) coded as a random effect. Using raw dietary carotenoids concentration value, model residuals were initially skewed and non-normal, so we transformed dietary carotenoid values using an ordered quantile transformation. R2 values are adjusted values, accounting for the additional terms encoded in the model. The blue line is a smoothed line of best fit and the grey shading represents the 95% confidence interval around this line. Each black dot represents a single experimental trial, with three copepods per trial.

4.4.3 Astaxanthin, dietary carotenoids, and respiration

Across all experiments, there was a positive relationship between respiration rate and astaxanthin concentration after controlling for sex and diet (Figure 4.5A, B), significant only in DNP-treated copepods (Figure 4.5B; *df*=74; *t*=0.52; β_1 = model estimate in μg astaxanthin mg^{-1} tissue, CI = confidence interval; $B_1 = 4.4e^{-3}$; CI=$6.1e^{-4}$ - $8.5e^{-3}$). This same effect was observed between dietary carotenoids and respiration rate in DNP-treated copepods (Figure 4.5C, D), again controlling for sex and diet (Figure 4.5D; *df*=55; *t*=2.49; β_1 = model estimate in ordered quantile of dietary carotenoid concentration, CI = confidence interval; $B_1 = 0.014$; CI=$7.8e^{-4}$ – 0.024). However, there was a significant relationship between the concentration of dietary

carotenoids and the concentration of astaxanthin in the tissues of control copepods only (Fig. 6A; *df*=79; *t*=3.41; β_1 = model estimate in μg astaxanthin mg^{-1} tissue, CI = confidence interval; B_1 = 0.12; CI=0.050 – 0.18). We observed no significant effect of increasing dietary carotenoid concentration on astaxanthin concentration in the tissues in DNP-exposed copepods (Fig. 6B).

4.5 Discussion

When treated with the mitochondria uncoupler and protonophore, 2,4-dinitrophenol, *T. californicus* copepods produced more of the red carotenoid pigment astaxanthin after seven days, but not after three days. These experiments provide further support for the hypothesis that metabolism of ketocarotenoids from yellow dietary pigments is intimately linked to energetic processes in the mitochondria. Here, we observed that acute exposure to DNP increased the metabolic rate in *T. californicus* copepods similar to observations in other taxa (Caldeira da Silva et al., 2008; Cantelmo et al., 1978; Geisler et al., 2017; Goldgof et al., 2014; Pinchot, 1967). As predicted if carotenoid metabolism is tied to mitochondrial function, we then observed that DNP-treated copepods produced more astaxanthin than did control copepods. This study provides the first experimental demonstration of a relationship between DNP-induced changes to mitochondrial metabolism and the accumulation of metabolized carotenoid pigments. Critically, it is the first chemical manipulation of mitochondrial function to affect metabolism of ketocarotenoids where results should not be confounded by an increase in the production of reactive oxygen species in the mitochondria.

An alternative hypothesis is that carotenoid-based coloration is a signal of condition because there are tradeoffs in the allocation of carotenoid resources between coloration and stress responses (Alonso-Alvarez et al., 2007; Alonso-Alvarez et al., 2008; Moller et al., 2000;

Simons et al., 2012) This is the Resource Tradeoff Hypothesis which proposes that carotenoids such as astaxanthin may serve a defensive role against oxidative stress such that carotenoid pigments may be a key, but limited, metabolic resource (Hill, 1999; Lozano, 1994; Moller et al., 2000). The shared pathway hypothesis and the resource tradeoff hypothesis are not mutually exclusive; carotenoid resource availability and mitochondrial energetics might both affect coloration (see Fig. 2 of Powers and Hill (2021)). For instance, our observation that copepods increased the concentration of astaxanthin in their tissues could indicate that the DNP, in stabilizing the mitochondrial membranes via uncoupling, freed up carotenoid resources that would normally be consumed during antioxidant activities. (Caramujo et al., 2012; Davenport et al., 2004; Weaver et al., 2018b).

Our experiments cannot fully refute the resource tradeoff hypothesis. However, for several reasons we think a carotenoid resource tradeoff alone cannot fully explain our results. Differences in astaxanthin concentrations between DNP-treated and control copepods did not appear to be due to differences in a greater uptake of dietary carotenoids. In both red stock copepods and colorless copepods and for both males and females, individuals exposed to DNP did not accumulate more dietary carotenoids than control copepods (Figs S6, S7). Moreover, we observed no relationship between dietary carotenoid concentration and astaxanthin concentrations in DNP-treated copepods (Fig. 6). Thus, the positive relationship between dietary carotenoids and respiration rate (Figure 4.5D) may have been driven by hydroxyechinenone, which is also a metabolic intermediate between β-carotene and astaxanthin (Weaver et al., 2018a). Further, DNP exposure did not result in an increase in mass compared in DNP treated copepods compared to controls, countering the idea that differences in astaxanthin concentrations

might simply be due to a difference in algae consumption and, by extension, dietary carotenoid resources.

Several lines of evidence support the hypothesis that DNP treatment stimulated increased metabolism of astaxanthin via increased mitochondrial metabolism. We observed an increase in the rate of oxygen consumption, and hence respiration, in copepods exposed to DNP compared to controls (Figures 4.2, 4.4). This effect was present in both sexes after 3 days exposure to a moderate dose of 10μM DNP. Excluding red stock female copepods (addressed below), the effect persisted in both red stock and colorless copepods when exposure was extended to seven days and the DNP dosage was lowered to 2μM (Figs 2, 4). We observed that, on average, copepods exposed to DNP over seven days accumulated more astaxanthin in their tissues compared to control copepods (Figs 3, 4), even though this effect was statistically significant in red stock males and colorless females only. This indicates that the red stock copepods exposed to DNP increased astaxanthin concentrations in their tissues beyond the normal levels found in copepods in routine culture conditions. Again, this effect was strongest in male red stock copepods (Fig. 3B). In comparison, colorless copepods exposed to DNP replenished their tissue concentrations of astaxanthin at a faster rate than control individuals, significantly in the case of the colorless females tested. We suspect that we did not see a significant increase in astaxanthin concentrations after three days exposure to DNP (Figure 3A) due to the base carotenoid conversion rate in the species requiring a full two weeks to reach levels comparable to wild individuals (Davenport et al., 2004). Three days may not have been enough time to detect measurable differences due to DNP exposure, a limitation addressed through the inclusion of 7-day exposure trials.

It previously was shown that hybridization had a greater negative impact on astaxanthin metabolism in colorless male *T. californicus* than in colorless females (Powers et al., 2021). Colorless copepods supplemented with DNP also replenished more astaxanthin over seven days than did colorless copepods not provided DNP. However, this effect was observed to be stronger in colorless females (Figure 4.4B). Colorless females—but not red stock females—supplemented with 2μM DNP for seven days showed an elevated oxygen consumption on average compared to controls (Figs 3B, 3C, 4). However, red stock females exposed to DNP showed oxygen consumption rates that had, on average, fallen below that of controls (Figure 2C). This creates an interesting pattern, because at higher concentrations of DNP (10μM) over a shorter period (3 days), red stock females showed significantly elevated respiration rates compared to controls (Figure 2A). When exposure to 10μM DNP was extended to one week, the respiration rate of red stock females was not different from controls (Figure 2B). These observations could indicate a hormetic effect of DNP in red stock females fed algae or may also be explained by the observations that female *T. californicus* copepods are more resistant to changes due to oxidative stress (Foley et al., 2019; Harada et al., 2019; Willett, 2010; Willett and Son, 2018). It is possible that colorless females raised on yeast were less able to compensate for the effects of DNP, possibly due to the relatively poor nutrition provided by yeast compared to algae (Powers et al., 2021). We think it unlikely that this result was due to DNP degrading on its own over the course of seven days. The half-life of DNP in water is over a year (Tratnyek and Hoigne, 1991), and even powerful bacterial degradation of DNP can still takes up to 68 days (Capel and Larson, 1995).

It has been observed that in low doses or relatively short exposures, DNP acts to increase the rate of respiration during oxidative phosphorylation, speeding up the rate at which redox

reactions occur to compensate for the lack of ATP produced (Goldgof et al., 2014; Leverve et al., 1998; Rognstad and Katz, 1969; Stier et al., 2014). This uncoupling does not result in an increase of ROS (Goldgof et al., 2014; Korde et al., 2005; Lozinsky et al., 2013; Stier et al., 2014), and thus, should not have required the consumption of astaxanthin to combat oxidative stress, as has been observed in *T. californicus* exposed to pro-oxidants (Weaver et al., 2018b). We propose that the acute doses of DNP that we tested uncoupled oxidative phosphorylation from ATP production, briefly increasing the rate at which redox reactions occurred in copepod mitochondria in compensation. In turn, this shift in redox environment may have also stimulated an increase in the rate of redox reactions required to metabolize β-carotene and hydroxyechinenone to astaxanthin. In hybrid inbred lines of *T. californicus*, we also observed an inverse relationship between astaxanthin accumulation and ATP production in colorless copepods (Powers et al., 2021); although in that previous study, respiration rate was not measured. In a separate analysis of *T. californicus* hybrids, however, increased respiration was associated with increased ketocarotenoid accumulation (Powers and Hill, 2021).

It appears that across diverse animal taxa, the enzymes responsible for carotenoid metabolism belong to the cytochrome p450 superfamily of oxygenases (Lopes et al., 2016; Mojib et al., 2014; Mundy et al., 2016; Twyman et al., 2018; Twyman et al., 2016; Weaver et al., 2020). The predicted cellular location of carotenoid oxygenases places them either directly in the IMM, or in the mitochondrially associated membranes (MAM) in the cytosol (Hill et al., 2019). However, a full description of the mechanism and subcellular location of carotenoid metabolizing enzymes in *T. californicus* is needed before we can fully understand the role of mitochondrial redox state in influencing the expression of ketocarotenoid-based coloration in copepods (Mojib et al., 2014; Weaver et al., 2020). These oxygenases perform their functions via

redox reactions with coupled reductases that utilize electron donors such as NADH, NADPH, or FADH in the same fashion as the enzymes of the mitochondrial ETS (Cojocaru et al., 2007; Munro et al., 2007; Strohmaier et al., 2019). Low doses of DNP result in an increase in electron donors, like NADH or NADPH (Leverve et al., 1998; Rognstad and Katz, 1969). This increase is observed to occur first in the cytosol, and then, subsequently in the mitochondria itself via the influx of NADH and ATP back into the mitochondria (Leverve et al., 1998; Rognstad and Katz, 1969). These changes lead to a decrease in the ratios of electron donors to acceptors in both the cytosol and mitochondria but an increase in reducing power (Leverve et al., 1998). An increase in the redox reaction rate of the mitochondria in the copepods is critically important given the predicted biochemistry of the enzymatic reactions required to metabolize dietary carotenoids to ketocarotenoids.

Recent evidence suggests that changes to mitochondrial energetics can affect carotenoid metabolism. It has been demonstrated that other mitochondria-targeted compounds, besides DNP, can affect the expression of ketocarotenoid-based coloration or the expression of carotenoid metabolizing enzymes. Cantarero et al. (2020a) demonstrated that exposure to the IMM-targeted superoxide dismutase mimetic, mitoTEMPO, successfully altered avian ketolase expression in zebra finches (*Taeniopygia guttata,* Reichenbach, 1862). These same researchers also successfully documented an effect of stabilizing or destabilizing the mitochondrial redox environment with mitoQ and dTTP, respectively, on the visible redness of bills of these same birds (Cantarero and Alonso-Alvarez, 2017). These compounds may have altered the normal ratios of ubiquinol and ubiquinone, redox cyclers responsible for the shuttling of electrons in the ETS, thereby altering the redox state of carotenoid metabolizing enzymes (Cantarero and Alonso-Alvarez, 2017). Caution should be taken to extrapolate these results to our observations

in *T. californicus* considering the biological differences between terrestrial and aquatic organism physiology. Moreover, the effect of mitochondria targeted compounds is likely highly contextual and, perhaps, species specific. Indeed, when Red Crossbills (*Loxia curvirostra,* Linnaeus, 1758) were exposed to mitoQ and mitoTEMPO, each compound affected the levels of ketocarotenoids in the blood and feathers differently (Cantarero et al., 2020b).

Our observations of an association between aerobic respiration and metabolism of carotenoids in copepods supports the hypothesis of an intimate link between ketocarotenoid metabolism and mitochondrial function and corroborates previous studies. In wild-caught house finches (*Haemorhous mexicanus,* Müller, 1776), Hill et al. (2019) observed a positive relationship between state 4 respiration (resting rate) and ketocarotenoid-based coloration as well as an inverse relationship between lipid damage, mitochondrial replacement, red coloration. In this study, the authors hypothesized that redder individuals that accumulated more ketocarotenoids in their feathers may have been more capable of withstanding higher levels of metabolic damage and energetic output before needing to replace old mitochondria with new copies. Similarly, in *T. californicus* hybrids fed a restrictive diet supplemented with *ad libitum* carotenoids, tissue concentrations of ketocarotenoids was negatively correlated with ATP production and not significantly related to offspring development time (Powers et al., 2021), indicating that redder individuals may have needed less ATP to maintain homeostasis. When the dietary stress was alleviated in this copepod study via reintroduction of algal nutrients, the negative correlation between ATP production and ketocarotenoid accumulation disappeared, and there was a positive correlation between ketocarotenoid concentrations and offspring development times (Powers et al., 2021). As noted previously, experimentation with *T.*

californicus fed algae ad libitum also resulted in a significant positive relationship between ketocarotenoid biosynthesis and respiration rate (Powers and Hill, 2021).

Observations in the house finch and *T. californicus* lend support to an evolving understanding of how metabolic rate relates to "quality" or "fitness", and an increasing understanding that metabolic rate itself may be highly context dependent (Koch et al., 2021). Indeed, it has been suggested that under conditions in which food is not limiting, it may be beneficial to operate at a higher metabolic rate (Norin and Metcalfe, 2019). Likewise, quantitative analyses of over one-hundred published studies have revealed that it is common to find positive relationships between metabolic rate and many components of fitness are observed (Arnold et al., 2021).

In the house finch and *T. californicus* studies discussed above, it was unclear how the interaction between reactive oxygen species produced via normal oxidative phosphorylation might influence the relationship between ketocarotenoid concentrations and mitochondrial function at a mechanistic level. In our study, however, we observe an overall relationship between respiration rate and ketocarotenoid biosynthesis without the confounding effects of increased ROS, despite a potential additional effect of sex on the relationship. A critical point is that we did not measure natural differences in metabolic efficiency in this study; the chemically induced uncoupling that we used forced an increase in respiration regardless of natural efficiency. With this design, we did not test for a correlation between mitochondrial efficiency and carotenoid ketolation; rather, we tested for a correlation between the rates of mitochondrial redox reactions and carotenoid ketolation, regardless of ATP production. As we extend conclusions to systems like the wild house finches or the interpopulation hybrids of *T. californicus*, we make the assumption that ATP production scales with respiratory rate naturally,

such that faster respiring individuals generally make more ATP. In this experiment we did not measure ATP production. Thus, we cannot make explicit conclusions about the ratio of energy produced to oxygen consumed or the incurrence of any compensatory mechanisms to restore ATP production in response to DNP exposure in the copepods. We can, however, conclude that the rate of mitochondrial redox reactions and the rate of carotenoid biosynthesis redox reactions are positively correlated, implying that redox capacity is reflected in ketocarotenoid accumulation as originally proposed by Völker (1957). Future studies may measure both ATP production and respiration rate to assess relationships between carotenoid ketolation and mitochondrial efficiency. Regardless, the results presented in this study suggest that a propensity for ketocarotenoid coloration to signal metabolic capacity is independent of a mediating influence of ROS, even though we cannot conclude that ROS has no effect on tissue carotenoid concentrations in wild populations.

Our observations of potential links between cellular respiration and carotenoid coloration in copepods hold important implications for the evolution of condition-dependent traits. Unlike with house finches, *T. californicus* copepods do not choose mates based on red carotenoid coloration (Powers et al., 2020). Thus, the condition-dependent carotenoid signal in these copepods appears to exist without a receiver, and it seems unlikely to have evolved in copepods because it is a condition-dependent signal. Rather, our observations with copepods may speak to a fundamental biochemical link between ketocarotenoid-based coloration and mitochondrial function—red carotenoid coloration in copepods evolved for UV protection (Weaver et al. 2018b), and its condition dependency is simply a property of the enzymatic pathways that produce the red pigments. The implications of this observation is that in some systems, such as songbirds, metabolically modified carotenoids have been co-opted as a social signal of individual

condition (Twyman et al., 2016) and that condition dependency of red carotenoid coloration does not evolve via sexual selection.

4.6 Supplemental Figures

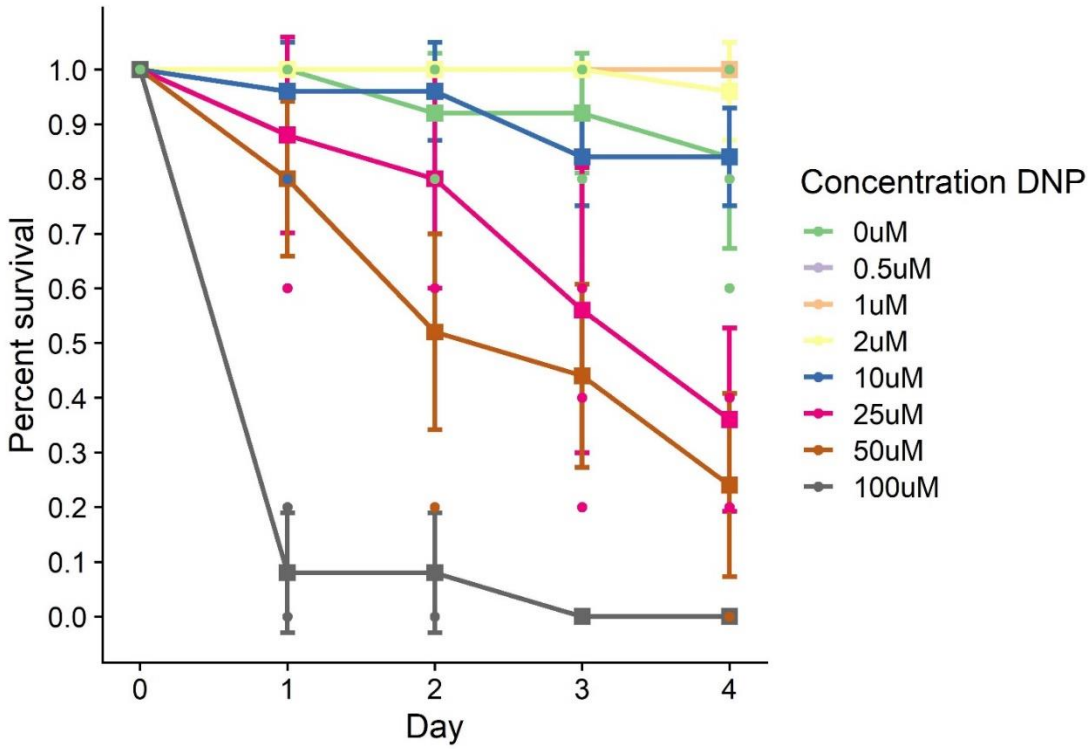

Figure S4.1: Average survival out of 5 copepods over 4 days exposure time to different DNP concentrations. Each average value (indicated by the squares) represents the average of 5 trials (25 copepods total for each tested concentration). Bars represent standard errors around the averages for each day. Small circles indicate individual trials, with most falling on the average (thus, hidden visually). The lilac (0.5µM), orange (1µM), and yellow lines (2µM) overlap.

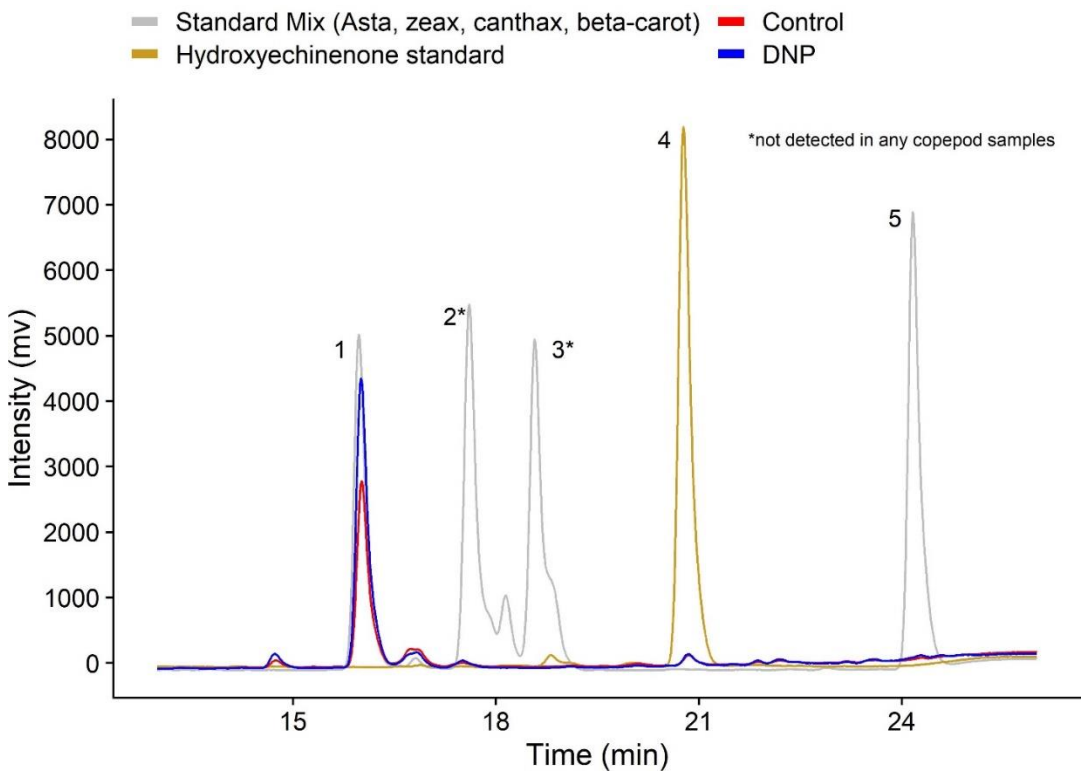

Figure S4.2: Representative HPLC chromatogram showing carotenoid standards (grey and yellow), a DNP-treated copepod sample (blue), and a control copepod sample (red). Peaks are 1) astaxanthin, 2) zeaxanthin, 3) canthaxanthin, 4) hydroxyechinenone, 5) β-carotene. β-carotene and hydroxyechinenone are present in the *Tetraselmis* algae used to feed copepods. In many samples, like the representative graph shown here, β-carotene was only detectable in trace amounts.

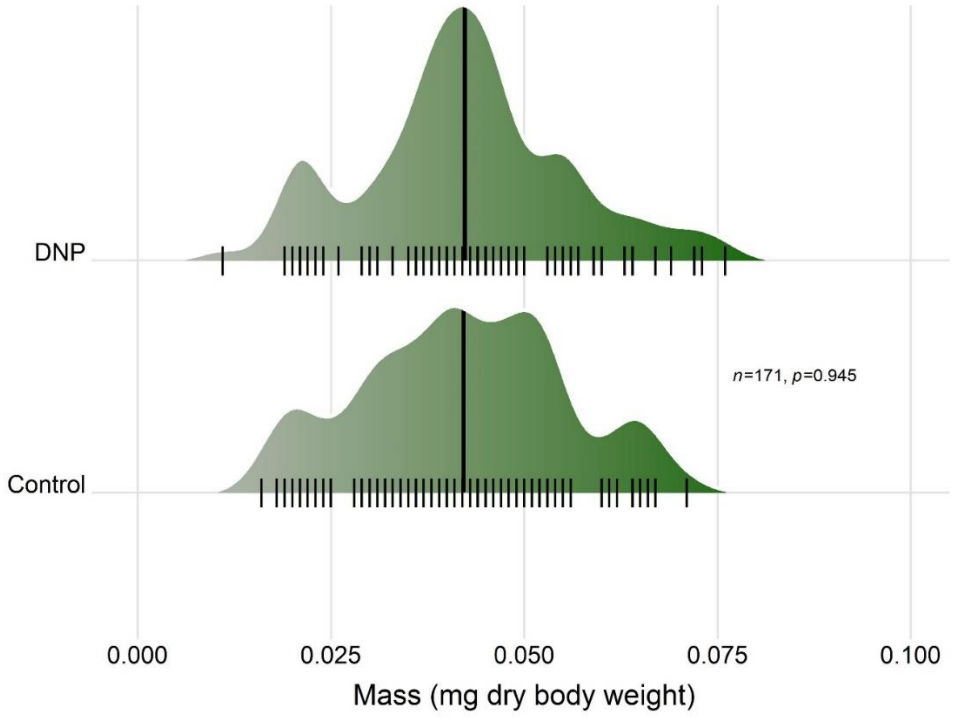

Figure S4.3: Ridgeline plot showing the distribution of dry mass measurements for DNP-treated and control copepods. The tall black line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear mixed effects model comparing the two treatment groups that included a fixed effect of sex and a random effect of diet (i.e., red stock or color-restored). The samples sizes indicated by *n* represents the number of experimental replicates analyzed in the model, with each replicate containing 3 individual copepods.

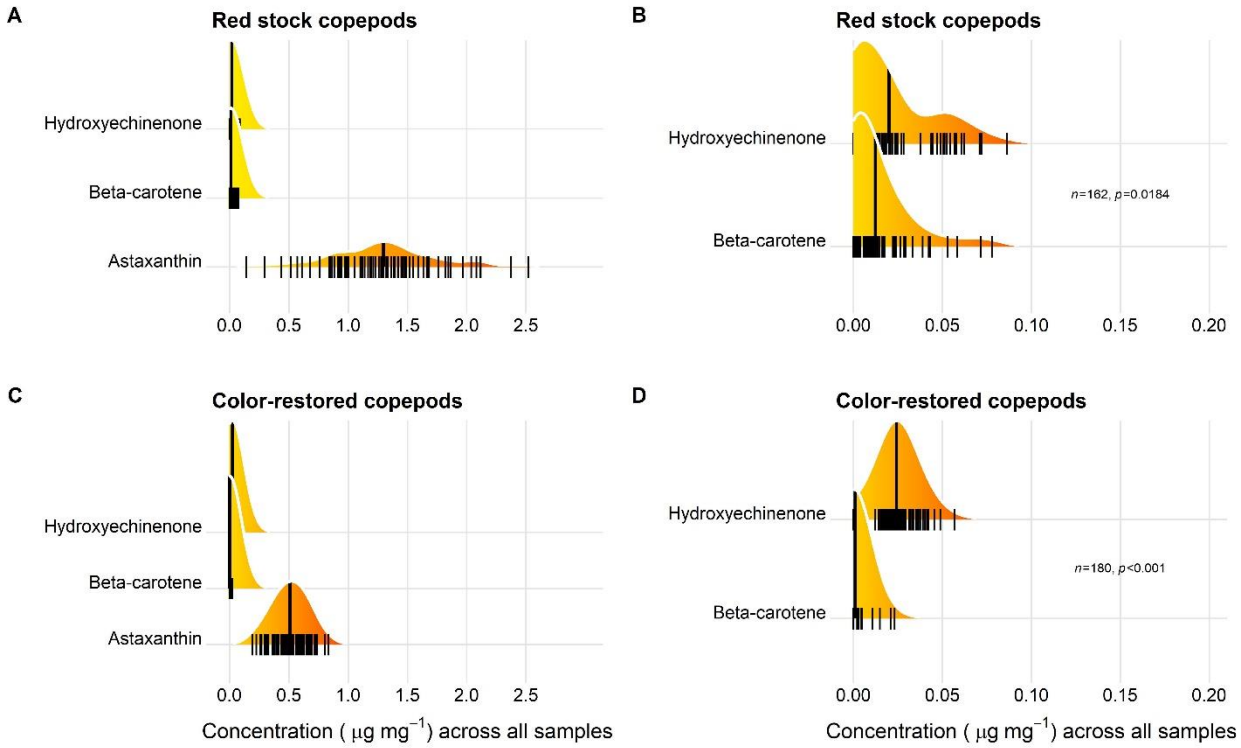

Figure S4.4: Ridgeline plots showing the distribution of carotenoid concentrations for all copepod samples in red stock copepods (AB) and color-restored copepods (CD). The same data is shown twice: panels C and D are the same hydroxyechinenone and β-carotene data shown without the swamping effect of plotting astaxanthin on the same graph. The concentration of astaxanthin was significantly higher than either hydroxyechinenone or β-carotene (p<0.0001 for each comparison). The tall black line represents the average for each carotenoid. Small black lines indicate individual trial replicates of 3 copepods each.

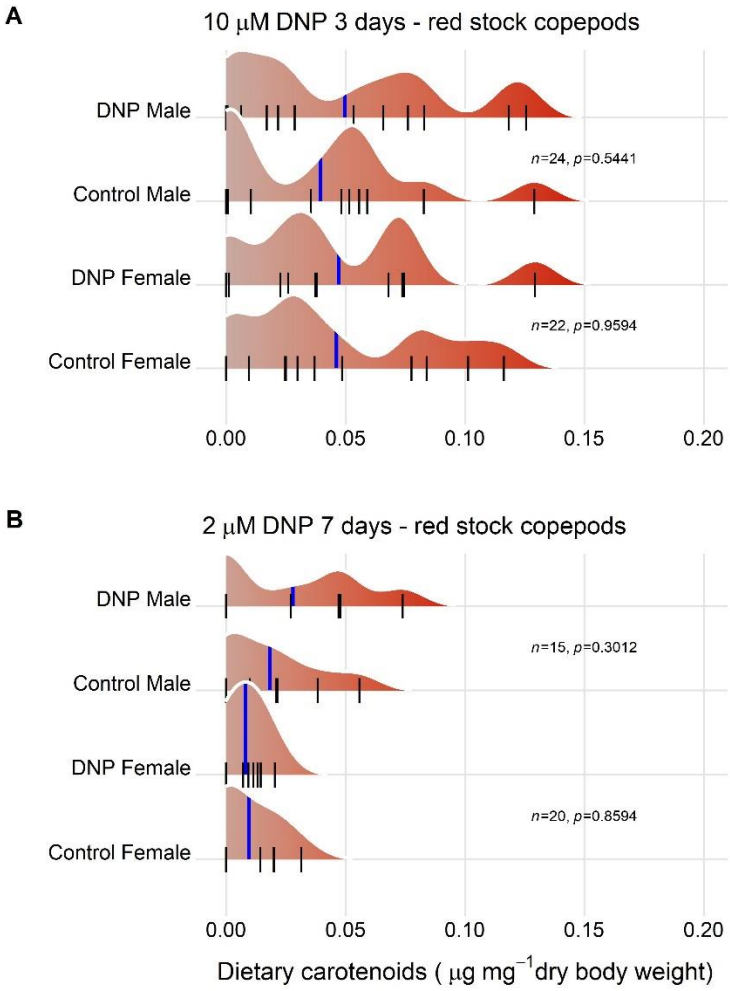

Figure S4.5: Ridgeline plot showing the distribution of dietary carotenoid concentration measurements for DNP-treated and control red stock copepods, separated by sex. Panels are separated by A) the dietary carotenoid concentrations after 3 days exposure to 10μM DNP and B) dietary carotenoid concentrations after 7 days exposure to 2μM DNP. The label at the top of each panel indicates the concentration of DNP tested and the exposure time. The blue line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods.

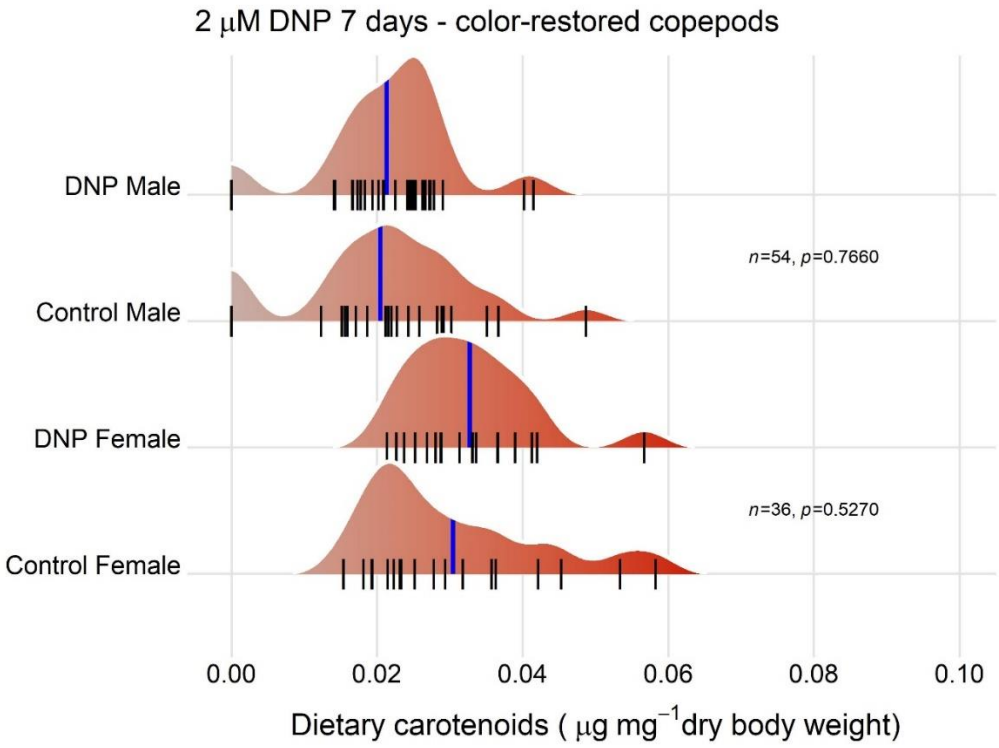

Figure S4.6: Ridgeline plot showing the distribution of dietary carotenoid concentration measurements for DNP-treated and control color-restored copepods, separated by sex. The blue line represents the group average. Small black lines indicate individual trial replicates of 3 copepods each. The p-value shown is reported from a linear model comparing the two treatment groups that included the interaction with sex. The samples sizes indicated by *n* represents the number of experimental replicates analyzed in each model, with each replicate containing 3 individual copepods. We detected no dietary carotenoids in the tissues of colorless males and females never given access to algae to restore their coloration.

Concluding Remarks

Our understanding of the role of carotenoid-based coloration as a condition-dependent, honest signal of individual quality continues to evolve. Previously, it was only assumed that carotenoid pigmentation could be thought of as a reliable fitness correlate simply due to limited-resource tradeoffs between cellular maintenance and visible coloration. However, the experimental results presented in this dissertation, as well as research presented by academics and scientists around the world, should indicate the story is not so simple. We have seen through experimentation with multiple species, including vertebrates and invertebrates, as well as aquatic and terrestrial animals, that we should consider at a more mechanistic level the elements of "fitness" that carotenoid-based traits signal.

In this dissertation, I have presented compelling evidence that the metabolism of dietary carotenoids to ketocarotenoid variants is metabolically tied to the function of mitochondrial organelles of the copepod, *T. californicus*. This offers support for the Shared-Pathway Hypothesis, which argues that observed relationships between carotenoid-based coloration and fitness-related traits is driven by shared biochemical pathways in or near the mitochondria. The relationship between mitochondrial function and carotenoid biosynthesis I observed in *T. californicus* is not straight-forward, but is an agreement with evidence collected from experimentation with many avian species. I argue that this suggest a fundamental connection between the metabolism of ketocarotenoid pigments and the metabolism of energy in the mitochondria that evolved prior to or separately from the need to produce reliable signals from mating. This is because, different from our observations in birds, we see no evidence that copepods are capable of visually perceiving differences in the ketocarotenoid-based coloration of potential mates. Importantly, I argue a fundamental relationship between carotenoid ketolation

and mitochondrial function need not rule out the influence of carotenoid resource tradeoffs in animal systems that produce carotenoid-based coloration, especially those that directly deposit dietary carotenoids into their colorful displays. Instead, I argue that shared biochemical pathways between carotenoid and energy metabolic processes may play a large (or larger) role in regulating the variation in color we see among individuals. Specifically, it appears that the rates at which the reduction-oxidation reactions occur in both processes are positively correlated.

I recommend care be taken to adjust hypotheses accordingly prior to further tests. As originally proposed, the Shared-Pathway Hypothesis argued solely from the perspective of efficiency, suggesting that carotenoid-based pigmentation could act as a signal of metabolic productivity – more ketocarotenoid pigment should signal more ATP per unit of oxygen and substrate consumed. However, the production of energy is not a simple process and is only one of the many metabolic pathways in which mitochondria play a vital role in the cell. Based on the experimental data collected in the experiments presented herein, and the growing body of literature documenting complex interactions between hormones, oxidative phosphorylation, and carotenoid ketolation, I recommend that the Shared-Pathway Hypothesis be revised so as to not exclude the many different ways in which mitochondrial function could be reflected in ketocarotenoid-based coloration. In illustration of this point, carotenoid biosynthesis rates may also reflect metabolic capacity; however, metabolic rate varies depending on the external conditions surrounding an organism. In many cases, increased metabolic rate may signal greater maximum energetic output, thus underscoring the need to carefully evaluate the context in which fitness-related traits are measured. Moving forward, as researchers look to further test and to tease apart subtleties in the mechanisms proposed Shared-Pathway Hypothesis, a careful consideration of mitochondrial physiology and dynamics is absolutely required.

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