

An *in vivo* test of the biologically relevant roles of carotenoids as antioxidants in animals

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Abstract

Carotenoids are well known for their contribution to the vibrant coloration of many animals and have been hypothesized to be important antioxidants. Surprisingly few examples of carotenoids acting as biologically relevant antioxidants *in vivo* exist, in part because experimental designs often employ dosing animals with carotenoids at levels that are rarely observed in nature. Here we use an approach that reduces carotenoid content from wild-type levels to test for the effect of carotenoids as protectants against an oxidative challenge. We used the marine copepod, *Tigriopus californicus* reared on a carotenoid-free or a carotenoid-restored diet of nutritional yeast and then exposed them to a prooxidant. We found that carotenoid-deficient copepods not only accumulated more damage, but also were more likely to die during an oxidative challenge than carotenoid-restored copepods. We suggest that carotenoid reduction, and not supplementation, better tests the proposed roles of carotenoids in other physiological functions in animals.

Introduction

In animals, two distinct, yet related, properties have been attributed to carotenoids: the production of vibrant coloration of integuments and service as biologically relevant antioxidants. Empirical evidence demonstrating the former is ubiquitous and definitively testable (McGraw et al., 2004; Meléndez-Martínez et al., 2006; Weaver et al., 2018a); in contrast, convincing evidence for the latter has been elusive in some systems (Britton, 1995; Costantini and Møller, 2008; Koch et al., 2018; Young and Lowe, 2001). From a fundamental biochemical perspective, carotenoids undeniably have the potential to function as antioxidants. The conjugated system of pi-bonds that makes up the core of carotenoid molecules has the capacity to accept electrons at a higher affinity than other cellular components (*i.e.*, an antioxidant) (Britton, 1995; Gutteridge and Halliwell, 2010). However, the most definitive demonstrations that carotenoids actually function as antioxidants in biological systems comes only from *in vitro* experiments. The relevance of carotenoids as antioxidants *in vivo* remains uncertain with inconsistent evidence in support of antioxidant function across many studies, particularly in vertebrate systems (Britton, 1995; Britton, 2008; Koch et al., 2018; Simons et al., 2012).

A major challenge in testing the role of carotenoids as protectants against cellular damage from oxidative stress is the difficulty of measuring the total amount of carotenoids available to an individual (Koch and Hill, 2018; Parker, 1996). Carotenoids are often measured only from the circulatory system (Hörak et al., 2006; Koch et al., 2018; Miki, 1991) which may not accurately represent levels in specific tissues or the animal as a whole (Pérez-Rodríguez, 2009). In wild animals, the identity and quantity of carotenoids in their diet is often unknown. Experimental designs to test the antioxidant role of carotenoids in animals *in vivo* often include carotenoid supplementation of a group of animals –sometimes those animals naturally have carotenoid-based traits, in other instances they do not– followed by exposure to some pro-oxidant (Aguilera and Amat, 2007; Isaksson and Andersson, 2008; Koch and Hill, 2017). Correlations among carotenoid levels of plasma or other circulatory fluid and the outcome of some measure of oxidative damage in carotenoid-supplemented animals are used to infer the antioxidant function of carotenoids by comparing them to unsupplemented control animals (Alonso- Alvarez et al., 2004; Babin et al., 2010; Miki, 1991).

In contrast to vertebrate systems, the role of carotenoids as antioxidants in invertebrates has received more consistent support from empirical evidence (Atarashi et al., 2017; Babin et al., 2010; Byron, 1981). Freshwater and marine copepods and cladocerans have been especially well studied where carotenoids are often found to provide protection from UV radiation likely through antioxidant mechanisms (Caramujo et al., 2012; Davenport et al., 2004; Hairston, 1976; Sommaruga, 2010). The antioxidant function of carotenoids is often assumed *a priori*, then measurements of carotenoids in tissues are related to some physiological endpoint, such as survival or lipid peroxidation. However, they are typically poor controls for the influence of other dietary components on the resistance to a stressor. Inference from studies that employ supplementation of high concentration carotenoid supplementation experimental design or do not isolate the effect of carotenoids from other dietary components are poorly suited to testing how animals may use carotenoids, specifically, as antioxidants at levels that are typical of their natural diet. An approach that may be more useful would be to reduce the amount of carotenoids in the diet or animal from normal levels and measure the effects of a lack of carotenoids during an oxidative challenge (Atarashi et al., 2017; Davenport et al., 2004).

Because nearly all animals obtain carotenoids exclusively from their diet (Britton and Goodwin, 1982; Parker, 1996), tight control of an animal's carotenoid consumption is possible in a laboratory setting by manipulating the amounts of carotenoids in their food (Caramujo et al., 2012). The marine copepod *Tigriopus californicus* typically consumes carotenoid-rich microalgae in the wild and displays a bright red-orange coloration that results from the bioconversion of dietary algal carotenoids to the red ketocarotenoid astaxanthin (Weaver et al., 2018a). In the laboratory, however, they can be raised on a nutritional yeast-only diet that reduces carotenoid accumulation in body tissues to trace levels while still providing complete nutrition (Davenport et al., 2004). Carotenoids can then be reintroduced into an experimental group of animals by supplementing carotenoids to their diet (Weaver et al., 2018a). Adding carotenoids at biologically relevant concentrations to a base diet that lacks carotenoids isolates the effect of carotenoids on the outcomes of interest. The result of this feeding scheme produces two distinct phenotypes: 1) yeast-fed copepods, that lack carotenoids and 2) carotenoid-restored copepods, that accumulate astaxanthin (Fig 1).

In this study, we tested the potential antioxidant role of carotenoids *in vivo* by exposing carotenoid-deficient and carotenoid-restored *Tigriopus californicus* to a prooxidant (*tert*-Butyl hydroperoxide), then measured survival and enzyme activity of aconitase. The cytotoxicity of prooxidant exposure is mediated by availability of reactants within the cellular targets of reactive oxygen species (ROS) (Kruszewski, 2003). Aconitase is a labile iron-sulfur (Fe-S) containing protein in high abundance within some eukaryotic tissues and has been shown to be sensitive to inactivation by ROS (Cairo et al., 2002; Talib et al., 2014). The release of Fe from aconitase may enhance cytotoxicity of prooxidants by providing a substrate for Fenton-like reactions which leads to production of highly damaging hydroxyl radicals (Jomova and Valko, 2011; Stohs and Bagchi, 1995).

We tested the hypothesis that carotenoids are relevant antioxidants *in vivo* in animals and predicted that carotenoid-restored copepods would show higher survival and aconitase activity following prooxidant exposure than carotenoid-deficient copepods.

Materials and methods

Copepod culturing and experimental design

We have continuously cultured laboratory populations of *Tigriopus californicus* on a carotenoid-free diet of nutritional yeast since 2015 (Weaver et al., 2018a). Just as with most other animal taxa, wild copepods obtain carotenoids exclusively from their diet, which consists of mostly unicellular algae. Our nutritional yeast diet contains inactive dry yeast and lacks carotenoids. As a result, copepods reared on this diet in the lab have only trace amounts of carotenoids in their system (Weaver et al., 2018a). We refer to these copepods as “yeast-fed copepods”. To produce copepods that contain carotenoids, we supplemented a random subset of yeast-fed copepods with 20 $\mu\text{g mL}^{-1}$ zeaxanthin, a carotenoid found in the wild-type algal diet. We have previously shown that zeaxanthin-supplemented yeast-fed copepods metabolize this dietary carotenoid into the red ketocarotenoid, astaxanthin (Weaver et al., 2018a) which they accumulate and deposit to their internal tissues and carapace to produce their characteristic orange-red color. We refer to these copepods as “carotenoid-restored copepods”.

We used *tert*-Butyl hydroperoxide (tBHP) as a general prooxidant (Koch and Hill, 2017) and employed a 2x2 (food x exposure) factorial design that resulted in four groups of copepods, 1) yeast-fed, 2) carotenoid-restored, 3) yeast-fed tBHP-exposed, and 4) carotenoid-restored tBHP-exposed (Fig 1).

Carotenoid analysis using high-performance liquid chromatography

We extracted carotenoids from copepods by sonicating in acetone and incubating at 4 C under nitrogen gas overnight. We separated extracted carotenoids from a 40 μ L sample injection using a Shimadzu HPLC system fit with a Sonoma C18 column (10 μ m, 250 x 4.6 mm, ES Technologies) and C18 guard cartridge. We used mobile phases A) 80:20, methanol: 0.5 M ammonium acetate, B) 90:10, acetonitrile:H₂O, and C) ethyl acetate in a ternary linear gradient following (Weaver et al., 2018a). We quantified astaxanthin by comparison to an authentic standard and report astaxanthin as ng copepod⁻¹ and as μ g mg⁻¹ dry mass.

Survival during tert-butyl hydroperoxide exposure

To test for the potential for astaxanthin to protect against mortality from an oxidative challenge, we exposed yeast-fed and carotenoid-restored copepods to the following concentrations of tBHP: none (0 mM), moderate (1.6 mM), or high (3.3 mM) for 3h and monitored individual survival from individual wells of a 24-well plate ($n=12$ per group). Survival was confirmed if the copepod was spontaneously swimming or if it swam in response to gentle prodding by a pipet tip.

Aconitase activity measurements

We exposed groups of ~80 yeast-fed and carotenoid-restored copepods to either 0 mM (control) or 1 mM tBHP for 1h, then measured total aconitase activity following the manufacturers protocol (cat# K716-100 Biovision, Milpitas, CA). Briefly, copepods were homogenized on ice and the supernatant was activated with cysteine-HCl and ammonium iron(II) sulfate, then reacted with citrate for 1h in the presence of a colorimetric probe. Absorbance was measured at 450nm using a BioTek spectrophotometer and compared to known concentrations of isocitrate standards. Control and tBHP exposure of yeast-fed and carotenoid-restored groups were

replicated in triplicate ($n = 3$ per group) and each sample was measured in duplicate. Total protein was measured using the Bradford method (Bradford, 1976). We subtracted background absorbance from no-substrate control wells to calculate sample aconitase activity, then standardized those values to total protein. We report aconitase activity as nmol of citrate converted to isocitrate $\text{min}^{-1} \text{mL}^{-1} \text{mg}^{-1}$ protein.

Total iron measurements

We used inductively-coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer, Inc. Weltham, MA) to quantify elemental mineral concentrations in each group of copepods (Cobine et al., 2013). For each sample ($n = 8$ per group) we digested 40 copepods in 100 μL concentrated nitric acid at 95 C for 1h, then brought the sample volume to 300 μL with milli-q purified water, then injected in the plasma for analysis. Each sample was measured twice and the average was taken. Metal concentrations of the sample were calculated based on known concentration of mineral standard solutions. Total iron content was normalized to phosphorous and corrected for background metal content. We report iron content results as $\text{ng } \mu\text{g}^{-1}$ phosphorous.

Data analysis

We compared mean aconitase activity and total iron content between tBHP exposed and control groups from yeast-fed and carotenoid-restored copepods using *t*-tests. Survival analysis was performed using logistic regression with generalized linear models. All analyses were performed in R, version 3.3.0 (R Core Team, 2017). Data and code are available in the online repository, Figshare (<https://doi.org/10.6084/m9.figshare.6587684.v1>).

Results and discussion

Whether carotenoids play a significant as antioxidants in animals, *in vivo*, has been a contentious issue in biomedical and ecological physiology and evolutionary biology research for the past several decades (Britton, 1995; Chew and Park, 2004; Gutteridge and Halliwell, 2010; Koch et al., 2018; Svensson and Wong, 2011). Experimental designs to test this idea often include mega-dosing of carotenoids in animal systems that far exceed biological levels (Koch et al., 2016).

Moreover, in vertebrates the effects of treatments are often assessed only in plasma, even though more metabolically active tissues such as liver, brain, or heart may be more relevant (Alonso-Alvarez et al., 2004; Koch et al., 2018). Here, we used a carotenoid-deficient yeast-fed and carotenoid-restored copepod system to test the protective effects of astaxanthin at biologically relevant levels on survival and oxidative damage from prooxidant exposure.

To ensure our system reflects natural abundance of carotenoids relevant to those found in normal diets, we determined the total astaxanthin in carotenoid-restored copepods supplemented with zeaxanthin for 48h. Carotenoid-restored copepods accumulated 7.9 ± 1.2 ng astaxanthin copepod⁻¹ (0.9 ± 0.14 $\mu\text{g mg}^{-1}$ dry mass, $n = 4$), whereas yeast-fed copepods contained only 0.7 ± 0.1 ng astaxanthin copepod⁻¹ (0.06 ± 0.005 $\mu\text{g mg}^{-1}$ dry mass, $n = 5$). The amount of astaxanthin in carotenoid-restored copepods is lower than the mean of wild-type algae-fed copepods reared under the same conditions (Weaver et al., 2018a), but is within a biologically relevant range for this species. Therefore, our system represents a manipulatable system where copepods have a minimum level of carotenoids.

To examine the role of astaxanthin as a protectant against ROS, we used tert-Butyl hydroperoxide (tBHP) as a prooxidant. Under normal conditions, unexposed yeast-fed copepods and carotenoid-restored copepods had similar aconitase activity (Fig 2a, mean $\pm$ s.d.: Yeast: 78.5 ± 10.9 , Carot: 80.9 ± 5.1 , $n = 3$, $t = -0.293$, $P = 0.78$). Exposure to tBHP decreased aconitase activity in carotenoid-restored copepods by 6 %, but this decrease was not statistically significant (Carot-tBHP: 75.83 ± 10.26 , $n = 3$, $t = -0.624$, $P = 0.55$). In contrast, yeast-fed copepods exposed to tBHP showed a 34% decrease in aconitase activity relative to unexposed yeast-fed copepods (Yeast-tBHP: 51.5 ± 11.7 , $n = 3$, $t = -3.35$, $P = 0.01$). These results show that tBHP reduced aconitase activity only in the yeast-fed copepods, suggesting that carotenoids are offering some protection against this oxidative challenge. Indeed, when we analyzed the effect of carotenoid-restoration on tBHP exposure we found that carotenoid-restored copepods had 32.03 % more aconitase activity than yeast-fed copepods ($n = 3$, $t = -3.01$, $P = 0.017$). Differences in aconitase activity were not due to differences in total iron content, as all groups has similar levels of iron (Fig 2b. mean iron ng μg^{-1} phosphorous $\pm$ s.d. Yeast: 4.5 ± 1.8 , Carot: 3.9 ± 1.4 , Carot-tBHP: 5.1 ± 2.7 , Yeast-tBHP: 3.9 ± 0.7 , all comparisons $P > 0.2$, $n = 8$ per group).

Astaxanthin has been shown to be one of the most likely carotenoids to provide biologically relevant protection against oxidative stress in marine organisms (Edge et al., 1997; Shimidzu et al., 1996). Survival following copper and UV light exposure was modulated by astaxanthin content of the meiobenthic copepod, *Amphiascoides atopus* (Caramujo et al., 2012).

Supplemented groups that contained more astaxanthin tended to have higher survival probability than copepods with less astaxanthin. However, it must be noted that in the experiments with *A. atopus* the groups also differed in other dietary components from their algal supplements that may have also impacted survival (Caramujo et al., 2012). In our study, the protective effects of astaxanthin, were observed as increased survival probability of tBHP-exposed copepods. We found that carotenoid-restored copepods were $10.0 (\pm 2.7, \text{mean} \pm \text{s.e.m.})$ and $33.0 (\pm 3.5)$ times as likely to survive than yeast-fed copepods after 3h exposure to 1.6 mM and 3.3 mM tBHP, respectively (1.6mM: $n = 24$, $z = 2.33$, $P = 0.02$; 3.3mM: $n = 24$, $z = 2.82$, $P = 0.005$, Fig. 3). These survival benefits are most likely due to the protective effect of astaxanthin, alone, because carotenoid-restored and yeast-fed copepods only differed by the presence or absence of zeaxanthin in their diet and the amount of astaxanthin in their tissues.

We found that deleterious effects of a general prooxidant, tBHP, were mitigated by the presence of the ketocarotenoid astaxanthin, suggesting that carotenoids may act as relevant antioxidants *in vivo* in a system that naturally accumulates carotenoids in its tissues. Carotenoid-restored copepods not only showed less damage to aconitase proteins (Fig 2a), but also had a higher survival probability (Fig 3) in the face of an oxidative challenge than carotenoid-deficient copepods. Astaxanthin in the carotenoid-restored copepods may have protected inactivation of aconitase or prevented subsequent hydroxyl radical production from an increase in the labile iron pool that results from aconitase inactivation by tBHP. Free radical scavenging by astaxanthin is among the most commonly cited mechanisms involved in the protective effects conferred by this carotenoid (Atarashi et al., 2017; Caramujo et al., 2012; Davenport et al., 2004; Liu and Osawa, 2007; Schneider et al., 2016). Regardless of the mechanisms at play, our results show that astaxanthin acts to protect enzyme activity of a sensitive marker of oxidative stress and supports the idea that species that naturally circulate or store carotenoids in tissues may do so in part because of their antioxidant capacity.

As the body of literature on the possible antioxidant function of carotenoids continues to grow, it is becoming increasingly apparent that perhaps physiological or life-history-based differences among taxa may obscure a general role for carotenoids in this arena (Pérez-Rodríguez, 2009). For example, results from studies on the relationship between carotenoid content and antioxidant function in birds and lizards are often equivocal (Costantini and Møller, 2008; Cote et al., 2010; Koch et al., 2018; Weaver et al., 2018b). In contrast, studies on zooplankton tend to support the idea that carotenoids act as antioxidants, *in vivo*. The differences among taxonomic groups in the relevance or strength of carotenoid antioxidant function is possibly carotenoid-specific. Studies on copepods, which accumulated large amounts of the red ketocarotenoid astaxanthin, often show that carotenoids provide protection against oxidative challenges such as ultra-violet light and xenobiotic exposure (Caramujo et al., 2012; Moeller et al., 2005). Lutein and zeaxanthin are commonly found in the circulatory system of vertebrates, whereas ketocarotenoids such as astaxanthin are less common (McGraw, 2006), and *in vitro* experiments demonstrate that astaxanthin has greater antioxidant potential than less polar carotenoids such as zeaxanthin and β -carotene (Edge et al., 1997; Miki, 1991).

In this study, we focused on understanding the role of carotenoids as antioxidants in an animal that naturally accumulates carotenoids in its tissues by reducing, rather than increasing, the amount of carotenoids normally accumulated. We suggest that this approach is more likely to test the biological relevance of carotenoids as antioxidants than the approach of many previous studies that drastically increases carotenoid content (Koch et al., 2016), often in animals that normally do not accumulate large quantities of carotenoids (Britton, 1995; Chew and Park, 2004; Miki, 1991). In addition, results from many of these studies have been based on sampling plasma from animals, as it is relatively easy and non-lethal. However the results from studies that measure markers of antioxidant activity and prooxidant damage from only plasma may not accurately represent the consequences of prooxidant exposure in other tissues (Pérez-Rodríguez et al., 2015).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.J.W.; Methodology: R.J.W., P.W. & P.A.C.; Formal analysis: R.J.W.
Investigation: R.J.W. & P.W. Resources: R.J.W. & P.A.C Writing - original draft: R.J.W;
Writing -review & editing: all authors Supervision: G.E.H; Project administration: G.E.H

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References

- Aguilera, E. and Amat, J. A.** (2007). Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). *Naturwissenschaften* **94**, 895–902.
- Alonso- Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. and Sorci, G.** (2004). An Experimental Test of the Dose- Dependent Effect of Carotenoids and Immune Activation on Sexual Signals and Antioxidant Activity. *Am. Nat.* **164**, 651–659.
- Atarashi, M., Manabe, Y., Kishimoto, H., Sugawara, T. and Osakabe, M.** (2017). Antioxidant protection by astaxanthin in the citrus red mite (Acari: Tetranychidae). *Environ. Entomol.* **46**, 1143–1150.
- Babin, A., Biard, C. and Moret, Y.** (2010). Dietary Supplementation with Carotenoids Improves Immunity without Increasing Its Cost in a Crustacean. *Am. Nat.* **176**, 234–241.
- Bradford, M. M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Britton, G.** (1995). Structure and properties of carotenoids in relation to function. *FASEB J.* **9**, 1551–1558.
- Britton, G.** (2008). Functions of intact carotenoids. In *Carotenoids: Natural functions* (ed. Britton, G., Liaaen-Jensen, S., and Pfander, H.), pp. 189–211. Basel: Birkhauser Verlag.
- Britton, G. and Goodwin, T.** (1982). *Carotenoid chemistry and biochemistry*. (ed. Britton, G.) and Goodwin, T.) New York: Pergamon Press Ltd.
- Byron, E. R.** (1981). Metabolic stimulation by light in a pigmented freshwater invertebrate. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 1765–1767.
- Cairo, G., Recalcati, S., Pietrangelo, A. and Minotti, G.** (2002). The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage. *Free Radic. Biol. Med.* **32**, 1237–1243.
- Caramujo, M.-J., de Carvalho, C. C. C. R., Silva, S. J. and Carman, K. R.** (2012). Dietary carotenoids regulate astaxanthin content of copepods and modulate their susceptibility to UV light and copper toxicity. *Mar. Drugs* **10**, 998–1018.
- Chew, B. P. and Park, J. S.** (2004). Carotenoid action on the immune response. *J. Nutr.* **134**, 257S–261S.

- Cobine, P. A., Cruz, L. F., Navarrete, F., Duncan, D., Tygart, M. and de la Fuente, L.** (2013). *Xylella fastidiosa* Differentially Accumulates Mineral Elements in Biofilm and Planktonic Cells. *PLoS One* **8**, e54936.
- Costantini, D. and Møller, A. P.** (2008). Carotenoids are minor antioxidants for birds. *Funct. Ecol.* **22**, 367–370.
- Cote, J., Meylan, S., Clobert, J. and Voituron, Y.** (2010). Carotenoid-based coloration, oxidative stress and corticosterone in common lizards. *J. Exp. Biol.* **213**, 2116–2124.
- Davenport, J., Healy, A., Casey, N. and Heffron, J.** (2004). Diet-dependent UVAR and UVBR resistance in the high shore harpacticoid copepod *Tigriopus brevicornis*. *Mar. Ecol. Prog. Ser.* **276**, 299–303.
- Edge, R., McGarvey, D. J. and Truscott, T. G.** (1997). The carotenoids as anti-oxidants--a review. *J. Photochem. Photobiol. B.* **41**, 189–200.
- Gutteridge, J. M. C. and Halliwell, B.** (2010). Antioxidants: Molecules, medicines, and myths. *Biochem. Biophys. Res. Commun.* **393**, 561–564.
- Hairston, N. C.** (1976). Photoprotection by carotenoid pigments in the copepod *Diaptomus nevadensis*. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 971–4.
- Hõrak, P., Zilmer, M., Saks, L., Ots, I., Karu, U. and Zilmer, K.** (2006). Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. *J. Exp. Biol.* **209**, 4329–38.
- Isaksson, C. and Andersson, S.** (2008). Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. *Proc. R. Soc. B-Biological Sci.* **275**, 309–314.
- Jomova, K. and Valko, M.** (2011). Advances in metal-induced oxidative stress and human disease. *Toxicology* **283**, 65–87.
- Koch, R. E. and Hill, G. E.** (2017). An assessment of techniques to manipulate oxidative stress in animals. *Funct. Ecol.* **31**, 9–21.
- Koch, R. E. and Hill, G. E.** (2018). Do carotenoid-based ornaments entail resource tradeoffs? An evaluation of theory and data. *Funct. Ecol.* 0–2.
- Koch, R. E., Wilson, A. E. and Hill, G. E.** (2016). The Importance of Carotenoid Dose in Supplementation Studies with Songbirds. *Physiol. Biochem. Zool.* **89**, 61–71.
- Koch, R. E., Kavazis, A. N., Hasselquist, D., Hood, W. R., Zhang, Y., Toomey, M. B. and Hill, G. E.** (2018). No evidence that carotenoid pigments boost either immune or

- antioxidant defenses in a songbird. *Nat. Commun.* **9**, 491.
- Kruszewski, M.** (2003). Labile iron pool: The main determinant of cellular response to oxidative stress. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* **531**, 81–92.
- Liu, X. and Osawa, T.** (2007). Cis astaxanthin and especially 9-cis astaxanthin exhibits a higher antioxidant activity in vitro compared to the all-trans isomer. *Biochem. Biophys. Res. Commun.* **357**, 187–193.
- McGraw, K. J.** (2006). Mechanics of carotenoid coloration. In *Bird Coloration, Volume 1: Measurements and Mechanisms* (ed. Hill, G. E. and McGraw, K. J.), p. Cambridge, Massachusetts: Harvard University Press.
- McGraw, K. J., Hudon, J., Hill, G. E. and Parker, R. S.** (2004). A simple and inexpensive chemical test for behavioral ecologists to determine the presence of carotenoid pigments in animal tissues. *Behav. Ecol. Sociobiol.* **57**, 391–397.
- Meléndez-Martínez, A. J., Britton, G., Vicario, I. M. and Heredia, F. J.** (2006). Relationship between the colour and the chemical structure of carotenoid pigments. *Food Chem.* **101**, 1145–1150.
- Miki, W.** (1991). Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **63**, 141–146.
- Moeller, R. E., Gilroy, S., Williamson, C. E., Grad, G. and Sommaruga, R.** (2005). Dietary acquisition of photoprotective compounds (mycosporine-like amino acids, carotenoids) and acclimation to ultraviolet radiation in a freshwater copepod. *Limnol. Oceanogr.* **50**, 427–439.
- Parker, R. S.** (1996). Absorption, metabolism, and transport of carotenoids. *Faseb J* **10**, 542–551.
- Pérez-Rodríguez, L.** (2009). Carotenoids in evolutionary ecology: Re-evaluating the antioxidant role. *BioEssays* **31**, 1116–1126.
- Pérez-Rodríguez, L., Romero-Haro, A. A., Sternalski, A., Muriel, J., Mougeot, F., Gil, D. and Alonso-Alvarez, C.** (2015). Measuring oxidative stress: the confounding effect of lipid concentration in measures of lipid peroxidation. *Physiol. Biochem. Zool.* **88**, 345–351.
- R Core Team** (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Schneider, T., Grosbois, G., Vincent, W. F. and Rautio, M.** (2016). Carotenoid accumulation in copepods is related to lipid metabolism and reproduction rather than to UV-protection.

- Limnol. Oceanogr.* **61**, 1201–1213.
- Shimidzu, N., Goto, M. and Miki, W.** (1996). Carotenoids as Singlet Oxygen Quenchers in Marine Organisms. *Fish. Sci.* **62**, 134–137.
- Simons, M. J. P., Cohen, A. A. and Verhulst, S.** (2012). What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds-A meta-analysis. *PLoS One* **7**, e43088.
- Sommaruga, R.** (2010). Preferential accumulation of carotenoids rather than of mycosporine-like amino acids in copepods from high altitude Himalayan lakes. *Hydrobiologia* **648**, 143–156.
- Stohs, S. J. and Bagchi, D.** (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* **18**, 321–336.
- Svensson, P. A. and Wong, B. B. M.** (2011). Carotenoid-based signals in behavioural ecology: a review. *Behaviour* **148**, 131–189.
- Talib, J., Cook, N., Pattison, D. and Davies, M.** (2014). Disruption of the iron-sulfur cluster of aconitase by myeloperoxidase-derived oxidants. *Free Radic. Biol. Med.* **75**, S27–S28.
- Weaver, R. J., Cobine, P. A. and Hill, G. E.** (2018a). On the bioconversion of dietary carotenoids to astaxanthin in the marine copepod, *Tigriopus californicus*. *J. Plankton Res.* **40**, 142–150.
- Weaver, R. J., Santos, E. S. A., Tucker, A. M., Wilson, A. E. and Hill, G. E.** (2018b). Carotenoid metabolism strengthens the link between feather coloration and individual quality. *Nat. Commun.* **9**, 73.
- Young, A. J. and Lowe, G. M.** (2001). Antioxidant and Prooxidant Properties of Carotenoids. *Arch. Biochem. Biophys.* **385**, 20–27.

Figures

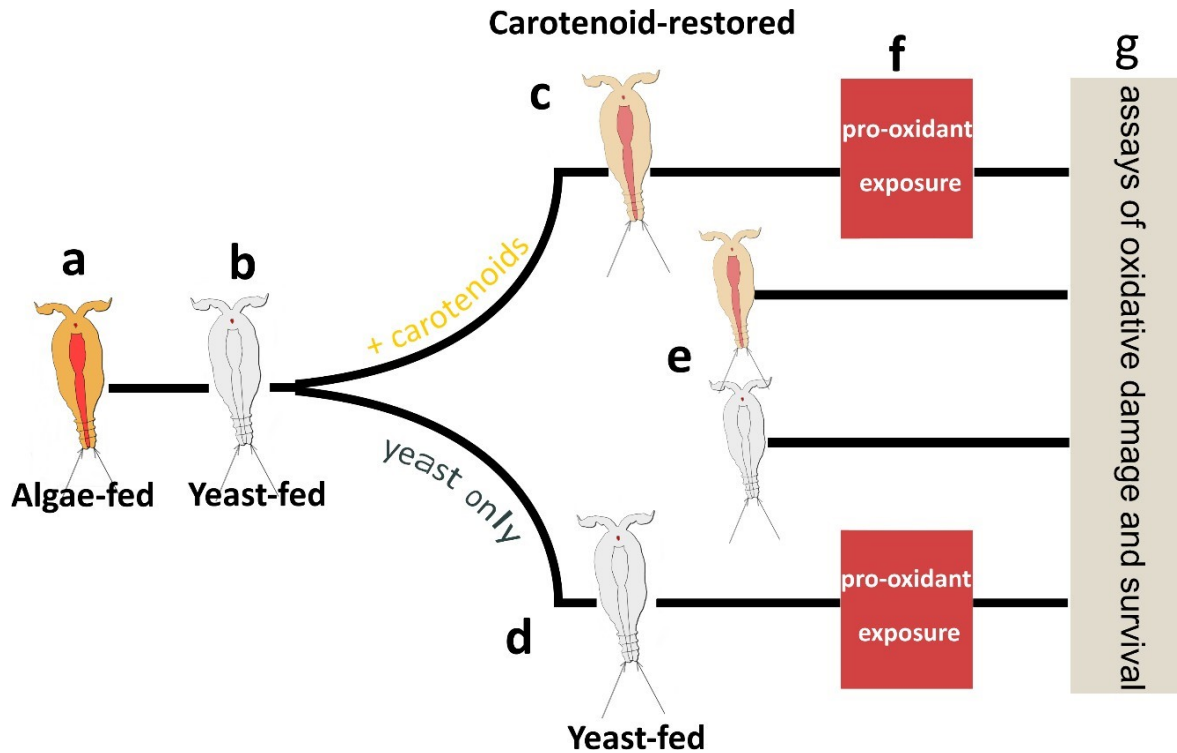


Figure 1.

Overview of the experimental design. Wild-type *Tigriopus californicus* copepods (A) were switched to a yeast-only diet that lacks carotenoids (B). Carotenoids were restored to a subset of copepods by supplementing the yeast diet with zeaxanthin (C), while another group of copepods remained on the yeast only diet (D). Copepods from both groups were exposed to the same oxidative challenges (F) or kept as unexposed controls (E) then assayed for survival, aconitase activity, and iron content (G).

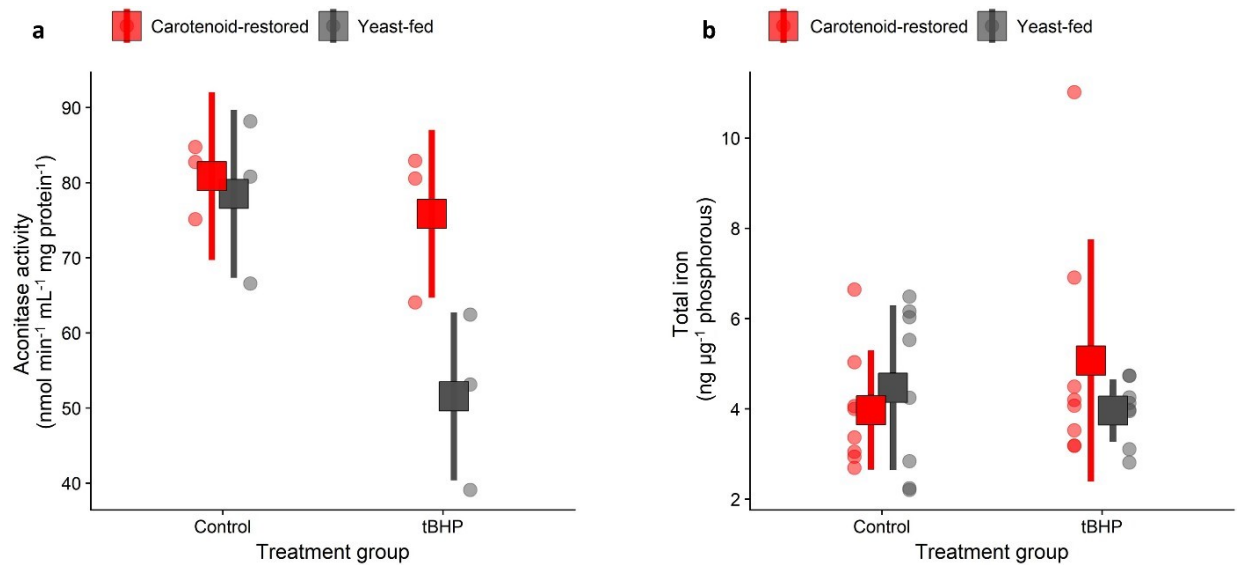


Figure 2.

Physiological responses of carotenoid-deficient and carotenoid-restored copepods to tert-butyl hydroperoxide exposure. (A) Aconitase activity ($n = 3$ replicates of 80 copepods per group) and (B) total iron content ($n = 8$ replicates of 40 copepods per group) of carotenoid-restored (red) and yeast-fed (gray) copepods either exposed to 1 mM tert-butyl hydroperoxide (tBHP) or unexposed controls. Carotenoid-restored copepods exposed to tBHP had greater aconitase activity than yeast-fed copepods (t-test; $P = 0.017$), but did not differ in iron content (t-test; $P > 0.2$). Squares and lines represent the mean $\pm$ s.e.m., while circles represent the individual samples within each group.

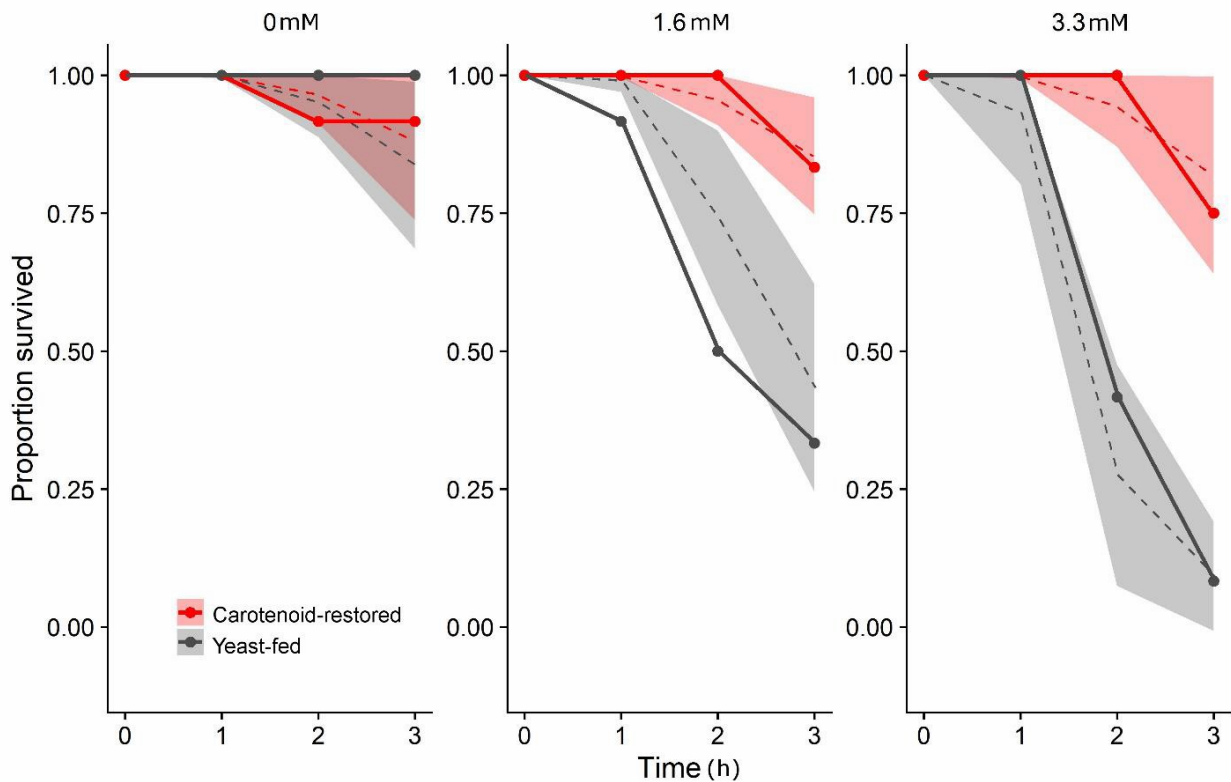


Figure 3.

Individual survival during tert-butyl hydroperoxide exposure. The proportion of copepods that survived exposure to no (0mM), moderate (1.6 mM), and high 3.3 mM) tert-butyl hydroperoxide over 3h ($n = 12$ per group). Carotenoid-restored copepods were more likely to survive than yeast-fed copepods after 3h exposure to 1.6 mM (generalized linear model; $P = 0.02$) and 3.3mM ($P = 0.005$) tBHP. Circles and solid lines represent raw counts and dashed lines and shaded area represent the model estimated mean $\pm$ 95% c.i. for survival of carotenoid-restored (red) and yeast-fed (gray) copepods.