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SHORT COMMUNICATION

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 carotenoid doses at levels that are rarely observed in nature. Here, we used 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 pro-oxidant. 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.

KEY WORDS: *Tigriopus*, Astaxanthin, Copepod

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 come 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, 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 (Hõrak 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 the 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 fluids and the outcome of some measure of oxidative damage in carotenoid-supplemented animals are used to infer the antioxidant function of carotenoids by comparison with data from 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, and carotenoids have often been 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 high-concentration carotenoid supplementation in the experimental design or do not isolate the effect of carotenoids from those of 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 is 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* (Baker 1912) 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

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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, which lack carotenoids, and (2) carotenoid-restored copepods, which accumulate astaxanthin (Fig. 1).

In this study, we tested the potential antioxidant role of carotenoids *in vivo* by exposing carotenoid-deficient and carotenoid-restored *T. californicus* to a pro-oxidant (*tert*-butyl hydroperoxide), then measuring survival and enzyme activity of aconitase. The cytotoxicity of pro-oxidant exposure is mediated by the 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 the cytotoxicity of pro-oxidants by providing a substrate for Fenton-like reactions, which leads to the 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 pro-oxidant exposure than carotenoid-deficient copepods.

MATERIALS AND METHODS

Copepod culturing and experimental design

We have continuously cultured laboratory populations of *T. 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 µg 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 in 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 pro-oxidant (Koch and Hill, 2017) and employed a 2×2 (food×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 copepods (Fig. 1).

Carotenoid analysis using high-performance liquid chromatography (HPLC)

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 µl sample injection using a Shimadzu HPLC system fitted with a Sonoma C18 column (10 µm, 250×4.6 mm, ES Technologies, North Chelmsford, MA, USA) and C18 guard cartridge. We used mobile phases (A) 80:20, methanol:0.5 mol l^{-1} ammonium acetate, (B) 90:10, acetonitrile: H_2O, and (C) ethyl acetate in a ternary linear gradient following Weaver et al. (2018a). We quantified astaxanthin by comparison with an authentic standard and report astaxanthin as ng per copepod and as µg mg^{-1} dry mass.

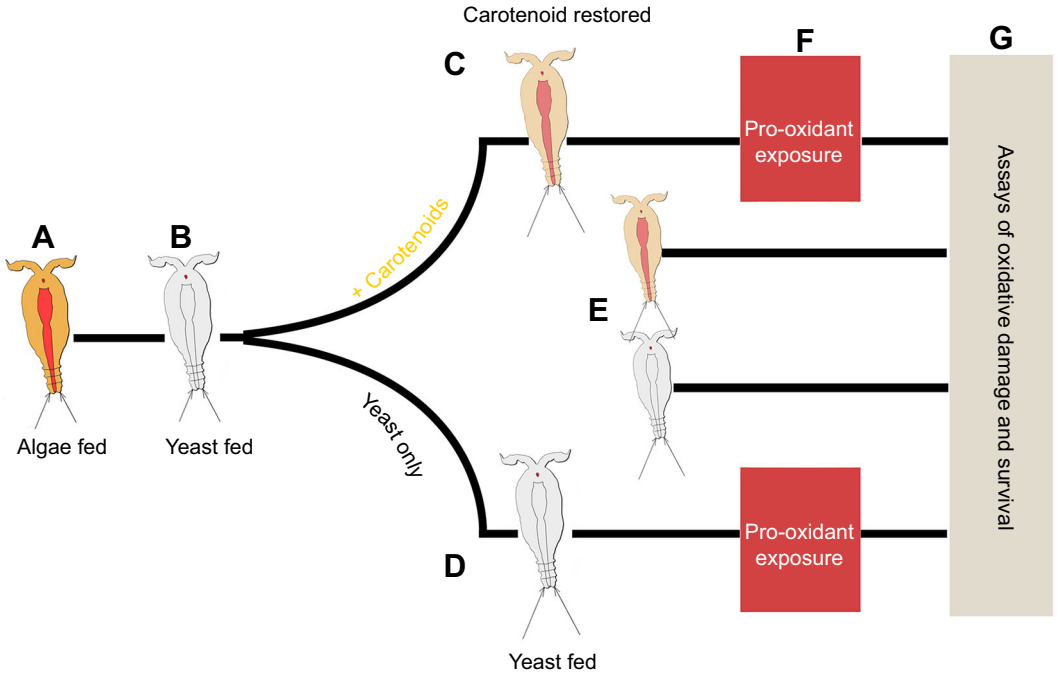

Fig. 1. Overview of the experimental design. Wild-type *Tigriopus californicus* copepods (A) were switched to a yeast-only diet that lacks carotenoids (B). Carotenoids were restored to a subset of copepods by supplementing the yeast diet with zeaxanthin (C), while another group of copepods remained on the yeast-only diet (D). Copepods from the two groups were exposed to the same oxidative challenge (F) or kept as unexposed controls (E), then assayed for survival, aconitase activity and iron content (G).

Survival during tBHP 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: zero, moderate (1.6 mmol l^{-1}) or high (3.3 mmol l^{-1}) for 3 h 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 measured total aconitase activity in groups of ∼80 yeast-fed and carotenoid-restored copepods exposed to 1 mmol l^{-1} tBHP for 1 h versus controls (not exposed), following the manufacturer's protocol (cat. no. K716-100 Biovision, Milpitas, CA, USA). Briefly, copepods were homogenized on ice and the supernatant was activated with cysteine-HCl and ammonium iron (II) sulfate, then reacted with citrate for 1 h in the presence of a colorimetric probe. Absorbance was measured at 450 nm using a BioTek spectrophotometer and compared with known concentrations of isocitrate standards. Control and tBHP exposure of yeast-fed and carotenoid-restored groups was 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 min^{-1} ml^{-1} mg^{-1} protein.

Total iron measurements

We used inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer, Inc., Weltham, MA, USA) 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 1 h, then brought the sample volume to 300 µl with Milli-Q purified water for analysis. Each sample was measured twice and the average was taken. Metal concentrations of the sample were calculated based on the 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 ng μg^{-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 (http://www.R-project.org/). 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 role 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 pro-oxidant exposure.

To ensure our system reflects the natural abundance of carotenoids relevant to that found in normal diets, we determined the total astaxanthin in carotenoid-restored copepods supplemented with zeaxanthin for 48 h. Carotenoid-restored copepods accumulated 7.9±1.2 ng astaxanthin per copepod (0.9±0.14 µg mg^{-1} dry mass, n=4), whereas yeast-fed copepods contained only 0.7±0.1 ng astaxanthin per copepod (0.06±0.005 µg 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 tBHP as a pro-oxidant. Under normal conditions, unexposed yeast-fed copepods and carotenoid-restored copepods had similar aconitase activity (Fig. 2A, mean±s.d.: yeast: 78.5±10.9 nmol min^{-1} ml^{-1} mg^{-1} protein, carotenoid: 80.9±5.1 nmol min^{-1} ml^{-1} mg^{-1} protein, 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 (carotenoid–tBHP: 75.83±10.26 nmol min^{-1} ml^{-1} mg^{-1} protein, 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 nmol min^{-1} ml^{-1} mg^{-1} protein, 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 offer 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 had similar levels of iron (Fig. 2B; mean±s.d.: yeast: 4.5±1.8 ng iron μg^{-1} phosphorous, carotenoid: 3.9±1.4 ng iron μg^{-1} phosphorous, carotenoid–tBHP: 5.1±2.7 ng iron μg^{-1} phosphorous, yeast–tBHP: 3.9±0.7 ng iron μg^{-1} phosphorous, 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±2.7 and 33.0±3.5 times (means±s.e.m.) more likely to survive than yeast-fed copepods after 3 h exposure to 1.6 and 3.3 mmol l^{-1} tBHP, respectively (1.6 mmol l^{-1} tBHP: n=24, z=2.33, P=0.02; 3.3 mmol l^{-1} tBHP: n=24, z=2.82, P=0.005, Fig. 3). These survival

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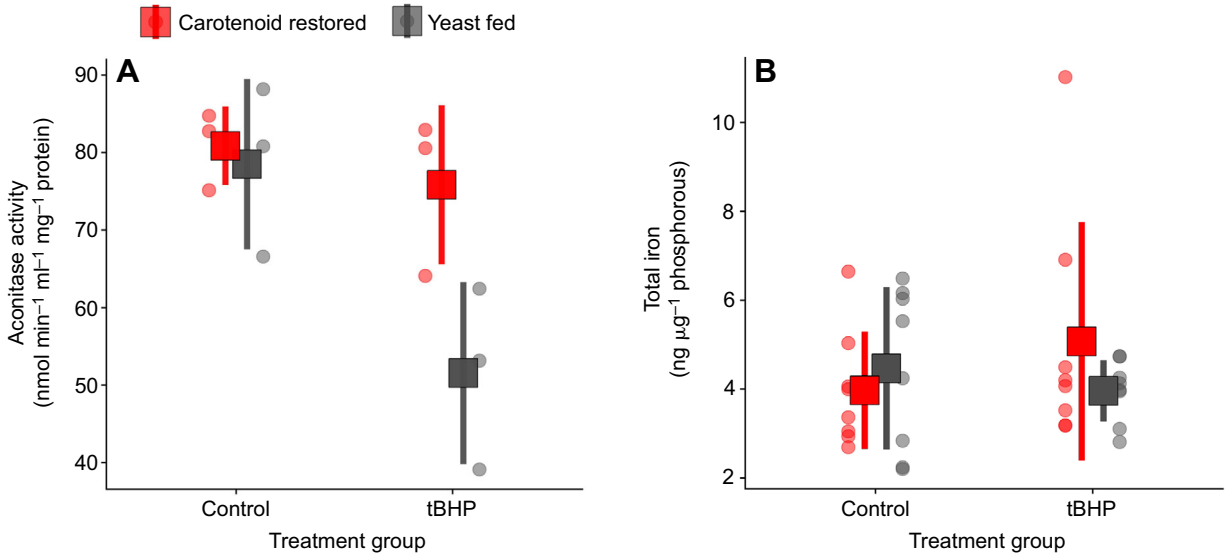

Fig. 2. Physiological responses of carotenoid-deficient and carotenoid-restored copepods to tert-butyl hydroperoxide exposure. (A) Aconitase activity (n=3 replicates of 80 copepods per group) and (B) total iron content (n=8 replicates of 40 copepods per group) of carotenoid-restored (red) and yeast-fed (gray) copepods exposed to 1 mmol l^{-1} tert-butyl hydroperoxide (tBHP) versus 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±s.d., while circles represent the individual samples within each group.

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 pro-oxidant, 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 prevented inactivation of aconitase or 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

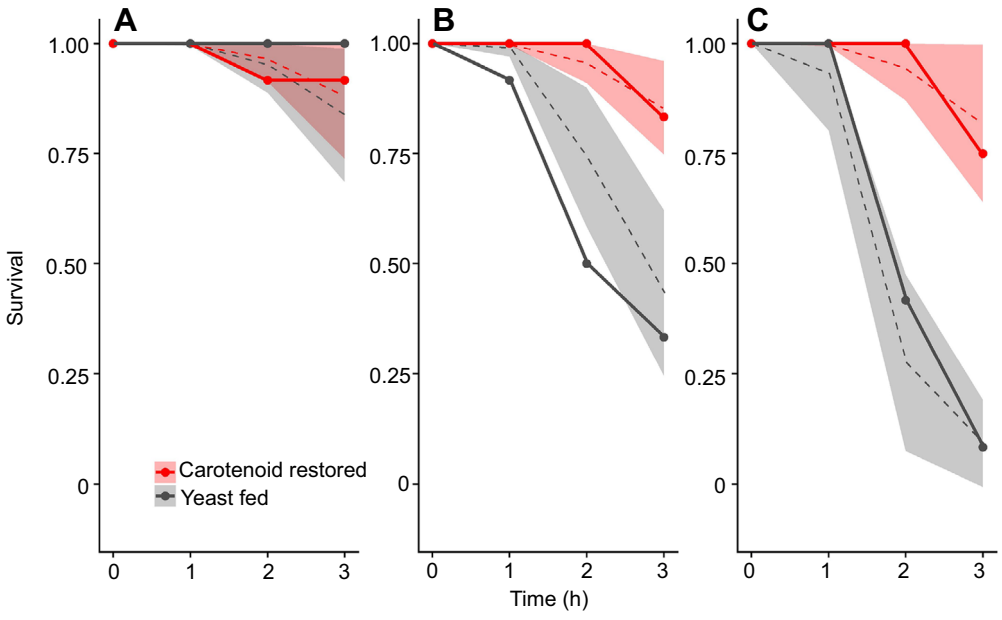

Fig. 3. Individual survival during tBHP exposure. The proportion of copepods that survived exposure to no (A), moderate (1.6 mmol l^{-1}; B) and high (3.3 mmol l^{-1}; C) tBHP over 3 h (n=12 per group). Carotenoid-restored copepods were more likely to survive than yeast-fed copepods after 3 h exposure to 1.6 mmol l^{-1} (generalized linear model; P=0.02) and 3.3 mmol l^{-1} (P=0.005) tBHP. Circles and solid lines represent raw counts and dashed lines and shaded areas represent the model-estimated mean±95% confidence interval for survival of carotenoid-restored (red) and yeast-fed (gray) copepods.

(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 UV 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 present. 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 pro–oxidant damage from only plasma may not accurately represent the consequences of pro-oxidant 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: R.J.W., P.W., G.E.H., P.A.C.; Supervision: G.E.H.; Project administration: G.E.H.; Funding acquisition: R.J.W., P.W.

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Data availability
Data and code are available from the figshare data repository: https://doi.org/10.6084/m9.figshare.6587684.v2

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