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Copper exposure reduces production of red carotenoids in a marine copepod

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abstract

Sub-lethal exposure to copper has been shown to modulate both mitochondrial function and antioxidant gene expression in zooplankton. To date, however, researchers have not identified a quantifiable phenotypic trait that reliably indicates such physiological responses to copper exposure. Red ketocarotenoids are abundant in marine zooplankton serving both physiological and coloration roles, and their production is sensitive to environmental stress. In this study the expression of mitochondrial gene cytochrome c oxidase I (COI) and antioxidant gene glutathione reductase (GR), and the production of red ketocarotenoid, astaxanthin, was measured in response to sub-lethal copper exposure. We found that mRNA of COI and GR was more abundant in copper-exposed copepods than controls, suggesting there was a physiological response to copper exposure. At the same time, copper-exposed copepods produced less astaxanthin than controls. We suggest that ketocarotenoid content of zooplankton has the potential to be a sensitive bioindicator of marine environmental pollution. Understanding how cellular responses to environmental stressors manifest in the phenotypes of marine animals will greatly increase our capacity to monitor marine ecosystem health.

1. Introduction

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

Copper toxicity compromises the health of marine animals by catalyzing the formation of reactive oxygen species (ROS) that damage cellular components (Jomova and Valko, 2011; Valko et al., 2006) and by depleting antioxidants including glutathione (Speisky et al., 2009). The damaging effects from copper-induced ROS include reduced energy metabolism and growth (Bancroft et al., 2007; Sabatini et al., 2009), decreased fecundity and longevity (Bielmyer et al., 2006; Munkittrick and Dixon, 1988), and ultimately disruptions to population and food web dynamics (Hamilton, 2004; Real et al., 2003). Free copper ions require existing ROS (*e.g.* superoxide or H_2O_2) or other reducing equivalents as redox partners to induce oxidative damage *via* hydroxyl radicals (Jomova and Valko, 2011), and mitochondria are the main site of ROS generation (Brookes, 2005; Kowaltowski et al., 2009). As a result, mitochondrial membranes in particular take the brunt of heavy-metal-induced oxidative damage through lipid peroxidation of the inner mitochondrial membrane (Gaetke and Chow, 2003), and oxidative stress has been shown to reduce the rates of oxidative phosphorylation and activity of complex IV (cytochrome c oxidase) of the electron transport system (Belyaeva et al., 2008; Krumschnabel et al., 2005; Sokol et al., 1993). Disruption of core

Abbreviations: COI, cytochrome c oxidase subunit 1; GR, glutathione reductase; UPLC, ultra-high performance chromatography; HPLC, high-performance liquid chromatography; GAPHD, glyceraldehyde 3-phosphate dehydrogenase; MCMC, Markov chain Monte Carlo iterative process.

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mitochondrial processes such as oxidative phosphorylation has systemic deleterious effects. For example, copper-exposed copepods show decreased growth and development rates (Lee et al., 2008a) and modulated expression of key genes involved in mitochondrial respiration and antioxidant defense (Ki et al., 2009; Lee et al., 2008b).

Aerobically respiring organisms have evolved a complex network of enzymatic and non-enzymatic antioxidants as an innate defense system to maintain ROS homeostasis and minimize oxidative damage. Isoforms of superoxide dismutase are enzymatic antioxidants that reduce the primary ROS generated, superoxide, to the less reactive secondary ROS, hydrogen peroxide (H_2O_2). Glutathione can reduce H_2O_2 to water, thus eliminating one of the substrates by which copper ions catalyze hydroxyl radical production. Through the acceptance of an electron from H_2O_2, glutathione becomes oxidized thus losing antioxidant potential. However, radical scavenging by glutathione is not a consumptive process; glutathione reductase (GR) regenerates glutathione to its reduced state, restoring its antioxidant capacity. Glutathione also directly binds with free copper ions, thus directly reducing glutathione availability, and creating a more oxidized cellular environment which favors the production of ROS (Freedman et al., 1989). Gene expression profiling of antioxidant defense response to heavy metal exposure has frequently included GR to understand physiological consequences of environmental stressors in organisms at the base of the marine food web (Ki et al., 2009; Kwok and Leung, 2005; Raisuddin et al., 2007; Rhee et al., 2013).

Because of the targeted effects of copper on mitochondrial function, we were intrigued by the potential of monitoring copper pollution *via* phenotypic traits that are sensitive to mitochondrial function (Hill, 2014). The development of a bioassay using a model species that reliably reflects copper contamination or other environmental pollutants would facilitate monitoring of marine food webs. The marine copepod *Tigriopus japonicus* is widely used in ecotoxicology (Raisuddin et al., 2007), and this species has three key attributes that make it an ideal system for studying the impact of copper pollution on marine ecosystem health. First, *T. japonicus* is easy to collect in large quantities along the rocky shores of the East Asian Pacific coast and the species does well in laboratory settings. Second, the toxicities of many environmental stressors have been studied using *T. japonicus* (Raisuddin et al., 2007). Third, the entire mitochondrial genome and many nuclear genes have been annotated (Jung et al., 2006). Here we propose that the orange-red color of *T. japonicus,* which results from metabolic conversion of dietary yellow algal carotenoids to the red ketocarotenoid, astaxanthin (Goodwin, 1986), holds potential to be an excellent bioassay of copper pollution. The red carotenoid astaxanthin comprises more than 95% of the carotenoids detected in *Tigriopus* copepods (R. Weaver *unpublished data*) and is almost exclusively responsible for their characteristic orange-red coloration. T. *japonicus* has been well studied in ecotoxicology, but the use of its red carotenoid production has not been investigated as a biomarker for environmental stress.

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

In this study, we tested the hypothesis that production of red ketocarotenoids is sensitive to environmental stressors by exposing *T. japonicus* copepods to copper (Cu^{2+} from $CuSO_4$) and measuring changes in expression of mitochondrial and antioxidant genes and in carotenoid content. We predicted that if environmental stressors induced mitochondrial dysfunction and increased ROS production then exposure to copper would decrease the production of red carotenoid pigments by *T. japonicus* copepods.

2. Materials and methods

2.1. Animal collection and culturing conditions

T. japonicus were collected from Hoping Dao Island, Keelung City, Taiwan (25°09′47.2″N, 121°45′48.2″E) and reared in the lab in 0.22 μm filtered artificial seawater (ASW, 35 psu salinity, pH 8.03) at 26C (±0.5), on a 14 h light: 10 h dark cycle. Copepods were fed live microalga *Tetraselmis chui* (Butcher) daily. This species of microalgae provides complete nutrition for *T. japonicus* as well as the yellow precursor carotenoids—β-carotene and [illegible]are necessary for the production of astaxanthin (Brown and Jeffrey, 1992; Goodwin and Srisukh, 1949). Importantly, *Tetraselmis chui* contains no red ketolated carotenoids. Therefore any astaxanthin detected in *T. japonicus* will have come from oxidation of dietary yellow carotenoids by the copepods. Copepods were acclimated to lab conditions for 2 weeks prior to exposure experiments.

2.2. Copper exposure

To test for the effect of environmental stressors on copepod carotenoid content we exposed *T. japonicus* to sub-lethal levels of copper in an acute 24 h exposure experiment. Copper test solutions were made from 1 M stock solution of $CuSO_4$ (Wako Pure Chemicals Osaka, Japan) and diluted to a concentration of 2 mg/L using filtered ASW, pH 8.03. This copper concentration has been shown to have low mortality, but still modulate antioxidant gene transcript levels in *T. japonicus* (Lee et al., 2007, 2008b; Rhee et al., 2013), which suggests that there are physiological consequences at this exposure level. All stock solutions were prepared the same day as the beginning of the exposure experiment.

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

To measure how carotenoid content and expression of antioxidant (glutathione reductase; GR) and mitochondrial (cytochrome

Table 1
Primers used for RT-qPCR.

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

F: forward primer; R: reverse primer.

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

2.3. Carotenoid analysis using ultra high performance liquid chromatography (UPLC)

At the time of sampling, copepods were homogenized in 500 μL HPLC-grade acetone and incubated in the dark at 4C for 24 h. After incubation, supernatant containing carotenoids was filtered through 0.2 μm hydrophilic polypropylene discs (Pall Corp), evaporated to dryness under vacuum at 40C then re-suspended in 100 μL acetone and kept at −80C until all samples were collected. All samples were run at the same time under the following conditions: 10 μL of sample was injected to a Waters Acquity H-Class BEH C18 (1.7 μm, 2.1 mm ⊠0 mm) UPLC column kept at 7C. The sample was delivered to the column using a linear gradient mobile phase consisting of A: 100% dH2O, B: Acetonitrile:Methanol, 70:30 v/v at flow rate of 0.2 mL min^{-1}. Initial ratio of 15% A: 85% B for 10 s, 100% B for 5.86 min, then returned to initial ratio over 4 min. Relative amounts of carotenoids were quantified by an ultraviolet/visible detector set to 450 nm by calculating the area under the curve of each peak. Free astaxanthin was identified by comparison of peak retention time to an analytical standard obtained under identical UPLC conditions (Sigma). Carotenoid extraction and processing were done under low light conditions to prevent carotenoid artefacts from photo-oxidation.

2.4. mRNA preparation and RT-qPCR

Whole adult copepods were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) on ice immediately after sampling. Total RNA was extracted and purified following manufacture's protocol and DNA contaminants were removed using DNase I (Promega, Madison, WI, USA). Total RNA quality and quantity was assessed by measuring absorbance at 260 and 280 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from 0.5 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA); total reaction volume was 20 μL. mRNA transcript abundance of GR and COI genes were measured using real time RT-PCR along with reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPHD; Table 1). SYBR Green (Invitrogen) was used as a fluorescent dye for the detection of target and reference gene PCR products. Amplification and detection was carried out on a Roche LightCycler 480 using 1 μL of cDNA with 0.2 μL of each primer set of target and reference genes were reacted under the following conditions: 1 cycle for 5 min at 95 °C followed by 45 cycles of 95 °C, 58 °C, and 72 °C for 10 s each. To confirm amplification of target genes melting curve analysis was conducted with 1 cycle of 95 °C for 5 s at a rate of 4.4 °C/s, 65 °C for 1 min at a rate of 2.2 °C/s, then increased to a hold temperature of 97 °C at a rate of 0.11 °C/s with 5 readings/°C. qPCR reactions for each of the five biological replicates per treatment were analyzed in triplicate in a 384-well format with no-template and no-reverse-transcription controls.

2.5. Statistical analyses

2.5.1. Modeling RT-qPCR data

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

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

2.5.2. Astaxanthin analysis

To evaluate how copper exposure affected the production of carotenoids we report carotenoid amounts as the mean relative change in carotenoids (with 95% confidence intervals) from time zero. Preliminary results found that free astaxanthin (*i.e.* not esterified) to be the dominant carotenoid of this species (95%) with mono- and di-esters of astaxanthin and unidentified minor carotenoids comprising approximately 5% of the total carotenoid content. Because relative amounts of total carotenoids and free astaxanthin were highly correlated (R^2 = 0.987 Fig. S1), we used only relative amount of free astaxanthin from time 0 h as the response variable in our models. We compared relative astaxanthin amount between treatment groups over time using a linear mixed effects model, including fixed effects of treatment and time

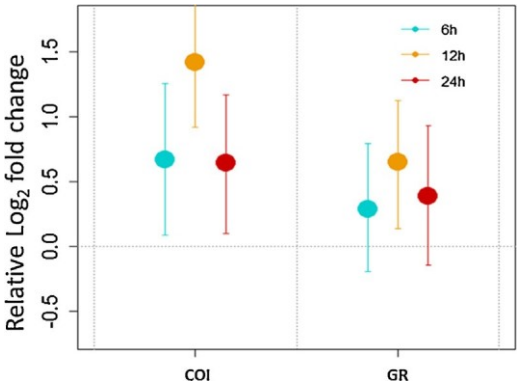

Fig. 1. Log_2 fold change in transcript abundance of cytochrome c oxidase 1 (COI) and glutathione reductase (GR) during 24 h copper exposure relative to control copepods. Abundance data were normalized to reference gene (GAPHD) transcript abundance. Mean ±95% credible intervals. Credible intervals that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

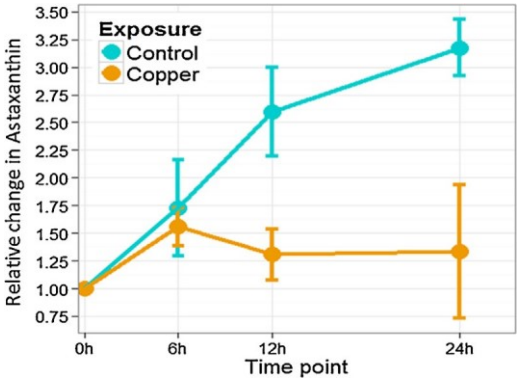

Fig. 2. Relative change in astaxanthin levels in copepods relative to time 0 h. Mean (±SE). Values greater than 1 indicate an increase in astaxanthin while a value less than 1 indicate decreased astaxanthin relative to time 0 h.

point as well as an interaction between treatment and time. We also included a random effect of beaker ID to account for repeated sampling from the same beaker.

3. Results

3.1. Changes in target gene mRNA abundance during 24 h Cu exposure

We found that COI transcript abundance of Cu-exposed copepods was significantly higher than control copepods at each time point (Fig. 1). GR mRNA was more abundant in Cu-exposed copepods at each time point but these increases were only significantly different from control copepods at 12 h (Table 2).

3.2. Changes in astaxanthin during 24 h Cu exposure

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

4. Discussion

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

Classical ecotoxicology endpoints for exposure to sub-lethal concentrations of toxicants focus mainly on population ecology dynamics such as growth rates and individual endpoints including larval development and mortality (Bechmann, 1994; Cairns Jr et al., 1981). More recently, transcriptomic analyses have been used to look at the effects of stressors on the up and down regulation of components of stress responses (Ki et al., 2009; Raisuddin et al., 2007). Behavioral endpoints such as swimming speed also link exposure and physiological responses to a functional consequence (Cohen et al., 2007; Garaventa et al., 2010; Rice et al., 1997). Carotenoid pigmentation is unique in that it reveals immediate functional consequences of pollutant exposure at a molecular level. Moreover, carotenoids can be quantified either through simple HPLC methods or by measure of the coloration of tissues (McKay, 2013; Saks et al., 2003).

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

The specific mechanism by which environmental copper is proposed to disrupt carotenoid pigmentation arises from the redox capacity of free intracellular copper ions. Physiological levels of copper are essential to cell function. Cellular copper ions are redox active in both oxidation states (Cu^{2+} or Cu^{1+}) and serve critical functional roles including facilitating electron transfer through cytochrome *c* oxidase in mitochondria during cellular respiration and cytosolic free radical defense, (*e.g.* Cu/Zn superoxide dismutase). Because of its high reactivity, copper is transported and bound to these proteins in tightly controlled processes through chaperones. Therefore intracellular copper is almost completely absent in its free form (Rae et al., 1999). However, when copper levels exceed biological demands or storage capacity, free copper ions accumulate in cells and react with H_2O_2 to form hydroxyl free radicals through Fenton-like reactions (Jomova and Valko, 2011; Macomber and Imlay, 2009; Valavanidis et al., 2006). Hydroxyl radicals preferentially damage lipid-bound proteins like those in the inner mitochondrial membrane which are integral to cellular respiration (Macomber and Imlay, 2009; Sokol et al., 1993).

In our study we found that copper exposure modulated COI transcript levels in a time-dependent manner suggesting that copper-induced ROS likely damaged these mitochondrial proteins causing an upregulation of COI gene transcription. The most pro-

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

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

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

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

nounced increase in COI mRNA was after 12 h of copper exposure with intermediate increases at 6 and 24 h (Fig. 2). This pattern of gene expression suggests that as antioxidant systems were upregulated (GR; Fig. 2), copper-induced ROS decreased, leading to less damage to mitochondrial membranes, which in turn, decreased the expression of mitochondrial proteins that were upregulated to replace damaged mitochondria. The North American sister species, *T. californicus*, had similar physiological responses to increased ROS production and mitochondrial dysfunction following interpopulation hybridization (Barreto and Burton, 2013; Barreto et al., 2015). Previous studies on copper toxicity have shown modulation of mitochondrial and antioxidant genes following copper exposure (Bremner, 1998; Craig et al., 2007; Ki et al., 2009; Rhee et al., 2013; Scott and Major, 1972). Although we did not measure ROS levels or mitochondrial function directly, our observation that COI and GR transcript levels were increased during Cu exposure suggests that mitochondria of *T. japonicus* copepods were damaged through lipid peroxidation from copper-induced hydroxyl radicals and that there was a physiological response to Cu exposure. At the same time we observed more than a 50% reduction in the amount of astaxanthin found in Cu exposed copepods relative to control copepods (Fig. 3) suggesting that a drastic phenotypic change over a relatively short period of time is acutely signaling physiological responses to an environmental stressor.

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

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

The ultimate goal of this study was to determine if red carotenoid production was sensitive to environmental stressors. Carotenoid content and color in other taxa have been used as bioindicators of environmental stress (Isaksson et al., 2005; Lopez-Antia et al., 2015; Vallverdú-Coll et al., 2016). In this study, we used copper exposure as a source of pro-oxidant stress, but our findings are consistent with those from studies that examined the effect of other pro-oxidants on carotenoids including lead (Vallverdú-Coll et al., 2016, 2015), testosterone (Alonso-Alvarez et al., 2008), and other pollutants (Isaksson et al., 2005; Pérez-Rodríguez et al., 2010). Isaksson and others found that great tits (*Parus major*) from an urban environment, likely with more pollution, had fewer carotenoids in their feathers, and had a relatively larger pool of oxidized glutathione than birds from rural sites (Isaksson et al., 2005). In a follow-up study in which oxidative stress was experimentally induced with the pro-oxidant paraquat, how-

ever, carotenoids levels of great tits were not affected by increased levels of free radicals (Isaksson and Andersson, 2008). It is interesting to note that this bird species has yellow feathers and does not modify dietary yellow carotenoids; instead they directly deposits them to feathers (Mcgraw, 2006). Alonso-Alvarez et al. (2008) found that testosterone-supplemented red-legged partridges *(Alectoris rufa*) had higher levels of yellow circulating carotenoids and decreased red coloration of their red ketocarotenoid-pigmented facial mask (De Blas et al., 2014). Conversely a recent study on the same species found an positive relationship between red coloration and pesticide-induced oxidative stress (Lopez-Antia et al., 2015). Thiram-exposed red-legged partridges had higher levels of circulating yellow carotenoids and redder beak coloration than unexposed partridges (Lopez-Antia et al., 2015). Clearly, more research is needed to deduce the complex relationships of oxidative stress, antioxidant systems, carotenoid metabolism, and carotenoid coloration.

Objectively measured carotenoid color values have been strongly linked to internal concentrations of carotenoids (Saks et al., 2003). Our observation that copper-exposed copepods had a physiological response that correlated with decreased red carotenoid production is encouraging for further tests on the efficacy of carotenoid color as a biomarker for environmental stress. Indeed carotenoid coloration has been shown to be sensitive to other environmental stressors such as UV-radiation (Brüsin et al., 2016; Caramujo et al., 2012), other heavy metals (Eeva et al., 2008; Isaksson et al., 2005), and parasites (Hill, 1995; McGraw and Hill, 2000). Future studies should address the functional role of carotenoids as antioxidants *in vivo* through carefully planned experiments to further elucidate the mechanisms by which carotenoid metabolism links to oxidative stress in animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2016.06.040.

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