Ecological Indicators 70 (2016) 393–400

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Copper exposure reduces production of red carotenoids in a marine copepod

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ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 16 June 2016
Accepted 20 June 2016
Available online 29 June 2016

Keywords:
Astaxanthin
ROS
Heavy metals
Carotenoids
Tigriopus

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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http://dx.doi.org/10.1016/j.ecolind.2016.06.040
1470-160X/

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 zeaxanthin—that 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