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Dietary antioxidants enhance immunocompetence in larval amphibians

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ABSTRACT

Dietary antioxidants have been shown to confer a variety of benefits through their ability to counter oxidative stress, including increased immunocompetence and reduced susceptibility to both infectious and non-infectious diseases. However, little is known about the effects of dietary antioxidants on immune function in larval amphibians, a group experiencing worldwide declines driven by factors that likely involve altered immunocompetence. We investigated the effects of dietary antioxidants (quercetin, vitamin E, and β-carotene) on two components of the immune system, as well as development and growth. *Lithobates pipiens* tadpoles fed diets with supplemental β-carotene or vitamin E exhibited an enhanced swelling response as measured with a phytohemagglutinin assay (PHA), but there was no induced antibody response. Effects were often dose-dependent, with higher antioxidant levels generally conferring stronger swelling that possibly corresponds to the innate immune response. Our results indicate that the antioxidant content of the larval amphibian diets not only had a detectable effect on their immune response capability, but also promoted tadpole growth (mass gain), although developmental stage was not affected. Given that many environmental perturbations may cause oxidative stress or reduce immunocompetence, it is critical to understand how nutrition may counter these effects.

1. Introduction

The effects of dietary antioxidants in the form of secondary plant metabolites have been well-characterized; we know much about their effects on human health and animal physiology (see reviews by Catoni et al., 2008; Birben et al., 2012). Dietary antioxidants largely consist of four main groups commonly found in food: vitamin C, vitamin E, carotenoids, and polyphenolic compounds (Catoni et al., 2008). These groups differ dramatically in antioxidant potency, environmental availability, and absorptive capacity. Polyphenolic acids/flavonoids are the most concentrated in many foods (e.g., seeds, fruits, leaves, algae, and arthropods), and have the highest antioxidant potency followed by carotenoids, vitamin E and vitamin C (Catoni et al., 2008). Among the reported benefits of dietary antioxidants are increases in immunocompetence (e.g., Bendich, 1989, 1996; Chew, 1993; Hughes, 1999), a reduction in both infectious and non-infectious diseases (e.g., Bendich, 1989; Horak et al., 2001), and slowing of aging (e.g., Ames et al., 1993; Driver and Georgeou, 2003).

The key health advantage of antioxidants lies in their ability to reduce chemically reactive forms of oxygen and nitrogen, often referred to as free radicals or reactive oxygen species (ROS), which are potentially damaging molecules that all living organisms need to deal with. Several key physiological functions (e.g., chemical signaling, detoxification, energy conversion, and the initial immune response) depend on free radicals (Chew and Park, 2004; Catoni et al., 2008), but an overproduction/overexposure causes oxidative stress, resulting in cell and tissue damage, premature aging, and degenerative diseases (Finkel and Holbrook, 2000; Catoni et al., 2008; Birben et al., 2012). A number of enzyme-based antioxidants and metabolites help counter-balance the damaging effects of free radicals; however, the concentrations of these internal compounds are frequently lower than the level required to combat free radicals, and in such cases, dietary sources of antioxidants are required (Chew and Park, 2004). The primary role of vitamin E appears to be as a potent lipid-soluble antioxidant, acting to maintain the integrity of long-chain polyunsaturated fatty acids in cell membranes (Traber and Atkinson, 2007). However, along with antioxidant properties, certain vitamins such as A, D, and E can also enhance immune function by stimulating the production of lymphocytes and antibodies (Mora et al., 2008; Kopena et al., 2014).

The immune-enhancing properties of one of the best-studied groups, carotenoids, appear related to their ability to quench ROS (Machlin and Bendich, 1987; Burton, 1989; Galano et al., 2010), with effects linked to bolstered immune function in vertebrates and invertebrates (Bendich, 1989, 1996; Chew, 1993; Fiedor and Burda, 2014). Carotenoids specifically protect macrophages from oxidative damage during the inflammatory response (Gruner et al., 1986), increase the

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number of circulating lymphocytes to significantly enhance the rejection response to foreign compounds (Seifter et al., 1981), and can generally stimulate the immune system (Fiedor and Burda, 2014). Although more recent work suggests that carotenoids, specifically β-carotene, play a substantial role in the evolution of animal signals associated with health in addition to acting as antioxidants or general immune effectors (e.g., Grether et al., 2004; Clotfelter et al., 2007; Simons et al., 2012), we still do not understand the overall importance of various dietary antioxidants for basic physiological processes and their role with respect to animal ecology (Catoni et al., 2008). This is especially timely given the emergence of ecoimmunology as a discipline and the increasing integration of immunology and physiology with respect to evolutionary dynamics and processes (Metcalfe and Alonso-Alvarez, 2010; Brock et al., 2014).

Natural dietary compounds, including antioxidants that confer beneficial health properties or immune-bolstering characteristics, may be an important area of investigation for ecologists, particularly those focused on the effects of stressors and how these are mitigated by organisms. In the context of eco-immunology, this is an important consideration because variation in diet among differing environments can have consequences for various organismal traits. For instance, amphibian populations are declining worldwide (Stuart et al., 2004), and there is considerable evidence suggesting these are related to complex interactions among multiple environmental stressors (Blaustein et al., 2011), including climate change, habitat alteration/destruction, contaminants, and diseases (e.g., Carey, 1993; Carey et al., 1999; Rohr et al., 2008; Blaustein et al., 2012). While various mechanisms are likely at play, many of these stressors have been linked with immunosuppression. For example, environmental changes may expose amphibians to new, highly virulent pathogens that kill them before they can mount an immune response, or directly decrease immunocompetence and make hosts more susceptible to existing pathogens (Carey et al., 1999; Kiesecker, 2002). If changes in immunocompetence are a common component underlying numerous environmental variables implicated in amphibian declines, it is also important to consider factors that can bolster their immune function and possibly allow them to counter the effects of environmental stress. In addition to better understanding how the availability of naturally-occurring dietary antioxidants may influence tadpole immunocompetence and survival in wild populations, ex situ conservation programs are playing an increasingly key role in combatting amphibian declines, thus maintaining both artificial and natural habitats that promote an optimal diet may be important.

Dietary antioxidants may be particularly significant for larval anurans because they likely encounter these potentially beneficial compounds in their food, but are also subject to a variety of environmental stressors known to have detrimental effects, such as pathogens and contaminants (Blaustein et al., 2011). The diet of larval anurans is diverse, but many are generalized herbivores/detritivores, scraping periphyton or detritus from the substrate or filter-feeding phytoplankton directly from the water column (Altig et al., 2007). The various forms of algae consumed by tadpoles are thus likely to contain a wide variety of dietary antioxidants, and are particularly rich in carotenoid pigments and polyphenolic compounds (Allen et al., 1964; Goiris et al., 2012; Christaki et al., 2013). Importantly, dietary antioxidants such as β-carotene, and vitamins E and C impact growth in other taxa (e.g., Sealey and Gatlin, 2002; Cucco et al., 2006). Rapid growth rates likely require high metabolic outputs, resulting in the generation of free radicals which are associated with a reduction in life span and lifetime reproductive output (Monaghan et al., 2009). Understanding the role of dietary antioxidants will aid understanding of how organisms maintain their growth while countering the negative effects of environmentally induced oxidative stress (i.e. overproduction of free radicals).

Because there is limited information available regarding dietary antioxidants and amphibian immune function, we investigated the effects of these compounds on two components of the immune system (innate and adaptive), as well as how these affected the development and growth of *Lithobates pipiens* (northern leopard frog) tadpoles – a species extirpated or in decline in much of its natural range (Corn and Fogleman, 1984; Rorabaugh, 2005). Tadpoles of this species are facultative suspension feeders that consume a variety of microalgae, but switch their diet to include other items when necessary (Hendricks, 1973; Seale, 1982). To do this, we selected a representative member of 3 of the 4 dietary antioxidant classes: vitamin E (tocopherols), quercetin (polyphenolics), and β-carotene (carotenoids). These were chosen because their antioxidant potencies (the relative strength of each antioxidant) are known to vary widely (e.g., Bramley et al., 2000; Gomez-Coronado et al., 2004, Konyalioglu et al., 2005), and they are found in natural algae species (e.g., Miranda et al., 1998; Mendiola et al., 2008). Asorbic acid (vitamin C) was excluded as amphibians can synthesize it (Chatterjee et al., 1974).

2. Materials and methods

2.1. General animal husbandry

Ten *L. pipiens* egg masses were purchased from the Carolina Biological Supply Company (Burlington, NC) and placed singly into 1.5 L glass bowls containing dechlorinated water, or in 300 mL bowls for smaller egg masses. These were then placed into a walk-in growth chamber at 15 °C on a 14:10 light:dark cycle. As hatchlings emerged from egg masses, they were housed in groups of no >15 hatchlings per tub (10.8 L Rubbermaid® dishpans, 37 × 32 × 14 cm, filled with 8 L of dechlorinated water with pH adjusted to 7). Prior to commencing experimental diets, tadpoles were maintained on ground Spirulina Algae Discs (Wardley®, Secaucus, NJ). Tanks were cleaned of feces every second day and a complete water change was performed weekly. All experiments were carried in accordance with guidelines set by the Canadian Council for Animal Care and with protocols approved by the University of Toronto.

2.2. Custom diet and experimental design

Each of the 3 chosen antioxidant compounds (vitamin E, quercetin, and β-carotene) was added in 3 concentrations (Table 1) to a customized diet (a mixture of ground Mysid shrimp, krill, shrimp, chironomids – bloodworms, and clam meat) that was dehydrated and used as our standard base; ~1 g of the dry powder was fed to the tadpoles by placing the food into their experimental tanks each day. All food (treatment and algae) was ground to a fine powder, before addition to tanks where it initially floated but then sank, allowing tadpoles to feed *ad libitum*. Purified quercetin, α-tocopherol (vitamin E), and β-carotene were obtained from a commercial supplier (Sigma Aldrich, Oakville, Canada). We chose our antioxidant concentrations based on Grether et al. (2004), who fed guppies experimental diets containing 0.79 μg/g carotenoids for a "low carotenoid" treatment and 2081 μg/g carotenoids for a "high carotenoid" diet. Because many dietary antioxidants are lipophilic

Table 1
Composition of experimental diets fed to tadpoles by each of the three major antioxidant groups (in μg/g) as determined by HPLC. Target indicates the desired amount of antioxidant above that in the regular diet comprised of algae.

Diet type	Target	quercetin	α-Tocopherol (vitamin E)	β-Carotene
Algae (*Spirulina* discs)	N/A	0.95	33.96	60.11
Quercetin Low (QL)	24	27.76	13.56	0.96
Quercetin Med. (QM)	500	542.66	15.13	0.15
Quercetin High (QH)	1200	857.99	15.32	0.14
Vitamin E Low (VL)	24	0.23	25.90	0.31
Vitamin E Med. (VM)	500	0.24	532.16	1.52
Vitamin E High (VH)	1200	0.27	868.33	0.85
β-Carotene Low (BL)	24	5.65	14.77	24.59
β-Carotene Med. (BM)	500	1.18	13.14	570.23
β-Carotene High (BH)	1200	0.73	13.95	865.14