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Cadmium- and calcium-mediated toxicity in rainbow trout (*Oncorhynchus mykiss*) *in vivo*: Interactions on fitness and mitochondrial endpoints

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ABSTRACT

Rainbow trout were exposed to sublethal waterborne Cd (5 and 10 μg L^{-1}) and dietary Ca (60 mg g^{-1}), individually and in combination, for 30 d to elucidate the interactive effects and evaluate the toxicological significance of mitochondrial responses to these cations *in vivo*. Indices of fish condition and mortality were measured and livers, centers of metabolic homeostasis, were harvested to assess mitochondrial function and cation accumulation. All indices of condition assessed (body weight, hepatosomatic index and condition factor) were reduced in all the treatment groups. Mortality occurred in the Cd-exposed groups with dietary Ca partly protecting against and enhancing it in the lower and higher Cd exposure, respectively. State 3 mitochondrial respiration was inhibited by 30%, 35% and 40% in livers of fish exposed to Ca, Cd and Cd + Ca, respectively, suggesting reduced ATP turnover and/or impaired substrate oxidation. While the phosphorylation efficiency was unaffected, state 4 and state 4+ (+ oligomycin) respirations were inhibited by all the exposures. Mitochondrial coupling was reduced and transiently restored denoting partially effective compensatory mechanisms to counteract Cd/Ca toxicity. The respiratory dysfunction was associated with accumulation of both Cd and Ca in the mitochondria. Although fish that survived acute effects of Cd and Ca exposure apparently made adjustments to energy generation such that liver mitochondria functioned more efficiently albeit at reduced capacity, reduced fitness was persistent possibly due to increased demands for maintenance and defense against toxicity. Overall, interactions between Cd and Ca on condition indices and mitochondrial responses were competitive or cooperative depending on exposure concentrations and duration.

1. Introduction

Cadmium is a non-essential metal that enters aquatic systems via multiple anthropogenic and natural release processes (Camusso et al., 1995; Frew et al., 1997; Vazquez-Sauceda, 2011). In contaminated environments, Cd is assimilated by resident organisms and accumulated to invoke a variety of toxico-pathological responses (Bertin and Averbeck, 2006) resulting in reduced fitness. For example, waterborne Cd can reduce growth and cause mortality (Versteeg and Giesy, 1986; Ricard et al., 1998; Hollis et al., 1999; McGeer et al., 2000) due to impaired ion balance (Wood, 2001) and/or mitochondrial dysfunction (Fernandez et al., 2003; Kurochkin et al., 2011). Typically, exposure to elevated waterborne Cd is accompanied by physiological acclimation with enhanced tolerance (McDonald and Wood, 1993; McGeer et al., 2000). This tolerance is primarily achieved through Cd sequestration by biomolecules such as metallothionein and/or by incorporation into insoluble granules rendering it unavailable for binding and impairment of sensitive cellular target sites (Hollis et al., 1999; Kamunde, 2009). The reduction in internal bioavailability together with induction of stress response can effectively protect chronically exposed organisms from Cd toxicity (Prakash and Rao, 1995; Campbell et al., 2005).

Calcium on the other hand is an indispensable second messenger controlling numerous physiological processes including regulation of energy metabolism (Clapham, 2007). To act as a second messenger Ca concentrations in the cytosol are maintained at very low levels (0.1–0.5 μM: Clapham, 2007). Regardless, elevated tissue Ca concentrations can result from consumption of food high in Ca (Ye et al., 2006), but there have been no specific studies in fish to measure cytosolic or organelle Ca concentration after exposure to elevated dietary Ca. Additionally, cytosolic Ca overload can result from mobilization of Ca stored in organelles such as the mitochondria and endoplasmic/sarcoplasmic reticulum via intra-cellular Ca-trigger-Ca-release mechanisms (Domingo and Demaurex, 2010). Conversely, elevated cytosolic Ca may lead to excessive Ca uptake by mitochondria with mitochondrial dysfunction and cell death (Duchen, 2000; Giorgi et al., 2008).

Several Ca uptake pathways including ion channels, exchangers and pumps (Rizzuto and Pozzan, 2006; Domingo and Demaurex,

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2010) are recognized in organisms, but remain to be fully characterized, particularly with regard to the mitochondria (Duchen, 2000; Rizzuto and Pozzan, 2006). These Ca uptake pathways also have been shown to mediate Cd uptake at gill epithelial surface in fish (Wood, 2001; Niyogi and Wood, 2004). While interactions between waterborne Cd and Ca at the gill surface have been extensively studied and shown to have significant toxicological implications (Verbost et al., 1987, 1989; Wood, 2001), interactions between dietary Ca and waterborne Cd have only recently come to light (Zohouri et al., 2001; Baldisserotto et al., 2004, 2005). It is nonetheless apparent from these studies that, similar to waterborne Ca and Cd interactions, dietary Ca inhibits Cd uptake at the gill and protects against toxicity of waterborne Cd (Baldisserotto et al., 2004). Interactions of Cd and Ca at internal target sites are, however, scarcely investigated in fish and it is unclear if they are consistent with those at epithelial (gill) surfaces. Our recent *in vitro* study demonstrated cooperative rather than competitive interactions between Cd and Ca at mitochondrial sites, resulting in enhancement of Cd/Ca toxicity (Adiele et al., 2010). At present hardly anything is known about the interactive effects of these two cations on mitochondrial function in fish during *in vivo* exposures.

The objective of this study was therefore to elucidate the interactions of waterborne Cd and dietary Ca at internal sites with special focus on mitochondrial bioenergetics in order to assess the relevance of mitochondrial endpoints of toxicity in real-world exposure conditions. We hypothesized that interactions occurring at uptake sites (gill/gut) determine the amount of Cd/Ca internalized and are the key drivers of the mitochondrial responses. Because impaired mitochondrial function affects an organism's ability to generate energy with implications for fitness and survival, indices of fish condition were also measured.

2. Materials and methods

All the experimental procedures that fish were subjected to were approved by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care.

2.1. Fish

Rainbow trout (approximately 60 g) procured from Ocean Trout Farm Inc., Brookvale, PE, were held in a 250-L tank in a flow-through system of aerated well water (temperature 10–11 °C, pH 7.5) at the Atlantic Veterinary College Aquatic Facility. Main water chemistry parameters in mg L^{-1} were: Na 47, Ca 59, Mg 28, K 2.3, Cl 137, sulfate 17, hardness 260 (as $CaCO_3$), and total alkalinity 145 (as $CaCO_3$). During the holding period fish were fed to satiation on alternate days with 3 mm^3 trout chow pellets (Corey Feed Mills, Fredericton, NB) containing, according to the manufacturer, crude protein 50% (minimum), crude fat 20% (minimum), crude fiber 1.4% (maximum), calcium 1.7% (actual), phosphorous 1.0% (actual), sodium 0.6% (actual), vitamin A 2500 IU kg^{-1} (minimum), vitamin D3 2400 IU kg^{-1} (minimum), and vitamin E 200 IU kg^{-1} (minimum). Background Cd concentrations measured in the feed and water were 0.78 μg g^{-1} and below our limit of detection (0.03 μg L^{-1}), respectively.

2.2. Experimental diet

Trout chow containing 1.7% (17 mg g^{-1}) Ca was ground into powder using a grinding mill (Retsch Inc., Newtown, PA) and was supplemented with 43 mg g^{-1} of Ca to achieve a nominal concentration of 60 mg g^{-1} Ca. This dietary Ca concentration falls within the range of 2–80 mg g^{-1} measured in freshwater crustacean zooplankton sampled from waters differing in Ca concentrations (Jeziorski and Yan, 2006). Briefly Ca (as $CaCl_2{\cdot}2H_2O$) was dissolved in 40% volume to total weight of filtered deionized (Milli-Q) water (Millipore, Bedford, MA) and mixed in a pasta maker (Pasta Products Inc. Agoura Hills, CA) for 30 min. Subsequently, 20% volume to diet weight of Milli-Q water was added and mixed for 15 min. The resulting food paste was re-pelleted by extrusion via a 3 mm die, air-dried and hand-broken into approximately 3 mm^3 pellets. Control diet was prepared in a similar manner without addition of Ca. All the experimental diets were stored at −20 °C prior to use. The actual concentration of Ca in the supplemented and control food measured by flame atomic absorption spectrometry (FAAS: AAnalyst800, Perkin–Elmer, Foster City, CA) following digestion with 15:1 solution of 70% HNO_3 (trace metal grade) and 30% H_2O_2 were 55 ± 0.4 and 18 ± 0.9 mg g^{-1}, respectively (n = 5 each).

2.3. Experimental procedure

Following a 14-d acclimation period fish (n = 328) were randomly distributed among 8 experimental tanks (n = 41/42) containing approximately 100 L of well water in a flow-through system (flow rates: 3.70–4.15 L min^{-1}) with constant aeration. Because of logistical constrains relating to isolating mitochondria and measuring their respiration the experiment was staggered, i.e., fish from all the 8 tanks were not sampled and tested on the same day. The 8 groups, nominal (actual) exposure concentrations of Cd and Ca, and the staggered sampling protocol are shown in Table 1. During acclimation, the fish were gradually introduced to the re-pelleted control diet at ratios (control diet:original diet) of 25:75%, 50:50%, 75:25% and 100:0% before initiation of the waterborne Cd and dietary Ca exposures.

Stock solutions of Cd were prepared in Milli-Q water and delivered from 4 Mariotte bottles to the 4 waterborne Cd exposures at flow rates of 2.3–2.6 mL min^{-1} after initial spiking with the Cd stock solution to attain the desired (nominally 5 or 10 μg L^{-1}) Cd concentration. These Cd concentrations are within the range of concentrations found in contaminated Canadian freshwaters (0.1–122 μg L^{-1}: CCME, 1999). In selecting the exposure concentrations and delivery method (waterborne and dietary), considerations were made of the high water hardness (260 mg L^{-1} as $CaCO_3$) and background dissolved organic carbon (1.8 mg L^{-1}) in AVC well water that potentially would decrease Cd bioavailability, uptake and toxicity (Kamunde, 2009; Kamunde and MacPhail, 2011). Indeed, exposure of rainbow trout to comparable Cd concentration in water of lower hardness and DOC content caused excessive mortality (Hollis et al., 1999). Fish were maintained at 12 h light:12 h darkness photoperiod and fed 2% body weight daily ration in two servings (1% body weight morning and evening) of the specified experimental diet. The ration was adjusted to account for biomass loss whenever mortality occurred or when fish were sampled. Tanks were flushed before and after feeding to minimize ammonia build-up. In-tank dissolved oxygen, pH and ammonia were measured twice weekly and were 10 ± 0.5 mg L^{-1}, 7.5 ± 0.2 and 0.07 ± 0.05 mg L^{-1} (n = 8), respectively. Cadmium and Ca concentrations in the experimental tanks also were measured twice weekly before and 1 h after feeding using atomic absorption spectrometry (AAS) to check tank dissolved Cd concentration and potential leaching of Ca from the food to water. The measured Cd in the low waterborne Cd, high waterborne Cd, low waterborne Cd plus dietary Ca and high waterborne Cd plus dietary Ca were 5.6 ± 0.2, 9.6 ± 0.9, 5.3 ± 0.2 and 10.9 ± 0.7 μg L^{-1} (n = 16), respectively. The measured Ca concentrations before and after feeding ranged from 59 to 64 mg L^{-1} and there was no evidence of leaching of Ca from the food to water. The exposure lasted for 30 d and