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Interactions of waterborne and dietary cadmium on the expression of calcium transporters in the gills of rainbow trout: Influence of dietary calcium supplementation

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Abstract

Recent studies have shown that dietary Ca^{2+} supplementation strongly inhibits uptake of Ca^{2+} and Cd at the fish gill. To better understand the influence of dietary Ca^{2+} on branchial Ca^{2+} transport, we examined the expression of two trout gill calcium transporters during waterborne and dietary Cd exposure, at two different levels of dietary Ca^{2+}. Quantitative polymerase chain reaction (PCR) was used to monitor epithelial calcium channel (ECaC) and sodium–calcium exchange (NCX) mRNA levels following 7–28 days of exposure to these treatments. In brief, juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to control, 3 μg/L waterborne Cd, 500 mg/kg dietary Cd, or a combined 3 μg/L waterborne plus 500 mg/kg dietary Cd exposure, supplemented with either 20 mg/g or 60 mg/g dietary calcium (Ca^{2+}). Two-way analysis of variance was used to discern the main effects of Cd exposure and dietary Ca^{2+} supplementation on ECaC and NCX mRNA levels. We found that dietary Ca^{2+} supplementation decreased significantly ECaC mRNA expression on days 14 and 21. In comparison, NCX mRNA levels were not influenced by dietary Ca^{2+} supplementation, but rather were significantly inhibited in the combined waterborne and dietary Cd exposure on day 7 alone. Statistical analysis found no interactive effects between Cd exposure and dietary Ca^{2+} exposure at any time point, except for day 28.This study provides evidence of the importance of nutritional status on the transcriptional regulation of ion transport at the fish gill. We discuss the importance of diet and nutritional status to the development of new regulatory approaches, such as the biotic ligand model, which currently do not account for the significance of diet on metal bioavailability in aquatic organisms.
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Keywords: mRNA levels; Epithelial calcium channel (ECaC); Sodium calcium exchanger (NCX); Biotic ligand model

1. Introduction

Cadmium is a class IIB metal that typically exists in a +2 oxidation state in water, which, based on its valence and relatively small size, can act as an ionic mimic of Ca^{2+} in biological systems (Zalups and Ahmad, 2003). Numerous studies have demonstrated the ability of Cd to inhibit active Ca^{2+} uptake in transporting epithelia (Lock et al., 1987; Hollis et al., 2000; Zalups and Ahmad, 2003). In the case of freshwater fish, waterborne Cd exerts the majority of its toxic effects at the gill, which has an implicated role in the uptake of Ca^{2+} from water (Perry and Wood, 1985; Perry and Flik, 1988; Shahsavarani et al., 2006). Cd is thought to be transported across the apical membrane of the fish gill via voltage-insensitive epithelial Ca^{2+} channels (ECaC) (Lock et al., 1987; Verbost et al., 1989), which belong to the vanilloid class of transient receptor potential proteins (Qiu and Hogstrand, 2004). Once internalized within the cell, Cd is initially sequestered by any of a number of intracellular ligands (Olsson and Hogstrand, 1987; Glynn, 1996), including Ca^{2+} binding proteins (Zalups and Ahmad, 2003), and some Cd may eventually be transported across the basolateral membrane via sodium–calcium exchangers (NCX) (Verbost et al., 1989).

Intracellular Cd interferes with plasma membrane Ca^{2+} ATPase (PMCA, also known as the high affinity Ca^{2+}-ATPase) at low nM concentrations, leading to possible accumulation of intracellular Ca^{2+} and the indirect blockade of Ca^{2+} and Cd

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entry into the cell due to the ensuing down-regulation of ECaC (Verbost et al., 1988). At each stage along this process, Cd is thought to interfere with the normal transport or handling of Ca^{2+}, leading to hypocalcaemia, and potentially death during more extreme exposures of Cd. Reciprocally, waterborne Ca^{2+} is a potent inhibitor of Cd bioavailability and toxicity in freshwater fish (Playle et al., 1993; Hollis et al., 1999, 2000; Niyogi and Wood, 2004; Franklin et al., 2005).

Little work has been done to characterize the mechanisms and toxicity of dietary Cd in freshwater fish. There is some evidence that dietary Cd inhibits the activity of intestinal NCX and PMCA (Schoenmakers et al., 1992). Furthermore, information is emerging that dietary Cd can accumulate readily on/in the fish gill (Szebedinszky et al., 2001), leading to morphological alterations in the tissue (Pratap and Wendelaar Bonga, 1993), and to inhibition of branchial Ca^{2+} uptake (Baldisserotto et al., 2004a, 2005) Although Cd uptake in the gastrointestinal tract of fish may occur through a Ca^{2+}-mediated pathway, little experimental evidence exists to support this mechanism at present (Wood et al., 2006; Baldisserotto et al., 2006). However, in mammalian enterocytes, Cd is transported via the divalent metal transporter 1 (DMT1) (Zalups and Ahmad, 2003), further supporting a linkage between Ca^{2+} and Cd uptake pathways in the gastrointestinal tract.

Fish, in contrast to other vertebrates, are unique in their ability to sequester Ca^{2+} from both the surrounding water and through their diet. The availability of Ca^{2+} in most natural waters, together with the favourable electrochemical gradient for Ca^{2+} entry across the apical membrane of the fish gill, provide a near infinite source of the ion for branchial uptake (Flik, 1990). It is known that stanniocalcin alters the permeability of the apical membrane to Ca^{2+} through second messenger regulated Ca^{2+} channels on the membrane (Flik, 1990; Wagner et al., 1993). Recent studies have shown that dietary Ca^{2+} supplementation strongly inhibits the uptake of Ca^{2+} and Cd at the fish gill (Baldisserotto et al., 2004a,b, 2005; Niyogi and Wood, 2004; Franklin et al., 2005), likely resulting from a transient increase in plasma Ca^{2+} balance (Baldisserotto et al., 2004b). This epithelial "cross-talk" is likely mediated through transient alterations in extracellular Ca^{2+}, as monitored by ion sensing receptors. It is thought that the calcitropic hormone, stanniocalcin, responds to changes in extracellular Ca^{2+} levels, by inhibiting the mechanisms of Ca^{2+} influx in the fish gill, and perhaps at the intestine. It is expected that the majority of the control on branchial Ca^{2+} uptake will be mediated by ECaC because of its proposed role as the rate limiting step in transcellular Ca^{2+} uptake. Nonetheless, branchial transporters such as NCX are also expected to be differentially regulated during perturbations in Ca^{2+} homeostasis, in order to restore intracellular Ca^{2+} activities.

The objectives of the study were twofold. First, we were interested in investigating the effects of chronic exposure of waterborne Cd, dietary Cd and a combined waterborne and dietary Cd exposure on ECaC and NCX mRNA levels. Second, we wanted to address the importance of dietary Ca^{2+} supplementation on the differential regulation of branchial ECaC and NCX transcription. The overall aim was to gain insight into the interactions between different routes of Cd exposure, and the confounding issue of how nutrient status alters the transcriptional regulation of two transporters critical in the maintenance of Ca^{2+} homeostasis in freshwater fish. This research will be put into context of current initiatives to establish a new regulatory framework for metals, based on an understanding of the influence of water chemistry on metal bioavailability in aquatic organisms.

2. Materials and methods

2.1. Animal husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*, 10–12 g) of both sexes were obtained from Humber Springs Trout Hatchery (Orangeville, ON). Fish were held in ∼200 L polyethylene tanks supplied with Lake Ontario dechlorinated tap water (hardness 140 mg/L as $CaCO_3$; and in mM: 1.0 Ca^{2+}, 0.2 Mg^{2+}, 0.6 Na^+, 0.2 K^+, 0.7 Cl^-, and 0.04 μg/L Cd, 3 mg/L dissolved organic carbon, and pH 8.0). Each tank received a constant supply of water at 1 L/min, which was maintained at a constant temperature of approximately 12 °C. Cd exposures were performed as outlined in Franklin et al. (2005) except for the inclusion of a set of combined waterborne and dietary Cd exposures as this was part of a larger study (see below). Fish were allowed to acclimate to laboratory conditions for at least two weeks prior to experimentation.

2.2. Exposure system and experimental design

Before the start of the experiment, fish were randomly divided amongst one of eight 200 L exposure tanks, with coarse mesh dividers used to split 90 fish into two equal groups of 45 fish per half-tank. In total, there were eight different experimental treatments, each replicated twice. Data collected from each replicate were later pooled. As noted in Franklin et al. (2005), one side of each tank housed animals fed the low dietary Ca^{2+} diet, whereas fish in the other side received the elevated Ca^{2+} diet. In all cases, the Cd regime (either dietary or waterborne) in both sides of each tank was identical. Water flow was sufficiently high to ensure that leaching of ions from the Ca^{2+}- and/or Cd^{2+}-supplemented food did not alter water chemistry. Fish were exposed for 28 days to waterborne Cd (3 μg/L), dietary Cd (500 mg/kg dry weight), a combined waterborne (3 μg/L) plus dietary Cd exposure (500 mg/kg dry weight), and an unexposed control (background of 0.04 ug/L waterborne Cd and 0.1 mg/kg dietary Cd). Fish in each treatment were fed at a ration of 2% body weight per day with either a control or Cd-laden diet containing either 20 mg/g Ca^{2+} or 60 mg/g Ca^{2+} as $CaCO_3$. Prior studies by Baldisserotto et al. (2004a,b, 2005) showed that the chosen levels were effective in altering gill Ca^{2+} and Cd uptake. The work presented here is part of a larger study investigating the effects of dietary Ca^{2+} supplementation on Cd bioaccumulation during waterborne Cd, dietary Cd and combined waterborne and dietary Cd exposure (data not shown). As such, water was spiked with non-radioactive Cd, whereas diets were made with radioactive ^{109}Cd (Franklin et al., 2005) to ascertain the route of Cd uptake during the combined waterborne and dietary metal exposure.

2.3. Tissue preparation for total RNA isolation

Gills were collected on days 7, 14, 21 and 28 of exposure, immediately flash frozen in liquid nitrogen, and then stored at −80 °C to await further processing. Quantitative PCR analyses were started at McMaster University and completed at Louisiana State University. Samples were shipped between the two sites using a dry-shipper at liquid nitrogen temperature. At the start of RNA isolation, samples were ground into a fine powder using a liquid nitrogen cooled mortar and pestle, after which, approximately 50–100 mg of gill tissue was transferred into 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA.). The solution was immediately vortexed, and then passed 10 times through a 20–1/2 gauge needle to promote complete homogenization. Samples were processed as outlined by the manufacturer with minor modifications. To increase the quality of the RNA, samples were taken through two sequential treatments with Trizol, by reconstituting the RNA pellet from the first treatment into RNAase-free water, and then repeating the Trizol isolation from the point of chloroform treatment onward. Following RNA isolation, each sample was dissolved in 50 μL RNAase-free water, and total RNA concentration quantified by UV spectroscopy using a micro-spectrophotometer (Nanodrop NA, Wilmington, DE). The A260:280 nm ratios ranged from 1.75 to 2.11, with a mean value of 2.02 ± 0.05. RNA integrity and purity were also assessed on a random batch of samples using the RNA 6000 Nano Labchip kit (Biorad) and Experion Automated Electrophoresis System according to the manufacturer's protocol. Distinct 18S and 28S ribosomal RNA peaks were seen in all samples tested, further verifying the quality of total RNA used in quantitative PCR analyses. Samples were stored in 0.1× volume of sodium acetate and 2.5× volume 75% ethanol until needed for cDNA synthesis.

First strand cDNA was synthesized from 2 μg total RNA in a total reaction volume of 20 μL. Samples were initially processed with 14.8 μL diethyl pyrocarbonate-treated H_2O, 1 μL oligo dT primers (0.5 μg/μL; Invitrogen), 0.8 μL (25 mM dNTP mix; Invitrogen), and 2 μL 10× Stratascript buffer (Stratagene, La Jolla, CA), and incubated at 65 °C for 5 min. After being allowed to incubate at room temperature for 10 min, 1 μL RNA guard RNAase inhibitor (Amersham) and 0.4 μL Stratascript reverse transcriptase (Stratagene, California) were added, and incubated at 42 °C for 60 min, and 90 °C for 5 min.

2.4. Quantitative polymerase chain reactions

The mRNA expression was quantified in triplicate for the Ef1α, or in duplicate for analyses of ECaC and NCX mRNA levels. First strand cDNA was diluted five times in autoclaved H_2O and quantified on an ABI Prism Series thermocycler using a SYBR Master Mix kit (Stratagene). Samples were processed with a final volume of 20 μL, containing 3.7 μL autoclaved H_2O, 10 μL of 10× SYBR Green master mix (Stratagene), 0.3 μL 15 μM 6-carboxyl-X-rhodamine (ROX) reference dye, 0.5 μL Forward primer, and 0.5 μL reverse primer (final concentrations of 200 nM each), and 5 μL of 1:5 diluted first strand cDNA. After an initial step of 94 °C for 5 min, samples were cycled 40 times at: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s. After a final 72 °C step for 5 min, samples underwent a melting curve analysis to determine quality of products according to the supplier's instructions.

The critical threshold (Ct) values, the cycle number at which cDNA amplification enters an exponential increase, were automatically calculated using preset algorithms within the software. Preliminary experiments were performed to calculate qPCR efficiencies for each gene product at varying concentrations of first strand cDNA over a concentration range spanning over two orders of magnitude. One control sample (no Cd exposure at low dietary Ca^{2+}) was randomly selected to develop a standard curve relating Ct to the relative quantity of cDNA. From this curve, efficiency (E) was calculated from the linear regression of Ct versus log cDNA for each gene of interest according to the formula:

$$E = [10^{(-1/\mathrm{slope})}] - 1 \quad (1)$$

Relative changes in mRNA levels were calculated using the delta-delta Ct method (Fleige et al., 2006) following efficiency correction as described by Pfaffl (2001) and summarized in the following formula:

$$R = \frac{(E_{\mathrm{target}})\Delta\mathrm{Ct\ \ target(control\text{-}treatment)}}{(E_{\mathrm{ref}})\ \Delta\mathrm{Ct\ \ ref(control\text{-}treatment)}} \quad (2)$$

Target refers to either ECaC or NCX, and ref refers to the reference gene Ef1α. E represents the efficiency of the PCR reaction according to Eq (1), which was calculated from the slopes of the Ct versus relative cDNA quantity in ln units at the start of the PCR reactions. Reaction efficiencies for the target and reference genes were between 90% and 110%, with 100% efficiency representing a doubling in the amount of cDNA after each cycle. ΔCt represents the differences in Ct between the target genes (ECaC or NCX) and the reference gene (EF1α). Ct for Ef1α amplification varied only moderately amongst the different treatments yielding mean Ct values of 20.6 ± 0.4, 21.3 ± 0.2, 21.8 ± 0.3 and 20.0 ± 0.4 on days 7, 14, 21 and 28, respectively. Ef1α was used as the reference gene based on its effectiveness as a quantitative PCR control in a study investigating ECaC gene expression in zebrafish (Craig et al., 2007), and based on the small variation observed in Ct amongst treatments over time in this study. Control RNA was isolated from animals exposed to no Cd and fed the low Ca^{2+} level diets. Relative ECaC or NCX mRNA levels in other treatments were expressed to these controls.

Primers for qPCR were developed using Primer 3 software, ensuring that a GC base content between 40% and 60% was maintained, with a theoretical annealing temperature of 60 °C, a length of approximately 21 nucleotides, and producing an amplicon of ∼200 bp (Table 1). All primers amplified single products as demonstrated by agarose gel electrophoresis and denaturation analysis following individual quantitative PCR runs.

2.5. Statistical analyses

Data are reported as the mean ± standard error. Homogeneity of variances was tested using the Levene's test. A two-way

Table 1
Sequences of forward and reverse primers used for quantitative PCR

Name	Direction	Sequence 5′→3′
ECaC-qPCR	Forward	GGACCCTTCCATGTCATTCTTATT
ECaC-qPCR	Reverse	ACAGCCATGACAACTGTTTCC
NCX-qPCR	Forward	GGCAAGATGGTCAACTCACA
NCX-qPCR	Reverse	GCTGGTGAGACAAGGCGTAG
Ef1α-qPCR	Forward	GAGACCCATTGAAAAGTTCGAGAAG
Ef1α-qPCR	Reverse	GCACCCAGGCATACTTGAAAG

analysis of variance (ANOVA) followed by a posthoc Dunnett's test were used to uncover main effects of Cd exposure or dietary Ca^{2+} supplementation, and the interactive effects of both independent factors on relative changes in ECaC or NCX mRNA levels. The fiducial level of significance was set at $p < 0.05$. Statistical analyses were performed using SPSS version 13.

3. Results

Statistical analyses of the data by two-way analysis of variance allowed us to assess the main effects of Cd exposure and dietary Ca^{2+} supplementation on ECaC and NCX mRNA expression. These analyses showed no interactive effects between Cd exposure and dietary Ca^{2+} supplementation on days 7, 14, and 21. As such, analyses allowed us to monitor independently the influence of each main factor on mRNA expression, without the confounding effect of interaction.

3.1. The influence of dietary Ca^{2+} on ECaC and NCX mRNA profiles

Elevating dietary Ca^{2+} from 20 mg/g to 60 mg/g significantly reduced ECaC mRNA levels after 14 (Fig. 1b) and 21 (Fig. 1c) days of exposure. These effects were attenuated partially by day 28 ($p = 0.06$). In comparison, dietary Ca^{2+} supplementation had no significant influence on NCX mRNA levels at any point during the experimental period (Fig. 2).

3.2. The influence of waterborne and dietary Cd exposures on ECaC and NCX mRNA levels

Messenger RNA transcript levels were also expressed relative to the unexposed controls at each of the two dietary Ca^{2+} levels tested. The statistical analyses performed allowed us to evaluate the effects of waterborne Cd, dietary Cd, or the combined waterborne and dietary Cd exposure on ECaC and NCX expression, without the confounding influence of dietary Ca^{2+} supplementation. Overall, Cd exposure over 28 days had only negligible effects on ECaC and NCX mRNA levels (Fig. 1a–d). The only significant effect was a reduction in NCX mRNA level in the combined waterborne and dietary Cd exposure at day 7 (Fig. 2a).

4. Discussion

The goal of the present study was to examine the transcriptional regulation of Ca^{2+} transporters in the gill epithelium of fish during waterborne Cd and dietary Cd exposure, and during

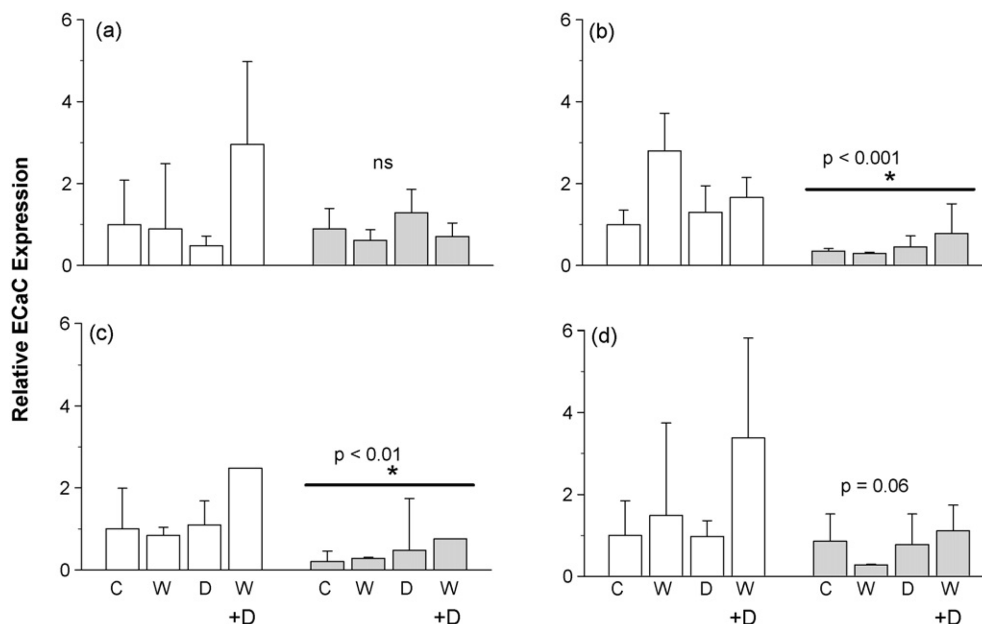

Fig. 1. The relative changes in ECaC mRNA levels in the gill epithelium of rainbow trout during control (C), waterborne Cd (3 μg/L) (W), dietary Cd (500 mg/kg) (D), or combined waterborne and dietary Cd (W + D) exposures, and fed a control (20 mg/g dry weight Ca^{2+}; open bars) or elevated Ca^{2+} (60 mg/g dry weight Ca^{2+}; gray bars) for (a) 7 days, (b) 14 days, (c) 21 days, or (d) 28 days. A two-way ANOVA was used to assess the main effects (factors) of Cd exposure and dietary Ca^{2+} supplementation, and potential interactive effects between factors on relative ECaC mRNA levels. Relative ECaC mRNA expression was determined relative to the no Cd controls fed the 20 mg/g Ca^{2+} diet. Values are mean ± standard error of the mean ($n = 5$). (*) represents a statistically significant difference in ECaC mRNA levels between the 20 mg/g and 60 mg/g dietary Ca^{2+} treatments. ($p < 0.05$). There was no significant effect of Cd exposure on gene expression.

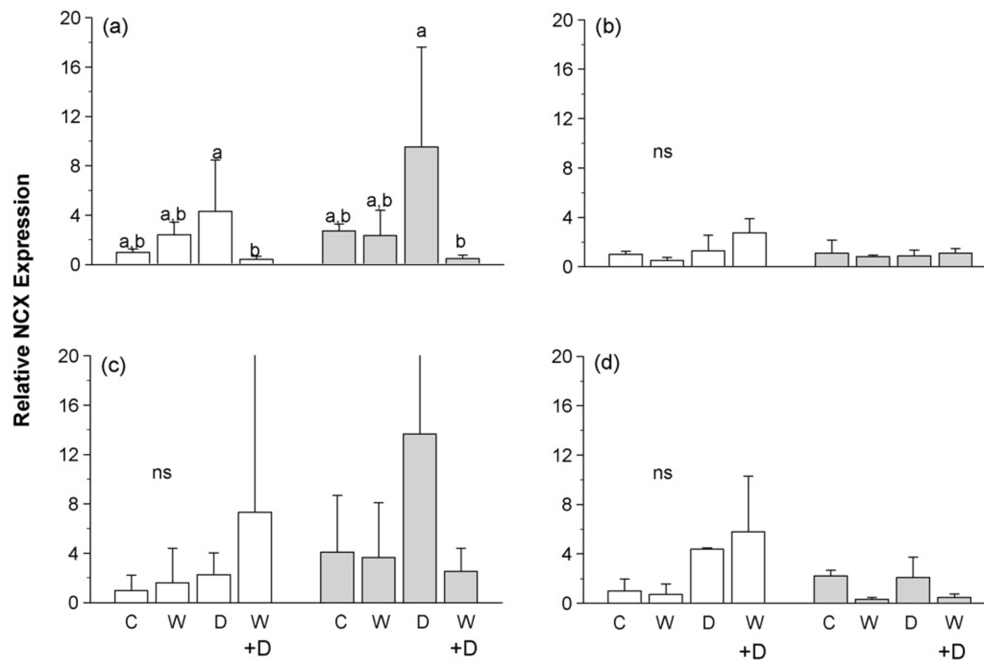

Fig. 2. The relative changes in NCX mRNA levels in the gill epithelium of rainbow trout during control (C), waterborne Cd (3 μg/L) (W), dietary Cd (500 mg/kg) (D), or combined waterborne and dietary Cd (W + D) exposures, and fed a control (20 mg/g dry weight Ca^{2+}; open bars) or elevated Ca^{2+} (60 mg/g dry weight Ca^{2+}; gray bars) for (a) 7 days, (b) 14 days, (c) 21 days, or (d) 28 days. A two-way ANOVA was used to assess the main effects (factors) of Cd exposure and dietary Ca^{2+} supplementation, and potential interactive effects between factors on relative NCX mRNA levels. Relative NCX mRNA expression was determined relative to the no Cd controls fed the 20 mg/g Ca^{2+} diet. Values are mean ± standard error of the mean ($n = 5$). Statistical differences between treatment means with regards to Cd exposure are denoted by different letters ($p < 0.05$). There was no significant effect of dietary Ca^{2+} supplementation on gene expression.

increased dietary Ca^{2+} supplementation. We hypothesized that increased dietary Ca^{2+} supplementation would elicit a hypercalcaemic response, leading to the differential regulation of ECaC and NCX mRNA expression. This hypothesis is supported by the fact ECaC expression in the gills of fish fed a diet rich in Ca^{2+} was significantly inhibited relative to that seen in fish maintained on a diet low in Ca^{2+}. This inhibition of ECaC expression is consistent with the significant and sustained reductions in waterborne Ca^{2+} and Cd uptake in the same group of animals maintained on Ca^{2+}-supplemented diets (Franklin et al., 2005), and further corroborates the previous findings by Baldisserotto et al. (2004a,b, 2005). We found that ECaC mRNA levels were significantly lowered by elevated dietary Ca^{2+} following 14 days of exposure (Fig. 1b–d). In fact, there is strong supporting evidence that dietary Ca^{2+} consistently reduces both Ca^{2+} and Cd uptake at the fish gill, despite producing no sustained alterations in plasma Ca^{2+}. In a recent study by Shahsavarani and Perry (2006), ECaC gene transcript in rainbow trout was significantly lowered 24 h following an artificial infusion of Ca^{2+}. These data support the role of an internal Ca^{2+} sensing mechanism for detecting subtle changes in extracellular Ca^{2+} balance (Radman et al., 2002), and altering the expression of Ca^{2+} transporting genes. This response would likely act in coordination with other regulatory mechanisms, such as post-translational control of existing Ca^{2+} transporting proteins.

The influence of dietary Ca^{2+} (at each of the Cd exposures tested) on NCX expression was less clear. There were no significant effects of dietary Ca^{2+} supplementation on NCX mRNA levels (Fig. 2). In light of the inconsistent dietary Ca^{2+} response, NCX is not likely an overly sensitive marker of dietary Ca^{2+} status. This finding may be partly explained by the presence of plasma membrane Ca^{2+}-ATPase (PMCA) on the basolateral membrane, representing an alternate route for Ca^{2+} efflux from the intracellular compartment in response to increased intracellular Ca^{2+} concentrations (Lock et al., 1987; Flik, 1990).

The present study provides the first experimental evidence on the influence of nutritional status on the transcriptional regulation of ECaC in the fish gill. Dietary Ca^{2+} supplementation reduces ECaC mRNA levels, although this effect was most pronounced in animals exposed to waterborne Cd, either singly or in combination with dietary Cd. In comparison, the inhibitory action of dietary Ca^{2+} supplementation on ECaC expression was either slightly attenuated (Fig. 1 b and c) or completely abolished (Fig. 1d) in animals concomitantly exposed to Cd via the diet (Fig. 1c). It is currently unclear what the physiological basis of this differential response to waterborne and dietary Cd. One explanation is that elevated dietary Cd inhibits the uptake of dietary Ca^{2+} at the gastro-intestinal tract intestine, preventing it from eliciting a hypercalcaemic response as seen with dietary Ca^{2+} exposure alone. This explanation would depend on Ca^{2+} and Cd^{2+} sharing one or more common uptake mechanisms in the GI tract, for which there is abundant circumstantial evidence, but only very limited mechanistic evidence in fish (reviewed by Wood et al., 2006). Accordingly, increasing the dietary content of Cd would likely compete with Ca^{2+} for transporter-mediated uptake in the gut, a response that is likely to lower extracellular Ca^{2+} levels to the point ECaC mRNA levels would increase. Nonetheless, these data support the notion of a

common uptake pathway for Ca^{2+} and Cd in the gastrointestinal tract.

The present study also attempted to address the influence of the route of Cd exposure on ECaC and NCX mRNA expression. In contrast to the pronounced inhibitory effect of elevated dietary Ca^{2+} on mRNA levels, ECaC and NCX mRNA levels were not affected by waterborne, dietary, or combined waterborne and dietary Cd exposure (Figs. 1, 2), except for a slight inhibition of NCX mRNA expression on day 7 in the combined Cd exposure. The lack of any major transcriptional alteration in ECaC and NCX expression is consistent with the lack of impairment in the unidirectional influxes of waterborne Ca^{2+} (Baldisserotto et al., 2004a) or Cd (Franklin et al., 2005) during similar experimental regimes. Although waterborne Cd is known to elicit inhibitory responses to unidirectional Ca^{2+} influx and plasma Ca^{2+} balance, the Cd concentrations typically required to produce these responses are significantly higher (10–50 μg/L Cd); (Baldisserotto et al., 2004a) than that used in the current study (3.0 μg/L). Similarly, we did not observe an inhibitory effect of dietary Cd (500 mg/kg Cd) on branchial Ca^{2+} uptake. However, this is in contrast to other studies, which have shown inhibitory effects of dietary Cd on Ca^{2+} uptake at concentrations as low as 300 mg/kg Cd (Baldisserotto et al., 2005).

4.1. Future perspectives

There is currently a regulatory initiative termed the biotic ligand model, which is being developed to characterize the modifying effects of water chemistry on metal bioavailability and toxicity (Playle et al., 1993; Playle, 1998; Niyogi and Wood, 2004). Water hardness, which effectively competes with Cd, and other chemical constituents, including the concentrations of inorganic ligands and dissolved organic carbon which may complex Cd, are known to play critical roles in altering Cd bioavailability. However, equally if not more important, is the role of physiology in controlling the ability of an aquatic animals to take up metals and other ions at the gill epithelium. Certainly, the current work supports the notion that increasing calcium concentrations in the diet over a physiologically relevant range will strongly influence metal uptake at the fish gill. More specifically, we have shown that nutritional status is an important transcriptional regulator of ECaC in the fish gill. Other factors, such as hormones (e.g., vitamin D-3, cortisol), and alterations in acid–base status are also identified as being important mitigating factors in controlling epithelial calcium uptake (Hoenderop et al., 2002; Shahsavarani and Perry, 2006). Clearly, the importance that diet plays in altering the bioavailability of metals needs to be considered, especially when comparing bioaccumulation data from several laboratories, where fish may have been maintained on distinct diets with varying components.

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