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The Protective Role of Dietary Calcium Against Cadmium Uptake and Toxicity in Freshwater Fish: an Important Role for the Stomach

Chris M. Wood,[A,D] *Natasha M. Franklin*,[A,B] *and Som Niyogi*[A,C]

[A] Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada.

[B] Present Address: CSIRO Land and Water, Bangor, NSW 2234, Australia.

[C] Present Address: Department of Biology, University of Saskatchewan, Saskatoon, SK S7N 5E2, Canada.

[D] Corresponding author. Email: woodcm@mcmaster.ca

Environmental Context. *Contamination of freshwater ecosystems by cadmium is of increasing concern with accumulation and toxicity in aquatic animals occurring through both waterborne and dietary routes. Increases in water calcium ('hardness') levels protect against waterborne uptake. Physiological research on freshwater fish has demonstrated that this occurs because cadmium moves through the calcium uptake pathway at the gills. Surprisingly, elevated dietary calcium also protects against waterborne exposure by down-regulating the calcium uptake pathway at the gills, and against dietary exposure by reducing cadmium uptake through the gastrointestinal tract. In both cases, the stomach is the critical site of action.*

Abstract. Waterborne cadmium causes toxicity in freshwater fish by inducing hypocalcaemia. Research on the rainbow trout (*Oncorhynchus mykiss*), a sensitive model species, has demonstrated that this occurs because Cd^{2+} ions compete with waterborne Ca^{2+} ions for the active branchial uptake pathway which normally ensures internal homeostasis of calcium levels. Therefore, increases in waterborne calcium concentrations ('hardness') protect against waterborne cadmium uptake and toxicity in both acute and chronic exposures. Increases in dietary calcium concentration also protect against waterborne exposure, because elevated gastrointestinal calcium uptake down-regulates the Ca^{2+} uptake pathway at the gills, thereby simultaneously reducing Cd^{2+} entry. Furthermore, dietary calcium also protects against dietborne cadmium exposure, although the physiological mechanisms appear to differ from those at the gills. Surprisingly, the principal site of this inhibitory action of dietary calcium on gastrointestinal cadmium uptake appears to be the stomach, which is also the major site of gastrointestinal calcium uptake, rather than the intestine as in mammals. These results underline the importance of considering not only water chemistry but also dietary chemistry in the environmental regulation of cadmium, and suggest that fish in the wild under chronic cadmium stress would benefit by switching to a more calcium-rich diet. While diet switching has been seen in the wild in fish under metal stress, its etiology remains unknown; to date, laboratory experiments have not been able to show that voluntary diet-switching of an adaptive nature actually occurs.

Keywords. aquatic chemistry — cadmium — calcium — fish — hardness — metal uptake

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Introduction

The presence of cadmium in the freshwater environment has long been of concern to environmental regulators because of its high level of acute toxicity in the dissolved phase, and water 'hardness' has long been known to play a powerful protective role against toxicity.[1] Most early studies failed to separate the effects of the greater alkalinity, pH, and magnesium levels that often co-vary with greater calcium in hard water, though Carrol et al.[2] is a notable exception. However, there is now wide-spread recognition that calcium is the true protective agent in water hardness, that waterborne cadmium toxicity is due mainly to the ionic form (Cd^{2+}) of cadmium, that acute toxicity is associated with hypocalcaemia in the extracellular fluids, and that toxicity to most aquatic organisms is inversely proportional to the dissolved calcium concentration of the exposure waters (cf. Wood[3] for review). Classic experiments performed almost two decades ago on freshwater rainbow trout (*Oncorhynchus mykiss*), a sensitive model species used for much cadmium research, illuminated the dominant physiological basis for this protection by calcium.[4] Cd^{2+} enters across the gills in competition with Ca^{2+} by the active Ca^{2+} uptake pathway which these organisms use both to supplement calcium uptake from the diet, and for rapid regulation of extracellular calcium homeostasis; a potent and largely irreversible inhibition of Ca^{2+} uptake develops. The apical entry step

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in the gill ionocytes is via a lanthanum-sensitive, voltage-insensitive Ca^{2+} channel, while the basolateral extrusion step is via a high-affinity Ca^{2+}–ATPase which Cd^{2+} selectively inhibits. The ionocytes of principal importance in this regard are so-called PNA^+ mitochondrial rich cells,[5] which are also thought to be the sites of Cl^- uptake, whereas the PNA^- cells are thought to be the sites of Na^+ uptake (PNA is peanut lectin agglutinin, referring to whether or not the cells bind this diagnostic tool).[5] At both steps, the Cd^{2+} versus Ca^{2+} interaction for common binding sites seems to occur, and recent kinetic analyses on whole trout[6] indicate that at least in the short term, the interactions are by direct competition. Thus, it is entirely logical that increasing waterborne calcium reduces cadmium uptake and toxicity.

Log K values for Ca^{2+} versus Cd^{2+} competition and binding site densities (βmax) in freshwater fish gills were first determined experimentally by Playle et al.[7] This led to the development of gill-binding models which are able to incorporate other protective agents in natural waters, both competing (e.g. H^+) and complexing (e.g. natural organic matter) moieties, into a geochemical modelling framework which predicts toxicity as a function of gill cadmium burden.[8] The principles behind these models have now been extended to many organisms other than fish, and many other metals; they are now known as 'biotic ligand models' (BLMs) and have been the subject of recent critical reviews.[9] In the present article, we further explore the nature of the calcium–cadmium interaction, extending our analyses from the gill to the gastrointestinal tract, feeding, behaviour, and regulatory issues relevant to fish populations exposed to metal contaminants in the wild.[10]

Calcium–Cadmium Interactions of Dietary Origin

Freshwater fish acquire calcium from both the water and the diet; the exact proportions depend on availability. While the hardness of most natural freshwaters is typically less than 500 ppm in traditional units of $CaCO_3$ equivalents (i.e. <5 mM calcium), the hardness of a typical fish diet is ~100-fold greater (50 000 ppm or 500 mM calcium) because of its high component of bone, scale, carapace, and shell. Therefore, an interesting recent finding is that waterborne cadmium uptake across the gills is also sensitive to the calcium level in the diet. In rainbow trout, very modest increases (1.5–3-fold) in dietary calcium concentration dramatically reduce both the acute cadmium uptake by the gills, and the longer term internal cadmium accumulation in a variety of organs.[11–15] In total, these studies have shown that the protective effect is independent of the anion (Cl^- or CO_3^{2-}) which accompanies Ca^{2+} in the dietary supplementation, and appears to occur because the fish down-regulates the active Ca^{2+} uptake pathway in the gill ionocytes, probably by closing the apical channels. Branchial Na^+ uptake is not affected, so the effect is specific. Branchial Ca^{2+} uptake is well known to be sensitive to plasma Ca^{2+} levels, probably via mobilization of the hormone stanniocalcin from the corpuscles of Stannius. Stanniocalcin activates a pathway which closes the apical Ca^{2+} channels if blood plasma Ca^{2+} concentration

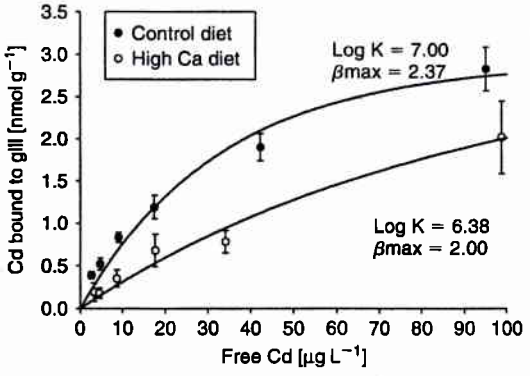

Fig. 1. The effect of elevated dietary calcium on the biotic ligand model properties of trout gills in vivo. Short-term (3-h) gill–cadmium binding profiles were determined using ^{109}Cd in juvenile rainbow trout after feeding with control (total calcium = ~20 g kg^{-1} food) or calcium-supplemented (~60 g kg^{-1} food, added as $CaCO_3$) diets for one week in moderately hard Lake Ontario water. Fish in both treatments were fed at a ration of 3% bodyweight per day. Gill-binding assays were carried out using synthetic soft water (hardness: 40–48 mg L^{-1} as $CaCO_3$, alkalinity: 34 mg L^{-1} as $CaCO_3$, pH: 7.3, and temperature: 12 ± 1°C). There was a significant overall effect of diet (2-way ANOVA). Data are expressed as the mean ± s.e. ($n = 7$).[17]

rises too high.[16] Thus, by taking more calcium from the diet and less from the water, the fish gains protection against the 'accidental' uptake of cadmium from the water. A decrease in the number and/or affinity of gill Cd^{2+}/Ca^{2+} binding sites (i.e. alterations in log K and βmax) would be predicted, and indeed both have recently been demonstrated[17] (Fig. 1). Similar protective effects of dietary calcium on waterborne zinc uptake,[18] and of dietary sodium on waterborne copper uptake[19] have been identified, acting through analogous mechanisms. There is a need to incorporate dietary ions into the waterborne BLM approach, but this has not yet been done in a formal manner.[20]

On a simple concentration basis (i.e. mg kg^{-1} of food or water) freshwater organisms are more tolerant of metals in food than in water by several orders of magnitude, but when the actual dose delivered to the uptake surface (gut or gills) is considered (i.e. concentration × mass flow of food or water), the exposures become more comparable.[21] It is now clear that, in nature, organisms accumulate metal from both sources.[22] Nevertheless, the dietary route of metal toxicity has received rather less attention and at present is not incorporated into environmental regulations in most jurisdictions, even though the capacity for dietary uptake is greater than for waterborne uptake. However, recently, there has been heightened interest in this topic as the regulatory focus has shifted from acute toxicity to chronic lifetime protection, where trophic transfer of metals may become more important. Meyer et al.[23] provide a comprehensive recent review.

Cadmium is taken up from the diet across the digestive tract of freshwater fish and accumulated in internal organs, particularly the gut tissues themselves, the kidney and the liver.[14,15,21,24–26] It is noteworthy that pre-exposure to elevated cadmium in the diet altered the BLM characteristics of the gills for waterborne Cd^{2+} (decreased log K,

but increased βmax), reduced its internal accumulation, and provided increased tolerance based on both 96-h LC_{50} data and physiological criteria.[25–27] Thus, the dietary and waterborne exposure pathways clearly interact.

In contrast to the gills, the gastrointestinal transport mechanism(s) involved is unknown, but in mammals, there is some evidence that Cd^{2+} may be taken up by the same transporters as used by several divalent nutrient metals such as Zn^{2+}, Fe^{2+}, and Ni^{2+} (DMT1, also known as DCT1 or Nramp2[28]), and Cu^{2+} (CTR1).[29] Both transporters are known to be expressed in the teleost intestine,[30] and there is indirect physiological evidence for the functioning of both[31] for the transport of other metals in the fish gut. Interestingly, Cooper et al.[31] reported very recently that zebrafish fed an iron-deficient diet exhibited increased intestinal uptake of cadmium, and up-regulation of DMT1, providing still more circumstantial evidence for the linkage. There is also evidence for the presence of L-type voltage-gated Ca^{2+} channels in fish enterocytes which are very different from the voltage-insensitive Ca^{2+} channels in the gills.[32] DMT1, CTR1, and these L-type Ca^{2+} channels are all apical pathways, and the basolateral transport mechanism is unknown, though there is long-standing evidence that cadmium may inhibit a basolateral high affinity Ca^{2+} ATPase in teleost enterocytes,[33] similar to its action in branchial ionocytes discussed earlier. Schoenmakers et al.[33] postulated that the Cd^{2+} export pathway from the enterocytes could be via a Na^+/Ca^{2+} exchange mechanism.

Recent Research Highlights on Calcium–Cadmium Interactions

Given the likely very different pathway(s) of cadmium uptake through the gut versus the gills, we have recently posed several questions:

(i) *Will elevated dietary calcium also protect against dietary cadmium uptake?* The answer is very definitely yes, as shown by two chronic feeding studies with juvenile trout on a commercial diet spiked with either 300[14] or 500 mg kg^{-1} cadmium,[15] whereas levels in control diets were less than 1 mg kg^{-1}. While these elevated concentrations were likely above the range of environmental relevance,[22] they were below the threshold for negative effects on growth. Whole body cadmium uptake from these diets was highly significant. However, internal organ-specific cadmium burdens over 28 days were reduced 40–50% by 2.5–3-fold elevations of calcium (as $CaCO_3$) in the diet. On a relative basis, these reductions were not as large as seen for waterborne cadmium exposures (up to 85%), but on an absolute basis they were much greater, because the fish can take up and tolerate much greater loads from the diet than from the water.

(ii) *How and where in the gastrointestinal tract does this protection by calcium occur?* In mammals, calcium and cadmium are taken up mainly through the intestine, so this was our initial point of focus. Intestinal sac preparations from trout chronically exposed to elevated dietary cadmium exhibited higher rates of cadmium uptake than did sacs from naïve fish, when assayed under identical conditions.[34] This response, while apparently maladaptive, was in agreement with an earlier in vivo catheterization study showing similarly greater cadmium uptake capacity in the gut of trout chronically exposed to dietary cadmium.[26] However, in trout which had been simultaneously exposed to both high dietary cadmium and calcium in combination for 30 days, this increase in intestinal cadmium transport rate was blocked, though the phenomenon was not seen in the posterior segment.[34] Thus, part of the protective effect is that chronic dietary calcium prevents the up-regulation of cadmium transport that would otherwise occur, but only in a portion of the system. Additional information on regional differentiation was obtained by analysis of tissue cadmium burdens in various sections of the gastrointestinal tract in these chronically exposed trout.[15] The only section in which there was clear evidence of protection was the stomach where tissue cadmium burdens were reduced by 50–70% at all time points (Fig. 2). Again, if anything, the posterior intestine showed the reverse. The absolute cadmium burdens were lower in the stomach tissue than in either part of the intestine (Fig. 2), but there is much more muscle tissue diluting the burden in the stomach than in the intestine, and the levels of calcium are much higher here, as discussed subsequently. A series of in vitro experiments have examined Ca^{2+} versus Cd^{2+} competition in various segments of the gastrointestinal tract of naïve trout.[35] Cadmium is taken up via the stomach in a saturable fashion, albeit at a lower concentration-dependent rate than via the various parts of the intestine, and again it is the only segment of the tract where calcium inhibits uptake. Raising mucosal calcium from 1 to 10 mM reduced cadmium uptake through the stomach by ~60%. While surprising, the response makes sense in light of recent evidence that virtually all of the calcium uptake in vivo occurs via the stomach rather than by the intestine in trout,[36] and dissolved calcium concentrations in stomach chyme are 5–10 fold higher (up to 50 mM) than in intestinal chyme. A similar trend applies to dissolved cadmium concentrations in chyme.[14] The Cd^{2+} and Ca^{2+} transport mechanism(s) remain(s) unknown, but clearly, future mechanistic focus should shift from the intestine to the stomach. For example, now that molecular tools are available for the epithelial calcium channel (ECaC) of the gill,[37] it would be of interest to see whether the same ECaC is also expressed in the stomach.

(iii) *Can fish under cadmium stress preferentially select a calcium-rich diet to gain protection?* Sherwood et al.[38] reported that yellow perch (*Perca flavescens*) in Canadian Shield lakes impacted by metal contamination (including cadmium) ate more invertebrates and less fish in their diets than did perch from pristine lakes. This strategy might increase the calcium content of their diet, though it would depend on the actual

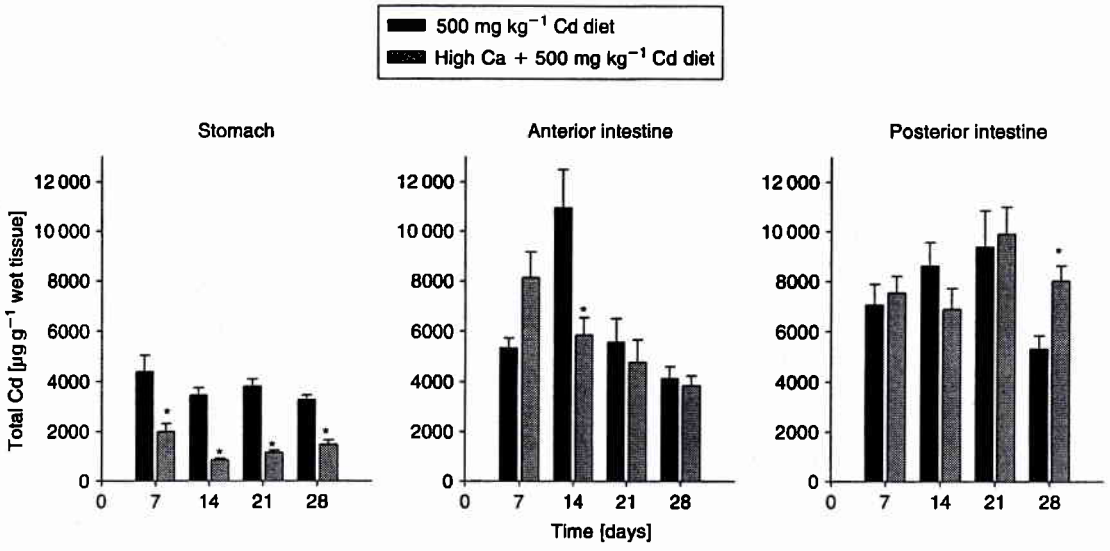

Fig. 2. The action of elevated dietary calcium on the accumulation of dietary cadmium in the tissues of various parts of the gastrointestinal tract in juvenile rainbow trout over a 28-day period. Diets were either an elevated cadmium (500 mg kg^{-1} food) + control calcium diet (20 g kg^{-1} food as $CaCO_3$; black bars), or an elevated cadmium (500 mg kg^{-1} food) + calcium-supplemented diet (60 g kg^{-1} food as $CaCO_3$; light bars). Fish in both treatments were fed at a ration of 3% bodyweight per day. The experiment was conducted at 12 ± 1°C in moderately hard Lake Ontario water. Data are expressed as the mean ± s.e. ($n = 7$). Asterisks indicate significant differences ($P < 0.05$). Data are from Franklin et al.[15]

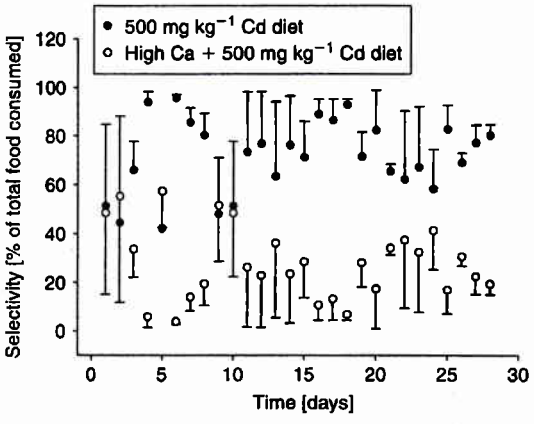

Fig. 3. Dietary preference of juvenile rainbow trout given a choice of two demand-feeders containing either an elevated dietary cadmium (500 mg kg^{-1} food) + control calcium diet (15 g kg^{-1} food as $CaCO_3$) (○), or elevated dietary cadmium (500 mg kg^{-1} food) + calcium-supplemented diet (60 g kg^{-1} food as $CaCO_3$) (•) over a 28-day period. The experiment was conducted at 12 ± 1°C in moderately hard Lake Ontario water. There was a significant overall effect of diet (2-way repeated-measures ANOVA). Data are expressed as the mean ± s.e. of two replicate tanks containing 30 fish each.[41]

composition of the invertebrates selected, which was not measured. We have therefore investigated whether voluntary switching to a diet of higher calcium content can be demonstrated using trout in the laboratory, because the phenomenon would be of considerable ecological relevance. An initial experiment with chronic waterborne cadmium exposure (3 μg L^{-1}) revealed no preference whatsoever for the high calcium diet in chronically exposed trout.[39] However, this may be explained by the fact that only 2 μg L^{-1} cadmium in the same water quality was required to eliminate social behaviours and alarm reactions involving chemosensory stimuli; this was associated with marked accumulation of cadmium in the water-facing olfactory organ.[40] Presumably, the fish could not smell or taste the elevated $CaCO_3$ in the food. However, because chemosensory responsiveness remained intact in trout under chronic dietary cadmium loading (300 mg kg^{-1}), a second series using a novel demand-feeding approach has examined dietary preference under this situation[41] (Fig. 3). Remarkably, the fish actively avoid eating the calcium-supplemented diet, preferring instead the food spiked with cadmium alone! The response is obviously maladaptive, similar to the up-regulation of cadmium absorptive capacity through the intestine mentioned earlier.[34] However, adding $CaCO_3$ to commercial trout food is not the same as altering a natural diet by increasing its complement of calcium-rich prey. Future studies should assess whether these same maladaptive responses occur in fish undergoing chronic cadmium stress at much lower concentrations in the wild, or whether the postulated protective effects occur. There is also a need to determine the degree to which the actual calcium content of fish diets varies across natural gradients of cadmium contamination.

Conclusions

These results underline the importance of considering not only water chemistry but also dietary chemistry in the environmental regulation of cadmium.

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