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Physiological response to a metal-contaminated invertebrate diet in zebrafish: Importance of metal speciation and regulation of metal transport pathways

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ABSTRACT

Dietary metal uptake in fish is determined by metal bioavailability in prey and the metal requirements of the fish. In this study zebrafish were fed the intertidal polychaete worm *Nereis diversicolor* (3% wet weight day^{-1}) collected from Ag, Cd and Cu-impacted Restronguet Creek (RC) or a reference site, Blackwater estuary (BW), for 21 days. On days 0, 7, 14 and 21 fish were fed a single meal of RC or BW *N. diversicolor* labeled with ^{110m}Ag or ^{109}Cd for measurements of metal assimilation efficiency (AE). Zebrafish intestines were also taken for mRNA expression analysis of copper transporter 1 (*ctr1*), divalent metal transporter 1 (*dmt1*) and metallothionein 2 (*mt2*). No significant difference was observed in the AE of ^{109}Cd in metal naïve fish at day 0 between RC and BW worms, 11.8 ± 2.1 and 15.3 ± 2.8%, respectively. However, AE of ^{110m}Ag was significantly greater in fish fed worms from BW compared to RC, 5 ± 1.2% and 1.6 ± 0.5%, respectively at day 0. Fractionation analysis of radiolabeled metal partitioned in *N. diversicolor* from RC revealed a greater proportion of Ag (40 ± 1.1%) in a fraction containing protein and organelle bound metal, associated with high trophic availability, compared to BW polychaetes (24 ± 2.5%). Lower AE of ^{110m}Ag from RC polychaetes is therefore unlikely due to speciation of ^{110m}Ag in *N. diversicolor* from RC, but to the high concentration of Cu, a potential Ag antagonist. Exposure to RC polychaetes significantly increased the AE of ^{110m}Ag (6.2 ± 1%), but not ^{109}Cd, from RC worms, after 21 days. AE of ^{110m}Ag and ^{109}Cd was unaffected by pre-exposure to BW. Elevated concentration of intestinal Cu and increased expression of *ctr1*, *dmt1* and *mt2* after 14 days exposure in fish fed worms from RC suggest altered Cu handling strategy of these fish which may increase AE of Ag via shared Ag and Cu transport pathways. These data suggest metal exposure history of invertebrates may affect metal bioavailability to fish, and fish may alter intestinal uptake physiology during chronic dietary exposure with implications for the assimilation and toxicity of dietary metals.

1. Introduction

Historically, waterborne metals were considered as the main determinants of metal toxicity in aquatic systems and branchial metal toxicity has been well characterised as a result (e.g. Wood, 2001). Consequently, it is data from aqueous metal toxicity studies which inform current environmental metals legislation (USA, USEPA, 1986; EU, 2006/11/EC). In contrast, dietary metal exposure has, until recently, been largely overlooked, although there is growing recognition that dietary metals may contribute more to total metal burdens and chronic toxicity in aquatic organisms than previously thought (Dallinger and Kautzky, 1985; Woodward et al., 1995; Bervoets et al., 2001; Clearwater et al., 2002; Luoma and Rainbow, 2005; Boyle et al., 2008, 2010). Understanding what factors affect intestinal metal uptake is therefore fundamental to correlating dietary metal exposure with metal uptake and toxicity and for the incorporation of dietary metals into environmental risk assessment (Croteau and Luoma, 2009).

Invertebrates exhibit different strategies for handling metals dependent on essential metal requirement and mechanisms of metal detoxification (Hare, 1992; Rainbow, 2002; Vijver et al., 2004). In turn, these processes affect bioavailability of metals to predators, including fish. For example, assimilation efficiencies (AEs) of Cd, Co and Zn by marine fish (*Menidia* sp.) were proportional to the quantity of each metal associated with the nonexoskeleton fraction of the zooplankton prey (Reinfelder and Fisher, 1994). Similarly, purified subcellular fractions of Cd bound to metallothionein-like proteins isolated from the amphipod *Gammarus pulex* were more efficiently assimilated by zebrafish than fractions containing Cd partitioned to insoluble granules and the exoskeleton (Khan et al., 2010).

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Whilst these and similar studies (e.g. Zhang and Wang, 2006) demonstrate the link between metal speciation in prey and bioavailability of metals to predators, data from laboratory studies of metal bioaccumulation (e.g. Kamunde et al., 2002a,b; Alves and Wood, 2006) and from studies of fish inhabiting metal-impacted environments (Couture et al., 2008; Goto and Wallace, 2009) indicate that elevated metal exposure may elicit a change in metal handling strategy of fish, including AE. For example, one-week exposure of black sea bream (*Acanthopagrus schlegeli*) to a diet supplemented with Ag (5.2 $\mu g\,g^{-1}$ as $AgNO_3$) or Cd (10.5 $\mu g\,g^{-1}$, as $CdCl_2$), increased AE of both metals (Long and Wang, 2005).

Populations of the polychaete worm *Nereis diversicolor* from Restronguet Creek (RC), Cornwall, UK and Blackwater estuary (BW), Essex, UK have different metal exposure histories and exhibit different metal handling strategies and tissue metal concentrations (Berthet et al., 2003; Mouneyrac et al., 2003; Rainbow et al., 2004). Previously, Boyle et al. (2008) observed no difference in Cu or Zn burdens of zebrafish fed *N. diversicolor* from RC for 68-days compared to zebrafish fed *N. diversicolor* from BW estuary despite 30-fold higher Cu and 3-fold higher Zn load in RC worms, indicative of regulation of Cu and Zn accumulation. Furthermore, Ag and Cd burdens were higher in zebrafish fed *N. diversicolor* from BW, despite lower concentrations of these metals in their diet.

To further explore these observations, this study investigates the effects of dietary exposure to *N. diversicolor* from RC and BW for 21 days, on the AE of ^{110m}Ag and ^{109}Cd in zebrafish. To gain a better understanding of metal acclimation at a mechanistic level and the interplay of essential and non-essential metal regulation, mRNA expression of metal import-proteins implicated in Cu acquisition are also investigated. Copper transporter 1 (Ctr1, Mackenzie et al., 2004; Minghetti et al., 2008) as well as the divalent metal transporter 1 (Dmt1) which has a broad substrate specificity that includes Fe and Cd (Cooper et al., 2007) have been implicated from gut sac metal uptake studies in Cu transport (Nadella et al., 2007).

2. Materials and methods

2.1. Experimental fish

One hundred and sixty zebrafish (0.38 ± 0.01 g; $n = 9$), obtained from Neil Hardy Aquatica Ltd (Carshalton, Surrey, UK), were randomly assigned to four exposure tanks containing 40 L of continuously aerated and filtered synthetic water (reverse-osmosis H_2O supplemented with sea salts, 60 mg Tropic Marin L^{-1} with the addition of 200 μM $CaCl_2$) under a photoperiod regime of 12 h light, 12 h dark and reared at $28 \pm 1\,^{\circ}C$. Zebrafish were acclimated to experimental conditions for a period of at least three weeks prior to commencing the experiment. Twenty L of synthetic water was renewed daily.

2.2. Diets

N. diversicolor were collected from intertidal mudflats in Blackwater estuary, Essex, UK (N 051 44 08, E 000 41 34) and Restronguet Creek, Cornwall, UK (N 050 12 36, W 005 05 41). *N. diversicolor* were depurated for 72 h in artificial seawater at a salinity of 15‰ at 15 °C. After depuration, polychaetes were rinsed with deionised water to remove external salt and stored at −80 °C until required.

2.3. Dietary metal exposure regime

Zebrafish were fed twice a day for a period of 21 days at a combined rate of 3% wet weight d^{-1} (adjusted for moisture content and following removal of head) either *N. diversicolor* from Blackwater estuary (2 tanks) or Restronguet Creek (2 tanks). Fish were weighed at day 0 and then following measurement of AE on days 7 and 14 and the dietary ration for remaining fish adjusted accordingly. There was no difference in water quality between the treatment groups; pH 6.10 ± 0.05, 6.17 ± 0.02; alkalinity 2.26 ± 0.12, 2.48 ± 0.11 mg L^{-1} (as $CaCO_3$); Ca hardness 27.1 ± 3.4, 29.9 ± 5.4 mg L^{-1} (as $CaCO_3$); all means ± standard deviation $n = 3$, RC and BW respectively; total Cu concentration 18.3 ± 8.7, 22.5 ± 10 $\mu g\,L^{-1}$, $n = 10$, means ± standard, RC and BW respectively; temperature $28 \pm 1\,^{\circ}C$ (continuous monitoring).

2.4. Radioisotopes

Radioisotopes were obtained from the following sources: ^{110m}Ag (as $AgNO_3$ in 0.5 M HCl) courtesy of Prof. P. S. Rainbow, Natural History Museum, London, UK, originally from Risø National Laboratory, Denmark; ^{109}Cd (as $CdCl_2$ in 0.1 M HCl) from Perkin Elmer, UK. The radioisotopes are essentially carrier-free and addition of metal to solution during radiolabeling is negligible.

2.5. Radiolabeling of N. diversicolor

N. diversicolor (0.22 ± 0.04 g Restronguet Creek; 0.26 ± 0.04 g Blackwater estuary $n = 8$) were individually labeled in solution as previously described by Rainbow et al. (2006) but with minor modification. Individual polychaetes (total number exposed 20–30) from each site were exposed to 0.125 MBq ^{110m}Ag or ^{109}Cd in 100 mL artificial seawater at a salinity of 15‰ (Tropic Marin) in individual plastic beakers at 7 °C. *N. diversicolor* had previously been depurated for 72 h (as described above) to prevent *N. diversicolor* ingesting radioisotope bound to gut contents which had been previously excreted. Rainbow et al. (2006) reported differences in AE from *N. diversicolor* that had been labeled from sediment or water, suggesting different partitioning behaviour depending on route of exposure. Consequently, worms were not fed during radiolabeling. After 14 days, each worm was briefly rinsed in either sodium thiosulfate (5 mM, Sigma–Aldrich, UK), to displace externally bound ^{110m}Ag, or EDTA (ethylenediaminetetraacetic acid, 5 mM, Sigma–Aldrich, UK) to chelate external ^{109}Cd. Polychaetes were then rinsed in deionised H_2O and stored at −20 °C until required.

2.6. Metal partitioning in N. diversicolor

Analysis of the compartmentalisation of ^{110m}Ag and ^{109}Cd in *N. diversicolor* was performed using a simplified version of a fractionation procedure outlined in Wallace et al. (2003) and Wallace and Luoma (2003). *N. diversicolor* ($n = 4$, per isotope and site of origin) were counted in a gamma counter with specific windows for ^{110m}Ag and ^{109}Cd (LKB wallac, 1282 compugamma, Turku, Finland). Worms were then homogenised by hand in deionised water (1:2, w/v) for 2 min and centrifuged at $1450 \times g$ for 15 min at 4 °C. This yielded a supernatant containing organelles, enzymes and metallothionein-like proteins (S1) and a pellet containing metal-rich granules and cell debris (P1). Fractions were counted as described for whole worms (above). Cumulative counts exceeded 80% of initial counts measured in each worm.

2.7. Assimilation efficiency

Assimilation efficiency of ^{110m}Ag and ^{109}Cd in zebrafish from radiolabeled *N. diversicolor* was measured using an established radiotracer technique (Wang and Fisher, 1999). To assess the effects of pre-exposure to dietary metals on assimilation, this was performed with fish fed non-radiolabeled RC or BW worms for 0, 7, 14 and 21 days. At each timepoint 10 fish per treatment (10 total day 0) were removed to individual 2 L beakers (AE chambers) filled with 1 L synthetic water and in waterbaths maintaining a constant