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Mechanisms of zinc toxicity in the galaxiid fish, *Galaxias maculatus*

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ABSTRACT

Zinc (Zn) is an essential metal, which is ubiquitous in aquatic environments occurring both naturally, and through anthropogenic inputs. This study investigated impacts of sub-lethal Zn exposure in the galaxiid fish *Galaxias maculatus*. Known as inanga, this amphidromous fish is widespread throughout the Southern hemisphere, but to date almost nothing is known regarding its sensitivity to elevated environmental metals. Fish were exposed to environmentally-relevant concentrations of Zn (control, 8, 270 and 1000 μg L^{-1}) over 96 h. End-points measured included those relating to ionoregulatory disturbance (whole body calcium and sodium influx), oxygen consumption (respirometry), oxidative stress (catalase activity and lipid peroxidation) and whole body accumulation of Zn. Zn exposure caused increases in catalase activity and lipid peroxidation, but only at the highest exposure level tested. Zn also significantly inhibited calcium influx, but stimulated sodium influx, at 1000 μg L^{-1}. The sub-lethal changes induced by Zn exposure in inanga appear to be conserved relative to other, better-studied species. These data are the first to explore the sensitivity of juvenile galaxiid fish to Zn, information that will be critical to ensuring adequate environmental protection of this important species.

1. Introduction

Anthropogenic activities, such as agriculture, urbanisation, and mining have resulted in elevated levels of trace elements, such as zinc (Zn), in natural waters. Zn is enriched in the aquatic environment through sources such as corrosion of galvanised products, breakdown of car tire rubber and urban runoff (Davis et al., 2001; Veleva et al., 2010; O'Sullivan et al., 2012). At low levels, Zn is an essential element playing an important role in many biochemical processes, with Zn-dependent proteins comprising around 10% of the proteome (Watanabe et al., 1997; Hogstrand, 2011). Fish take up Zn across the gills (Hogstrand et al., 1994) and also via the gastrointestinal tract (Glover and Hogstrand, 2002; Bury et al., 2003). Zn then enters the bloodstream and is transported to the liver, where the metal-binding protein metallothionein facilitates Zn donation to metalloenzymes (Valavanidis et al., 2006; Hogstrand, 2011). Metallothionein can also act to sequester potentially toxic levels of Zn from interfering with sensitive cellular entities (Valavanidis et al., 2006; Hogstrand, 2011), however, at higher levels of exposure, homeostatic regulation of Zn can be overwhelmed and toxicity may result (Spry and Wood, 1995; Hogstrand et al., 1996; Loro et al., 2014).

The major mechanism of waterborne Zn toxicity occurs at the site of absorption, manifested by the free ion form (Zn^{2+}). As a divalent cation Zn^{2+} disrupts the absorption of calcium (Ca) by competing with this important ion for uptake, at least in studied model species such as the rainbow trout (*Oncorhynchus mykiss*; Hogstrand et al., 1995, 1996, 1998, 1994). This results in hypocalcaemia and eventually causes fish death (Spry and Wood, 1995; Hogstrand et al., 1996; Bury et al., 2003). While interference with Ca homeostasis appears to be the main mode of Zn toxicity, effects on other biochemical and physiological processes have also been noted. For example, Zn has been shown to inhibit the basolateral Na^+/K^+-ATPase (NKA; Loro et al., 2014), the enzyme that is primarily responsible for the transport of ions across the fish gill, thus ensuring ionic and acid–base homeostasis (Evans et al., 2005). Furthermore, at high exposure levels Zn is known to cause branchial mucus secretion, a mechanism of toxicity that increases diffusive distance and impairs both ion regulation, but also other vital gill-based processes such as oxygen (O_2) uptake (Skidmore, 1970). In addition to ionoregulatory and respiratory effects of Zn, impacts on oxidative stress markers have also been recognised. For example, killifish (*Fundulus heteroclitus*) exposed to Zn exhibited a decrease in tissue catalase (CAT) activity (an enzyme that degrades H_2O_2 formed through reactive O_2 species), and an increase in lipid peroxidation (a marker of oxidative damage; Loro et al., 2012). However, there is little information as to whether Zn involvement in oxidative stress is a widespread phenomenon in fish (Lushchak, 2011). The median lethal concentrations (LC_{50}) for freshwater fish exposed to Zn range from 66 to 40 900 μg L^{-1} (Eisler, 1993), although sub-lethal effects have been noted in sensitive species, such as brown trout (*Salmo trutta*), at Zn exposure levels of 5 μg L^{-1} (Sayer et al., 1989). This is of concern as these concentrations are lower than those commonly measured in urban streams (O'Sullivan et al., 2012).

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The Biotic Ligand Model (BLM) is a regulatory tool that provides a site-specific assessment of metal toxicity. Using the relationships between water chemistry, metal accumulation, and toxicity, these models can be used to predict effect levels of Zn in a given water (Santore et al., 2002). BLM's have been developed for a number of metals and are in regulatory use worldwide (e.g. Bodar et al., 2005; United States Environmental Protection Agency, 2007). In Australia and New Zealand (NZ), the BLM approach is an approved method for water quality assessment but is not specifically mandated (ANZECC/ARMCANZ, 2000). BLM approaches have been developed using a few model species (e.g. rainbow trout and fathead minnows), and as such may not necessarily be applicable to other species, particularly if the mechanisms of metal toxicity differ (Niyogi and Wood, 2004). Consequently, more research is required to establish whether mechanisms of metal toxicity are conserved between species. Such data will validate the use of BLM's in settings, and for species, outside of those that were used to develop and calibrate the models.

Galaxias maculatus (commonly known as inanga (NZ), jolly tail (Australia) or puye (South America)) is one of the world's most widely distributed freshwater fish species (McDowall, 1990), although it remains restricted to temperate Southern hemisphere waters (McDowall, 2006). This species is of significant value, being the predominant component of the culturally and economically important NZ whitebait fishery (McDowall, 2006), and a potential aquacultural species in South America (Mardones et al., 2008). Inanga display a number of physiological characteristics that are quite distinct from those of more commonly studied Northern hemisphere fishes. For example, inanga are scaleless and in aquatic settings the skin accounts for almost 40% of total O_2 uptake (Urbina et al., 2014). The importance of the skin in transport processes usually associated with the gill, means that the skin could act as an alternative locus of metal toxicity and/or a rescue pathway supplementing transport processes impacted by metal actions at the branchial epithelium.

Inanga are one of the few truly amphidromous fish (McDowall, 2007). They hatch on spring tides in estuarine nurseries, migrate out to the ocean as larvae, where they develop into juvenile fish through the winter, before migrating back to freshwater in the spring (McDowall, 1990, 2007; Watanabe et al., 2014). The migration of juvenile inanga through estuaries is likely to expose them to high levels of environmental contaminants (Harley and Glover, 2014), and as adults, inanga inhabit near-coastal streams with significant potential for contamination by urban or mining effluents. For example, levels of Zn as high as 270 μg L^{-1} have been recorded in urban streams of the Canterbury region of NZ (O'Sullivan et al., 2012), while levels as high as 1280 μg L^{-1} have been reported in acid-mine impacted streams of the West Coast, known to be an important inanga habitat (Harley et al., 2015). Although limited to certain metals, and life stages, previous research has shown that inanga are significantly impacted by exposure to metals (Barbee et al., 2014; Harley and Glover, 2014; Harley et al., 2015), but physiological mechanisms of metal toxicity remain unknown. Among other impacts such as altered land-use, introduced species, and overfishing, pollution is considered one factor responsible for the decline in inanga populations (Rowe et al., 1999, 2007).

The goals of the current study were to investigate Zn toxicity to inanga. Assessing the impacts metal toxicants have on inanga will provide insight into their sensitivity, thus contributing information vital for the monitoring and protection of this species in NZ and worldwide. It will also confirm that modelling approaches based on physiological mechanisms of uptake and toxicity are applicable to species outside those in which the models have been tested and calibrated. In the current study fish were exposed for 96 h to concentrations of Zn representing a regulatory level (8 μg L^{-1}; value considered to be protective to 95% of freshwater biota; ANZECC/ARMCANZ, 2000), an elevated environmental level (270 μg L^{-1}; O'Sullivan et al., 2012), and an extreme environmental level (1000 μg L^{-1}; Harley et al., 2015). End-points examined included whole body Zn accumulation, markers of oxidative stress (CAT activity, lipid peroxidation), ionoregulatory dysfunction (Ca and Na influx), and respiratory toxicity (O_2 consumption).

2. Materials and methods

2.1. Animal collection and holding

Inanga were caught using seine nets from near-coastal streams in the Canterbury region of the South Island of NZ. The average concentration of Zn at the collection sites was 1.9 (±0.3) μg L^{-1} (n = 3). Fish were placed into aerated plastic containers and transported back to the aquarium facility at the University of Canterbury, before being housed in 500-L aquaria receiving flow-through freshwater and constant aeration. They were held under constant temperature (15 °C) and light (12 h dark: 12 h light) conditions. Fish were acclimated for one month prior to experimentation and during this time were fed daily (Nutrafin® Max, USA). Feeding ceased 48 h prior to, and during, experimentation. The University of Canterbury Animal Ethics Committee approved all procedures.

2.2. Zn exposure

For biochemical and O_2 consumption analysis, a total of 32 inanga (mean ± SEM, 1.34 ± 0.20 g) were randomly distributed (n = 8) to one of four Zn exposures (nominally: control (no added Zn), 8, 270 or 1000 μg L^{-1}) for 96 h. Exposures were conducted in plastic containers (4.5 L) that were acid washed before exposures. Desired Zn levels were achieved by spiking chambers with stock solutions (1 or 10 g L^{-1} $ZnSO_4$) to 2 L of aquarium water (pH 6.7; total hardness 0.70 mmol L^{-1}; total alkalinity 0.519 mmol L^{-1}; electrical conductivity 18.8 mS m^{-1}; total Ca 0.57 mmol L^{-1}; total magnesium 0.14 mmol L^{-1}; total potassium 0.29 mmol L^{-1}; total Na 0.37 mmol L^{-1}; chloride 0.31 mmol L^{-1}; dissolved organic carbon <0.2 mg C L^{-1}). Waters were left for 24 h to equilibrate, before addition of fish (one fish per chamber). Exposure chambers were continually aerated, and maintained under constant temperature (15 ± 1 °C) and light (12 h dark: 12 h light) regimes. A complete water change was performed at 48 h, with water that had been equilibrated for 24 h.

A second exposure was conducted for the Ca and Na influx experiments. This exposure was conducted in an identical manner to that described above, except in this instance just two concentrations were tested (control and 1000 μg L^{-1}). A total of 16 fish were exposed for each influx (mean ± SEM; Ca influx; 0.91 ± 0.05 g, Na influx; 0.51 ± 0.11 g; both n = 8), with two fish per exposure chamber.

Water samples were taken for Zn analysis at four time points (fish addition, before and after the water change, and at the conclusion of the exposure). These values were averaged across each replicate, and then replicates were averaged to provide the measured Zn exposure concentration. Water was sampled by passing it through a Millex 0.45 μm filter (Millipore Ltd, Cork, Ireland) using a syringe (Chirana, Slovakia) without a rubber stopper to avoid Zn contamination. Water samples (15 mL) were acidified with 20 μL of ultrapure 70% nitric acid (HNO_3), and stored at 4 °C before being analysed by Inductively Coupled Plasma Mass Spectometry (ICP-MS) as described below.

2.3. Oxygen consumption

O_2 consumption was measured in fish via closed box respirometry (Urbina et al., 2012; Urbina and Glover, 2013). At cessation of the Zn exposure, fish were placed individually into 0.25 L Schott glass bottles and covered with plastic mesh so water could flow in. Chambers were submerged in a controlled temperature water bath (15 ± 1 °C) for the duration of the experiment. Fish were acclimated for 1 h prior to the chambers being sealed with a rubber bung. Attached to the bung was a syringe filled with water and a three-way tap to take samples. Fish naturally depleted O_2 in the chamber and measurement of this is a