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Zinc bioaccumulation and ionoregulatory impacts in *Fundulus heteroclitus* exposed to sublethal waterborne zinc at different salinities

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ABSTRACT

Exposure of *Fundulus heteroclitus* to an environmentally relevant Zn concentration (500 μg L^{-1}) at different salinities (0, 3.5, 10.5, and 35 ppt) revealed the following effects: (i) plasma [Zn] doubled after exposure at 0 ppt, a response which was eliminated at 35 ppt. Tissue [Zn] also increased in gill, liver, intestine, and carcass at 0 ppt. (ii) Both branchial and intestinal $Ca2^+$ ATPase activities decreased in response to Zn at 0 ppt and were elevated at 35 ppt. Plasma [Ca] decreased by 50% at 0 ppt and by 30% at 3.5 ppt and increased by 20% at 35 ppt. Gill [Ca] decreased by 35% at 0 ppt and increased by about 30% at all higher salinities. (iii) Branchial Na^+,K^+ ATPase activity decreased by 50% at 0 ppt, increased by 30% and 90% at 10.5 and 35 ppt respectively. Intestinal Na^+,K^+ ATPase activity was reduced by 30% at 0 ppt. (iv) Plasma [Na] decreased by 30% at 0 ppt in Zn-exposed. Zn exposure also disturbed the homeostasis of tissue cations (Na^+, K^+, Ca^{++}, Mg^{++}) in a tissue-specific and salinity-dependent manner. (v) Drinking rate was not altered by Zn exposure. In toxicity tests, acute Zn lethality (96-h LC50) increased in a close to linear fashion from 9.8 mg L^{-1} at 0 ppt to 75.0 mg L^{-1} at 35 ppt. We conclude that sublethal Zn exposure causes pathological changes in both Ca^{++} and Na^+ homeostases, and that increasing salinity exerts protective effects against both sublethal and lethal Zn toxicities.

1. Introduction

Pollution of natural waters by metals released by domestic, industrial, and agricultural processes is an environmental problem of global significance. Zinc (Zn) is introduced into aquatic systems through industrial process, such as smelting and use of fertilizers in agriculture (Eisler, 1993). Most metals originate from land-based sources and enter the sea through rivers, and many sewage treatment plants discharge metals into rivers and estuaries (Wood, 2012). Therefore the toxic effects of metals in intermediate salinities are of particular concern. In addition to copper (Cu), Zn is of special interest because it is both an abundant metal toxicant and an essential micronutrient with important properties indispensable for life. Zn plays a critical role in cellular metabolism, serving as a co-factor in a number of enzymatic reactions and as an intracellular signaling agent. Zn also acts as an antioxidant and a vital constituent of >200 enzymes (Bury et al., 2003; Hogstrand, 2012). However, at higher concentrations, metals such as Zn and Cu are able to disrupt physiological and biochemical mechanisms causing both ionoregulatory disturbance and oxidative damage in fish (e.g. Spry and Wood, 1985; Gioda et al., 2007; Lushchak, 2011; Loro et al., 2012).

Most research to date on the sublethal physiological effects of Zn has been conducted on freshwater fish. This information has been reviewed by Wood (2001) and Hogstrand (2012). There is a general consensus that the principal toxic effect is hypocalcemia, caused by Zn interfering with active calcium (Ca) uptake at the gills. There is "ionic mimicry" between Zn and Ca (Bury et al., 2003). The result is both competition by Zn with Ca for an apical calcium channel and inhibition of abasolateral Ca^{2+}ATPase in the gill ionocytes (e.g. Spry and Wood, 1985, 1989; Hogstrand et al., 1994, 1996). Far less information exists on its action in marine fish, and almost none at intermediate salinities. In fresh water, Ca concentrations are low, generally less than 2 mmol L^{-1}, whereas 100% sea water contains about 10 mmol L^{-1} Ca. Therefore one might predict that Zn toxicity would decrease as salinity increased, especially as the availability of free Zn^{2+} decreases because of increased complexation by the anions present in sea water. However, Hogstrand (2012) noted that tabulations of acute Zn LC50s (USEPA, 1987; Eisler, 1993) reveal generally similar values between fresh and sea water. There are several possible reasons for this apparent discrepancy. The first is that different species have been generally tested at different

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salinities, so that the comparisons are confounded. The second is that fish drink very little in fresh water, but tend to drink to an increasing extent as salinity increases (e.g. Scott et al., 2006, 2008), such that the gut becomes another potential route of Zn uptake and site of Zn toxicity. However it is not known whether Zn exposure itself alters drinking rate.

The Atlantic killifish (*Fundulus heteroclitus*) lives in estuaries and salt marshes of the eastern coast of North America. One special characteristic of the killifish is its extensive range of tolerance to environmental variables, including salinity (Griffith, 1974; Burnett et al., 2007). Indeed this species has become a model organism to study some aspects of physiology and toxicology across a range of salinities from fresh water to > 100% seawater (e.g. Marshall et al., 1999; Marshall, 2003; Blanchard and Grosell, 2006; Grosell et al, 2007; Wood and Grosell, 2009; Genz et al., 2011). Bielmyer et al. (2012) have exploited this euryhalinity to show that the acute toxicity of Zn^{2+} to 7–8 day old killifish larvae decreases dramatically (i.e. 96 h LC50 increases greatly) as salinity increases, and that much of this protective effect can indeed be explained by the increase in Ca^{2+} concentration in the exposure medium. In a parallel study to the present investigation, we showed that increased salinity also protected against oxidative stress caused by sublethal Zn exposure in adult killifish (Loro et al., 2012). Furthermore, Shyn et al. (2012) have reported that increased salinity tended to reduce Zn bioaccumulation in adult killifish.

In the present study, we explore more deeply the potential interactions between waterborne Zn exposure and environmental salinity in adult killifish. Our focus was on ionoregulatory parameters (plasma and tissue ions, gill and gut Na^+,K^+ ATPase, Ca^{2+}ATPase activities, drinking rates) and tissue-specific Zn bioaccumulation after 96-h exposures to an environmentally relevant sublethal Zn concentration (500 μg L^{-1}; Eisler, 1993; Hogstrand, 2012) in killifish acclimated to different salinities ranging from fresh water to 100% sea water (0, 3.5, 10.5 and 35 ppt). We also measured acute 96-h lethality at higher Zn concentrations at these same salinities. Our first hypothesis was that acute 96-h Zn LC_{50} in adult *Fundulus heteroclitus* would increase with salinity in a similar manner to that reported in larval killifish by Bielmyer et al. (2012). Secondly we postulated that disturbances in ionoregulatory parameters at the sublethal Zn concentration (500 μg L^{-1}), especially those involving Ca homeostasis, would progressively decrease as salinity increased. Our third hypothesis was that increasing salinity would decrease Zn bioaccumulation and alter tissue-specific Zn distribution, reflecting the increasing importance of Zn uptake through the gut due to greater drinking.

2. Materials and methods

2.1. Animal collection and acclimation

Adult Atlantic killifish of the northern subspecies *Fundulus heteroclitus macrolepidotus* of both sexes (mass: 2–6 g; length: 4–6 cm) were beach-seined from local tidal flats in New Hampshire (USA) by Aquatic Research Organisms (ARO) Ltd. (Hampton, NH, USA). In the laboratory, they were held at 18.5 °C and a salinity of 3.5 ppt for several weeks under static conditions, with aeration and filtration, and a 12 L:12 days photoperiod. Saline waters were made by the addition of Instant Ocean® sea salt (Aquarium Systems Inc., Mentor, OH, USA) to fresh water, considering 35 g/L as 35 ppt of salinity. The major ion composition of the fresh water is represented by the values for salinity 0 in Table 1. The fish were then acclimated to four different salinities (0, 10, 30 and 100% = 0, 3.5, 10.5, and 35 ppt) in similar 250-L static tanks for 7 days prior to experiments. Major ion composition was again similar to values reported in Table 1. A single satiation meal consisting of a mix of 50% commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secausus, NJ, USA) and 50% frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA) was administered once per day. Feeding was suspended for 2 days prior to the start of experiments and throughout the 96-h exposures.

Table 1
Acute (96 h) LC_{50} values (mg L^{-1}) and 95% confidence intervals (CI) for waterborne Zn toxicity in adult *F. heteroclitus* at different salinities, expressed as nominal concentrations, measured total concentrations, and measured dissolved concentrations. Capital letters indicate significant differences ($P \leq 0.05$) among LC_{50} values at different salinities. Means sharing the same letter are not significantly different.

Salinity (ppt)	Nominal	Total	Dissolved
0	10.3 ^{A} (6–14)	10 ^{A} (6–13)	9.8 ^{A} (6–12)
3.5	22 ^{B} (13–30)	21 ^{B} (13–29)	21 ^{B} (13–28)
10.5	37 ^{C} (29–48)	36 ^{C} (29–47)	36 ^{C} (29–46)
35	76 ^{D} (59–95)	75 ^{D} (58–94)	75 ^{D} (58–93)

Procedures were approved by the Universidade Federal of Santa Maria (UFSM) Animal Care Committee and complied with the laws of Brazil.

2.2. Acute Zn toxicity tests

Acclimated killifish were kept under control conditions at the four different salinities as described above (Section 2.1) and exposed for 96 h to a range of waterborne Zn concentrations. Killifish (N = 12) were exposed to Zn in pre-cleaned (1% HNO_3) aquaria, each containing 8 L of experimental media. The media were allowed to pre-equilibrate for 6 h prior to introduction of killifish. Temperature (22 °C) and photoperiod (12 L:12 days) were fixed and the aquaria were continuously aerated.

Different Zn concentrations (nominal values) were tested at the four different salinities: 0 ppt (fresh water) — 1, 2.5, 5, 10, 15, 20, 30, and 40 mg/L^{-1}; 3.5 ppt — 10, 20, 30, 40 and 50 mg/L^{-1}; 10.5 ppt — 30, 40, 50, 60, 70 and 80 mg/L^{-1}; 35 ppt — 70, 80, 90, 120, 140 and 160 mg/L^{-1}. Stock solutions were made by dissolving Zn (as $ZnSO_4.7H_2O$; Sigma-Aldrich) in acidified Milli-Q water® and were added inappropriate amounts to the different salinity waters. Every 12 h, living fish were counted, dead fish removed, and test media were completely renewed. No food was provided during the toxicity tests. Zn concentrations (both total and dissolved) in water samples from toxicity tests were measured every day as described in Sections 2.3 and 2.6, allowing comparison of LC_{50} values calculated as described in Section 2.7 on the basis of nominal Zn, measured total Zn, and measured dissolved Zn concentrations.

2.3. Sublethal Zn exposures and in vivo accumulation tests

After acclimation, fish were divided into eight groups and transferred to separate 8-L aquaria pre-cleaned with 1% HNO_3. There were four control groups of salinity (0 ppt = fresh water, 3.5 ppt, 10.5 ppt and 35 ppt = 100% seawater), and four experimental groups at these same salinities exposed to nominally 500 μg Zn L^{-1} (as $ZnSO_4.7H_2O$; Sigma-Aldrich, St. Louis, MO, USA) for 96 h, each with two replicates of N = 6. To prevent Zn loss, no filters were used, so every day, 80% of the water was renewed. Daily water samples were taken for major ion and Zn measurements, which are reported in Table 1. Samples for measurements of dissolved Zn and various ions were obtained by passage through 0.45 μM syringe filters (Acrodisc syringe filter; Pall Life Sciences, Houston, TX, USA). Unfiltered water samples were also taken for total Zn measurements. Water dissolved organic carbon (DOC), pH, and alkalinity values, and the resulting effects of water chemistry on calculated Zn speciation were measured in a parallel series of experiments reported previously (Loro et al., 2012) After 96-h exposure, all fish were euthanized with a lethal dose of NaOH-neutralized MS-222 (Syndel Laboratories Ltd., Qualicum Beach, B.C., Canada), and a blood sample was taken by blind caudal puncture using a gas-tight 100-μL Hamilton syringe with a needle modified to sample at the correct depth. The plasma was separated by centrifugation (10,000 ×*g*, 2 min) and frozen for