



## 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<sup>-1</sup>) 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 Ca<sup>2+</sup> 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<sup>+</sup>,K<sup>+</sup> ATPase activity decreased by 50% at 0 ppt, increased by 30% and 90% at 10.5 and 35 ppt respectively. Intestinal Na<sup>+</sup>,K<sup>+</sup> 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<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup>, Mg<sup>++</sup>) 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<sup>-1</sup> at 0 ppt to 75.0 mg L<sup>-1</sup> at 35 ppt. We conclude that sublethal Zn exposure causes pathological changes in both Ca<sup>++</sup> and Na<sup>+</sup> homeostases, and that increasing salinity exerts protective effects against both sublethal and lethal Zn toxicities.

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### 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<sup>2+</sup>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<sup>-1</sup>, whereas 100% sea water contains about 10 mmol L<sup>-1</sup> Ca. Therefore one might predict that Zn toxicity would decrease as salinity increased, especially as the availability of free Zn<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> 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<sup>+</sup>, K<sup>+</sup> ATPase, Ca<sup>2+</sup> ATPase activities, drinking rates) and tissue-specific Zn bioaccumulation after 96-h exposures to an environmentally relevant sublethal Zn concentration (500 µg L<sup>-1</sup>; 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 LC50 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<sup>-1</sup>), 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., Secaucus, 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<sub>50</sub> values (mg L<sup>-1</sup>) 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<sub>50</sub> values at different salinities. Means sharing the same letter are not significantly different.

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

Procedures were approved by the Universidade Federal de 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<sub>3</sub>) 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<sup>-1</sup>; 3.5 ppt – 10, 20, 30, 40 and 50 mg/L<sup>-1</sup>; 10.5 ppt – 30, 40, 50, 60, 70 and 80 mg/L<sup>-1</sup>; 35 ppt – 70, 80, 90, 120, 140 and 160 mg/L<sup>-1</sup>. Stock solutions were made by dissolving Zn (as ZnSO<sub>4</sub>·7H<sub>2</sub>O; Sigma-Aldrich) in acidified Milli-Q water® and were added in appropriate 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<sub>50</sub> 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<sub>3</sub>. 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<sup>-1</sup> (as ZnSO<sub>4</sub>·7H<sub>2</sub>O; 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

later analysis. Gills, liver, and intestine were then dissected out, and these collected organs, together with the carcass, were blotted dry, weighed, and completely digested in 65% HNO<sub>3</sub> (Suprapur®, Merck, Haar, Germany) at 60 °C for 2 days in sealed vials. Digested samples were centrifuged (10,000 ×g, 10 min) and the supernatant was collected to measure Zn and ions.

#### 2.4. Drinking rate measurements

In a separate series, drinking rates were measured according to methodology described previously (Scott et al., 2006). After acclimation, killifish (N = 6 at each salinity) were exposed to either control conditions or 500 µg Zn L<sup>-1</sup> for 96 h at the four different salinities, exactly as described above (Section 2.2). At the end of 4 days, fish were moved to static polyethylene chambers containing 200 mL of the appropriate water (control or 500 µg Zn L<sup>-1</sup>) and allowed to settle for 2 h. At the start of each measurement period 8 µCi (0.29 MBq) of radiolabeled polyethylene glycol MW 4000 ([<sup>3</sup>H]PEG-4000, NEN Life Science Products Inc., Boston, MA, USA), a drinking rate marker which is negligibly absorbed through the digestive tract, was added to the chamber. Water samples (5 mL) were taken at 0, 3 and 6 h for radioactivity measurements. After 6 h, fish were killed with MS-222, rinsed in clean water, and weighed. The gastrointestinal tract was exposed and ligated at both ends (anterior esophagus and rectum). The entire gastrointestinal tract was removed, weighed, digested in HNO<sub>3</sub>, and then centrifuged, as for tissue samples in Section 2.3, before measurement of [<sup>3</sup>H]PEG-4000 radioactivity in the supernatant. Drinking rate was expressed as the volume ingested (from radioactivity counts of the tract digest and the water samples), relative to body mass and [<sup>3</sup>H]PEG-4000 exposure time.

#### 2.5. Na<sup>+</sup>, K<sup>+</sup> ATPase and Ca<sup>2+</sup> ATPase activities

A separate series focused on the effects of salinity and Zn exposure on the activities of these two transport enzymes in gill and intestine. Again, killifish were acclimated to the various salinities (N = 6 at each salinity) and then exposed to either control conditions or 500 µg Zn L<sup>-1</sup> for 96 h exactly as described above (Section 2.3). Gill and intestine Na<sup>+</sup>, K<sup>+</sup> ATPase activities were assayed using a modification of the method described by Bianchini and Castilho (1991). Na<sup>+</sup>, K<sup>+</sup> ATPase activity was determined as the difference between phosphate liberated from ATP in the presence of K<sup>+</sup> (medium A) and in the absence of K<sup>+</sup> with 1 mM of ouabain (medium B). The gill and intestine homogenate was prepared according to Loro et al. (2012). For each assay, 20 µL of the homogenate fraction was added and mixed to 2.0 mL of assay media containing the following final concentrations. The medium A was: 77 mM NaCl, 19 mM KCl, 6 mM MgCl<sub>2</sub>, 3 mM ATP, and buffer Tris–HCl 0.1 M at pH 7.6. The medium B was: 96 mM NaCl, 6 mM MgCl<sub>2</sub>, 3 mM ATP, 1 mM ouabain, and buffer Tris–HCl 0.1 M at pH 7.6. The reaction started with the addition of the homogenate and was incubated at 30 °C for 30 min. The reaction was stopped by adding 0.2 mL of trichloroacetic acid (20%) to the reaction medium. Phosphate concentration in the reaction medium was determined using a modification of the assay described in Ames (1966) by reacting 0.5 mL of the sample with 1 mL of a 1:6 (v/v) mixture of 10% ascorbic acid and 0.42% of ammonium molybdate in 0.5 mM H<sub>2</sub>SO<sub>4</sub> at 30 °C for 30 min, followed by measurement of absorbance at 620 nm. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as the standard. Ca<sup>2+</sup> ATPase activity was measured in the same homogenate fraction of gill and intestine by the method of Vajreswari et al. (1983) in a medium containing 80 mM NaCl, 5 mM MgCl<sub>2</sub>, 3 mM ATP, 20 mM Tris–HCl (pH 7.4), 0.5 mM CaCl<sub>2</sub> and 1 mM of ouabain in a final volume of 0.5 mL. The reaction was stopped by adding 0.2 mL of trichloroacetic acid (20%) to the reaction medium. Inorganic phosphate production and protein content were measured as for Na<sup>+</sup>, K<sup>+</sup> ATPase activity.

#### 2.6. Analytical techniques

Concentrations of Zn and major cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>) in water samples, plasma, and tissue digest supernatants were analyzed on a Varian FS220 flame atomic absorption spectrophotometer (Varian, Mulgrave, Victoria, Australia), using certified standards (Fisher Scientific, Toronto, ON, Canada). In saline waters, Zn measurements were performed using the lanthanum oxide and Na<sub>2</sub>CO<sub>3</sub> precipitation method of Toyota et al. (1982). Cl<sup>-</sup> concentrations in higher salinities (10.5 and 35 ppt) were measured with a CMT10 chloridometer (Radiometer, Copenhagen, Denmark) and for salinities of 0 and 3.5 ppt were determined by the colorimetric technique of Zall et al. (1956). SO<sub>4</sub> concentrations were determined by a turbidometric method as described by Cabrera et al. (2006).

For [<sup>3</sup>H]PEG-4000 analyses in the drinking rate experiments (Section 2.4), 1 mL gut tissue supernatant was added to 5 mL Ultima Gold AB scintillation fluid (Perkin-Elmer, Waltham, MA, USA), and 5 mL water was added to 5 mL Opti-phase scintillation fluid (Perkin-Elmer). [<sup>3</sup>H]-PEG-4000 radioactivity was determined using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer). Quench curves constructed from tissue digests were used to correct tissue radioactivity measurements to the same counting efficiency as water samples by the external standard ratio method.

#### 2.7. Statistical analyses

Data have been expressed as mean ± standard error of mean (N). All data were tested and exhibited normal distribution. Comparisons among mean values were performed using one-way analysis of variance (ANOVA) followed by the Fisher's LSD test to verify differences among control groups at different salinities, and among Zn exposure groups at different salinities. Differences between Zn-exposed and control animals at the same salinity were assessed using Student's two-tailed unpaired *t*-test. In all cases, the significance level adopted was 95% (α = 0.05). In the acute toxicity tests (Section 2.2), the trimmed Spearman–Karber test was used to calculate Zn concentrations causing mortality of 50% of the killifish (96-h LC50) and their corresponding 95% confidence intervals (C.I.), in each of the different salinities.

### 3. Results

#### 3.1. Acute Zn toxicity

Salinity exerted a marked protective effect against acute Zn toxicity in adult killifish (Table 1). The acute concentration (96-h LC50) of dissolved Zn increased 8-fold from 9.8 mg L<sup>-1</sup> (95% C.I. = 6.0–2.0) to 75.0 mg L<sup>-1</sup> (95% C.I. = 57.5–93.0) as salinity increased from 0 to 35 ppt. These effects were consistent, regardless of whether exposure values were expressed as nominal, total, or dissolved Zn concentrations (Table 1).

#### 3.2. Water chemistry in sublethal Zn exposures

Total and dissolved Zn concentrations were evaluated every day for all experiments. Zn concentrations were always close to the nominal value of 500 µg Zn L<sup>-1</sup> at all salinities tested and dissolved concentrations were only marginally below total concentrations (Table 2). The measured Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cl<sup>-</sup>, and SO<sub>4</sub> concentrations differed significantly among the four salinities, with the exception of total Zn and dissolved Zn which showed only minor variation (Table 2). Alkalinity, pH, and DOC concentrations were measured in a parallel study (Loro et al., 2012). Alkalinity (68, 78, 108, and 130 mg L<sup>-1</sup> as CaCO<sub>3</sub> at 0, 3.5, 10.5, and 35 ppt) and pH values (7.61, 7.85, 8.10, and 8.35 respectively) increased with salinity, whereas DOC concentrations did not (2.48, 2.55, 2.65, and 2.75 mg C L<sup>-1</sup> respectively). The differences in water

**Table 2**

Exposure water concentrations of Zn and major ions at different salinities (ppt). Data are expressed as means  $\pm$  SEM. Letters indicate significant differences ( $P \leq 0.05$ ) among salinities. Means which share the same letter are not significantly different.

Parameter	Salinity	Salinity	Salinity	Salinity
	0	3.5	10.5	35 ppt
Total Zn ( $\mu\text{g L}^{-1}$ )	497 $\pm$ 1.2 <sup>b</sup>	486 $\pm$ 1.5 <sup>a</sup>	500 $\pm$ 0.8 <sup>b</sup>	485 $\pm$ 1.7 <sup>a</sup>
Dissolved Zn ( $\mu\text{g L}^{-1}$ )	486 $\pm$ 0.9 <sup>b</sup>	476 $\pm$ 1.3 <sup>a</sup>	490 $\pm$ 1.5 <sup>b</sup>	475 $\pm$ .6 <sup>a</sup>
Na (mmol L <sup>-1</sup> )	0.86 $\pm$ 0.04 <sup>a</sup>	85 $\pm$ 2.0 <sup>b</sup>	192 $\pm$ 4.5 <sup>c</sup>	495 $\pm$ 9.0 <sup>d</sup>
Cl (mmol L <sup>-1</sup> )	0.98 $\pm$ 0.2 <sup>a</sup>	52 $\pm$ 2.5 <sup>b</sup>	155 $\pm$ 9.5 <sup>c</sup>	532 $\pm$ 11 <sup>d</sup>
K (mmol L <sup>-1</sup> )	0.06 $\pm$ .01 <sup>a</sup>	0.94 $\pm$ 0.15 <sup>b</sup>	3.1 $\pm$ 0.60 <sup>c</sup>	10.50 $\pm$ 1.6 <sup>d</sup>
Ca (mmol L <sup>-1</sup> )	0.47 $\pm$ 0.01 <sup>a</sup>	2.2 $\pm$ 0.15 <sup>b</sup>	4.63 $\pm$ .20 <sup>c</sup>	12.8 $\pm$ 0.95 <sup>d</sup>
Mg (mmol L <sup>-1</sup> )	0.37 $\pm$ 0.01 <sup>a</sup>	6.03 $\pm$ 0.45 <sup>b</sup>	17 $\pm$ 1.20 <sup>c</sup>	54.7 $\pm$ 2.60 <sup>d</sup>
SO <sub>4</sub> (mmol L <sup>-1</sup> )	0.00 $\pm$ 0.00 <sup>a</sup>	7.30 $\pm$ 0.50 <sup>b</sup>	12.50 $\pm$ 0.90 <sup>c</sup>	25.5 $\pm$ 0.95 <sup>d</sup>

chemistry caused differences in Zn speciation among salinities which have been reported previously (see Table 1 of Loro et al., 2012). Most notably, free Zn<sup>2+</sup>, the dominant form at 0 ppt, decreased markedly as salinity increased, being replaced by hydroxide, carbonate, and chloride complexes.

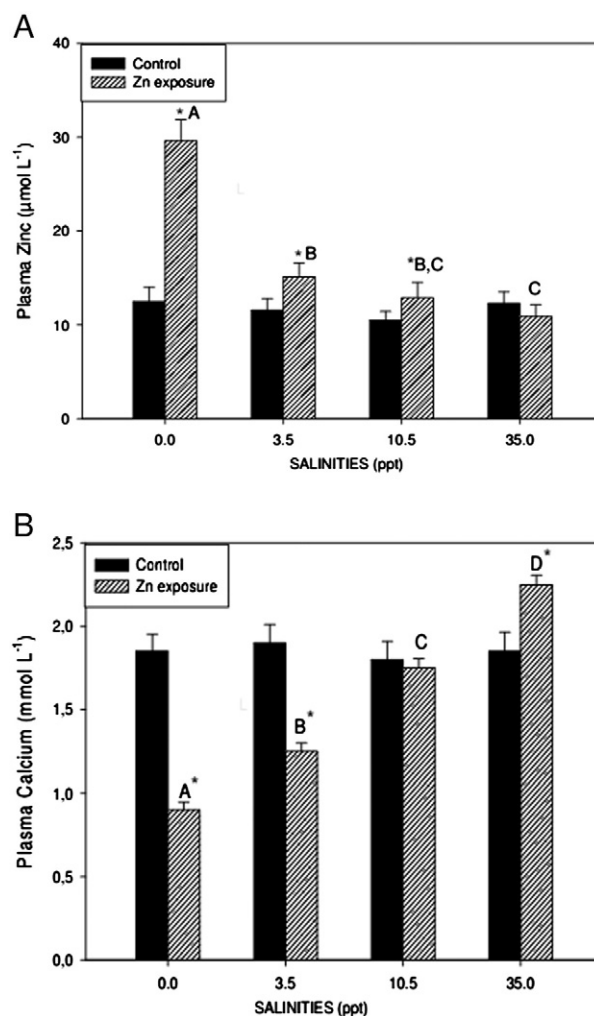
### 3.3. Plasma Zn, Ca, and Na concentrations in sublethal Zn exposures

Plasma Zn concentrations were unaffected by salinity in control animals, averaging about 12  $\mu\text{mol L}^{-1}$  (Fig. 1A). When killifish were exposed to waterborne Zn (500  $\mu\text{g L}^{-1}$  ~ 8  $\mu\text{mol L}^{-1}$ ) for 96 h, plasma Zn concentration more than doubled to about 30  $\mu\text{mol L}^{-1}$  in the 0 ppt exposure group. This effect was progressively attenuated at 3.5 and 10.5 ppt, and was eliminated at 35 ppt.

Plasma Ca<sup>2+</sup> concentrations were similarly unaffected by salinity in control animals (Fig. 1B), averaging about 1.8 mmol L<sup>-1</sup>, approximately 150-fold greater than plasma Zn<sup>2+</sup> levels. Zn exposure caused a very pronounced drop in plasma Ca by almost 1 mmol L<sup>-1</sup> (i.e. 50%) at 0 ppt. This decrease was slightly less at 3.5 ppt (30%), disappeared at 10.5 ppt, and transitioned to a 0.4 mmol L<sup>-1</sup> increase in plasma Ca<sup>2+</sup> concentration at 35 ppt. Because sample volumes were limited, Na<sup>+</sup> was the only other ion measured in blood plasma. Plasma Na<sup>+</sup> concentrations were not affected by salinity in control animals. However, at 0 ppt, plasma Na<sup>+</sup> exhibited a highly significant 30% reduction after 96 h of Zn<sup>2+</sup> exposure, an effect which did not occur at the other salinities (Table 3).

### 3.4. Tissue Zn, Ca, Na, K, and Mg concentrations in sublethal Zn exposures

Zn<sup>2+</sup> concentrations in the various tissues sampled were between 0.8 and 1.4  $\mu\text{mol g}^{-1}$  wet mass under control conditions (Fig. 2). There were some minor variations among tissues (carcass levels tended to be higher than other tissues) and a few small differences associated with salinity in the control treatments. However, when killifish were exposed to 500  $\mu\text{g Zn L}^{-1}$  in fresh water (i.e. 0 ppt) for 96 h, Zn concentrations increased by 40%–80% in all four tissues. In all cases, this bioaccumulation was abolished at salinities of 3.5, 10.5, and 35 ppt. Tissue Ca<sup>2+</sup> concentrations were more than 100-fold greater than tissue Zn concentrations and showed much greater variation among tissues, with gill and carcass levels being approximately 5-fold higher than liver and 10-fold higher than intestine. This likely reflected the presence of bone, cartilage, and/or scales in gill and carcass samples (Fig. 3). Ca concentrations were significantly reduced by about 35% in gills of killifish exposed to Zn at 0 ppt. However, at salinities ranging from 3.5 to 35 ppt, Zn exposure caused significant increases of gill Ca concentration by about 30% (Fig. 3). Zn exposure did not significantly alter Ca concentrations in the carcass, liver or intestine at any salinity, with all three tissues showing the same trend as the controls with increasing salinity (Fig. 3).



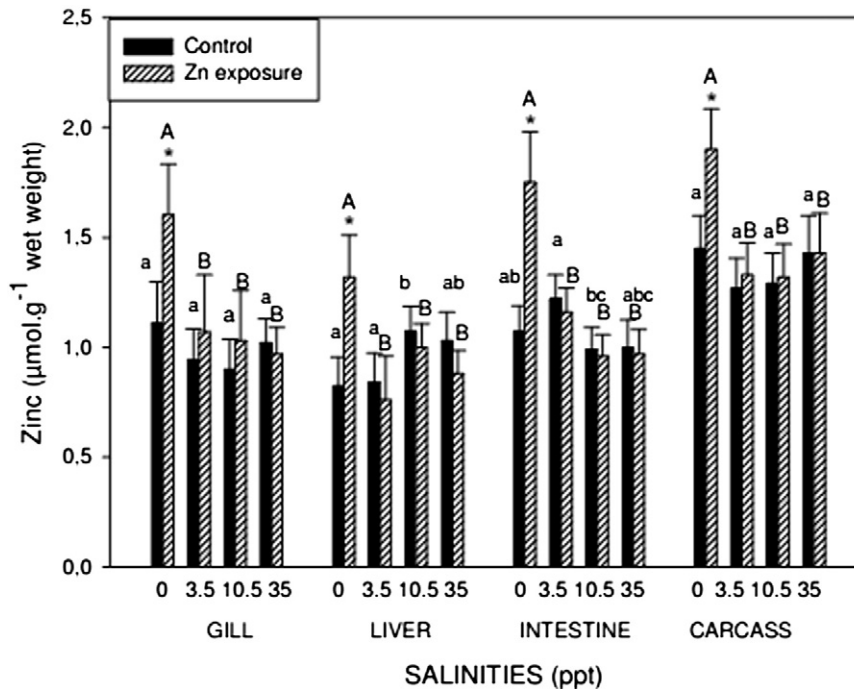
**Fig. 1.** (A) Plasma zinc ( $\mu\text{mol L}^{-1}$ ) and (B) plasma calcium ( $\text{mmol L}^{-1}$ ) in *F. heteroclitus* exposed to 500  $\mu\text{g Zn L}^{-1}$  at different salinities for 96 h. Means  $\pm$  1 SEM. Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.

Na<sup>+</sup> concentrations of the gill tissue in control animals were independent of salinity in the 0–10.5 ppt range, but increased significantly at 35 ppt (Table 3). After 96 h of Zn exposure, gill tissue Na<sup>+</sup> increased significantly at all salinities in the 3.5–35 ppt range, but remained unchanged at 0 ppt. In controls, liver Na<sup>+</sup> concentrations were significantly lower at 0 ppt than at the three higher salinities. Zn exposure substantially increased liver Na<sup>+</sup> only at 3.5 and 10.5 ppt. Na<sup>+</sup> levels of the intestinal tissue in control animals decreased at the highest salinity, an opposite trend to that seen at the gills and liver. Furthermore, Zn exposure raised intestinal Na<sup>+</sup> concentration at 0 ppt, but lowered it at 3.5 and 10 ppt, again different from the trends seen in the gills and liver. In the carcass, Na<sup>+</sup> concentration exhibited some inconsistent variations with salinity, but Zn<sup>2+</sup> exposure consistently lowered carcass Na<sup>+</sup> levels at salinities of 0 to 10.5 ppt (Table 3).

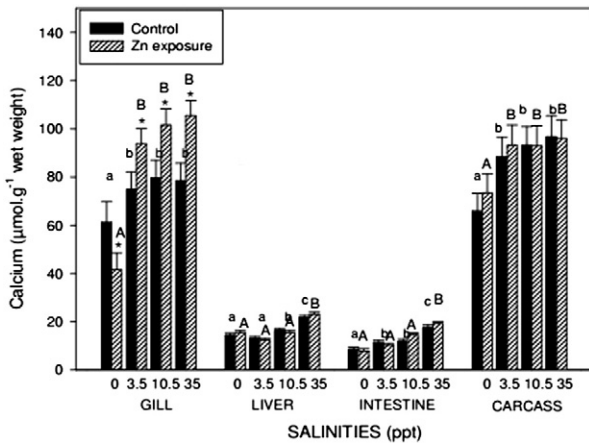
In controls, tissue K<sup>+</sup> concentrations did not change consistently with salinity in gills or intestine, but tended to be greater at higher salinities in liver and carcass (Table 3). Tissue K<sup>+</sup> levels were generally unresponsive to Zn<sup>2+</sup> exposure but there were a few significant increases – at 0 ppt in gills, at 3.5 and 35 ppt in intestine, and at 3.5 and 10.5 ppt in carcass. Tissue Mg<sup>2+</sup> concentrations tended to increase with salinity in gills, intestine, and carcass, but there was no consistent pattern in liver. Zn<sup>2+</sup> exposure caused some minor but significant variations in tissue

**Table 3**  
Concentrations of cations ( $\mu\text{mol g}^{-1}$  wet mass) in different tissues of killifish exposed to  $500 \mu\text{g Zn L}^{-1}$  for 96 h at different salinities. Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.

Na <sup>+</sup> Levels									
Gill		Liver		Intestine		Carcass			
Sal (ppt)	Control	Zn	Control	Zn	Control	Zn	Control	Zn	
0	139 ± 5 <sup>a</sup>	146 ± 7 <sup>A</sup>	88 ± 3 <sup>a</sup>	93 ± 8 <sup>A</sup>	78 ± 0.8 <sup>b</sup>	89 ± 0.9 <sup>C*</sup>	89 ± 0.8 <sup>ab</sup>	78 ± 0.6 <sup>A*</sup>	
3.5	125 ± 4.5 <sup>a</sup>	149 ± 6 <sup>A*</sup>	115 ± 4 <sup>bc</sup>	175 ± 6 <sup>B*</sup>	82 ± 0.9 <sup>b</sup>	69 ± 0.8 <sup>B*</sup>	93 ± 0.9 <sup>b</sup>	85 ± 0.6 <sup>B*</sup>	
10.5	134 ± 4 <sup>a</sup>	156 ± 5 <sup>A*</sup>	125 ± 6 <sup>c</sup>	180 ± 5 <sup>B*</sup>	78 ± 1.4 <sup>b</sup>	69 ± 1.3 <sup>B*</sup>	99 ± 0.9 <sup>c</sup>	91 ± 0.7 <sup>C*</sup>	
35	168 ± 4.5 <sup>b</sup>	207 ± 6 <sup>B*</sup>	106 ± 6 <sup>b</sup>	101 ± 4 <sup>A</sup>	67 ± 0.7 <sup>a</sup>	64 ± 1.2 <sup>A</sup>	86 ± 1.1 <sup>a</sup>	90 ± 0.8 <sup>C</sup>	
K <sup>+</sup> Levels									
Gill		Liver		Intestine		Carcass			
Sal (ppt)	Control	Zn	Control	Zn	Control	Zn	Control	Zn	
0	74 ± 1.6 <sup>ab</sup>	81 ± 1.5 <sup>B*</sup>	99 ± 5 <sup>a</sup>	95 ± 8 <sup>A</sup>	81 ± 1.8 <sup>b</sup>	79 ± 1.5 <sup>A</sup>	82 ± 0.8 <sup>b</sup>	85 ± 1.2 <sup>B</sup>	
3.5	76 ± 0.9 <sup>b</sup>	80 ± 1.6 <sup>B</sup>	135 ± 6 <sup>b</sup>	135 ± 6 <sup>B</sup>	81 ± 0.9 <sup>b</sup>	104 ± 1.4 <sup>B*</sup>	70 ± 0.9 <sup>a</sup>	75 ± 0.9 <sup>A*</sup>	
10.5	75 ± 1.1 <sup>ab</sup>	78 ± 1.1 <sup>AB</sup>	109 ± 7 <sup>a</sup>	112 ± 8 <sup>AB</sup>	76 ± 0.8 <sup>a</sup>	79 ± 1.4 <sup>A</sup>	89 ± 1.2 <sup>c</sup>	97 ± 1.1 <sup>C*</sup>	
35	72 ± 0.9 <sup>a</sup>	74 ± 1.1 <sup>A</sup>	102 ± 6 <sup>a</sup>	98 ± 9 <sup>A</sup>	80 ± 1.1 <sup>ab</sup>	85 ± 1.2 <sup>A*</sup>	94 ± 1.5 <sup>c</sup>	90 ± 1.3 <sup>B</sup>	
Mg <sup>+</sup> Levels									
Gill		Liver		Intestine		Carcass			
Sal (ppt)	Control	Zn	Control	Zn	Control	Zn	Control	Zn	
0	68 ± 1.1 <sup>b</sup>	65 ± 0.8 <sup>B</sup>	32 ± 0.4 <sup>c</sup>	30 ± 0.5 <sup>B*</sup>	22 ± 0.3 <sup>a</sup>	25 ± 0.4 <sup>B*</sup>	67 ± 1.1 <sup>a</sup>	66 ± 1.1 <sup>A</sup>	
3.5	56 ± 0.6 <sup>a</sup>	50 ± 0.8 <sup>A*</sup>	29 ± 0.3 <sup>b</sup>	26 ± 0.6 <sup>A*</sup>	21 ± 0.3 <sup>a</sup>	23 ± 0.3 <sup>A*</sup>	65 ± 0.9 <sup>a</sup>	62 ± 0.9 <sup>A*</sup>	
10.5	79 ± 0.8 <sup>c</sup>	72 ± 1.1 <sup>C*</sup>	25 ± 0.4 <sup>a</sup>	27 ± 0.5 <sup>A*</sup>	26 ± 0.3 <sup>b</sup>	26 ± 1.3 <sup>BC</sup>	78 ± 0.8 <sup>b</sup>	75 ± 0.9 <sup>B*</sup>	
35	81 ± 1.8 <sup>c</sup>	79 ± 1.5 <sup>D</sup>	35 ± 0.5 <sup>d</sup>	36 ± 0.6 <sup>C</sup>	26 ± 0.2 <sup>b</sup>	27 ± 0.3 <sup>C*</sup>	90 ± 1.1 <sup>c</sup>	93 ± 1.2 <sup>C</sup>	
Plasma Na <sup>+</sup> Levels									
Sal (ppt)	Control				Zn				
0	115 ± 8.5 <sup>a</sup>				80 ± 5.5 <sup>A*</sup>				
3.5	115 ± 9 <sup>a</sup>				119 ± 11 <sup>B</sup>				
10.5	114 ± 8 <sup>a</sup>				116 ± 9 <sup>B</sup>				
35	118 ± 9 <sup>a</sup>				115 ± 8 <sup>B</sup>				



**Fig. 2.** Zinc concentrations ( $\mu\text{mol g}^{-1}$  wet mass) in tissues of *F. heteroclitus* exposed to  $500 \mu\text{g Zn L}^{-1}$  at different salinities for 96 h. Means  $\pm$  1 SEM ( $n = 6$ ). Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.



**Fig. 3.** Calcium concentrations ( $\mu\text{mol g}^{-1}$  wet mass) in tissues of *F. heteroclitus* exposed to  $500 \mu\text{g Zn L}^{-1}$  at different salinities for 96 h. Means  $\pm$  1 SEM ( $n = 6$ ). Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.

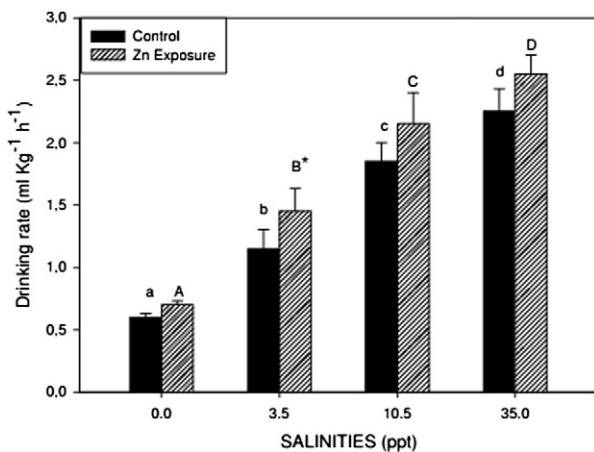
$\text{Mg}^{2+}$ , but the overall changes were small, and variable in direction among tissues and salinities (Table 3).

### 3.5. Drinking rate in sublethal Zn exposures

In control animals, drinking rate increased progressively from about  $0.6$  to  $2.3 \text{ mL kg}^{-1} \text{ h}^{-1}$  as salinity increased from 0 to 35 ppt (Fig. 4). Drinking rates tended to be slightly higher in Zn-exposed fish, but the only significant elevation was at 3.5 ppt (Fig. 4).

### 3.6. $\text{Na}^+, \text{K}^+$ ATPase and $\text{Ca}^{2+}$ ATPase activities in sublethal Zn exposures

In control fish,  $\text{Na}^+, \text{K}^+$  ATPase activities were higher in the gill than in the intestine, but did not vary significantly with salinity in either tissue (Fig. 5). Branchial  $\text{Na}^+, \text{K}^+$  ATPase activity was reduced by almost 50% by 96 h of Zn exposure at 0 ppt. This effect was eliminated at 3.5 ppt, and changed over to significant stimulations of about 30% and



**Fig. 4.** Drinking rate of *F. heteroclitus* acclimated to different salinities and exposed to  $500 \mu\text{g Zn L}^{-1}$  for 96 h. Means  $\pm$  1 SEM ( $n = 6$ ). Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.

90% at 10.5 and 35 ppt, respectively.  $\text{Zn}^{2+}$ -exposure also reduced intestinal  $\text{Na}^+, \text{K}^+$  ATPase activity by about 30% at 0 ppt, but no effects were recorded at the higher salinities (Fig. 5).

As for  $\text{Na}^+, \text{K}^+$  ATPase activity,  $\text{Ca}^{2+}$  ATPase activity was also higher in the gills than in the intestine, and was unaffected by salinity in either tissue under control conditions (Fig. 6). Zn exposure reduced branchial  $\text{Ca}^{2+}$  ATPase activity by about 20% at 0 ppt, and stimulated it by about 20% at 35 ppt. There were no effects of Zn at intermediate salinities. An almost identical pattern was seen in the intestine (reduction at 0 ppt, elevation at 35 ppt with constancy at intermediate salinities), but the changes were somewhat smaller (Fig. 6).

## 4. Discussion

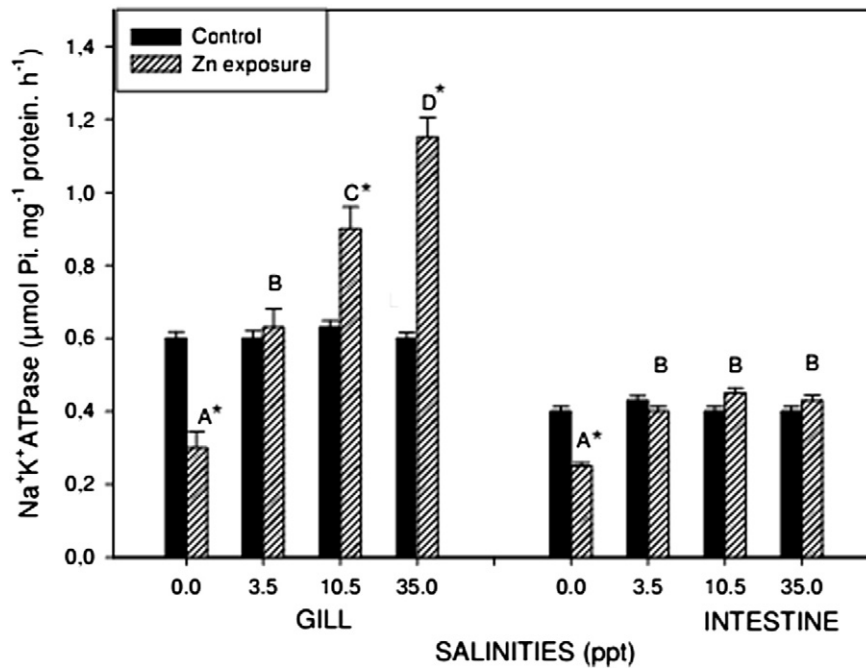
### 4.1. Acute Zn toxicity

Our first hypothesis, that acute Zn toxicity in adult killifish would decrease with increasing salinity was strongly supported (Table 2). Fig. 7 illustrates that this was the same general trend as reported for larval killifish (7–8 days old) by Bielmyer et al. (2012), but the adult animals of the present study were clearly far more resistant. This trend of greater toxicity in early life stages has been well-established for many metals, and at least in part reflects the inverse relationship between toxicity and body size for toxicants which act by causing ionoregulatory dysfunction (Bianchini et al., 2002; Grosell et al., 2002). Interestingly, Bielmyer et al. (2012) demonstrated that up to about this same threshold, the protective effect of increasing salinity could largely be explained by the greater Ca concentration of the water, while the present physiological data on sublethal Zn toxicity point to hypocalcemia as a key toxic mechanism in this same low salinity range, in agreement with much previous literature (reviewed by Hogstrand, 2012). While undoubtedly less effective than Ca, the greater concentrations of other cations (Na, Mg, K) at higher salinities also likely offer protection by competing with Zn for binding sites at the gills (Alsop and Wood, 1999). Furthermore, changes in speciation occur with increasing salinity (see Table 2 in Loro et al., 2012) as free  $\text{Zn}^{2+}$  is replaced by hydroxide, carbonate, and chloride complexes which likely reduce the ability of Zn to interact with gill sites (Santore et al., 2002).

The demonstration in both the present study and that of Bielmyer et al. (2012) that increasing salinity greatly protects against acute Zn toxicity (Fig. 7) runs counter to tabulations which show generally similar LC50 values between fresh and sea water (USEPA, 1987; Eisler, 1993). The likely explanation is that the comparisons in these two studies on *Fundulus heteroclitus* are not confounded by differences in species or life stages.

### 4.2. Sublethal Zn exposure concentration

A recent summary of regulatory guidelines for waterborne Zn revealed levels of  $4\text{--}216 \mu\text{g L}^{-1}$  in various jurisdictions (Hogstrand, 2012). The concentration of Zn ( $500 \mu\text{g L}^{-1}$ ) used in our exposures was above this range but nevertheless environmentally relevant based on measured levels found at contaminated sites (Eisler, 1993; Hogstrand, 2012). Clearly, this exposure level was sublethal, representing only 5% of the measured 96-h LC50 value at 0 ppt, and much less at higher salinities (Table 2), and no deaths occurred in our experiments. Nevertheless,  $500 \mu\text{g Zn L}^{-1}$  was sufficient to provoke substantial disturbances in internal physiology which provided valuable diagnostic information on the toxic mechanisms at different salinities (see Section 4.4). In particular, these findings supported our second original hypothesis that Zn-induced disturbances in ionoregulatory parameters, especially those involving  $\text{Ca}^{2+}$  homeostasis, would progressively decrease as salinity increased. However the discovery of several reversed ionoregulatory effects at high salinities was unexpected (see Section 4.4).

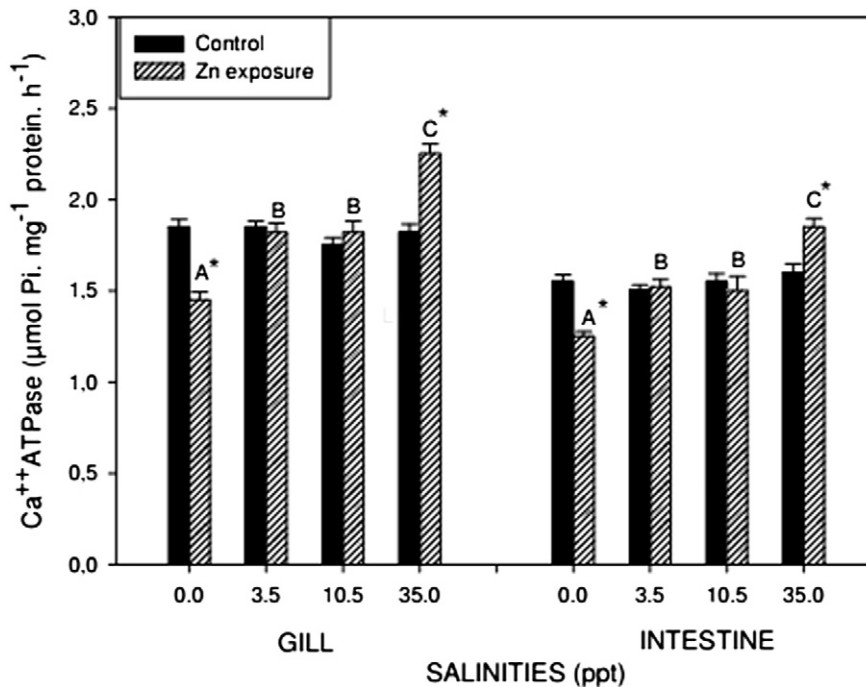


**Fig. 5.** Na<sup>+</sup>K<sup>+</sup> ATPase activity (μmol Pi mg<sup>-1</sup> protein h<sup>-1</sup>) in gill and intestine of *F. heteroclitus* exposed to 500 μg Zn L<sup>-1</sup> at different salinities for 96 h. Means ± 1 SEM (N = 6). Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.

#### 4.3. Zn bioaccumulation during sublethal Zn exposures

While there was clear evidence of net Zn accumulation in plasma (Fig. 1A) as well as in gill, liver, intestine, and carcass (Fig. 2) at 0 ppt, this was greatly attenuated (Fig. 1A) or prevented (Fig. 2) at higher salinities. In general, these Zn accumulation data agree well with the

recent findings of Shyn et al. (2012) on adult *Fundulus heteroclitus* in fresh water versus full strength sea water. These authors reported that within this Zn concentration range, significant Zn accumulation occurred only in freshwater animals, and that it was necessary to increase Zn exposure levels to 1000 μg L<sup>-1</sup> in order to see significant Zn accumulation in seawater animals (and only in intestine and liver). Clearly,



**Fig. 6.** Ca<sup>2+</sup> ATPase activity (μmol Pi mg<sup>-1</sup> protein h<sup>-1</sup>) in gill and intestine of *F. heteroclitus* exposed to 500 μg Zn L<sup>-1</sup> at different salinities for 96 h. Capital letters indicate significant differences ( $P \leq 0.05$ ) among the Zn-exposed groups at different salinities. Lower case letters indicate significant differences ( $P \leq 0.05$ ) among the control groups at different salinities. Means sharing the same letters are not significantly different. Asterisks indicate significant differences ( $P \leq 0.05$ ) between the Zn-exposed group and the corresponding control group at the same salinity.

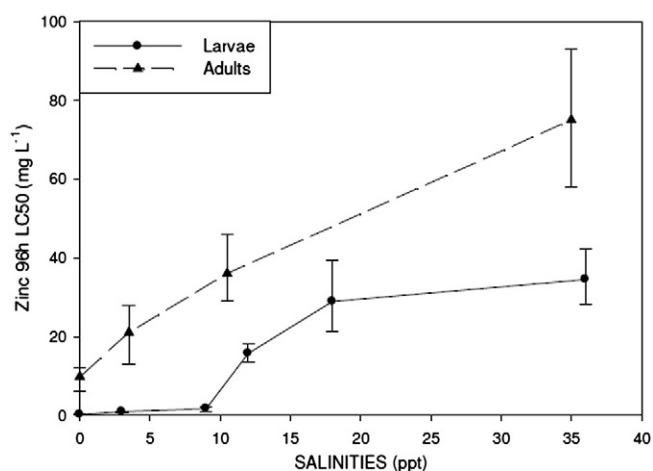


Fig. 7. A comparison of 96-h LC50 values as a function of salinity in *F. heteroclitus* between larvae (7–8 days old; data from Bielmyer et al., 2012) and adults (data from present study). Means  $\pm$  95% C.I.

increasing salinity greatly decreased the bioavailability of Zn for bioaccumulation, and the threshold for this protective effect was as low as 3.5 ppt (10% sea water; Figs. 1A, 2). This conclusion was in accord with the first part of our third original hypothesis.

However, the other part of this third hypothesis was not supported. We had predicted that a marked change in the tissue-specific distribution of Zn would be seen at higher salinities due to greater importance of Zn uptake via the intestinal tract as a result of increased drinking. This did not occur. Certainly, a higher drinking rate was recorded at higher salinities, though it was only marginally affected by Zn exposure (Fig. 4), and the 0 and 35 ppt values were in agreement with previous reports for freshwater and seawater killifish (Scott et al., 2006, 2008). This internal distribution shift (greater metal accumulation in the gut tissue at higher salinity) has been seen for silver (Ag) distribution in toadfish (Wood et al., 2004) and for both Cd and Zn distribution in black sea bream (Zhang and Wang, 2007). However, these studies used radiolabeled metals and did not assess total gut tissue metal burdens. Preferential accumulation of total Zn in the intestinal tissue at higher salinity did not occur in the present study (Fig. 2), presumably because the Zn exposure level was not high enough and/or because of homeostatic mechanisms that prevented net (as opposed to radiolabeled) Zn accumulation. As an essential element, Zn is well regulated by most organisms, including fish (Hogstrand, 2012).

At 0 ppt, where plasma and tissue Zn levels increased markedly, it is possible that internal Zn accumulation contributed to toxicity, but not at higher salinities where there was little or no Zn buildup (Figs. 1A, 2). Essential metals are generally well-tolerated by tissues, so resulting damage would likely be indirect, such as greater oxidative stress due to ROS production promoted by Zn, as seen in parallel exposures (Loro et al., 2012). The greater significance of internal Zn bioaccumulation is that it is evidence for a more intense and effective competition of Zn with Ca for branchial uptake mechanisms at 0 ppt.

#### 4.4. Mechanisms of toxicity during sublethal Zn exposures

As outlined in the Introduction, previous studies have indicated that the toxic mechanism of waterborne Zn exposure in fresh water is competitive blockade of active Ca uptake at the gills, resulting in hypocalcemia in the plasma (Hogstrand, 2012). This appears to reflect both competition by Zn with Ca for an apical calcium channel and inhibition of a basolateral Ca<sup>2+</sup> ATPase in the gill ionocytes (e.g. Spry and Wood, 1985, 1989; Hogstrand et al., 1994, 1996). The present findings of plasma hypocalcemia (Fig. 1B), internal Zn accumulation (Figs. 1A, 2), and decreased branchial Ca<sup>2+</sup> ATPase activity in Zn-exposed killifish at 0 ppt are all in accord with this explanation. However, it is notable

that branchial Na<sup>+</sup>,K<sup>+</sup> ATPase activity was also inhibited (Fig. 5), and that plasma Na level also decreased at 0 ppt (Table 2). This suggests that hyponatremia also contributes to Zn toxicity in freshwater killifish. A transitory loss of whole body Na<sup>+</sup> has been reported in at least one previous sublethal Zn exposure study, on rainbow trout (McGeer et al., 2000), as well as other indirect evidence of disturbances in Na<sup>+</sup> regulation (Spry and Wood, 1985; Heath, 1987). More studies will be required to determine whether Zn specifically inhibits Na uptake pathways and the basolateral Na<sup>+</sup>,K<sup>+</sup> ATPase enzyme in the gills of killifish, or whether these effects on Na<sup>+</sup> regulation are due to generalized gill damage, resulting loss of ionocytes, and increased diffusive permeability (Mallatt, 1985).

Both Ca<sup>2+</sup> ATPase (Fig. 6) and Na<sup>+</sup>,K<sup>+</sup> ATPase (Fig. 5) activities in the intestine were also reduced in Zn-exposed killifish at 0 ppt. As this occurred at the salinity where drinking rate was lowest (Fig. 4), it is difficult to attribute these effects to direct inhibitory actions of imbibed waterborne Zn. Perhaps the basolateral location of these enzymes rendered them susceptible to inhibition by the elevated plasma Zn (Fig. 1A) and tissue Zn levels (Fig. 2) which occurred in this exposure.

Further studies are needed to explore this possibility, and to determine whether inhibition of these intestinal transport enzymes contributed to the observed systemic hypocalcemia and hyponatremia. Most ionoregulatory effects seen at 0 ppt were attenuated or prevented at 3.5 ppt in accord with preceding theory, but it was surprising to see that some were actually reversed at the two higher salinities, particularly at 35 ppt. For the first time, this provides some insight into the toxic mechanisms of Zn in sea water. Recently, Hogstrand (2012) concluded: “It is fair to say that almost nothing is known about the mechanism of acute Zn toxicity to seawater fish”. Loro et al. (2012) clearly demonstrated that oxidative stress was minimal at higher salinities, and therefore unlikely to be involved. The present data suggest that hypercalcemia (Fig. 1B), in combination with elevated concentrations of Ca<sup>2+</sup> (Fig. 3) and Na<sup>+</sup> (Table 2) in gill tissue may be at least part of the toxic mechanism of Zn at higher salinities. However it is unclear how Zn exerts this action.

In contrast to freshwater fish, where the active uptake mechanisms for Ca<sup>2+</sup> have been well characterized, there is very little information at present available on Ca regulation in seawater teleosts (reviewed by Flik et al., 1995; Evans et al., 2005).

For example, it is unclear whether the active mechanisms for Ca<sup>2+</sup> uptake present in the ionocytes of the freshwater gill are completely turned off in seawater acclimated fish. Furthermore the transepithelial potential (TEP) across the gills is positive (inside) in seawater killifish (Wood and Grosell, 2009), and therefore should oppose Ca<sup>2+</sup> influx from the much higher Ca levels in the external water. However, it is unclear whether this TEP is sufficient to totally negate net influx, or whether an active Ca extrusion mechanism is also present. One possible interpretation of the current results is that Zn exposure inhibits active Ca excretion across seawater ionocytes (i.e. a reciprocal effect to the inhibition of active Ca uptake across freshwater ionocytes), and that the observed increases in branchial Ca<sup>2+</sup> ATPase and Na<sup>+</sup>,K<sup>+</sup> ATPase activities are compensatory responses to help restore active Ca<sup>2+</sup> excretion. Alternately, the Zn<sup>2+</sup> exposure may increase the diffusive permeability of the gills and/or lower the TEP so as to favor greater Ca<sup>2+</sup> uptake, again necessitating these compensatory active excretion mechanisms. A third possibility however is that these enzymes are inappropriately activated so as to promote inwardly directed Ca transport, while a fourth is that Zn somehow interferes with Ca<sup>2+</sup> regulation at another site(s) such as gut or kidney. There is an obvious need for future research on these possibilities.

#### 5. Concluding remarks

Regardless of which of these possibilities, if any, proves true, it is clear that excess Zn<sup>2+</sup> interferes with Ca<sup>2+</sup> regulation in both seawater and freshwater killifish, with resulting opposite effects at salinity



extremes, and gradations of these responses at intermediate salinities.  $Zn^{2+}$  and  $Ca^{2+}$  homeostasis are clearly interrelated, and  $Zn^{2+}$  may also interfere with  $Na^+$  regulation. These disturbances are pathological, and are likely proximate causes of toxicity. Overall however, increasing salinity exerts marked protective effects against both sublethal and lethal  $Zn^{2+}$  toxicity.

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