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Research article

The roles of calcium and salinity in protecting against physiological symptoms of waterborne zinc toxicity in the euryhaline killifish (*Fundulus heteroclitus*)

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ABSTRACT

In fresh water, environmental Ca ameliorates Zn toxicity because Ca^{2+} and Zn^{2+} compete for uptake at the gills. Zn toxicity is also lower in sea water, but it is unclear whether this is due to increased Ca^{2+} concentration, and/or to the other ions present at higher salinity. Using the euryhaline killifish, we evaluated the relative roles of Ca^{2+} (as $CaNO_3$) versus the other ions contributing to salinity in protecting against physiological symptoms of Zn^{2+} toxicity. Killifish were exposed to a sublethal level of Zn (500 μg/L, as $ZnSO_4$) for 96 h in either fresh water (0 % salinity) at low (1 mmol/L) and High Ca (10 mmol/L) or 35 ppt sea water (100 % salinity) at low (1 mmol/L) and High Ca (10 mmol/L). At 0 % salinity, High Ca partly or completely protected against the following effects of Zn seen at Low Ca: elevated plasma Zn, hypocalcaemia, inhibited unidirectional Ca^{2+} influx, inhibited branchial Na^+/K^+ATPase and Ca^{2+}ATPase activities, and oxidative stress in gills, liver, intestine, and muscle. At 100 % salinity, in the presence of 1 mmol/L (Low Ca), Zn caused no disturbances in most of these same parameters, showing that the "non-Ca" components of sea water alone provided complete protection. However, for a few endpoints (inhibited intestinal Ca^{2+}ATPase activity, oxidative stress in gill and liver), High Ca (10 mmol/L) was needed to provide full protection against Zn in 100 % salinity. There was no instance where the combination of 100 % salinity and High Ca failed to provide complete protection against Zn-induced disturbances in sea water.

1. Introduction

Zinc is an essential metal with important cellular functions, and is also the most abundant intracellular trace element (Maret, 2013). The catalytic role of Zn is essential for critical biological functions catalyzed by >300 enzymes (McCall et al., 2000; Lall and Kaushik, 2021). However, high levels of Zn in water can be extremely toxic to aquatic organisms such as fish (Hogstrand, 2011). There is now abundant evidence that the toxicity of most metals to fish decreases as salinity increases so that lethality at a given concentration is much greater in fresh water than in sea water (Hall and Anderson, 1995; Bielmyer et al., 2013; Bielmyer-Fraser et al., 2018; Blewett and Leonard, 2017). This is certainly true for acute Zn toxicity (Bielmyer et al., 2012; Loro et al., 2014; Park et al., 2014; Pérez-López et al., 2020; Horie and Takahashi, 2021). In part, the protective effect of salinity is explained by changes in Zn speciation, such that the relative concentration of the most toxic species, the free Zn^{2+} cation, decreases as salinity increases (Loro et al., 2014). However, it is clear from these and other studies (Zhang and Wang, 2007) that other factors must also be involved, such as differences in physiology and/or greater competition from cations at higher salinity. For example, much lower Zn bioaccumulation in seawater versus freshwater-acclimated specimens of the same species was more pronounced than could be explained by complexation alone (Shyn et al., 2012; Loro et al., 2014).

The euryhaline killifish (*Fundulus heteroclitus*) is a model teleost in both physiology and toxicology (e.g. Wood and Marshall, 1994; Burnett et al., 2007). Griffith (1974) has shown that *F. heteroclitus* is capable of acclimating to any salinity from 0 ppt (freshwater) to 120 ppt (almost 400 % seawater!). The killifish is native to the eastern coast of North America, living naturally in coastal freshwater streams, brackish water

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estuaries, full strength seawater, and hypersaline tidal flats and lagoons, though spawning generally occurs in the intertidal zone (brackish water or seawater). This species has contributed to the above findings on salinity-dependent Zn toxicity (Bielmyer et al., 2012; Shyn et al., 2012). In two previous studies (Loro et al., 2012; Loro et al., 2014), we have documented some of the physiology behind this salinity-dependent toxicity in *F. heteroclitus*. Loro et al. (2012) demonstrated that 100 % salinity, which completely protected against a rise in plasma Zn concentration (Loro et al., 2014), also greatly protected against the internal oxidative stress caused by a 96-h exposure to a sublethal concentration of Zn (500 μg/L, <5 % of LC50). Interestingly, at intermediate salinities between freshwater and 100 % seawater, oxidative stress was observed in internal organs, as well as small but significant increases in plasma Zn levels. This suggests that oxidative stress in internal organs is a response to elevated plasma Zn concentration. Loro et al. (2014) demonstrated that 100 % sea water protects not only against zinc bioaccumulation, but also against multiple indices of ionoregulatory disturbance, most importantly decreases in plasma calcium concentrations and Ca^{2+} ATPase activities. This fits well with the general consensus that the principal toxic effect, at least in freshwater fish (Spry and Wood, 1989; Hogstrand et al., 1994; Hogstrand et al., 1996), involves ionic mimicry (Bury et al., 2003) such that Zn^{2+} competes with Ca^{2+} for active uptake mechanisms in the gills (Hogstrand, 2011). The protective action could be explained by the fact that as a salinity increases, water calcium concentration also increases in parallel.

Against this backdrop, the current study continues our investigations of the physiological toxicity of waterborne Zn (500 μg/L) to *F. heteroclitus* in 100 % sea water versus fresh water, with a focus on oxidative stress, Zn bioaccumulation, Ca uptake rates, and ionoregulatory disturbance. Our goal was to separate the protective roles of Ca from those of all other normal components of 100 % seawater. For convenience, these components are referred to as "salinity" throughout this paper. Therefore, we independently manipulated salinity (0 % versus 100 %) and Ca concentration [1 mmol/L (typical freshwater level) versus 10 mmol/L (typical seawater level) as $CaNO_3$]. Our overall hypothesis was that Zn bioaccumulation, inhibition of Ca uptake, hypocalcemia, oxidative stress in the gills, and disturbances in gill ionoregulatory enzymes, which seem to be direct effects of external Zn exposure, would be governed primarily by the Ca concentration of the external medium, whereas internal responses (primarily oxidative stress in tissues other than gills) would be a function of plasma Zn levels.

2. Material and methods

2.1. Animals and acclimation

Adult killifish (*Fundulus heteroclitus*) of both sexes (weight: 3.6 ± 0.6 g; length: 4.3 ± 0.8 cm) were obtained from Aquatic Research Organisms (ARO) Ltd. (Hampton, New Hampshire, USA) and were maintained in 10 % SW (3.5 ppt) at 18–20 °C in the animal holding facilities at McMaster University, Hamilton, ON, Canada. Killifish were maintained in aquaria receiving recirculating water passing through charcoal filters. Depending on experimental design, fish were acclimated for 2 weeks to fresh water of 0 % salinity (McMaster University dechlorinated tapwater: Na^+ = 0.5, Cl^- = 0.7, Ca^{2+} = 0.98, Mg^{2+} = 0.15, K^+ = 0.05 mmol/L, hardness = 140 mg/L $CaCO_3$, pH = 8.0, or 100 % salinity (sea water) that was created by mixing distilled water with commercial sea salt (Instant Ocean™, Spectrum Brands, Blacksburg, VA, USA) to achieve a salinity of 35 ppt. Fish were maintained at room temperature (19 °C) on a 12 h light:12 h dark photoperiod with O_2 levels at >80 % air saturation and were fed to satiation once daily with a mix of commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secaucus, NJ, USA) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA). Fish were fasted for 48 h prior to and during all experimental periods.

2.2. Experimental design

The experiments were designed to understand the separate and interactive influences of salinity (0 % and 100 %) and Low (1 mmol/L)) and High (10 mmol/L) Ca levels on the physiological disturbances associated with zinc toxicity to killifish. All experimental procedure were in accordance with guidelines for the care and use of laboratory animals. Freshwater-acclimated killifish were used for the tests conducted at 0 % salinity, and seawater-acclimated killifish were used for the tests at 100 % salinity. In all cases the experimental fish were exposed to 500 μg/L of zinc (as $ZnSO_4{\cdot}7H_2O$; Sigma-Aldrich, St. Louis, MO, USA) for 96 h, as in our previous studies (Loro et al., 2012; Loro et al., 2014). In the present experimental design, Control fish (no zinc exposure) were exposed to fresh water (0 % salinity) or sea water (100 % salinity), prepared as described above. Experimental fish were exposed to 0 % salinity or 100 % salinity, each containing either Low Ca (nominally 1 mmol/L, the same as in Control fresh water) or High Ca (nominally 10 mmol/L, the same as in Control sea water) plus zinc (nominally 500 μg/L). The measured zinc and calcium concentrations during the exposures are shown in Table 1.

The fresh water used for 0 % salinity (Low Ca or High Ca) was soft water obtained from reverse osmosis facilities at McMaster University, Hamilton, ON, Canada, which was supplemented by the addition of either 1 mmol/L or 10 mmol/L of reagent grade $Ca(NO_3)_2$. The background levels of major ions in this soft water were: Ca^{2+} (0.061); Mg^{2+} (0.01), Na^+ (0.04), K^+ (0.003), and Cl^- (0.04) mmol/L; pH was 7.34, hardness was 7.8 mg $CaCO_3$./L, and O_2 levels were maintained at >80 % air saturation. Water samples for cations for all experimental procedures were analyzed using a Varian FS220 flame atomic absorption spectrophotometer (Varian, Mulgrave, Victoria, Australia), using commercial standards (Fisher Scientific, Toronto, ON, Canada). Cl^- concentrations were measured by a colorimetric assay (Zall et al., 1956), again using commercial standards. The sea water used for 100 % salinity (Low Ca or High Ca) was made up by adding reagent grade salts (Table 2) to the same reverse osmosis water according to the formulation of Kester et al. (1967) to reach 35 ppt sea water composition (100 % salinity). Calcium was again added as reagent grade $Ca(NO_3)_2$.. Note that $NaHCO_3$ was omitted from the original recipe of Kester et al. (1967) to avoid the precipitation of calcium carbonate. The conditions in the experimental waters, as measured in the exposure tanks, were: 18.6 ± 0.3 °C (SD), dissolved oxygen >80 % air saturation, and pH ranging from 7.81 ± 0.02 (0 % salinity) up to 8.20 ± 0.03 (100 % salinity). All exposures were carried out in triplicate with 6 fish in each 4-L aquarium. The water was renewed (80 %) every 24 h to maintain zinc concentrations.

2.3. Calcium and zinc in water and blood plasma

Total [Ca] in water and plasma samples was measured via atomic absorption spectrophotometry (SpectrAA 220FS, Varian, Agilent, Santa

Table 1
Measurements of zinc (nominally 0 μg/L in Control and 500 μg/L in all exposures) and calcium (nominally 1 mmol/L in Low Ca treatments and 10 mmol/L in High Ca treatments). All water measurements were taken in triplicate and represent the averages obtained at the beginning (0 h) and end (96 h) of the exposure periods.

		Control		Exposure		
Time	0 %	100 %	0 %, Low Ca	0 %, High Ca	100 %, Low Ca	100 %, High Ca
				Zinc (μg/L)		
0 h	0.003	0.005	496.3	499.7	495.3	497.5
96 h	0.003	0.003	485.3	495.7	484.3	491.4
				Calcium (mmol/L)		
0 h	0.98	9.98	0.98	9.98	0.98	9.96
96 h	0.97	9.90	0.97	9.55	0.92	9.65

Table 2
Salt composition of the artificial sea water (mmol/L) used in the zinc exposures (100 % salinity).

Salt (mmol/L)	100 % Low Ca	100 % High Ca
NaCl	409	409
Na_2SO_4	28	28
KCl	9	9
KBr	0.82	0.82
H_3BO_3	0.42	0.42
NaF	0.071	0.071
$SrCl_2$	0.15	0.15
$MgCl_2{\cdot}6H_2O$	53.7	53.7
$Ca(NO_3)_2$	0.98	9.98

Clara, CA, USA). The total [Zn] in water and plasma was analyzed using a Varian FS220 flame atomic absorption spectrophotometer (Varian, Mulgrave, Victoria, Australia). [Zn] and [Ca] were monitored daily (three samples per treatment). In both cases, commercial standards were used as described above.

2.4. Unidirectional calcium influx and intestinal calcium intake rates

Adult killifish were acclimated for 2 weeks as described in *Section 2.1*. After exposure to zinc in 0 or 100 % salinity plus Low or High Ca levels or to the 0 % salinity and 100 % salinity Control conditions for 4 days, we measured unidirectional calcium influx rate and intestinal calcium intake rate using radio-labelled $^{45}Ca^{2+}$. The term "intake rate" is used for the $^{45}Ca^{2+}$ accumulation in the intestine due to uncertainties about the route of accumulation (see Discussion). To assess Ca^{2+} influx and intake rates in different treatment groups, fasted fish from each experimental condition were placed individually into plastic containers fitted with aeration devices and containing 400 mL of their respective exposure water for overnight settling (n = 6 per treatment). The following day, water was completely replaced and fish were allowed to settle for an additional 1 h. After this period, $^{45}Ca^{2+}$ (Perkin Elmer, Waltham, MA, USA) was added to each container. This marked the beginning of the flux period. In order to maintain the proportion of total Ca^{2+} that was radiolabeled relatively constant across all treatments, different amounts of radioisotope (1 or 10 μCi, for 1 mmol/L calcium or 10 mmol/L calcium exposures respectively) were added to each container. After radioisotope addition, water samples (5.5 mL) were taken at 0, 3, and 6 h for the measurement of $^{45}Ca^{2+}$ radioactivity and total calcium concentration ([Ca]). Following collection of final water samples at 6 h, fish were rinsed 3 times with non-radioactive water of the respective salinity supplemented with 100 mmol/L $Ca(NO_3)_2$ to displace any loosely bound $^{45}Ca^{2+}$. They were then euthanized with an overdose of neutralized MS-222 and weighed.

To quantify intestinal calcium intake rates, the entire intestinal tract was ligated at either end (esophagus and rectum) and removed, and the carcass and the intestinal tract were weighed separately, and then digested separately. The tissues were digested in approximately 3 volumes of 1 N HNO_3 (exact volume noted) in sealed vials for 48 h at 65 °C. Radioactivity (β emissions) of $^{45}Ca^{2+}$ in water and digest samples, and the total calcium concentration ([Ca]) of water samples were measured subsequently. β -radioactivity of $^{45}Ca^{2+}$ in water and digest samples was measured using a scintillation counter (Tri-Carb 2900TR Liquid Scintillation Analyzer, Perkin Elmer) after mixing 5 mL of water sample, or 0.1–2 mL of digest with 10 mL of scintillation cocktail (Ultima Gold AB, Perkin Elmer) and incubating in the dark for 3 h to minimize chemiluminescence. The counting efficiency of digest samples was corrected to the same efficiency as that of water samples using a quench curve constructed from various amounts of digest. Unidirectional Ca^{2+}influx rate (nmol/g/h) was calculated using the following equation:

$$Ca^{2+}\ \text{influx} = R_{fish} / SA_{water} / (wt \times t) \quad (1)$$

where R_{fish} is the radioactivity (counts per minute; cpm) in the entire carcass digest, SA_{water} is the average specific activity (cpm/nmol) of the water over the period of the experiment, wt is the weight of the whole fish (g), and t is time (h).: The "intestinal Ca^{2+} intake" rates (i.e., not "influx") (nmol/g/h) was calculated using the following equation:

$$\text{Intestinal } Ca^{2+}\ \text{intake} = R_{intestine} / SA_{water} / (wt \times t) \quad (2)$$

where $R_{intestine}$ is the radioactivity (cpm) in the entre intestine digest, SA_{water} is the (cpm/nmol) of the water over the period of the experiment, wt is the weight of the whole fish, and t is the time (h).

2.5. Oxidative parameters and ATPase measurements

After 96 h of exposure, 6 fish per treatment were euthanized with a lethal dose of NaOH-neutralized MS-222 (Syndel Laboratories Ltd., Vancouver, B.C., Canada). Gill, liver, intestine and muscle were quickly removed by dissection, frozen in liquid nitrogen, and stored at −80 °C for later enzyme assays. Tissue samples were prepared by homogenization (1:5, weight:volume) in a buffer containing Tris-HCl (100 mmol/L, pH 7.75), EDTA (2 mmol/L) and Mg^{2+} (5 mmol/L). The homogenates were centrifuged at 10,000 ×*g* for 20 min at 4 °C.

Reactive oxygen species (ROS) were determined in the supernatant fraction of gill, liver, intestine, and muscle by the method of Viarengo et al. (1999). ROS was expressed as area of ROS mg^{-1} of protein. In the same supernatant, antioxidant capacity against peroxides (ACAP) was determined using the method of Amado et al. (2009). ACAP measurements were quantified as the relative area of ROS/mg protein. This method provides an inverse index such that high values for ACAP represent low values of antioxidant capacity against peroxides, and vice versa.

For the assay of Na^+/K^+-ATPase or Ca^{2+}ATPase, we used the same homogenate as described above for ROS and ACAP measurements. The Na^+/K^+-ATPase activity was assayed by measuring the amount of inorganic phosphate (Pi) liberated following the hydrolysis of ATP by ATPase as described by Bianchini and Carvalho de Castilho (1999). Ca^{2+}-ATPase activity was determined by the method of Vajreswari et al. (1983). Both ATPase activities were expressed as micromoles Pi liberated per minute per milligram protein (μmol Pi·min^{-1} mg $protein^{-1}$). The protein concentration was determined spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as the standard.

2.6. Statistical analyses

Data have been expressed as means ± standard deviation of mean (n = 6). All data were tested and exhibited normal distributions. Comparisons of mean values were performed using two-way analysis of variance (ANOVA) considering calcium and salinity as factors, followed by Tukey's test. The significance level adopted was 95 % (α = 0.05).

3. Results

3.1. Zinc and calcium in experimental water and killifish blood plasma

Water zinc and calcium concentrations measured during the exposures are summarized in Table 1. Zinc concentrations for all experimental groups were close to nominal, and in general declined by <5 % over the 4-day exposures, regardless of salinity or calcium levels. Zinc levels in the Control waters were very low. As shown in Fig. 1, the concentration of Zn in blood plasma (about 15 μmol L^{-1}) in the Control treatment increased more than two-fold after waterborne Zn exposure (nominally 500 μg/L = 7.6 μmol L^{-1}) in water with 0 % salinity and 1 mmol/L Ca. This level of Zn accumulation was partially attenuated but not eliminated when the Ca concentration was raised to 10 mmol/L in 0 % salinity water. Control plasma Zn levels were the same in 100 % salinity as in 0 % salinity, but the responses to Zn exposure were very

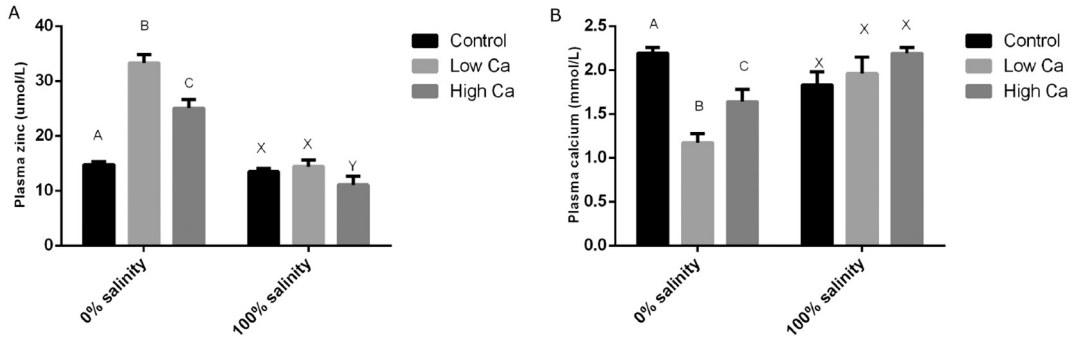

Fig. 1. **A** Zinc levels in blood plasma under Control conditions and after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). Different capital letters represent significantly different within the same salinity ($P < 0.05$).
B Calcium levels in blood plasma under Control conditions and after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). Different capital letters represent significantly different within the same salinity ($P < 0.05$).

different. The 100 % salinity water completely protected against Zn buildup in the plasma., regardless of whether it contained Low Ca or High Ca.

Fig. 1B shows that when fish were exposed to Zn in water with 0 % salinity and Low Ca (1 mmol/L), plasma calcium concentrations (about 2.2 mmol/L) were reduced by about 40 %. Raising the water Ca level to 10 mmol/L prevented about half of this reduction, but it remained significant ($P \leq 0.05$). In 100 % salinity, Control plasma calcium levels were similar to those in 0 % salinity. However, during waterborne Zn exposure, 100 % salinity completely protected against hypocalcaemia, regardless of whether it contained Low Ca or High Ca. Thus, the overall responses in plasma calcium concentrations reciprocally mirrored those in plasma Zn concentrations, despite the fact that plasma calcium levels (Fig. 1B) were in the millimolar range, whereas plasma Zn levels (Fig. 1) were in the micromolar range.

3.2. Unidirectional calcium influx and intestinal calcium intake rates

As shown in Fig. 2, unidirectional calcium influx rate represents the uptake into the whole body, except for the intestinal tract. Under Control conditions in 100 % salinity, this calcium influx rate (about 53 nmol g^{-1} h^{-1}) was about 3-fold higher than under Control conditions (about 19 nmol g^{-1} h^{-1}) in 0 % salinity. Zn exposure at 0 % salinity inhibited calcium influx by about 30 % at 1 mmol/L Ca. However, when waterborne Ca was raised to 10 mmol/L in the presence of Zn at 0 % salinity, calcium influx rate was significantly stimulated to about two-fold Control levels. A qualitatively similar pattern was seen at 100 % salinity, with about a 55 % lower calcium influx rate in the presence of Zn at Low Ca (1 mmol/L) but a significant 1.2-fold stimulation in the presence of Zn at High Ca (10 mmol/L) However, note that at Low Ca (1 mmol/L) in the presence of Zn, the calcium influx rate was approximately equal to that in the Control treatment at 0 % salinity (no Zn present) where the calcium concentration was also 1 mmol/L. Thus, there was in fact no apparent inhibition of unidirectional calcium uptake caused by 100 % salinity at Low Ca (Fig. 2)..

The calcium uptake into the intestine (Fig. 2B) measured with radiolabelled ^{45}Ca has been termed “intake rate” rather than “influx rate” because of uncertainties about its route of appearance (see Discussion). These intestinal intake rates were much higher in 100 % salinity, than in 0 % salinity, regardless of the water calcium concentration or presence/absence of Zn. The intake rates were also about 20–30 % of the unidirectional calcium influx rates in the 0 % salinity exposures, and about 40–80 % in the 100 % salinity exposures (Fig. 2B versus Fig. 2). When fish were exposed to Zn in 0 % salinity water at Low Ca (1 mmol/L), there was a substantial 60 % inhibition of the intestinal calcium intake rate, but this was completely prevented at High Ca (10 mmol/L) (Fig. 2B). In contrast, at 100 % salinity, there was no inhibition of intestinal calcium intake by waterborne Zn exposure at Low Ca (1 mmol/L), and indeed there was a significant 2-fold stimulation at High Ca (10 mmol/L).

3.3. ROS and ACAP measurements

As illustrated in Fig. 3, under Control conditions, relative ROS formations were similar in the various tissues regardless of salinity. In 0 % salinity water at Low Ca^{2+} (1 mmol/L), Zn exposure caused an

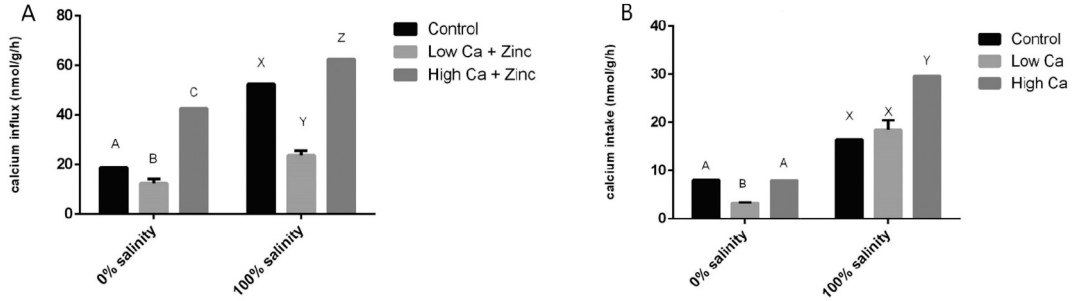

Fig. 2. **A** Unidirectional calcium influx rates (nmol/g/h) under Control conditions and after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). Different capital letters represent significantly different within the same salinity ($P < 0.05$).
B intestinal calcium intake rates (nmol/g/h) under Control conditions and after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). Different capital letters represent significantly different within the same salinity ($P < 0.05$).

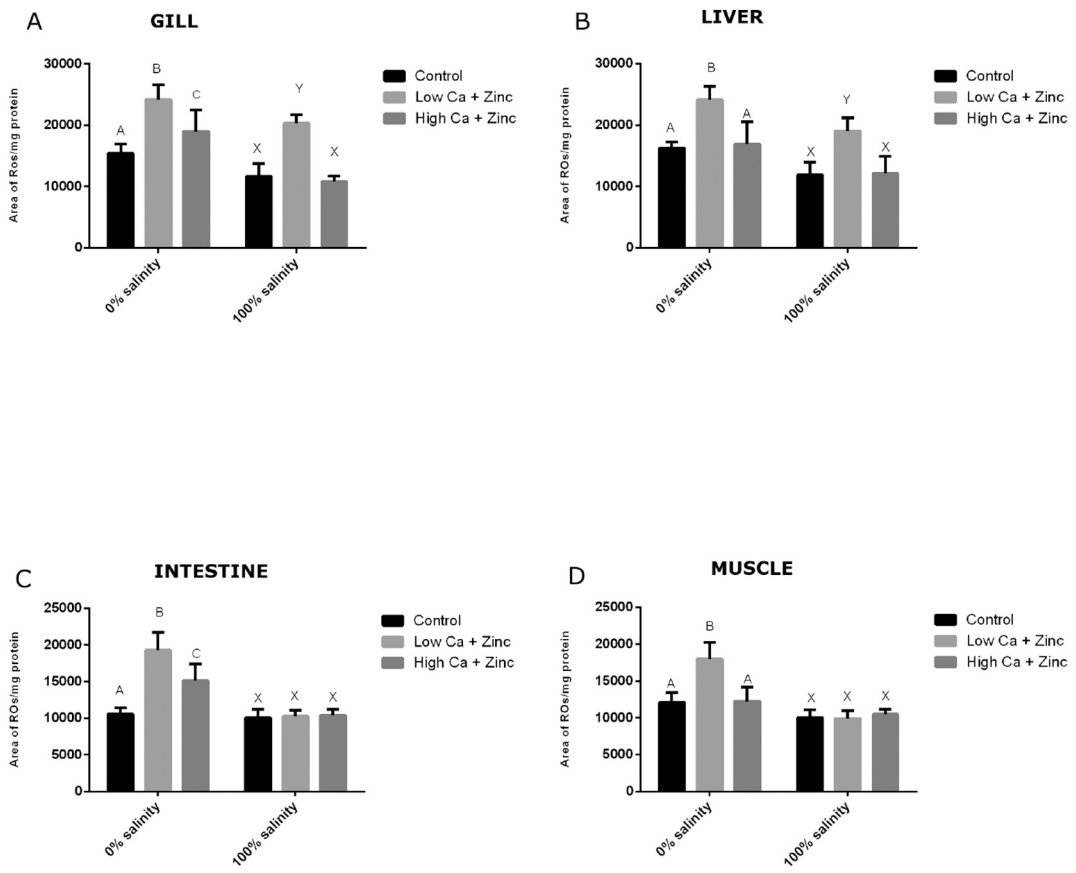

Fig. 3. Reactive oxygen species (ROS) in tissues of killifish: (A) gill, (B) liver, (C) intestine and (D) muscle under Control conditions after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). The values are expressed as the ROS formation (area of ROS per mg of protein). Different capital letters represent significantly differences within the same salinity ($P < 0.05$) for each tissue.

approximate doubling of relative ROS formation in all four tissues, and these effects were attenuated by High Ca (10 mmol/L), though the effects remained significant in gill and intestine. In 100 % salinity water at Low Ca (1 mmol/L), Zn exposure caused significant increases in ROS formation only in gills and liver, whereas at High Ca (10 mmol/L) these effects were prevented, and ROS was restored to Control levels. Intestine and muscle tissues at 100 % salinity and Low Ca did not show any difference from Control, and there were no additional effects of High Ca at 100 % salinity.

Fig. 4 shows the protection against peroxide formation (ACAP) in various tissues. As noted in Methods, this is an inverse index such that increases of ACAP represent decreases of antioxidant capacity, and vice versa. In general, ACAP levels were similar under Control conditions in 0 % and 100 % salinity, and amongst tissues, with white muscle exhibiting somewhat lower capacities (i.e. higher ACAP values) than gill, liver, or intestine. In 0 % salinity water, Zn exposure caused a reduction in protection (i.e. higher ACAP values) in all tissues at Low Ca (1 mmol/L), and this effect was prevented entirely at High Ca (10 mmol/L). In 100 % salinity at Low Ca (1 mmol/L), Zn exposure induced this effect only in the liver. Gill, intestine, and muscle were unaffected. High Ca (10 mmol/L) completely prevented this effect at 100 % salinity in the liver, and significantly reduced the ACAP values (i.e. increased protection) in the other tissues to varying extents.

3.4. ATPase measurements

Na^+/K^+-ATPase activities in gills were approximately twice as high as those in the intestine, but there were no differences between 0 % salinity and 100 % salinity under Control conditions (Fig. 5). In 0 % salinity water, exposure to Zn caused 50–60 % inhibitions of Na^+/K^+-ATPase activities in both tissues at Low Ca (1 mmol/L), and these effects were completely prevented at High Ca (10 mmol/L). In contrast, 100 % salinity completely protected Na^+/K^+-ATPase against zinc in both tissues, regardless of whether it contained Low Ca or High Ca.

Fig. 6 shows Ca^{2+}ATPase activities in gill and intestine. Under Control conditions, Ca^{2+}ATPase activities in gills and intestine were similar and there were no differences between 0 % and 100 % salinity. In 0 % salinity water with Low Ca (1 mmol/L), exposure to Zn caused approximately 30 % inhibitions in both tissues, and these were reversed to significant stimulations by High Ca (10 mmol/L). In 100 % salinity water with Low Ca (1 mmol/L) inhibition of Ca^{2+}ATPase activity by Zn did not occur in the gills but was still seen in the intestine, whereas High Ca (10 mmol/L) caused stimulation in the intestine.

4. Discussion

4.1. Overview

In interpreting our results, it must be remembered that 100 % salinity contained all components of normal sea water at normal concentrations (Table 2) *except* Ca which was at either 1 mmol/L in Low Ca (typical freshwater level) or 10 mmol/L (normal seawater concentration) in High Ca treatments. We had originally hypothesized that Zn bioaccumulation, inhibition of calcium uptake, hypocalcaemia, and disturbances of branchial ionoregulatory enzymes, as direct responses to external Zn exposure, would be governed primarily by the Ca concentration of the external medium acting as a competitor against Zn. We had also posited that oxidative stress in internal organs would be governed

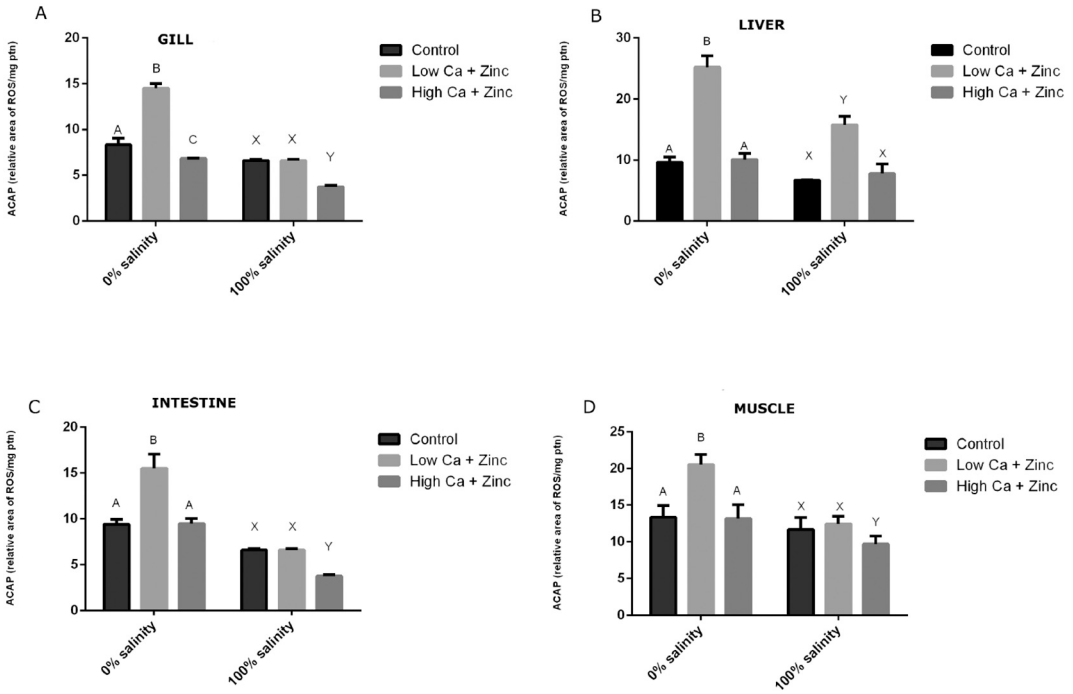

Fig. 4. Antioxidant capacity against peroxides (ACAP) in tissues of killifish: (A) gill, (B) liver, (C) intestine and (D) muscle under Control conditions after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). The values are expressed as the ROS formation (relative area of ROS) in relation to Control. Different capital letters represent significantly differences within the same salinity ($P < 0.05$) for each tissue.

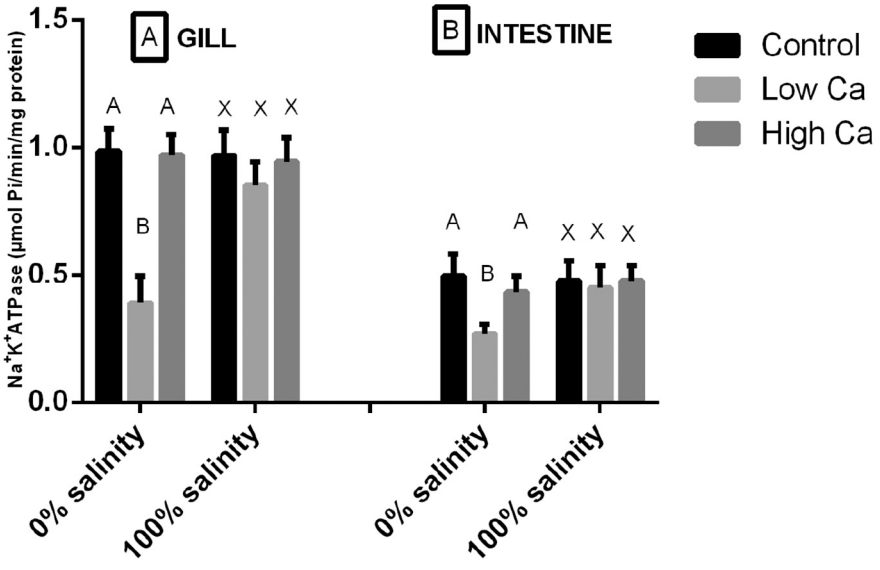

Fig. 5. Na^+/K^+ATPase activity (μmol Pi/min/mg protein) in gills (A) and intestine (B) of killifish under Control conditions after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). Different capital letters represent significantly differences within the same salinity ($P < 0.05$) for each tissue.

primarily by plasma Zn levels. Our results actually show a more complex pattern which is partly supportive of our ideas. In agreement with our hypotheses, Ca was clearly a very important protective agent for all endpoints. In freshwater (0 % salinity). High Ca (10 mmol/L) always provided partial or complete protection against the negative effects of waterborne Zn, but in sea water, we had underestimated the importance of 100 % salinity alone, which was usually, but not always, fully protective. However, there was no instance where the combination of 100 % salinity and High Ca failed to provide complete protection against Zn-induced disturbances in sea water. Finally, there was some evidence that responses in internal organs were a function of plasma Zn concentrations.

4.2. *Protection by salinity- the role of speciation*

Part of the protective effect of salinity can be attributed to salinity-dependent changes in Zn speciation. The free zinc cation (Zn^{2+}), generally considered the most toxic form of zinc (Hogstrand, 2011),

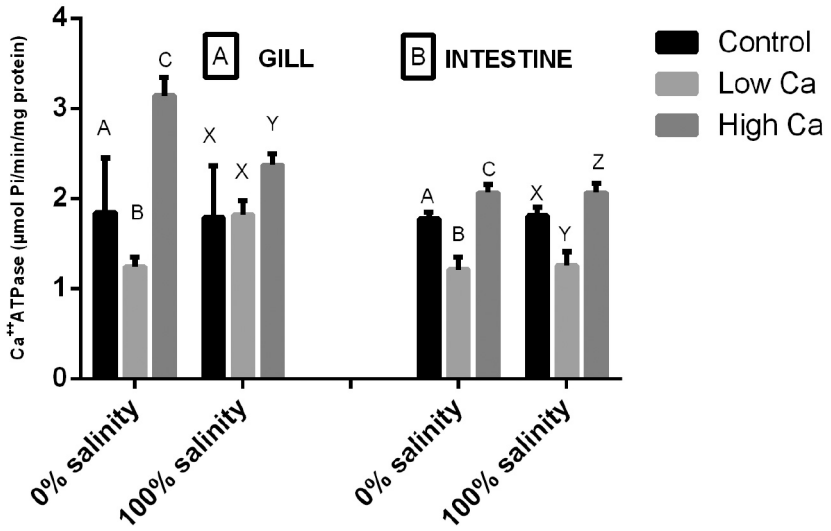

Fig. 6. Ca^{2+}ATPase activity (μmol Pi/min/mg protein) in gills (A) and intestine (B) of killifish under Control conditions and after exposure to Zn (nominally 500 μg/L^{-1}) in water with 0 % salinity or 100 % salinity and with levels of calcium designated as either Low (1 mmol/L) or High (10 mmol/L). Different capital letters represent significantly differences within the same salinity ($P < 0.05$) for each tissue.

decreases from about 79 % of the total in 0 % salinity to 26 % of the total in 100 % salinity [see speciation table in Loro et al., 2014]. Changing Ca from 1 to 10 mmol/L has negligible effect on this speciation. Furthermore, by the Debye–Hückel relationship, the higher ionic strength will tend to decrease the chemical activity of all charged zinc species, which may provide further protection. These speciation effects and/or additional competitive effect(s) of the other cations present in high concentrations in 100 % salinity (e.g. Na^+, Mg^{2+}, K^+; Alsop and Wood, 1999) can explain the complete protection offered by 100 % salinity alone for most endpoints. The exceptions were ROS generation in gill (Fig. 3A) and liver (Fig. 3B), loss of anti-oxidant capacity (ACAP) in liver only (Fig. 4B), and loss of Ca^{2+}ATPase activity in intestine only (Fig. 6). For all of these, High Ca was additionally needed to provide full protection. Three of these four exceptions were internal endpoints, where direct contact with the environment would be lacking, so the influence of 100 % salinity on zinc speciation or the effects of ions other than Ca^{2+} in seawater could be less important. Note that the apparently depressed calcium influx rate in 100 % salinity with Low Ca relative to the 100 % salinity control rate (Fig. 2) cannot be considered an exception, because this treatment had only 10 % (1 mmol/L) of the control substrate concentration (10 mmol Ca/L) for the calcium uptake system in sea water. Indeed, the calcium influx rate in the 100 % salinity Low Ca treatment (1 mmol/L) in the presence of Zn was approximately equal to that in the 0 % Control treatment (1 mmol/L) in the absence of Zn – i.e. no inhibition (Fig. 2).

4.3. Protection by High Ca

In fresh water, there is abundant evidence that Zn^{2+} and Ca^{2+} cations share a common uptake mechanism on the gills, such that Ca^{2+} inhibits Zn^{2+} uptake (Spry and Wood, 1989; Everall et al., 1989; Bentley, 1992; Hogstrand et al., 1995; Hogstrand et al., 1996; Hogstrand et al., 1998), and vice versa (Hogstrand et al., 1994; Hogstrand et al., 1995; Hogstrand et al., 1996). Thus, waterborne Zn exposure causes hypocalcaemia as seen in both our earlier investigation (Loro et al., 2014) and the present study (Fig. 1B) on *F. heteroclitus* in fresh water, and this is associated with an inhibition of both unidirectional calcium uptake (Fig. 2) and Ca^{2+}ATPase activity (Fig. 6A) at the gills. Furthermore, increased concentrations of Ca in fresh water greatly reduce Zn toxicity (Bentley, 1992; Alsop and Wood, 1999; Bielmyer et al., 2012), a concept that is incorporated into environmental criteria (e.g. CCME (Canadian Council of Ministers of the Environment), 2022; US EPA (United States Environmental Protection Agency), 2022) and regulatory models (e.g. Santore et al., 2002; De Schamphelaere and Janssen, 2004; DeForest and Van Genderen, 2012). Finally, it should be noted that in *F. heteroclitus*, a significant portion of calcium uptake occurs via the mitochondria-rich cells of the opercular epithelia (Marshall et al., 1995), which was not separated from the uptake at the gills in our experiments, so competitive effects of Ca^{2+} were likely occurring at both sites.

In freshwater trout, the affinity of the joint Zn^{2+}/Ca^{2+} transporter is about 10- to 50-fold higher for Zn^{2+} than for Ca^{2+} (Spry and Wood, 1989; Hogstrand et al., 1998), so in the present killifish, the 10-fold increase from Low Ca (1 mmol/L) to High Ca (10 mmol/L) can explain both the prevention of inhibition of unidirectional Ca influx (Fig. 2) as well as the partial protections against lowered plasma Ca concentration (Fig. 1B) and elevated plasma Zn concentration (Fig. 1). However, it is curious that in this treatment, the protections against disturbances in plasma Ca and Zn concentrations were only partial, despite the fact that branchial Ca influx rate actually increased above control levels (Fig. 2). These results suggest the additional involvement of other transport pathways, such as ZIP and ZnT transporters for Zn (Zheng et al., 2008; Hogstrand, 2011), and the respective efflux pathways for both cations; the latter remain poorly understood.

In sea water, under control conditions, the unidirectional calcium influx rate was 2- to 3- fold higher than in fresh water (Fig. 2), in agreement with two previous studies on *F. heteroclitus* (Prodocimo et al., 2007; Zimmer et al., 2019). At noted in *Section 4.2*, this reflects the 10-fold higher substrate (Ca) concentration in sea water. Both of these earlier studies reported that unidirectional calcium efflux rate, which was not measured in the present study, remains unchanged from freshwater levels, so it is unclear how calcium homeostasis is achieved in seawater-acclimated killifish. Nevertheless, it is interesting that in the presence of Zn, High Ca at 100 % salinity actually stimulated unidirectional calcium influx rate, as it did in 0 % salinity (Fig. 2). If the same Zn^{2+}/Ca^{2+} transport system is involved in sea water as in fresh water, this could explain the small but significant depression of plasma Zn level (Fig. 1), despite the elevated Zn concentration in the environment. High Ca in 100 % salinity also protected against ROS generation in gill (Fig. 3A) and liver (Fig. 3B), loss of anti-oxidant capacity (ACAP) in liver only (Fig. 4B), and loss of Ca^{2+}ATPase activity in intestine only. The point was made in *Section 4.1* that except for increased gill ROS caused by Zn exposure, these were internal effects where the tissues were not directly exposed to seawater. Additionally, for two other internal endpoints, ACAP in intestine (Fig. 4A) and ACAP in muscle (Fig. 4B), High

Ca at 100 % salinity exerted additional protective action above the complete protection provided by 100 % salinity alone. The resulting depressed Zn concentration in blood plasma (Fig. 1) may explain these internal effects.

4.4. Intestinal calcium intake rates

Interpretation of these rates is complicated by the fact that there are two possible routes of "intake": (1) direct via drinking of the medium, and (2) indirect via transport of radioactive calcium taken up at the gills through the bloodstream, which then equilibrates with the intestinal tissue, as discussed by Zimmer et al. (2019). Earlier we measured drinking rates in killifish exposed to 500 μg/L Zn under conditions comparable to those of the present experiments (Loro et al., 2014). Zn did not significantly change the drinking rates from those under control conditions, which averaged about 0.0007 ml/g/h in freshwater, and 0.0025 ml/g/h in full strength sea water.

Therefore, in Control fresh water or 0 % salinity with Low Ca (both containing 1 mmol/L Ca), the calcium intake rates by drinking would be about 0.7 nmol/g/h, amounting to only about 10 % of the observed rate (Fig. 2B). Therefore, most of the intake must have occurred via the indirect route, and the inhibitory effect of Zn was explained by its inhibitory action on branchial calcium uptake (Fig. 2), thereby reducing intestinal intake indirectly. However, at 0 % salinity with High Ca (10 mmol/L), the protective effect could be completely explained by the 10-fold increase in calcium concentration in the ingested water which would raise intake via the direct route (drinking) by 10-fold.

In Control sea water or 100 % salinity with High Ca, the calcium intake rates by drinking would be 25 nmol/g/h, more than enough to explain the control intestinal intake rate (~16 nmol/g/h), and sufficient to account for most of rate in 100 % salinity with High Ca (~29 nmol/g/h) (Fig. 2B). The unchanged rate in 100 % salinity with Low Ca (1 mmol/L) is difficult to explain, because the presence of Zn markedly depressed branchial calcium uptake (Fig. 2) which would reduce the indirect route, and the 10-fold lower calcium content of the medium would be expected to greatly reduce direct intake via drinking. Perhaps the abnormal composition of this seawater, in combination with Zn, results in stress-induced high drinking rates (Tytler et al., 1990). High NaCl in itself is known to stimulate drinking (Grosell, 2010). Drinking rates as high as 0.01 ml/g/h have been reported in stressed killifish at intermediate salinities (Blewett et al., 2013).

4.5. Oxidative stress responses

Our original demonstration that sublethal Zn exposure induces ROS generation and degrades anti-oxidant defense capacity in killifish (Loro et al., 2012) has been confirmed in the present study (Figs. 3, 4), and is in accord with findings of Zn-induced oxidative stress in several other fish species (e.g. Atli and Canli, 2010; Zheng et al., 2016; MacRae et al., 2016; Kim et al., 2019). The exact mechanism(s) is/are unclear, but there is evidence that ROS generation is associated with Zn entry into mitochondria (Sharaf et al., 2017). If this is the case, then oxidative stress in internal organs would be seen only in treatments where extracellular (i.e. plasma) Zn concentrations were elevated. This was generally true in the present study, but higher levels of ROS (Fig. 3B) and ACAP (depressed detoxification capacity; Fig. 4B) in the liver despite unchanged plasma Zn concentrations (Fig. 1) in the Low Ca treatment at 100 % salinity were an exception. Nevertheless, High Ca at 100 % salinity fully protected against ROS and ACAP disturbances in all tissues (Figs. 3, 4).

4.6. Conclusions

Our results confirm the key importance of water calcium concentration in protecting against Zn uptake and associated symptoms of ionoregulatory dysfunction and oxidative stress in *F. heteroclitus* exposed to sublethal Zn in fresh water. In sea water, the situation is more complex. For many endpoints (plasma zinc and calcium concentrations, oxidative stress in the intestine and muscle, gill ionoregulatory enzyme activities), the "non -calcium" components of 100 % salinity are sufficient to provide complete protection in killifish exposed to the same sublethal concentration of Zn. However, for several indices (oxidative stress in gills and liver, Ca^{2+}ATPase activity in the intestine), the presence of the naturally high calcium levels (10 mmol/L) in sea water are additionally required to provide full protection. These results help elucidate the mechanisms involved in a growing body of evidence that seawater protects against Zn uptake and physiological symptoms of Zn toxicity in euryhaline fishes (Zhang and Wang, 2007; Loro et al., 2012; Bielmyer et al., 2012; Shyn et al., 2012; Loro et al., 2014).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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