USING DEVLOPING ZEBRAFISH (*DANIO RERIO)* TO EXAMINE THE EFFECTS OF EXPOSURE TO ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF ZINC: THE IMPLICATIONS FOR ASSESSESSING ZINC CONTAMINATION IN FRESHWATER SYSTEMS

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Abstract

Increasing concentrations of zinc (Zn) in freshwater aquatic systems around the world have created concern for the many inhabitants of these environments. Zebrafish throughout various developmental stages were exposed to sublethal environmentally relevant concentrations of waterborne and dietary Zn. My research showed that exposures to Zn significantly decreased the survival of the larvae, while also disrupting the homeostasis of several essential trace metals and major ions (i.e., copper, nickel, manganese, calcium). Furthermore, Zn transporters which displayed spatial-specific expression were shown to exhibit complex regulatory patterns dependent on exposure route of Zn and developmental age. Overall, my research showed that developing fish are sensitive to the elevated levels of Zn seen in polluted environments and that future ecological risk management would benefit from incorporating the impacts and parameters assessed in our study (i.e. life stage and exposure route) when developing new environmental guidelines.

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Statement of Contributions

The projects presented in this thesis were conceptualized by Pankaj Puar, Dr. Som Niyogi, and Dr. Raymond W.M. Kwong. All experiments were conducted by Pankaj Puar. All formal analysis and investigation was conducted by Pankaj Puar. Pankaj Puar also carried out all statistical analysis with the exception of the repeated measures test as described in Chapter 3 which was completed with assistance from Dr. Mohammad Naderi. This thesis was prepared by Pankaj Puar with input from Dr. Raymond W.M. Kwong and Dr. Som Niyogi.

Organization of thesis

This thesis is comprised of four separate but related chapters written by the author, Pankaj Puar. **Chapter 1** is included as a general introduction which provides relevant background for the thesis in its entirety. **Chapter 2** is organized as an independent research chapter which investigates the physiological implications of environmental Zn pollution and Zn exposures to aquatic life at the very early life stage**. Chapter 3** is a follow-up study to Chapter 2 and is structured as an independent research article which compares the implications of environmentally relevant waterborne and dietary Zn exposures (individually or simultaneously) at various developmental timepoints. **Chapter 4** is the final chapter of this thesis and is used as a point of general discussion to synthesize, analyze, and compare the findings of both studies as well as discuss the overall implications and significance of the research.

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Chapter 2 and Chapter 3 presented in this thesis have each been published in a peer reviewed journal. Permission from the editorial manager and all co-authors was collected prior to inclusion of the journal articles in the thesis. The published articles have been referenced below:

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Chapter I:

General Introduction

1.1 General introduction and exploring the fundamental importance of zinc

There exist eleven metals found in trace quantities throughout the body that are essential for the optimal functioning of vertebrate species. These trace metals include iron (Fe), zinc (Zn), copper (Cu), selenium (Se), manganese (Mn), iodine (I), molybdenum (Mo), fluorine (F), cobalt (Co), chromium (Cr), and vanadium (V) (Fraga, 2005; Zhao et al., 2014). Of these eleven classified trace metals, Zn has been recorded to be the second most abundant in the body and is unique as it exists and carries out its cellular functions in a single valance state (Zn^{2+}) (Zhao et al., 2014). Zn is of particular interest due to its fundamental importance in the body, complex transport mechanisms, and increasing environmental concern, all of which will be discussed in detail throughout this review chapter.

The first scientific evidence demonstrating the importance of Zn on the cellular level was uncovered in 1940 with the isolation of the Zn-dependent enzyme, carbonic anhydrase. In the present date over 300 enzymes and 2000 transcription factors have been identified that rely on Zn for their functioning, highlighting the prominent role of Zn in the body (Vallee and Auld, 1990; Zhao et al., 2014). A few other examples of Zn-dependent enzymes include alkaline phosphatase, alcohol dehydrogenase, and acid phosphatase (Blesbois and Mauger, 1989; Prasad et al., 1979; Rosser and George, 1986; Watanabe et al., 1997). Though Zn has long been recognized to participate in the catalytic component of enzyme performance, substantial evidence has provided a rationale for the involvement of Zn in structural stabilization as well (Andreini et al., 2011; Gamsjaeger et al., 2007; Krishna et al., 2003; Maret, 2012; B. L. Vallee

and Falchuk, 1993). The structural and scaffolding functions of Zn allow for important protein-protein interactions and protein-nucleic acid interactions collectively facilitating efficient cellular functioning (Macdonald, 2000; Maret, 2000; Wu and Wu, 1987).

Zn is also powerful antioxidant in the body. Zn can inhibit the production of reactive oxygen species through the structural role of Zn in antioxidant proteins, as well as through the direct binding of Zn to -thiol subgroups which then protects these proteins from oxidation (Olechnowicz et al., 2018; Oteiza, 2012). Furthermore, Zn plays an important anti-inflammatory role through the regulation of pro-inflammatory cytokines and inhibition of Nuclear factor kappa B (NF-kB), an important immune response transcription factor (Fernández-Sánchez et al., 2011; Haase and Rink, 2014).

In addition, a new and emerging facet surrounding role of Zn in the nervous system is now being recognized. In the nervous system, Zn is found in high quantities within glutamatergic neurons and is released together with glutamate into the synaptic cleft during the signalling process (Cole et al., 1999; Sensi et al., 2009). Synaptically released Zn may influence both excitatory and inhibitory synapses in the brain through the interaction with γ-Aminobutyric acid type A ($GABA_A$) and N-methyl-D-aspartate (NMDA) receptors (Sensi et al., 2009). It has even been argued that Zn may act as an independent neurotransmitter, and may have its own neuronal receptors in the brain (Besser et al., 2009). However, these suggestions remain speculative at this point and still require further investigation before any definite conclusions can be reached.

Collectively, the role and functioning of Zn in the body is enormous and goes well beyond those points mentioned here. In particular, Zn can be appreciated for its critical role in cellular homeostasis, gene regulation, immune defence, response to oxidative stress, cell division, DNA transcription, protein translation, gene expression, and membrane structure (Chen

et al., 2002; Dalton et al., 2000; Hershfinkel et al., 2010; Zheng et al., 2010a, 2010b). These widespread functions of Zn in the cell provide substantial support and clear evidence for the fundamental importance of Zn in organisms (Ho et al., 2012; Zhao et al., 2014).

1.2 Mechanisms of Zinc homeostasis

Though Zn is a nutrient of fundamental importance, the concentrations of Zn in the body of vertebrate's, including fish, must be tightly regulated to remain in the optimal range for proper organism health and functioning.

To understand how Zn is regulated, its distribution within the body must also be understood. At the cellular level, Zn is primarily found to be conjugated to proteins, with free unbound Zn not readily available and maintained in the femto-molar to the pico-molar range (Zhao et al., 2014). When analyzing the tissue distribution of Zn, the highest concentrations of Zn in vertebrates can be found in the eye, brain, bone, liver, kidney, and muscle (Eisler, 1993; NAS, 1979). Although Zn is more concentrated in these specific tissues, in fish it appears that there exists no clear storage organ for Zn, with an exception in female fish where Zn can be stored in the liver before distribution into the yolk of eggs for embryonic development (Montorzi et al., 1994; Thompson et al., 2003). Relatedly, it is important to note that tissue concentrations of Zn in freshwater (FW) organisms are elevated when localized near point sources of Zn contamination. For example, tissue concentrations of common carp (*Cyprinus Carpio*) residing in Zn polluted waters in Utah measured upwards of 168 mg Zn/kg wet weight against an average of 63 mg Zn/kg wet weight at non-polluted sites (Lowe et al., 1985). This potential of Zn to accumulate within the body of fish highlights the need for efficient homeostatic mechanisms to counteract prospective Zn overload and prevent any negative consequences associated with it.

At the cellular level, Zn homeostasis is largely controlled by two families of transporters, "Zn Transport proteins" (ZnT, SLC30a) and "Zrt-Irt like proteins" (ZIP, SLC39a). ZnT's are a class of Zn transporters that function to facilitate cytosolic Zn efflux and are localized to the membranes of intracellular organelles and vesicles. In contrast, ZIPs function to primarily mediate cytosolic influx of Zn from the extracellular space, intracellular vesicles, and organelles. The specific function of different ZIP transporters is discussed in more detail in section 1.3. Apart from Zn specific transport routes, evidence has shown that Zn uptake and homeostasis in fish may also be mediated by ZIP independent pathways. One such elucidated pathway involves the calcium (Ca) specific, brachial epithelial Ca channel (ECaC/TRPV6) which will be discussed in depth in section 1.8. It has also been suggested that Zn homeostasis involves amino acid specific pathways, as studies have reported the intestinal absorption of Zn bound to L-histidine and L-cysteine to be as efficient as unbound Zn (Glover and Hogstrand, 2002a). Supporting this idea, the presence of histidine and cysteine bound Zn have been shown to strongly influence the distribution of newly accumulated Zn in the body. In one study, histidine bound Zn promoted the accumulation and pooling of Zn in the intestinal tissue, whereas cysteine bound Zn promoted Zn distribution to the blood (Glover and Hogstrand, 2002a), providing strong support for the potential involvement of amino acid pathways in regulating Zn homeostasis.

When discussing the cytosolic storage and maintenance of Zn during periods of Zn spill-over (i.e when the concentrations of free Zn in the cell go above the normal level), there are small ligands in the cytoplasm such as metallothionein (MT) and glutathione that act as low molecular weight sequestering molecules and homeostatic regulators that maintain cytosolic free Zn^{2+} concentrations in the picomolar-femtomolar range. Increases in the cytosolic concentration of Zn above this range can have many deleterious effects (e.g., cellular apoptosis, non-specific

enzyme activation, tissue necrosis) (Nriagu, 2007.; Stefanidou et al., 2006). MT is a cysteine-rich (>20%) protein ubiquitously expressed throughout the body and contains 7 binding spots capable of binding and releasing Zn through the oxidation and reduction of cysteine sulfur residues (Maret, 2000). Overall, MT functions as a cellular safeguard to regulate concentrations of free Zn in the cell to match the needs of the given cellular environment by binding Zn when concentrations of Zn in the cell rise and releasing Zn when cellular concentrations fall. Expression of MT is often controlled by the influence of cellular levels of Zn^{2+} on metal-responsive transcription factor-1 (Mtf-1) (Chen et al., 2002; Dalton et al., 2000). Mtf-1 itself contains six Zn finger domains which form tight associations with Zn when the concentration of Zn in the cell rise. Once associated with Zn, Mtf-1 enters an active form capable of interacting with metal-responsive element and effectively promotes the transcription of metallothionein, which then acts to lower and safeguard cytosolic Zn concentrations (Kimura et al., 2009).

Controlling and regulating the uptake of Zn from the environment to meet the needs at the organism level is an important component of homeostasis that must be considered. In teleost fish, the brachial system and the gastrointestinal tract combine to be responsible for the majority of environmental Zn absorption, except Zn absorption via the skin during early development (Alderdice, 1988; Kaneko et al., 2002; Pan et al., 2005). Interestingly, in FW fish the gill has been shown to have a much greater affinity but far lower maximum transport capacity for Zn than the gut (Bury, 2003; Glover and Hogstrand, 2002b; Hogstrand et al., 1998; Niyogi et al., 2007; Spry and Wood., 1989). Conversely, while the gill may display a higher affinity for Zn, the anterior region of the intestine has been shown to be the predominant site for Zn absorption and is the largest contributor to whole body Zn contents in FW fish (Hogstrand et al., 1996; Shears and Flethcher, 1983). On the other hand, Niyogi et al (2007) suggested that the gill,

relative to the gut, plays a larger role in regulating Zn uptake in fish during conditions of elevated waterborne Zn. In this study, yellow perch (*Perca flavescens*) in Zn contaminated waters exhibited changes in the kinetics of Zn absorption at the gill while no apparent changes were observed at the gut. Although no responses were demonstrated in the gut in this study by Niyogi et al (2007), other studies have however reported decreases in Zn absorption at the anterior region of the intestine during periods of dietary Zn loading. This suggests that potential Zn regulatory patterns exist at the level of the gut as well (Glover and Hogstrand, 2002b; Hardy et al., 1987; Shears and Flethcher, 1983).

Whereas the topics of cellular Zn handling and uptake from the environment have been studied quite intensively over the years, these aspects only constitute half the picture of maintaining Zn homeostasis. The matter of Zn excretion in relation to homeostasis in aquatic organisms is a topic that is of importance but unfortunately has received little attention. Much of what is known about excretion in FW fish has been extrapolated from mammalian models, where even there the topic has been sparsely investigated. Zn is largely excreted through the gastrointestinal tract via intestinal sloughing (Handy, 1996; Niyogi et al., 2007; Shears and Fletcher, 1983), while loss of Zn through other means such as perspiration and urine has been considered to be minimal (<4%) (Hardy et al., 1987). The excretion of Zn through intestinal sloughing has been linked to the transporter ZIP5 which is localized on the basolateral surface of absorptive enterocytes at the intestine (Dufner-Beattie et al., 2004; Wang et al., 2004). ZIP5 functions to pump Zn ions into enterocyte cells which can then be shed into the intestinal lumen and excreted, effectively lowering contents of Zn in the body (Dufner-Beattie et al., 2004; Geiser et al., 2013). Additionally, ZIP5 has been shown to be upregulated during periods of Zn overload to increase excretion of Zn and maintain internal Zn contents in the homeostatic range (Geiser et

al., 2013; Weaver et al., 2007). Importantly, ZIP5 has recently been identified in the zebrafish genome, which now suggests the potential involvement of ZIP5 in the excretion of Zn in FW fish. Aside from excretion at the gut, other evidence has been presented for alternative excretion routes of Zn in FW fish. One study in 1987 administered radioactive ^{65}Zn through the diet to rainbow trout and then examined the fish for 72 hours. Upon investigation, the researchers found significant traces of ^{65}Zn leaving from the head region of fish with no seen regurgitation, suggesting a potential branchial excretion route of Zn (Hardy et al., 1987). While some evidence has been presented on the potential mechanisms of Zn excretion in aquatic organisms, the topic remains quite understudied and warrants further investigation before any conclusions can be drawn on the roles and mechanisms of Zn excretion.

1.3 Function of ZIP Transporters: What is known?

As described previously, the uptake of Zn at the cellular level is mediated and controlled by the ZIP family of transporters. The first members of the ZIP family were identified in 1996 when the cation transporter, "iron-regulated transporter-1" (IRT-1) was isolated from the roots of plants (Eide et al., 1996) and a group of "Zn regulated transporters" (ZRT) from yeast (*Saccharomyces cerevisiae*) (Zhao and Eide, 1996). Since these early discoveries in 1996, over 100 transporters across a variety of phylogenetic levels have been recognized and grouped into the family based on structural and functional similarities to these originally founded transporters, giving rise to the family name "ZRT and IRT-like proteins" (ZIP) (Guerinot, 2000).

Within the human genome, 14 ZIP isoforms have been identified and they are known to transport Zn (Cousins et al., 2006). Similarly, 12 of those 14 mammalian ZIP orthologues have also been recognized in the zebrafish genome (*Danio rerio*), suggesting that ZIP are conserved

between humans and fish (Bury, 2003; Feeney et al., 2005). The 14 ZIP homologs expressed in human (and 12 in zebrafish) are classified according to their Human Genome Organization Nomenclature Committee designation of "solute carrier family 39" (SLC39) and are referred to as ZIP 1 through 14. When examining the structural properties of ZIP, it can be noted that ZIPs range from 300-500 amino acids in length, with the variety in length predominately a result of variation in the region between transmembrane domains II and IV of the protein (Guerinot, 2000). Furthermore, the various ZIPs have been categorized into four subfamilies based on structural similarities: ZIPI (ZIP1, ZIP2 and ZIP3), ZIPII (ZIP9), gufA (ZIP11) and LIV-1 (ZIP4-8, ZIP10, and ZIP12-14) (Gaither and Eide, 2001; Jeong and Eide, 2013). Interestingly, only members of the LIV-1 sub-family possess a HEXXH binding motif which functions as a metal binding site that is absent in other ZIP. Moreover, all ZIPs consist of eight transmembrane domains with transmembrane domain IV and V predicted to line the cavity through which substrates pass, with both the N and C terminus of all ZIP facing the extracellular space (Guerinot, 2000; Jeong and Eide, 2013). The driving force for Zn transport through ZIP remains elusive, however, it is hypothesized to be stimulated by a bicarbonate gradient relaying on a metal-bicarbonate symport mechanism (Gaither and Eide, 2000, 2001; Girijashanker et al., 2008). Transport of Zn through ZIP has also been hypothesized to rely on a variety of other factors such as pH and phosphorylation as a post-translational modification and should be considered when addressing ZIP activity and functioning (Jenkitkasemwong et al., 2012; Thingholm et al., 2020).

When assessing the expression patterns within a cell, different ZIPs have been found to be localized in different subcellular compartments and membranes. All ZIPs, except for ZIP 7, 11, and 13, are localized on the plasma membrane, aiding in the influx of Zn from the

extracellular environment (Jeong and Eide, 2013; Kimura and Kambe, 2016). ZIP7 is the only ZIP known to be localized in the endoplasmic reticulum (ER) and is used for Zn release into the cytosol upon signalling events, while ZIP 9, 11, and 13 are reported to be responsible for the efflux of stored Zn from the Golgi apparatus (Bafaro et al., 2017). ZIP 8 has also been described to be expressed on the surface of lysosomes and endosomes, with the ability to transport Zn into the cytosol from these cellular vesicles (Hogstrand, 2012; Jeong and Eide, 2013; Kimura and Kambe, 2016). A summary of the cellular localization pattern of the various ZIP is shown in Figure 1.1.

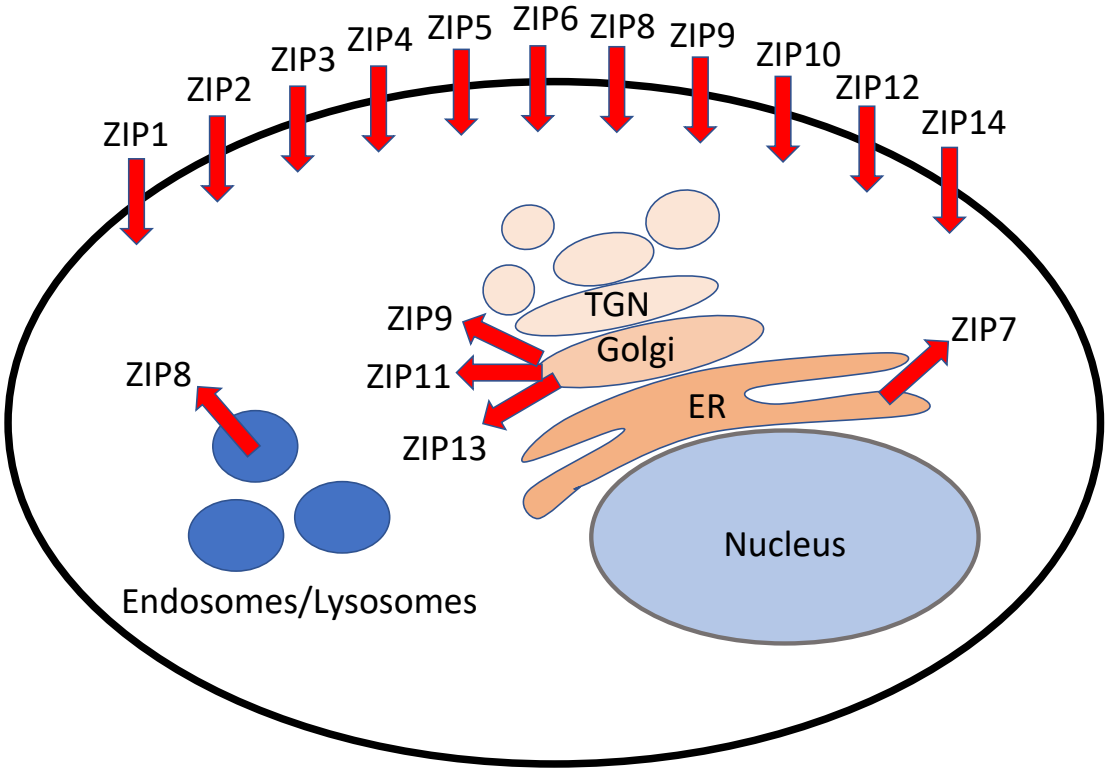

Figure 1.1. Summary of the cellular localization pattern of various ZIP within a cell. The arrow of direction indicates the facilitated flow of Zn by the transporter**.** All relevant cytosolic organelles have been labelled. Abbreviations are as follows ZIP, Zrt-Irt-Like Proteins; ER, Endoplasmic Reticulum; Golgi, Golgi Apparatus; TGN, Trans Golgi Network. Figure has been adapted from Kimura and Kambe (2016).

It has been well established through many studies that Zn is required ubiquitously throughout the body, however, it is important to note that not all ZIP appear to be equally expressed in all tissue of the body. Studies with mammals and adult fish have shown that different ZIPs display tissue-specific localization patterns, suggesting that each ZIP may play a unique role in fulfilling the needs of the tissue in which it is expressed (Bafaro et al., 2017; Feeney et al., 2005; Jeong and Eide, 2013; Kimura and Kambe, 2016; Ohashi et al., 2019). Whether this tissue-specific expression is also the case for fish during development, and whether the pattern of expression remains consistent from development to adulthood is a question that still remains elusive. A summary of what is known about the tissue distribution of the various ZIPs in mammals (adult mice) and fish (adult zebrafish) is presented in Table 1.1.

Table 1.1. Comparing the tissue localization pattern of ZIP in adult mice and zebrafish

Gene	Tissue Localization (Adult Mice)	Tissue Localization (Adult Zebrafish)	References
ZIP1 **ZIP3** **ZIP10**	Ubiquitous	Ubiquitous	Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006; Feeney et al., 2005; Gao et al., 2017; Ohashi et al., 2019
ZIP2	Liver, Skin, Ovary, Dendritic cell, Prostate	Not expressed in zebrafish genome	Ohashi et al., 2019; Peters et al., 2007
ZIP4	Intestine, Epidermis, Kidney	Eye, Ovary, Intestine	Bin, et al., 2017a; Dufner-Beattie et al., 2007; Feeney et al., 2005; Ohashi et al., 2019
ZIP5	Intestine, Kidney, Pancreas	Localization not measured	Feng et al., 2017; Geiser et al., 2013; Ohashi et al., 2019
ZIP6	Ubiquitous	Gill, Kidney, Brain, Eye, Ovary	Feeney et al., 2005; Gerold et al., 2016; Hogstrand et al., 2013;
ZIP7	Ubiquitous	Gill, Kidney, Muscle, Liver, Eye, Ovary, Intestine	Bin, et al., 2017b; Feeney et al., 2005; Ohashi et al., 2016, 2019
ZIP8	Ubiquitous	Intestine	Feeney et al., 2005; Li et al., 2016; Ohashi et al., 2019;
ZIP9	Ubiquitous	Localization not measured	Ohashi et al., 2019; Thomas et al., 2018
ZIP11	Ubiquitous	Localization not determined	Ohashi et al., 2019; Wu et al., 2015
ZIP12	Brain, Pulmonary Venue, Smooth muscle	Not expressed in zebrafish genome	Chowanadisai, 2014; Ohashi et al., 2019
ZIP13	Bone, Hard and connective tissues	Ubiquitous	Feeney et al., 2005; Fukada et al., 2008; Ohashi et al., 2019
ZIP14	Liver, Intestine, pancreas, heart, brain	Localization not measured	Ohashi et al., 2019; Tuschl et al., 2016

Among all of the ZIPs characterized, ZIP4 has arguably been the most thoroughly investigated. ZIP4 is found to be highly expressed in enterocytes on the epithelial surface of the small intestine and is recognized to play a critical role in the intestinal uptake of Zn in mammals (Dufner-Beattie et al., 2003, 2004; Wang et al., 2002). Interestingly, it has been shown that the daily supplementation of 25mg Zn in humans for 14 days was associated with the downregulation of *zip4* mRNA and protein expression in the small intestine (Cragg et al., 2005). Similarly, additional studies in mice have also demonstrated the capability of dietary Zn supplementation to stimulate the internalization and ubiquitin-mediated degradation of the ZIP4 protein at the level of the intestine, effectively reducing the cellular uptake of Zn during periods of Zn loading (Dufner-Beattie et al., 2007; Mao et al., 2007; Weaver et al., 2007). On the other hand, dietary restriction of Zn in mice has been shown to promote the localization of ZIP4 to the apical membrane in enterocytes to effectively increase the intestinal uptake capacities of Zn during periods of need and deprivation (Dufner-Beattie et al., 2003, 2007; Weaver et al., 2007). Together, these results suggest that ZIP4 plays an important role in regulating dietary Zn uptake and maintaining internal Zn homeostasis in cases of both excess and inadequate dietary Zn availability in mammals. Although ZIP4 has also been shown to be expressed in fish, it is still unclear whether ZIP4 can provide the same levels of regulation in response to Zn supplementation or deprivation to help fish meet their nutritional needs.

With regards to the specific functions of other ZIP transporters, ZIP7 has been associated with the promotion of myoblast proliferation, differentiation, and maturation by stimulating the PI3K/Akt cascade pathway in muscle cells (Mnatsakanyan et al., 2018). Knockdown of ZIP7 has reportedly been linked with reduction of myofibril Zn content, subsequently leading to decreased PI3K/Akt pathway activation, a reduced number of myofibers, decreased myotube development,

delayed growth, and developmental malformations (Mnatsakanyan et al., 2018; Yan et al., 2012). ZIP6 and ZIP10 play important roles in immune function, where ZIP10 has been recognized to be critical for the survival of ß-Cells (Hojyo et al., 2014; Miyai et al., 2014), while ZIP6 is observed to be involved with antigen presentation to CD4 T-cells during the immune response (Yu et al., 2011).

Two ZIP transporters of particular interest are ZIP8 and ZIP14 as these two transporters have been shown through *Xenopus laevis* oocyte overexpression studies to be capable of transporting the divalent metals Fe, Mn, and Cd, in addition to Zn (Jenkitkasemwong et al., 2012; Pinilla-Tenas et al., 2011; Wang et al., 2012). This multiple metal transport capability of ZIP8 and ZIP14 is in contrast to most other ZIP transporters which are capable of transporting Zn only, and is due to a replacement of the first histidine (H) for a glutamic acid (E) in the HEXXH Zn binding motif of ZIP8 and 14 (Wang et al., 2012). Interestingly, the Mn transport function of ZIP14 has been elucidated to be essential for maintaining Mn homeostasis under normal conditions in vertebrate's. A study using zebrafish demonstrated that knockout of ZIP14 led to insufficient Mn cycling eventually causing excessive accumulation of Mn in the brain. This accumulation of Mn in the brain ultimately led to alterations in fish behaviour that resembled overall motor deficits and behavioural characteristics associated with that of dystonia Parkinsonism (Tuschl et al., 2016). The mechanism of ZIP14's role in Mn homeostasis has now been linked to the facilitation of Mn transport from the liver and pancreas into the bile for excretion with the help of ZNT10 (Aydemir and Cousins, 2018; Tuschl et al., 2016). Aside from Mn transport and expression in the liver, ZIP14 has also been found to be expressed in the intestinal villi and has been linked with intestinal Zn regulation as well (Aydemir and Cousins, 2018)

On the other hand, mutations of ZIP8 in humans have been associated with decreased levels of both Zn and Mn in the blood, suggesting that like ZIP14, ZIP8 also has a role in maintaining both Mn and Zn homeostasis during normal conditions. In support of this, a study by Lin et al (2017) elucidated the functioning of ZIP8 in Mn homeostasis through the use of ZIP8 global knockout, ZIP8 liver-specific knockout, and ZIP8 liver-specific overexpression mice models. The group found that ZIP8 performs a vital function through expression at the hepatocyte canalicular membrane in the liver to aid in the reabsorption of Mn from the bile to limit Mn excretion and maintain whole body Mn homeostasis (Lin et al., 2017). Furthermore, the loss-of-function of ZIP8 has also been associated with many diseases and disorders including intellectual disability, delayed development, stunted growth, organ hypoplasia, and cerebellar atrophy, all of which highlight the critical role of ZIP8 under normal conditions (Li et al., 2016; Lin et al., 2017; Park et al., 2015). Whether ZIP8 plays the same role in Mn homeostasis in fish as in mammals still remains elusive, and could provide potentially valuable insight into the roles and regulations of Mn contents in the body.

While much progress has been made with regards to the specific functions of the different ZIP transporters, many unanswered questions still remain, and the function of many transporters still continue to be unknown. How the different ZIPs co-ordinate with one another and respond during periods of stress (e.g., excess Zn), how the expression of these transporters change during development from early life stages to adulthood, and why the need for such a complex transport system involving multiple transporters in the body (12-14 isoforms), are among some of most interesting questions to be addressed.

1.4 Risks and consequences associated with Zinc deficiency

With today's knowledge, it can be recognized that Zn plays a fundamental role across many phylogenetic levels, however the critical importance of Zn has not always been appreciated. Although characterized to be essential in mammals (rats) since 1934, the essential role of Zn in humans was not considered until much later in the 1960s (Todd et al., 1934). In 1963, Prasad et al identified men in Iran who were exhibiting abnormal phenotypes that included growth retardation, testicular atrophy, and rough skin, as well as mental deficits, all of which were attributed to be a result of Zn deficiency due to insufficient dietary Zn uptake (Prasad et al., 2001; Prasad et al., 1963). Similar consequences associated with Zn deficiency (i.e. embryonic abnormalities, testicular atrophy, growth defects) have also been reported in other animal models such as mice and calves further demonstrating the implications associated with decreased levels of Zn intake (Blamberg et al., 1960; Miller and Miller, 1960).

Since these early studies, many more consequences of inadequate Zn availability have come to light. Suboptimal Zn uptake has now been linked to poor pregnancy outcomes, premature birth, low birth weights, and increased congenital malformations (Hess and King, 2009; Ho et al., 2012; Meadows et al., 1983; Scholl et al., 1993; Shah and Sachdev, 2006). Other long term health complications associated with Zn deficiency include increased susceptibility to diabetic stress, impaired learning and memory, gastrointestinal dysfunction, and compromised immune health (Halas et al., 1986; Ho et al., 2012; Skrovanek, 2014; Uriu-Hare et al., 1989). Furthermore, insufficient Zn uptake in animals has also been characterized to result in growth inhibition (Blamberg et al., 1960; Prasad et al., 1963; Yan et al., 2012), which is suggested to result from a decreased availability of Zn to participate in RNA transcription and protein synthesis as an enzymatic cofactor.

In fish specifically, Zn deficiency has been associated with anorexia, poor growth, bone deformations, reduced survival, cataracts disease, and an exaggerated startling response (Eid and Ghonim, 1994; Gatlin and Wilson, 1983). In one study, rainbow trout (*Oncorhynchus mykiss*) fed Zn deficient diets ranging from 1 to 3 mg Zn/kg (dry weight) demonstrated decreased rates of growth, decreased survival, and visible signs of fin erosion when compared to fish fed with control diets (15-30mg/kg food) (Spry et al., 1988). Interestingly, in this study replacement of deficient Zn diets with control diets was found to alleviate and reverse the detrimental symptoms experienced by the fish.

Overall, the studies examining and demonstrating the implications associated with Zn deficiency have made very apparent the vital importance of maintaining an adequate intake of Zn in the body.

1.5 Zinc overload and toxicity: How much Zinc is too much Zinc?

Although the previous section described the many detrimental effects associated with suboptimal Zn availability, it must also be noted that when introduced in quantities above the nutritional threshold, Zn overexposure can cause many toxic effects as well.

In mammals, Zn poisoning is most often associated as an occupational hazard and has been reported to result in anorexia, depression, diarrhoea, convulsions, and in severe cases death (Ogden et al., 1988). Excess Zn intake in aquatic animals such as fish and frogs has been found to be consequential and is linked with many teratogenic deformities (Fort et al., 1989; Küçükoğlu et al., 2013; Salvaggio et al., 2016; Stebler et al., 1988). The risks associated with Zn exposure is far greater for aquatic organisms than terrestrial organisms due to the extreme sensitivity of the gills in addition to the extra stress of aquatic metal contaminations (see section 1.6).

Furthermore, when comparing mammals to aquatic organisms during early development, Zn toxicity has been found to be much less severe in mammals due to the protective developmental mechanisms available to these species (e.g., maternal liver and metalloenzymes). These same mechanisms are often not available to oviparous aquatic organisms, leaving their embryos vulnerable to potential developmental defects during exposure to elevated environmental levels of Zn (Küçükoğlu et al., 2013; Nagel, 2002).

In previous studies, high waterborne metal concentrations have been reported to induce deleterious effects in the gill of fish, including inflammation and increased mucus secretion which consequentially impair gas exchange and produce potentially severe downstream events (McDonald and Wood, 1993; Skidmore and Tovell, 1972). One of the first studies assessing the toxicity of Zn in fish found that adult common carp (*Cyprinus carpio*) acutely exposed (3 hours) to extremely high levels of Zn (20 mg/L) displayed an increased hematocrit value, elevated plasma glucose levels, decreased leukocyte number, and immature erythrocyte release into the bloodstream. These changes together indicated a high-stress response as well as a disturbance in specific immune functions in the carp as a result of acute Zn exposure (Witeska and Kosciuk, 1962). Other studies with zebrafish (*Danio rerio*) found that exposure to extremely high levels of Zn (0.5-200 mg/L) during development resulted in lateral curvature of the spine, severe edema, and increased vascular stress (Küçükoğlu et al., 2013; Salvaggio et al., 2016). These morphological defects associated with the exposures caused the loss of swimming and feeding ability in the larvae which eventually led to decreased survival. Similarly, another study using extremely high levels of Zn demonstrated that zebrafish larvae acutely exposed to ≥ 1.0 mg Zn/L (as Zn chloride) during early development displayed abnormal embryogenesis and delayed

hatching, with many embryos exhibiting mortality without ever hatching from the chorion at 11 or 12 days post fertilization (Küçükoğlu et al., 2013).

Interestingly, the impacts of Zn exposure on reproductive capabilities have also been investigated. It was observed that after a 9-day exposure to 5 mg/L Zn, adult zebrafish displayed decreased and delayed spawning and in instances where spawning did occur the Zn-exposed mating pairs exhibited decreased egg production and reduced embryo viability. Interestingly, the negative impacts of Zn overexposure on reproduction were shown to be readily reversed by returning the exposed fish to a normal background level of waterborne Zn (Speranza and Seeley, 1977). Similarly, additional studies have investigated the reproductive sensitivity of other fish species to Zn (Driessnack et al., 2017). One study using fathead minnow (*Pimephales promelas)* found that sublethal doses of Zn (150µg/L) decreased egg production by over 80% but interestingly had no effect on survival of the adults (Brungs, 1969), suggesting that reproduction may be one of the most sensitive endpoints of chronic sublethal Zn exposure in adult FW fish (Brungs, 1969; Driessnack et al., 2017; Speranza and Seeley, 1977; Suter et al., 1987).

Importantly, the main toxic action pathway associated with acute Zn exposures has been linked to Zn-induced hypocalcaemia (Hogstrand, 2012). Hypocalcaemia has the potential to induce abnormal bone development, muscle spasms, seizures, and impaired cardiac function, which together can adversely affect the physiological performance of fish and potentially prove to be lethal (Catalano et al., 2012; De Schamphelaere and Janssen, 2004; Hogstrand et al., 1995; Hogstrand et al., 1996; Hogstrand et al., 1998; Hogstrand, 2012; Niyogi et al., 2006; Spry and Wood, 1985; Yamaguchi et al., 1983).

These studies briefly summarized here provide a snapshot of the significant impacts and consequences associated with the contamination of Zn in the aquatic ecosystem. It must be noted

that although these studies discussed here provide valuable information on Zn toxicity, the exposure levels used were often far above the environmentally relevant exposure levels of Zn. Therefore, we suggest that there is a clear need to study the chronic and developmental effects of exposure to environmentally relevant levels of elevated Zn to provide a more meaningful insight into the impacts of Zn contamination in the natural aquatic environment.

1.6 Zinc in the environment: Environmental risk and potential areas of concerns

Zn is an important natural constituent of all aquatic environments around the world. Under normal conditions, global FW environments are reported to contain Zn levels that fluctuate anywhere from as low as 0.1 µg/L up to 50 µg/L, with an average background concentration of 12 µg/L seen in Canadian surface waters (Environment and Climate Change, 2019; Feeney et al., 2005). Naturally, Zn can enter the aquatic environment in low concentrations through rock and soil as sulphide ores and carbonates (Environment and Climate Change, 2019). However, as a result of numerous anthropogenic activities and increased use of Zn in our everyday lives (e.g., steel galvanization, alloys, rubbers, paint, agricultural feeds, vitamins), Zn contamination in aquatic ecosystems around the world has increased substantially. This increased Zn content has now led to Zn being labelled as a priority aquatic contaminant with concentrations reaching upwards of 5 mg/L in metal-contaminated waters (detailed below).

The input of Zn into the global environment is estimated to be upwards of 8.8 million metric tons yearly, >90% of which is a result of anthropogenic related activities (Eisler, 1993). The corrosion of Zn galvanized metals and Zn alloys has been recognized to be the largest contributor (>30%) of total Zn addition to surface waters and aquatic systems in recent years (Bodar et al., 2005). Other anthropogenic factors such as wastewater treatment, drainage of

agricultural soils, effluents from fish farms, domestic and industrial sewage, steel production, burning of waste materials, fertilizer leaching, road runoff, and mining have all also contributed to the growing environmental concern of increased Zn contents in FW habitats (Eisler, 1993; Legret and Pagotto, 2006; Llobet et al., 1988; Mirenda, 1986; Preciado and Li, 2006). The increased concentration of Zn in aquatic systems has generated a great level of concern as metal ions such as Zn are considered persistent contaminants, signifying that they cannot be degraded but rather continue to exist and transform between different chemical species within the polluted environmental compartment.

The Canadian water quality guidelines (CWQG) for the protection of aquatic life sets the limit of acute Zn exposures in natural FW environments to be 37 µg/L (Canadian Council of Ministers of the Environment, 2018) and is based upon a Zn species sensitivity distribution while other regulatory agencies such as The United States Environmental Protection Agency dictate 66 µg/L as the highest continuous (chronic) concentration acceptable in natural environments with water hardness values of 50 mg/L (United States Environmental Protection Agency, 1996). Unfortunately, numerous cases of extremely high levels of Zn far exceeding those threshold concentrations set out by government regulatory agencies have been recorded in waters around the world. At these sites, Zn reaches levels so high that chemical migration barriers are generated for aquatic species living in the region. One such case was recorded at the Boulder River watershed in Montana, USA where due to mining activities at the Comet Mine, the levels of Zn reached an alarming 5.7 mg/L (water hardness = 135 mg/L as $CaCO_3$). In this river, cutthroat trout (*Oncorhynchus clarkii*) populations were found to reside upstream from the mine but interestingly no populations were located downstream. In fact, transplanted populations of

cutthroat trout placed downstream from the mine suffered almost 100% mortality within 5 days of transplantation (Farag et al., 2003; Hogstrand, 2012).

A case similar to the Boulder River watershed was noted at the Hayle River in the United Kingdom which hosts populations of brown trout (*Salmo trutta*). Unfortunately, the river flows through a region where Zn mining activities were prevalent in the 16th century and now contains levels of Zn recorded to reach upwards of 1 mg/L (Hardness = 100 mg/L as $CaCO_3$). Still, hundreds of years later, no fish populations have been recorded within a 2 km stretch downstream from the old mining site (Brown, 1977).

Another case associated with ecological Zn pollution includes the disappearance of the stone loach (*Noemacheilus barbatulus*) in streams of the United Kingdom. This disappearance is now recognized to be a result of industrial wastewater contamination raising Zn concentrations in the streams to an alarming 5 mg/L (De and Flook, 1975).

More recent examples associated with Zn contamination in aquatic environments include a fish kill at the Mokelumne River in California where Zn concentrations were found to be upwards of 1.4mg/L, the Northern Egyptian lakes with levels as high as 460 µg/L, as well as in the French lot river where levels reached upwards of 1 mg/L (Andres et al., 2000; Saeed and Shaker, 2008; United States Fish and Wildlife, 1998). Furthermore, a Canadian study performed from 2011-2015 compared multiple mining facilities by measuring the surface water concentrations of local water bodies after mining against reference concentrations. Here, mining was found to elevate Zn concentrations in nearby aquatic systems by up to 200 µg/L (Environment and Climate Change, 2019). In line with these data, the average predicted environmental concentration (PEC) as a result of metal mining in Canada is estimated to be 253

µg/L (Environment and Climate Change, 2019), which is significantly above those safe levels recommended by regulatory agencies.

These past and continuing experiences related to the increased concentrations of Zn in the natural environment provide substantial evidence for the need to bring awareness to the topic of Zn pollution and the requirement to investigate further the impacts of waterborne Zn exposure on the wildlife populations affected in these environments. Some examples of concentrations of waterborne Zn recorded in polluted FW environments have been summarized in Table 1.2.

Table 1.2. Elevated levels of Zn seen in metal polluted FW environments globally

Location	Concentration (µg/L)	Reference
French Lot River, France	1000	(Andres et al., 2000)
Boulder River, USA	5700	(Farag et al., 2003; Hogstrand, 2012)
Hayle River, UK	>1000	(Brown, 1977)
Mokelumne River, USA	1300	(United States Fish and Wildlife, 1998)
United Kingdom Rivers	5000	(De and Flook, 1975)
Northern Egyptian Lakes, Egypt	460	(Saeed and Shaker, 2008)
Canadian Reference Mining Facility, Canada	205	(Environment and Climate Change, 2019)

*It must be considered that various water parameters which may impact Zn bioavailability are not reported here.

Although assessing the concentrations of dissolved Zn in aquatic systems is of extreme importance and should be continually monitored, it is critical to understand that much of the Zn that enters these environments is also sequestered into the sediment, creating yet another area of

concern (Eisler, 1993; Environment and Climate Change, 2019). Zn sequestered in the sediment can directly be consumed by aquatic organisms that feed in the sediment or can also be accumulated by benthic invertebrates. Many invertebrates due to their sedentary nature act as filters for the aquatic environment, passing water through them, and in the process accumulate large concentrations of pollutants (including Zn). Tissue concentrations of Zn found in a variety of invertebrate species in Zn contaminated aquatic systems have been recorded to reach upwards of 5000 mg Zn/kg (summarized in Table 1.3). This accumulated Zn within benthic invertebrate species poses a major exposure risk to the carnivorous and omnivorous fish that feed upon them by significantly increasing the dietary Zn load for fish far beyond the nutritional level (most teleost fish require a dietary intake of Zn between 15 -30 mg Zn/kg dry mass of diet with up to 90 mg Zn/kg dry mass of diet being considered adequate for other species) (Clearwater et al., 2002). More interestingly, field studies have also demonstrated that metal-contaminated invertebrates fed to rainbow trout induced toxic effects at lower metal concentrations than required in laboratory prepared diets, suggesting that Zn in these invertebrates is more bioavailable than Zn salts commonly used in prepared diets (e.g. $ZnSO_4 \cdot 7H_2O$) (Clearwater et al., 2002; Woodward et al., 1995). Whether this increased dietary Zn exposure due to bioaccumulation can elicit deleterious downstream consequences for the exposed fish is a question that remains poorly understood as few studies have directly assessed the implications of environmentally relevant dietary Zn loading in fish (Ogino and Yang, 1978; Watanabe et al., 1997).

Table 1.3. Tissue concentration of invertebrate species collected from Zn contaminated aquatic systems.

Species	Location	Tissue Concentration (mg Zn/kg BW DW)	Reference
Crassostrea Commercialis	Chesapeak Bay, Mexico	3975	(Presley et al., 1990)
Pooled Mussel species	Diasnshan Lake, China	684.9	(Wu et al., 2017)
Crassostrea iridescens	Mazatlan Bay, Mexico	1160	(Soto-Jiménez et al., 2001)
Crassostrea gigas	Amurskiy Bays, Sea of Japan	5280	(Shulkin et al., 2003)

Although many countries have begun to recognize the impacts of environmental Zn pollution, a major lack of quality criteria overall can still be seen. Unfortunately, many developing countries rely on the water quality guidelines set by industrial nations irrespective of potentially different environmental conditions and stressors. These countries do not take into account various influencing parameters which could have a large impact on Zn bioavailability and toxicity even at low soluble concentrations of Zn (e.g., water hardness and pH; discussed in section 1.7), resulting in non-specific, non-representative guidelines. Furthermore, guidelines directly addressing the dietary concerns of Zn exposure for aquatic populations are non-existent all together, alarming the need for significant attention. Given this information, it is of extreme importance for countries to begin to implement assessment measures to ensure that set criteria are accurate and reasonable to safeguard the resident aquatic organisms in these environments. One such approach supports the inclusion and use of the biotic ligand model (BLM) to account for the differences in Zn availability and toxicity based on water chemistry to generate region-specific criteria/guidelines for Zn (Niyogi and Wood, 2004).

1.7 Zinc Speciation in aquatic systems: Chemistry and modulating factors

In natural waters, Zn may either remain dissolved in the water column or be transferred to the bottom sediment. In the dissolved form, the speciation of Zn is highly dependent on the composition and chemistry of the water, and more specifically is modulated by the levels of dissolved organic matter (DOM), anionic ligands (e.g., Cl^-, CO_3^{-2}, SO_4^{-2}), and pH of the water (Ho et al., 2012; Huang et al., 2010; Niyogi and Wood, 2004). Understanding the speciation of Zn, and more specifically the conditions in which free Zn^{2+} becomes more abundant is an important aspect of evaluating the implications of Zn pollution. This is because free Zn^{2+} is believed to the most bioavailable and toxic form of Zn to aquatic organisms (Bury, 2003).

At low water pH (below pH 7) and low DOM level, the formation of free Zn^{2+} is promoted and is often present as the predominant form of inorganic Zn (Eisler, 1993). In contrast, the presence of Zn hydroxides and Zn carbonates become increasingly abundant as pH rises to about pH 8 at which point they begin to dominate over free Zn^{2+} (Bervoets and Blust, 2000; Evans, 2000; Qiu and Hogstrand, 2005).

Moreover, increasing levels of alkalinity and water hardness (dissolved calcium and magnesium) have been documented to exhibit a protective effect on FW fish by reducing lethal concentration 50 (LC_{50}) values and reducing short-term Zn uptake during acute exposures. For example, the 96h LC_{50} in soft water (11.7 mg/L as $CaCO_3$) was measured to be 2500 µg/L in larval zebrafish, whereas no significant mortalities were observed in hard water (141 mg/L as $CaCO_3$) up to a Zn concentration of 2230 µg/L (Alsop and Wood, 2011). Comparably, the 120h LC_{50} for juvenile rainbow trout in soft water (20 mg/L as $CaCO_3$) was reported to be 162 µg/L, while it was 869 µg/L in hard water (120 mg/L as $CaCO_3$) (Alsop et al., 2009). More specifically, these hardness cations (i.e Ca^{2+} and Mg^{2+}) are able to compete with Zn^{2+} for binding

at the gill, effectively reducing the uptake and toxicity of Zn during exposure (Niyogi and Wood, 2004; Zitko and Carson, 1976). In contrast, alkalinity serves a protective effect during periods of exposure through the promotion of inorganic complexations of Zn^{2+} with HCO_3^- and OH^- to lower the amounts of free Zn^{2+} available (Niyogi and Wood, 2004; Pagenkopf, 1983).

In addition, DOM is known to bind Zn with moderately high affinity (Hoffmann et al., 2007; Hogstrand, 2012), and in instances when considerable concentrations of DOM are present, organic species of Zn tend to dominate over inorganic forms thereby limiting the bioavailability and free ionic activity of Zn^{2+}. Conversely, under conditions of high DOM but low pH, H^+ ions compete with Zn^{2+} for binding sites on DOM, limiting the organic complexation of Zn^{2+} and thereby rendering it more bioavailable.

1.8 The interaction of Zn with other major ion and trace metals

Though previous sections in this review have highlighted the ability of Zn to be transported through specific Zn transport pathways (i.e. ZIPs), one important aspect of Zn exposure that often remains overlooked is the interactions of Zn with other metals. The excess availability of Zn in the environment may potentially impact the uptake and transport kinetics of other essential metals, consequentially negatively impacting the physiological condition of the exposed organisms.

The interaction of Zn and Ca has been well characterized in recent years. Spry and Wood (1989) first demonstrated that increased concentrations of waterborne Ca were correlated with decreased branchial Zn uptake in adult rainbow trout. Comparably, a follow-up study investigating the influence of waterborne Zn [150 µg/L] on the unidirectional Ca uptake in juvenile rainbow trout found that the presence of elevated Zn increased the K_m (inverse of

affinity) of branchial Ca transport by 300% with a marginal effect on J_{max} (maximum transport rate) (Hogstrand et al., 1994). Importantly, these findings suggested a competitive interaction between Zn^{2+} and Ca^{2+}, likely through a shared transport mechanism at the gills of fish (Hogstrand and Wood, 1996; Hogstrand et al., 1994, 1998). The epithelial calcium channel (ECaC/Trpc6) has now been identified to be responsible for this competitive interaction between Zn and Ca, as EcaC has been found to be localized at the apical gill membrane and permeable to both Zn^{2+} and Ca^{2+} (Qiu and Hogstrand, 2004; Shahsavarani et al., 2006; Shahsavarani and Perry, 2006). Although most studies have mainly investigated the interaction of these two metals at the gill, an interaction between brachial and gastrointestinal uptake of Zn and Ca was also previously reported, as dietary supplementation of Ca was found to decrease branchial uptake of both Zn and Ca (Niyogi and Wood, 2006). While these studies summarized here provided important insights into the interaction of Zn and Ca, they were all carried out in limited number of species and also almost exclusively through waterborne exposures. Whether these interactions vary among species, developmental life stage (embryonic vs larval vs adult), or route of exposure (waterborne vs. dietary) remains to be investigated.

Aside from the well-documented interactions with Ca, Zn has also been noted to interact with Cu in both mammals and fish. In mammals, chronic dietary Zn supplementation has been correlated with the onset of Cu deficiency (Gyorffy and Chan, 1992). Similarly, the inhibitory effects of Zn and Cu supplementation on the uptake of one another have also been reported in the intestine of rainbow trout (Glover and Hogstrand, 2003; Ojo et al., 2009). It was found that a 10-fold increase in dietary Zn concentration over Cu inhibited the uptake of Cu at the mid and posterior regions of the gut but interestingly did not impact absorption of Cu at the anterior region of the gut where Cu uptake is the greatest (Ojo et al., 2009). Furthermore,

supplementation with 20-fold excess Cu over Zn has been shown to result in a complete inhibition of Zn transport through the Zn specific transporter ZIP3 in pufferfish (*Takifugu rubripes*) (Qiu and Hogstrand, 2005). These results lend the possibility of a potential competitive interaction between Zn and Cu via specific Zn transporters (i.e ZIP3).

Zinc may also disrupt the homeostasis of other essential elements through interactions that are yet to be characterized. The knowledge of such interactions is critical in order to understand the true impacts of Zn contaminations on the health and long-term sustenance of aquatic organisms in the natural environment.

1.9 Rationale and Objectives of my thesis

1.9.1 Study Rationale

The scientific information presented above has made apparent the vital physiological role played by Zn in organisms, while also providing evidence for the rising levels of Zn in FW systems globally. Understanding how aquatic organisms are affected by elevated levels of Zn is a crucial aspect of understanding the implications of Zn contamination in natural environments. Though recent studies have provided important information on Zn toxicity, these studies have often used extremely high levels of Zn exposure that are generally far above the Zn concentrations reported in the natural environment. In my study, sub-lethal environmentally relevant Zn exposure concentrations were used to better understand the impacts of Zn contamination on fish health in the natural environment. It is also important to note that although the dietary route of Zn exposure poses a significant risk to aquatic organisms including fish, the implications of dietary Zn exposure has been investigated sporadically. In addition, in Canada and many other jurisdictions, the water quality guidelines for the protection of aquatic life against the toxicity of most metals including Zn are based solely on waterborne exposures, with

little to no attention given towards dietary exposures. Thus, my research was also aimed to characterize the negative consequences of elevated dietary Zn exposure in fish – information that may aid future ecological risk assessment of Zn in aquatic ecosystems and development of water quality guidelines for Zn that can account for potential dietary Zn toxicity to aquatic organisms.

Moreover, to date most of the investigations into the effects of elevated Zn exposures have been focused on adult fish, and the implications of chronic environmentally relevant Zn exposures on early life stages (when many species display the greatest sensitivity to metal exposures) remains largely unknown. Thus, my study also aimed specifically to focus on understanding the effects of elevated Zn exposure in fish during development.

A final aim of my study was to address the current knowledge gap on the physiological basis for the complex transport mechanisms of Zn uptake in fish. In comparison to other trace metals and major ions, cellular Zn uptake occurs via a very intricate pattern being regulated by over 12 specific Zn transporters. The exact functionality of each transporter remains poorly understood and thus warrants further investigation. In my thesis, I hoped to provide new insights into the different functions of these transporters from a physiological point of view while addressing the need for such a complex system.

1.9.2 Model Organism

For the current study, zebrafish (*Danio rerio)* was selected as the model organism of choice. Zebrafish carry many advantages as a model organism for various reasons which include: "high fecundity, small size, rapid generation time, and ease of handling" all of which collectively allow for high throughput and high sample size experiments (Lawrence, 2007). Furthermore, zebrafish is a highly conserved vertebrate model with a readily available sequenced genome database. Access to this gene database will allow for development of specific primer sets that can

be used for the analysis of many Zn transport related genes. Additionally, zebrafish also display optical transparency during embryogenesis, this benefit will allow for whole-mount *in-situ* hybridization techniques to visualize the expression of these various Zn transport genes on the whole organism. Finally, the clear classification and characterization of developmental timepoints in zebrafish allows for the investigation of the impacts of Zn exposure during different early life stages to understand how these sensitivities to Zn change at different life stages.

1.9.3 Hypothesis and Objectives

Hypothesis I: The various *zip* transporters are expressed in a tissue-specific manner in early-life stage zebrafish and this expression pattern differs from that in adults

Objective*:* Examine the tissue localization of the various *zip* transporters during both early development and at the adult stage to better understand the specific function and role of each transporter in maintaining whole body Zn contents and identifying how this pattern changes across different life stages.

Methods: Casper line of zebrafish will be collected at 5 days post fertilization (dpf) and analyzed to develop a tissue distribution profile of *zip* using whole-mount *in-situ hybridization* techniques. Adult zebrafish will be collected, dissected, and tissues will be analyzed for *zip* expression using droplet digital PCR (ddPCR) technologies. Expression of *zip* between these life stages will then be compared and contrasted.

Hypothesis II: Fish at the early life stages are sensitive to the levels of elevated waterborne Zn observed in metal-contaminated environments.

Objective: Examine the effects of environmentally relevant concentrations of waterborne Zn on the physiological processes in zebrafish during the early life stage to understand possible consequences of Zn pollution to aquatic species. Specific considerations for this study include deleterious effects on physiological condition, alteration of metal homeostasis and possible interactions of Zn with other metals, and Zn transporter regulation as a compensatory response to better understand the role of each transporter in maintaining Zn homeostasis under stress.

Methods: Zebrafish larvae will be exposed to 261 µg/L (4µM) Zn from 0-5 dpf and compared to controls to examine changes in terms of standard body length, hatching rate, and survival. Larvae will also be collected during this exposure regime and analyzed via inductively coupled plasma mass spectrometry (ICP-MS) to examine whole body levels of various major ion and trace metals in response to sublethal Zn exposure to identify potential interactions of Zn with a spectrum of other essential metals. ZIP mRNA expression will be assessed using RNA extraction and ddPCR technologies to determine patterns of regulation in response to exposures.

Hypothesis III: Increased dietary Zn exposure (as observed in metal-contaminated environments) alone or in combination with elevated waterborne Zn will have deleterious impacts on the physiological conditions of fish during development.

Objective: Evaluate the effects of dietary Zn exposure with or without excess waterborne Zn on the physiological conditions of developing fish at different early life stages to understand the possible negative impacts of dietary Zn exposure on fish during development and provide a rationale for the potential need of regulatory guidelines that account for dietary Zn toxicity.

Methods: Zebrafish larvae will be exposed to an elevated Zn diet containing 1500 mg Zn/kg food and/or with waterborne Zn 261 µg/L (4µM) Zn from 5 dpf to 28 dpf, and evaluated for effects on body length, feed consumption, survival, and physiological status using a variety of laboratory techniques.

Hypothesis IV: Increased dietary Zn exposure in the presence or absence of elevated waterborne Zn will influence the homeostasis of other major ions and trace metals while also modulating the expression of various ZIP transporters in response to elevated Zn. More specifically, the responses elicited from Zn supplementation will be exposure route specific (i.e., waterborne vs dietary vs combined exposure).

Objective: Evaluate the effects of dietary Zn exposure (with or without excess waterborne Zn) on whole body metal homeostasis to understand the interactions of Zn with other metals at the gut and how these interactions differ from those interactions at the gill. Furthermore, assessment of the response of each *zip* transporter in maintaining Zn homeostasis under different conditions of stress (i.e., waterborne Zn vs dietary Zn vs combined exposure) at different developmental timepoints will also be compared and assessed.

Methods: Zebrafish larvae will be treated with an elevated Zn diet containing 1500 mg Zn/kg food and/or with waterborne Zn 261 µg/L (4µM) Zn from 5 dpf to 28 dpf. Throughout this exposure regime larvae will be collected and analyzed for whole body concentrations of metals and major ions using ICP-MS to assess potential interactions of Zn with a spectrum of other essential metals. Furthermore, during the study, larvae at different developmental stages will be collected and ddPCR technologies will be used to determine changes in ZIP mRNA expression in response to different routes of Zn exposure.

Overall Study Hypothesis: I hypothesize that fish at the developmental stages will be sensitive to exposures of environmentally relevant concentrations of Zn through both the waterborne and dietary routes triggering physiological compensations and responses, and that this sensitivity will shift with developmental age.

Overall Study Objective: In my study I aim to use zebrafish as a model organism to test the effects of Zn exposure (waterborne and dietary) using environmentally relevant concentrations to understand the implications of Zn pollution on resident aquatic organisms. I intend to survey a wide variety of endpoints at multiple developmental ages to determine what compensations are made by fish to survive and maintain internal homeostasis during periods of elevated Zn exposures, and assess how these responses change over development.

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CHAPTER II:

Regulation of metal homeostasis and zinc transporters in early life-stage zebrafish following sublethal waterborne zinc exposure

2.1. Abstract

In the present research chapter, the effects of exposure to a sublethal concentration of zinc (Zn) on metal and ion homeostasis, and the regulation and the localization of various Zn transporters (i.e., the Zrt-Irt Like Protein (ZIP) family of Zn transporters), were investigated in zebrafish (*Danio rerio*) during early development. Exposure to an elevated level of Zn [4 μM (high) vs. 0.25 μM (control)] from 0 day post-fertilization (dpf) resulted in a significant increase in the whole body content of Zn at 5 dpf. A transient decrease in the whole body calcium (Ca) level was observed in 3 dpf larvae exposed to high Zn. Similarly, whole body nickel (Ni) and copper (Cu) contents were also reduced in 3 dpf larvae exposed to high Zn. Importantly, the magnitude of reduction in whole body Ni and Cu contents following Zn exposure was markedly higher than that in Ca content, suggesting that internal Ni and Cu balance were likely more sensitive to Zn exposure in developing zebrafish. Exposure to high Zn altered the mRNA expression levels of specific zip transporters, with an increase in *zip1* (at 3 dpf) and *zip8* (at 5 dpf), and a decrease in *zip4* (at 5 dpf). The expression levels of most *zip* transporters tended to decrease from 3 dpf to 5 dpf with the exception of *zip4* and *zip8*. Results from *in-situ* hybridization revealed that several *zip* transporters exhibited distinct spatial distribution (e.g., *zip8* in the intestinal tract, *zip14* in the pronephric tubules). Overall, our findings suggested that exposure to sublethal concentrations of Zn disrupts the homeostasis of essential metals during early development and that different ZIP transporters may play unique roles in regulating Zn homeostasis in various organs in developing zebrafish.

2.2. Introduction

Zinc (Zn) is an essential trace metal that plays a critical role in many physiological functions, including immune defense, response to oxidative stress, as well as acting as a secondary messenger (Tammy and Bettger, 1990; Stefanidou et al., 2006; Ho et al., 2012; Zhao et al., 2014). Intracellular Zn levels are typically low, being maintained in the femtomolar-picomolar range (Zhao et al., 2014). However, it is important to note that Zn can be toxic when its concentration in the body exceeds the normal homeostatic range (Skidmore and Tovell, 1972; McDonald and Wood, 1993; Niyogi et al., 2008; Salvaggio et al., 2016).

In freshwater (FW) environments, Zn levels are generally in the range of 0.1 μg/L to 50 μg/L (Feeney et al., 2005). However, anthropogenic activities such as steel production, burning of waste materials, metal mining, and fertilizer leaching have been reported to be major sources responsible for increased Zn levels in various FW environments around the world (Legret and Pagotto, 2006; Preciado and Li, 2006; Hogstrand, 2012). For example, several studies have reported that Zn concentrations in FW systems could reach over 500μg Zn/L (e.g., French lot river, 1000 μg/L; Northern Delta Lakes in Egypt, 460 μg/L) (Andres et al., 2000; Feeney et al., 2005; Saeed and Shaker, 2008; Salvaggio et al., 2016; Zheng et al., 2016). In waters, the toxicity of Zn is highly dependent on the amount of free Zn^{2+}, which is the most bioavailable form. The speciation and the bioavailability of Zn are known to be modulated by water chemistry such as pH and hardness (Niyogi and Wood, 2004; Huang et al., 2010; Ho et al., 2012).

It has been documented that waterborne Zn toxicity in fish varies with developmental age. Zn appears to be less toxic when fish are still in the embryonic stage, likely due to the reduced absorption in the presence of the chorion (Pickering and Vigor, 1965; Kazlauskienė and Stasiūnaitė, 1999). In larval zebrafish (Danio rerio), the 96 h LC_{50} of Zn in soft water (11.7 mg

$CaCO_3$/L) was reported to be 2.5 mg Zn/L, while the 96h LC_{50} in hard water (141mg $CaCO_3$/L) was believed to be much greater with concentrations of 2.3mg Zn/L resulting in no significant mortalities (Alsop and Wood, 2011). Exposure to > 0.5 mg Zn/L was also shown to cause skeletal deformities, reduced growth, and increased mortality in larval zebrafish (Salvaggio et al., 2016). Several studies have also documented the lethal effects of Zn in adult and juvenile rainbow trout (Oncorhynchus mykiss) (Mcgeer et al., 2000a; McGeer et al., 2000b; De Schamphelaere and Janssen, 2004). However, the sublethal effects of Zn exposure on early life stages of fish have remained poorly understood.

In mammals, cellular Zn homeostasis is largely maintained by two families of Zn transporters, Zrt-Irt-like-proteins (ZIP, SLC39a) and Zn transport proteins (ZnT, SLC30a). ZIP transporters are primarily involved in the apical uptake of Zn while ZnT's are involved in the basolateral extrusion. In humans, 14 members of the ZIP family have been identified, with 12 of these transporters also being present in zebrafish. Previous studies have examined the transcriptional regulation of Zn transporters and their tissue distribution in adult zebrafish (Feeney et al., 2005; Zheng et al., 2008, 2010). However, their potential modulation by Zn exposure in developing fish has yet to be investigated.

In addition to ZIP transporters, it has also been suggested that Zn shares an apical surface transporter with calcium (Ca) (Hogstrand et al., 1998; Qiu and Hogstrand, 2004). The acute toxicity of Zn has been linked to impaired Ca transport via competition, leading to hypocalcemia (Hogstrand et al., 1994, 1995; De Schamphelaere and Janssen, 2004). Moreover, chronic exposure of rainbow trout to 250 μg Zn/L for 30 d has been demonstrated to disrupt and reduce the carcass concentrations of Ca as well as Na and Cu during the first week of exposure (Hilmy et al., 1987; McGeer et al., 2000; Alsop and Wood, 2011).

Although previous studies have provided important information on the toxic effects of Zn exposure on adult fish, the regulation of Zn homeostasis during early development is not completely understood. Additionally, the sublethal effects of Zn exposure and the regulation of ZIP transporters in the early life stages of fish also remain largely unknown. In the present study, we used larval zebrafish as a model organism to test the hypothesis that fish at early developmental ages are vulnerable to Zn exposure; exposure to sublethal concentrations of waterborne Zn adversely affects the homeostasis of Zn and potentially other essential metals or ions (e.g., Ca). We also hypothesized that different zip transporters are expressed in various organs in larvae and that their expression levels are modulated by Zn exposure.

2.3. Methods

2.3.1 Animals

Adult zebrafish (TL strain) were maintained in recirculating systems (Aquaneering, CA, USA) containing 7.55 ± 0.85 μg/L Zn. The fish were kept at pH 7.4, 28 °C with a 14h:10 h light: dark cycle. Water hardness and alkalinity were 150 mg/L and 65 mg/L (as $CaCO_3$), respectively. All exposure experiments were performed in this condition except mentioned otherwise. Adults used for breeding were fed daily with a mixed diet consisting of brine shrimp and a commercial diet (Zeigler, PS, USA). Fish were bred by placing female and male pairs in breeding traps overnight with traps being opened and embryos collected the following morning. The embryos were transferred to Petri dishes (50 embryos/dish) kept in an incubator at 28°C until exposure. The measured concentrations of major ions and trace metals in the exposure waters are summarized in Tables 2.1 and 2.2 (methods detailed below). All exposure experiments were performed between 0 and 5 days post-fertilization (dpf) and the procedures were conducted in

compliance with the guidelines outlined by the Canadian Council of Animal Care and the York University Animal Care Committee (2017−2).

Table 2.1. Ionic composition of the exposure waters.

	Major ion (mg/L)				Trace metals (µg/L)					
	Na	Mg	K	Ca	Mn	Fe	Co	Ni	Cu	Se
Concentration	105.0 ± 2.83	6.38 ± 0.04	4.70 ± 0.04	3.85 ± 0.02	0.34 ± 0.03	2.35 ± 0.33	0.07 ± 0.03	0.30 ± 0.04	14.49 ± 3.78	0.05 ± 0.00

Values are mean ± SEM from four independent measurements.

Table 2.2. Measured Zn concentrations in the exposure waters

	Low Zn treatment	High Zn treatment
Target concentration	0.25 µM (16.4 µg/L)	4 µM (261.6 µg/L)
Measured concentration	0.36 ± 0.04 µM (23.5± 2.6 µg/L)	4.46 ± 0.07 µM (291.6 ±4.6 µg/L)

Zn concentrations were analyzed using ICP-MS. The detection limit of Zn was measured to be 0.016 µg/L. Values are mean ± SEM from two independent measurements.

2.3.2 Assessment of physiological conditions

At 8 h post-fertilization (hpf), larvae were transferred to 50 mL Petri dishes (25 larvae/dish) containing either 16.4 µg/L Zn (0.25 µM Zn; as $ZnSO_4 \cdot 7H_2O$) or 261 µg/L Zn (4 µM Zn). Each Petri dish was considered as one replicate, with a total of four replicate dishes (N=4) per treatment. Dishes with 0.25 µM Zn were considered as controls (normal Zn concentration regularly found in FW environments). Dishes with 4 µM Zn were considered as high Zn treatments; this concentration was chosen based on Zn levels reported in Zn-polluted sites (see introduction). The fish were monitored for mortality and the number of larvae that had hatched from the chorion, with water being changed daily. At 5 dpf, larvae were also collected

for measurement of standard body length (from the tip of the snout to the base of the caudal peduncle) using the Leica MZ10F modular stereo microscope and the Leica Application Suite X software.

2.3.3 Exposure regime for assessment of metal body burden and zip mRNA expression level

A separate experiment was performed to collect samples for ICP-MS analysis and ddPCR analysis. At 8 hpf, embryos were transferred to 8 separate 2 L plastic tanks (350 embryos/tank) containing 1 L of water each. Four tanks were supplemented with 0.25 μM Zn and another 4 tanks with 4 μM (each tank was considered as one replicate; N = 4 per treatment). Water was changed daily (static renewal with > 90 % water change) during the experiment.

2.3.4 ICP-MS analysis

At 1, 3, and 5 dpf, 25 larvae from each replicate tank were collected and were pooled as one sample (N=1, with a total of N=4 per treatment). These ages were chosen in efforts to examine possible temporal effects of Zn exposure at different developmental stages (1 dpf, pre-hatching; 3 dpf, post-hatching; 5 dpf, free-swimming and prior to the beginning of exogenous feeding).

Methods for sample preparation for ICP-MS analysis are detailed in the Supplementary Material section with the detection limits presented in Table S2.1. In brief, larvae were acid digested with 6 N HNO_3 and the digested samples were diluted with 2% HNO_3. Water samples collected from the exposure tanks were acidified using 2% HNO_3. Both tissue and water samples were passed through a 0.45 μm nylon filter and subsequently analyzed by inductive coupled plasma mass spectrometry (ICP- MS; Agilent 8800 ICP-QQQ-MS) in the Water Quality Center at Trent University.

2.3.5 ddPCR analysis

To investigate possible modulation in the mRNA expression levels of *zip* transporters following Zn exposure, larvae at 3 and 5 dpf were collected (N = 4 per treatment, where each replicate consisted of 25 pooled larvae) and analyzed using droplet digital PCR (ddPCR). Complementary DNA (cDNA) preparation and ddPCR analysis were performed as described previously (Chandrapalan and Kwong, 2020) and the methods are detailed in the Supplementary Material. Briefly, 1 μg of extracted RNA was converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). The ddPCR reaction mixtures were then prepared using EvaGreen™ supermix (Bio-Rad). Droplets were analyzed using the QX200 Droplet Reader (Bio-Rad) and the QuantaSoft™ software. The primer sets used for the analyses are summarized in Table 2.3. The mRNA expression levels of various *zip* were normalized to *elf1* (housekeeping gene) expression levels. The interactive effects between Zn treatments and exposure time on *elf1* were tested using a two-way ANOVA analysis ($F_{(1,11)} = 0.06$, $P = 0.8$).

Table 2.3. Primer sets used for digital droplet PCR analysis.

Gene	Accession Number	FWD primer (5'-3')	REV primer (5'-3')
Zip1	NM_212583.2	ACGACGAATGCCAAGGTGTT	GATGCCGATGCCAAGAGGAG
Zip4	NM_001130777.1	AGCTACCACACGAGTTAGGTGA	CAGCCCGACGTACAGGAAGA
Zip6	NM_001001591.1	TACATCGCATGGCTTGGAGG	ATGAGGTGGAGGAGAGCGTC
Zip7	NM_130931.3	TGGTCTGTGGGTGCTAGGTG	TTGGAGCAGAGGGAGAGTGG
Zip8	XM_009307205.3	GCTCGGACACAGTCATTTCCC	AACGCACACGCATTCTGCTC
Zip9	NM_001013540.1	GACCAGCGTCCAGCTTATTGT	GCCTCTTTACTGCTCTGACTGA
Zip10	NM_200671.1	TTCACCAGTACGGCCTGAGC	CCCACATCCAAACCCATCCTGA
Zip13	NM_001005306.3	GGCAGAGAACGCCACTACCT	TGCAGAAGAGAGTTCCTTGGGT
Zip14	NM_001326699.1	ACAACGCAAGCATTTCCGTC	CTGTTCGCTCATCCCGTACA
Elf1a	NM_131263	AAGACAACCCCAAGGCTCTCA	CCTTTGGAACGGTGTGATTGA

2.3.6 Preparation of RNA probes and whole-mount in situ hybridization

cDNA prepared from wildtype zebrafish larvae at 5 dpf (pooled from 25 larvae) was used to generate the template for RNA probe synthesis (i.e., *zip1, zip7, zip8, zip9, zip13, and zip14*). The sizes of the PCR templates ranged between 400–700 bp. Multiple attempts were also made to prepare PCR templates for other *zip* transporters but were unsuccessful (e.g., unable to obtain a specific PCR product). PCR was performed using OneTaq DNA polymerase (New England Biolabs) with the following conditions: Initial denaturation at 95 °C for 3 min, and then 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 1 min extension at 68 °C, followed by a final 10 min extension at 68 °C. Each PCR product was designed to contain a T7 promoter site on the 5′ end of the reverse strand. Primer sets used for probe generation are summarized in Table 2.4.

Table 2.4. Primer sets used for RNA probe preparation.

Gene	Accession Number	FWD primer (5'-3')	REV primer (5'-3')
Zip1	NM_212583.2	CCGTGCTGAGTTTCGTGAGC	CGTCTGCAAGCCAATAGCCA
Zip7	NM_130931.3	GTTGCTAGACGTGGATGCGG	CCATGTTCCGTTTGCCTGCT
Zip8	XM_009307205.3	GTGAGAGTCCAGCGGTTGAG	CGAGATAGCAGCACATCGCT
Zip9	NM_001013540.1	ATGGTGTTGCTTTGGGAGCG	AGTGTCAGGGCAGTCCATCC
Zip13	NM_001005306.3	CCCTTACACACCGCCAGAGA	AGAGCCATGACGCCTATCCC
Zip14	NM_001326699.1	TCTGTACTCCAACGCCCTCT	GCAGTGAAGGAGGCTCCAAT

To facilitate *in vitro* transcription, the T7 promoter sequence (underlined, 5'-GCGTAATACGACTCACTATAGG-3') were added on the 5' end of the REV primer (not shown in the table).

DIG-labelled RNA probes complementary to mRNA sequences of interest were synthesized using 200 ng of purified PCR product, T7 RNA polymerase (New England Biolabs), and DIG RNA Labelling Mix (Sigma-Aldrich), following the manufacturer's guidelines. RNA

products were cleaned up using the Monarch™ Total RNA Miniprep Kit (New England Biolabs) and concentrations measured using a plate reader (BioTek). The quality of the RNA probes was checked on a 2% agarose gel after denaturing at 70 °C for 5 min.

In situ hybridization was performed as previously described (Kwong et al., 2014; Thisse and Thisse, 2008) with minor modifications. Casper line of zebrafish larvae were collected at 5 dpf, fixed overnight with 4% paraformaldehyde (PFA), and then dehydrated with 100% methanol. Dehydrated larvae were then rehydrated, digested with proteinase k (10 µg/ mL) for 30 min and then subsequently hybridized overnight at 60 °C with DIG-labelled RNA probe (100 ng of RNA probe in 500 µL hybridization buffer). After serial washing with hybridization buffer and phosphate buffered saline plus 0.1 % tween (PBST), the larvae were incubated with an anti-DIG antibody (1:2000 dilution) overnight at 4 °C. The next day larvae were washed and incubated in the dark with staining solution comprised of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, and periodically monitored every 20 min for staining intensity. Once optimal staining was obtained, the reaction was terminated with the use of 1 mM ethylenediaminetetraacetic acid (EDTA) in PBST. The embryos were then fixed overnight with 4% PFA and equilibrated in 85 % glycerol for later imaging. Images were captured with the use of a Leica MZ10 F modular stereo microscope and the Leica Application Suite X software.

2.3.7 Statistical analysis

All statistical analyses were performed using the Sigmaplot™ software (Systat System, Point Richmond, CA, USA). All data (whole body metal and ion levels, mRNA expression levels) were analyzed using a two-way analysis of variance (ANOVA) with developmental ages (1, 3, and 5 dpf) and exposure conditions (normal and high Zn) as two independent variables, followed by a post hoc Holm-Sidak test. If the data did not meet the assumption of normality or

equal variance, data transformation with either log or square-root was performed. A significance level of $p < 0.05$ was taken and data were presented as mean ± standard error of the mean (SEM).

2.4. Results

2.4.1 Effects of sublethal Zn exposure on physiological condition

Sublethal Zn exposure (4 μM) did not affect cumulative hatching rate nor mortality of zebrafish larvae (Supplementary Fig S2.1A and S2.1B). Zn exposure also had no effect on the standard body length of larvae at 5 dpf (Supplementary Figure S2.1C).

2.4.2 Zn body burden in zebrafish larvae exposed to normal and high Zn

The effects of Zn exposure on whole body Zn content in developing zebrafish at 1, 3, and 5 dpf are presented in Fig. 2.1. Results from a two-way ANOVA revealed that there was a significant interactive effect between developmental age and Zn treatment (Supplementary Table S2.2). A temporal change in whole body Zn content was observed in larvae exposed to high Zn, with a significant decrease at 3 dpf but no difference between 1 and 5 dpf. At 5 dpf, exposure to high Zn also resulted in a significant increase in Zn accumulation when compared to larvae exposed to normal Zn.

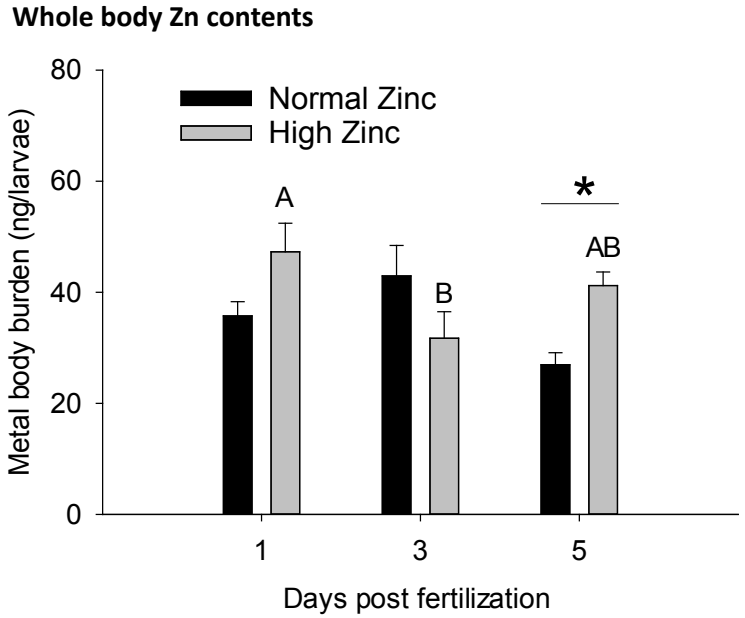

Figure 2.1. Whole body contents of Zn in developing zebrafish following sublethal waterborne Zn exposure. Whole body Zn contents in developing zebrafish at 1, 3, and 5 days post fertilization (dpf) following exposure to normal (0.25 μM) or high (4 μM) level of waterborne Zn. Values are presented as mean ± SEM, N = 4 (4 replicate tanks per treatment, where each replicate consisted of 25 pooled larvae). Bars not sharing the same letter represent a statistical difference between developmental ages within the same treatment group (i.e., capital letters, comparison among different developmental ages following high Zn exposure). Bars labelled with an asterisk represent a statistical difference between treatment groups within the same developmental age (two-way ANOVA followed by a post hoc Holm-Sidak test; $p < 0.05$).

2.4.3 Major ion contents in zebrafish larvae exposed to normal and high Zn

Whole body ion content in developing zebrafish exposed to normal or high Zn was determined at 3 and 5 dpf. Results from two-way ANVOA showed no interactive effect between developmental age and Zn treatment for any major ions tested (Na, Mg, K, and Ca; Supplementary Table S2.2). Exposure to high Zn did not affect whole body contents of Na, Mg, and K. In both normal and high Zn treatments, whole body contents of Na were lower in 3 dpf larvae when compared to 1 or 5 dpf larvae (Fig. 2.2A). Mg content decreased from 1 dpf to 3 dpf and then remained lower at 5 dpf (Fig. 2.2B). K and Ca contents increased substantially from 1

dpf to 5 dpf (Fig. 2.2C and 2.2D). Larvae exposed to high Zn exhibited a significant reduction in Ca level at 3 dpf when compared to larvae in the normal Zn treatment group. Such a reduction was not observed at 1 or 5 dpf.

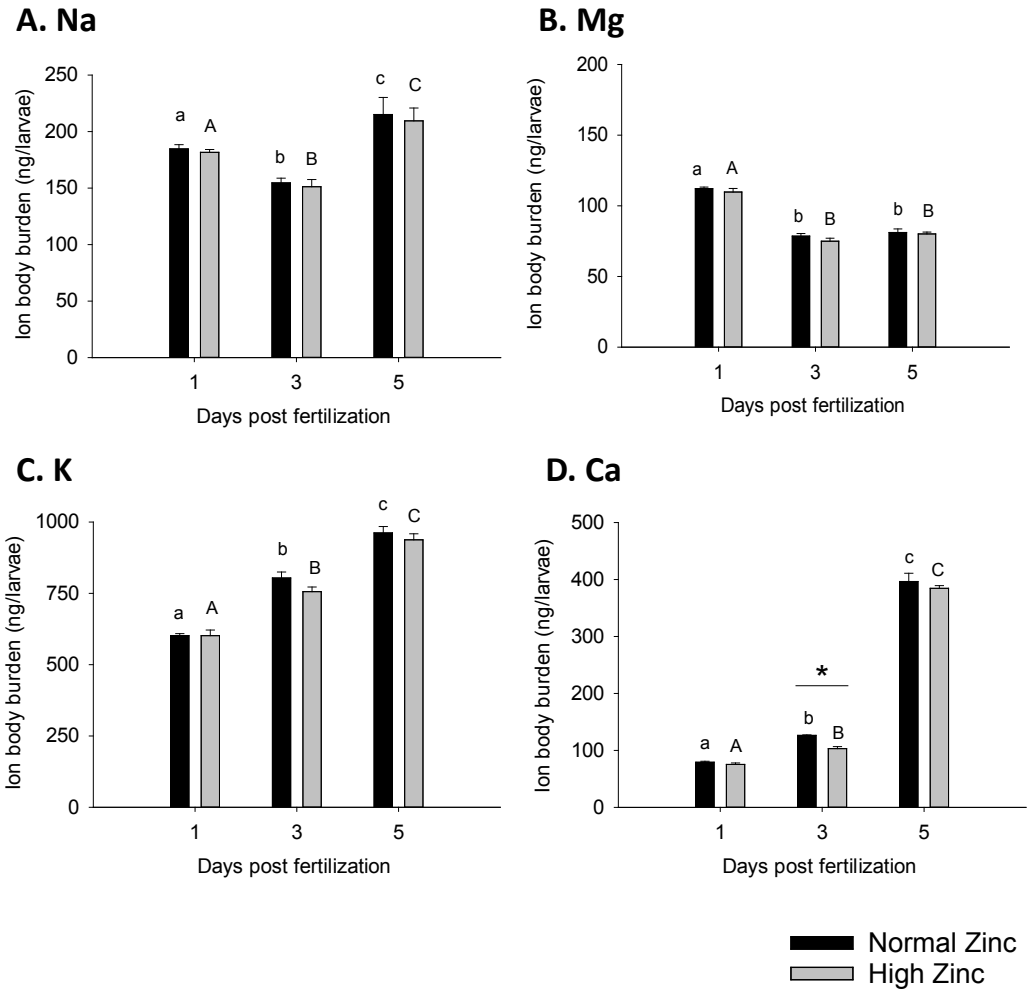

Figure 2.2. Whole body contents of major ions in developing zebrafish following sublethal waterborne Zn exposure. Whole body contents of (A) Na, (B) Mg, (C) K, and (D) Ca in developing zebrafish at 1, 3, and 5 days post fertilization (dpf) following exposure to normal (0.25 μM) or high (4 μM) level of waterborne Zn. Values are presented as mean ± SEM, N = 4 (4 replicate tanks per treatment, where each replicate consisted of 25 pooled larvae). Bars labelled with different letters represent a statistical difference between developmental ages within the same treatment group (i.e., small letters, comparison among different developmental ages following normal Zn exposure; capital letters, comparison among different developmental ages following high Zn exposure). Bars labelled with an asterisk represent a statistical difference between Zn treatments within the same developmental age (two-way ANOVA followed by a post hoc Holm-Sidak test; $p < 0.05$).

2.4.4 Trace metal body burden in zebrafish larvae exposed to normal and high Zn

To evaluate whether Zn exposure also affected the whole body contents of other essential trace metals, ICP-MS analysis was performed on 1, 3, and 5 dpf larvae exposed to normal or high Zn. Results from two-way ANOVA indicated that developmental age and Zn treatment had a significant interactive effect on the whole body contents of Ni and Cu (Supplementary Table S2.2), but not for other metals (i.e., Mn, Fe, Co, Se). No significant difference in Mn, Fe, and Co contents was observed between normal and high Zn treatments at any of the three ages examined (Fig. 2.3A to 2.3C). Larvae exposed to high Zn exhibited a significant decrease in Se content from 1 dpf to 5 dpf, which did not occur in larvae exposed to normal Zn (Fig. 2.3D). Additionally, a reduction in whole body Ni and Cu levels was observed in 3 dpf larvae exposed to high Zn, which was then recovered by 5 dpf (Fig. 2.3E and 2.3 F).

2.4.5 mRNA expression levels of *zip* transporters in developing zebrafish exposed to normal and high Zn

ddPCR technologies were employed to evaluate the changes in the mRNA expression levels of various *zip* transporters at 3 and 5 dpf following normal or high Zn treatment (Fig. 2.4). Results from two-way ANOVA analysis indicated that developmental age and Zn treatment had a significant interactive effect on the expression of *zip8* only (Supplementary Table S2.3). Exposure to high Zn resulted in an increase in the mRNA expression levels of *zip1* at 3 dpf relative to that in normal Zn treatment (Fig. 2.4A). The mRNA expression level of *zip8* was also found to increase in 5 dpf larvae exposed to high Zn (Fig. 2.4E). In contrast, mRNA level of *zip4* was significantly decreased by high Zn treatment at 5 dpf (Fig. 2.4B). No significant change in the mRNA expression levels of *zip6, zip7, zip9, zip10, zip13*, and *zip14* was observed between

normal and high Zn exposure at 3 or 5 dpf. Their expression levels were generally decreased from 3 to 5 dpf irrespective of Zn exposure levels (Fig. 2.4C, D, F, G, H, and I).

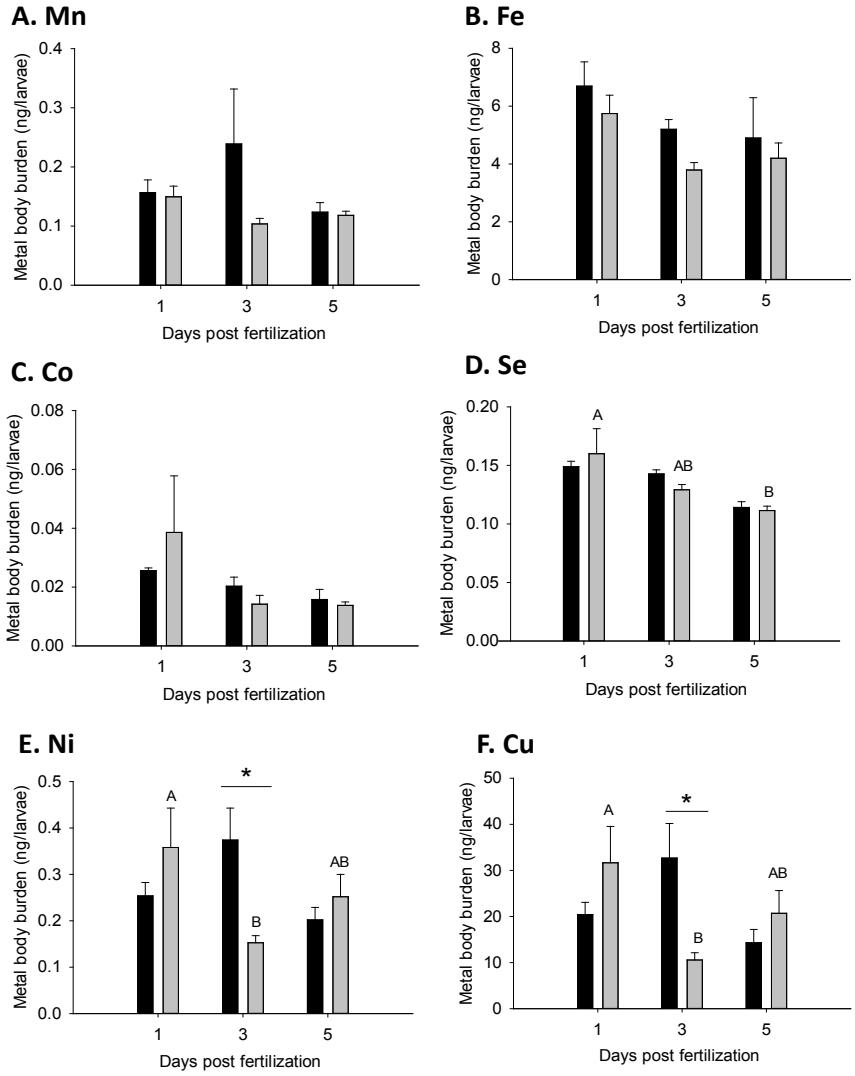

Figure 2.3. Whole body contents of trace metals in developing zebrafish following sublethal waterborne Zn exposure . Whole body contents of (A) Mn, (B) Fe, (C) Co, (D) Se, (E) Ni, and (F) Cu in developing zebrafish at 1,3, and 5 days post-fertilization (dpf) following exposure to normal (0.25 μM) or high (4 μM) level of waterborne Zn. Values are presented as mean ± SEM, N = 4 (4 replicate tanks per treatment, with each replicate consisting of 25 pooled larvae). Bar labelled with different letters represents a statistical difference between developmental ages within the same treatment group (i.e., capital letters, comparison among different developmental ages following high Zn exposure). Bars labelled with an asterisk represent a statistical difference between Zn treatments within the same developmental age (two-way ANOVA followed by a post hoc Holm-Sidak test; $p < 0.05$).

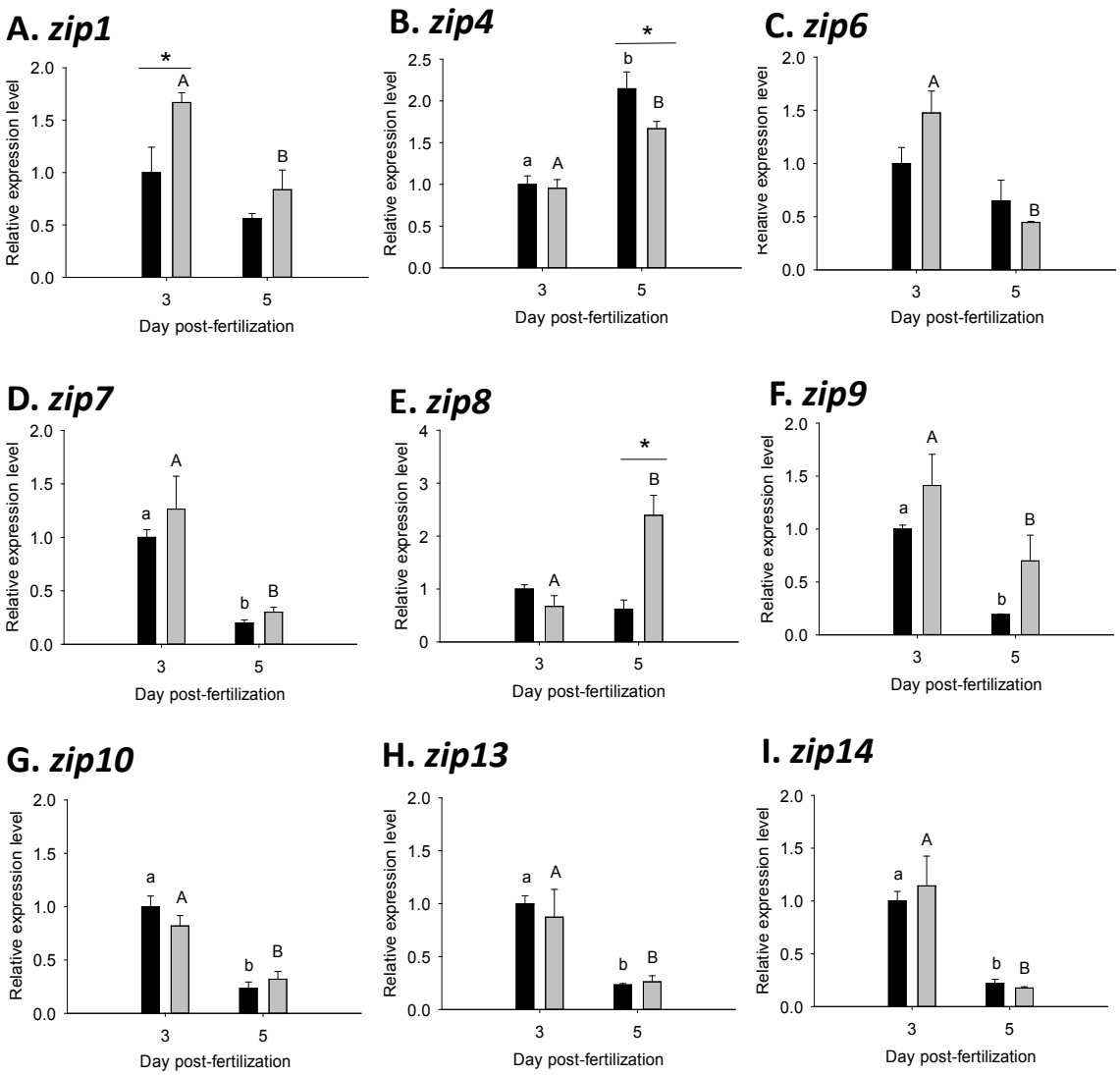

Figure 2.4. The mRNA expression levels of various *zip* transporters in developing zebrafish following sublethal waterborne Zn exposure. The mRNA expression levels of (A) *zip1*, (B) *zip4*, (C) *zip6*, (D) *zip7*, (E) *zip8*, (F) *zip9*, (G) *zip10*, (H) *zip13*, and (I) *zip14* in developing zebrafish at 3 and 5 days post fertilization (dpf) following exposure to normal (0.25 μM) or high (4 μM) level of waterborne Zn. Values are presented as mean ± SEM, N = 4 (4 replicate tanks per treatment, with each replicate consisting of 25 pooled larvae). The mRNA expression levels were normalized by elf1a and were expressed relative to larvae exposed to normal Zn at 3 dpf. Bars labelled with different letters represents a statistical difference between developmental ages within the same treatment group (i.e., small letters, comparison among different developmental ages following normal Zn exposure; capital letters, comparison among different developmental ages following high Zn exposure). Bars labelled with an asterisk represent a statistical difference between Zn treatments within the same developmental age (two-way ANOVA followed by a post hoc Holm-Sidak test; $p < 0.05$).

2.4.6 Localization of selected *zip* transporters in developing zebrafish

In situ hybridization was conducted to assess the mRNA expression patterns of several *zip* transporters in developing zebrafish at 5 dpf (Fig. 2.5). The results showed that various *zip* transporters appeared to be expressed in a tissue-specific manner. *Zip1, zip8, zip9,* and *zip13* were found to be abundantly expressed in the ethmoid cartilage of larvae. *Zip1* was also expressed in the branchial regions. *Zip7* was primarily expressed in the otolith and the heart, while *zip8* and *zip9* were expressed in the gut regions. *Zip14* was predominantly expressed in the head and the pronephric tubules.

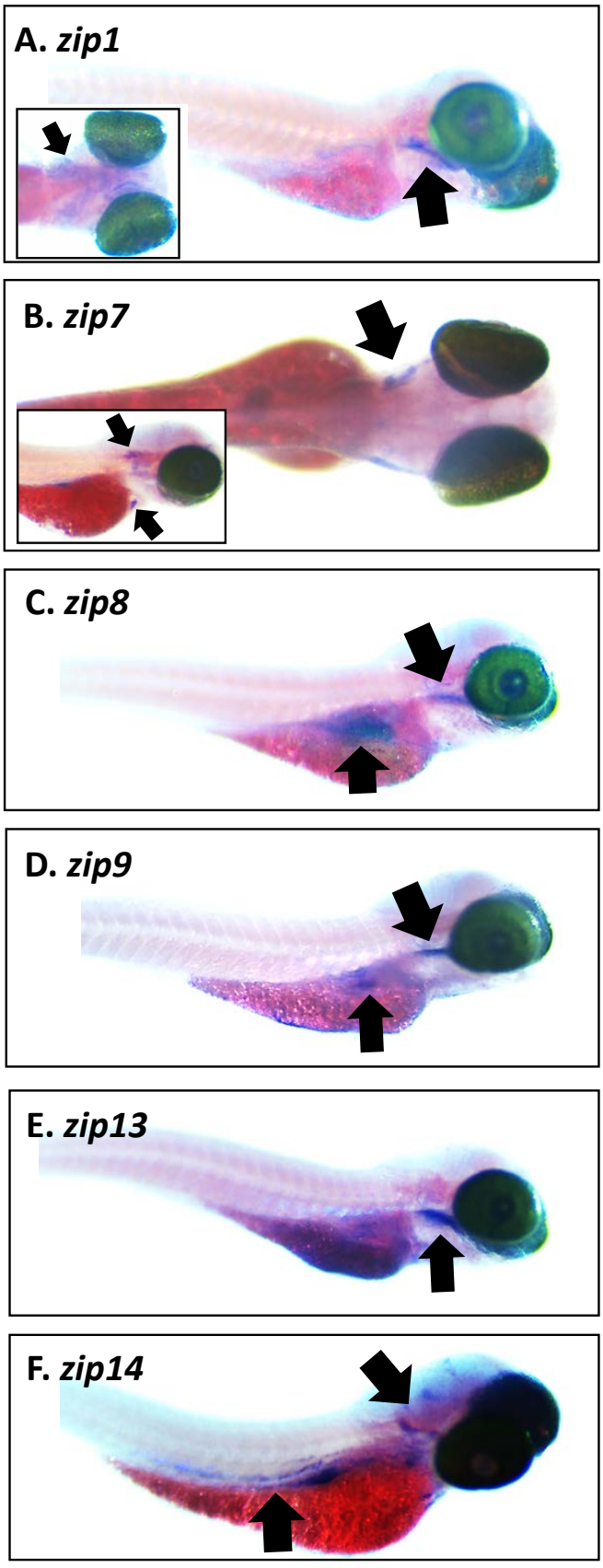

Figure 2.5. Whole mount in situ hybridization of various *zip* transporters in developing zebrafish. Localization of (A) *zip1*, (B) *zip7*, (C) *zip8*, (D) *zip9*, (E) *zip13*, and (F) *zip14* mRNA in developing zebrafish at 5 days post-fertilization. Insets in (A) and (B) are ventral views and side views of the larvae, respectively. An arrow indicates regions where strong mRNA signals were detected in the larvae.

2.5 Discussion

2.5.1 Overview

In the present study, the regulation of Zn and other essential trace metals following exposure to an environmentally-relevant concentration of Zn [4 μM (high Zn) vs 0.25 μM (normal Zn)], and the mRNA expression levels and localization of selected *zip* transporters, were investigated in developing zebrafish. The results indicated that exposure to high Zn led to a transient reduction in whole body content of Ca. A transient dysregulation in whole body Ni and Cu contents was also observed in larvae exposed to high Zn. Using ddPCR technologies, we found that Zn exposure modulated the mRNA expression levels of various *zip* transporters in a time-dependent manner. Further analysis using in situ hybridization revealed that some of these *zip* transporters were expressed in specific regions, suggesting possible tissue-specific involvement of ZIP in Zn homeostasis.

2.5.2 Effects of Zn exposure on Zn and Ca body burden

It is well documented that waterborne exposure to elevated levels of Zn (0.5 mg Zn/L - 200 mg Zn/L) adversely affects the hatching, survival, and development of fish (Speranza and Seeley, 1977; De Schamphelaere and Janssen, 2004; Brun et al., 2014; Salvaggio et al., 2016). However, the effects of exposure to environmentally-relevant concentrations of Zn on developing fish have only been sporadically examined. In the present study, we observed that exposure to normal Zn (0.25 μM; 16.4 μg Zn/L) or high Zn (4 μM; 261 μg/L) did not affect the general physiological conditions of larvae, including hatching rate, mortality, and body length. We also observed that whole body Zn level between 1–5 dpf was relatively steady in larvae rearing in normal Zn condition. A previous study has also shown that Zn content over early

development (0–5 dpf) in zebrafish remained unchanged (Ho et al., 2012), although Zn concentration in the water was not reported in that study. Here, we also observed that exposure to high Zn starting from 0 dpf significantly increased whole body Zn content at 5 dpf. Notably, high Zn exposure did not increase whole body Zn burden at 1 and 3 dpf, suggesting a delayed accumulation of Zn. This delay in Zn accumulation was presumably due to the presence of the chorion at early life stages (e.g., 0–2 dpf) which reduced Zn absorption by acting as a protective barrier (Guadagnolo et al., 2001; Finn, 2007).

In adults, Zn is known to compete with Ca for absorption via Ca transport pathways (e.g., epithelial Ca channel; ECaC) at the gills, and exposure to elevated levels of Zn results in a disruption in Ca balance, ultimately leading to hypocalcemia (Hogstrand et al., 1994, 1998; Qiu and Hogstrand, 2004). Our results also demonstrated that zebrafish larvae exposed to high Zn exhibited a reduction in whole body content of Ca at 3 dpf. In freshwater rainbow trout, a 29-day exposure to Zn (150 μg/L; 2.3 μM) was shown to reduce plasma Ca level after 1 day of exposure but its level returned back to the control level within the first week (Hogstrand et al., 1995). On day 29, the plasma Ca level in Zn-exposed fish was even found to be higher than that in the control fish (Hogstrand et al., 1995). In the present study, we also showed that the whole body Ca level of larvae exposed to high Zn was restored back to the control level at 5 dpf, indicating a possible compensation for Ca absorption following high Zn treatment. It is important to note that zebrafish at early life stages do not have a functional gill for ionic regulation; the regulation of epithelial Ca transport occurs primarily in the skin of the yolk sac (i.e., via Na^+ /K^+ -ATPase-rich cells; NaRCs) (Pan et al., 2005; Kwong et al., 2014; Lin and Hwang, 2016). Therefore, as opposed to the gills in adults, it is possible that the interactive effects between Zn and Ca occurred in the skin in larval zebrafish.

2.5.3 Effects of Zn exposure on the homeostasis of other essential trace metals

The present study showed that exposure to high Zn led to a transient decrease in whole body contents of Ni and Cu at 3 dpf. Notably, a reduction in Ca content by high Zn also occurred at 3 dpf, but not at other developmental ages tested (discussed above). These findings suggested that the dysregulation in Ni, Cu, and Ca balance by Zn exposure likely occurred via similar mechanisms. Although the precise mechanism for their interactions was unclear, it was possible that the disruption in Ni and Cu balance was associated with the impairment in Ca transport. For example, a previous study with zebrafish larvae (4–7 dpf) has demonstrated that exposure to Ni or Cu reduced Ca uptake (Alsop and Wood in 2011). On the other hand, an increase in water Ca level has been shown to reduce Cu accumulation in zebrafish gills (Craig et al., 2010; Alsop and Wood, 2011). Therefore, it seems likely that the Ca transport pathways are involved in the regulation of Ni and Cu homeostasis in zebrafish. Interestingly, Cu exposure has been found to decrease mRNA expression levels of the Zn transporter *zip8* (Komjarova and Bury, 2014), while the present study showed that Zn exposure increased *zip8* expression at 5 dpf (discussed later), which suggested a potential Zn-Cu interaction on the regulation of *zip8*.

2.5.4 Localization of various zip transporters and effects of Zn exposure on zip expression

In addition to the Ca transport pathways, the uptake and the cellular homeostasis of Zn in fish may be regulated by the ZIP family of transporters (Feeney et al., 2005; Ho et al., 2012). In the present study, we observed that in both normal and high Zn treatments, the mRNA expression levels of several *zip* transporters (e.g, *zip7, zip9, zip10, zip 13,* and *zip14*) were generally decreased from 3 to 5 dpf. The expression levels of other *zip* transporters (e.g., *zip1, zip4, zip6,* and *zip8*) were either increased or remained unchanged between 3 and 5 dpf. Ho et al. (2012) previously reported that the mRNA levels of most *zip* transporters were increased over

early development. The apparent discrepancy in *zip* expression levels observed between the present study and that of Ho et al. (2012) could be due to the differences in water chemistry in the two studies. Water parameters such as levels of Zn, water hardness, and pH, were not reported by Ho et al. (2012) and these parameters could influence Zn bioavailability and *zip* expression. Here, we observed that exposure to high Zn modulated the mRNA levels of several *zip* transporters in a time-dependent manner. Specifically, high Zn exposure resulted in an increase in the mRNA levels of *zip1* and *zip8* at 3 dpf and 5 dpf, respectively. In contrast, mRNA level of *zip4* was decreased by high Zn exposure at 5 dpf. Further analysis using in situ hybridization revealed that *zip1* was abundantly expressed in the ethmoid cartilage of the head. In mammals, ZIP1 protein plays a critical role in chondrocyte development, and overexpression of ZIP1 has been shown to increase osteoblast differentiation (Tang et al., 2006). Therefore, the increased expression of *zip1* by high Zn exposure could be a response to promote skeletal development while the larvae were experiencing a disruption in Ca balance. In zebrafish larvae, knockdown of *zip7* resulted in delayed growth and developmental malformation (Yan et al., 2012). *Zip7* knockdown also affected the mRNA levels of other *zip* transporters, including *zip6* (Yan et al., 2012). Our study showed that exposure to different levels of Zn did not affect the expression levels of *zip7*, although a reduced expression from 3 dpf to 5 dpf was observed in both normal and high Zn treatments. In adult zebrafish, *zip7* is expressed in various tissues (Feeney et al., 2005). In contrast, the present study revealed that the expression of *zip7* appeared to be restricted to the otoliths and the heart, suggesting that *zip7* might play a specific role in these tissues during early development.

A previous study examining the tissue distribution of *zip* transporters in adult zebrafish has shown that *zip4* was expressed in the intestine and the eyes (Feeney et al., 2005).

Furthermore, it has been reported that waterborne Zn exposure resulted in a reduction in *zip4* expression (Feeney et al., 2005). Similarly, the present study showed that the mRNA level of *zip4* decreased at 5 dpf after high Zn exposure. Although the localization of *zip4* in larval zebrafish is currently unknown, the results suggested that the mRNA level of *zip4* was inversely regulated by waterborne Zn exposure in both larval and adult zebrafish. On the other hand, the present study showed that *zip8* mRNA was abundantly expressed in the intestinal tract of larvae. *Zip8* mRNA level was also found to be significantly increased by high Zn exposure at 5 dpf, suggesting its possible contribution to the increased whole body Zn burden in 5 dpf larvae. However, it is noteworthy that drinking in larval zebrafish was found to be minimal (Kwong et al., 2013), and therefore it was unlikely that *zip8* was involved in the intestinal absorption of Zn at this developmental age. In mammals, ZIP14 protein is known to transport not only Zn but also Mn and Fe (Pinilla-Tenas et al., 2011). Our results showed that Zn exposure did not affect *zip14* expression level. Whole body Fe and Mn contents also were not influenced by Zn exposure. A previous study with 4 dpf larval zebrafish has shown that *zip14* was expressed in the pronephric tubules (Tuschl et al., 2016). Interestingly, in addition to the pronephric tubules, we found that *zip14* was also expressed in specific regions of the head in 5 dpf larvae, indicating its spatial-temporal difference in expression pattern during development. Together, our results indicated that in contrast to adult zebrafish where most *zip* transporters were expressed ubiquitously (Feeney et al., 2005), *zip* transporters in larval fish appeared to be expressed in a tissue-specific manner. These findings suggest the unique role of the various ZIP transporters in the regulation of Zn homeostasis in different tissues during early development in zebrafish.

2.6 Conclusion and future perspectives

The present study demonstrated that exposure to an environmentally-relevant concentration of Zn resulted in an elevated Zn body burden in the early life stages of zebrafish. Exposure to high Zn appeared to result in a transient disruption in the homeostasis of Ni, Cu, and Ca. Additionally, we found that various *zip* transporters were expressed in different regions of larvae and that exposure to Zn led to differential changes in the expression levels of *zip* transporters. In the early life stages of zebrafish, different ZIP proteins are suggested to play different roles in development. Future studies should address how changes in *zip* expression by Zn exposure affect developmental processes in larval fish. The effects of Zn exposure on the protein abundance and the functional regulation of ZIP transporters also warrant further investigation. On the other hand, our results suggested that future toxicological assessment of Zn exposure in developing fish could consider the evaluation of not only Zn and Ca balance but also the homeostasis of other essential trace metals. The long-term consequences of the transient disruption in metal and ionic balance by Zn exposure during early development, and the molecular mechanisms underlying the interactions between Zn and other essential metals, also warrant further investigation.

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2.9 Supplementary Materials

2.9.1 Supplementary Materials and Methods

ICP-MS analysis

Following euthanization with ice, larvae (25 larvae were pooled as one replicate, N=1) were gently washed twice with MilliQ water in a Petri dish and were transferred to a 1.5 mL centrifuge tube. These larvae were then dehydrated for 48 h at 65ºC followed by an acid digestion in 250 µL of 6N HNO_3 (TraceMetal™ grade, Fisher Chemical) for another 48 h. Both the yolk and the chorion (at 1 dpf) were included for acid digestion. The digested tissue was quickly centrifuged and 100 µL of the supernatant was collected and diluted with 2% HNO_3 to a final volume of 5 mL. Water samples collected from the exposure tanks were also acidified using 2% HNO_3. Tissue and water samples were then passed through a 0.45 µm nylon filter, and subsequently analyzed by inductive coupled plasma mass spectrometry (ICP-MS; Agilent 8800 ICP-QQQ-MS) in the Water Quality Center at Trent University. Calibration standards were prepared by serial dilution of a 100 ng/mL multi-element solution in 2% HNO_3. The NIST SRM 1640a (Trace Elements in Natural Water) was used for QA/QC and the measured concentrations were within 5% of the certified values. The detection limits for each of the metals analyzed are summarized in Supplementary Table 2.1.

ddPCR analysis

Total RNA was extracted using an RNA extraction kit which included an on-column DNase I treatment to eliminate genomic DNA (Monarch Total RNA Miniprep Kit, New England Biolabs). RNA concentration and integrity were measured using a plate spectrophotometer (BioTeK, Synergy LX), with all RNA samples having a 260 nm/280 nm ratio of 2.00 or above. 1 µg of total RNA was converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). The ddPCR reaction mixtures were prepared using EvaGreen™ supermix following the manufacture's

guidelines. PCR conditions were as follows: 5 min 95ºC, followed by 40 cycles of 3 min denaturation at 95ºC and 1 min annealing/extension at 58ºC. Subsequently, signal stabilization was performed which consisted of 5 min at 4 ºC followed by 5 min at 90 ºC. Droplets were then read using the QX200 Droplet reader (Bio-Rad) and analyzed using the QuantaSoft™ software. The primer sets used for the analyses are summarized in Table 2.3. The expression levels of various *zip* were normalized to *elf1* (housekeeping gene) expression levels. The expression stability of *elf1* among treatments and across developmental ages was tested by a two-way ANOVA ($P>0.05$ for both factors). The sequences of all the PCR amplicons were confirmed using the Sanger sequencing (Sick Kids hospital, Toronto, ON) prior to ddPCR analysis.

2.9.2 Supplementary Tables and Figures

Supplementary Table S2.1. Detection limit of metal concentrations measured by ICP-MS analysis

Metal	Detection Limit (ppb)
Na	0.503
Mg	0.048
K	0.098
Ca	0.039
Mn	0.010
Fe	0.023
Co	0.002
Ni	0.028
Cu	0.007
Zn	0.016

Detection limit is defined as 3x the standard deviation of the blank. Measurement was performed using the Alignment 8800 ICP-QQQ-MS.

Supplementary Table S2.2. Statistical output of Two-way Analysis of Variance for major ion and trace metals in larvae exposed to high waterborne Zn during early development

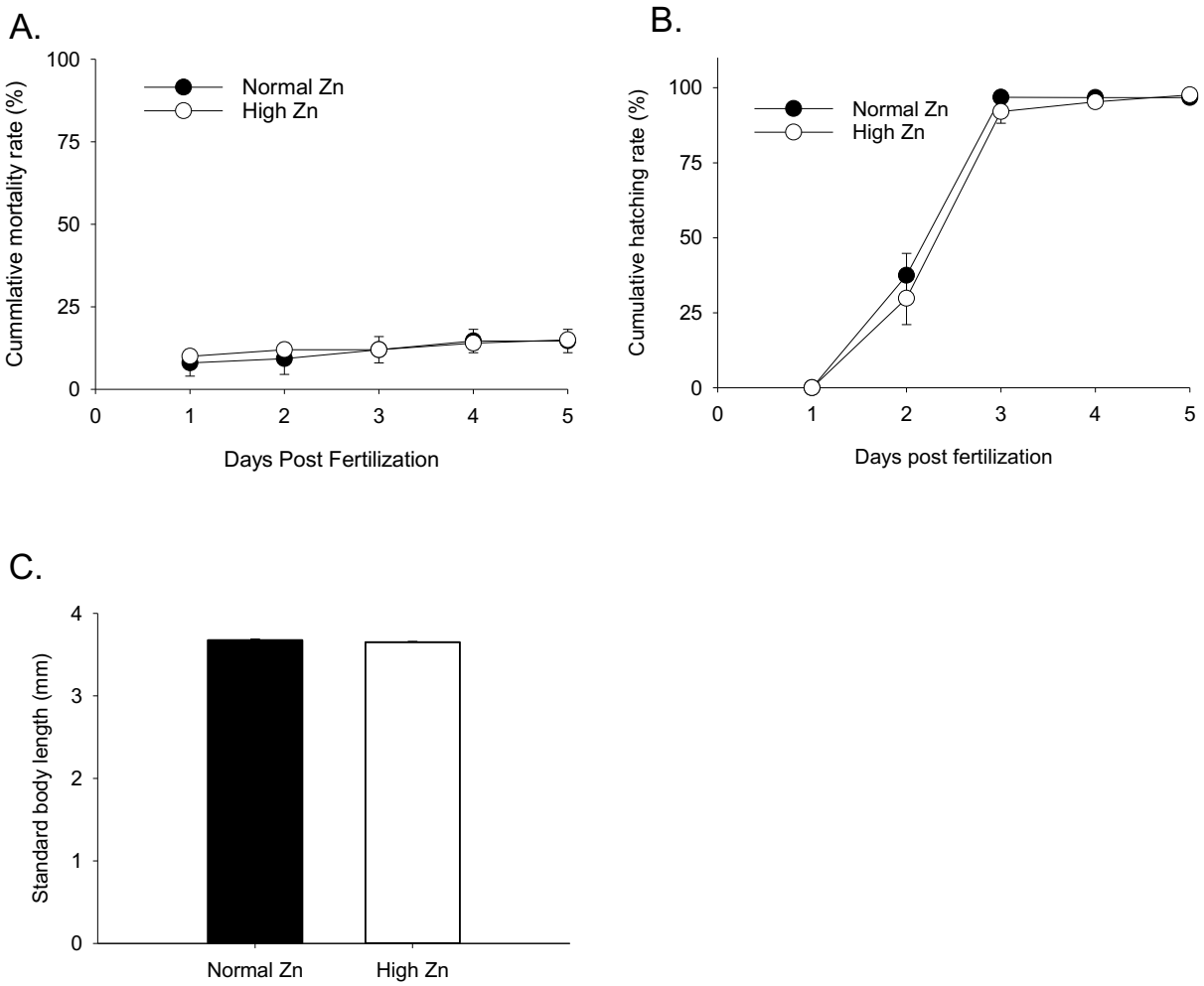
Major Ion/ Trace Metal	F- Value	P-Value
Na	0.0130	0.987
Mg	0.206	0.816
K	0.825	0.454
Ca	1.008	0.386
Mn	1.690	0.212
Fe	0.111	0.896
Co	0.761	0.481
Ni	5.690*	0.012*
Cu	6.058*	0.010*
Se	0.836	0.450
Zn	5.682*	0.013*

F-value and P- value presented for the interactive effect between days' post fertilization (1,3,5 dpf) and waterborne Zn supplementation (Normal and high Zn). Asterisk used to represent values found to be statistically significant ($p<0.05$).

Supplementary Table S2.3. Statistical output of Two-way Analysis of Variance for *zip* transporter mRNA in larvae exposed to high waterborne Zn during early development

Zip Transporter	F- Value	P-Value
Zip1	1.619	0.232
Zip4	2.205	0.168
Zip6	3.993	0.074
Zip7	0.352	0.566
Zip8	24.734*	<0.001*
Zip9	0.0610	0.811
Zip10	2.436	0.150
Zip13	0.414	0.534
Zip14	0.520	0.487

F-value and P- value presented for the interactive effect between days' post fertilization (3 and 5 dpf) and waterborne Zn supplementation (Normal and high Zn). Asterisk used to represent values found to be statistically significant ($p<0.05$).



Supplementary Figure S2.1. The effects of sublethal Zn exposure on the physiological condition of developing zebrafish. (A) cumulative mortality rate, (B) hatching rate, and (C) standard body length (at 5 dpf) in developing zebrafish exposed to normal (0.25 μM) and high (4 μM) level of waterborne Zn. Values are reported as mean ± SEM, N=4 (4 replicate dishes with each dish containing 25 larvae).

CHAPTER III:

Using zebrafish as a model to assess the individual and combined effects of sub-lethal waterborne and dietary zinc exposure during development

3.1. Abstract

The present research used zebrafish (5–28 days post-fertilization; dpf) as a model organism to investigate the effects of chronic exposure to environmentally relevant sub-lethal concentrations of waterborne (261 μg/L) and dietary zinc (Zn) (1500 mg Zn/kg dw), either independently or simultaneously, during development. The results showed that whole body contents of Zn were increased in all Zn treatment groups, with the highest accumulation of Zn observed in larvae simultaneously exposed to elevated waterborne and dietary Zn. In addition, exposure to elevated levels of Zn, either through the water or the diet, led to a decrease in whole body calcium (Ca) contents at 28 dpf. The findings also suggested that exposure to elevated levels of Zn resulted in a significant reduction in whole body manganese (Mn) contents. More importantly, the magnitude of decrease in Mn contents by Zn exposure was markedly higher than that in Ca and appeared to mirror the increases in whole body Zn accumulation. These results indicate that Mn regulation is more sensitive than Ca to disruption by Zn exposure in developing fish. Further examination of the Zrt-Irt-Like Protein (ZIP) family of transporters using droplet digital PCR technologies revealed that several *zip* transporters exhibited temporal and exposure route-specific changes following Zn exposure. In particular, the level of *zip4* was influenced by Zn exposure regardless of the exposure routes, while changes in *zip7* and *zip8* levels were predominantly driven by waterborne exposure. Overall, our findings demonstrated that zebrafish during the developmental periods are sensitive to elevated levels of Zn seen in the environment,

particularly following co-exposures to waterborne and dietary Zn. Future toxicological assessment of elevated Zn exposure should consider both the exposure routes and the life stages of fish.

3.2. Introduction

Zinc (Zn) is an abundant trace metal in the body that plays a vital role in the survival of all vertebrates (Ho et al., 2012; Stefanidou et al., 2006; Tammy and Bettger, 1990; Zhao et al., 2014). However, when introduced in quantities above the nutritional level, Zn can become toxic and detrimental (Hogstrand, 2012). Additionally, Zn is considered as a priority aquatic contaminant in many metal-contaminated waters. In clean freshwater (FW) environments, the levels of Zn typically range from 0.1 μg/L to 50 μg/L (Feeney et al., 2005). However, elevated concentrations of dissolved Zn that far surpass the nutritional level have been recorded in many Zn-impacted aquatic environments. These elevated levels of Zn in aquatic ecosystems primarily result from anthropogenic activities, such as urban runoff, mining activities, galvanization of steel products, and fertilizer application and leaching (Hogstrand, 2012; Legret and Pagotto, 2006; Preciado and Li, 2006). In some Zn-contaminated environments, concentrations of Zn in the surface water have been recorded to reach over 5 mg/L, posing a serious concern for aquatic species inhabiting those environments (Andres et al., 2000; Feeney et al., 2005; Saeed and Shaker, 2008; Salvaggio et al., 2016; Zheng et al., 2016).

The adverse effects associated with waterborne Zn exposure in coldwater fish species such as rainbow trout (*Oncorhynchus mykiss*) are well documented (De Schamphelaere and Janssen, 2004; Mcgeer et al., 2000a; McGeer et al., 2000b). Exposure to high concentrations of waterborne Zn (0.5–200 mg/L) has also been shown to be detrimental to other fish species such as zebrafish (*Danio rerio*), resulting in teratogenic effects and mortality during development

(Salvaggio et al., 2016). It is well documented that the toxic effect of acute waterborne Zn exposure in FW fish is primarily associated with the competitive inhibition of Ca uptake by Zn at the apical surface of the gill, resulting in hypocalcaemia (De Schamphelaere and Janssen, 2004; Hogstrand et al., 1994, 1998). Waterborne Zn exposure has also been reported to result in the transient reduction in whole body nickel (Ni) and copper (Cu) contents in zebrafish during early development (Puar et al., 2020). Although the competitive interaction of Zn and Ca in adult fish is well characterised, the effects of Zn exposure on the homeostasis of other major ion and trace metals in developing fish remain largely unknown.

In FW fish, the effects of waterborne Zn exposure have been studied more extensively than the effects of dietary Zn exposure, despite the fact that the gastrointestinal tract is an important site for Zn absorption (Hogstrand, 2012). This knowledge gap has resulted in most of our current understanding of gastrointestinal uptake of Zn in fish being predominantly based upon extrapolation from mammalian studies, and even there the knowledge is somewhat incomplete. It is important to note that although elevated dissolved Zn can cause toxicity to aquatic organisms, much of the Zn in metal-impacted aquatic ecosystems is also sequestered into the sediment where it is bioaccumulated by benthic invertebrates which then becomes a significant source of Zn to fish that feed upon them (Baudrimont et al., 2005; Besada et al., 2011; Birch and Apostolatos,2013;Shulkin et al., 2003;Soto-Jim´enez et al., 2001; Tsangaris et al., 2013; Wu et al., 2017). The Zn levels in benthic invertebrates from metal-contaminated aquatic ecosystems have been recorded to be in the range of 700–5300 mg Zn/kg dry weight (dw), which is 20–60 fold higher than the suggested dietary Zn requirement (90 mg Zn/kg dw) for teleost fish (Luo et al., 2011; Watanabe et al., 1997).

In vertebrates, uptake and cellular homeostasis of Zn are largely regulated by two families of Zn transporters; (i) the Zinc Transport Proteins (ZnT; SLC30), which are often associated with the basolateral extrusion of Zn, and (ii) the Zrt-Irt-Like Proteins (ZIP; SLC39), which mediate apical Zn uptake and facilitate intracellular Zn release from organelles. Previous studies have shown that 12 members of the ZIP family are found in zebrafish (Feeney et al., 2005; Puar et al., 2020; Zheng et al., 2008) and their mRNA expression levels appear to respond differently to waterborne Zn exposure at different life stages (Feeney et al., 2005; Puar et al., 2020). Additionally, exposure to elevated levels of dietary Zn has been found to modulate the mRNA expression levels of several *zip* transporters at the gill of adult zebrafish (Zheng et al., 2008, 2010). Nevertheless, these studies were conducted with adult fish while the effects of exposure to elevated Zn, either via water or diet, during the developmental periods where intensive growth occurs, have yet to be further explored. Understanding how *zip* transporters are modulated by Zn exposure may provide new insights into the pathways that regulate Zn balance and the mechanisms that underly Zn toxicity and tolerance. Importantly, in Zn-contaminated aquatic environments, fish are often exposed simultaneously to elevated levels of Zn via both water and diet. Advancing our understanding of the effects of combined waterborne and dietary exposure in developing fish is important for predicting the risks of Zn exposure. In fact, most regulatory guidelines around the world consider only the impacts of waterborne Zn when assessing the risk of Zn contamination in aquatic ecosystems, with little to no attention given toward dietary Zn exposure and its associated risk to aquatic organisms including fish (Clearwater et al., 2002).

In the present study, we used developing zebrafish as a model organism to test the hypothesis that chronic exposure to environmentally relevant levels of Zn via water and/or diet

would lead to increased Zn body burden and disrupt the homeostasis of other trace metals and major ions during development. We predicted that exposure to elevated levels of Zn via both routes would result in an additive effect on metal and ion dysregulation. We also hypothesized that mRNA expression levels of different *zip* transporters vary in different transport epithelia of zebrafish and that their expressions are differentially modulated depending on the route of exposure (waterborne *vs* dietary). To our knowledge, this is the first study to investigate the interactive effects of environmentally relevant levels of waterborne and dietary Zn on metal/ ion homeostasis and *zip* regulation in developing zebrafish.

3.3. Materials and Methods

3.3.1. Animals

Adult zebrafish of the Tüpfel long-fin (TL) strain were held on a recirculating system (Aquaneering, CA, USA) under conditions of pH 7.4, temperature 28 ˚C, and a 14 h light:10 h dark photoperiod. The concentrations of major ions and trace metals in the water of the recirculating system are summarized in Table 3.1. The Zn level in the recirculating system was measured to be 25.4 ± 3.7 μg/L (n = 3), while water hardness and alkalinity were kept at 150 mg/L and 65 mg/L (as $CaCO_3$), respectively. Adult fish were fed three times daily; once with a commercial diet (Zeigler, PS, USA) and twice with a live brine shrimp diet. The same conditions were maintained for all adult fish used unless stated otherwise.

For breeding purposes, female and male zebrafish were separated for 1 week (8–10 fish/tank) and then placed in breeding traps overnight on a recirculating system. Breeding traps were opened the following morning, embryos were collected, and then transferred to 50 mL Petri plates (30 embryos/plate) filled with recirculating system water. Fish in the Petri plates were

maintained in an incubator at 28 °C, with water being changed daily until 5 days post fertilization (dpf). The larvae were then transferred to larger containers and subjected to various exposure conditions described below (section 3.3.2). Untreated adult fish from the recirculating system were also collected and used for assessing the mRNA expression profile of *zip* transporters (outlined in section 3.3.3). All experiments were designed and conducted in compliance with the guidelines outlined by the Canadian Council of Animal Care and were approved by the York University Animal Care Committee (2017-R2).

Table 3.1. Ionic composition of the exposure waters.

	Major ion (mg/L)				Trace metals (µg/L)					
	Na	Mg	K	Ca	Mn	Fe	Co	Ni	Cu	Se
Concentration	102.4 ± 1.70	8.98 ± 0.04	5.13 ± 0.04	5.38 ± 0.02	0.20 ± 0.01	1.23 ± 0.22	0.036 ± 0.01	0.50 ± 0.03	19.36 ± 6.78	0.06 ± 0.02

Values are mean ± SEM from four independent measurements.

3.3.2. Exposure regime for developing zebrafish

Beginning 5 dpf, free-swimming larvae were transferred to 2L plastic tanks (120 larvae/tank; 16 tanks total) containing 1L of water each and were maintained in an incubator at 28°C until 28 dpf. Each tank was assigned to a treatment group with a total of 4 replicate tanks (n = 4) per treatment. To test the effects of the different routes of Zn exposure, we employed a fully factorial experimental design consisting of 4 treatment groups: (A) control water and control diet (regular Zn concentration), (B) control water and elevated Zn diet, (C) elevated waterborne Zn and control diet, and (D) elevated waterborne Zn and elevated Zn diet. The elevated Zn diet was prepared by the addition of Zn (as $ZnSO_4 \cdot 7H_2O$) dissolved in Milli-Q water to a commercially

available zebrafish larvae diet (GEMMA Micro 75, Skretting; comprised of 59% protein; 14% lipids; 0.2% fibre; 14% ash; 1.3% phosphorus; 1.5% calcium and 0.7% sodium). The mixture was then dehydrated at 65 °C for 48 h and subsequently ground with a mortar and pestle into a granular powder. The feed was stored at 4 °C until use. Control diets were prepared in the same manner with the addition of Milli-Q water to the diet with no additional Zn. Control diets had ~200 mg Zn/kg dw, while the high Zn diets contained ~1500 mg Zn/kg dw. The Zn concentration in the control diet used in the study represented dietary Zn levels commonly found in commercially available feeds which are based upon the required daily Zn intake of fish. The concentration used for the elevated Zn diet was selected to mimic the levels of Zn found in benthic invertebrates collected from Zn-contaminated sites. Water in the experimental tanks was either left untreated from the recirculating system as control or supplemented with 261 µg Zn/L (4 µM Zn). The chosen Zn level was within the range of dissolved Zn levels reported in Zn-contaminated aquatic ecosystems (see the introduction for details). The measured concentrations of Zn in the water and diets are reported in Table 3.2.

Table 3.2. Measured Zn concentrations in experimental waters and diets

Sample	Measured Zn Concentration
Control Water	25.37 ± 3.7 µg/L (0.39 ± 0.06 µM)
High Zn Water	290 ± 7.1 µg/L (4.43 ± 0.11 µM)
Control Diet	196.9 ± 18.2 mg Zn/ kg dw food
High Zn Diet	1488.0 ± 48.8 mg Zn/ kg dw food

Concentrations of Zn were analyzed using ICP-MS analysis. The detection limit of Zn was measured to be 0.016 µg/L. Values are mean ± SEM from three independent measurements. Concentration of Zn in diet is expressed as dry weight (dw).

Larvae were fed once daily with the granular powder diet for 1 h and all unconsumed food was then pipetted out. >90% of water was changed daily (i.e., static renewal) for both waterborne and dietary exposure experiments. To test for possible Zn leaching from the diet into the water, Zn levels were measured in the exposure waters 1 h after feeding before water change (see below for details on sample preparation and methods). No significant leaching of Zn from the respective diets into the exposure waters was observed (Supplementary Table S3.1).

Physiological assessment of developing zebrafish during Zn exposure

Larvae were monitored daily for mortality prior to feeding. At 7 dpf and 28 dpf, 10 larvae from each replicate tank were collected, euthanized with buffered tricaine methanesulfonate (MS-222), and measured for standard body length (SL) as described previously (Chandrapalan and Kwong, 2020; Puar et al., 2020). The SL of 10 larvae collected from the same tank was averaged and was considered as n = 1, with a total of n = 4 from 4 replicate tanks.

Larvae at 28 dpf were analyzed for food consumption to determine any differences in the amount of food intake between the different treatment groups. A total of 8 larvae from each replicate tank were moved to a 6-well plate containing the respective treatment water and allowed to acclimate for 4 h at 28°C. The respective diets for each replicate were placed into separate weighing plates and measured. Following acclimation, respective diets for each treatment group were added in small amounts and fish were allowed to feed for 1 h. Uneaten food from each replicate was then pipetted on to separate weight plates, dehydrated overnight at 65 °C, and added back to the original plate containing the unused food. These plates were then weighed once again and the differences between initial weight and final weight were taken as the amount of food intake.

Assessment of zip mRNA expression levels in Zn exposed larvae using ddPCR

Changes in mRNA expression levels of various zip transporters in response to waterborne and/or dietary Zn exposure were examined using droplet digital PCR (ddPCR; QX200 system, Bio-rad). At 14 and 28 dpf, 10 larvae pooled together from each replicate tank (n = 1) were collected and euthanized with an overdose of buffered MS-222 (a total of n = 4 per treatment group). The methods for cDNA preparation and ddPCR analysis were carried out as described previously (Puar et al., 2020) (outlined in Supplementary Materials and Methods 3.9.1). The primer sets used for the analysis are summarized in Supplementary Table S3.2. Normalization of the expression levels of the various *zip* transporters was performed against the housekeeping genes elf1, rps-18, and rpl-13a. Verification of the reliability and the stability of the housekeeping genes was tested among treatments and developmental age using a three-way ANOVA analysis ($p > 0.05$ for all factors) prior to use for normalization. The specificity of each primer set used in this experiment was confirmed via Sanger sequencing (Sick Kids hospital, Toronto, ON).

Assessment of major ion and trace metal body burden following Zn exposure

The effects of waterborne and/or dietary Zn exposure on whole body contents of major ions (Na, K, Ca, and Mg) and trace metals (Ni, Cu, Zn, Fe, Co, and Mn) in larvae were evaluated using inductively coupled plasma mass spectrometry (ICP-MS). At 7, 14, and 28 dpf, 15 larvae pooled as a single replicate (n = 1; a total of n = 4 per treatment) were collected and analyzed by ICP-MS. Detailed procedures for sample preparation and ICP-MS measurement are provided in Supplementary Materials and Methods 3.9.1.

3.3.3. mRNA expression profile of ZIP transporters in adult zebrafish tissue

Adult zebrafish from the recirculating system were collected and euthanized with an overdose of buffered MS-222. To measure the mRNA expression levels of the various *zip* transporters, the major transport epithelial tissues (gill, intestine, intestinal bulb, and kidney) and liver were sampled immediately following euthanization. Each tissue was pooled from 4 to 6 different adults as a single replicate with a total of 4 replicates for each tissue. The methods for cDNA preparation and ddPCR analysis were carried out as described previously (Puar et al., 2020) and are outlined in Supplementary Materials and Methods 3.9.1.

3.3.4. Statistical analysis

The statistical software Sigmaplot^{TM} (Version 14, Systat System, Point Richmond, CA, USA) was used to conduct all data analysis. The effects of Zn exposure on food intake at 28 dpf were analyzed using a one-way analysis of variation (ANOVA) followed by a post-hoc Holm-Sidak test. The mRNA expression profile of the various zip transporters in the tissue of adults was also analyzed using a one-way ANOVA. An arcsine square root transformation was carried out on mortality data (i.e., percentage) prior to a two-way repeated-measures test. Standard body length, major ion body burden, trace metal body burden, and mRNA expression levels of *zip* transporters in response to Zn treatment were analyzed using a three-way ANOVA, with waterborne Zn treatment, dietary Zn treatment, and developmental age, as three independent variables, followed by a post-hoc Holm-Sidak test. When data did not meet the assumptions of normality and equal variance, data were transformed either by the use of log or square root functions. All analysis performed used a significance level of $p < 0.05$, and all data have been presented as mean ± standard error of the mean (SEM).

3.4 Results

3.4.1. Effects of chronic Zn exposure on physiological condition

The amount of food intake was examined on 28 dpf larvae, and no significant differences were observed between the various treatment groups (Fig. 3.1A). To evaluate the possible effects of waterborne and dietary Zn exposure on the growth of developing zebrafish, the standard body length (SL) was measured at 7 and 28 dpf. A significant increase in SL was observed from 7 dpf to 28 dpf in all treatment groups (Fig. 3.1B). On day 28, the SL of larvae exposed to elevated dietary Zn was higher than that of the other treatment groups. Results from a three-way ANOVA suggested that there were no interactive effects between waterborne treatment, dietary treatment, or developmental age on larval SL (Supplementary Table S3.3).

Mortality was measured daily for the duration of the exposure. There was sphericity for the interaction term, as assessed by Mauchly's test of sphericity ($p > 0.05$). Results from a two-way repeated-measures ANOVA suggested a significant effect of time and time × treatment interaction on cumulative mortality, but it did not show a significant main effect of treatment. Therefore, simple main effects were determined. As reported in Supplementary Table S3.4, there were significant cumulative mortalities among treatment groups from day 16/17 onward. Higher cumulative mortalities in larvae exposed to elevated Zn were observed (Fig. 3.1C). In particular, larvae that were exposed simultaneously to elevated waterborne Zn and dietary Zn exhibited the highest cumulative mortality rates when compared to the other treatment groups.

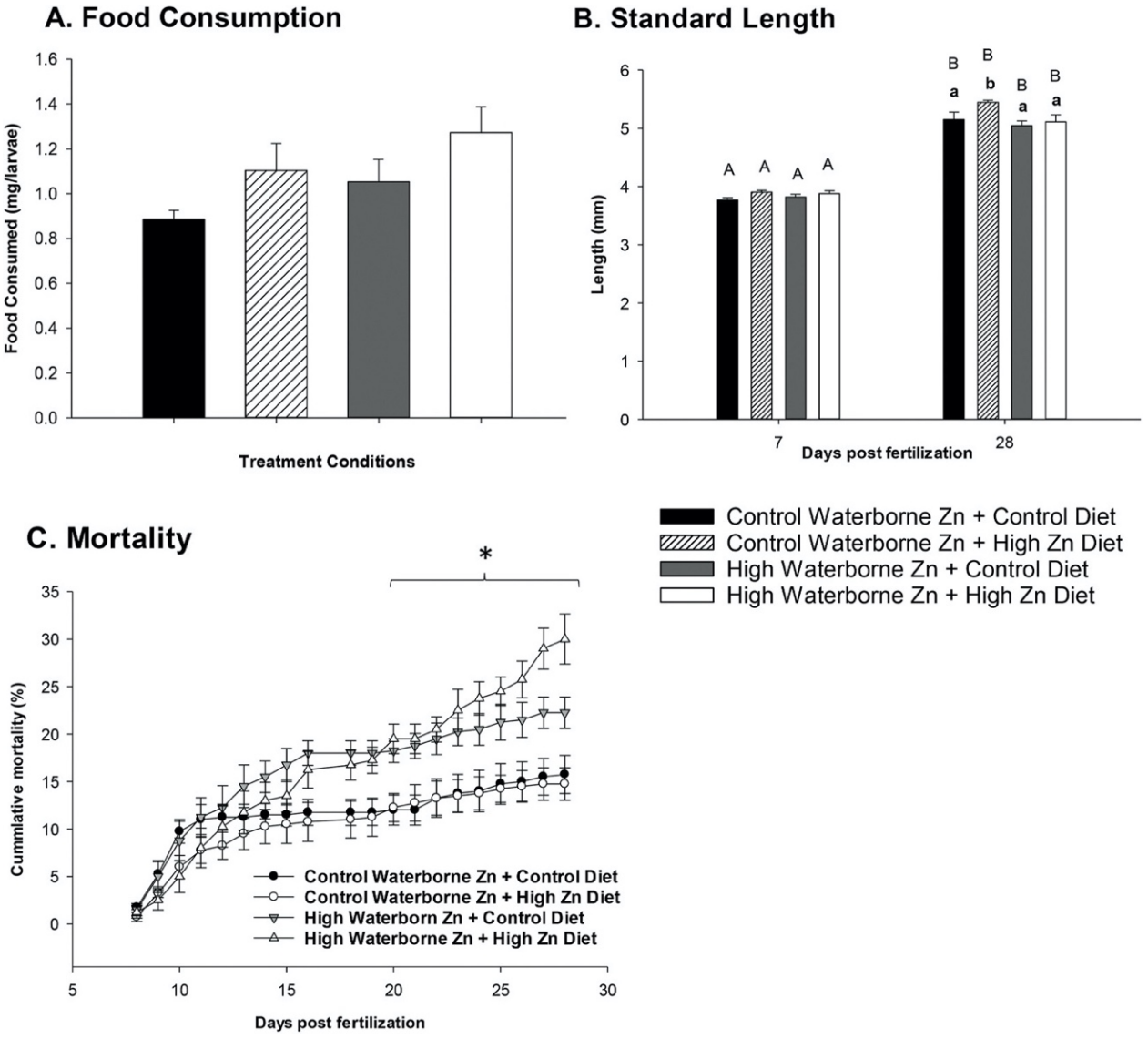


Figure. 3.1. The effects of waterborne and dietary Zn exposure on the physiological condition of developing zebrafish. (**A**) Food consumption in 28 days post- fertilization (dpf) larvae after Zn exposure. Data are presented as mean ± SEM. (**B**) The standard body length of larvae at 7 and 28 dpf following exposure to different combinations of waterborne and dietary Zn. Ten fish were measured from each replicate tank (as n = 1) with a total of n = 4 for each treatment group. Capital letters are used to signify a significant difference within the same treatment group across developmental age. Lower case letters are used for comparison between treatments within the same developmental age. Data are presented as mean ± SEM. (**C**) Cumulative mortality (%) in larvae from 5 dpf to 28 dpf exposed to elevated Zn. An asterisk is used to depict the developmental ages at which differences in mortalities were observed between the different treatment groups.

3.4.2. Zn body burden in developing zebrafish after waterborne and dietary Zn exposure

To investigate the impacts of waterborne and/or dietary Zn exposures on whole-body Zn content, an ICP-MS analysis was performed on larvae at 7, 14, and 28 dpf. Analysis using a three-way ANOVA showed a significant interactive effect between waterborne Zn treatment and developmental age, as well as between dietary Zn treatment and developmental age. No interactive effect was found between waterborne treatment and dietary treatment (Supplementary Table S3.5). At 7 dpf, no differences in whole-body Zn contents were observed between the different treatment groups (Fig. 3.2). Exposure to both elevated waterborne Zn and elevated dietary Zn resulted in the highest increase in Zn contents at both 14 and 28 dpf. Larvae exposed to elevated waterborne Zn alone, and larvae fed on an elevated Zn diet alone, also experienced increases in Zn contents at 14 and 28 dpf, but the increases were significantly lowered than that in larvae co-exposed to elevated waterborne and dietary Zn.

3.4.3. Whole-body major ion contents in developing zebrafish after waterborne and dietary Zn exposure

The body burden of all major ions examined (Na, Mg, K, and Ca) was increased over development from 7 dpf to 14 dpf and then to 28 dpf in all treatment groups (Fig. 3.3A–D). Results from a three-way ANOVA showed no interactive effect between developmental age, waterborne Zn treatment, and dietary Zn treatment for Na, Mg, and K. However, a significant interaction was found for Ca between developmental age and waterborne Zn treatment (Supplementary Table S3.5). The results also indicated that whole-body content of Ca in 28 dpf decreased significantly in all three Zn treatment groups compared to the control (Fig. 3.3D).

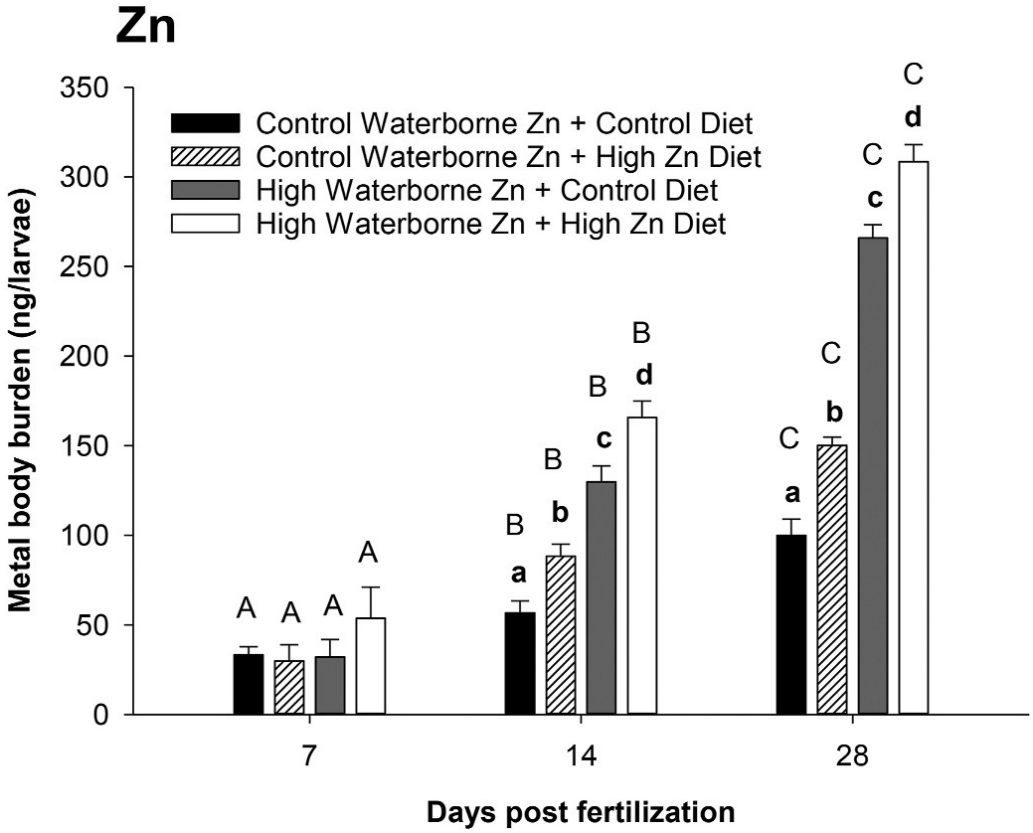


Figure. 3.2. Accumulation of Zn in zebrafish larvae after exposure to environmentally relevant concentrations of waterborne and/or dietary Zn. Whole-body Zn levels in 7, 14, and 28 days post-fertilization (dpf) larvae. Data are presented as mean ± SEM (n = 4, with 15 pooled larvae per n). Bars with different capital letters indicate a significant difference within the same treatment group across developmental age. Bars with different lowercase letters indicate a significant difference between treatment groups within the same developmental age.

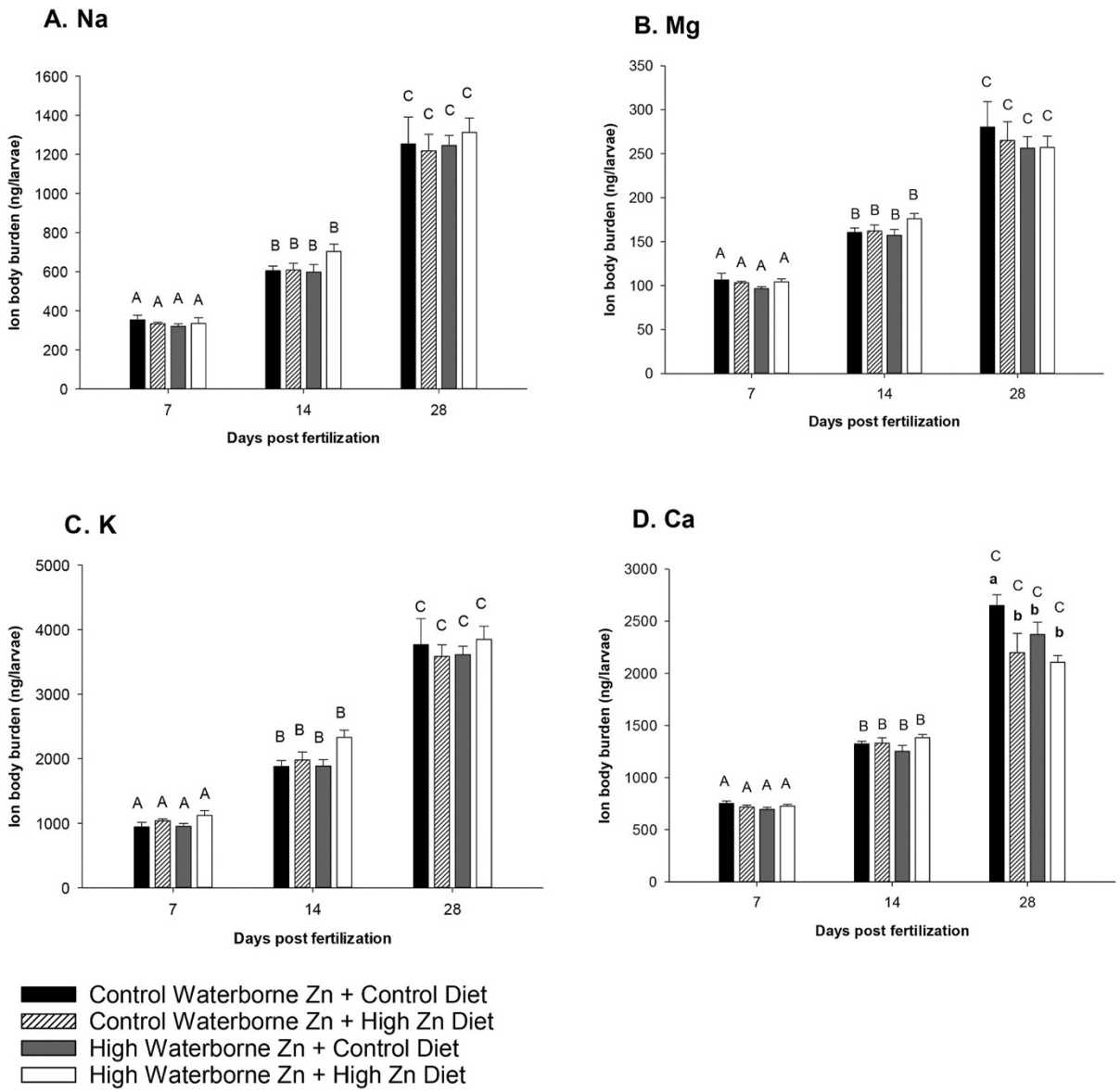


Figure. 3.3. Influence of waterborne and/or dietary Zn exposure on body burden of major ions in developing zebrafish. Whole-body levels of **(A)** Na, **(B)** Mg, **(C)** K, and **(D)** Ca in 7, 14, and 28 days post-fertilization (dpf) larvae. Data are presented as mean ± SEM (n = 4, with 15 pooled larvae per n). Bars with different capital letters indicate a significant difference within the same treatment group across developmental age. Bars with different lowercase letters indicate a significant difference between treatment groups within the same developmental age.

3.4.4. Whole-body trace metal contents in developing zebrafish after waterborne and dietary Zn exposure

Mn and Fe contents were increased from 7 to 28 dpf across all treatment groups (Fig. 3.4A and B). At 14 dpf, larvae fed on an elevated Zn diet (singly or in combination with high waterborne Zn exposure) experienced a decrease in whole-body Mn level when compared to the control (Fig. 3.4A). At 28 dpf, exposure to elevated Zn (waterborne and/or dietary) also resulted in a decrease in Mn content, with the highest reduction occurred in larvae exposed to both elevated waterborne and dietary Zn (Fig. 3.4A). Results from a three-way ANOVA indicated a significant interaction between developmental age and waterborne Zn treatment, as well as between developmental age and dietary Zn treatment on whole-body Mn contents (Supplementary Table S3.5). Results also showed an interactive effect between waterborne Zn treatment and dietary Zn treatment on Mn contents (Supplementary Table S3.4).

Larvae exposed to waterborne Zn (on control or elevated Zn diet) experienced a decrease in whole-body Co level at 14 dpf (Fig. 3.4C). Results from a three-way ANOVA indicated an interactive effect between developmental age and waterborne Zn treatment on Co body burden (Supplementary Table S3.5). No significant changes in Ni or Cu body burden were found during development or across treatment groups (Fig. 3.4D and E).

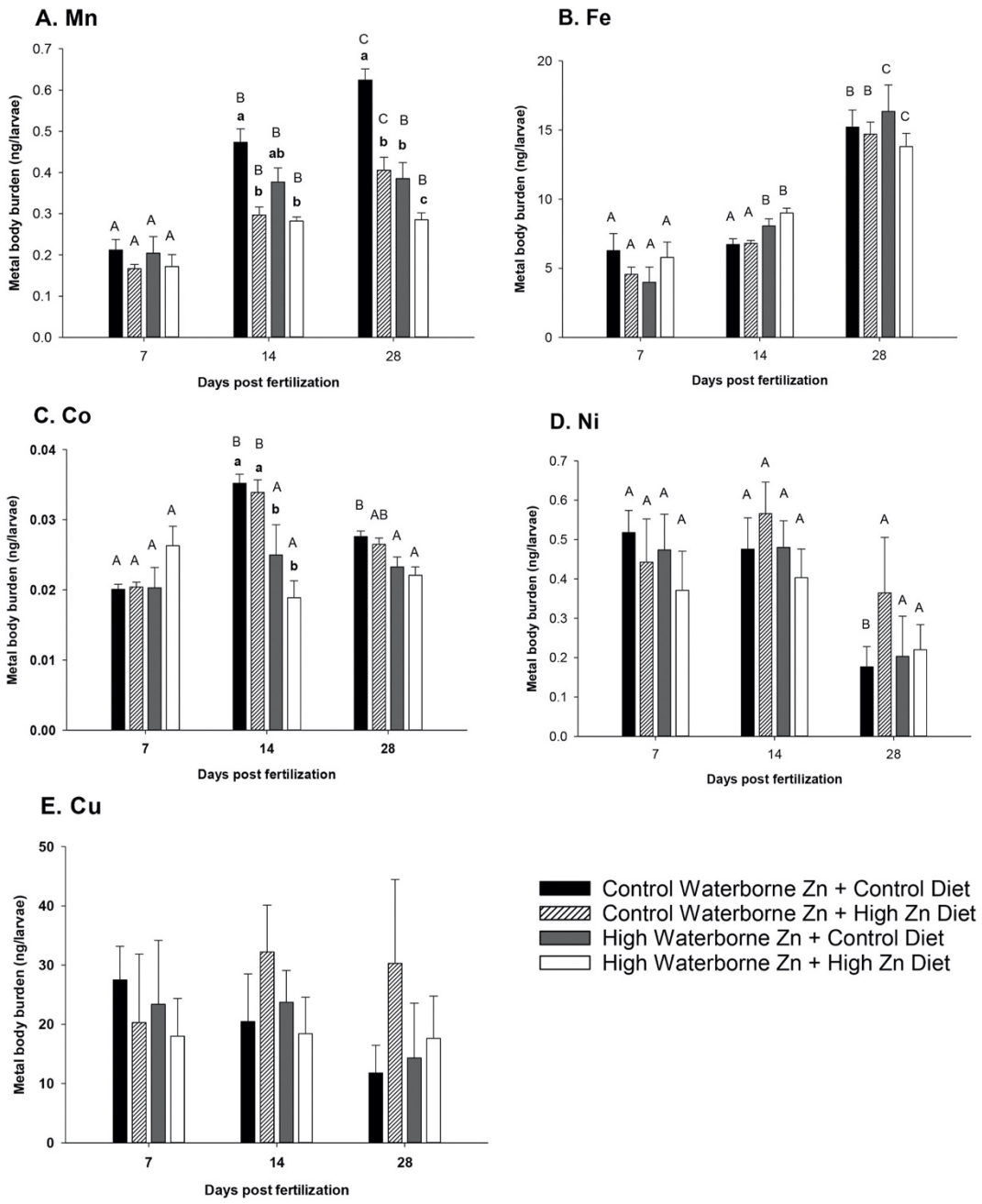


Figure. 3.4. Influence of waterborne and/or dietary Zn exposure on body burden of trace metals in developing zebrafish. Whole- body levels of **(A)** Mn, **(B)** Fe, **(C)** Co, **(D)** Ni, and **(E)** Cu in 7, 14, and 28 days post-fertilization (dpf) larvae. Data are presented as mean ± SEM (n = 4, with 15 pooled larvae per n). Bars with different capital letters indicate a significant difference within the same treatment group across developmental age. Bars with different lowercase letters indicate a significant difference between treatment groups within the same developmental age.

3.4.5. Tissue expression profile of *zip* transporters in adult zebrafish

The mRNA expression levels of various *zip* transporters in different tissues of adults were measured by ddPCR. This dataset was used to analyse and indicate (i) the expression levels of various *zip* in each tissue (Fig. 3.5), and (ii) the expression levels of each *zip* across all the tissues where it is found to be expressed (Fig. 3.6). The results showed that *zip1, zip6*, *and zip7* were abundantly expressed in the gill (Fig. 3.5A), intestine (Fig. 3.5B), and kidney (Fig. 3.5D). *Zip13* was also found to be highly expressed in the gill and kidney. In the intestinal bulb, no statistical difference in the mRNA levels of various *zip* was observed (Fig. 3.5C). In the liver, *zip7* was the most abundantly expressed transporter among all the *zip* transporters analyzed, while *zip4, zip10,* and *zip14* showed the lowest levels of expression (Fig. 3.5E).

The results also demonstrated that *zip1, zip4, zip6,* and *zip7* exhibited the highest expression levels in the intestine (Fig. 3.6A–D). *Zip4* was also highly expressed in the intestinal bulb (Fig. 3.6B). *Zip8, zip10,* and *zip14* were found to be abundantly expressed in the intestinal bulb (Fig. 3.6E, G, I), while *zip9* showed higher abundance in the gill, intestine, and liver (Fig. 3.6F). *Zip13* appeared to be highly expressed in the gill, intestinal bulb, and kidney (Fig. 3.6H).

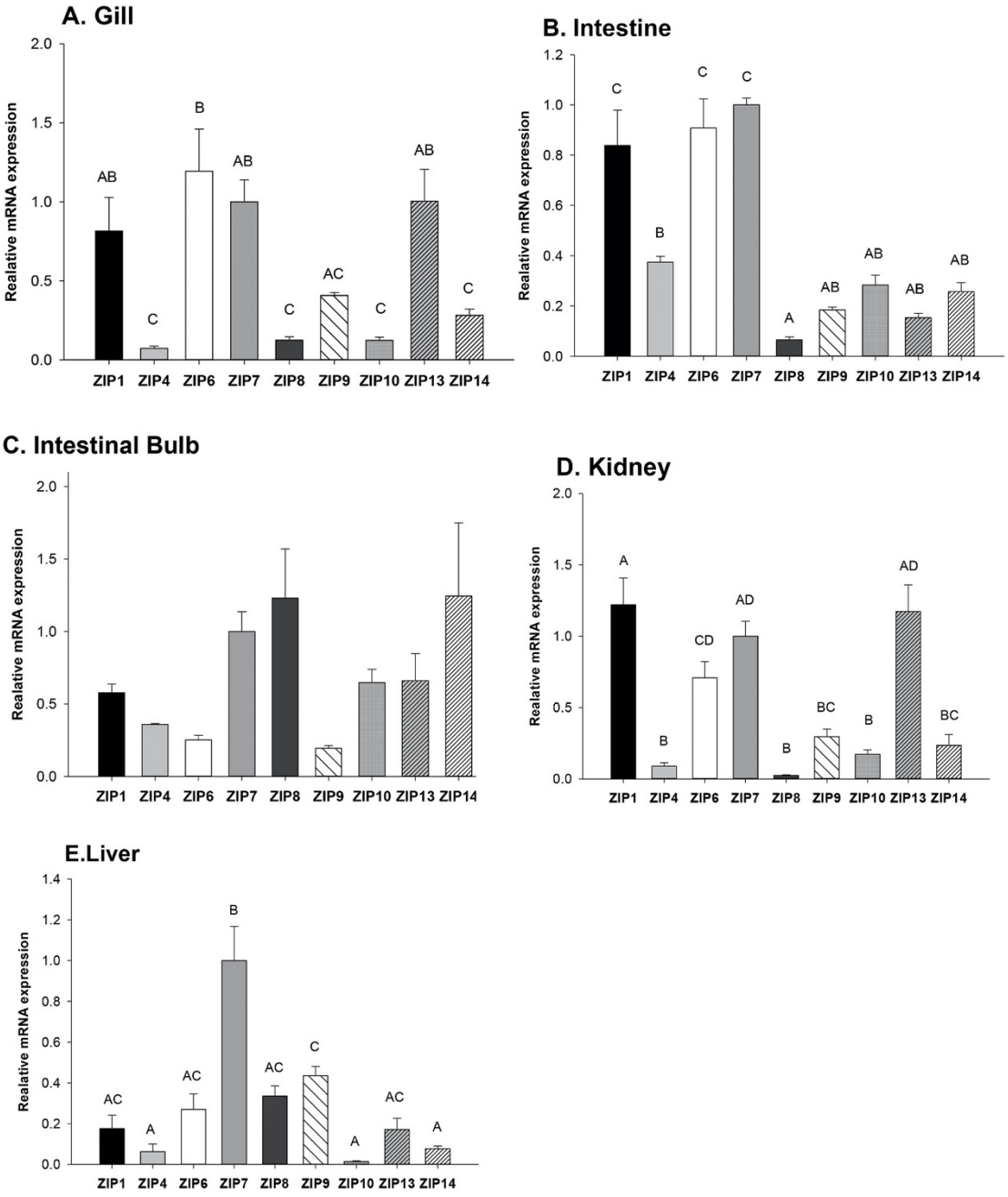


Figure. 3.5. The mRNA expression levels of *zip* transporters in various tissues in adult zebrafish. The relative mRNA expression levels of various *zip* transporters in the **(A)** gill, **(B)** intestine, **(C)** intestinal bulb, **(D)** kidney, and **(E)** liver of adult zebrafish. Data are presented as mean ± SEM (n = 4, with each replicate containing tissues pooled from 4 to 6 adults). Bars sharing any letter in common (regardless of the presence of other letters) represent a lack of statistical difference. Expression levels were normalized to the expression of *elfa, rpl13a,* and *rps18*, and are presented relative to expression levels of *zip7*.

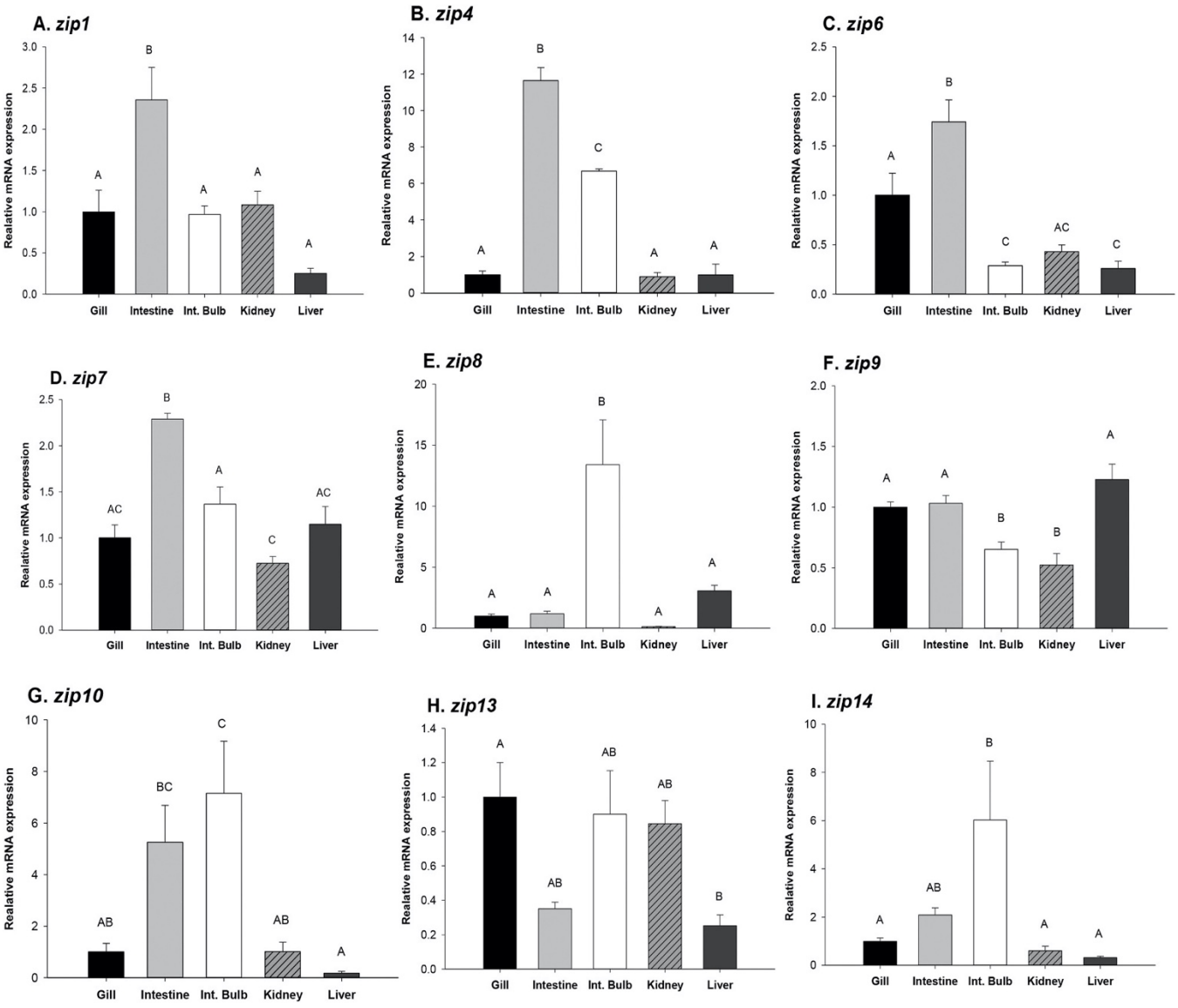


Figure. 3.6. The tissue distribution profile of *zip* transporters in adult zebrafish. The relative mRNA expression levels of **(A)** *zip1*, **(B)** *zip 4*, **(C)** *zip 6*, **(D)** *zip 7*, **(E)** *zip 8*, **(F)** *zip 9*, **(G)** *zip 10*, **(H)** *zip 13*, and **(I)** *zip14* in various tissues in adult zebrafish. Data are presented as mean ± SEM (n = 4, with each replicate contained tissues pooled from 4 to 6 adults). Bars sharing any letter in common (regardless of the presence of other letters) represent a lack of statistical difference. Expression levels were normalized to the expression of *elfa, rpl13a,* and *rps18*, and are presented relative to expression levels in the gill.

3.4.6. mRNA expression levels of *zip* transporters in developing zebrafish after waterborne and dietary Zn exposure

To understand the regulation of the various *zip* transporters in response to waterborne and/or dietary Zn exposure during development, mRNA expression levels of the various transporters were measured at 14 dpf and 28 dpf. mRNA expression levels of *zip6, zip10,* and *zip14* were not changed by various Zn treatments nor over development (Fig. 3.7C, G, and I). Results from a three-way ANOVA showed that developmental age and waterborne Zn treatment had an interactive effect on the expression levels of *zip7* and *zip14* (Supplementary Table S3.6). Results also showed an interactive effect of developmental age and dietary Zn treatment on the expression of *zip6* and *zip9.* An interactive effect between waterborne Zn treatment and dietary Zn treatment was found on *zip4* expression. In addition, an interactive effect between developmental age, waterborne Zn, and dietary Zn was also observed on *zip1* expression. For the majority of the *zip* transporters analyzed, expression levels remained unchanged over development, except for *zip13* which was found to increase over development across all treatment groups (Fig. 3.7).

Expression levels of *zip4* were found to be reduced at 14 dpf in all treatment groups exposed to elevated Zn (waterborne and/or dietary Zn). Similar reductions were also observed at 28 dpf, but only in those treatment groups that were exposed to elevated waterborne Zn, irrespective of dietary Zn levels (Fig. 3.7B). A significant increase in *zip7* (at 28 dpf) and *zip8* (at both 14 and 28 dpf) expressions were observed in larvae treated with elevated waterborne Zn with or without co-exposure to elevated dietary Zn; exposure to elevated dietary Zn alone did not affect *zip7* and *zip8* expression levels (Fig. 3.7D and E). The expression levels of *zip13* in both 14 dpf and 28 dpf larvae were reduced by Zn exposure, particularly in larvae that were co-exposed to both elevated waterborne Zn and dietary Zn (Fig. 3.7H).

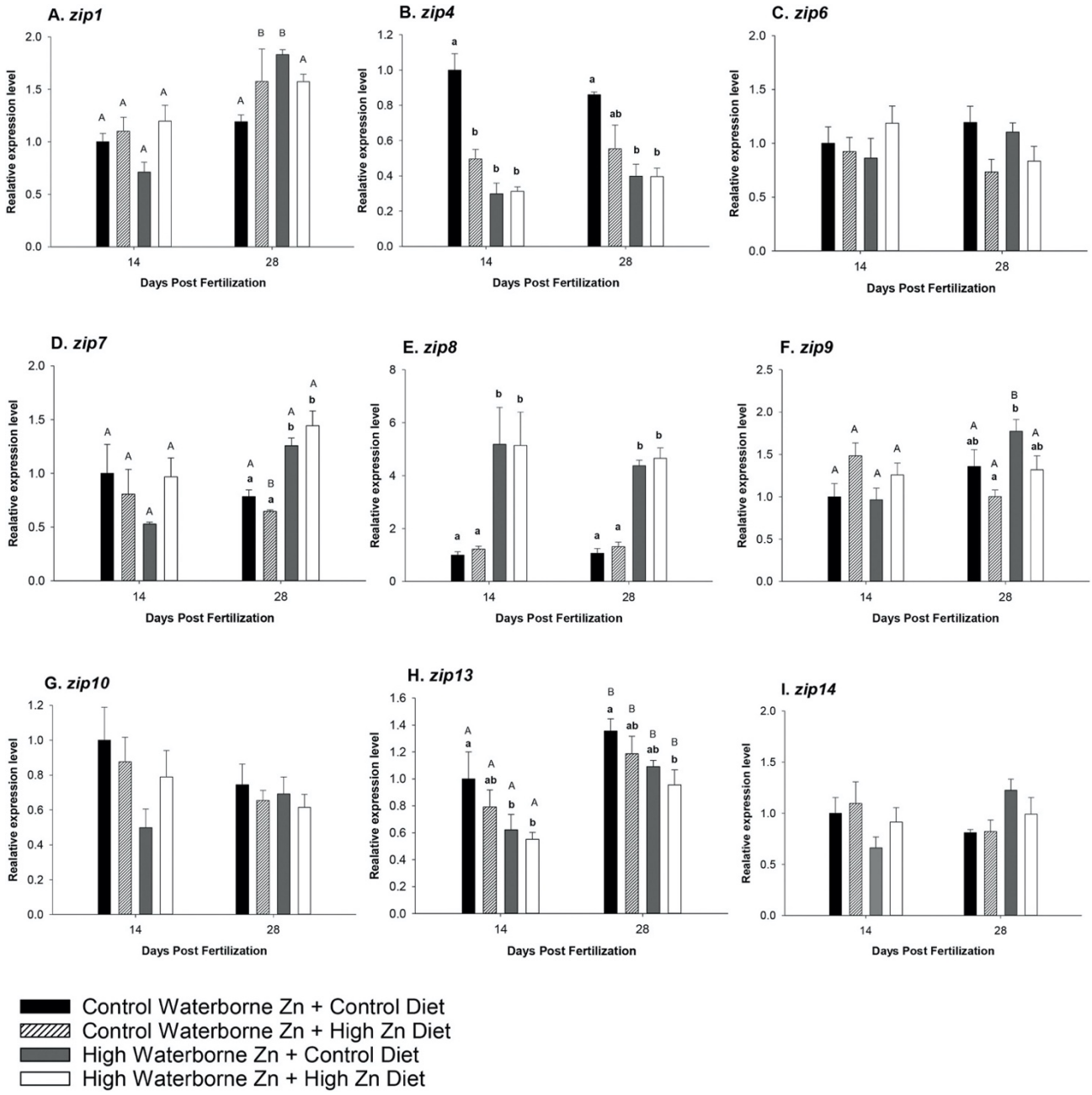


Figure. 3.7. Influence of waterborne and/or dietary Zn exposure on mRNA expression levels of various *zip* transporters in developing zebrafish. The relative mRNA expression levels of **(A)** *zip1*, **(B)** *zip 4*, **(C)** *zip 6*, **(D)** *zip 7*, **(E)** *zip 8*, **(F)** *zip 9*, **(G)** *zip 10*, **(H)** *zip 13*, and **(I)** *zip 14* in 14 and 28 days post fertilization (dpf) larvae. Data are presented as mean ± SEM (n = 4, with 15 pooled larvae per n). Bars with different capital letters indicate a significant difference within the same treatment group across developmental age. Bars with different lowercase letters indicate a significant difference between treatment groups within the same developmental age. Expression levels were normalized to the expression of *elfa, rpl13a,* and *rps18*, and are presented relative to larvae exposed to control waterborne Zn and control dietary Zn at 14 dpf.

3.5 Discussion

3.5.1. Effects of waterborne and dietary Zn exposure on physiological condition

In the present study, we observed that various exposure regimes did not affect the amount of food consumed by 28 dpf larvae (~5% of their wet body weight), suggesting that the increased Zn levels present in the diet or the water had no impact on the feeding behaviour of larvae. Interestingly, our results showed that larvae fed on a high Zn diet exhibited a longer body length at 28 dpf when compared to other treatment groups; however, we could not rule out the possibility that larvae which survived during the exposure period would have become more tolerant to Zn toxicity and in turn more resilient to any impacts on growth. On the other hand, we observed that exposure to a high Zn diet alone did not affect cumulative mortality when compared to control, whereas the cumulative mortality was markedly higher in larvae exposed to elevated waterborne Zn, or co-exposed to both elevated waterborne and dietary Zn (~7.5% higher than waterborne exposure alone, and ~15% higher than control). These results suggested that co-exposure via both routes was the most detrimental to the survival of larvae. Previous studies using similar concentrations of waterborne and dietary Zn did not report their adverse effects on mortality in adult zebrafish (Spry et al., 1988; Zheng et al., 2008, 2010). Therefore, it is likely that zebrafish during the developmental periods are more sensitive to Zn exposures, particularly when the levels of Zn in water and diet are both elevated.

3.5.2. Interactions of waterborne and dietary Zn exposure on whole body Zn content

The present study showed that chronic exposure to elevated levels of Zn via water and/or diet significantly increased whole-body Zn content in developing zebrafish. We found that the increased accumulation of Zn was not observed until 14 and 28 dpf. The delayed accumulation

of Zn was similar to the findings previously reported in early life stage zebrafish where embryos exposed to a sublethal concentration of Zn (260 μg/L) exhibited an increase in whole-body Zn content after 5 days of exposure (Puar et al., 2020). These results suggest a low uptake during the early phases of Zn exposure and thereby a delay in Zn accumulation. Additionally, our results showed that whole-body Zn contents continued to increase from 14 dpf to 28 dpf following the exposure, leading to a sustained disruption in Zn homeostasis. This observation suggests that larval zebrafish had limited capacity to reduce Zn uptake and accumulation and were unable to acclimate to elevated concentrations of Zn. Furthermore, our results demonstrated that the highest degree of Zn accumulation was observed in larvae simultaneously exposed to elevated waterborne and dietary Zn. In rainbow trout, it has also been shown that combined exposure to elevated levels of waterborne and dietary Zn resulted in the highest level of Zn accumulation in comparison to exposure to elevated waterborne Zn alone (Spry et al., 1988). These results indicate the additive influence of the gill and the intestine in the uptake and accumulation of Zn during periods of elevated Zn availability.

3.5.3. Interaction of waterborne and dietary Zn exposure on Ca homeostasis

It is well documented that a competitive interaction between Zn and Ca can occur at the gills of adult FW fish, likely through the Zn permeable epithelial Ca channel (ECaC) (Alsop et al., 2011; Hogstrand et al., 1994, 1995). In the present study, waterborne Zn was found to disrupt whole body Ca balance, providing evidence that this interaction between waterborne Zn and Ca might also occur in developing zebrafish. Interestingly, a previous study with winter flounder (*Pseudopleuronectes americanus*) has shown that Zn accumulation in the intestine was not influenced by Ca (Shears and Flethcher, 1983). In rainbow trout, high concentrations of Ca (100 mM) have also been found to have no impact on intestinal Zn uptake (Ojo and Wood, 2008).

These studies have suggested a lack of competition between Zn and Ca at the gut in those fish species. In contrast, our study demonstrated that exposure to a high Zn diet significantly reduced whole body Ca content in developing zebrafish, suggesting possible Zn/Ca interactions at the uptake sites of the intestine. Notably, in contrast to other fish species such as pufferfish (*Fugu rubripes)* and rainbow trout, in which intestinal *ecac* mRNA expression has been found to be extremely low (Qiu and Hogstrand, 2004; Shahsavarani et al., 2006), *ecac* mRNA is highly expressed in the gut of zebrafish (Pan et al., 2005). Therefore, it seems possible that the interaction between Zn and Ca in the gut of zebrafish may occur at least in part through ECaC, and the apparent species-specific Zn/Ca interaction in the gut was possibly owing to the differences in ECaC expression and its functional characteristics.

Our results showed that a disruption in Ca balance by Zn exposure occurred during a later stage of development (28 dpf), regardless of the route of exposure. This delayed onset of Ca disruption observed here in comparison to previous transient disruptions seen at 0 to 5 dpf could be due to the transition of the site of Ca/Zn interactions during development (i.e., from the skin at early life stage to gill and gut later) and the differences in their uptake capacity at the different sites (Puar et al., 2020). Notably, whole-body Ca content was found to increase significantly over development (from 7 to 28 dpf), possibly owing to increased Ca uptake to meet the requirement for growth (e.g., bone development). Therefore, the increased Ca uptake at 28 dpf may also contribute to the increased sensitivity to Zn disruption at that developmental stage. On the other hand, our research revealed that despite different exposure regimes affecting Zn accumulation differently (e.g., co-exposure to elevated waterborne and dietary Zn resulted in the highest Zn body burden), the degree of Ca disruption was found to be comparable among treatments. This

finding raised the possibility that the increased Zn loading in larvae could be a result of uptake through other transport pathways which were independent of Ca uptake (discussed below).

3.5.4. Influence of Zn exposure on whole body homeostasis of other essential trace metals

The present study demonstrated that whole-body Mn homeostasis was highly sensitive to Zn exposure in developing zebrafish. In particular, we observed that the greatest reduction in Mn content occurred in larvae simultaneously exposed to elevated waterborne and dietary Zn when compared to larvae exposed to waterborne or dietary Zn alone. This result indicated an additive effect of the exposure route on the disruption of Mn homeostasis. With initial impairment beginning at 14 dpf and becoming more pronounced at 28 dpf, the disruption in Mn balance appeared to be a long-term chronic condition associated with chronic Zn exposure. Long-term Mn deficiency has been linked to increased inflammation and antioxidant system dysfunction (Jiang et al., 2017); the disruption in Mn homeostasis by Zn exposure could be one potential mechanism for deleterious effects associated with chronic Zn exposure in developing fish.

Our results showed that whole-body contents of Fe, Ni, and Cu were not influenced by Zn exposure. Interestingly, we observed a transient decrease in whole-body Co content in 14 dpf larvae treated with elevated waterborne Zn, irrespective of dietary Zn exposure. To our knowledge, no study has demonstrated Zn/Co interactions in fish; nevertheless, Co like Zn is a Ca analogue, and therefore a competitive interaction of Zn and Co uptake might have occurred in the present study. In vertebrates, Co is critically involved in a variety of biological functions including the metabolism of vitamins and amino acids (Czarnek et al., 2015). The impacts of the transient disruption in Co balance by Zn exposure in developing fish warrants further investigation.

3.5.5. Responses of *zip* transporters to waterborne and dietary Zn exposure during development and their tissue distribution profile in adults

In the present study, we found that mRNA expression of most *zip* transporters remained relatively unchanged over development from 14 to 28 dpf, with the exception of *zip13*. Interestingly, this differed from those patterns reported in the early developmental stages of zebrafish (from 3 to 5 dpf), which suggested a possible age-specific regulation of *zip* expression in zebrafish (Feeney et al., 2005; Puar et al., 2020). In mammals, *zip4* is highly expressed on the apical membrane of enterocytes in the small intestine and is one of the critical Zn transporters responsible for regulating intestinal Zn uptake (Dufner-Beattie et al., 2004; Mao et al., 2007; Wang et al., 2002; Weaver et al., 2007). Similarly, the present study found that *zip4* was predominantly expressed in the intestine and intestinal bulb, supporting its possible involvement in mediating intestinal Zn transport in zebrafish. A recent study has shown that waterborne Zn exposure reduced *zip4* expression in 5 dpf larvae (Puar et al., 2020). In agreement with this finding, the present study also demonstrated that exposure to elevated Zn, either via water or diet, significantly reduced *zip4* expression at both 14 and 28 dpf. These results indicated the presence of a negative-feedback regulatory response of *zip4* to Zn exposure, irrespective of the route of exposure. Considering that *zip4* expression levels were markedly higher in the intestine/intestinal bulb than the gill, the findings suggested that *zip4* may play a role in adjusting dietary Zn uptake when environmental Zn levels become elevated.

In the present study, we observed that exposure to elevated waterborne Zn, with or without the co-exposure to elevated dietary Zn, resulted in a significant increase in *zip7* at 28 dpf. Interestingly, the increase was not observed in larvae fed on an elevated Zn diet alone, suggesting that the increased *zip7* expression was primarily driven by waterborne Zn exposure. Additionally, our study demonstrated that *zip7* was ubiquitously and abundantly expressed in all

the major transport epithelia examined in adults, including the gill, intestine, and intestinal bulb. Notably, waterborne Zn exposure was found to have no effect on *zip7* expression in 3 and 5 dpf zebrafish larvae (Puar et al., 2020), while combined exposure to both elevated waterborne and dietary Zn reduced *zip7* expression in the intestine of adult zebrafish (Feeney et al., 2005). Therefore, it seems likely that the role of *zip7* in regulating Zn homeostasis could be developmental stage-specific and temporal in nature.

A recent study has demonstrated an increase in *zip8* expression levels in 5 dpf larvae after waterborne Zn exposure (Puar et al., 2020). The present study also showed that *zip8* levels were significantly increased by Zn exposure at 14 dpf and remained elevated at 28 dpf. These results suggest that the increases in *zip8* expression occurred as a long-term response to Zn exposure. Importantly, our results also revealed that exposure to elevated dietary Zn alone did not influence *zip8* expression levels, suggesting that the increased *zip8* expression levels were mainly induced by waterborne Zn exposure. In mammals, ZIP8 and ZIP14 are known to transport Mn in addition to Zn (Jenkitkasemwong et al., 2012), and a loss-of-function mutation in ZIP8 has been associated with Mn dysregulation (Lin et al., 2017). Therefore, our findings seemed to support the idea that the increasing *zip8* levels by Zn exposure could be a compensatory response to restore whole-body Mn contents. Nevertheless, our results also suggested that such increases were not sufficient to compensate for the Mn dysregulation as Mn levels remained low. On the other hand, the present study showed that *zip14* was highly expressed in the intestinal bulb; however, its levels were not influenced by Zn exposure. Collectively, the findings raised the possibility that elevated levels of Zn inhibited Mn absorption through Zn transport pathways, potentially via ZIP8 and/or ZIP14.

Our study showed that exposure to elevated Zn significantly decreased *zip13* expression. Interestingly, among all the *zip* transporters examined, only *zip13* expression exhibited a clear inverse relationship with whole body Zn contents (e.g., larvae experiencing higher Zn accumulation also exhibited a lower *zip13* expression level). Notably, our study also revealed that *zip13* was highly expressed in the gills and in the intestinal bulb, suggesting a possible role for *zip13* in mediating Zn absorption. These findings suggested that the regulation of *zip13* expression was dependent on whole-body Zn contents, and the decreased expression in *zip1*3 by Zn exposure could be a response to reduce Zn uptake to help counteract Zn accumulation associated with waterborne and dietary Zn exposure.

3.6 Conclusion and future perspectives

The present study demonstrated that chronic exposure to environmentally relevant concentrations of waterborne and/or dietary Zn increased the whole-body contents of Zn. Larvae exposed to both routes exhibited the highest cumulative mortality and Zn accumulation, indicating that the co-exposure is more detrimental to the physiological performance of larvae. Our study also showed that Zn exposure disrupted Ca, Mn, and Co homeostasis in larvae, with Mn appearing to have an inverse relationship with that of Zn, suggesting their interactions at uptake sites. The results also suggested that Mn balance is a sensitive endpoint in assessing the sublethal effects of Zn exposure in developing fish. Additionally, our study revealed that mRNA levels of *zip4*, *zip7*, *zip8*, and *zip13* were modulated by Zn exposure, with *zip7* and *zip8* levels appearing to be primarily influenced by waterborne Zn exposure. In metal-contaminated environments, fish are often exposed to metals via both water and diet; our study demonstrated the importance of assessing the effects of both routes of exposure in fish when they are

undergoing intensive growth. Findings from the present study also raised several key questions that should be addressed in future studies, including the mechanism underlying the Zn and Mn interactions, regulation of the ZIP transporters at the protein level, and functional significance of the various ZIP transporters. Advancing these pieces of knowledges are important for identifying more sensitive endpoints for ecological risk assessment and for improving our risk prediction associated with Zn contamination.

3.7 Acknowledgements

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3.9 Supplementary Materials

3.9.1 Supplementary Materials and Methods

mRNA expression analysis using droplet digital PCR

Total RNA was first extracted from collected larvae (or tissue) using an RNA extraction kit (Monarch Total RNA Miniprep Kit, New England Biolabs) which included an on-column DNase I treatment to prevent carryover of any possible genomic DNA contamination. A plate spectrophotometer (Take3, Biotek Synergy LX) was used to measure RNA concentration and purity (260 nm/280 nm > 2.00). 1 µg of total RNA was then converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's guidelines. Droplet digital PCR (ddPCR) was then performed to measure the absolute mRNA expression levels of various target genes using the QX200 Droplet Digital PCR system (Bio-rad). The ddPCR reaction mixtures were prepared with EvaGreen™ supermix (Bio-Rad) following the manufacturer's guidelines. PCR conditions were as follows: 5 min 95ºC, followed by 40 cycles of 3 min denaturation at 95ºC and 1 min annealing/extension at 58ºC. Subsequently, signal stabilization was performed which consisted of 5 min at 4 ºC followed by 5 min at 90 ºC. Droplets were then read using the QX200 Droplet Reader (Bio-Rad) and analyzed using the QuantaSoft™ software.

Major ion and trace metal analysis

A more detailed summary of the procedures required for analysis of major ion and trace metal using ICP-MS can be found elsewhere (section 2.3.4). In brief, collected larvae were washed twice with fresh Milli-Q water, transferred to 1.7 mL Eppendorf tubes, dehydrated for 48h at 65°C on a heat block with the lid open, and then digested with TraceMetal grade 6N HNO_3 for another 48h at 65°C with the lid closed. Following digestion, 100 µL of supernatant

from the tissue digest was then diluted to a final volume of 5 mL in 2% HNO_3. Water samples from the recirculating system as well as the exposure waters were also acidified to contain 2% HNO_3. Each sample was passed through a 0.45 µm nylon filter into a 15 mL centrifuge tubes and analyzed by ICP-MS (Agilent 8800 ICP-QQQ-MS) at the Trent University Water Quality Center. Detection limits and QA/QC analysis for ion and trace metal analysis have been reported previously (Supplementary Table S2.1).

3.9.2 Supplementary Tables

Supplementary Table S3.1. Concentration of Zn in exposure waters 1 hour after addition of diet

Treatment Group	[Zn] (µg/L)
Control waterborne Zn + Control Zn diet	37.4 ± 1.2
Control waterborne Zn + High Zn diet	62.5 ± 11.9
High waterborne Zn + Control Zn diet	254.9 ± 11.8
High waterborne Zn + High Zn diet	270.1 ± 3.2

Data are presented as mean ± SEM (n=4)

Supplementary Table S3.2. Primer sets used for droplet digital PCR analysis.

Gene	Accession Number	FWD primer (5'-3')	REV primer (5'-3')
Zip1	NM_212583.2	ACGACGAATGCCAAGGTGTT	GATGCCGATGCCAAGAGGAG
Zip4	NM_001130777.1	AGCTACCACACGAGTTAGGTGA	CAGCCCGACGTACAGGAAGA
Zip6	NM_001001591.1	TACATCGCATGGCTTGGAGG	ATGAGGTGGAGGAGAGCGTC
Zip7	NM_130931.3	TGGTCTGTGGGTGCTAGGTG	TTGGAGCAGAGGGAGAGTGG
Zip8	XM_009307205.3	GCTCGGACACAGTCATTTCCC	AACGCACACGCATTCTGCTC
Zip9	NM_001013540.1	GACCAGCGTCCAGCTTATTGT	GCCTCTTTACTGCTCTGACTGA
Zip10	NM_200671.1	TTCACCAGTACGGCCTGAGC	CCCACATCCAAACCCATCCTGA
Zip13	NM_001005306.3	GGCAGAGAACGCCACTACCT	TGCAGAAGAGAGTTCCTTGGGT
Zip14	NM_001326699.1	ACAACGCAAGCATTTCCGTC	CTGTTCGCTCATCCCGTACA
Elf1a	NM_131263	AAGACAACCCCAAGGCTCTCA	CCTTTGGAACGGTGTGATTGA
Rps18	NM_173234.1	CCCTCGTCATCCCAGAGAAGT	CGCCTTCCAACACCCTTAATAG
Rpl13a	NM_212784.1	GTATTTGGCTTTCCTCCGCA	ACCATGCGCTTTCTCTTGTC

ZIP, Elf1a, Rps18, and rpl13a are abbreviated for Zrt-Irt-Like Protein, Eukaryotic translation initiation factor 1, Ribosomal Protein S18, and Ribosomal protein L13a, respectively.

Supplementary Table S3.3. Results from three-way Analysis of Variance for standard body length of zebrafish larvae

Interaction	Degrees of Freedom	F- Value	P-Value
DPF x Waterborne	1	3.951	0.068
DPF x Diet	1	0.671	0.421
Waterborne x diet	1	2.131	0.157
DPF X Waterborne X diet	1	0.48	0.495

Interactions between waterborne Zn treatment, dietary Zn treatment, and developmental ages (days post fertilization; DPF).

Supplementary Table S3.4. Mean cumulative mortality of fish in different treatment groups across the exposure period.

DPF	Mean ± SEM	F	Sig	Differed from control
8	A=1.75 ± 0.25 B=0.75 ± 0.47 C=1.5 ± 0.64 D=1.25 ± 0.62	0.66	0.59	
9	A=5.2±1.31 B=3.2 ± 0.47 C=5 ± 1.68 D=2.5 ± 1.04	1.22	0.34	
10	A= 9.75 ± 1.2 B= 6 ± 1.22 C=8.75 ± 2.05 D=5± 1.68	1.98	0.17	
11	A=11±1.58 B=7.75±1.37 C=11.25 ± 2.05 D=8 ± 2.08	1.09	0.38	
12	A=11.25±1.37 B=9.5 ± 1.65 C=14.5±2.25 D=11.75±2.25	0.82	0.503	
13	A=11.5± 1.37 B=9.5±1.65 C=14. 5±2.25 D=11.75±2.25	1.16	0.36	
14	A=11.5± 1.19 B=10.25±1.79 C=15.5±1.60 D=13±1.91	1.84	0.19	
15	A=11.5± 1.19 B=10.5±2.02 C=16.75±1.75 D=13.5±1.70	2.64	0.97	
16/17	A=11.75±1.37 B=10.75±2.05 C=18±1.29 D=16.25±1.6	4.23	0.029	
18	A=11.75±1.37 B=11±1.95 C=18±1.29 D=16.75±1.6	4.96	0.018	

19	A=11.75±1.37 B=11.25±2.01 C=18±1.29 D=17.25±1.37	5.31	0.015	
20	A=12±1.58 B=12.25±1.49 C=18.25±1.25* D=19.5±1.55*	7.1	0.005	C: p=0.048 D: p=0.017
21	A=12±1.58 B=12.75±1.93 C=18.75±1.31 D=19.5±1.55*	5.92	0.01	D: p=0.028
22	A=12.75±1.65 B=13.25±1.79 C=19.5±1.65 D=20.5±1.32*	6.32	0.008	D: p=0.024
23	A=13.25±1.7 B=13.5±1.7 C=20.25±1.4 D=22.5±2.21*	6.93	0.006	D: p=0.015
24	A=13.5±1.84 B=13.75±1.75 C=20.5±1.65 D=23.75±1.75*	8.41	0.003	D: p=0.007
25	A=14.25±1.75 B=14.25±1.43 C=21.25±1.88* D=24.5±1.50*	9.71	0.002	C: p=0.048 D: p=0.004
26	A=14.5±1.70 B=14.5±1.65 C=21.5±1.84 D=25.75±1.93*	9.60	0.002	D: p=0.004
27	A=15±1.58 B=14.75±1.70 C=22.25±1.65 D=29±2.16*	14.42	0.001	D: p=0.001
28	A=15.25±1.7 B=14.75±1.70 C=22.25±1.65 D=30±2.64*	13.22	0.001	D: p=0.001

* denotes significant differences from control values ($p<0.05$). Letters A, B, C, and D are used to denote each specific treatment condition of control waterborne and control dietary Zn, control waterborne and high dietary Zn, high waterborne and control dietary Zn, and high waterborne and high dietary Zn, respectively.

Supplementary Table S3.5. Results from three-way Analysis of Variance for whole body major ion and trace metal contents in zebrafish larvae

Major Ion/ Trace Metal	Interaction	Degrees of Freedom	F- Value	P-Value
Zn	DPF x Waterborne*	2	68.434	<0.001*
	DPF x Diet*	2	4.339	0.021*
	Waterborne x diet	1	0.472	0.496
	DPF X Waterborne X diet	2	0.824	0.447
Na	DPF x Waterborne	2	0.347	0.709
	DPF x Diet	2	0.251	0.779
	Waterborne x diet	1	1.424	0.241
	DPF X Waterborne X diet	2	0.111	0.895
Mg	DPF x Waterborne	2	0.724	0.492
	DPF x Diet	2	0.481	0.622
	Waterborne x diet	1	1.044	0.314
	DPF X Waterborne X diet	2	0.0206	0.980
K	DPF x Waterborne	2	0.211	0.811
	DPF x Diet	2	0.573	0.569
	Waterborne x diet	1	2.198	0.147
	DPF X Waterborne X diet	2	0.315	0.732
Ca	DPF x Waterborne	2	1.545	0.227
	DPF x Diet*	2	8.723	<0.001*
	Waterborne x diet	1	1.948	0.171
	DPF X Waterborne X diet	2	0.144	0.866
Mn	DPF x Waterborne*	2	10.488	<0.001*
	DPF x Diet*	2	5.131	0.011*
	Waterborne x diet*	1	4.832	0.034*
	DPF X Waterborne X diet	2	0.912	0.411
Fe	DPF x Waterborne	2	1.533	0.230
	DPF x Diet	2	1.250	0.299
	Waterborne x diet	1	0.491	0.488
	DPF X Waterborne X diet	2	2.052	0.144
Co	DPF x Waterborne*	2	10.212	<0.001*
	DPF x Diet	2	1.956	0.157
	Waterborne x diet	1	0.00674	0.935
	DPF X Waterborne X diet	2	1.151	0.328
Ni	DPF x Waterborne	2	0.0185	0.982
	DPF x Diet	2	1.186	0.317
	Waterborne x diet	1	1.439	0.238
	DPF X Waterborne X diet	2	0.216	0.807
Cu	DPF x Waterborne	2	0.0180	0.982
	DPF x Diet	2	1.015	0.373
	Waterborne x diet	1	1.058	0.311
	DPF X Waterborne X diet	2	0.370	0.693

Interactions between waterborne Zn treatment, dietary Zn treatment, and developmental ages (days post fertilization; DPF). Asterisk (*) is used to denote significant interaction ($p<0.05$).

Supplementary Table S3.6. Results from three-way Analysis of Variance for *zip* expression levels in zebrafish larvae

Zip transporter	**Interaction**	**Degrees of Freedom**	**F- Value**	**P-Value**
Zip1	DPF x Waterborne	1	3.327	0.086
	DPF x Diet	1	1.035	0.323
	Waterborne x diet	1	0.321	0.578
	DPF X Waterborne X diet	1	5.076	0.038*
Zip4	DPF x Waterborne	1	1.397	0.253
	DPF x Diet	1	0.649	0.431
	Waterborne x diet*	1	13.385	0.002*
	DPF X Waterborne X diet	1	0.885	0.359
Zip6	DPF x Waterborne	1	0.0693	0.795
	DPF x Diet*	1	4.907	0.039*
	Waterborne x diet	1	1.768	0.199
	DPF X Waterborne X diet	1	0.229	0.638
Zip7	DPF x Waterborne*	1	10.556	0.004*
	DPF x Diet	1	0.162	0.691
	Waterborne x diet	1	3.874	0.062
	DPF X Waterborne X diet	1	0.405	0.531
Zip8	DPF x Waterborne	1	0.399	0.535
	DPF x Diet	1	0.0235	0.880
	Waterborne x diet	1	0.0118	0.915
	DPF X Waterborne X diet	1	0.0178	0.895
Zip9	DPF x Waterborne	1	4.335	0.051
	DPF x Diet*	1	11.135	0.003*
	Waterborne x diet	1	0.370	0.550
	DPF X Waterborne X diet	1	0.0345	0.855
Zip10	DPF x Waterborne	1	1.843	0.189
	DPF x Diet	1	0.843	0.369
	Waterborne x diet	1	1.363	0.256
	DPF X Waterborne X diet	1	1.204	0.285
Zip13	DPF x Waterborne	1	0.928	0.349
	DPF x Diet	1	0.848	0.370
	Waterborne x diet	1	0.349	0.562
	DPF X Waterborne X diet	1	0.0266	0.872
Zip14	DPF x Waterborne*	1	6.826	0.017*
	DPF x Diet	1	1.815	0.193
	Waterborne x diet	1	0.0446	0.835
	DPF X Waterborne X diet	1	0.907	0.352

Interactions between waterborne Zn treatment, dietary Zn treatment, and developmental ages (days post fertilization; DPF). Asterisk (*) is used to denote significant interaction ($p<0.05$).

CHAPTER IV:

General Discussion

4.1 General Review of Discussion

4.1.1 Overview

My study demonstrates that chronic exposure to sub-lethal environmentally relevant concentrations of Zn increases whole body Zn contents in zebrafish during development regardless of developmental stage or exposure route (waterborne and/or dietary). The highest degree of Zn accumulation was observed in larvae co-exposed to elevated waterborne and dietary Zn suggesting an additive influence of the intestine and the gill on whole body Zn contents. In addition, I observed dysregulations in Ca homeostasis in response to Zn exposures at various developmental stages in larval fish, and notably the patterns of disruption differed depending on life-stage of the fish. Furthermore, the degree of Ca disruption was not associated with Zn exposure route, as fish appeared to be sensitive to both waterborne and dietary routes of Zn. Zn exposure was also found to disrupt Mn homeostasis, and co-exposure to elevated waterborne Zn and dietary Zn was found to exhibit the strongest disruptive effect. These findings indicate that Mn regulation could be a sensitive physiological parameter for assessing chronic Zn toxicity in developing fish. Interestingly, these changes in Mn contents were not noted at the 0-5 dpf stages, while interactions with Ni and Cu were noted at these stages only (0-5 dpf). Together, these results suggest a life-stage specific sensitivity to Zn exposures with regards to metal homeostasis. Additionally, I identified that mRNA expression levels of several Zn transporters were modulated by Zn exposures, and that these regulations were dependent on the route of exposure and developmental age. These transporters also appeared to display temporal and

spatial differences in expression levels across different life-stages. Collectively, my study demonstrated that environmentally relevant concentrations of Zn (both waterborne and dietary) have the potential to cause toxic effects in fish during the early life stages, which should be taken into consideration when developing water quality guidelines to better protect aquatic ecosystems.

4.1.2 Physiological performance of fish exposed to elevated Zn

Physiological performance of organisms is an important factor that must be considered during periods of potentially toxic exposures. In the present study, exposures to environmental concentrations of Zn did not affect the hatching rate or standard body length of larval zebrafish. However, larvae co-exposed to waterborne and dietary Zn displayed a diminished rate of survival. In agreement with my observation, a previous study also reported survival to be a more sensitive endpoint than growth in juvenile rainbow trout exposed to Zn, with concentrations as low as 150 μg/L of Zn reducing survival of exposed juveniles (De Schamphelaere and Janssen., 2004). This is in contrast to other studies that did not observe similar consequences of Zn exposures at comparable exposure concentrations in adult zebrafish and adult rainbow trout (Feeney et al., 2005; Spry et al., 1988; Zheng et al., 2008, 2010). This suggests that fish during the developmental phases are more sensitive than their adult counterparts to exposures of Zn, which may impact the recruitment and long-term sustenance of fish populations in Zn-contaminated aquatic ecosystems.

4.1.3 Zn overload: Accumulation of Zn due to environmentally relevant Zn exposures

In the present study, a consistent pattern of increasing whole body Zn accumulation in zebrafish across different developmental stages was recorded following exposure to environmentally relevant levels of Zn (via both waterborne and/or dietary). Specifically, during

early life stages (0-5 dpf) and at older developmental timepoints (5-28dpf) zebrafish larvae exposed to sub lethal waterborne concentrations of Zn and/or excess dietary Zn suffered from significantly higher Zn body burden. Highest levels of accumulation were noted in larvae simultaneously exposed to both waterborne and dietary Zn, suggesting an additive interaction between branchial and intestinal Zn uptake. This finding is in agreement with previous findings in rainbow trout which also showed combined exposures of waterborne and dietary Zn to cause greater levels of accumulations than waterborne exposures alone (Spry et al., 1988). Furthermore, in the present study it was noted that the accumulation of Zn increased in magnitude over time. Similarly, studies conducted with rainbow trout and embryonic orange-spotted grouper (*Epinephelus coioides*) also reported bioaccumulation of Zn in a dose dependent manner, suggesting the potential of Zn bioaccumulation to span across multiple species of fish (Mcgeer et al., 2000; Zeng et al., 2021). These findings suggest that fish during development have a limited capacity to regulate excess Zn in their body and cannot acclimate to environmentally relevant expsoures of Zn. This inability to regulate environmental levels of Zn may result in potentially detrimental downstream consequences, leading to Zn overload which may be one possible mechanism responsible for the diminished survival of fish co-exposed to waterborne and dietary Zn as seen in my study (De Schamphelaere and Janssen; Küçükoğlu et al., 2013; Salvaggio et al., 2016).

4.1.4 The interaction of Zn and Ca during development

The competitive interaction between waterborne Zn and Ca has been well characterized in cold water teleost such as rainbow trout and this interaction is believed to occur through the brachial epithelial Ca channel (ECaC) (Hogstrand et al., 1994, 1995, 1998; Qiu and Hogstrand, 2004). Notably, cold-water fish are often shown to be most sensitive and least resilient to

changes in their environment. In addition, previous studies have seldom assessed the Zn-Ca interaction at the early life stage. The limited scope of these studies has restricted the generalizability of the previous findings surrounding this Zn-Ca relationship. In the present study, I examined the interactions of Zn and Ca during periods of Zn exposure (waterborne and/or dietary) in a temperate fish (zebrafish) across different early-life stages. Interestingly, a dysregulation in Ca homeostasis was noted at both the early and later developmental stages, suggesting that Zn-Ca related interactions span across both species and life stages.

Notably, in my study I also demonstrated that in addition to waterborne sensitivities to Zn, dietary Zn exposures can also disrupt whole body Ca contents to the same degree. This was in contradiction to previous studies which have suggested a lack of interaction between Zn and Ca at the intestine of other species (e.g., rainbow trout and winter flounder) (Ojo and Wood, 2008; Shears and Flethcher, 1983). It is important to note here that ECaC has been shown to be absent in the gut of these species (Qiu and Hogstrand, 2004; Shahsavarani et al., 2006) but it is known to readily express in the intestine of zebrafish (Pan et al., 2005). With this knowledge, I suggest that the sensitivity to dietary Zn seen in my study to likely involve ECaC and its expression in the intestine of zebrafish.

When assessing the pattern of interaction between Zn and Ca within my two studies (Chapter 2 and 3), I noted that larvae at different developmental stages displayed unique sensitivities to waterborne Zn exposures. More specifically, from 0-5 dpf, waterborne Zn exposure led to a transient and acute dysregulation in whole body Ca contents. Similarly, in previous studies, Ca contents in adult fish have been shown to be disrupted during the early phases of Zn exposures followed by the restoration back to normal levels (Hogstrand et al., 1995; Spry and Wood, 1989). In contrast, at later development stages from 5-28 dpf, larvae

experienced a delayed dysregulation in Ca contents which occurred only after 4 weeks of exposure. I suggest this life stage dependent sensitivity to Zn to be due to differences in uptake capabilities of the different ionic transport sites and their change in transporter expression pattern over development. More specifically, zebrafish do not have functioning ionic regulation of Ca at the brachial filaments until at least 5 dpf (Pan et al., 2005). Therefore, it is very likely that in early life stage exposures the interaction of Zn and Ca occurs at the skin of the yolk sac which is the major uptake site for Ca (via ECaC) prior to development of the gills (Pan et al., 2005), while in contrast occurring at the level of the gill during exposures at later development stages.

Collectively, these novel findings in the present study suggest that not only are larvae equally sensitive to both waterborne and dietary Zn exposures with regards to Ca contents, but that this sensitivity changes over development. The observations of Zn-induced Ca imbalance during development have significant implications as hypocalcaemia is believed to be the main toxic mechanism of Zn in fish and is associated with growth retardation, bone malformities, and diminished cardiac function (Catalano et al., 2012; De Schamphelaere and Janssen, 2004; Hogstrand et al., 1995; Hogstrand et al., 1996; Hogstrand et al., 1998; Hogstrand, 2012; Niyogi and Wood, 2006; Spry and Wood, 1985; Yamaguchi et al., 1983).

4.1.5 Effects of Zn exposure on whole body major ion and trace metal homeostasis

Aside from the well documented interactions of Zn with Ca, I demonstrated in my study that Zn can also interact and disrupt the homeostasis of a variety of trace metals during development (e.g., Ni, Cu, Mn). More interestingly, these interactions appeared to be both life-stage (early development vs later development vs adults) and exposure route specific (waterborne vs dietary).

Specifically, larvae exposed to elevated concentrations of waterborne and/or dietary Zn from 5-28 dpf suffered a dysregulation (decreased) in Mn contents that amplified in severity up until the end of the study. This continued disturbance suggests Mn imbalance to be a long-term consequence of Zn exposures during development. More importantly, Zn-induced decrease in Mn contents appeared to be more pronounced than Ca contents, suggesting Mn balance to be a more sensitive endpoint for assessing Zn toxicity than Ca balance during development. Suboptimal Mn contents have been shown to elicit detrimental consequences (Jiang et al., 2017), suggesting that this sensitivity of Mn contents could be a potential toxic mechanism of Zn exposure in fish during development. Interestingly, the magnitude of decrease in Mn contents was highest during combined exposures to waterborne and dietary Zn. Though the exact mechanism for the interaction of Zn and Mn remains elusive, the function and activity of two transporters (ZIP8 and ZIP14) could provide valuable insights into the relationship of these two metals (see section 4.1.5).

Interestingly, exposures to sub lethal concentrations of waterborne Zn from 0-5 dpf did not affect Mn contents (as observed at 14 and 28dpf) but did in fact reduce the whole body content of both Ni and Cu. Interestingly, these Zn-induced disruptions in Mn, Ni, and Cu homeostasis have not been reported in previous studies with adult fish models (i.e. zebrafish or rainbow trout), emphasizing the life-stage specific nature of these interactions. Notably, the patterns of dysregulation of Ni and Cu homeostasis in response to Zn at the early developmental stage followed the same patten observed with Ca. Though not much investigation has been done on the homeostatic relationship of these metals, previous studies in zebrafish showed that waterborne Ni or Cu could reduce Ca uptake at 4 to 7dpf (Alsop and Wood, 2011), while Ca exposures were found to reduce uptake of Cu (Alsop and Wood, 2011; Craig et al., 2010).

Overall, these observations along with the finding of the present study provide some support for the presence of a shared transport mechanisms for Zn, Ca, Ni, and Cu during early development, however, this must be further investigated before any conclusions can be made.

4.1.6 Maintaining balance: Temporal and spatial localization of ZIP transporters and regulation in response to Zn exposures

Aside from those pathways through which Zn may interact with other metals (e.g., ECaC), Zn uptake is primarily mediated through the ZIP family of transporters. In my studies, I examined the expression of the various *zip*'s at the early life stage using *in-situ* hybridization assays, and at the adult stage through the generation of a tissue-specific expression profile using droplet digital PCR technologies. I noted that the various *zip* transporters display a tissue-specific localization that changes in pattern over development from early-life stages to adults, and that Zn exposures modulate the expression of these *zip* in both temporal and treatment-dependent manners. This suggests that each ZIP plays a unique life-stage dependent role in maintaining Zn homeostasis in its respective tissue. For example, at 1 dpf *zip7* is expressed in the eyes only (Yan et al., 2012); at 5 dpf *zip7* expression is restricted to the heart and the otolith; while at the adult stage *zip7* is highly expressed in all major transport epithelia (intestine, intestinal bulb, gill, kidney). Interestingly, in our study *zip7* was found to be upregulated in response to both waterborne, as well as combined waterborne and dietary exposures of Zn at 28 dpf only, but not in response to dietary exposures or at any other developmental timepoint. In contrast, in adult zebrafish, *zip7* has been shown to be downregulated in the intestine in response to combined waterborne and dietary Zn exposures (Feeney et al., 2005), further strengthening the argument for the temporal and treatment specific regulation of ZIP transporters.

In mammals, ZIP4 is highly expressed at the apical surface of intestinal enterocytes and has been shown to undergo significant levels of regulation at both the mRNA and protein level to either increase or decrease intestinal Zn absorption depending on the exposure conditions (Dufner-Beattie et al., 2003, 2004, 2007; Mao et al., 2007; Weaver et al., 2007). Interestingly, in the present study, *zip4* was shown to also be expressed in the intestine and intestinal bulb of zebrafish, and was downregulated in response to waterborne and/or dietary Zn exposures. This suggests that similar to mammals, ZIP4 may also be involved in reducing intestinal absorption of Zn in fish during periods of Zn loading. Interestingly, though *zip4* expression was decreased in response to Zn exposures, *zip13* was the only ZIP to display a clear inverse relationship with whole body Zn contents. *Zip13* has been shown to be highly expressed in the intestine, intestinal bulb, and gill (Feeney et al., 2005) of zebrafish, suggesting that *zip13* also has a prominent role in maintaining whole body Zn contents during periods of overload. Notably, Zn accumulation was still pronounced in these larvae during exposures, indicating that any regulation occurring in *zip4* and *zip13* was insufficient to completely ameliorate the effects of elevated Zn exposure.

ZIP8 is a unique Zn transporter as it has been shown through *xenopus* oocyte overexpression studies to be capable of transporting Mn and Fe in addition to Zn (Jenkitkasemwong et al., 2012; Wang et al., 2012). Other studies in mice models have uncovered that ZIP8 plays a critical role at the hepatocyte canalicular membrane in the reabsorption of Mn from the bile to limit Mn excretion (Lin et al., 2017). Interestingly, in the present study, waterborne Zn exposures were found to upregulate the expression of *zip8* at those same developmental timepoints that larval fish suffered from Mn dysregulations. This suggests that upregulation in *zip8* could be a possible compensatory response to restore Mn homeostasis via increased reabsorption during periods of waterborne Zn-induced Mn disruptions. However,

further experimental analysis is needed before any conclusions can be made on the exact nature of the involvement of ZIP8 in Zn and Mn balance (see section 4.3).

The effects and impacts of Zn exposures during development as discussed here in section 4.1 have been summarized and presented in Figure 4.1.

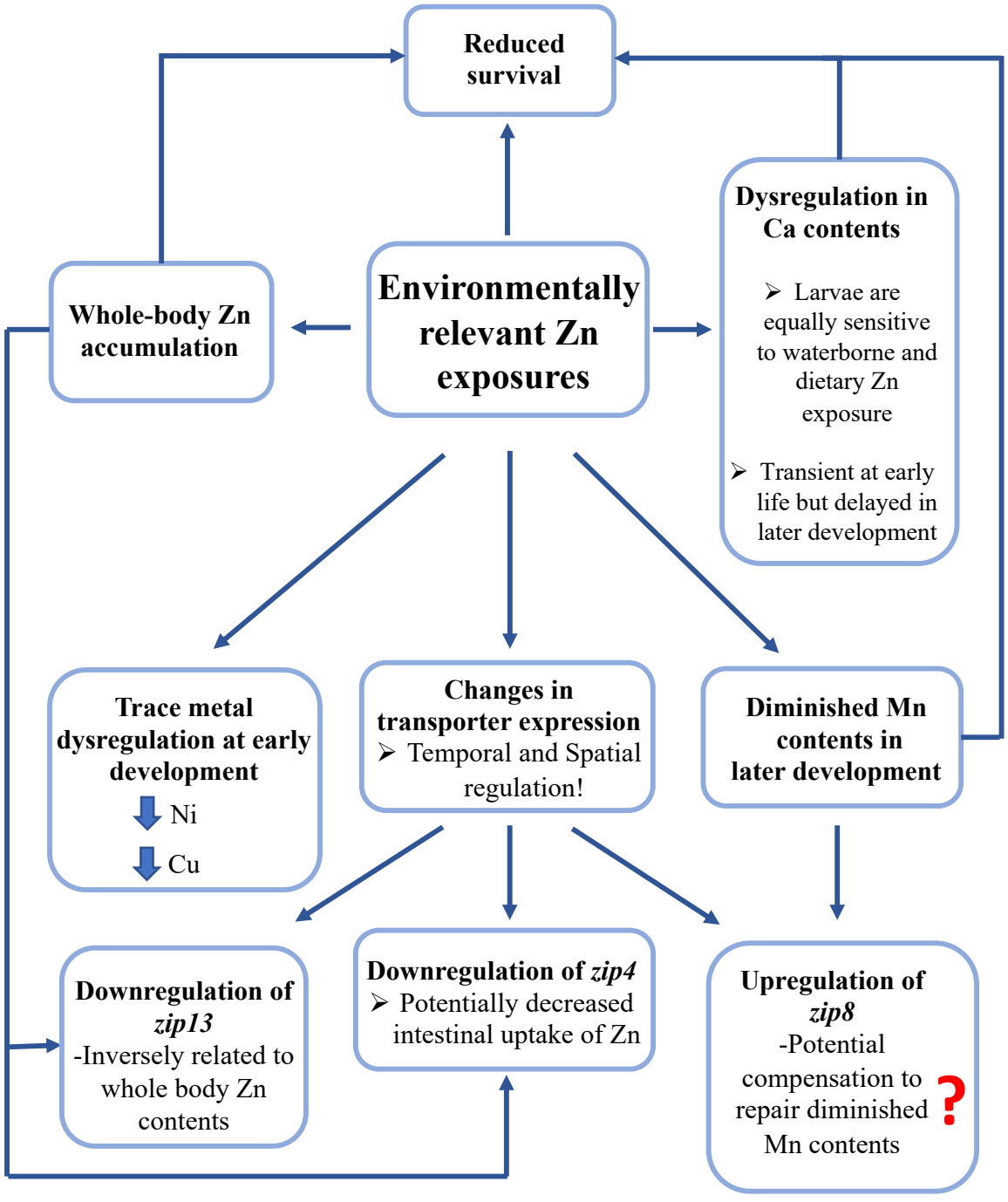

Figure 4.1. A summary of the impacts of environmentally relevant Zn exposures during development. The effects reported here have been summarized from my findings discussed in this thesis. All experiments were conducted using zebrafish during the developmental stages (0-28 days post fertilization). A wide variety of parameters were assessed in my experiments in response to waterborne (261 µg/L) and/or dietary Zn (1500 mg Zn/ kg food) and are discussed in more detail in Chapter 2 and Chapter 3 of this thesis.

4.2 Implications and Significance of the Research

My studies provide substantial evidence that larval fish at different developmental ages are sensitive to the concentrations of Zn recorded in polluted aquatic environments. In particular, these sensitivities include disruptions in major ion and trace metal homeostasis (Ca, Zn, Mn, Ni, and Cu) during development. Many previous studies have suggested that their disruptions may induce a variety of adverse effects in fish, including increased inflammation, impaired gas exchange, growth retardation, decreased immune function, impaired cardiac function, abnormal development, reduced reproductive performance, as well as stimulated cellular apoptosis and tissue necrosis (Brungs, 1969; De Schamphelaere and Janssen, 2004; Driessnack et al., 2017; Hogstrand et al., 1995; Hogstrand et al., 1996; Hogstrand, 2012; Hogstrand et al., 1998; Hojyo et al., 2016; Jiang et al., 2017; Küçükoğlu et al., 2013; Witeska and Kosciuk, 1962; McDonald and Wood, 1993; Niyogi and Wood, 2006; Salvaggio et al., 2016; Speranza and Seeley, 1977; Spry and Wood, 1985). Notably, although developing fish undergo varying degrees of regulation to counteract these effects, such regulatory processes are not sufficient to completely ameliorate the effects of elevated Zn exposure rendering them vulnerable. With this knowledge, I suggest that my findings could be used to develop an adverse outcome pathway (AOP) linking Zn exposure to its apical toxicity. AOPs are a central element of toxicological frameworks that are built to support environmental risk assessment of contaminants (Villeneuve et al., 2014). My findings in this thesis will help begin to organize information on the toxic mechanisms of Zn into models that clearly link Zn exposures to key toxicity initiation events (metal and ion dysregulation, transporter regulation) that ultimately impair the overall physiological performance of freshwater fish. This will help to build a conceptual framework that may be further developed through

additional experiments and extensive literature reviews to assist in future environmental regulatory decision making processes.

Notably, most regulatory decision making today relies on species sensitivity distributions (SSD) that are used to set safe limits in surface waters. In comparison to standardized toxicity tests that use a single surrogate species (e.g., rainbow trout), a SSD fits single species data to a cumulative probability distribution that can be used to develop environmental guidelines that are more representative and protective of all species in complex aquatic systems (Raimondo et al., 2008). Importantly, though fish in natural environments are often co-exposed to waterborne and dietary Zn, dietary Zn exposure remains an overlooked aspect of environmental metal pollution and are not typically included as part of a SSD (Bossuyt et al., 2005). Furthermore, although a SSD can assess the impacts of exposures across a wide variety of taxa and species, they often do not include life-stage specific differences in sensitivity to metals within a given species. Notably, the research presented here provides strong indication that larval fish are just as sensitive to dietary exposures of Zn as they are to waterborne exposures, and that these sensitivities to Zn change over development with larval fish displaying greater sensitivities than their later life-stage counterparts. With this in mind, although zebrafish are not currently used as a model species in a regulatory context within North America, the findings presented in this thesis provide important implications to suggest that Zn SSD should be expanded to incorporate those parameters assessed in our study to involve both the assessment of multiple exposure routes of Zn (e.g., both waterborne and dietary) and also the various life-stages of fish, which can then be used to generate a more reliable and more protective ecological risk assessment framework.

4.3 Conclusion and Future perspectives

Although my studies presented here have provided a great deal of insights into the implications of elevated Zn exposure in fish during development, there are still some gaps in the knowledge that exist.

First, the current study examined the regulation of Zn transporters in response to Zn exposures at the whole-body level only. Though this has provided important information with regards to the regulatory patterns of ZIP, future studies should examine the regulation in specific tissue to better and more accurately understand how this regulation varies between tissues during development. Secondly, due to a lack of availability for antibodies, I assessed the regulation of transporters at the transcriptional level only. Therefore, I suggest that future studies should examine the potential regulation of ZIP at the protein level through the development of ZIP antibodies to better understand the complex regulatory patterns of fish during Zn exposures. In addition, the current study did not assess any possible regulation in basolateral Zn efflux transporters (e.g., ZnT). How ZnT's contribute to maintaining whole body homeostasis during periods of elevated Zn exposure should also be addressed in future studies.

Next, in metal contaminated environments, multiple metals are often found to be elevated together as they originate from similar anthropogenic sources of pollution. More specifically, elevated levels of Zn are often accompanied with elevated levels of Cd in contaminated aquatic systems due to common anthropogenic routes (ATSDR, 2012; Driessnack et al., 2017; Thornton, 1992). Since the present study assessed the impacts of Zn alone, I suggest that future studies should assess the impacts of Zn and Cd co-exposure on the physiology of fish.

Next, though my study has provided clear evidence for a relationship of Zn with Ni and Cu at the early life stage, the exact mechanisms responsible for such interaction remains elusive.

Whether these metals interact through a shared transport pathway warrants further investigation and could provide great insight into metal homeostasis in FW fish.

Finally, though I have provided some support for the relationship of Mn transport involving ZIP8 during periods of Zn exposure, further evidence needs to be collected before any conclusions can be drawn. Currently through my time in the lab I have utilized cutting-edge CRISPR-cas9 technologies to generate a new and novel ZIP8 knockout line in zebrafish. I hypothesize that ZIP8$^{-/-}$ mutants will experience dysregulations in whole-body Zn and Mn contents and that other Zn specific and Mn specific transporter mechanisms will be upregulated in order to compensate for loss of ZIP8 transport ability. Furthermore, I also hypothesize that during periods of elevated Zn exposure (both waterborne and dietary), ZIP8$^{-/-}$ mutant larvae will experience greater disruptions in Mn homeostasis than wildtype larvae due to the lack of regulation through ZIP8. With the study currently in progress, I hope to provide further insight into this novel and newly discovered relationship between Zn and Mn, while also uncovering further the physiological role of ZIP8 in maintaining whole-body Zn and Mn contents.

Overall, our study on the impacts of environmentally relevant exposures to Zn on developing fish showed significant cause for concern. These impacts were assessed across a variety of developmental stages using a multitude of parameters. The adverse effects associated with Zn exposures in the natural environment should bring much needed attention to the requirement of developing better regulatory strategies for the protection of aquatic life.

4.4 References

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