INFLUENCE OF DIETARY IRON EXPOSURE ON THE PHYSIOLOGICAL REGULATION OF IRON AND OTHER TRACE METALS DURING DEVELOPMENT IN ZEBRAFISH (Danio rerio)

Theanuga Chandrapalan

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Graduate Program in Biology
York University
Toronto, Ontario

July 2019

© Theanuga Chandrapalan, 2019
Abstract

Iron (Fe) is an essential trace metal for development; however, its level in the body must be maintained within physiological range. In the present study, the effects of dietary Fe on growth, trace metal homeostasis, and the expression of various metal transporting genes were investigated during development in zebrafish (Danio rerio). The results demonstrated that growth and whole body Fe content were substantially higher in 14 dpf larvae fed high Fe diets. Interestingly, prolonged exposure to high Fe increased mortality but did not affect the levels of Fe and other trace metals (e.g., Zn$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, Ni$^{2+}$) in 28 dpf larvae. Molecular analysis revealed that exposure to high dietary Fe induced differential changes in the mRNA expression levels of various metal transporters (e.g., dmt1, zip8, and zip14). These findings suggested that the maintenance of metal homeostasis following Fe exposure was likely associated with the dynamic regulation of metal transporting proteins.
Acknowledgments

I would like to thank my supervisor Dr. Raymond Kwong for giving me this opportunity to not only learn but also to strengthen my passion for research. I am always grateful for his guidance which has been crucial in the completion of my studies and it goes without saying that it has been a great privilege to learn from him.

I also thank my advisor Dr. Scott Kelly for his continuous support during my thesis and sharing his expertise in this field. I appreciate all your feedback which always gets me thinking.

Thank you to Janet Fleites for teaching me everything that I know about zebrafish husbandry and spending endless mornings with me in the fish room! You have been a big support for me.

I am also grateful to Dr. Andrew Donini for always being welcoming and allowing me to use his lab equipment for my experiments.

To my peers in the comparative physiology group, I thank them for making the past two years even more enjoyable. A special thanks to Chun for his time and support and Andrea for her guidance when I first started this program.

Lastly, I also thank my parents who have sacrificed their lives back home in order to give me the opportunities that I have never dreamed of before.
### Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>viii</td>
</tr>
</tbody>
</table>

### CHAPTER 1: General Introduction ......................................................... 1

1.1. Importance of iron metabolism in vertebrates................................. 1

1.2. Current understandings of cellular Fe regulation .......................... 3

1.2.1. Ferric reductase ............................................................................ 5

1.2.2. Divalent metal transporter 1 ....................................................... 6

1.2.3. Iron-regulated transporter 1 ....................................................... 9

1.2.4. Ferritin ....................................................................................... 10

1.2.5. Transferrin ................................................................................ 12

1.2.6. Hepcidin ..................................................................................... 13

1.3. Routes of Fe absorption in fishes .................................................. 13

1.3.1. Waterborne .................................................................................. 14

1.3.2. Dietary ....................................................................................... 15

1.4. Fe and divalent metal interaction .................................................... 17

1.4.1. Zinc ............................................................................................ 19

1.4.2. Copper ....................................................................................... 19

1.4.3. Calcium ..................................................................................... 19

1.5. Metal toxicity and environmental concerns ...................................... 20

1.6. Research objectives ......................................................................... 20

1.6.1. Rationale ..................................................................................... 20

1.6.2. Experimental model ..................................................................... 21

1.6.3. Hypotheses and objectives .......................................................... 21

1.7. References ..................................................................................... 24
Chapter 2: Influence of dietary iron exposure on trace metal homeostasis and expression of metal transporters during development in zebrafish

2.1. Introduction

2.2. Materials and methods

   2.2.1. Animal husbandry
   2.2.2. Expression profile of Fe-regulatory genes in adult zebrafish
   2.2.3. Diet preparation and dietary Fe exposure regime
   2.2.4. Metal analysis
   2.2.5. Evaluation of growth
   2.2.6. Droplet digital PCR analysis of metal-regulatory genes
   2.2.7. Statistical analysis

2.3. Results

   2.3.1. Expression profile of major Fe-regulatory genes in various tissues of adults
   2.3.2. Physiological conditions of larval zebrafish exposed to dietary Fe
   2.3.3. Whole body Fe burden in larval zebrafish exposed to dietary Fe
   2.3.4. mRNA transcript levels of Fe-regulatory genes in larval zebrafish following exposure to dietary Fe
   2.3.5. Whole body trace metal and ion content in larval zebrafish exposed to dietary Fe
   2.3.6. mRNA transcript levels of various metal/ion transporters in larval zebrafish following exposure to dietary Fe

2.4. Discussion

   2.4.1. Fe-regulatory gene expression in adult tissues
   2.4.2. Fe homeostasis in larval zebrafish exposed to dietary Fe
   2.4.3. Transcriptional regulation by dietary Fe
   2.4.4. Trace metal homeostasis in larval zebrafish exposed to dietary Fe

2.5. Conclusions and Perspectives

2.6. References

2.7. Appendix
List of Tables

Chapter 2: Influence of dietary iron exposure on trace metal homeostasis and expression of metal transporters during development in zebrafish

Table 1. Primer sets used for RT-PCR and droplet digital PCR for iron-regulatory and metal transport genes. ........................................................................................................................................................................ 43

Table 2. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of trace metal (Zn, zinc; Cu, copper; Mn, manganese; Ni, nickel; Co, cobalt) concentration (ng/larvae) in zebrafish larvae exposed to different levels of dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) .................................................................................................................................................. 59

Table 3. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of major ions (Na, sodium; K, potassium; Mg, magnesium; Ca, calcium) concentration (ng/larvae) in zebrafish larvae exposed to different levels of dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) ........................................................................................................................................ 60
List of Figures

CHAPTER 1: General Introduction

Figure 1. Importance of Fe homeostasis................................................................. 2
Figure 2. Current model for the cellular absorption of Fe in vertebrates......................... 4
Figure 3. Divalent metal transporter 1 (DMT1).......................................................... 8
Figure 4. Iron response protein (IRP) regulation of IREG1 and Ferritin.......................... 12
Figure 5. Interactions between divalent metal transport pathways................................ 18

Chapter 2: Influence of dietary iron exposure on trace metal homeostasis and expression of metal transporters during development in zebrafish

Figure 1. The expression profile of iron-regulatory genes (dmt1, ireg1, frrs1a, frrs1b, fh1a, fh1b, tf, and hep) in the tissues (gill, intestine, intestinal bulb, kidney, brain, liver, ovaries, and testis) of adult zebrafish. ............................................................................ 51

Figure 2. Cumulative mortality (%) in larvae exposed to dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) from 5 to 28 dpf. .............................................. 52

Figure 3. Standard length (mm; SL) of zebrafish larvae exposed to different dietary Fe levels (Low Fe (black), 157 mg Fe/kg; Medium Fe (light grey), 490 mg Fe/kg; High Fe (dark grey), 1795 mg Fe/kg). .................................................................................................................. 53

Figure 4. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of total Fe body burden (ng Fe/larvae) in zebrafish larvae (5 (pre-feeding; white bar), 14, and 28 dpf) exposed to different levels of dietary Fe (control (black), diet 1 (grey), and diet 2 (dark grey))........... 54

Figure 5. Expression of iron-regulatory genes following dietary Fe exposure (low Fe (black circle), medium Fe (white circle), and high Fe (black triangle) at 5 (pre-feeding), 14 and 28 dpf. ................................................................................................................................. 55

Figure 6. Expression of trace metal transporter genes following dietary Fe exposure (low Fe (black circle), medium Fe (white circle), and high Fe (black triangle) at 5 (pre-feeding), 14 and 28 dpf............................................................... 57
List of Abbreviations

µg = microgram
ANOVA = Analysis of variance
ATPase = Adenosine triphosphatase
Ca = Calcium
Cd = Cadmium
Co = cobalt
CTR1 = Copper transporter 1
Cu = Copper
CuATPase = Copper ATPase
Dcytb = Duodenal cytochrome b
ddPCR = Droplet digital polymerase chain reaction
DMT1 = Divalent metal transporter 1
DNA = Deoxyribonucleic acid
dpf = Days post fertilization
ECaC = Epithelial calcium channel
EF1α = Elongation factor 1α
Fe = Iron
Frrs = Ferric reductase
FTH = Ferritin
Hep = Hepcidin
ICP-MS = Inductively coupled plasma mass spectrometry
IRE = Iron response element
IREG1 = Iron regulated transporter 1
IRP = Iron regulatory protein
K = Potassium
M = Molar concentration
Mg = Magnesium
Mn = Manganese
mRNA = Messenger ribonucleic acid
MS-222 = Tricaine methanesulfonate
N = Normality
Na = Sodium
NADH = Reduced nicotinamide adenine dinucleotide
NADPH = Reduced nicotinamide adenine dinucleotide phosphate
Ni = Nickel
NTC = No template control
Pb = Lead
PCR = Polymerase chain reaction
RNA = Ribonucleic acid
RT-PCR = Reverse transcriptase polymerase chain reaction
SEM = Standard error of the mean
SL = Standard length
SLC11 = Solute carrier 11
Tf = Transferrin
UTR = Untranslated region
ZIP = Zrt- and Irt-like protein
Zn = Zinc
CHAPTER 1: General Introduction

1.1. Importance of iron metabolism in vertebrates

Iron (Fe) is an essential trace element that is vital for growth and survival (Wood et al., 2012). It is involved in many biological processes including oxygen transport, DNA synthesis, and cellular respiration. Fe is also an integral component in enzymes and proteins (Muckenthaler and Lill, 2012). Accordingly, when there is an imbalance of Fe in the body, it has adverse effects on the organism often leading to the development of disorders such as anemia and Fe overload (Figure 1) (Andrews, 2000; Steinbicker and Muckenthaler, 2013). Low Fe in the body affects hemoglobin count and negatively impacts normal development. In contrast, excess Fe generates free radical species and induces cellular oxidative damage (Aisen et al., 2001). Therefore, Fe levels in the body must be tightly regulated and maintained within physiologically safe concentrations.

Organisms have evolved sophisticated mechanisms of Fe regulation that function in the transport, storage, and metabolism of Fe (Andrews and Schmidt, 2007; Chen and Paw, 2012). In general, these mechanisms are thought to be highly conserved among vertebrate groups, with some of the same proteins identified in aquatic and terrestrial vertebrates including the sea lamprey, hagfish, zebrafish, mice, and humans (Aisen et al., 1972; Andersen et al., 1998; Zhao et al., 2014). Similarly, molecular controls of vertebrate Fe metabolism can be influenced by various factors including Fe, nitric oxide, and oxidative stress (H$_2$O$_2$) (Hentze and Kühn, 1996). In the current chapter, the focus will be directed specifically on the molecular regulation of systemic Fe concentrations by Fe associated regulatory and transport proteins to elucidate how each of these individual components orchestrates the overall maintenance of Fe metabolism and homeostasis.
Figure 1. Importance of Fe homeostasis. Fe imbalances have adverse effects on the organism leading to the development of disorders of Fe metabolism. Low Fe concentrations lead to the development of Fe deficiency while high Fe results in Fe overload. It is important to maintain Fe levels in the body within physiologically safe concentrations.
1.2. Current understandings of cellular Fe regulation

Current understanding of the molecular mechanisms regulating cellular Fe transport is gained mostly from studies using mammalian models. These studies have led to the development of a proposed model for cellular Fe metabolism (Figure 2). This model includes six major proteins: ferric reductase (Frrs), divalent metal transporter 1 (DMT1), iron-regulated transporter 1 (IREG1; also called ferroportin), ferritin (Fth), transferrin (Tf), and hepcidin (Hep). Frrs is an apical membrane-bound enzyme that catalyzes the conversion of insoluble Fe\(^{3+}\) (ferric) to the soluble Fe\(^{2+}\) (ferrous) (McKie et al., 2001; Raja et al., 1992). This increases the bioavailability of Fe which can then be assimilated into the cell. In mammals, DMT1 is expressed prominently in the brush border membrane of the duodenum and is proposed to be the major site of dietary Fe absorption (Gunshin et al., 1997; Tandy et al., 2000).

Once Fe enters the cell, it can be stored in Fth (Chasteen and Harrison, 1999), utilized for cellular processes (Kaplan and Ward, 2013; Lill and Kispal, 2000), or be exported out into the bloodstream by IREG1 (McKie et al., 2001). In the bloodstream, Fe\(^{2+}\) is converted back to Fe\(^{3+}\) which is bound to Tf and circulates in the plasma (McKie, 2001; Raja et al., 1992). Lastly, Hep is a peptide hormone which is thought to be the major hormonal regulator of systemic Fe homeostasis (Ganz, 2005; Nemeth et al., 2004). The processes of cellular Fe metabolism are summarized in Figure 2 and a brief overview of each protein will be outlined in the following sections. It is important to note that while these six proteins are highlighted due to their major role in Fe absorption, there are also several other key factors that aid in the maintenance of overall Fe homeostasis.
Figure 2. Current model for the cellular absorption of Fe in vertebrates. Ferric reductase reduces Fe\(^{3+}\) to soluble Fe\(^{2+}\) that can be transported into the cell by divalent metal transporter 1 (DMT1). This Fe can be stored intracellularly by binding to ferritin, metabolized within the cell (mitochondrial usage, Fe-protein synthesis, Fe-enzyme complexes), or be exported out of the cell via iron-regulated transporter 1 (IREG1). The expression of IREG1 is modulated by the peptide hormone hepcidin, which is synthesized in the liver. In the plasma, Fe\(^{2+}\) is converted back to Fe\(^{3+}\) which remains bound to transferrin while circulated throughout the body.
1.2.1. Ferric reductase

Ferric reductase (Frrs) is an enzyme that catalyzes the reduction of Fe$^{3+}$ to the soluble Fe$^{2+}$ (McKie et al., 2001). This enzyme is important for Fe absorption because dietary Fe in the form of ferric salts or insoluble oxides must first be reduced to Fe$^{2+}$ for bioavailability and cellular uptake. As such, this step is required prior to Fe uptake via DMT1 and is considered the rate-limiting step in Fe absorption (Ekmekcioglu et al., 2018). Frrs enzymes have been identified in the duodenum of mammals as duodenal cytochrome b (Dcytb) and this enzyme is located on the brush border membrane of enterocytes that aid in the dietary uptake of Fe (McKie et al., 2001). The study by McKie et al. (2001) also demonstrated that Frrs can be modulated by dietary Fe levels. For example, when mice were fed a diet deficient in Fe, an upregulation in Dcytb mRNA and protein expression was observed, potentially to increase Fe uptake (McKie et al., 2001).

Frrs functions by using protons donated from NADH/NADPH to catalyze electron transfer to Fe$^{3+}$ (Ekmekcioglu et al., 2018). Also, in human epithelial colorectal adenocarcinoma cell line (Caco-2), Fe uptake was stimulated by ascorbic acid in a dose-dependent manner by aiding in this reduction process by Frrs (Han et al., 1995). In mammals, ascorbic acid is typically secreted in the duodenum as biliary and pancreatic secretions. However, this differs from fishes as the enzyme gluconolactone oxidase responsible for the biosynthesis of ascorbic acid is absent (Dabrowski, 1990). Therefore, in fishes, the mechanism of Fe$^{3+}$ reduction via ascorbic acid is dependent on the dietary intake of ascorbate (Wood et al., 2012). More importantly, although Frrs activity has been reported in fishes, there has been no fish homolog to mammalian Dcytb cloned as of yet. Nonetheless, the evidence and importance for Frrs activity have been highlighted in rainbow trout (Oncorhynchus mykiss) with studies demonstrating a double in intestinal Frrs activity upon exposure to a Fe-deficient diet (Carriquiriborde, 2003).
1.2.2. **Divalent metal transporter 1**

Divalent metal transporter 1 (DMT1) is a member of the solute carrier family 11 (SLC11) of the proton-coupled divalent metal transporters (SLC11A2) ([Figure 3A](#)) (Neves et al., 2011). This protein was identified by two independent groups, Gunshin *et al.* (1997) (via positional cloning from rats) and Fleming *et al.* (1997) (G185R mutation in microcytic (*mk*) mice) as an apical Fe transporter. Since then, four isoforms of DMT1 have been identified with splice variations: 1) exon 16 that gives rise to two variants that differ in their 3’ untranslated region (UTR), resulting in either the presence or absence of an iron response element (IRE) (Lee et al., 1998); and 2) the presence of two transcriptional start sites on exon 1A and 1B, respectively (Hubert and Hentze, 2002). All four isoforms have been reported with equal Fe transport capabilities. However, the IRE-containing variants show post-transcriptional regulation by iron regulatory proteins (IRPs) (Mackenzie et al., 2007). In the absence of Fe, IRP is bound to the IRE which aids in the increased stability of the *dmt1* mRNA, protecting it from endonuclease activity ([Figure 3B](#)) (Anderson et al., 2012). In contrast, when Fe is present, it binds IRPs either mediating its conversion into an active aconitase or promoting ubiquitination and proteasomal degradation of the IRP (Kaplan and Ward, 2013). Thereby, this process leaves IRE unoccupied and *dmt1* susceptible to degradation ([Figure 3C](#)). Nevertheless, it is the IRE-containing variant that is thought to be the predominant isoform involved in intestinal Fe absorption (Mackenzie et al., 2007).

The role of DMT1 in Fe transport has also been characterized by using two mammalian models that have mutations encoding this gene leaving it non-functional; Belgrade (*b*) rat and microcytic (*mk*) mouse. Both animals had difficulties with Fe uptake resulting in anemia and significant growth retardation (Fleming *et al.*, 1997a; Fleming *et al.*, 1998; Veuthey and Wessling-
Likewise, the *chardonnay (cdy)* zebrafish mutants developed hypochromic, microcytic anemia from a mutation that produced truncated DMT1 mRNA (Donovan et al., 2002). These studies across organisms further consolidate the conserved function of DMT1 in Fe transport and homeostasis (Zhao et al., 2014).

Lastly, although DMT1 is considered to be the major Fe transporter, studies have also found that it is functionally capable of transporting other divalent metals such as zinc (Zn), manganese (Mn), cobalt (Co), copper (Cu), cadmium (Cd), nickel (Ni), and lead (Pb) (Gunshin et al., 1997). Studies also illustrate the complex interactions between Fe transport pathways and divalent metal transport, these will be examined in more detail later. With regards to DMT1 transport mechanisms, it has been well characterized that this protein is a symporter which transports one molecule of Fe$^{2+}$ with a proton (H$^+$) (Gunshin et al., 1997; Mackenzie and Hediger, 2004). Finally, DMT1 is involved in both transferrin bound (erythrocytes) and non-transferrin bound (enterocytes) Fe transport.
Figure 3. Divalent metal transporter 1 (DMT1). A) Transports one molecule of Fe^{2+} into the cell with every proton (H^{+}). Proposed to transport other divalent metals such as zinc (Zn), copper (Cu), nickel (Ni), manganese (Mn), cobalt (Co), cadmium (Cd), and lead (Pb). DMT1 variants with the presence of an iron response element (IRE) near the 3’ untranslated region (UTR) are regulated by the binding of iron regulatory proteins (IRPs). B) When there is low availability of Fe, IRPs are bound to the IRE and increase stability of the mRNA. C) In the presence of high Fe, IRP is not bound to the IRE which leaves the transcript unoccupied and susceptible to degradation.
1.2.3. Iron-regulated transporter 1

Iron-regulated transporter 1 (IREG1 or ferroportin) was identified as a basolateral membrane protein that is involved in the efflux of Fe from cells into the blood (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2001). For example, McKie et al. (2000) had located this protein in the basolateral membrane of the duodenal mucosa of mice and Donovan et al. (2000) identified this gene as responsible for the development of hypochromic anemia as a result of low circulating Fe in the zebrafish mutant *weissherbst*. Under conditions of high Fe, both *ireg1* and its protein abundance significantly increase (Donovan et al., 2000; McKie et al., 2001). These findings agree with the presence of an IRE in the *ireg1* mRNA, which is post-transcriptionally regulated by IRP and systemic Fe conditions (Abboud and Haile, 2000; McKie et al., 2001).

In contrast to *dmt1*, the IRE in *ireg1* is situated at the 5’ UTR near the transcriptional start site (Figure 4) (Lymboussaki et al., 2003). Accordingly, IRP binding to the IRE hinders the binding of transcription factors required for protein synthesis (Anderson et al., 2012). Conversely, in Fe sufficient conditions, Fe binds the IRP which leaves the IRE unoccupied and ready for translation. However, this implies that under conditions of low Fe, IREG1 expression is essentially repressed and Fe efflux cannot meet systemic Fe demands. Accordingly, an *ireg1* transcript variant lacking the IRE was later identified by Zhang et al. (2009). This transcript was found localized to duodenal cells of mice and was upregulated under Fe deficient conditions. The presence of these two *ireg1* variants provides a solution for the maintenance of systemic Fe homeostasis during times of high and low Fe conditions.

In addition to IREG1 regulation via IRPs, IREG1 is also regulated by Hep which affects its localization to the cell membrane (discussed in more detail below) (Nemeth et al., 2004). Recently, the possibility of IREG1 regulation via metal exposure was also investigated and Cu and
Zn were identified to stimulate expression levels in the human intestinal Caco-2 TC7 cell line (Tennant et al., 2002; Yamaji et al., 2001). Currently, the mechanism of how IREG1 transports Fe is unknown. However, Fe export by IREG1 requires the presence of ferroxidases (Ward and Kaplan, 2012). For example, it is believed that IREG1 transports Fe in the form of Fe$^{2+}$ because Fe export has been linked to the presence of membrane-bound multicopper oxidases such as ceruloplasmin and/or hephaestin (Vulpe et al., 1999; Ward and Kaplan, 2012). These enzymes located on the basolateral membrane facilitates the oxidation of Fe$^{2+}$ exported out by IREG1 into Fe$^{3+}$ which can then be bound to transferrin molecules (Ward and Kaplan, 2012). Further, defective Fe transport was observed in mice lacking hephaestin (Vulpe et al., 1999). Overall, the role of IREG1 in Fe efflux is vital for the maintenance of systemic Fe homeostasis.

### 1.2.4. Ferritin

Ferritin molecules are intracellular storage proteins that help sequester Fe in the cytoplasm. They have been identified across species, with conserved three-dimensional structures (Harrison et al., 1991; Miguel et al., 2009; St. Pierre et al., 1992). These proteins are comprised of 24 subunits arranged in a manner to form a hollow shell in the center that can accommodate up to 4500 Fe$^{3+}$ molecules (Ford et al., 1984; Harrison et al., 1991). Ferritin is composed of a mix of light (L) and heavy (H) chain subunits (Harrison and Arosio, 1996). The L-chain ferritin is involved in core formation while H-chains function in the oxidation of Fe$^{2+}$. Accordingly, their relative abundance was shown to differ among tissues. For example, in tissues where there is a higher need for prolonged Fe storage, such as the liver, there was a greater level of L-chain ferritin (Arosio et al., 1978). However, in tissues requiring relatively low Fe storage, there is a higher level of H-chain ferritin. Fe stored in ferritin is believed to be in the oxidized form (Fe$^{3+}$) which is transported out via endosomes and degraded in lysosomes (Harrison and Arosio, 1996).
Different types of ferritin with specialized functions have been identified including mitoferrin and hemosiderin. Mitoferrin is the mitochondrial form of ferritin that acts as a protectant against the formation of reactive oxygen species that accompany metabolic reactions in the mitochondria (Levi et al., 2001). Hemosiderin functions in sequestering excess Fe and for degradation (Wixom et al., 1980). Also, ferritin molecules not bound to any Fe is referred to as apoferritin (Macara et al., 2015). Lastly, ferritin mRNA also contains an IRE in the 5’ UTR which allows for post-transcriptional regulation by IRP binding and modulation by Fe (Aziz and Munro, 1987; Muckenthaler et al., 1998). Regulation of ferritin by IRP is similar to the regulation of ireg1 (Figure 4). Overall, ferritin is an important factor in Fe metabolism; protecting the cell against free Fe and providing a safe place for its storage.

A. **Low Fe**

B. **High Fe**
Figure 4. Iron response protein (IRP) regulation of IREG1 and Ferritin. A) In Fe-deficient conditions, IRP is bound to the IRE which is situated near the transcriptional start site. This hinders the binding of transcription factors required for translation. B) In the presence of Fe, it binds IRPs which leaves the IRE unoccupied and ready for translation.

1.2.5. Transferrin

Free Fe cannot be transported throughout the body, in order to overcome this obstacle, specialized Fe transport proteins called transferrin (Tf) are utilized (Huebers and Finch, 1987). These proteins bind Fe$^{3+}$ and circulate it in the blood to different parts of the body. They play a crucial role in distributing Fe from sites of absorption to tissues in need, contributing to the maintenance of systemic Fe homeostasis. Tf has two high-affinity binding sites that allow for the binding of two Fe molecules and this occurs in a reversible manner allowing for the efficient transport and release of Fe. Also, the binding of Fe to Tf is pH-dependent with association favored at pH greater than 7 and dissociation favored at acidic pH below 6.5 (Huebers and Finch, 1987). This is important because during the disassociation of Fe three protons are required and these are typically donated from bicarbonate (Chung, 1984).

In the absence of Fe, Tf allows binding of various other metals such as Cu, Co, and Mn (Aasa et al., 1963; Aisen et al., 1969). Tf itself is synthesized in the liver and is composed of a single polypeptide chain. Tf mRNA and protein expression have also been shown to increase during Fe deficiency but the exact mechanisms by which Fe modulates Tf expression is currently unknown (Tong et al., 2002). On the contrary, Tf receptors are known to contain IRE elements and are appropriately modulated by IRPs (Idzerda et al., 2006). Most cells have Tf receptors on their cell membrane which allows for the binding of Tf-bound Fe and receptor-mediated endocytosis into the cell (Karin and Mintz, 1981).
1.2.6. Hepcidin

Hepcidin, also known as hepcidin antimicrobial peptide (HAMP), is the hormonal regulator of Fe homeostasis and it is synthesized in the liver. Hepcidin secretion is dependent on Fe levels and it is released into the blood when there is a high level of Fe in the body. In studies of mice with a mutation for the hepcidin gene, the animals were reported to develop Fe overload and severe hemochromatosis (Lesbordes-Brion et al., 2006). Hepcidin regulation of Fe metabolism occurs by affecting IREG1 expression. Studies have demonstrated that hepcidin promotes the internalization of IREG1 from cell membranes and subsequent proteolysis in lysosomes (Nemeth et al., 2004). This prevents Fe efflux into the blood lowering circulating levels of Fe (Nemeth et al., 2004). However, as a hormonal regulator, its mode of action takes about 1-4 h to affect IREG1 expression (De Domenico et al., 2005; De Domenico et al., 2007). Still, how Fe modulates hepcidin expression remains unclear. Further, other regulators of hepcidin expression have also been identified. For example, in hypotransferrinemic (hpfx) mice Tf injection was able to restore Fe levels in the body and normalize hepcidin expression (Bartnikas et al., 2011). Interestingly, hepcidin is also regulated by inflammation which acts as a defense mechanism in order to lower the availability of Fe and protect the organism during the invasion by pathogens (Krause et al., 2000; Skaar, 2010).

1.3. Routes of Fe absorption in fishes

Unlike mammals, fishes can acquire Fe from the gastrointestinal tract as well as the gills. However, even as one of the most abundant metals on Earth, the availability of Fe in the aquatic environment is low (Bury, 2003a; Watanabe et al., 1997). This is due to the insoluble nature of Fe at circumneutral pH where Fe$^{2+}$ usually exists as Fe oxides (Stumm and Morgan, 1996) and as complexes with organic matter such as humic acids, fulvic acids, and siderophores (Hamilton-
Ta
yor et al., 2002; Rozan et al., 2000) and inorganic matter such as sulphides (Witter et al., 2000). As a result, fishes acquire the majority of their Fe intake from their diets. An estimate of Fe requirement is currently unknown; however, it has been suggested that fishes in aquaculture require 30-170 mg Fe/kg feed for normal growth (Andersen, 1997; Watanabe et al., 1997).

Previous studies on fishes have highlighted the negative effects of low Fe (i.e. anemia and reduced growth) (Watanabe et al., 1997) and elevated Fe (i.e. tissue damage and increased mortality) on the optimal function and survival of the organism (Bury, 2003b; Dalzell and Macfarlane, 1999; Standal et al., 1997). Lastly, although there is no specialized route for Fe excretion, fishes still lose Fe through defecation and sloughing of the epithelial mucosa (Andrews, 2000). In zebrafish, the daily loss of Fe ranges around 28.4 µg/kg/day and this must be accounted for by new Fe uptake. Therefore, it is important for fishes to tightly regulate waterborne and dietary intake of Fe in order to maintain systemic Fe levels without inducing the toxicity associated with Fe imbalance.

1.3.1. Waterborne

In fishes, waterborne Fe uptake occurs through the gills (Andersen, 1997; Bury and Grosell, 2003; Roeder and Roeder, 1966). A study by Andersen (1997), provided the first direct evidence for uptake of Fe via the gill epithelium using radiolabelled $^{59}$Fe, however, the cellular location of DMT1 is still currently unknown (Bury, 2003b). In waterborne studies, Fe as ferrous FeSO$_4$ was in general found to be more bioavailable than ferric salts and resulted in greater Fe accumulation in the organism (Roeder and Roeder, 1966). This study also highlighted the nutritive role of Fe with observations of its stimulatory effect on fish growth. As for the mechanism of Fe transport, it is thought to be similar to other tissues, where Fe is transported through the apical Fe transporter, DMT1, accompanied with the ferroxidase, ferric reductase (Bury and Grosell, 2003).
On the other hand, modulation in transport kinetics has been reported with higher affinity recorded at lower Fe concentrations (<40 nM Fe) (Bury and Grosell, 2003).

Furthermore, when zebrafish were exposed to various divalent metals (Cu, Zn, Mn, Co, Ni, Pb, and Cd), the only metal that disrupted Fe uptake through the gills was Cd. This illustrates that Cd likely shares the same uptake route via DMT1 through the gills. Moreover, although waterborne Fe uptake is not the major route of absorption, it is still sufficient to account for the daily loss rates of Fe in fishes (Bury and Grosell, 2003). However, exposure to high Fe (~2 mg/L) has adverse effects on fish resulting in gill damage and disruption to ion regulation (Peuranen et al., 1994). Lastly, most literature pertaining to Fe regulation in fishes have been selective to waterborne studies, but the importance of dietary Fe has been highlighted in fishes and there is still a need for more studies on this topic.

1.3.2. Dietary

Dietary Fe uptake occurs throughout the intestine. In some family of fishes such as the cyprinids which include carps and minnows (i.e. zebrafish), there is an absence of a distinct stomach and instead, the swelling of the anterior region of the intestine forms the intestinal bulb allowing for the temporary storage of food before digestion (Wallace et al., 2005). In general, the intestine in fishes is a long tube-like structure that can be divided into three subregions (anterior intestine, mid-intestine, and posterior intestine) depending on functional segregation as a result of differences in structural properties such as the abundance of intestinal villi and types of epithelial cells present (enterocytes, goblet cells, enteroendocrine cells, and Paneth cells). However, it seems that Fe uptake in fishes occurs throughout the entire intestinal tract with studies also demonstrating the stimulatory effects of Fe on digestive and absorptive enzyme activities along the intestine (Kwong and Niyogi, 2008; Ling et al., 2010). Furthermore, Fe uptake in the intestine occurs
through enterocytes with apical DMT1 and ferric reductase which transport Fe from the intestinal epithelium into the body for assimilation (Kwong et al., 2010). Also, as a proton symporter, the slightly acidic nature of the intestinal lumen favours the function of DMT1 in Fe uptake (Bury, 2003b).

As mentioned above, feeds containing ~30-170 mg Fe/kg were recommended for normal growth and function in fishes (Bury, 2003b; Watanabe et al., 1997). However, many studies on adult fishes have explored the effects of varying dietary Fe concentrations on Fe burden, transcriptional regulation, and even the protective role of a high Fe diet against toxic metal uptake. For example, in rainbow trout a high Fe diet (1975 mg Fe/kg) was shown to increase tissue accumulation which resulted in an 86-fold increase in Fe in the intestine, followed by a 6.5-fold increase in the liver. High dietary Fe also resulted in an elevation of Fe in the blood which was accompanied by increases in plasma transferrin level (Carriquiriborde, 2003; Kwong et al., 2013). On the contrary, in a study by Cooper et al. (2006) which explored the effects of a low Fe diet (33 mg Fe/kg) on zebrafish, it was demonstrated that the fish exhibited an increase in DMT1 and IREG1 expression in the intestine. Interestingly, there was also a noted increase in DMT1 expression in gills, which may indicate a compensatory mechanism for increasing Fe uptake with an upregulation of waterborne intake (Cooper et al., 2006). Similar responses were observed in marine medaka (Oryzias melastigma) where exposure to a low Fe diet was shown to increase the absorption of waterborne Fe (Wang and Wang, 2016). Lastly, evidence for the protective effect of dietary Fe on Cd accumulation was demonstrated in zebrafish and rainbow trout where exposure to dietary Fe was shown to reduce Cd accumulation in tissues (Cooper et al., 2006; Kwong et al., 2011). In summary, although the importance of dietary Fe in fishes have been demonstrated in
multiple studies, information on the regulatory mechanisms involved in this process and the effects of dietary Fe during early development remains limited.

1.4. Fe and divalent metal interaction

There are complex interactions between metal transport pathways, and the Fe transport pathway is no exception (Figure 5). Limitations in the sensitivity of metal transporters in discriminating among metals of similar sizes and characteristics enable multiple metal substrates for some transporters. This includes the major Fe transporter, DMT1 which has been demonstrated to mediate the absorption of various other divalent metals, including the essential metals Zn, Cu, Mn, Ni and Co, and the non-essential metals Cd and Pb (Bannon et al., 2003; Bressler et al., 2004; Garrick et al., 2003; Tallkvist et al., 2001). Although the majority of these studies were conducted in mammals, recently similar results have also been proposed in models of fishes (Bury and Grosell, 2003; Kwong and Niyogi, 2009). An in vitro examination of the inhibition of intestinal Fe absorption in rainbow trout demonstrated that Ni/Pb, Cd/Cu, and Zn (in the order of magnitude) all competed with Fe uptake via DMT1 (Kwong and Niyogi, 2009). However, direct in vivo evidence for the role of DMT1 in the transport of other metals in fishes has yet to be proven. Instead, research continues on the modulation of Fe uptake by metals exposures. These studies have elucidated complex metal-metal and metal-protein interactions between Fe transport pathways. For example, Zn and Cu not only interact with Fe uptake via DMT1, but they also affect DMT1 expression (Iyengar et al., 2009; Tennant et al., 2002; Yamaji et al., 2001). Likewise, calcium (Ca) interaction with Fe has also been highlighted across studies (Shawki and Mackenzie, 2010; Thompson et al., 2010). A brief summary of these interactions will be discussed in the sections below.
**Figure 5. Interactions between divalent metal transport pathways.** The major Fe transporter, divalent metal transporter 1 (DMT1), has been proposed to be able to transport other divalent metals such as zinc (Zn), copper (Cu), nickel (Ni), manganese (Mn), cobalt (Co), cadmium (Cd), and lead (Pb). Similarly, these divalent metals have interactive effects on DMT1 protein expression and Fe uptake. Zn stimulates DMT1 and iron-regulated transporter 1 (IREG1) expression and the Zn transporters, Zrt- and Irt-like proteins (ZIP), ZIP 8 and ZIP 14 can also mediate the uptake of Fe. Cu inhibits DMT1 and stimulates IREG1 expression. Copper transporter 1 (CTR1) transports Cu into the cell, which is exported out via the basolateral copper ATPase (CuATPase). Ca and Fe can be transported via the epithelial calcium channel (ECaC) and Ca is a non-competitive inhibitor of Fe uptake. Green arrows with a positive sign (+) are used to denote stimulatory effects and red arrows with a negative sign (-) are used to denote inhibitory effects. Red arrows with dashed lined denote non-competitive inhibition.
1.4.1. **Zinc**

In agreement with Zn transport via DMT1, this metal also inhibited Fe absorption in the intestine of rainbow trout (Kwon and Niyogi, 2009). When the overall magnitude of inhibition by divalent metals was examined, Zn was on the lower end (Ni > Cu > Zn). This may be related to the presence of the family of zinc-specific transporters called Zrt- and Irt-like proteins (ZIP) (Zheng et al., 2008). To emphasize further the complex interactions between metal transport pathways, two proteins of the ZIP family, ZIP 8 and ZIP 14, can also mediate the uptake of Fe (Ho et al., 2012). Lastly, Zn exposure has stimulatory effects on the mRNA and protein expression of DMT1 in the Caco-2 TC7 cells (Yamaji et al., 2001).

1.4.2. **Copper**

Cu is a competitive inhibitor of Fe uptake via DMT1 and significant increases in Cu accumulation were also observed in rats reared under Fe-deficient conditions (Jiang et al., 2013). Similarly, elevation in dietary Cu negatively affects protein and mRNA expression of DMT1, potentially due to the shared route of uptake (Sharp, 2004). However, Cu specific transporters also exist including copper transporter 1 (CTR1) located on the apical membrane for metal uptake and copper ATPase (CuATPase) for the basolateral absorption of Cu into the blood (Komjarova and Bury, 2014; Mackenzie et al., 2004; Ravia et al., 2005). Interestingly, the complex interactions of Fe and Cu also extend to the basolateral Fe exporter, IREG1. With Cu stimulating IREG1 expression in Caco-2 cells (Tennant et al., 2002).

1.4.3. **Calcium**

Radiotracer and voltage clamp studies on *Xenopus* oocytes expressing DMT1 RNA demonstrated that Ca was not a substrate for DMT1 but that it is a non-competitive inhibitor of Fe
uptake via DMT1 (Shawki and Mackenzie, 2010). This supports the negative implications of dietary calcium on Fe bioavailability (Hallberg et al., 1991). Lastly, the calcium-specific transporter, epithelial calcium channel (ECaC) functions in the transport of calcium in fishes (Pan et al., 2005). Further, characterization of piscine ECaC in *Fugu rubripes* (pufferfish) demonstrated that this protein was not only permeable to Ca but also to Zn and Fe (Qiu and Hogstrand, 2004).

1.5. Metal toxicity and environmental concerns

Generally, the Fe level in natural freshwaters is below 1 mg/L (Xing and Liu, 2011). Environmental sources of Fe include weathered rocks and soil, and anthropogenic sources include wastewater and acid mine drainage (Vuori, 1995). In Fe-contaminated waters, the Fe levels in invertebrates (i.e., prey of fishes) range between 160-12,650 mg/kg dry weight (Winterbourn et al., 2000). With elevated Fe having adverse effects on fishes including tissue damage, oxidative stress, and even increased mortality. Conversely, low Fe also produces negative effects such as the development of Fe deficiency, leading to anemia, decreased hemoglobin and erythroid count, and reduced growth rate (Watanabe et al., 1997). However, the likelihood for the development of anemia in feral fishes is low. Similarly, due to the availability of Fe in aquatic environments, dietary Fe is the primary source of Fe for Fishes (Bury, 2003a; Watanabe et al., 1997). Yet, the current understanding of dietary Fe in relation to metal uptake, homeostasis, and toxicity in fishes is limited.

1.6. Research objectives

1.6.1. Rationale

Fe plays vital roles in the body and its importance has been highlighted in many areas of literature. However, there is still much that remains unknown with regards to the mechanisms
regulating Fe homeostasis during development. In fishes, studies on the effects of Fe during early development is limited with the majority of studies selectively examining the effects of waterborne Fe. Therefore, the current understanding of the effects of dietary Fe is limited. This is important because it is the diet that accounts for the majority of Fe uptake in fishes. Furthermore, although the Fe uptake pathways have been proposed to interact with multiple other essential (Zn, Cu, and etc.) and non-essential (Cd) metals, there is still limited in vivo evidence on the effects of dietary Fe and metal accumulation in fishes. Therefore, examining the effects of dietary Fe exposure on developing fishes will not only provide insight into the regulation of Fe during early development but it will also highlight any differences in the mechanisms employed by adults.

1.6.2. **Experimental model**

The model organism utilized in this study is the freshwater zebrafish and this organism was optimal to answer my research questions because: 1) several major Fe regulatory genes have been cloned and identified in zebrafish; 2) it is a widely used vertebrate model with conserved processes of Fe metabolism that can be compared with a wide array of organisms (van der Vorm and Paw, 2017; Wood et al., 2012); 3) it is a well-characterized developmental model with embryonic and larval stages defined; and 4) easy breeding and husbandry with one breeding pair yielding 200-300 embryos and larvae reaching only a few millimeters in size.

1.6.3. **Hypotheses and objectives**

The goal of my thesis was to examine the effects of dietary Fe exposure on the homeostasis of Fe and other trace metals in developing zebrafish and this was broken down into four main hypotheses and objectives (the focus of chapter 2):
**Hypothesis 1:** Fe-regulatory genes in zebrafish are differentially expressed in different organs of adults.

**Objective:** Examination of the expression of Fe-regulatory genes in the tissues of adults.

Tissues from the gill, intestine, intestinal bulb, kidney, brain, ovaries, and testis of adult zebrafish were collected and RT-PCR analysis was performed to examine the expression profile of Fe-regulatory genes.

**Hypothesis 2:** Exposure to an elevated level of dietary Fe affects the physiological condition of larval zebrafish.

**Objective:** Evaluation of the effects of dietary Fe on physiological condition of larval zebrafish.

Zebrafish larvae were exposed to three different concentrations of dietary Fe from 5 to 28 days post fertilization (dpf). Physiological parameters such as growth, morphology, and mortality were examined during dietary Fe exposure. Growth was estimated using standard body length (SL; mm), which is the distance from the snout of the head to the base of the caudal fin. Changes in morphology were monitored by examining patterns of pigmentation and fin development. Lastly, mortality rates were measured daily for the whole duration of the feeding exposure.

**Hypothesis 3:** Whole body levels of Fe and other metals are influenced by dietary Fe in zebrafish.

**Objective:** Evaluation of the effects of dietary Fe on the whole body accumulation of Fe and other trace metals and ions.
The effects of dietary Fe exposure on whole body metal accumulation was measured using inductively coupled plasma mass spectrometry (ICP-MS). This allowed for the evaluation of trace metal body burden including Fe and other divalent trace metals.

**Hypothesis 4:** Fe-regulatory and metal transporter genes are expressed during early development and mRNA transcript levels are modulated by Fe exposure in zebrafish.

**Objective:** Examination of the expression of Fe-regulatory and metal transport genes in larvae exposed to different dietary Fe levels.

Larval zebrafish were also collected following dietary Fe exposure for droplet digital PCR (ddPCR) analysis to examine the mRNA abundance of Fe-regulatory and metal transporter genes during development and following Fe exposure.
1.7. References


(Oncorhynchus mykiss) fed low and high iron diets. J. Exp. Biol. 207, 75–86.

Chasteen, N. D. and Harrison, P. M. (1999). Mineralization in Ferritin: An Efficient Means of


on gastrointestinal and branchial assimilation of both iron and cadmium in zebrafish (Danio

Dabrowski, K. (1990). Gulonolactone oxidase is missing in teleost fish. The direct

Dalzell, D. J. B. and Macfarlane, N. a a (1999). The toxicity of iron to brown trout and effects

De Domenico, I., Ward, D. M., Nemeth, E., Vaughn, M. B., Musci, G., Ganz, T. and
Acad. Sci. 102, 8955–8960.

Ganz, T., Musci, G. and Kaplan, J. (2007). The Molecular Mechanism of Hepcidin-


Chapter 2: Influence of dietary iron exposure on trace metal homeostasis and expression of metal transporters during development in zebrafish

2.1. Introduction

Iron (Fe) is an essential trace metal for all vertebrates; however, at high levels Fe is potentially toxic. It may result in Fe overload, leading to oxidative damage (i.e., induced by the generation of free radicals) and mortality (Bury, 2003a; Dalzell and Macfarlane, 1999). Several recent studies have shown that increasing level of Fe in freshwater ecosystems is an emerging issue of environmental concern in many geographic regions (Björneräs et al., 2017; Kritzberg and Ekström, 2012; Sarkkola et al., 2013). In Fe-contaminated aquatic environments, elevated accumulation of Fe in invertebrates may contribute to increased dietary Fe loading in higher trophic animals including fish (Brown, 1977; Rainbow, 2002; Winterbourn et al., 2000). Although the gills of fish have the capacity to absorb Fe from water (Andersen, 1997; Bury and Grosell, 2003; Cooper and Bury, 2007), diet is considered as the major route of Fe exposure. This is because dissolved ferrous iron (Fe$^{2+}$) concentration is usually quite low under circumneutral pH and ferric iron (Fe$^{3+}$) forms insoluble Fe oxides (Stumm and Morgan, 1996).

The molecular mechanisms regulating cellular Fe transport and metabolism in fish are thought to be similar to higher vertebrates such as mammals (Zhao et al., 2014). In enterocytes, the apical ferric reductase (Frrs) reduces Fe$^{3+}$ to Fe$^{2+}$ (McKie et al., 2001), whereas the divalent metal transporter 1 (DMT1) mediates the absorption of Fe$^{2+}$ into the cell (Donovan et al., 2002; Fleming et al., 1997b; Gunshin et al., 1997; Tandy et al., 2000). Intracellularly, Fe can either be stored by binding to ferritin (FTH) or be exported via the basolateral iron-regulated transporter 1 (IREG1; also called ferroportin) (Andrews and Schmidt, 2007; Chasteen and Harrison, 1999; McKie et al., 2001). Fe in the blood is bound to transferrin (Tf) and is transported throughout the
body. Systemic Fe level can also be regulated by the peptide hormone hepcidin (Hep) via modulation of IREG1 expression (Ganz, 2005; Nemeth et al., 2004).

Several studies in fish have demonstrated modulation in Fe absorption and expression of Fe-regulatory genes in response to changes in dietary Fe level. For example, an increase in waterborne Fe uptake has been reported in developing medaka (Oryzias melastigma) fed a low Fe diet (Wang and Wang, 2016). Adult zebrafish (Danio rerio) exposed to a low Fe diet exhibited an increase in mRNA levels of both dmt1 and ireg1 in the gill and intestine (Cooper et al., 2006). Interestingly, in adult zebrafish and rainbow trout (Oncorhynchus mykiss), exposure to a high Fe diet also increased mRNA expression level of dmt1 in the intestine (Craig et al., 2008; Kwong et al., 2013). How the various Fe-regulatory proteins co-ordinate to maintain Fe homeostasis, particularly during the nutritional transition period from yolk resorption to exogenous feeding, remains unclear.

In mammals, DMT1 has been proposed to mediate the uptake of several divalent metals in addition to Fe$^{2+}$, including Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ (Bannon et al., 2003; Bressler et al., 2004; Garrick et al., 2003; Tallkvist et al., 2001). Studies with Xenopus oocyte or HEK293 cell line have shown that the Zn$^{2+}$ transporter ZIP 8 and ZIP 14 may also facilitate the uptake of Fe$^{2+}$ (Liuzzi et al., 2006; Wang et al., 2012). These complex interplays between metal absorption and the involvement of multiple metal transporters may have important implications for the homeostasis and toxicity of metals. The interaction of Fe with other divalent metals have also been demonstrated in fish using isolated intestine or Xenopus oocyte expressing piscine DMT1 (Cooper and Bury, 2007; Kwong and Niyogi, 2009; Nadella et al., 2007). However, the physiological significance of such interaction under in vivo condition, and the influences of dietary Fe on the regulation of various metals, still remain poorly understood. Understanding the fundamental
mechanisms regulating the metal transport pathways may help develop a more reliable model to predict the physiological effects of dietary metal exposure.

In the present study, we investigated the effects of dietary Fe exposure on the physiological performance and homeostatic regulation of divalent metals during the early transition period of development (from larvae stage to juvenile), using zebrafish as a model species. Specifically, we examined i) the expression profile of major Fe-regulatory genes in different organs in adults, ii) the effects of dietary Fe exposure on physiological conditions and metal homeostasis over development, and iii) the regulation of Fe-regulatory and various metal transport-related genes following dietary Fe exposure. Our results demonstrated that larval fish are sensitive to the effects of dietary Fe. Exposure to high dietary Fe led to a significant elevation in mortality and induced differential changes in the expression pattern of various metal transporters during development.
2.2. Materials and methods

2.2.1. Animal husbandry

Adult zebrafish (*Danio rerio*; Tüpfel long fin strain) were maintained in a recirculating water system (Aquaneering, CA, USA) at 28°C and were subjected to 14:10 light/dark photoperiod. Fish were fed twice daily with brine shrimp (rinsed in deionized water) in the morning and commercial zebrafish diet (Zeigler, PA, USA) in the afternoon. Embryos were collected and transferred to 50 mL Petri dishes until use (detailed below). The experiments were conducted in compliance with the Canadian Council of Animal Care guidelines and the York University Animal Care Committee protocol.

2.2.2. Expression profile of Fe-regulatory genes in adult zebrafish

To examine the mRNA expression profile of various Fe-regulatory proteins in adults, gill, intestine, intestinal bulb, kidney, brain, liver, ovaries, and testis were collected following euthanization using an overdose of tricaine methanesulfonate (MS-222). Tissues from 3 to 5 adults were pooled as one sample (N=1). Total RNA was extracted from the samples using an RNA extraction kit (GeneJet, Thermo Fisher Scientific). Genomic DNA was removed using DNase I (NEB) and total RNA concentrations were quantified using a NanoDrop (ThermoScientific). cDNA was synthesized from 1 μg of total RNA using iScriptTM cDNA synthesis kit (Bio-Rad). RT-PCR was performed to examine the expression of divalent metal transporter 1 (*dmt1*), iron-regulated transporter 1 (*ireg1*), ferric reductase 1a and 1b (*frrs1a,frrs1b*), ferritin 1a and 1b (*fth1a,fth1b*), transferrin (*tf*), and hepcidin (*hep*). Primer sets are summarized in Table 1. The PCR condition was: 3 min initial denaturation at 95°C, 30 cycles of 95°C for 30 s, 58°C for 60 s, 72°C for 45 s, and a final extension at 72°C for 10 min. Elongation factor 1 alpha (*ef1α*) was selected as
an internal control. The PCR amplicons were visualized using agarose gel electrophoresis. Purified PCR products were sent out for sequencing (The Centre for Applied Genomics, The Hospital for Sick Children) to confirm that all amplicons were the gene of interest.
Table 1. Primer sets used for RT-PCR and droplet digital PCR for Fe-regulatory and metal transport genes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Abbreviation</th>
<th>Accession Number</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divalent metal transporter 1</td>
<td>dmt1</td>
<td>NM_001040370.1</td>
<td>F: 5’-CCAGCAAAACAACGAGACCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CAGGAAACCCCTCCATCAAAAC-3’</td>
</tr>
<tr>
<td>Iron-regulatory protein 1</td>
<td>ireg1</td>
<td>HM068067.1</td>
<td>F: 5’-CCTAAACACTGAACCCCCCGAT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CGAAGGACCAAAGACCAACTCT-3’</td>
</tr>
<tr>
<td>Ferric reductase 1 alpha</td>
<td>frrs1a</td>
<td>XM_021468500.1</td>
<td>F: 5’-GTGCAAGTTCTCCGCATCCA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-GGAGGGCCGATAATGACTTG-3’</td>
</tr>
<tr>
<td>Ferric reductase 1 beta</td>
<td>frrs1b</td>
<td>XM_017353998.2</td>
<td>F: 5’-ATGGATGGGGAACGATGACG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CTCTGATAGCCTCCAAGCCCT-3’</td>
</tr>
<tr>
<td>Ferritin 1 alpha</td>
<td>fth1a</td>
<td>XM_017356903.2</td>
<td>F: 5’-GCTGGGCATCTCAACACAACG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CTTTGCCGATTCTTCCAACCAGC-3’</td>
</tr>
<tr>
<td>Ferritin 1 beta</td>
<td>fth1b</td>
<td>NM_001004562.1</td>
<td>F: 5’-TCAAGGAGCTGTCGGATTGG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CCCTGCATATGGCTGACTTGA-3’</td>
</tr>
<tr>
<td>Transferrin</td>
<td>tf</td>
<td>NM_001291499.1</td>
<td>F: 5’-GTTCAGGAGGGACCGCAAGT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-ACTCTGGGATTTTCCAACCAGC-3’</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>hep</td>
<td>NM_205583.2</td>
<td>F: 5’-TCCCTTCATACACAGCGAGAC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CTTTGGCCGCTCTGTCG-3’</td>
</tr>
<tr>
<td>Zrt- and Irt-like protein 8</td>
<td>zip 8</td>
<td>XM_009307205.3</td>
<td>F: 5’-TGGAAGATGAAGCAATCCGC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-CTGACACCTGACAGCCGAGC-3’</td>
</tr>
<tr>
<td>Zrt- and Irt-like protein 14</td>
<td>zip 14</td>
<td>NM_001326699.1</td>
<td>F: 5’-GCAGAGGGAAGGATGGAAGAAGAC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-TGCGCATGAGTCAGAAGCC-3’</td>
</tr>
<tr>
<td>Copper transporter 1</td>
<td>ctrl1</td>
<td>NM_001320405.1</td>
<td>F: 5’-CCATCTCCTGCTCAGACGA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-GCCATCTCTCCTGTTGTT-3’</td>
</tr>
<tr>
<td>Copper-transporting P-type ATPase</td>
<td>cu-ATPase</td>
<td>NM_001042720.1</td>
<td>F: 5’-CATCAAGGGAGGAGAGGAGCA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-ATCGCCAGCAAGAGCG-3’</td>
</tr>
<tr>
<td>Epithelial calcium channel</td>
<td>ecac</td>
<td>XM_005173469.3</td>
<td>F: 5’-GTCTCGGTGTCCTCCTGAAATC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-GCATTGTTCTCTTAGTGGC-3’</td>
</tr>
<tr>
<td>Elongation factor 1 alpha</td>
<td>ef1a</td>
<td>AM422110.2</td>
<td>F: 5’-CCTCTTGGTGCTTTGGCTGT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: 5’-GAGGTTGGGAAAAACAGCC-3’</td>
</tr>
</tbody>
</table>

All primers were designed in this study.
2.2.3. **Diet preparation and dietary Fe exposure regime**

Diets with different concentrations of Fe were prepared as previously described by Kwong et al. (2013) with slight modifications. In brief, an appropriate amount of Fe (as FeSO₄·7H₂O) was dissolved in deionised water and mixed with a standard zebrafish diet (GEMMA Micro 75, Skretting; comprised of 59% protein; 14% lipids; 0.2% fiber; 14% ash; 1.3% phosphorus; 1.5% calcium; and 0.7% sodium). The resulting paste was dried at 65°C for 2 days. The food was then ground to small particles and stored at 4°C until use. Control diet was prepared in an identical manner in the absence of additional FeSO₄. The concentrations of Fe in the diets were measured using inductively coupled plasma mass spectrometry (ICP-MS) in the Water Quality Center at Trent University. The measured Fe content in the diets were: 157 mg Fe/kg (control diet), 490 mg Fe/kg, and 1795 mg Fe/kg. For clarity, these diets are referred as low Fe diet, medium Fe diet, and high Fe diet, respectively.

Larval zebrafish were fed with the experimental diets from 5 to 28 dpf. The fish were fed daily and water in the holding tanks were changed 1 h post-feeding. To determine possible leaching of Fe into the water during feeding, water samples were taken and measured for Fe. To evaluate physiological condition following dietary Fe exposure, larvae were monitored from 5 (pre-feeding) to 28 dpf. Two replicate tanks were used per dietary treatment with the tank holding density (after 5 dpf) of 150 larvae per 600 mL of water. Mortality was recorded daily and due to the high number of mortality, this experiment was repeated to collect tissues for metal burden and droplet digital PCR (ddPCR) analysis. Thirty fish were randomly sampled from each tank, with 20 fish pooled as one sample (N=1) for trace metal analysis and remaining 10 fish were pooled (N=1) for ddPCR analysis.
2.2.4. **Metal analysis**

Diets and fish samples were first dried at 65°C overnight and then digested with 6N nitric acid (HNO$_3$) for 48 h. The samples were diluted in 2% HNO$_3$ and filtered (0.45 µm; Basix™, Fisher Scientific). Trace metal levels were measured using ICP-MS (Trent University). Water samples were also analyzed with ICP-MS after acidification with 2% HNO$_3$.

2.2.5. **Evaluation of growth**

To examine the effects of dietary Fe exposure on growth, standard body length (SL) was measured at 5, 14 and 28 dpf. Ten larvae were randomly collected from each replicate tank and were anaesthetized in buffered MS-222 (0.168 g/L). Images were captured using a camera-equipped stereomicroscope (Leica), and distance from the tip of the snout to the base of the caudal peduncle was measured.

2.2.6. **Droplet digital PCR analysis of metal-regulatory genes**

To evaluate the effects of dietary Fe exposure on the mRNA transcript abundance of metal transporter genes, ddPCR analysis was performed using a QX200 Droplet Digital™ PCR System (Bio-Rad). Reaction mix was prepared using ddPCR™ EvaGreen® Supermix, followed by the manufacturer’s guideline. PCR condition was as follows: 5 min enzyme activation at 95°C, followed by 40 cycles of 30 sec denaturation at 95°C and 1 min annealing/extension at 58°C. Signal stabilization was performed at the end of the cycle (4°C for 5 min and then 90°C for 5 min). Subsequently, samples were processed using a QX200 Droplet Reader (Bio-rad). Data acquisition and analysis were performed using QuantaSoft™ Software (Version 1; Bio-rad). All data were normalized to the expression of $efl\alpha$ and this gene was not influenced by dietary Fe treatment, however, there seems to be statistically significant ($P = 0.048$; two-way analysis of variance...
(ANOVA)) changes in expression during development. Therefore, the temporal change in mRNA level over different developmental ages should be interpreted with caution.

2.2.7. **Statistical analysis**

All statistical analyses were performed using Sigmaplot® Software (Version 14; Systat Software Inc., Point Richmond, CA, USA). The effects of dietary Fe exposure on mortality, standard body length, Fe accumulation, and mRNA expression level, were analyzed using two-way ANOVA with a threshold value of $p < 0.05$ followed by a Holm-Šidák post-hoc test (dietary Fe levels and developmental ages were considered as two independent variables). Whole body trace metal and ion accumulation were analyzed using one-way ANOVA with a threshold value of $p < 0.05$. Data are presented as the mean ± standard error of the mean (SEM).
2.3. Results

2.3.1. Expression profile of major Fe-regulatory genes in various tissues of adults

Examination of adult zebrafish for Fe-transport and Fe-metabolism associated genes showed a broad organ distribution of \(dmt1\), \(frrs1b\), and \(fth1a\), whereas other genes examined showed an organ specific expression profile (Figure 1). While \(frrs1b\) was detected in all adult zebrafish organs, especially with a predominance in the kidney, \(frrs1a\) was only detected to a low extent in the intestine and intestinal bulb. Similarly, in contrast to \(fth1a\), \(fth1b\) expression was localized to the kidney and brain. \(ireg1\) was expressed in all tissues but the ovaries. However, there were more Fe-associated genes expressed in the ovaries than in the testis. \(tf\) was prevalent in the intestinal bulb and liver, but is also detected in the intestine and kidney at a lower expression. Finally, \(hep\) was primarily expressed in the liver of zebrafish but expression in the intestine and intestinal bulb was also detected. Overall, Fe-regulatory genes showed differential expression throughout the body of adult zebrafish.

2.3.2. Physiological conditions of larval zebrafish exposed to dietary Fe

To evaluate the effects of dietary Fe on physiological condition in larval zebrafish, the cumulative mortality (\%) was recorded daily during the course of the experiment (from 5 to 28 dpf) (Figure 2). Feeding of larvae with different Fe diets did not significantly affect the mortality during the first 10 days of exposure (e.g., 5 to 14 dpf). However, an increase in mortality was observed during 15-28 dpf in all treatments. Cumulative mortality of larvae fed a high Fe diet was substantially higher when compared to the other two dietary treatments. In fact, the high Fe treated fish exhibited \(~25\%\) higher mortality than the low and medium Fe treated fish.
The effects of dietary Fe on larval growth was measured with SL. Larvae were measured before (5 dpf) and after dietary Fe treatments (14 and 28 dpf) (Figure 3). A significant increase in SL was observed over development in all treatment groups. At 14 dpf, fish fed with the high Fe diet exhibited the greatest increase in SL. This increase in body length for high Fe diet diminishes at 28 dpf, where, the highest SL was observed in fish fed with the medium Fe diet. In all cases, fish exposed to low dietary Fe appeared to have the shortest SL length over the course of the experiment. No gross morphological difference (i.e. pigmentation and fin development) was observed during exposure to the three dietary treatments (data not shown).

2.3.3. Whole body Fe burden in larval zebrafish exposed to dietary Fe

Whole body Fe content in larval zebrafish exposed to Fe diets was examined using ICP-MS. Compared to fish at 5 dpf (pre-feeding), exposure to a low or medium Fe diet did not affect whole body Fe content at 14 or 28 dpf (Figure 4). However, a significant increase in whole body Fe content was observed in larvae fed with high Fe diet at 14 dpf. Such an increase by high dietary Fe was not observed at 28 dpf. Hence, no significant effect of dietary Fe was observed following prolonged Fe exposure in all treatments.

2.3.4. mRNA transcript levels of Fe-regulatory genes in larval zebrafish following exposure to dietary Fe

The effects of dietary Fe exposure on mRNA transcript abundance of various Fe-transport and Fe-metabolism genes were examined using ddPCR (Figure 5). Results from two-way ANOVA revealed that there were temporal changes in the mRNA transcript abundance of all the genes tested. For dmt1, transcript levels were comparable in the medium Fe treatment from 14 to 28 dpf. However, for both high and low Fe treated fish, there was a significant increase in transcript
levels during this period. Further, at 14 dpf, larvae fed a low Fe diet exhibited a significant reduction in \textit{dmt1} expression when compared to larvae fed a medium or high Fe diet. Interestingly, with prolonged exposure at 28 dpf, the medium Fe treatment had the lowest \textit{dmt1} levels.

Both \textit{fth1a} and \textit{fth1b} transcripts were substantially higher in 14 dpf larvae in the high Fe treatment but no significant effect of Fe was observed between the three treatments at 28 dpf. Further, within the high Fe treatment, there was a significant decline in \textit{fth} expression from 14 to 28 dpf. Modulation in \textit{ireg1} and \textit{tf} expressions by Fe were only observed following prolonged dietary exposure at 28 dpf. For \textit{ireg1}, transcript levels changed throughout development in the high and low Fe treatments while the medium Fe treatment was comparable at 14 and 28 dpf. Conversely, \textit{tf} expression increased throughout development for the low and high Fe treatments. Significant increases in expression were also noted within 28 dpf in the low Fe treatment. Exposure to different dietary Fe levels did not affect the transcript levels of \textit{hep} at any developmental timepoint and although there was a decline in expression from 5 dpf, all expression was comparable between 14 and 28 dpf.

2.3.5. \textbf{Whole body trace metal and ion content in larval zebrafish exposed to dietary Fe}

Whole body trace metal contents in larvae exposed to different dietary Fe levels are summarized in \textbf{Table 2}. Whole body Zn$^{2+}$ content increased with increasing developmental ages (14 to 28 dpf). Larvae fed with a low Fe diet exhibited a significant reduction in the whole body levels of Zn$^{2+}$ at 14 dpf. Exposure to different dietary Fe levels did not affect the levels of Cu$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$. Their contents also remained unchanged over development (5 to 28 dpf). Whole body contents of major ions in larvae following dietary Fe exposure are summarized in \textbf{Table 3}. An increase in whole body Ca$^{2+}$ content was observed over the period of 5 to 28 dpf. Compared to 5 dpf (pre-feeding), larvae fed a low Fe diet displayed a significant reduction in
whole body K⁺ and Mg²⁺ content at 14 dpf. Nevertheless, these reductions were not observed in 28 dpf larvae. Whole body Na⁺ content remained unchanged over development and following dietary Fe exposure.

2.3.6. mRNA transcript levels of various metal/ion transporters in larval zebrafish following exposure to dietary Fe

The mRNA transcript levels of Zn transporters, zip 8 and zip 14; Cu transporters, ctr1 and cuATPase; and epithelial Ca channel, ecac, were examined using ddPCR (Figure 6). Results from two-way ANOVA revealed that there were temporal changes in the mRNA transcript levels of all the genes tested. The expression level of zip 8 was significantly increased in 28 dpf larvae fed with the low Fe diet. However, during development a significant increase in zip 8 expression was only noted in the low Fe treatment (from 14 to 28 dpf). In contrast, zip 14, ctr1, and cuATPase increased from 14 to 28 dpf in the high and low Fe-exposed larvae. Interestingly, within 28 dpf, the expression of these three transporters were the greatest in the high Fe treated fish. Similarly, a substantial increase in ecac was also observed in 14 dpf larvae exposed to high dietary Fe and this was maintained during prolonged exposure. For the low and medium Fe treatments, ecac expression was comparable both between the Fe treatments and during development. Lastly, mRNA transcript levels of zip 8, zip 14, ctr1, and cuATPase were comparable between the treatments at 14 dpf, regardless of dietary Fe concentration.
Figure 1. The mRNA expression profile of major Fe-regulatory proteins in different organs of adult zebrafish. *dmt1*, divalent metal transporter 1; *ireg1*, iron-regulated transporter 1; *frrs1a*, ferric reductase 1 alpha; *frrs1b*, ferric reductase 1 beta; *fth1a*, ferritin 1 alpha; *fth1b*, ferritin 1 beta; *tf*, transferrin; *hep*, hepcidin. Elongation factor 1 alpha (*ef1a*) was used as an internal control. Each sample was collected from at least 3 adults. NTC, no template control.
Figure 2. Cumulative mortality rate (%) in larvae between 5 to 28 days post fertilization (dpf) exposed to dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg). There was a significant difference in mortality between all three dietary Fe treatment (Two-way ANOVA, P<0.05). 150 fish from each treatment were monitored daily for mortality, with a total of 2 replicate tanks per treatment (N=2). Data are means ± SEM.
Figure 3. Standard body length (mm) of developing zebrafish at 5, 14, and 28 dpf following dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg). Samples at 5 dpf were collected before dietary Fe exposure (pre-feeding). Bars labelled with different letters represent a statistical difference (capital letters: comparison among dietary treatments; lowercase letters: comparison over development) (Two-way ANOVA, followed by a post-hoc Bonferroni multiple comparisons test, P<0.05). 10 fish were collected from each tank for measurement, with a total of 3 replicate tanks per treatment (N=3). Data are means ± SEM.
Figure 4. Whole body Fe body burden (ng Fe/kg) in zebrafish larvae exposed to different dietary Fe levels (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg). Each replicate consisted of 20 pooled larvae, with a total of 3 replicate tanks per treatment (N=3). Data are means ± SEM. Bars labeled with different letters represent a statistical difference among treatments. Asterisks represent a statistical difference over developmental ages (Two-way ANOVA followed by a post-hoc Bonferroni multiple comparisons test, P<0.05).
**Figure 5.** The mRNA expression levels of various Fe-regulatory proteins in zebrafish larvae exposed to different dietary Fe levels (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) at 5 (pre-feeding), 14 and 28 days post-fertilization (dpf). Ten fish were collected for each replicate, with a total of 5-6 replicates per treatment (N=5-6). Data are normalized by elongation factor 1a (ef1α) and are expressed relative to larvae at 5 dpf. Values are means ± SEM. Statistical significance is denoted by letters (capital letters: comparison among dietary treatments; lowercase letters: comparison over development) (Two-way ANOVA followed by a post-hoc Bonferroni multiple comparisons test, P<0.05). Divalent metal transporter 1, *dmt1*; iron-regulated transporter 1, *ireg1*; ferritin 1a, *fth1a*; ferritin 1b, *fth1b*; transferrin, *tf*; hepcidin, *hep*. Note that for some data points the error bar is too small to be seen.
Figure 6. The mRNA expression levels of various metal/ion transporters in zebrafish larvae exposed to different dietary Fe levels (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) at 5 (pre-feeding), 14 and 28 days post-fertilization (dpf). Ten fish were collected for each replicate, with a total of 5-6 replicates per treatment (N=5-6). Data are normalized by elongation factor 1a (ef1α) and are expressed relative to larvae at 5 dpf. Values are means ± SEM. Statistical significance is denoted by letters (capital letters: comparison among dietary treatments; lowercase letters: comparison over development) (Two-way ANOVA followed by a post-hoc Bonferroni multiple comparisons test, P<0.05). Zrt- and Irt-like protein 8, zip 8; Zrt- and Irt-like protein 14, zip 14; copper transporter 1, ctr1; copper ATPase, cuATPase; epithelial calcium channel, ecac. Note that for some data points the error bar is too small to be seen.
Table 2. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of trace metal (Zn$^{2+}$, zinc; Cu$^{2+}$, copper; Mn$^{2+}$, manganese; Ni$^{2+}$, nickel; Co$^{2+}$, cobalt) concentration (ng/larvae) in zebrafish larvae exposed to different levels of dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Days post fertilization</th>
<th>Treatment</th>
<th>Whole body metal burden (ng/larvae)</th>
<th>Zn$^{2+}$</th>
<th>Cu$^{2+}$</th>
<th>Mn$^{2+}$</th>
<th>Ni$^{2+}$</th>
<th>Co$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pre-feeding</td>
<td>19.77 ± 1.42$^{AB}$</td>
<td>0.52 ± 0.10</td>
<td>0.32 ± 0.04</td>
<td>0.11 ± 0.06</td>
<td>0.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Low Fe</td>
<td>14.83 ± 1.31$^{A}$</td>
<td>0.49 ± 0.05</td>
<td>0.37 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium Fe</td>
<td>17.56 ± 0.88$^{AB}$</td>
<td>0.54 ± 0.06</td>
<td>0.31 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High Fe</td>
<td>18.80 ± 2.11$^{AB}$</td>
<td>0.50 ± 0.06</td>
<td>0.38 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Low Fe</td>
<td>23.23 ± 1.09$^{B}$</td>
<td>0.76 ± 0.25</td>
<td>0.34 ± 0.02</td>
<td>0.01 ± 0.00</td>
<td>0.02 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium Fe</td>
<td>22.94 ± 1.71$^{AB}$</td>
<td>0.53 ± 0.22</td>
<td>0.31 ± 0.04</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High Fe</td>
<td>23.28 ± 2.52$^{AB}$</td>
<td>0.36 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SEM, n=3. Values labelled with different letters represent a statistical difference within dietary treatment (One-way ANOVA, P<0.05).
Table 3. Inductively coupled plasma mass spectrometry (ICP-MS) analysis of major ions (Na\(^+\), sodium; K\(^+\), potassium; Mg\(^{2+}\), magnesium; Ca\(^{2+}\), calcium) concentration (ng/larvae) in zebrafish larvae exposed to different levels of dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Days post fertilization</th>
<th>Treatment</th>
<th>Na(^+)</th>
<th>K(^+)</th>
<th>Mg(^{2+})</th>
<th>Ca(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Pre-feeding</td>
<td>393.81 ± 40.56</td>
<td>359.97 ± 35.78(^{AB})</td>
<td>55.32 ± 12.28(^{A})</td>
<td>278.81 ± 17.06(^{A})</td>
</tr>
<tr>
<td>14</td>
<td>Low Fe</td>
<td>306.45 ± 40.17</td>
<td>182.40 ± 17.78(^{A})</td>
<td>23.30 ± 2.53(^{B})</td>
<td>301.01 ± 22.31(^{A})</td>
</tr>
<tr>
<td></td>
<td>Medium Fe</td>
<td>434.90 ± 59.65</td>
<td>245.32 ± 23.66(^{AB})</td>
<td>28.81 ± 1.55(^{AB})</td>
<td>367.27 ± 26.59(^{AB})</td>
</tr>
<tr>
<td></td>
<td>High Fe</td>
<td>399.29 ± 11.90</td>
<td>254.47 ± 31.67(^{AB})</td>
<td>27.43 ± 2.70(^{AB})</td>
<td>385.67 ± 26.20(^{AB})</td>
</tr>
<tr>
<td>28</td>
<td>Low Fe</td>
<td>410.21 ± 15.70</td>
<td>389.16 ± 17.03(^{B})</td>
<td>34.81 ± 1.75(^{AB})</td>
<td>549.87 ± 23.92(^{B})</td>
</tr>
<tr>
<td></td>
<td>Medium Fe</td>
<td>359.69 ± 57.28</td>
<td>360.76 ± 53.66(^{AB})</td>
<td>32.91 ± 4.90(^{AB})</td>
<td>506.60 ± 83.13(^{B})</td>
</tr>
<tr>
<td></td>
<td>High Fe</td>
<td>381.39 ± 45.73</td>
<td>320.90 ± 49.82(^{AB})</td>
<td>31.09 ± 3.74(^{AB})</td>
<td>459.18 ± 60.33(^{B})</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n=3. Values labeled with different letters represent a statistical difference within dietary treatment (One-way ANOVA, P<0.05).
2.4. Discussion

Several previous studies have explored the interplay of metal exposure and consequent metal burden, compensatory metal assimilation, and gene expression regulation in adult fishes (Cooper et al., 2006; Craig et al., 2008; Kwong et al., 2013; Wang and Wang, 2016). These studies have highlighted the adaptations that allow fishes to encounter and survive the dynamic nature of their environment. Still, questions regarding how fishes respond to metal exposure, especially during the dynamic period of early development and how these responses compare to adults remain unexplored. Further, recent studies have also demonstrated the importance of dietary metal exposure in metal toxicity and accumulation in fishes (Bury, 2003b). Although Fe is an essential metal with vital functions in oxygen transport and redox reactions (Bury, 2003b; Hentze et al., 2004), there is also a multitude of adverse effects involving Fe toxicity and deficiency (Donovan et al., 2002; Meneghini, 1997; Payne et al., 1998).

Increasing level of Fe in the freshwater ecosystems has also been recognized as an emerging issue of environmental concern in many geographic regions (Björnerås et al., 2017; Kritzberg and Ekström, 2012; Sarkkola et al., 2013). In Fe-contaminated environments, the Fe level in natural fish diet could reach over 12,000 mg/kg dry weight (Winterbourn et al., 2000). In the present study, we observed that larvae fed a high Fe diet (~1,800 mg Fe/kg) exhibited a transient increase in growth. However, exposure to such a diet also increased whole body Fe content and mortality. Further molecular analysis showed that dietary Fe exposure led to temporal changes in the mRNA levels of various metal transporters (e.g., Fe^{2+}, Zn^{2+}, and Cu^{2+} transporters). Understanding the interplays among metal exposure, physiological performance, and regulation of metal transporters may help develop a model to assess the physiological effects of dietary metals.
2.4.1. **Fe-regulatory gene expression in adult tissues**

Ubiquitous expression of *dmt1*, *frrs1b*, and *fth1a*, which function in Fe transport and storage, may be important for increasing the availability of Fe throughout the body, especially as all cells require access to Fe (Watanabe et al., 1997). Conversely, greater expression of *frrs1a*, *fth1b*, *tf*, and *hep* in digestive organs such as the intestine/intestinal bulb and liver is important for Fe absorption and metabolism. Generally, the gills, intestine, and liver are known to be the major sites of Fe uptake and metabolism in fishes (Carriquiriborde, 2003; Watanabe et al., 1997). Further, the liver is also the site of *tf* and *hep* synthesis (Nemeth and Ganz, 2009). Lastly, in the reproductive tissues, there was a greater number and level of Fe-associated genes expressed in the ovaries than the testis. Although literature on the expression of Fe-associated genes in the reproductive tissues of fishes is lacking, there is an interesting difference in the bioaccumulation of Fe noted in the tissues of *tilapia sparrmanii* (banded tilapia) exposed to acute (72 h) and chronic (four week) Fe (du Preez et al., 1993). This study reported that Fe concentration in the ovaries was higher than in the testis of all fish which may indicate the greater involvement of the ovaries in Fe metabolism. The differential expression of Fe-regulatory genes in various tissues of the body not only indicate the requirement for Fe in all tissues but also highlight the intricate segregation of Fe-regulatory processes throughout the body.

2.4.2. **Fe homeostasis in larval zebrafish exposed to dietary Fe**

Currently, there is much that remains unknown regarding the nutritional requirement of Fe for developing fishes and how dietary Fe levels may affect the physiological condition in these larvae. Commercial fish feeds typically contain 30-170 mg Fe/kg food (Cooper et al., 2006). Previous studies with rainbow trout have shown that dietary Fe up to ~2000 mg Fe/kg food does not appear to induce toxic stress responses (i.e. oxidative stress) in these fish (Carriquiriborde,
Furthermore, in Fe-contaminated waters, the Fe levels in invertebrates (i.e., prey of fishes) range between 160-12,650 mg/kg dry weight (Winterbourn et al., 2000). Therefore, diets in this study were prepared in order to both mimic environmentally relevant concentrations and to examine the effects of high Fe during the period of early life development where fishes are likely to be more susceptible to metal toxicity. Three dietary concentrations were chosen for this study: low Fe (157 mg Fe/kg), medium Fe (490 mg Fe/kg), and high Fe (1795 mg Fe/kg). Following Fe exposure, observations about mortality, growth (i.e. SL), developmental parameters (pigment pattern, fin development), and whole body Fe burden were recorded.

**Mortality**

Formulated dry feeds have been shown to be sufficient in the rearing of zebrafish larvae and these diets are proven to be adequate sources of nutrition (i.e. essential amino acids, fatty acids, minerals, and trace elements) for normal development (Kaushik et al., 2011). However, in the feeding protocol established by Certal and Farias (2016), it was found that zebrafish fed a dry diet only, similar to the current experimental conditions, generally had the lowest survival rates (~40%) as opposed to larvae fed a mix of dry and live diets. Similarly, in this study, there was a general increase in mortality (%) noted in all dietary Fe treatments between 15 to 28 dpf. This increase may be associated with an increase in dietary requirements that occur during development. However, the overall mortality was still greater in the high Fe treated fish. Which could potentially be due to the greater energetic costs that accompany the maintenance of metal homeostasis during elevated Fe exposure (Couture and Pyle, 2008). Interestingly, this also demonstrates that larval zebrafish are more sensitive to Fe exposure than adult fish who can tolerate up to 2000 mg Fe/kg (Carriquiriborde, 2003; Kwong et al., 2013). Hence, these results indicated that larval fish are likely more susceptible to the toxic effects of dietary Fe.
During larval development, zebrafish larvae typically reach ~5.44 mm in length by 15 dpf (Singleman and Holtzman, 2014). Similarly, in a study examining early larval development in zebrafish, Biga and Goetz (2006) reported larval lengths ranging from 3.448 ± 0.133 mm at hatching to a max of 4.692 ± 0.133 mm 4 weeks post-hatching. These SL values were in general agreement with the present study, however, an additional stimulatory effect of Fe was observed in the Fe supplemented larvae. For example, high Fe treated fish at 14 dpf had the highest SL and they also had the greatest whole body Fe concentrations. Similarly, developing medaka fed a Fe enriched diet (~335 mg Fe/kg) also displayed increases in body length (Wang and Wang, 2016). Interestingly, however, zebrafish larvae fed a medium Fe diet (~490 mg Fe/kg) exhibited the greatest increase in body length at 28 dpf, suggesting that moderate enrichment of diets with Fe could stimulate the growth of larvae. Conversely, the lowest growth was observed in the low Fe treated fish under all conditions. Therefore, it seems that a moderate level of dietary Fe supplementation has stimulatory effects on growth which is in agreement with the nutritive role that Fe plays in the body (Bury, 2003b).

In adult fish, exposure to high Fe diets has been reported to increase whole body or tissue Fe burden (Carriquiriborde, 2003; Cooper et al., 2006; Craig et al., 2008; Kwong et al., 2013). Interestingly, in the present study, there was only a transient increase in Fe noted in the high Fe fed larvae at 14 dpf. With prolonged exposure, this elevation was diminished, and all larvae were comparable at 28 dpf. This is quite surprising as the high Fe diet contained ~11.4 times more Fe than the low Fe diet so in order to prevent Fe accumulation in the body these fish must employ...
tight regulation of Fe uptake and metabolism. These results suggest possible physiological adjustments on Fe assimilation following prolonged exposure in the high Fe treated fish.

2.4.3. **Transcriptional regulation by dietary Fe**

Since elevated levels of Fe in the body may negatively affect the physiological function of fishes (e.g., induction of oxidative stress), it was hypothesized that there is a feedback inhibitory mechanism which reduces the absorption of excess Fe during exposure to a high Fe diet. This study aimed to evaluate the transcriptional responses of major Fe-regulatory genes in larval zebrafish following exposure to dietary Fe in hopes to i) identify genes that are responsive to dietary Fe exposure and; ii) elucidate the relationship between Fe accumulation and regulation of Fe-regulatory genes.

*mRNA transcript abundance of Fe-regulatory genes*

Some Fe-regulatory genes contain IREs which are post-transcriptionally regulated by systemic Fe levels (e.g., stability of mRNA, activation/suppression of translation). In the present study, *dmt1*, *ireg1*, *fth1a*, *fth1b*, and *tf* seemed to be transcriptionally regulated by dietary Fe. With most of the changes in mRNA transcript levels observed at 14 dpf, which coincided with the changes in whole body Fe burden. Here, *dmt1* transcript was elevated in Fe supplemented larvae. Therefore, it was possible that the increased Fe body burden was owing to an increase in Fe absorption via DMT1. Similarly, adult zebrafish and marine medaka also exhibited increases in *dmt1* expression following elevation in Fe exposure (Cooper et al., 2006; Craig et al., 2008; Wang and Wang, 2016). Interestingly, larvae exposed to the medium Fe diet had comparable *dmt1* expression throughout Fe exposure from 14 to 28 dpf. However, *dmt1* transcripts in high and low Fe treated larvae increased from 14 to 28 dpf. This elevation in *dmt1* expression may have been
instrumental in the maintenance of whole body Fe and other trace metals (i.e. Zn, Cu) during continued high Fe exposure. Equally, it could have also contributed to the maintenance of whole body Fe levels during low Fe exposure in which the diet only contained ~150 mg Fe/kg.

Unlike dmt1, fth1a and fth1b transcripts were significantly increased in 14 dpf larvae fed a high Fe diet. This distinction is probably associated with the critical role that FTH plays in storing excess amount of Fe in the cell, preventing the free ionic form of Fe from acting on other cellular components (Andersen, 1997; Arosio and Levi, 2002). Notably, the increased expression of fth appeared to correlate with the elevated level of whole body Fe content at 14 dpf. The induction in fth1a and fth1b expression by exposure to high dietary Fe was likely a protective mechanism in handling increased cellular Fe loading. Likewise, the expression of these two genes were comparable between the three dietary Fe treatments at 28 dpf where the larvae also had comparable levels of whole body Fe. Accordingly, there was also a decline in fth1a and fth1b transcripts from 14 to 28 dpf in the high Fe treated fish. In mammals, serum FTH level is commonly used as a marker for Fe overload (Jacobs et al., 1972; Piperno, 1998). Whether FTH level could also be used as a relevant biomarker for the evaluation of Fe exposure in larval fish warrants further investigations.

Similarly, in rainbow trout exposed to elevated Fe, these fish seemed to regulate systemic Fe levels by sequestering Fe in the blood with an increase in tf and consequent loading into the liver (Andersen, 1997). Likewise, decreases in ireg1 transcripts can further aid in the reduction of systemic Fe levels by reducing Fe absorption from sites such as the intestine and gills (Donovan et al., 2000). Accompanied increases in hep expression in zebrafish embryos and Dicentrarchus labrax (sea bass) liver also occur during Fe overload (Fraenkel et al., 2005; Rodrigues et al., 2006). However, a similar change in tf and ireg1 expression was not observed at 14 dpf in the present
study. Instead, there was modulation in gene expression observed following prolonged Fe treatment at 28 dpf. Here tf and ireg1 abundance increased from 14 dpf to 28 dpf, which potentially could contribute to the increased demands for Fe during development and the maintenance of Fe levels at 28 dpf. Still, hep expression was not affected by dietary Fe treatment at any developmental timepoint instead there was a decline in expression levels in all treatments from 5 dpf.

These differences could be attributed to potential variations between the sensitivity (i.e. toxicity) of adults and developing zebrafish to Fe exposure. For example, variations in metal sensitivities have also been reported between embryonic and larval stage of fishes. With the embryos less sensitive to metal exposure due to the protective nature of their chorion (Guadagnolo et al., 2001). Different species of fishes also respond to metal exposure differently. For example, the salmonids (i.e. rainbow trout) are more sensitive (96 h LC50) to metals such as Zn and Cd than cyprinids (i.e. zebrafish) (Alsop and Wood, 2011). Interestingly, the expression of most Fe-regulatory genes examined (dmt1, ireg1, fth1a, tf, and hep) were comparable throughout Fe treatment from 14 to 28 dpf in the medium Fe exposed larvae. This could indicate that the medium Fe diet contained an optimal level of Fe for developing zebrafish.

**Gene expression over development**

During development, temporal changes in the mRNA transcript abundance of Fe-regulatory genes were evident. Due to the vital nature of Fe for both developmental and cellular processes, the expression of these Fe-regulatory genes would be crucial from early development (Bury, 2003b; Watanabe et al., 1997). Additionally, zebrafish depend on nutrition from the egg yolk for early embryonic growth, and as such the expression of Fe transport proteins would be essential very early into development. In fact, the importance of IREG1 was highlighted in the export of Fe from the yolk for nutrition and growth in zebrafish (Donovan et al., 2000). Similarly,
there were dynamic changes in gene expression observed during development in the present study and this may be related to increased demands for protein synthesis that accompany development.

2.4.4. Trace metal homeostasis in larval zebrafish exposed to dietary Fe

The Fe uptake pathways interact with multiple metals. DMT1 for example, is considered the major Fe transporter but it has also been proposed to function as a potential route of uptake for other metals including Zn, Cu, Mn, Ni and Co, and the non-essential metals Cd and Pb (Bannon et al., 2003; Bressler et al., 2004; Garrick et al., 2003; Tallkvist et al., 2001). These assumptions were further supported in a study which examined the intestinal absorption of Fe in rainbow trout where uptake was shown to be inhibited by Ni, Pb, Cd, Cu, and Zn (in order of greatest inhibition to least) (Kwong and Niyogi, 2009). In addition to these competitive inhibitors, Ca was identified as a non-competitive inhibitor of Fe uptake via DMT1 (Shawki and Mackenzie, 2010). Further, several studies have also demonstrated that Fe exposure affects the whole body levels of other metals/ions including Zn, Cu, and Ca (Craig et al., 2009; Liuzzi et al., 2006; Wang et al., 2012). Hence, exposure to Fe could have the potential to inhibit the absorption of other divalent metals and consequently affect whole body metal homeostasis of Fe as well as these metals.

Therefore, in order to fully assess these complex metal-protein and metal-metal interactions, this study also examined the effects of dietary Fe exposure on i) the mRNA expression levels of Zn transporters (zip 8 and zip 14), Cu transporters (ctr1 and cuATPase), and Ca channel (ecac) and ii) whole body burden of Zn$^{2+}$, Cu$^{2+}$, and Ca$^{2+}$ in larval zebrafish. The ZIP8 and ZIP14 of the ZIP family of transporters were chosen for this study because their functions are known to be influenced by Fe (Pinilla-Tenas et al., 2011; Wang et al., 2012).
mRNA expression of metal transporters

Exposure to high Fe may affect the dynamics of all divalent metals that utilize DMT1 as a route of uptake. Accordingly, there may be compensatory modulation in the expression of other transport proteins. In the present study, zip 8, zip 14, ctrl, cuATPase, and ecac expression were all modulated following Fe exposure. Interestingly, most modulation in gene expression as observed following prolonged dietary Fe exposure at 28 dpf and the only gene that was affected at 14 dpf was ecac. To begin, the mRNA expression of the Zn transporters, zip 8 and zip 14, which are also known to transport Fe$^{2+}$, were elevated in the low Fe treated fish throughout development (Pinilla-Tenas et al., 2011; Wang et al., 2012). It is possible that this increase in expression was a compensatory response to maintain whole body Fe levels when the diet contained less Fe. Conversely, there was also a significant increase in zip 8 and zip 14 expressions in the high Fe treated fish throughout development. It was possible that the elevated level of Fe in the high Fe diet potentially inhibited Zn$^{2+}$ absorption, and the increased zip 8 and zip 14 expressions could be a compensatory mechanism to maintain Zn$^{2+}$ uptake levels. Therefore, dynamic changes in the expression of these Zn transporters was observed throughout dietary Fe treatment and further demonstrate the complex interactions between Zn and Fe.

Similarly, ctrl and cuATPase expression were not affected by dietary Fe treatment at 14 dpf but with prolonged exposure at 28 dpf there was a significant effect of dietary Fe concentrations on transcript levels. For both Cu transporters, there was an increase in abundance in the high Fe treatment at 28 dpf. The expression of these two Cu transporters was also examined in a study by Craig et al. (2008) where exposure of adult zebrafish to a dietary Fe did not produce a significant change in both ctrl and cuATPase gene expression. However, previous studies have demonstrated that Cu is not only a competitive inhibitor of Fe uptake via DMT1 but it also has
negative feedback on the expression of dmt1 (Tennant et al., 2002). Further, cuATPase also seems to be upregulated during Fe deficiency and the link between Fe and Cu homeostasis has been highlighted in many mammalian dietary studies (Collins et al., 2005; Evans, 1973; Gulec and Collins, 2014; Ravia et al., 2005). Unfortunately, the same cannot be said for studies on the expression of these transporters under conditions of high Fe for comparison. Hence, the expression of ctr1 and cuATPase has produced some conflicting results across studies. However, the increase in ctr1 and cuATPase expression observed in this study within the high Fe treatment may have aided to increase Cu\textsuperscript{2+} absorption via CTR1/CuATPase when competition with Fe for uptake via DMT1 was elevated. Future inquiry assessing the protein expression of these transporters could provide an additional perspective to these results. Likewise, a previous study has shown that piscine ECaC could also mediate the uptake of Fe\textsuperscript{2+} (Qiu and Hogstrand, 2004). Therefore, it was possible that the increased ecac expression was a compensatory response to increase Ca\textsuperscript{2+} absorption when the larvae were on a high Fe diet. Lastly, similar to the expression of Fe-regulatory genes, the expression of zip 8, zip 14, ctr1, cuATPase, and ecac were all comparable throughout Fe treatment from 14 to 28 dpf within the medium Fe exposed larvae. This lack of compensatory gene expression evident in the other two dietary Fe treatments may further support the possibility that the medium Fe diet contained the optimal Fe range for developing zebrafish.

**Expression of metal transporters over development**

Zn, Cu, and Ca are vital nutrients for development (Davis D. and Gatlin, 1996; Zhao et al., 2014). Accordingly, the increases in zip 8, zip 14, ctr1, cuATPase, and ecac expression observed during the development of zebrafish, may suggest the increased need for the uptake of these metals. For example, significant increases in zip 8 expression was also identified during embryonic development in zebrafish, with gene expression detected as early as 12 hpf (Ho et al., 2012).
Similarly, ZIP 14 was identified as a regulator of systemic growth in mice; controlling G-protein coupled receptor-mediated signaling (Hojyo et al., 2011). Disruptions to the zip 14 gene via genetic knockout also resulted in growth defects and impaired gluconeogenesis in the mutants. Likewise, it is well known the importance of Ca in developmental processes. In fact, its importance is highlighted as early as egg fertilization, with Ca signaling playing crucial roles in the process of egg activation (Webb and Miller, 2000). Lastly, the Cu transporter gene ctr1 was cloned in zebrafish and it was found ubiquitously expressed during early embryogenesis (Mackenzie et al., 2004). The expression of Cu transporters is vital for the uptake of Cu from the yolk sac prior to exogenous feeding. After 24 hpf, ctr1 mRNA becomes localized to the intestinal region of zebrafish larvae which later functions in the dietary uptake of Cu.

Effects of dietary Fe exposure on whole body metal burden

Of all the essential metals (Zn, Cu, Mn, Ni, and Co) and major ions (Na, K, Mg, and Ca) examined, the only metal that had increased due to dietary Fe exposure was Fe itself. Although changes in Zn$^{2+}$, Ca$^{2+}$, K$^+$, and Mg$^{2+}$ levels in larvae following Fe exposure were noted over development, there was not a statistically significant effect of dietary Fe concentration. Instead, these changes may support the roles that these metals play during development. However, it is surprising how even with complex interactions between Fe uptake pathways and various divalent metals, these larvae were able to maintain metal homeostasis with little change. This further highlights the tight regulation of metal homeostasis in these fish.
2.5. Conclusions and Perspectives

In conclusion, metal homeostasis is tightly regulated in larval zebrafish and exposure to Fe supplemented diets had little effect on whole body metal concentrations (essential metals and major ions). Temporal effects of dietary Fe on growth were evident which hint at the nutritive role that Fe may play in the body. However, there was also evidence of dysregulation in whole body Fe homeostasis at 14 dpf and an associated increase in mortality during prolonged elevation in Fe. This suggested that fish during the early transition period of development (e.g., the beginning of exogenous feeding to the juvenile stage) are more susceptible to the effects of dietary Fe exposure. Further, high dietary Fe induced differential changes in the expression levels of multiple metal-regulatory proteins, including the Fe transporter \textit{dmt1}, the Zn transporters \textit{zip 8} and \textit{zip 14} and the Cu transporters \textit{ctr1} and \textit{cuATPase}. Studies from mammalian models have suggested that these metal transporters play specific roles in immune response, organogenesis, and other cellular functions (Kuo et al., 2001; Liu et al., 2013). How dietary Fe influences these processes during development remains to be investigated. Because of the very small size of zebrafish larvae, possible tissue-specific modulation of metal-regulatory genes by dietary Fe could not be evaluated in the present study. Recent advances on nano-scale sampling (Dickmeis et al., 2019) may prove useful to quantify molecular changes in different tissues of larvae.

Future studies on protein expression will also provide a crucial perspective to the present results which mainly focused on the mRNA expression levels. Furthermore, the addition of a Fe-deficient diet could also provide a vital comparison for the results obtained for low and Fe supplemented treatments. In the present study, the lack of modulation for Fe-regulatory and metal transport genes during medium Fe treatment hints at the possibility that this diet contained an optimal range of Fe for developing zebrafish.
Lastly, the understanding of Fe regulation in fishes is still limited. For example, DMT1 has been proposed to transport various divalent metals with some evidence to support these claims. However, direct *in vivo* evidence for the involvement of DMT1 in these processes in fish is still lacking. In the future, the development of a genetic model with a knockout of DMT1 could provide a valuable *in vivo* model to study the function and involvement of this protein in Fe metabolism. Similarly, the subcellular localization of Fe transport proteins in the gills of fish have also not been characterized. The localization of these proteins could provide insight into the differences in dietary versus waterborne Fe regulation in fishes.

In conclusion, this study illustrates the homeostatic mechanisms employed by larval zebrafish in high Fe environments during early development and their sensitivity to Fe toxicity.
2.6. References


2.7. Appendix

Appendix 1. P-values for two way analysis of variance (ANOVA) of cumulative mortality (%) in larvae during development (5 to 28 days post fertilization (dpf)) exposed to dietary Fe (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Fe treatment</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Interaction</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Appendix 2. P-values for two way ANOVA of standard body length (mm) of developing (5, 14, and 28 dpf) zebrafish following dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Fe treatment</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.009</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Appendix 3. P-values for two way ANOVA of whole body iron body burden (ng Fe/kg) in developing (5, 14, and 28 dpf) zebrafish exposed to different dietary iron levels (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>0.013</td>
<td>Yes</td>
</tr>
<tr>
<td>Fe treatment</td>
<td>0.188</td>
<td>No</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.112</td>
<td>No</td>
</tr>
</tbody>
</table>
Appendix 4. P-values for two way ANOVA of the expression levels of elongation factor 1a (ef1a) throughout development (5, 14, and 28 dpf) and between dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) for droplet digital PCR (ddPCR).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>0.048</td>
<td>Yes</td>
</tr>
<tr>
<td>Fe treatment</td>
<td>0.552</td>
<td>No</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.530</td>
<td>No</td>
</tr>
</tbody>
</table>

Appendix 5. P-values for two way ANOVA of the expression levels of Fe regulatory genes\(^1\) throughout development (5, 14, and 28 dpf) and following dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) for droplet digital PCR (ddPCR).

<table>
<thead>
<tr>
<th>Sources of Variation</th>
<th>dmt1</th>
<th>ireg1</th>
<th>fth1a</th>
<th>fth1b</th>
<th>tf</th>
<th>hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe treatment</td>
<td>0.084</td>
<td>0.163</td>
<td>0.003</td>
<td>0.005</td>
<td>0.008</td>
<td>0.958</td>
</tr>
<tr>
<td>Interaction</td>
<td>&lt;0.001</td>
<td>0.108</td>
<td>0.114</td>
<td>0.037</td>
<td>0.017</td>
<td>0.996</td>
</tr>
</tbody>
</table>

\(^1\)Divalent metal transporter 1, dmt1; iron regulatory protein 1, ireg1; ferritin 1a, fth1a; ferritin 1b, fth1b; transferrin, tf; hepcidin, hep. Note for some data points the error bar is too small to be seen.
Appendix 6. P-values for two way ANOVA of the expression levels of metal transport related genes\(^1\) throughout development (5, 14, and 28 dpf) and between dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg) for droplet digital PCR (ddPCR).

<table>
<thead>
<tr>
<th>Sources of Variation</th>
<th>zip 8</th>
<th>zip 14</th>
<th>ctr1</th>
<th>cuATPase</th>
<th>ecac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe treatment</td>
<td>0.330</td>
<td>0.066</td>
<td>0.049</td>
<td>0.121</td>
<td>0.004</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.256</td>
<td>0.208</td>
<td>0.023</td>
<td>0.225</td>
<td>0.186</td>
</tr>
</tbody>
</table>

\(^1\) Zrt- and Irt-like protein 8, zip 8; Zrt- and Irt-like protein 14, zip 14; copper transporter 1, ctr1; copper ATPase, cuATPase; epithelial calcium channel, ecac.

Appendix 7. P-values for one way ANOVA of the whole body trace metal (\((\text{Zn}^{2+}, \text{zinc}; \text{Cu}^{2+}, \text{copper}; \text{Mn}^{2+}, \text{manganese}; \text{Ni}^{2+}, \text{nickel}; \text{Co}^{2+}, \text{cobalt}\)) concentrations (ng/larvae) throughout development (5, 14, and 28 dpf) and between dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Metals during development</th>
<th>Low Fe</th>
<th>Medium Fe</th>
<th>High Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(^{2+})</td>
<td>0.014</td>
<td>0.104</td>
<td>0.389</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>0.331</td>
<td>0.684</td>
<td>0.459</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>0.187</td>
<td>0.928</td>
<td>0.109</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>0.388</td>
<td>0.315</td>
<td>0.275</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>0.704</td>
<td>0.729</td>
<td>0.408</td>
</tr>
</tbody>
</table>
Appendix 8. P-values for one way ANOVA of the whole body ion (Na⁺, sodium; K⁺, potassium; Mg²⁺, magnesium; Ca²⁺, calcium) concentration (ng/larvae) throughout development (5, 14, and 28 dpf) and between dietary Fe treatment (Low Fe, 157 mg Fe/kg; Medium Fe, 490 mg Fe/kg; High Fe, 1795 mg Fe/kg).

<table>
<thead>
<tr>
<th>Metals during development</th>
<th>Low Fe</th>
<th>Medium Fe</th>
<th>High Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>0.370</td>
<td>0.757</td>
<td>0.973</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.021</td>
<td>0.066</td>
<td>0.187</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.019</td>
<td>0.102</td>
<td>0.169</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.002</td>
</tr>
</tbody>
</table>