Zinc Acquisition and Handling in the Pacific Hagfish (*Eptatretus stoutii*)

by

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Abstract

Acting as a co-factor for hundreds of enzymes, zinc (Zn) is an essential trace metal required for organismal success across all phyla. When aquatic organisms such as fish are Zn deficient, growth, metabolism, and health are all negatively impacted. However, Zn in excess may also impair overall piscine survival. The early diverging Pacific hagfish (*Eptatretus stoutii*) was used as a research organism in this thesis.

Similar to other fish, hagfish may acquire environmental nutrients across their hindgut and gills. However, they have also demonstrated a capacity for nutrient uptake across their skin. I investigated where hagfish accumulate Zn, how they acquire Zn^{2+} across epithelia, and what potential cellular mechanisms may be present.

Hagfish exposed to 2 μM and 100 μM Zn for 6, 24, and 48 hours demonstrated significant Zn accumulation in the gills, as well as within the blood plasma and the foregut. In a separate experiment, uptake kinetics for the hindgut, gill, and skin were biphasic. At higher concentrations of Zn, uptake was dominated by diffusive pathways, whereas saturable, sigmoidal uptake was observed at lower Zn concentrations. Measured affinity constants revealed the hindgut to have the lowest affinity for Zn, with the highest affinity occurring in the gill.

The sigmoidal nature of the uptake curves led me to test Zn^{2+} uptake in the presence of putative metal competitors in an attempt to elucidate which protein transporters are used for Zn^{2+} transport in the hagfish. Within the hindgut and skin, no significant effect on Zn^{2+} uptake occurred when metals were in concentrations 2X and 5X that of Zn. Zn^{2+} uptake significantly increased within the gill in the presence of lanthanum, a known calcium channel inhibitor.

Metallothionein (MT) levels within hagfish exposed to 38 μM Zn for a period of 0, 1, 3, 5, and 7 days were quantified, with the liver and intestine having the highest concentrations of MT, however, an induction of MT production was not demonstrated.

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List of Abbreviations

%	percent
°C	degrees Celsius
[x]	concentration of x
µCi	microcurie
µg	microgram
µL	microliter
µM	micromolar
AAS	atomic absorption spectrophotometry
Ag/Ag^{+}	silver
$AgNO_3$	silver nitrate
ANOVA	analysis of variance
BMSC	Bamfield Marine Sciences Centre
Ca/Ca^{2+}	calcium
$CaCl_2\ 2H_2O$	calcium chloride dihydrate
C_{AG}	silver concentration
C_{BKG}	background concentration
Cd/Cd^{2+}	cadmium
Cl^{-}	chloride
cm^2	centimeter squared
CPM	counts per minute
Co^{2+}	cobalt

Cu/Cu^{2+}	copper
DMT-1	divalent metal transporter 1
ECaC	epithelial calcium channel
Fe/Fe^{2+}	iron
g	gram
g	gravity
h	hour
HF	hagfish
Hg/Hg^{2+}	mercury
HNO_3	nitric acid
J_{max}	maximal rate of uptake
K^+	potassium
KCl	potassium chloride
kg	kilogram
K_m	affinity constant
L	liter
La/La^{3+}	lanthanum
m	meter
M	molar
Mg^{2+}	magnesium
$MgCl_2$ $6H_2O$	magnesium chloride hexahydrate
$MgSO_4$ $7H_2O$	magnesium sulfate heptahydrate
mL	milliliter

mM	millimolar
MT	metallothionein
N	normal
N	number
Na^+	sodium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4 H_2O	sodium dihydrogen phosphate monohydrate
Ni/Ni^{2+}	nickel
NaOH	sodium hydroxide
nM	nanomolar
nmol	nanomoles
Pb/Pb^{2+}	lead
PE	polyethylene
RNA	ribonucleic acid
SAct	specific activity
SDF	sample dilution factor
SEM	standard error of the mean
SO_4^{2+}	sulfate
S_v	sample volume
TMS	tricaine methanesulfonate
V_T	total volume
w/v	weight per volume

X	times
Zn/Zn^{2+}	zinc
$^{65}ZnCl_2$	radioactive zinc chloride
$Zn(OH)_2$	zinc hydroxide
ZIP	zrt, irt-related protein
ZnT	zinc transporter

Chapter 1 - General Introduction

Importance of Zinc

Zinc (Zn) is the second most distributed trace element after iron in the body of organisms and is essential across all phyla (Vallee and Auld, 1990b; Saper and Rash, 2009). The specific chemical properties of Zn allow for the formation of sturdy complexes with organic molecules, which in turn have many necessary functions for biological systems in all organisms (Brown *et al*., 2001). Zn is a crucial component for the metabolic activity of approximately 300 enzymes. In fact, Zn is the only metal required for the function of at least one enzyme in each of the six classes, many of which are responsible for the synthesis of key biomolecules and whole-body homeostasis (Vallee and Auld, 1990a; Vallee and Auld, 1990b; Bhowmik *et al.,* 2010). The first Zn enzyme discovered was carbonic anhydrase, responsible for CO_2 equilibration within erythrocytes and tissues (Keilin and Mann, 1940). Other Zn metalloenzymes range from superoxide dismutase which protects against the toxicity of free oxygen radicals (Brown *et al*., 1983), aspartate transcarbamylase which initiates the first step of pyrimidine biosynthesis required for both DNA and RNA (Kantrowitz *et al*., 1980), and carboxypeptidase (Vallee and Neurath, 1954) which is involved with digestion and wound healing (Skidgel, 2014). Part of the effectiveness of Zn as an essential trace metal is derived from its general lack of redox cycling, allowing it to be transported throughout the body of an organism without the risk of oxidant damage, unlike other divalent metals like iron or copper (Brown *et al*., 2001).

The first study to demonstrate that Zn was required for growth, and therefore survival of even the smallest organisms, was in *Aspergillus niger*, the fungus responsible for black mould (Raulin, 1869). Since then, the focus on Zn deficiency and its negative effects have increased significantly (Prasad, 1990). In fish, it was found that feeding rainbow trout diets

containing low Zn concentrations (1-4 µg Zn/g) impaired both growth and survival. Zn deficient fish were less than half the size of control fish and were susceptible to nodular gill disease and fungal infection development (Spry *et al.,* 1988). Spry *et al.* (1988) further observed that Zn deficiency significantly decreased plasma Zn concentrations and hematocrit. Fish fed low Zn diets have also demonstrated a reduction in the ability to digest nutrients such as proteins, most likely due to a decrease in Zn-associated protease activity (Ogino and Yang, 1978). Additionally, Zn is required for proper piscine development, with physical signs of Zn deficiency in fish being lens cataracts, as well as erosion of the fins and skin (Ogino and Yang, 1978; Spry *et al.,* 1988).

Zinc Toxicity

While Zn is necessary for organismal survival, it may also act as an environmental toxicant when concentrations exceed organism and environmental specific thresholds. The toxicity of Zn to aquatic organisms is dependent on both the physical and chemical form of the metal, determined by abiotic factors like pH, amount of dissolved oxygen, or salinity (Eisler, 1993). In marine environments, Zn either exists as a solid precipitate, in a dissolved state or adsorbed to organics or particles (Eisler, 1993). When at a pH of approximately 8, the dominant soluble Zn species is $Zn(OH)_2$ (62%), followed by free Zn^{2+} ions (17%). When pH lowers to 7, Zn^{2+} present as free ions increases to 50% (Eisler, 1993). As salinity decreases, the concentration of free Zn^{2+} ions also increases (Eisler, 1993).

Unlike with dietborne Zn, where elevated concentrations have seemingly little effect on fish homeostasis, the gills are quite sensitive. One of the main impairments to fish from high concentrations of Zn is its potential to compete with calcium (Ca) present in the water.

At Zn concentrations less than 15 μM there was evidence of ionic mimicry present in the gills of rainbow trout, as Ca^{2+} uptake was essentially eliminated, indicating a strong competition with Zn^{2+} for Ca^{2+} homeostasis *via* a common uptake pathway (Spry and Wood, 1985). Spry and Wood (1985) also reported impaired ionoregulation (branchial net loss of Na^{+}, Cl^{-}, and K^{+} ions), as well as acid-base disturbances leading to metabolic acidosis. At Zn concentrations similar to Spry and Wood (1985), McRae *et al*. (2016) also saw a reduction in Ca^{2+} uptake and ionoregulation disturbances in inanga (*Galaxias maculatus*). Elevated water [Zn] is also associated with pathological alteration in gill histology including lamellar lifting and coalescing (Skidmore and Tovell, 1972; Matthiessen and Brafield, 1973). This gill tissue damage has been demonstrated to negatively impact oxygen transfer, causing both hypoxia and a reduction in blood pH (Spry and Wood, 1984). Furthermore, while itself does not experience redox cycling, the highest level of Zn tested (15 μM) was associated with increased lipid peroxidation in the liver of *G. maculatus* (McRae *et al.,* 2016). Given the negative effects that occur when Zn is present in both low and high concentrations, aquatic organisms have highly regulated systems to acquire Zn from the environment.

Sources of Zinc in the Ocean

Zn exists in the aquatic environment *via* contributions from both natural environmental cycling and increased inputs through anthropogenic activities (see below). The dominant natural source of Zn to the environment is eroded soil particles, or "windblow dusts" (Nriagu, 1979). These airborne dusts make up approximately 60% of global natural zinc emissions produced each year, reaching emission levels of ~$25X10^{6}$ kg/year (Nriagu, 1979). Additional main sources of Zn include both volcanic ash and plant exudates, which

annually produce ~7.0X10^6 kg and ~9.4X10^6 kg of Zn, respectively (Nriagu, 1979). Processes related to production and commercial use (e. g., manufacturing, steel galvanization, urban runoff; Nriagu and Pacyna, 1988; Councell *et al.,* 2004) or other industrial materials (e. g. rubber, paint; Councell *et al.,* 2004; Hogstrand, 2011) are also key contributors of anthropogenic Zn release into the aquatic environment. When combined with all other anthropogenic sources, Zn levels can reach ~226X10^6 kg/year (Nriagu and Pacyna, 1988) although these values are outdated and need revision for current anthropogenic activities.

The natural oceanic profile of Zn within the water column is that [Zn] increases with depth. In the upper 20 m of open oceans, Zn concentrations are generally < 1 nM (Eisler, 1993) while in the deep Pacific Ocean, ~10 nM has been measured and ~2-3 nM is found at depth in the Atlantic Ocean (Biller and Bruland, 2012). Coastal regions and estuaries may have high Zn levels, reaching to ~75 μM, primarily due to anthropogenic inputs (Hogstrand, 2011).

In general, Zn from the surface precipitates depositing the metal in the sediments at the bottom of the ocean. When in proximity to highly urbanized areas, sediment cores have been shown to have Zn levels of approximately 1.5 mM. Zn may also be naturally introduced to sediment *via* active oceanic ridges. As a geochemically volatile element, Zn can migrate from the mantle to the surface through mass flow (Horowitz, 1970). The East Pacific-Antarctic Ridge system has been found to have the highest concentration of Zn within sediments cores (~3 mM) compared to both the Indian Ocean Ridge (~2 mM) and the North Atlantic Ridge (~1 mM; Horowitz, 1970).

Aquatic animals may further have access to oceanic Zn through feeding behaviours, such as the consumption of marine mammals which are known to sequester Zn in higher concentrations. The bottom-feeding Gray whale (*Eschrichtius robustus*), which can ingest substantial amounts of sediment, is a prime example. If the liver of this whale is preyed upon, either hunted or *via* a whale fall, its predators could be exposed to elevated Zn levels (100-640 µg/g dry weight; Varanasi *et al.,* 1994; Méndez *et al.,* 2002). The kidney is another organ that had high Zn concentrations (~120-440 µg/g dry weight), as well as the heart and muscle (Varanasi *et al.,* 1994; Méndez *et al.,* 2002). Other cetaceans, as well as pinnipeds and sirenians, have also been found to have elevated Zn in their tissues (Thompson, 1992).

Hagfish as a Study Organism

Evolution

Diverging from the remainder of the vertebrate lineage 500 million years ago, hagfishes have remained morphologically similar to their fossil records dating back to the late Paleozoic era, approximately 305 million years (Bardack, 1991; Janvier, 2007). Hagfish have been grouped together with lamprey, to represent extant agnathan fishes in the clade Cyclostomata. Cyclostomes notably differ from vertebrate fishes (gnathostomes) in their absence of a hinged jaw, paired fins, and a mineralized skeleton, which makes evolutionary inferences using fossil records difficult (Shimeld and Donoghue, 2012). However, there is strong evidence that suggests hagfishes are indeed vertebrates, such as their cartilaginous vertebral elements arising from the sclerotome, reminiscent of gnathostomes (Ota *et al.*, 2013). Regardless of the similarities between hagfish and vertebrates, the classification of hagfish remains a subject of debate.

Currently, there are two main arrangements for the phylogeny of hagfish, lamprey, and gnathostomes. The first has lamprey being more closely related to the gnathostomes, placed in the clade Vertebrata, and hagfish representing a distantly related out-group (Shimeld and Donoghue, 2012). This paraphyletic arrangement relies on morphological differences and is therefore dubbed the "morphology-based craniate hypothesis" (Miyashita *et al.,* 2019). While there have been several morphological studies in favor of paraphyly (Hardisty, 1982; Janvier, 1996; Donoghue *et al.,* 2000), there is a large amount of molecular support in favour of cyclostome monophyly. This competing hypothesis has lamprey and hagfish grouped together, therefore having these sister taxa being equally related to gnathostomes (Shimeld and Donoghue, 2012). Phylogenetic comparisons of 18S ribosomal RNA sequences between invertebrates, hagfish, lamprey, and gnathostomes was in favour of cyclostome monophyly (Stock and Whitt, 1992). This was supported further through RNA and genomic analyses, showing lamprey and hagfish to share 4 microRNA families not seen in jawed fish (Heimberg *et al.,* 2010). A recent study completed by Myashita *et al.* (2019) on a newly discovered fossil hagfish also presented support of monophyly using only morphological data.

If Cyclostomata is paraphyletic, this would allow us to assume hagfishes are the earliest-diverging extant fishes, and therefore useful in examining traits of an ancestral vertebrate. However, if Cyclostomata is monophyletic, it could indicate that hagfish characteristics are specialized, rather than primitive (Miyashita *et al.,* 2019). Regardless of its phylogeny, the hagfish remains a particularly interesting organism to study from both an evolutionary and physiological standpoint.

Habitat and Behaviour

There are at least 60 species of hagfish within five genera, with the first species (*Myxina glutinosa*) being originally classified among the worms (Linnaeus, 1758; Fernholm, 1998). Aside from approximately 10 species, the majority of species have been named between the 20th and 21st century. Hagfishes are exclusively marine stenohaline iono- and osmoconformers with only Mg^{2+}, Ca^{2+}, and SO_4^{2+} being regulated (Currie and Edwards, 2010). Generally living in cold waters along the bottom of the ocean floor, the global distribution of hagfish is quite extensive except in the Polar seas. However, species have been found off the northern coast of Norway, and as far south as the southern parts of New Zealand, and the Falkland Islands (Fernholm, 1998). The depth distribution of hagfishes is also quite variable, occurring at all depths, with some being seen as deep as 5000 m (Martini, 1998). Along the ocean floor, many species of hagfish have been known to seek shelter among the substrate. The most common preferred substrate for hagfish appears to be areas with silty, sandy, or muddy bottoms, in which they can easily form protective burrows. *Eptratretus* species exist in a larger range of substrates compared to other hagfish species, with some being found burrowed in loose gravel or among boulders in rocky areas (Martini, 1998).

Hagfishes are most often considered to be opportunistic scavengers but have also been shown to actively search out prey, including benthic polychaetes, euphausiids, cephalopods, and small fishes (Johnson, 1994). If abiotic conditions are suitable (e. g. temperature, salinity), hagfish may even hunt at the surface (Martini, 1998). However, when examining hagfish feeding in deep waters, carrion such as large fishes or marine mammals may become a more significant source of food (Smith, 1985). Hagfish scavenge on dead

organisms by entering *via* openings such as the mouth, anus, or open injury, consuming flesh from within (Linnaeus, 1758; Martini, 1998).

Zinc Acquisition Pathways in Fish

Hindgut

Similar to mammals, fish utilize the large surface area of the intestinal tract to acquire dietary Zn, and acts as the dominant absorption pathway under normal conditions (Hogstrand, 2011). In general, the daily piscine dietary Zn requirements range from 15-30 μg/g dry weight (Ogino and Yang, 1978; Gatlin and Wilson, 1983). Studies using both marine and freshwater fish have indicated the possibility for varying capacities involving Zn^{2+} transport based on the location of occurrence along the intestinal tract (including the stomach; Pentreath, 1976; Shears and Fletcher, 1983, Ojo and Wood, 2007). Compared to branchial Zn^{2+} uptake, studies have shown that intestinal uptake may have a lower affinity, but a higher capacity for Zn^{2+} transport (Bury *et al.,* 2003). Zn^{2+} transport within the intestinal tract first involves the binding of the metal to the mucus present within the lumen. Glover and Hogstrand (2002) suggest that at low Zn concentrations within the intestine, mucus may enhance Zn^{2+} uptake by trapping the metal close to the epithelial cells of the intestine. Conversely, at high concentrations mucus secreted intestinally can prevent excess Zn^{2+} from entering the uptake pathway, therefore protecting against any possible toxic effects that could occur (Glover and Hogstrand, 2002; Clearwater *et al.,* 2002). After the metal transfers from the mucosal epithelium into the enterocytes across the apical membrane, it is either

sequestered within the cell or moved out of the cell into the fish *via* the basolateral membrane (Ojo and Wood, 2007).

Gill

There is a consensus that the intestine serves as the primary pathway used by fish for Zn^{2+} uptake in the environment. However, uptake of Zn^{2+} across the gills is independent of intestinal acquisition (Spry *et al.,* 1988; Bury *et al.,* 2003). This is of particular importance when dietary sources of Zn are scarce or episodic, as fish gills may become the sole contributor of Zn homeostasis for the animal when required (Spry *et al.,* 1988). Unlike in intestinal Zn^{2+} acquisition where fish fed Zn diets above 1000 μg/g had seemingly no negative consequences, fish are much more sensitive to waterborne Zn^{2+} *via* the effects on gills (Wekell *et al.,* 1983). As previously mentioned, at Zn^{2+} levels lower than 15 μM, Spry and Wood (1985) reported acid-base and ionoregulatory disturbances in rainbow trout (Spry and Wood, 1985). It has been demonstrated that Zn^{2+} and Ca^{2+} strongly compete with each other, and therefore its mode of uptake and also the mode of toxicity may be associated with apical Ca^{2+} channel function (discussed below; Qui and Hogstrand, 2004; Hogstrand, 2011).

Skin

A potential third general uptake route that aquatic organisms may use to acquire nutrients is the skin. Although this mechanism is most typically used by marine invertebrates (Gomme, 2001), it has also been seen in one group of fishes: the hagfishes. Hagfishes are stenohaline osmo- and ionoconformers with blood osmolality and ion concentrations similar to seawater (Sardella *et al.,* 2009). Therefore, with the skin of hagfish not acting as a barrier

for osmo- and ionoregulation, its leading role may then be an enlarged exchange surface, similar to the intestine and gills (Glover *et al*., 2015). Furthermore, since hagfishes osmoconform, they do not need to drink unlike marine teleost fishes (Glover *et al.,* 2017). This finding supports the idea of hagfish using their skin as a strategy to increase Zn bioavailability. The specific lifestyle of hagfishes may put them at risk of increased Zn exposure. As previously mentioned, hagfishes are bottom-dwellers that burrow in sediment, exposing them to increased levels of Zn. Furthermore, during feeding, they target the liver and other visceral organs first (Weinrauch *et al*., 2018), organs known to accumulate high levels of Zn. As hagfish enter into decaying organisms to feed, this also exposes the skin and gills to a "nutrient soup" containing higher levels of Zn (Martini, 1998). While there has been previous research on hagfish absorption of organic (e.g., amino acids; Glover *et al*., 2011a) and inorganic (e.g., phosphate, nickel; Schultz *et al*., 2014; Glover *et al.*, 2015) nutrients *via* the skin, an in-depth look at the relative importance of different organs in Zn^{2+} uptake has not yet been investigated.

Tissue Specific Zn Distribution and Transport

Organ Storage

Once fish absorb trace elements, the elements are transferred from the point of entry into the blood and subsequently distributed around the body. Unlike iron (Fe) or copper (Cu), there is no specific plasma protein known for Zn (Hogstrand, 2011). Instead, the majority of Zn^{2+} within blood plasma is bound to albumin (about 67%), and the remaining Zn is bound to α_2-macroglobulin and amino acids (Eisler, 1993; Hogstrand, 2011).

Compared to marine plants and invertebrates, marine fishes have lower concentrations of Zn in their tissues, generally ranging from 10-40 μg/g wet weight (Hogstrand and Wood, 1996; Hogstrand, 2011). When fish are exposed to Zn in the environment (either dietary or waterborne), the highest amount of Zn accumulation occurs in the skin, muscle, and bone (Hogstrand and Wood, 1996). When combined, the accumulation in these tissues may make up approximately 50%-60% of the fish's Zn content (Glynn, 1991). The gill, liver, and kidney of fishes may also have elevated levels of Zn; however, the turnover rate is significantly faster than elimination from the rest of the body. The majority of Zn was lost from the gills, liver, and kidney of minnows after 2.5 days, 6.5 days, and 1.8 days, respectively (Glynn, 1991). The highest levels of Zn in tissues is found in the eye of fish, with concentrations reaching as high as 30 mg/g dry weight in the choroid of perch (Eckhert, 1983). This is especially true for nocturnal fishes, where the light reflecting tapetum lucidum may contain extremely high levels of Zn (Hogstrand, 2011). It should be noted that Zn concentration in tissues of fish may change depending on diet, age, the state of reproduction, and distance from point sources of Zn (Eisler, 1993).

Metallothionein Proteins

Due to the possibility of Zn^{2+} acting as a toxicant in fish, the cellular handling of the metal must be effective in removing all free Zn^{2+} present in the cell. Therefore, the use of protein transporters and ligands must be used to keep Zn concentrations tightly regulated. One main metal-binding protein found within the cytosol of cells is metallothionein (MT). This small, ubiquitous, cysteine-rich protein can bind up to seven Zn^{2+} atoms at one time (Stillman, 1995; Hogstrand, 2011). Along with Zn^{2+}, MT is also able to bind other metals

including Cu^{2+}, Cd^{2+}, Ag^{+}, and Hg^{2+}, with varying degrees of affinity. However, under control conditions, the metals found *in vivo* bound to MT are Zn^{2+} and Cu^{2+} (Hogstrand and Wood, 1996). A key feature of MT is the ability of this protein to be induced by the presence of excess metals. In both *in vivo* and *in vitro* studies, it has been demonstrated that the level of hepatic MT present in fish increases after exposure to metals (Roch *et al.,* 1982; Hogstrand *et al.,* 1989; Hogstrand and Haux, 1990). Other metal-binding proteins such as glutathione may also play a role in mitigating potential risks of Zn toxicity, but the exact extent of this is not yet known.

Protein Transporters

Zinc Specific Transporters

There are two main families of proteins responsible for Zn-specific transport, the first being Zn transporter proteins (Slc30; ZnTs), and the second being Zrt, irt-related proteins (Slc39; ZIPs). The first Zn^{2+} transporter to be characterized in fish was ZnT-1 (Slc30a1), sharing important features with its mammalian counterpart in rats (Palmiter and Findley, 1995; Feeney *et al.,* 2005). Of the 10 ubiquitously expressed Slc30 paralogues in mammals, 8 have been in found in fish genomes, specifically in those of the Japanese pufferfish, spotted green pufferfish, and zebrafish (Feeney *et al.,* 2005). For the most part, ZnTs have 6 transmembrane domains and are located within the membranes of organelles, with two notable exceptions (Hogstrand, 2011). ZnT-1 is the only ZnT protein associated with Zn extrusion, exporting Zn^{2+} out of the cell into the blood (Cousins and McMahon, 2000; Hogstrand, 2011). Conversely, ZnT-5 (SLC30A5), the largest ZnT protein with 12

transmembrane domains, has been associated with cellular Zn^{2+} uptake into the cytosol (Jackson *et al.,* 2007).

While there are 14 ZIPs found in mammalian genomes, 13 have been found in fish, most of which have eight transmembrane domains (Feeney *et al*., 2005). Whereas ZnTs generally move Zn^{2+} out of the cytosol, ZIP paralogues are complementary, responsible for bringing Zn^{2+} into the cell (Feeney *et al.,* 2005). Most ZIP proteins are located within the plasma membrane, mediating Zn^{2+} uptake from the external environment. However, Zn^{2+} is released into the cell *via* ZIP7 located in the endoplasmic reticulum, and *via* ZIP9 and ZIP13 located in the Golgi apparatus (Hogstrand, 2011).

Calcium Channels

In fish, one of the main non-specific pathways Zn may use to enter the cell is the apically located membrane epithelial calcium channel (ECaC; Trpv6), part of the transient receptor potential family of proteins (Hoenderop and Bindels, 2008). Primarily expressed in the gill, Ca^{2+} uptake employing ECaC was substantially inhibited when in the presence of Zn, strongly indicating Ca^{2+} mimicry (Hogstrand *et al.,* 1995; Qiu and Hogstrand, 2004). Additionally, early research demonstrated that within invertebrates, voltage-gated Ca^{2+} channels were permeable to certain divalent cations including Zn^{2+} and that blockers of Ca^{2+} channels inhibited Zn^{2+} uptake (Fukuda and Kawa, 1977; Vercauteren and Blust, 1999).

Divalent Metal Transporter 1

A highly conserved metal transporter existing in all animals, plants, fungi, and prokaryotes, is the divalent metal transporter 1 (Gunshin *et al.*, 1997; Cellier *et al.,* 2001). Part of the NRAMP (natural resistance-associated macrophage protein; NRAMP2) or *slc11*

gene family (*slc11a2*), DMT-1 is a 561-amino acid, apically located divalent metal symporter, powered by a H^+ electrochemical gradient (Gunshin *et al.,* 1997; Mackenzie and Hediger, 2004). While DMT-1 is mainly known for intestinal Fe^{2+} uptake mechanisms, it is ubiquitously expressed. Both essential (Cu^{2+}, Ni^{2+}, Zn^{2+}) and non-essential (Cd^{2+}, Pb^{2+}) metals have been shown to inhibit intestinal and branchial Fe^{2+} absorption in fish (Cooper *et al*., 2007; Kwong and Niyogi, 2009). Therefore, DMT-1 could be playing a role in Zn^{2+} uptake, as it is suggested other divalent cations may compete for this transporter.

Thesis Objectives

Zinc as a trace element is essential to the survival of all organisms, given its role as a co-factor for numerous vital enzymes responsible for whole-body homeostasis. Alternatively, Zn may also act as an environmental toxicant when fish are exposed to higher environmental concentrations, and therefore transport across epithelia must be tightly regulated. In aquatic organisms like fish, they have additional uptake pathways (e. g. Ca^{2+} homeostasis) not present in mammals that may be impacted by Zn. The overarching goal of this thesis is to investigate both whole animal Zn^{2+} acquisition and distribution, as well as intracellular Zn^{2+} handling and transport, using the Pacific hagfish (*Eptatretus stoutii*) as a study organism.

My first objective is to examine the whole-body accumulation and distribution of Zn^{2+} within hagfish at three different timepoints when exposed to both low and high Zn concentrations. It is *hypothesized* that over time Zn will accumulate at the site of entry (e. g. within the gills), as well as within blood plasma bound to proteins and within the liver, a common tissue of interest for metal accumulation.

My second objective is to investigate the role of the hagfish hindgut, gill, and skin in Zn^{2+} acquisition from the external environment. Because of potential for either Zn^{2+} deficiency or toxicity, it is *hypothesized* that Zn^{2+} will be taken up across each of these tissues in a regulated fashion.

My third objective is to elucidate which protein transporters are potentially present and used for Zn^{2+} movement into and out of the cell of the hindgut, gill, and skin. Using putative metal competitors of Zn (Cd^{2+}, Cu^{2+}, La^{3+}, Ni^{2+}, Pb^{2+}) in excess of Zn^{2+} both individually and as a mixture (Cu^{2+}, La^{3+}, Ni^{2+}), I will be able to able to differentiate the specific subtypes. I *hypothesize* that Zn^{2+} uptake will be hindered by each these metals, with differential inhibition by metal type, thus giving insight into Zn cellular transport in the hagfish.

Finally, my fourth objective is to measure the levels of metallothionein present in tissues of hagfish when exposed to Zn for 0, 1, 3, 5, and 7 days. It is *hypothesized* that Zn exposure will induce increased production of metallothionein. Additionally, I *hypothesize* that the liver will contain the highest level of these proteins due to its role in metal detoxification, as seen in other organisms. To our knowledge, this is the first study where hagfish metallothionein levels will be examined.

Chapter 2 – Accessing essential trace metals in the marine environment: the characterization of zinc acquisition and cellular handling using three absorptive surfaces in the Pacific hagfish (*Eptatretus stoutii*)

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Introduction

Zinc (Zn) is a micronutrient required for the survival of even the most basal organisms (Raulin, 1869; Prasad, 1990). As an essential trace metal, Zn is a crucial component for the metabolic activity of approximately 300 enzymes, many of which are responsible for the synthesis of critical biomolecules and general whole-body homeostasis (Vallee & Auld, 1990; Bhowmik *et al*., 2010). Zn deficiency in fish has been shown to cause growth retardation, as well as metabolic challenges (e. g., reduced nutrient digestibility; Ogino and Yang, 1978). Additionally, low [Zn] has been shown to negatively impact fish immunity (Kiron *et al.,* 1993). While Zn is necessary for organismal survival, it may also act as an environmental toxicant. Ca^{2+} homeostasis, acid-base balance, and ion regulation are all impaired by excess environmental Zn^{2+} (Spry and Wood, 1985; McRae *et al.,* 2016). Furthermore, while itself does not undergo redox cycling, high levels of Zn^{2+} (15.3 μM) have been associated with hepatic lipid peroxidation (McRae *et al.,* 2016). Due to the detrimental effects when Zn is both lacking and in excess, it must be tightly regulated within the body through both acquisition and cellular handling (Bury *et al.,* 2003).

To ensure survival, fish can acquire Zn from their environment using two primary sources: dietborne sources taken up by their intestinal tract, or waterborne sources taken up across their gills. In general, intestinal uptake plays a larger role in overall Zn^{2+} acquisition compared to branchial uptake (Hogstrand, 2011). Hagfish (of the family *Myxinidae)* are unique among fishes in that they acquire waterborne nutrients utilizing their integument, an ability typically only seen in invertebrates (Gomme, 2001). Although this third route of nutrient absorption may be beneficial when dietborne Zn is scarce, it could also increase the risk of toxicity to the organism.

The lifestyle of hagfish allows for multiple potential sources by which these fish might access Zn. Hagfish are a widespread group of benthic fish which typically dwell along the seafloor. Additionally, some species have been known to burrow within sediment (Martini, 1998). As Zn enters marine waters, the natural oceanic profile within the water column increases with depth, with 10 nM measured in the deep Pacific Ocean and ~2-3 nM found at depth in the Atlantic Ocean (Biller and Bruland, 2012). In general, Zn from the surface precipitates, depositing the metal in the sediments at the bottom. Coastal regions and estuaries may have higher Zn levels, reaching ~75 nM (Hogstrand, 2011). Furthermore, while it is accepted that hagfish may actively hunt live prey, they are primarily recognized as opportunistic scavengers which enter and consume the dead and decaying flesh of large marine organisms (Martini, 1998; Tamburri and Barry, 1999). Whales, in particular, are known to accumulate high levels of Zn in their tissues and have had measured concentrations exceeding 100 µg/g dry weight (Méndez *et al.,* 2002).

While there has been previous research on hagfish absorption of organic (e.g., amino acids; Glover *et al*., 2011a; Glover *et al.,* 2011b) and inorganic (e.g., phosphate, nickel, iron; Schultz *et al*., 2014; Glover *et al.*, 2015; Glover *et al.,* 2016) nutrients *via* the intestine, gill, and skin, the uptake of Zn^{2+} has not yet been investigated. The present study examines both Zn acquisition and handling within the Pacific hagfish (*Eptatretus stoutii*) through a series of *in vivo* and *in vitro* techniques. Distribution of absorbed Zn within hagfish tissues at differing concentrations and time points were measured *via* whole animal exposures. Concentration-dependent kinetics of Zn^{2+} uptake were determined for the hindgut, gill, and skin, while putative metal competitors were used to elucidate potential pathways Zn^{2+} may utilize to enter the cell. One way in which hagfish may handle elevated cellular levels of Zn^{2+}

is through the presence of metal-binding proteins such as metallothionein (MT; Hogstrand, 2011). Therefore, a time-dependent *in vivo* exposure was conducted to measure metallothionein levels within hagfish tissues, as well as observe a potential MT induction. To our knowledge, this is the first study in which hagfish MT levels are measured.

Methods and Materials

Animals

Pacific hagfish (*Eptatretus stoutii*; $N = 130$; 82.5 ± 2.9 g; mean ± SEM) were caught with baited traps from Barkley Sound near Bamfield (Vancouver Island, Canada) during Summer 2017 and 2018. Once collected, fish were taken to Bamfield Marine Sciences Centre (BMSC) and transferred to outdoor holding tanks (500 L) with constant flow-through seawater (12 °C (2017); 10 °C (2018)). Hagfish were fasted for a minimum of 7 days prior to the start of experimentation. Additional animals were transported to the University of Alberta and placed in a recirculating artificial seawater system (Instant Ocean SeaSalt; Spectrum Brands, Blacksburg, VA, USA). Hagfish acclimated to this system for a minimum of 2 months prior to being used for experiments. All animal collections and procedures were approved by the Department of Fisheries and Oceans (Collection Permit No. XR-136-2017; XR-112-2018), BMSC Animal Care (No. RS-17-03 (2017); RS-18-14(2018)), and University of Alberta Animal Care (AUP00001126). Hagfish were euthanized by anesthetic overdose using $NaHCO_3$ buffered 4 g/L tricaine methanesulfonate (TMS; AquaLife, Syndel Laboratories Ltd., Nanaimo, BC, Canada).

Solutions

Hagfish saline (in mM: NaCl, 490; KCl, 8; $CaCl_2$ $2H_2O$, 5; $MgSO_4$ $7H_2O$, 3; $MgCl_2$ $6H_2O$, 9; NaH_2PO_4 H_2O, 2; $NaHCO_3$, 8; HEPES, 20; Glucose, 5; pH 7.68) was used for all serosal solutions. Mucosal solutions were made of HF saline (seawater for skin transport assays) containing $ZnCl_2$ (0-500 μM) and radiolabeled $^{65}ZnCl_2$ (0.05-0.1 μCi/mL; PerkinElmer Inc., Waltham, MA, USA). For metal competition experiments, mucosal

solutions also contained 2X or 5X metal competitors ($CdCl_2$ H_2O, $CuCl_2$, $LaCl_3$ $7H_2O$, $NiCl_2$ $6H_2O$, $PbCl_2$; 10-200 μM). Quench correction was applied to all tissues prior to any calculations. All chemicals and reagents were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated.

In vivo Exposures

Hagfish were individually placed in sealed chambers with 500 mL of aerating seawater and either 2 or 100 μM Zn spiked with $^{65}ZnCl_2$ to a final concentration of ~0.15 μCi/mL. Water samples (100 μL) were taken at the start and end of each exposure and radioactivity measured was used to calculate mean specific activity. Each chamber was temperature controlled in a wet table and left for a period of 6, 24, or 48 hours, after which hagfish were euthanized. A 1 mL blood sample was taken from the caudal sinus which was then divided into plasma and blood cell fractions *via* centrifugation (12,000 *g* for 2 minutes). Once dissected longitudinally, bile was withdrawn from the gallbladder using a syringe. The brain, heart, and kidneys were dissected in full, along with the hindgut which was divided into 4 even sections. A section of liver, foregut, and muscle were also taken. Approximately 4-5 gills were removed (excluding the first pair of gill pouches due to morphological differences) and a similarly sized dorsal section of skin was also removed from the anterior, medial, and posterior region of the hagfish. Each tissue was washed with an isotope displacement solution (1 g/L Zn in 1% HNO_3 HF saline), blotted dry, weighed (wet weight) and depending on tissue type, either 2 or 4 mL of 2 N HNO_3 were added to all tissues in a scintillation vial which were subsequently digested for 48 hours at 65 °C. Scintillation fluor was added to the samples (UltimaGold for tissues, Optiphase for plasma, bile, and water;

PerkinElmer Inc., Waltham, MA, USA) prior to being measured on a Beckman Coulter LS6500. Accumulation of Zn within tissues was calculated as:

$$Tissue\ accumulation\ (nmol/g) = \frac{CPM/SAct}{mass}$$

Where CPM are quench corrected counts per minute, SAct is the specific activity (CPM/nmol), and mass is the weight of each tissue (g).

In vitro Transport Assays

Intestinal Assays

Intestinal Zn fluxes were conducted using a gut-sac method previously described for hagfish (Glover *et al.*, 2011b; Schultz *et al.*, 2014). After euthanasia, a longitudinal incision along the ventral side of the fish was made exposing the hindgut (posterior to the gallbladder), which was subsequently removed and divided into segments that were randomized between test concentrations. One end of each segment was tied shut with suture thread, and the other was secured in place with a sampling port made from flared PE-50 tubing. The sac was filled until turgid (0.2-1.18 mL) with HF saline spiked with Zn (5-500 μM), and radiolabeled $^{65}ZnCl_2$ (0.05 μCi/mL). Gut sacs were placed in 10 mL of aerating serosal solution in a wet table for temperature regulation. After a 2-hour flux period (Appendix 1A), gut sacs were blotted dry, and samples were taken of both mucosal and serosal fluid. Intestinal tissue was washed with displacement solution to remove excess adsorbed isotope. The mucosal surface was then gently scraped with a microscope slide, and surface area was determined using Image J Software (National Institutes of Health, Bethesda, MD, USA). Tissue and mucosal scrapings were placed in a scintillation vial with 2-4 mL 2

N HNO_3 and allowed to digest for 48 hours at 65 °C. Scintillation fluor was added to the tissue digests and both mucosal and serosal fluid samples (UltimaGold for tissue, Optiphase for water samples) prior to being measured on a Beckman Coulter LS6500. Uptake was expressed per unit area exposed (nmol/cm^2/h).

Gill Perifusions

Gill perifusions were performed using a modified protocol from Forster & Fenwick (1994), as previously described by Glover *et al*. (2011a). Starting at the second pair of gills, hagfish gill pouches were removed from the fish and the afferent and efferent water ducts were cannulated with flared PE-50 tubing secured in place with suture thread. A peristaltic pump connected to the afferent cannula was used to perfuse mucosal Zn solutions (2.5-100 μM) spiked with $^{65}ZnCl_2$ (0.1 μCi/mL) through each gill pouch at a mean rate of 6.1 mL/h, similar to flow rates previously recorded (Glover *et al.*, 2011a; Schultz *et al.,* 2014). Each trial ran for a minimum of 3 to a maximum of 4 hours (Appendix 1B), with perifusates collected at every 30-minute interval. During perifusion, each gill pouch was placed in 5 mL temperature-controlled, aerating Zn-free HF saline. At the end of each trial, food colouring dissolved in HF saline was used to validate the preparation prior to the gill being flushed with displacement solution, blotted dry, and weighed. For each fraction, and the whole gill, ^{65}Zn activity was counted on a Cobra Quantum gamma counter. The initial 30-minute perifusate fraction was discarded, with the disappearance of $^{65}ZnCl_2$ between afferent solutions and remaining efferent fractions representing Zn uptake into the gill. Uptake was averaged over the final 2.5-3.5 hours of perifusion, divided by gill wet weight and converted to an hourly rate expressed as nmol/g/h.

Skin Assays

Skin Zn fluxes were performed following a modified Ussing chamber technique as described previously (see Glover *et al.,* 2011a for chamber diagram). Briefly, sections of skin randomly assigned to test concentrations were dissected from dorsal sections posterior to the final gill pore and fitted over the opening 20 mL scintillation vials. The skin was secured by screwing the lid (with a hole cut out of the middle) with the mucosal surface facing the outside, excess skin around the lid was removed, and any slime present was gently wiped away. Ten mL of aerating Zn-free HF saline was added to the serosal side (inside the vial), and the apparatus was inverted into a temperature controlled, aerating seawater bath containing both radiolabeled $^{65}ZnCl_2$ (0.05 μCi/ml) and Zn (2.5-500 μM). Fluxes were run for 2 hours (Appendix 1C), after which mucosal and serosal fluid samples were taken. The skin was removed, washed with displacement solution, measured for surface area, digested, and counted along with fluid samples taken, all as previously described for the intestinal fluxes. Uptake was expressed per unit area exposed (nmol/cm^2/h).

In vivo Metallothionein Induction

Hagfish were placed individually in sealed chambers with 1 L of aerating seawater and 50 μM Zn. Each chamber was placed in a wet table to be temperature controlled and left for a period of 0, 1, 3, 5, or 7 days (based on a previous MT induction study completed with marine teleosts; Hogstrand and Haux, 1990), with 50% water replacements occurring every 48 hours to replenish the metal. Water samples were taken for measurement of [Zn] at the beginning and end of each water change. Once complete, hagfish were euthanized and dissected longitudinally. A section of the liver and muscle was removed, along with

approximately 6-8 gill pouches (excluding the first pair). The hindgut was dissected out and divided into 4 even sections, and a similarly sized dorsal section of skin from the anterior, medial, and posterior region of the hagfish was also taken. All tissues were frozen in liquid nitrogen and stored at -80 °C until analyses could be completed.

Levels of metallothionein proteins present were quantified *via* silver saturation, modified from the protocol described by Scheuhammer and Cherian (1991). Briefly, 0.2-0.5 g of frozen tissue was ground, to which 4 volumes of 0.25 M sucrose was added. The solution was then homogenized for 1-2 minutes and centrifuged (18,000 *g* for 20 minutes at 4 °C), after which the supernatant was collected. Approximately 50-400 µL of supernatant (or sucrose as blank) was transferred to a 1.5 mL microfuge tube, to which 0.5 M glycine (pH 8.5 with 4 N NaOH) was added to make 0.8 mL, followed by 0.5 mL $AgNO_3$ (20 µg Ag^+/mL glycine). Tubes incubated for 15 minutes, after which 0.1 mL 2% (w/v) hemoglobin solution was added (crude bovine hemoglobin in 30mM Tris buffer, pH 8.0; Eaton and Cherian, 1991) and mixed. Tubes were heated in a boiling water bath for 2 minutes and centrifuged (1,200 *g* for 5 minutes). An additional 0.1 mL of hemoglobin solution was added, with the heating and centrifuging steps repeated. The supernatant was then centrifuged (15,000 *g* for 5 minutes), with the final supernatant being collected and measured for [Ag] by atomic absorption spectrophotometry (AAS), using an air-acetylene flame (iCE 3000 Series, Thermo Fisher Scientific, Waltham, MA, USA). Amount of metallothionein present in each tissue was calculated as:

$$\mu g\ Metallothionein/g\ tissue = \frac{\left(C_{Ag}\ - C_{BKG}\right) \times\ 3.55\ \times V_T \times SDF}{S_V}$$

Where C_{Ag} is the concentration of silver in the final supernatant, C_{BKG} the concentration of silver of the blank in the final supernatant, V_T the total volume of the assay, SDF the sample

dilution factor, and S_V the sample volume. The constant 3.55 is used as 1 µg Ag represents 3.55 µg MT (Scheuhammer and Cherian, 1991).

Statistical Analyses

All data are presented as means ± SEM and calculated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). All *in vivo* exposure results were evaluated using a one-way ANOVA followed by a Tukey multiple comparisons test when differences occurred. If assumptions of normality (D'Agostino-Pearson) and equal variances (Brown-Forsythe) were not met, a Kruskal-Wallis test with Dunn's test was used. Concentration-dependent kinetic curves were fitted to linear, hyperbolic, or sigmoidal equations to determine which had the highest R^2 value. Metal competition experiments were analyzed using a one-way ANOVA with Dunnett's multiple comparisons test when differences occurred. Metal mixture results were analyzed *via* unpaired t-tests, or Mann-Whitney U tests when assumptions of normality (D'Agostino-Pearson) and homogeneity of variance (F-test of equality of variances) were violated. Differences were considered statistically significant at $p < 0.05$.

Results

In vivo Zn Exposures

Of all samples collected, only plasma, gill, foregut, hindgut, heart, and brain showed measurable Zn accumulation across time and within both 2 and 100 μM tested concentrations. No differences were found across each section of the hindgut and was therefore grouped together (data not shown). See Appendix 2 for Zn accumulation within all tissues.

At the low exposure concentration (2 μM), Zn accumulation followed similar patterns for both 6-hour (Figure 1A) and 48-hour (Figure 1C) exposure times, with the gill and foregut having the highest levels of Zn accumulation. At 24 hours, the gill had Zn levels approximately 20-fold higher than all other tissues (Figure 1B). At the 100 μM concentration, the gill continued to have significantly high Zn accumulation, along with plasma at all three time points (Figure 2).

Due to varying scales over time and to allow for direct comparison of accumulation over time for each tissue at each concentration, the data was reorganized and presented in Figures 3 and 4. At low [Zn], both the plasma and heart demonstrated substantial increases in Zn accumulation as time of exposure increased (Figure 3A, 3E). The foregut, hindgut, and brain showed no statistically significant increases (Figure 3C, 3D, 3F) due to high variability at the 24-hour time period. The gill peaked in Zn accumulation at 24 hours with an approximate 20-fold increase (Figure 3B). Neither the brain nor the hindgut demonstrated changes in Zn accumulation over time at 100 μM (Figure 4D, 4F). All remaining tissues experienced the highest accumulation of Zn at 48 hours, the largest change occurring within plasma (approximately 20-fold increase; Figure 4A).

In vitro Transport Assays

Intestinal Assays

Zinc uptake within the hindgut of the Pacific hagfish was considered to have occurred if Zn^{2+} appeared in the mucosal scrapings, underlying tissue, and in serosal fluid. Rate of uptake was linear/diffusive at high concentrations (Figure 5A) but had saturation at Zn concentrations ≤ 250 µM (Figure 5B). The saturable component best fit a sigmoidal distribution ($R^2 = 0.5767$), with an apparent affinity constant (K_m-like) of 65.5 ± 14.7 µM and a maximal rate of Zn uptake (J_{max}) of 0.15 ± 0.02 nmol/cm^2/h.

The addition of metals to mucosal solutions (Cd^{2+}, Cu^{2+}, La^{3+}, Ni^{2+}, or Pb^{2+}) as potential competitors to Zn^{2+} uptake elicited no inhibition of Zn^{2+} uptake at either 2 µM with 5X competing metals (10 µM; Figure 6A) or 100 µM with 2X competing metals (200 µM; Figure 6B) when compared to the control (Zn^{2+} only). Similarly, a mixture of potentially competing metals (Cu^{2+}, La^{3+}, and Ni^{2+}) at 10 µM and 200 µM had no effect on Zn^{2+} uptake in the hindgut (Figure 7).

Gill Perifusions

The rate of Zn^{2+} uptake by the hagfish gill increased with increasing exposure concentrations, and experienced both a linear/diffusive (Figure 8A) in high [Zn] and saturable component (Figure 8B) when [Zn] was low. The saturable pathway was seen at Zn concentrations ≤ 25 µM, and the data was best fitted with a sigmoidal distribution ($R^2 = 0.5236$). The apparent K_m was 15 ± 5.7 µM and had a J_{max} of 880.7 ± 355.4 nmol/g/h.

The addition of La (10 µM) to mucosal Zn (2 µM) caused a significant increase in the rate of uptake across the gill ($p = 0.0039$; Figure 9A), while the other metals did not cause

any statistically significant changes. High [Zn] (40 μM) with 5X competing metals (200 μM) did not elicit a response compared to the control group (Zn^{2+} alone; Figure 9B), nor did the metal mixture of Cu^{2+}, La^{3+}, and Ni^{2+} at both low and high concentrations (Figure 10).

Skin Assays

Zinc uptake across the skin of the hagfish increased with increasing exposure concentrations and was considered to have occurred if Zn^{2+} appeared in serosal fluid. Similar to both the hindgut and gill, rate of uptake in the skin was linear/diffusive at high concentrations (Figure 11A) but also had saturation at Zn concentrations ≤ 100 μM (Figure 11B). The saturable component best fit a sigmoidal distribution (R^2 = 0.8889), with an apparent K_m of 25.1 ± 3.9 μM and a J_{max} of 1.2 ± 0.12 nmol/cm^2/h.

The addition of Cu^{2+}, Cd^{2+}, La^{3+}, Ni^{2+}, or Pb^{2+} to mucosal solutions elicited no statistically significant changes to Zn^{2+} uptake at either 2 μM Zn with 10 μM competing metals (Figure 12A) or 100 μM Zn with 200 μM competing metals (Figure 12B). Additionally, the metal mixture of Cu^{2+}, La^{3+}, and Ni^{2+} at 10 μM and 200 μM had no effect on Zn^{2+} uptake across the skin (Figure 13).

In vivo Metallothionein Induction

Water chemistry analysis using AAS determined nominal 50 μM water samples taken to be 38 ± 2.4 μM over the 7-day period. Hagfish gill, liver, hindgut, and skin all demonstrated measurable levels of MT (Figure 14) while in muscle samples no MT was detected within any replicate across all time points tested and are therefore not shown. No differences were found across each section of the hindgut or skin, and therefore each tissue

was placed together as a single group (data not shown). For all days of exposure, liver had the highest level of MT (~500-600 µg MT/g of tissue). The general pattern of MT levels tissues across time was as followed: Liver > Hindgut > Gill ~ Skin (Figure 2-14).

Similar to *in vivo* Zn accumulation data above, due to varying scales over time and to allow for direct comparison of MT induction over time for each tissue, the data was reorganized and is presented in Figure 15. When observing MT levels at 1, 3, 5, and 7 days of fish exposed to 38 µM, no significant induction of MT was seen for any tissue compared to control (0 day; Figure 15).

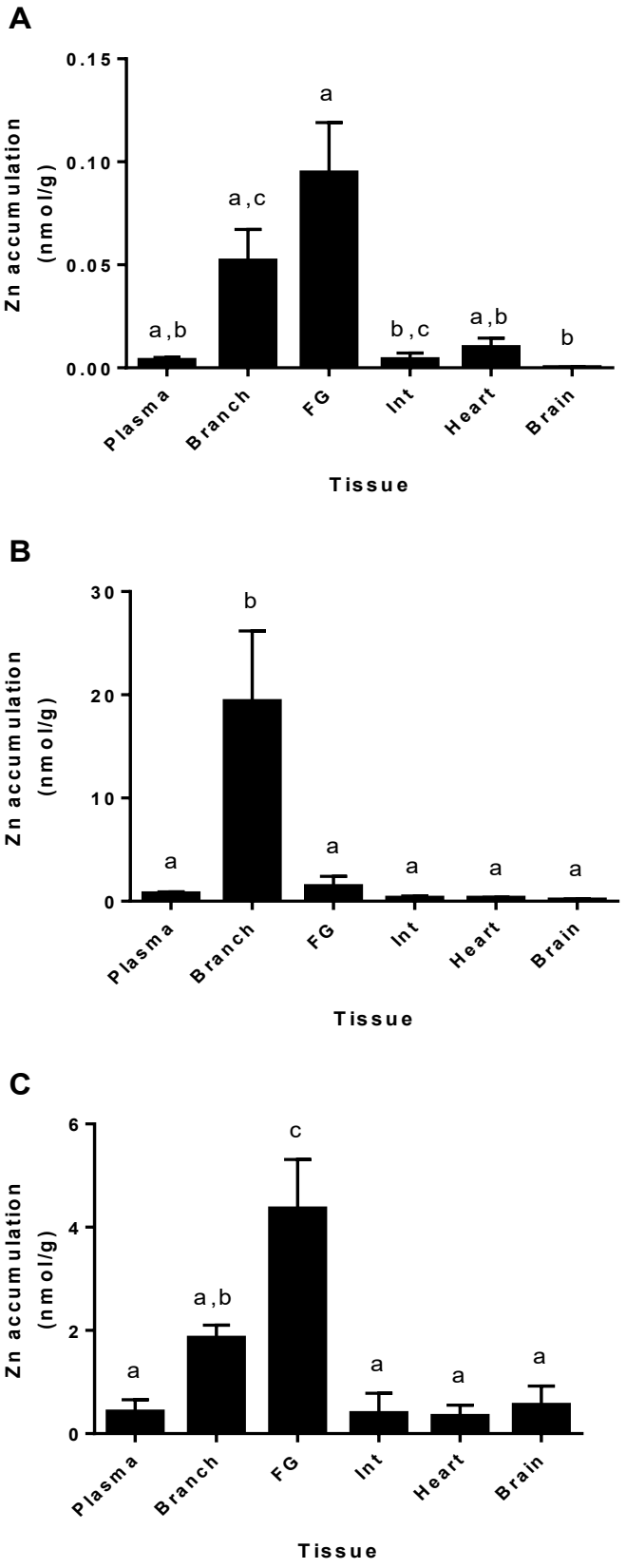

A
0.15
0.10
0.05
0.00
Zn accumulation (nmol/g)
a,b
a,c
a
b,c
a,b
b
Plasma
Branch
FG
Int
Heart
Brain
Tissue
B
30
20
10
0
Zn accumulation (nmol/g)
a
b
a
a
a
a
Plasma
Branch
FG
Int
Heart
Brain
Tissue
C
6
4
2
0
Zn accumulation (nmol/g)
a
a,b
c
a
a
a
Plasma
Branch
FG
Int
Heart
Brain
Tissue

Figure 1. Accumulation of Zn in tissues of the Pacific hagfish following exposure to 2 μM for 6 h (A), 24 h (B) or 48 h (C). Bars represent means ± SEM of 4-5 replicates. Bars sharing letters are not significantly different ($p < 0.05$), determined using a Kruskal-Wallis test with a Dunn's multiple comparisons test (A) or a one-way ANOVA with a Tukey multiple comparisons test (B, C). Data was analyzed using GraphPad Prism 6.

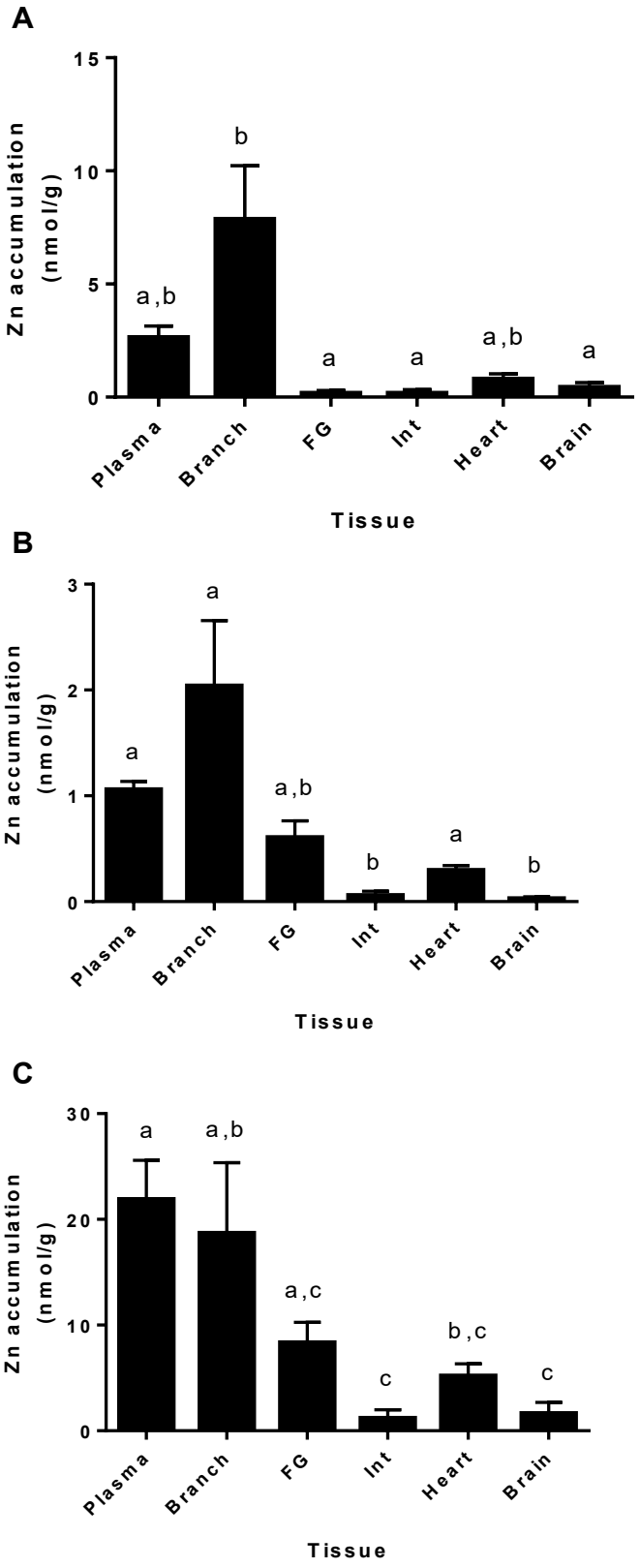

A
Zn accumulation (nmol/g)
15
10
5
0
a,b
b
a
a
a,b
a
Plasma
Branch
FG
Int
Heart
Brain
Tissue
B
Zn accumulation (nmol/g)
3
2
1
0
a
a
a,b
b
a
b
Plasma
Branch
FG
Int
Heart
Brain
Tissue
C
Zn accumulation (nmol/g)
30
20
10
0
a
a,b
a,c
c
b,c
c
Plasma
Branch
FG
Int
Heart
Brain
Tissue

Figure 2. Accumulation of Zn in tissues of the Pacific hagfish following exposure to 100 µM for 6 h (A), 24 h (B) or 48 h (C). Bars represent means ± SEM of 4-5 replicates. Bars sharing letters are not significantly different ($p < 0.05$), determined using a Kruskal-Wallis test with a Dunn's multiple comparisons test (A, B) or one-way ANOVA with a Tukey multiple comparisons test (C). Data was analyzed using GraphPad Prism 6.

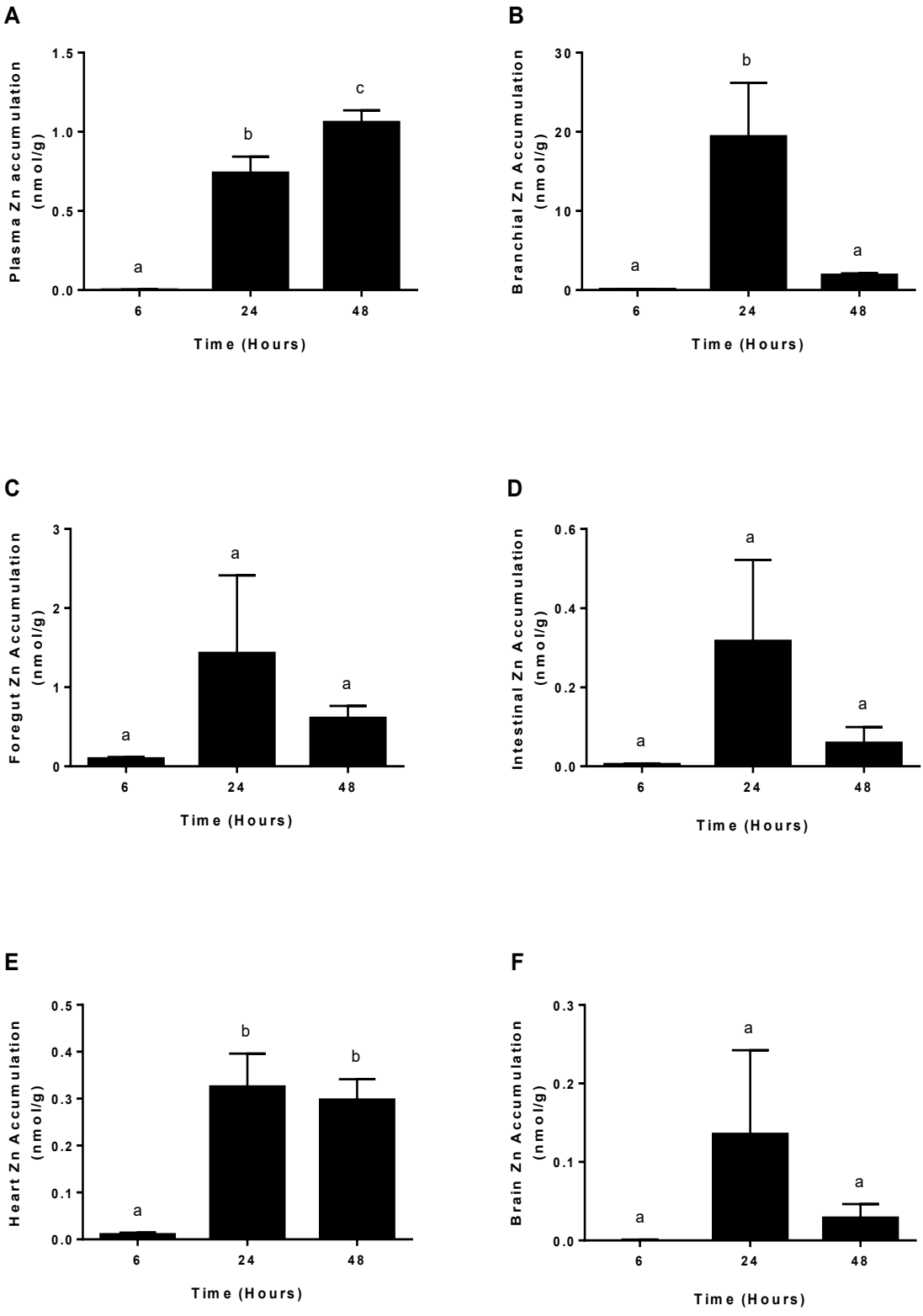

A
Plasma Zn accumulation (nmol/g)
1.5
1.0
0.5
0.0
a
b
c
6
24
48
Time (Hours)
B
Branchial Zn Accumulation (nmol/g)
30
20
10
0
a
b
a
6
24
48
Time (Hours)
C
Foregut Zn Accumulation (nmol/g)
3
2
1
0
a
a
a
6
24
48
Time (Hours)
D
Intestinal Zn Accumulation (nmol/g)
0.6
0.4
0.2
0.0
a
a
a
6
24
48
Time (Hours)
E
Heart Zn Accumulation (nmol/g)
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
6
24
48
Time (Hours)
F
Brain Zn Accumulation (nmol/g)
0.3
0.2
0.1
0.0
a
a
a
6
24
48
Time (Hours)

Figure 3. Accumulation of Zn in the plasma (A), gill (B), foregut (C), hindgut (D), heart (E), and brain (F) of Pacific hagfish following exposure to 2 μM for 6 h, 24 h, and 48 h. Bars represent means ± SEM of 4-5 replicates. Bars sharing letters are not significantly different ($p < 0.05$), determined using a one-way ANOVA with a Tukey multiple comparisons test (A, B, C, F) or a Kruskal-Wallis test with a Dunn's multiple comparisons test (D, E). Data was analyzed using GraphPad Prism 6.

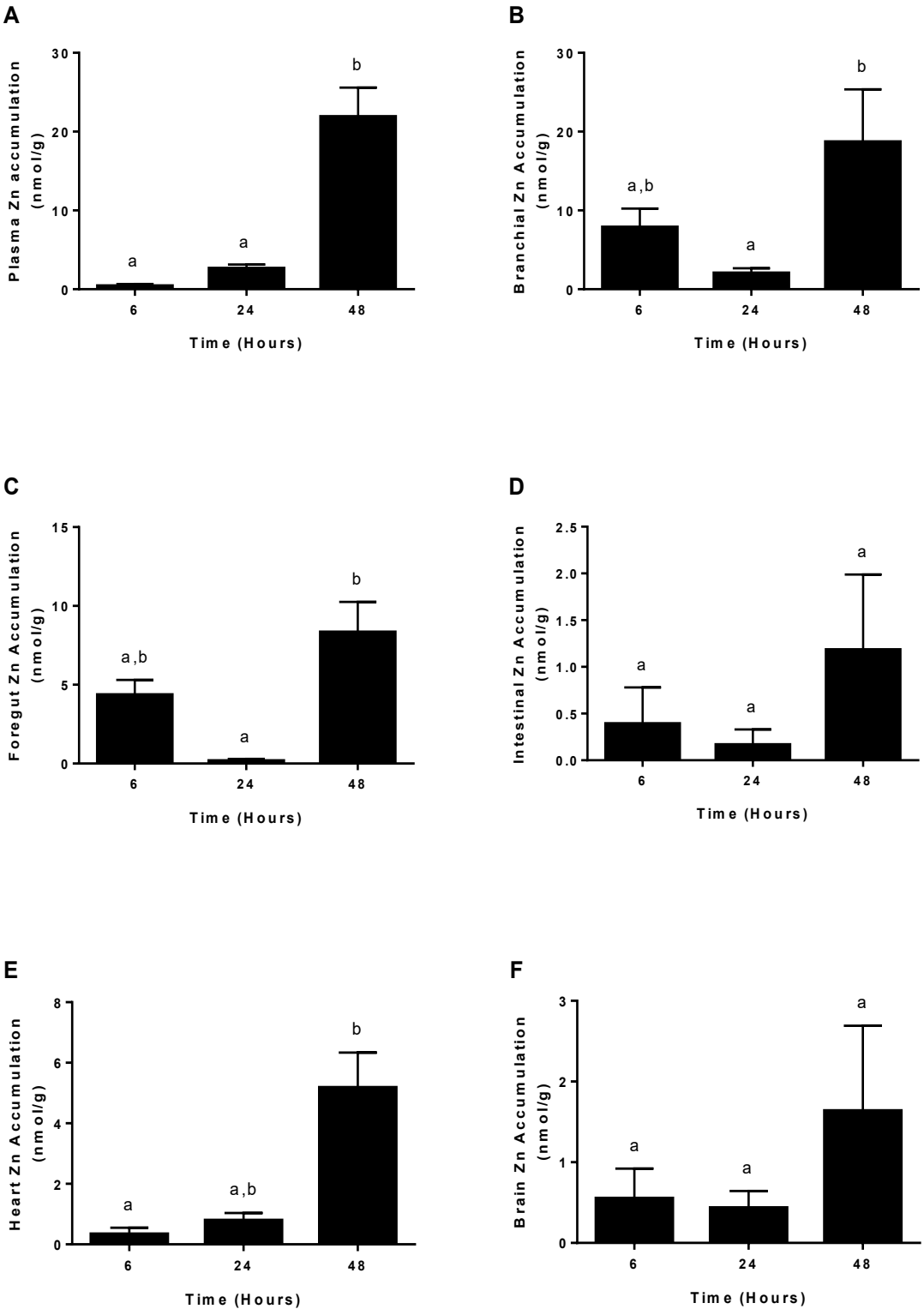
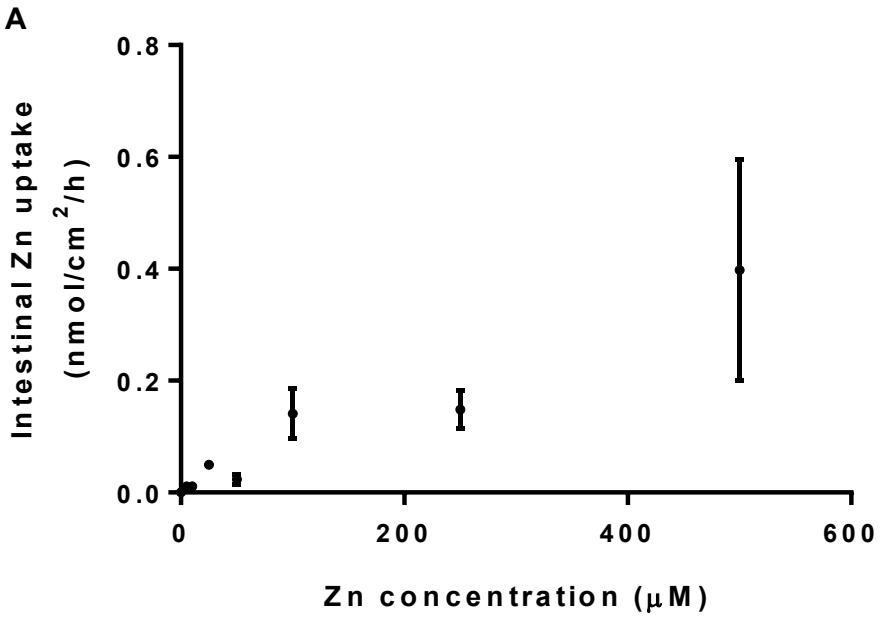

A
Plasma Zn accumulation (nmol/g)
30
20
10
0
a
a
b
6
24
48
Time (Hours)
B
Branchial Zn Accumulation (nmol/g)
30
20
10
0
a,b
a
b
6
24
48
Time (Hours)
C
Foregut Zn Accumulation (nmol/g)
15
10
5
0
a,b
a
b
6
24
48
Time (Hours)
D
Intestinal Zn Accumulation (nmol/g)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
6
24
48
Time (Hours)
E
Heart Zn Accumulation (nmol/g)
8
6
4
2
0
a
a,b
b
6
24
48
Time (Hours)
F
Brain Zn Accumulation (nmol/g)
3
2
1
0
a
a
a
6
24
48
Time (Hours)

Figure 4. Accumulation of Zn in the plasma (A), gill (B), foregut (C), hindgut (D), heart (E), and brain (F) of Pacific hagfish following exposure to 100 μM for 6 h, 24 h, and 48 h. Bars represent means ± SEM of 4-5 replicates. Bars sharing letters are not significantly different ($p < 0.05$), determined using a one-way ANOVA with a Tukey multiple comparisons test (A, B, C, F) or a Kruskal-Wallis test with a Dunn's multiple comparisons test (D, E). Data was analyzed using GraphPad Prism 6.


A
Intestinal Zn uptake
(nmol/cm2/h)
0.8
0.6
0.4
0.2
0.0
0
200
400
600
Zn concentration (μM)

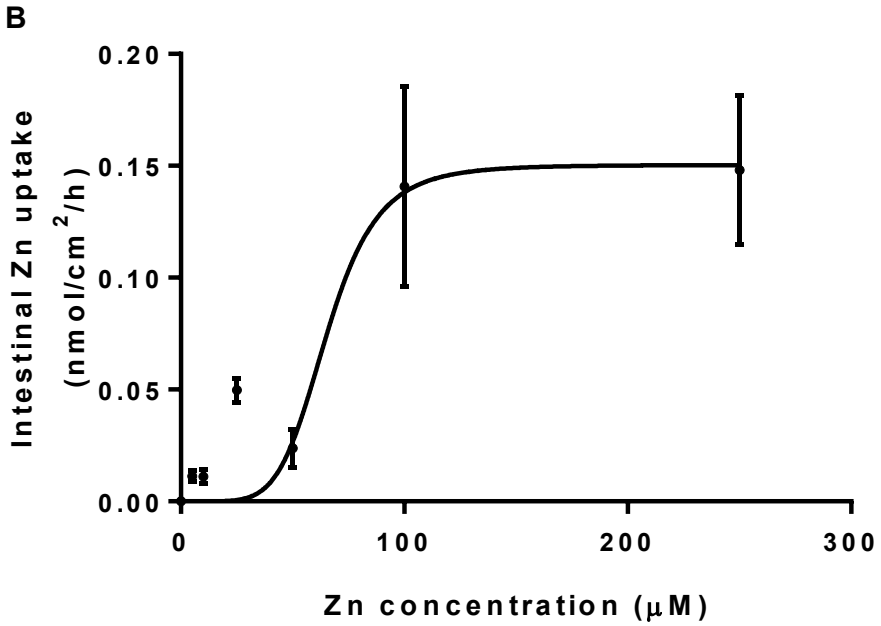
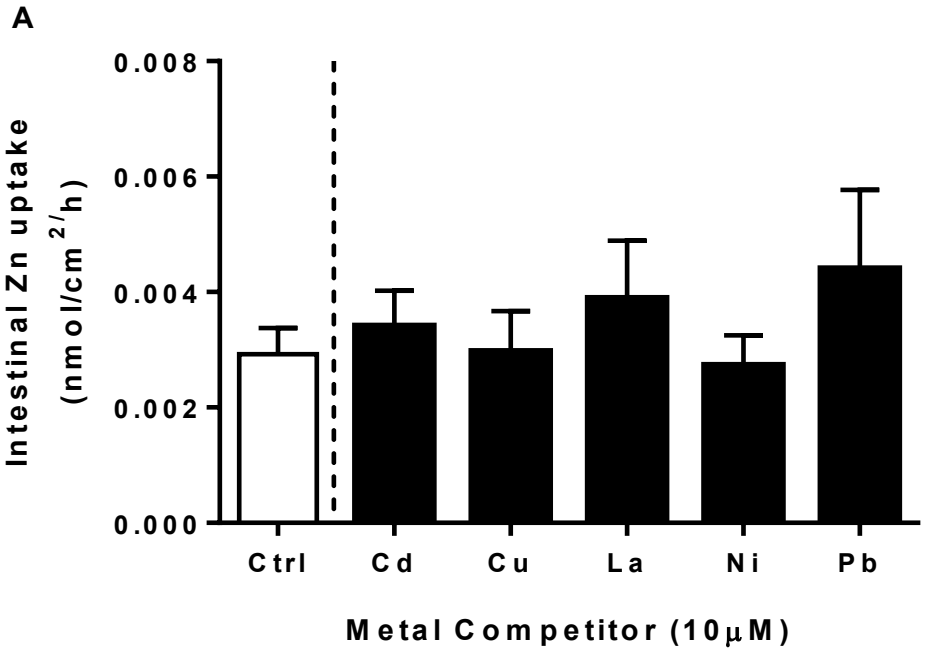

B
Intestinal Zn uptake
(nmol/cm2/h)
0.20
0.15
0.10
0.05
0.00
0
100
200
300
Zn concentration (μM)

Figure 5. Concentration-dependent Zn^{2+} uptake across the hindgut of the Pacific hagfish determined using *in vitro* gut sacs. A: Zn^{2+} uptake rates at concentrations ranging from 5 to 500 μM. B: Enlarged view of Zn^{2+} uptake rates at concentrations from 5 to 250 μM. At high [Zn] uptake was linear indicating diffusion, with a saturable sigmoidal component at low [Zn]. Values represent means ± SEM of 5 preparations. Kinetic analysis was conducted using GraphPad Prism 6.


A
0.008
0.006
0.004
0.002
0.000
Intestinal Zn uptake
(nmol/cm2/h)
Ctrl
Cd
Cu
La
Ni
Pb
Metal Competitor (10μM)

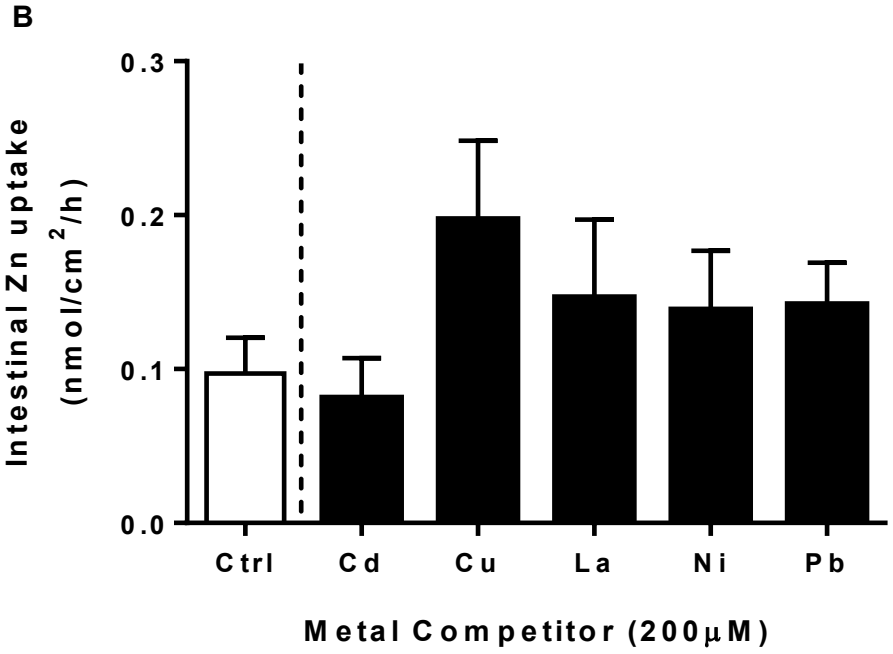

B
0.3
0.2
0.1
0.0
Intestinal Zn uptake
(nmol/cm2/h)
Ctrl
Cd
Cu
La
Ni
Pb
Metal Competitor (200μM)

Figure 6. The effect of A: 10 μM or B: 200 μM cadmium, copper, lanthanum, nickel, and lead on intestinal Zn^{2+} uptake rates using an *in vitro* gut sac technique. Bars represent means ± SEM of 5-8 preparations. No statistical significance was seen compared to the control (A: 2 μM Zn alone; B: 100 μM Zn alone) as determined using a one-way ANOVA analyzed in GraphPad Prism 6.

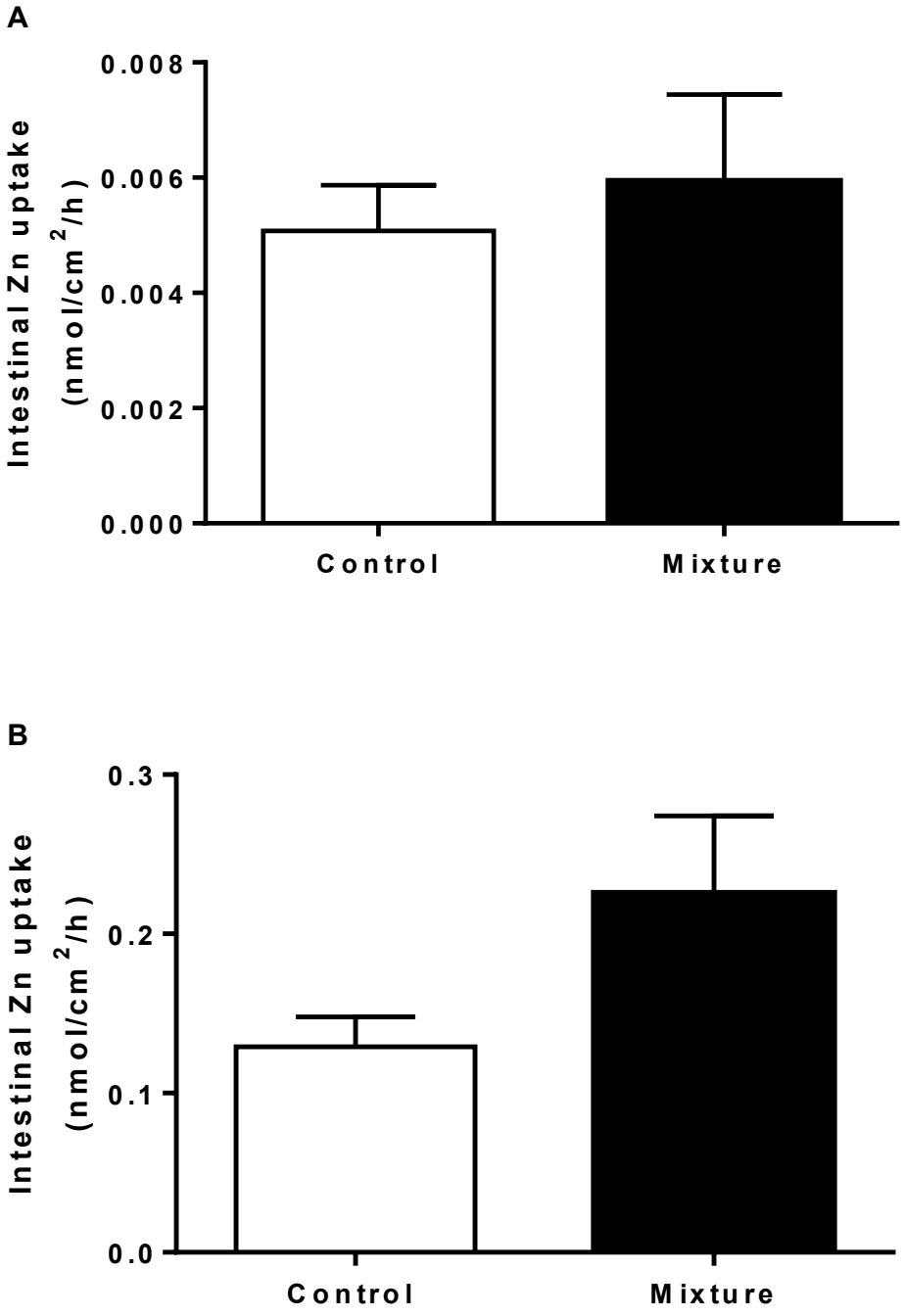
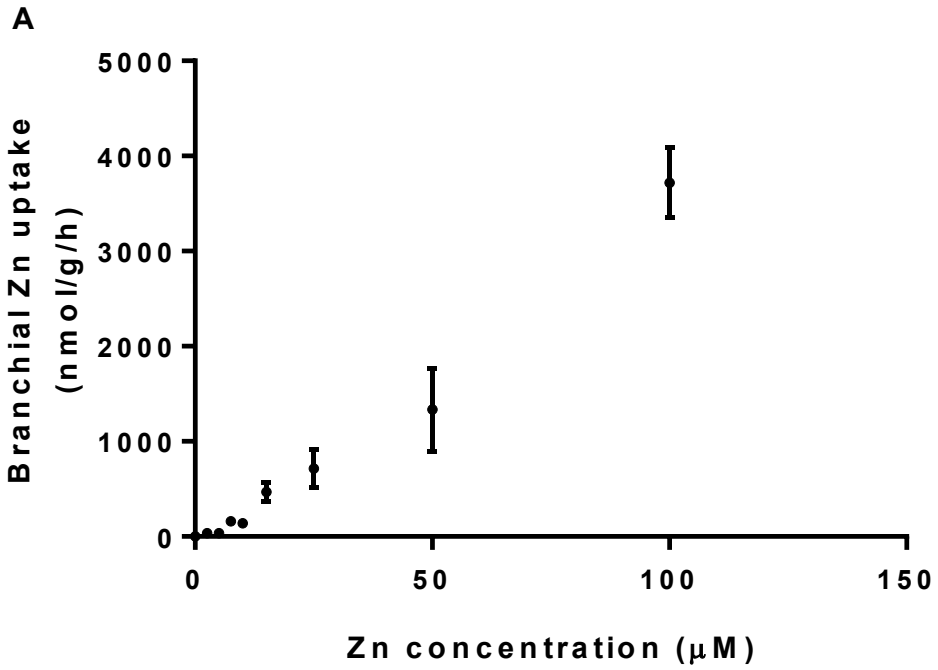

A
Intestinal Zn uptake
(nmol/cm2/h)
0.008
0.006
0.004
0.002
0.000
Control
Mixture
B
Intestinal Zn uptake
(nmol/cm2/h)
0.3
0.2
0.1
0.0
Control
Mixture

Figure 7. The effect of a copper, lanthanum, and nickel mixture on Zn^{2+} uptake across the hindgut using an *in vitro* gut sac technique. Either A: 10 µM or B: 200 µM of each metal was added to the mucosal Zn solution. Bars represent means ± SEM of 7-9 preparations. No statistical significance was seen compared to the control (A: 2 µM Zn alone; B: 100 µM Zn alone) as determined using an unpaired *t*-test (A) or a Mann-Whitney U test (B) in GraphPad Prism 6.


A
Branchial Zn uptake (nmol/g/h)
5000
4000
3000
2000
1000
0
0
50
100
150
Zn concentration (μM)

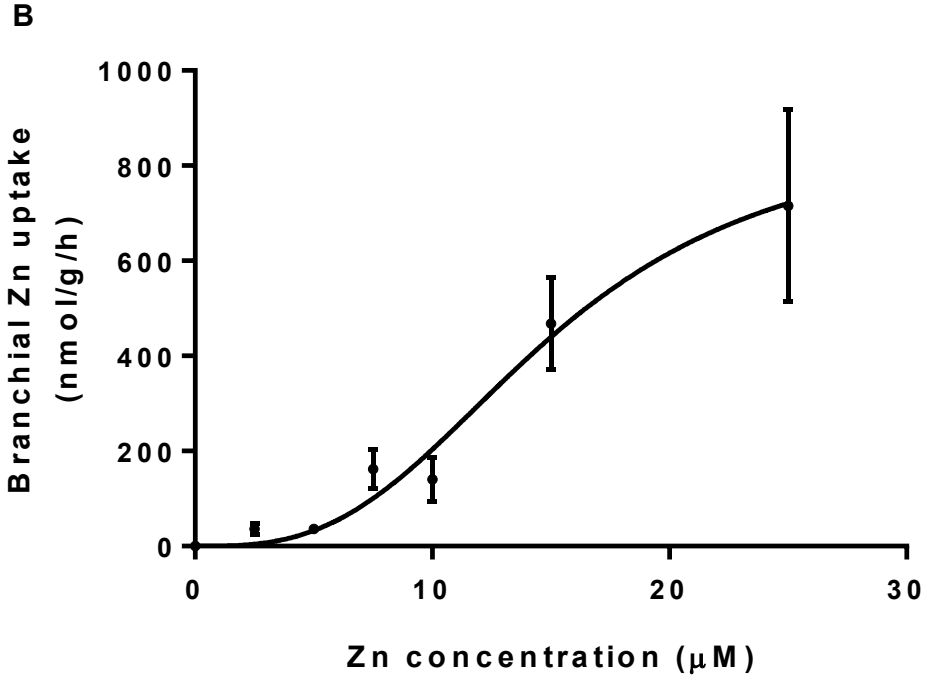

B
Branchial Zn uptake (nmol/g/h)
1000
800
600
400
200
0
0
10
20
30
Zn concentration (μM)

Figure 8. Concentration-dependent Zn^{2+} uptake in Pacific hagfish gills as measured by gill perifusion. A: Uptake rates at high Zn concentrations ranging from 2.5 to 100 μM. B: Enlarged view of uptake rates at Zn concentrations from 2.5 to 25 μM. Uptake of Zn at high concentrations was linear indicating diffusion, with a saturable sigmoidal component at low [Zn]. Values represent means ± SEM of 4-8 preparations. Kinetic analysis was conducted using GraphPad Prism 6.

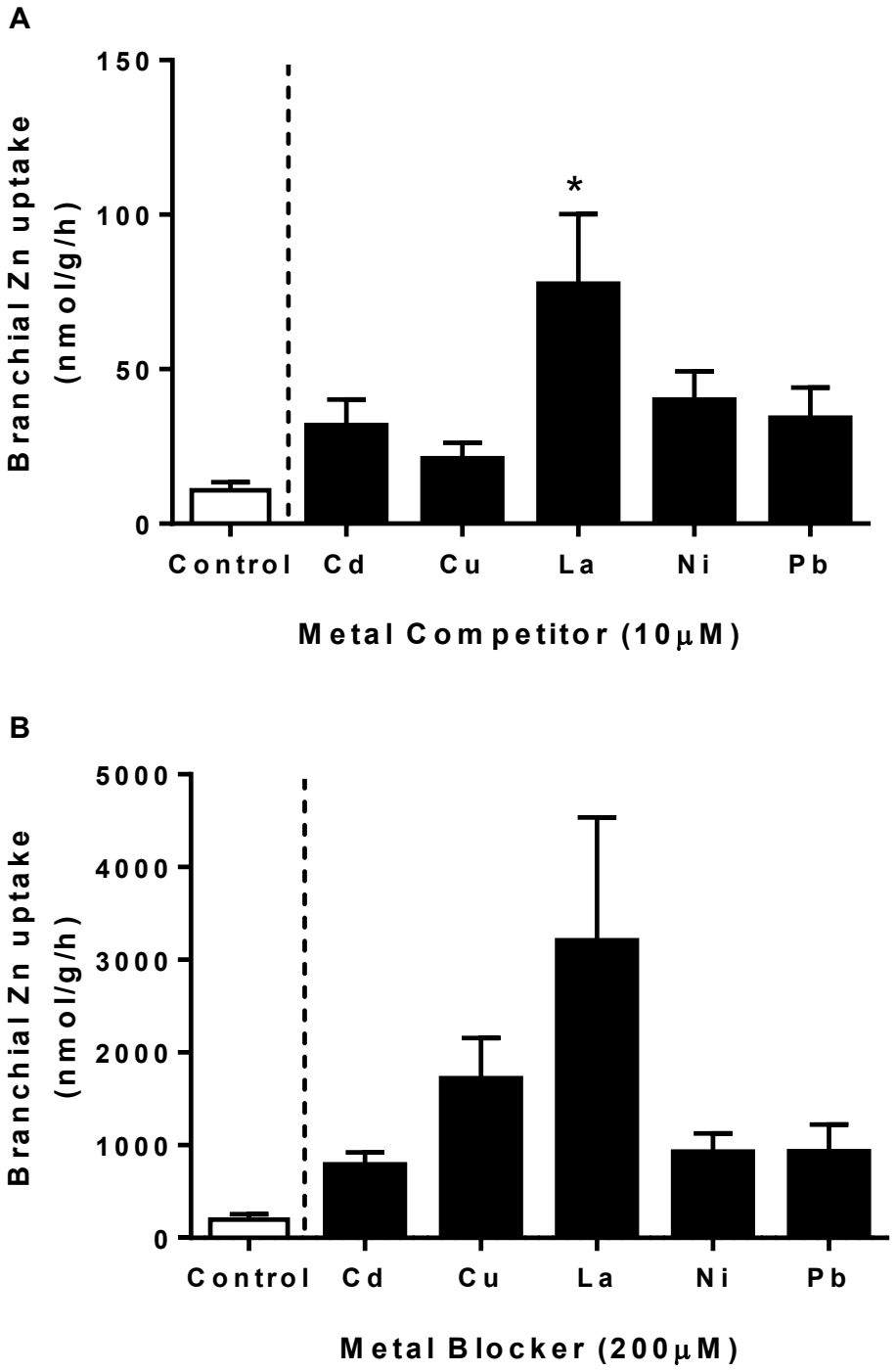
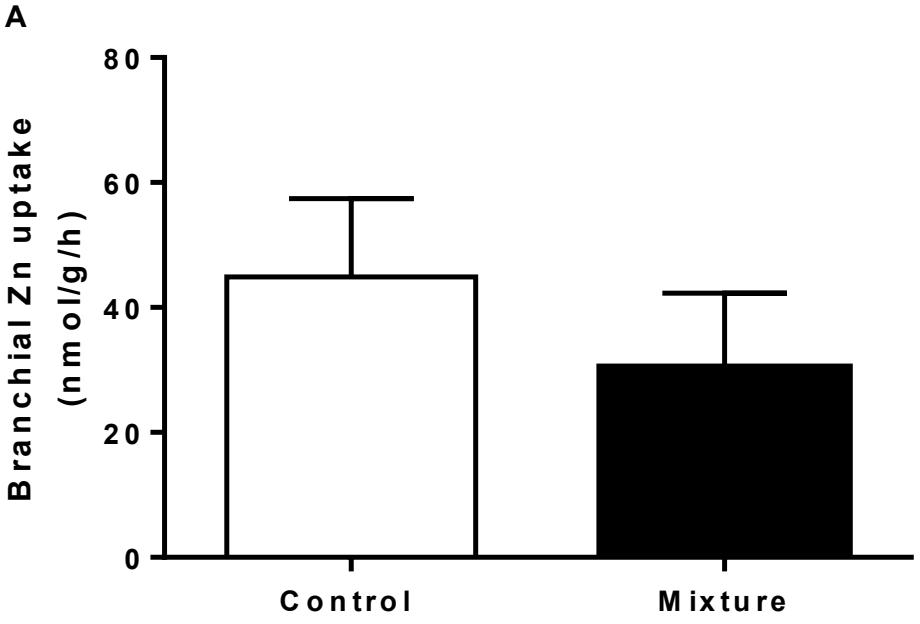

A
Branchial Zn uptake (nmol/g/h)
150
100
50
0
*
Control
Cd
Cu
La
Ni
Pb
Metal Competitor (10μM)
B
Branchial Zn uptake (nmol/g/h)
5000
4000
3000
2000
1000
0
Control
Cd
Cu
La
Ni
Pb
Metal Blocker (200μM)

Figure 9. The effect of A: 10 μM or B: 200 μM cadmium, copper, lanthanum, nickel, and lead on Zn^{2+} uptake across perifused hagfish gills. Bars represent means ± SEM of 5-6 preparations. Statistical significance compared to the control (2 μM Zn alone) was only seen in A (denoted by an asterisk, $p = 0.0039$) determined by a one-way ANOVA with a Dunnett's multiple comparisons test. No statistical significance was seen in B comparing to the control (40 μM Zn alone) using a Kruskal-Wallis test. Data was analyzed in GraphPad Prism 6.


A
Branchial Zn uptake (nmol/g/h)
80
60
40
20
0
Control
Mixture

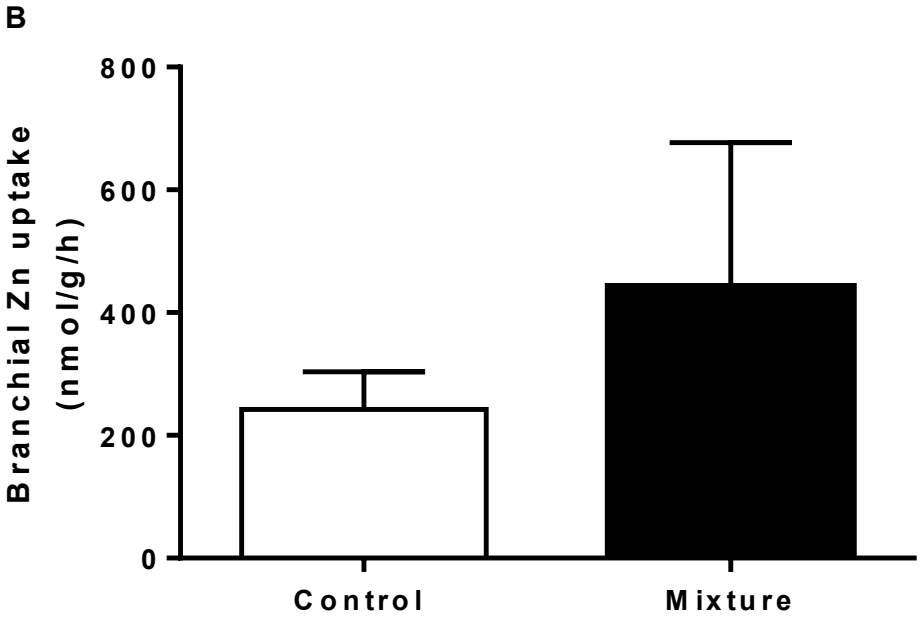

B
Branchial Zn uptake (nmol/g/h)
800
600
400
200
0
Control
Mixture

Figure 10. The effect of a copper, lanthanum, and nickel mixture on branchial Zn^{2+} uptake across the gills using an *in vitro* perifusion technique. Either A: 10 µM or B: 200 µM of each metal was added to the mucosal Zn solution. Bars represent means ± SEM of 3-5 preparations. No statistical significance was seen compared to the control (A: 2 µM Zn alone; B: 40 µM Zn alone) as determined using an unpaired *t*-test (A) or a Mann-Whitney U test (B) in GraphPad Prism 6.

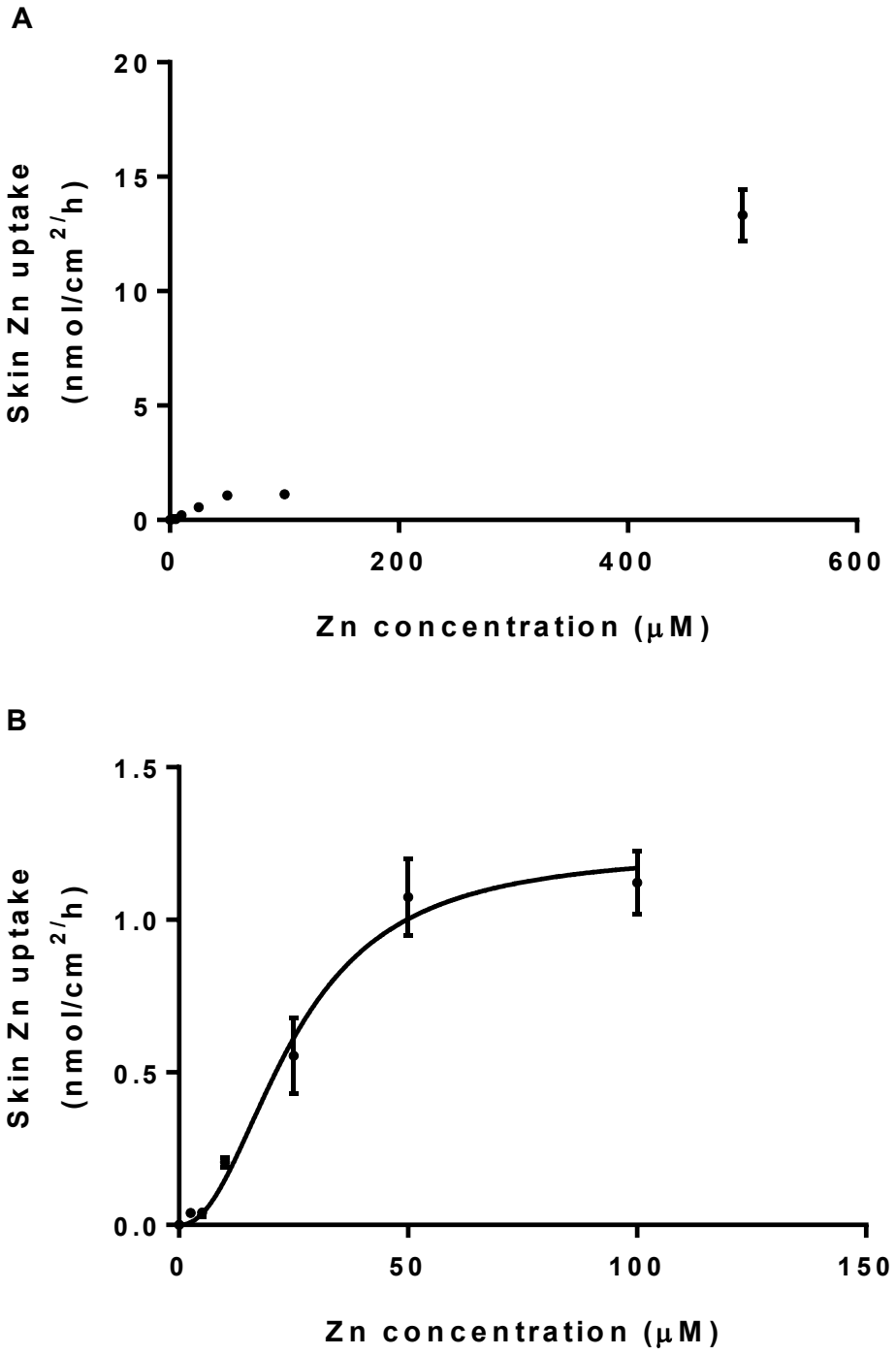
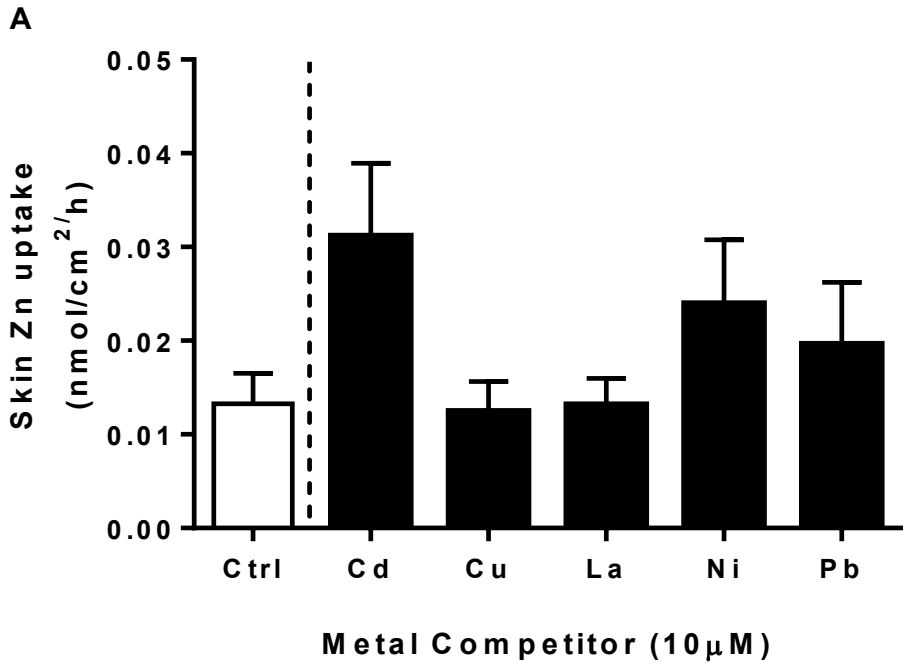

A
Skin Zn uptake
(nmol/cm2/h)
20
15
10
5
0
0
200
400
600
Zn concentration (μM)
B
Skin Zn uptake
(nmol/cm2/h)
1.5
1.0
0.5
0.0
0
50
100
150
Zn concentration (μM)

Figure 11. Concentration-dependent Zn^{2+} uptake across skin of the Pacific hagfish using a modified Ussing chamber. A: Zn^{2+} uptake rates at high concentrations ranging from 2.5 to 500 μM. B: Enlarged view of Zn^{2+} uptake rates at low concentrations from 2.5 to 100 μM. Linear uptake of Zn at high concentrations indicates diffusion, with a second saturable sigmoidal transport pathway at low [Zn]. Values represent means ± SEM of 4-5 preparations. Kinetic analysis was conducted using GraphPad Prism 6.


A
0.05
0.04
0.03
0.02
0.01
0.00
Skin Zn uptake
(nmol/cm2/h)
Ctrl
Cd
Cu
La
Ni
Pb
Metal Competitor (10μM)

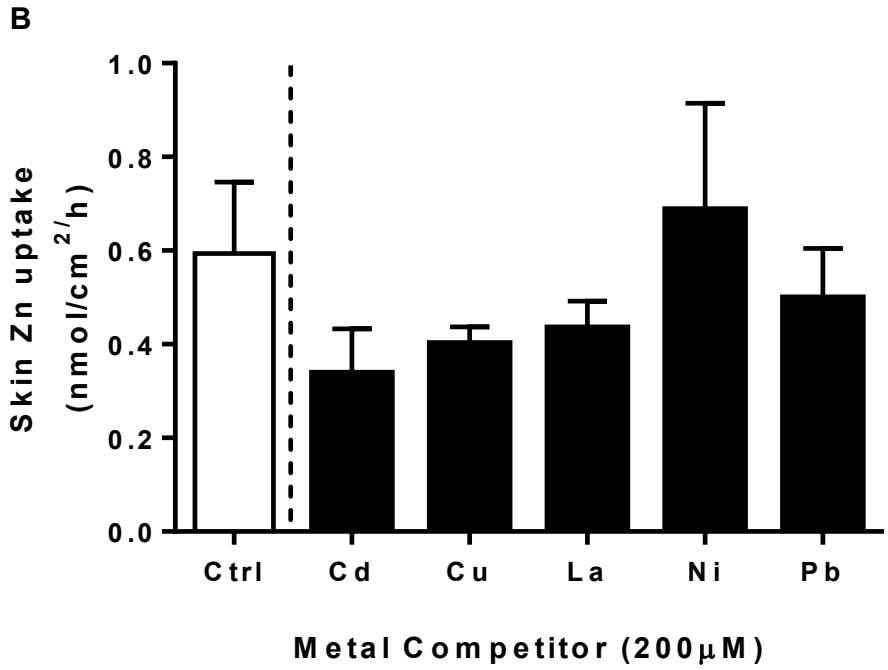
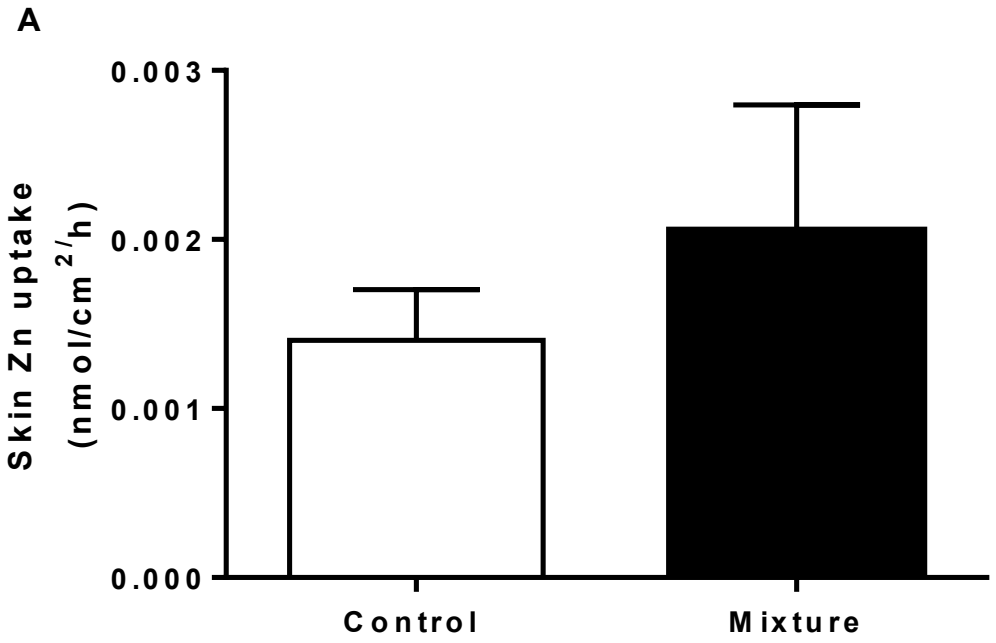

B
1.0
0.8
0.6
0.4
0.2
0.0
Skin Zn uptake
(nmol/cm2/h)
Ctrl
Cd
Cu
La
Ni
Pb
Metal Competitor (200μM)

Figure 12. The effect of A: 10 μM or B: 200 μM cadmium, copper, lanthanum, nickel, and lead on Zn^{2+} uptake rates across hagfish skin using a modified Ussing chamber. Bars represent means ± SEM of 4-7 preparations. No statistical significance was seen compared to the control (A: 2 μM Zn alone; B: 100 μM Zn alone) as determined using a one-way ANOVA analyzed in GraphPad Prism 6.


A
Skin Zn uptake
(nmol/cm2/h)
0.003
0.002
0.001
0.000
Control
Mixture

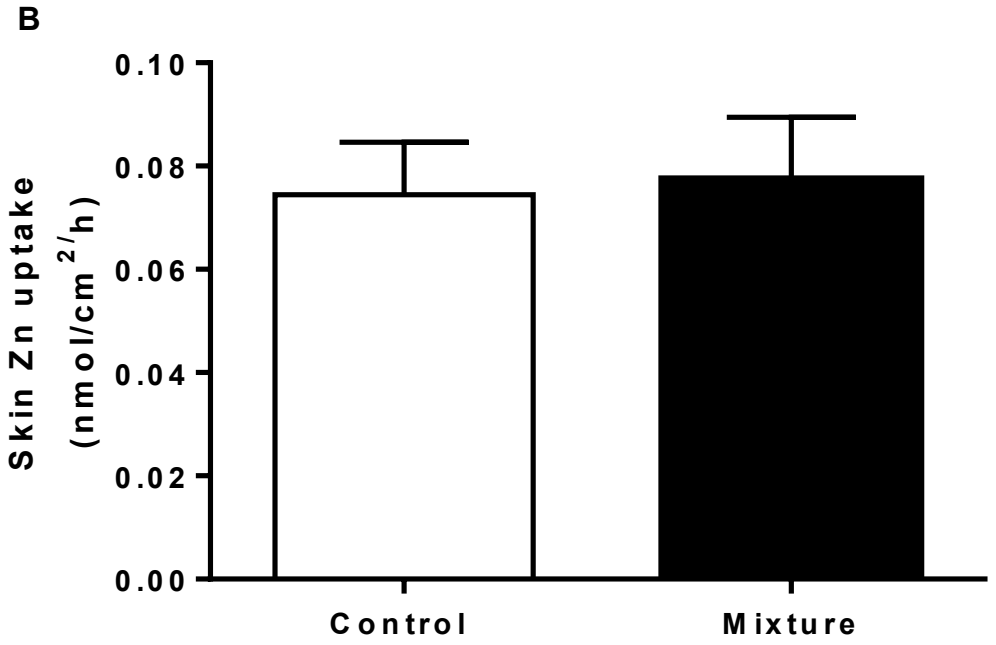

B
Skin Zn uptake
(nmol/cm2/h)
0.10
0.08
0.06
0.04
0.02
0.00
Control
Mixture

Figure 13. The effect of a copper, lanthanum, and nickel mixture on Zn^{2+} uptake rates across the skin using a modified *in vitro* Ussing chamber technique. Either A: 10 µM or B: 200 µM of each metal was added to the mucosal Zn solution. Bars represent means ± SEM of 5-6 preparations. No statistical significance was seen compared to the control (A: 2 µM Zn alone; B: 100 µM Zn alone) as determined using an unpaired *t*-test in GraphPad Prism 6.

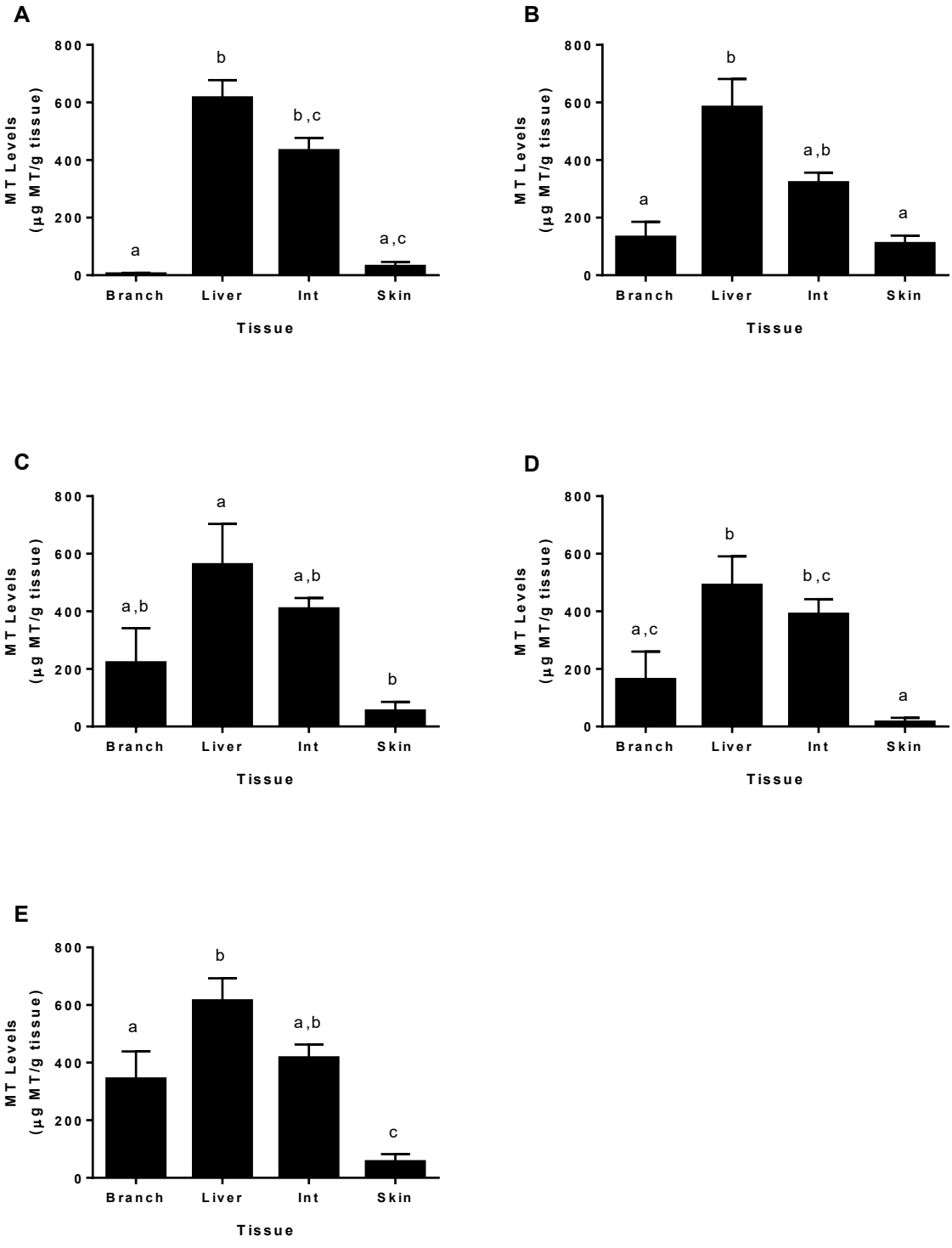

A
MT Levels (µg MT/g tissue)
800
600
400
200
0
a
b
b,c
a,c
Branch
Liver
Int
Skin
Tissue
B
a
b
a,b
a
C
a,b
a
a,b
b
D
a,c
b
b,c
a
E
a
b
a,b
c

Figure 14. Levels of MT in tissues of Pacific hagfish following exposure to 38 μM Zn for 0 (A), 1 (B), 3 (C), 5 (D), and 7 (E) days. Bars represent means ± SEM of 5-6 replicates. Bars sharing letters are not significantly different ($p < 0.05$), determined using a Kruskal-Wallis test with a Dunn's multiple comparisons test (A, B) or a one-way ANOVA with a Tukey multiple comparisons test (C, D, E). Data was analyzed using GraphPad Prism 6.

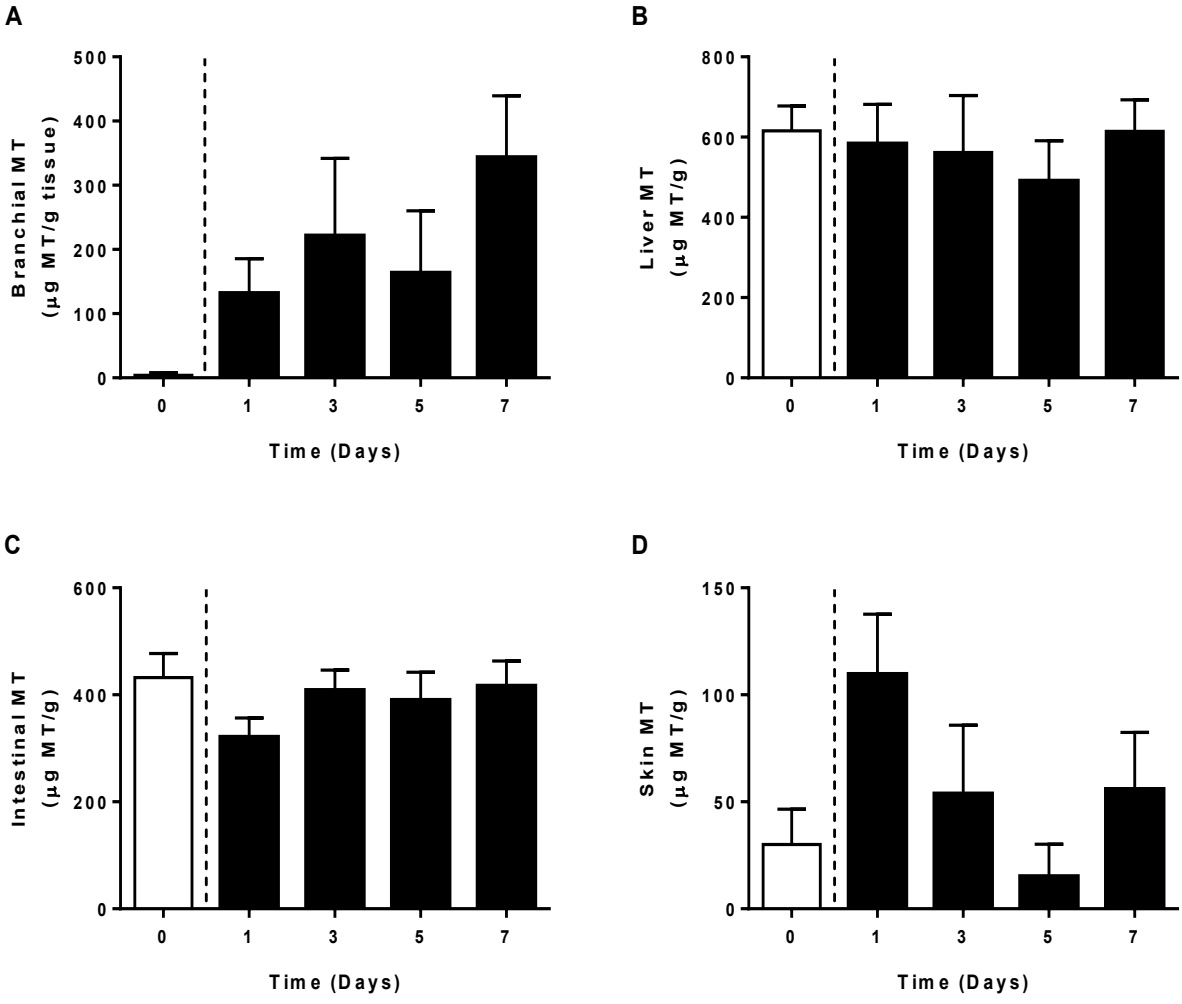

A
Branchial MT
(µg MT/g tissue)
500
400
300
200
100
0
0
1
3
5
7
Time (Days)
B
Liver MT
(µg MT/g)
800
600
400
200
0
0
1
3
5
7
Time (Days)
C
Intestinal MT
(µg MT/g)
600
400
200
0
0
1
3
5
7
Time (Days)
D
Skin MT
(µg MT/g)
150
100
50
0
0
1
3
5
7
Time (Days)

Figure 15. Levels of MT in the gill (A), liver (B), hindgut (C), and skin (D) of Pacific hagfish following exposure to 38 μM Zn for 1, 3, 5, and 7 days. Bars represent means ± SEM of 5-6 replicates. No statistical significance was seen compared the control (0 days) determined using a one-way ANOVA. Data was analyzed using GraphPad Prism 6.

Discussion

When hagfish were exposed to Zn in both a time- and concentration-dependent manner, accumulation was highest at a point of entry (gills), followed by the plasma and foregut. Zn^{2+} acquisition was biphasic for the hindgut, gill, and skin, demonstrating saturation of the transporter(s). Sigmoidal uptake kinetics for each epithelium indicates the potential for multiple transporters being present, but a lack of inhibition in Zn^{2+} uptake in the presence of putative competitors does not allow for differentiation of the types of transporter(s) present. While hagfish MT was found to be expressed at the highest levels in the visceral organs tested, I did not find induction of MT over the course of a 7-day Zn exposure suggesting either an insensitivity for induction by Zn or that our sampling regime did not capture any changes.

Whole-body Zn Accumulation

The general pattern of accumulation observed for the *in vivo* Zn exposures in the Pacific hagfish was as follows: Gill > Plasma ~ Foregut > Heart > Brain ~ Intestine, and in this next section I will focus primarily on the first three tissues and their role in Zn handling.

The elevated accumulation of Zn seen in the gills can be assumed as a reflection of the proximity of the gill to the concentrations of Zn in the water, rather than a storage site *per se* (Roméo *et al.,* 1999). The gill pouches of hagfish are in direct contact with the environment and exhibit similar characteristics of teleost fish gills (e. g. high surface area, thin diffusion distance; Weinrauch *et al.,* 2015), making them an ideal site for Zn accumulation. When the freshwater common minnow was exposed to Zn at a comparable concentration and time point (1.5 μM; 24 hours) to those used in my study (2 μM; 24 hours),

there was significant branchial Zn accumulation (Glynn, 1990). However, Zn accumulation in the minnow gill ranged from 740-2370 nmol/g whereas mean Zn accumulation in the Pacific hagfish gill in my study did not exceed 20 nmol/g. The substantial difference between gill accumulation of these two fish may be due to the sedentary lifestyle of hagfish, which allows them to have the lowest ventilation rate of any vertebrate (Forster, 1990; Weinrauch *et al.,* 2015). In this study, accumulation of Zn over time in the gill of the Pacific hagfish peaked at 24 hours with an approximately 20-fold increase compared accumulation at 6 and 48 hours. This may either be explained by the gills inducing a rapid removal of Zn^{2+} from the gills to the plasma and the rest of the organism to counteract the import and potential toxicity, or by reducing apical uptake mechanism. Future research should investigate the relative involvement of these two possible scenarios. In freshwater minnows, approximately 70% of accumulated Zn was lost from the gills by 60 hours (Glynn, 1990) supporting my suggestion of increased Zn handling capacity.

The levels of Zn in hagfish blood plasma seen in this study were the second highest site of accumulation when exposed to the higher concentrations of Zn. The majority of Zn within blood plasma is bound to albumin, as well as α_2-macroglobulins and amino acids (Hogstrand, 2011). Due to the elevated accumulations of Zn seen in gill tissue compared to that of the plasma circulating through it, it is possible a positive concentration gradient may be formed allowing for loss from the gills to the plasma. Previously, Zn^{2+} transport into the plasma for both teleosts and invertebrates was thought to be passive (Bryan, 1971; Pentreath, 1973) but more recent research has identified a basolateral Zn transporter that provides a regulated export pathway (Feeney *et al.,* 2005). Congruent with my own results, this may

also be why plasma Zn accumulation was not as substantial at the low Zn concentration tested.

The results of this study also demonstrated that Zn noticeably accumulated within the foregut of the Pacific hagfish, despite not having an apparent discernable pattern. Hagfish are osmoconformers, and therefore do not need to drink seawater. However, when Glover *et al*. (2017) measured drinking rate in hagfish, approximately 33% of hagfish tested consumed minimal amounts of seawater. Therefore, sporadic accumulation of Zn within the hagfish foregut is not a surprising observation. Moreover, given that intestinal mucous cells are restricted to the foregut compared to the hindgut (Weinrauch *et al.,* 2015), it is possible that the Zn accumulation in the foregut may simply be adsorption by the mucus in this region, despite washing in displacement solution.

Intestinal Zn^{2+} Uptake

Zn^{2+} uptake in hagfish hindgut was found to consist of a saturable, sigmoidal component at low Zn, environmentally relevant concentrations (≤ 250 μM), and a linear component at higher concentrations indicate a diffusive mechanism. Kinetic analysis of the sigmoidal curve yielded a maximal rate of Zn^{2+} uptake (J_{max}) of 0.15 ± 0.02 nmol/cm^2/h, and an affinity constant of 65.5 ± 14.7 μM. Saturable Zn^{2+} intestinal uptake was demonstrated in gulf toadfish, a marine teleost (Glover *et al.,* 2003) while similar saturable Zn^{2+} uptake was reported in the intestine of rainbow trout. However, rainbow trout were shown to have a lower affinity (309 μM) but higher capacity (933 nmol/kg/h) for Zn^{2+} (Glover and Hogstrand, 2002) compared to that seen in this study. The apparent higher affinity within the hindgut of the Pacific hagfish may be of particular importance owing to their scavenging lifestyle. Hagfish can experience extended periods of fasting, up to at least 11 months (Foster and

Moon, 1986). When encountering episodic carrion or other sources of food, hagfish would ideally acquire the maximum allowable amount of dietary Zn when it is available. Whales and other marine mammals tend to accumulate large amounts of Zn within their tissues (Thompson, 1992; Varanasi *et al.,* 1994; Méndez *et al.,* 2002) and under these conditions, having a low capacity pathway may protect hagfish against potential Zn toxicity. Furthermore, the euryhaline black sea bream intestine played a larger role for Zn^{2+} uptake in marine environments compared to freshwater (Zhang and Wang, 2007). When examining Zn^{2+} uptake in mammals, a similar range of affinities in the intestine of both rats and humans has been demonstrated (24 μM-226 μM), which includes the affinity constant for hagfish intestinal Zn^{2+} uptake presented in this study (Steel and Cousins, 1985; Hoadley *et al.,* 1987; Oestreicher and Cousins, 1989; Fleet *et al*., 1993).

The apparent conservation of Zn^{2+} uptake across species is likely due to the actions of transport proteins compatible with Zn (Glover and Hogstrand, 2002). The sigmoidal kinetics seen in the hindgut suggests that either multiple transport pathways may be present or that cooperative binding may be occurring (Schultz *et al.*, 2014). In order to elucidate which protein transporters may be present and used for Zn^{2+} uptake in the hindgut of hagfish, a variety of metal competitors (Cd^{2+}, Cu^{2+}, La^{3+}, Ni^{2+}, or Pb^{2+}) were introduced within the gut sacs. My results demonstrating lack of competitive inhibition are contradictory to what was documented in winter flounder, a marine teleost. In flounder, the uptake of Zn^{2+} was significantly depressed by each of Cd^{2+}, Cu^{2+}, and Ni^{2+}, as well as other divalent metals not examined in our study (Shears and Fletcher, 1983). However, in flounder, Ca^{2+}, which can serve as a potential competitive inhibitor, did not inhibit Zn^{2+} uptake. This can perhaps explain my lack of significant inhibition of Zn^{2+} transport using La^{3+} as a potential

competitive Zn^{2+} inhibitor since La^{3+} is similarly considered a Ca^{2+} channel blocker (Perry and Flik, 1988). Moreover, the lack of competition with Zn^{2+} in the presence of Ca^{2+} or a Ca^{2+} channel blocker is consistent with the general absence of ECaC expression within the intestine of fish (Qiu and Hogstrand, 2004).

Branchial Zn^{2+} Uptake

Using *in vitro* gill perifusions, Zn^{2+} uptake across the gills of the Pacific hagfish increased linearly with increasing concentrations of Zn > 25 μM. This is indicative of diffusive uptake mechanisms dominating Zn^{2+} transport capacity at these higher Zn concentrations. Kinetic analysis yielded a maximal rate of Zn^{2+} uptake (J_{max}) of 880.7 ± 355.4 nmol/g/h, and an affinity constant of 15 ± 5.7 μM. For comparison, branchial Zn^{2+} acquisition in rainbow trout was determined to have an affinity range of 3.6 μM- 7.9 μM, and an accompanying J_{max} of 240-410 nmol/kg/h (Spry and Wood, 1989; Hogstrand *et al*. 1998). Hagfish having a lower affinity for Zn in their gills compared to rainbow trout is congruent with what was seen in the black sea bream. Zhang and Wang (2007) found that the gills were sensitive in response to salinity, playing a larger role in Zn^{2+} uptake at lower salinities. Additionally, when hagfish are burrowing in sediment or feeding, they may be exposed to elevated concentrations of Zn not seen in freshwater species, therefore not requiring as high an affinity for Zn.

Similar to the hindgut, putative metal competitors of possible cellular Zn transporters were used to differentiate transporter properties. In my study, I found that at low Zn concentrations (2 μM), the addition of 10 μM La significantly increased branchial Zn^{2+} uptake while at high concentrations of both Zn and La, this did not occur. Unlike my results, there have been numerous studies demonstrating that Ca inhibits Zn^{2+} uptake indicating Zn

may use La-sensitive Ca^{2+} channels (Spry and Wood, 1989; Hogstrand *et al.,* 1996). However, the majority of studies looking at Zn^{2+} uptake at the gill have been performed in rainbow trout. For saltwater fish, only a few studies have examined Zn^{2+} uptake directly. Zhang and Wang (2007) demonstrated that black sea bream acclimated to freshwater had significantly reduced Zn^{2+} uptake in the presence of La^{3+} but did not occur in fish acclimated to saltwater water. It was suggested that apical Ca^{2+} channels are not active at the gill in seawater environments, and that any Zn^{2+} uptake occurred paracellularly (Zhang and Wang; 2007). The increase in Zn^{2+} uptake seen in the hagfish may be due to the fact that hagfish are osmoconformers and while they do regulate plasma Ca^{2+} (5.1 mM) the levels in plasma are approximately half of that of seawater (8.4 mM; Currie and Edwards, 2010) meaning there is likely the absence of substantial active Ca^{2+} uptake at the gill. Alternatively, if Ca^{2+} channels are present, they may not be functional, meaning Zn^{2+} must be moving through either other transcellular pathways or paracellularly. Similar to my results showing an increase in Zn^{2+} uptake in the presence of La^{3+}, the presence of La concentrations as low as 1 μM stimulated both Co^{2+} and Pb^{2+} influxes in freshwater teleost fishes (Comhaire *et al.,* 1998; Rogers and Wood, 2004). Rogers and Wood (2004) suggested that stimulation in uptake may be due to a disruption of the apical membrane integrity, increasing the diffusive component of uptake.

Skin Zn^{2+} Uptake

Using modified Ussing-style chambers, *in vitro* uptake of Zn^{2+} across the skin was observed in a saturable fashion at concentrations ≤ 100 μM. With an apparent K_m of 25.1 ± 3.9 μM and J_{max} of 1.2 ± 0.12 nmol/cm^2/h, it can be inferred that the skin more closely

resembled the uptake kinetics of the gill compared to the hindgut. Thus, hagfish appear to have the potential to take up significant amounts of Zn^{2+} at a high affinity across multiple extraintestinal epithelia. As mentioned previously, this may be of great importance when taking advantage of sporadic dietary sources of Zn^{2+}, such as a carrion fall, or when encountering long periods of fasting where the benthic sediment may provide a source of Zn. This has been suggested for hagfish Ni^{2+} uptake (Glover *et al.,* 2015) when hagfish skin has contact with sediment acting as an additional source of essential metals, further explaining why the affinity for Zn^{2+} in hagfish skin was slightly lower than that of the gill.

Uptake across the skin of the Pacific hagfish has also been observed for Ni^{2+} and Fe^{2+} (Glover *et al*., 2015; Glover *et al.,* 2016). Similar to what was seen for Zn in this study, uptake of these metals was found to have both saturable and linear components, indicating active transport at lower metal concentrations and more passive mechanisms at higher concentrations. The affinity constants for Ni^{2+} and Fe^{2+} were calculated to be 38 μM and 9.4 μM, respectively. The relative similarities between the K_m for Ni^{2+}, Zn^{2+}, and Fe^{2+} indicate that these trace metals may be using a similar transport pathway to enter the skin, potentially by the action of DMT-1. However, in this study, potential metal competition with Ni^{2+} in 2X and 5X excess of Zn did not hinder the rate of Zn^{2+} acquisition.

Metallothionein Induction

In general, the liver of fish is the organ most studied concerning metal detoxification and the induction/presence of MT but other tissues also express MT for metal handling (Hogstrand and Haux, 1991). It is thought the binding of excess metals present in the cytosol by MT proteins can act as a detoxifying agent to maintain homeostasis (Hogstrand and Haux,

1991; Khang and Wang, 2005). Previous exposure to metals in both freshwater and marine fish species have shown that MT induction is tissue-specific, with the highest levels most often found in the viscera (particularly the liver and kidneys; Hogstrand and Haux, 1996; Hollis *et al.,* 2001; Long and Wang; 2005). This trend was observed for Pacific hagfish exposed to Zn, with the liver demonstrating the highest levels of MT followed closely by the hindgut.

In vivo exposure to a measured Zn concentration of 38 µM did not induce any significant induction of MT in any organ observed for the Pacific hagfish after 7 days. This is in contrast to what was seen previously in two species of marine teleosts (Zhang and Wang, 2005). When exposed to Zn concentrations as low as 2.6 µM for 7 days, the body of the black sea bream, and the viscera and carcass of the crescent grunter, had significantly higher MT concentrations than those of control. After a 3-week exposure, a similar trend was seen by Zhang and Wang (2005) at Zn concentrations as low as < 0.25 µM. Moreover, plaice injected intraperitoneally with Zn demonstrated a 15-fold increase in hepatic MT (20 µg MT/g to 300 µg MT/g), which remained elevated for the month following the injection (Overnell *et al.,* 1987). However, similar to this study using the Pacific hagfish, the gills of the crescent grunter did not see any induction of MT after 7 days.

An explanation for the lack of MT induction seen in the Pacific hagfish may be a product of their lifestyle. As hagfish may be often exposed to high concentrations of Zn *via* the water or through feeding habits, maintaining a naturally high level of MT could act as a protective measure against excess free Zn^{2+} in the cell. Therefore, it is possible a much higher concentration of Zn is required to observe an induction, or that the times where MT was measured in my study did not capture an induction. Conversely, while MT is a main Zn^{2+}

binding ligand, in rainbow trout that only 30% of cytosolic Zn^{2+} was bound to MT (Hogstrand *et al.,* 1991), thus other metal-binding ligands (e. g. glutathione; Hogstrand, 2011) might be playing a larger role than MT in metal homeostasis.

Chapter 3 – General Conclusions

Summary

Zinc is a crucial micronutrient required for overall organismal success. However, it must be tightly regulated within the body to mitigate the negative consequences of deficiency and toxicity. The overarching goal of this thesis was to examine the acquisition and cellular handling of Zn^{2+} using the Pacific hagfish (*Eptatretus stoutii*) as a study organism. Within this thesis, I have determined for the Pacific hagfish the accumulation points of waterborne Zn, established uptake kinetics of Zn^{2+} across three epithelia, and investigated possible cellular mechanisms for Zn^{2+} transport and storage.

The first part of my study consisted of *in vivo* whole animal exposures at three timepoints (6, 24, and 48 hours), at both low (2 μM) and high (100 μM) Zn concentrations. As predicted, I observed preferential Zn accumulation within the gills for all treatments. Hagfish gill pouches are directly in contact with the marine environment. This coupled with a large surface area and a small diffusion distance makes them an ideal site for Zn accumulation. Blood plasma was also observed to accumulate significant levels of Zn at higher Zn concentrations. As gills are highly vascularized, it is possible that a positive concentration gradient was formed, transporting Zn^{2+} from the gills into the blood. Unlike what I had hypothesized, the foregut of the Pacific hagfish also had elevated levels of Zn accumulated within the tissue. While hagfish are osmoconformers it is likely that the hagfish drank small amounts of seawater, allowing for Zn adsorption to the mucous cells present in this region.

The second part of my study was two-fold. First, I was able to demonstrate *in vitro* that the Pacific hagfish is capable of acquiring Zn^{2+} across their hindgut, gills, and skin. Uptake kinetics were biphasic for all epithelia and conformed to a sigmoidal distribution at

lower Zn concentrations, with diffusive mechanisms dominating higher Zn concentrations. This indicated that saturable Zn^{2+} uptake was occurring, presumably involving multiple different transporters.

In order to elucidate which transporters are potentially involved with Zn^{2+} uptake in the hagfish, putative metal competitors (Cd^{2+}, Cu^{2+}, La^{3+}, Ni^{2+}, or Pb^{2+}) were introduced in excess of Zn^{2+}. Within the hindgut, I demonstrated a lack of competitive inhibition for all metals and metal mixtures tested, which is contradictory to what has been observed in other fish. Within the gills, I was able to elicit a significant uptake of Zn^{2+} in the presence of La^{3+}, a Ca^{2+} channel blocker. While hagfish are able to regulate the levels of Ca^{2+} in their plasma, it is only approximately half of that of seawater indicating there is most likely an absence of Ca^{2+} channels at the gill. Furthermore, apical disruption may be occurring, causing an increase of the diffusive mechanisms present. Similar to the hindgut, Zn^{2+} uptake within the skin of the Pacific hagfish was not inhibited by the metal competitors being present. However, the apparent affinity constant for skin Zn^{2+} uptake closely resembled those of both Ni^{2+} and Fe^{2+}, indicating that these trace metals may all share a similar transport pathway for entry into the skin, perhaps using DMT-1.

The last part of my thesis consisted of exposing hagfish *in vivo* to 38 μM Zn for a period of 7 days in an attempt to induce the production of metallothionein proteins. While I was not able to capture an induction of MT in the hagfish, I was able to quantify the levels of MT within hagfish tissues, with the highest concentration occurring in the liver. As hagfish may often be exposed to elevated concentrations of Zn in their environment, maintaining a natural level of MT higher than other marine fishes may aid in mitigating the potential risk of excess intracellular Zn^{2+}.

Future Directions

My thesis establishes the location of Zn accumulation and distribution in the Pacific hagfish, as well as the mechanisms of Zn^{2+} uptake across the hindgut, gill, and skin. However, these acquisition experiments were completed strictly using starved hagfish. While these studies may be indicative of when hagfish are encountering long periods of fasting, it does not allow me to infer the effects that may arise after feeding. In marine teleosts, Zn accumulation occurs at a faster rate when fish are fed compared to those acquiring Zn^{2+} only from the water (Willis and Sunda, 1984). Therefore, it would be of value to complete the experiments presented in this thesis on hagfish that have been provided access to food compared to those that have been fasted.

Zn^{2+} acquisition across the hindgut, gill, and skin was examined in the presence of putative metal competitors, selected for their potential ability to use or block Zn^{2+} transport proteins (ZIPs, ZnTs, DMT-1, Ca^{2+} channels). The general lack of effect of Zn^{2+} uptake in the Pacific hagfish indicated that more than one of these transporters might be used to acquire Zn^{2+} from the environment. Therefore, the use of molecular techniques may further provide me with information on which Zn^{2+} transport proteins are present in the hagfish. Thus far, searching the hagfish gill and slime gland transcriptomes available in the Goss lab provided sequences for DMT-1, which I subsequently verified with PCR.

Finally, the hagfish metallothionein study did not effectively capture an induction of MT over the course of 7 days. While an MT induction was determined in marine teleosts exposed to concentrations of Zn at least 13-fold lower than used in my study (Zhang and Wang, 2005), the authors used an exposure period of 7 and 21 days. Therefore, it is possible the timescale used for my experiment was not of adequate length. Furthermore, I may not

have used a concentration of Zn high enough to produce an induction of MT. With this knowledge, I would further investigate the levels of MT in hagfish exposed *in vivo* to Zn concentrations higher than those used in this study (38 μM) for an extended period of exposure (14, 21, and 30 days).

The work presented in this thesis has contributed to the overall understanding of the acquisition and handling of essential trace metals in a unique research organism, the hagfish. Studying physiology in early diverging fishes provides increased knowledge of the evolution of nutrient uptake, as well as a better understanding on how fish may need to differ to succeed in adverse environmental conditions.

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Appendix

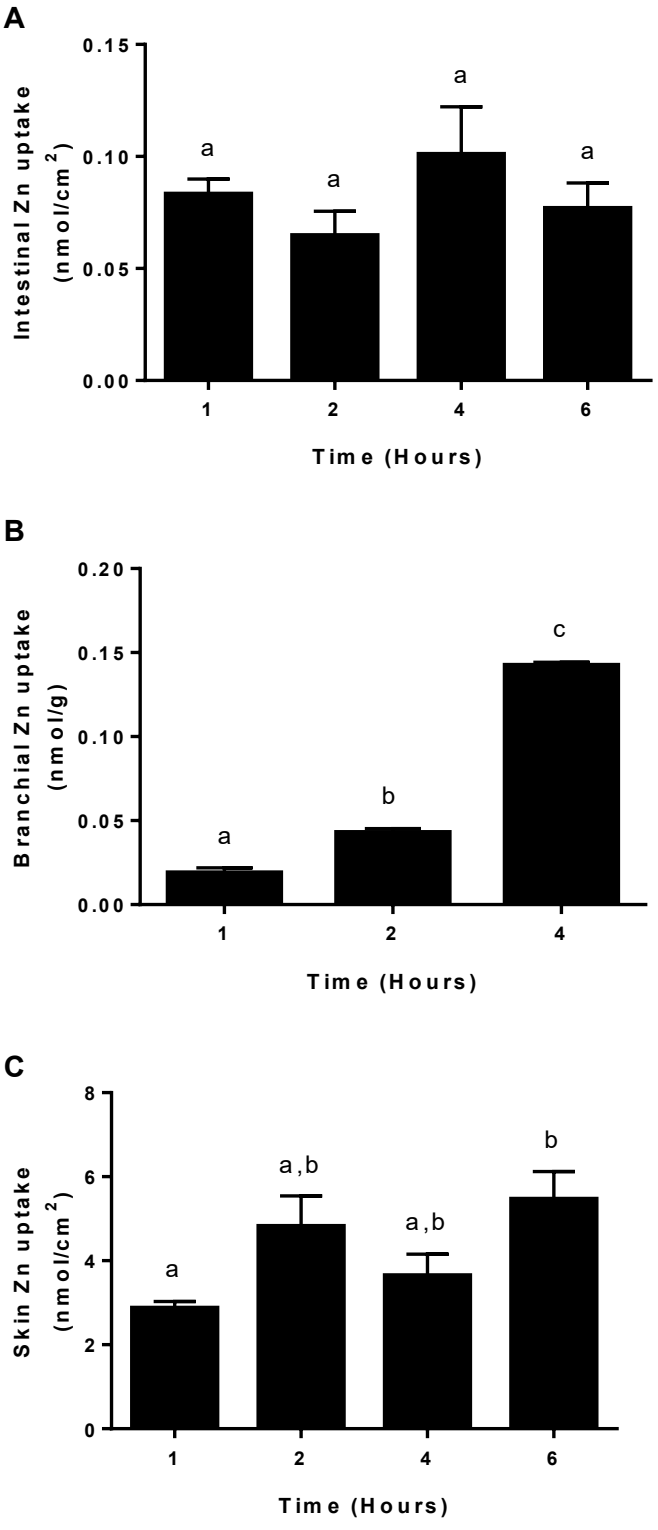
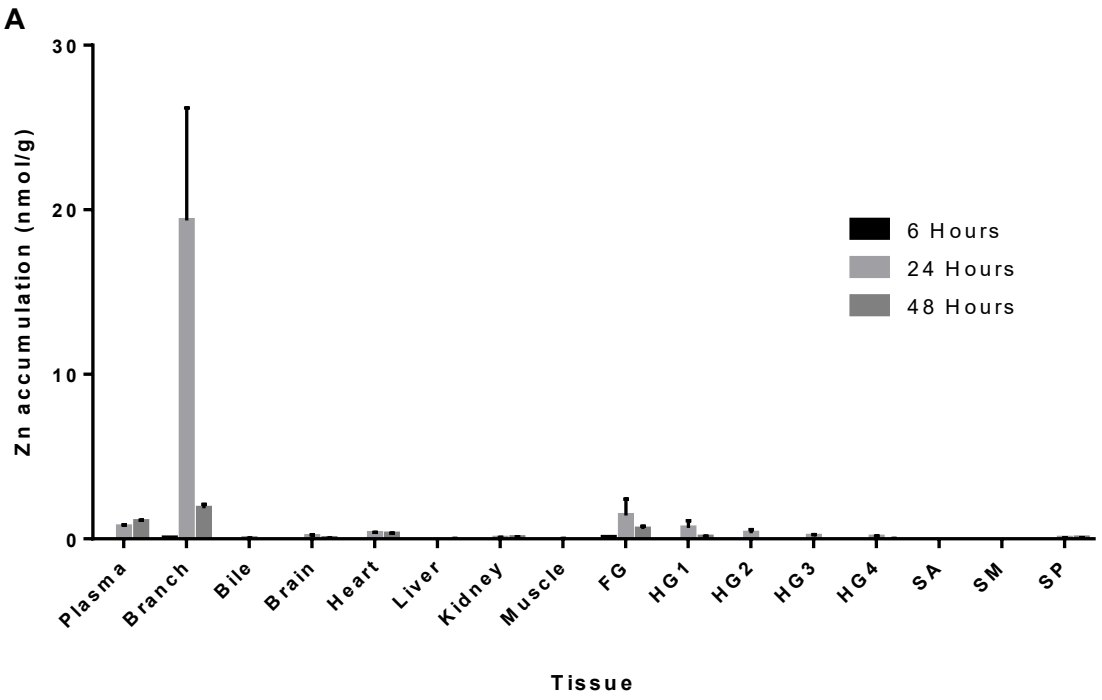

A
Intestinal Zn uptake (nmol/cm2)
0.15
0.10
0.05
0.00
a
a
a
a
1
2
4
6
Time (Hours)
B
Branchial Zn uptake (nmol/g)
0.20
0.15
0.10
0.05
0.00
a
b
c
1
2
4
Time (Hours)
C
Skin Zn uptake (nmol/cm2)
8
6
4
2
0
a
a,b
a,b
b
1
2
4
6
Time (Hours)

Figure 16 (Appendix 1). *In vitro* time course experiments for the Pacific hagfish in the hindgut (A), gill (B), and skin (C). Bars represent means ± SEM of 3-4 preparations. Bars sharing letters are not significantly different ($p < 0.05$), determined using a one-way ANOVA with a Tukey multiple comparisons test. Data was analyzed using GraphPad Prism 6.


A
30
20
10
0
Zn accumulation (nmol/g)
6 Hours
24 Hours
48 Hours
Plasma
Branch
Bile
Brain
Heart
Liver
Kidney
Muscle
FG
HG1
HG2
HG3
HG4
SA
SM
SP
Tissue

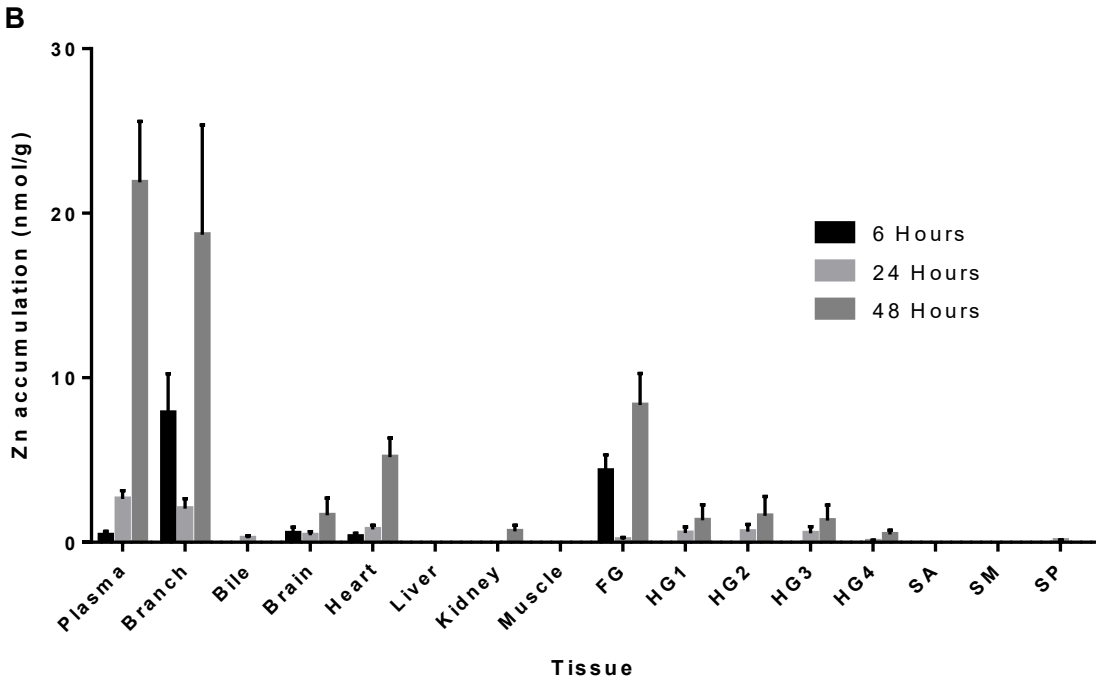

B
30
20
10
0
Zn accumulation (nmol/g)
6 Hours
24 Hours
48 Hours
Plasma
Branch
Bile
Brain
Heart
Liver
Kidney
Muscle
FG
HG1
HG2
HG3
HG4
SA
SM
SP
Tissue

Figure 17 (Appendix 2). Accumulation of Zn in tissues of the Pacific hagfish following exposure to 2 μM (A) and 100 μM (B) for 6 h, 24 h, or 48 h. Bars represent means ± SEM of 4-5 replicates. Data was analyzed using GraphPad Prism 6.