Biochemical and Physiological Effects of Organic and Inorganic Zinc in a Commercial Strain of Diploid and Triploid Rainbow Trout (*Oncorhynchus mykiss*)

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Kristen A. Meiler

Major Professor: Vikas Kumar, Ph.D.
Committee Members: Madison Powell, Ph.D.; Kenneth Cain, Ph.D.
Department Administrator: Robert J. Collier, Ph.D.

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Authorization to Submit Thesis

This thesis of Kristen A. Meiler, submitted for the degree of Master of Science with a Major in Animal Science and titled "Biochemical and Physiological Effects of Organic and Inorganic Zinc in a Commercial Strain of Diploid and Triploid Rainbow Trout (*Oncorhynchus mykiss*)," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Vikas Kumar, Ph.D.

Committee Members: _____ Date: _____
Madison Powell, Ph.D.

_____ Date: _____
Kenneth Cain, Ph.D.

Department Administrator: _____ Date: _____
Robert J. Collier, Ph.D.

Abstract

Zinc is an essential micro-mineral for fin fish and is required for a wide range of physiological functions. Bioavailability of minerals in diets depends on quality and source. Finding chelates that are more bioavailable to fish can help decrease production costs and metal waste pollution. In aquaculture, triploid fish can be utilized for their sterility to decrease risk of genetic pollution and for their potential for faster growth. However, zinc requirements may differ between ploidies due to physiologic differences and to our knowledge there is no information regarding zinc requirements for triploid Rainbow Trout. These experiments aim to determine the effects of supplemental zinc in a commercial strain of genetically similar diploid and triploid Rainbow Trout (*Oncorhynchus mykiss,* average initial weight: 53.07 ± 4.35g) using various levels (63, 93, 123, 153, 183 mg kg^{-1} Zn) of both organic and inorganic zinc. A total of 11 diets were formulated to be isonitrogenous (42% crude protein) and isolipidic (21% lipid) and were fed in triplicate for 64 days. Results showed that neither ploidy (diploid vs. triploid) nor type of mineral (organic vs. inorganic) significantly influenced the growth performance of fish, distal intestine histology, or cataract formation/lens histology. However, there were significant interactions in nutrient retentions and vertebral zinc content for both ploidies. Additionally, there were significant differences in oxidative stress related gene expression patterns in hepatocytes, supporting evidence for a mechanism of zinc's role in the antioxidant defense system of Rainbow Trout. Gene expression was generally higher in response to organic zinc in triploids than in diploids. These findings also establish a relationship between dietary zinc and dose on the retention of zinc and other macronutrients, such as lipid and protein, and the oxidative stress response in diploid and triploid Rainbow Trout. Increased supplementation of organic zinc may aid diploid and triploid Rainbow Trout in utilization of macronutrients in feed and increased capacity to combat oxidative stress.

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Chapter 1. Review of Literature

1.1 Introduction

Global aquaculture production has increased exponentially and now provides more than half of the fish and shellfish consumed by humans (FAO, 2018). In 2016, global fish supply, including that from capture fisheries and aquaculture, peaked at 171 million tonnes, with aquaculture contributing 47% of total production (53% if non-food uses were excluded) (FAO, 2018). In 2015, fish accounted for 17% of total global animal protein consumption. Fish production is predicted to continue to expand and is estimated to reach 201 million tonnes by 2030, with aquaculture predicted to reach 109 million tonnes in that same period (37% growth) (FAO, 2018). In this rapidly growing field, there has been increasing interest in various aspects of fish nutrition to ensure production of fast growing, healthy fish, which provide human consumers with sustainable and nutritionally superior food.

There are nearly 600 species of organisms utilized in aquaculture to date, with finfish species accounting for approximately 60% of annual production (FAO, 2018). The production of Rainbow Trout (*Oncorhynchus mykiss*), a commercially important, fast growing cold-water species, accounts for approximately 2% of the total farmed fish and ranks as the 17th most cultured finfish species (FAO, 2018). Rainbow Trout are native to many Pacific draining rivers and lakes in North America. Currently, more than half of U.S. states farm Rainbow Trout, however approximately 50% of the Rainbow Trout sold for food is still imported (USDA, 2018). As such, there is potential to increase domestic production to meet the current demand. The U.S. produces over 20,000 metric tons of Rainbow Trout annually and is a prominent exporter and importer of the species worldwide. In 2017, Rainbow Trout sales in the U.S. reached 109 million dollars. Approximately 46% of this

production occurred in Idaho, and the vast majority of overall production originated in the Pacific Northwest (USDA, 2018). This expansion is largely attributable to an increase in production and distribution channels, in tandem with a decrease of waste in the production pipeline (FAO, 2018).

One of the main concerns aquaculturists face is a reliance on fishmeal (FM) and fish oil (FO). These two ingredients are typically required for meeting the dietary needs of carnivorous species. Small, pelagic species such as mackerel, herring, sardines, and anchovies are primarily used to source FM and FO. These dietary ingredients provide essential amino acids, fatty acids, and micronutrients in most aquaculture diets, particularly those which are fed to carnivorous species. The use of FM and FO from capture fisheries has risen significantly in recent years, with an increase of approximately 19% from 2016 (FAO, 2018). However, the availability and sustainability of these sources has decreased steadily, thus creating an urgent need for sustainable and affordable alternatives. Subsequently, there is increasing pressure on fish nutritionists to explore the use of alternative ingredients, while ensuring diets still provide adequate nutrition to cultured fish in order to maintain maximum health benefits and rapid growth. However, as sustainability and inclusion of alternative protein sources increases, so does the need to reassess nutritional feed profiles.

Alternative sources of proteins such as plant meals most often have a less desirable balance of amino and fatty acids and may also include antinutritional factors (ANFs) that can make minerals unavailable to fish. This has been reviewed by a number of studies which include minerals that support and promote growth (Hamre et al., 2020; Silva et al., 2019; Welker et al., 2015). As such, there has been interest in utilizing organic mineral chelates in order to increase the bioavailability of these minerals (Apines et al., 2003; Ashmead, 1991;

Glover and Hogstrand, 2002; Paripatananont and Lovell, 1995). This increased bioavailability can subsequently affect macronutrient metabolism, zinc status of the fish, growth, and ability to combat oxidative stress (Apines et al., 2003; Glover and Hogstrand, 2002; Lall, 2002; Mertens et al., 2015; Prabhu et al., 2014).

1.2 Zinc

Zinc is a trace mineral that plays a complex role in interconnecting organisms to their environments. Elemental zinc is one of fifteen trace elements essential for the maintenance of life (Lall, 2002). It is present in all organs, tissues, and fluids with structural, catalytic, and regulatory functions (Eide, 2006; Maret and Krezel, 2007). Zinc plays an important role in cellular metabolism, formation of skeletal structures, maintenance of colloidal systems, acid-base regulation, immunity enhancement, stress reduction, enzyme cofactors, and in the formation of nucleoproteins (Lall, 2002; Watanabe et al., 1997). Additionally, zinc is beneficial for the metabolism of Vitamin A and has been shown to reduce susceptibility to certain diseases, such as bacterial kidney disease (Blazer, 1992; Lall et al., 1985).

Zinc is also an integral part of some 20 different metalloenzymes and acts as a catalyst for regulating other zinc dependent enzymes (Lall, 2002; Watanabe et al., 1997). As an example, zinc plays a key role in the enzymatic function of carbonic anhydrase (CO_2 formation), alcohol dehydrogenase (alcohol metabolism), carboxypeptidase (protein digestion), alkaline phosphatase (hydrolysis of phosphate esters), polymerase (synthesis of RNA/DNA chains), and collagenase (wound healing) (Lall, 2002). Zinc also plays a pivotal role in many metabolic pathways by helping promote and regulate carbohydrate, lipid, and protein metabolism (Glover and Hogstrand, 2002; Kucukbay et al., 2006; Watanabe et al.,

1997). Therefore, zinc type (organic or inorganic) and level is likely to influence the balance of anabolism and catabolism. Furthermore, zinc may influence bone mineralization either as a divalent cation, which acts on mineral accumulation, or as a cofactor involved in relevant enzymatic processes (Apines et al., 2003). For example, in humans, bone formation is related to zinc's role in bone alkaline phosphatase, osteoblast tyrosine kinase, and RNA synthetase, and perhaps even enhances bone growth in collaboration with the IGF-1 endocrine axis (Apines et al., 2003). With such a wide range of biological functions, zinc requirements depend on many different factors such as fish species, size, life stage, and reproductive status (Lall, 2002).

Essential elements, such as zinc, are those consistently present in all living tissue within a zoological group and will produce deficiency symptoms when the element is missing. The growth and health responses of an animal to supplementation of a limiting essential nutrient can be affected by changes in both feed intake and metabolic efficiency (Cowey, 1992). Signs of deficiency will occur if the essential element is removed, and will disappear when physiological levels of the element are restored (Forstner and Wittmann, 1983). Unlike terrestrial animals, fish have the unique advantage of being able to obtain zinc not only from dietary pathways, but also at the gills via uptake from their external aquatic environment (Glover and Hogstrand, 2002).

Divalent ions, such as zinc, have a unique ability to form a variety of complexes, allowing them to interact with many different cellular components and feed ingredients. However, this flexibility provides an opportunity for competition with other molecules as effectors in biological processes (Bury et al., 2003). Zinc and other trace metals can become toxic to organisms when their nutritional supply becomes extreme (Forstner and Wittmann,

1983). In fish, metal accumulation is largely dependent on dose and duration of supply, water temperature, water chemistry and hardness, interactions with other metals, as well as fish age and physiologic status (Kargin and Cogun, 2014). Zinc homeostasis is regulated by excretory functions in congruence with gastrointestinal and gill uptake, with an optimum level that is species and situation specific (Watanabe et al., 1997). In Rainbow Trout, the optimal level of dietary zinc depends on feed ingredients but is generally accepted to be around 30 mg kg^{-1} when fed purified diets (NRC, 2011).

If supplemented dietary and waterborne zinc are not adequate, deficiency can be indicated by retarded growth, short-body dwarfism, cataracts, fin and skin erosion, reduced body and bone zinc, and mortality (Lall, 2002). In one example of zinc deficient Rainbow Trout fry, negative interactions of zinc and iron were suggested, as a severe reduction in the zinc content of the vertebrae was found in association with an increase in iron content of various tissues (Ogino and Yang, 1978). Deficiency can also activate the hypothalamus-pituitary-interrenal (HPI) axis causing chronic production of glucocorticoids, primarily cortisol, resulting in alterations to metabolism and potentially cell death (Fraker et al., 2000). Additionally, zinc deficiency can result in decreased digestibility of other nutrients such as proteins and carbohydrates, possibly due to a decrease in the activity of carboxypeptidase (Watanabe et al., 1997). Another common sign of long-term zinc deficiency is the development of cataracts as observed in a number of studies involving either zinc deficient diets, or an increased presence of ANFs which can negatively influence zinc metabolism (Ketola, 1979; Lall, 2002).

1.3 Fish adaptations

There is a close relationship between how and what fish eat that allows them to be more successful in the utilization of different food types. Fish species have evolved anatomical and physiological features such as unique mouths, buccal cavities, pharynx, and digestive tract structures, as well as varied chemical and enzymatic methods based on their life history which often dictate food selection and obtainment (De Silva and Anderson, 1995; Karachle and Stergiou, 2012). For example, herbivorous fish that consume macrophytes have structural adaptations that allow them to ingest and masticate plant material in a way that makes it more suitable for their particular digestive processes (De Silva and Anderson, 1995; Karachle and Stergiou, 2012). Additionally, fish such as tilapia possess bi- and tri-cuspid teeth that help to cut and grind plant tissues before reaching the intestine (De Silva and Anderson, 1995). Herbivorous fish also have longer intestines than carnivorous fish that promote an increased opportunity for digestion to help break down plant material in association with their microbiome (Karachle and Stergiou, 2012). This consideration is especially important to nutritionists and we must study bioavailability of nutrients in alternative plant proteins and how these may impact various fish species. Furthermore, there are correlations that have been observed between nutritional composition of food and subsequent effects on body composition of the fish (Jobling, 2001; Lie et al., 1988) which may impact consumer satisfaction.

Metal bioavailability and toxicity is largely contingent on differences in fish physiology and is highly variable among the nearly 30,600 species of fish that have been described so far. Stark differences occur between freshwater and marine species, especially as marine fish have vastly different osmoregulatory functions that likely affect nutrient and

metal uptake (Clearwater et al., 2002). However, these requirements are more seriously considered in aquaculture and fisheries species with significant human importance. Accumulations of zinc are observed at the highest levels in the digestive tract followed by the gills, kidney, skeletal tissue, liver, and spleen (Clearwater et al., 2002). There are a number of different reliable ways to determine zinc retention in teleost fish. Zinc can be retained for a long period in otoliths, lenses, and caudal fins and these can be used as biomarkers of environmental pollution (Dove and Kingsford, 1998). Alternatively, vertebral zinc content can also act as a good indicator, as reported by Satoh et al. (1989), who observed that vertebral zinc content was significantly reduced as ingredients antagonistic of zinc were added.

Sugiura et al. (2000) claim that increased amounts of other minerals, especially other divalent cations that might occupy similar uptake pathways, can decrease the bioavailability of zinc. Watanabe et al. (1997) state that phosphorous likely inhibits zinc availability, and a 1:1 ratio of phosphorous and calcium is observed to work best in some diets to decrease the negative effects on zinc metabolism. Also, Clearwater et al. (2002) state that excessive dietary zinc could compete with the absorption of iron, calcium, and copper. However, Kargin and Cogun (2014) assert that uptake of dietary zinc can be increased if it is paired with other metals, such as cadmium. These are of important consideration especially to wild species because environmental presence of metals rarely occurs independently. Further research is required in order to more confidently assess the interactions of zinc with other elements.

1.4 Gastrointestinal zinc uptake

The fish gut appears to be the primary pathway for zinc absorption, while the gills supplement acquisition when dietary zinc is scant (Bury et al., 2003; Spry et al., 2011). Diet-borne metals refer specifically to gastrointestinal metal exposure that is separate from water-to-gill exposure (Clearwater et al., 2002). Digestion of food occurs mainly in the acidic stomach (if present), and absorption occurs in the more alkaline environment of the intestine (Clearwater et al., 2002). In the digestive tract, mineral absorption in fish is thought to be similar to that in mammals, where the anterior intestine is the most important region through proteins similar to those found in mammals (comparable to the jejunum region in humans) (Bury et al., 2003; Clearwater et al., 2002). Data suggest there might be several different, parallel uptake pathways for Zn^{2+} including one facilitated by amino acids and another for inorganic Zn^{2+} (Glover and Hogstrand, 2002).

While gastrointestinal mineral absorption has not yet been fully delineated, information from other organismal systems suggests proteins from the ZIP family (derivative of Zrt, Irt-like proteins) are homologous to those observed in teleost species (Bury et al., 2003). Other molecules that may play a role in zinc uptake in the intestine include metallothionein, glutathione, and Zinc transporter-1 (or a homolog to ZNT1 found in mammals) (Bury et al., 2003). While it is important to understand these processes, much of the current literature focuses on feed efficiency rather than on specific mechanisms. Future studies that are able to contribute to our understanding of these mechanisms might prove beneficial to natural resource scientists and production specialists alike. Once better understood, nutritionists will be able to more effectively formulate specific diets with the unique goals of the project in mind, while simultaneously decreasing waste. Knowledge of

critical differences in fish metabolism will in turn help advance feed efficiency and development of increasingly necessary, sustainable dietary formulations. Finding the optimum levels of essential nutrients that allow maximum health benefits at a minimum cost is vital. Insufficient or excess amounts of these nutrients can be expensive and detrimental to the animal (Ashmead, 1991).

1.5 Gill zinc uptake

The gills both take up environmental zinc and excrete excess zinc when nutritional levels become too high (Bury et al., 2003). In a hypothetical representation presented by Bury et al. (2003), zinc enters the cell via the mucus layer either alone through a putative lanthanum-sensitive epithelial channel (ECaC) or bound to an aquatic ligand. Next, zinc transverses a zinc-regulated transporter (ZTL) or Zrt, Irt-like protein (ZIP). Excess cytoplasmic zinc is bound to metallothionein (MT), and finally is transferred basolaterally via zinc-transporter 1 (Znt-1) (Bury et al., 2003).

Absorption of zinc at the gills is thought to occur through a series of channels and bound transporters that are especially important when dietary sources are limited (Bury et al., 2003; Clearwater et al., 2002; Hogstrand et al., 1996). This is largely governed by water hardness as calcium can act as a competitive inhibitor to zinc (Bury et al., 2003; Lall, 2002). Hogstrand et al. (1996) suggested that the transcellular carrier-mediated branchial influx of zinc exhibits Michaelis-Menten characteristics that link zinc with calcium transport. The capacity of water hardness and high calcium levels to inhibit environmental zinc uptake indicate that the apical entry and pathway for these two elements is at least partially the same (Hogstrand et al., 1996). However, if water-borne zinc concentrations are high and

dietary concentrations are low, more than 50% of the necessary requirements can be fulfilled through gill uptake (Clearwater et al., 2002; Prabhu et al., 2014).

1.6 Zinc Toxicity

It is relevant to note that more often than not the chance of zinc toxicity is low for Rainbow Trout, specifically. Studies have shown that Rainbow Trout can tolerate extremely high levels of water-borne zinc (up to 1,700-1,900 mg kg^{-1}) and whole-body zinc without apparent signs of toxicity (Lall, 2002; Spry et al., 2011). However, other species are more susceptible and apt to be affected from higher levels of soluble zinc in the environment. Zinc can be absorbed or ingested by animals through a variety of different mechanisms, with great differences occurring based on life history and the animal's environment. Effects of metal pollution in the environment can be compounded in response to organic matter and other nutrients which promote biological growth and ultimately oxygen depletion (Abel, 2002; Forstner and Wittmann, 1983). Due to this, decreasing the amount of waste caused by overloading essential metals in fish feeds in order to combat ANFs should be an important consideration for aquaculture facilities and feed manufacturers for reasons pertaining to both cost and pollution. Glover and Hogstrand (2002) artfully state that the key goal is to maintain adequate uptake of essential nutrients while simultaneously regulating the accumulation of potential heavy metal toxicants.

1.7 Oxidative stress

Many animals undergo oxidative stress, which can result from an excess production of reactive oxygen species (ROS). This may occur in tandem with a reduction in an organism's capacity to mitigate deleterious effects of ROS with their own endogenous antioxidant defenses (Sevcikova et al., 2010). Free radicals, or ROS, are always present and

are produced as intermediary metabolites or byproducts of aerobic cellular metabolism within the electron transport chain. In fact, the main endogenous source of ROS is through mitochondrial respiration within the cells. Some ROS can be beneficial to the organism and can help with processes such as defense against pathogens (Evans and Halliwell, 2001). However, many of these effects can be harmful, as free radicals possess a very reactive unpaired electron. Free radicals commonly found in the cells include the superoxide anion, the hydroxyl radical, and hydrogen peroxide. As a result, all aerobic organisms possess antioxidant defense mechanisms to protect their cells and tissues from damage, including antioxidant enzymes and adaptive genetic responses (Tovar-Ramírez et al., 2010). Further, the antioxidant ability of a fish is dependent upon the dietary antioxidant levels, which can significantly affect recommended inclusion levels (Prabhu et al., 2014). It has recently been shown that nutritional supplementation can be an effective way to increase an organism's antioxidant capacity (Wu et al., 2017). Zinc, in particular, has been shown to be an antioxidant mineral through both its direct interaction with various enzymes involved in antioxidant pathways (i.e. CuZn superoxide dismutase) and through its indirect role in gene regulation (Banni et al., 2011; Kokou et al., 2020). Furthermore, decreased cellular zinc concentrations have been observed in cases regarding excess oxidative damage (Mertens et al., 2015).

Oxidative damage from ROS can lead to the presence of cataracts, protein and lipid peroxidation, DNA oxidation, and altered gene expression (Wu et al., 2017). Oxidative stress is the consequence of an imbalance between the production of ROS and the ability of the organism to combat them (Sevcikova et al., 2010). In the early 1970's, there was a pervasive incidence of cataracts that occurred in Rainbow Trout fed practical diets. This

eventually led to an increase in research on the issue and the realization that zinc was more unavailable in white fish meal due to the high levels of hydroxyapatite, a large inorganic mineral composed of calcium and phosphate ($Ca_5(PO_4)_3$) found in bone (Lall, 2002; Watanabe et al., 1997). Cataracts are often categorized by the formation and presence of bladder cells (atypical lens fibers) and morgagnian globules that undergo granulation and liquefication (Cogan, 1962). The liquefied portion leaks out of the lens and causes an immune response while the retained nuclei of the cells precipitate and fall to the bottom of the capsular envelope (Cogan, 1962; Spector, 1995). This causes the formation of a typical cataract resulting in opacity of the retinal lens. One study observed elevated levels of ROS associated with the presence of cataracts in a significant amount of cases (Spector, 1995). The most effective way to combat these cataract-causing ROS is through enzymatic activity (Spector, 1995). Free radicals can also induce lipid peroxidation by degrading the polyunsaturated fatty acids in cell membranes as a part of a self-propagating chain reaction (Lall and Lewis-McCrea, 2007; Mylonas and Kouretas, 1999). Because of this self-propagating nature, the oxidation of a very small number of cells or lipid molecules can initiate wide-spread tissue damage resulting in a decrease in viability (Mylonas and Kouretas, 1999).

Antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase which can scavenge ROS to protect the animal. This suite of enzymes typically represents the first line of defense against cellular oxidation. Superoxide dismutase (SOD) is a metalloenzyme that catalyzes the dismutation (or simultaneous oxidation and reduction) of the superoxide anion to molecular oxygen and hydrogen peroxide. Catalase (CAT) can then reduce hydrogen peroxide molecules to water and

dioxygen. Glutathione peroxidase (GPX) can also reduce or decompose hydrogen peroxide using the glutathione peptide as a co-substrate (Tovar-Ramírez et al., 2010). Glutathione reductase (GSR) helps to maintain glutathione levels, which is helpful to maintaining a reducing environment within a cell to combat oxidative stress. The upregulation of oxidative stress related genes suggests that fish possess an adaptive mechanism against oxidative stress that is influenced by exogenous factors (Wu et al., 2017). An increase in transcription could contribute to a concurrent increase in translation and activation of enzymes and therefore, a higher ability of the organism to combat ROS (Banni et al., 2011). Zinc can bind to metal regulatory transcription factors thus stimulating the transcription of specific target genes (Banni et al., 2011; Sevcikova et al., 2010).

Proteins related to production of the aforementioned antioxidant enzymes are coded for by similarly named genes; *SOD*, *CAT*, *GPX*, and *GSR*, respectively. Metals can also interact within some tissues by increasing the production of related proteins, such as metallothionein (MT), which is a family of metal binding proteins (Walker et al., 2007). Metallothioneins are involved in the regulation and detoxification of both essential and non-essential metals, including zinc (Sevcikova et al., 2010). Studies have found that metallothionein promotors can be activated in a dose-dependent manner by metals like zinc via activation of metal response elements (Walker et al., 2007). Other genes related to the oxidative stress response in Rainbow Trout include Glutathione S-Transferase (*GST*), which plays a detoxification role by catalyzing the conjugation of compounds with reduced glutathione, and the Liver X Receptor (*LXR*), involved in cholesterol catabolism and *de novo* fatty acid biosynthesis in the liver (Cruz-Garcia et al., 2009). However, zinc's regulation of

gene expression is not constrained to genes directly involved in zinc homeostasis alone (Banni et al., 2011).

The gastrointestinal tract and gills are the primary tissues involved in zinc uptake, and the liver can play an important role in zinc homeostasis as well (Prabhu et al., 2014). The liver is the main metabolic organ in teleost fish and is frequently challenged by free radicals due to its high-level metabolic turnover. The liver has been reported to have a more highly developed antioxidant response than other organs in fishes (Vijayan et al., 2003). Oxidative stress is closely related to the progression of various liver diseases and high amounts of ROS have been shown to decrease the viability and function of hepatocytes in some animals (Wu et al., 2017). Altered gene expression profiles are one strategy by which hepatocytes may mitigate oxidative stress, and many oxidative stress biomarkers have been well delineated in fish. As such, measurement of hepatic gene expression has become a common method among researchers interested in evaluating oxidative stress.

1.8 Bioavailability

Bioavailability of zinc depends on its chemical form as well as other dietary components present in the diet, particularly protein source (Watanabe et al., 1997). Zinc sulphate ($ZnSO_4$), an inorganic metal salt, is a common form for zinc supplementation in aquaculture diets because it can be effectively utilized by many fish species, including Rainbow Trout (Lall, 2002; Prabhu et al., 2014). Most sources indicate a requirement of about 30 mg kg^{-1} of zinc for diploid Rainbow Trout when using a purified diet (NRC, 2011). However, often purified diets are not fiscally feasible to produce for large-scale production facilities. With inclusion of alternative protein sources, often inclusion levels of minerals such as zinc must be increased proportionally. For example, excessive amounts of minerals

(such as Ca and P) found in bone meal or phytic acid from plant meals can reduce zinc bioavailability, generating additional negative consequences (Apines et al., 2003; Spry et al., 2011). As such, in Rainbow Trout feed, up to 80 mg kg^{-1} of zinc must be included in semi-purified diets and up to three times this amount must be included in practical diets (Prabhu et al., 2014). A meta-analysis of many mineral requirement studies reports significant variations of recommended inclusion levels for a specific species even within diet types (Prabhu et al., 2014). The great differences in 'requirements' and the range of estimates reported by the NRC (15 mg kg^{-1} up to 80 mg kg^{-1} zinc (NRC, 2011)) can be attributed to an error in the estimation of the true bioavailability of the zinc present in various dietary formulations. However, many studies report signs of deficiency even when zinc was included in the diet at levels above NRC recommendations, which is likely due to the inhibitory action of the other ingredients present in the diet (Kucukbay et al., 2006).

Evidence indicates that zinc is more bioavailable when derived from animal protein sources than from plant sources (Sugiura et al., 1998). Plant sources contain different constituents, such as phytic acid, that can chelate divalent minerals, such as zinc, making it unavailable to the fish (Satoh et al., 1989; Sugiura et al., 1998; Watanabe et al., 1997). This most likely occurs as insoluble phytates are formed in the intestinal lumen (Watanabe et al., 1997). Satoh et al. (1989) also suggested that a cause could be the formation of a casein phytate complex when fish were fed a fish- and soybean-meal mix, which would render the ingredients only partially digestible by proteolytic pepsin enzymes (6.6% less so than casein alone).

Commercial diets prepared with phytic acid now often include increased levels of zinc to ensure adequate uptake (Watanabe et al., 1997). However, other possible and more

reasonable solutions to this issue exist. One of these solutions is through heat treatment of plant ingredients. Watanabe et al. (1997) observed treatment of soybean meal at 150°C increased the overall response performance parameters measured by decreasing the content of phytic acid from 1.3% to less than 1%. This is advantageous because this decrease in phytic acid led to an overall increase in zinc bioavailability, and presumably an increase in bioavailability of other divalent minerals. While plant feed sources are more sustainable than protein sources created from wild fish, additional steps are necessary for them to be as useful. This is particularly true for diets of more carnivorous species, in order to ensure adequate digestibility of those ingredients in order to meet nutritional requirements. A better understanding of these dynamics will result in decreases to production costs as current formulation strategies typically involve an over supplementation of minerals in order to ensure requirements are met.

Plant feedstuffs are of great interest due to their economic and environmental sustainability. However, plant protein sources are currently the most common problematic protein source regarding fish feed due to potential inhibitory or antinutritional effects. The wide-spread manifestation of cataracts in Rainbow Trout from 1973-1974 was likely due to a decrease in bioavailability of zinc in white fish meal (Lall, 2002). White fish meal is derived from hard tissues, such as bone, resulting in increased amounts of hydroxyapatite (mainly from tricalcium phosphate) (Watanabe et al., 1997). Tricalcium phosphate decreases the bioavailability of zinc and other minerals, and again much higher levels of supplementary zinc are required to ensure adequate nutrition when compared to semi-purified diets (Satoh et al., 1989). If adequate zinc is not available, fish will become deficient, resulting in decreased growth, skeletal deformities, cataract formation, and

eventually mortality (Satoh et al., 1989). As global availability and sustainability of fish-meal decreases, it is important to understand the differences in the nutritional and the anti-nutritional contents of alternative protein sources and how these interact with mineral availability. More recently, the use of organically chelated minerals, as opposed to inorganic constituents, has started to gain attention in fish feed production, especially in diets that have a high percentage plant meal. Organic minerals have the potential to outcompete with mineral inhibitors at the site of uptake.

1.9 Organic zinc

Organic, or chelated zinc, enters the body bound to another ion or molecule, while inorganic forms of zinc are unbound or in metal salt form. Studies have shown than certain forms of chelated zinc may be more bioavailable to fish (Apines et al., 2003; Ashmead, 1991; Glover and Hogstrand, 2002; Paripatananont and Lovell, 1995). Inclusion of a zinc amino acid chelate, characteristically similar to di- or tri-peptides, can have a two-fold or greater uptake efficiency compared to the metal salt form of zinc (Ashmead, 1991). This characteristic allows for an increase in intact absorption of the mineral in the gastrointestinal tract due to the chelate's structural stability and low molecular weight, similar to small peptides (Ashmead, 1991). Increases in availability have been inferred based on an increase in whole-body and bone zinc concentrations and retention values in fish supplemented with an amino acid chelated zinc (Apines et al., 2003; Glover and Hogstrand, 2002). Paripatananont and Lovell (1995) used a form of amino acid chelated zinc in a feeding study in channel catfish and observed that organic chelates resulted in greater deposition of zinc in body tissues at three times the rate of non-chelated zinc. This greater bioavailability could

result in a reduction of the amount of zinc that must be added to diets in order to meet the nutritional requirements of the fish (Apines et al., 2003).

There is evidence for the presence of several different uptake pathways for zinc including one that is amino acid modulated as well as one pathway for free zinc ions (Glover and Hogstrand, 2002). Amino acid chelates are predicted to work in a number of different ways to increase zinc bioavailability in the fish gut. First, certain amino acids may act as a shuttle for zinc compounds with low binding affinity to alternative uptake surfaces with notably higher affinity (Glover and Hogstrand, 2002). Alternatively, amino acid chelates may create a substrate that is more suitable for transport across the epithelial surface (Glover and Hogstrand, 2002). Ashmead (1991) suggests enzymatic breakdown of the chelated molecule in a mucosal cell occurs first, which then triggers the active transport of the chelate from the lumen to the cytoplasm. A pH change would then facilitate the breaking of the link between the transport molecule and the chelate, and the chelate would then be able to migrate into systemic circulation (Ashmead, 1991).

Unlike metal salts, amino acid chelates do not need to be ionized before they are able to be absorbed by the fish (Ashmead, 1991). They may endure as intact molecules and are relatively stable, so fats and other fibers do not often interfere with uptake (Ashmead, 1991). Moreover, zinc chelated to amino acids may be able to reach a steady state in the lumen even faster than free zinc. Histidine and cystine are known to have high binding affinities for zinc and can help increase bioavailability by stripping chelated zinc from alternative dietary binding constituents, such as phytic acid (Glover and Hogstrand, 2002). Specifically, histidine can act as a donor molecule to facilitate the release of zinc through action of the L-histidine-zinc chelate transferred intact across the gut epithelium (Glover and Hogstrand,

2002). This study further suggests that some uptake mechanisms are stereospecific and are competitive for the specific transporter involved, because stereoisomers (D-histidine and D-cysteine) showed no significant differences from the control (with $ZnSO_4$) (Glover and Hogstrand, 2002). However, specific transporters and pathways have yet to be completely described. Further, authors suggest the chelation might stay intact until it arrives at the cellular site where the element is needed (Paripatananont and Lovell, 1995). This concept has allowed scientists to hypothesize and test different forms of chelated zinc (and other minerals) and potential effects of increased bioavailability. This may include decreased operational costs attributed to decreasing waste zinc that passes through the fish without being absorbed.

There is support of the idea that uptake is more dependent on the chemical nature of the chelated species rather than the presence of chelation itself (Glover and Hogstrand, 2002). For example, if the chelated compound is not able to resist dissociation within the intestine, it will not be useful to the fish in the chelated form (Ashmead, 1991). As such, there are large variations reported in the success of using minerals chelated to organic molecules in supplementation studies (Apines et al., 2003; Ashmead, 1991; Glover and Hogstrand, 2002; Paripatananont and Lovell, 1995; Prabhu et al., 2014). These variations in chelated organic mineral bioavailability are likely due to varying qualities of the chelation, production methods, proportions of the various forms of the mineral under study, and varying presence of antagonistic ingredients (Prabhu et al., 2014). Often, more than one amino acid is used in metal chelation in order to hinder competition of insoluble complexes (Ashmead, 1991). As a result, ingredients and chelated compounds must be carefully

considered when formulating a diet with the intention of decreasing supplemental levels of zinc.

1.10 Effects of ploidy on mineral requirements

Mineral requirements of fish are highly variable and dependent on species, life stage, water quality (and mineral content of water), genetics, as well as many other factors (Lall, 2002). Interest in nutritional mineral requirements began relatively recently compared to the practice of aquaculture. Nutritionists are continuing to define and refine recommendations to ensure fish are receiving the best possible feed for optimal performance in their unique circumstance. In aquaculture, induced triploidy, a genetic manipulation resulting in an extra set of chromosomes in each somatic cell, is often utilized in the production of trout and salmon (Thorgaard, 1983). This extra set of chromosomes renders the fish sterile. Triploids have been thought to have the capability for faster growth, especially during later life stages because triploid fish do not expend energy on reproductive physiology (Thorgaard, 1983). In fish, triploidy can be induced by either hydrostatic pressure, thermal, or chemical shock (Tiwary et al., 2004). Increased demand and lack of adequate indoor water availability makes production in natural rivers and lakes an attractive and necessary option. In natural waters, however, the stocking of fertile diploid fish is declining because of the heightened risk of genetic pollution to natural populations, thus making sterile triploids an increasingly popular mitigation option (Cotter et al., 2000).

Studies on dietary zinc requirements have been done on many different fish including hybrid tilapia, *Oreochromis niloticus* x *O. aureus* (26-29 mg kg^{-1} diet) (Lin et al., 2008), Siberian sturgeon, *Acipenser baerii* (29.15 mg kg^{-1} diet) (Moazenzadeh et al., 2017), Jian carp, *Cyprinus carpio* var. Jian, (48.7 mg kg^{-1} diet) (Tan et al., 2010), and diploid

Rainbow Trout, *Oncorhynchus mykiss* (80 mg kg^{-1} diet) (Welker et al., 2015). However, there are currently no reports on dietary zinc requirements for triploid Rainbow Trout. This requires attention as fish farmers in the U.S. are showing more interest in utilization of the triploid strain, particularly among Steelhead Trout producers who raise their fish to later life stages. Some research suggests nutritional and mineral requirement differences between ploidies may exist (Fjelldal et al., 2016).

Scientists speculate on reasons for these differences. One theory is that triploids may have dissimilar mineral requirements simply due to their differing physiology, specifically that triploids are not allocating these nutrients toward reproductive behaviors or gamete production (Maxime, 2008). Alternatively, some evidence suggests triploids may have higher dietary requirements for certain minerals to prevent onset of skeletal deformities as a result of an increased growth rate (Fjelldal et al., 2016). There is further evidence supporting muscle fiber dynamic differences, where triploids exhibit reduced hyperplasia compensated for by increased hypertrophic growth rates compared to their diploid counterparts, resulting in larger cells and more numerous cell nuclei (Maxime, 2008).

Induction of triploidy itself may alter nutrient requirements in fish for continued normal function. At the induction of triploidy, a polar body (which is typically extruded in normal development) is retained within the egg, resulting in an increase in the number of chromosomes (3X rather than 2X). Due to the odd number of chromosomes in triploid Rainbow Trout, there may be a direct impact on the nutrient/mineral requirements of fish (Maxime, 2008; Ren et al., 2017). For example, some proteins contain structural “zinc fingers”. Zinc finger proteins are transcription factors that require one or more zinc ions for fold stabilization within the protein. Any of these reasons may contribute to the notion that

triploids have potentially different nutrient requirements, and these must be studied especially as use of triploids of all species continues to increase.

1.11 Objectives

It is important to fully understand the effects of zinc, as well as other essential elements, in order to gain a better understanding of their impacts in aquaculture and to procure the best and most efficient diets possible. As aquaculture continues to grow providing an important food source for humans, minimizing cost and waste as well as providing species with the healthiest conditions possible is paramount. Additionally, action must be taken to preserve the natural environment, and a better understanding of how each element interacts within an ecosystem is crucial to prevent further damage. Based upon the findings of the current literature review, to date no studies have been conducted to examine diploid versus triploid Rainbow Trout performance in response to semi-purified diets containing organic or inorganic zinc, and how such supplementation may affect growth and dietary retention in these fish. An outline of the thesis is presented in Figure 1.1. The objectives of this study are to perform a comparative analysis of organic and inorganic zinc among a commercial strain of diploid and triploid Rainbow Trout on:

1. Growth performance, chemical composition of whole-body, distal intestinal histology, cataract formation and lens histology, and zinc dependent enzyme activity in plasma.
2. Gene expression patterns relating to oxidative stress in hepatocytes.

I hypothesize the organic zinc supplementation will result in increased growth and nutrient utilization in Rainbow Trout and triploids will show benefits from higher inclusion levels than diploids.

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Chapter 2. Dietary Effects of Organic and Inorganic Zinc on Growth Performance, Physiological, and Histological Parameters in a Commercial Strain of Diploid and Triploid Rainbow Trout (*Oncorhynchus mykiss*)

2.1 Abstract

Zinc is an important micro-mineral for finfish required in trace amounts and is involved in a wide variety of biochemical processes. In aquaculture, triploid fish are utilized for their sterility to decrease the risk of genetic pollution to wild populations and for their potential for faster growth. However, to our knowledge there is no information regarding zinc requirements for triploid Rainbow Trout, despite reports from previous studies that mineral requirements may differ. Hence, this study was designed to determine the effects of organic and inorganic zinc on a commercial strain of genetically similar diploid and triploid Rainbow Trout (*Oncorhynchus mykiss*). Eleven dietary treatments, including one basal diet (33 mg kg^{-1}, Zn_{33}) and 10 experimental diets, supplemented with incremental levels of inorganic ($ZnSO_4$) and organic (Alltech) zinc supplemented to the basal diet (Zn_{63}, Zn_{93}, Zn_{123}, Zn_{153}, Zn_{183}) were fed for 9 weeks. Twenty-two experimental groups (a unique combination of ploidy, zinc type, and dose level) were run in triplicate, with 11 fish per tank (66 L), with an average starting weight of 53.07 ± 4.35g. Results showed neither ploidy (diploid vs. triploid) nor type of mineral (organic vs. inorganic) significantly influenced growth performance. There was a significant interaction between zinc type and dose in diploids for lipid retention, energy retention, and vertebral zinc content. In triploids, there was a significant interaction between zinc type and dose for lipid retention, energy retention, and protein retention, and vertebral zinc content. Dietary zinc had no significant effects on cataract formation or histology of the distal intestine. There were no significant differences of oxidative stress related enzyme activity (CuZnSOD;

GPX) in plasma. In conclusion, the results showed that interactions between type of zinc and dose had a significant effect on nutrient retention, representing a potential for increased capacity to utilize feed ingredients.

2.2 Introduction

Zinc is an essential trace mineral for finfish that is required for life. It is typically found in the bones and other tissues of fish used for FM production and is lacking in many plants used as alternative protein sources. A mineral analysis of feeds used in our lab showed that zinc content in soybean meal compared to FM was about 1:3 (unpublished data). Zinc plays a role in metabolism, enzymes, hormones, and the stress response along with many other physiological functions (Lall, 2002; Watanabe et al., 1997). The function of enzymes related to zinc include that of carbonic anhydrase (CO_2 formation), CuZnSOD (combatting oxidative stress), carboxypeptidase (protein digestion), alkaline phosphatase (hydrolysis of phosphate esters), polymerase (synthesis of RNA/DNA chains), and collagenase (wound healing). Zinc also plays a pivotal role in the metabolism of macronutrients; carbohydrates, proteins, and lipids (Lall, 2002). While zinc is always essential, nutritional requirements for different species of fish depend on many factors such as age, size, life stage, water hardness, and presence of antagonistic ingredients in non-purified diets. If there is not enough zinc in the diet, deficiency symptoms can include decreased growth, short-body dwarfism, cataracts and other oxidative stress related consequences, and eventually mortality (Banni et al., 2011; Kucukbay et al., 2006). Minerals can accumulate in the bone and other tissues and as such, these parameters provide a good indication of the zinc-status of the animal (Lall, 2002; Satoh et al., 1989).

The availability of zinc and other divalent cations is contingent largely upon the presence of antinutritional factors (ANFs) (Ketola, 1979; Lall, 2002). These can be components of alternative protein sources, such as phytic acid in plant meals and hydroxyapatite in white bone meals, that can form insoluble complexes with minerals, like zinc (Watanabe et al., 1997). These complexes are commonly known as chelations, or bonds between a molecule and a metal ion. These complexes are difficult for fish, especially carnivorous species, to break, making the minerals unusable (Karachle and Stergiou, 2012). The primary uptake mechanism for zinc in fish is through the gastrointestinal tract, while they are also able to uptake zinc through the gills if dietary zinc is limited (Bury et al., 2003; Lall, 2002). Metal homeostasis is primarily regulated by the gills (Watanabe et al., 1997). Although not yet clearly described, data suggest that there might be multiple uptake mechanisms present in the fish gut for different chemical forms of zinc. Pathways may include one for free, inorganic zinc and one facilitated by amino acids (Glover and Hogstrand, 2002).

In its inorganic form, zinc sulphate ($ZnSO_4$) is commonly used in fish feeds and is readily available to fish, such as Rainbow Trout (Prabhu et al., 2014). For Rainbow Trout, in purified diets, the NRC (2011) recommends including zinc at a minimum of 30 mg kg^{-1} to ensure adequate nutrition. Even still, these numbers are variable as deficiencies have been reported at inclusion levels above what the NRC (2011) recommends (Kucukbay et al., 2006). As more complex protein sources are included in semi-purified and practical diets, supplementation levels can increase up to 240 mg kg^{-1}, according to a meta-analysis on mineral requirements of fish (Prabhu et al., 2014). This is because ANFs present in the diets used can bind the zinc, making it unavailable to the fish (Prabhu et al., 2014; Watanabe et

al., 1997). Additionally, requirement levels may vary depending on specific goals. Requirement levels for basal maintenance are different than requirement levels for growth and reproduction (Prabhu et al., 2014). This is especially important as many plant proteins have naturally low levels of zinc and also include high levels of phytic acid (Glover and Hogstrand, 2002; Thompson and Erdman Jr., 1982). A few ways to increase bioavailability in the presence of ANFs include: heat treatment, which has been shown to decrease phytic acid content (Watanabe et al., 1997), increased inclusion of $ZnSO_4$ (Prabhu et al., 2014), or inclusion of organic forms of zinc, which is stabilizing, making it unavailable to chelate with ANFs (Apines et al., 2003; Glover and Hogstrand, 2002).

Organic zinc is bound with other ingredients that are known to be available to the species in question before addition to the diet. One of the more common practices includes binding zinc to one or more amino acids (Ashmead, 1991; Glover and Hogstrand, 2002). For example, one study has shown that zinc bound to amino acids such as histidine and cystine is more available to fish because the amino acids can facilitate the release of zinc for transfer across the gut epithelium (Glover and Hogstrand, 2002). This claim of increased availability can be supported by a significant increase in zinc deposition in various body tissues (Paripatananont and Lovell, 1995). Therefore, it is important to consider not only the presence of ANFs in alternative ingredients, but also the chemical form of supplemental zinc and its ability to competitively withstand binding with other ingredients.

Most of the current research focuses on diploid fish although there is evidence that supports variability in zinc requirements in Rainbow Trout between ploidies. Fjelldal et al. (2016) suggest that different amounts may be required to prevent the onset of skeletal deformities and other consequences in triploid fish. Triploid trout possess an extra set of

chromosomes most commonly induced by thermal, chemical, or pressure shock causing them to retain a polar body during development (Tiwary et al., 2004). Triploids can be utilized for their sterility in order to decrease the risk of genetic pollution when stocked in natural watersheds (Tiwary et al., 2004). Differences in ploidy can potentially include variable growth rates, especially after reaching reproductive maturity, as triploids are not utilizing nutrients for gamete production (Maxime, 2008). Additionally, muscle fiber dynamics differ between diploids and triploids, with triploids having reduced hyperplasia and increased hypertrophic growth rates compared to their diploid counterparts (Maxime, 2008). Differences in nutrient requirements may also be a consequence of varied genetic material (Ren et al., 2017). This is directly related to some transcription factors and proteins which require zinc for structure and function.

To our knowledge, no studies have been done to examine the effects of organic and inorganic zinc on diploid and triploid Rainbow Trout performance and there is no current information on zinc requirements for triploids. Therefore, the aim of the present study is to determine differences between supplemental levels of either organic or inorganic zinc in Rainbow Trout feed. The objectives of this study are two-fold, focusing on both on the differences between inorganic and organic zinc inclusion as well as potential differences between ploidies of genetically similar Rainbow Trout. We examined growth parameters, proximate composition, intestinal histology, formation of cataracts, zinc dependent enzyme activity, and mineral content of both diploid and triploid fish supplemented with either inorganic zinc sulphate or organic zinc bound to amino acids.

2.3 Materials and methods

2.3.1 Feed formulation

A basal control diet (without supplemental zinc) plus 10 experimental diets were formulated similarly except for level and source of supplemental zinc ($ZnSO_4$ and an organic amino acid chelate). Diets were formulated to be isonitrogenous and isolipidic, containing 42% crude protein and 21% lipid. All ingredients used were dried and ground to a particle size of <500µm prior to mixing and pelleting. Diets were produced by compression pelleting at the Hagerman Fish Culture Experiment Station. Both organic and inorganic zinc were added via premixes based on α-cellulose (40,000 ppm basis). Inorganic Zn premix ($ZnSO_4 \cdot 7H_2O$), sourced from Trouw Nutrition (Nutreco, Amersfoort, Netherlands) was added 'wet' by dissolving in water. Mineral mixtures were added to a quantity of α-cellulose, mixed, dried at 40°C in a fan assisted oven for 18 hours, sieved, re-powdered with a blender and then transferred to a zip-tie bag. Alpha-cellulose was added to the required final weight and the bag was inflated and shaken until thoroughly mixed. This method was used in the Zn free premix as well, which supplied the following inorganic minerals in mg kg^{-1} to each diet: Cu, 1.54 ($CuSO_4 \cdot 5H_20$); I, 10 (KIO_3), Mn, 20 ($MnSO_4 \cdot H_2O$) (Trouw Nutrition). Zn variable premix organic Zn (Bioplex-Zn) was added dry to the required concentration and thoroughly mixed in a 'zip tie' bag. Refer to Table 2.1 for ingredient and chemical compositions of the experimental diets for zinc requirements. Feed was pelleted in accordance with the size of the fish, increased as fish increased in size, and all pellet sizes were kept consistent for the diets throughout the experiment (2.5mm for 22 days, 3.5mm for remainder of trial).

2.3.2 Fish handling and sampling

Eggs were sourced from a commercial trout farm using a single parental cross. Half of the fertilized eggs were thermal shocked to induce triploidy (accomplished by the farm where the eggs were sourced). Diploid and triploid fish were kept separate until they reached an average weight of 53.07 ± 4.35g. Eleven fish were randomly distributed by ploidy into 66 (60 L) plastic tanks utilizing a flow through system supplied by well water with an average temperature of 13.5ºC. Tanks were randomly assigned one of the 11 diets (3 tanks/diet for each ploidy) at the Aquaculture Research Institute, Moscow, Idaho. Water quality parameters including ammonia nitrogen and nitrite (API, Chalfront, PA), pH (LaMotte, Chestertown, MD), DO (YSI, Yellow Springs, OH), and temperature were tested regularly and as needed according to manufacturer's recommendations. Fish were fed two times per day, 6 days per week to apparent satiation for 64 days. Feed consumption and mortalities were recorded daily. All rearing and sampling protocols were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2018-60).

Sampling occurred on days 64 and 65, fish were not fed for 24 hours prior to sampling of relevant tanks. Three fish per tank were euthanized using an overdose of tricaine methanesulfonate (MS-222; Western Chemical Inc, Ferndale, WA) (300 mg L^{-1}) for whole body proximate analysis. Another 3 fish were anesthetized in 80 mg L^{-1} MS-222 and measured for total length and weight. Caudal blood (1.5 mL) was drawn in two of these fish with heparinized syringes and centrifuged to obtain plasma to be analyzed for zinc dependent enzymes (ZnCu Superoxide Dismutase and Glutathione Peroxidase). Fish were then euthanized using an overdose of MS-222 (300 mg L^{-1}) and tissues removed for vertebrae and liver mineral analysis. Distal intestine samples were taken, washed with

phosphate buffered saline (PBS), and preserved until setting and sectioning for histology. Two additional fish per tank were euthanized with an overdose of MS-222 (300 mg L^{-1}), their heads were removed and wrapped in gauze soaked in saline (to protect the lenses) and shipped to Texas A&M, College Station, Texas, for cataract scoring.

2.3.3 Chemical and trace element composition of feed and tissue

Feed samples and fish tissue were analyzed for proximate composition (%) and energy content (cal./gram). Fish were pooled by tank, pureed, and homogenized using an industrial food processor, dried in a convection oven at 105°C for 24 hours (to determine moisture level), and ground with mortar and pestle for further analysis. Crude protein (total nitrogen x 6.25) was determined with a LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, MI) per manufacturer's instructions. Crude lipid was analyzed with an ANKOM XT15 extraction apparatus (ANKOM Technology, Macedon, NY) per manufacturer's instructions with petroleum ether as the extracting solvent, and ash by incineration at 550°C in a muffle furnace. Energy content was determined with a Parr Adiabatic Calorimeter (Parr Instruments, Moline, IL) per manufacturer's instructions. Mineral analysis of vertebrae and liver samples were conducted by Alltech (Nicholasville, KY) (zinc, manganese, iron, copper, and selenium).

2.3.4 Growth performance and nutrient utilization analyses

Fish in each tank were bulk weighed and feed consumption data was used to calculate weight gain, feed conversion ratio, specific growth rate, and individual weight and length of 3 fish was used to calculate condition factor (K).

- *Weight gain (%): (final wt.−initial wt.) × 100/initial wt.*
- *Specific growth rate (%/day): ($\log_e$ final wt.−$\log_e$ initial wt.)/days.*

- *Feed conversion ratio: dry feed weight/wet weight gain.*
- *Condition factor (K): weight/length*^{3}.
- *Thermal growth coefficient (TGC): ((*$^3\sqrt{}$*final wt.) – (*$^3\sqrt{}$*initial wt.))/(∑daily temp.) × 100*
- *Hepatosomatic index (HSI): (liver wt./body wt.) × 100*

Nutrient retention was determined from dietary and whole-body element concentration relative to feed and element intake concentration.

- *Nutrient/element retention (%): 100 x (final body wt.× [final whole-body nutrient] – initial body wt.× [initial whole-body nutrient]) / (feed consumed × [dietary nutrient]))*

2.3.5 Determination of antioxidant enzymes activity

Activities of zinc dependent enzymes were assayed in duplicate using samples from two fish and pooled by tank. Heparinized syringes were used to collect blood from fish. Samples were centrifuged and plasma was pipetted out and immediately put on ice. For each enzyme analysis, samples were diluted the appropriate amount. Cayman Chemical SOD determination kit (Item No. 706002, Cayman Chemical) was used to measure plasma Cu-ZnSOD (superoxide dismutase). Cayman Chemical GPX determination kit (Item No. 703102, Cayman Chemical) was used to measure plasma GPX (glutathione peroxidase). Both assays were performed per manufacturer's instructions.

2.3.6 Distal intestine histology

Samples from the distal intestine were taken from three fish per tank. Approximately one centimeter was removed and washed with PBS and placed in 2mL Formalin for 24 hrs. After this, the samples were removed from Formalin and put in 2mL Isopropanol (100%)

until setting in paraffin wax. For setting, tissues were cut with a scalpel and paraffin cassettes were made using a Tissue Tek tissue processor and embedding station (Sakura Finetechnical, Co., Tokyo, Japan). Cassettes were sent to the University of Arkansas at Pine Bluff for staining and photographing.

There, a rotary microtome (HM 340 E; Thermoscientific, Walldorf, Germany) was used to cut 5µm serial sections, then placed in a warm water bath and attached to microscope slides. Slides were vertically placed in metal trays (24 slide capacity) and manually stained. The staining protocol was performed as follows: Xylene - 5 min., Xylene – 2 min., 100% ethanol - 2 min., 70% ethanol - 2 min., 50% ethanol - 2 min., running tap water - 30 sec. followed by rinsing with distilled water, Hematoxylin - 6 min., running tap water - 30 sec. followed by rinsing with distilled water, Eosin – 2.5 min., running tap water - 30 sec. followed by rinsing with distilled water, 50% ethanol - 2 min., 70% ethanol - 2 min., 100% ethanol - 2 min., Xylenes - 2 min., Xylenes - 1 min. Two drops of DPX were then put on a cover slip, and the slide (with the stained sample) was brought down facing the DPX at an angle optimal for the DPX to catch the slide. A pencil was used to remove any air bubbles, and the slides were died for 3 days at 80ºC. The photos of the distal intestine sections were taken at 200x magnification using a light microscope (Leica DM 2500 LED, Wetzlar, Germany).

2.3.7 Cataract formation

Dr. Erin Scott performed the post-mortem ophthalmic examination. One hundred thirty-two eyes of 66 fish were examined for cataracts using slit lamp biomicroscopy at 16x magnification (SL-17 portable slit-lamp biomicroscope, Kowa Optimed Inc., Torrance, CA, USA). Cataract examination was done by a board-certified veterinary ophthalmologist (Dr.

Erin Scott, Department of Small Animal Clinical Sciences, Texas A &M University, College Station, Texas).

One eye of four fish (two diploids; two triploids) from diets including the basal and inclusion of both inorganic and organic Zn_{63}, Zn_{123}, and Zn_{183} was randomly selected for histologic evaluation. Both eyes were collected from one diploid fish fed organic Zn_{63} with bilateral lenticular lesions. Therefore, a total of 29 eyes from 28 fish were fixed in 10% neutral buffered formalin and submitted to the Comparative Ocular Pathology Laboratory of Wisconsin (COPLOW) for histopathological evaluation. The fixed globes were sectioned in the vertical plane adjacent to the optic nerve, embedded in paraffin blocks, and 5-µm serial sections were obtained through the lenses. Slides were stained with hematoxylin and eosin (H & E) and the lenses were examined for histological evidence of cataract formation by board a certified veterinary pathologist (Dr. Leandro Teixeira, Department of Pathobiology, University of Wisconsin-Madison, Madison, Wisconsin).

2.3.8 Statistical analysis

Tank means were used as units of observation for statistical analysis. Parameters were analyzed using R (R Core Team, 2018) and tested for normality and homogeneity of variance prior by using the residuals and normal Q-Q plots and by using Bartlett's Test. To maintain balance in the statistical design and to focus on the differences between the organic and inorganic zinc, the basal control tanks were removed from analysis and used as a reference. A two-way ANOVA analysis for each ploidy (main effects of type and level of zinc, plus an interaction of the two main effects) was performed using a generalized linear model. If significant differences were found, data were subjected to a least squared means comparison *post hoc* analysis using the emmeans package (Lenth, 2020) with a significance

level of $\alpha < 0.05$. Planned pairwise comparisons consisted of zinc inclusion levels within a diet type and of similar inclusion levels across diet types. In order to focus on these comparisons of interest, and due to the high number of treatments, differing inclusion levels across diet types were omitted from this report.

2.4 Results

2.4.1 Growth performance and nutrient utilization

Refer to Tables 2.2 and 2.3 for feed and whole-body proximate composition. There were no significant differences within either ploidy group for any of the growth parameters (PWG, SGR, FCR, K, or TGC). For reference, refer to Figure 2.1 for a plot comparing weight gain (%) for all treatments for both diploids and triploid Rainbow Trout. Results of statistical analysis and p-values for growth parameters can be found in Table 2.4. There were also no significant differences observed for either ploidy regarding protein efficiency ratio. However, diploids had a significantly different HSI depending on dose of zinc concentration ($p<0.001$, Figure 2.2). Pairwise comparisons showed fish fed 63 mg kg^{-1} and 153 mg kg^{-1} of $ZnSO_4$ had a significantly higher HSI than those fed 183 mg kg^{-1} $ZnSO_4$ ($p=0.001$ and $p=0.040$, respectively).

In the diploid fish, a significant interaction between type of zinc and dose was observed when compared to lipid and energy retention. Lipid retention was significantly affected due to this interaction ($p=0.001$), however the interaction was disordinal. An interaction plot (Figure 2.3) shows inclusion levels up to 93 mg kg^{-1} of zinc were not statistically different between diet types. However, $ZnSO_4$ inclusion of 123 mg kg^{-1} yielded significantly higher lipid retention than organic zinc ($p=0.049$). Contrarily, the inclusion of organic zinc at 153 mg kg^{-1} yielded significantly higher lipid retention than $ZnSO_4$

(p=0.010). Pairwise comparisons also revealed: fish fed 123 mg kg^{-1} $ZnSO_4$ had a significantly higher lipid retention than fish fed 153 mg kg^{-1} $ZnSO_4$ (p=0.003), fish fed 183 mg kg^{-1} organic zinc had significantly higher lipid retention than fish fed 123 mg kg^{-1} organic zinc (p=0.015), and fish fed 183 mg kg^{-1} $ZnSO_4$ had significantly higher lipid retention than fish fed 153 mg kg^{-1} $ZnSO_4$ (p=0.048).

An interaction between type of zinc and dose also had a significant impact on energy retention in both diploid and triploid fish (p=0.025 and p=0.036, respectively). The interaction plot for diploids can be found in Figure 2.4, pairwise comparisons revealed a trend at level 153 mg kg^{-1} zinc inclusion for greater energy retention in fish fed organic zinc (p=0.079) than diploid fish fed $ZnSO_4$. Similarly, in Figure 2.5, at 153 mg kg^{-1} zinc inclusion, the fish fed organic zinc had significantly greater lipid retention than those fed $ZnSO_4$ (p=0.002).

Triploid fish also showed a significant disordinal interaction between type of zinc and dose on the lipid retention response (p<0.001). An interaction plot (Figure 2.6) shows a similar trend to the aforementioned interactions. There are no statistically significant differences between the diet types up to inclusion levels of 123 mg kg^{-1}. However, at inclusion of levels 153 and 183 mg kg^{-1} organic zinc, lipid retention was significantly higher in triploid fish than those fed $ZnSO_4$ (p=0.022 and p=0.014, respectively). Pairwise comparisons also revealed: fish fed 183 mg kg^{-1} organic zinc had a significantly higher lipid retention than fish fed 123 mg kg^{-1} organic zinc (p=0.002), fish fed 153 mg kg^{-1} organic zinc had significantly higher lipid retention than fish fed 123 mg kg^{-1} organic zinc (p=0.022), and fish fed 183 mg kg^{-1} organic zinc had significantly higher lipid retention than fish fed 63 mg kg^{-1} organic zinc (p=0.039). In triploids, there was also a significant interaction effect

between type of zinc and dose on protein retention ($p=0.041$, Figure 2.7). Pairwise comparisons revealed: fish fed 153 mg kg^{-1} organic zinc had significantly higher protein retention than fish fed 93 mg kg^{-1} organic zinc ($p=0.011$).

Vertebrae and liver samples were analyzed for minerals, their mineral compositions are shown in Table 2.5 and p-values from the two-way ANVOA can be found in Table 2.6. A significant interaction between type of zinc and dose was observed in vertebral zinc content of the diploid fish ($p<0.001$, Figure 2.8). Pairwise comparisons revealed: fish fed 153 mg kg^{-1} $ZnSO_4$ had a trend of higher vertebral zinc content than fish fed 153 mg kg^{-1} organic zinc ($p=0.053$). A significant interaction between type of zinc and dose was also observed in vertebral zinc content of triploid fish ($p=0.045$, Figure 2.9). Pairwise comparisons revealed: fish fed 123 mg kg^{-1} $ZnSO_4$ had significantly higher vertebral zinc content than fish fed 63 mg kg^{-1} $ZnSO_4$ ($p=0.003$).

There were no significant differences in hepatic content of copper and selenium in any treatments. However, the diploid fish did show a significant interaction between type of zinc and dose on hepatic iron levels ($p=0.040$, Figure 2.10). Pairwise comparisons revealed: fish fed 153 mg kg^{-1} organic zinc had significantly higher hepatic iron content than fish fed 63 mg kg^{-1} organic zinc ($p=0.033$). There was also a significant interaction between type of zinc and dose on the hepatic zinc content in the diploids ($p=0.048$, Figure 2.11). Pairwise comparisons revealed: fish fed 93 mg kg^{-1} organic zinc had a trend of higher liver iron content than fish fed 93 mg kg^{-1} $ZnSO_4$ ($p=0.056$). Finally, there was an observed dose-dependent effect on manganese content in the liver of the diploids ($p=0.048$, Figure 2.12). Pairwise comparisons did not reveal any significant comparisons of interest.

2.4.2 Antioxidant enzyme activity, cataract formation, and lens and distal intestine histology

There were no significant differences observed for either ploidy in CuZnSOD or GPX activity with any dietary regime. There were no significant differences in cataract formation or lens histology for any of the treatments. However, one fish did develop bilateral incipient cataracts, and tissue damage was reiterated in the lens histology, as shown in Figures 2.14.1-2 and 2.15.1-4. Figure 2.14.2 shows the right eye of a cataractous fish (a diploid supplemented with 63 mg kg^{-1} organic zinc). Opacity within the eye lens is apparent in the photo along with clinical signs of cataracts described in both eyes in the lens histology (Figures 2.15.1-4), such as mild liquefaction and morgagnian globules in the lens.

There were no significant differences in distal intestine histology measures, indicating that neither type of zinc nor dose had a negative effect. Histology of the distal intestine is shown in Figures 2.13.1-4. Frames 1 and 2 refer to the diploid and triploid Rainbow Trout fed the basal diet (Zn_{33}), respectively. Frames 3 and 4 refer to the diploid and triploid Rainbow Trout fed 183 mg kg^{-1} organic zinc, respectively. The serosa, muscularis, submucosa, lamina propria, goblet cells, absorptive vacuoles, and the epithelial layers are labelled for each sample. Due to the lack of significant differences, the histology figures are representative of all samples we examined.

2.5 Discussion

We demonstrate the presence of a relationship between inclusion level of zinc and source (either organic or inorganic) that has a distinct effect on nutrient retention ability of both diploid and triploid Rainbow Trout. However, no significant differences were found in antioxidant enzyme activity, cataract formation, or lens and distal intestine histology. Additionally, no significant differences were observed in growth parameters: PWG, SGR,

FCR, K, or TGC. This is reflected in various other studies examining inclusion of organic in comparison with inorganic minerals (without inhibitors), which report inconclusive results (Apines et al., 2003; Benfey, 1998; Kokou et al., 2020; Kucukbay et al., 2006; Maxime, 2008; Sarker and Satoh, 2008; Tiwary et al., 2004). As such, it has been speculated that weight gain or other growth parameters might not be the best response unit of measurement for studies examining microminerals (Prabhu et al., 2014).

Nutrient retention, however, was significantly affected by type of zinc and dose in both diploid and triploid Rainbow Trout. Significant differences were observed in lipid and energy retention in diploids, and in lipid, protein, and energy retention in triploids. Lipid and energy retention values in both diploid and triploid fish revealed fish had significantly higher retention when supplemented with greater inclusion of organic zinc. This is likely correlated with zinc's role in macronutrient metabolism and involvement with various metabolic enzymes (Glover and Hogstrand, 2002; Kucukbay et al., 2006; Watanabe et al., 1997). The ability of fish to more efficiently uptake and utilize organic zinc results in significantly better exploitation of macronutrients in feed.

In this study, a significant interaction between type of zinc and dose was observed on vertebral zinc analysis for both diploids and triploids. Additionally, all vertebral zinc content levels in treatment groups were greater than those seen in the control fish. On average, vertebral zinc content was 2.3x in the treatment groups than the controls in the diploid fish, and 1.8x higher in the treatment groups than the control in the triploid fish. According to a meta-analysis, vertebral zinc content was the defining criteria for many different micronutrient studies (Prabhu et al., 2014). It has been shown in a previous study that vertebral zinc content was significantly reduced as levels of phytic acid were increased in

the diets (Apines et al., 2003). Additionally, bone zinc content was often higher when zinc was chelated with amino acids (Apines et al., 2003). Due to the interaction effects, concrete conclusions are difficult to make from this data alone. Further research on vertebral zinc content will be able to provide more specific differences between the various chemical forms of zinc inclusion, particularly between various body tissues. However, speculation indicates inclusion levels above 33 mg kg^{-1} (found in the basal diet and similar to NRC (2011) recommendations) may allow trout to store zinc without depleting bone storage, which would have a great impact on the chronic zinc status of the fish.

Between the fish fed 93 mg kg^{-1} of zinc, the organic supplemented group had a significantly higher hepatic zinc content than the inorganic supplemented group. This has been observed in another study, with a 36% increase in liver zinc level when animals were fed an organic chelate rather than a mineral salt (Ashmead, 1991). Zinc is often utilized in the oxidative stress response of fish (Banni et al., 2011; Kucukbay et al., 2006). The liver is a metabolically active organ and has a well-developed antioxidant response. Increased levels of hepatic zinc content in some of the treatments indicate greater utilization in organic zinc as the mineral often is not separated from the amino acid in the chelate until it reaches the tissue where it will be used (Ashmead, 1991). However, as supplementation of both types of dietary zinc increased in this study, hepatic zinc content trended downward. It has been theorized that the availability of zinc in the liver may be controlled at least in part by metallothionein (MT) activity (Coyle et al., 1995). Additionally, zinc accumulation is unlikely in the absence of MT (Coyle et al., 1995), so *MT* expression and protein activity may be an important consideration when further evaluating this trend.

When hepatic iron content increased, zinc content decreased (Figures 2.10 and 2.11). This may be indicative of an interaction between these two divalent cations that has been similarly observed in other studies (Lall, 2002; Ogino and Yang, 1978). Ogino and Yang (1978) observed an increase in iron content in the liver that was congruent with a decrease in zinc content. These two divalent cations may occupy similar pathways, and iron may act as a competitive inhibitor to zinc uptake. Iron is a metalloenzyme involved in succinate dehydrogenase (aerobic oxidation of carbohydrates), cytochromes (electron transfer), catalase (reduction of hydrogen peroxide), as well as being essential for cellular respiration (Lall, 2002). However, rather than as a result of deficiency, iron overload is typically the cause of oxidative stress, or iron overload in congruence with this interaction, as a cause for zinc deficiency (Evans and Halliwell, 2001). Regardless, the interaction and relationship between iron and zinc has been documented and was observed in the present study as seen in hepatic mineral content. The antagonistic relationship between the two minerals may arise from the competition for similar uptake pathways or binding sites.

Selenium is another metalloenzyme, and is involved with glutathione peroxidase (removal of hydrogen peroxide) and type I and III deiodinases (conversion of thyroxide to the active form) (Lall, 2002). Selenium's role in the oxidative stress response helps to protect nucleic acids, proteins, and lipids against free radicals (Kokou et al., 2020). There is also evidence that even under non-stressful conditions, selenium is related to oxidative stress related genes, including isoforms of glutathione peroxidase (*GPX*) 1 (Pacitti et al., 2013). In the present study, hepatic selenium content was not significantly different between zinc type and inclusion level, and neither was GPX enzyme activity. The has been differentially observed by Pacitti et al. (2013) with significant changes in selenium content influencing

significant changes in antioxidant capacity. It is possible that no significant differences in GPX activity were observed in this study because selenium is more directly linked to GPX activity than zinc.

Hepatic copper content was also not significantly different among treatments in this study. Copper is another divalent cation and limiting trace mineral (Lall, 2002). Copper has a role in many metabolic processes aside from enzymatic function, and is a metalloenzyme involved in cytochrome oxidase (terminal oxidase), lysyl oxidase (lysine oxidation), ceruloplasmin ferroxidase (iron utilization and copper transport), and CuZnSOD, along with zinc (Lall, 2002). The lack of significance observed in the present study is contrary to another which found a reduction in hepatic copper levels with increased inclusion of dietary zinc (Knox et al., 1984). As copper is very important in many metabolic processes, this results from the present study could be beneficial. As observed, uptake and accumulation of copper was not negatively affected by type nor inclusion level of zinc.

In the present study, there was a significant effect of dose on manganese content in the livers of the diploid fish, the only significant main effect without an interaction. Manganese is a limiting trace mineral involved with fatty acid and cholesterol biosynthesis as well as MnSOD (similar function to CuZnSOD), pyruvate carboxylase (pyruvate metabolism), and is closely involved in carbohydrate, lipid, and protein metabolism (Evans and Halliwell, 2001; Lall, 2002). Even though there was a significant effect of dose, based on the plot of manganese content (Figure 2.12) there is no trend between level of zinc inclusion or type of zinc on the hepatic manganese content. However, manganese content in experimental fish appears to be lower than manganese content in the control. Future research may also consider activity of MnSOD in comparison with CuZnSOD in studies like this.

Oxidative stress related enzymes, such as CuZnSOD, have been observed to be significantly higher in animals fed amino acid chelated zinc (Apines et al., 2003). The lack of significant differences seen in the present study may indicate that there was no need for an adaptive increase in enzymatic activity from the fish in order to combat oxidative stress. For example, some studies have reported that in order to combat increasing levels of ROS in tissues, the organism may respond adaptively through an increased production of ROS-scavenging enzymes, such as SOD (Banni et al., 2011; Liu et al., 2007). The notion that the fish in this study were not under oxidative stress was further reiterated by the fact that there were no significant instances of cataracts observed. It has been pointed out that in a significant majority of cataract cases, there has been a congruent increase in ROS (Spector, 1995). Alternatively, it has been noted in another study that deficiency of copper, rather than zinc, had a greater effect on the activity of CuZnSOD (Evans and Halliwell, 2001).

Having zinc that is more available to fish becomes increasingly important as inclusion of ANFs increases (Glover and Hogstrand, 2002; Watanabe et al., 1997). This study is similar to others in that supplemental zinc did not significantly affect growth parameters, distal intestine histology parameters, oxidative stress related enzyme activity, or cataract formation and lens histology. A lack of significant differences in distal intestine histology can be a positive indication none of the diets fed in this study caused any sign of enteritis, which is important as higher levels of organic zinc had other benefits for fish. Novel findings include that there appears to be a difference in lipid, protein, and energy retention that takes place between 123 and 153 mg kg^{-1} zinc inclusion between the different types of zinc. There is also a significant interaction between type of zinc and dose on the vertebral zinc content in both diploids and triploids. This indicates a potential change in the

way that zinc is taken up, metabolized, or stored by fish at these higher inclusion levels. More research is needed to clearly describe what this change may be. Another important consideration would be to see if differences in ploidy and/or sex exist if the fish are stocked together (aggressiveness, competition for food, etc.) (Galbreath et al., 1994; Sheehan et al., 2011). This study supports that there is a difference between $ZnSO_4$ and organic zinc supplementation for Rainbow Trout, and more research is required to determine interaction effects between type of zinc and dose in order to determine optimal dose depending on specific goals.

2.6 Conclusions

Significant interactions between type of zinc and dose were observed on the response parameters of lipid, energy, and protein retention. More research is required to describe this relationship, but of particular interest are responses between organic and inorganic zinc at inclusion levels between 123 mg kg^{-1} and 153 mg kg^{-1}. Vertebral zinc content, and hepatic mineral content of fish used in this study also varied depending on the type and dose of zinc. Furthermore, no negative consequences were observed in regard to growth and proximate composition, zinc dependent enzyme activity, distal intestine histology, or cataract formation and lens histology. These results, particularly the variations in nutrient retention, indicate that there are differences in uptake and/or utilization between inclusion of organic zinc and $ZnSO_4$.

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Chapter 3. Oxidative Stress Related Gene Expression Patterns in Diploid and Triploid Rainbow Trout (*Oncorhynchus mykiss)* fed Diets with Organic and Inorganic Zinc

3.1 Abstract

In Rainbow Trout, zinc is among the most important essential micro-minerals involved in many biological processes. Zinc plays a significant role in oxidative stress, bone mineralization, and growth. The bioavailability of zinc depends largely on feed ingredients and their compositions. Divalent ions, such as zinc, have a unique ability to form complexes with various nutrients in feed. Organic minerals have been observed to be more bioavailable to fish, resulting in a reduction in the amount required to be effective. This study examined gene expression response patterns in both diploid and triploid Rainbow Trout fed to satiation for 9 weeks with inclusion of either inorganic ($ZnSO_4$) or organic (amino acid chelated) zinc in incremental levels (Zn_{63}, Zn_{123}, Zn_{183}) to an otherwise sufficient basal diet (Zn_{33}). Hepatic genes related to oxidative stress were analyzed and included superoxide dismutase (*SOD1*; *SOD2*), glutathione peroxidase (*GPX1a*; *GPX1b1*; *GPX1b2*), catalase (*CAT*), glutathione reductase (*GSR*), glutathione S-transferase (*GST*), liver X receptor (*LXR*), and the zinc storage protein, metallothionein-A (*MTA*). Results from this study revealed that dose and type of zinc as well as ploidy level had an effect on expression of genes related to oxidative stress. Organic zinc promoted higher levels of gene expression and increased instances of dose effects. Additionally, gene expression was more upregulated in response to organic zinc in triploids than in diploids. These findings also establish a relationship between dietary zinc and oxidative stress defense, supporting previous research that shows adequate levels of dietary zinc are important for fish health.

3.2 Introduction

Zinc is an essential trace mineral for teleost fish and all vertebrates and is found in all organs, tissues, and fluids. It has structural and catalytic functions and also plays a regulatory role in multiple metalloenzymes as a specific cofactor and catalyst (Watanabe et al., 1997). In teleost fish, zinc can be absorbed either from food in the gastrointestinal tract or from the environment through the gills. For diploid Rainbow Trout, dietary zinc is included at about 30 mg kg^{-1} using a purified diet, 80 mg kg^{-1} for semi-purified diets, and up to three times this value using practical diets (Lall, 2002; NRC, 2011; Prabhu et al., 2014; Welker et al., 2015). However, a meta-analysis of various studies reported considerable variations in recommendations even within diet types, and largely attributes these to perturbances in the environment or within the organism and the consequential effects on the elements in question (Prabhu et al., 2014). Additionally, some sources indicate triploids might have different dietary requirements for zinc either due to an increased growth rate or due to a difference in genetic make-up of the fish (Maxime, 2008; Ren et al., 2017).

Bioavailability of zinc in both ploidies is dependent largely upon type/source of protein, the chemical form of the element, and the presence of anti-nutritional factors (ANFs) (Glover and Hogstrand, 2002; Watanabe et al., 1997). Zinc and other divalent ions are more easily absorbed from animal protein sources compared to plant protein sources, due to the presence of ANFs (like phytic acid) in plant proteins which can strongly chelate zinc to form insoluble complexes in the intestinal lumen (Lall, 2002; Thompson and Erdman Jr., 1982; Watanabe et al., 1997). This poses a problem for fish farmers as production shifts toward increased inclusion of plant ingredients in fish diets for sustainability reasons. As

such, increased levels of limiting nutrients must be included proportionally or alternative methods must be developed to compensate for reduced bioavailability in plant-based diets.

Zinc deficiency can result in reduced growth, reduced bone/body zinc levels, reduced capacity to combat oxidative stress, and eventually mortality (Banni et al., 2011; Kucukbay et al., 2006). Oxidative stress is the result of an organism's inability to counteract reactive oxygen species (ROS) with their endogenous antioxidant defense systems (Sevcikova et al., 2010). For example, ROS include free radicals with a very reactive unpaired electron, such as the superoxide anion and the hydroxyl radical, as well as hydrogen peroxide. If the organism is unable to combat ROS reactivity, negative consequences such as cataract formation, lipid and protein peroxidation, and alterations in gene expression can occur (Sevcikova et al., 2010). ROS produced by aerobic cellular metabolism can readily attack polyunsaturated fatty acids or lipid membranes and activate a self-propagating, destructive chain reaction (Mylonas and Kouretas, 1999). The destruction of these cellular components can be very dangerous and initial oxidation of only a small portion of cells can ultimately lead to wide-scale tissue damage.

The antioxidant defense mechanisms of an organism include exogenous effects from nutritional supplementation as well as endogenous enzymatic and genetic responses. It has recently been shown that dietary supplementation can be an effective method for improving the antioxidant capacity of an organ or tissue (Wu et al., 2017), and zinc is an established antioxidant. Antioxidants can either donate or accept electrons to eliminate the unpaired condition of ROS. Endogenously, antioxidant enzymes include; Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPX), and Glutathione Reductase (GSR). SOD is a metalloenzyme that catalyzes the dismutation of the superoxide anion to molecular

oxygen and hydrogen peroxide. CAT and GPX can then reduce hydrogen peroxide to water and dioxygen, and GSR helps to maintain glutathione levels and a reducing environment within a cell. Proteins related to the production of these enzymes are coded for by similarly named genes, such as superoxide dismutase (*SOD*), catalase (*CAT*), glutathione peroxidase (*GPX*), and glutathione reductase (*GSR*). *GPX1* has three isoforms that have been identified (*GPX1*, *GPX1b1*, and *GPX1b2*) (Pacitti et al., 2013). There are many other genes that are involved in the oxidative stress response. Glutathione S-Transferase (*GST*) plays a detoxification role by catalyzing the conjugation of compounds with reduced glutathione. The liver X receptor (*LXR*) is a transcription factor involved in the catabolism of cholesterol and *de novo* fatty acid biosynthesis in the liver (Cruz-Garcia et al., 2009). Finally, Metallothionein-A (*MTA*) codes for a zinc transport and storage protein. Metals have the ability to increase transcription, including genes that code for metal-binding proteins such as metallothionein (MT) (Walker et al., 2007). Often, promotors can be dose-dependently activated by DNA metal response elements in the promotor region of genes such as *MT*s which initiate the transcription of MT (Walker et al., 2007). Genetic responses to oxidative stress can be adaptive, an increase in the transcription of antioxidant related genes can subsequently increase the antioxidant response capacity of the animal (Wu et al., 2017).

Currently, research efforts are geared toward increasing plant-based ingredients in aquafeed as aquaculture continues to expand globally. However, increased utilization of plant-based ingredients is not without challenges. Plant-based ingredients that include ANFs often directly or indirectly impede mineral availability, especially zinc, in diets of cultured fish (Glover and Hogstrand, 2002; Thompson and Erdman Jr., 1982; Watanabe et al., 1997). Nevertheless, several strategies have been developed for making zinc more available to fish

fed plant-based diets. One of these possibilities includes the intentional formation of zinc chelates with other dietary ingredients in order to compete with inhibitory binding agents and enhance bioavailability. For example, amino acids and small peptides have been studied as a possible means to increase dietary mineral bioavailability because of their high binding affinity to minerals (Apines et al., 2003; Glover and Hogstrand, 2002). These amino acids may act either as a shuttle to transport zinc toward uptake surfaces with higher affinity, or the chelate may be more capable of intact transport across the epithelial surface (Glover and Hogstrand, 2002). The chemical nature and quality of the chelation seems to be most important in uptake efficiency (Glover and Hogstrand, 2002; Prabhu et al., 2014).

Induced triploidy, a genetic manipulation resulting in an extra set of chromosomes in each somatic cell rendering the fish sterile, is often utilized in trout and salmon aquaculture (Thorgaard, 1983). This is a popular mitigation option in natural water sheds where risk of escape and potential genetic impacts to wild fish exist (Cotter et al., 2000). Due to the variations in physiology, differences in mineral requirements between ploidies may exist (Fjelldal et al., 2016; Tiwary et al., 2004). This may be due either to an increase in growth potential after sexual maturity, or inherent genetic differences due to the odd number of chromosomes (Maxime, 2008; Ren et al., 2017; Thorgaard, 1983). For example, some proteins contain structural “zinc fingers”. Many zinc finger proteins are transcription factors that require one or more zinc ions for fold stabilization within the protein motif or require zinc for activation. However, there are currently no report on dietary zinc requirements for triploid Rainbow Trout.

The liver is the major metabolic organ in fish, and oxidative stress plays a pivotal role in the progression of hepatic diseases (Vijayan et al., 2003; Wu et al., 2017).

Furthermore, because the liver is constantly challenged by ROS, it is reported to have more highly developed antioxidant defenses than other organs by up to 10-fold (Wu et al., 2017). Hence, supplementation of the antioxidant, zinc, may support an enhanced oxidative stress response capacity in the liver. The objective of this study was to compare the effects of supplementary $ZnSO_4$ and organic zinc chelated to amino acids on the expression in oxidative stress related genes (*SOD1*, *SOD2*, *GPX1a*, *GPX1b1*, *GPX1b2*, *CAT*, *GSR*, *GST*, *LXR*, and *MTA*) in hepatic cells of diploid and triploid Rainbow Trout.

3.3 Materials and Methods

3.3.1 Experimental diets

A basal control diet (without supplemental zinc) plus 6 experimental diets were similar in formulation except for level and source of supplemental zinc (organic amino acid chelated and inorganic zinc sulphate; $ZnSO_4$). Diets were formulated to be isonitrogenous (42% crude protein) and isolipidic (21% lipid) (Table 3.1). Diets were dried and ground, and produced by compression pelleting at Hagerman Fish Culture Experiment Station. All ingredients used were ground to a particle size of <500μm prior to mixing and pelleting and both types of zinc were added via premixes based on α-cellulose (40,000 ppm basis). Inorganic Zn premix ($ZnSO_4 \cdot 7H_2O$), sourced from Trouw Nutrition (Nutreco, Amersfoort, Netherlands) was added 'wet' by being dissolved in water. These were added to a quantity of α-cellulose, mixed, dried at 40°C in a fan assisted oven for 18 hours, sieved, re-powdered with a blender and then transferred to a zip-tie bag. Alpha-cellulose was added to the required final weight and the bag was inflated and shaken until thoroughly mixed. This method was used in the Zn free premix as well, which supplied the following inorganic minerals in mg kg^{-1} to each diet: Cu, 1.54 ($CuSO_4 \cdot 5H_20$); I, 10 (KIO_3), Mn, 20

($MnSO_4 \cdot H_2O$), inorganic minerals were also sourced from Trouw Nutrition. Zn variable premix organic Zn (Bioplex-Zn) was added dry to the required concentration and thoroughly mixed in a 'zip tie' bag. Proximate compositions of the basal and experimental diets are presented in Table 3.2. Feed was pelleted in accordance with the size of the fish, increased as fish increased in size, and all pellet sizes were kept consistent for the diets throughout the experiment (2.5mm for 22 days, 3.5mm for remainder of trial).

3.3.2 Fish and feeding trial and sample collection

All fish eggs were sourced from a commercial trout farm from the same parental fish and half of the eggs were thermal shocked to achieve triploidy (accomplished by the farm where the eggs were sourced). Diploid and triploid fish were kept separate until they reached an average weight of 53.07 ± 4.35g. Eleven fish were randomly distributed by ploidy into 42 (60 L) plastic tanks utilizing a flow through system supplied by well water with an average temperature of 13.5ºC. Tanks were randomly assigned one of the 7 diets (3 tanks/diet for each ploidy) at the Aquaculture Research Institute, Moscow, Idaho. Water quality parameters including ammonia nitrogen and nitrite (API, Chalfront, PA), pH (LaMotte, Chestertown, MD), DO (YSI, Yellow Springs, OH), and temperature were tested regularly and as needed according to manufacturer's recommendations. Fish were fed two times per day, 6 days a week to apparent satiation for 64 days. Feed consumption and mortalities were recorded daily. All rearing and sampling protocols were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC-2018-60).

Due to the high number of tanks, sampling occurred on days 64 and 65, fish were not fed for 24 hours prior to sampling of relevant tanks. Tanks anesthetized in 100 mg L^{-1} tricaine methanesulfonate (MS-222) and bulk weighed and 3 fish were collected per tank

measured for total length and weight. Three fish per tank were euthanized using an overdose of tricaine methanesulfonate (MS-222; Western Chemical Inc, Ferndale, WA) (300 mg L^{-1}) and tissue was removed for gene expression analysis of the liver. Samples were immediately suspended in 0.5mL RNA*later* and stored at 4°C overnight, and then at -20°C until RNA isolation.

3.3.3 Gene expression analysis

Total RNA was isolated from 50 – 100 mg of liver tissue and homogenized in 1 ml TRIzol (Invitrogen, Carlsbad, CA) per manufacturer's suggested protocol using a 5 mm steel bead and a multi-tube shaker. The RNA pellet was then washed twice with 75% ethanol and resuspended in nuclease-free water. For the RT reaction, 1 µl of RNA was DNase treated (Promega, Madison, WI) and was reversed transcribed using random primers (Invitrogen) and M-MLV reverse transcriptase (Promega) per manufacturer's protocol. The cDNA was diluted 1:4 with nuclease-free water, and 2 µl were used in a 15 µl PCR reaction also containing 825 nM forward and 825 nM reverse primers and 7.5 µL SYBR Green Master Mix (Applied Biosystems, Foster City, CA) (Primer sequences for target genes: Table 3.2). The real-time PCR was performed with an ABI7900 Sequence Detection System (Applied Biosystems) with the following protocol. Temperature was held at 50°C for 2 minutes followed by 95°C for 10 minutes. Forty PCR cycles of 95°C (15 sec) and 60°C (1 min) were completed, followed by melt curve analysis to confirm a single PCR product in each reaction. Real-time QPCR data were analyzed using the efficiency corrected relative expression method with β-actin as a reference gene (Pfaffl, 2001).

3.3.4 Statistical analysis

Tank means were used as units of observation for statistical analysis. Parameters were tested for normality and homogeneity of variance prior to three-way ANOVA. Data were analyzed using the General Linear Model procedure in the Statistical Analysis System (SAS Institute, Cary, NC, V9.4). If required, data was transformed to achieve normal distribution. Control tanks were removed from analysis to perform the ANOVA but included in subsequent figures as a reference. There was never a three-way interaction between ploidy, type of zinc, and dose, or a two-way interaction between ploidy and dose, so these were removed from the final model (Montgomery, 2013; Nelder, 1994). If significant differences were observed, data were subjected to Tukey's HSD *post hoc* test to separate the means at a significance level of $\alpha<0.05$.

3.4 Results

All p-values from the ANOVA can be found in Table 3.3. There were no significant differences in growth or mortality between any of the treatments. Refer to Chapter 2 for a comprehensive report.

3.4.1 SOD1 and SOD2 expression

All inclusion levels displayed significantly higher expression for these genes in the organic zinc supplemented groups compared with $ZnSO_4$. There was a significant interaction between dose and type of zinc observed in both *SOD1* and *SOD2* (p=0.004 and p=0.004) (Figure 3.1). There was also a significant interaction between ploidy and type of zinc observed in *SOD2* (p=0.015). Organic zinc supplementation resulted in significantly higher expression of the *SOD* genes than $ZnSO_4$ supplementation for both ploidies, and triploids exhibited higher expression within organic zinc supplemented group, which can be

seen in Figure 3.2. No differences in expression were observed by ploidy in fish fed inorganic zinc-based diets.

3.4.2 GPX1a, GPX1b, and GPX1b2 expression

For all three isoforms, the main effect of type of zinc was significant ($p<0.001$ for each, Figure 3.3). At all inclusion levels, fish showed significantly higher expression of all *GPX* genes when diets were supplemented with organic zinc. Additionally, the main effect of ploidy was significant in *GPX1a* ($p=0.005$). Both ploidies had significantly higher expression of *GPX1a* when given organic zinc and triploids had significantly higher expression than diploids within the organic zinc diets (Figure 3.4). No significant interactions were observed either between dose and zinc type or ploidy and zinc type in *GPX1a*, *GPX1b1*, and *GPX1b2* expression.

3.4.3 CAT, GSR, and GST expression

The main effect of type of zinc was significant for all three genes ($p<0.001$ for each, Figure 3.5). At all inclusion levels, expression was higher in all three genes in fish fed supplemental organic zinc when compared to the inorganic fed groups (Figure 3.5). The main effect of dose was also significant for *CAT*, *GSR*, and *GST* expression ($p<0.001$, $p<0.001$, and $p=0.002$, respectively). There was no significant interaction effect between type of zinc and dose or ploidy and type of zinc on the expression of *CAT*, *GSR*, and *GST* (Table 3.3).

3.4.4 LXR expression

At each inclusion level, fish fed organic zinc had significantly higher levels of *LXR* expression. Both ploidies had significantly higher expression when fed the organic zinc and triploids had significantly higher expression than diploids within the organic zinc diets

(Figure 3.6). There was a significant interaction between ploidy and type of zinc in *LXR* expression (p=0.009) but no interaction effect was observed between type of zinc and dose (Table 3.3).

3.4.5 MTA expression

The main effects for dose (p=0.004) and ploidy (p=0.005) were significant (Table 3.3). Fish fed the organic zinc showed a declining dose-dependent response of *MTA* expression while no trend was observed in fish fed $ZnSO_4$. No significant differences were observed between ploidies in fish fed $ZnSO_4$. Contrary to other genes in this study, diploids fed organic zinc had significantly higher *MTA* expression than the triploids. These results can be observed in Figure 3.7. No significant interactions were observed for either type of zinc and dose or ploidy and type of zinc on the expression of *MTA*.

3.5 Discussion

We demonstrate evidence of the effects of supplemental dietary zinc on the antioxidant defense system in the hepatic cells of diploid and triploid Rainbow Trout. Additionally, our research shows evidence for significant differences in genetic responses between ploidy types. The results obtained from this study were similar for many of the genes measured and offer an ideal platform to gain important information about the regulation of genes related to the antioxidant response in Rainbow Trout hepatocytes.

In this study, statistical interactions were observed for both ploidy and type of zinc as well as dose and type of zinc in the *SOD* genes. There was a trend of an increasing dose effect seen in the gene expression of fish fed the organic zinc, while expression remained relatively constant in fish fed the inorganic zinc. This is further supported with no differences seen between ploidies fed inorganic zinc and significantly higher expression in

both ploidies fed organic zinc. Furthermore, triploids exhibited significantly higher *SOD* expression than diploids for both genes measured. This might suggest that the organic zinc supplementation has the ability to initiate a greater genetic response. However, more research is required, especially on inherent genetic differences between diploids and triploids to confirm this. Zinc has a known antioxidant capacity in part by being a crucial part of the enzyme CuZnSOD. The upregulation of the *SOD* genes may indicate a greater capacity for enzyme function as well (Banni et al., 2011). However, this activity should be measured in the hepatocytes in order to determine tissue specific transcription and enzymatic relationships. The differences observed between the ploidies may be due to the induced variations in genetic material and effects of these differences on expression (Maxime, 2008; Ren et al., 2017). The gene expression in the triploids seems to be more significantly upregulated by the organic zinc supplementation. As a result, lower inclusion of organic zinc may have similar beneficial effects on *SOD* expression as higher inclusion of $ZnSO_4$. However, studies must also take precautions to not elicit an oxidative stress response due to treatments. Future research should consider indicators of oxidative stress and measure these responses in addition to the genetic response.

There were no significant interactions observed for any of the *GPX* genes, but type of zinc influenced expression at all inclusion levels. There was not a dose-dependent response, but organic zinc stimulated significantly higher expression levels at all doses than $ZnSO_4$. Similar to *SOD*, *GPX1a* was not differentially expressed by fish fed inorganic zinc but expression was significantly higher in triploid fish fed organic zinc. An increase in the transcription of oxidative stress related genes would help increase the organism's capacity to scavenge ROS from the cells (Banni et al., 2011). However, under non-stressful conditions,

it has been observed that even when *GPX1* transcription was altered, there were no concurrent changes in enzymatic activity (Pacitti et al., 2013). Another study has also observed inconsistencies between mRNA expression and activities of antioxidant enzymes, including *SOD*, *GPX*, and *CAT* (Zheng et al., 2016). Future studies that can compare mRNA and enzymatic expression under both stressful and non-stressful conditions will offer unique insight on this. However, the upregulation in expression for both the *SOD* and *GPX* genes when supplemented with organic zinc may support an increase in the function of the adaptive genetic stress response in animals.

The main effects of type of zinc and dose were significant regarding differences in expression of *CAT*, *GSR*, *GST*, and *LXR* genes studied. In each of these genes, fish fed organic zinc had significantly higher expression at all dose levels. Another study used zinc as a pretreatment for oxidative stress and observed inhibited cytotoxicity and apoptosis through the indirect antioxidant actions of zinc (Chung et al., 2005). However, neither *CAT*, *GSR*, or *GST* were differentially expressed between the two ploidies. The increased expression of these genes (*CAT*, *GSR*, and *GST*) in organic zinc fed groups suggests increased potential defense mechanisms against oxidative stress.

The only gene that responded differently with respect to expression observed in the other genes examined as well as contrary to what we expected was *MTA*. This study observed a dose-dependent decrease in *MTA* expression which is conflicting to the responses seen in other studies (Kucukbay et al., 2006; Walker et al., 2007). However, Walker et al. (2007) examined expression in gill tissues rather than hepatic tissue. The differences between the study performed by Walker et al. (2007) and the current study may be due to differences in uptake and transport mechanisms between dietary and water-borne zinc. MTs

are involved in essential metal regulation in fish and zinc has an essential function in gene regulation (Sevcikova et al., 2010). Banni et at. (2011) did however observe a decrease in *MT* mRNA abundance in the livers of zinc supplemented fish when exposed to a stressor, and another study revealed similar results in rats (Banni et al., 2010). However, Coyle et al. (1995) observed a correlative relationship between hepatic zinc content and *MT* expression in rats. This study theorized that the availability of zinc in the liver may be controlled at least in part by metallothionein (MT) activity (Coyle et al., 1995). So, further research is necessary (especially in fish) in order to describe the relationship of *MT* expression and zinc availability.

The up- and downregulatory patterns of oxidative stress related gene expression observed in this study has been similarly observed in other animals (Banni et al., 2010; Coyle et al., 1995). A decrease in the ability to respond to oxidative stress has been reported to decrease liver function in fish and the upregulation of related genes could indicate an adaptive mechanism to benefit normal function (Wu et al., 2017). Although the genes studied in this experiment were primarily related to zinc function and homeostasis, zinc's regulation of other genes is not constrained to only these. Zinc acts as a stabilizer for membranes, and may displace bound transition metal ions in order to prevent lipid peroxidation (Evans and Halliwell, 2001). Additionally, zinc may play a role in regulation by binding to the metal regulatory transcription factors and metal response elements of proteins such as structural zinc fingers (Banni et al., 2011). More research is required to more clearly describe the relationship in gene expression of *SOD*, *GPX*, *CAT*, *GSR*, *GST*, *LXR*, and *MTA* especially in the presence of inclusion of organic zinc. Nevertheless, zinc may directly or indirectly act as a functional signal to facilitate the oxidative stress response

(Banni et al., 2011). This study supports potential beneficial effects of organic zinc on the antioxidant defense system in the liver of Rainbow Trout, with a differential response between ploidies.

3.6 Conclusions

Significant main effects of either type of zinc, dose, and ploidy were observed among genes examined in this study along with significant interaction between some factors. Supplementation of organic zinc stimulated an increase in expression of oxidative stress-related genes (*SOD*, *GPX*, *CAT*, *GSR*, *GST*, and *LXR*) in fish when compared with those fed $ZnSO_4$. Furthermore, triploid fish often tended to show more upregulated expression than diploids regarding supplemental organic zinc. This increase in expression may be related to the fish's ability to uptake the different forms of zinc. More research is required to determine the relationship between zinc accumulation and *MT* expression in trout hepatocytes. Based on the results obtained in the present study, type of zinc supplementation, dose, and ploidy level have a significant effect on the oxidative stress related gene expression patterns of Rainbow Trout. Further research is required to understand interactions between type of zinc and dose along with their effects on both diploids and triploids.

3.7 References

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Chapter 4. Summary and Implications

Aquaculture production is expected to continue to increase along with the growing world population. Carnivorous fish are highly sought-after food fish and among the top carnivorous species is Rainbow Trout. As this growth continues, to ensure sustainability of natural resources, we must develop successful alternative protein sources that provide adequate nutrition to carnivorous species. This is especially important due to the inherent anatomical and physiological differences that are present between different species. However, as alternative protein sources are included at increasing levels, the availability of certain essential micro-minerals, such as zinc, is altered. Zinc is required for life at various levels and requirements are dependent upon species, age, water chemistry, presence of other ingredients in feed, and many other factors. Zinc plays a role in metabolism, various metalloenzymes, the oxidative stress response, as well as many different physiological functions. Because it is an essential nutrient, if it is not present, deficiency symptoms will incur, such as reduced growth, lipid and protein peroxidation, decreased metabolism, formation of cataracts, and eventually mortality.

In teleost fish, the main uptake route of microminerals is through gastrointestinal absorption from the diet. However, this route provides opportunity for zinc and other divalent cations to chelate with ingredients in the feed. Some of these chelates, such as with phytic acid and tricalcium phosphate, can make zinc and other minerals unavailable to the consumer. This is a problem especially in carnivorous species. This unavailability can rapidly lead to deficiency symptoms. To combat this, increased amounts are often be added to the feed, which can be costly and wasteful. This can also result in an increase of metals in natural waters when ingredients pass through the fish without being utilized. Depending on

the species of the fish, goals of the industry, and the ingredients present in the feed, different levels of target minerals must be added to ensure adequate nutrition. While some of these chelates are less available to fish, there is evidence that supports intentional chelations may be more available to certain species. One of the most common forms of this is chelations of minerals to one or more amino acids. Reports of successful use of amino acid chelates vary, often due to the quality and source of the chelation in congruence with the specific goals of the study. Availability and requirements may also be affected by ploidy, as different ploidies have different genetics and niche roles, which are likely to increase after reaching the age of sexual maturity.

For the aforementioned reasons, we chose to investigate the differences of varying levels and sources of supplemental zinc on diploid and triploid Rainbow Trout. Zinc types included an inorganic zinc sulphate ($ZnSO_4$) and an organic amino acid chelate. Fish were all from the same parental cross, allowing a narrow focus on response variables with as little external or genetic variation as possible. No significant differences in growth parameters or proximate composition of fish, activity of zinc dependent enzymes, distal intestine histology, or cataract formation and lens histology were observed. The lack of differences in growth parameters have been similarly observed in other studies, especially when diploids and triploids were kept separate. The similarity of zinc dependent and oxidative stress related enzyme activity may indicate that these fish were not under oxidative stress, which is reaffirmed in the lack of cataracts present. Finally, the lack of differences in the distal intestine histology indicates that none of the supplemental levels of zinc affected the integrity of the distal intestine.

Significant differences in several nutrient retention parameters were observed. For

example, in diploids, significant differences in both lipid and energy retention due to an interaction between type of zinc and dose was observed. At levels of zinc inclusion of 153 mg kg^{-1} and higher, organic zinc resulted in higher retention than $ZnSO_4$. There was also a significant interaction between type of zinc and dose on the response parameters of lipid, energy, and protein retention in triploids observed. There was a similar trend to diploids at higher inclusion levels. In addition to this, at all levels and types of zinc inclusion and for both ploidies, vertebral zinc content was notably higher than in the control fish. Based on the results found in this part of the study, there was a distinct interaction between type of zinc and dose, particularly at inclusion levels of 123 and 153 mg kg^{-1}. Further research is required to describe this interaction and potential benefits of organic zinc inclusion at specific levels on nutrient retention.

Furthermore, there were significant differences in oxidative stress related gene expression patterns based on type of zinc, inclusion level, and/or ploidy observed in all of the genes selected for this study. Gene expression was significantly upregulated in fish supplemented with organic zinc for genes; *SOD1*, *SOD2*, *GPX1a*, *GPX1b1*, *GPX1b2*, *CAT*, *GSR*, *GST*, and *LXR*. Triploids also exhibited significantly higher expression than diploids for genes; *SOD1*, *SOD2*, *GPX1a*, and *LXR*. The only gene that was markedly differentially expressed than the others was *MTA*. In *MTA* expression, we observed a slight declining dose effect for expression in fish supplemented with organic zinc. This may provide insight into the declining hepatic zinc content. Speculations predict zinc availability in the liver is largely reliant on MT protein activity. As a part of the *MT* family, *MTA* plays a known in zinc transport and storage. The results observed in this study of the similarly decreasing hepatic zinc content and hepatic *MTA* expression with increased inclusion levels may reflect

on this relationship. Additionally, it was the one gene that was more highly expressed in diploids supplemented with organic zinc than triploids. More research will provide insight on further describing the relationship between zinc and *MT* gene expression. The increased expression observed in triploids may be due to physiological differences, perhaps including the variation in genetic material found.

Future research should include a digestibility or requirement study of the organic zinc, especially in the presence of ANFs. Organic zinc is typically more expensive than inorganic and should be more successful than $ZnSO_4$ in order to be cost effective. Additionally, describing specific uptake pathways for organic zinc will allow nutritionists to formulate diets with specific goals in mind. Based on the evidence observed in this study, there is an interaction effect on various response variables that is affected or regulated by type of zinc, level of supplemental zinc, and/or ploidy of fish.

The hypotheses that organic zinc supplementation would result in increased growth and nutrient utilization in Rainbow Trout and the triploids will show benefits from higher inclusion levels than the diploids were partially supported. There were no effects on growth performance and further research is necessary to describe relationship affecting nutrient utilization. However, this study does establish a strong relationship between type of zinc, dose, and ploidy that has a significant effect on the oxidative stress related gene expression patterns in Rainbow Trout hepatocytes. This will help provide a platform of knowledge to allow future studies to establish this mechanism without overlooking important, significant interaction effects.

Tables and Figures

Table 2.1 Ingredient and chemical composition of basal and experimental diets

Ingredients (%)	Diets										
	33 mg kg^{-1} Zn (Control)	63 mg kg^{-1} $ZnSO_4$	93 mg kg^{-1} $ZnSO_4$	123 mg kg^{-1} $ZnSO_4$	153 mg kg^{-1} $ZnSO_4$	183 mg kg^{-1} $ZnSO_4$	63 mg kg^{-1} Alltech Organic Zinc	93 mg kg^{-1} Alltech Organic Zinc	123 mg kg^{-1} Alltech Organic Zinc	153 mg kg^{-1} Alltech Organic Zinc	183 mg kg^{-1} Alltech Organic Zinc
Casein, dried	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Fishmeal, de-boned	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Corn gluten yellow, 75% CP	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Soy meal, sol ext	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Soy protein concentrate	16.12	16.12	16.12	16.12	16.12	16.12	16.12	16.12	16.12	16.12	16.12
Choline chloride	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Dicalcium phosphate	3.40	3.40	3.40	3.40	3.40	3.40	3.40	3.40	3.40	3.40	3.40
Stable C (35%) vitamin	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Fish oil	18.64	18.64	18.64	18.64	18.64	18.64	18.64	18.64	18.64	18.64	18.64
Wheat flour	16.94	16.94	16.94	16.94	16.94	16.94	16.94	16.94	16.94	16.94	16.94
Vitamin Premix, ARS 702	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Zn free mineral[3]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
a-cellulose	3.00	2.40	1.80	1.20	0.60	0.00	2.40	1.80	1.20	0.60	0.00
Bioplex-Zn mixture[1]	0.00	0.00	0.00	0.00	0.00	0.00	0.60	1.20	1.80	2.40	3.00
Inorganic Zn mixture[2]	0.00	0.60	1.20	1.80	2.40	3.00	0.00	0.00	0.00	0.00	0.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1]Zn variable premix organic Zn, 5000 ppm. Bioplex; Alltech, Nicholasville, KY 40356
[2]Zn premix inorganic ($ZnSO_4 \cdot 7H_2O$), Trouw Nutrition, Nutreco, Amersfoort, Netherlands
[3]Zn free mineral (mg kg^{-1}): Cu, 1.54 ($CuSO_4 \cdot 5H_20$); I, 10 (KIO_3), Mn, 20 ($MnSO_4 \cdot H_2O$), Trouw Nutrition, Nutreco, Amersfoort, Netherla

Table 2.2 Proximate composition (%) and gross energy (calories) of basal and experimental diets

	Diets										
	33 mg kg^{-1} Zn (Control)	**63 mg kg^{-1} $ZnSO_4$**	**93 mg kg^{-1} $ZnSO_4$**	**123 mg kg^{-1} $ZnSO_4$**	**153 mg kg^{-1} $ZnSO_4$**	**183 mg kg^{-1} $ZnSO_4$**	**63 mg kg^{-1} Alltech Organic Zinc**	**93 mg kg^{-1} Alltech Organic Zinc**	**123 mg kg^{-1} Alltech Organic Zinc**	**153 mg kg^{-1} Alltech Organic Zinc**	**183 mg kg^{-1} Alltech Organic Zinc**
Moisture	6.24 ± 0.39	7.32 ± 0.90	8.62 ± 1.44	6.90 ± 0.50	8.33 ± 1.17	8.12 ± 0.93	7.58 ± 0.37	8.75 ± 1.36	10.44 ± 1.89	8.46 ± 1.01	7.77 ± 0.91
Ash	5.27 ± 0.14	5.37 ± 0.01	5.27 ± 0.05	5.25 ± 0.01	5.27 ± 0.02	5.25 ± 0.04	5.29 ± 0.07	5.33 ± 0.00	5.37 ± 0.06	5.28 ± 0.04	5.12 ± 0.02
Lipid	21.82 ± 0.11	21.58 ± 0.01	21.39 ± 0.23	21.51 ± 0.29	21.81 ± 0.04	21.39 ± 0.20	21.45 ± 0.18	21.60 ± 0.03	21.46 ± 0.09	19.26 ± 0.20	18.62 ± 0.03
Protein	43.29 ± 0.37	42.90 ± 0.07	43.51 ± 0.06	43.74 ± 0.32	42. 40 ± 0.05	42.54 ± 0.37	42.03 ± 0.01	42.73 ± 0.24	41.70 ± 0.16	41.91 ± 0.04	42.12 ± 0.08
Energy	5471.0 ± 6.0	5452.0 ± 2.0	5458.0 ± 1.0	5444.0 ± 1.0	5432.5 ± 5.5	5401.0 ± 1.0	5336.0 ± 2.0	5379.0 ± 2.0	5309.0 ± 0.5	5176.0 ± 0.0	5111.5 ± 7.5

Table 2.3.1 Proximate composition (%) and gross energy (calories) of whole body of fish

		Diets										
		33 mg kg^{-1} Zn (Control)	**63 mg kg^{-1} $ZnSO_4$**	**93 mg kg^{-1} $ZnSO_4$**	**123 mg kg^{-1} $ZnSO_4$**	**153 mg kg^{-1} $ZnSO_4$**	**183 mg kg^{-1} $ZnSO_4$**	**63 mg kg^{-1} Alltech Organic Zinc**	**93 mg kg^{-1} Alltech Organic Zinc**	**123 mg kg^{-1} Alltech Organic Zinc**	**153 mg kg^{-1} Alltech Organic Zinc**	**183 mg kg^{-1} Alltech Organic Zinc**
Moisture	**2X**	71.2 ± 0.5	70.2 ± 0.2	70.1 ± 0.1	69.6 ± 0.5	70.4 ± 0.2	69.0 ± 0.2	69.9 ± 0.3	69.8 ± 0.3	70.0 ± 0.3	70.4 ± 0.2	70.1 ± 0.2
	3X	71.5 ± 0.2	70.5 ± 0.2	70.7 ± 0.2	70.4 ± 0.2	70.9 ± 0.1	70.5 ± 0.3	70.2 ± 0.4	70.2 ± 0.2	70.6 ± 0.1	70.5 ± 0.2	70.1 ± 0.1
Ash	**2X**	2.1 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.1
	3X	2.2 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.2 ± 0.0	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 01	2.2 ± 0.1	2.1 ± 0.1
Lipid	**2X**	10.4 ± 0.5	11.2 ± 0.1	11.1 ± 0.1	11.8 ± 0.4	10.5 ± 0.1	12.2 ± 0.2	11.4 ± 0.3	11.1 ± 0.4	10.9 ± 0.4	10.8 ± 0.2	10.9 ± 0.2
	3X	9.8 ± 0.3	10.8 ± 0.1	10.6 ± 0.2	10.9 ± 0.1	10.6 ± 0.1	10.4 ± 0.2	10.8 ± 0.3	11.6 ± 0.2	10.7 ± 0.2	10.6 ± 0.3	11.0 ± 0.1
Protein	**2X**	15.5 ± 0.0	16.2 ± 0.2	16.1 ± 0.1	16.2 ± 0.1	16.6 ± 0.3	16.3 ± 0.2	16.0 ± 0.2	16.6 ± 0.1	16.9 ± 0.2	16.0 ± 0.1	15.6 ± 0.2
	3X	16.3 ± 0.2	16.0 ± 0.1	16.2 ± 0.2	16.3 ± 0.2	15.7 ± 0.1	16.4 ± 0.2	16.3 ± 0.1	15.3 ± 0.1	16.1 ± 0.3	16.4 ± 0.2	16.5 ± 0.2
Energy	**2X**	6492.2 ± 60.6	6549.3 ± 16.0	6510.5 ± 20.3	6629.8 ± 40.1	6464.3 ± 43.5	6686.3 ± 28.9	6554.8 ± 28.9	6531.3 ± 36.4	6471.3 ± 74.9	6405.8 ± 103.3	6479.8 ± 17.7
	3X	6357.8 ± 41.9	6488.8 ± 31.7	6486.2 ± 21.5	6513.8 ± 26.0	6499.5 ± 23.1	6464.7 ± 15.0	6580.8 ± 42.6	6593.8 ± 32.0	6509.7 ± 32.8	6455.3 ± 32.1	6542.0 ± 14.7

Table 2.3.2 P-values from two-way ANOVA by ploidy for the proximate composition and gross energy content of the whole body of the fish

	p-value					
	Diploids			Triploids		
Parameter	Dose	Zn Type	Dose * Zn Type	Dose	Zn Type	Dose * Zn Type
Moisture	0.163	0.310	0.172	0.646	0.967	0.641
Lipid	0.289	0.379	0.172	0.478	0.713	0.982
Protein	0.587	0.267	0.184	0.897	0.394	0.398
Ash	0.543	0.502	0.358	0.612	0.327	0.412
Energy	0.496	0.442	0.153	0.998	0.059	0.193

Table 2.4 P-values from two-way ANOVA by ploidy for the growth performance, nutrient retention, and body indices

	p-value					
	Diploids			Triploids		
Parameter	**Dose**	**Zn Type**	**Dose * Zn Type**	**Dose**	**Zn Type**	**Dose * Zn Type**
SGR	0.345	0.789	0.938	0.810	0.595	0.230
PWG	0.336	0.721	0.935	0.241	0.591	0.241
TGC	0.462	0.359	0.894	0.699	0.731	0.699
Efficiency	0.523	0.856	0.586	0.187	0.443	0.300
Lipid Retention	**0.001**	0.996	**0.000042**	0.621	0.472	**0.00079**
Protein Retention	0.853	0.685	0.916	0.316	0.919	**0.041**
Energy Retention	**0.023**	0.290	**0.025**	0.504	0.420	**0.036**
K	0.375	0.284	0.213	0.899	0.120	0.634
HSI	**0.00043**	0.544	0.139	0.329	0.119	0.099
FCR	0.228	0.555	0.554	0.365	0.199	0.259

Table 2.5 Mineral content in vertebrae and liver mineral of diploid and triploid Rainbow Trout (diploids: 2X; triploids: 3X) fed with experimental diets expressed as parts per million (ppm) or parts per billion (ppb). N=3, mean ± standard error

		Diets										
		33 mg kg^{-1} Zn (Control)	**63 mg kg^{-1} $ZnSO_4$**	**93 mg kg^{-1} $ZnSO_4$**	**123 mg kg^{-1} $ZnSO_4$**	**153 mg kg^{-1} $ZnSO_4$**	**183 mg kg^{-1} $ZnSO_4$**	**63 mg kg^{-1} Alltech Organic Zinc**	**93 mg kg^{-1} Alltech Organic Zinc**	**123 mg kg^{-1} Alltech Organic Zinc**	**153 mg kg^{-1} Alltech Organic Zinc**	**183 mg kg^{-1} Alltech Organic Zinc**
Vert. Zn [ppm]	**2X**	121.5 ± 29.7	247.9 ± 25.2	256.5 ± 24.5	244.9 ± 4.1	328.1 ± 7.7	281.8 ± 22.4	286.6 ± 33.4	291.9 ± 32.7	336.1 ± 12.3	235.8 ± 2.6	286.7 ± 6.5
	3X	152.7 ± 5.6	196.4 ± 27.1	286.4 ± 28.1	338.6 ± 10.7	287.2 ± 19.2	296.2 ± 22.5	253.9 ± 35.8	302.7 ± 36.2	245.1 ± 11.4	287.1 ± 32.8	272.8 ± 10.3
Liver Mn [H2] [ppm]	**2X**	6.0 ± 0.2	5.9 ± 0.1	6.2 ± 0.3	5.1 ± 0.4	5.1 ± 0.7	5.6 ± 0.2	5.2 ± 0.4	5.6 ± 0.4	4.8 ± 0.3	5.6 ± 0.2	5.2 ± 0.2
	3X	4.7 ± 0.4	5.8 ± 0.4	5.1 ± 0.1	4.7 ± 0.7	5.0 ± 0.5	5.4 ± 0.0	5.5 ± 0.2	5.9 ± 0.4	4.2 ± 0.6	4.8 ± 0.5	5.1 ± 0.1
Liver Fe [H2] [ppm]	**2X**	430.0 ± 17.5	361.0 ± 9.6	404.9 ± 35.0	416.2 ± 12.9	349.5 ± 42.3	471.9 ± 10.2	310.4 ± 30.4	430.0 ± 25.4	386.3 ± 38.9	449.6 ± 31.4	409.9 ± 38.5
	3X	442.5 ± 15.4	520.9 ± 57.6	552.5 ± 58.3	521.5 ± 19.4	466.1 ± 65.0	514.9 ± 32.7	448.8 ± 13.9	468.6 ± 66.1	492.1 ± 26.9	551.8 ± 68.0	460.1 ± 28.2
Liver Cu [H2] [ppm]	**2X**	303.9 ± 38.6	274.7 ± 14.2	335.6 ± 16.8	314.4 ± 7.1	319.0 ± 42.1	363.9 ± 33.9	246.1 ± 28.3	287.4 ± 31.0	297.4 ± 28.8	338.2 ± 27.2	320.7 ± 38.7
	3X	294.4 ± 13.5	306.6 ± 5.9	311.1 ± 15.8	341.6 ± 23.3	312.9 ± 46.1	373.1 ± 48.1	311.9 ± 14.4	301.8 ± 26.0	331.4 ± 21.2	321.5 ± 23.2	294.2 ± 13.8
Liver Zn [H2] [ppm]	**2X**	125.5 ± 6.1	117.4 ± 5.1	112.1 ± 2.5	122.5 ± 3.2	117.4 ± 3.7	112.3 ± 0.8	112.7 ± 2.4	132.0 ± 5.1	120.3 ± 6.4	116.4 ± 4.4	114.8 ± 7.4
	3X	102.5 ± 1.3	112.4 ± 4.5	129.4 ± 2.5	110.2 ± 2.0	120.7 ± 1.8	119.2 ± 10.1	120.7 ± 10.5	117.2 ± 4.3	111.5 ± 1.7	115.0 ± 2.9	126.3 ± 10.3
Liver Se [H2] [ppb]	**2X**	5192.6 ± 325.2	5349.4 ± 451.4	4807.1 ± 211.9	4991.0 ± 504.2	4403.6 ± 169.5	5118.8 ± 441.4	4769.6 ± 376.1	5033.9 ± 257.7	5004.4 ± 67.1	4457.5 ± 438.0	4589.7 ± 447.7
	3X	4854.5 ± 201.7	4797.2 ± 342.2	5717.3 ± 414.0	5071.0 ± 175.7	4754.6 ± 426.3	5797.6 ± 643.9	4849.7 ± 521.4	5034.2 ± 246.1	4931.4 ± 380.6	5171.6 ± 138.7	5276.0 ± 192.2

Table 2.6 P-values from two-way ANOVA by ploidy for mineral content of vertebrae and liver tissue

	p-value					
	Diploids			Triploids		
Parameter	Dose	Zn Type	Dose * Zn Type	Dose	Zn Type	Dose * Zn Type
Vert. Zn	**0.025**	0.188	**0.00051**	**0.002**	0.108	**0.045**
Liver Mn	**0.048**	0.160	0.333	0.352	0.532	0.533
Liver Fe	**0.030**	0.230	**0.040**	0.794	0.289	0.400
Liver Cu	0.274	0.482	0.782	0.362	0.890	0.482
Liver Zn	0.451	0.456	**0.048**	0.197	0.343	0.412
Liver Se	0.432	0.261	0.732	0.142	0.922	0.610

Table 3.1 Ingredient and chemical composition of basal and experimental diets

	Diets						
Ingredients (%)	**33 mg kg^{-1} Zn (Control)**	**63 mg kg^{-1} $ZnSO_4$**	**123 mg kg^{-1} $ZnSO_4$**	**183 mg kg^{-1} $ZnSO_4$**	**63 mg kg^{-1} Alltech Organic Zinc**	**123 mg kg^{-1} Alltech Organic Zinc**	**183 mg kg^{-1} Alltech Organic Zinc**
Casein, dried	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Fishmeal, de-boned	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Corn gluten yellow, 75% CP	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Soy meal, sol ext	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Soy protein concentrate	16.12	16.12	16.12	16.12	16.12	16.12	16.12
Choline chloride	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Dicalcium phosphate	3.40	3.40	3.40	3.40	3.40	3.40	3.40
Stable C (35%) vitamin	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Fish oil	18.64	18.64	18.64	18.64	18.64	18.64	18.64
Wheat flour	16.94	16.94	16.94	16.94	16.94	16.94	16.94
Vitamin Premix, ARS 702	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Zn free mineral[3]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
a-cellulose	3.00	2.40	1.20	0.00	2.40	1.20	0.00
Bioplex-Zn mixture[1]	0.00	0.00	0.00	0.00	0.60	1.80	3.00
Inorganic Zn[2]	0.00	0.60	1.80	3.00	0.00	0.00	0.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1]Zn variable premix organic Zn, 5000 ppm. Bioplex; Alltech, Nicholasville, KY 40356
[2]Zn premix inorganic ($ZnSO_4 \cdot 7H_2O$), Trouw Nutrition, Nutreco, Amersfoort, Netherlands
[3]Zn free mineral (mg kg^{-1}): Cu, 1.54 ($CuSO_4 \cdot 5H_20$); I, 10 (KIO_3), Mn, 20 ($MnSO_4 \cdot H_2O$), Trouw Nutrition, Nutreco, Amersfoort, Netherlands

Table 3.2 Primer sequences used for reverse transcription reaction

Gene	Forward Primer	Reverse Primer	Accession Number	Product length
SOD1	TGGTCCTGTGAAGCTGATTG	TTGTCAGCTCCTGCAGTCAC	AF469663.1	201
SOD2	TCCCTGACCTGACCTACGAC	GGCCTCCTCCATTAAACCTC	CA352127.1	201
GPX1a	AATGTGGCGTCACTCTGAGG	CAATTCTCCTGATGGCCAAA	HE687021	131
GPX1b1	CGAGCTCCATGAACGGTACG	TGCTTCCCGTTCACATCCAC	CA357669.1	183
GPX1b2	TCGGACATCAGGAGAACTGC	TCCTTCCCATTCACATCCAC	HE687023	121
CAT	TGATGTCACACAGGTGCGTA	GTGGGCTCAGTGTTGTTGAG	BX087110.3	195
GSR	CTAAGCGCAGCGTCATAGTG	ACACCCCTGTCTGACGACAT	CA368976.1	108
GST	TCGCTGACTGGACGAAAGGA	CGAAGGTCCTCAACGCCATC	BX302932.3	196
LXR	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	FJ470291.1	171
MTA	ATCTTGCAACTGCGGTGGAT	CAAGTCTTGCCCTTGCACAC	XM_021597409	135

Table 3.3 Proximate composition (%) and gross energy content (calories) of basal and experimental diets

	Diets						
	33 mg kg^{-1} Zn (Control)	**63 mg kg^{-1} $ZnSO_4$**	**123 mg kg^{-1} $ZnSO_4$**	**183 mg kg^{-1} $ZnSO_4$**	**63 mg kg^{-1} Alltech Organic Zinc**	**123 mg kg^{-1} Alltech Organic Zinc**	**183 mg kg^{-1} Alltech Organic Zinc**
Moisture	6.24 ± 0.39	7.32 ± 0.90	6.90 ± 0.50	8.12 ± 0.93	7.58 ± 0.37	10.44 ± 1.89	7.77 ± 0.91
Ash	5.27 ± 0.14	5.37 ± 0.01	5.25 ± 0.01	5.25 ± 0.04	5.29 ± 0.07	5.37 ± 0.06	5.12 ± 0.02
Lipid	21.82 ± 0.11	21.58 ± 0.01	21.51 ± 0.29	21.39 ± 0.20	21.45 ± 0.18	21.46 ± 0.09	18.62 ± 0.03
Protein	43.29 ± 0.37	42.90 ± 0.07	43.74 ± 0.32	42.54 ± 0.37	42.03 ± 0.01	41.70 ± 0.16	42.12 ± 0.08
Energy	5471.0 ± 6.0	5452.0 ± 2.0	5444.0 ± 1.0	5401.0 ± 1.0	5336.0 ± 2.0	5309.0 ± 0.5	5111.5 ± 7.5

Table 3.4 P-values from three-way ANOVA of hepatic gene expression for oxidative stress related genes

	p-value				
Gene	**Ploidy**	**Dose**	**Zn Type**	**Ploidy * Zn Type**	**Dose * Zn Type**
SOD1	**0.012**	**0.002**	**<0.001**	0.154	**0.004**
SOD2	0.124	**0.020**	**<0.001**	**0.015**	**0.004**
GPX1a	**0.005**	0.071	**<0.001**	0.438	0.501
GPX1b1	0.089	0.098	**<0.001**	0.071	0.108
GPX1b2	0.299	0.714	**<0.001**	0.337	0.920
CAT	0.053	**<0.001**	**<0.001**	0.239	0.093
GSR	0.125	**<0.001**	**<0.001**	0.652	0.382
GST	0.124	**0.002**	**<0.001**	0.764	0.800
LXR	0.075	**0.007**	**<0.001**	**0.009**	0.210
MTA	**0.005**	**0.004**	0.203	0.088	0.058

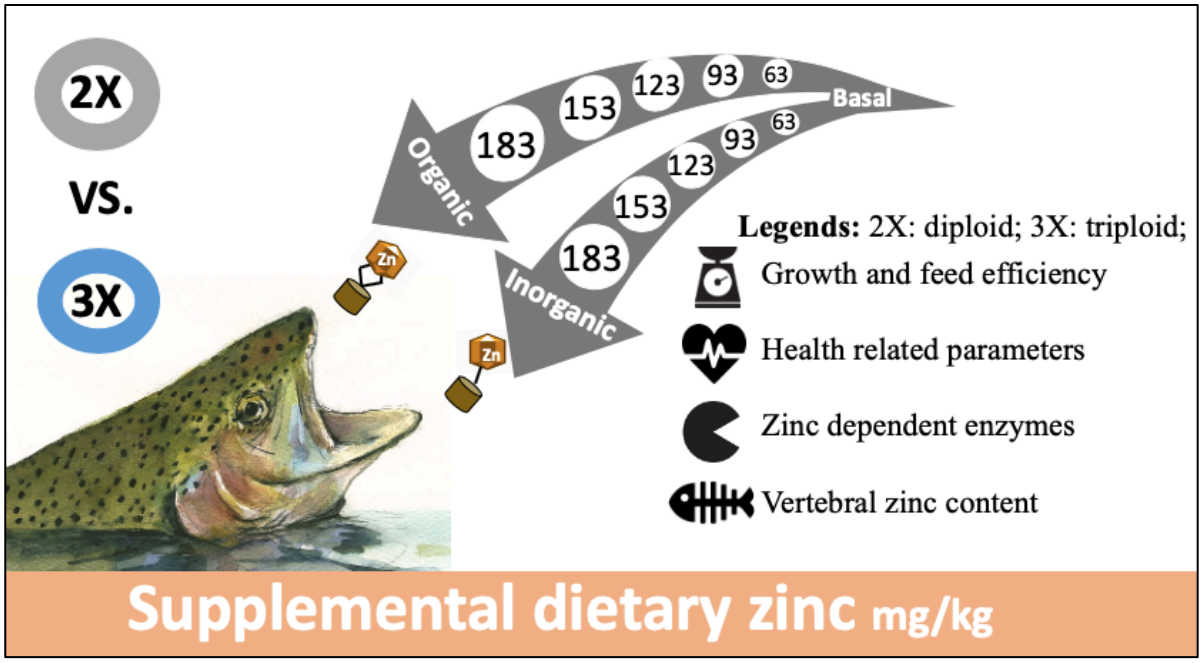


Figure 1.1 Proposed graphic for thesis study objectives. Supplementing organic and inorganic dietary zinc to diploid (2X) and triploid (3X) Rainbow Trout. Response parameters include growth performance and nutrient utilization, mineral content, and activity of zinc dependent enzymes.

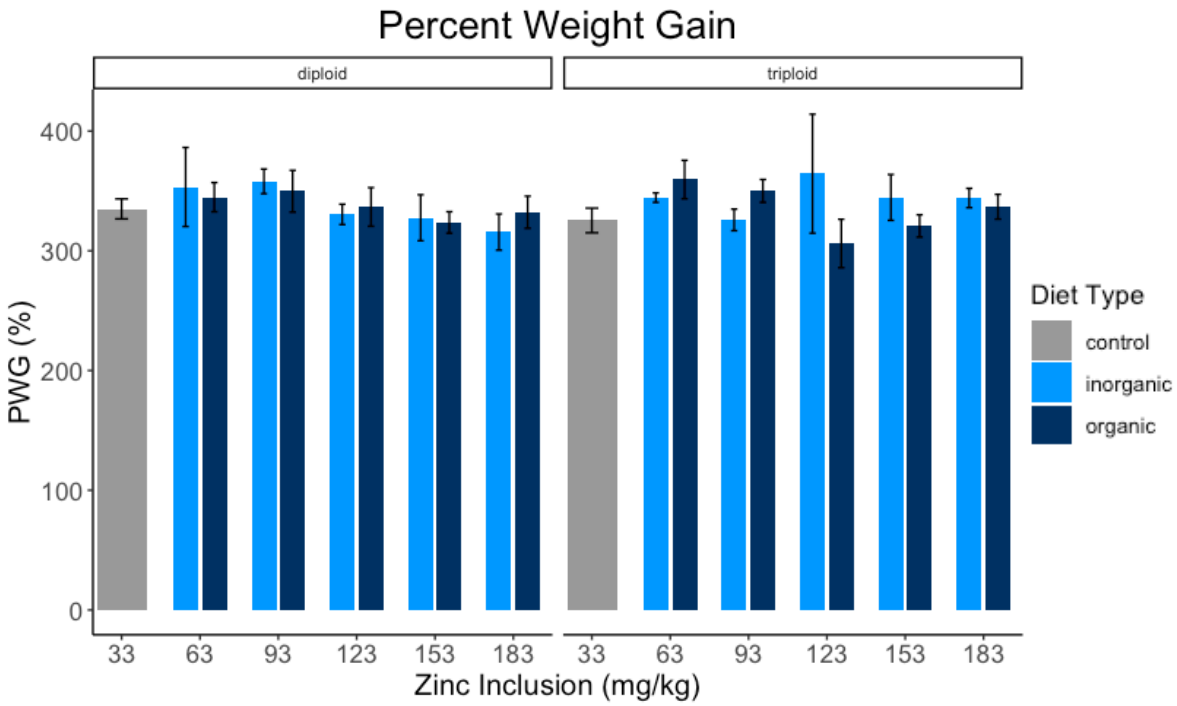


Figure 2.1 Weight gain (%) of diploid and triploid Rainbow Trout fed with organic and inorganic based diets. Data presented in a bar graph (N=3; mean ± standard error)

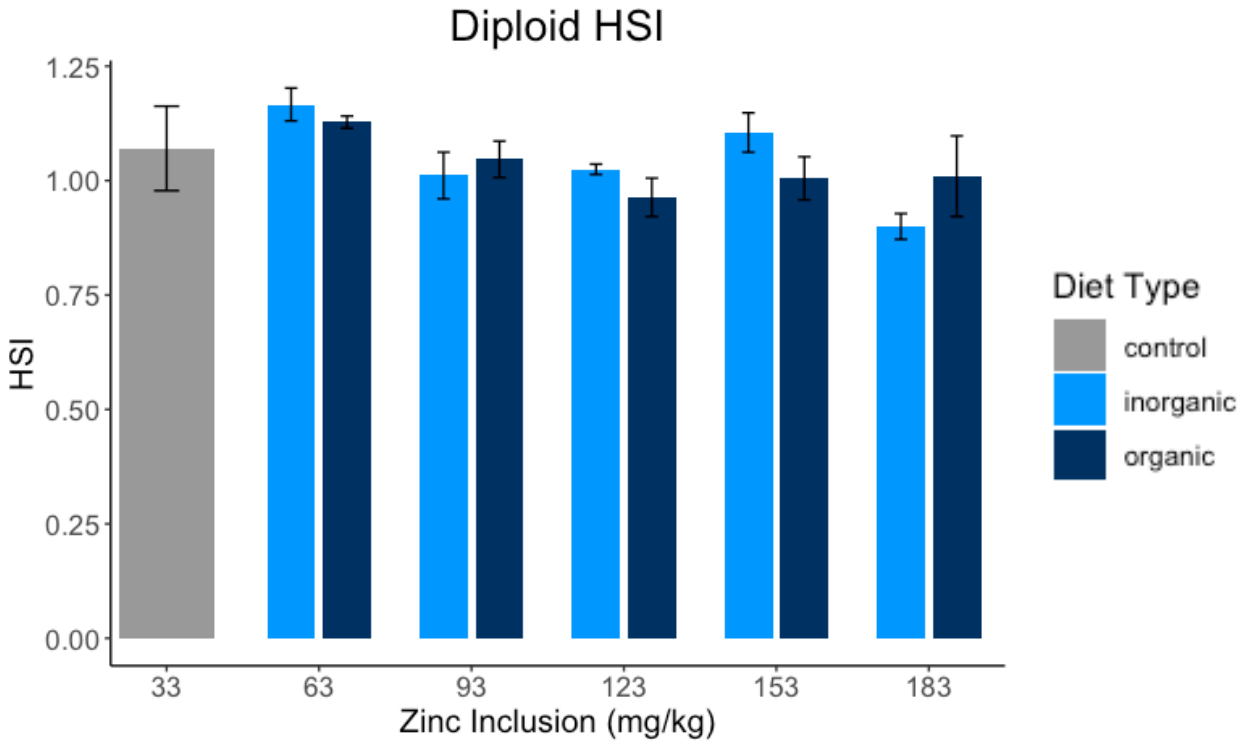


Figure 2.2 Hepatosomatic index (HSI) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in a bar graph (N=3; mean ± standard error). No significant interaction was found, but the main effect of dose was significant ($p<0.001$).

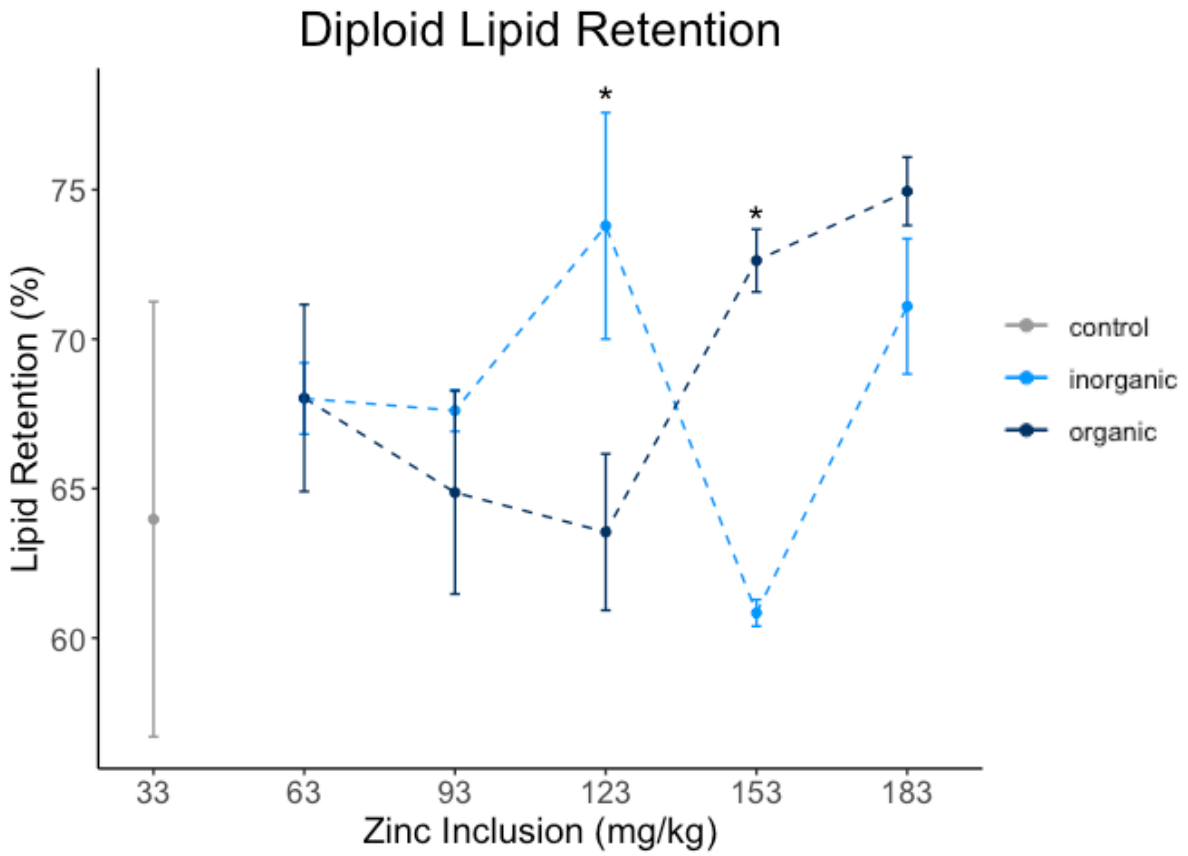


Figure 2.3 Lipid retention (%) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed ($p<0.001$; dose: $p=0.001$ and zinc type: $p=0.996$).

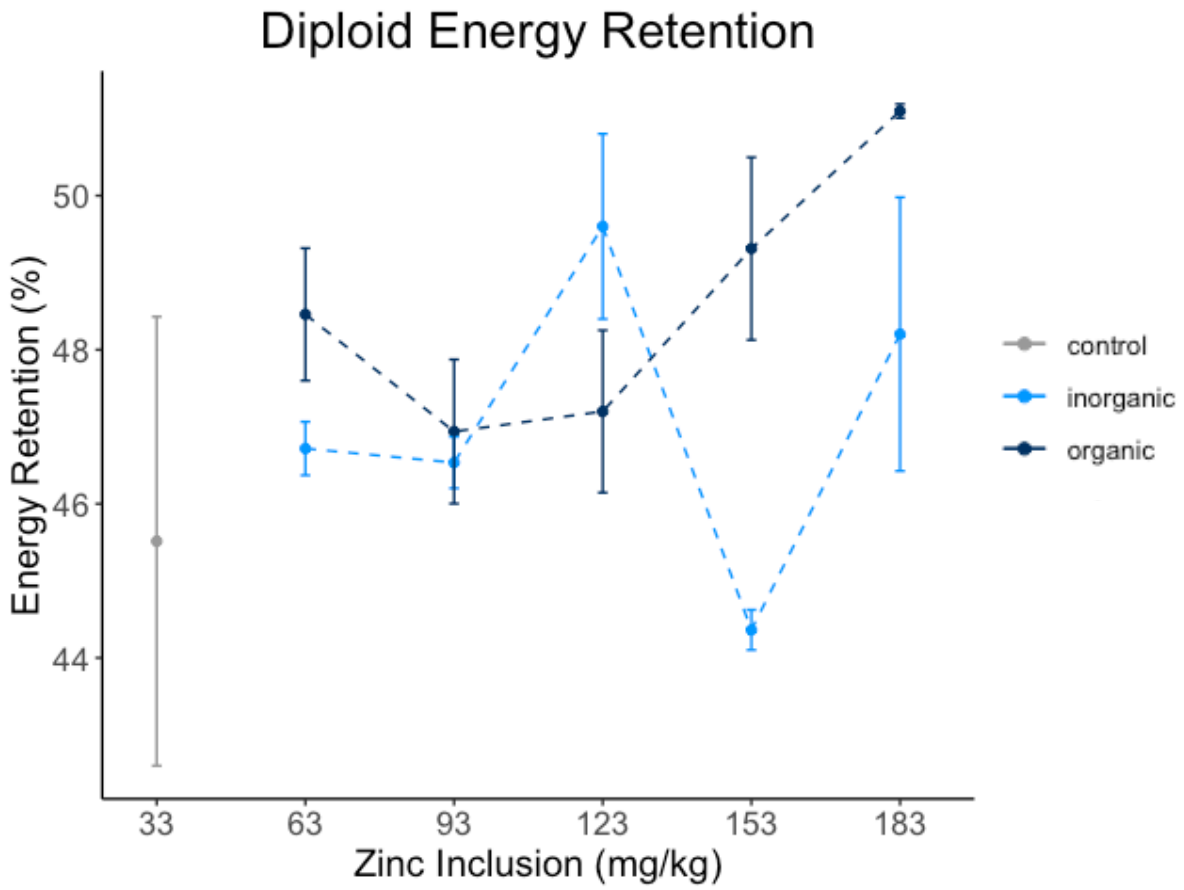


Figure 2.4 Energy retention (%) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed (p=0.025; dose: p=0.023 and zinc type: p=0.290).

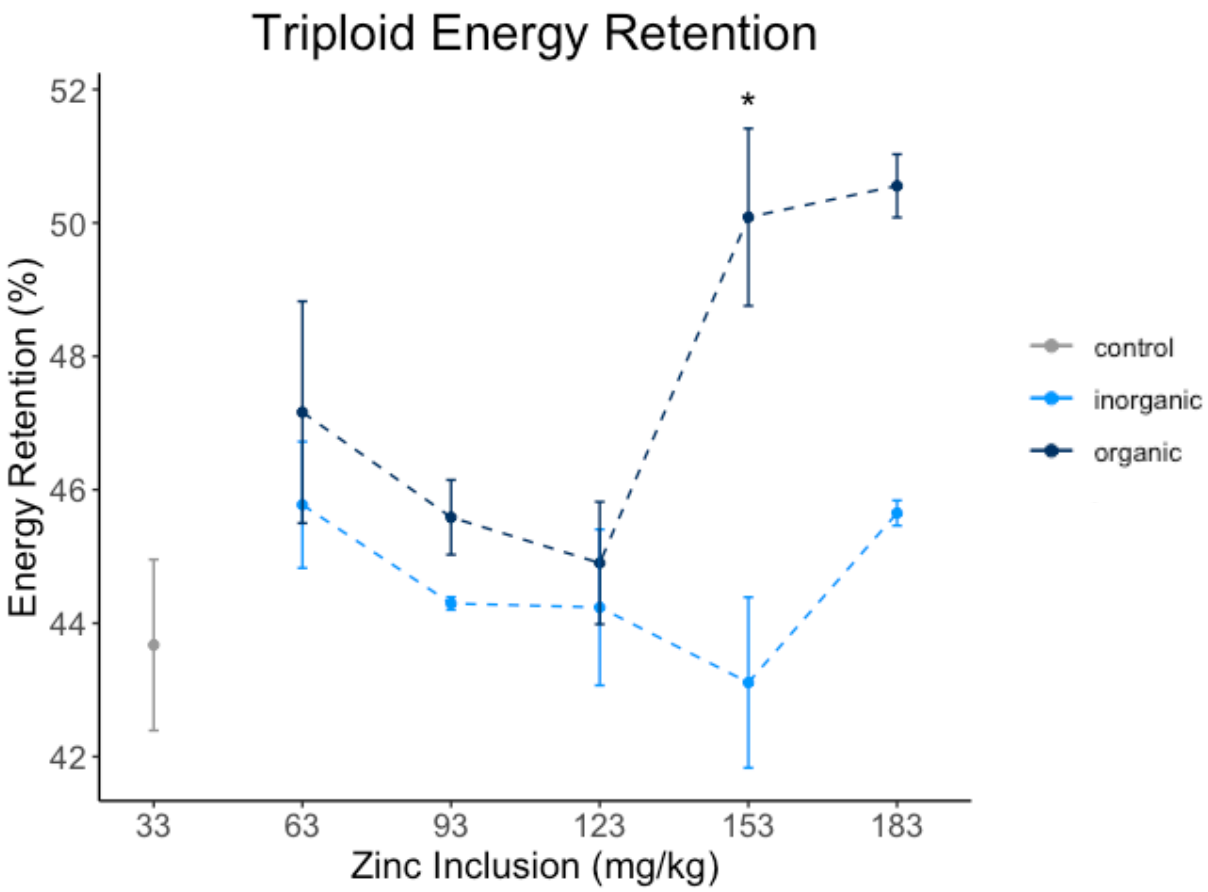


Figure 2.5 Energy retention (%) of triploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed (p=0.036; dose: p=0.504 and zinc type: p=0.420).

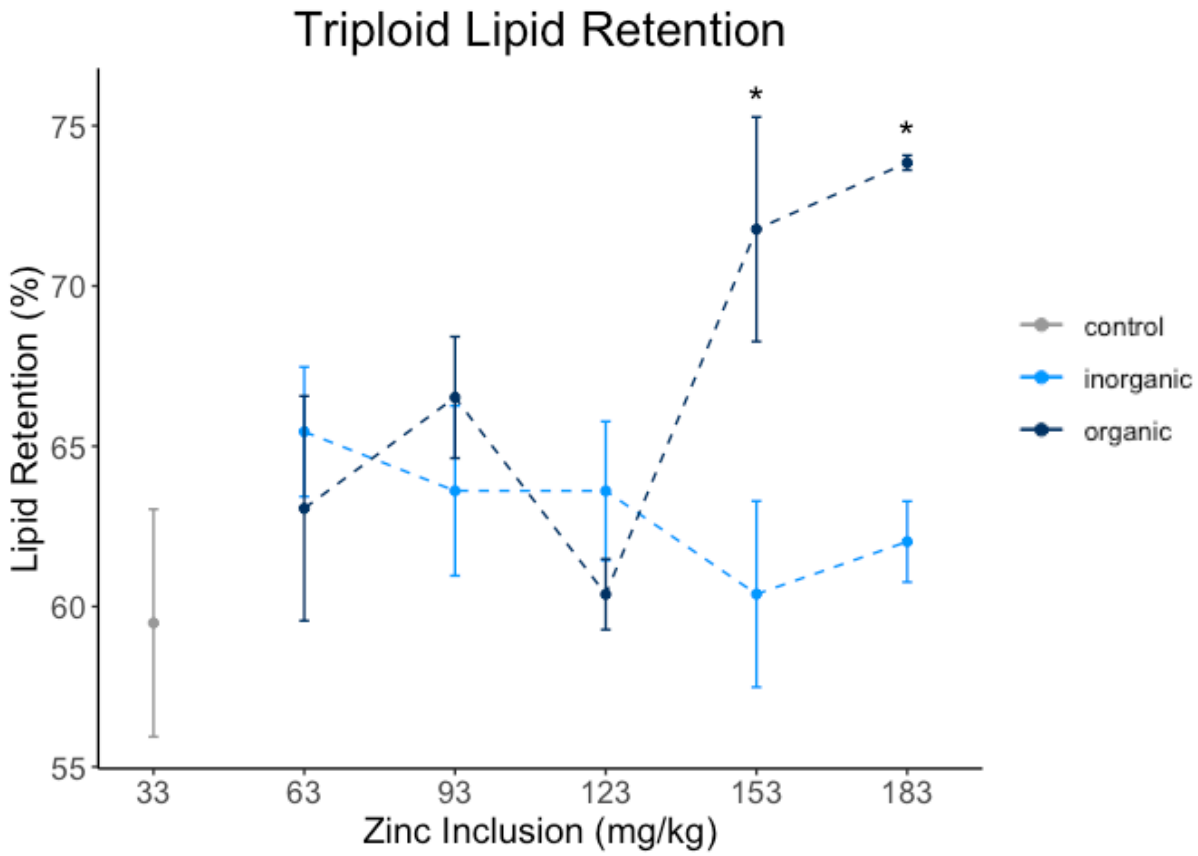


Figure 2.6 Lipid retention (%) of triploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed ($p<0.001$; dose: $p=0.621$ and zinc type: $p=0.472$).

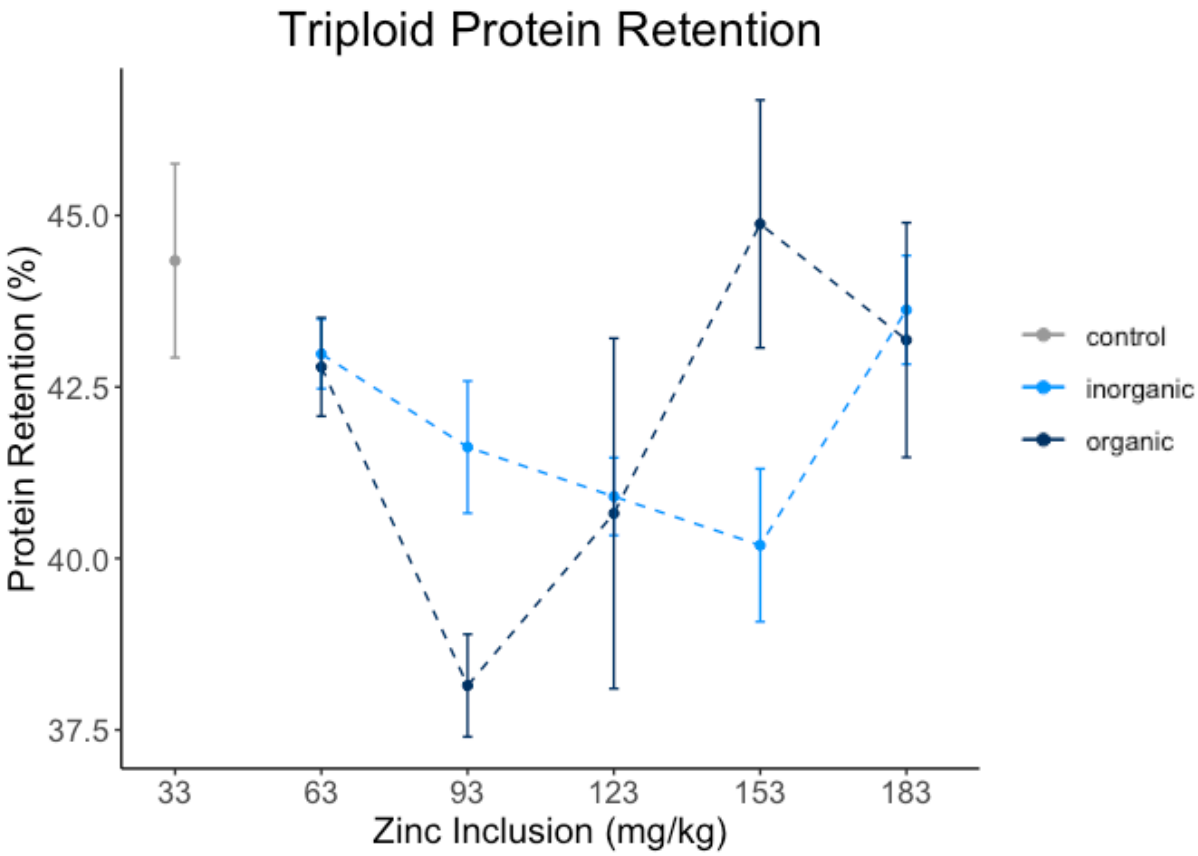


Figure 2.7 Protein retention (%) of triploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed (p=0.041; dose: p=0.316 and zinc type: p=0.919).

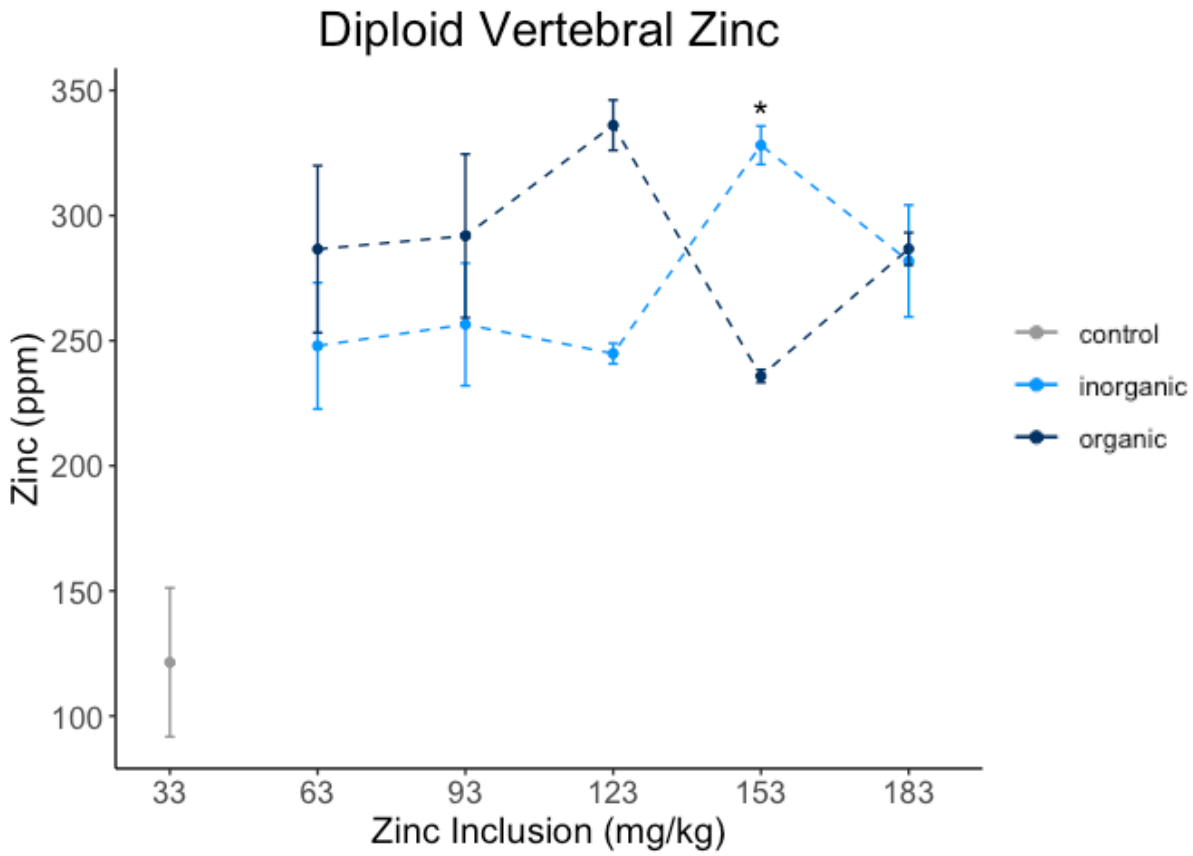


Figure 2.8 Vertebral zinc content (ppm) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed ($p<0.001$; dose: $p=0.025$ and zinc type: $p=0.188$).

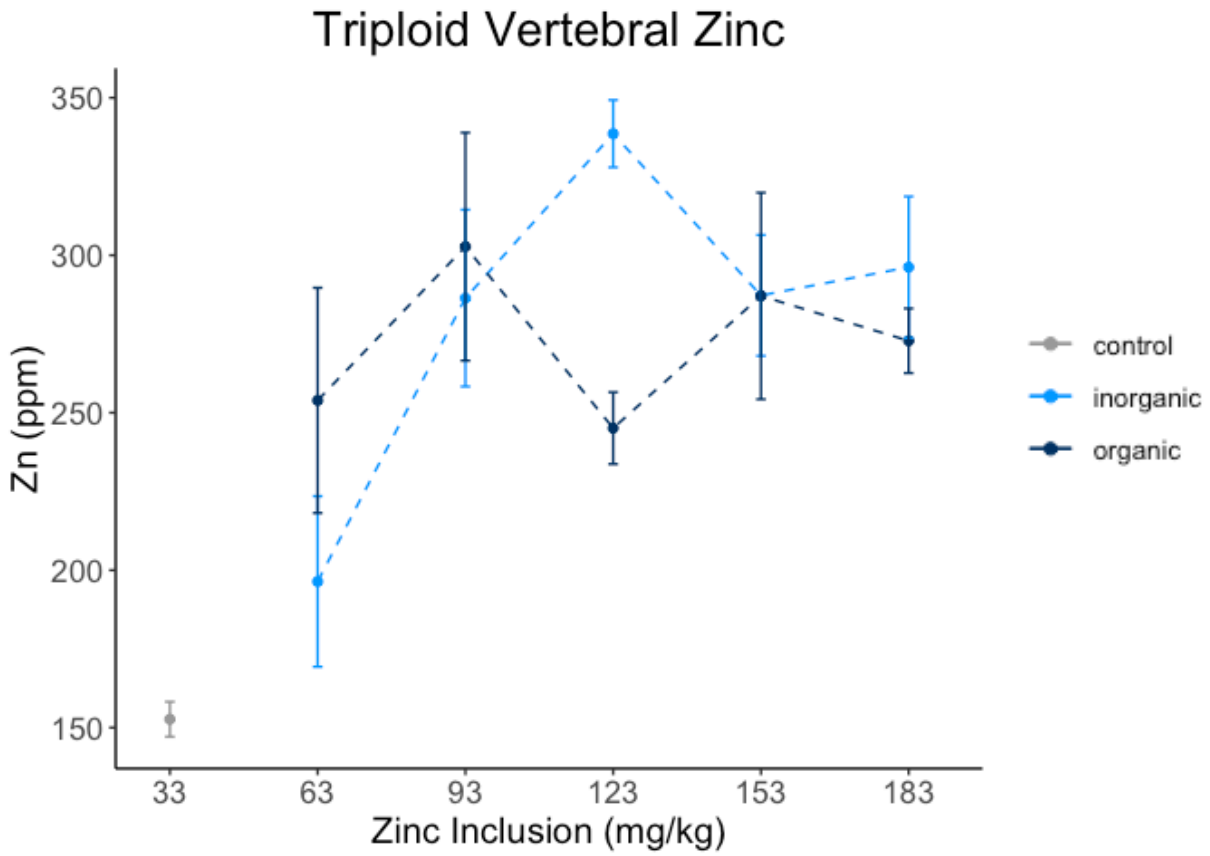


Figure 2.9 Vertebral zinc content (ppm) of triploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed (p=0.045; dose: p=0.002 and zinc type: p=0.108).

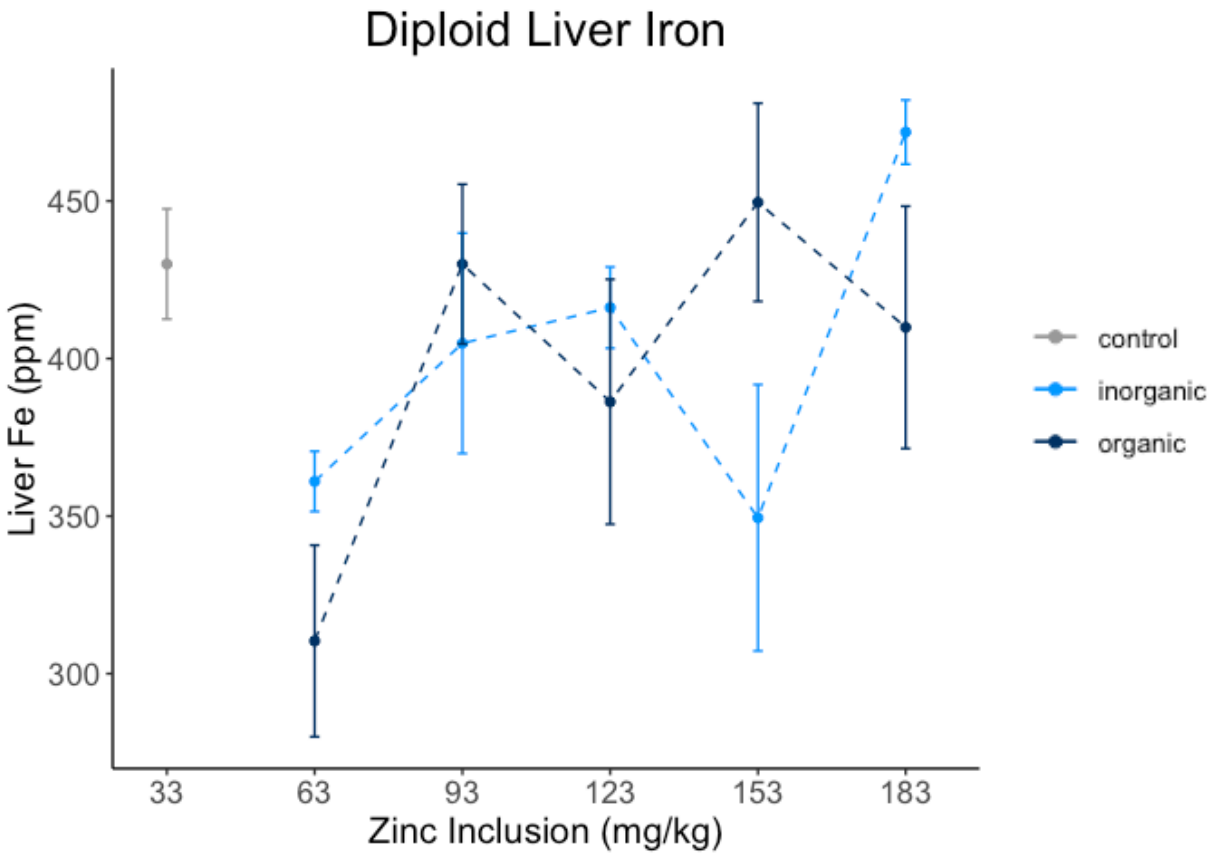


Figure 2.10 Hepatic iron content (ppm) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed (p=0.040; dose: p=0.030 and zinc type: p=0.230).

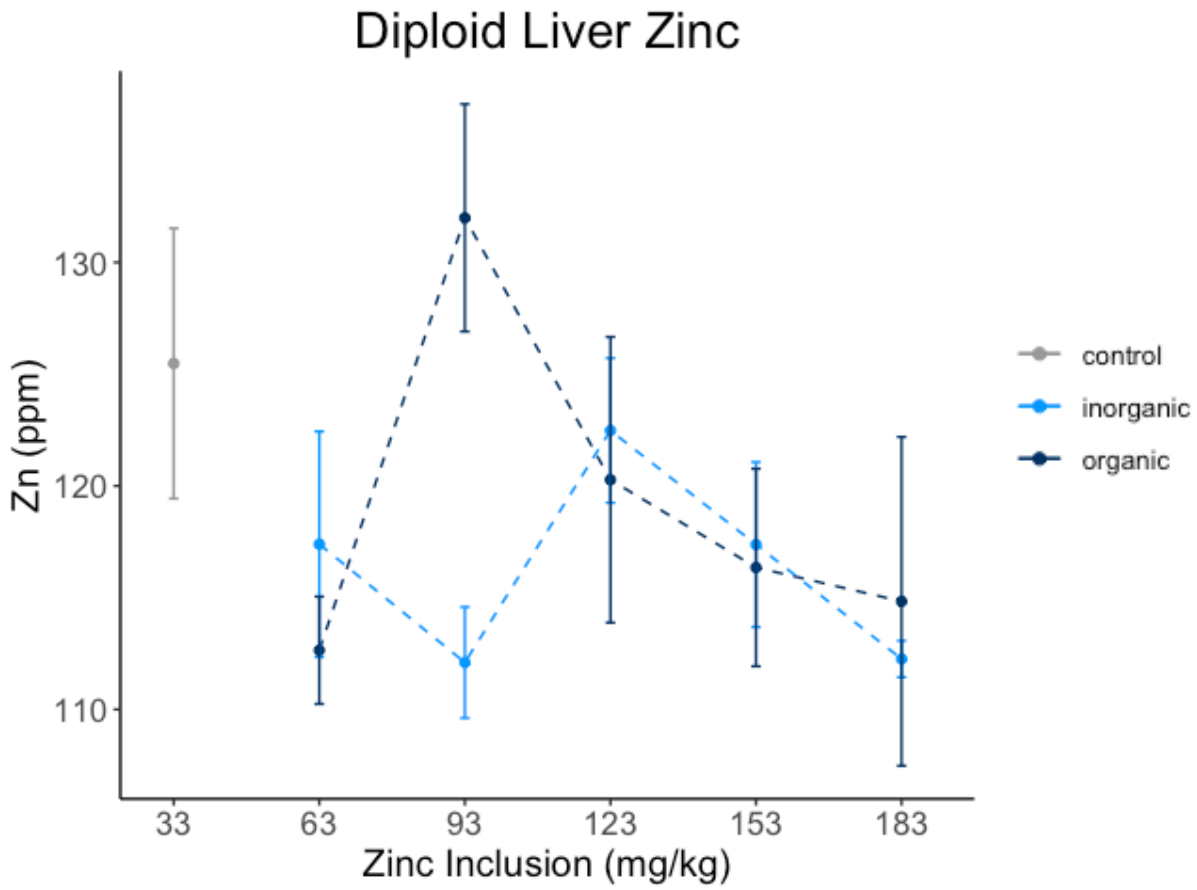


Figure 2.11 Hepatic zinc content (ppm) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in an interaction plot between zinc type and dose in mg kg^{-1} (N=3; mean ± standard error). A significant interaction between type of zinc and dose was observed (p=0.048; dose: p=0.451 and zinc type: p=0.456).

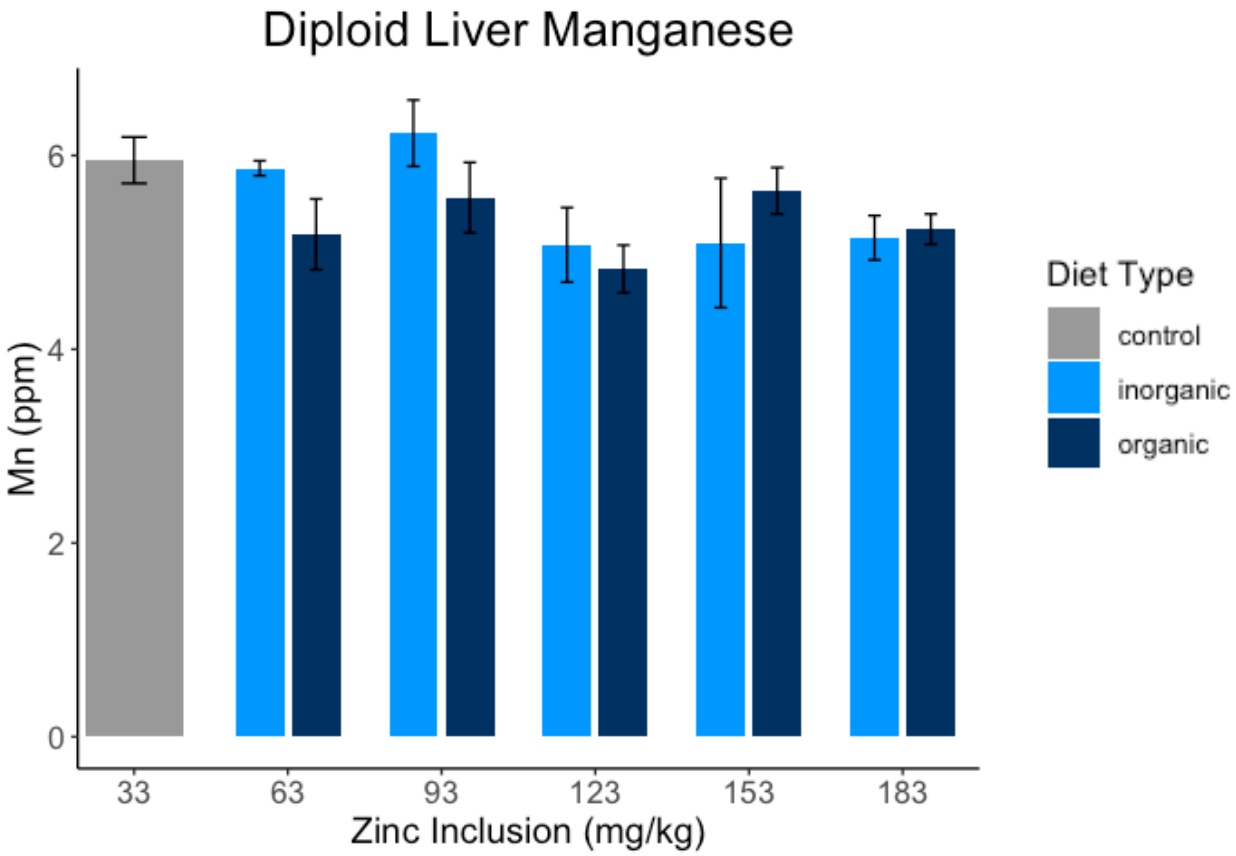


Figure 2.12 Hepatic manganese content (ppm) of diploid Rainbow Trout fed with organic and inorganic based diets. Data presented in a bar graph (N=3; mean ± standard error). No significant interaction was found, but the main effect of dose of zinc was significant (p=0.048).

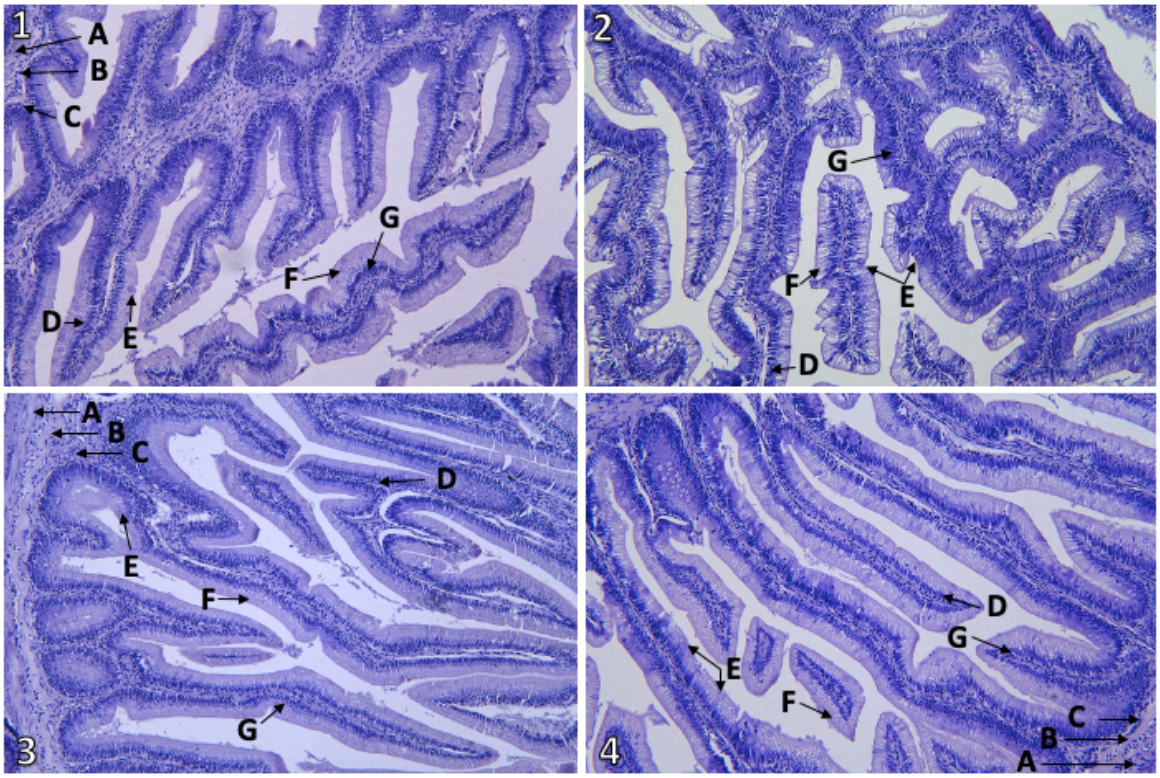
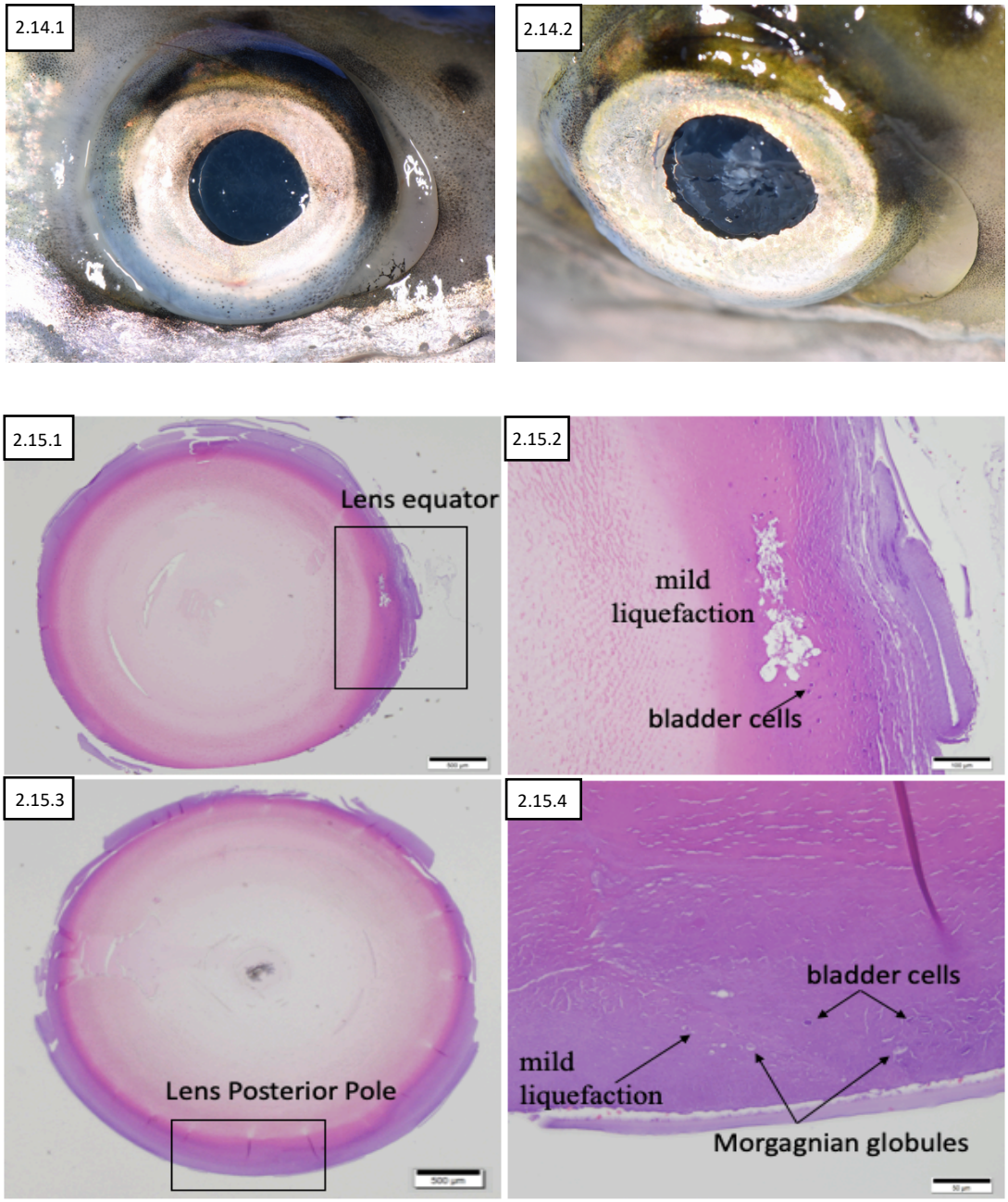


Figure 2.13.1-4 Distal intestinal histology (magnification: 200x) for diploid (2.13.1) and triploid (2.13.2) fish fed the control diet (Zn_{33}) and diploid (2.13.3) and triploid (2.13.4) fish fed the highest inclusion level of organic zinc (Zn_{183}). There were no significant differences or trends between treatments. Letters represent the serosa, muscularis, submucosa, lamina propria, goblet cells, absorptive vacuoles, and epithelial layers.



Figures 2.14.1-2 and 2.15.1-4 Photographs of a normal/control fish eye (2.14.1) and the right eye of a cataractous fish showing typical opacity (2.14.2). Lens histology of the right and left eye of diploid fish fed organic Zn_{63} which developed bilateral incipient cataracts Figures 2.15.1-4 shows the lens histology of the right and left eye of the cataractous fish. Signs of typical morgagnian cataracts (liquefaction, bladder cells, and morgagnian globules) are labeled.

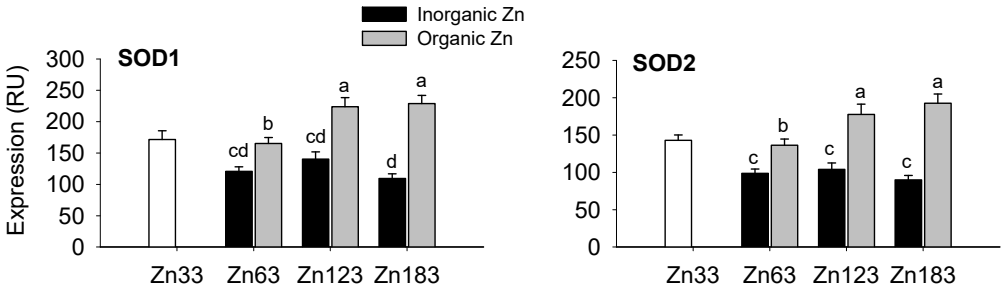


Figure 3.1 Hepatic gene expression patterns of superoxide dismutase (*SOD1* and *SOD2*). An interaction between type of zinc and dose was observed for both *SOD1* (p=0.004) and *SOD2* (p=0.004).

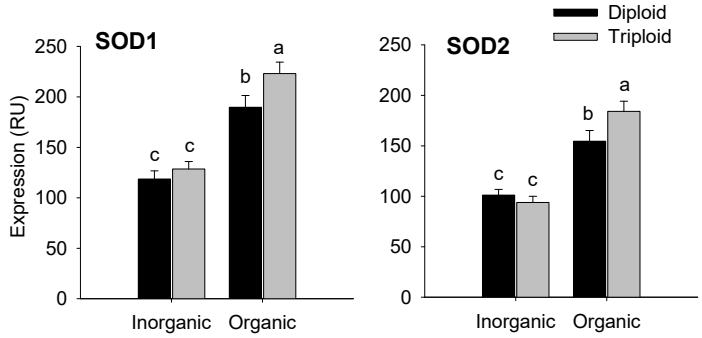


Figure 3.2 Hepatic gene expression patterns of superoxide dismutase (*SOD1* and *SOD2*). An interaction between ploidy and type of zinc was observed for both *SOD1* (p=0.012) and *SOD2* (p=0.015).

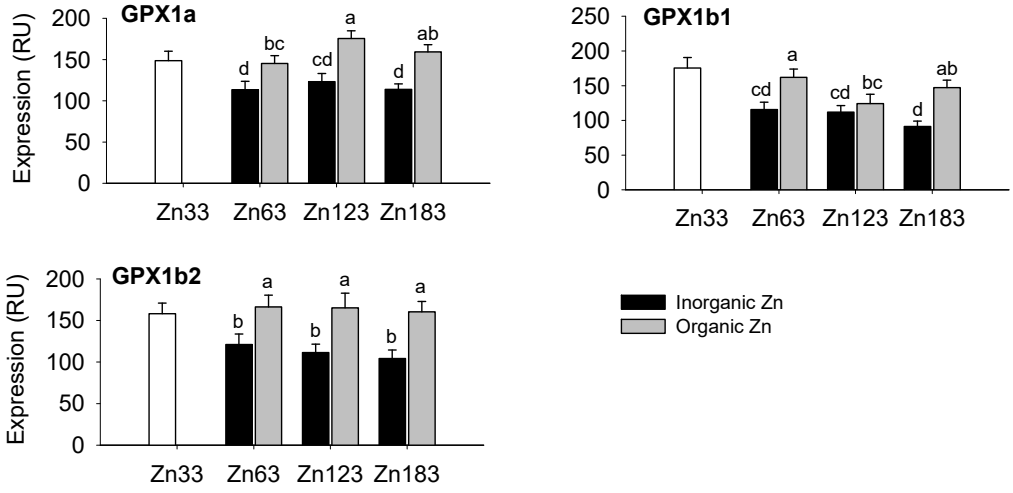


Figure 3.3 Hepatic gene expression patterns of glutathione peroxidase (*GPX1a*, *GPX1b1*, and *GPX1b2*). An interaction between of type of zinc and dose was observed for all three genes; $p<0.001$.

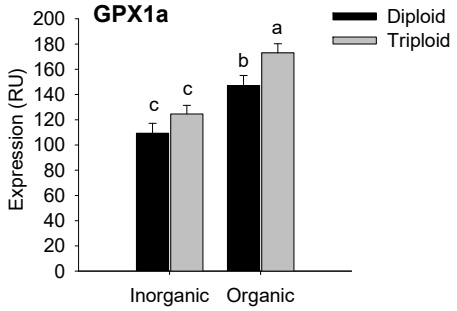


Figure 3.4 Hepatic gene expression patterns of glutathione peroxidase (*GPX1a*). No significant interaction effect was seen, but main effects of ploidy ($p=0.005$) and zinc type ($p<0.001$) were significant.

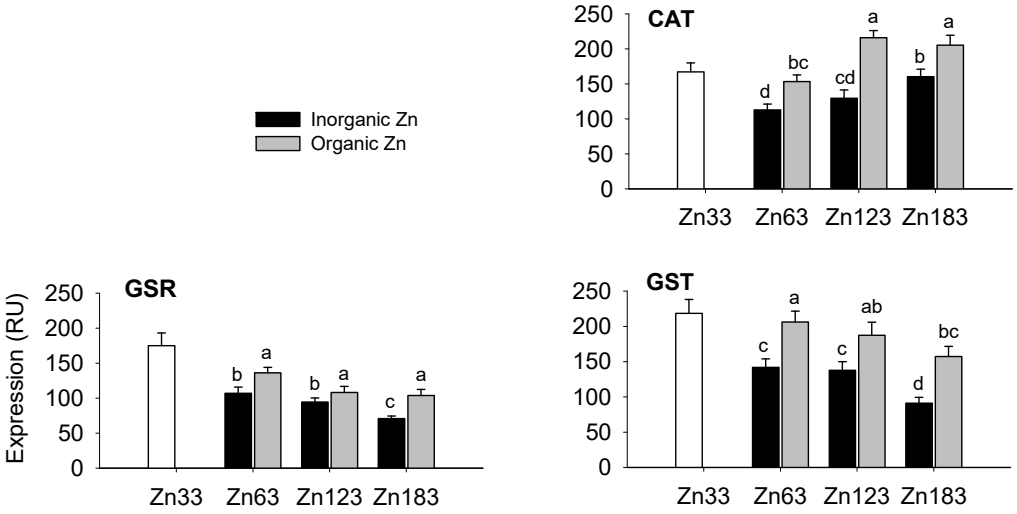


Figure 3.5 Hepatic gene expression patterns of catalase (*CAT*), glutathione reductase (*GSR*), and glutathione S-transferase (*GST*). A significant effect of type of zinc was seen for all three genes; p<0.001 for all. A significant effect of dose was all seen for all three genes; p<0.001 for *CAT* and *GSR*, p=0.002 for *GST*.

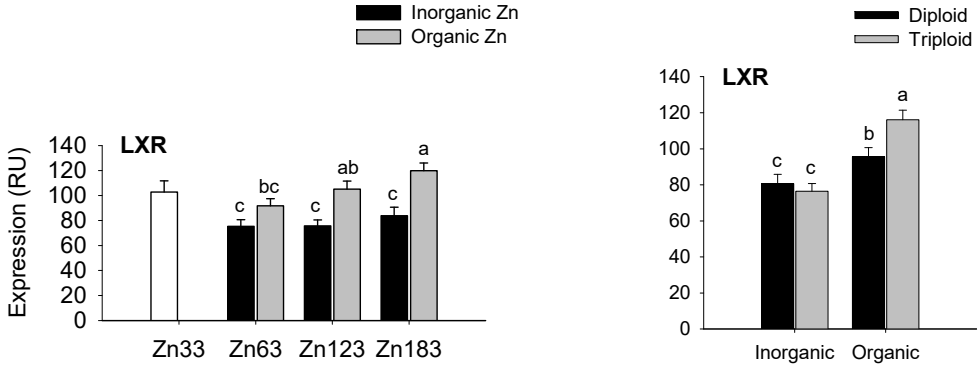


Figure 3.6 Hepatic gene expression patterns of liver X receptor (*LXR*). There was not a significant interaction between dose and zinc type but both main effects of dose (p=0.007) and zinc type (p<0.001) were significant. A significant interaction effect of ploidy and zinc type was all seen (p=0.009).

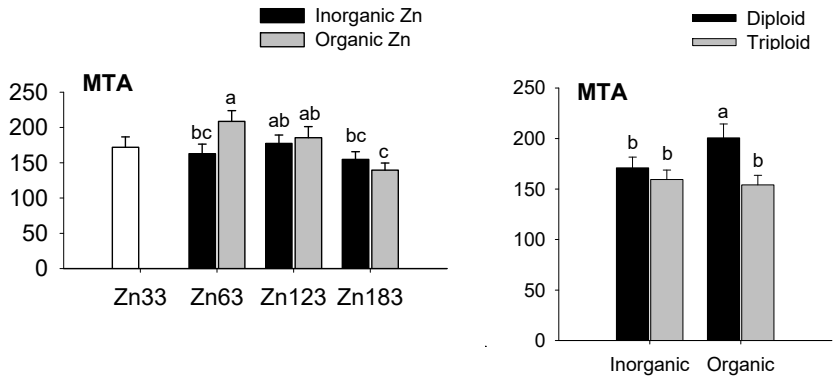


Figure 3.7 Hepatic gene expression patterns of metallothionein-A (*MTA*) with interactions of dose and type of zinc as well as ploidy and type of zinc. Significant main effects of dose (p=0.004) and ploidy (p=0.005) were observed.

Appendices

University of Idaho
Institutional Animal Care and Use Committee

Date: November 02, 2018
To: Vikas Kumar
From: University of Idaho
Institutional Animal Care and Use Committee
Re: IACUC-2018-60 *Dietary requirements and metabolic roles of organic and inorganic mineral (Zinc) in a commercial strain of Rainbow trout*

Your animal care and use protocol for the project shown above was reviewed and approved by the Institutional Animal Care and Use Committee on 11/02/2018.

The original approval date for this protocol is: 11/02/2018
This approval will remain in effect until: 11/01/2019
The protocol may be continued by annual updates until: 11/01/2021

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Craig McGowan, IACUC Chair