ANTIOXIDANT PROTEIN 1, ATPASE COPPER TRANSPORTING ALPHA POLYPEPTIDE, AND COPPER TRANSPORTER 1 mRNA TISSUE DISTRIBUTION AND INTESTINAL ONTOLOGY BASED ON DEVELOPMENTAL STAGE IN BROILER CHICKENS.

by

JAMES ANDREW WILSON

(Under the Direction of Adam J. Davis)

ABSTRACT

Although copper is an essential micronutrient for poultry that serves as a critical cofactor for many enzymes involved in energy metabolism, the neutralization of free-radicals, tissue development and neuroendocrine function, very little research has been conducted on the proteins involved in cellular absorption and transport of copper in poultry. Based on mammalian research, antioxidant protein 1 (ATOX1), ATPase copper transporting alpha polypeptide (ATP7A), and copper transporter 1 (CTR1), are three critical proteins involved in copper absorption and utilization. The goal of the current research was to determine the mRNA expression of these 3 copper transport proteins in chicken digestive, reproductive, and muscle tissues, and to determine if ileum expression varied in broilers at 0, 14 and 42 days of age. Total RNA was extracted from broiler, broiler breeder hen and broiler breeder rooster tissues. Extracted RNA samples were DNase treated and then prepared for 2-step Real Time RT-PCR analysis for ATOX1, ATP7A, CTR1, and glyceraldehyde 3-phosphate dehydrogenase (control) mRNA expression using TaqMan minor groove binding probes and primers designed for each

gene. Expression of ATOX1, ATP7A and CTR1 mRNA was detected in all tissues examined and each was individually expressed equivalently in the duodenum, jejunum and ileum. The expression of CTR1 mRNA was significantly greater ($P < 0.05$) in ileum tissue of day of hatch broilers than 42 day old broilers. The expression of ATOX1, ATP7A did not differ in ileum samples collected from broilers at day 0, 14 and 42 days of age. In immature testes the expression ATOX1, ATP7A, and CTR1 was significantly greater than in mature testes. Similarly, the mRNA expression of ATP7A and CTR1 was greater in granulosa cells of large white follicles than in granulosa or theca cells of the three largest most mature follicles. The results suggest as previously seen in mammals, that ATOX1, ATP7A and CTR1 play a critical role in the absorption, transport and utilization of copper in chicken tissues. In addition, the results suggest that further research is needed to determine the potential importance of enhanced copper utilization by immature testes and undeveloped follicles relative to mature testes and follicles.

INDEX WORDS: testes, granulosa, theca, broiler breeder

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DEDICATION

It is with the utmost sincerity that I dedicate this dissertation to my loving wife Madison, without your love and support I never would have been able to complete this stage of life. I would also like to thank my amazing parents who have always pushed me to further myself in all aspects of life, and lastly but certainly not least I want to thank my heavenly Father for completing this chapter of my life.

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CHAPTER 1

INTRODUCTION

1.1 COPPER USE IN POULTRY

In the 1920's copper was deemed necessary for animal physiology as a mineral required for hemoglobin synthesis as reviewed by Leeson (2009). Since then, research has identified copper as a cofactor for numerous enzymes and its necessity for many physiological functions in animal species. Thus, dietary supplementation of this essential nutrient provides a range of benefits to avian species. The first and most obvious example is through general performance parameters of poultry such as growth rate or egg production. The poultry National Research Council (NRC, 1994) recommended feeding broilers 8 mg/kg and laying pullets between 4-5 mg/kg, but they also indicated that sufficient research data was not available, such as research for the copper requirement in laying hens beyond 18 weeks of age, to allow solid and complete recommendations for poultry production. Because the poultry NRC requirements have not been updated since 1994, and because of the lack of research for the original recommendations, dietary copper supplementation for poultry is often at much higher levels in commercial settings. Leeson (2009) stated that the average broiler diet contained nearly 150% of the NRC requirements. Dozier et al. (2003) estimated that broiler diets are supplemented at even higher levels, with typical diets containing 125 mg/kg of copper. These authors indicate the reasoning for the industry's extraneous addition of dietary copper is due to the belief that high levels of copper will provide additional growth and performance benefits in poultry. For the use of copper sulfate, the most widely utilized copper source, a therapeutic dose is considered to be between

125-250 mg/kg, which can elicit growth promotion and antibacterial effects (Forouzandeh et al., 2021).

Countries within the European Union are limited to the amount of copper that can be fed to livestock. According to the European Food Safety Authority (2012), the maximum content a complete feed can have is no greater than 25 mg/kg of copper, which limits producers in these countries from the potential benefits obtained from feeding therapeutic levels of dietary copper. The limitations of copper usage within the European Union were set based on the potential for copper pollution within the environment. Through the use of predictive modeling, Monteiro et al. (2010) found that manure application on topsoil could have lasting effects for decades to centuries. Within their study, they discovered that applied copper would remain as a component of the topsoil for decades and would slowly accumulate with each subsequent application of livestock manure. The results of this study emphasized the need for the reduction of dietary copper levels in food animal production to slow or prevent the further accumulation of copper within the topsoil, since copper can require centuries for natural remediation (Monteiro et al., 2010). Dozier et al. (2003) found that a reduction in dietary copper in poultry from as little as 12 mg/kg to 4 mg/kg can reduce copper excretion by 35%, further strengthening the European Union's argument for the benefits of decreasing copper in food animal production diets.
This being said, Cobb-Vantress only recommends 15 mg/kg of copper in broilers and 10-15 mg/kg in breeders to reach peak performance (Cobb-Vantress, 2018; Cobb Vantress, 2019). However, when broilers have been fed higher levels of copper from various sources within this therapeutic zone (125-250 mg/kg), improved growth rates have sometimes been seen when compared to similar treatments fed lower copper amounts (Skrivan et al. 2000, Forouzandeh et al. 2021). Arias and Koutsos (2006) saw improvements in growth when feeding 188 mg/kg of

copper as CuSO4 or TriBasic Copper Chloride (TBCC), but these differences were only seen when birds were raised on recycled litter. Thus, one explanation for the discrepancy between obtaining normal growth in broilers fed dietary copper levels near NRC recommendations versus therapeutic levels is that higher dietary copper concentrations provide benefits to broiler performance only during times of stress, such as when birds are challenged from the microbes present in recycled litter.

When graded levels of copper were fed to rats, it could be seen how copper played a role in the development of bone structure (Roughead and Lukaski, 2003). These researchers discovered that dietary copper levels were the primary determinant of blood insulin-like growth factor-1 (IGF-1) concentrations in their study, with high copper producing the greatest levels IGF-1 in the serum. Additionally, it was found that the same high copper treatments resulted in increased bone weight, density, and strength in the rats. Roughead and Lukaski (2003) hypothesized that the increased IGF-1 from feeding higher dietary copper levels, was the primary reason for the increased bone mass in the rats. If this trend is similarly applicable in avian species, the inclusion of increased dietary copper levels could provide better bone health in fast growing broilers or helping prevent cage layer fatigue in hens.

The copper recommendations for laying hens and broiler breeders are not well researched with the NRC (1994) only providing recommendations for laying hen pullets through 18 weeks of life. When graded levels of copper were fed to breeder hens from 3.5 up to 83.5 (mg/kg), no changes were seen in the amount of egg production or egg weight across the 6 treatments (Gou et al., 2020). Based on the numerous factors that were investigated such as; shell strength, shell thickness, yolk color score, Haugh unit, hatchability, chick weights, and antioxidant parameters, the recommended copper level for breeder hens established by this research was between 15.7

and 21.2 mg/kg. Berwanger et al. (2017) completed a similar experiment, but began with copper deficient hens at 24 weeks of age. The researchers concluded that copper supplementation of 12.5 mg/kg was sufficient to alleviate deficiency symptoms and provide the best sum of results in egg production, hen blood parameters, egg composition, and chick health parameters.

In copper deficient hens, the first signs of deficiency are generally associated with abnormal formation of eggs. Baumgartner et al. (1978) examined the effects of feeding 16-week old laying hens a diet supplemented with only 0.72 mg/kg of copper. Despite being fully calcified, eggs produced by these hens became blunted in shape on the pointed end, lost their pink hue, and had a wrinkled appearance. It was determined that the causative agent was malformation of the shell membrane, which provided a poor framework for normal calcification. Nys et al. (2004) also confirmed that proper formation of the shell membrane is necessary to provide a scaffold for normal calcification of the shell, and without sufficient dietary copper the shell membrane lacks the proper collagen crosslinking for normal formation and integrity of the egg shell membranes. Physical alterations between normal and copper deficient eggshell membranes were able to be visually captured under scanning electron microscope inspection (Baumgartner et al., 1978). The mammillary layer normally contains a high density of fibers, knots, and nodules within the crisscrossing filaments of the shell membranes, but these crosslinks are scarcely seen in the case of the eggs produced by copper deficient hens. These crosslinks also provide a rigidity against turgor pressures during egg development (Baumgartner et al., 1978). Eggs from copper deficient hens were found to have increased weights, but upon egg composition measurements, the only fraction that was different was that of water. Baumgartner et al. (1978) hypothesized that the malformed shell membranes were unable to

resist the inflow of water during development resulting in heavier eggs as well as thinner shells due to the increased surface area of the egg.

The NRC recommendations have not been updated since 1994, and the research that was conducted to achieve the various recommendations was limited in their scope of copper sources to develop the requirements in poultry. Further research implies that copper may need to be supplemented at levels above the NRC recommendations since the copper level needed for cessation of deficiency symptoms may be lower than the level needed for optimal proper growth of modern poultry strains. While increasing levels of copper appear to produce increased performance in poultry, environmental concerns of copper excretion begin to come into effect when setting an optimal level for copper supplementation in food animal diets.

1.2 COPPER AT THERAPEUTIC LEVELS

Unfortunately, copper can also be a highly toxic compound in animal tissues if ingested in excess quantities. Because of copper's unique ability to easily change its oxidative state, it is also known to catalyze surrounding substrates to create harmful hydroxyl radicals and other reactive oxygen species in cells (Oguz et al., 2014). Goff (2018) even characterizes copper as potentially one of the most toxic required minerals because of the relatively small 4-to-10-fold difference between what is required and what causes harm in organisms. Chiou et al. (1999) began to see differences in broilers when copper levels as low as 100 mg/kg was fed. In their experiment, the duodenal muscle wall thickened numerically for 100 mg/kg and significantly for the 250 and 500 mg/kg treatments. Intake of dietary copper levels at 250 mg/kg began to cause oral lesions affecting the mouth, tongue and pharynx, while ingestion of the 500 mg/kg diet resulted in gizzard erosion (Chiou et al., 1999). Mehring et al. (1960) examined graded levels of copper supplementation beginning at 16 mg/kg and increasing up to 1180 mg/kg across twelve

treatments. In their investigation, they found that drastic reductions in body weight began with the 500 mg/kg level, and this closely aligned with the data from Chiou et al., (1999) who also reported that a dietary copper level of 500 mg/kg significantly depressed growth.

With the risk of copper toxicity and research indicating that the requirements for copper supplementation are low, why are therapeutic levels of copper added to production animal diets? One of the primary reasons for supplementing livestock with extraneous amounts of copper higher than what has been established as the minimal requirement, is the increased growth when compared to diets that provide only the amounts to prevent deficiency symptoms. It is suspected that the growth promoting effects of therapeutic levels of dietary copper result from similar mechanisms by which antibiotic growth promoters (AGP) can positively influence growth in some circumstances. With mounting pressures from consumers to end the use of AGP and sub-therapeutic antibiotics in diets of livestock, there may be an increase in the incidence of enteric diseases throughout the poultry industry (Bortoluzzi et al., 2020). The specific mechanism of action for the effects of high copper diets may be from improving various aspects of immune function and limiting the proliferation of pathogenic bacteria in challenged animals. Growth efficiency is better in animals utilizing digested nutrients for maintenance and growth than in animals that have to divert nutrients from these functions to meet immunological challenges of pathogenic GI bacteria. Goff (2018) speculates that the required amount of copper to prevent deficiency may not be what is needed for optimal immune function and growth. When the effects of commensal bacterial involvement were investigated in rats, it was found that conventional rats produced spontaneous bactericidal plaques against E. coli, increased activation and differentiation of B-cells, and increased homing of mesenteric node cells for mucosal surfaces when compared to rats raised under germfree conditions (Tlaskalova-Hogenova et al.1983). It

could be seen that a healthy population of nonpathogenic bacterium stimulated healthy immune responses of the host against infection.

When investigating how copper effects bacterium, Tong et al. (2005) found that copper utilizes its oxidative ability to disrupt bacterial membranes. Specifically, the investigators found that copper treatment of *E. coli* and *S. choleraesuis* resulted in damaged cell walls, vacuole development, nutrient leakage, concentrated cytoplasm, and an increased gap between the membrane and cell wall of the bacterium. The physical alterations induced by copper addition can be attributed to this divalent cation adhering to the negatively charged plasma membrane and slowly diffusing into the cell (Tong et al., 2005). This then leads to a change in cell permeability, resulting in the leakage of minerals and the degradation of the cells ion gradient (Tong et al., 2005). Additionally, once inside the bacterium copper will bind to sulfur containing compounds such as amino acids, which can disrupt protein structure and prevent their proper function (Tong et al., 2005). When copper treatment on bacterium's respiration pathways was investigated, an increase in respiration enzyme activity was seen by the addition of 10-30 mg/kg of copper but it was fully inhibited when 40-50 mg/kg was applied (Tong et al., 2005). Lastly, addition of the higher levels of copper to E. coli and S. choleraesuis cultures resulted in an increase in the number of intracellular enzymes that were detected outside of the cell (Tong et al., 2005). These findings suggest either a drastic change in the permeability of the bacterium cellular membrane or damage to the cell membrane resulting in cytoplasmic leakage of enzymes (Tong et al., 2005).

The antibacterial effects seen by Tong et al. (2005) are likely to be a mechanism of action that helps to improve the performance of animals fed therapeutic levels of dietary copper. When determining if there were differences in the growth promotive effects of high copper diets versus

AGPs, Weeks and Sullivan (1972) reported there was no difference in performance between a copper only, AGP only, or a copper and AGP supplemented diet (Weeks and Sullivan, 1972). The results of this experiment imply that copper acts in a similar fashion to AGPs. Therefore, if both are applied to a diet, no additional benefits should be seen since both provide antimicrobial protection in the GI system. Pang et al. (2009) fed challenged broilers a diet containing therapeutic levels of copper and reported that the high levels of dietary copper reduced both *E. coli* and *lactobacilli* populations in the Gi tract. Copper supplemented at 250 mg/kg was also seen to significantly reduce populations of *S. typhimurium* by 18% compared to inoculated broilers without copper addition (Leyva-Diaz et al., 2021).

While copper is known to possess bactericidal effects for some harmful GI bacteria, it has also been found to allow some commensal bacterial populations to flourish. Forouzandeh et al. (2021) discovered that when a dietary supplementation of 150 mg/kg of copper in the form of copper oxide was fed to necrotic enteritis challenged broilers, there was a significant increase in the alpha diversity indices of bacterial diversity and population homogeneity among bacterial families in the copper – supplemented birds relative to control birds not given the therapeutic dietary copper. Additionally, the investigators found that the primary families of bacteria that are known to be pathogenic were reduced in proliferation (*Strepotococcaceae*) while known commensal bacterial families were increased in prevalence i.e. *Enterococaccaceae*, *Peptostrptococcaceae*, and *Clostridiaceae* in the broilers given the therapeutic dietary copper supplement. It is these changes in bacterial populations that the researchers suspected resulted in the increased growth of the broilers fed 150 mg/kg of Cu_2O.

Another possible immunological effect of feeding therapeutic copper levels are changes in the levels of immunoglobulins and other immune system proteins. Wu et al. (2020) found

increased levels of IL-6, IgA, and IgG when copper levels higher than NRC (1994) recommendations were fed (9 vs 20-30 mg/kg) to broilers. The increase in IL-6 could result in drastic immune benefits as this is the primary cytokine for B lymphocyte maturation and growth (Wu et al., 2020). When low levels of copper were fed in turkeys (2 mg/kg), it resulted in decreased levels of ceruloplasmin, IgY and Il-6, again indicating the need for copper in proper immune function (Jankowski et al. 2019).

Copper has also been found to provide protection against bacterial strains that have current multi-drug antibiotic resistances (Benhalima et al., 2019). This antimicrobial effect of copper was further investigated to find that of the different copper sources tested, copper sulfate was the most effective at preventing bacterial proliferation. However, it should be noted that antimicrobial mineral resistance genes have risen within bacterial populations associated with animal production (Argudin et al., 2017).

Despite copper's potential toxic effects when fed in high levels to food animals, it would appear that it can also provide distinct benefits. With the decreasing popularity of AGP supplementation, there are fewer options to prevent the colonization and infection of pathogenic microbes in the intestinal tract. Furthermore, therapeutic copper supplementation may provide some level of relief from pathogenic bacteria by increasing immune function, reducing dietary pathogens, and diversifying commensal microbiota. However, caution should be advised with the addition of copper within therapeutic ranges, because although it can help to reduce pathogens, accumulated tissue/cellular copper can elicit oxidative stress in animals.

1.3 COPPER AND ANTIOXIDANT FUNCTION IN POULTRY

Because of copper's major involvement in both the protection from and potential generation of free radicals and other oxidative molecules, many researchers have investigated

how supplementing higher levels of copper will affect the antioxidant status of poultry. When dietary copper was supplemented between 2 and 20 mg/kg in turkeys, Jankowski et al. (2019) found few changes in antioxidant parameters within the blood. The lowest dietary treatment of copper increased the activity of super oxide dismutase (SOD) and glutathione peroxidase as well as the circulating levels of malondialdehyde (MDA). It was hypothesized that feeding 10-20 mg/kg of supplemental copper provided some level of antioxidant activity in the blood compared to feeding 2 mg/kg (Jankowski et al., 2019). When indices of oxidant activity were investigated in the liver, Jankowski et al. (2019) found that increasing dietary copper concentrations above that recommended by the NRC (1994) resulted in significantly elevated levels of SOD activity as well as decreased catalase activity and total glutathione amounts compared to the lower dietary levels. In conclusion, Jankowski et al. (2019) stated that both the high and low levels of copper resulted in increased oxidant activity, resulting in the best copper level for developing turkeys to be 10 mg/kg. Wu et al. (2020) conducted a similar experiment in broiler chickens with copper supplemented at 0, 10, or 20 mg/kg above the basal amount of 9 mg/kg. In this study, serum indices showed that supplemental copper provided increased antioxidant protection over that of the basal level copper alone. Significantly higher activity of ceruloplasmin, SOD, and glutathione peroxidase were found in supplemented diets implying better antioxidant activities than the basal copper level (Wu et al., 2020). Similar to the results of Wu et al. (2020), Ognik et al., (2018) found that as copper content increased above recommended levels, there was an increase in antioxidant enzymes providing better protection against Reactive Oxygen Species (ROS). When copper was supplemented to chicken embryos on day 1 of incubation, Oğuz et al. (2014) found major signs of oxidative damage to developed embryos at day 21 of incubation. Fifty μg of copper injected into the air sac of embryos resulted in significant reduction of brain

volume by day 21 based on the Cavalieri brain volume estimation method (Oğuz et al., 2014). Additionally, specific indicators of oxidative damage were found in the form of increased formation of MDA and decreased level of glutathione, showing that oxidative stress was indeed a major factor in the neurodegenerative brain development (Oğuz et al., 2014). Based on these results it can be seen that an essential amount of copper is needed to prevent oxidative damage within the body, but if there is an excess of copper ions it can begin to catalyze the development of ROS while the body attempts to increase production of storage proteins and other copper chaperones to prevent the oxidative damage.

The adaptability of copper to alter its ionic state makes it invaluable cofactor in enzymes throughout the body. However, copper's ease in transitioning between ionic states also allows for harmful oxidative processes to occur on unwanted targets if cellular copper levels reach excessive concentrations. Free ionic copper will induce the development of free radicals causing tissue damage throughout the body, but is also vital at lower regulated concentrations as a cofactor for proteins that neutralize ROS. Therefore, a fine balance between deficiency and toxicity is needed in order to maintain suitable copper homeostasis.

1.4 COPPER PROTEIN COMPLEXES

The first of the four major proteins that copper is intimately involved with, is found in nearly all cells in the body and is called cytochrome c oxidase. Utilizing copper's ability to easily switch between two different oxidation states, cytochrome c oxidase is responsible for the end step of cellular respiration. This process uses two copper atoms and two heme groups to successfully shuttle electrons from Krebs cycle products along with protons from the surrounding mitochondrial environment to reduce oxygen to form water (Linder 1991, Linder

and Hazegh-Azam 1996). Without the presence of copper this integral mitochondrial protein would not function, and the aerobic production of ATP would not be possible.

Next, copper can be found as a major component of super oxide dismutase (SOD) which utilizes minerals with easily changed oxidation states as the backbone for the dismutase function. Because of this, cells can produce copper/zinc, manganese, or iron forms of SOD for different situations (Linder and Hazegh-Azam 1996). Copper/zinc-SOD can be found in cells throughout the body and within cells it is located primarily in the cytoplasm, the nuclear matrix, and lysozymes. Within these regions it performs the integral task of neutralizing harmful ROS and thereby protecting the cell from oxidative damage (Linder 1991). Within the Cu/Zn-SOD protein, the copper ion is buried within in a highly hydrophilic tunnel and a charged O^{2-} superoxide anion will be channeled towards the positively charged copper ion (Linder 1991). Once the radical molecule reaches the copper active site, the cupric ion is reduced to a charge of +1 and the neutral O_2 is then released. When a second superoxide anion is presented to the SOD, it interacts with the now cuprous ion along with two protons to form hydrogen peroxide and revert SOD back to its original cupric state (Linder 1991; Surai 2016). Because of the protective nature of this metalloprotein, it is usually associated with tissues that produce high levels of oxygen radicals such as the liver, kidney, adrenal glands, and blood cells (Linder and Hazegh-Azam 1996; Linder 1991; Surai 2016). With the common production of superoxide anions as part of normal metabolism many different triggers serve to alter SOD expression. High levels of O_2, the presence of mineral ions, and increased superoxide radicals can all increase the production of SOD in tissues (Surai 2016). On the other hand, the primary depressant of SOD production is a deficiency of copper needed for the appropriate manufacture of the enzyme (Linder 1991).

As copper enters cells, it is quickly bound to a protein to prevent potential oxidative damage. One of the primary proteins that adhere to copper ions within the cell is metallothionein (MT). This metalloprotein is the major intracellular storage site for divalent metal ions, preventing their potential toxic effects from improper catalysis of cellular components (Linder and Hazegh-Azam 1996). Metallothionein is made up of a high number of cysteine molecules which aid directly in mineral binding and allowing MT to bind between eleven and twelve copper ions between the two domains of the protein (Linder 1991). The increased negative charge of MT caused by binding to these minerals is offset by the large number of lysine and other basic amino acids found within MT (Linder and Hazegh-Azam 1996). The expression of MT is upregulated when an increased amount of divalent metal ions such as cadmium, zinc, or copper, are found within the cell (Linder and Hazegh-Azam 1996). In addition, to its function as a divalent mineral storage protein, MT also confers some level of metalloenzyme ability within the cell. Similar to SOD, MT has some ability to scavenge O^{2-} radicals from the cell when carrying copper ions. Felix et al. (1993) investigated the mechanism and effectiveness of this "Cu(I)-thionein" enzyme to eliminate superoxide anions. It was concluded that Cu(I)-thionein could effectively eradicate O^{2-} radicals at the cost of converting a cuprous copper to a cupric form. Once converted to Cu^{2+} the copper dissociates from MT and is free in the cytosol to induce further MT synthesis. This could further explain the observation behind increased MT production during periods when the body is under oxidative stress.

Ceruloplasmin is another major copper containing protein, whose primary function is that of a copper shuttle throughout the body. Ceruloplasmin is predominantly produced and secreted by the liver, but, it has also been found to be produced in the uterus, testis, yolk sac, and brain (Linder 1991). It is the main transporter of copper within plasma (Linder 1991). As the intestine

absorbs copper from the diet, it is quickly taken up by the liver which binds copper to apoceruloplasmin during ceruloplasmin synthesis. It is the presence of ceruloplasmin that is necessary for the adequate distribution of copper to other tissues, as experiments have been conducted in aceruloplasminemia mice that indicate little copper uptake in the tissues of these mice despite plasma copper being available (Linder and Hazegh-Azam 1996). In humans, ceruloplasmin has been found to bind six copper ions (Linder 1991; Linder and Hazegh-Azam 1996), and although a ceruloplasmin is a highly conserved protein, in other species the amount of copper bound to ceruloplasmin varies with five copper ions bound in chickens and with as many as eight or nine copper ions bound in geese (Linder, 1991; Linder, 2016).

In addition to ceruloplasmin's primary function as a copper shuttle, it also participates in iron metabolism within the body. Ceruloplasmin is thought to act as a ferroxidase by converting Fe(II) to Fe(III) which is then available to be bound to transferrin and transported to other tissues (Frieden 1980). This was further investigated by Harris et al. (1999), which involved the development of aceruloplasminemia mice. In these transgenic mice it was found that the concentrations of iron and ferritin within the liver and spleen were three to six times higher compared to control mice. Additional experiments indicated that injection of ceruloplasmin into aceruloplasminemia mice led to an increase in iron in the serum, again pointing to the necessity of ceruloplasmin as a ferroxidase in iron metabolism and efflux from hepatocytes (Harris et al., 1999). When higher levels of copper were supplemented in broiler diets, decreased levels of hepatic iron were detected in these broilers compared to control-fed broilers (Wu et al., 2020). This reduction in hepatic liver iron was hypothesized by Wu et al. (2020) to be caused by an increased amount of ceruloplasmin production which allowed for additional release of Fe(II) from the liver.

The final function of ceruloplasmin is participating in some level of antioxidant protection through free radical scavenging. This is completed by ceruloplasmin catalyzing the oxidation of Fe(II), to quickly alter and reduce O_2 and four protons into water (Frieden 1980). The Fe(II) atom used can be a lone ion or part of a complex, and because of ceruloplasmin catalyzing the reaction, there is little chance for a radical oxygen or superoxide anion to be formed (Frieden 1980). It is surmised that this function of ceruloplasmin is why inflammatory hormones; IL-1, IL-6, TNF, increases the short-term expression of ceruloplasmin. With inflammation there will be increased numbers of immune cells which will produce ROS as an offense against pathogens within the body. As a result, it would be beneficial for the organism to increase production of radical scavengers to prevent self-inflicted oxidation of body cells during an immune response (Linder and Hazegh-Aam 1996).

In a smaller capacity, but not any less important, copper is also found to be a component of lysyl oxidase. This enzyme is an extremely important catalyst within the body, particularly during periods of growth and development. While this enzyme may not be as plentiful or contain as large a share of the bodies active copper pool compared to the four previously mentioned proteins, it still serves highly important functions within the body. This enzyme partakes in the catalyzing of cross-links in synthesized collagen and tropoelastin fibers. Without the presence and function of this enzyme, these important connective tissues would not be properly formed leading to poor tensile strength of the structure (Linder and Hazegh-Azam 1996). Lysyl oxidase functions by catalyzing an oxidative deamination which produces ammonia, peroxide, and the aminoadipic semialdehyde bond from lysine ε-amino sidechains of collagen and tropoelastin. This deamination reaction can potentially be catalyzed by other amine oxidases, but lysyl oxidase is found to have a high affinity for the lysine side chains of newly

produced collagen and elastin. In times of copper deficiency when lysyl oxidase activity is reduced, aortic aneurisms, spinal curvature, and defective alveoli can be seen from the improper foundation of connective tissue. Fortunately, in the event of copper deficiency, the activity of lysyl oxidase is quickly restored by reintroduction or increased supplementation of dietary copper (Linder 1991).

Another less abundant copper associated protein is dopamine-β-monooxygenase which is involved in the production of catecholamines that are required for neuronal transmissions. Dopamine-β-monooxygenase can be found in various parts of the brain, associated with noradrenergic receptors, or within the adrenal medulla coupled with chromaffin granules. Within the neurons, dopamine-β-monooxygenase catalyzes the reaction of hydroxyphenethylamine, or dopamine, with oxygen to produce norepinephrine and water (Linder 1991; Linder and Hazegh-Azam 1996). Norepinephrine can be converted to epinephrine by the enzyme phenylethanolamine *N*-methyltransferase. Thus, without dopamine-β-monooxygenase activity there would be no production of epinephrine or norepinephrine in the body. These crucial neurotransmitters and hormones are responsible for a host of diverse body functions such as cognitive ability, reproductive success, as well as regulating stress and blood pressure (Vendelboe et al., 2016).

The last of the copper containing proteins identified through copper deficiency research is tyrosinase or monophenol monooxygenase. This enzyme is responsible for the production of melanin in the body and is therefore associated with the melanosomes within melanocytes distributed in the skin, hair, and eyes (Linder 1991; Linder and Hazegh-Azam 1996). The specific distribution of melanocytes and by extension tyrosinase activity is determined by copper content of the body as well as by strict genetic patterns that determine body coloration as in the

case of zebras (Linder 1991; Sanchez-Ferrer et al., 1994). Tyrosinase converts tyrosine first into DOPA using half of an O_2 and then in step two converts DOPA to dopaquinone utilizing the second half of O_2 (Linder 1991; Sanchez-Ferrer et al., 1994). Dopaquinone undergoes several reactions to subsequently form melanin.

1.5 SUMMARY

As a cofactor for several essential enzymes, copper is essential for normal cellular respiration and structural integrity in animals. In domestic animal production, there typically is not enough copper present in the primary feed ingredients used to construct animal diets to meet the needed levels of copper for the optimal wellbeing and growth. Therefore, copper is added to animal diets in trace amounts to meet dietary requirements. In poultry production, it has become common to supplement copper to diets at rates that are 10 to 20 times more the NRC recommendation. The benefits of this is high level of copper supplementation is not proven and increases the potential of creating a copper toxicity in the animals fed these high levels of copper. Furthermore, when the feces generated by the birds fed excessive amounts of copper is applied to soil as a fertilizer, the high levels of copper accumulate in the soil and can cause toxicity issues for plants growing in this soil.

CHAPTER 2

COPPER ABSORPTION

2.1 MAMMALIAN COPPER TRANSPORT

The process of copper absorption begins within the stomach compartment of mammalian species. The acidic environment of the stomach and upper intestinal tract provides a vital environment for the start of copper digestion. As the acidity and initial digestion that occurs here allows copper that is complexed with other macromolecules in ingested food to dissociate and become free ionic copper (Crisponi et al., 2010; Goff, 2018; Olukosi et al., 2018). Once free, ionic copper can travel into the duodenum and the rest of the small intestine for absorption by enterocytes. Because of the positive ionic charge of copper and the large potential difference across the tight junctions of intestinal cells, copper is unable to diffuse freely from the lumen of the GI tract across the intestinal enterocytes and instead must be shuttled into enterocytes by copper specific transporters (Goff, 2018).

The predominant absorptive method for copper across the luminal membrane in mammals is through copper transporter 1 (CTR1), a membrane spanning transporter that exists as a homotrimer within cells (Crisponi et al., 2010; Hashimoto and Kimbe, 2015; Nose et al., 2006). To demonstrate the necessity of CTR1 in copper absorption, Nose et al. (2006) bred CTR1 knockout mice that do not express CTR1. The resulting mice showed drastic reduction of tissue copper levels, extreme weight loss, and 100% lethality compared to the control (Nose et al., 2006). However, conflicting reports about the exclusivity of CTR1 as the predominant and only luminal copper transporter have arisen. Zimnicka et al. (2011) suggest that copper uptake is

mediated by an anion transporter that is reliant on Cl^- ions for transport within the enterocyte. In Caco-2 intestinal cell line, Arredondo et al. (2003) reported that copper is able to utilize the Divalent Metal Transporter 1 (DMT1) to cross the luminal membrane. While this effect was seen during in vitro experiments, DMT1 is a nonspecific metal transporter which is prone to competitive inhibition of substrates which may lead to poor copper absorption by this transporter in vivo when other ions such as iron or zinc are present in the digesta.

Many different factors can affect the basolateral absorption of copper into the enterocyte such as dietary copper form, complexes within the GI system, or antagonistic molecules. The primary inhibitor of copper absorption are divalent ions such as zinc, iron or molybdenum (Crisponi et al., 2010; Goff, 2018; Lee et al., 2002; Leeson, 2009). Each of these minerals are able to exist in a divalent cationic state which allows them to be absorbed by the nonspecific DMT1 protein (Arredondo and Nunez, 2005; Goff, 2018). Because of this, high levels of any of these ions is able to competitively inhibit the absorption of the other from the diet. Additionally, zinc can further inhibit copper absorption by preventing the transport of copper from enterocytes. Increased cellular levels of zinc will stimulate the production of MT which will bind and store both copper and zinc ions in the cell (Crisponi et al., 2010; Goff, 2018). If high levels of MT are produced in intestinal enterocytes, copper and zinc will be sequestered in the enterocyte rather than being transported across the enterocyte and secreted into the blood stream. Furthermore, the sequestered copper will be lost when the enterocytes are sloughed off from the end of the villi into the lumen of the intestine (Crisponi et al., 2010; Goff 2018).

However, CTR1, has a specific affinity for the cuprous form of copper, but the predominant form of copper from dietary sources is the divalent cupric form of copper (Hashimoto and Kambe, 2015). A metalloreductase has been hypothesized to be associated with

the function of CTR1 to convert divalent copper to Cu^{1+} for transport into the enterocyte (Hashimoto and Kambe, 2015).

Once across the enterocyte luminal membrane copper must be immediately bound to prevent potential oxidative damage to the cell. Within enterocytes, copper is usually bound to antioxidant protein 1 (ATOX1) and this chaperone protein shuttles the ion to the proper locations within the cell (Lutsenko et al., 2007). For export of copper out of the enterocyte, ATOX1 must ferry copper to the enterocyte Golgi apparatus which contains ATPase copper transporting alpha polypeptide (ATP7A) (Lutsenko et al., 2007; Lutsenko et al., 2008). This transporter is usually found associated with the membrane of the Golgi apparatus until such a time as copper concentrations become elevated within the enterocyte. At that point, ATP7A is packaged into vacuoles that ultimately fuse with the basolateral membrane allowing for ATP7A to protrude into the surrounding blood stream. Then ATP is utilized to dispel the 6 copper molecules attached to each ATP7A into the blood stream or extracellular fluid for other cell types (Fontaine and Mercer, 2007; Lutsenko et al., 2008). During expulsion of the copper ions from ATP7A, copper appears to change from a cuprous to cupric oxidation state. Like CTR1 and the metalloreductase, Lutsenko et al. (2007) suggest that a copper oxidase is associated with ATP7A to again alter the charge of the copper ion.

Based on research in rats with radiolabeled copper, after exiting enterocytes copper rapidly binds to albumin and transcuprein, with roughly 85% bound to albumin, about 15% to transcuperin with a small fraction associated with histidine or unbound (Crisponi et al., 2010; Linder and Hazegh-Azam, 1996; Weiss and Linder, 1985). Over the next two hours the radiolabeled copper disassociated from the blood protein transporters in parallel with an accumulation of copper in the liver and kidney (Weiss and Linder, 1985). Copper homeostasis is

regulated almost exclusively by the liver through a process of excretion (Linder and Hazegh-Azam, 1996; Lutsenko et al., 2008). This is evident based on the fact that there is little to no regulation of plasma copper entering the liver, while copper excretion and distribution to other tissues from the liver is highly regulated (Lutsenko et al., 2008). Entrance into hepatocytes by copper that was bound to albumin, histidine, or transcuprein is thought to be conducted by CTR1 or other members of the SLC31 gene family (Crisponi et al., 2010; Roberts and Sarkar, 2008). As is the case with enterocytes, copper must be reduced to cuprous copper in order to cross through CTR1 into the hepatocyte. Ohgami et al. (2006) reported that a six-transmembrane epithelial antigen of the prostrate protein (STEAP) may be responsible for reducing copper from a charge of 2+ in the plasma to a 1+ for cellular entry. When STEAP 2, 3, and 4 proteins were overexpressed in a human embryonic kidney cell line there was an increase in the conversion of copper from 2+ to 1+ and subsequent translocation of copper into the cells (Ohgami et al., 2006).

Once within the hepatocyte, a variety of different chaperone proteins can bind to copper and shuttle it where it is needed. For example, COX17 directs copper to the production of cytochrome oxidase, CCS delivers copper for SOD manufacturing, and ATOX1 delivers copper to ATP7B (Dirksen et al., 2017; Roberts and Sarkar 2008). Within the Golgi apparatus, ATP7B provides multiple substantial routes for copper transport such as providing the copper for incorporation into ceruloplasmin which is then used to transport copper all over the body, or facilitating the deposition of copper into lysosomes in hepatocytes for subsequent biliary excretion (Dirksen et al., 2017; Gross et a., 1989; Roelofsen et al., 2000; Wijmenga and Klomp, 2004). Additionally, the importance of ATP7B in copper excretion has been well established in patients with the genetic disorder Wilson's disease where mutations in the ATP7B gene lead the protein to have reduced or no functionality that results in hepatic copper toxicity (Roberts and

Sarkar 2008). Finally, Weiss and Linder (1985) proposed that ceruloplasmin plays a large role in copper excretion by transporting copper back from peripheral tissues to the liver for excretion.

2.2 COPPER TRANSPORTER IN AVIAN SPECIES

While the absorptive pathway of copper is fairly well understood in mammalian species, little research on copper absorption and transport has been conducted in avian species. Goff (2018) provides a comprehensive review of the currently understood mechanisms for copper absorption in livestock animals, but all sources cited when referencing the proteins associated with copper transport were based in mammalian species not used in food animal production.

2.3 SUMMARY

Intestinal absorption and cellular transport of copper is highly regulated given copper's natural ability to oxidase cellular components and thus harm the cell. In mammalian species cellular copper transport is well understood with CTR1 being the primary protein regulating copper transport into a cell. Once copper enters the cell several proteins such as ATOX1, bind copper to prevent it from causing oxidative damage and to shuttle it to various locations with the cell. One of the primary locations that copper is shuttled is the Golgi apparatus. Within the Golgi apparatus copper binds to ATP7A or ATP7B so that it can be expelled from the cell. In avian species, the cellular transport mechanisms of copper have not been investigated, but are assumed to be similar to those of mammalian species.

CHAPTER 3

STATEMENT OF PURPOSE

The necessity of copper in poultry diets for proper growth and development has been established for several decades. While rough estimates have been determined for the specific copper requirement for different varieties of poultry, it is common practice within the poultry industry to feed in excess of established requirements to insure adequate amounts of copper are taken up by the animal from the diet. This is due to several factors including poor bioavailability of inorganic copper salts, antagonisms with other dietary nutrients, the low cost of inorganic copper salts, and growth promoting effects when therapeutic levels of copper are supplied in the diet. The widespread practice of over supplementation of copper can have numerous consequential effects resulting in potential toxicosis of animals, development of bactericidal mineral resistant bacteria, and environmentally harmful levels of copper excretion. Countries within the European Union have already restricted their supplementation of copper to minimize animal excretion of copper.

Additionally, the nutrient requirements for copper in poultry were established prior to the advent and popularization of organically chelated copper complexes. These organic copper compounds have increased bioavailability compared to inorganic salts. It is hypothesized that these organic minerals are able to employ alternative methods of absorption to account for their increased bioavailability and decreased levels of use compared to traditional inorganic salts. However, the biology of copper absorption and metabolism are poorly understood in avian species. Based on mammalian research, ATOX1, ATP7A, and CTR1 are three critical proteins

involved in copper absorption and utilization. As a component of the apical membrane of enterocytes CTR1 allows entry of free cuprous ions into the cell. Because ionic copper has a strong potential to oxidize its surroundings, it is usually complexed with a chaperone protein such as ATOX1 that shuttles the movement of copper ions in the cell. As a component of the Golgi apparatus membrane vesicles, ATP7A, binds up to 6 cuprous ions. When formed vesicles fuse with the basolateral membrane of a cell, the copper ions are released into extracellular fluids allowing, for the utilization of copper by other tissues.

Therefore, the goals of the current research are to (1) subclone and sequence ATOX1, ATP7A and CTR1 in broilers so that real time RT-PCR primers and probes can be made, (2) determine the relative mRNA expression of ATOX1, ATP7A and CTR1 in broiler digestive, reproductive, and muscle tissues and to determine if ileum expression varied in broilers at day 0, 14 and 42 days of age, (3) determine if different dietary concentrations and sources of copper altered broiler performance and processing yields when fed from 0 to 49 days of age.

CHAPTER 4

MATERIALS AND METHODS

4.1 EXPERIMENT 1- CLONING ATOX1, ATP7A, AND CTR1

Primers for reverse transcriptase polymerase chain reactions

Reverse transcriptase polymerase chain reaction (RT-PCR) primers were designed based on potential identified sequences for Chicken CTR1 (GenBank accession # NM_001305660.1), ATOX1 (GenBank accession # CR353280.1), ATP7A (GenBank accession # NM_001389516.1), that were associated with sequencing the chicken genome (International Chicken Genome Sequencing Consortium, 2004). The forward primer for CTR1 was 5'-TTATGAAGCCAGGCCCACCC-3' while the reverse primer was 5'-GTGAAGCTGTTCAGCCACCTTT-3' and predicted an 819-base pair RT-PCR product that corresponded to bases 2270-3088 of the GenBank sequence. The forward primer for ATOX1 was 5'-TGCTCTTACGGGCATGCAAT-3'while the reverse primer used was 5'-GATGTCGGAAAGGAGAGGGC-3' and predicted a 1010-base pair PCR product that corresponded to bases 72-1082 of the GenBank sequence. The forward primer for ATP7A was 5'-GCAACGCTGCAGGAAGCAAT-3' while the reverse primer was 5'-GCTGGCGTTCATCTCGTTGG-3' and predicted a 2200-base pair PCR product that corresponds to bases 306-2505 of the GenBank sequence. The primers for RT-PCR were designed using the National Center for Biotechnology Information's Primer-BLAST program, and the designed primers were manufactured by the Invitrogen brand of ThermoFisher Scientific, (Waltham, MA).

RT-PCR

Using TaqMan Reverse Transcription kit (Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA), reverse transcription cDNA synthesis was performed following the manufacturer's instructions on diluted RNA samples isolated from the ileum of 42 day old broilers. Seven hundred thirty ng of cDNA was used for polymerase chain reaction (PCR) and PCR was completed following a protocol previously described (Davis and Johnson, 1998). An annealing temperature of 60°C was used for all PCR reactions.

Cloning and sequencing

Ten μL of each PCR reaction was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide to visualize RT-PCR products. The size of the RT-PCR products was estimated using a 100 base pair DNA ladder (Invitrogen brand, ThermoFisher Scientific, Waltham, MA) that was electrophoresed as a sample on the gel. Putative mRNA transcripts of gene products identified in the chicken genome based on having a high degree of homology with a corresponding verified mammalian mRNA sequence of the protein of interest, often have minor sequence errors as individual chicken gene products were not verified in the sequencing of the chicken genome. Therefore, in the present research, the RT-PCR products were subcloned in order to have enough of each product to sequence.

For subcloning, RT-PRC products of the appropriate predicted size (ATOX1 -1010 base pairs, ATP7A 2200 base pairs, and CTR1 819 base pairs) were excised from the 1.5% agarose gel. The RT-PCR generated cDNA products were isolated from the excised gel pieces using a PureLink Quick Gel Extraction Kit (Invitrogen brand, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Isolated cDNA products for each copper transport protein of interest was subsequently cloned into a pCR®2.1 vector/plasmid using the TOPO-TA

cloning kit (Invitrogen brand, ThermoFisher Scientific, Waltham, MA). The plasmid preparations for each copper transporter of interest was then transfected into One Shot® TOP10 chemically competent *E. coli* (Invitrogen brand, ThermoFisher Scientific, Waltham, MA) which were then grown on agar plates overnight. Five transformed colonies for each cDNA subclone of interest were each placed into individual culture tubes containing 5 mL of Luria-Bertani (LB) broth and grown overnight.

A PureLink Quick Plasmid Miniprep Kit (Invitrogen brand, ThermoFisher Scientific, Waltham, MA) was used to isolate plasmid from each of the 5 mL cultures following the manufacturer's instructions. Isolated plasmid from each preparation was then digested with EcoRI restriction enzyme (Fisher Scientific, Pittsburg, PA) according to the manufacturer's directions to cut the cloned RT-PCR product from the vector. Each digest reaction and the 100 base pair DNA ladder (Invitrogen brand, ThermoFisher Scientific, Waltham, MA) was electrophoresed on 1.5% agarose gels to confirm the presence and size of each subcloned RT-PCR product. The isolated plasma preparation for each cDNA subclone of interest with the highest concentration of plasmid was then sent to Eurofins Genomics (Louisville, KY) for sequencing using M13 forward and reverse primers which bind to pCR®2.1 vector just ahead and behind the inserted cDNA product.

Internal primers for sequencing ATP7A

While the length of ATOX1 (1010 base pairs) and CTR1 (819 base pairs) cDNA clones were small enough to obtain their full sequence using the M13 forward and reverse primers, this was not possible with the much larger, ATP7A (2200 base pairs) cDNA clone. Therefore, once partial sequence information was obtained from the first set of primers in the ATP7A cDNA clone, additional internal primers were then developed from the sequence information to obtain

the complete sequence of the ATP7A cDNA clone. The first internal primer set was 5'-GCTGTTGACCTGGAAAGGCTG-3' for the forward primer and 5'-CAGTCAACTCTCAGATGCTCACC-3' for the reverse primer. The last internal primer set for ATP7A was 5'-TGTGCTTCGTGTGTAGCCAAC-3' for the forward primer and 5'-GGAGGCCATTCACTCGTCTATG-3' for the reverse. The internal primers were manufactured by Eurofins Genomics (Louisville, KY).

4.2 EXPERIMENT 2- TISSUE DISTRIBUTION OF ATOX1, ATP7A, AND CTR1 mRNA

The purpose of the experiment was to investigate the mRNA expression ATOX1, ATP7A, and CTR1 in various tissues of broilers.

Animals

Cobb 500 X Cobb 500 fast feathering male broilers which had been hatched and vent sexed at the University of Georgia Poultry Research Center were reared from hatch to 35 days of age in floor pens using standard corn/soybean meal based starter, grower and finisher diets. The floor pens were in an environmentally controlled facility. Ambient temperature was set to 34 °C on day 1 and decreased daily by 0.28 °C until 24 °C was reached and then maintained for the duration of the experiment. A typical broiler industry lighting program was implemented with a lighting intensity of 20 lux for 24 hours (0 to 4 days of age), 20 lux for 20 hours (5 to 7 days of age), 10 lux for 16 hours (8 to 14 days of age), and 2 lux for 16 hours (15 to 35 days of age). Birds were provided with water and feed ad libitum.

Tissue collection

At the end of the experimental duration, 6 birds were killed by cervical dislocation for tissue collection. Approximately 150 mg samples of duodenum, jejunum, ileum, gizzard, liver, spleen, kidney, gastrocnemius (leg muscle), pectoralis major (breast muscle), and heart tissue

were excised. Tissue from two birds were combined into single samples to produce 3 total replicate samples of each tissue type (n = 3). Once the samples were isolated, the tissues were homogenized for 30 seconds using a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA) in 3 mL of guanidium isothiocyanate solution. The separate homogenized tissue samples were then frozen and stored at -80°C for future RNA extraction. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

4.3 EXPERIMENT 3- THE mRNA EXPRESSION OF ATOX1, ATP7A, AND CTR1 IN IMMATURE AND MATURE BROILER TESTES

The purpose of this experiment was to determine if ATOX1, ATP7A, and CTR1, mRNA was expressed in the testicular tissues of sexually immature male broilers and sexually mature broiler breeder rosters.

Animals

Off-sex male broiler chicks from the Cobb 500 female parent line were obtained at day of hatch from the Cleveland, Georgia hatchery and housed at the University of Georgia Poultry Research Center. The chicks were reared in an environmentally controlled complex until they reached 42 days of age. During the starter, grower, and finisher phases birds were fed standard corn/ soybean based diets. The bird management with regard to feed and water access, temperature and lighting was as described in Experiment 2. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

For samples of mature testes, 62 weeks of age Ross broiler breeder roosters were obtained from a commercial broiler breeder farm in Northeast Georgia.

Tissue collection

Twelve male broilers were killed by cervical dislocation at 42 days of age for testes tissue collection. From each bird 300 mg of sexually immature testicular tissue was collected from the left testicle.

Twelve, 62 weeks of age Ross broiler breeders were killed by carbon dioxide asphyxiation for sample collection. The left testicle was removed from each rooster and cut in half. A sample of 300 mg of tissue was taken from the center of the testicle and collected.

A PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA) was utilized to homogenize each immature and mature testicular sample in 3 ml of guanidium isothiocyanate for 30 seconds. All samples were then frozen and stored at -80°C for future RNA extraction. Once RNA extraction had been performed, the RNA from 2 roosters from each age group were combined to create a total of 6 replicates (n= 6) for each age group.

4.4 EXPERIMENT 4- THE HEPATIC mRNA EXPRESSION OF ATOX1, ATP7A, AND CTR1 IN FED AND FASTED BROILERS

The purpose of this experiment was to determine if ATOX1, ATP7A, and CTR1, mRNA expression was altered in broilers that had been fasted for 48 hours.

Animals

Off-sex male broiler chicks from the Cobb 500 female parent line were obtained at day of hatch from the Cleveland, Georgia hatchery and housed at the University of Georgia Poultry Research Center. The broilers were reared as described in Experiment 3. At 42 days of age, one pen of 12 birds continued with free access to feed and water, while another pen of 12 birds were given free access to water but were feed deprived for the following 48 hour period.

Tissue collection

At the end of the fasting period, 12 birds from each treatment group were killed by cervical dislocation. A sample of 150 mg of liver was removed from each bird and paired with another sample to provide 6 samples of 300 mg for each treatment (n = 6). Samples were then placed in 3 mL of guanidium isothiocyanate and homogenized for 30 seconds using a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). Each sample was then frozen and stored at -80°C for future RNA extraction.

4.5 EXPERIMENT 5- OVULATORY FOLLICULAR TISSUE EXPRESSION OF THE mRNA FOR ATOX1, ATP7A, AND CTR1

The purpose of this experiment was to determine if ATOX1, ATP7A, and CTR1, mRNA expression was present in the granulosa or theca cells of developing preovulatory follicles of broiler breeder hens.

Animals

Cobb 500 fast feathering broiler breeder pullets were reared in floor pens from day 1 of age at the University of Georgia Poultry Research Center. They were provided a standard broiler breeder pullet diet on a skip a day feed restriction program. Ten percent of the pullets were randomly selected and weighed once per week in the rearing phase to determine feed allocation. This method was used to ensure that the body weight gain of the pullets matched the recommended guidelines of the primary breeder (Cobb-Vantress 2005a). From placement on day 1 until 21 weeks of age, the pullets received 8 hours of light. The lighting program was adjusted to provide 14 hours of light per day for photostimulation at 21 weeks of age. At time of photostimulation, the hens were provided a standard broiler breeder layer diet on an every day basis. The amount of feed provided to the hens every day was determined using the guidelines

of the primary breeder (Cobb-Vantress 2005a; Cobb-Vantress 2005b), which are based on the weekly body weight measurements and egg production rates of the hens. At 42 weeks of age, a group of 40 hens were removed from floor pens and placed into individual cages, where egg laying was monitored, and feed continued to be provided on a daily basis. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue collection

When hens reached between 45 and 52 weeks of age, they were killed via cervical dislocation for tissue sampling. The hens were killed approximately 2 to 4 hours before ovulation based on egg laying records and the presence of a hard-shelled egg in the uterus. The entire ovary was carefully excised from each killed broiler breeder hen. From each ovary, the four largest hierarchical follicles, F1, F2, F3, and F4, the small yellow follicles (SYF, >5 to 12 mm in diameter), and the large white follicles (LWF, <2 to 5 mm in diameter) were collected. Manual removal of the granulosa cell layer from the theca cell layer was performed on the hierarchical follicles (F1-F4) (Huang and Nolbandov, 1979). For the prehierarchical follicles (SYF and LWF), the theca and granulosa cell layers were separated enzymatically (Davis et al., 2000). Tissue samples from two individual hens were combined to create a single replicate for each follicle size and tissue type. The theca layers collected from the hierarchical follicles and the theca layers enzymatically separated from the individual pools of prehierarchical follicles were placed into 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disruptor (Fisher Scientific, Pittsburg, PA). The single cell layer of granulosa tissue from each hierarchical follicle and the granulosa cells enzymatically separated from the individual pools of prehierarchical follicles were placed into 2 mL of guanidinium isothiocyanate solution and vortexed for 20 seconds. The tissue solutions were stored at -80ºC

for subsequent RNA extraction. This collection procedure was repeated until a total of 3 replicate follicular tissue sets were obtained (n=3).

4.6 EXPERIMENT 6- ILEAL mRNA EXPRESSION OF ATOX1, ATP7A, AND CTR1 IN DIFFERENT AGE BROILERS

The purpose of this experiment was to determine if ATOX1, ATP7A, and CTR1, mRNA expression in ileum tissue differed in broilers during the first few weeks of age.

Animals

Off-sex male broiler chicks from the Cobb 500 female parent line were obtained at day of hatch from the Cleveland, Georgia hatchery and housed at the University of Georgia Poultry Research Center. The broilers were reared as described in Experiment 3. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

Tissue collection

A total of twelve chicks at 0 days of age were killed by cervical dislocation and 100 mg section of the ileum was excised approximately 4 cm distal to Meckel's diverticulum. The tissue from 2 birds was combined and placed in a 15 mL sterile tube containing 3mL of RNA*later* (Ambion Inc, Austin, TX). On day 14, and 42 of age, 6 birds were killed by cervical dislocation and a 300 mg section of ileum was excised approximately 4 cm distal to Meckel's diverticulum and immediately placed in a 15 mL tube containing 3 mL of RNA*later*. Subsequently, the tissue samples were removed from the RNA*later* solution and placed in 3 mL of guanidinium isothiocyanate solution and homogenized using a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA) for 30 seconds. Post homogenization, sample solutions were frozen at -80°C for future RNA extraction.

4.7 EXPERIMENT 7- THE INFLUENCE OF DIETARY COPPER SOURCE AND CONCENTRATION ON BROILER PERFORMANCE

This floor pen experiment was completed to investigate if different sources and concentrations of dietary supplemental copper influenced broiler live performance from 0 to 49 days of age. Off-sex male broiler chicks from the Cobb 500 female parent line were obtained at day of hatch from the Cleveland, Georgia hatchery. Chicks were sorted by weight to remove extreme outliers as well as any noticeable physical deformities before the remaining chicks were assigned to the 96 pens (23 birds per pen). Prior to placing chicks, each of the 96 pens were assigned to a dietary treatment such that there were 12 replicate pens per treatment (6 replicate pens per room).

This experiment was conducted in a facility with 2 identical rooms. Each room contained 48 (1.52m by 1.22m) floor pens. Each pen was equipped with 5 nipple drinkers originating from a common water line and 1 pan feeder that occupied 0.92m^2 of floor space. The litter for each pen consisted of 5 cm of new pine shavings. A lighting program was implemented with a light intensity of 20 lux for 24 hours (0 to 4 days), 20 lux for 20 hours (5 to 7 days), 10 lux for 18 hours (8 to 14 days), and 2 lux for 18 hours (15 to 49 days). To ensure proper lighting was applied, parameters were validated using a Light ProbeMeter™ (model 403125, Extech Instruments Corp. Waltham, MA).

For each room, a computerized environmental controller regulated 2 gas-fired furnaces, an exterior evaporative cooling system present on both sides of the room for air intake by ceiling air inlet vents, six 45.7 cm sidewall circulation fans, and at the end of each room two 91.4 cm exhaust fans and one 61 cm exhaust fan. Ambient temperature was set at of 34 °C at day one and decreased by 0.28 °C each day until 24°C was achieved and maintained through the end of

the experiment. Temperature and relative humidity were recorded by HOBO External Temp/RH data loggers spaced throughout each room (ONSET Computer Corporation, Bourne, MA). No significant differences in temperature and humidity were detected between the 2 rooms throughout the experiment.

The experiment consisted of 8 dietary treatments (**Table 4.1**) that consisted of 3 copper sources [copper sulfate monohydrate, basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN), or AvailaCu (Zinpro Corporation, Eden Prairie, MN)] and 6 different dietary copper inclusion rates. The dietary copper supplements were added to a basal corn and soybean meal - based starter, grower, finisher and withdrawal diet (**Table 4.2**) that contained a copper free trace mineral premix (Table 4.2). The starter treatment diets were fed from 0 to 14 days of age, the grower treatment diet were fed from 14 to 28 days of age, the finisher treatment diets were fed from 28 to 42 days of age, and the withdrawal treatment diets were fed from 42-49 days of age. The starter diets were in crumble form while the grower, finisher and withdrawal diets were in pellet form. Diets were formulated on a digestible amino acid basis. Feed and water were provided ad libitum throughout the duration of the experiment. All animal procedures were approved by the University of Georgia Animal Care and Use Committee.

For each room, humidity, temperature, water consumption and pen mortality were recorded 2 times per day. Birds and feed were weighed on a pen basis on days 0, 14, 28, 42 and 49 to determine body weight, feed intake, body weight gain, and feed conversion. On day 49, the mean bird weight for each pen was determined and then 8 broilers within 300 grams of the average pen weight were selected from each of the pens and placed in a coop for overnight feed

Table 4.1. Dietary copper supplementation in parts per million for each dietary treatment during each phase of the 49-day broiler experiment (Experiment 7).

Copper source	Dietary treatment							
	1	2	3	4	5	6	7	8
Starter[1]								
Cu chloride[2]	7	7	7	125	0	0	200	0
Cu sulfate[3]	125	0	0	0	0	0	0	0
Availa Cu[4]	0	14	7	0	14	7	0	21
Total supplemented Cu	132	21	14	125	14	7	200	21
Total dietary Cu[5]	145	34	18	116	17	11	183	22
Grower[6]								
Cu chloride[2]	7	7	7	125	0	0	150	0
Cu sulfate[3]	125	0	0	0	0	0	0	0
Availa Cu[4]	0	14	7	0	14	7	0	14
Total supplemented Cu	132	21	14	125	14	7	150	14
Total dietary Cu[5]	148	22	15	116	16	9	153	16
Finisher[7]								
Cu chloride[2]	7	7	7	125	0	0	125	0
Cu sulfate[3]	125	0	0	0	0	0	0	0
Availa Cu[4]	0	14	7	0	14	7	0	7
Total supplemented Cu	132	21	14	125	14	7	125	7
Total dietary Cu[5]	123	16	12	121	9	14	121	14
Withdrawal[8]								
Cu chloride[2]	7	7	7	125	0	0	125	0
Cu sulfate[3]	125	0	0	0	0	0	0	0
Availa Cu[4]	0	14	7	0	14	7	0	7
Total supplemented Cu	132	21	14	125	14	7	125	7
Total dietary Cu[5]	163	18	12	123	9	8	123	8

[1]Starter diet was fed from 0 to 14 days of age.
[2]Copper in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
[3]Copper in the form of copper sulfate monohydrate.
[4]Copper in the form of AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[5]Analyzed Cu levels of the diet.
[6]Grower diet was fed from 14 to 28 days of age.
[7]Finisher diet was fed from 28 to 42 days of age.
[8]Withdrawal diet was fed from 42 to 49 days of age.

Table 4.2. Composition of the broiler diets (Experiment 7).

Ingredient	Starter[1]	Grower[2]	Finisher[3]	Withdrawal[4]
Corn	52.56	56.33	60.59	64.19
Soybean meal (46% CP)	35.98	30.41	24.39	19.06
Corn distillers dried grains with solubles	4.00	6.00	8.00	10.00
Soybean oil	3.83	3.90	3.90	3.98
Defluorinated phosphate	0.97	0.69	0.41	0.13
Calcium carbonate	0.82	0.86	0.89	0.93
Vitamin mix[5]	0.27	0.57	0.57	0.57
Salt	0.39	0.39	0.39	0.39
DL- Methionine (99.0%)	0.34	0.30	0.26	0.21
L-Lysine, HCl (78.8%)	0.22	0.23	0.26	0.26
Cu free mineral mix[6]	0.01	0.01	0.01	0.01
L-Threonine (98.0%)	0.06	0.06	0.07	0.06
Monensin[7]	0.05	0.05	0.05	0.00
Sand/Cu[8]	0.05	0.05	0.05	0.05
Titanium dioxide	0.03	0.03	0.03	0.03
Choline chloride (60%)	0.02	0.03	0.04	0.04
Phytase[4]	0.01	0.01	0.01	0.01
Calculated analysis				
AME (kcal/kg)	3025	3075	3125	3175
Crude protein (%)	21.97	20.27	18.41	16.79
Calcium (%)	0.95	0.85	0.65	0.65
Available phosphorus (%)	0.48	0.43	0.38	0.33
Digestible total sulfur amino acids (%)	0.91	0.84	0.76	0.68
Digestible lysine (%)	1.20	1.09	0.98	0.87
Digestible threonine (%)	0.78	0.72	0.66	0.59

[1]Starter diet was fed from 0 to 14 days of age.
[2]Grower diet was fed from 14 to 28 days of age.
[3]Finisher diet was fed from 28 to 42 days of age.
[3]Withdrawal diet was fed from 42 to 49 days of age.
[5]Vitamin premix (DSM custom vitamin premix. DSM Nutritional Products, Inc. Parsippany, NJ) provides the following per kilogram of diet: vitamin A, 12,500 IU; vitamin D_3, 2,500 IU; vitamin E, 25 IU; vitamin B_{12}, 0.03 mg; riboflavin, 10 mg; niacin, 100 mg; d-panthotenic acid, 25 mg; choline chloride, 434 mg; menadione sodium bisulfate, 2.5 mg; folic acid, 1.25 mg; pyridoxine HCl, 5 mg; thiamin mononitrate, 5 mg; d-biotin, 0.25 mg; and ethoxyquin, 283 mg.
[6]Copper free trace mineral premix contained 47.53% rice hulls, 14.52% ferric chloride hexahydrate, 16.88% zinc hydroxychloride, 19.59% manganese hydrochloride, 0.26% sodium selenite mix (with the mix containing 11.4% sodium selenite), 0.22 calcium iodate and 1% mineral oil. and provides the following milligrams per kilogram of diet zinc, 100; manganese, 120, iron, 30; iodine, 1.4, and selenium 0.3.
[7]Coban 90 (Elanco Animal Health, Greenfield, IN), added to supply Monensin at 0.1 g/kg finished feed.

[8]Copper in the form of copper sulfate monohydrate, basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN), or AvailaCu (Zinpro Corporation, Eden Prairie, MN). See Table 4.1 for further details.
[9]Quantum blue (5,000 FTU/g, AB Vista Plantation, FL), added to supply 500 FTU/kg finished feed.

withdrawal. These selected birds had their individual weights recorded, and were leg banded for identification. On day 50 of age, the selected birds were processed at the University of Georgia's Pilot Processing Plant as described by Hidalgo et al. (2004). After a 4 hour period of static chilling in an ice bath, carcasses were drained, weighed and further processed to determine pectoralis major, pectoralis minor, leg quarter, and wing yields. Based on the scoring system of Tijare et al., (2016) cases of woody breast were classified. Incidents of green muscle and white striping of the pectoral muscles were also classified using the methodology of Bilgili and Hess, (2008) and Kuttappan et al. (2012), respectively.

4.8 RNA EXTRACTION

Using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski, 1987), total RNA was extracted from the tissue samples. Upon completion of RNA extraction, samples of RNA were stored at -80°C. Each RNA sample's integrity was determined by the presence of intact bands for 28S and 18S rRNA on a 1.5% agarose gel stained with ethidium bromide.

4.9 REAL TIME RT-PCR

To remove any potential genomic DNA contamination, the extracted RNA samples were DNase treated using the TURBO-DNA-free kit (Life Technologies brand, ThermoFisher Scientific, Waltham, MA) following the manufacturer's instructions. Primers and TaqMan minor groove binding probes (MGB) were designed from the obtained cDNA sequences (Experiment 1) for ATOX1, ATP7A, and CTR1. For an endogenous control, an MGB primer and probe set was designed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession # M11213). The primer and probes were designed using Primer Express software version 2.0 (Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA) and manufactured by Applied Biosystems (ThermoFisher Scientific, Waltham, MA).

The forward primer sequence for ATOX1 was 5'-CCCAGCATGATGCTGACATG-3' while the reverse primer sequence was 5'-GGTTTTTGTGGACAGGATTTCAG-3'. The probe sequence for ATOX1 was 5'-CACCCTCCTTACTGTGAA-3'. The forward primer sequence for ATP7A was 5'- GTGGACAGTCAACTCTCAGATGCT-3' while the reverse primer sequence was 5'- AGGAAATGGAGGCCATTCACT-3'. The probe sequence for ATP7A was 5'- CCAACATCTCAACATGAG-3'. The forward primer sequence for CTR1 was 5'-GAGCTGTGAGGACATCTTAGTGCTT-3' while the reverse primer sequence was 5'-GTGTTGGAGTTGGCCGTGC-3'. The probe sequence for CTR1 was 5'-CAGTGAAACAGAGCAGCTA-3'. The forward primer for GAPDH was 5'GACGTGCAGCAGGGAACACTA-3' and the reverse primer was 5'CCTCTGTCATCTCTCCACAGC-3'. The probe sequence for GAPDH was 5'TGACCACTG TCCATGCCAT-3'. Each probe was labeled at the 5' end with FAM (6-carboxyfluorescein), the reporter dye, and at the 3' end with TAMRA (6-carboxy-N, N, N', N'-tetramethylrhodamine), the quencher dye. Validation of the primer and MGB probe sets for real-time PCR was completed by determining the optimal amplification efficiency and primer/probe concentration as described by the manufacturer (Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA).

Next, reverse transcription production of cDNA was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA) and following the manufacturers protocol. The real time RT-PCR reaction for all tissue samples utilized 100 ng of cDNA in a volume of 25 µL containing 1x TaqMan Universal Master Mix (Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA), 900 nM of the primer being tested, and 25 nM of the respective MGB probe. The reactions were completed by an ABI

7500 Thermocycler (Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA). The thermocycler conditions were 10 minutes at 95°C and 40 cycles each of 15 seconds at 95°C and 1 minute at 60°C. The reactions for all tissues were performed in duplicate for ATOX1, ATP7A, CTR1, and GAPDH assays. The CT (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction using the Sequence Detection Software (version 1.2.2, Applied Biosystems brand, ThermoFisher Scientific, Waltham, MA), and quantification was completed using the 2-ΔΔCT method (Livak and Schmittgen, 2001). The CT values for ATOX, ATP7A and CTR1 were determined for each sample and, subsequently, normalized to the GAPDH CT value from the same sample (thus for example ATOX CT - GAPDH CT = ΔCT). After the ΔCT values for all reactions were obtained for an experimental replicate, the ΔCT values for each individual ATOX reaction were compared to the sample within the replicate that had the highest mRNA expression for ATOX using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Therefore, all data for ATOX is expressed as the fold-difference relative to the sample with the highest expression. This relative expression procedure was repeated for ATP7A and CTR1.

4.10 STATISTICS

The experimental data were subjected to ANOVA according to the General Linear Model (GLM) procedure. Tukey's multiple comparison procedure (Neter et al. 1990) was used to determine significant differences between tissue mRNA expression and production values. Significant differences were determined by a $P < 0.05$. All statistical procedures were completed with the Minitab statistical software package (Releases 17, State College, PA).

CHAPTER 5

RESULTS

5.1 EXPERIMENT 1- CLONING ATOX1, ATP7A, AND CTR1

The RT-PCR primers designed based on putative mRNA transcripts identified in the chicken genome for ATOX1, ATP7A and CTR1 were successful for the creation of cDNA products for these 3 genes (GenBank sequence upload pending). The created cDNA products for ATOX1, ATP7A and CTR1 were successfully subcloned and expressed so that they could be sequenced. Using the sequencing information obtained from these products allowed for the design and synthesis of MGB primers and probe for ATOX1, ATP7A and CTR1 for subsequent real time RT-PCR mRNA expression analyses of these 3 copper transport proteins in various tissues of broilers and broiler breeders.

5.2 EXPERIMENT 2

All tissues examined expressed the mRNA transcripts for ATOX1, ATP7A and CTR1 (**Table 5.1**). As the primary transporter of copper into cells, it was not surprising that intestinal expression of CTR1 was greater ($p<0.05$) in duodenum, jejunum and ileum tissue than most other tissues examined (Table 5.1) as this would help insure adequate absorption of dietary copper. In contrast, the mRNA expression of the copper chaperone protein ATOX1 and the cellular copper exporter protein ATP7A were more evenly expressed across all tissues (Table 5.1).

Table 5.1. The relative fold expression of ATOX1, ATP7A and CTR1 mRNA in tissues of 35 day old broilers[1] (Experiment 2).

Tissue	ATOX1	ATP7A	CTR1
Gizzard	0.479 ± 0.146^{abcd}	0.163 ± 0.071^{bcd}	0.029 ± 0.010^{d}
Proventriculus	0.476 ± 0.081^{abcd}	0.495 ± 0.153^{abcd}	0.306 ± 0.095^{cd}
Duodenum	0.647 ± 0.115^{ab}	0.457 ± 0.030^{abcd}	0.804 ± 0.183^{ab}
Jejunum	0.762 ± 0.234^{a}	0.719 ± 0.281^{ab}	0.970 ± 0.030^{a}
Ileum	0.576 ± 0.032^{abc}	0.577 ± 0.073^{abcd}	0.745 ± 0.095^{ab}
Liver	0.093 ± 0.025^{cd}	0.113 ± 0.015^{cd}	0.480 ± 0.043^{bc}
Kidney	0.149 ± 0.026^{cd}	0.778 ± 0.144^{a}	0.070 ± 0.002^{d}
Cardiac muscle	0.230 ± 0.072^{bcd}	0.193 ± 0.040^{abcd}	0.069 ± 0.011^{d}
Pectoralis major muscle	0.002 ± 0.000^{d}	0.005 ± 0.001^{d}	0.000 ± 0.000^{d}
Gastrocnemius muscle	0.013 ± 0.004^{d}	0.019 ± 0.007^{d}	0.002 ± 0.001^{d}
Fat	0.161 ± 0.043^{cd}	0.205 ± 0.092^{abcd}	0.068 ± 0.019^{d}
Spleen	0.907 ± 0.047^{a}	0.653 ± 0.121^{abc}	0.094 ± 0.009^{d}

[1] The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyeraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). $^{a-d}$Values within a column with different superscripts for a given tissue differ, ($P < 0.05$).

5.3 EXPERIMENT 3

Testicular tissue from immature broilers have greater expression of ATOX1, ATP7A and CTR1 mRNA than testicular tissue from sexually mature roosters (**Table 5.2**).

5.4 EXPERIMENT 4

Fasting market age broilers did not influence the mRNA expression of ATOX1, ATP7A and CTR1 (**Table 5.3**).

5.5 EXPERIMENT 5

Although ATOX1, ATP7A and CTR1 mRNA was expressed in granulosa and theca cells in all hierarchical and nonhierarchical follicles examined (**Table 5.4**), the overall expression of ATOX1, ATP7A and CTR1 was significantly greater ($P < 0.000$) in the nonhierarchical follicles than the hierarchical follicles with the mean ± SEM expression of ATOX1 0.183 ± 0.031 and 0.5882 ± 0.092, of CTR1 0.437 ± 0.038 and 0.851 ± 0.045, and of ATP7A 0.150 ± 0.018 and 0.544 ± 0.092 in hierarchical and nonhierarchical follicles, respectively.

Because the mRNA expression of ATOX1, ATP7A and CTR1 in the testes and preovulatory follicles was done independently from the initial tissue distribution experiment the relative expression of the mRNA for ATOX1, ATP7A and CTR1 between the reproductive tissues and digestive tissues was unknown. Therefore, select tissues from each experiment (Experiments 2, 3 and 5) were analyzed together to determine the relative mRNA expression of ATOX1, ATP7A and CTR1 among these tissues. The mRNA expression of CTR1 is greater in jejunum than in immature testicular or prehierarchical follicular tissue (**Table 5.5**). The expression of the mRNA for ATOX1 and ATP7A does not differ between jejunum, immature testicular and prehierarchical follicular tissue (Table 5.5).

Table 5.2. The relative fold expression of ATOX1, ATP7A and CTR1 mRNA in immature and mature testes from 6 week old broilers and 62-week old broiler breeder roosters[1] (Experiment 3).

Tissue	ATOX1	ATP7A	CTR1
Immature testes	0.199 ± 0.014^{a}	0.391 ± 0.126^{a}	0.685 ± 0.099^{a}
Mature testes	0.060 ± 0.020^{b}	0.028 ± 0.006^{b}	0.177 ± 0.030^{b}

[1]The values are means ± SEM, n = 6 replicates with each replicate consisting of pooled tissue from two birds. The mRNA expression data were normalized with glyeraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). [a-b]Values within a column with different superscripts for a given tissue differ, ($P < 0.05$).

Table 5.3. The relative fold expression of ATOX1, ATP7A and CTR1 mRNA in hepatic tissue from 44 day old broilers with free access to food or from 44 day old broilers fasted from day 42 to 44 of age[1] (Experiment 4).

Feeding state	ATOX1	ATP7A	CTR1
Fed	0.311±0.090	0.207±0.097	0.550±0.104
Fasted	0.425±0.145	0.470±0.174	0.583±0.130

[1]The values are means ± SEM, n = 6 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyeraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$).

Table 5.4. The relative fold expression of ATOX1, ATP7A, and CTR1 mRNA in theca or granulosa collected from the four largest hierarchical follicles (F1 through F4), the small yellow follicles, and the largest white follicles from 45 to 52 week old broiler breeder hens[1] (Experiment 5).

Tissue	ATOX1	ATP7A	CTR1
F1 theca	0.438 ± 0.114^{abc}	0.250 ± 0.051^{bc}	0.592 ± 0.010^{bcd}
F1 granulosa	0.092 ± 0.007^{c}	0.124 ± 0.043^{c}	0.289 ± 0.045^{d}
F2 theca	0.299 ± 0.124^{abc}	0.168 ± 0.059^{c}	0.441 ± 0.092^{cd}
F2 granulosa	0.110 ± 0.027^{c}	0.145 ± 0.067^{c}	0.292 ± 0.051^{d}
F3 theca	0.129 ± 0.033^{c}	0.124 ± 0.060^{c}	0.384 ± 0.100^{cd}
F3 granulosa	0.130 ± 0.003^{c}	0.162 ± 0.045^{c}	0.444 ± 0.053^{cd}
F4 theca	0.066 ± 0.015^{c}	0.059 ± 0.020^{c}	0.312 ± 0.044^{d}
F4 granulosa	0.201 ± 0.048^{bc}	0.170 ± 0.017^{c}	0.742 ± 0.124^{abc}
Small yellow theca	0.185 ± 0.027^{bc}	0.201 ± 0.063^{c}	0.689 ± 0.097^{abc}
Small yellow granulosa	0.756 ± 0.159^{a}	0.671 ± 0.195^{ab}	0.926 ± 0.074^{ab}
Large white theca	0.653 ± 0.175^{ab}	0.473 ± 0.073^{abc}	0.833 ± 0.087^{ab}
Large white granulosa	0.759 ± 0.121^{a}	0.830 ± 0.170^{a}	0.958 ± 0.028^{a}

[1]The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two broiler breeder hens. The mRNA expression data were normalized with glyeraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$).
[a-d]Values within a column with different superscripts for a given tissue differ, ($P < 0.05$).

Table 5.5. The relative fold expression of ATOX1, ATP7A and CTR1 mRNA in tissues with high expression levels of these mRNA transcripts[1].

Tissue	ATOX1	ATP7A	CTR1
Jejunum	0.733 ± 0.267	0.533 ± 0.261^{ab}	1.000 ± 0.000^{a}
Liver	0.087 ± 0.031	0.086 ± 0.031^{b}	0.501 ± 0.038^{b}
Large white granulosa	0.357 ± 0.035	0.961 ± 0.039^{a}	0.227 ± 0.075^{c}
Immature testes	0.416 ± 0.105	0.341 ± 0.125^{ab}	0.194 ± 0.068^{c}

[1]The values are means ± SEM, n = 3 replicates with each replicate consisting of pooled tissue from two birds. The mRNA expression data were normalized with glyeraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). $^{a\text{-}c}$Values within a column with different superscripts for a given tissue differ, ($P < 0.05$).

5.6 EXPERIMENT 6

The mRNA expression of CTR1 decreased with age in broilers (**Table 5.6**), while ATOX1 and ATP7A mRNA expression did not differ in broilers at 0, 14 and 42 days of age.

5.7 EXPERIMENT 7

During the starter period, broilers fed a diet containing 125 ppm copper sulfate combined with 7 ppm copper chloride had lower body weight gain and greater feed to gain values than the chicks fed any of the other dietary copper supplements or concentrations (**Table 5.7)**. During the grower phase (day 14 to 28 days of age) the broilers fed the diet containing 125 ppm copper sulfate combined with 7 ppm copper chloride gained as much weight as the broilers fed the other dietary treatments (**Table 5.8**). However, given their lower performance in the starter period, in the overall period from 0 to 28 days of age, the broilers fed a diet containing 125 ppm copper sulfate combined with 7 ppm copper chloride had a greater feed to gain value than the broilers fed the other dietary treatments except for treatment 3 which had a dietary copper supplement that consisted of 7 ppm from copper chloride and 7 ppm from Availa copper (**Table 5.9**). In the subsequent finisher (day 28 to 42 of age) and withdrawal (day 42 to 49 of age) phases, as well as the overall (0 to 42 and 0 to 49 days of age) periods there were no differences in body weight, body weight gain or feed to gain among the broilers fed any of the dietary copper treatments (**Tables 5.10 to 5.13**).

Table 5.6. The relative fold expression of ATOX1, ATP7A and CTR1 mRNA in ileum tissue collected from broilers at day 0,14 and 42 of age[1] (Experiment 6).

Day of age	ATOX1	ATP7A	CTR1
0	0.441 ±0.076	0.461±0.063	0.734±0.065[a]
14	0.480±0.125	0.652±0.134	0.538±0.126[ab]
42	0.454±0.032	0.350±0.046	0.410±0.018[b]

[1]The values are means ± SEM, n = 6 replicates with each replicate consisting of pooled tissue from two broilers. The mRNA expression data were normalized with glyeraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta Ct}$). [a-b]Values within a column with different superscripts for a given tissue differ, ($P < 0.05$).

Table 5.7. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of cooper from 0 to 14 days of age[1] (Experiment 7).

Dietary treatment	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
		g/bird		g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	495 ± 4^{b}	450 ± 4^{b}	1.300 ± 0.006^{a}	580 ± 3^{b}	1.8
2 CuCl (7ppm) + AvailaCu (14ppm)	541 ± 3^{a}	496 ± 3^{a}	1.194 ± 0.006^{c}	588 ± 4^{ab}	1.8
3 CuCl (7ppm) + AvailaCu (7ppm)	535 ± 4^{a}	490 ± 4^{a}	1.195 ± 0.004^{bc}	582 ± 6^{ab}	2.5
4 CuCl (125ppm)	548 ± 4^{a}	503 ± 4^{a}	1.201 ± 0.005^{bc}	600 ± 4^{a}	0.7
5 AvailaCu (14ppm)	536 ± 2^{a}	491 ± 2^{a}	1.188 ± 0.004^{c}	583 ± 3^{ab}	0.0
6 AvailaCu (7ppm)	538 ± 5^{a}	492 ± 5^{a}	1.194 ± 0.004^{c}	585 ± 5^{ab}	1.1
7 CuCl (200ppm)	537 ± 4^{a}	491 ± 4^{a}	1.217 ± 0.007^{b}	592 ± 4^{ab}	1.4
8 AvailaCu (21ppm)	549 ± 4^{a}	503 ± 4^{a}	1.179 ± 0.004^{c}	594 ± 5^{ab}	1.4

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments. $^{a\text{-}c}$Values with different superscripts for a given parameter differ, ($P < 0.05$).

Table 5.8. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of copper from 14 to 28 days of age[1] (Experiment 7).

Dietary treatments	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
	g/bird			g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	1929 ± 13[b]	1434 ± 10[ab]	1.346 ± 0.005[ab]	1910 ± 12	1.4
2 CuCl (7ppm) + AvailaCu (14ppm)	1974 ± 11[ab]	1434 ± 10[ab]	1.354 ± 0.006[a]	1934 ± 9	1.1
3 CuCl (7ppm) + AvailaCu (7ppm)	1952 ± 14[ab]	1416 ± 11[b]	1.360 ± 0.009[a]	1903 ± 19	1.8
4 CuCl (125ppm)	1993 ± 17[a]	1445 ± 15[ab]	1.352 ± 0.005[ab]	1948 ± 16	1.1
5 AvailaCu (14ppm)	1982 ± 10[ab]	1445 ± 9[ab]	1.350 ± 0.003[ab]	1933 ± 11	1.8
6 AvailaCu (7ppm)	1979 ± 16[ab]	1440 ± 12[ab]	1.343 ± 0.007[ab]	1922 ± 14	1.4
7 CuCl (150ppm)	2001 ± 13[a]	1467 ± 11[a]	1.328 ± 0.005[b]	1924 ± 14	1.4
8 AvailaCu (14ppm)	1975 ± 18[ab]	1426 ± 15[ab]	1.339 ± 0.003[ab]	1895 ± 20	1.4

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments. [a-b]Values with different superscripts for a given parameter differ, ($P < 0.05$).

Table 5.9. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of copper from 0 to 28 days of age[1] (Experiment 7).

Dietary treatments	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
	g/bird			g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	1929 ± 13[b]	1884 ± 13[b]	1.335 ± 0.004[a]	2468 ± 16	3.3
2 CuCl (7ppm) + AvailaCu (14ppm)	1974 ± 11[ab]	1929 ± 11[ab]	1.311 ± 0.005[b]	2498 ± 10	2.9
3 CuCl (7ppm) + AvailaCu (7ppm)	1952 ± 14[ab]	1906 ± 14[ab]	1.316 ± 0.006[ab]	2456 ± 21	4.3
4 CuCl (125ppm)	1993 ± 17[a]	1948 ± 17[a]	1.312 ± 0.004[b]	2530 ± 17	1.8
5 AvailaCu (14ppm)	1982 ± 10[ab]	1937 ± 10[ab]	1.308 ± 0.003[b]	2497 ± 13	1.8
6 AvailaCu (7ppm)	1979 ± 16[ab]	1934 ± 16[ab]	1.304 ± 0.006[b]	2485 ± 18	2.5
7 CuCl (150ppm)	2001 ± 13[a]	1956 ± 13[a]	1.299 ± 0.004[b]	2490 ± 18	2.9
8 AvailaCu (14ppm)	1975 ± 18[ab]	1929 ± 18[ab]	1.296 ± 0.002[b]	2468 ± 23[a]	2.9

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments. [a-b]Values with different superscripts for a given parameter differ, ($P < 0.05$).

Table 5.10. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of cooper from 28 to 42 days of age[1] (Experiment 7).

Dietary treatments	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
		g/bird		g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	3510 ± 36	1584 ± 27	1.827 ± 0.012	2826 ± 42	0.7
2 CuCl (7ppm) + AvailaCu (14ppm)	3574 ± 34	1596 ± 28	1.845 ± 0.021	2805 ± 42	0.7
3 CuCl (7ppm) + AvailaCu (7ppm)	3559 ± 29	1608 ± 26	1.846 ± 0.018	2860 ± 32	0.4
4 CuCl (125ppm)	3558 ± 38	1595 ± 28	1.842 ± 0.021	2827 ± 46	1.1
5 AvailaCu (14ppm)	3557 ± 31	1577 ± 25	1.875 ± 0.018	2846 ± 37	1.4
6 AvailaCu (7ppm)	3546 ± 29	1571 ± 32	1.888 ± 0.021	2830 ± 34	1.4
7 CuCl (125ppm)	3568 ± 26	1577 ± 29	1.849 ± 0.025	2827 ± 34	1.4
8 AvailaCu (7ppm)	3542 ± 31	1568 ± 19	1.857 ± 0.015	2848 ± 36	1.4

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments.

Table 5.11. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of copper from 0 to 42 days of age[1] (Experiment 7).

Dietary treatments	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
	g/bird			g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	3510 ± 36	3465 ± 36	1.550 ± 0.005	5239 ± 46	4.0
2 CuCl (7ppm) + AvailaCu (14ppm)	3574 ± 34	3528 ± 34	1.538 ± 0.007	5270 ± 39	3.6
3 CuCl (7ppm) + AvailaCu (7ppm)	3559 ± 29	3513 ± 29	1.546 ± 0.007	5237 ± 45	4.7
4 CuCl (125ppm)	3558 ± 38	3542 ± 38	1.538 ± 0.006	5311 ± 57	2.9
5 AvailaCu (14ppm)	3557 ± 31	3512 ± 31	1.549 ± 0.007	5286 ± 48	3.3
6 AvailaCu (7ppm)	3546 ± 29	3501 ± 29	1.549 ± 0.006	5249 ± 43	4.0
7 CuCl (125ppm)	3568 ± 26	3523 ± 26	1.532 ± 0.008	5288 ± 53	4.3
8 AvailaCu (7ppm)	3542 ± 31	3497 ± 31	1.536 ± 0.006	5265 ± 53	4.3

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments.

Table 5.12. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of cooper from 42 to 49 days of age[1] (Experiment 7).

Dietary treatments	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
	g/bird			g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	4071 ± 56	550 ± 27	2.814 ± 0.089	1427 ± 31	2.2
2 CuCl (7ppm) + AvailaCu (14ppm)	4126 ± 45	553 ± 31	2.887 ± 0.138	1417 ± 27	2.2
3 CuCl (7ppm) + AvailaCu (7ppm)	4143 ± 52	582 ± 31	2.817 ± 0.204	1464 ± 36	2.5
4 CuCl (125ppm)	4180 ± 54	589 ± 31	2.738 ± 0.105	1468 ± 26	1.4
5 AvailaCu (14ppm)	4130 ± 49	563 ± 29	2.942 ± 0.159	1436 ± 31	2.9
6 AvailaCu (7ppm)	4105 ± 54	564 ± 36	3.065 ± 0.197	1415 ± 42	2.9
7 CuCl (125ppm)	4104 ± 43	542 ± 26	2.883 ± 0.123	1453 ± 25	1.8
8 AvailaCu (7ppm)	4054 ± 40	514 ± 22	3.065 ± 0.106	1385 ± 21	4.0

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments.

Table 5.13. Body weight, body weight gain and feed efficiency of broilers fed different sources and dietary levels of cooper from 0 to 49 days of age[1] (Experiment 7).

Dietary treatments	Body weight	Body weight gain	Feed to gain	Feed Intake	Mortality
		g/bird		g/bird	%
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	4071 ± 56	4025 ± 56	1.697 ± 0.008	6609 ± 81	6.2
2 CuCl (7ppm) + AvailaCu (14ppm)	4126 ± 45	4081 ± 45	1.684 ± 0.007	6656 ± 66	5.8
3 CuCl (7ppm) + AvailaCu (7ppm)	4143 ± 52	4098 ± 52	1.689 ± 0.009	6621 ± 82	7.2
4 CuCl (125ppm)	4180 ± 54	4135 ± 54	1.681 ± 0.009	6754 ± 78	4.3
5 AvailaCu (14ppm)	4130 ± 49	4084 ± 49	1.699 ± 0.010	6670 ± 70	6.2
6 AvailaCu (7ppm)	4105 ± 54	4060 ± 54	1.700 ± 0.010	6613 ± 90	6.9
7 CuCl (125ppm)	4104 ± 43	4059 ± 43	1.686 ± 0.009	6675 ± 52	6.2
8 AvailaCu (7ppm)	4054 ± 40	4009 ± 40	1.695 ± 0.007	6500 ± 82	8.3

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens for the dietary treatments.

For processing there were no differences detected for the loss of weight after the fasting period preceding processing (**Table 5.14**), in whole carcass yields (**Table 5.15**) or in carcass part yields (**Table 5.16**) between any of the dietary treatments. The overall incidence of breast defects (woody, green muscle and white striping) was negligible and did not vary by treatment. In the processed birds, no white striping was detected, woody breast was found in one processed bird from each of the following treatments 2, 3, 6 and 8, and green muscle was present in one processed bird from treatments 1, 3, 5 and 6 and was present in 2 processed birds in treatment 2.

Table 5.14. Processing yields from 50 day old broilers fed different dietary sources and dietary levels of cooper from 0 to 49 days of age[1]. (Experiment 7).

Dietary treatments	Live weight (day 49 of age)	Fasted weight (day 50 of age)	Loss of weight
	g/bird		%[2]
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	4074 ± 57	3849 ± 53	5.70 ± 0.14
2 CuCl (7ppm) + AvailaCu (14ppm)	4131 ± 46	3884 ± 43	5.99 ± 0.19
3 CuCl (7ppm) + AvailaCu (7ppm)	4152 ± 50	3901 ± 47	5.95 ± 0.12
4 CuCl (125ppm)	4181 ± 56	3935 ± 50	5.86 ± 0.16
5 AvailaCu (14ppm)	4141 ± 49	3901 ± 44	5.80 ± 0.11
6 AvailaCu (7ppm)	4125 ± 56	3898 ± 55	5.58 ± 0.15
7 CuCl (125ppm)	4107 ± 42	3863 ± 39	5.93 ± 0.17
8 AvailaCu (7ppm)	4054 ± 38	3826 ± 37	5.60 ± 0.13

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens with 7 birds per pen selected for processing for all treatments.
[2]As a percent of live weight.

Table 5.15. Processing yields from 50 day old broilers fed different dietary sources and dietary levels of cooper from 0 to 49 days of age[1]. (Experiment 7).

Dietary treatments	Hot carcass		Chilled carcass		Frame	
	g/bird	%[2]	g/bird	%[2]	g/bird	%[2]
1 CuCl (7ppm) + $CuSO_4$ (125ppm)	2984 ± 41	77.59 ± 0.19	2986 ± 37	77.60 ± 0.22	747 ± 11	19.43 ± 0.15
2 CuCl (7ppm) + AvailaCu (14ppm)	3004 ± 32	77.38 ± 0.15	3001 ± 32	77.29 ± 0.26	754 ± 7	19.40 ± 0.12
3 CuCl (7ppm) + AvailaCu (7ppm)	3021 ± 34	77.44 ± 0.16	3028 ± 33	77.63 ± 0.23	762 ± 11	19.56 ± 0.17
4 CuCl (125ppm)	3064 ± 42	77.75 ± 0.15	3067 ± 42	77.73 ± 0.20	768 ± 10	19.54 ± 0.13
5 AvailaCu (14ppm)	3019 ± 34	77.31 ± 0.14	3023 ± 31	77.42 ± 0.29	756 ± 8	19.39 ± 0.09
6 AvailaCu (7ppm)	3020 ± 44	77.58 ± 0.10	3022 ± 44	77.61 ± 0.16	764 ± 11	19.61 ± 0.11
7 CuCl (125ppm)	2997 ± 32	77.58 ± 0.18	2989 ± 30	77.38 ± 0.21	752 ± 8	19.48 ± 0.11
8 AvailaCu (7ppm)	2973 ± 27	77.70 ± 0.18	2975 ± 23	77.70 ± 0.26	755 ± 9	19.71 ± 0.11

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).
AvailaCu (Zinpro Corporation, Eden Prairie, MN).
[1]The values are means ± SEM, n = 12 replicate pens with 7 birds per pen selected for processing for all treatments.
[2]As a percent of live fasted weight.

Table 5.16. Processing yields from 50 day old broilers fed different dietary sources and dietary levels of cooper from 0 to 49 days of age[1]. (Experiment 7).

Dietary treatments	Pectoralis major		Pectoralis minor		Total white meat[2]		Wings		Leg quarters	
	g/bird	%[3]	g/bird	%[3]	g/bird	%[3]	g/bird	%[3]	g/bird	%[3]
1	834 ± 13	21.64 ± 0.14	150 ± 3	3.90 ± 0.06	986 ± 15	25.59 ± 0.14	303 ± 4	7.87 ± 0.03	944 ± 10	24.57 ± 0.19
2	839 ± 13	21.60 ± 0.13	150 ± 3	3.86 ± 0.06	984 ± 14	25.32 ± 0.12	306 ± 4	7.88 ± 0.04	943 ± 10	24.28 ± 0.17
3	855 ± 14	21.89 ± 0.17	153 ± 3	3.92 ± 0.05	1008 ± 16	25.82 ± 0.20	306 ± 3	7.84 ± 0.03	940 ± 7	24.12 ± 0.17
4	868 ± 18	21.97 ± 0.19	152 ± 2	3.86 ± 0.04	1020 ± 20	25.84 ± 0.19	307 ± 3	7.80 ± 0.05	955 ± 10	24.30 ± 0.17
5	851 ± 14	21.79 ± 0.20	152 ± 2	3.90 ± 0.06	1000 ± 16	25.61 ± 0.23	306 ± 3	7.85 ± 0.04	939 ± 9	24.06 ± 0.14
6	842 ± 18	21.59 ± 0.22	150 ± 3	3.85 ± 0.06	992 ± 21	25.44 ± 0.25	303 ± 3	7.80 ± 0.06	951 ± 12	24.45 ± 0.09
7	835 ± 14	21.58 ± 0.19	150 ± 2	3.89 ± 0.04	985 ± 16	25.47 ± 0.20	303 ± 3	7.86 ± 0.04	943 ± 9	24.41 ± 0.20
8	832 ± 10	21.71 ± 0.18	149 ± 1	3.89 ± 0.04	980 ± 11	25.60 ± 0.19	302 ± 3	7.88 ± 0.04	929 ± 7	24.28 ± 0.19

Treatment 1 CuCl (7ppm) + $CuSO_4$ (125ppm), treatment 2 CuCl (7ppm) + AvailaCu (14ppm), treatment 3 CuCl (7ppm) + AvailaCu (7ppm), treatment 4 CuCl (125ppm), treatment 5 AvailaCu (14ppm), treatment 6 AvailaCu (7ppm), treatment 7 CuCl (125ppm), treatment 8 AvailaCu (7ppm).

CuCl in the form of basic copper chloride (IntelliBond C, Micronutrients USA LLC, Indianapolis, IN).

AvailaCu (Zinpro Corporation, Eden Prairie, MN).

[1]The values are means ± SEM, n = 12 replicate pens with 7 birds per pen selected for processing for all treatments.

[2]Pectoralis major plus pectoralis minor

[3]As a percent of live fasted weight

CHAPTER 6

DISCUSSION

6.1 TISSUE DISTRIBUTION OF COPPER TRANSPORTERS

This research is the first to characterize the presence and tissue expression of the mRNA for ATOX1, ATP7A, and CTR1 in broilers. In the current research, CTR1 mRNA expression was most abundant in intestinal tissue which is not surprising given it is the primary transporter of copper across intestinal enterocytes in mammalian species. Ahn et al. (2017) similarly reported that in horses the duodenum was one of the tissues with the highest CTR1 mRNA expression. In addition, the current research indicated that hepatic expression of CTR1 mRNA was also abundant compared to nondigestive tissues. Because the liver is the site of copper incorporation into the various different metalloproteins it would make sense that CTR1 is expressed in high concentrations to allow for mineral acquisition by hepatic cells (Linder, 2016; Lutsenko et al., 2008). In mammals, liver is often found as the tissue with the highest expression of CTR1 mRNA (Ahn et al., 2017; Lee et al., 2000; Zhou and Gitschier, 1997). In lizards, liver and intestinal tissue also express the highest levels of CTR1 mRNA based on Northern blot analysis. As was found in the current research some level of CTR1 mRNA expression was detected in all tissues examined in these other species with muscle tissues expressing very low levels of CTR1 mRNA (Lee et al., 2000; Zhou and Gitschier, 1997).

In the current research, CTR1 mRNA expression in the ileum decreased in day 42 of age broilers relative to day of hatch broilers. This finding needs further investigation to determine if protein expression mirrors the mRNA expression pattern. If the protein expression does mirror

the mRNA expression it would indicate that intestinal absorption capability could decrease with age which might also correlate with a lowered requirement for copper in older broilers that are no longer growing rapidly.

In the current research ATOX1 mRNA was expressed abundantly in the spleen and tissues from the GI tract but was detected in all tissues examined. In humans, it was found that ATOX1 mRNA expression is widely distributed across all tissues with more abundant expression found in the kidney, liver, and spleen (Hatori and Lutsenko, 2013; Klomp et al., 1997). In mice and rats, ATOX1 expression was usually seen predominantly in the liver and kidney followed by brain and small intestine (Hamza et al., 2000; Hiromura and Sakurai, 1999; Naeve et al., 1999). Given this protein is needed to protect cells from copper oxidation and to shuttle copper to its needed locations within the cell, it would make sense that ATOX1 is widely produced by all tissue types.

Differences in the tissue expression of ATP7A mRNA in broilers were less pronounced than what was observed for CTR1. In humans, based on Northern blot analyses, ATP7A expression was relatively constant across tested tissues except for the liver where expression was reduced or even undetectable (Chelly et al., 1993; Turner and Horn, 1997; Vulpe et al., 1993). Similar results were found in sea bream (Minghetti et al., 2010). A possible explanation for the low expression of ATP7A within the liver may be due to the presence of an alternative copper efflux transporter ATP7B which is expressed predominantly in the liver (Wijmenga and Klomp,2004). Antithetical to the findings in humans, horses have been found to have the highest expression of ATP7A within the liver followed by gonadal tissue when investigated utilizing quantitative PCR (Ahn et al., 2017). However, when further examination of ATP7A was performed, Ahn et al. (2017) found based on Western blot analyses that ATP7A protein

expression is greatest in the heart and gonadal tissues with the liver expressing very little ATP7A protein. In broilers, further research is needed to determine if ATP7B is expressed by any tissues and if the expression of ATP7A mRNA mirrors protein expression levels.

6.2 COPPER REQUIREMENTS IN BROILER CHICKENS

Despite the recommendation of the NRC (1994) that the requirement for copper is just below 10 mg/kg , it is common practice for commercial broiler diets to have copper concentrations between 100-150 mg/kg (Dozier et al., 2003; Forouzandeh et al., 2021; Leeson, 2009) based on the perception and limited in vivo data (Arias and Koutsos, 2006) that these higher dietary inclusion levels promote better performance especially when the birds are exposed to microbial challenges. While it is relatively cheap to provide this extra dietary copper, there are environmental concerns stemming from this practice. Because copper within the body is readily utilized and incorporated into proteins in a highly regulated manner, very little excess copper is ever stored (Linder and Hazegh-Azam, 1996) especially because excessive cellular copper not bound to proteins can cause oxidative stress. As a result, the majority of extraneous copper is excreted from the body (Linder and Hazegh-Azam, 1996). This can lead to copper contents in manure reaching excessive levels, particularly if copper in the diet is fed in high amounts. Xiong et al. (2010) found that copper levels in animal manure was significantly greater than the levels of copper found within the feed. After a 4 year application of poultry manure, He et al. (2017) reported an increase in the copper concentration of 72.1-88.7% in the top 15 cm of soil. These high excretion levels and potential environmental pollution has led to several countries banning therapeutic levels of copper from poultry diets (EFSA, 2012).

The easiest way to prevent potential repercussions from high copper excretion is to limit the copper fed to poultry. In addition, some sources of copper are more bioavailable than others.

When copper sulfate availability was set at 100%, tribasic copper chloride (TBCC) and copper methionine complex (Cu-Met) were found to be 128% and 138% more available than copper sulfate, respectively (Wu et al., 2020). Luo et al. (2005) found TBCC to be 8% more available than copper sulfate in broilers, and Mintrex, a chelated copper compound, was found to be about 11% more bioavailable than copper sulphate (Wang et al., 2007). It is hypothesized that organic copper sources that consist of copper linked to amino acids (typically methionine) are absorbed bound to the amino acids, utilizing enterocyte amino acid transporters (Nollet et al., 2007). Gao et al. (2014) conducted experiments in human Caco-2 cells and utilized carboplatin, which inhibits the ability of CTR1 to transport copper across membranes. While the absorption of copper supplied by copper sulfate was inhibited, cellular uptake of complexed copper - methionine was not. Thus, diets that are supplemented with a mixture of organic copper and inorganic copper may lead to maximal absorption of copper which could reduce the amount of copper needed in the diet to meet animal requirements.

In the current broiler growth trial experiment, 2 inorganic sources of copper (copper sulfate and copper chlroide) and one organic source of copper (AvailaCu) were utilized with the organic source being utilized alone or in combination with an inorganic source. In addition, these various copper sources were included in the diets to provide a range of supplemented levels spanning from the 8 mg/kg requirements indicated by the NRC (1994) to therapeutic levels of the inorganic sources. Despite the wide range of dietary inclusion rates, overall live performance (0-49 days of age) and carcass yields did not vary across the treatments. Overall mortality for the treatments ranged from 4 to 8 percent with most of the mortality being associated with yolk sac infections, femur head necrosis and air sacculitis. Based on the mortality incidence, the

broilers faced health challenges and adding therapeutic levels of dietary copper did not improve mortality rates or live performance of the broilers.

The only significant treatment effects seen in the production data was for the broilers in treatment 1, which were supplemented with 125 mg/kg copper sulfate plus 7 mg/kg TBCC actually had decreased body weight gains and increased feed to gain values relative to the other treatments within the starter period. In subsequent research by our laboratory, a decrease in weight gain and an increase in feed to gain values has been replicated in broilers from 0 to 21 days of age when supplementing diets with sulfate forms of trace minerals compared to those bound to chloride (data not shown). Thus, the causative agent of this decreased performance is the presence of the excess sulfates associated with the minerals and not the minerals themselves.

Based on the current research it would be difficult to justify feeding copper to broilers in excess of the NRC recommendation. This agrees with the previous research of Nollet et al. (2007) in which there was no difference in growth performance of broilers when 12 mg/kg, 37 mg/kg, 70 mg/kg, and 45 mg/kg of Cu, Zn, Mn and Fe, respectively as inorganic salts were fed compared to broilers fed a diet containing 2.5 mg/kg copper and 10 mg/kg of the other three minerals in an organic form. Nollet et al. (2007) did find a significant decrease in the amount of mineral excretion when the organic forms were utilized. Similarly, in other research when NRC recommended levels of inorganic minerals are applied compared to graded levels of organics, low supplementation of organic minerals provide the same level of growth as the inorganic levels (Aksu et al., 2010; Leeson and Caston, 2008).

Given the potential for the copper associated with organic sources of dietary copper such as AvailaCu in which copper is linked to amino acids being absorbed into enterocytes with the amino acid raher than through CTR1, further research is needed to determine if the poultry NRC

requirements could actually be lowered. If cooper enters the enterocyte through both amino acid transporters and CTR1 then the absorptive capacity for copper would be increased and possibly more efficient which could mean less copper needs to be present in the diet.

6.3 COPPER TRANSPORTERS IN IMMATURE GONADAL TISSUE

In the current research, immature testes and follicular tissue from immature unselected preovulatory follicles had greater mRNA expression for ATOX1, ATP7A, and CTR1 than mature testes or granulosa and theca tissue from selected hierarchical preovulatory follicles. In mammals, it has been found that immature oocytes utilize the production of ROS as a signal for maturation (Agarwal et al., 2005; Behrman et al., 2001; Dubela et al., 2004; Takami et al., 1999). As the corpus luteum regresses it begins to produce ROS which may serve as a signal for immature follicles to begin resumption of meiotic processes for the next ovulation (Behrman et al., 2005). Additionally, increased levels of antioxidants within the developing tissue have been shown to prevent maturation. Takami et al. (1999) describes that one of the main signals for resumption of meiosis is from gonadotropin. The investigators continue by detailing that gonadotropin will first act by reducing the levels of ascorbic acid in the follicles, a known antioxidant, prior to the return of follicular meiosis. Lastly, they describe how in cases with ascorbic acid deficiency, infertility occurs in females from premature meiotic processes. In rats, oocytes have been treated with various antioxidants and have delayed follicular maturation (Dubela et al., 2004; Takami et al., 1999). In both examples the prevention of ROS in the oocytes led to a delay in maturation and meiotic processes. During the investigation by Dubela et al. (2004), it was found that the theca interstitial cells utilized in the experiment would spontaneously produce ROS, leading to the cells' maturation. It was also found that applying treatments that induced super oxide radical formation resulted in faster rates of maturation than

the control theca interstitial cells alone (Dubela et al., 2004). While these researchers all relayed that ROS or some level of oxidative stress is necessary for follicular development, excess amounts can also produce harmful effects. Karuputhula et al., (2013) investigated women with various abnormal follicular development and found that excessive oxidative stress and ROS production was a common thread within these abnormalities. Thus, while specific research has not been conducted to investigate copper transporter activity within the avian ovary, mammalian research into oxidative stress of ovarian tissue and the current results may suggest that copper in its role in regulating oxidative stress may be critical in early follicular growth and selection. In all vertebrate females the fate of over 90percent of all preovulatory follicles is atresia. Thus, if a brief down regulation of copper transport capacity and the activity of copper containing enzymes that neutralize ROS is needed for the initiation of follicular maturation and selection, then examining and interpreting the expression of copper transport proteins in small follicles could be misleading. Because these follicles are small in size theca and granulosa tissue is collected from all of the small yellow and large white follicles. However, if 90 percent of these follicles are destined for atresia in the next 24-48 hours versus the one follicle selected to move forward for maturation, then the mRNA expression of the copper transporters in a pool of tissue isolated from all of these follicles is going to be dominated by the ones destined for atresia and not from the one selected. Thus, future research needs to focus on individual follicles to determine if copper transport is different in one of these follicles to be selected versus the others.

While it is possible that the abundant mRNA expression of the copper transporting proteins in the small follicles seen in the present research is because it reflected a prevention of ROS formation in all but one selected preovulatory follicle, another explanation is also logical. The blood supply to the individual follicles stops at the theca layer in avian species. Thus, for

substances such as copper to be deposited in the ovum, they have to be transported through the granulosa layer and then through the plasma layer of the ovum. Furthermore, granulosa cells develop very early in primordial follicles and then do not multiply. Instead as the follicle grows and develops the granulosa cells simply rearrange from multiple layers to a single layer. Thus, the mRNA expression and subsequent protein expression for copper transport proteins would likely be high in the small follicles as they are programmed for subsequent copper absorption and this was observed in the current research. Furthermore, as an egg laying species copper transport would be more essential in avian species than in species that gestate as all of the copper needed for the development of the embryo would have to be present at the end of egg formation. In addition, egg yolk has a very high lipid content that is susceptible to oxidative breakdown during an extended incubation period at high temperatures. Thus, is not surprising that female birds go to great lengths to provision their egg yolks with antioxidants. In addition, the primary site of copper storage in the egg is within the yolk. Therefore, future research needs to determine if maximizing the copper content of egg yolks in incubated eggs enhances subsequent chick quality and growth.

Studies have been conducted to investigate the effect of antioxidant loading in developing embryos on the effect of hatchability and chick development. In these studies, it was found that natural antioxidants from maternal sources rapidly deplete after hatch, requiring the new chick to rely on antioxidant enzymes for protection against oxidative stress (Surai, 2002). Therefore, developing embryos and new chicks may rely on the inclusion of minerals, i.e. copper, for the production of antioxidant enzymes during the early stages of growth.

Cellular copper levels in the testes also have to be tightly regulated to prevent deficiency or toxicosis that results in physiological damage (Herman et al., 2020). In mice testes, ATP7A mRNA expression peaks 20 days after birth and is significantly lower 30 days after birth (mice become sexually mature at about 35-40 days of age) and while protein expression also peaks with the peak in mRNA expression, protein expression is maintained despite the lower mRNA expression levels through 180 days of age (Ogorek et al., 2017). Similarly, CTR1 and ATOX1 protein expression peak at about 18 days of age and are maintained thereafter (Ogorek et al., 2017). In rodents, it was found that ROS were needed for male gonadal development as it was in the ovary (Fisher and Aitken, 1997). A strain of knockout mice was developed by Ghaffari et al. (2019) that were missing CTR1, but only in germ cells of the testicular tissues. In this experiment, the researchers found significantly smaller testes weight compared to control mice 28 days after birth. With further investigation it was determined that the copper deficient testes contained a lack of postmeiotic germ cells and spermatocyte differentiation with an increase in spermatozoa defects (Ghaffari et al., 2019). To further investigate the effect of ATP7A in the developing gonad, Kowal et al. (2010) utilized a strain of ATP7A deficient mice that poorly expresses ATP7A within the testes and other tissues. With decreased ability to excrete copper due to lower ATP7A, high concentrations of copper within the testes resulted in numerous signs of poor fertility such as decreased live spermatozoa, decreased sperm motility, abnormal sperm morphology, and diminished membrane integrity within the tail section of the sperm. Collectively these results all heavily suggest that maintaining tight control of copper homeostasis within testicular tissue is vital for normal spermatogenesis and fertility, but as was the case for the role of copper in follicular development much more research is necessary to determine the specific roles of copper in testicular development and spermatogenesis.

In summation, the present research is the first to characterize the mRNA expression of ATOX, ATP7A and CTR1 fin avian tissues. The mRNA expression profiles of these 3 key cellular copper transporters suggests that copper plays essential roles in spermatogenesis and follicular development and selection. Finally, the current research suggests that the current NRC guidelines for dietary copper content are correct and that the industry trend to use therapeutic levels of dietary copper are unfounded until conclusive research proves otherwise, especially given that copper toxicity can occur.

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