

University of São Paulo
“Luiz de Queiroz” College of Agriculture

Intestinal integrity, oxidative stress, and immune competence of broilers exposed to heat stress and supplemented with Zn amino acid complex

Alvaro Mario Burin Junior

Thesis presented to obtain the degree of Doctor in
Science. Area: Animal Science and Pastures

Piracicaba
2019

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Doctor of Veterinary Medicine

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RESUMO

Integridade intestinal, estresse oxidativo e competência imunológica de frangos de corte em estresse por calor e suplementados com complexo Zn aminoácido

Para determinar o efeito da suplementação dietética de complexo zinco-aminoácido (ZnAA; Availa Zn) na integridade intestinal, metabolismo oxidativo e desempenho de frangos de corte criados sob estresse cíclico crônico por calor, dois experimentos foram projetados; o Experimento 1 foi dividido em dois ensaios idênticos repetidos ao longo do tempo para avaliar parâmetros biológicos e o Experimento 2 consistiu em um ensaio em aviário experimental para determinar os resultados de desempenho. Nos dois ensaios do Experimento 1, os tratamentos seguiram um arranjo fatorial 4×2 , incluindo quatro níveis de ZnAA suplementar (dieta 0, 20, 40 e 60mg / kg) e duas condições ambientais (termoneutro - TN - e estresse térmico cíclico crônico - HS). Os tratamentos dietéticos foram aplicados a partir do alojamento. Aos 21d de idade, um total de 12 aves por combinação de tratamento foram distribuídos aleatoriamente em três gaiolas em salas com controle ambiental. A temperatura do TN foi de 24 ° C e o HS foi ajustado para 33°C. Para o Experimento 2, 1.800 pintos de corte foram distribuídos em 4 tratamentos (níveis de ZnAA) em 10 repetições de 45 aves cada. Todos os pintos utilizados foram machos da linhagem Cobb 500. A dieta não suplementada (0 mg ZnAA / kg) foi baseada em milho, farelo de soja e óleo de soja e foi adequada em todos os nutrientes, exceto Zn. As aves foram alimentadas com tratamentos dietéticos a partir do alojamento e o estresse térmico, iniciado aos 21d e com duração de 21 dias. Todas as avaliações foram realizadas em 42d. O HS prejudicou praticamente todos os parâmetros avaliados. HS resultou em aumento da temperatura corporal e frequência respiratória; pior desempenho e parâmetros imunológicos; aumento dos produtos de oxidação e atividade de enzimas antioxidantes no sangue (eritrócitos), fígado e intestino (jejuno); parâmetros histomorfométricos comprometidos, bem como integridade intestinal. A suplementação dietética de ZnAA provou ser uma excelente ferramenta para frangos de corte sob estresse oxidativo (causado pelo estresse térmico). Melhorou linearmente o peso corporal e a taxa de conversão alimentar; peso relativo de bursa aumentou linearmente, bem como quantificação de gama-globulinas; produtos de oxidação linearmente diminuídos no sangue e fígado; aumentou quadraticamente a capacidade antioxidante no sangue e influenciou os parâmetros histomorfométricos de maneira diferenciada para aves termoneutras ou estressadas pelo calor. Sob estresse térmico, o ZnAA melhorou a integridade intestinal medida pelo FITC-d e melhorou a quantificação das proteínas ocludina e ZO-1 (Western Blot). Na avaliação de desempenho (Experimento 2), o ZnAA suplementar aumentou linearmente o peso corporal e o ganho de peso corporal para o período experimental total (1 a 42). O tratamento com HS aplicado neste estudo revelou ser um modelo disruptor da barreira intestinal e gerador de ROS (espécies reativas de oxigênio). A suplementação de ZnAA, por sua vez, mostrou potencial para melhorar a competência imune de frangos de corte criados nas duas condições ambientais testadas, para diminuir os efeitos do estresse oxidativo e melhorar a integridade intestinal medida pelo FITC-d e quantificação de ocludina e ZO-1, especialmente para aves em estresse térmico.

Palavras-chave: Avicultura, Estresse, Metabolismo oxidativo, Nutrição mineral, Permeabilidade intestinal

ABSTRACT

Intestinal integrity, oxidative stress, and immune competence of broilers exposed to heat stress and supplemented with Zn amino acid complex

To determine the effect of zinc-amino acid complex (ZnAA; Availa Zn) dietary supplementation on intestinal integrity, oxidative metabolism and performance of broilers raised under chronic cyclic heat stress, two experiments were designed; Experiment 1 was divided in two identical trials repeated over time to assess biological parameters and Experiment 2 consisted of a floor pen trial to determine performance results. For both trials of Experiment 1 the treatments followed a 4×2 factorial arrangement, including four levels of supplemental ZnAA (0, 20, 40 and 60mg/kg diet) and two environmental conditions (thermoneutral – TN - and a chronic cyclic heat stress – HS). The dietary treatments were applied from placement. At 21d of age, a total of 12 birds per treatment combination were randomly allotted to three battery cages in environmentally controlled rooms. TN temperature was 24 °C and HS was set to depart 9 °C. For Experiment 2, 1,800 broiler chicks were allotted into 4 treatments (levels of ZnAA) in 10 replicates of 45 birds each. All chicks used were males from Cobb 500 strain. The unsupplemented diet (0 mg ZnAA/kg) was based on corn, soybean meal, and soybean oil and was adequate in all nutrients, except Zn. As for Experiment 1, birds were fed dietary treatments from placement and heat stress protocol started at 21d of age and lasted 21 days. All the assessments were performed at 42d. HS impaired virtually all parameters assessed. HS resulted in increased body temperature and respiratory frequency; worsened performance and immunological parameters; increased oxidation products and antioxidant enzyme activity in blood (erythrocyte), liver and intestine (jejunum); impaired histomorphometry parameters as well as gut integrity. Dietary ZnAA supplementation proved to be an excellent tool for broilers under oxidative stress (caused by heat stress). It has linearly improved body weight and feed conversion ratio; linearly improved bursa relative weight and gamma-globulins quantification; linearly decreased oxidation products in blood and liver; quadratically increased antioxidant capacity in blood and influenced histomorphometric parameters differentially for thermoneutral or heat stressed birds. Under heat stress, ZnAA improved gut integrity measured by FITC-d and enhanced occludin and ZO-1 proteins quantification (Western Blot analysis). In the performance assessment (Experiment 2), supplemental ZnAA linearly increased body weight and body weight gain for the total experimental period (1 to 42). The HS treatment applied in this study has revealed to be a model of gut barrier disruptor and ROS (reactive oxygen species) generator. ZnAA supplementation, in turn, showed potential to improve immune competence of broilers raised under both environmental conditions tested, to diminish the effects of oxidative stress and improve intestinal integrity measured by FITC-d and quantification of occludin and ZO-1, especially for heat stressed birds.

Keywords: Boiler performance, Gut permeability, Mineral nutrition, Oxidative metabolism, Stress

1. INTRODUCTION

In spite of the volume of investments and the technology evolution for housing environmental control, heat stress remains one important factor of performance and revenue impairment to the poultry production industry. Zinc arises as an alternative to mitigate the deleterious effects of heat stress.

Heat stress (HS) is a worldwide concern in poultry production. Feed intake, nutrient digestibility, growth rate, and mortality are negatively influenced by HS. Furthermore, authors have reported immune impairment in broilers under heat exposure, presenting a reduction in the weight of lymphoid organs, titers of total antibodies, IgG and IgM, and reduction in the phagocytic capacity of macrophages (Bartlett and Smith, 2003).

Heat stress is also known to cause damage to intestinal health and integrity, by diverting blood flow to the periphery, leading to hypoxia associated with increased serum markers of endotoxemia and inflammation (Hall et al. 2001). Hence, HS is also an inducer of oxidative stress (OS), due to an increase in the demand for energy in the cell, which is closely associated with a greater generation of free radicals (Akbarian et al. 2016; Xie et al. 2015). In turn, increased production of reactive oxygen species (ROS) is associated with the pathogenesis of gastrointestinal diseases, resulting in damage to the integrity of intestinal epithelial cells (Bhattacharyya et al. 2014; Song et al. 2014).

Zinc (Zn) is an essential micromineral for animals, including broilers. Its functions are related to a wide variety of metabolic and structural activities, participating as cofactor or activator of more than 300 enzymes (Roohani et al. 2013). Zn supplementation in broilers has important objectives related to different areas of the organism, including productive performance (Pimentel et al, 1991, Ao et al., 2007), immune competence and development of lymphoid organs (Bartlett and Smith, 2003; Bun et al., 2011; Kakhki et al., 2016), antioxidant system (Powell, 2000; Bun et al., 2011), and, more recently, influence on intestinal integrity and histomorphometry (Miyoshi et al., 2016; Shao et al. 2014).

There is a growing interest on the negative effects of HS and its interaction with OS, adversely affecting intestinal integrity, which jeopardizes broiler health and productivity. In this scenario, Zn appears as a potential alternative to minimize the aforementioned negative effects, reducing oxidative damage and enhancing intestinal integrity.

This study aimed to assess the effects of zinc-amino acid complex supplementation in the diet on intestinal integrity, oxidative metabolism and performance of broilers raised under chronic cyclic heat stress, used as a model of gut barrier disruptor and ROS (reactive oxygen species) generator.

2. LITERATURE REVIEW

2.1. HEAT STRESS

Due to the presence of feathers and the absence of sweat glands, the mechanisms by which birds regulate their body temperature are limited. To compensate, birds use behavioral and physiological mechanisms to maintain their temperature and homeostasis. Mack et al. (2013) showed that birds change their normal behavior in situations of hyperthermia decreasing feed intake, increasing water consumption, and spending more time resting and with wings raised. Respiratory rate is increased aiming more heat exchange through evaporative losses, while blood flow is diverted to the peripheral blood vessels in order to dissipate heat (Han et al., 2010). There is also a distinct reduction in the productive capacity of birds under thermal stress, not only due to reduced feed intake, but also related to the inflammatory process and immune activation that is initiated as a consequence of HS (Klasing et al., 1987).

The behavioral and metabolic responses to HS are regulated by two pathways of stress response: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenomedullary axis (Post et al., 2003), where catecholamines are secreted. In the HPA axis, specific neurons produce corticotropin-releasing hormone (CRH) in response to a stimulus such as heat stress. CRH stimulates the anterior pituitary lobe to secrete the adrenocorticotropic hormone (ACTH), which is responsible for inducing the production and secretion of glucocorticoid hormones in the adrenal glands' cortex (Frankel, 1970).

Bartlett and Smith (2003) reported pronounced effects of immunosuppression in broilers submitted to chronic HS. There was a reduction in lymphoid organ weight, total antibody titers, IgG and IgM and reduction in phagocytic capacity of macrophages. Changes in electrolyte balance and blood pH, reduced energy availability to cells, increased serum corticosterone levels, and impaired intestinal epithelial cell integrity are also described (Quinteiro-Filho et al., 2012; Song et al., 2014).

The epithelium is the greater interface of the animal with the environment, and as a consequence, it is continually exposed to dietetic antigens and microorganisms (Ménard et al., 2010; Turner 2009). The junctional complexes between enterocytes (mainly tight junctions) have the primary function of regulating the paracellular flux, including avoidance of bacterial translocation. Hence, the integrity of the cells forming the intestinal epithelium is of fundamental importance, since it acts as a selective barrier against immunogenic agents and microorganisms (Jacobi 2012; Moran 2016; Ruth and Field 2013).

HS is known to cause damage to intestinal health and integrity by diverting blood flow to the periphery promoting hypoxia associated with increased serum endotoxemia and inflammation markers (Hall et al., 2001). HS has also been associated to increased oxidative stress due to increased demand for energy in the cell, which is closely associated with higher generation of ROS (Akbarian et al., 2016).

As causes for reduced intestinal quality, it has been postulated that hyperthermia is related to processes of cellular hypoxia and metabolic stress in the intestine and liver, caused by reduced blood supply to these organs, associated with high oxygen consumption and stimulation of metabolism. (Hall et al., 1999). Already in 2001, Hall and colleagues proposed that events secondary to the hypoxia process promote mitochondrial ROS production and stimulate the activity of oxidative enzymatic catalysts. The excess of these free radicals causes damage to cellular integrity, increasing intestinal permeability toxins and pathogenic bacteria, which accentuates local inflammation processes. Consequently, there is more stimulus for macrophages to produce NO (nitric oxide) through iNOS (inducible nitric oxide synthetase), aggravating the condition (Hall et al., 2001; Bhattacharyya et al., 2014).

Quinteiro-Filho et al. (2012) concluded that heat stress was able to generate a mild inflammatory bowel response through a change in the lamina propria lymphocytic cell population when compared to thermoneutral birds, characterizing a lympho-plasmacytic enteritis in the jejunum with the presence of heterophile infiltrates. According to Bhattacharyya et al. (2014), the presence of an inflammatory process in the gastrointestinal tract produces cytokines and other mediators that potentiate the production of ROS and, consequently, aggravate the gastrointestinal tract injury. These changes facilitate colonization of pathogenic bacteria present in the gastrointestinal tract, which in turn promote the activation of the immune response by increasing the toll-like receptor (TLR) signaling pathway, leading to the development of intestinal inflammation and further damage to the epithelium (Peterson et al., 2010; Lillehoj and Lee, 2012).

Song et al. (2014), in a study with broiler chickens reared under thermoneutral or HS conditions, showed that the intestinal integrity was significantly lower in the birds under HS when compared to those raised under thermal comfort. These results can be explained by the lower gene expression levels for occludin and ZO-1 observed for birds that suffered HS compared to the thermoneutral control. In addition, birds subjected to HS had shorter intestinal villi length and greater crypt depth compared with thermoneutral control (Song et al., 2014; Quinteiro-Filho et al. 2012).

2.2. MINERAL NUTRITION FOR POULTRY: EMPHASIS ON ZINC

O Trace minerals such as zinc (Zn), manganese (Mn), copper (Cu), iron (Fe), iodine (I) and selenium (Se) are required for numerous biological functions, sustaining adequate growth, production/reproduction and health status. In commercial broiler production, diets are supplemented with trace minerals, since feedstuffs do not contain sufficient concentration. Typically, the trace minerals are supplemented as inorganic sources, such as sulphates or oxides. Trace minerals need to be solubilized in the gastro-intestinal (GI) tract before being absorbed. The low pH of the gizzard facilitates solubilization in the gut of birds (Schlegel et al., 2010) but trace minerals, especially those present in plant feedstuffs, or free ions may be chelated to molecules, being excreted instead of absorbed. Zinc is especially susceptible to phytate and fiber, which could drastically limit its bioavailability (Wedekind et al., 1992; Linares et al., 2007).

The trace mineral requirements for broiler chickens are based on levels recommended by the NRC (1994), some of them referring to 1950 data, and therefore nutritionists often use higher levels of minerals, usually based on their own practical knowledge (Leeson, 2005). Mondal et al. (2010) added that the levels recommended by the NRC may not offer the optimum mineral input to meet the maximum productive potential of modern strains, and that this larger amount of minerals used by industry can often exceed the mineral amount needed for the production meeting the chicken requirement. In addition, considering the high variability or even low bioavailability of different mineral sources, the increased safety margin in micro-mineral supplementation results in a high level of mineral excretion (Leeson, 2005), causing environmental contamination (Aksu et al., 2011).

Mineral utilization by the metabolism fundamentally depends on minerals' absorption. Alternatively to traditional and inexpensive minerals salts, organic compounds are available nowadays. They are formed by the metal complexed to amino acids or chelated to proteins or carbohydrates. Organic microelements have been primarily defined by AAFCO (2005) as: "Specific Metal Amino Acid Complex", resulting from complexing a soluble metal salt with a specific amino acid; "Metal Amino Acid Complex", resulting from complexing a soluble metal salt with amino

acids; “Metal Amino Acid Chelate”, resulting from the reaction of a metal ion from a soluble salt with amino acids in a molar ratio of 1 mol of the metal to 1 – 3 (preferably 2) moles of amino acids, which form coordinated bonds (molecular weight of the hydrolyzed chelate should exceed 800 Daltons); “Proteinate Metal”, resulting from the chelation of a soluble salt with amino acids and/or partially solubilized protein; “Polysaccharide Metal Complex”, resulting from complexing a soluble salt with a solution of polysaccharides declared as integral part of an specific complex. Solubility, stability to physiological pH’s and absorption rate of the organic ligand are mandatory to adequate absorption and utilization of the minerals by the organism.

Different sources are absorbed by different pathways. In order to fully understand the potential of each source, it is mandatory to address different absorption mechanisms. Minerals are released from the diet as free ions to the GI tract. Free ions then bind to specific transporters that take them up into enterocytes in the duodenum and jejunum. Although major sites of zinc absorption may exist, it is likely that zinc absorption occurs along the entire GI tract to some extent. In mammals, there are two families of zinc transporters: Zrt- and Irt-like protein (ZIP) and the zinc transporter (ZnT). There are at least 14 different ZIP and 10 different ZnT transporters in human cells. ZIP and ZnT transporters appear to have opposite roles in controlling cellular zinc levels. ZIP transporters increase intracellular zinc levels by promoting uptake (import) of zinc from the extracellular environment such as the lumen of the gastrointestinal system (Figure 1; upper left), or by mediating the release of zinc from intracellular stores. In contrast, ZnT transporters mediate the efflux (export) of zinc from cells into the extracellular fluid such as blood plasma (Figure 1; lower left) or sequester zinc into intracellular vesicles (Hashimoto and Kambe, 2015). The evaluation of Zn transporters in avian species is relatively new to the literature; however, Zn transporter function is highly conserved between species (Wang and Zhou, 2010).

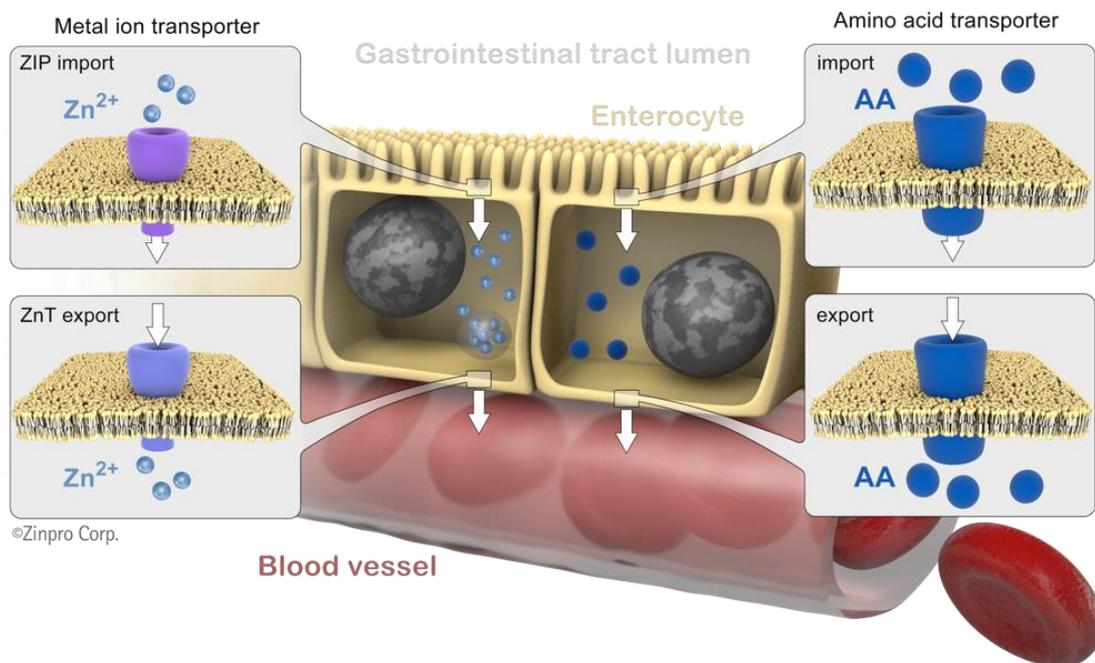


Figure 1. Comparison of metal ion transporter pathway and the metal-amino acid complex pathway. Courtesy of Zinpro Corporation.

Zinc deficiency may be caused by low levels of dietary zinc or, more likely, caused by the presence of antagonists of zinc uptake in the diet. For example, high levels of phytic acid may bind zinc in a form that becomes unavailable for zinc transporters. In addition, high levels of other divalent metals such as calcium, copper and iron may out-compete zinc for its transporter (Underwood and Suttle, 2001; Wedekind et al., 1994). Therefore, the bioavailability of zinc (and other trace metals) always has to be calculated considering the presence of other dietary components. In conclusion, there are three main impairers to adequate mineral absorption: chelation with dietary compounds, antagonistic behavior of cationic ions and metal ion transporter availability.

Conceptually, organic trace minerals would be able to avoid these aforementioned issues and deliver adequate mineral concentration to the bloodstream by being stable at physiological pH's (no free ions) and absorbable through an alternative pathway, such as the amino acids pathway. Sauer et al., (2017), conducted a study with human defective enterocytes. They have used Caco-2 cells and enterocytes differentiated from human induced pluripotent stem cells from a control and Acrodermatitis enteropathica (AE) patient. This condition is characterized by a mutation in ZIP4 transporter (Zinc uptake), rendering them inoperant causing a serious, and if left untreated, lethal recessive genetic disorder of Zn uptake (Wang et al., 2002; Andrews, 2008). Control and defective Caco-2 cells were exposed to an inorganic zinc supplement and a Zn- amino acid complex (ZnAA; Zinpro Corporation). Uptake of free zinc from inorganic sources was significantly impaired in cells with genetic mutation of the most important zinc transporter ZIP4. However, with the ZnAA source, zinc uptake in these cells was similar to cells with intact ZIP4, meaning that zinc from ZPM sources could enter the cells independent from the functional classic zinc importer in cells with non-functional ZIP4. Further, exposure of cells modeling enterocytes to inorganic zinc in the presence of high levels of phytic acid, folic acid, or calcium and copper supplement resulted in a decrease of zinc uptake as those factors antagonize absorption of free zinc. In contrast, treatment of these cells with zinc from ZnAA source and the antagonists showed much less effect on zinc uptake as ZnAA were hardly affected by the inhibitors. In addition, experiments utilizing amino acid blocking techniques have confirmed that zinc from CuAA (copper-amino acid complex; Zinpro Corporation) source utilize amino acid transport pathways. These experiments used very high concentrations of amino acids to saturate/block AA transport. In the presence of these high levels of amino acids, uptake of copper from CuAA source was reduced due to the competition between amino acids and copper from CuAA for uptake. This confirms that ions complexed to amino acids use amino acid transport pathways. Conversely, in these experiments the uptake of free inorganic zinc was not affected due to free inorganic zinc utilizing ZIP transporters (versus amino acid transporters) (Gao et al., 2014).

Zinpro's proprietary compounds are characterized by AAFCO (2005) as metal- amino acid complexes. The metal is between the carboxyl group and the alpha nitrogen, with a free side group to increase the stability but is not required for binding purposes. It forms 5 membered ring, complexes that are known to keep stable in the stomach acid (Sauer et al., 2017).

2.3. ZINC REQUIREMENT

Historically, we have formulated diets to meet broilers requirement aiming adequate productivity and no signs of deficiencies of any kind. As described by Leeson and Summers (2001), requirement would be "the minimum amount of the nutrient required to produce the best weight gain, feed efficiency, etc. and the lack of any signs of nutritional deficiency," which are often referred to as the "minimum nutrient needs" (Applegate and Angel, 2014).

The last NRC update (National Research Council) for poultry, dates of 1994 (25 years old now). Trace mineral requirements of poultry were measured through a dose response approach, using as criterion growth rate and tissue composition (NRC, 1994). The choice of the criteria is crucial. Performance is generally optimized at lower dietary levels than tissue contents or for additional functions such as oxidative metabolism or immune properties (Nys et al. 2018). Zinc is related to a wide variety of metabolic and structural activities, participating as cofactor or activator of more than 300 enzymes (Roohani et al. 2013). Thus, it participates not only on performance, but immune competence (Bartlett and Smith, 2003; Bun et al., 2011; Kakhki et al., 2016), antioxidant system (Powell, 2000; Bun et al., 2011), and intestinal integrity and histomorphometry (Miyoshi et al., 2016; Shao et al. 2014). This represents a problem from the standpoint of requirement determination.

Another limitation is the ability to precisely assess bioavailability, which also impacts requirements determination. The composition of the diet can also influence bioavailability. The NRC recommendation for dietary Zn in broilers was based on 9 studies and it is at least 40 mg of Zn per kg of diet. Eight of these studies have used purified or semi-purified diets to assess growth requirement. Wedekind et al. (1992) has estimated the bioavailability of Zn-methionine relative to Zn sulfate using three different diets: purified; semi-purified; and a practical diet of corn and soybean meal and found 117, 177 and 206% for these different diets, respectively. This led the industry to adopt mineral safety margins, avoiding sub-nutrition. The safety margins generate further problems with mineral antagonism and results in high mineral excretion levels (Leeson, 2005) contributing to environmental contamination (Aksu et al., 2011). Over-supplementing Zn is not a viable strategy for establishing a Zn requirement. Instead a balance should be struck between providing adequate Zn for biological functions while limiting Zn excretion into the environment. Accurate assessment of bioavailability of fed Zn source would allow nutritionists to optimize trace mineral supplementation. In recent years, organic zinc sources have been used increasingly due to their potentially higher zinc bioavailability (Kidd et al., 1996; Salim et al., 2010). Burrell et al. (2004) assessed the supplementation of zinc sulfate (ZnSO₄) and ZnAA and confirmed better zinc utilization, meaning same performance at lower levels. This results in less environmental contamination.

2.4. ZINC AND IMMUNITY

The essentiality of Zn in all aspects of immunity and its critical function (catalytic or structural) associated to enzymes to the integrity of cells involved in the immune response has been reported (Sherman, 1992). Recently, studies have been consistently reporting results of Zn supplementation on immunocompetence parameters. The immune systems are highly proliferative, and thus particularly susceptible to Zn deficiency (Prasad, 2013). Sunder et al. (2008) found higher antibody titers in birds supplemented with Zn levels greater than 80 mg/kg of feed. The heterophil:lymphocyte ratio and relative weight of lymphoid organs also responded to this level of supplementation. Bartlett and Smith (2003) provided diets for broiler chickens at three Zn levels (34, 68 and 181 mg/kg of feed) associated to HS. The researchers showed that the higher dose Zn was effective in increasing humoral response and macrophage activity but it was unable to improve these parameters when animals underwent HS. These results were also found by Kakhki et al. (2016) at the maximum feed level of 100 mg Zn / kg.

The effects of Zn on immune function are complex. The adaptive immune response is orchestrated by highly specialized cells, the T and B lymphocytes. B lymphocytes play a role in the humoral immune response by the production of antibodies specifically directed against an antigen, whereas T-lymphocytes are involved in cell-mediated immune responses by activation of other immune cells (T helper lymphocytes) and by the production of

toxic granules in cytotoxic T lymphocytes (Bonaventura et al., 2015). It has been established that it impacts T cell balance, specifically altering the CD4+T helper (Th) cells. Examples of these category of cells are Th1, Th2, Th17, and regulatory T-cells (Hönscheid et al., 2011). In general, Th1 cells are considered part of cell-mediated immunity and produce cytokines (INF γ , IL2, and TNF β) responsible for the activation of phagocytic cells, e.g. macrophages. In contrast, Th2 cells produce cytokines which activate antibody responses (IL-4, IL-5, IL-10, and IL-13) which in turn downregulate phagocytic responses. Zn deficiency is thought to negatively impact stem cell production in the thymus. Low Zn status decreases the ratio of CD4+ to CD8+ T-cells. Typically, there is little to no impact on Th2 cytokine expression, however Th1 cytokines decrease with Zn deficiency (Prasad et al., 1997). B cell development and functions are less affected by Zn changes than T cells. Still, Zn deficiency causes a reduction of B cells, affecting the development of immature and pre-mature B cells (Stefanidou et al., 2006) and affecting antibody production (DePasquale et al., 1984). More recently, the relationship of Zn status and different transcription factors such as NF- κ B and protein kinase c (PKC) have been reported (Haddad, 2009; Suzuki et al., 2006). In conclusion, Zn has major effects on both the innate and acquired immune system as well as its deficiency contributes and results from inflammation (Bonaventura et al., 2015).

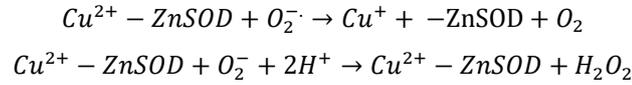
2.5. ZINC AND OXIDATIVE METABOLISM

As described by Goff (2018), oxidative stress is a term used when the generation of free radicals in the tissues of the body exceeds the ability of the various systems of the body to neutralize the free radicals (primarily reactive oxygen species - ROS) or exceeds the ability of the body to repair the damage done by unquenched free radicals.

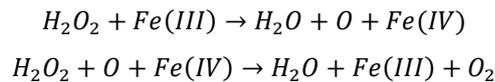
The main types of ROS are oxygen superoxide (O $_2^-$), hydroxyl (OH \cdot) and hydrogen peroxide (H $_2$ O $_2$). A free radical is any atom or molecule capable of existing independently that contains one or more unpaired electrons. The unpaired electron makes free radicals unstable and very reactive; they tend to remove an electron from a neighboring compound or donate their extra electron to some other molecule or atom (Goff, 2018). Less common, but even more destructive, are the free ionized metals in bodily fluids, particularly those trace minerals that fall into the category of transition metals in the periodic table of the elements (Valko et al., 2016). Primary targets that ROS try to steal electrons from, or donate electrons to, include nitric oxide (creating reactive nitrogen species, such as peroxynitrite) and the carbon-carbon double bonds found in PUFA within the lipid bilayer of cell membranes. Peroxidation of cell membrane lipids by reactive oxygen species can cause cell membranes to lose integrity and become leaky (Dizdaroglu and Jaruga, 2012).

Under normal conditions, oxygen consumed during cellular respiration may be reduced (receive an electron) to form a radical superoxide. Then, mitochondrial SOD transforms it into hydrogen peroxide, being converted to water by CAT and GPx. However, when ischemia/reperfusion (I/R) process is generated as a result of heat stress, superoxide production intensifies and exceeds SOD action capacity, which results in mitochondrial dysfunction. Further, in the presence of transition metals, such as iron and copper ions, H $_2$ O $_2$ may be reduced to hydroxyl, which is the most reactive ROS, extremely harmful to cell membranes. This is called the Fenton's reaction. In an attempt to minimize the presence of free ions in body fluids, the organism produces proteins that form reversible bonds to metal ions avoiding further oxidation, such as metallothionein, albumin and transferrin (Sitar et al., 2013).

The process in which Cu-ZnSOD performs the dismutation or removal of the superoxide anion from fluids may be described by two equations to transform O_2^- into less harmful oxygen and hydrogen peroxide molecules:



The same equations could be used to describe the chemistry of a superoxide dismutase that utilizes Mn as the metal and serves as the primary antioxidant enzyme within mitochondria (Cu-ZnSOD is found in the cytosol). The Mn is substituted for Cu, and the Mn valence state would waver between +3 and +2 instead of the +2 and +1 for Cu. The hydrogen peroxide generated by the action of the SODs can be subsequently converted to water and oxygen by catalase, an Fe-containing enzyme that is abundant in mammalian tissue. The Fe⁺³ incorporated into the catalase is used to donate an electron to one molecule of H₂O₂, becoming Fe⁺⁴ temporarily. Catalase temporarily holds the unstable oxygen atom created in the process. The Fe⁺⁴ now takes an electron away from a second molecule of H₂O₂. This allows the unstable oxygen atom held on the catalase to react with the second H₂O₂ to generate water and an oxygen molecule. It also returns the catalase Fe⁺⁴ to the Fe⁺³ state (McCord and Fridovich, 1988).



Two molecules of reduced glutathione (GSH) will each donate a hydrogen atom to an H₂O₂ molecule, forming 2 water molecules. The sulfhydryl groups of the 2 oxidized glutathione molecules join together, forming glutathione disulfide. Selenium is a cofactor necessary for function of glutathione peroxidase (GPx). This enzyme takes oxidized glutathione disulfide and reduces the sulfur-sulfur bond, regenerating 2 GSH molecules. The reduced glutathione can then be used to break down another hydrogen peroxide molecule.

Zn is also considered an indispensable component for the antioxidant system of animals (Naz et al., 2016). It is a cofactor of the enzyme SOD, which acts on the conversion of superoxide anions to hydrogen peroxide (Niles et al., 2008). In addition, Zn increases the synthesis of metallothionein, a cysteine-rich protein that acts as a free radical scavenger (Sahin et al., 2005); and prevents the formation of hydroxyl radicals (OH \cdot) by competing for transition metal binding sites such as iron and copper from the hydrogen peroxide (H₂O₂) molecule (Naz et al., 2016). Bun et al. (2011) reported that supplementation of organic Zn up to 40 mg/kg of feed was effective in reducing lipid peroxidation levels measured by malondialdehyde (MDA) concentration, and increased levels of GPx and hepatic SOD.

Nuclear factor-erythroid 2 (Nrf2) is one of the most important cellular defense factors against oxidative stress (He et al., 2009). It regulates intracellular antioxidants and other proteins to neutralize reactive oxygen and/or nitrogen species (ROS and/or RNS), such as superoxide dismutase (SOD) and glutathione S-transferase (He et al., 2009; Tan et al., 2011). Studies have indicated the induction of Nrf2 expression and function by zinc (Li et al., 2014).

2.6. ZINC AND INTESTINAL INTEGRITY

A single layer of epithelial cells separates the luminal content of the lamina propria and the interior of the organism (Lerner and Matthias, 2015). Therefore, the integrity of the cells is essential as the gut has simultaneous responsibility for nutrient absorption and acting as a selective barrier against immunogenic agents and microorganisms (Ruth and Field, 2013; Moran, 2016). Tight junctions (TJs) are proteins responsible to control permeability and selectivity, regulating the flow of solutes between enterocytes (Turner, 2009; Ménard et al., 2010). There are three main families: claudins, occludins and junctional adhesion molecules (JAMs) (Figure 2). Claudins are the main determinants of this selective function exerted by TJs, and some of them act as occlusive proteins, while others act as paracellular channels, due to their different affinities for electric charges (Lu et al., 2013). In total, 24 members of the claudin family have been identified (Lal-Nag and Morin, 2009; Lu et al., 2013). Occludins interact directly with claudins and actin (Ménard et al., 2010), demonstrating their role as regulators of paracellular permeability. JAMs, and zonula occludens 1 and 2 proteins (ZO1 and ZO2), are considered essential for the structure and maintenance of TJ, interacting with claudins, occludins and actin (Groschwitz and Hogan, 2009; Turner, 2009). In situations where the intestinal barrier is damaged, leads to increased intestinal permeability and bacterial translocation, ultimately resulting in systemic release of inflammatory mediators, activating the immune system (Andrade et al., 2015). Disproportionate levels of molecules such as ROS (reactive oxygen species) represent a major problem for homeostasis and cause damage to different body tissues, including the intestinal epithelium mucosa (Bhattacharyya et al., 2014). Heat stress (HS) is known to damage intestinal health and integrity by diverting blood flow to the periphery promoting hypoxia associated with increased serum endotoxemia and inflammation markers (Hall et al., 2001; Akbarian et al., 2016).

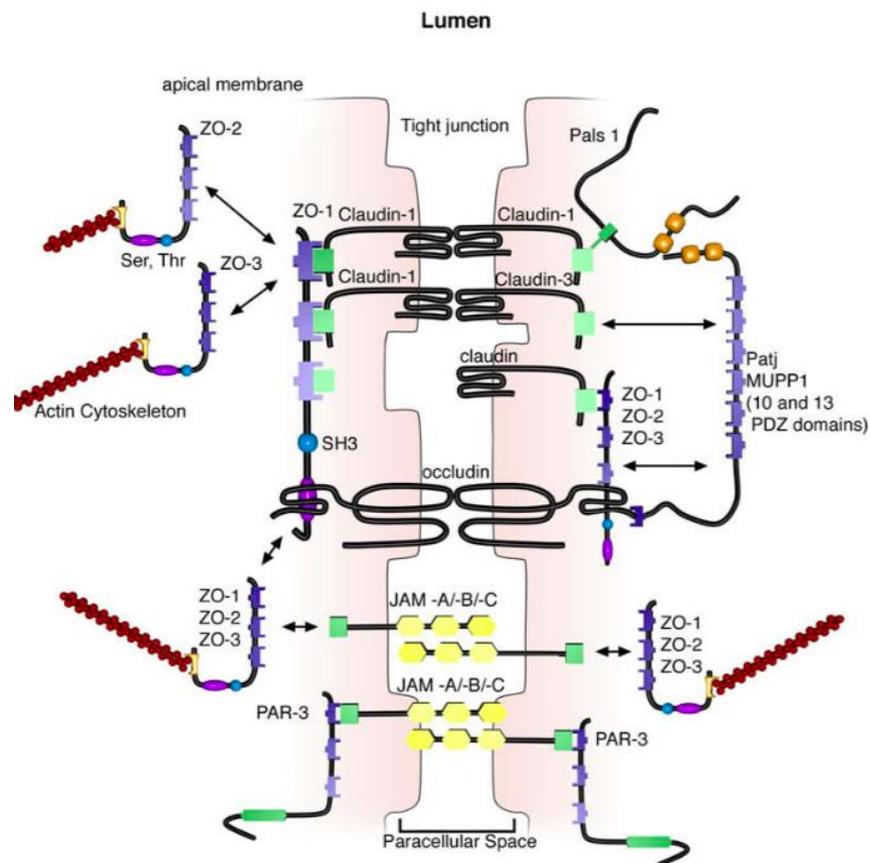


Figure 2. Interaction between different proteins comprising junctional complexes. Source: Groschwitz e Hogan, 2009.

Cell culture (Caco-2 cells) in Zn-deficient media presented reduced transepithelial electrical resistance (TER) values in relation to cultured cells in Zn-supplemented media (Finamore et al. 2008), an indication of reduced intestinal integrity. These results were attributed to a lower amount of the proteins occludin and zonula occludens (ZO-1), which compose the tight junctions, measured by Western blot. In addition, Zn deficiency increased neutrophil translocation, a result attributed to the increased expression of inflammatory cytokines (IL-8). The authors concluded that Zn-deficiency can cause damage to intestinal integrity and increase production of inflammatory cytokines, which increases neutrophil migration, in turn, exacerbating the inflammatory process and damage to the intestinal epithelium. Similarly, Miyoshi et al. (2016) reported decreased TER in cultured cells in Zn-deficient media, and increased FITC-FD4 flux. Both results indicate increased intestinal permeability. Furthermore, the gene expression of occludin and claudin-3 was lower for cells in Zn-deficient medium compared to the medium supplemented with Zn. In an experiment using *Salmonella Typhimurium* infection as an intestinal damage model in broilers, there was a reduction in the expression of claudin-1 and occludin. However, birds fed diets supplemented with 80 and 120 mg of Zn/kg of feed had a significant increase in the expression of these proteins, as well as significant effects on villus height and villus:crypt ratio in the ileum compared to birds not supplemented or supplemented with lower levels (Zhang et al. 2012).

Consolidating the important role of Zn addressed previously on performance, immune competence, oxidative metabolism, studies showed that, in stressful situations, the Zn requirement increases and its retention in the body decreases (Belay and Teeter 1996). In addition, Bartlett and Smith (2003) and Sahin and Kucuk (2003)

showed that the level of Zn in the blood plasma of heat-stressed chickens was significantly reduced compared to the control group.

This study aimed to assess the effects of zinc-amino acid complex supplementation on intestinal integrity, oxidative metabolism and performance of broilers raised under chronic cyclic heat stress, used as a model of gut barrier disruptor and ROS (reactive oxygen species) generator.

3. MATERIAL AND METHODS

3.1. EXPERIMENT 1

A group of 400 one-day-old Cobb 500 chicks originated from 40-weeks old broiler breeders were randomly allotted to four groups (100 birds each). Chicks were vaccinated for Marek's disease, infectious bursal disease and bronchitis at the hatchery. Each of these groups were fed diets containing different levels of zinc (0, 20, 40 and 60 ppm) supplemented exclusively in the form of metal-amino acid complex (Availa® Zn; ZnAA). From d1, these groups received the dietary treatments aforementioned and were raised under optimal temperature and humidity conditions and continuous light in floor pens with fresh rice hulls bedding until 21 days of age.

On d21, all birds of each dietary treatment were individually weighed, and 24 chicks were selected within the range of $\mu \pm 5\%$. They were randomly assigned according to their dietary treatment to two different environmental conditions (thermoneutral –TN and heat stress – HS). In each environment there was a metallic battery with 12 cages. The experimental treatments consisted of a 4×2 factorial arrangement including four levels of Zn supplementation and two environmental conditions (Table 1). A total of 12 birds per treatment combination were randomly allotted to 3 cages (replicates) with 4 birds per cage. For sample collection, two birds per cage were randomly taken and each individual chicken corresponded to an experimental unit. This trial was identically repeated over time (as a block), resulting in 12 replicates/treatment combination.

Birds were kept in wire cages (70 cm \times 70 cm \times 45 cm) in temperature-controlled rooms at either 24 ± 1 °C all day (TN) or 32 ± 1 °C (HS) for 12 h/d. During the whole experimental period birds were provided with ad libitum access to feed and water. The lighting program consisted of 18 h of light from d21 to d42 for both environments. The temperature protocol may be seen in detail in Figure 1 and was carried out from d21 to d42. Relative humidity was kept constant for both environmental conditions (45-55%).

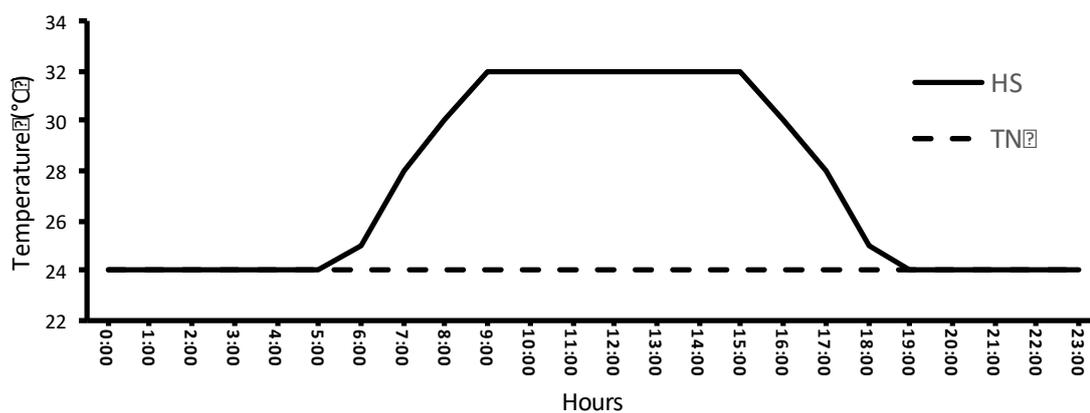


Figure 3. Description of the chronic cyclic heat stress protocol (HS) and thermoneutral temperature (TN) from 21 to 42 days. From 08:00 to 11:00 AM, the temperature gradually rises from 24°C to 32°C. From 11:00 AM to 05:00 PM, the temperature reaches the plateau, ranging from 32°C to 33°C. From 05:00 to 08:00 PM, the temperature gradually decreases from 32°C to 24°C. From 08:00 PM to 08:00 AM, the temperature was kept at 24°C.

Table 1. Treatments for Experiment 1

Treatment combination	ZnAA supplementation, mg/kg ¹	Environment ²
1	0	Thermoneutral
2	20	Thermoneutral
3	40	Thermoneutral
4	60	Thermoneutral
5	0	Heat Stress
6	20	Heat Stress
7	40	Heat Stress
8	60	Heat Stress

¹ Zinc source was Availa®Zn (Zinpro Corp., Eden Prairie, MN, United States). The Zn level of the basal diet (0 mg/kg of Zn) was approximately 30 mg/kg of Zn provided by the feed ingredients.

² Birds subjected to chronic cyclic heat stress during the period from 21 to 42 days of age. The thermoneutral group was kept under optimal temperature according to the strain recommendations.

3.2. EXPERIMENT 2

A total of 1,800 one-day-old Cobb 500 chicks from 35-week old broiler breeders were obtained from a commercial hatchery, individually weighed and assigned to one of five body weight categories. The goal was to ensure similar initial body weight for all replicates. Each group of 45 birds were randomly allocated to 40 floor-pens (3.0 × 1.34 m) resulting in a density of 11.25 birds/m². Chicks were vaccinated for Marek's disease, infectious bursal disease and bronchitis at the hatchery. The experiment was conducted in a completely randomized design comprising 4 dietary treatments with 10 replicates each (pen as the experimental unit). Each of these groups were fed diets containing different levels of zinc (0, 20 40 and 60 mg/kg) supplemented exclusively in the form of metal-amino acid complex (Availa® Zn; ZnAA). From d1, these groups received the dietary treatments aforementioned and were raised under optimal temperature and humidity conditions and continuous light and fresh rice hulls bedding. During the whole experimental period birds were provided with ad libitum access to feed and water. From d21 to d42, it was applied exactly the same heat stress protocol of Experiment 1 (Figure 3). Relative humidity was monitored over the period and varied within levels considered adequate (45-55%). These variables were monitored every 10 minutes with the aid of 4 digital loggers strategically distributed along the poultry house at 30 cm of the floor. The poultry house was equipped with fans, nebulizers and infrared heating lamps. A programmable electronic system controlled these equipment's as needed to reach the desired temperature (Figure 3). Curtain management was also performed if necessary.

The growth performance (feed intake and weight gain) of the animals was evaluated weekly by weighing birds and leftover feed by replicate (45 birds). Mortalities were recorded daily, and feed conversion ratio were calculated considering the weight of dead birds.

3.3. EXPERIMENTAL DIETS

Mineral premix was exclusively formulated and mixed for Experiments 1 and 2 according to the different zinc levels for the dietary treatments. Mineral sources used were in the form of metal-amino acid complex (Availa® Zn) for zinc; sulfates for manganese, iron and copper; and potassium iodate for iodine. Selenium was supplemented as sodium selenite via vitamin premix. To assure mixing quality, a filler composed by ground rice hulls, limestone and

mineral oil was included by the minimum of 37%, varying according to the inclusion of Availa® Zn. We considered that the amount of calcium contained in the filler was relevant, therefore, the variable inclusion of filler was accounted for in the formulation, resulting in variable inclusion of limestone in the diets. A specially built mixer was used to produce the mineral premix (Figure 4). Table 2 shows the premix composition for each dietary treatment.



Figure 4. Premix mixer (Source: Personal Archive).

Table 2. Mineral premix composition

ZnAA supplementation (ppm)	Premix inclusion (kg/ton) ¹	Mineral sources (kg/ton) ²	Filler inclusion (kg/ton)	Filler amount (%)	Limestone amount (kg/ton) ³	Calcium supplied (kg/ton) ⁴
0	1.5	0.442	1.058	70.5	0.349	0.132
20	1.5	0.609	0.891	59.4	0.294	0.111
40	1.5	0.776	0.724	48.3	0.239	0.090
60	1.5	0.942	0.558	37.2	0.184	0.069

¹ Premix inclusion is a result of the sum of mineral sources and filler inclusion.

² Sum of ZnAA (Availa Zn), manganese sulfate, iron sulfate, copper sulfate and potassium iodate.

³ Limestone inclusion in the filler was 33%.

⁴ Calcium concentration in limestone was 37.7%.

The birds were fed a starter diet until 21d of age, followed by a grower diet from d21 to d35 and a finisher diet from d35 to d42. Ingredients and chemical composition of the diets for Experiment 1 are shown in Table 3 (starter), Table 4 (grower) and Table 5 (finisher). For Experiment 2, the analyzed levels are presented in Table 6. The corn-soybean meal-based diets were formulated to be isonutritive, despite the variable inclusions of limestone. Phytase (Smizyme PT 5000, Salus Group) was included at 100 g ton⁻¹ to provide 500 FYT and considered to deliver 0.12% of calcium and 0.12% of available phosphorus. For the starter period, which birds were raised in floor pens, sodium salinomycin 12% (Salus Group) was included at 550 g/ton, which corresponds to 66 mg/kg of salinomycin. For Experiment 2, the only difference was that salinomycin was also included in the grower phase at the same level. Finisher diet was salinomycin-free.

Table 3. Composition, calculated and analyzed nutritional levels of the **starter diets** (1 to 21d) for Experiments 1 and 2.

Ingredients, %	ZnAA supplementation, ppm							
	0		20		40		60	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Corn (7.5% CP)	49.99		49.98		49.97		49.96	
Soybean Meal (46% CP)	42.34		42.34		42.34		42.34	
Soybean oil	4.15		4.15		4.15		4.15	
Dicalcium phosphate	1.08		1.08		1.08		1.08	
Limestone ¹	0.97		0.98		0.99		1.00	
Salt	0.52		0.52		0.52		0.52	
DL-Met (98%)	0.33		0.33		0.33		0.33	
Vitamin mix ²	0.15		0.15		0.15		0.15	
L-Lys HCl (78%)	0.11		0.11		0.11		0.11	
Choline chloride (60%)	0.08		0.08		0.08		0.08	
L-Thr (98%)	0.06		0.06		0.06		0.06	
Salinomycin ³	0.055		0.055		0.055		0.055	
Phytase ⁴	0.01		0.01		0.01		0.01	
Mineral mix ^{1,5} + 0 ppm Zn	0.15		-		-		-	
Mineral mix + 20 ppm Zn	-		0.15		-		-	
Mineral mix + 40 ppm Zn	-		-		0.15		-	
Mineral mix + 60 ppm Zn	-		-		-		0.15	
Total	100.0		100.0		100.0		100.0	
<i>Calculated Levels, % or as noted</i>								
ME (kcal/kg)	3025		3025		3025		3025	
CP	23.5		23.5		23.5		23.5	
Ca	0.92		0.92		0.92		0.92	
Av. P	0.43		0.43		0.43		0.43	
Supplemental Zn (mg/kg)	0		20		40		60	
Na	0.22		0.22		0.22		0.22	
Dig. Lys	1.26		1.26		1.26		1.26	
Dig. Met + Cys	0.94		0.94		0.94		0.94	
Dig. Thr	0.85		0.85		0.85		0.85	
<i>Analyzed Levels, % or as noted</i>								
CP	24.5	24.6	24.5	24.4	23.9	24.6	24.7	24.4
EE	5.5	5.5	5.7	5.3	5.5	5.3	5.6	5.3
Ca	0.82	0.76	0.79	0.74	0.72	0.69	0.73	0.72
P	0.59	0.61	0.61	0.58	0.58	0.60	0.59	0.56
Zn (mg/kg)	37	38	58	47	70	70	122	91
Fe (mg/kg)	210	170	242	177	217	168	241	174
Cu (mg/kg)	14.6	10.9	17.0	10.2	16.3	10.6	16.5	11.1
Mn (mg/kg)	97	89	109	87	103	81	106	88

¹ Mineral premixes with different inclusions of Zn (as Availa Zn[®], Zinpro Corporation, Eden Prairie, US) were prepared with variable amount of filler containing calcium, which was accounted in feed formulation.

² The vitamin premix provided the following per kg of diet: retinol, 13,500 IU; cholecalciferol, 3,750 IU; tocopherol, 30.0 IU; menadione, 3.75 mg; thiamin, 3.0 mg; riboflavin, 9.0 mg; pyridoxine, 4.5 mg; cyanocobalamin, 22.5 µg; niacin, 50 mg; pantothenic acid, 20 mg, folic acid, 2.25 mg; biotin 0.15 mg, selenium, 0.38 mg.

³ Salinomycin 12% at 550g / ton (66 mg/kg of salinomycin).

⁴ Phytase at 100g / ton to provide 500 FYT, delivering 0.12% of calcium and 0.12% of available phosphorus.

⁵ The mineral premix provided the following per kg of diet: Manganese (MnSO₄), 70 mg; iron (FeSO₄), 50 mg; copper (CuSO₄), 10 mg; iodine, 1 mg; and zinc (metal-amino acid complex) according to treatments.

Table 4. Composition, calculated and analyzed nutritional levels of the **grower diets** (21 to 35d) for Experiments 1 and 2.

Ingredients, %	ZnAA supplementation, ppm							
	0		20		40		60	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Corn (7.5% CP)	49.02		49.01		49.00		48.99	
Soybean meal (46% CP)	40.98		40.98		40.98		40.98	
Soybean oil	6.87		6.87		6.87		6.87	
Dicalcium phosphate	0.93		0.93		0.93		0.93	
Limestone ¹	0.86		0.87		0.88		0.89	
Salt	0.50		0.50		0.50		0.50	
DL-Met (98%)	0.32		0.32		0.32		0.32	
Vitamin mix ²	0.12		0.12		0.12		0.12	
L-Lys HCl (78%)	0.13		0.13		0.13		0.13	
Choline chloride (60%)	0.06		0.06		0.06		0.06	
L-Thr (98%)	0.05		0.05		0.05		0.05	
Phytase ³	0.01		0.01		0.01		0.01	
Mineral mix ⁴ 1,4 + 0 ppm Zn	0.15		-		-		-	
Mineral mix + 20 ppm Zn	-		0.15		-		-	
Mineral mix + 40 ppm Zn	-		-		0.15		-	
Mineral mix + 60 ppm Zn	-		-		-		0.15	
Total	100.0		100.0		100.0		100.0	
Calculated Levels, % or as noted								
ME (kcal/kg)	3200		3200		3200		3200	
CP	22.8		22.8		22.8		22.8	
Ca	0.83		0.83		0.83		0.83	
Av. P	0.38		0.38		0.38		0.38	
Supplemental Zn	0		20		40		60	
Na	0.21		0.21		0.21		0.21	
Dig. Lys	1.23		1.23		1.23		1.23	
Dig. Met + Cys	0.91		0.91		0.91		0.91	
Dig. Thr	0.81		0.81		0.81		0.81	
Analyzed Levels, % or as noted								
CP	23.2	24.1	23.7	23.7	23.4	22.9	23.7	21.7
EE	8.1	8.2	8.2	8.1	8.1	8.2	8.3	8.2
Ca	0.64	0.68	0.72	0.64	0.69	0.70	0.59	0.69
P	0.58	0.62	0.59	0.56	0.56	0.60	0.58	0.60
Zn (ppm)	38	41	48	56	69	70	75	104
Fe (ppm)	165	179	172	178	164	186	169	181
Cu (ppm)	12.6	11.7	12.8	13.5	12.8	11.8	14.3	11.4
Mn (ppm)	95	87	91	93	87	87	85	88

¹ Mineral premixes with different inclusions of Zn (as Availa Zn[®], Zinpro Corporation, Eden Prairie, US) were prepared with variable amount of filler containing calcium, which was accounted for in feed formulation.

² The vitamin premix provided the following per kg of diet: retinol, 10,800 IU; cholecalciferol, 3,000 IU; tocopherol, 24.0 IU; menadione, 3.0 mg; thiamin, 2.4 mg; riboflavin, 7.2 mg; pyridoxine, 3.6 mg; cyanocobalamin, 18.0 µg; niacin, 40 mg; pantothenic acid, 14 mg; folic acid, 1.8 mg; biotin 0.12 mg, selenium, 0.30 mg.

³ Phytase at 100g / ton to provide 500 FYT, delivering 0.12% of calcium and 0.12% of available phosphorus.

⁴ The mineral premix provided the following per kg of diet: Manganese (MnSO₄), 70 mg; iron (FeSO₄), 50 mg; copper (CuSO₄), 10 mg; iodine, 1 mg; and zinc (metal-amino acid complex) according to treatments.

Table 5. Composition, calculated and analyzed nutritional levels of the **finisher diets** (35 to 42d) for Experiments 1 and 2.

Ingredients, %	ZnAA supplementation, ppm							
	0		20		40		60	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp2	Exp 1	Exp 2
Corn (7.5% CP)	59.00		58.99		58.98		58.97	
Soybean meal (46% CP)	32.77		32.77		32.77		32.77	
Soybean oil	5.76		5.76		5.76		5.76	
Limestone ₁	0.74		0.74		0.75		0.75	
Dicalcium phosphate	0.55		0.55		0.55		0.55	
Salt	0.47		0.47		0.47		0.47	
DL-Met (98%)	0.25		0.25		0.25		0.25	
L-Lys HCl (78%)	0.16		0.16		0.16		0.16	
Vitamin mix ₂	0.07		0.07		0.07		0.07	
Choline chloride (60%)	0.04		0.04		0.04		0.04	
L-Thr (98%)	0.03		0.03		0.03		0.03	
Phytase ₄	0.01		0.01		0.01		0.01	
Mineral mix _{1,4} + 0 ppm Zn	0.15		-		-		-	
Mineral mix + 20 ppm Zn	-		0.15		-		-	
Mineral mix + 40 ppm Zn	-		-		0.15		-	
Mineral mix + 60 ppm Zn	-		-		-		0.15	
Total	100.0		100.0		100.0		100.0	
<i>Calculated Levels, % or as noted</i>								
ME (Kcal/Kg)	3250		3250		3250		3250	
CP	19.9		19.9		19.9		19.9	
Ca	0.66		0.66		0.66		0.66	
Av. P	0.32		0.32		0.32		0.32	
Supplemental Zn	0		20		40		60	
Na	0.20		0.20		0.20		0.20	
Dig. Lys	1.07		1.07		1.07		1.07	
Dig. Met + Cys	0.79		0.79		0.79		0.79	
Dig. Thr	0.70		0.70		0.70		0.70	
<i>Analyzed Levels, % or as noted</i>								
CP	20.2	20.2	20.2	19.8	21.2	19.5	19.8	19.8
EE	7.3	7.3	7.5	7.3	7.3	7.3	7.3	7.4
Ca	0.52	0.57	0.50	0.59	0.49	0.53	0.49	0.57
P	0.48	0.49	0.48	0.51	0.48	0.51	0.48	0.51
Zn (ppm)	29	28	54	50	73	68	81	77
Fe (ppm)	182	172	150	151	135	152	150	157
Cu (ppm)	10.5	11.9	12.7	10.3	12.1	10.2	10.8	10.7
Mn (ppm)	78	89	83	88	80	102	80	89

¹ Mineral premixes with different inclusions of Zn (as Availa Zn[®], Zinpro Corporation, Eden Prairie, US) were prepared with variable amount of filler containing calcium, which was accounted for in feed formulation.

² The vitamin premix provided the following per kg of diet: retinol, 6,300 IU; cholecalciferol, 1,750 IU; tocopherol, 14.0 IU; menadione, 1.75 mg; thiamin, 1.40 mg; riboflavin, 4.20 mg; pyridoxine, 2.10 mg; cyanocobalamin, 10.50 µg; niacin, 25 mg; pantothenic acid, 8 mg; folic acid, 1.05 mg; biotin 0.07 mg, selenium, 0.175 mg.

³ Phytase at 100 g / ton to provide 500 FYT, delivering 0.12% of calcium and 0.12% of available phosphorus.

⁴ The mineral premix provided the following per kg of diet: Manganese (MnSO₄), 70 mg; iron (FeSO₄), 50 mg; copper (CuSO₄), 10 mg; iodine, 1 mg; and zinc (metal-amino acid complex) according to treatments.

Table 6. Analyzed nutritional levels of starter, grower and finisher diets for Experiment 2.

<i>Analyzed Levels, % or as noted</i>	ZnAA supplementation, ppm			
	0	20	40	60
<i>Starter</i>				
CP	24.1	23.6	24.5	24.5
EE	5.6	5.8	5.5	5.7
Ca	0.79	0.76	0.78	0.78
P	0.70	0.71	0.69	0.69
Zn (ppm)	37	52	78	84
Fe (ppm)	207	220	201	167
Cu (ppm)	7.6	7.7	7.6	7.7
Mn (ppm)	92	96	90	83
<i>Grower</i>				
CP	24.2	23.2	23.5	23.7
EE	8.1	8.2	8.0	7.9
Ca	0.74	0.76	0.72	0.77
P	0.64	0.63	0.65	0.64
Zn (ppm)	35	44	56	86
Fe (ppm)	128	147	145	144
Cu (ppm)	7.0	7.2	7.1	7.0
Mn (ppm)	83	86	81	79
<i>Finisher</i>				
CP	20.5	19.9	20.5	20.2
EE	6.2	6.8	7.1	6.9
Ca	0.57	0.57	0.55	0.59
P	0.54	0.55	0.54	0.53
Zn (ppm)	34	50	67	77
Fe (ppm)	139	120	124	119
Cu (ppm)	6.8	7.8	7.7	7.3
Mn (ppm)	84	81	78	79

3.4. BIOLOGICAL SAMPLING

At 42d of age, two birds per cage (6 birds/treatment combination) were punctured in the ulnar vein for peripheral blood collection with the aid of vacutainer tubes (treated with clot activator or EDTA). For serum, blood was allowed to clot for two hours at room temperature and EDTA treated tubes were refrigerated. After centrifugation at $3,000 \times g$ for 15 minutes, serum and plasma were aliquoted and stored at a -80°C . Still from the EDTA treated tube, three hundred μL of erythrocytes were diluted with 1,500 μL of Triton-x (5 mL L-1) homogenized and centrifuged at $12,000 \times g$ at 4°C . The hemolysate was aliquoted into multiple microtubes and stored at -80°C until final analyses.

Afterwards, the same birds had their individual weight recorded and were killed by cervical dislocation for tissue collection. Bursa, spleen and three thymic lobes on the left side of the neck were removed, stripped of adhering tissue and weighed individually. Relative organ weights were calculated as percentages of body weight. Fragments of enteric tissue (jejunum), liver (lateral part of the right lobe) and breast muscle (left side) were collected and stored at -80°C until further processing. Additional collections of jejunum for Western Blot analyses and of jejunum and ileum for histomorphometry were performed. Histological samples were cut open, intestinal content

was gently removed by trained personnel and subsequently fixed in 4% paraformaldehyde solution (for 48 hours), dehydrated in ethanol solutions, and stocked in 70% ethanol. All samples that required freezing, were immediately submerged into liquid nitrogen after collection. The right leg and whole liver were removed from the body and frozen at -20°C .

3.4.1. Quantification of serum total protein (tp) and electrophoretic profile

TP was determined in serum by the biuret method. Twenty μL of sample were homogenized with 980 μL of NaOH and 200 μL of reactive biuret. After 20 minutes of reaction in a dark environment, the absorbance reading in a spectrophotometer with wavelength set at 545 nm was performed. A standard curve relating concentrations of standard bovine serum albumin (Sigma Chemicals Company -USA) to absorbance values was performed. The concentrations of TP are expressed in g dL^{-1} . The electrophoretic analysis was performed with 25 μL of serum in a film of cellulose acetate in gel form (Cellogel). After 18 min of electrophoresis at 200 volts, the film was stained with Ponceau S dissolved in 1 L of 5% TCA (trichloroacetic acid) solution. After the staining, a reading of the protein fractions in a densitometer at a wavelength of 520 nm was performed. Serum proteins were divided into the following fractions: albumin, globulins, and gamma globulins. The relative percentage of each protein fraction was calculated in the software Celm SE-250 from the area under the curve created by the protein band.

3.4.2. Serum lysozyme determination

The quantification of lysozyme was performed in serum by turbidimetric assay (Parry et al. 1965), using as substrate 0.75 mg mL^{-1} of lyophilized cells of *Micrococcus lysodeikticus* Gram-positive bacteria, diluted in 0.1 M citrate, phosphate buffer pH adjusted to 5.8. After dispensing 175 μL of bacterial solution into microplate wells and 25 μL of each serum sample, the absorbance reading at 450 nm was registered after 0 and 20 minutes of incubation. The results are presented as U mL^{-1} , wherein 1.0 U mL^{-1} of lysozyme corresponds to the amount of enzyme that reduces the optical density by 0.001 units per minute.

3.4.3. Lipid peroxidation

The rate of lipid peroxidation was evaluated in erythrocytes and tissues by the measurement of reactive substances to thiobarbituric acid (TBARS), according to a protocol described by Buege and Aust (1978). This method is based on the reaction of two molecules of thiobarbituric acid with one molecule of malondialdehyde (MDA), producing a complex of pink coloration that may be quantified by spectrophotometer reading at a wavelength of 532 nm. This reaction occurs at an acidic pH at temperatures between 90 and 100°C . An aliquot of 100 μL of sample was homogenized in 200 μL of a solution composed by trichloroacetic acid (15%), thiobarbituric acid (0.375%) and hydrochloric acid (0.25 N), heated for 15 minutes in boiling water, then cooled in water and centrifuged for 10 minutes at $1,300 \times g$. The supernatant was used to quantify the TBARS in spectrophotometer at a wavelength of 532 nm. The results obtained were compared with a standard MDA curve. For erythrocytes the values are expressed in $\text{ng of TBARS hemoglobin}^{-1}$, and for tissues, in $\text{ng of TBARS protein}^{-1}$.

3.4.4. Superoxide dismutase (SOD) activity

SOD activity was determined in hemolysate of erythrocytes and enteric, hepatic and muscular tissues. This method uses a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and xanthine. Samples (8 μL) were added in duplicate into microplate wells. Aliquots of 200 μL of the reaction solution (19.3 mL of 50 mmol sodium phosphate buffer (pH 8.0); 0.1 mmol diethylenetriamine-pentaacetic acid; 0.1 mmol xanthine; 100 μL tetrazolium salt 10 mmol; and 5 μL xanthine oxidase 4.5 $\mu\text{m mL}^{-1}$) were added to the wells and the plate was placed in the reader at room temperature to be agitated. Thereafter, an initial absorbance reading was performed with wavelength set at 450 nm. The rate of increase in absorbance was followed over a period of 5 min, followed by an endpoint at a fixed time after the initial reading. Such a protocol allows verification that the assay is performed within the linear part of the reaction, and that the subtraction of the value from the end point gives more accurate numbers for the rates of increase in absorption. The level of SOD in the cell extracts was determined by the ratio of inhibition by the measured SOD standard at the same time. For blood, values are expressed as U mg^{-1} of hemoglobin and for tissues, U mg^{-1} of protein.

3.4.5. Glutathione peroxidase (GPx) activity

GPx was determined in hemolysate of erythrocytes, enteric, hepatic and muscular tissues. The reaction was carried out in a medium containing 300 μL of a solution composed by 48 mmol phosphate buffer (pH 7.7); 0.38 mmol EDTA; 0.95 mmol azide (to inhibit catalase); 1 mmol glutathione; 0.12 mmol nicotinamide adenine dinucleotide phosphate (NADPH); 3.2 U glutathione reductase; 0.02 mmol DL-dithiothreitol, and 0.0007% hydrogen peroxide (Wendel, 1981). The decay of the absorbance was recorded for 5 minutes in a spectrophotometer with wavelength set at 340 nm. For blood, values are expressed as U mg^{-1} of hemoglobin and for tissues, U mg^{-1} of protein. One unit catalyzes the oxidation by H_2O_2 of 1 mole of reduced glutathione to oxidized glutathione per minute at 25 $^\circ\text{C}$, pH 7.0.

3.4.6. Catalase (CAT) activity

The activity of CAT was determined in hemolysate of erythrocytes, enteric, hepatic and muscular tissues according to the methodology described by Iwase et al. (2013). In this analysis, 100 μL of sample or standard (catalase from bovine liver, Sigma) was incubated with 100 μL of Triton-x 1% and 100 μL of 30% oxygen peroxide. Within 15 minutes, the height of the O_2 foam formed was measured with a digital caliper. The specificity of the reaction was tested with samples containing 10 μM sodium azide. A calibration curve was drawn with defined units of catalase activity. For blood, values are expressed as U. mg^{-1} of hemoglobin and for tissues, U mg^{-1} of protein. A catalase unit is responsible for the consumption of 1 μmol H_2O_2 per minute.

3.4.7. Capacity of oxygen radicals' absorption (orac)

Samples of plasma, enteric, hepatic and muscular tissues were analyzed for the absorption capacity of oxygen radicals. In this analysis, fluorescein is used as a probe to detect damages caused by the radicals generated by the 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) throughout the reaction. The fall of fluorescence generates a decay curve that is then analyzed by measuring the area produced in relation to the curve of a control ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid - TROLOX) that does not have antioxidant agents that could impede the process. The analyzes were performed in triplicate using kinetic mode methodology using 528 nm emission absorbance and 485 nm excitation lasting two hours at 37°C. For plasma, values are expressed as U mg⁻¹ of hemoglobin and for tissues, U mg⁻¹ of protein.

3.4.8. Determination of intestinal integrity

To detect enteric leakage, chickens were dosed with 4.16 mg/bird of FITC-d (MW 3,000–5,000; Sigma Aldrich Co., St. Louis, MO) by oral gavage. After 2.5 h, blood was collected and allowed to sit for 3 h for clotting at room temperature and centrifuged (500 × *g* for 15 min) to separate serum. Fluorescence levels of diluted serum (1:1 in PBS) were measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm. FITC-d concentration per mL of serum was calculated based on a standard curve.

3.4.9. Histomorphometric analyses of the intestinal epithelium

Specimens for light microscopy were taken from two sites (jejunum and ileum) of the small intestine. The samples of jejunum and ileum stocked in 70% ethanol were included in paraffin and the histological blocks were cut on a microtome to give 5- μ m cuts. Fifteen sections from each bird and intestinal segment were stained with 1% Alcian blue (Ab; pH=2.5)/periodic acid-Schiff (PAS) to identify neutral (pink) and acidic (blue) mucins. Then, the number of goblet cells containing neutral and acidic mucins were counted using a light microscope (Top Light B2, BEL Engineering) coupled to a BEL Micro Image Explorer analysis system (BEL Engineering SRL). The following morphometric analyzes were performed in 15 villi randomly chosen from different well-orientated parts of the sections: villus height, crypt depth and the ratio between villus height and crypt depth.

For the quantification of the different mucin chemotypes containing goblet cells, 10 villi were randomly chosen from different well-orientated parts of the sections, where the goblet cells showed clear staining characteristics in sections. There was no subdifferentiation for acidic mucins (sialomucins and sulfomucins).

3.4.10. Western blot

Approximately 50 mg of ileum specimens (four randomly selected samples per treatment) were lysed with 500 μ l RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) to analyze claudin-1, occludin and zonula occludens (ZO-1) quantities. Total protein concentration was assessed by a BCA protein assay kit (Thermo scientific) and equal protein amounts of boiled

samples were separated by electrophoresis (Criterion™ Gel, 4–20% Tris-HCL, Bio-Rad Laboratories Inc.) and electro-transferred onto nitrocellulose membrane 0.45 µm (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with PBS supplemented with 0.05% Tween-20 (PBST) and 5% milk proteins and incubated overnight at 4°C with antibodies for claudin-1, occludin and ZO-1 (Thermo scientific, Rockford, IL, USA). After washing in PBST, the membranes were incubated with appropriate reagent for Western blot detection (VECTASTAIN, Vector Laboratories, Burlingame, CA, USA) and digital images were analyzed for signal intensities were quantified using the ImageJ 1.47 software. The signal intensities were normalized to beta-actin and then transformed to percentage within each run (all treatment combinations repeated once). The average of the 4 runs (4 animals per treatment combination) are presented.

3.4.11. Zinc concentration in diets, tibia and liver

The right tibia of the birds at 42 days were collected, identified and stored in a freezer at -20°C for later processing. After removal of the adherent tissue, the tibia was subjected to fat extraction by treatment with petroleum ether for 8 hours and then oven dried for 12 hours, weighed and calcined in a muffle oven at 540°C for 6 hours for the determination of bone ash. Liver was oven dried for 12 hours. Samples of tibia and liver were ground into fine particles using a coffee grinder (from the lowest zinc treatments to the highest). Ground tibias and livers samples were weighed (0.2 g) and then microwave digested with 6 mL of 20% HNO₃ before being submitted to the ICP - OES (induced coupled plasma) analysis (AOAC, 1995).

To determine the amount of zinc in the feed, 1 gram per sample was dissolved in 50% nitric acid (HNO₃) and subjected to digestion. Then, 2% HNO₃ is added to the solution which was weighed to obtain the dilution factor. The mineral extract was then carefully filtered using Whatman (42) filters (Whatman International Ltd., Maidstone, England) and the filtrate used for analysis of zinc concentration by atomic absorption spectrometry (Solaar Spectrometer, Thermo Fisher Scientific).

3.5. STATISTICAL ANALYSES

The results are reported as means with their standard errors and all the data were analyzed using the MIXED procedure of SAS software (SAS Institute, Inc.). Data were subjected to two-way ANOVA in a 2 x 4 factorial arrangement with environmental conditions and zinc-amino acid complex level (ZnAA) as the main effects and their interactions. As the exact same experiment was repeated over time (blocking factor), this effect was included in the model as a random effect. Interaction between treatments and blocks were tested for all variables and as no significant interaction was observed it was excluded from the model. Therefore, the following model was conceived:

$$y_{ijk} = \mu + D_i + C_j + (DC)_{ij} + \delta_k + \varepsilon_{ijk}$$

Where:

y_{ijk} = observation in i^{th} diet, j^{th} environmental condition and k^{th} block;

μ = the overall mean;

D_i = the effect of i^{th} diet, $i = 4$;

C_j = the effect of j^{th} environmental condition, $j = 2$;

$(DC)_{ij}$ = the effect of the ij^{th} interaction of diet x environmental condition;

δ_k = random effect of k^{th} block, $k = 2$;

ε_{ijk} = random error y_{ijk} .

Polynomial contrasts for the ZnAA levels were conducted using CONTRAST statements of SAS. When interactions were significant, polynomial contrasts were used to determine the linear and quadratic responses of simple means to dietary ZnAA levels within the heat stress (HS) and thermoneutral (TN) groups. When interactions were not significant, the polynomial contrasts were conducted over main effect means for ZnAA levels (averaged over environmental condition treatments). The equations were obtained using PROC REG.

All data was tested for normality of residuals and homoscedasticity through Shapiro-Wilk test and histogram analyses, and Brown–Forsythe test, respectively. Observations were marked as outliers and excluded from the data set before statistical analyses if the residue of one observation exceeded the parameter residual mean by more or less than 2 standards deviations (or until reach normality).

FITC-d data did not meet the assumption for the aforementioned statistical model. Therefore, a non-parametric procedure was implemented. The chosen method was Conover Scores. Conover scores are based on the squared ranks of the absolute deviations from the sample means (Conover, 1999).

4. RESULTS AND DISCUSSION

4.1. EXPERIMENT 1

4.1.1. Water and environmental characterization

The quantifications of zinc results in the diets are presented in Tables 3, 4, 5, and 6. Table 7 shows the results for water analyses before the beginning of the trial to assure no mineral contamination existed.

Table 7. Water analyses for each environment and different tests with the battery cages.

Item	Unit	Tap water Heat stress room	Tap water Thermoneutral room	Galvanized steel wire ¹	Stainless steel drinker ²
Zinc	mg L ⁻¹	0.05	0.004	0.536	0.017
Iron	mg L ⁻¹	0.01	0.02	-	-
Calcium	mg L ⁻¹	15.0	17.7	-	-
pH	-	7.6	7.9	-	-

¹The wired floor of the cages was washed, and the water was collected to quantify zinc content.

²Drinkers were filled with tap water and left for 24 hours to quantify zinc content.

The series of charts below (Figures 5, 6 and 7) shows the expected *vs.* observed temperatures for both environmental conditions. Measurements of temperature (°C) and relative humidity (%) were acquired by data loggers placed at the same position in both rooms, every 10 minutes. Data were compiled by hour and plotted by week, from d21 to d42. The observed relative humidity for the experimental period in both environments for experiment 1 and 2 (repeated over time) and their combination are shown on Table 8.

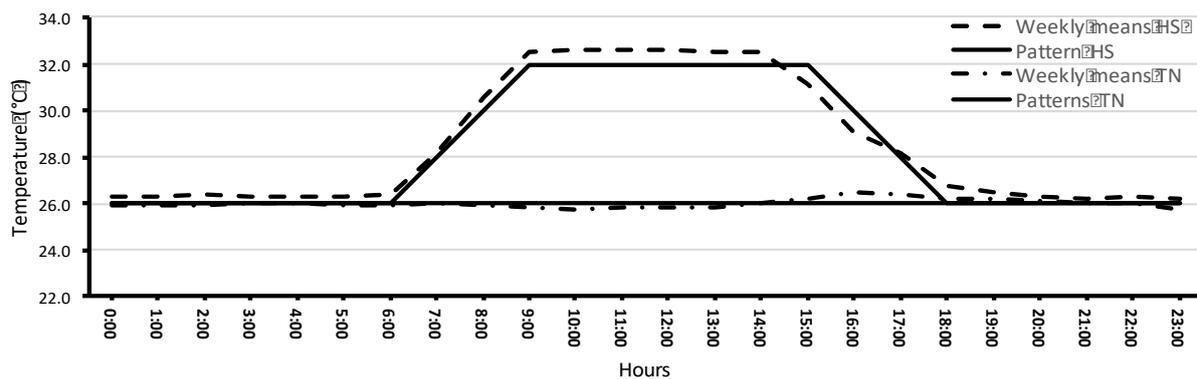


Figure 5. Average of observed temperatures for heat stress and thermoneutral environments and the expected patterns from d22 to d28.

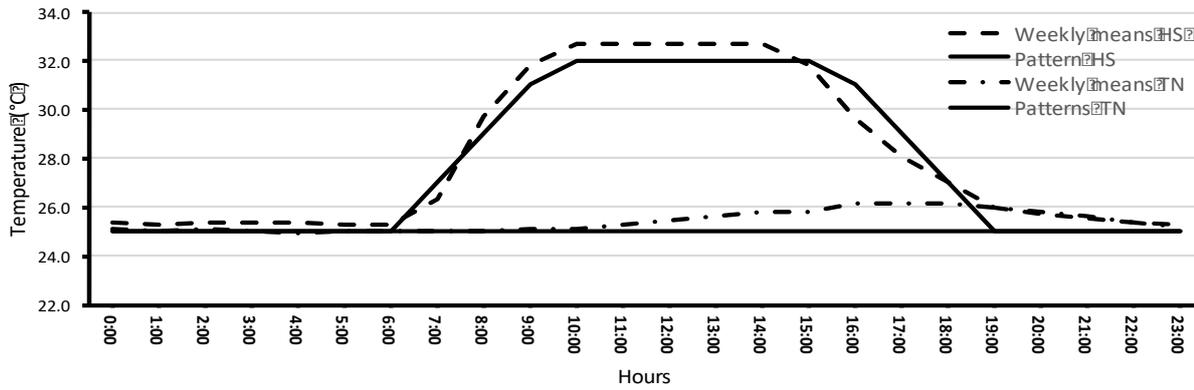


Figure 6. Average of observed temperatures for heat stress and thermoneutral environments and the expected patterns from d28 to d35.

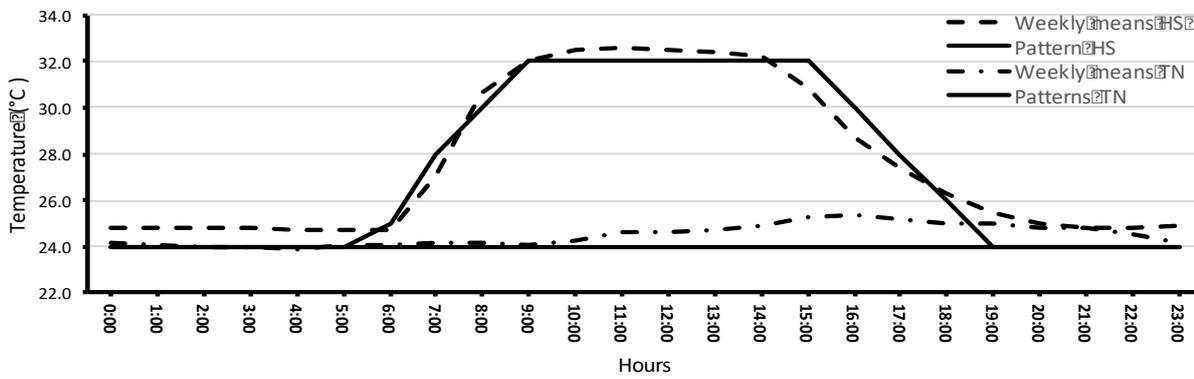


Figure 7. Average of observed temperatures for heat stress and thermoneutral environments and the expected patterns from d35 to d42.

Table 8. Relative Humidity (%) for the experimental period for both experiments and its combination.

	d21 to d42	
	Heat Stress	Thermoneutral
Trial 1	64.5	47.3
Trial 2	67.0	47.0
Combined	65.8	47.2

4.1.2. Physiological response to heat stress and zinc supplementation

At d38, two birds per cage (24 birds/environmental condition) were used to assess respiratory rate (respiratory movements min⁻¹) and rectal temperature (°C) with the aid of a digital veterinary thermometer. Figure 8 shows the results averaged over ZnAA levels (as no statistical effects were detected).

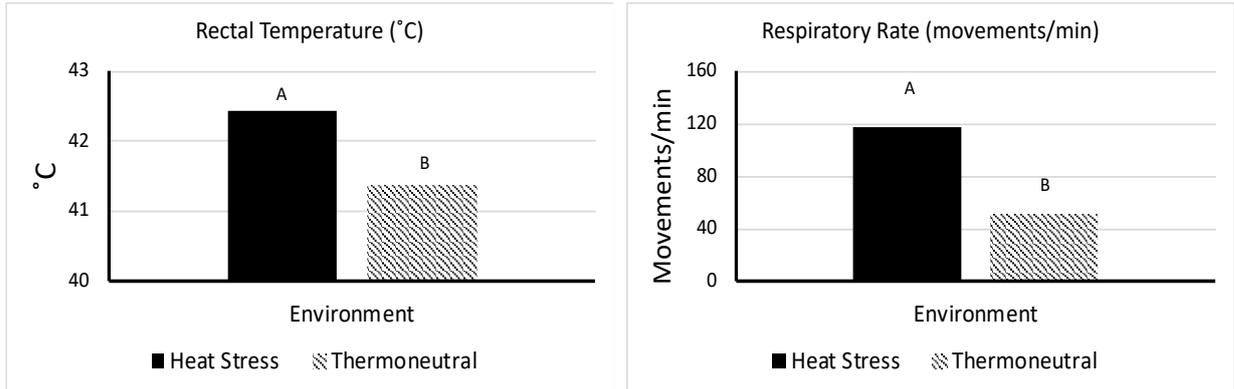


Figure 8. Respiratory rate (movements min⁻¹) and rectal temperature (°C) at d38.

Heat stress is one of the most important environmental stressors in poultry husbandry worldwide. Feed intake, growth rate, mortality, egg production, hatchability, and other important traits governing the economic success of the poultry industry are adversely affected by severe heat stress.

Performance results for experiments 1 and 2 from 21 to 42 days of age are summarized in Table 9. As the birds received the experimental diets from 1d, on the day of selection and relocation (21d), higher body weight (iBW) may be observed as ZnAA level increased. At 42d, heat stress negatively impacted all performance variables ($p < 0.05$). The reduction in performance for heat stressed birds compared to thermoneutral birds, was 9.0% for body weight, 13.7% for body weight gain, 9.8% for feed intake and a 3.6% worse feed conversion ratio. Azad et al. (2010) found similar performance results for a very similar cyclic chronic heat stress protocol. These results, combined to increased rectal temperature and respiratory rate, confirms that stress has been generated and birds were far out the comfort zone. Higher zinc levels linearly improved feed conversion ratio (FCR; Figure 9). Aligned with the significant result of the interaction for FCR ($p = 0.086$), contrast analyses showed a significant interaction ($p = 0.028$) between environment and ZnAA level. The sliced results (Figure 10) shows that birds raised under optimal temperature responded linearly, improving FCR as ZnAA increased. Stressed birds, conversely, had a positive quadratic response, the lowest value being at 60 ppm of ZnAA. Despite no apparent reason for intermediate levels (20 and 40 ppm) performed worse, maybe under heat stress conditions, birds require more zinc to perform satisfactorily.

Table 9. Growth performance of broilers fed increasing levels of ZnAA and raised under different environment conditions from 21 to 42 days.

Item ¹	iBW	BW	BWG	FI	FCR
Environment ²					
TN	-	3480 _A	2434 _A	3675 _A	1.508 _B
HS	-	3166 _B	2099 _B	3315 _B	1.568 _A
ZnAA levels					
0 ppm	1030 _B	3283	2253	3493	1.560
20 ppm	1050 _A	3315	2263	3506	1.548
40 ppm	1054 _A	3337	2277	3504	1.522
60 ppm	1055 _A	3358	2274	3478	1.522
SEM	45	91	53	105	0.009
ANOVA					
Environment	-	<0.001	<0.001	<0.001	<0.001
ZnAA level	<0.001	0.430	0.942	0.947	0.095
Environment x ZnAA level	-	0.199	0.168	0.198	0.086
Contrast					
Linear	<0.001	0.106	0.572	0.775	0.016
Quadratic	0.032	0.869	0.839	0.597	0.662

¹ iBW: initial body weight in grams (at 21d); BW: body weight in grams; BWG: body weight gain in grams; FI: feed intake in grams; FCR: feed conversion ratio in g:g.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: cyclic heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$), $n = 6$ replicates of 4 birds each per treatment combination.

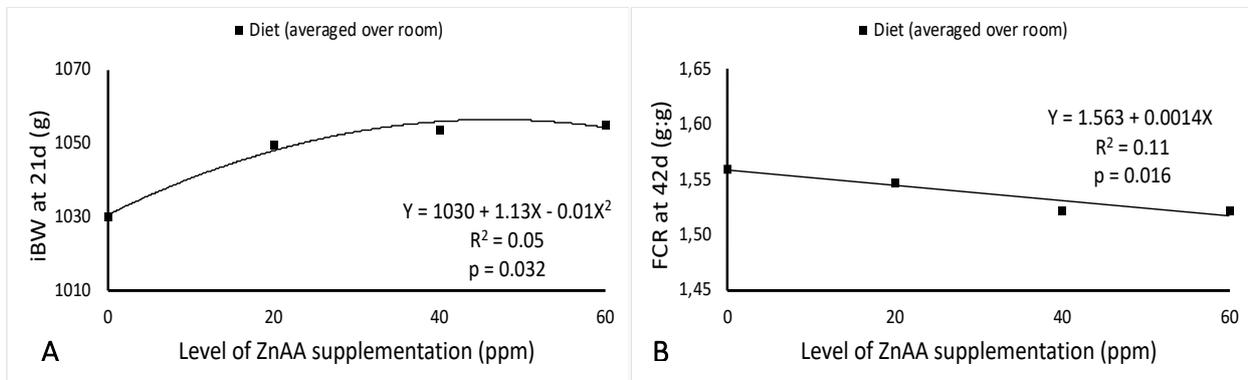


Figure 9. Growth performance of broilers fed increasing levels of ZnAA and raised under different environment conditions from 21 to 42 days. A) Regression analyses for body weight (g) at 21d and B) for FCR (g:g) at 42d. Results averaged over environment.

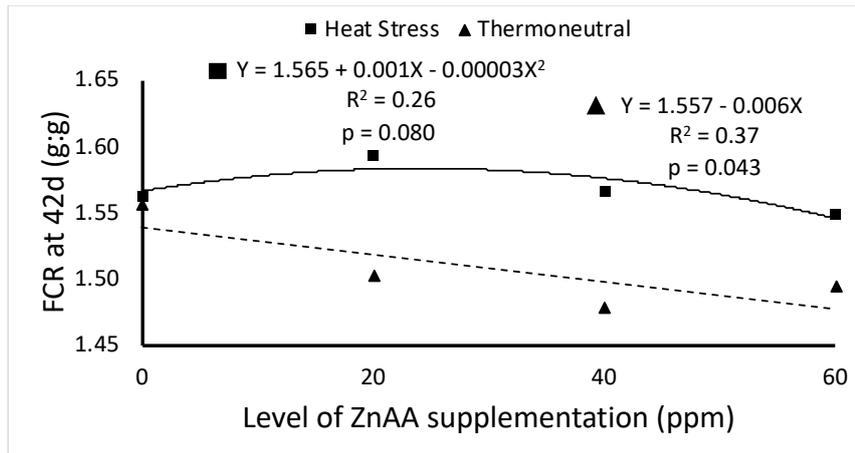


Figure 10. Regression analyses for FCR (g:g) within each environmental condition.

4.1.3. Immune competence

Relative weight of lymphoid organs was obtained dividing the weight of the organ by live weight, multiplied by 100. The size and development of lymphatic organs are directly correlated with the health status of animals (Abdukalykova and Ruiz-feria, 2006). In this study, heat stress significantly reduced relative weight of cloacal bursa, spleen and thymus ($p < 0.05$) in Table 10. It is noteworthy to mention that the reduction in lymphoid organs occurred even in the situation of smaller bodyweight for the HS chickens (see Table 9). The reason for that might be explained by the hypothalamic-pituitary-adrenal (HPA) axis activation. Corticosterone has been reported as a performance and immune system modulator for broilers. Shini et al. (2008) reported worsened performed parameters for corticosterone injected broilers, as well as lymphoid organ involution; these results have been confirmed by Quinteiro-Filho et al. (2010) and Sohail et al. (2012). Similarly to our results, Bartlett and Smith (2003) reported reduction in the relative weight of lymphoid organs for broilers that underwent heat stress. Increasing levels of ZnAA had a positive linear impact on relative weight of cloacal bursa; this response to ZnAA is illustrated in Figure 11.

Table 10. Relative weight of lymphoid organs (%) of broilers fed increasing levels of ZnAA and raised under different environment conditions at 42d.

Item ¹	Cloacal bursa	Spleen	Thymus
Environment ²			
TN	0.147 _A	0.096 _A	0.085 _A
HS	0.115 _B	0.078 _B	0.066 _B
ZnAA levels ³			
0 ppm	0.117	0.083	0.076
20 ppm	0.131	0.084	0.075
40 ppm	1.134	0.090	0.073
60 ppm	1.143	0.090	0.079
SEM	0.008	0.006	0.003
ANOVA			
Environment	<0.001	<0.001	0.002
ZnAA level	0.075	0.609	0.904
Environment x ZnAA level	0.754	0.347	0.388
Contrast			
Linear	0.011	0.227	0.789
Quadratic	0.711	0.976	0.544

¹ Results expressed as a percentage of the body weight.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). n = 12 replicates per treatment combination.

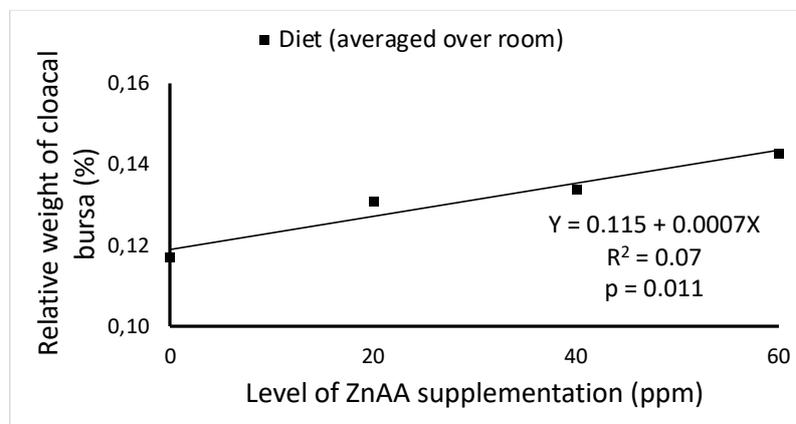


Figure 11. Regression analyses for relative weight (%) of cloacal bursa at 42d. Results averaged over environment.

Anti-microbial products, such as lysozymes, are part of innate immune response. They are secreted by epithelial cells in the gut and help to eliminate bacteria that threaten to cross the mucus barrier (White, 1995). Furthermore, immune competence and health status in terms of infection and inflammation alter production and release of plasma/serum proteins. Antibody response, an important element of adaptive immune system, is associated with the production of γ -globulins, whilst albumin determination decreases during inflammatory process (Kutlu and Forber, 1993; Al-Khalifa, 2016). Neither environment nor ZnAA level provoked an alteration on lysozyme activity. However, heat stressed birds presented reduced albumin concentration compared to thermoneutral animals, indicating they were possibly undergoing an inflammatory process. In turn, ZnAA level affected γ -globulins concentration, which may be confirmed by total globulins results. The positive quadratic response was similar for these parameters and infers on the capacity of immunoglobulin production and, therefore,

immune competence of birds (Table 11; Figure 12). Similar results have been obtained by Sunder et al. (2008), Bartlett and Smith, (2003) and Kakhki et al., (2016), who reported higher relative weight of lymphoid organs and higher humoral response measured by antibody against SRBC (sheep red blood cells).

Table 11. Lysozyme activity and blood proteinogram of broilers fed increasing levels of ZnAA and raised under different environment conditions at 42d.

Item ¹	Lysozyme	Albumin	γ -globulins	Globulins
Environment ²				
TN	351.3	1.141 _A	0.571	2.006
HS	381.0	1.087 _B	0.599	2.041
ZnAA levels ³				
0 ppm	352.8	1.127	0.533	1.886
20 ppm	408.9	1.143	0.625	2.153
40 ppm	356.1	1.072	0.645	2.126
60 ppm	346.8	1.115	0.536	1.930
SEM	10.7	0.014	0.017	0.036
ANOVA				
Environment	0.147	0.053	0.403	0.627
ZnAA level	0.112	0.303	0.036	0.017
Environment x ZnAA level	0.120	0.346	0.361	0.495
Contrast				
Linear	0.444	0.385	0.849	0.747
Quadratic	0.118	0.614	0.004	0.001

¹ Lysozyme results expressed as U mL⁻¹. Albumin, gamaglobulin and globulins expressed as g dL⁻¹.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: cyclic heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). $n = 12$ replicates per treatment combination.

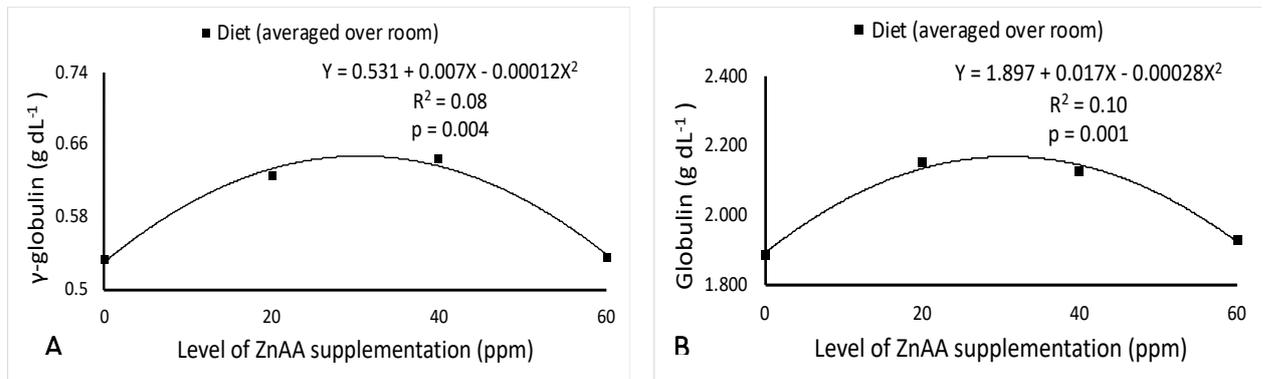


Figure 12. Blood proteinogram of broilers fed increasing levels of ZnAA and raised under different environment conditions at 42d. A) Regression analyses for serum γ -globulins and B) for serum total globulins at 42d. Results averaged over environment.

4.1.4. Oxidative status

To regulate the core temperature, broilers undergo a series of actions (systemic and behavioral) controlled by the central nervous system (CNS). One particularly important for this study, is the process of ischemia/reperfusion (I/R) that takes place in the gut when blood is diverted to the periphery to allow temperature exchange. I/R results in a local deprivation of oxygen and energy (Cronje, 2007), process which generates reactive

oxygen species (ROS) within gut cells leading to impaired cell structure and membrane integrity (Ashraf et al., 2013; Belhadj Slimen et al., 2016; Bhattacharyya et al., 2014; Takizawa et al., 2012).

Oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage”, concept originally conceived by Helmut Sies in 1985. An important source of ROS is when oxygen it is not converted into water by the enzyme cytochrome c oxidase (complex IV in the mitochondrial respiratory chain); instead, it turns into a superoxide anion ($O^{\bullet-}$). Superoxide anions reduces Fe^{3+} , enabling it to enter into Fenton-type reactions that leads to hydroxyl radical generation (OH^{\bullet} ; Habibian and Sadeghi, 2015). This radical is extremely reactive and is responsible to initiate peroxidative chain reactions (Ghazi et al., 2012). It is important to state that superoxide radical is generated during normal metabolism; nevertheless, it is increased by heat stress exposure (Akbarian et al., 2016; Habibian and Sadeghi, 2015). Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced (Ayala et al., 2014).

To control ROS generation, cells have evolved a defense system, which includes both non-enzymatic (e.g. tocopherols, vitamin C and glutathione) and enzymatic compounds [e.g. SOD (superoxide dismutase); GPx (glutathione peroxidase) and CAT (catalase)]. Cells are equipped with efficient dismutation pathways; i.e. Cu-ZnSOD acts in the intermembrane space (also present in cytosol) and MnSOD acts in the matrix to reduce superoxide to hydrogen peroxide (H_2O_2) (Akbarian et al., 2016). This conversion retards reduction of Fe^{3+} to Fe^{2+} , which catalyzes the formation of OH^{\bullet} . Consecutively, CAT and GPx convert H_2O_2 to water (Habibian and Sadeghi, 2015).

The next set of tables (Tables 12 to 15) summarize the results of five different variables assessed in four different tissues. The variables aim to measure the extent of: lipid peroxidation, quantifying malondialdehyde content (MDA); total anti-oxidant capacity through oxygen radical absorbance capacity (ORAC); and the activity of the anti-oxidants enzymes, SOD, GPx and CAT. These variables were measured in blood, intestine, liver and breast muscle.

Heat stressed broilers increased their activity of glutathione peroxidase ($p < 0.05$) and MDA concentration ($p = 0.053$) compared to thermoneutral broilers (Table 12; Figure 13). Malondialdehyde is the main product of polyunsaturated fatty acids (PUFA) peroxidation (Belhadj et al., 2014; Habibian and Sadeghi, 2015). Exposure to heat stress increased lipid peroxidation, as a consequence of the increased generation of ROS, as indicated by the concentration of MDA. As expected and reported previously by Belhadj et al. (2016) and Altan et al. (2003), the antioxidants enzymes can diminish detrimental effects of ROS and, in order to do that, their activity are usually increased under heat exposure. In spite of that, no changes in SOD and CAT were observed. Contrarily, Song et al. (2017) reported a reduced anti-oxidant enzymatic activity in the jejunum of broilers exposed to a very similar heat protocol to ours, while MDA levels were higher for heat stressed birds compared to control.

ZnAA supplementation influenced all variables in blood. It has linearly decreased MDA quantification ($p < 0.05$), along with a linear decrease on SOD ($p < 0.05$) and a negative quadratic response for GPx ($p < 0.05$); contrarily, a positive quadratic response has been observed for ORAC ($p < 0.05$), whilst CAT activity linearly increased ($p < 0.05$) as supplementation increased. As zinc plays a role as a cofactor in intracellular and extracellular Cu-ZnSOD enzymes (Bou et al., 2009), it has been hypothesized that the activity of this enzyme may be modulated by increasing levels of zinc supplementation. However, controversial results have been published. Ivanišinová et al. (2016) and Liao et al. (2013) have found that Cu-ZnSOD activity did not change when zinc was supplemented up to 140 ppm and using different sources (none of them was Availa Zn). Nevertheless, enhanced SOD activity have been reported by Ma et al. (2011) when zinc glycinate was added up to 120 ppm and by Bun et al., (2011) who used Mintrex Zn up to 60 ppm. This has not been confirmed by this study and it seems SOD activity was more reliant on

the general condition or state of the pro/antioxidant balance than the direct dietary modulation exerted by zinc itself. In a review of the role of zinc in oxidative stress, Naz et al. (2016) divided zinc action according to the duration of the heat exposure. According to them, under a chronic stimulus, its effect involves indirect protection from pro-oxidants due to induction of other substances, e.g. metallothionein (MT), which are cysteine rich proteins that serve as antioxidants by scavenging ROS (Ruttikay-Nedecky et al., 2013). Liu et al. (2015) tested different zinc sources (including Availa Zn) with levels up to 180 ppm and showed that MT content and Cu-Zn SOD activities in the breast and thigh muscles of broilers up-regulated the mRNA expressions of MT and Cu-Zn SOD in the liver and thigh muscle. The result of increased MT for Availa Zn treatments is not expected and it causes strangeness, as the mechanism Availa Zn is absorbed does not participate in the pool of cellular zinc. As we have used phytase and our basal zinc level was about 35 ppm, this could have increased MT. Therefore, the lower amounts of MDA and higher total antioxidant capacity (ORAC) obtained for higher levels of ZnAA supplementation may be explained by this. Unfortunately, MT levels have not been measured, and neither was SOD in earlier ages.

Table 12. MDA content, ORAC and activity of antioxidant enzymes in the **blood** of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	MDA*	ORAC	SOD	GPx	CAT
Environment ²					
TN	10.89 _B	8369	165.5	25.5 _B	1.63
HS	11.54 _A	8226	171.2	31.7 _A	1.62
ZnAA levels ³					
0 ppm	11.73	7738	176.8	29.7	1.39
20 ppm	11.69	8842	183.4	27.9	1.62
40 ppm	10.78	8687	155.7	27.7	1.74
60 ppm	10.66	7924	157.6	29.1	1.75
SEM	0.17	572	22.60	2.7	0.39
ANOVA					
Environment	0.053	0.675	0.526	<0.001	0.873
ZnAA level	0.041	0.059	0.076	0.077	0.083
Environment x ZnAA level	0.497	0.291	0.816	0.862	0.076
Contrast					
Linear	0.008	0.787	0.036	0.432	0.018
Quadratic	0.903	0.007	0.791	0.013	0.303

¹ MDA results expressed as nmol mg⁻¹ of hemoglobin (determined using thiobarbituric acid reacting substances test, TBARS); ORAC as μmol mg⁻¹ of hemoglobin; anti-oxidants enzymes as U mg⁻¹ of hemoglobin. * Results are expressed in the form: $\mu \times 10^3$.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ (p < 0.05). n = 12 replicates per treatment combination.

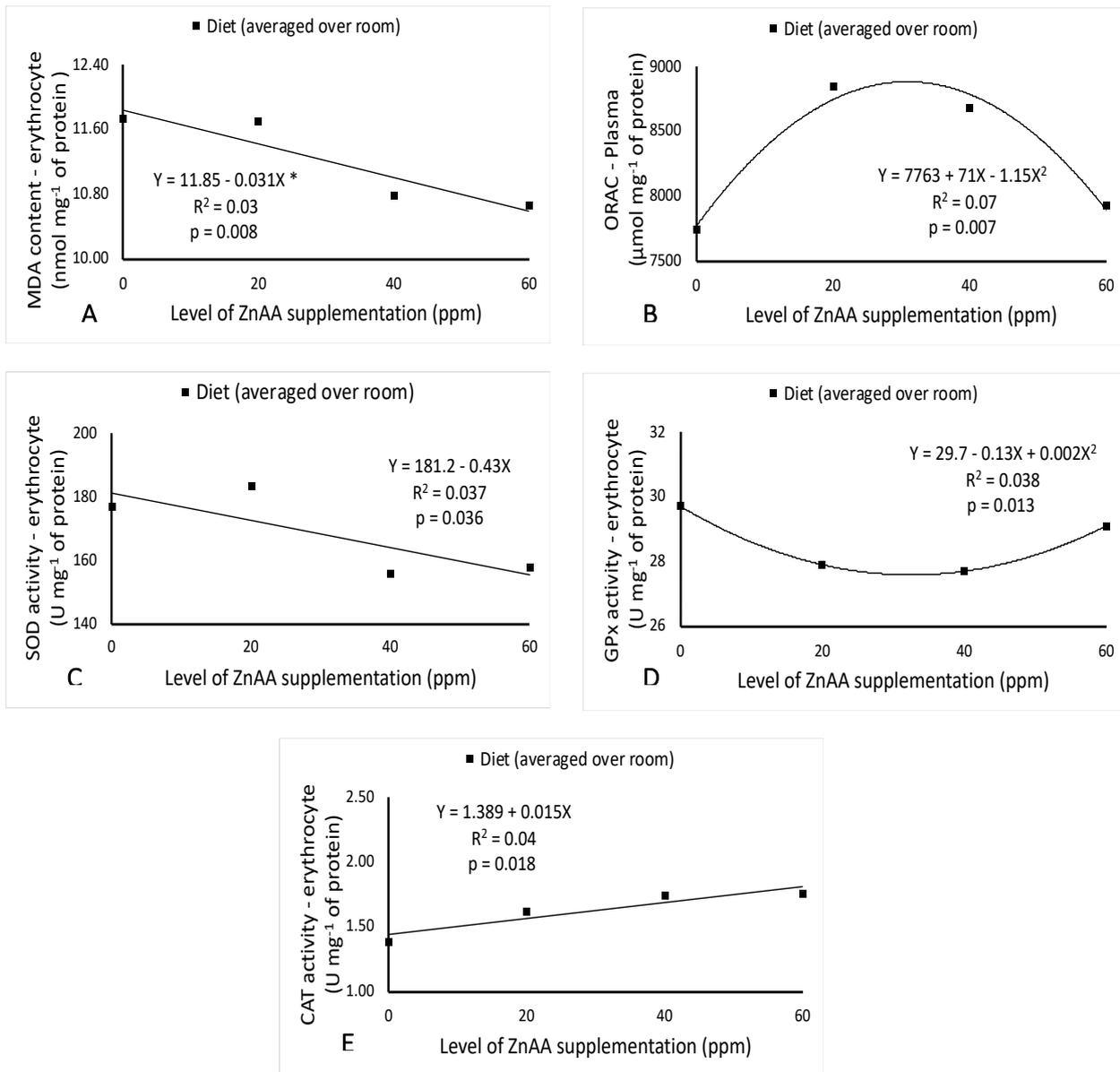


Figure 13. MDA content, ORAC and activity of antioxidant enzymes in the **blood** of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d. A) Regression analyses for malondialdehyde content (MDA) on erythrocyte; B) for ORAC in plasma; C) for superoxide dismutase (SOD) activity; D) glutathione peroxidase (GPx) activity and E) catalase (CAT) in erythrocyte at 42d. Results averaged over environment.

Results for intestine (jejunum), liver and breast muscle (*longissimus pectoralis major*) are presented in Tables 13, 14 and 15, respectively. For intestine and liver, MDA quantification has not changed between heat stressed and thermoneutral broilers. SOD activity in jejunum and GPx activity in liver were higher for heat stressed broilers compared to thermoneutral ($p < 0.05$). The hypothesis is that, as anti-oxidants enzymes (SOD in jejunum and GPx in liver) increased their activity, MDA quantification responded and, seemingly, the broilers succeeded in preventing the progress of oxidative stress. Hence, MDA concentration has not been affected by the environment. This result was corroborated by a higher total antioxidant capacity ($p < 0.05$) measured by ORAC (in liver). Similar results have been reported by Xie et al. (2015). They did not observe any alterations in lipid or protein peroxidation in broiler breeders submitted to both acute and chronic heat stress. Contrasting to these results, Mujahid et al. (2007) observed increased plasma and mitochondrial MDA for heat stressed birds compared to control. Altan et al. (2003) also

reported a higher MDA concentration for heat stressed birds, associated with an increase in SOD, GPx and CAT. Song et al. (2017) also reported a higher MDA concentration, however, associated with a lowered antioxidant enzymes activity. This shows that the observed responses might vary considerably, especially regarding the duration and intensity of stress exposure.

A different scenario took place in breast muscle. Birds raised under thermoneutral condition have shown a 2.15-fold increase of MDA concentration compared to heat stressed birds ($p < 0.05$). That could be a consequence of higher activity of antioxidant enzymes, as observed and discussed for liver and intestine. However, SOD and CAT activities also presented higher in thermoneutral broilers. This expose an incapacity of these enzymes to cope with oxidative stress in thermoneutral broilers, or less likely, for some reason that have not been understood yet, the oxidative stimulus in the *pectoralis major* for thermoneutral broilers was even higher than for stressed broilers. The higher metabolic activity and tissue synthesis may be implied and act as a confounding factor.

ZnAA supplementation affected MDA quantification and GPx activity ($p = 0.083$ and $p < 0.05$, respectively) in liver (Table 14). Increasing levels of ZnAA linearly decreased MDA quantification on liver and GPx activity presented a negative quadratic response, replicating the results observed for erythrocytes (Figure 14). No interaction between the main effects environment and ZnAA level were observed for any variable assessed in blood (erythrocyte), intestine, liver and breast muscle.

Table 13. MDA content, ORAC and activity of antioxidant enzymes in the **intestine** (jejunum) of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	MDA	ORAC	SOD	GPx	CAT
Environment ²					
TN	0.155	422.6	468.9 _B	12.1	11.50
HS	0.126	467.0	548.7 _A	12.7	12.11
ZnAA levels ³					
0 ppm	0.133	434.1	486.3	12.3	12.42
20 ppm	0.154	393.6	505.8	12.3	12.87
40 ppm	0.121	514.6	540.8	12.9	11.56
60 ppm	0.154	436.8	502.2	12.1	10.37
SEM	0.016	76.1	30.37	0.3	5.55
ANOVA					
Environment	0.188	0.357	0.002	0.255	0.732
ZnAA level	0.638	0.333	0.505	0.666	0.771
Environment x ZnAA level	0.514	0.291	0.775	0.753	0.930
Contrast					
Linear	0.759	0.552	0.467	0.994	0.359
Quadratic	0.789	0.699	0.260	0.402	0.650

¹MDA results expressed as nmol mg^{-1} of protein (determined using thiobarbituric acid reacting substances test, TBARS); ORAC as $\mu\text{mol mg}^{-1}$ of protein; antioxidant enzymes as U mg^{-1} of protein.

²Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). $n = 12$ replicates per treatment combination.

Table 14. MDA content, ORAC and activity of antioxidant enzymes in the **liver** of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ₁	MDA	ORAC	SOD	GPx	CAT
Environment ₂					
TN	0.044	202.5 _B	1262	6.28 _B	5.13
HS	0.041	227.3 _A	1268	8.92 _A	5.49
ZnAA levels					
0 ppm	0.046	214.1	1286	8.39	5.86
20 ppm	0.044	217.9	1337	7.17	5.50
40 ppm	0.038	213.7	1218	7.19	4.59
60 ppm	0.042	213.9	1219	7.65	5.28
SEM	0.001	23.5	161.7	0.24	0.99
ANOVA					
Environment	0.104	<0.001	0.950	<0.001	0.467
ZnAA level	0.092	0.965	0.158	0.025	0.334
Environment x ZnAA level	0.895	0.833	0.553	0.620	0.936
Contrast					
Linear	0.083	0.878	0.109	0.126	0.233
Quadratic	0.172	0.787	0.509	0.009	0.299

¹ MDA results expressed as nmol mg⁻¹ of protein (determined using thiobarbituric acid reacting substances test, TBARS); ORAC as μmol mg⁻¹ of protein; antioxidants enzymes as U mg⁻¹ of protein.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ (p < 0.05). n = 12 replicates per treatment combination.

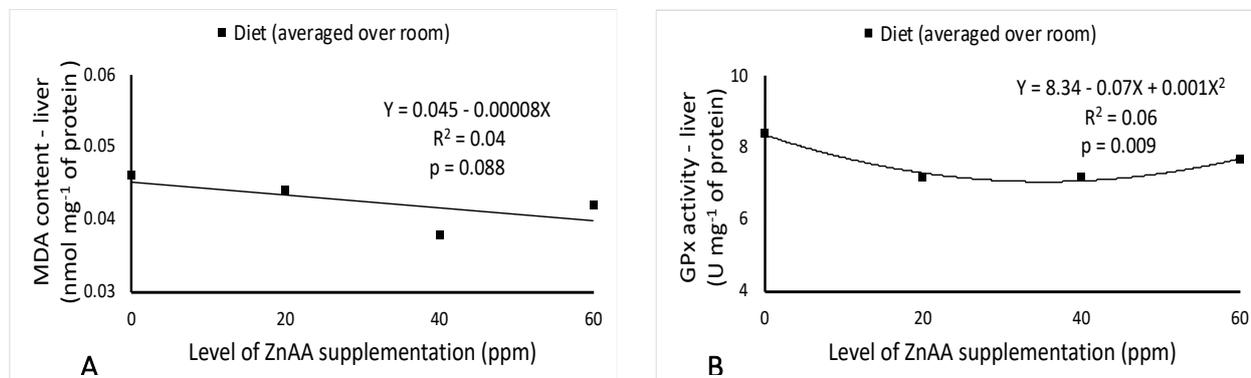


Figure 14. MDA content and GPx activity in the **liver** of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d. A) Regression analyses for MDA concentration and B) GPx activity in liver at 42d. Results averaged over environment.

Table 15. MDA content, ORAC and activity of antioxidant enzymes in the **breast muscle** of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	MDA	ORAC	SOD	GPx	CAT
Environment ²					
TN	0.028 ^A	407.2	90.2 ^A	5.65	0.73 ^A
HS	0.013 ^B	418.7	74.5 ^B	5.64	0.46 ^B
ZnAA levels ³					
0 ppm	0.023	400.1	77.7	5.95	0.61
20 ppm	0.020	405.8	83.0	5.25	0.63
40 ppm	0.022	428.7	84.3	5.77	0.55
60 ppm	0.016	417.2	84.5	5.59	0.58
SEM	0.002	76.2	10.0	0.16	
ANOVA					
Environment	<0.001	0.544	0.003	0.980	<0.001
ZnAA level	0.660	0.722	0.775	0.454	0.780
Environment x ZnAA level	0.116	0.831	0.271	0.893	0.263
Contrast					
Linear	0.661	0.388	0.355	0.680	0.504
Quadratic	0.268	0.652	0.624	0.406	0.954

¹MDA results expressed as nmol mg⁻¹ of protein (determined using thiobarbituric acid reacting substances test, TBARS); ORAC as μmol mg⁻¹ of protein; antioxidant enzymes as U mg⁻¹ of protein.

²Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ (p < 0.05). n = 12 replicates per treatment combination.

4.1.5. Histomorphometry, gut integrity and tight junction proteins quantification

Histomorphometry parameters for jejunum and ileum, FITC-d permeability assay and Western blot of claudin-1, occludin and zonula occludens-1 in the ileum of broilers raised under a cyclic chronic heat stress protocol fed increasing levels of ZnAA have been evaluated. Intestinal epithelium is a monolayer that keeps noxious agents as pathogens and toxins apart from the organism (Lerner and Matthias, 2015). Hence, its microarchitecture and integrity plays a vital role in the adequate absorption capacity as well as a barrier against immunogenic agents and pathogens (Jacobi and Odle, 2012; Ménard et al., 2010; Moran, 2016; Ruth and Field, 2013). A number of publications have demonstrated the negative impact of stressors, i.e. heat stress, on mucosal epithelium resulting in worsened histomorphometric parameters (Burkholder et al., 2008; Liu et al., 2016; Sohail et al., 2012; Song et al., 2014; Yi et al., 2016) and impaired barrier function (Song et al., 2014; Varasteh et al., 2015; Wu et al., 2018; C. Zhang et al., 2017). Inversely, zinc supplementation is reported to improve the histomorphometry of the small intestine (Shao et al. 2014), as well as to improve the barrier integrity through modulation of tight junction proteins (Miyoshi et al., 2016).

Table 16 present the results for jejunum and ileum histomorphometry. There was a main effect of heat stress reducing villus height in the ileum; however, in the jejunum the interaction between environments and dietary ZnAA indicated that Zn levels did not affect villus height in TN but increased villus height under HS condition (p < 0.05), Figure 15). In jejunum, but not in the ileum, crypt depth was greater (p<0.05) for thermoneutral compared to heat stressed broilers. Villus height:crypt depth ratio (V:C ratio) was impacted only in the ileum (p<0.05); V:C ratio was lower for HS birds, confirming its the detrimental effect on epithelial dynamics. Similarly, Sohail et al. (2012), using a chronic heat exposure, reported that heat treated birds had lower villus height, lower crypt depth and lower

villus surface area compared to control. Song et al. (2014) used a cyclic chronic heat exposure very similar to ours and reported shortened villus, deeper crypts, and lower V:C ratio for heat stressed compared to thermoneutral broilers. Altogether, the results on histomorphometry found, indicate that zinc requirement may be altered depending on the environmental conditions the broilers are submitted. ZnAA supplementation linearly improved villus height of broilers raised under heat stress. Similarly, Shah et al. (2019) reported that supplemental ZnSO₄ up to 60 ppm improved villus height and V:C ratio compared to control. Zhang et al. (2012) reported that Zn supplemented at the rate of 80 and 120 mg/kg resulted in a higher V:C ratio in broilers challenged with *Salmonella* Typhimurium.

Table 16. Jejunum and ileum histomorphometry of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	Jejunum			Ileum		
	Villus height	Crypt depth	V:C ratio	Villus height	Crypt depth	V:C ratio
Environment ²						
TN	1154 ^A	150.3 ^A	8.03	1063 ^A	130.2	7.83 ^A
HS	1073 ^B	135.8 ^B	8.48	988 ^B	132.3	6.92 ^B
ZnAA levels ³						
0 ppm	1075	138.3	7.99	1001	128.4	7.47
20 ppm	1141	144.2	8.44	1032	131.7	7.25
40 ppm	1131	141.3	8.37	1032	135.6	7.13
60 ppm	1106	148.5	8.22	1038	129.4	7.66
SEM	19.6	3.30	0.18	15.0	2.37	0.15
ANOVA						
Environment	0.035	0.028	0.218	0.009	0.643	0.003
ZnAA level	0.626	0.726	0.819	0.810	0.666	0.614
Environment x ZnAA level	0.025	0.281	0.506	0.839	0.165	0.508
Contrast						
Linear	0.841	0.352	0.694	0.392	0.731	0.755
Quadratic	0.704	0.919	0.415	0.665	0.289	0.228

¹ Villus height and crypt depth results expressed as μm ; villus: crypt ratio (V:C ratio) as arbitrary unit; surface of absorption as μm^2 (15 villi counted per replicate).

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). n = 12 replicates per treatment combination.

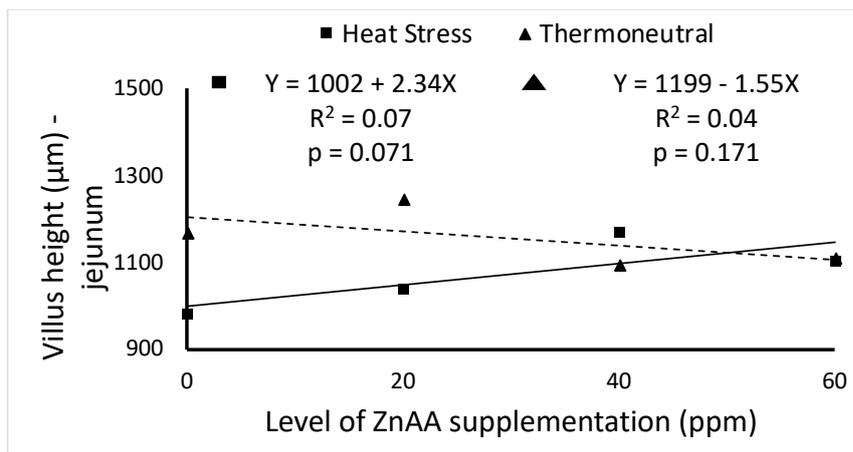


Figure 15. Regression analyses for villus height (μm) on jejunum at 42d. Results averaged over environment. Results of dietary Zn within each environmental condition.

The intestinal epithelium is covered with mucus secreted by goblet cells (GC), which can protect the intestinal mucosa from pathogen attacks and environmental toxins and play an important role in repairing injury of intestinal mucosa (Liu et al., 2016). Therefore, GC are important for intestinal mucosa barrier function. Conversely, excessively increased or hypersecretional GC tends to cause diarrhea and catarrhal inflammation.

Liu et al. (2016) reported that heat stress significantly decreased goblet cell numbers in the jejunum and ileum of black-boned chickens. Similarly, Zhang et al. (2017) indicated that GC numbers and the mRNA level of the mucin-2 gene in the jejunum were decreased by heat stress. However, other authors also have reported an increase in the number of GC in animals under heat stress (Hu et al., 2017). The results of the present study show interactions between the main effects for neutral and acidic-secreting mucus GC, as well as total GC in the jejunum and ileum. Results for ZnAA supplementation within each environmental condition are depicted in Figure 16. In the jejunum, thermoneutral animals had a negative quadratic response ($p < 0.05$) (Table 17), which means intermediate levels of supplemental ZnAA (20 and 40 ppm) had lower counts of GC. The only significant result in the heat stressed group ($p < 0.05$) was observed for neutral GC, which linearly decreased as Zn increases. In the ileum, the significant effects were observed only for heat stressed broilers. Heat stressed animals had a positive quadratic response ($p < 0.05$), which means intermediate levels of supplemental ZnAA (20 and 40 ppm) had higher counts of GC, while thermoneutral kept the behavior observed in the jejunum. The increase of acidic mucins acts in a protective role for the immature immune system (Cebra, 1999), while the production of neutral mucins could serve as a protective mechanism against invasion by pathogenic bacteria (Dean-Nystrom and Samuel, 1994).

Table 17. Goblet cells in the jejunum and ileum of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	Jejunum			Ileum		
	Neutral	Acidic	Total	Neutral	Acidic	Total
Environment ²						
TN	61	81 _B	144	64	80	141
HS	63	87 _A	152	64	82	145
ZnAA levels ³						
0 ppm	64	87	152	66	80	144
20 ppm	61	81	145	65	86	148
40 ppm	60	83	147	67	83	149
60 ppm	63	85	148	58	75	130
SEM	1.4	1.4	2.9	1.3	1.3	2.6
ANOVA						
Environment	0.340	0.022	0.180	0.894	0.520	0.356
ZnAA level	0.698	0.414	0.835	0.051	0.009	0.017
Environment x ZnAA level	0.012	0.003	0.017	0.003	<0.001	0.001
Contrast						
Linear	0.678	0.624	0.696	0.056	0.112	0.065
Quadratic	0.266	0.149	0.446	0.119	0.003	0.015

¹ Results expressed as the mean of the number of cells per villi (10 villi counted per replicate).

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). n = 12 replicates per treatment combination.

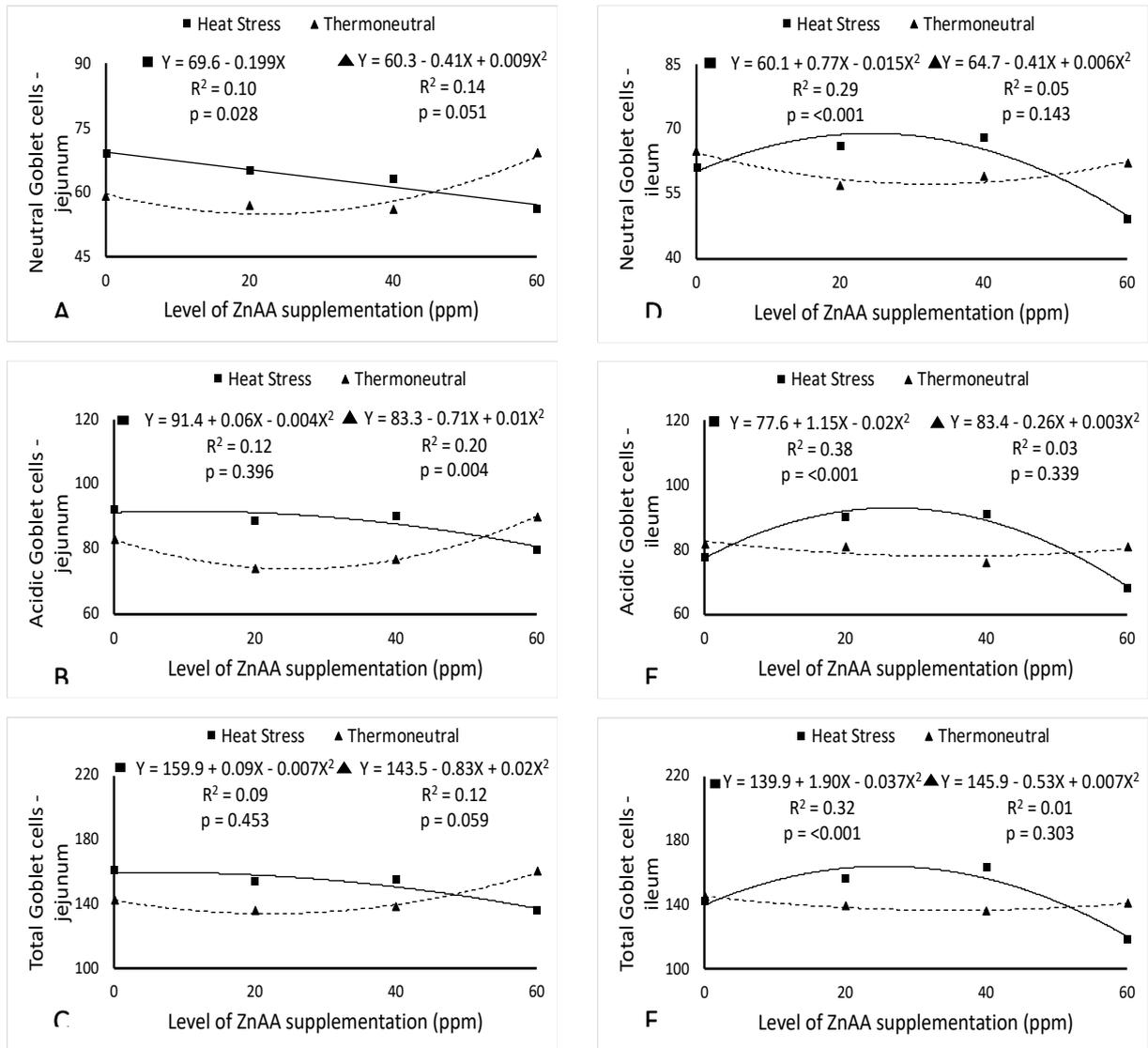


Figure 16. Goblet cells in jejunum and ileum of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d. A) Regression analyses for neutral mucin goblet cells in jejunum; B) acidic mucin goblet cells in jejunum; C) total goblet cells in jejunum; D) neutral mucin goblet cells in ileum; E) acidic mucin goblet cells in ileum, and F) total goblet cells in ileum. Results within each environmental condition.

The epithelium is permeable to nutrients and macromolecules but provides an effective barrier against luminal antigenic agents, such as bacteria, toxins, and feed-associated antigens. Impairment of this barrier function leads to increased permeability to luminal antigens, which gain access to sub epithelial tissues and result in inflammation, malabsorption, and potentially systemic disease (Moesser et al., 2007). Lambert (2009) reported that ischemia of gut could lead to intestinal hypoxia, which likely results in reduced cellular viability and increased paracellular permeability. Moreover, Hall et al. (2001) reported that reduced blood flow could also result in oxidative and nitrosative stress, which can damage cell membranes and open tight junctions. Researchers have reported the beneficial effects of zinc supplementation (Zhang et al., 2012) and detrimental effects of zinc deficiency (Finamore et al., 2008) on intestinal permeability, measured by FITC-D, TER (transepithelial electrical resistance) and quantification of tight junction proteins (TJ), such as claudin-family proteins, occludin and zonula occludens.

The results of Western Blot analyses for claudin-1, occludin and zonula occludens (ZO-1), and determination of FITC-D in the blood of inoculated birds are summarized in Tables 18 and 19, respectively. Heat

stress did not change the protein expression in ileum ($p>0.05$). Researchers have reported that heat stress reduced the gene expression of these proteins (mRNA) (Song et al., 2014; Zhang et al., 2017). Contrarily, Pearce et al. (2013), using Western Blot analysis in the ileum of pigs, reported that tight junction proteins, i.e, occludin and claudin-3, were upregulated when under heat exposure. They discuss that the upregulation of these TJ proteins may indicate a barrier enhancement effect during HS in an attempt to compensate for increased permeability. Therefore, it may be that the signal as mRNA might not be entirely transcript into proteins.

Recently, Vicuña et al. (2015) reported that broilers receiving an oral gavage with FITC-D, and then had the FITC-D circulating levels determined could be used for rapid evaluation of gastrointestinal permeability. The results in the present study show that heat stress impaired gut integrity ($p<0.05$; Figure 17 A). This result corroborates with Zhang et al. (2017) who reported higher FITC-D values for broilers raised under heat exposure compared to the control. Higher FITC-D determination indicates a higher permeability, or a leaky gut.

A number of scientific papers have reported the beneficial effects of zinc supplementation on the expression of tight junction proteins (Finamore et al., 2008; Mercado et al., 2013; Zhang et al., 2012). In the present study, no significant effects were observed for Claudin-1. Nevertheless, for occludin and ZO-1, interaction between environment and ZnAA level may be observed ($p=0.057$ and $p=0.059$, respectively). The interaction results are presented in Figure 18. For both proteins (occludin and ZO-1), a positive quadratic response is observed for broilers exposed to heat stress. It means that the intermediate levels of supplemental zinc (20 and 40 ppm) enhanced the protein expression, resulting in greater intestinal cohesion. Similarly, Zhang and Guo (2009) have reported that zinc supplemented pigs had higher gene and protein expression of occludin and ZO-1 proteins, and no significant effect for claudin-1 protein. Back to the present study, an opposite behavior may be seen for occludin when broilers were raised under thermoneutral condition. Apparently, it has caused a downregulation of this protein expression as zinc was added, with exception to the highest level (60 ppm). These results are logical when analyzed conjunctly with FITC-D determination. FITC-D results show that zinc supplemented broilers presented lower levels of the marker on the blood, meaning a less permeable gut, which is desirable. This result was observed specially for broilers raised under heat exposure; for thermoneutral birds, no differences between zinc supplementation have been seen.

Table 18. Western blot analyses for tight junction proteins Claudin-1, Occludin and Zonula occludens (ZO-1) in the ileum of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	Claudin -1	Occludin	Zonula occludens-1
Environment ²			
TN	14.21	10.99	11.37
HS	10.78	14.20	13.62
ZnAA levels			
0 ppm	10.91	13.36	15.58
20 ppm	12.88	9.23	12.48
40 ppm	11.25	16.51	16.68
60 ppm	14.94	11.28	5.24
SEM	1.36	2.31	2.03
ANOVA			
Environment	0.269	0.469	0.523
ZnAA level	0.772	0.683	0.123
Environment x ZnAA level	0.888	0.057	0.059
Contrast			
Linear	0.448	0.956	0.100
Quadratic	0.779	0.899	0.243

¹ Results expressed as relative signal intensity in percentage (%), normalized to beta actin.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). $n = 3$ replicates per treatment combination.

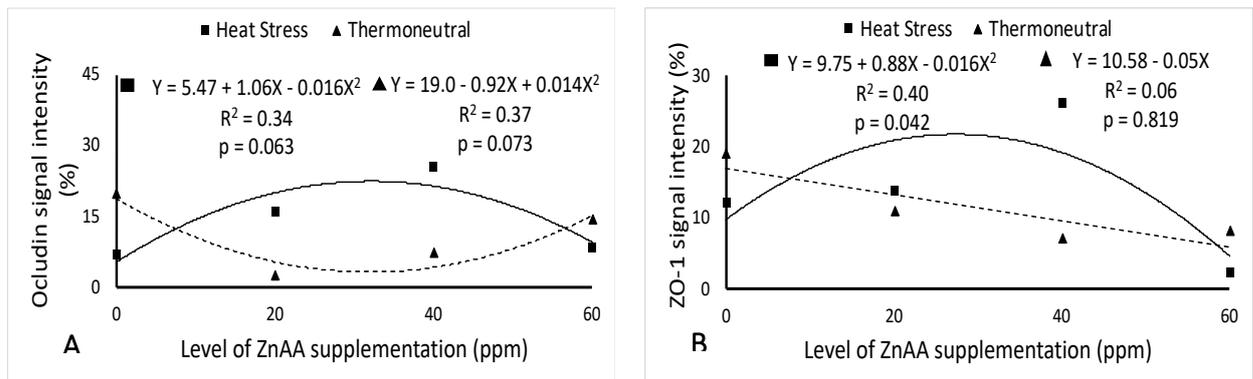


Figure 17. Tight junction protein quantification by Western Blot in the ileum of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d. A) Regression analyses for Occludin (%); B) Regression analyses for ZO-1 (%). Results within each environmental condition.

Table 19. FITC-D determination of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

ZnAA levels	Thermoneutral ²		Heat Stress ²		<i>P</i> - value
	Serum FITC ₁	SEM	Serum FITC-1	SEM	
0 ppm	393 _b	8.50	588 _{Aa}	116.0	<0.001
20 ppm	405 _a	11.0	371 _{Ba}	8.10	0.479
40 ppm	381 _a	9.40	371 _{Ba}	10.8	0.783
60 ppm	414 _a	16.70	370 _{Ba}	10.0	0.415
<i>P</i> - value	0.538		<0.001		

¹ Results expressed as ng of FITC-D ml⁻¹ of blood.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Different uppercase letters differ within the column and different lower-case letter differ within the row. $n = 12$ replicates per treatment combination.

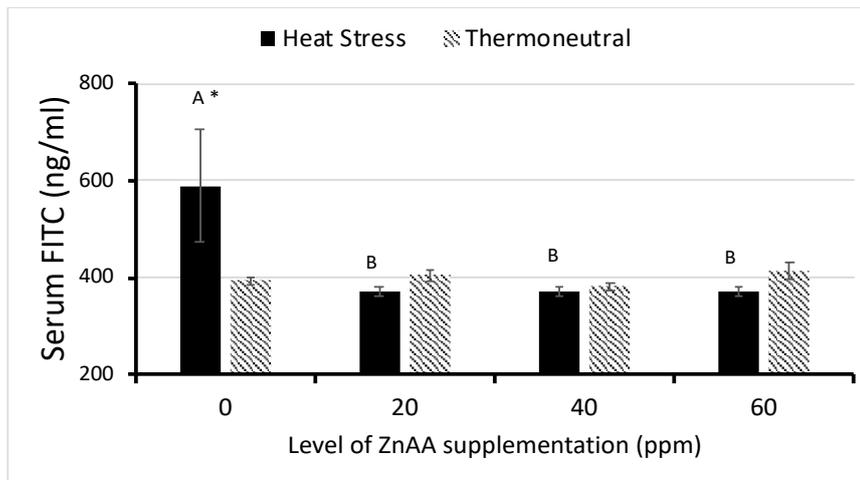


Figure 18. FITC-D determination of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d (ng of FITC-D ml⁻¹ of blood. Asterisk (*) means that level 0 ppm differs between environment.

4.1.6. Zinc determination

Table 20 summarizes the results for absolute and relative tibia weight, and zinc quantification in tibia and liver. HS reduced absolute tibia weight ($p < 0.05$) compared to broilers raised under TN conditions. On the other hand, HS increased relative tibia weight ($p < 0.05$); this effect was probably due to differences in allometric growth between bones and body mass. There was no significant impact of environment on either tibia or liver quantification.

Supplemental zinc, however, influenced all variables. A positive linear effect for absolute and relative tibia weight may be observed ($p = 0.081$ and $p = 0.087$, respectively). The regression analyses are presented in Figure 19 (A and B). A positive linear response was also observed for tibia zinc ($p = 0.073$). For zinc quantification in liver, a positive quadratic response was observed ($p < 0.05$). The regression analyses for zinc determination on both tissues are presented in Figure 19 (C and D).

Table 20. Tibia weight and zinc determination on tibia and liver of broilers fed increasing levels of ZnAA and raised under different environmental conditions at 42d.

Item ¹	Tibia weight	Relative tibia weight	Tibia zinc	Liver zinc
Environment ²				
TN	14.56 ^A	0.441 ^B	146.0	30.5
HS	13.45 ^B	0.465 ^A	148.8	30.6
ZnAA levels ³				
0 ppm	13.78	0.441	140.9	28.9
20 ppm	13.87	0.452	149.0	34.8
40 ppm	13.82	0.455	150.6	30.7
60 ppm	14.54	0.462	149.1	27.6
SEM	0.16	0.005	1.46	1.05
ANOVA				
Environment	<0.001	0.006	0.393	0.958
ZnAA level	0.186	0.379	0.153	0.068
Environment x ZnAA level	0.738	0.754	0.168	0.269
Contrast				
Linear	0.081	0.087	0.073	0.374
Quadratic	0.251	0.813	0.137	0.029

¹ Tibia weight results expressed as the absolute weight in grams; Relative tibia weight expressed as a percentage in relation to live body weight; Tibia and liver zinc expressed as zinc concentration in ppm.

² Environmental conditions from 21 to 42 d. TN: thermoneutral 24 ± 1 °C all day; HS: heat stress 32 ± 1 °C for 12 h/d.

³ Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). $n = 12$ replicates per treatment combination.

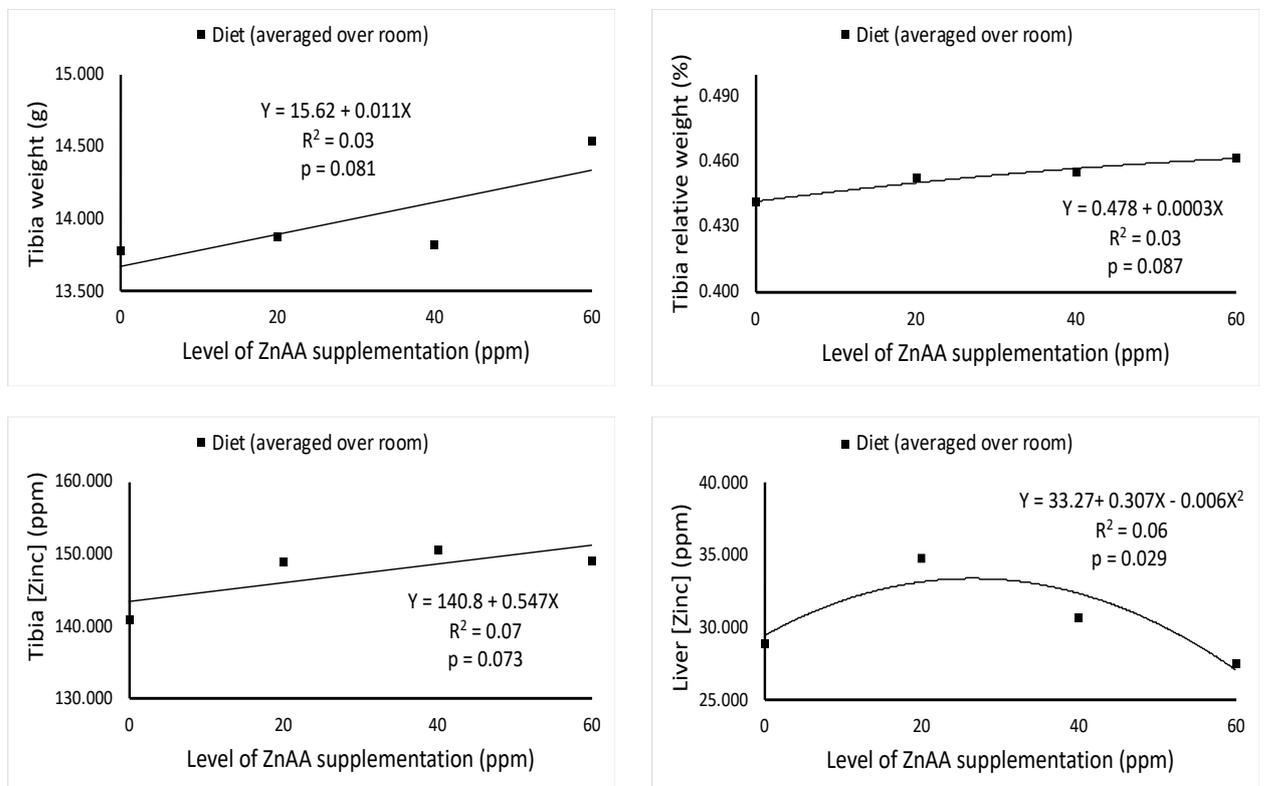


Figure 19. A) Regression analyses for absolute tibia weight; B) tibia relative weight (%); C) tibia zinc determination (ppm) and D) liver zinc determination (ppm). Results averaged over environmental condition.

4.2. EXPERIMENT 2

Figure 20 summarizes the observed temperatures from 21 to 42d (heat stress period) for Experiment 2. Four data loggers placed at the same height in four different locations in the poultry house assessed the temperature (°C) and relative humidity (%) every 10 minutes. Data was averaged by hour and plotted for the whole experimental period. The average relative humidity observed was 63.7%. As the experiment was conducted in a poultry house with partially controlled environment, and not in environmentally controlled rooms like in Experiment 1, the observed temperatures did not fit exactly to the expected temperatures but served well to the purpose of simulating cyclic heat stress (Figure 20). As we were working in a poultry house, it was harder to precisely control the increments of temperature compared to the climate room, and that is why the shape of the observed curve does not conform exactly to the expected temperatures.

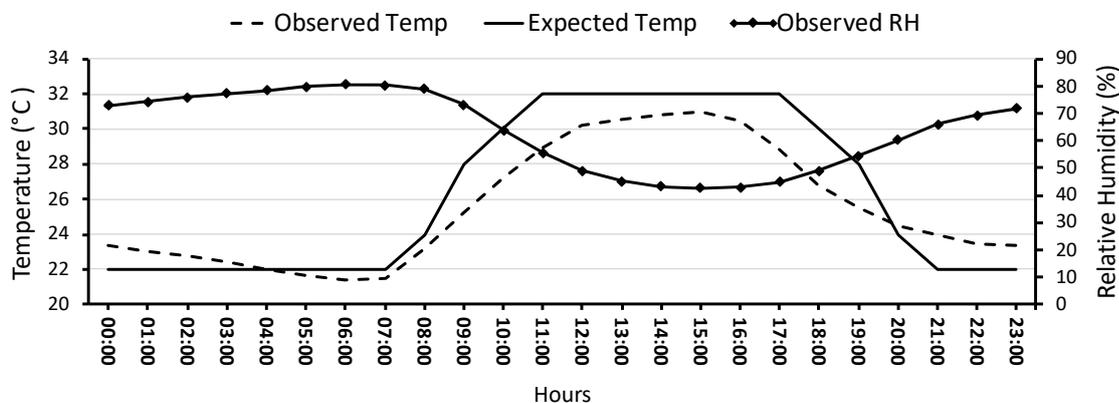


Figure 20. Average of observed temperatures (°C) and relative humidity (%) from 21 to 42d.

The aim of this second experiment was to exclusively assess performance and validate the results that were obtained in the previous trials for broilers raised under the same heat stress protocol implemented for Experiment 1. Diets were the same used for Experiment 1 (see section “Experimental Diets”). Results of the analyzed nutritional levels are presented in Table 6 (above). One experimental poultry house has been used in this experiment, therefore, no thermoneutral control was assessed. Birds received the experimental diets from 1d and the heat protocol started on 21d and lasted 21d. The variables body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were assessed weekly and are presented by period (1 to 21d; 21 to 42d; and 1 to 42d). Mortality was recorded daily and used to correct weekly body weight gain. For the starter period (1 to 21d; Table 21) significant responses to ZnAA levels have been observed for all performance parameters assessed. BW and BWG were increased linearly with ZnAA supplementation ($p < 0.072$ and $p < 0.068$, respectively). Feed intake responded quadratically ($p < 0.05$), with higher intake observed for intermediate levels (20 and 40 ppm of supplemental ZnAA). FCR was also linearly improved with increasing levels of zinc ($p < 0.05$). Regression analyses for the starter phase are presented in Figure 21. For the final period (21 to 42d), during which the birds were submitted to the heat stress protocol, results are presented in Table 22. A positive linear response for BW at 42d was observed ($p < 0.05$) as ZnAA supplementation increased (Figure 22). Although non significantly ($p = 0.102$), the maximum zinc supplementation resulted in around 40 g (2.2%) advantage for BWG. Feed intake and feed conversion ratio have not been altered.

Table 21. Growth performance of broilers fed increasing levels of zinc from 1 to 21d

ZnAA level ²	BW ₁	BWG ₁	FI ₁	FCR ₁
0 ppm	971	924	1168	1.264
20 ppm	1005	960	1195	1.245
40 ppm	989	944	1192	1.258
60 ppm	997	951	1159	1.219
SEM	4.3	4.4	6.5	0.006
ANOVA	0.019	0.015	0.131	0.023
Contrast				
Linear	0.072	0.068	0.623	0.017
Quadratic	0.118	0.091	0.026	0.371

¹ BW: body weight in grams; BWG: body weight gain in grams; FI: feed intake in grams; FCR: feed conversion ratio (g:g).

² Supplemental zinc level as metal-amino acid complex.

Means lacking a common superscript differ ($p < 0.05$). $n = 10$.

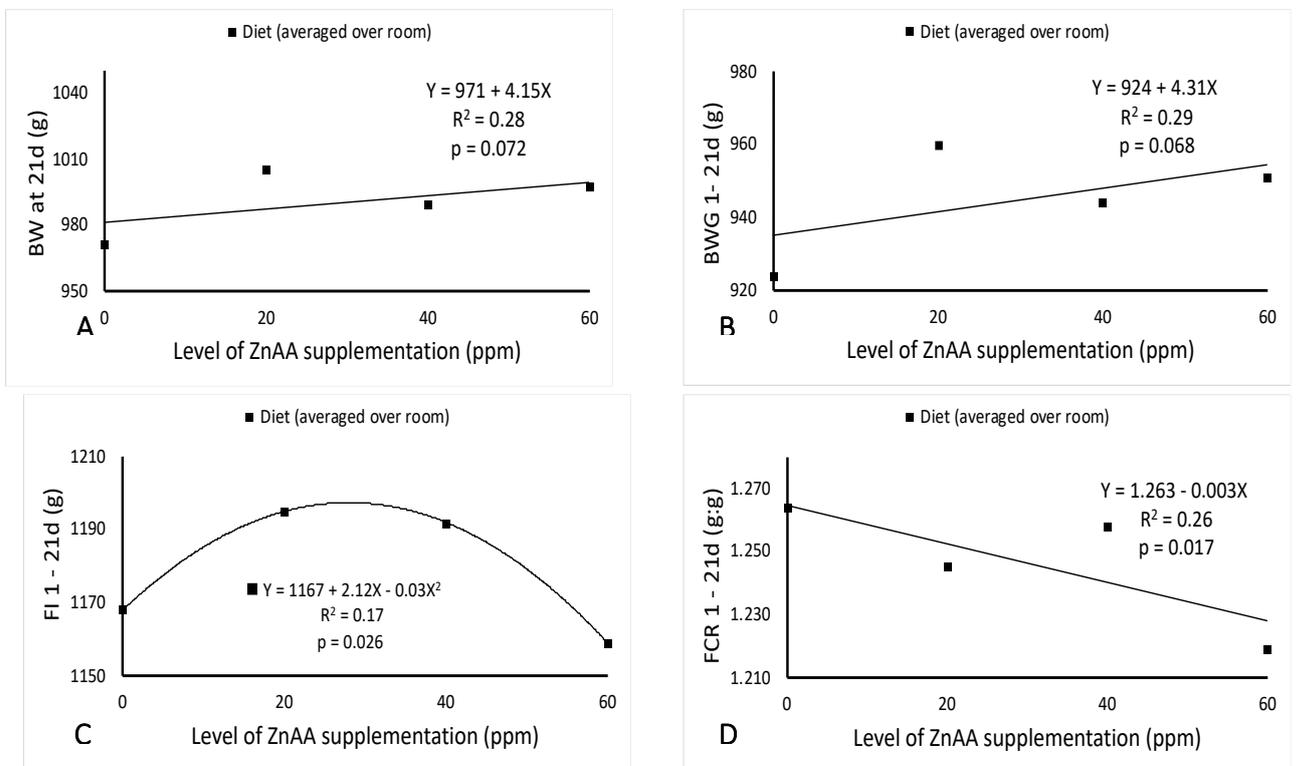


Figure 21. Growth performance of broilers fed increasing levels of zinc from 1 to 21d. A) Regression analyses for body weight (g); B) body weight gain (g); C) feed intake (g); D) feed conversion ratio (g:g). Results averaged over environmental condition.

Table 22. Growth performance of broilers fed increasing levels of zinc raised under heat stress from 21 to 42d.

ZnAA level	BW ₁	BWG ₁	FI ₁	FCR ₁
0 ppm	2854	1869	3114	1.666
20 ppm	2889	1870	3125	1.663
40 ppm	2887	1901	3117	1.640
60 ppm	2920	1910	3158	1.654
SEM	10.2	10.7	11.8	0.005
ANOVA	0.129	0.386	0.539	0.421
Contrast				
Linear	0.028	0.102	0.244	0.241
Quadratic	0.954	0.848	0.535	0.450

¹BW: body weight; BWG in grams: body weight gain in grams; FI: feed intake in grams; FCR: feed conversion ratio (g:g).

²Supplemental zinc level as metal-amino acid complex. Environmental conditions from 21 to 42 d. HS: heat stress 32 ± 1 °C for 12 h/d.

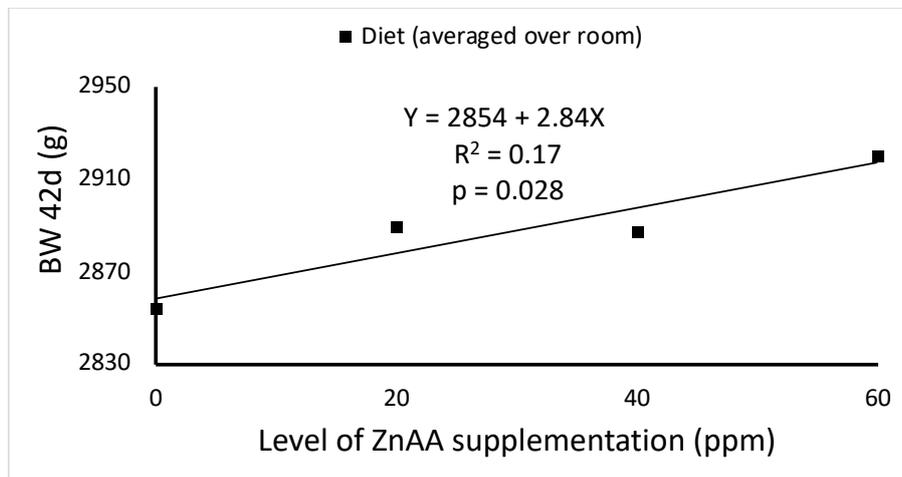


Figure 22. Regression analyses for BW (g) of broilers fed increasing levels of zinc raised under heat stress from 21 to 42d.

Results for the entire period (1 to 42d) are shown in Table 23. ZnAA supplementation linearly increased body weight and body weight gain ($p < 0.05$; Figure 22 and 23, respectively). The highest supplemental level (60 ppm) generated 26 grams increment over the 40-ppm level and 60 grams increment over 0-ppm ZnAA supplementation level (~35 ppm of zinc in the diet; Table 6). Mortality has not been changed by zinc supplementation; however, number are higher than usual due to the excruciating heat stress protocol implemented (Table 24).

Table 23. Growth performance of broilers fed increasing levels of zinc raised under heat stress from 1 to 42d

ZnAA level ²	BW ₁	BWG ₁	FI ₁	FCR ₁
0 ppm	2854	2799	4283	1.530
20 ppm	2889	2832	4321	1.526
40 ppm	2887	2833	4315	1.524
60 ppm	2920	2859	4324	1.513
SEM	10.2	10.3	14.1	0.003
ANOVA	0.129	0.213	0.719	0.434
Contrast				
Linear	0.028	0.048	0.361	0.126
Quadratic	0.954	0.875	0.615	0.670

¹BW: body weight; BWG in grams: body weight gain in grams; FI: feed intake in grams; FCR: feed conversion ratio (g:g).

²Supplemental zinc level as metal-amino acid complex. Environmental conditions from 21 to 42 d. HS: heat stress 32 ± 1 °C for 12 h/d.

Means lacking a common superscript differ ($p < 0.05$). $n = 10$.

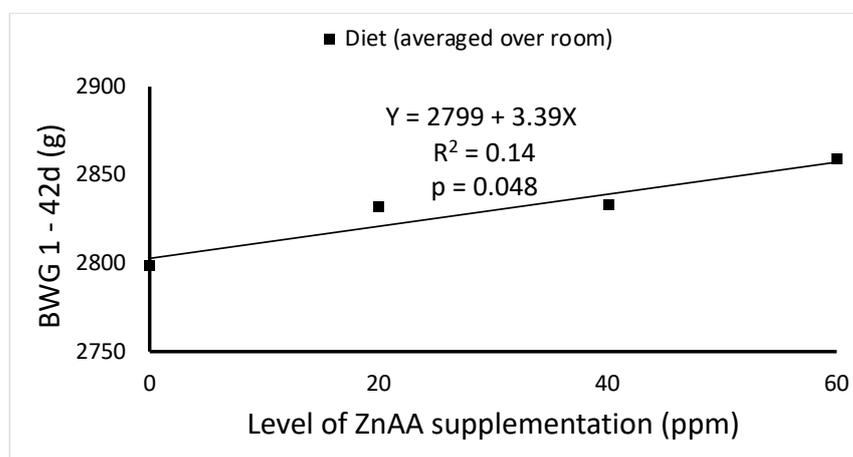


Figure 23. Regression analyses for BWG (g) of broilers fed increasing levels of zinc raised under heat stress from 1 to 42d.

Table 24. Mortality (%) of broilers fed increasing levels of zinc raised under heat stress from 1 to 42d within three periods.

Item	1 to 21 days	21 to 42 days	1 to 42 days
ZnAA level ¹			
0 ppm	0.67	6.91	7.56
20 ppm	0.44	7.78	8.22
40 ppm	0.44	5.56	6.00
60 ppm	0.44	5.58	6.00
SEM	0.003	0.021	0.020
ANOVA	0.914	0.963	0.928
Contrast			
Linear	0.631	0.631	0.534
Quadratic	0.720	0.828	0.810

¹Supplemental zinc level as metal-amino acid complex. Environmental conditions from 21 to 42 d. HS: heat stress 32 ± 1 °C for 12 h/d.

Means lacking a common superscript differ ($p < 0.05$). $n = 10$.

5. CONCLUSION

The heat stress protocol implemented was very effective, causing impairment on performance, immune suppression, oxidative stress and poor gut health. Availa Zn supplementation improved performance parameters, enhanced immune parameters, diminished oxidative stress and upregulated occludin and ZO-1 protein expression. Using these data, a more extensive study on Availa Zn requirement may be carried out. The ideal level ranges from 40 and 60 ppm depending on the parameter studied. As linear results were observed, future studies should include higher levels.

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