# *In ovo* supplementation of chelated minerals (zinc, iron, and manganese) for enhanced embryonic development, nutrient absorption, and modulation of the immune system in chicken embryos

By

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**Abstract**

*In ovo* administration of chelated (organic) minerals offers a promising strategy to enhance immunity, mitigate stress, and boost nutrient absorption in embryos, potentially optimizing post-hatch health and performance in poultry compared to inorganic minerals. The first objective of this study was to determine the optimal individual *in ovo* doses of amino acid-chelated zinc (Zn), iron (Fe), and manganese (Mn). The second objective was to test the combined effects at three levels (0.5×, 1×, and 2×) based on the optimal doses identified in the first objective and compare them with their inorganic counterparts at the same levels.

In the first experiment, 104 commercial fertilized broiler eggs were randomly assigned to 13 treatment groups (n = 8 per group). The control group received distilled water, while the remaining 12 groups were injected with four different doses of each chelated mineral, respectively. The doses were 20, 40, 60, 80 µg/egg for Zn, 100, 300, 500, 700 µg/egg for Fe, and 10, 20, 30, 40 µg/egg for Mn. Gene expression analysis was performed using qRT-PCR. and statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test in GraphPad Prism 8 software ($p < 0.05$). The expression of the mTOR in the liver showed that the injection of Zn at 40, 60, and 80 µg/egg showed higher expression ($p < 0.05$) compared to the control, indicating its role in promoting embryonic growth. Furthermore, expression of SGLT1, PepT1, and IL-22 resulted in the upregulation ($p < 0.05$) compared to the control at 40 µg Zn/egg, indicating Zn at this level enhances glucose and peptide absorption and promotes tissue regeneration in the intestine. Similarly, Fe treatment at 500 µg/egg showed upregulation ($p < 0.05$) of mTOR in the liver, IL-22 in the intestine, and downregulation of proinflammatory cytokine IL-2 in the spleen, compared to the control. Manganese at 20 µg/egg resulted in the higher expression of SGLT1, PepT1, and IL-22 in the intestine ($p < 0.05$), while downregulated the expression of proinflammatory cytokines IL-1β and IL-2 and upregulated anti-inflammatory cytokine IL-10 in the spleen ($p < 0.05$), compared to the control. In conclusion,

the optimal *in ovo* doses were identified as 40 μg/egg for Zn, 500 μg/egg for Fe, and 20 μg/egg for Mn.

In the second experiment, 56 fertile eggs were randomly divided into seven treatments, with 8 eggs per treatment. Treatments included a control group, three inorganic minerals (IM), and three organic chelated minerals. The three levels of inorganic and chelated minerals were 0.5× (20 μg Zn, 250 μg Fe, and 10 μg Mn per egg), 1× (40 μg Zn, 500 μg Fe, and 20 μg Mn per egg), and 2× (80 μg Zn, 1000 μg Fe, and 40 μg Mn per egg). Results indicated that organic minerals, especially at lower doses (0.5× and 1×), significantly enhanced mTOR expression in the liver, PepT1, SGLT1, and IL-22 in the intestine ($p < 0.05$). In contrast, inorganic minerals required higher doses (2×) to achieve similar PepT1 expression but showed no significant impact on SGLT1 and IL-22. Inorganic minerals also induced a proinflammatory response at higher doses (2×). In conclusion, combined organic minerals, particularly at lower doses (0.5× and 1×), were found to be more effective than inorganic minerals in supporting embryonic growth, nutrient absorption, immune modulation, and oxidative stress management.

**Résumé**

L'administration in ovo de minéraux chélatés (organiques) représente une stratégie prometteuse pour améliorer l'immunité, réduire le stress et stimuler l'absorption des nutriments chez les embryons, optimisant ainsi potentiellement la santé et les performances post-éclosion des volailles par rapport aux minéraux inorganiques. La première objectif de cette étude était de déterminer les doses individuelles optimales in ovo de zinc (Zn), fer (Fe) et manganèse (Mn) chélatés à des acides aminés. Le deuxième objectif était d'évaluer les effets combinés de ces minéraux à trois niveaux (0,5×, 1× et 2×) basés sur les doses optimales identifiées, puis de les comparer à leurs équivalents inorganiques aux mêmes concentrations.

Dans la première expérience, 104 œufs fécondés de poulets à griller ont été répartis aléatoirement en 13 groupes (n = 8 par groupe). Le groupe témoin a reçu de l'eau distillée, tandis que les 12 autres groupes ont été injectés avec quatre doses distinctes de chaque minéral chélaté : 20, 40, 60 ou 80 µg/œuf pour le Zn ; 100, 300, 500 ou 700 µg/œuf pour le Fe ; et 10, 20, 30 ou 40 µg/œuf pour le Mn. L'expression génique a été analysée par qRT-PCR, et les données statistiques ont été traitées par ANOVA bidirectionnelle suivie du test post hoc de Tukey (GraphPad Prism 8 ; $p < 0,05$). L'expression de mTOR dans le foie a révélé que les doses de Zn à 40, 60 et 80 µg/œuf induisaient une expression significativement plus élevée ($p < 0,05$) que le témoin, soulignant son rôle dans la promotion de la croissance embryonnaire. De plus, à 40 µg/œuf, le Zn a entraîné une régulation positive de SGLT1, PepT1 et IL-22 dans l'intestin ($p < 0,05$), indiquant une amélioration de l'absorption du glucose et des peptides, ainsi qu'une régénération tissulaire. Le Fe à 500 µg/œuf a augmenté l'expression de mTOR dans le foie et d'IL-22 dans l'intestin ($p < 0,05$), tout en réduisant l'expression de la cytokine pro-inflammatoire IL-2 dans la rate. Le Mn à 20 µg/œuf a augmenté l'expression de SGLT1, PepT1 et IL-22 dans l'intestin ($p < 0,05$), tout en diminuant les cytokines pro-inflammatoires

IL-1β et IL-2 et en augmentant l'anti-inflammatoire IL-10 dans la rate. Les doses optimales identifiées étaient de 40 µg/œuf pour le Zn, 500 µg/œuf pour le Fe et 20 µg/œuf pour le Mn.

Dans la deuxième expérience, 56 œufs fertiles ont été divisés en sept groupes (n = 8 par groupe), incluant un témoin, trois groupes de minéraux inorganiques (MI) et trois groupes de minéraux organiques (MO) aux niveaux 0,5× (20 µg Zn, 250 µg Fe, 10 µg Mn/œuf), 1× (40 µg Zn, 500 µg Fe, 20 µg Mn/œuf) et 2× (80 µg Zn, 1000 µg Fe, 40 µg Mn/œuf). Les résultats ont montré que les minéraux organiques, particulièrement aux doses 0,5× et 1×, augmentaient significativement l'expression de mTOR dans le foie, ainsi que celle de SGLT1, PepT1 et IL-22 dans l'intestin ($p < 0,05$). En revanche, les minéraux inorganiques nécessitaient une dose élevée (2×) pour atteindre une expression similaire de PepT1, sans effet significatif sur SGLT1 et IL-22. De plus, les MI à 2× ont déclenché une réponse pro-inflammatoire.

En conclusion, les minéraux organiques combinés, particulièrement aux doses 0,5× et 1×, se sont avérés plus efficaces que leurs équivalents inorganiques pour soutenir la croissance embryonnaire, l'absorption des nutriments, la modulation immunitaire et la gestion du stress oxydatif. Ces résultats soulignent l'avantage des formes chélatées pour une supplémentation in ovo optimisée en aviculture.

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**Contributions of Authors**

This thesis follows McGill University's manuscript guidelines and includes two manuscripts that are intended for submission to a journal named Poultry Science.

**Authors of manuscript 1 (Chapter 4)** entitled "*In ovo* supplementation of chelated minerals (Zn, Fe & Mn) independently to determine the optimal dose" are Atul Jadhav, Ayobami Aboderin, Lim Mei Lynn, Bushansingh Baurhoo, Raj Duggavathi, and Alexander Yitbarek.

Atul Jadhav: Conceptualization, execution of the experiment, mineral injections, necropsies, data processing, qRT-PCR procedure, results interpretation, and writing the manuscript.

Alexander Yitbarek, Bushansingh Baurhoo, and Raj Duggavathi: Experimental design, materials and methods selections, experimental supervision, comprehensive discussions, results interpretation, revision, editing, and manuscript formatting.

Ayobami Aboderin and Lim Mei Lynn: Mineral injections, necropsies, and sample collections.

**Authors of manuscript 2 (Chapter 6)** entitled "Combined effect of the optimized chelated mineral doses of zinc, iron, and manganese and their comparison with their inorganic counterparts" are Atul Jadhav, Ayobami Aboderin, Lim Mei Lynn, Bushansingh Baurhoo, Raj Duggavathi, and Alexander Yitbarek.

Atul Jadhav: Conceptualization, execution of the experiment, mineral injections, necropsies, data processing, qRT-PCR procedure, results interpretation, production of tables and figures, and writing the manuscript.

Alexander Yitbarek, Bushansingh Baurhoo, and Raj Duggavathi: Experimental design, materials and methods, experimental supervision, results interpretation and discussion, revision, editing, and formatting of the manuscripts.

Ayobami Aboderin and Lim Mei Lynn: Mineral injections, necropsies, and sample collections.

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**List of Abbreviations**

| | |
|---|---|
| Zn | Zinc |
| Fe | Iron |
| Mn | Manganese |
| UN | United Nations |
| HF | Hatchability of Fertile Eggs |
| AGPs | Antibiotic Growth Promoters |
| SOD | Superoxide Dismutase |
| GPx | Glutathione Peroxidase |
| GIT | Gastrointestinal Tract |
| MD | Marek's Disease |
| NRC | National Research Council |
| eGH | Endogenous Growth Hormones |
| IGFs | Insulin-like Growth Factors |
| ROS | Reactive Oxygen Species |
| RNS | Reactive Nitrogen Species |
| NRAMP | Natural Resistance Protein |
| NO | Nitrous Oxide |
| IRPs | Iron Regulated Proteins |
| FCR | Feed Conversion Ratio |
| NK | Natural Killer |
| STAT | Signal Transducer & Activator of transcription |
| AMPs | Antimicrobial Peptides |
| FWM | First Week Mortality |
| OC | Optimum Concentration |
| CORT | Corticosterone |
| Cu | Copper |
| RTase | Reverse Transcriptase |
| mTOR | Mechanistic Target of Rapamycin |
| IM | Inorganic Minerals |
| OC | Optimum Concentration |
| RT-qPCR | Reverse Transcriptase Quantitative Polymerase Chain Reaction |

| | |
|---|---|
| 0.5×-In | 0.5 time optimum concentration of inorganic minerals |
| 1×-In | 1 time optimum concentration of inorganic minerals |
| 2×-In | 2 time optimum concentration of inorganic minerals |
| 0.5×-Ch | 0.5 time optimum concentration of chelated (organic) minerals |
| 1×-Ch | 1 time optimum concentration of chelated (organic) minerals |
| 2×-Ch | 2 time optimum concentration of chelated (organic) minerals |
| PepT | Peptide transporter |
| SGLT | Sodium Dependent Glucose Transporter |
| IL | Interleukin |
| Th | Helper T cells |
| MT | Metallothionein |
| JAK | Janus Kinase |
| ED | Embryonic Day |
| GSH | Glutathione Secreting Hormone |

# 1 Chapter 1: Introduction

The United Nations (UN) has predicted that the global population will reach 9.8 billion by 2050, and the demand for animal-derived protein is expected to double (Henchion et al., 2017). This increasing demand will require a significant rise in agricultural production. Some studies estimated that food production must increase by 50% in order to meet global needs (Lombardi et al., 2021). Poultry products are becoming popular and affordable options for meeting dietary protein needs, as chicken meat and eggs are widely available and relatively cheap. Not only in developing countries but also in developed countries, such as the United States and Canada, poultry is becoming the main source of protein for many people. However, in the poultry production chain, various hotspots require solutions, including preventing early mortality, producing healthy chicks, and developing alternatives to antibiotic growth promoters (AGPs) to produce chicken and eggs sustainably (Tabashsum et al., 2023).

Early chick mortality is a major problem the poultry industry is facing. Embryonic development is a complex process during which an embryo undergoes rapid growth and development, leading to metabolic stress and dysregulation of autophagy. This can ultimately result in embryonic mortality, or if hatched, chicks will be weak and may become more susceptible to external stressors or pathogens, leading to early mortality. In the current broiler production chain, chicks generally do not have access to feed and water for approximately 48 to 72 hours during transportation from the hatchery to the production house, which negatively affects their health and development (Dieryck et al., 2022). Additionally, they need to transition from using lipid-rich yolk reserves to digesting solid, complex feed (Ravindran and Abdollahi, 2021). Chicks are vulnerable during the initial days of post-hatching, where the immunity and gastrointestinal tract development is not completed until 2 weeks after hatch (Uni et al., 2012). Despite the quality of the chicks and effective management efforts, a certain level of early chick mortality is unavoidable, with a typical rate ranging from 1% to 5% on a well-managed poultry

farm. This rate may be reduced with *in ovo* nutritional interventions. Early chick mortality can be attributed to various factors, including genetic predispositions, management practices, disease, and nutritional deficiencies or excess (Yerpes et al., 2020).

A fresh, fertile egg from the healthy broiler breeder contains approximately 15.62% protein, 27.32% lipids, 0.42% carbohydrates, 53.32% water, and 3.32% other minerals (Yadgary et al., 2010). However, towards the end of the incubation period, about the $15^{th}$ day of incubation, the reserves of essential minerals such as Zn, Fe, and Mn in fertile broiler eggs decrease by up to 70% (Uni et al., 2012). Zinc supports the immune system and acts as an antioxidant, helping enzymes like superoxide dismutase (SOD) protect cells from damage (Marreiro et al., 2017). Iron helps in oxygen transport and cellular respiration (Abbaspour et al., 2014). Manganese is important for embryonic and bone development and acts as a cofactor for metabolic enzymes (Li and Yang, 2018). Additionally, Mn is vital for proper embryonic development, as deficiencies in this mineral can lead to embryonic abnormalities and reduced hatchability (Korver, 2023).

The significant reduction in these minerals during the final stages of embryogenesis can result in the reduction of hatchability percentages, limitation of embryonic growth, and underdevelopment of the skeleton and immune systems (Wilson, 1997; Angel, 2007; Dibner et al., 2007; Yair and Uni, 2011). Consequently, post-hatch chicks will be more susceptible to microbial infections or environmental stressors, contributing to increased early mortality. This indicates that the administration of nutrients during the embryonic stage can improve the chick's gut physiology and health towards the end of incubation and right after hatching. Therefore, *in ovo* nutrition is a strategic approach to enhancing a chick's immune responses, nutrient absorption, gut physiology, and overall health and development of newly hatched chicks and eventually prevents the negative effects of the lack of essential nutrients (Das et al., 2021).

*In ovo* administration of chelated minerals offers better nutrition to the developing embryos if administered at proper concentration. Deficiency, as well as excess of minerals, can have negative effects on the hatchability and overall health of the embryos. Many studies have shown the potential of *in ovo* delivery for chelated minerals, although those studies were not conducted to optimize the mineral doses, especially Zn, Fe, and Mn, to support embryonic health and development. A study (Oliveira et al., 2015) used two different dosages of organic zinc, manganese, and copper in combination. Dose-1 was 27.2, 13, and 1.5 μg/egg, and dose-2 was 81.6, 39, and 4.1 μg/egg, respectively. They observed that the hatchability of fertile eggs (HF) for dose-2 was significantly lower, and dose-1 was intermediate compared to the non-injected control group. Another study showed that *in ovo* feeding, nano minerals did not harm the developing embryo and did not influence the hatchability, with no significant effect on dressing percentage or breast muscle yield, except for the highest breast muscle percentage observed in the nano form of copper at 12 μg per egg. The study concluded that nano forms of zinc, copper, and selenium can be prepared at laboratory conditions and that *in ovo* feeding of these minerals does not harm the developing embryo or affect hatchability (Joshua et al., 2016). However, excessively high doses of chelated/organic minerals can also have a negative impact on the hatchability and quality of chicks, as was shown in another study that injected chelated minerals (Zn, Mn, and Cu) into eggs on the 17th day of incubation (Peebles et al., 2021). This indicates that the effect of the minerals on the growth and development of chicken embryos, hatchability, and post-hatch health is dose-dependent. Currently, no scientific studies are showing the safe dosage of chelated minerals for *in ovo* administration effective for preventing early mortality and improving immune responses, gut health, and growth of the chicken embryos.

Therefore, this study evaluated four different dosages of each chelated mineral (Zn, Fe, and Mn) to determine the optimal dose that positively influences chicken embryonic growth and

nutrient absorption, modulates immune responses, and reduces oxidative stress. Furthermore, using the safest and most effective dose identified, a follow-up study was conducted to assess the combined effects at 0.5×, 1×, and 2× concentrations of these minerals and compare their efficacy with their inorganic counterpart at the same level of concentration.

# 2 Chapter 2: Literature Review

## 2.1 Chicken embryonic development

Chicken embryonic development is a complex and highly coordinated process that takes place over a 21-day incubation period and occurs in three major phases, including the establishment of germ, embryo maturation, and emergence from the eggs, respectively. Each phase lasts approximately 7 days (Moran, 2007). The first structure formed inside the egg soon after its placement in the incubator is the two-layered embryonic disc on the yolk, where the embryo development begins. The first visible change is the primitive streaks, which direct the head and tail regions. The vascular system is the first and most important system that develops during the first day of incubation. Subsequently, during the second week of the incubation, major organs of the embryo start developing, such as the alimentary tract, beak, head, eyes, trunk, tail, limbs, lungs, etc. (Davey and Tickle, 2007). On day 15$^{th}$ of incubation, the embryo's gut (small intestine) is drawn into the abdominal cavity, which will help the chicks absorb the nutrients from the yolk after hatching. Most of the major organ systems development has been completed around this day, and the embryos are growing rapidly and preparing themselves for hatching. During the later stages, the embryo consumes most of the nutrition from the egg, and a deficiency of any nutrients at this point prevents embryonic growth and development. Furthermore, additional nutrients are stored as a nutritional reserve and will be utilized during hatching or after hatching for the development of the gastrointestinal tract (GIT) and other functional systems (Givisiez et al., 2020). On day 20$^{th}$ of incubation, the chicks are fully grown and are in preparation to begin hatching (Poultry Hub Australia, 2021).

## 2.2 *In ovo* nutrition

*In ovo* nutrition is a technique for administering bioactive compounds such as minerals, amino acids, prebiotics, probiotics, symbiotics, creatine, and growth hormones to developing embryos, generally between 12 to 20 days of incubation. This can be achieved using the

appropriate hypodermic needle, injecting through the desired route and on the day of incubation (Das et al., 2021). This technique was first used for the vaccination against Marek's disease (MD) by Sharma and Burmester in 1981. Subsequently, the success of this technique was further investigated for nutritional effect on embryonic growth and development and post-hatch chick health and development. For instance, a study (Sun et al., 2018b) demonstrated that *in ovo* administration of organic Zn had higher levels of DNA methylation and H3K9 acetylation than inorganic Zn, confirming organic Zn can improve embryonic growth and development. Another study proved that *in ovo* feeding of Fe methionine decreased embryonic mortality and increased hatchability percentages with higher body weights of hatched chicks (Hassan et al., 2022). *In ovo* injection of Mn can boost the antioxidant capacity of the developing embryos with higher hatchability and enhanced performance in challenging conditions (Geng et al., 2022).

### 2.3 *In ovo* application sites

Researchers have used various routes for *in ovo* administration of different compounds depending on the research objectives and expected outcomes. The most common routes used for various studies include air sac, albumen, yolk sac, extra-embryonic membrane, amnion, chorioallantois membrane, and allantoic fluid. However, it is important to consider the route and time of *in ovo* injection as it affects the output of the studies. For instance, a study (Ohta and Kidd, 2001) showed that the hatchability was increased when the amino acid was injected through the yolk and extra-embryonic membrane compared to the chorioallantois membrane or into the amniotic cavity. Another study (Wakenell et al., 2002) showed that the MD vaccine was not effective when administered through air cells but was found effective when injected through amnion. However, the amnion is the most preferred route of administration of nutritional compounds such as minerals, as the amniotic fluid is completely ingested by the

embryo towards the last stage of the incubation. Therefore, substances administered to this site will be ingested and utilized by the embryo (Uni et al., 2012; Peebles, 2018).

### 2.4 *In ovo* application timelines

The time of injection varies depending on the type of bioactive substance injected into the eggs. Generally, *in ovo* stimulants such as prebiotics, probiotics, symbiotics, and nutrients like vitamins, minerals, amino acids, carbohydrates, and hormones are administered at an early embryonic developmental stage starting from day $12^{th}$, while the vaccine is administered in the later stages especially on day $18^{th}$ of incubation when the eggs are transferring from setter to hatcher in commercial hatchery settings (Das et al., 2021). However, it is observed that the embryo consumes most of the essential nutrients, about 70%, around the $15^{th}$ day of incubation, and any nutritional injection would benefit the embryo at this time point (Yair and Uni, 2011). Despite this, it is still unclear when the embryo fully benefits from the supplementation.

### 2.5 Mineral nutrition

#### 2.5.1 Inorganic minerals

Inorganic minerals are the most used source of minerals in poultry nutrition. Minerals such as Zn, Fe, and Mn are crucial for poultry nutrition, promoting embryonic and post-hatch growth and development, immunity, and intestinal health (Richards et al., 2010). These minerals are typically administered in poultry feed in the form of oxides, sulfates, and carbonates, which significantly reduces their bioavailability. Due to their low bioavailability, inorganic minerals are often supplied in poultry diets at levels much higher than the National Research Council's (NRC) recommended requirements for birds. This excessive supplementation leads to high excretion in poultry waste, contributing to significant environmental pollution (Mézes et al., 2012).

### 2.5.2 Chelated (organic) minerals

Chelated minerals form when a metal binds to an organic ligand through coordinated bonds, with proteins and carbohydrates being the most common organic components (Vieira, 2008). These minerals are absorbed through the amino acid or peptide transport system, which enhances their bioavailability (Shah, 1981). Chelated minerals offer several advantages over inorganic ones as they are more efficiently absorbed and utilized by birds, requiring smaller doses to achieve the same nutritional benefits. Consequently, organic minerals can replace inorganic minerals at lower doses without negatively affecting broiler or layer production (Bhagwat et al., 2021). Studies showed that chicks receiving chelated trace minerals (Zn, Fe, and Mn) showed higher body weight gain, higher mineral retention in tissues, improved immunity, and better feed efficiency compared to those fed inorganic trace minerals at the same dose (Ghasemi et al., 2020).

## 2.6 Zinc (Zn)

Zinc plays a crucial role in chicken embryonic growth and development. It is involved in several enzymatic reactions and regulates many cellular processes, including DNA synthesis, cell proliferation, apoptosis, organ development, and embryo development (Beyersmann and Haase, 2001; Costa et al., 2023). Recent studies have shown that Zn plays an essential role in neuronal cell signalling in which Zn is stored and released from synaptic vesicles, which are then received by postsynaptic cells, inducing the intercellular communication necessary for cell growth, functioning, and development (Frederickson et al., 2000; Krall et al., 2021; Zhang et al., 2022). Zinc increases mitogenic signalling pathways by activating and releasing growth hormone (GH) and insulin-like growth factors (IGFs), which initiate several signalling cascades such as MAPK/ERK, PI3K/AKT, and PKC to promote cell growth and proliferation (Haase and Maret, 2005; Ohashi et al., 2015; Guo et al., 2020).

A study (Joshua et al., 2016) showed that the *in ovo* injection of nano form of Zn neither harmed the developing embryo nor affected the hatchability, suggesting it can be beneficial to chicken embryos. However, the optimum concentration of Zn is crucial as the higher concentration can negatively affect the hatchability and increase embryonic mortality as shown in (Sun et al., 2018b) where doses of 150–200 µg Zn/egg were harmful to the embryonic development. On the other hand, Zinc deficiency impairs skeletal development, resulting in weak chicks with stunted growth, retarded feathering, and frizzled feathers (Kienholz et al., 1961). An organic form of zinc has a better effect on the growth performance of the broilers compared to the inorganic form, as indicated in the study done by (Khan et al., 2024).

Zinc is a mineral that is crucial in poultry immunity, which is involved in the development of lymphoid organs, maturation, differentiation, and proliferation of T lymphocytes, and proper functioning of the heterophils. It plays a key role in activating both humoral and cell-mediated immune responses to fight against invading pathogens by improving the balance between Th1 and Th2 immune responses. This mineral was found to increase the number of CD4+ & CD8+ lymphocytes (Jarosz et al., 2017). The CD4+ lymphocytes, also known as helper T cells, activate the macrophages and dendritic cells, which target the intracellular pathogens and regulate the release of IL-2 interleukin to modulate immune responses (Luckheeram et al., 2012). Whereas CD8+ cells, also known as cytotoxic T cells, are responsible for cell-mediated immunity targeting and eliminating infected or abnormal cells (Topchyan et al., 2023). However, the inorganic form or the deficiency of the zinc can have no response or lead to a reduction in these lymphocytes, which causes delayed immune response as shown in the studies by (Jarosz et al., 2017) and (Cui et al., 2004), respectively. Another study (Gul and Alhidary, 2024) evaluated different doses (50 mg & 60 mg per kg feed) and forms (organic and inorganic) of the zinc and concluded that the broiler

birds supplemented with 50 mg/kg organic Zn exhibited enhanced humoral immune response and reduced malondialdehyde (MDA) levels.

The reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as oxides, hydrogen peroxides, nitric oxides, etc., are the normal products of cellular metabolism. They play a dual role in the body, depending on the level of their production. Generally, lower levels of ROS/RNS are beneficial as they regulate the physiological roles in cellular responses to anoxia to defend against the pathological agents and function as a signaling pathway in cell proliferation (Valko et al., 2007). However, the overproduction of ROS/RNS causes potential biological damage to cell structure, DNA, proteins, and lipids, impairing their functions through oxidative stress and nitrosative stress. This condition arises due to the reduced production of antioxidant enzymes, such as superoxide dismutase (SOD), which catalyses the conversion of highly reactive superoxide radicals into hydrogen peroxide. The resulting hydrogen peroxide is subsequently detoxified by glutathione peroxidase (GPx), converting it into water molecules, thereby mitigating oxidative stress (Valko et al., 2007).

Zinc is redox-inert, which means it is not an antioxidant itself, as it cannot neutralize free radicals as it does not donate or accept electrons (Maret, 2019). However, Zn can bind to the sulfur (thiolate) of the cysteine to produce Zn thiolate. Whenever Zn thiolate reacts with the ROS, thiolate binds to it, and Zn is released as a free element. Such oxidative release of Zn sends the signals that trigger an antioxidant response against the ROS. In cases of deficiency or excess, Zn acts as a pro-oxidant, leading to oxidative stress (Lee, 2018a; Maret, 2019). Additionally, Zn is a cofactor in Zn-Cu-superoxide dismutase, which plays a crucial role in the elimination of ROS (Prasad, 1997).

### 2.7 Iron (Fe)

Iron is a crucial mineral that plays a pivotal role in various physiological processes, including DNA metabolism, oxygen transport, red blood cell production, cellular energy

production, and others (Luckheeram et al., 2012). Additionally, Fe acts as a cofactor of many enzymatic processes, such as acetyl coenzyme A, succinodehydrogenase, xanthine oxidase, etc. Iron is absorbed in the small intestine, primarily in the duodenum. Ferric iron ($Fe^{3+}$) requires conversion into a more absorbable form of ferrous iron ($Fe^{2+}$) through the action of an enzyme known as duodenal cytochrome b (DCYTB). Once converted, ferrous Fe enters intestinal cells (enterocytes) via a transporter called divalent metal-ion transporter-1 (DMT1). Subsequently, it can either be stored within the cells or transported into the bloodstream via another protein, ferroportin (Fpn) (Donovan et al., 2000; McKie et al., 2000). Before iron's blood circulation, it must be reverted to its ferric form by enzymes such as hephaestin or ceruloplasmin. In this form, it binds to a protein called transferrin, which facilitates its distribution throughout the body, particularly to the bone marrow, where new red blood cells (erythrocytes) are synthesized (Horton, 1983). Old red blood cells are subsequently broken down by macrophages, facilitating Fe recycling. This recycled Fe can be stored within ferritin, released back into the bloodstream, or utilized in various metabolic processes. The hormone hepcidin, produced by the liver, regulates Fe levels by binding to ferroportin and causing its degradation. When Fe levels are elevated, hepcidin inhibits Fe release from enterocytes and macrophages, thereby reducing the amount available in the blood (Park et al., 2001; Ganz, 2013).

Iron has been found to regulate the mechanistic/mammalian target of the rapamycin (mTOR) signaling pathway, which controls cellular growth, as well as protein and lipid synthesis, thereby promoting cell survival and growth. This is done through two complexes, mTORC1 and mTORC2 (Saxton and Sabatini, 2017). mTORC1 promotes anabolic processes and acts like a switch that gets turned on or off by signals from the environment. However, when mTORC1 is overactive, it can lead to abnormal cell growth, resulting from mutations in genes such as PTEN, PI3K, or TSC1/2 that typically regulate mTORC1 (Shimobayashi and

Hall, 2014; Saxton and Sabatini, 2017). Excess of Fe leads to apoptosis through regulation of the PI3K/AKT/mTOR axis as shown in the study (Sun et al., 2022).

Nutritional immunity is the host's defence strategy that involves regulating the levels of essential minerals, such as iron, zinc, manganese, and copper, to prevent the growth of invading pathogens and deprive them of these minerals. Iron plays a critical role in nutritional immunity. Immune cells, such as neutrophils and macrophages, make Fe unavailable to these pathogens either intracellularly or through iron-binding proteins (Hood and Skaar, 2012). Calprotectin and lactoferrin are the proteins secreted by macrophages and neutrophils, and they act as a first line of defence against the infection. These proteins bind to the free Fe and make it unavailable to the pathogens. Natural resistant protein 1 (Nramp1) delivers the Fe into the cytoplasm, which can starve intracellular pathogens such as *salmonella* for Fe (Forbes and Gros, 2001). However, free or excess Fe can be toxic, leading to the formation of hydroxyl radicals, which causes oxidative stress. Hence, the proper concentration of Fe is necessary as there is no excretory mechanism in the human or animal body for its excretion. Therefore, its regulation becomes critical (Wardman and Candeias, 1996).

Excess concentration of Fe will have a negative effect on the cellular membrane, protein, and DNA, as it is believed to produce more reactive oxygen species (ROS). Iron takes part in the Haber-Weiss reaction, in which superoxide ($O_2^-$) and hydrogen peroxides ($H_2O_2$) convert into highly reactive hydroxy radicals ($OH^-$) (Halliwell and Gutteridge, 1984). Inside the cell, Fe is always found chelated or bound to a protein called ferritin. In uncertain conditions, ferritin releases Fe, leading to oxidative stress. Additionally, the enzyme in the liver, cytochrome P450, can also trigger the release of Fe from ferritin, increasing the radical formations (Puntarulo and Cederbaum, 1988; Puntarulo, 2005). Nitrogen oxide (NO), produced from the enzymatic conversion of amino acids l-arginine and l-citrulline catalyzed by NO synthases, plays a crucial role in Fe homeostasis and host defense against oxidative stress. Macrophages also produce

NO through an expression of cytokine-inducible NOS (iNOS), which results in a higher output of NO (Bogdan, 2015). NO has a high affinity for Fe and targets iron-sulfur clusters in the metabolic enzymes of pathogens and disrupts their functions (Nathan and Hibbs, 1991). It influences cellular Fe homeostasis by regulating Fe regulatory proteins (IRPs). These proteins control ferritin expression and regulate Fe homeostasis (Weiss et al., 1993). Additionally, chickens have developed complex antioxidant systems to counter against oxidative stress. The system consists of internally synthesized antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxide (GPx). These antioxidants work together to neutralize the ROS and RNS (Surai et al., 2019).

### 2.8 Manganese (Mn)

Manganese in chicken embryonic development plays a crucial role in proper bone formation and acts as a cofactor for glutamine synthetase, pyruvate carboxylase, and superoxide dismutase. Additionally, it helps in the metabolism of carbohydrates, amino acids, lipids, and proteins (Horning et al., 2015; Mezzaroba et al., 2019; Kulshreshtha et al., 2021). Manganese is primarily absorbed in the intestine through divalent metal transporter 1 (DMT1), however, the exact mechanism is not yet fully understood. It enters the body via the SLC39A14 importer, while excess Mn is removed by the SLC30A10 exporter, which transports it into bile for excretion (Chen et al., 2015; O'Neal and Zheng, 2015).

*In ovo* organic Mn had better hatchability percentages with a significant increase in the final weights of the chicks as showed in the study (Ghane-Khoshkebijari et al., 2024) in which they found that the eggs supplemented with 13 Mn µg/egg /egg and 26 Mn µg/egg /egg had a better effect on blood parameters such as lower level of triglycerides, cholesterol, and lipoproteins in contrast to the normal saline-injected group. Another study was conducted with different levels of organic as well as inorganic Mn on the broiler performance metrics. This study concluded that broilers fed with organic Mn had higher average daily gain and lower feed

conversion ratio (FCR) compared to the inorganic one (de Carvalho et al., 2021). Furthermore, in challenging conditions such as chicks infected with *Eimeria acervuline*, Mn was found to improve the growth of chicks, as indicated in the study (Brown and Southern, 1985). These studies suggest that the organic form with a proper concentration of Mn in chicken will have a better effect on the growth parameters, including body weight gain, hatchability percentages, and blood biochemistry. However, more studies are needed to confirm its effect in a dose-response manner to better understand its concertation and its effect on chicken embryonic growth and development.

Manganese plays a role in nutritional immunity by restricting its availability to bacteria, preventing them from using it for essential functions such as combating oxidative stress, enzyme activity, and virulence (Hood and Skaar, 2012). Additionally, Mn plays an important role in the immune systems of vertebrates, including poultry, humans, and rodents. Scientists confirmed that Mn can influence immune cell activity and the response to the infection. Manganese helps immune cells, such as macrophages, natural killer (NK) cells, and neutrophils, to secrete signaling cytokines such as IL-1β, IL-6, and TNF-α, which help coordinate the body's defence against invading pathogens (Mokgobu et al., 2015). For instance, in poultry, Mn supplementation improved immune response and increased resistance against *Eimeria acervuline* (Brown and Southern, 1985). The optimum concentration of Mn is relatively beneficial to chicken embryos as it helps in bone formation and cellular metabolism and acts as a cofactor for many metabolic processes. Despite the beneficial effects, higher concertation leads to oxidative stress, which impairs mitochondrial functions and thus negatively affects embryonic development. Mitochondria are mainly responsible for the majority of the energy production, consequently generating more reactive oxygen species (ROS), leading to oxidative stress. However, mitochondria have a built-in antioxidant system that helps to maintain the balance between the antioxidants and ROS. This balance is crucial

for the normal functioning of the cells (Dikalov, 2011). One of the key antioxidants is manganese superoxide dismutase (MnSOD), which plays a major role in neutralizing superoxide radicals to less reactive hydrogen peroxide. Further, this molecule broke down into the water with the help of glutathione peroxide (GPx) using glutathione (GSH) as a reducing agent (Bottje, 2019). If the levels of superoxide or hydrogen peroxides get too high, it will damage the protein in the mitochondria and subsequently react with other metals such as iron or copper, producing extremely harmful hydroxy radicals. In broilers, increased oxidative stress is linked to reduced energy efficiency, as shown in a study by (Ojano-Dirain et al., 2005).

## 2.9 Mineral interaction

### 2.9.1 Interaction of zinc and iron

The study (Bafundo et al., 1984) investigated the interaction between Zn and Fe in growing chicks. Two trials were conducted to examine this interaction, particularly when the chicks were challenged with *Eimeria acervuline*. In the first trial, excess Fe supplementation did not reduce Zn levels in infected chicks. However, in the second trial, excess Zn supplementation reduced Fe levels and slowed chick growth. In conclusion, high Zn levels impair Fe utilization in chicks, whereas high Fe levels do not affect Zn.

Iron has an additive effect on zinc, as shown in the study, (Ramadan et al., 2010) which found that layers supplemented with both Fe and Zn had significantly higher egg mass, improved FCR, and better blood health compared to those receiving Fe alone. Another study (Ullah et al.,) noted a similar observation: when birds were supplemented with high levels of Zn and Fe, they significantly enhanced the egg Zn and Fe content without affecting other production-related parameters.

### 2.9.2 Interaction of zinc and manganese

Zinc and manganese have interactive effects that maintain the homeostasis of these minerals. The study (Nishito et al., 2024) showed that Mn treatment decreased the expression

of Zn-regulating proteins, metallothionein (MT), and ZNT1, and lowered Zn enzyme activities, likely due to reduced Zn levels. Additionally, when the Mn efflux transport protein was lost, MT and ZNT1 expression and Zn enzyme activity decreased, but extracellular Mn levels remained unchanged. This reduction didn't happen when copper (Cu) concentrations were supplemented or in cells lacking Cu efflux proteins. This confirms that Zn and Mn maintain homeostasis by interacting with each other. Zinc and manganese also interact with the hormones found in anabolic processes, potentially enhancing protein synthesis and muscle growth (Thornton-Kurth et al., 2024).

### 2.9.3 Interaction of iron and manganese

The interaction between Fe and Mn is unidirectional in chicks. The study (Baker and Halpin, 1991) revealed that dietary Fe had little impact on Mn status, regardless of how much Fe was added. However, when Mn was supplemented at extremely high levels (1,000 mg/kg), it reduced blood haemoglobin concentration, but this only occurred when the dietary Fe level was low or matched the chick's requirement for Fe. Additionally, increasing Fe supplementation up to 2,500 mg/kg did not affect the chicks' performance. However, feeding 5,000 mg Fe/kg resulted in reduced weight gain and lower bone ash concentration.

## 2.10 Immune markers, nutrient transporters, antioxidants, and growth hormones

Interleukin-1β (IL-1β) is a proinflammatory cytokine that mediates inflammatory response. When a cell is triggered by a signal, certain molecules inside the cell come together to form a complex called the inflammasome. This complex includes an adaptor molecule (ASC), a pathogen recognition receptor that recognizes harmful signals (like NLR), and a molecule called pro-caspase-1. Once these components join, caspase-1 is activated, which then helps release a protein called IL-1β. IL-1β plays a key role in the immune response by causing inflammation (Lopez-Castejon and Brough, 2011). Interleukin-2 (IL-2) is a cytokine that plays a critical role in T cell activation, proliferation, differentiation, and regulation. The IL-2

receptor (IL-2R) is central to mediating the effects of IL-2. It consists of three subunits: α (CD25), β (CD122), and γ (CD132). Upon IL-2 binding, the receptor activates JAK (Janus Kinase) tyrosine kinases, which in turn activate STAT5 (Signal Transducer and Activator of Transcription 5) transcription factors. These signaling pathways promote the transcription of genes that regulate T-cell survival, differentiation, and proliferation. The secretion of IL-2 is triggered when a T cell is activated by an antigen, leading to the production and release of IL-2 via the ER-Golgi pathway (Ross and Cantrell, 2018; Volkó et al., 2019). Interleukin-10 (IL-10) is an anti-inflammatory cytokine that helps in the reduction of inflammation. When the cell is infected, IL-10 can slow down the activity of certain immune cells, like Th1 cells, NK cells, and macrophages, which are important for fighting off infections. However, these same cells can also cause damage to tissues, so IL-10 helps prevent this damage by reducing their secretion. This means IL-10 can have two effects: first, it may slow down the body's ability to fight the infection, and second, it helps protect the body from potential damage caused by an overactive immune response (Couper et al., 2008). Interleukin-22 (IL-22) is a signaling protein (cytokine) that helps tissues respond to inflammation. It activates a pathway called STAT3, which supports cell growth, prevents cell death, and helps produce antimicrobial peptides (AMPs) that protect and heal tissues (Kim et al., 2012).

Superoxide dismutase (SOD) is an antioxidant enzyme that protects the cells from oxidative damage by neutralizing harmful free radicals such as oxide, into less harmful hydrogen peroxides (Chen et al., 2023). Glutathione peroxide (GPx) is a selenium-containing antioxidant enzyme that catalyzes the reduction of hydrogen peroxide and lipid peroxides to water and lipid alcohols (Ighodaro and Akinloye, 2018). SOD and GPx work together to manage oxidative stress by neutralizing reactive oxygen species as well as reactive nitrogen species in water.

The mechanistic target of rapamycin (mTOR) is a central regulator of cell growth and protein metabolism in poultry. It participates in cellular homeostasis by coordinating anabolic and catabolic processes. Furthermore, it regulates autophagy, a multistep catabolic process that plays an important role in maintaining homeostasis by removing or recycling damaged cells and organelles and degrading proteins in all eukaryotic cells (Gómez-Virgilio et al., 2022). However, mTOR regulates the overactivation of autophagy.

Endogenous growth hormone (eGH) plays an important role in regulating metabolic growth, development, and reproduction. This hormone directly or indirectly influences the growth of various physiological systems such as the digestive, reproductive, endocrine, and immune systems in chicken embryonic development (Bahadoran et al., 2019). Insulin-like growth factors (IGF-1 and IGF-2) are the polypeptide hormones that are important in chicken embryonic growth and development. IGF-1 especially stimulates muscle cell proliferation and differentiation, leading to increased muscle mass. Furthermore, IGFs play a crucial role in metabolism by influencing nutrient uptake and utilization, which ultimately support embryonic growth and development (Duclos, 2005; Karabag et al., 2019).

SGLT1 is a sodium-dependent glucose transporter that facilitates glucose absorption, while PepT1 helps in the absorption of short-chain peptides in the intestinal lumen (Daniel and Zietek, 2015).

# 3 Chapter 3: Rationale, hypothesis, and objectives

## Rationale

Chicken embryonic development is a complex process in which the embryo continuously undergoes metabolic changes. This results in significant metabolic stress on the developing embryos, which can lead to embryonic mortality and if hatched, the chicks may be weak and more susceptible to early mortality (Givisiez et al., 2020). Chelated (organic) minerals are effective in mitigating oxidative stress, regulating immune responses, and promoting embryonic growth. However, these minerals work best when administered at optimal concentrations, as both deficiency and excess can negatively affect embryos, leading to increased oxidative stress or embryonic mortality. Furthermore, the inorganic form of these minerals showed no change or reduced effect on the growth metrics, immune response, and embryonic development compared to the organic form (Ghasemi et al., 2020; Kong et al., 2022).

Therefore, it is crucial to optimize the levels of organic minerals (Zn, Fe & Mn) to determine the most effective concentration that positively influences embryonic development. There is a paucity of research on the *in ovo* delivery of chelated minerals, and our research aimed to address this gap by conducting two experiments.

## Hypothesis

*In ovo* supplementation of chelated Zn, Fe, and Mn at optimum concentrations or levels enhances embryonic growth, increases nutrient absorptions, modulates immune responses, and reduces oxidative stress in chicken embryos compared to inorganic minerals.

## Objectives

1) To determine the optimal *in ovo* doses of chelated Zn, Fe, and Mn that positively influence chicken embryonic development, nutrient metabolism, immune modulation, and reduce oxidative stress.

2) To compare the combined effects at 0.5×, 1×, and 2× of optimized chelated Zn, Fe, and Mn supplementation with their inorganic counterpart at the same concentration levels to determine their relative efficacy in supporting embryonic growth, immune, and oxidative stress regulation in chicken embryos.

# 4 Chapter 4: *In ovo* supplementation of chelated minerals (Zn, Fe & Mn) independently to determine the optimal dose

## 4.1 Abstract:

This study aimed to determine the optimal *in ovo* doses of chelated Zn, Fe, and Mn that positively affect embryonic health and development. 104 fertilized broiler eggs were randomly assigned to 13 treatment groups (n = 8 each). The control group received distilled water, while the other 12 groups were injected with four different doses of each mineral. The tested doses were 20, 40, 60, 80 µg/egg for Zn, 100, 300, 500, 700 µg/egg for Fe, and 10, 20, 30, 40 µg/egg for Mn. Minerals were injected into the amnion on ED 15, and samples were collected on ED 20. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

Results showed that Zn treatments at 40, 60, and 80 µg/egg significantly upregulated mTOR expression ($p < 0.05$) in the liver. This indicates that Zn may help in cellular development and protein synthesis, thereby promoting embryonic development. The 40 µg Zn/egg dose showed the highest expression of SGLT1 and PepT1($p < 0.05$) in the intestine, enhancing glucose and protein absorption. Iron treatment at 500 µg/egg resulted in significant upregulation of mTOR ($p < 0.05$) in the liver, indicating cellular growth involvement. Among manganese treatments, 20 µg Mn/egg significantly increased SGLT1 and PepT1 expression ($p < 0.05$) in the intestine, suggesting better nutrient absorption. Additionally, 20 µg Mn/egg dose significantly downregulated IL-1β and IL-2 while upregulating IL-10 ($p < 0.05$) in the spleen, suggesting this dose is optimum as it improved immune homeostasis. In conclusion, the optimal *in ovo* doses identified for enhancing nutrient absorption, immune, and oxidative stress regulation were 40 µg Zn/egg, 500 µg Fe/egg, and 20 µg Mn/egg. Further studies will focus on the combined effects of these minerals.

Keywords: Optimum concentration, In ovo chelated minerals, embryonic growth, immune response, oxidative stress

## 4.2 Introduction

The development of a chicken embryo is a complex process that takes place over a 21-day incubation period. However, the rapid pace of growth places a high demand on the embryo's metabolism. Reduced levels of essential minerals such as Zn, Fe, and Mn exacerbate this metabolic load, leading to increased production of reactive oxygen species, increased inflammation, impaired immune regulation, and disrupted embryonic growth and development. Consequently, this can result in embryonic mortality or weak embryos that are more susceptible to environmental and pathological challenges after hatching. Chicks face new challenges during their first week of life. They need to transition from utilizing lipid-rich yolk reserves to digesting solid complex feed (Uni et al., 2012). The gastrointestinal tract (GIT) is still in the developmental stage and needs to mature fast to aid in nutrient digestion and absorption processes. Additionally, the gut microbiota begins to develop during the first week, which is crucial for both digestion and immune function (Al Hakeem et al., 2023). Unfortunately, delays in feeding, often due to transportation from the hatchery to the broiler-rearing farms, cause stress and negatively impact gut health and immune development (Kadam et al., 2009; Ballou et al., 2016). These early stressors and first-week challenges can make chicks more vulnerable to infections, slow down their growth, and increase mortality rates, all of which can significantly impact economic broiler production (Klasing, 2007). First-week mortality (FWM) is still a challenge; even in a well-managed farm, a mortality rate ranging from 1% to 5% is unavoidable. If the rates are higher, this usually means something is wrong and needs to be fixed (Yerpes et al., 2020).

*In ovo* feeding of Zn, Fe, and Mn offers promising solutions to mitigate embryonic as well as post-hatched first-week challenges in chicks as they provide essential minerals during

embryonic development. This will also help chicks fight against the environmental challenges in post-hatch life. The embryo starts consuming these minerals significantly after the 11th day of incubation, and by the 15th day, the levels of these minerals drop significantly when embryonic growth and development reach their peak (Yair and Uni, 2011). However, organic forms of these minerals are better than their inorganic counterparts as they have high bioavailability and hence require less concentration without compromising their beneficial effects (Bhagwat et al., 2021). These minerals play a crucial role in embryonic cell proliferation, thereby promoting growth, facilitating cell signaling, regulating nutrient metabolism, modulating immune responses, and managing oxidative stress (Hänsch and Mendel, 2009; Lv et al., 2023).

Zinc has been shown to increase the hatchability percentages at moderate levels. However, it can adversely affect embryonic viability if administered in excess, as shown in the study (Sun et al., 2018a) in which they tested a dose of 50 to 250 µg Zn/egg and concluded that up to 150 µg Zn/egg dose was safe as it did not affect the hatchability, whilst the dose 200 µg Zn/egg was harmful as it significantly increased embryonic mortality. In another study (Ciszewski et al., 2023), *in ovo* supplantation, zinc glycine has been shown to increase the CD8+ cells during the first week after hatching, suggesting Zn is crucial for post-hatch cellular immunity. Furthermore, Zn increases the activity of antioxidant enzymes such as GSH-Px and SOD, which are crucial to eliminating ROS and preventing oxidative stress, as confirmed in the study (Shokraneh et al., 2020). However, in cases of deficiency or excess, Zn acts as a pro-oxidant, leading to oxidative stress (Lee, 2018b; Maret, 2019).

Iron is a crucial mineral that plays a pivotal role in various physiological processes, including DNA metabolism, oxygen transport, red blood cell production, cellular energy production, and others (Crielaard et al., 2017). *In ovo* injection of Fe at 75 ppm was shown to improve embryonic growth and development, as well as the post-hatch performance of the

chicks, as noted by (Mogahid et al., 2019). Additionally, *in ovo* Fe-methionine has been shown to influence post-hatch production performance in broilers, leading to increased weight gain, improved live body weight, and enhanced FCR during the first three weeks post-hatch, as indicated in (El-Said, 2019). However, Fe deficiency leads to anaemia, while excess Fe can cause liver damage and oxidative stress (Swanson, 2003).

Manganese is an essential mineral for embryonic growth and development as it plays a crucial role in bone formation and cartilage development. (Geng et al., 2022) Confirmed that a dose of 50 µg Mn/egg was harmful, as it significantly reduced hatchability, while 25 µg Mn/egg had a slight impact on hatchability but did not affect embryonic mortality. Additionally, 25 µg Mn/egg resulted in higher expression of Mn-SOD, indicating its role in regulating oxidative stress.

In conclusion, Zn, Fe, and Mn are essential for chicken embryos, but their optimal concentrations are crucial. Due to limited data on the best-chelated mineral doses for *in ovo* administration, this study aimed to determine the most effective dose that supports embryonic growth, regulates immune modulation, and balances oxidative stress in chicken embryos. The research tested four doses for each mineral: Zn (20, 40, 60, 80 µg/egg), Fe (100, 300, 500, 700 µg/egg), and Mn (10, 20, 30, 40 µg/egg).

## 4.3 Material and methods

### 4.3.1 Experimental design and egg incubation

A total of 104 fertile broiler eggs from commercial broiler breeders (Cobb), approximately equal in size and weight, were purchased from the commercial hatchery (Ramsay Hatchery, Saint-Félix de Valois, Quebec, Canada). These eggs were then randomly divided into 13 treatment groups in two incubators, with each group consisting of 8 eggs. The standard incubation condition, temperature of 99.5°F (37.5°C), and relative humidity of 50%-

60% were maintained throughout the incubation period in a digital incubator (Digital 1502 Sportsman Incubator, USA).

### 4.3.2 Preparation of mineral solution

For this study, the water-soluble chelated minerals (zinc, iron, and manganese) were supplied by Belisle Solution, Nutrition, St-Mathias, Québec, Canada. Each mineral was provided in powder form with a concentration of 150 mg per gram. To prepare the injection solutions for each treatment group, the right amount of chelated mineral powder was measured and dissolved in distilled water to achieve the final concentrations specified in the following table 4.1. Each egg was injected with 0.2 ml of the prepared solution.

### 4.3.3 *In ovo* mineral injection

*In ovo* administration of minerals was done on the 15th day of incubation through the amnion. The amnion site was identified through candling, and a pinpoint mark was made on each egg. Then, a small hole was created at the marked location using an egg driller. 0.2 ml of respective mineral solution was injected using a 24G hypodermic needle (BD Microlance Hypodermic Needle 24G, Violet, 25mm), following the method described (Bhanja et al., 2014). Treatment group 1 served as a positive control group that received the distilled water, while the remaining 12 treatment groups were assigned to receive four different doses of each chelated Zn, Fe, and Mn, respectively.

| Treatments | Nutrient | Organic mineral concentration in diluent (µg/ml) | Volume of solution injected per egg (ml) | Total amount of organic mineral injected into each egg (µg) |
|---|---|---|---|---|
| Treatment-1 (Control) | Normal saline | - | 0.2 | - |
| Treatment-2 | Zn | 100 | 0.2 | 20 |
| Treatment-3 | Zn | 200 | 0.2 | 40 |
| Treatment-4 | Zn | 300 | 0.2 | 60 |
| Treatment-5 | Zn | 400 | 0.2 | 80 |
| Treatment-6 | Fe | 500 | 0.2 | 100 |
| Treatment-7 | Fe | 1500 | 0.2 | 300 |
| Treatment-8 | Fe | 2500 | 0.2 | 500 |
| Treatment-9 | Fe | 3500 | 0.2 | 700 |
| Treatment-10 | Mn | 50 | 0.2 | 10 |
| Treatment-11 | Mn | 100 | 0.2 | 20 |
| Treatment-12 | Mn | 150 | 0.2 | 30 |
| Treatment-13 | Mn | 200 | 0.2 | 40 |

Table 4.1: Mineral treatment solutions

### 4.3.4 Tissue sample collection

After 5 days of mineral injection (on the 20$^{th}$ day of incubation), using sterile forceps, the eggs were opened through the air sac. 8 embryos per treatment group were humanely euthanized by the cervical dislocation method, as per standard ethical guidelines. Approximately 200 mg of tissue samples were collected from the liver, spleen, and intestines of each embryo and placed into 2 ml Eppendorf tubes containing 0.5 ml of RNA*later* solution (Invitrogen™ RNAlater™ Stabilization Solution). The samples were then immediately stored at -20°C for further use.

### 4.3.5 RNA extraction

RNA extraction was done using TRIzol reagent (QIAzol Lysis Reagent). Tissue samples from the liver, spleen, and intestines were thawed at room temperature, and

approximately 100 mg of tissue was transferred into tubes containing 1 ml of TRIzol reagent and 8 to 10 silica beads (3 mm size). The samples were homogenized using a cryo-homogenizer (Precellys Evolution Touch Homogenizer, Bertine Technologies, France) at 4000 rpm for 30 seconds, followed by a 5-minute incubation at room temperature. After incubation, 0.2 ml of chloroform was added, and the tubes were shaken vigorously for 15 seconds, then left to stand for 2 to 3 minutes, and then centrifugated at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase was carefully transferred to a new tube, avoiding the interphase, and 0.5 ml of isopropanol was added to precipitate the RNA. The mixture was incubated at room temperature for 10 minutes and then centrifuged again at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the RNA pellet was washed twice with 1 ml of 75% ethanol, followed by centrifugation at 7500 rpm for 5 minutes. After removing the supernatant, the RNA pellet was briefly air-dried for approximately 2 hours. The dried RNA was resuspended in 100 µl of RNase-free water (UltraPure™ DNase/RNase-Free Distilled Water, Thermo Fisher Scientific, USA) and allowed to dissolve for 10 to 15 minutes before vertexing. The concentration and quality of the RNA were measured using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000 Spectrophotometer), with purity assessed by the 260/280 nm absorbance ratio, and the results were recorded.

### 4.3.6 Preparation of cDNA

cDNA was synthesized following the manufacturer's instructions (Advanced cDNA Synthesis Kit 100 reactions, Wisent). The cDNA reaction mixture was prepared by adding 4 µL of 5X cDNA Master Mix and 1 µL of reverse transcriptase (RTase) to each reaction well. A variable volume of mRNA specific to each sample was used (500 ng of RNA template added per reaction well). The final reaction volume was adjusted to 20 µL by adding RNase-free water (UltraPure™ DNase/RNase-Free Distilled Water, Thermo-Fisher Scientific, USA). The

thermal cycling protocol consisted of incubation at 42°C for 30 minutes, followed by 85°C for 10 minutes (T100 Thermal Cycler, Bio-Rad, Dorval, QC, Canada).

### 4.3.7 Standardization of primers for qPCR

The primers used in this research study were selected from previous publications. The specificity of these primers was verified using the NCBI BLAST program. The primers were purchased from Integrated DNA Technologies, Coralville, IA, USA, and received in lyophilized form. Upon receipt, the primers were reconstituted according to the manufacturer's instructions. A primer mix was then prepared by adding 10 µL of the forward primer and 10 µL of the reverse primer, followed by 180 µL of RNase-free water to achieve a 1:10 dilution, resulting in a final primer concentration of 10 µM.

To optimize the annealing temperature, each primer pair underwent a thermal gradient PCR at temperatures ranging from 55°C to 65°C (CFX384 Touch Real-Time PCR Detection System, Bio-Rad, Mississauga, ON, Canada). The primers, along with their annealing temperature and gene bank access ID, are detailed in Table 4.2.

| Genes | Pair | Anneal. temp. (°C) | Gene bank access ID | References |
|---|---|---|---|---|
| **Reference gene** | | | | |
| chBACTN-F | CAACACAGTGCTGTCTGGTGGTA | 57 | NM_205518.2 | (Yitbarek et al., 2012) |
| chBACTN-R | ATCGTACTCCTGCTTGCTGATCC | | | |
| **Target genes** | | | | |
| chIL1β -F | GGAGGTTTTTGAGCCCGTC | 58 | DQ393267.1 | (Dunislawska et al., 2017) |
| chIL1B-R | TCGAAGATGTCGAAGGACTG | | | |
| chIL2-F | GCTTATGGAGCATCTCTATCATCA | 58 | AF000631.1 | (Slawinska et al., 2019) |
| chIL2-R | GGTGCACTCCTGGGTCTC | | | |
| chIL10-F | CATGCTGCTGGGCCTGAA | 60 | AJ621254.1 | (Rothwell et al., 2004) |
| chIL10-R | CGTCTCCTTGATCTGCTTGATG | | | |
| chMTOR-F | TACGCGCCATTGTATTTGCT | 60 | XM_417614.8 | (Hao et al., 2021) |
| chMTOR-R | GCTAGATTTTCTCGGCCGGT | | | |
| chEGH-F | TCCCAGGCTGCGTTTTGTTACTC | 60 | NM_204359.2 | (Lei et al., 2007) |
| chEGH-R | ACGGGGGTGAGCCAGGACTG | | | |
| chIGFI-F | ACTGTGTGGTGCTGAGCTGG | 62 | NM_001004384. | (Vaccaro et al., 2022) |
| chIGFI-R | AGCGTGCAGATTTAGGTGGCTT | | | |
| chIGFII-F | ACACAAGCAAGGAGGGGATG | 60 | NM_001030342. | (Liu et al., 2016) |
| chIGFII-R | CCCGGCAGCAAAAAGTTCAA | | | |
| chIL22-F | TCAACTTCCAGCAGCCCTACAT | 59 | AJ617782.1 | (Yitbarek et al., 2018) |
| chIL22-R | TGATCTGAGAGCCTGGCCATT | | | |
| chPEPT1-F | CCCCTGAGGAGGATCACTGTT | 60 | KF366603.1 | (Mott et al., 2008) |
| chPEPT1-R | CAAAAGAGCAGCAGCAACGA | | | |
| chSGLT1-F | TGTCTCTCTGGCAAGAACATGT | 61 | AJ236903.1 | (Mott et al., 2008) |
| chSGLT1-R | GGGCAAGAGCTTCAGGTATCC | | | |
| chSOD1-F | AGGGGGTCATCCACTTCC | 57 | NM_205064.2 | (Mountzouris et al., 2020) |
| chSOD1-R | CCCATTTGTGTTGTCTCCAA | | | |
| chGPX1-F | GCTGTTCGCCTTCCTGAGAG | 61 | NM_001277853. | (Hassanpour et al., 2021) |
| chGPX1-R | GTTCCAGGAGACGTCGTTGC | | | |

Table 4.2: Primer pair sequences

### 4.3.8 Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR procedure)

qPCR reactions were carried out in a total volume of 10 µL, consisting of 5 µL of wisent Master Mix, 1 µL of cDNA template, 1 µL of primer mix, and 3 µL of RNase-free water according to the kit's protocol (Advanced qPCR MasterMix Hi-Rox, Wisent). A real-time PCR system (CFX384 Touch Real-Time PCR Detection System, Bio-Rad, ON, Canada) was used to perform the amplification in a 384-well plate, with each sample run in duplicate. A no-template control (NTC) was included for each gene to ensure specificity. The qPCR protocol involved an initial pre-denaturation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 25 seconds. The temperature curve was set according to the instrument's protocol. The beta-actin gene was used as the reference gene for normalization. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Melting curve analysis confirmed the absence of non-specific products or primer dimers, showing the accuracy of the mRNA quantification**.**

## 4.4 Statistical analysis

The experimental design followed a randomized complete block structure, with the control group injected with water and 12 treatment groups injected with respective doses of each mineral, randomly distributed into two incubators. Two-way ANOVA was performed using GraphPad Prism 8 software (Minerals and doses were two independent factors). Pairwise comparisons of treatment means were calculated using Tukey's multiple comparison test. All results are presented as mean ± SEM, and differences between means were considered statistically significant at $p < 0.05$.

## 4.5 Results

### 4.5.1 Expression pattern of genes in zinc (Zn) treatments

The expression of mTOR, eGH, IGF-1, IGF-2, SOD, and GPx was assessed in the liver of chicken embryos on ED20. Three doses of Zn (40, 60, and 80 µg Zn/egg) significantly ($p < 0.05$) upregulated mTOR expression, whereas 20 µg Zn/egg did not show any changes compared to the control (Fig.4.1A). Conversely, eGH expression was significantly ($p < 0.05$) downregulated at all Zn levels. Additionally, 20 and 60 µg Zn/egg significantly ($p < 0.05$) reduced IGF-1 expression, while IGF-2 expression remained unchanged across all Zn treatments compared to the control (Fig. 4.1B, 4.1C, 4.1D). SOD expression showed a significant ($p < 0.05$) decrease at 20 µg Zn/egg, whereas other Zn levels had no effect compared to the control group (Fig. 4.1H). Similarly, GPx expression remained almost identical to that of the control group (Fig. 4.1I). Similarly, the expression of IL-1β, IL-2, and IL-10 was analyzed in the spleen tissue of chicken embryos administered with different doses of Zn. No significant changes were observed in IL-1β and IL-10 expression. However, IL-2 expression was significantly ($p < 0.05$) lowered at 60 and 80 µg Zn/egg compared to the control group (Fig. 4.1E, 4.1F, 4.1G). Finally, the expression of IL-22, PepT1, and SGLT1 was analyzed in the intestinal samples. IL-22 expression was significantly ($p < 0.05$) higher at 20 and 40 µg Zn/egg, whereas it was downregulated at 60 and 80 µg Zn/egg compared to the control group. PepT1 expression was not affected by any Zn doses compared to the control group. Additionally, 40 µg Zn/egg significantly ($p < 0.05$) increased SGLT1 expression, while 20, 60, and 80 µg Zn/egg significantly ($p < 0.05$) downregulated it compared to the control group (Fig. 4.1J, 4.1K, 4.1L).

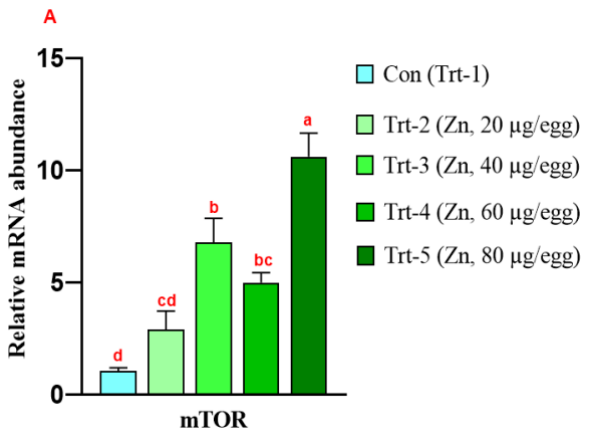
### 4.5.2 Expression pattern of genes in Iron (Fe) treatments

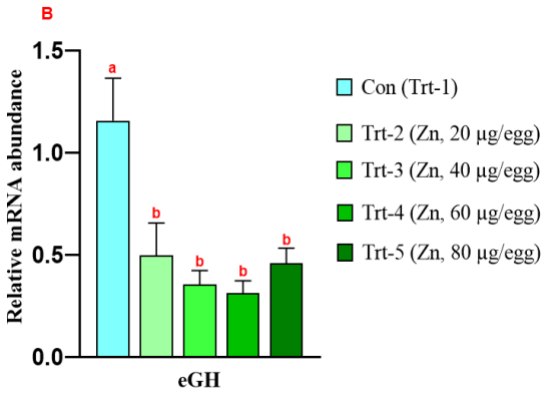
The expression of mTOR, eGH, IGF-1, IGF-2, SOD, and GPx was analyzed in the liver of chicken embryos at D20 across different levels of Fe treatments. mTOR expression was

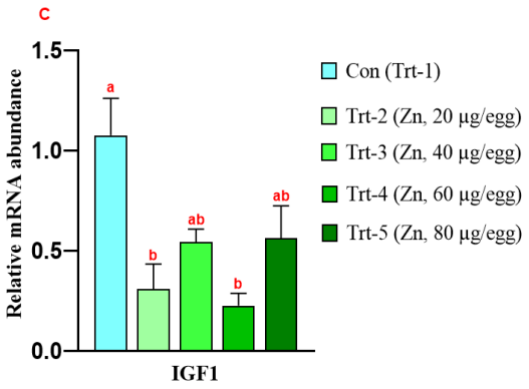
significantly ($p < 0.05$) increased at 500 μg Fe/egg, while the other Fe levels did not affect its expression ($p > 0.05$) compared to the control group. eGH expression showed a significant ($p < 0.05$) decrease at all Fe doses, whereas IGF-1 and IGF-2 expression remained unchanged across all treatments ($p > 0.05$) compared to the control group. Similarly, SOD expression was significantly ($p < 0.05$) downregulated at all Fe levels, while GPx expression did not show any significant changes compared to the control ($p > 0.05$) (Fig. 4.2A, 4.2B, 4.2C, 4.2D, 4.2H, 4.2I). Iron treatments did not significantly ($p > 0.05$) affect the expression of IL-1β and IL-10. Conversely, IL-2 expression was significantly ($p < 0.05$) lowered at all Fe doses compared to the control group in the spleen (Fig. 4.2E, 4.2F, 4.2G). The expression of IL-22 was significantly ($p < 0.05$) higher at 500 μg Fe/egg, whereas its expression was significantly ($p < 0.05$) lower at 100, 300, and 700 μg Fe/egg. There were no significant ($p > 0.05$) changes in the expression patterns of PepT1 and SGLT1 at any Fe concentration in the intestine compared to the control group (Fig. 4.2J, 4.2K, 4.2L).

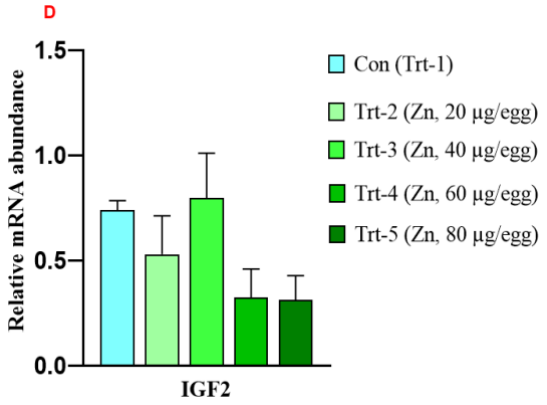
### 4.5.3 Expression pattern of genes in Manganese (Mn) treatments

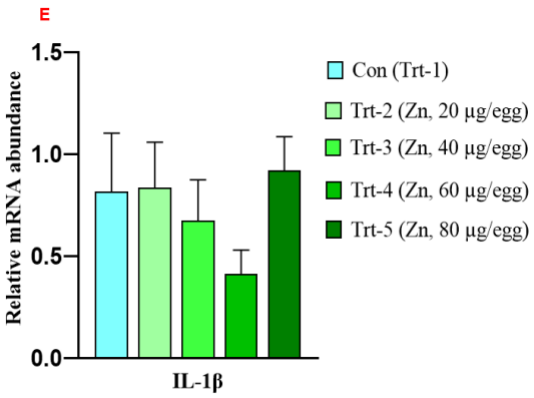
The expression of mTOR, eGH, IGF-1, IGF-2, SOD, and GPx was analyzed in the liver of chicken embryos at D20 across different levels of Mn treatments. mTOR and IGF-2 expression did not show any changes at any manganese dose ($p > 0.05$). However, eGH, SOD, and GPx were significantly ($p < 0.05$) downregulated across all Mn treatments. Additionally, 30 μg Mn/egg significantly ($p < 0.05$) reduced IGF-1 expression compared to the control group (Fig. 4.3A, 4.3B, 4.3C, 4.3D, 4.3H, 4.3I). The expression of IL-1β and IL-2 was significantly ($p < 0.05$) downregulated across all manganese treatments, while 20 μg Mn/egg significantly ($p < 0.05$) upregulated IL-10 expression in the spleen (Fig. 4.3E, 4.3F, 4.3G). The expression of IL-22 was significantly ($p < 0.05$) higher at all Mn doses. Additionally, PepT1 and SGLT1 expression was significantly ($p < 0.05$) upregulated at 20 μg Mn/egg compared to the control group (Fig. 4.3J, 4.3K, 4.3L).

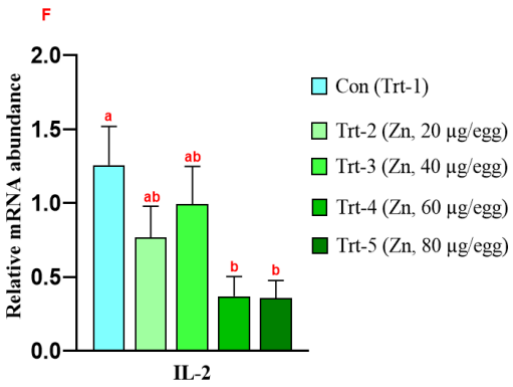

A
Relative mRNA abundance
15
10
5
0
d
cd
b
bc
a
mTOR
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)

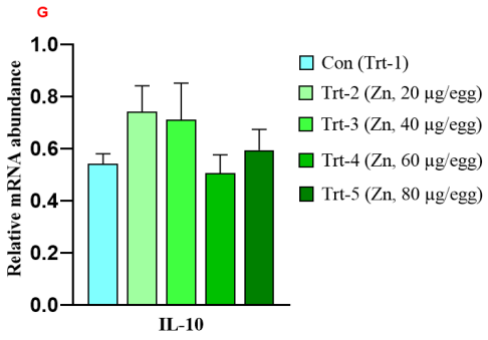

B
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
b
b
b
eGH
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)

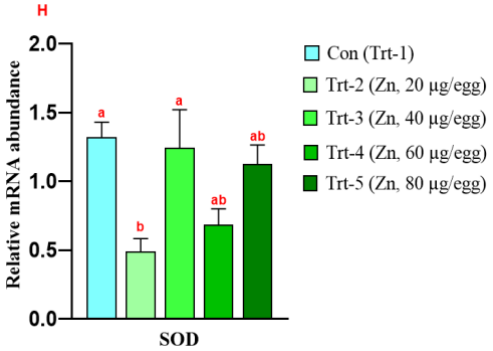

C
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
ab
b
ab
IGF1
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)


D
Relative mRNA abundance
1.5
1.0
0.5
0.0
IGF2
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)


E
Relative mRNA abundance
1.5
1.0
0.5
0.0
IL-1β
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)


F
Relative mRNA abundance
2.0
1.5
1.0
0.5
0.0
a
ab
ab
b
b
IL-2
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)


G
Relative mRNA abundance
1.0
0.8
0.6
0.4
0.2
0.0
IL-10
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)


H
Relative mRNA abundance
2.0
1.5
1.0
0.5
0.0
a
b
a
ab
ab
SOD
Con (Trt-1)
Trt-2 (Zn, 20 μg/egg)
Trt-3 (Zn, 40 μg/egg)
Trt-4 (Zn, 60 μg/egg)
Trt-5 (Zn, 80 μg/egg)

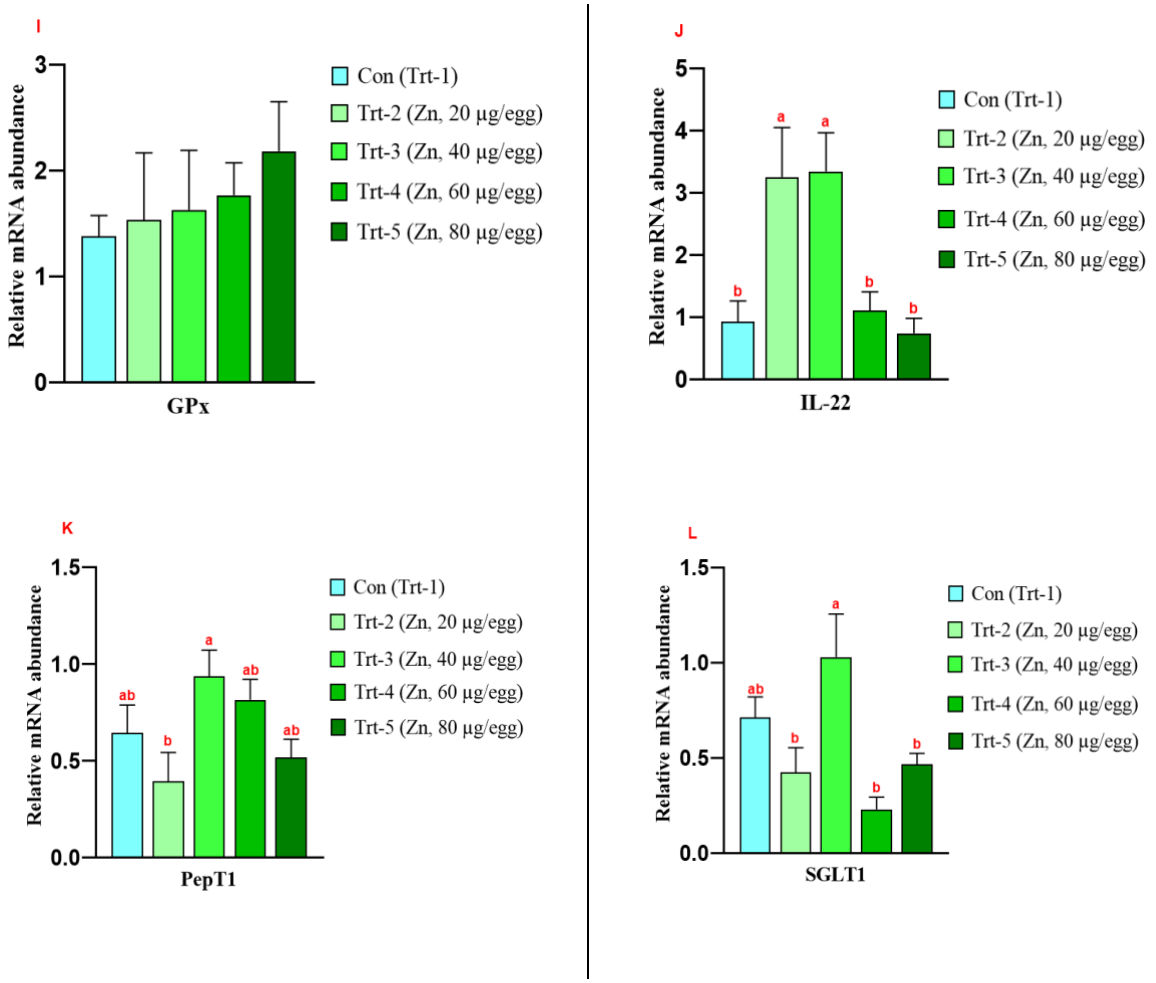
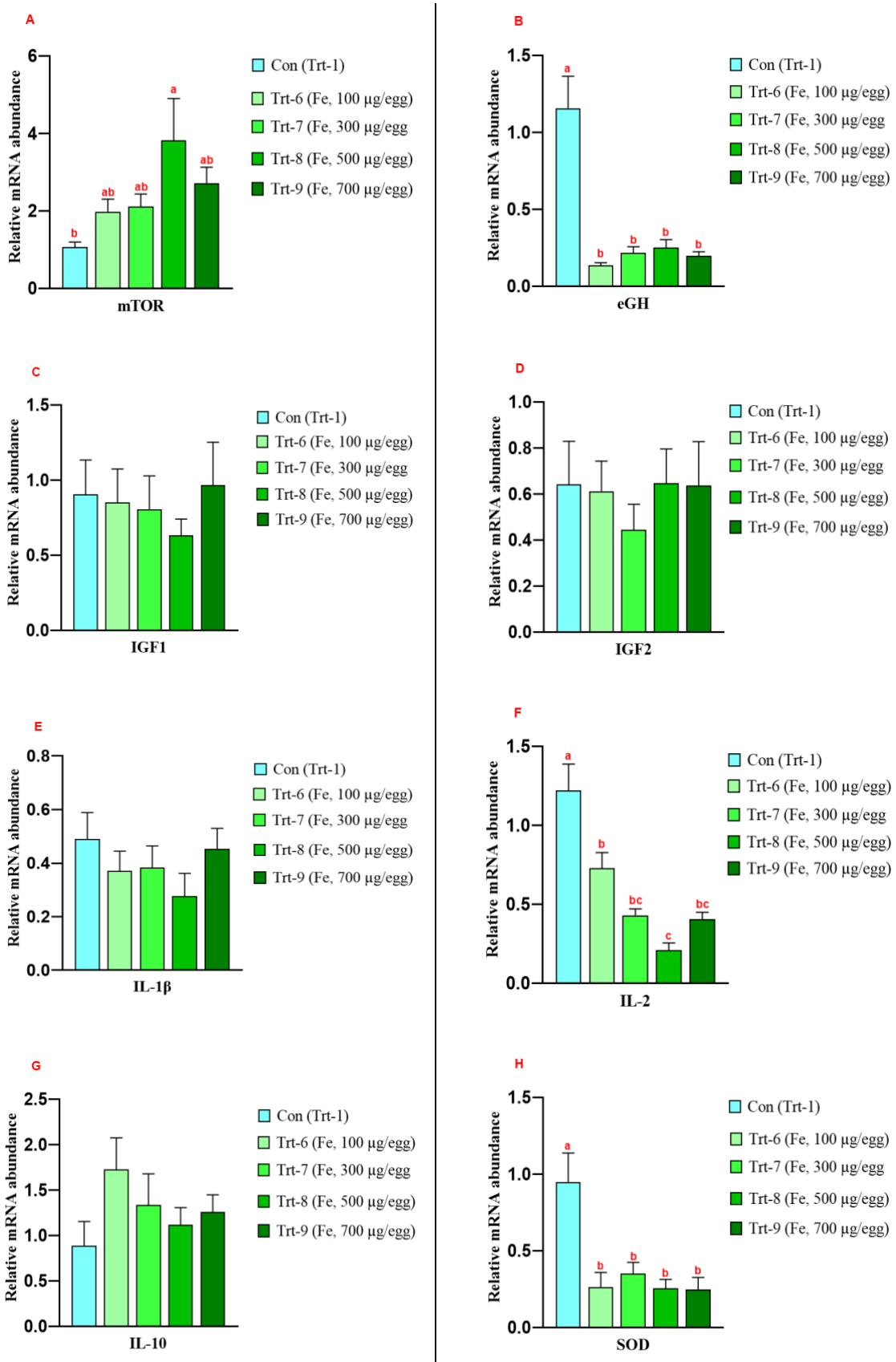



Figure 4.1 (A-L): Expression pattern of genes in Zinc treatments: Relative mRNA abundance of mTOR, eGH, IGF1, IGF2, SOD, GPx in the liver, IL-1β, IL-2, IL-10 in the spleen, and IL-22, PepT1, SGLT1 in the intestine of chicken embryos was analyzed on embryonic day 20. Different letters above bars indicate statistical differences ($P<0.05$) between zinc treatments and the control group


A
Relative mRNA abundance
6
4
2
0
b
ab
ab
a
ab
mTOR
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
B
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
b
b
b
eGH
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
C
Relative mRNA abundance
1.5
1.0
0.5
0.0
IGF1
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
D
Relative mRNA abundance
1.0
0.8
0.6
0.4
0.2
0.0
IGF2
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
E
Relative mRNA abundance
0.8
0.6
0.4
0.2
0.0
IL-1β
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
F
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
bc
c
bc
IL-2
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
G
Relative mRNA abundance
2.5
2.0
1.5
1.0
0.5
0.0
IL-10
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)
H
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
b
b
b
SOD
Con (Trt-1)
Trt-6 (Fe, 100 μg/egg)
Trt-7 (Fe, 300 μg/egg
Trt-8 (Fe, 500 μg/egg)
Trt-9 (Fe, 700 μg/egg)

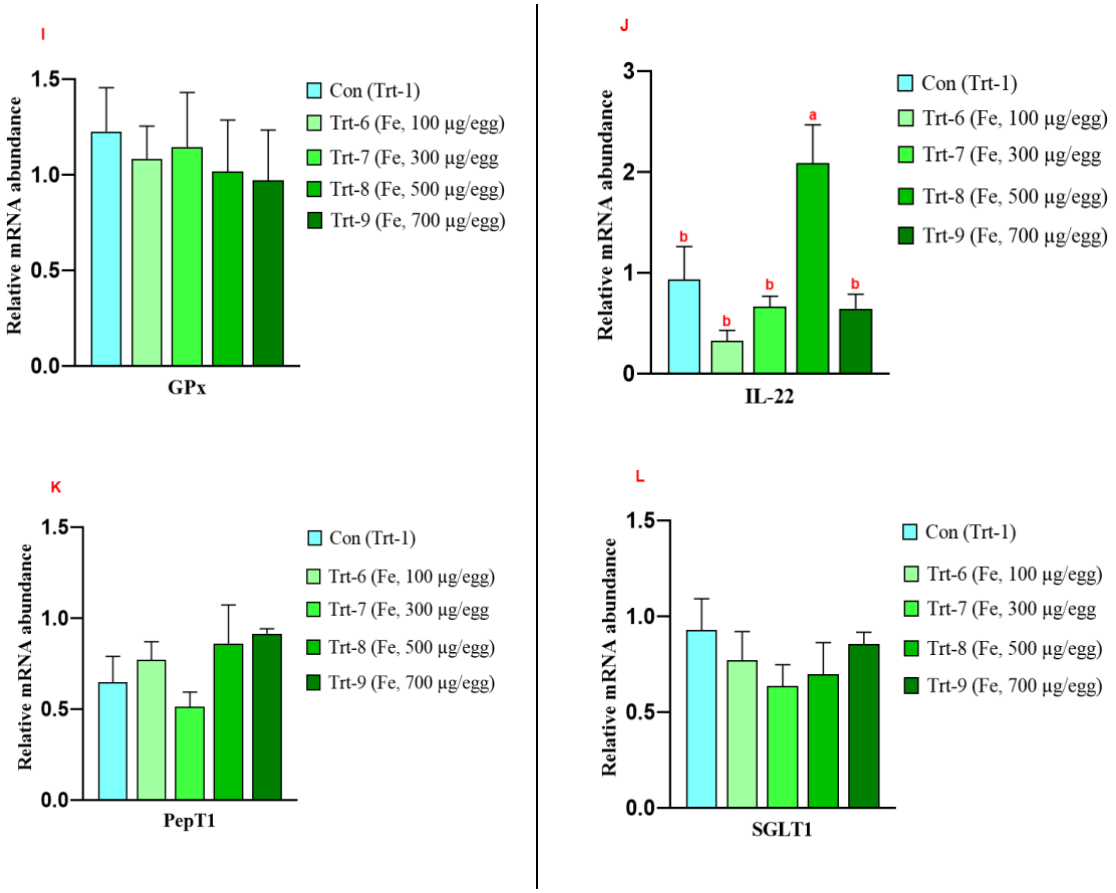
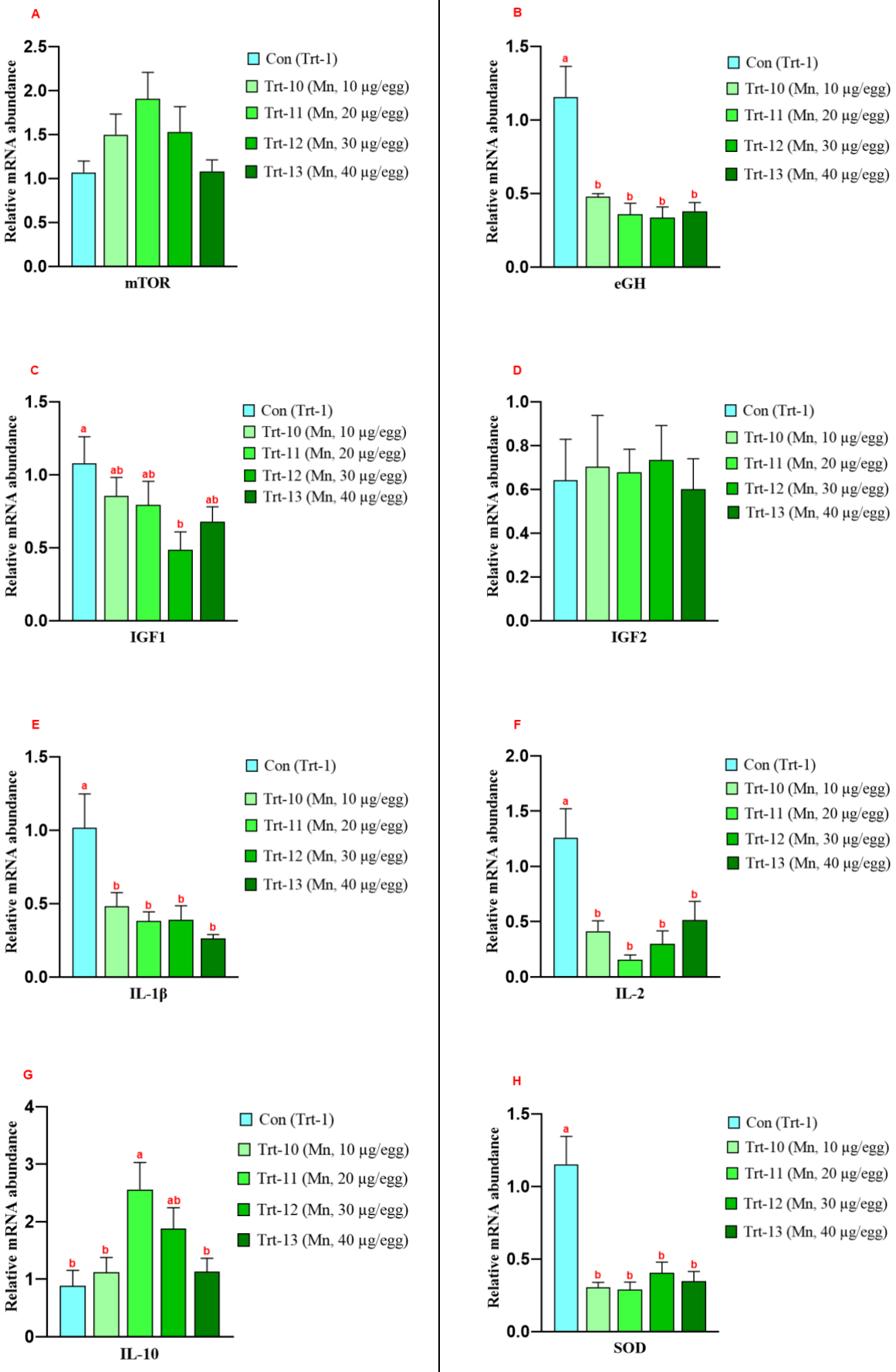



Figure 4.2 (A-L): Expression pattern of genes in Iron treatments: Relative mRNA abundance of mTOR, eGH, IGF1, IGF2, SOD, GPx in the liver, IL-1β, IL-2, IL-10 in the spleen, and IL-22, PepT1, SGLT1 in the intestine of chicken embryos were analyzed on embryonic day 20. Different letters above bars indicate statistical differences ($P<0.05$) between iron treatments and the control group.


A
Relative mRNA abundance
2.5
2.0
1.5
1.0
0.5
0.0
mTOR
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
B
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
b
b
b
eGH
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
C
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
ab
ab
b
ab
IGF1
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
D
Relative mRNA abundance
1.0
0.8
0.6
0.4
0.2
0.0
IGF2
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
E
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
b
b
b
IL-1β
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
F
Relative mRNA abundance
2.0
1.5
1.0
0.5
0.0
a
b
b
b
b
IL-2
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
G
Relative mRNA abundance
4
3
2
1
0
b
b
a
ab
b
IL-10
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)
H
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
b
b
b
b
SOD
Con (Trt-1)
Trt-10 (Mn, 10 μg/egg)
Trt-11 (Mn, 20 μg/egg)
Trt-12 (Mn, 30 μg/egg)
Trt-13 (Mn, 40 μg/egg)

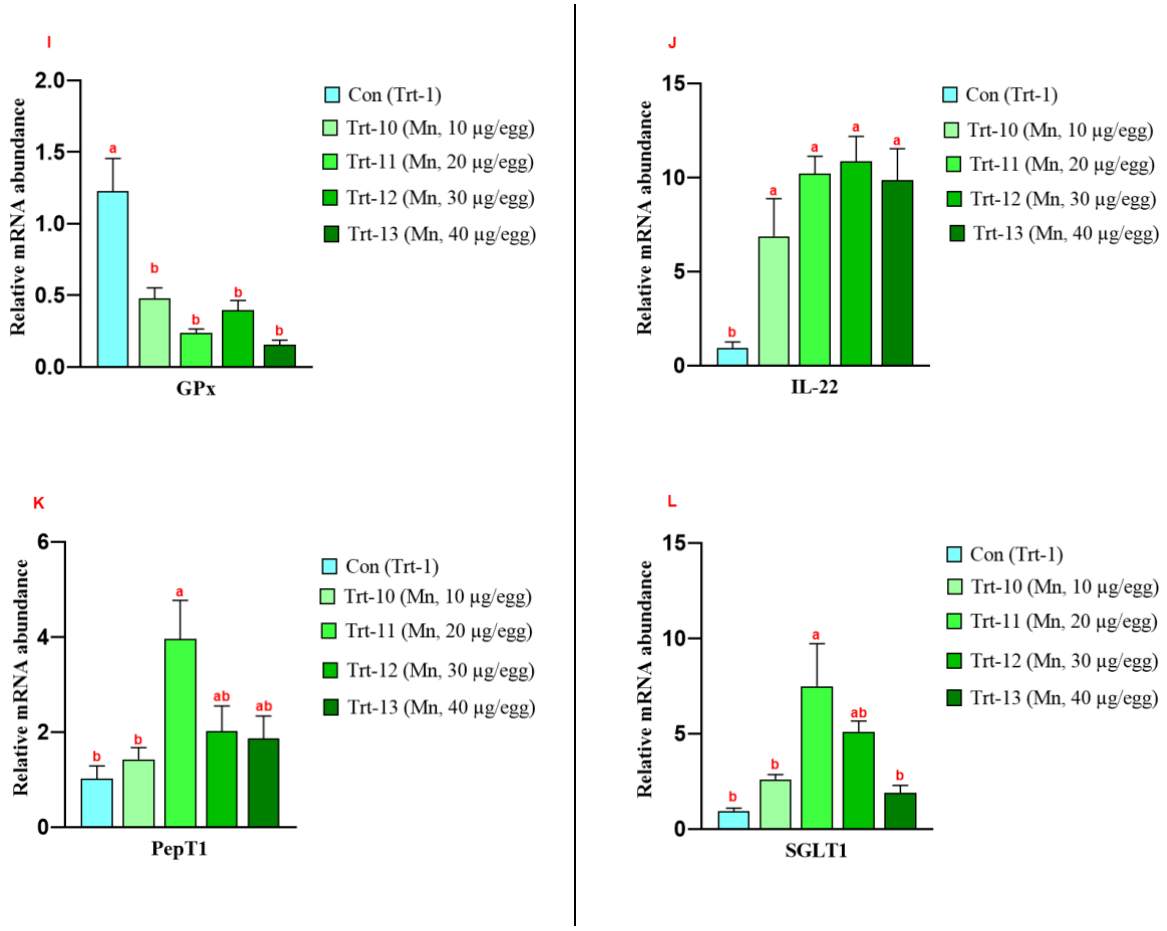



Figure 4.3 (A-L): Expression pattern of genes in Manganese treatments: Relative mRNA abundance of mTOR, eGH, IGF1, IGF2, SOD, GPx in the liver, IL-1β, IL-2, IL-10 in the spleen, and IL-22, PepT1, SGLT1 in the intestine of chicken embryos were analyzed on embryonic day 20. Different letters above bars indicate statistical differences ($P<0.05$) between manganese treatments and the control group.

## 4.6 Discussion

Autophagy is a multistep catabolic process that plays an important role in maintaining homeostasis by removing or recycling damaged cells, and organelles and degradation of proteins in all eukaryotic cells (Gómez-Virgilio et al., 2022). In chicken embryonic development, this process is essential as cells undergo differentiation to form organs that generate metabolic stress. Autophagy helps to keep the embryo healthy by recycling cellular components (Agnello et al., 2015). However, its regulation is important since it is primarily a catabolic process that restricts cellular proliferation and conserves energy rather than being utilized for cell growth and protein synthesis when over-activated. Mechanistic/mammalian target of rapamycin (mTOR) regulates autophagy, specifically when nutrients are abundant, through mTORC1 regulates cell growth, boosts protein synthesis, and reduces stress (Saxton and Sabatini, 2017). In our study, 40, 60 & 80 µg Zn/egg doses showed upregulation of mTOR expression, suggesting that Zn plays a significant role in activating the mTOR pathway. This is consistent with previous research highlighting zinc's involvement in promoting cellular growth and protein synthesis (Costa et al., 2023). mTOR promotes cellular proliferation and nutrient uptake under nutrient abundance conditions. These results agree with existing studies that indicate zinc's role in maintaining proper cellular function and supporting growth during embryonic development (Mnatsakanyan et al., 2019). Additionally, Zn plays a crucial role in carbohydrate metabolism by influencing intracellular Zn levels and increasing lactate and glucose production (Brand and Kleineke, 1996). Our finding confirmed that 40 µg Zn/egg showed the highest expression of glucose transporter (SGLT1), confirming this concentration is optimum for carbohydrate transportation and metabolism. Furthermore, Zn deficiency can impair protein metabolism by reducing ornithine transcarbamylase activity and decreasing ammonia detoxification, subsequently causing hyperammonemia (Katayama, 2020). In our study, 40 µg Zn/egg indicates higher protein uptake as confirmed by the higher expression of

protein transporter (PepT1). These finding further confirms that 40 µg Zn/egg is the optimal concentration for nutrient metabolism in chicken embryos. Interestingly, there were significant downregulation or no significant changes in the expression of endogenous growth hormone (eGH), insulin-like growth factor 1 (IGF1), and insulin-like growth factor 2 (IGF2) in all the Zn treatments. This observation aligns with the findings of (Liu et al., 2016) who demonstrated that IGF2 expression declines as the embryo progresses into the later stages of development. In this study, we collected and analyzed the liver samples at ED 20. This may reflect the fact that organogenesis is largely complete by this stage of embryonic development, and the demand for these growth factors decreases. This is again supported by the study (Liu et al., 2016) where they showed the IGF1 expression decreased as embryos reached the hatching time. Zinc also has a profound effect on immune modulation. IL-22 is an inflammatory cytokine that helps regulate inflammation and tissue repairs. It activates STAT3 signaling cascades, which prevent tissue damage and help in tissue regeneration (Kim et al., 2012). In our study, 40 and 60 µg. Zn/egg expressed the highest IL-22 gene, indicating Zn at this level plays a critical role in regulating inflammation and tissue damage. Furthermore, all Zn treatments were safe and did not cause inflammation or oxidative stress, as confirmed by our results. There was no significant embryonic stress or inflammation as the expression of pro-inflammatory cytokines IL-1β and IL-2 remained unchanged or was significantly downregulated. Additionally, the expression of oxidative stress markers such as SOD (Superoxide Dismutase) and GPx (Glutathione Peroxidase) were downregulated. These enzymes play essential roles in managing oxidative stress by neutralizing reactive oxygen species (ROS) when their levels increase (Prasad, 2014). In conclusion, 40 µg Zn/egg was identified as the optimal concentration for enhancing mTOR activation, improving glucose and protein absorption, modulating the immune response, and preventing oxidative stress.

In the iron treatments, we observed a significant upregulation of mTOR expression at 500 µg Fe/egg. As mTOR is a central regulator of anabolic processes, its activation by Fe at this concentration suggests that it plays a pivotal role in Fe homeostasis through modulation of Fe regulatory proteins 1 & 2 and changes in the cellular Fe flux (Bayeva et al., 2012). It is crucial as the mTOR signaling pathway controls cellular growth, as well as protein and lipid synthesis, thereby promoting cell survival and embryonic growth and development (Saxton and Sabatini, 2017). Iron is essential for energy metabolism; however, both Fe deficiency and excess can be harmful to cells. Therefore, Fe levels are tightly regulated by the liver hormone hepcidin, which controls Fe balance (Fillebeen et al., 2020). Our study found no effect of Fe concentration on glucose transporters, possibly because the Fe concentrations used were neither too low nor too high. Similarly, no changes were observed in the protein uptake. There were no significant changes in growth-related genes such as IGF1 and IGF2. However, the eGH expression was significantly lowered in all the iron treatments. This may again reflect the timing of embryonic development, as these hormones are more active during the earlier stages of organogenesis, and their expression naturally declines in later stages (Liu et al., 2016). In terms of immune regulation, our study found that 500 µg Fe/egg significantly increased the expression of the IL-22 cytokine, indicating that this concentration is optimal for immune regulation and tissue repair. Furthermore, all tested Fe doses were safe and did not induce stress, as confirmed by the lower expression of the pro-inflammatory cytokine IL-2. Additionally, the significant downregulation of SOD expression further supports the absence of oxidative stress at any Fe dose, as increased levels of this marker is typically observed in response to cellular damage (Jomova et al., 2024). In conclusion, 500 µg Fe/egg was identified as the optimal concentration. At this concentration, the mTOR expression was high, and Fe maintained the immune homeostasis by decreasing the proinflammatory cytokine and antioxidant activity by the downregulation of IL-2 and SOD expression, respectively.

Manganese in chicken embryonic development plays a crucial role in proper bone formation and acts as a cofactor for glutamine synthetase, pyruvate carboxylase, and superoxide dismutase. Additionally, it helps in the metabolism of carbohydrates, amino acids, lipids, and proteins (Horning et al., 2015; Mezzaroba et al., 2019; Kulshreshtha et al., 2021). Our study found that 20 µg Mn/egg significantly increased glucose transportation (SGLT1) as well as protein transportation (PepT1), indicating this dose works best in terms of nutrient absorption. Similarly, another study reported that the eggs injected between 13 and 26 µg Mn/egg had a better effect on embryonic growth, increased final weight of the chicks, better hatchability, and better blood biochemistry in the chicks (Ghane-Khoshkebijari et al., 2024). However, our study showed that administering 30 and 40 µg Mn/egg reduced the expression of both transporters, which shows that it may not have a beneficial effect at higher doses. We found no significant changes in mTOR activity at any Mn concentration, indicating that the tested levels were not sufficient to induce mTOR-regulated activity. Growth hormones such as eGH, IGF-1, and IGF-2 showed either reduced expression or no changes across all manganese treatments. This is because insulin-like growth factors (IGFs) are most active between days 15 and 18 of embryonic development. Their expression and requirement naturally start declining after day 18 and significantly decrease by day 20 of incubation (Liu et al., 2016). Our study showed similar findings, as we collected and analyzed samples for these growth hormones on day 20 of embryonic development. Manganese induces the expression of IL-22 cytokine, which shows that it has a protective role in inflammatory or infectious conditions (Seth and Dubey, 2023). In this study, all tested levels of Mn showed significantly higher expression of IL-22, indicating that Mn may help chicks combat challenging or pathological conditions. However, we did not assess its role under such conditions. This finding opens new avenues for exploring Mn as a potential mineral to mitigate pathological conditions in chickens. Manganese was also found to regulate immune responses by downregulating pro-inflammatory cytokines and

upregulating anti-inflammatory cytokines. Our study confirmed that none of the tested manganese levels induced inflammatory cytokine expression. However, 20 µg Mn/egg significantly reduced the expression of pro-inflammatory cytokines such as IL-1β and IL-2 while upregulating the anti-inflammatory cytokine IL-10, suggesting that this concentration is optimal for immune homeostasis. Furthermore, all tested manganese doses were safe and did not induce the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), as confirmed by the lower expression of antioxidant enzymes such as SOD and GPx. These enzymes are activated in response to oxidative stress; SOD converts harmful ROS/RNS, such as superoxide, hydroxides, and nitrites, into less harmful hydrogen peroxide, which is then converted into water by GPx. This process mitigates oxidative stress; however, enzyme levels typically increase under stressful conditions (Ighodaro and Akinloye, 2018). Since none of the tested Mn doses triggered oxidative stress, they can be considered safe. In conclusion, 20 µg Mn/egg was determined to be the optimal dose, as it significantly improved glucose and protein utilization, maintained immune homeostasis by downregulating pro-inflammatory cytokines (IL-1β and IL-2) and upregulating the anti-inflammatory cytokine (IL-10) and did not induce oxidative stress, as confirmed by the lower expression of antioxidant enzymes SOD and GPx.

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# 5 Chapter 5: Connecting statement

In modern commercial poultry production, supplementing minerals individually is rare. Additionally, inorganic minerals are increasingly being replaced by organic minerals due to their higher bioavailability, lower required doses, and greater effectiveness (Nollet et al., 2007). In contrast, inorganic minerals must be supplemented at higher concentrations to meet requirements since these minerals are provided in inorganic salt forms, such as oxides, sulfates, or hydroxides, which reduce their bioavailability, necessitating higher amounts. As a result, their excretion is higher in poultry, potentially causing environmental pollution (Giordano et al., 1975; Bao and Choct, 2009).

However, there is currently no available data on the optimal concentrations of these minerals when used in combination. Therefore, this research was conducted to evaluate the combined effects of zinc, iron, and manganese at different concentrations, previously determined in our earlier study. In this experiment, we are testing these minerals in combination at 0.5×, 1×, and 2× of the optimized concentrations and comparing their effects with the same minerals in inorganic form. This study is crucial for understanding whether these minerals have synergetic or antagonistic effects when supplemented together at different levels.

# 6 Chapter 6: Combined effect of the optimized chelated mineral doses of zinc, iron, and manganese and their comparison with their inorganic counterpart.

## 6.1 Abstract

Studying *in ovo* administration of chelated minerals offers a promising strategy to enhance immunity, mitigate stress, and boost nutrient absorption in embryos, potentially optimizing post-hatch health and performance in poultry. This study evaluated the combined effects of amino acid-chelated zinc, iron, and manganese on embryonic development, nutrient absorption, immune responses, and oxidative stress in broiler embryos, comparing these with their inorganic counterparts. Individual and optimal doses were determined prior to this experiment. 56 fertile eggs were randomly divided into 7 treatments, with 8 eggs per treatment. Treatments included a control group, three inorganic minerals (IM), and three organic chelated minerals. The three levels of inorganic and chelated minerals were 0.5× (20 µg Zn, 250 µg Fe, and 10 µg Mn per egg), 1× (40 µg Zn, 500 µg Fe, and 20 µg Mn per egg), and 2× (80 µg Zn, 1000 µg Fe, and 40 µg Mn per egg). Minerals were injected into the amnion on embryonic day 15. Tissue samples from the liver, spleen, and intestine were collected on embryonic day 20, and extracted mRNA were analyzed for gene expression using quantitative real-time PCR (qRT-PCR). Statistical analysis was conducted using two-way ANOVA in GraphPad Prism 8, with Tukey's post hoc test for multiple comparisons ($p < 0.05$ considered significant). Findings from the current study showed that lower doses (0.5× and 1×) of organic minerals at 0.5× and 1× levels significantly upregulated mTOR in the liver ($p < 0.05$) and enhanced PepT1 and SGLT1 expressions at 1× and 2× levels in the intestine ($p < 0.05$). The inorganic minerals at 0.5× indicated an upregulation of mTOR, while a higher dose of 2× was necessary for PepT1 expression and had no significant effect on SGLT1 at any tested levels. Organic minerals increased IL-22 expression at 1× and 2× ($p < 0.05$), supporting intestinal immunity and tissue regeneration in the intestine, while inorganic minerals had no effect. IL-1β remained

unchanged, whereas a high dose of inorganic minerals showed a pro-inflammatory trend. IL-2 was upregulated only at the highest inorganic dose (2×) in the spleen ($p < 0.05$), indicating T-cell activation. No significant embryonic mortality or oxidative stress was observed in either of the mineral treatments ($p > 0.05$). In conclusion, organic minerals at lower doses may promote embryonic development, nutrient absorption, and immune modulation without stress or inflammation.

**Keywords***: In ovo* chelated minerals, *In ovo* inorganic minerals, Embryonic development, Nutrient absorption, Immune modulation.

## 6.2 Introduction

Trace minerals are essential nutritional elements supplied in poultry diets as well as *in ovo* in micro quantities. Trace minerals, especially zinc (Zn), iron (Fe), and manganese (Mn), play crucial physiological, catalytic, and regulatory roles. Their inclusion is essential during embryonic development for proper growth, immunity, and overall chick health (Byrne and Murphy, 2022). Zinc is involved in immune regulation and antioxidant defense and serves as a cofactor for various enzymes and hormones (Kidd et al., 1996). Iron plays a crucial role in cellular oxygen transport and cell proliferation and supports embryonic growth (Milanović et al., 2008). Manganese is an important constituent of metalloenzymes and aids in nutrient metabolism, including glucose, fatty acids, and proteins (Crowley et al., 2000). Zn, Fe, and Mn exhibit additive and regulatory roles when administered together. Iron has an additive effect on zinc, as demonstrated in a study (Ramadan et al., 2010), which found that layers supplemented with both Fe & Zn had significantly higher egg mass, an improved feed conversion ratio (FCR), and better blood health compared to those receiving Fe alone. A similar observation was reported (Ullah et al., 2010) where birds supplemented with high levels of Zn and Fe showed significantly enhanced egg Zn and Fe content without negatively affecting other production-related parameters. Zinc and manganese interact to maintain homeostasis. They also interact

with the hormones found in anabolic processes, potentially enhancing protein synthesis and muscle growth (Thornton-Kurth et al., 2024). The interaction between Fe and Mn in chicks appears to be unidirectional. A study (Baker and Halpin, 1991) revealed that dietary Fe had little impact on manganese status, regardless of the Fe concentration in the diet.

In the majority of cases, these trace minerals are administered *in ovo* using inorganic compounds such as oxides, sulfates, phosphates, tetrahydrates, or chlorides due to their relative cost-effectiveness and availability in commercial settings. However, inorganic minerals are unstable, undergo rapid dissociation, and interact with other nutrients, leading to significant losses and incomplete absorption in the gastrointestinal tract of birds (Bortoluzzi et al., 2020). On the other hand, organic or chelated minerals are gaining attention in poultry nutrition because they follow a different absorption pathway and have lower interactions with other compounds. Chelated minerals are metal ions bound to organic ligands such as amino acids, peptides, or carbohydrates, forming more stable molecules with greater bioavailability. These minerals are absorbed more efficiently through organic ligands, providing better nutrient availability and, therefore, requiring smaller doses (Mellor, 1964; Kratzer and Vohra, 1986). Amino acid-chelated minerals are widely used *in ovo* and in-feed applications due to their enhanced stability, bioavailability, and digestibility (Umar Yaqoob et al., 2020).

However, the current literature lacks information on the combined mineral requirements during the incubation period that positively impact developing embryos by enhancing growth, modulating the immune response, and managing oxidative stress. In our first study, we determined the individual optimum concentrations (OC) of chelated minerals as follows: zinc (40 µg/egg), iron (500 µg/egg), and manganese (20 µg/egg). In this study, we investigated the combined effects of these minerals at 0.5×, 1×, and 2× of their optimum concentrations and compared them with the same concentration levels (0.5×, 1×, and 2× ) of inorganic minerals to evaluate their relative efficacy and impact on chicken embryos.

## 6.3 Materials and methods

### 6.3.1 Experimental design and egg incubation

This experiment was conducted in lab MS1-123, Department of Animal Science, McGill University, Macdonald Campus, Canada. A total of 56 fertile eggs from commercial broiler breeders (Cobb), with an average weight of 60 g, were purchased from a commercial hatchery (Ramsay Hatchery, Saint-Félix-de-Valois, Quebec, Canada). The eggs were then randomly assigned to seven treatment groups, with each group consisting of 8 eggs. Standard incubation conditions were maintained throughout the incubation period, with a temperature of 99.5°F (37.5°C) and a relative humidity of 50%–60%, using a digital incubator (Digital 1502 Sportsman Incubator, USA).

### 6.3.2 Preparation of mineral solution

The water-soluble chelated minerals (Zn, Fe, and Mn) were supplied by Belisle Solution, Nutrition, St-Mathias, Québec, Canada. Each chelated mineral was provided in powder form at a concentration of 150 mg per gram. Inorganic minerals (zinc oxide, iron sulfate, and manganese tetrahydrate) were purchased from Fisher Scientific, Canada, and received in powder form with 99.5% purity. These inorganic minerals were also water-soluble. To prepare the injection solutions for each treatment group, the appropriate amount of chelated mineral powder was weighed and dissolved in distilled water to achieve the final concentrations specified in Table 6.1. Each egg was injected with 0.3 mL of the prepared solution.

### 6.3.3 *In ovo* mineral injection

*In ovo* administration of minerals was performed on the 15th day of incubation through the amnion. The amnion site was identified using candling, and a pinpoint mark was made on each egg. A small hole was then created at the marked location using an egg driller. Subsequently, 0.3 mL of the respective mineral solution (0.1 ml from each stock solution) was injected using a 24G hypodermic needle (BD Microlance Hypodermic Needle 24G, Violet, 25

mm). The procedure followed the method described by (Bhanja et al., 2014). Treatment Group 1 served as the positive control and received distilled water. The remaining six treatment groups were divided into two categories: three groups received inorganic mineral doses, and three groups received chelated mineral doses. Each mineral type was administered at 0.5×, 1×, and 2× of the previously identified optimum concentration of chelated minerals. The optimum concentrations (OC) were as follows: Chelated zinc: 40 µg/egg, Chelated iron 500 µg/egg, and Chelated manganese: 20 µg/egg.

| **Treatment Dosages** | **Stock solution (µg /0.1ml)** | | | ***In ovo* dose (ml)** |
|---|---|---|---|---|
| Treatment-1 (Control) | Distilled water | | | 0.3 |
| | Inorganic Minerals | | | |
| | Zinc oxide | Iron sulfate | Manganese tetrahydrate | |
| Treatment-2 (0.5×-In) | 20 | 250 | 10 | 0.3 |
| Treatment-3 (1×-In) | 40 | 500 | 20 | 0.3 |
| Treatment-4 (2×-In) | 80 | 1000 | 40 | 0.3 |
| | Amino acid chelated (organic) minerals | | | |
| | Zinc | Iron | Manganese | |
| Treatment-5 (0.5×-Ch) | 20 | 250 | 10 | 0.3 |
| Treatment-6 (1×-Ch) | 40 | 500 | 20 | 0.3 |
| Treatment-7 (2×-Ch) | 80 | 1000 | 40 | 0.3 |

Table 6.1: Mineral treatments*:* 0.5×-In= Inorganic minerals at 0.5 times optimum concentration; 1×-In= Inorganic minerals at 1 times optimum concentration; 2×-In= Inorganic minerals at 2 times optimum concentration; 0.5×-Ch= Chelated minerals at 0.5 times optimum concentration; 1×-Ch= Chelated minerals at 1 times optimum concentration; 2×-Ch= Chelated minerals at 2 times optimum concentration.

### 6.3.4 Tissue sample collection

After 5 days of mineral injection (On the 20$^{th}$ day of incubation), using sterile forceps, the eggs were opened through the air sac. 8 embryos per treatment group were humanely euthanized by the cervical dislocation method, as per standard ethical guidelines. Approximately 200 mg of tissue samples were collected from the liver, spleen, and intestines of each embryo and placed into 2 ml Eppendorf tubes containing 0.5 ml of RNA*later* solution (Invitrogen™ RNAlater™ Stabilization Solution). The samples were then immediately stored at -20°C for further use.

### 6.3.5 RNA extraction

RNA extraction was performed using the TRIzol reagent (QIAzol Lysis Reagent). Tissue samples from the liver, spleen, and intestines were thawed at room temperature, and approximately 100 mg of tissue was transferred into tubes containing 1 mL of TRIzol reagent along with 8–10 silica beads (3 mm in size). The remaining extraction steps were carried out according to the kit's instructions. The concentration and quality of the extracted RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000). RNA purity was determined by measuring the absorbance ratio at 260/280 nm, and the results were recorded.

### 6.3.6 Preparation of cDNA

cDNA was synthesized following the manufacturer's instructions (Advanced cDNA Synthesis Kit 100 reactions, Wisent). The cDNA reaction mixture was prepared by adding 4 µL of 5X cDNA Master Mix and 1 µL of reverse transcriptase (RTase) to each reaction well. A variable volume of mRNA specific to each sample was used (500 ng of RNA template added per reaction well). The final reaction volume was adjusted to 20 µL by adding RNase-free water (UltraPure™ DNase/RNase-Free Distilled Water, Thermo-Fisher Scientific, USA). The

thermal cycling protocol consisted of incubation at 42°C for 30 minutes, followed by 85°C for 10 minutes (T100 Thermal Cycler, Bio-Rad, Dorval, QC, Canada).

### 6.3.7 Standardization of primers for qPCR

The primers used in this research study were selected from previous publications. The specificity of these primers was verified using the NCBI BLAST program. The primers were ordered from Integrated DNA Technologies, IA, USA, and received in lyophilized form. Upon receipt, the primers were reconstituted according to the manufacturer's instructions. A primer mix was then prepared by adding 10 µL of the forward primer and 10 µL of the reverse primer, followed by 180 µL of RNase-free water to achieve a 1:10 dilution, resulting in a final primer concentration of 10 µM.

To optimize the annealing temperature, a thermal gradient PCR was performed for each primer pair, with temperatures ranging from 55°C to 65°C (CFX384 Touch Real-Time PCR Detection System, Bio-Rad). The primers, along with their annealing temperature and gene bank access ID, are detailed in the following Table 6.2.

| Genes | Pair | Annealing temp. (°C) | Gene bank access ID | References |
|---|---|---|---|---|
| **Reference gene** | | | | |
| chBACTN-F | CAACACAGTGCTGTCTGGTGGTA | 57 | NM_205518.2 | (Yitbarek et al., 2012) |
| chBACTN-R | ATCGTACTCCTGCTTGCTGATCC | | | |
| **Target genes** | | | | |
| chIL1β-F | GGAGGTTTTTGAGCCCGTC | 58 | DQ393267.1 | (Dunislawska et al., 2017) |
| chIL1β -R | TCGAAGATGTCGAAGGACTG | | | |
| chIL2-F | GCTTATGGAGCATCTCTATCATCA | 58 | AF000631.1 | (Slawinska et al., 2019) |
| chIL2-R | GGTGCACTCCTGGGTCTC | | | |
| chIL10-F | CATGCTGCTGGGCCTGAA | 60 | AJ621254.1 | (Rothwell et al., 2004) |
| chIL10-R | CGTCTCCTTGATCTGCTTGATG | | | |
| chMTOR-F | TACGCGCCATTGTATTTGCT | 60 | XM_417614.8 | (Hao et al., 2021) |
| chMTOR-R | GCTAGATTTTCTCGGCCGGT | | | |
| chIL22-F | TCAACTTCCAGCAGCCCTACAT | 59 | AJ617782.1 | (Yitbarek et al., 2018) |
| chIL22-R | TGATCTGAGAGCCTGGCCATT | | | |
| chPEPT1-F | CCCCTGAGGAGGATCACTGTT | 60 | KF366603.1 | (Mott et al., 2008) |
| chPEPT1-R | CAAAAGAGCAGCAGCAACGA | | | |
| chSGLT1-F | TGTCTCTCTGGCAAGAACATGT | 61 | AJ236903.1 | (Mott et al., 2008) |
| chSGLT1-R | GGGCAAGAGCTTCAGGTATCC | | | |
| chSOD1-F | AGGGGGTCATCCACTTCC | 57 | NM_205064.2 | (Mountzouris et al., 2020) |
| chSOD1-R | CCCATTTGTGTTGTCTCCAA | | | |
| chGPX1-F | GCTGTTCGCCTTCCTGAGAG | 61 | NM_001277853. | (Hassanpour et al., 2021) |
| chGPX1-R | GTTCCAGGAGACGTCGTTGC | | | |

Table 6.2: Primer pair sequences

### 6.3.8 Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR procedure)

RT-qPCR reactions were carried out in a total volume of 10 µL, consisting of 5 µL of wisent Master Mix, 1 µL of cDNA template, 1 µL of primer mix, and 3 µL of RNase-free water according to the kit's protocol (Advanced qPCR MasterMix Hi-Rox, Wisent). A real-time PCR system (CFX384 Touch Real-Time PCR Detection System, Bio-Rad) was used to perform the amplification in a 384-well plate, with each sample run in duplicate. A no-template control (NTC) was included for each gene to ensure specificity. The qPCR protocol involved an initial pre-denaturation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 25 seconds. The temperature curve was set according to the instrument's protocol. The beta-actin gene was used as the reference gene for normalization. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Melting curve analysis confirmed the absence of non-specific products or primer dimers, showing the accuracy of the mRNA quantification.

## 6.4 Statistical analysis

The experimental design followed a randomized complete block design with seven treatments randomly distributed in an incubator. The control group received distilled water, while the remaining six treatments included three inorganic mineral treatments and three organic mineral treatments. Two-way ANOVA was performed using GraphPad Prism 8 software (minerals source and inclusion levels were the two independent factors). Pairwise comparisons of treatment means were calculated using Tukey's multiple comparison test. All results are presented as mean ± SEM, and differences between means were considered statistically significant at $p \leq 0.05$.

## 6.5 Results

### 6.5.1 Expression pattern of genes

The expression levels of mTOR, SOD, and GPx were analyzed in the liver of chicken embryos on day 20 of incubation (D20) following *in ovo* administration of inorganic (0.5×-In, 1×-In and 2×-In) and chelated minerals (0.5×-Ch, 1×-Ch and 2×-Ch). mTOR expression was significantly upregulated ($P < 0.05$) in both the 0.5×-In and 0.5×-Ch groups compared to the control, and 0.5×-Ch mTOR expression was significantly higher than 0.5×-In. Additionally, 1×-Ch showed significant upregulation compared to both 1×-In and control. However, higher levels of both mineral types (2×) did not show any differences in mTOR expression ($P > 0.05$) (Fig. 6.1A). No significant differences ($P > 0.05$) were observed in SOD and GPx expression across any of the mineral treatment groups (Fig. 6.1H & Fig. 6.1I).

The expression of PepT1 was significantly upregulated ($P < 0.05$) in 2×-In, 1×-Ch, and 2×-Ch groups compared to the control in the intestine. Similarly, 1×-Ch exhibited significantly higher PepT1 expression than the 1×-In ($P < 0.05$), whereas 0.5×-Ch showed no significant change compared to the control but had significantly lower expression than both 1×-Ch and 2×-Ch ($P < 0.05$). Additionally, 0.5×-In and 1×-In exhibited significantly lower PepT1 expression than 2×-In ($P < 0.05$) (Fig. 6.1B). The expression of SGLT1 was significantly upregulated in the 1×-Ch and 2×-Ch organic mineral groups compared to the control ($P < 0.05$). Additionally, SGLT1 expression in the 2×-Ch group was significantly higher than in the 2×-In group ($P < 0.05$). However, no significant differences were observed in SGLT1 expression across the inorganic mineral treatments compared to the control (Fig. 6.1C).

The expression of IL-22 was significantly higher ($P < 0.05$) in 1×-Ch and 2×-Ch compared to the control and all inorganic mineral treatment groups in the intestine (Fig. 6.1D). Similarly, IL-2 expression was significantly increased ($P < 0.05$) in the 2×-In group compared to the control and all organic mineral treatment groups in the spleen (Fig. 6.1F). However, no

significant differences ($P > 0.05$) were observed in IL-1β expression across any of the treatment groups (Fig. 6.1E). The expression of IL-10 showed significant increase in 1×-In, 1×-Ch, 2×-In and 2×-Ch compared to the control while there were no changes between 1×-In & 1×-Ch, as well as 2×-In & 2×-Ch. Similarly, no changes were observed in the expression between 0.5×-In & 0.5×-Ch (Fig. 6.1G).

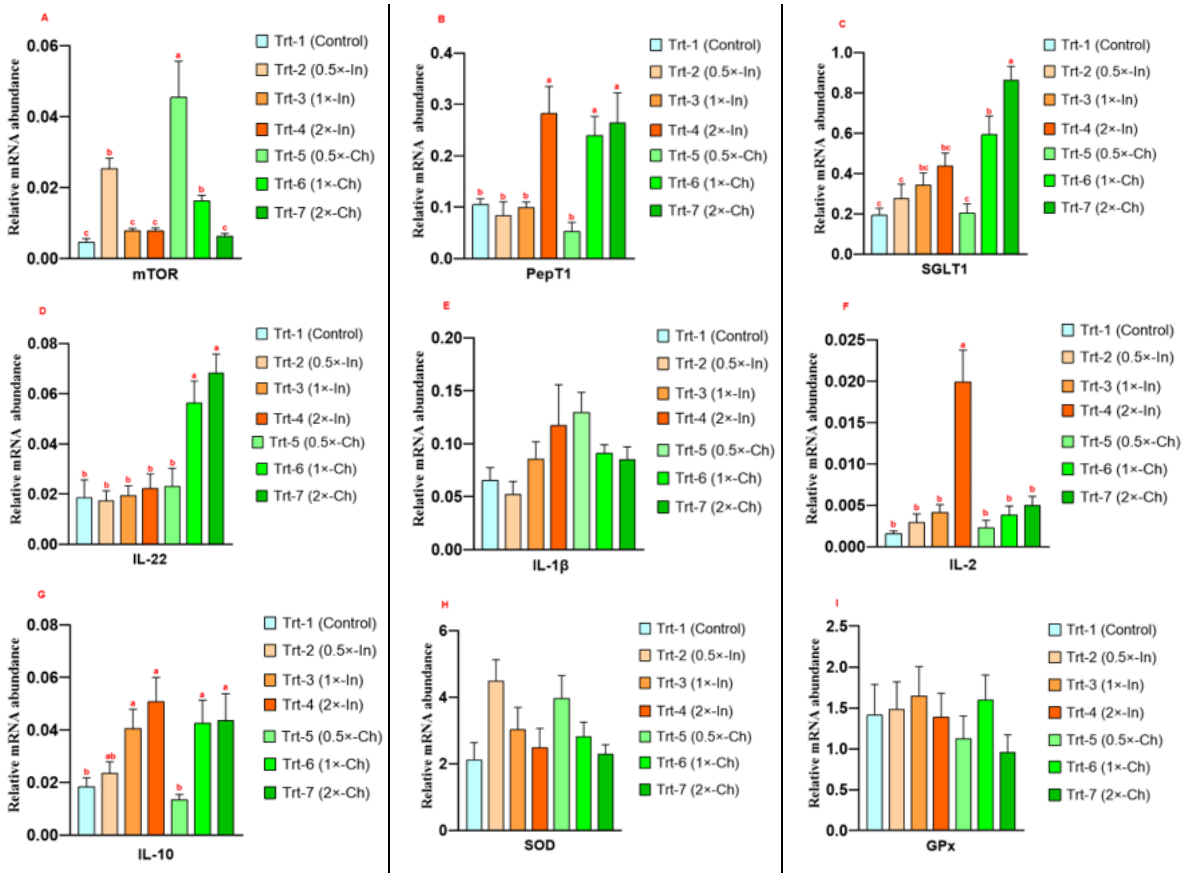


Figure 6.1(A-I): Expression pattern of genes: The relative mRNA abundance of mTOR, SOD, GPx in the liver, IL-1β, IL-2, and IL-10 in the spleen, and IL-22, PepT1, and SGLT1 in the intestines of chicken embryos were analyzed on embryonic day 20. Different letters above the bars indicate statistically significant differences ($P < 0.05$) among the inorganic, organic, and control groups. The treatments were categorized as follows: Trt-1 = Control (Distilled Water), Trt-2 = 0.5× Inorganic (In), Trt-3 = 1× Inorganic, Trt-4 = 2× Inorganic, Trt-5 = 0.5× Chelated (Ch), Trt-6 = 1× Chelated, and Trt-7 = 2× Chelated

## 6.6 Discussion

*In ovo* administration of organic minerals has gained attention in recent years due to its potential to enhance embryonic growth, reduce metabolic stress, improve hatchability, regulate immune responses, and increase the weight of day-old chicks. This study evaluated the combined effects of amino acid-chelated Zn, Fe, and Mn at different concentrations, comparing them with their inorganic counterparts. Previously, we identified the optimal individual concentrations as 40 µg/egg for Zn, 500 µg/egg for Fe, and 20 µg/egg for Mn. We tested three levels of these minerals: 0.5× (20, 250, and 10 µg/egg of zinc, iron, and manganese, respectively), 1× (40, 500, and 20 µg/egg of zinc, iron, and manganese, respectively), and 2× (80, 1000, and 40 µg/egg of zinc, iron, and manganese, respectively). Both chelated and inorganic mineral groups were assessed for relative efficacy, with treatments labeled as 0.5×-Ch, 1×-Ch, and 2×-Ch for chelated minerals and 0.5×-In, 1×-In, and 2×-In for inorganic minerals.

Our result showed that mTOR expression was significantly upregulated in 0.5×-Ch, 1×-Ch, and 0.5×-In compared to the control group. However, 0.5×-Ch and 1×-Ch increased their expression significantly compared to the 0.5×-In and 1×-In, respectively. This indicates that the supplementation of organic minerals could have a positive effect on embryonic growth. mTOR pathway is crucial for cell growth and protein metabolism. Additionally, mTOR controls cell growth by regulating autophagy (Zhao et al., 2007, 2015). Growth factors and nutrients help cells build proteins and prevent excessive protein breakdown by activating mTOR. However, when a cell lacks nutrients or growth factors, the mTOR activity decreases, which slows down cell growth by increasing protein breakdown through both autophagy and the ubiquitin-proteasome system (UPS) (Zhao et al., 2015). None of our treatment groups showed a significant downregulation of mTOR activity compared to the control. However, higher doses of both organic and inorganic minerals did not lead to an increase in mTOR expression

compared to their lower doses. This suggests that lower doses are more effective in activating mTOR, whereas higher doses, especially of inorganic minerals, do not inhibit mTOR activity but also do not enhance its expression. Egg white or albumin is the only source of protein to the developing embryos. From embryonic day 15, proteins are broken down into peptides and amino acids in the gut (Chen et al., 2002). PepT1 is a transporter present on the brush border membrane of the enterocytes and helps in the absorption of amino acids in the enterocytes (Miska et al., 2014). In our study, peptide transporter (PepT1) expression increased linearly in the intestine as the concentration of both minerals increased. This suggests that higher mineral levels enhance amino acid absorption. Both inorganic minerals at 2×-In and organic minerals at 1×-Ch and 2×-Ch showed a significant increase in PepT1 expression. However, the effect of 1×-Ch was comparable to 2×-In, indicating that the embryo requires twice the amount of inorganic minerals (2×-In) to achieve the same PepT1 expression as with 1×-Ch. Glucose is the major energy source for the developing embryos, which generates energy in the form of ATPs through aerobic oxidation. Like PepT1, the receptors for glucose transporters are present on the brush border of the enterocytes, and the uptake of glucose is mediated by the sodium-dependent glucose transporter (SGLT1) in the chicken embryos (Garriga et al., 1999; Barfull et al., 2002; Wright, 2013). Our study demonstrated that organic mineral supplementation, particularly at 1×-Ch and 2×-Ch doses, significantly upregulated the expression of glucose transporters. This suggests that these minerals play a crucial role in enhancing energy production during embryonic development. In contrast, no significant changes were observed in SGLT1 expression across inorganic mineral treatments, indicating their limited effect on glucose transport. These findings highlight the superior efficacy of organic minerals in promoting both protein and glucose absorption during the embryonic stage, which may contribute to improved metabolic efficiency and overall development. In conclusion, our study demonstrated that organic minerals, particularly at lower doses (0.5×-Ch and 1×-Ch),

significantly upregulated mTOR expression, indicating their positive role in embryonic growth and protein metabolism. Additionally, PepT1 and SGLT1 expression increased with organic mineral supplementation, enhancing amino acid and glucose absorption. In contrast, inorganic minerals had a limited effect on glucose transport and required higher doses for comparable PepT1 expression. These findings highlight the superior efficacy of organic minerals in optimizing nutrient utilization and metabolic efficiency during embryonic development.

Interleukin-1β (IL-1β) is a proinflammatory cytokine crucial in mediating inflammatory responses and initiating immune defense mechanisms (Lopez-Castejon and Brough, 2011). In this study, none of the mineral treatments resulted in a significant upregulation of IL-1β expression, indicating that the tested mineral levels did not induce inflammation or cause stress in developing embryos. However, higher doses of inorganic minerals exhibited an upward trend in IL-1β expression, suggesting a potential proinflammatory effect at elevated concentrations. In contrast, organic minerals showed a downregulating trend as the concentration increased, implying their potential role in mitigating inflammation and supporting immune homeostasis. Interleukin-2 (IL-2) is a cytokine that plays a critical role in T cell activation, proliferation, differentiation, regulation, and clearance of intracellular pathogens (Stepaniak et al., 1999). The expression of IL-2 is upregulated mainly in viral infections such as Newcastle disease virus, which clears the intracellular viral loads as shown in the study (Susta et al., 2015). In our study, the embryos were not challenged with any viral strain; therefore, no significant changes in IL-2 expression were expected. Consistently, we did not observe significant differences across the mineral treatment groups, except for 2×-In, which showed a significant upregulation of IL-2 expression. Our study suggests that high doses of inorganic minerals may induce stress in chicken embryos, leading to T-cell activation and increased interleukin-2 (IL-2) production. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that helps reduce inflammation. In this study, we found that the expression of IL-10

was significantly higher at higher doses (1× and 2×) of both organic and inorganic minerals. This indicates that these minerals may reduce stress or inflammation by modulating immune responses. Notably, IL-10 expression significantly increases when embryos are challenged with *Eimeria* infections, as indicated in studies by (Wu et al., 2016). Interleukin-22 (IL-22) is a signaling protein (cytokine) that helps tissues respond to inflammation. It is a key cytokine involved in epithelial cell proliferation, tissue repair, and mucosal immunity (Rutz et al., 2013). Our results demonstrated that higher concentrations of organic minerals (1×-Ch and 2×-Ch) significantly upregulated IL-22 expression, suggesting their potential role in supporting cell growth and tissue regeneration. In contrast, no significant changes in IL-22 expression were observed across inorganic mineral treatments, indicating that they may not be sufficient to promote cell growth and tissue regeneration. In summary, our study showed that organic minerals, particularly at higher doses, helped mitigate inflammation and supported cell growth by regulating cytokines like IL-10 and IL-22. In contrast, inorganic minerals at higher doses (2×) induced stress, leading to IL-2 upregulation. Overall, organic minerals appear more effective in modulating immune responses and promoting tissue regeneration in developing embryos.

In our study, we observed a downward trend in the expression of SOD in both organic and inorganic mineral treatments, indicating that these minerals did not induce the production of reactive oxygen species (ROS) or cause oxidative stress. SOD is an antioxidant enzyme that neutralizes harmful ROS, such as superoxide and hydroxide, converting them into less harmful hydrogen peroxide, which is further reduced to water by the enzyme glutathione peroxidase (GPx). Together, these enzymes manage oxidative stress, and their expression typically increases under stress conditions. However, our mineral treatments did not lead to increased expression of SOD or GPx, suggesting no induction of stress.

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# 7 Chapter 7: General Discussion

Late embryonic mortality and first-week chick mortality are major challenges currently faced by the poultry industry. Late embryonic mortality primarily results from the failure to transition from allantoic to pulmonary respiration, essential nutrient deficiencies, and metabolic stress (Amer, 1962). The first week of a broiler chick's life is a critical period, during which various external as well as internal factors can adversely affect their health and development. This initial phase, encompassing incubation and the first-week post-hatch, constitutes approximately 30% of the broiler's total lifespan (Yassin et al., 2009). After hatching, chicks face new challenges during their first week of life. In the current broiler production chain, chicks do not have access to feed for about 48-72 hours during transportation between the hatchery and production house, negatively impacting chick health and development. Also, they need to transition from utilizing lipid-rich yolk reserves to digesting solid complex feed (Uni et al., 2012). Chicks are vulnerable during the initial days of post-hatching, where the immunity and gastrointestinal tract development is not completed until 2 weeks after hatch. Despite the quality of the chicks and robust management efforts, a certain level of early chick mortality is unavoidable, with a typical rate ranging from 1-5% in a well-managed poultry farm. *In ovo* nutrition presents a strategic approach to enhancing embryonic growth, immunity, and nutrient absorption, ultimately supporting hatchlings in overcoming post-hatch challenges. Despite extensive testing of various bioactive compounds, including probiotics, prebiotics, amino acids, vitamins, and carbohydrates, no single product has effectively addressed these challenges. However, chelated minerals, particularly zinc, iron, and manganese, have shown promise in enhancing immunity, improving nutrient uptake, regulating stress, and promoting embryonic growth, ultimately leading to healthier day-old chicks. Zinc supports the immune system and acts as an antioxidant, helping enzymes like superoxide dismutase (SOD) protect cells from damage (Marreiro et al., 2017). Iron plays a role in oxygen

transport and cellular respiration (Abbaspour et al., 2014). Manganese is important for embryonic development and bone development and acts as a cofactor for metabolic enzymes (Li and Yang, 2018). However, these minerals are beneficial to the chicken embryos when received in proper concentrations. Otherwise, they may have negative effects, resulting in embryonic abnormality or death. Also, Zn, Fe, and Mn have synergistic as well as regulatory roles when administered together (Ramadan et al., 2010; Nishito et al., 2024). Current literature lacks sufficient information on the optimum concentration of these minerals for *in ovo* administration and their combined effects on chicken embryonic growth, nutrient absorptions, and immune modulation through oxidative stress regulations. Therefore, we conducted two research trials in which, first, we identified the optimum concentration of individual chelated minerals, and in the second trial, we tested their combined effect at 0.5×, 1×, and 2× levels of optimum concentration and compared them with their inorganic counterpart at the same levels.

In the first study, we found that Zn at 40, 60, and 80 µg/egg and iron at 500 µg/egg showed a significant increase in the mTOR expression. mTOR pathway functions as a regulator of autophagy, which is a crucial cellular process to recycle cells and conserve energy during stress or nutrient-deficient conditions. In nutrient abundance conditions, mTOR suppresses autophagy, supports protein and lipid synthesis, and increases nutrient uptake. However, it is crucial to regulate its expression, as suppressed autophagy leads to the accumulation of damaged protein, mitochondria, and cellular debris inside the cells. This condition significantly increases metabolic imbalances and causes developmental abnormalities (Deleyto-Seldas and Efeyan, 2021). Zinc at 80 µg/egg showed higher expression than other doses. This may indicate that the higher doses of Zn over-activate mTOR and hence suppress autophagy. However, further studies are needed to better understand the cellular mechanism and zinc's role in mTOR regulations. IL-22 cytokine expression was significantly increased at 40, 60 µg/egg zinc, 500 µg/egg iron, and 20, 30, 40 µg/egg manganese. At these levels, these minerals can respond to

tissue response during inflammation or stress, which helps in tissue damage and repair (Kim et al., 2012). This indicates the potential that all these minerals at these levels can induce intestinal immunity and tissue regeneration. This cytokine expression will be better understood when studied using challenge models such as necrotic or subclinical necrotic enteritis (AlAneed et al., 2024). The results of this study can be helpful in the post-hatch challenge models, especially on gut health issues. Manganese at 20 µg/egg showed strong immune regulation as indicated by the downregulation of pro-inflammatory cytokines such as IL-1β and IL-2 while upregulation of anti-inflammatory cytokine IL-10. Previous studies also noted similar findings of immune homeostasis by manganese (Wu et al., 2021). This finding suggests that manganese can help improve the inflammatory response against bacterial infections like *Salmonella* (Zhang et al., 2020).

In the second experiment, we found that chelated minerals at 1× and 2× levels significantly increased peptide and glucose absorption in the intestine. This indicates that these minerals have higher bioavailability and synergistic effects on nutrient absorption. This is crucial as per the hatchery practices; chicks have to wait approximately 48-72 hours without access to feed (Careghi et al., 2005). These results show that these minerals, in combination, help to grow chicks and prepare them for after hatching nutrient deficient time. This will benefit poultry farmers as chicks will grow fast and achieve the market weight without significant delay. This means that the administration of cheated minerals, especially Zn, Fe, and Mn, during embryonic stages profoundly helps in the economic poultry production, and chances of early mortality might also be reduced, further contributing to poultry welfare. On the other hand, inorganic minerals did not show any significant changes in glucose absorption. For amino acid absorption, 2× inorganic minerals were needed. This means double the quantity of inorganic minerals needed to gain the same benefits that 1× chelated minerals could have in terms of protein absorption. This suggests that inorganic minerals might not have a synergistic

effect and need more concentration to see the same. Additionally, this confirms that they have less bioavailability compared to chelated minerals. Furthermore, mTOR expression was significantly increased in the 0.5× and 1× concentrations of chelated minerals, indicating their roles in protein, lipid, and nucleotide synthesis. When 0.5× and 1× inorganic minerals effect compared to 0.5× and 1× chelated minerals effect, both the levels of chelated minerals significantly upregulated mTOR expression. This again proves that chelated minerals have a synergistic effect and more bioavailability than inorganic minerals. This finding was consistent with the earlier studies showing organic minerals have a better effect on growth parameters and hatchability (Oliveira et al., 2015). IL-22 expression was significantly higher in 1× and 2× levels of organic minerals. This is crucial in terms of intestinal immunity and tissue regeneration. This finding suggests that these minerals, combined, improve intestinal health and show potential benefits to improve intestinal health in enteric challenges. Furthermore, supplying chelated minerals during the embryonic phase might help chicks fight against the enteric pathogen by preventing tissue damage and repairs (Kim et al., 2012). For instance, salmonellosis in chickens, caused by salmonella bacteria that enter the intestine through the ingestion of contaminated food or water, results in inflammation or damage and disrupts the intestinal flora of birds. Therefore, intestinal or mucosal immunity plays a crucial role in reducing these infections in poultry (Shaji et al., 2023). Chelated zinc, iron, and manganese can be used as a preventive solution in the poultry industry and avoid potential economic loss associated with it. Salmonellosis is a major problem in poultry, which causes significant economic losses. They also act as a reservoir for this and can spread disease to humans (Shaji et al., 2023). Our results showed the chelated minerals as a potential solution to prevent Salmonellosis in chickens. Furthermore, chelated minerals were found to balance immune homeostasis as they reduce inflammation, which can be confirmed by reduced expression of pro-inflammatory cytokines such as IL-2, the downward trend of IL-1β, and increased

expression of anti-inflammatory cytokine-like IL-10. This means chelated minerals have the potential to maintain immune regulation whenever there is a stressful condition or inflammation in the embryos. This finding suggests that the chicks may be able to tolerate some extent of stress after hatching, such as heat stress, with higher levels of mineral concentration (1× and 2×). Heat stress is one of the biggest problems faced by poultry farmers due to significant environmental changes. This affects not only physiological changes like oxidative stress or compromised immune response but also increases mortality and reduces weight gain, water intake, and feed efficiency. Some of the researchers tried various nutritional strategies to overcome this problem, like wet or dual feeding, supplementing fat, vitamins, and minerals to the diet, and observed some improvement in the conditions (Wasti et al., 2020). However, supplementing chelated minerals may provide a better solution as they support immune homeostasis with increased nutrient absorption. Similar observations were reported (Baxter et al., 2020) in which they found that supplementing water amino acid-chelated trace minerals positively responds to heat stress by reducing the stress hormone, corticosterone (CORT), in the minerals-supplemented groups.

Overall, it is observed that the chelated minerals supplementation during embryonic stages combined is more effective than individual administration, which significantly increases nutrient absorptions, increases lipid and protein metabolism, balances immune responses, and reduces oxidative stress in the chicken embryos. This indicates the potential of chelated minerals, particularly zinc, iron, and manganese, to overcome major problems in the poultry industry. However, more studies are needed to better understand the effect of these minerals in post-hatch challenging conditions.

# 8 Chapter 8: Conclusion

Our study demonstrated that administering 40 µg of zinc (Zn), 500 µg of iron (Fe), and 20 µg of manganese (Mn) per egg was the most effective dosage for enhancing embryonic growth, regulating immune balance, and managing oxidative stress in chicken embryos. Notably, higher doses did not harm the embryos or induce stress, indicating that all tested doses were safe.

Additionally, the combined effect of chelated minerals provided more significant benefits in terms of growth and immune parameters, supporting the notion that chelated minerals offer additive or synergistic effects on chicken embryos. Furthermore, our findings showed that organic minerals (specifically, bioplex amino acid chelated minerals) exhibited relatively higher bioavailability and had a greater impact on chicken embryonic growth, immune modulation, and oxidative stress regulation compared to inorganic minerals.

Our studies highlighted the potential benefits of supplementing chelated minerals during embryonic growth. Future studies may focus on embryonic challenges or post-hatch conditions such as salmonellosis, heat stress, or subclinical necrotic enteritis models to assess the efficacy of these minerals in addressing these challenges.

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