

Effects of organic Iron source and L-Arginine on blood physiology linked to ascites in broiler chickens

by

**Anatha Erasmus
u17020001**

Submitted in partial fulfilment of the requirements for the degree
of

Magister Scientiae Agriculture

MSc (Agric) Animal Science: Animal Nutrition MSc (Agric)

in the Faculty of Natural and Agricultural Sciences

Department of Animal and Wildlife Sciences

University of Pretoria

Pretoria

2022

Supervisor: Dr T. Nkukwana

Co-supervisor: Dr. P.W. Plumstead

Table of Contents

Declaration	i
Acknowledgements	ii
Abstract	iii
List of Abbreviations	iv
List of Tables	v
List of Figures	vi
Chapter 1: General Introduction	7
Aim	8
Hypothesis	8
Chapter 2: Literature Review	9
2.1 Physiological mechanism of breathing	9
2.2 Blood physiology	9
2.2.1 Erythrocyte formation	9
2.2.2 Function of iron in erythropoiesis	10
2.2.3 Hemoglobin	11
2.2.4 Hematocrit	11
2.2.5 Method to measure blood parameters: i-Stat	12
2.3 Factors affecting blood physiology	12
2.3.1 Dietary electrolyte balance (DEB)	12
2.3.2 The Bohr effect and blood pH	13
2.3.3 Metabolic acidosis and alkalosis	14
2.4 Physiology of ascites	14
2.4.1 Effect of ascites on erythropoiesis	16
2.4.2 Effect of ascites on hematocrit	16
2.4.3 Effect of ascites on thyroid hormones	16
2.5 Ascites contributing factors	17
2.5.1 Nutrition	17
2.5.2 Environmental	17
2.5.3 Genetic aspects	19

2.5.4 Ascites related diseases and conditions	22
2.5.4.1 Sudden death syndrome	22
2.5.4.2 Cholangiohepatitis	22
2.5.4.3 Aspergillosis	22
2.5.4.4 Anaemia	23
2.5.5 Gender	23
2.5.6 Hatchery related ascites development (Early onset)	23
2.6 Gut health	24
2.6.1 Iron on gut health	24
2.6.2 Necrotic enteritis	25
2.7 Strategies to alleviate ascites	26
2.7.1 Growth manipulation	26
2.7.2 Nutrition	26
2.7.2.1 Fatty acids	27
2.7.2.2 Bicarbonate	27
2.7.2.3 Arginine	27
2.7.2.4 Iron	28
2.7.3 Environmental	29
2.7.4 Medicinal	30
2.8 Conclusion	30
Chapter 3: Material and methods	31
3.1 Introduction	31
3.1.1 Facilities and husbandry	31
3.2 Pen and house design	32
3.3 Dietary treatments and formulations:	32
3.3.1 Chemical analyses:	32
3.4 Measurements	35
3.4.1 Production performance	35
3.4.2 Blood sampling	35
3.5 Statistical analyses	36
Chapter 4: Results	37
4.1 Performance parameters	37
4.1.1 Average daily gain (g/bird)	37
4.1.2 Body weight	38

4.1.3 Feed intake	39
4.1.4 Feed conversion ratio (mortality corrected)	41
4.1.5 Ascites mortalities	42
4.2 Blood parameters	43
Chapter 5: Discussion	47
5.1 Experimental diets	47
5.2 Performance parameters	48
5.3 Blood parameters	50
Chapter 6: General Conclusion	51
Chapter 7: Critical review and recommendations	52
Chapter 8: References	53
Appendix 1	64

Declaration

I, Anatha Erasmus, declare that the dissertation, which I hereby submit for the degree MSc (Agric) Animal Science: Animal Nutrition at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date:

Acknowledgements

Firstly, I would like to thank my parents, Renatha, and André Erasmus, for your motivation and support throughout my studies and for believing in me. I cannot begin to explain the appreciation and love I have for you. I will forever be thankful for the opportunities you gave me and thank you to my family, for the inspiration and kind words. To my brother, Anlou Erasmus, thank you for understanding me when others don't, your kind words and phone calls when I needed reassurance.

Thank you, Dr. Nkukwana for all your support, guidance, and input in making this achievement possible. Mostly, thank you for your time and patience throughout this journey. Thank you for your kindness and willingness to help whenever I needed it. A great thank you to Prof. van Marle-Koster for your understanding and support.

Thank you, Dr. Plumstead for guiding me throughout this entire journey with such compassion and patience. I have learned so much from your knowledge and wisdom that no one could ever replace.

Thank you to the support and kindness of Chemuniqué, especially Terry Wiggil, for believing in me and giving me the opportunity and funding to complete my MSc. You have provided me with an incredible amount of knowledge and skills, and I will forever be grateful.

Dillan, my love, thank you for being by my side throughout this journey and your never-ending love. I would not have been here if not for you. Through the highs and lows, your encouragement and support through all the long nights, I would not trade it for the world. You believed in me at times where I could not believe in myself, and no words can explain what you have done for me. I love you so very much.

Lastly, to my Father in Heaven, you have taught me patience, endurance, and kindness throughout this journey. Thanks to You, I have accomplished one of my greatest goals for which I have worked so hard.

“Success is not final; failure is not fatal. It is the courage to continue that counts.” – Winston S. Churchill.

Abstract

Ascites or “water belly” is a multifactorial metabolic disease that occurs due to a disparity between the oxygen required and the oxygen supplied. Broilers today present with significantly higher growth rates than in the past and as a result, it increased workload of the heart to sustain the oxygen demand of the muscles and organs. Along with the high oxygen demand, a subsequent increase in the metabolic rate causes an increase in the susceptibility to ascites due their lack of pulmonary capacity. Therefore, by focusing on blood parameters to identify the causative factors for ascites syndrome one can potentially enable the prediction of ascites in juvenile broilers. The aim of this study was to investigate the effects of supplementing Arginine (dArg/dLys) and different iron sources (Fe; Availa®Fe; FeSO₄) to alleviate the occurrence of ascites in broilers. Arginine and iron respectively have been widely researched, however the interaction of the two was not a focus point. Performance parameters: body weight (BW), average daily gain (ADG), feed intake (FI), feed conversion ratio (FCR) and ascites mortality and several blood parameters such as hemoglobin (Hb), hematocrit (Hct), saturated oxygen (SO₂), partial pressure of carbon dioxide (pCO₂), partial pressure of oxygen (pO₂), pH and bicarbonate (HCO₃) were measured. This study was a 2 x 3 factorial design, with three different sources of iron, and two different levels of Arginine making up six dietary treatments. A total of 4800 Ross-308 male broilers were randomly allocated to one of the six dietary treatments resulting to 18 replicates per treatment. Previous research determined that approximately 97-136mg Fe/kg for day 1-to-21-day old broilers and 104-110mg Fe/kg for day 22–42-day-old broilers are required to support the full performance in broilers. These iron values are 20-30 mg Fe/kg above the NRC recommendation of 80 mg Fe/kg in broiler maize-soybean diets from 1 to 21 days of age. The results from this study showed that Fe source did significantly affect blood parameters such as pH, pCO₂, HCO₃ and TCO₂, however only numerical differences were found in Hb and Hct. Nevertheless, the Hb and Hct results still showed a trend towards higher levels in broilers supplemented with organic Fe. Arginine supplementation had no significant effect on blood parameters, although significant effects were seen in ADG, FI and FCR. Significant interaction effects were also observed between organic Fe and level of dArg/dLys. Results from this study showed a significant increase in broiler performance when fed organic Fe with the low level of dArg/dLys in contrast to the high level of dArg/dLys. Overall, broilers fed supplemental Fe regardless of source had a significant improvement in blood parameters.

List of Abbreviations

%	Percentage
ANOVA	Analysis of Variance
BW	Body Weight
ADG	Average Daily Gain
FCR	Feed Conversion Ratio
FI	Feed intake
Arg	Arginine
dArg/dLys	Digestible Arginine to digestible Lysine ratio
Fe	Iron
FeS	Iron sulphate
AvailaFe	Organic iron
Hct	Hematocrit
Hb	Hemoglobin
SO ₂	Saturated oxygen
CO ₂	Carbon dioxide
O ₂	Oxygen
pCO ₂	Partial pressure of carbon dioxide
pO ₂	Partial pressure of oxygen
HCO ₃	Bicarbonate
NRC	National Research Council
pH	Potential Hydrogen
H ⁺	Hydrogen ion
NO	Nitric Oxide
NC	Negative control

List of Tables

Table 1 The effects of feed restriction and cold environmental temperatures on ascites mortality, hematocrit value, heart weight relative to body weight, and RV/TV ratio in broilers (Bolukbasi <i>et al.</i> , 2004).	26
Table 2 Lighting program used in trial.....	31
Table 3 Ingredient composition (%) of the different dietary phases	33
Table 4 Ingredient composition of the broiler vitamin premix per kg of premix.....	33
Table 5 Treatment description including Fe source and level of dArg/dLys	34
Table 6 Analysed iron content in feed (mg/kg).....	34
Table 7 Analysed proximate composition for each dietary phase (as is)	34
Table 8 Interaction and main effects on cumulative average daily gain (grams) on broilers from 0-35 days of age ¹	37
Table 9 Interaction and main effects on average daily gain (grams) per week (phase) on broilers from 0-35 days of age ¹	38
Table 10 Interaction and main effects on weekly (phase) body weights in broilers from 0-35 days of age ¹	39
Table 11 Interaction and main effects on cumulative feed intake on broilers from 0-35 days of age (in grams) ¹	40
Table 12 Interaction and main effects on average feed intake on broilers from 0-35 days of age (in grams) ¹	40
Table 13 Interaction and main effects on cumulative feed conversion ratio, mortality corrected in broilers from 0-35 days of age ¹	41
Table 14 Interaction and main effects on weekly (phase) feed conversion ratio in broilers from 0-35 days of age ¹	42
Table 15 Interaction and main effect on ascites mortality in broilers from day 0-35 ¹	43
Table 16 Interaction and main effects on blood parameters in broilers on day 7 ¹	44
Table 17 Interaction and main effects on blood parameters in broilers on day 7 continued ¹	45
Table 18 Interaction and main effects on blood parameters in broilers on day 30 ¹	45
Table 19 Interaction and main effects on blood parameters in broilers on day 30 ¹	46

List of Figures

Figure 1: The process of hematopoiesis and erythropoiesis. Any stage that requires erythropoietin (EPO) is shown. HSC, hematopoietic stem cells; MPP; myeloid precursor progenitor; CLP, common lymphoid precursors; CGMP, common granulocyte or myeloid precursors; EP, common erythroid progenitor; MKP, megakaryocyte precursor; BFU-e, burst forming unit erythroid; CFU-e, colony-forming unit erythroid (Lin, 2009).....	10
Figure 2 The development of ascites syndrome in broilers (Adapted from Baghbanzadeh & Decuypere, 2008).....	21

Chapter 1: General Introduction

Broilers have been intensively selected for increased growth traits and this resulted in an indirect selection for higher rates of protein accretion, and a demand for more O₂ (Decuypere *et al.*, 2005). Broiler body weights have increased from 2kg in 64 days to 2kg in 35 days between 1979 and 2006 (Bao *et al.*, 2007). Due to genetic selection, broilers today have growth rates of 5% per year (Julian, 2000), and can achieve market weight 60% faster than the older strains of 40 years ago (Baghbanzadeh & Decuypere, 2008). Modern broilers weigh 42g at hatch and can achieve weights of 2.8kg within 42 days. This amounts to an average daily gain (ADG) of 66g (Kenyon, 2019). However, the development of major organs and systems does not develop at the same rate as the bodyweight, which leads to the impaired ability to regulate a metabolic balance during extreme conditions (Luger *et al.*, 2003). Physiologically, the fast growth and increased feed conversion increase the metabolic rate, thereby restricting blood flow to the lungs, subsequently reducing the oxygenation of tissues (Julian, 1993). Modern broilers have the cardiac capacity of broilers 40 years ago, which forces the cardiopulmonary system to reach its physiological limit much quicker.

This metabolic disorder is termed ascites or “water belly,” a multi-factorial, non-infectious disease that occurs when there is a disparity between the O₂ required and the O₂ supplied to the tissues (Julian, 2000; Peacock *et al.*, 1990). It is characterized by an increase in the workload of the cardiopulmonary system, hypoxemia, accumulation of fluid in the abdomen, right ventricular hypertrophy, a flaccid heart, and death (Aftab & Khan, 2005; Decuypere *et al.*, 2005; Baghbanzadeh & Decuypere 2008). Hypoxia occurs due to diminished partial pressure of O₂, which occurs with increasing altitude, or reduced O₂ percentage in the air capillaries of the lung. The acclimatization response to hypoxia generally involves increases in hematocrit (Hct) and in hemoglobin (Hb) concentration, a complicated adaptive erythropoietic response (Monge C & León-Velarde, 1991).

Healthy meat-type chickens have approximately 1-3 mL of fluid present in the pericardial sac at the age of 6 weeks, and any quantity above 4 mL of fluid is perceived abnormal (Julian, 1993). Ascites is the result of many mortalities and condemnations in the modern poultry industry (Aftab & Khan, 2005). Broilers that have developed ascites syndrome never recover and the result is either condemnation of the carcass or death that brings economic strain on the broiler industry (Julian, 1998). Thus, early detection is economically worthwhile, since mortalities only occur in the last half of the growth period (Scheele *et al.*, 2003). Mortalities caused by this metabolic disorder can be as high as 17.3% in extreme cases (Tekeli, 2014). It is estimated that in 40 billion broilers produced per annum (Baghbanzadeh & Decuypere, 2008), the mortality due to ascites is up to 5% for broilers and 20% for roaster birds (Balog, 2003).

Aim

The aim of this study was to investigate the effects of supplementing Arginine (dArg/dLys) and different iron sources (Fe; Availa®Fe; FeSO₄) to alleviate the occurrence of ascites in broilers. Specific objectives included comparing the effects of organic vs. inorganic iron on blood physiology and performance of broilers; and evaluating the effects of an increase in Arginine to Lysine ratio on broiler performance.

Hypothesis

H1: Organic iron will increase parameters such as hematocrit, hemoglobin, SO₂, pCO₂ and pH used for ascites identification.

H0: Organic iron will decrease parameters such as hematocrit, hemoglobin, SO₂, pCO₂ and pH used for ascites identification.

H1: An increase in Arginine to Lysine ratio will increase broiler growth performance.

H0: An increase in Arginine to Lysine ratio will decrease broiler growth performance.

Chapter 2: Literature Review

2.1 Physiological mechanism of breathing

Breathing introduces fresh air into the lungs and the heart subsequently pumps O₂-poor blood to the lungs for the removal of CO₂ (Powell, 2000). In birds, the transportation of O₂ and CO₂ in the systemic capillary is done by simple diffusion (Smith *et al.*, 2000). Oxygen diffuses to the blood inside the air sacs, oxygenating the blood as it returns to the heart by way of pulmonary circulation. Lastly, as O₂ diffuses out of the capillaries to the surrounding metabolizing tissues, CO₂ diffuses out of the cells by the same pathway in the opposite direction (Powell, 2000). Prolonged extraction of O₂ from arterial blood with the inability to raise cardiac output as well as O₂ delivery, results in the substantial desaturation of venous blood (Olkowski *et al.*, 2005).

2.2 Blood physiology

2.2.1 Erythrocyte formation

Hematopoiesis is the process of forming all cellular and cell-derived blood constituents. The stem cells for red blood cells colonize in the yolk sac and hematopoietic activity reaches maximum at around 10-15 days of embryonation. This process takes place inside the vascular sinuses of the embryo and once hatched, takes place inside the marrow of long bones. Red blood cell precursors are one of the main constituents of the circulating blood inside the embryo (Bounous & Stedman, 2010). These cellular precursors further differentiate into progeny cells that eventually develop into mature erythrocytes (Lin, 2009). These erythrocyte progenitors, called burst forming unit-erythroid cells (BFU-e), commit towards the formation of mature erythrocytes and require the enzyme erythropoietin (EPO) to complete the process (Olver *et al.*, 2010). These BFU-e cells further differentiate into colony-forming unit-erythroid cells (CFU-e), again with the help of EPO. Erythropoietin binds to the receptors found on the surface of the erythrocyte and this prevents the cell from degrading. Erythropoietin expression among these BFU-e and CFU-e cells are strictly restricted and indicates that the signalling for this enzyme is critical for erythropoiesis (Lin, 2009).

Mature peripheral erythrocyte development from pluripotent stem cells found inside bone marrow is a process regulated by erythropoietin, triiodothyronine, corticosterone, and other growth factors (Baghbanzadeh & Decuyper, 2008). Avian blood is different compared to mammalian blood in that the erythrocytes are oval and nucleated, blood glucose is two times that of mammals and plasma protein content is lower. Immature erythrocytes at the reticulocyte stage are capable of absorbing Fe whereas mature erythrocytes cannot, as they already contain Fe (Lin, 2009). There is an association between stress responses and abnormal erythropoiesis, which result in the production of many immature erythrocytes or stress reticulocytes (Olver *et al.*, 2010), which can influence the onset of ascites syndrome (Luger *et al.*, 2003).

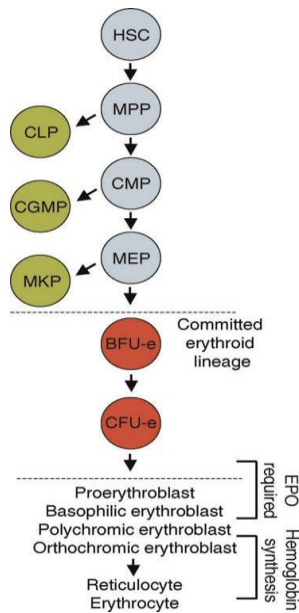


Figure 1: The process of hematopoiesis and erythropoiesis. Any stage that requires erythropoietin (EPO) is shown. HSC, hematopoietic stem cells; MPP, myeloid precursor progenitor; CLP, common lymphoid precursors; CGMP, common granulocyte or myeloid precursors; EP, common erythroid progenitor; MKP, megakaryocyte precursor; BFU-e, burst forming unit erythroid; CFU-e, colony-forming unit erythroid (Lin, 2009).

2.2.2 Function of iron in erythropoiesis

One of the most essential minerals needed for cellular metabolism, regulation of oxygenation, growth, and enzyme functioning is Fe. Erythrocytes consist of approximately 50-70% of the body's Fe concentration and is incorporated into heme, which is the reason for the significant changes in red blood cell production and function when Fe concentrations fluctuate. During heme synthesis, Fe is transported into the mitochondrial matrix to be used for iron-sulphur clusters or stored in mitochondrial ferritin (Arvedson & Sasu, 2009).

The Fe that is absorbed by the immature erythrocytes are temporarily stored, will remain as non-heme, or can be converted to heme within the erythrocyte. Iron is incorporated into heme molecules, thereafter heme is converted to Hb by complexed globin chains (Lin, 2009). Ferriredutase in the duodenum converts ferric Fe (Fe^{3+}) to ferrous Fe (Fe^{2+}) before being absorbed by the divalent metal transporter-1 (DMT1), compared to heme Fe that is absorbed through a heme carrier protein-1 (HCP1) (Theil, 2004; Kim *et al.*, 2018). Dietary Fe is stored primarily in reticuloendothelial cells of the spleen, liver, and bone marrow. The demands and rates for Fe requirements can be modulated through different pathways according to the Fe status of the body (Lin, 2009).

Transferrin is a serum protein that binds to two Fe atoms and mediates the transport of Fe to organs and tissues (Lin, 2009). The affinity for an Fe atom increases when another Fe atom binds to transferrin. In acidic conditions (pH of 6) these Fe atoms are released from the iron-transferrin complex to become free Fe in the plasma, whereas alkaline conditions allow the binding of these Fe atoms to transferrin. (Laurell, 1951).

2.2.3 Hemoglobin

Hemoglobin is a large iron-porphyrin-protein molecule that consists of four individual polypeptide chains that each have an iron-containing protein that binds to O_2 in the presence of Fe, in the ferrous (Fe^{++}) form, and can be found inside erythrocytes (Olver *et al.*, 2010). Iron in the ferric (Fe^{+++}) form is unable to bind to O_2 (Powell, 2000). Hemoglobin synthesis occurs inside the mitochondrial matrix and the synthesis of the polypeptide chains occur in the ribosomes and polyribosomes inside the cytoplasm (Olver *et al.*, 2010). The iron required by the Hb molecule is then obtained from ferritin which is the storage form of Fe (Olver *et al.*, 2010) and transferrin must transport Fe from the endosome into the matrix (Arvedson & Sasu, 2009). A single Hb molecule consists of four Fe atoms and therefore, four Fe-transferrin complex molecules should be proteolyzed to release sufficient Fe for one Hb molecule (Laurell, 1951). The turnover of Hb occurs shortly after the destruction of red blood cells. During hemolysis, the red blood cells are phagocytized, and this releases Hb into circulation in its free form. Free Hb can be oxidized to methemoglobin to be excreted or undergo further hydrolysis to release the inner Fe components. The half time for the clearance of free Hb is around 20-30 minutes (Olver *et al.*, 2010).

Normal broiler Hb levels are in the range of 7 to 13g/dL and are known to be affected by level and source of Fe concentration (Bounous & Stedman, 2010). In humans it takes approximately 14 days for supplemented iron to be incorporated into Hb however, this information is lacking in broilers. Nevertheless, studies have shown that broilers who received supplemented Fe, displayed higher values of hematological parameters such as Hb and Hct at day 21 of age (Shinde *et al.*, 2011). The most common avian response to high altitude is the production of more erythrocytes (polycythemia) and increasing the circulating Hct and Hb to compensate for the increased O_2 -carrying capacity. This increases the blood viscosity which leads to an increase in the resistance to the flow of blood and, an increase in the cardiac output (Smith *et al.*, 2000). Hemoglobin is also a contributing factor to internal viscosity (Smith *et al.*, 2000).

2.2.4 Hematocrit

Hematocrit is the suspension of erythrocytes in a homogenous plasma and is the apparent viscosity of the blood. Typical Hct values for broilers can range anything from 22-35% and they are sensitive to changes in dietary Fe levels (Bounous & Stedman, 2010). According to Poiseuille's law, there is an inverse relationship between blood flow and the radius of the vessel. Viscosity is dependent on the fraction of total volume used by erythrocytes, the velocity of blood flow, and the vessel radius (Smith *et al.*, 2000). A minor difference in vessel radius will have significant effects on blood flow and subsequently on resistance. Recent studies have shown that higher levels of Hct aids in the alleviation of hypoxemia, but ascitic broilers present an increased Hct along with an increase in the viscosity of the blood, contributing to the failure of the right ventricle to drive blood through vasoconstricted vessels

(Shlosberg *et al.*, 1998). Improved erythropoiesis and reduced plasma volumes can also lead to elevations in Hct values (Maxwell *et al.*, 1990).

Respiratory and nutritional deficiencies caused by ascites can be diagnosed by analysing the erythrocytic series. Any animal's natural response to stress is to increase plasma serum glucose, Hct and to reduce lymphocyte number and glycogen levels (Morita *et al.*, 2009). Elevations in Hct values occur because of reductions in plasma volume. This is caused by seepage of blood plasma from the blood into the pericardium, or due to enhanced erythropoiesis (Baghbanzadeh & Decuypere, 2008).

2.2.5 Method to measure blood parameters: i-Stat

The i-STAT is a portable device can be used to analyse blood at the point of care. This device can perform tests including Hct, Hb, white-blood-cell count, activated clotting time, and activated partial thromboplastin time (Martin, 2010). A single-use cartridge is used to perform these analyses. Calibration, and quality control of these cartridges also contain sensors to analyse pH, pCO₂, and pO₂. It functions by inserting a glucose strip into the i-STAT analyser and adding a single drop of whole blood onto the target area of the strip (Abbott group, 2014). This device was produced to allow on-site access to perform hematology tests with immediate availability. Electrolytes, urea, and blood gasses are performed with traditional electrode technology within the cartridge. Hematocrit determination is done based on plasma conductivity and Hb is determined from the Hct (Martin, 2010).

2.3 Factors affecting blood physiology

2.3.1 Dietary electrolyte balance (DEB)

Information on the ideal dietary electrolyte balance (DEB) that could effectively correct acid-base imbalance in broiler chickens under severe heat stress condition is limited. Electrolytes are compounds that either dissolve into cations or anions in solution and possess the ability to conduct electric currents (Mushtaq *et al.*, 2013). The minerals sodium (Na), potassium (K), and chloride (Cl) make up the dietary electrolytes of the bird and play a key role in acid-base homeostasis. Sodium has a significant effect on the stimulation of feed intake after hatching and stimulates the secretion of digestive enzymes (Mushtaq *et al.*, 2013). The NRC recommends between 0.15-0.2% Na in the diet. Potassium works together with Na and Cl to maintain growth performance and nutrient utilization. Chloride competes with bicarbonate ions inside the extracellular fluid to maintain their electrical conductivity. The recommended inclusion levels for Cl are 0.15-0.20% within the broiler diet, reducing the level as the bird ages (NRC, 1994).

A broiler's acid-base balance is the main factor regulating the blood pH and pH of bodily tissues. Any change in DEB depends on the metabolic rate, respiration, and ion exchange (Olanrewaju *et al.*, 2007). There are three systems to maintain and regulate the acid-base balance in the body namely

the buffer, respiratory and renal systems. The buffer system will firstly be activated, followed by the respiratory system to activate changes in pH and lastly, the renal system will compensate for any decrease in CO₂ (Mushtaq *et al.*, 2013). Any changes in pH will alter the binding of Ca²⁺ to proteins during respiratory alkalosis or acidosis, which ultimately causes low blood Ca²⁺ (Olanrewaju *et al.*, 2013).

2.3.2 The Bohr effect and blood pH

This phenomenon called the Bohr effect comes from the conformational change of Hb from oxyhemoglobin to deoxyhemoglobin together with allosteric interactions among H⁺/CO₂ binding sites and O₂ (Jensen, 2004). In stable conditions, hydrogen ions are passively distributed across erythrocyte membranes. The intracellular pH is dependent on the extracellular pH changes, O₂ saturation, and the organic phosphate content inside the erythrocyte. Therefore, as Hb converts between oxy- and deoxyhemoglobin in the venous transport, it takes up CO₂ and distributes O₂ in the capillaries (Jensen, 2004).

The Bohr effect has a fundamental role in the O₂ equilibrium curve when it is at a constant pH or pO₂. The protons that are bound to the O₂-linked groups found on HB lowers the affinity through electrostatic interactions. These Bohr groups take up protons during deoxygenation and release them during oxygenation. Therefore, even at constant pO₂ or pH, the O₂-binding is affected by the binding of these protons (Malte *et al.*, 2021). Any change in pH and pCO₂ influences the blood's affinity for O₂ (Hilpert *et al.*, 1963; Isaacks *et al.*, 1986). When CO₂ enters the capillaries, it decreases the blood affinity for O₂, and this results in the release of O₂. Inside the lung capillaries, the outflow of CO₂ subsequently increases the hemoglobin-oxygen binding capacity (Jensen, 2004).

The Bohr effect can be calculated as the change in the log of pO₂ at a stated O₂ saturation per unit of change in pH ($\Delta \log pO_2 / \Delta pH$) (Hilpert *et al.*, 1963). High blood pH levels improve the loading of O₂ to Hb inside the lungs due to the Bohr effect (Baghbanzadeh & Decuypere, 2008). Malte *et al.*, (2021) explained that we should not assume that capillary pH is the factor that determines the O₂ affinity through the Bohr effect, but we should view pH by the number of O₂-reliant protons that are bound to Hb during O₂ offloading. The Bohr effect along with a low RQ can increase the blood pH that flows through the capillaries. This proton preload determines the O₂ affinity (P₅₀) right at the beginning of the gas exchange (Malte & Lykkeboe, 2018).

The Henderson Hasselbach equation describes the relationship between the pCO₂, pH, and the bicarbonate concentration in the blood as $pH = pK_a + \log([HCO_3^-]/aP_{CO_2})$. An increase in ventilation will reduce the pCO₂ and increase pH and the opposite effects will take place when ventilation decreases (Powell, 2000). Increases in H⁺ concentrations will result in slight increases in vascular resistance and amplify it under hypoxic conditions (Wideman *et al.*, 2003). Squires and Julian, (2001)

confirmed that diets high in chloride aggravated pulmonary hypertension by increasing the right: total (RV: TV) ventricle ratio weight by increasing vascular resistance however, diets high in bicarbonate reduced the RV: TV weight.

2.3.3 Metabolic acidosis and alkalosis

Respiratory alkalosis occurs when CO₂ and O₂ balance in the blood are not in equilibrium. Metabolic acidosis is a condition in which an animal produces too much acid; or with an increased production or ingestion of organic acids, or excessive loss of bicarbonate (HCO₃). Reduced efficiency of the kidneys to excrete hydrogen ions (H⁺) or to re-absorb HCO₃ can also induce metabolic acidosis. During hot weather, excessive panting (hyperventilation) will cause respiratory alkalosis, which partly cancels out the negative effects of metabolic acidosis. Metabolic acidosis causes the arteries and veins to constrict, increasing the arterial pressure whereas alkalosis causes the arteries and veins to dilate, which decreases the pulmonary artery pressure (Owen *et al.*, 1994; Singh *et al.*, 2011). Fluctuations in blood pH affect the supply of O₂ to the tissues as abnormal blood pH levels affect the affinity for O₂ to bind with Hb pressure (Baghbanzadeh & Decuypere, 2008).

Fast-growing broilers with high metabolic rates are prone to metabolic acidosis when they are fully on their dietary feeds (Julian, 1993). A high blood pH will increase the affinity of Hb for O₂, which is already low in fully fed broilers due to metabolic acidosis, by supplementing bicarbonate into the broilers diet it can potentially alleviate the high O₂ requirements of fast-growing birds, on condition that the reduced pH present in the muscle that enables O₂ unloading is unaffected (Baghbanzadeh & Decuypere, 2008).

2.4 Physiology of ascites

The main factors that contribute to the decreased O₂ supply in broilers are the rapid growth and metabolism of the bird (Balog, 2003). The events that led up to ascites syndrome start with a lack of O₂ which simultaneously results in pulmonary and vascular vasoconstriction. Pulmonary hypertension results in increased blood pressure to transport higher volumes of blood through the capillaries of the lungs to maintain the O₂ demand (Julian, 2000; Wideman *et al.*, 2003). When the demand for O₂ in the myocardium increases, or if there is a reduction in the supply of O₂, then the result is a compensatory increase in coronary blood flow (Smith *et al.*, 2000).

Broiler lungs lack the capacity to sustain such a high O₂ demand necessary for rapid growth, and this leads to the impairment of energy regulation under extreme circumstances such as high altitude and low temperatures (Julian, 2000; Baghbanzadeh & Decuypere 2008). When the pressure on the right ventricle continues to rise, the pressure is diverted towards the pulmonary arteries and arterioles in the lung. This causes more pressure and force to pump blood through the heart and lungs and the right atrioventricular valve losses its effectiveness (Julian, 1998; Balog, 2003). The decrease in effectiveness

causes the valve to leak, overload in pressure in the right ventricle, and subsequently a dilation in the right ventricle. The resultant increase in sinusoid pressure in the vessels and liver induces plasma leakage into the peritoneal cavity (Julian, 1998; Squires and Julian, 2001).

An increased amount of protein present in the oedema fluid will cause clumps of fibrin which will indicate vascular damage (Julian, 1993). This fluid is not present in the air sacs, but the pressure that the fluid exerts on the air sacs causes these respiratory signs (Julian, 1998). Valvular leakage causes the kidneys to produce erythropoietin with a subsequent increase in erythrocyte production causing blood viscosity to increase dramatically (Julian, 2000). Pulmonary circulation cannot withstand the required cardiac output and pressures that are low enough to avoid pulmonary hypertension and hypoxemia (Chapman & Wideman, 2001).

The general morphology of the modern broiler contributes largely to the susceptibility of ascites. Their small stature, breast mass, pressure from the abdomen on their air sacs, and small lung capacity all influence this incidence (Julian, 1998; Balog, 2003). Birds have lungs that closely adapt to the shape of their body cavity, and they lack the function of expansion such as in mammalian lungs (Julian, 2000; Balog, 2003). According to Frank-Starling's Law of the heart, the ability of the heart to change the force of contraction and subsequently the stroke volume depends on the venous return. Once the venous return has increased, the filling of the ventricle along its passive pressure curve increases resulting in an increased end-diastolic volume (Chau-Berlinck & Monteiro, 2017). High pulmonary arterial pressures induce reactive oxygen species (ROS) production which impairs endothelial nitric oxide production. Nitric oxide (NO) is responsible for endothelial vasodilation and if the bioavailability decreases, it increases endothelial damage and high arterial pressures (Fathi, 2016).

As reported by Balog, (2005) and Julian, (2005) the occurrence of ascites might arise from multiple physiological changes that result in higher production or a decline in the elimination of peritoneal lymph. Any obstruction in the removal of lymph, plasma pressure, increased vascular permeability secondary to failure of the right ventricle, or liver damage can all result in ascites (Julian, 1993). There is a reduction of plasma proteins such as albumin in ascites-sensitive broilers due to the obstruction of lymph return caused by valvular insufficiency. This is the consequence of the loss of protein lymph coming from the liver or because of ventricular hypertrophy (Balog, 2003).

The clinical signs displayed in birds proven to have ascites and they were depression, respiratory distress, mild diarrhoea, reduced growth rate, distended abdomen (Das & Deka, 2019), cyanosis of the skin of the comb and body (Julian, 1993), liver cirrhosis and lung oedema although Scheele *et al*, (2003) referred to it as the high-altitude disease. During necropsy, they also found a swollen liver, yellow fluid in the abdominal cavity, and a mild form of pericarditis and hypertrophy. The abdominal fluid of ascitic broilers was analysed and Luger *et al*, (2003) found that the composition

of that fluid resembled that of plasma, indicating that the selective permeability of blood vessels was reduced.

2.4.1 Effect of ascites on erythropoiesis

A bird's capillary size is relatively small and incapable of expanding to allow an increase in blood flow (Julian, 1993). The reduction in erythrocyte deformability then increases the vascular resistance further and leads to an increase in blood afterload causing excess pressure on the right ventricle. Ventricular hypertrophy causes alterations, thickening, and leaking of the atrioventricular valves deeming them less efficient (Baghbanzadeh & Decuypere 2008). The increase in resistance to blood flow is especially important at high altitudes where hypoxemia is involved towards the contribution of pulmonary hypertension, compared to an increase in blood flow being the factor contributing to this condition at low altitudes (Julian, 1993). The partial saturation of Hb is also one of the major causes of pulmonary hypertension at high altitudes (Julian, 1993).

2.4.2 Effect of ascites on hematocrit

The Hct of ascitic broilers increased by up to 80% due to the enhanced erythropoiesis, and not due to the reduction in plasma volume. This increase in the Hct of ascitic broilers is due to an increased O₂ demand of the tissues because of a higher metabolic rate and a reduced O₂ saturation. Therefore, this increase in the number of erythrocytes is to compensate for the lack of O₂ present in the tissues (Luger *et al.*, 2003). A decrease in O₂ saturation causes an increase in blood viscosity which in return enhances cardiac workload to supply enough O₂ to meet the body's requirement (Tekeli, 2014). Balog, (2003) reported that an index ratio of 0.27 of RV: TV indicates ascites, and this is in accordance with the results of Tekeli, (2014) that found an increase of the right ventricle in ascitic broilers from 1.60mm to 4.92mm.

2.4.3 Effect of ascites on thyroid hormones

Thyroid hormone T₃ (triiodothyronine) is deionized to T₄ (thyroxine) which has considerable involvement in erythropoiesis regulation and differentiation as well as regulating the metabolic rate (Luger *et al.*, 2002; Malan *et al.*, 2003). Lower metabolic heat production is associated with reduced activity of the thyroid hormones (Decuypere *et al.*, 2000). Ascitic birds cannot produce sufficient amounts of T₄ as the conversion process is impaired due to hypoxia. The low Hb concentration in ascitic broilers and high Hct indicate the importance of T₃ in the Hb accumulation stage of red blood cell differentiation (Luger *et al.*, 2002).

2.5 Ascites contributing factors

2.5.1 Nutrition

Any nutritional factors such as high feed intakes, high nutrient-dense diets, and feed form can influence the incidence of ascites (Singh *et al.*, 2011). High protein and nutrient-dense diets contribute to ascites by increasing the bird's metabolic rate. Increased feed intake can also trigger ascites as it increased the O₂ requirement (Julian, 2000). Diets that are high in protein increase the deamination of excess nitrogen into uric acid which requires a lot of energy (Julian, 2000). A reduction in the energy level of the feed will ultimately mimic a high-energy diet since the bird will consume more feed to meet its requirements, and this will cause a marked increase in the O₂ demand (Julian, 2000). Broilers that receive pelleted diets have a much higher incidence of ascites compared to mash diets since birds consume more feed in a pelleted form (Decuyper *et al.*, 2000). Diets containing low chloride or high bicarbonate levels cause a decrease in the occurrence of pulmonary hypertension. On the contrary, feeding diets high in chlorine increases the prevalence of ascites (Bahgbanzadeh & Decuyper, 2008).

2.5.2 Environmental

Several factors are contributing to ascites and can be grouped in factors affecting the O₂ supply and factors affecting the O₂ requirement. High altitude, poor ventilation, or infectious diseases affect the O₂ supply whereas low environmental temperatures, nutrition, lighting programs, and stress affect the O₂ requirement of the bird because these factors all increase the metabolic rate (Decuyper *et al.*, 2000). The ability of a broiler to oxygenate its blood is highly dependent on the affinity of O₂ to Hb, the thickness of the blood-gas barrier as well as the amount of O₂ present in the air (Balog, 2003).

High altitudes cause a decrease in pO₂ and when birds are exposed to such low O₂ levels, pulmonary vasoconstriction occurs, and vascular resistance increases (Julian, 1993; Wideman, 1997). At high altitudes, arterial blood tends to be alkalotic and hypocapnic. CO₂ causes vasodilation due to the linking of increased aerobic metabolism inside the myocardium, with an increased rate of O₂ supply which is accomplished by the decrease in coronary resistance and increase in coronary blood flow (Smith *et al.*, 2000). At these high altitudes, the reduced O₂ supply induces erythrocyte proliferation, and therefore the Hct value and blood viscosity increase (Maxwell *et al.*, 1998). Hassanzadeh *et al.*, (2004) and Yilmaz-Dikmen *et al.*, (2014) found that when chicks were hatched at high altitudes followed by low altitudes, they displayed significantly higher levels of plasma T₄ concentrations than high altitude grown birds.

Today's modern broilers develop hypoxemia and cyanosis as a result of rapid growth. This is accompanied by low arterial pO₂ and low oxygen-haemoglobin saturation levels (Peacock *et al.*, 1989; Olkowski *et al.*, 2005; Druyan & Moschandreu, 2012). Hypoxemia occurs when there is a reduction in the pO₂ such as with cold temperatures, high altitudes, and decreased O₂ supply to the lungs. Hypoxia

is instantly recognized by the sensors found in the pulmonary arterioles which causes them to constrict to redirect blood to the oxygenated regions of the lung, and this can be attributed to the inability of the pulmonary vasculature to accommodate the cardiac output (Wideman, 2000; Khajali & Wideman, 2016). This results in little O_2 available to bind to haemoglobin (Druyan & Moschandreou, 2012). Peacock *et al.*, (1989) noted that pulmonary blood flow falls, and cardiac output decreases in hypoxemic birds. The rapid fall in blood flow prevents the red blood cells to remain at the gas exchange surface which in turn limits haemoglobin to saturate with O_2 (Wideman, 2000). This suggests that in fast-growing broilers a lack in performance of the heart is a factor that limits blood oxygenation. During rapid growth, there is an increased requirement for O_2 , if there is insufficient O_2 transport to tissues it may lead to hypoxemia (Olkowski *et al.*, 2005). The susceptibility of broilers to heart failure and hypoxemia related to fast growth decreases considerably when growth is controlled by dietary restriction (Reeves *et al.*, 1991; Olkowski *et al.*, 2005).

Birds that developed congestive heart failure are from populations of fast-growing broilers and had severely dilated cardiomyopathy (Olkowski *et al.*, 2005). Hypoxemia is associated with an increased O_2 demand due to a higher metabolic rate and is not likely constrained by ventilation or diffusion problems. Rapid growing birds are unable to increase their blood flow in the presence of hypoxemia, hence increased ventilation does not improve the arterial blood pO_2 (Peacock *et al.*, 1989; Olkowski *et al.*, 2005). Due to the lower CO_2 and arterial blood pO_2 , the body compensates by increasing the number of circulating erythrocytes and the ability to deliver more O_2 bound to haemoglobin. In an effort to satisfy their O_2 demand, they compensated for insufficient blood flow by extracting more O_2 from the arterial blood (Luger *et al.*, 2003). Birds with low SO_2 had remarkably high Hct values, which is known to aggravate pulmonary hypertension (Peacock *et al.*, 1989).

In ascitic broilers, it has been concluded that there is a persistent increase in corticosterone concentration due to the continual stress response which induces erythropoiesis however, the production of red blood cells in the presence of hypoxia decreases their deformability (Julian, 2000). These elevated levels of corticosterone are responsible for the impairment of red blood cell differentiation due to a lowered concentration of thyroid hormones which increased the immature red blood cell production without increasing the Hb concentration, therefore the development of hypoxemia was aggravated. With analyses of blood smears, it was shown that ascitic broilers have 23.5% immature red blood cells compared to 2.3 and 7.2% in healthy broilers (Luger *et al.*, 2003). If hypoxemia were to be the main cause of ascites, then it can be expected that arterial desaturation would be the first step to the onset of this syndrome and be predictive of it (Peacock *et al.*, 1990).

Ascites can be experimentally induced by subjecting the bird to temperatures colder than the comfort zone and simulating high altitude (Beker *et al.*, 2003). Cold stress increases the metabolic O_2 requirements along with a marked increase in cardiac output resulting in pulmonary hypertension (Stolz

et al., 1992; Julian, 1993). Cold stressing broilers cause a further imbalance between the O₂ demand and supply, and this is the reason for the further strain on the cardiovascular system (Luger *et al.*, 2001). Birds that are exposed to these cold environmental temperatures have significantly higher plasma corticosterone concentrations at three weeks of age and most of the broilers that experienced this died at four and five weeks of age (Luger *et al.*, 2003). Cold stressed birds that developed ascites cannot produce sufficient amounts of T₄ concentrations to compensate for the decrease caused by the cold. This provides an explanation for the lower T₃ in ascitic broilers as T₄ gets deionized to T₃ (Luger *et al.*, 2001)

Modern-day broilers are reared on near-continuous lighting programs to increase feed intake and growth rates (Singh *et al.*, 2011). Recent studies have shown that different lighting programs have significant effects on skeletal abnormalities as well as growth performance. Olanrewaju *et al.*, (2013) concluded that short photoperiods decreased the bird's respiration rate, pCO₂ increased and H⁺ ions started accumulating resulting in a pH reduction of the blood. This suggests that the lungs failed to eradicate all the CO₂ as quickly as it was being produced, leading to a state of respiratory acidosis. Birds that are reared in short photoperiods experience decreases in blood osmolality and can therefore be attributed to a reduction in blood electrolytes in comparison to those reared in long photoperiods (Olanrewaju *et al.*, 2013). The mechanism behind the manipulation of lighting schedules is the change of the growth curve during the starter period with subsequent compensatory growth in the finishing period (Decuyper *et al.*, 2000).

Poor air quality and ventilation result in lower environmental O₂ and higher toxic fumes, this affects the respiratory system and encourages ascites development (Wideman, 1998). This is due to the failure of the pulmonary system to deal with the significant increases in cardiac output because of hypoxia-induced increases in the metabolic rate (Bottje *et al.*, 1998). Ensuring proper ventilation and improved air quality to reduce the amount of CO₂, dust, and NH₃ can reduce the incidence of ascites. Air contaminated with pollutants causes oxidative stress on antioxidants found in the lining of the lung (Bottje *et al.*, 1998). Any factor that causes a reduction in respiratory efficiencies such as dust, diseases, and poor ventilation can predispose broilers to ascites (Singh *et al.*, 2011). Broilers exposed to poor air quality developed greater amounts of osseous nodules in their lungs and ascitic birds had an even higher amount of these nodules (Balog, 2003).

2.5.3 Genetic aspects

Broilers have been genetically selected for an increased growth rate without the occurrence of physiological or metabolic disorders (Pakdel *et al.*, 2004). The lungs of broilers that are subjected to fast growth are incapable of growing at the same rate, which increases the susceptibility to hypoxia and ascites (Afolayan *et al.*, 2016). Thyroid hormones are important for the regulation of the metabolic rate in the broiler. Early genetic selection for a better feed conversion ratio (FCR) can potentially cause

hypothyroidism because the thyroid hormone production is decreased or the peripheral metabolism of T_4 changed (Decuypere *et al.*, 2000).

Birds with slower growth rates or higher FCR had fewer cases of ascites compared to broilers with joint fast growth and low FCR (Baghbanzadeh & Decuypere, 2008). Selecting for lower FCR could potentially indirectly select for smaller lung weights or impaired breathing and therefore lead to elevated CO_2 levels in the blood (Scheele *et al.*, 2003). They conducted a study between ascites sensitive and ascites resistant lines and concluded that the progeny of the high risk displayed higher pCO_2 levels in venous blood at 6 weeks compared to the ascites resistant line as their pCO_2 blood levels were genetically predetermined.

Several studies have shown that slow-growing broilers have a higher O_2 saturation compared to fast-growing broilers which suggest that broilers do not saturate their Hb fully at low altitudes (Julian & Mirsalimi, 1992). Problems related to fast-growing broilers can be eliminated by slowing down the growth rate of the bird, but it does not necessarily reduce the incidence of diseases or ascites (Julian, 1998). Fast-growing broilers require higher amounts of specific nutrients to maintain their growth and performance because they are more prone to skeletal deformities (Havenstein *et al.*, 1994). These faster growth rates resulted in smaller thoracic cavity volumes compared to slower-growing birds and this gave rise to the increase in pCO_2 in the venous blood (Hassanzadeh *et al.*, 2014).

Slow-growing birds have significantly higher lung weights compared to faster-growing ascites susceptible broilers (Malan *et al.*, 2003). Unpublished studies by Olkowski concluded that fast-growing broilers exhibited higher shunt ratios suggesting that a substantial portion of the cardiac output bypassed the blood gas exchange in the lung. Some hypoxemic birds have an increased shunting of blood, and this indicates that a portion of CO_2 received from the right side of the heart bypassed the capillaries in the lungs (Olkowski *et al.*, 2005). These shunts are a result of congenital heart diseases such as intraventricular septal defects (Julian, 1993). In fast-growing broilers, the incidence of nodular structures inside the lung and the frequency thereof was much higher compared to normal birds. These nodules inhabit the parabronchial air spaces explaining why blood-gas exchange efficiency was compromised (Olkowski *et al.*, 2005).

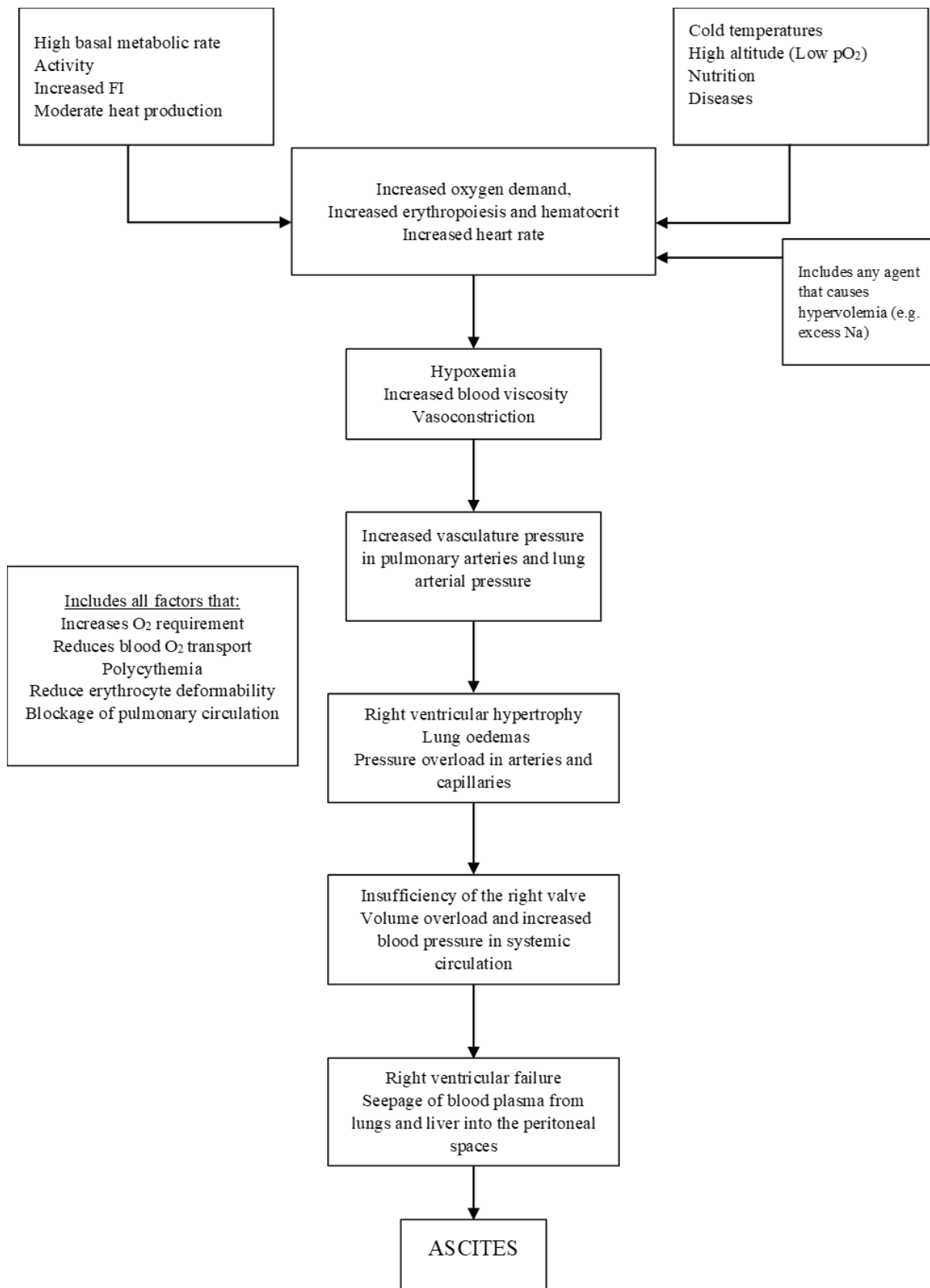


Figure 2 The development of ascites syndrome in broilers (Adapted from Baghbanzadeh & Decuyper, 2008).

2.5.4 Ascites related diseases and conditions

2.5.4.1 Sudden death syndrome

Sudden death syndrome (SDS) is a non-infectious metabolic disorder that results in the death of healthy birds without any distinct cause (Afloyan *et al.*, 2016). The pathogenesis of sudden death syndrome is not understood, but evidence has shown that it correlates to cardiac-related issues associated with rapid growth in broilers, which may predispose birds to ascites, especially at high altitudes. The susceptibility of the broiler heart to fail is related to the high metabolic rate of this fast growth (Olkowski *et al.*, 2008). Sudden death syndrome along with heart failure is affected by stress factors such as environmental stress or stress from human interaction for example blood withdrawals and weighing of birds (Olkowski *et al.*, 2008). Many of the SDS cases are also related to the broiler's diet. Due to the sudden heart failure, it is suggested that there is an involvement of the type of fat and the electrolyte balance in the diet (Hulan *et al.*, 1979).

A positive genetic correlation exists between sudden death syndrome and ascites, and that the incidence of both can be reduced by selecting against them and considering them both as a single trait (Moghadam *et al.*, 2005). Olkowski *et al.*, (2008) conducted a stress test to evaluate the susceptibility of broilers to sudden death and cardiac arrhythmia. They administered epinephrine and determined that 17-35% of their broiler flock displayed cardiac arrhythmia and 27 birds from the 158 birds died due to sudden death between the age of 10 to 28 days. Their ECG readings all show undulating waves indicating ventricular fibrillation. Histopathological examination showed that broilers that died of SDS had degenerative changes in their left ventricle, something that did not occur in healthy broilers. Therefore, they concluded that any changes in the architecture of the cardiomyocytes are a predisposing factor to arrhythmias in broilers.

2.5.4.2 Cholangiohepatitis

An increase in pressure on the sinusoids and capillary bed of the liver is one of the major causes of the fluid accumulation in peritoneal cavities which results in ascites (Julian, 1990a). Any form of hepatocyte necrosis and liver fibrosis causes vessel obstruction in the liver. Infections such as cholangiohepatitis caused by *Clostridium perfringens* result in liver damage that subsequently results in ascites syndrome. Although ascites was present in these broilers diagnosed with cholangiohepatitis, they did not have right ventricular failure (Julian, 1993).

2.5.4.3 Aspergillosis

Another naturally occurring infection that plays a role in the onset of ascites is aspergillosis caused by a non-contagious fungal species *Aspergillus fumigatus* (Julian, 1993; Beernaert *et al.*, 2010). This disease causes impairment of the immune response, and the main route of infection is caused by

the inhalation of the spores. Even though this is mainly a disease in the respiratory tract all organs are susceptible to spores (Beernaert *et al.*, 2010). Spore inhalation can enter the systemic circulation and cause small granulomas in the lung along with lung infection. (Julian & Groyo, 1990). Birds display signs such as difficult and open mouth breathing along with non-productive coughing. Thereafter, death occurred within two to five days after the presence of the first clinical sign (Zafra *et al.*, 2008). Infections such as aspergillosis that can cause fibrinous pneumonia, restrict bronchi, and air capillary development in young chicks, can ultimately result in pulmonary hypertension through hypoxia-induced polycythaemia followed by an increase in blood viscosity (Julian & Groyo, 1990). Vaccination programs to ensure protective immunity is especially useful in the prevention of aspergillosis (Beernaert *et al.*, 2010).

2.5.4.4 Anaemia

Limited erythrocyte production caused by an iron deficiency occurs when there is a failure of iron mobilization from the tissues (Arvedson & Sasu, 2009). Anaemia or iron deficiency hinders the oxygen-carrying capacity of the blood and ultimately results in an increase in blood flow. This explains the reduction in growth rate and a further reduction in O₂ requirement as the metabolic rate decreases (Julian, 1993). If a bird has anaemia, there is a subsequent increase in the volume of the blood, and it reduces the carrying capacity of O₂ which increases the heart rate (Julian, 1993).

2.5.5 Gender

Ascites development is more common in male broilers than in female broilers because female broilers display slower growth rates. Male broilers exhibit higher metabolic rates in contrast to female broilers therefore, male broilers present with a lower blood pH due to the development of respiratory acidosis (Van As *et al.*, 2010). In the study done by Van As *et al.* (2010), they concluded that male broilers had a significantly higher incidence of ascites in contrast to female broilers (30% vs. 17%). Similar results were found in a study done by Closter *et al.* (2012), who found that male broilers had higher incidences of ascites compared to female broilers because of their higher heritability estimates for growth rates. Further conclusions were made to analyse male and female broilers separately due to their differences in genetic correlations (Closter *et al.*, 2012).

2.5.6 Hatchery related ascites development (Early onset)

In the last 7 days of incubation rapid embryo growth takes place, and the O₂ consumption increases with over 60% during the period of breathing and hatching (Decuyper *et al.* 2000). Therefore, lengths and severity of O₂ deprivation influence post-hatching characteristics associated with ascites development. In a study done by Rouwet *et al.*, (2002), they found that chicken embryos subjected to chronic O₂ deprivation had structural and functional abnormalities in their cardiovascular system.

2.6 Gut health

Modern broilers are reared in environments where they are constantly exposed to high stocking densities which result in a higher microbial load on the broilers due to the higher biological pressure on their environment. The broiler gut plays an integral part in the proper physiological development of the bird, and it can be defined as the ability of the gastrointestinal tract to maintain homeostasis and fight against infectious and non-infectious diseases (Kogut *et al.*, 2017). Improvement and maintenance of the GIT are essential to improve the welfare and production of animals especially now that commercial farms have moved to AGP-free. Gut health entails the macro and microstructural integrity along with the balance of the immune status of the bird (Choct, 2009). The gastrointestinal tract of the bird is the organ with the most interaction with the environment and should therefore be able to reduce the exposure to pathogenic microbes and still allow nutrient absorption (Turner, 2009). The mucus layer of the gut inhibits microorganisms from entering the intestinal epithelium and is therefore the first line of defence (Kogut *et al.*, 2017).

To ensure a stable and healthy ecosystem of bacteria in the intestinal tract, the bird must establish a diversity of bacteria. These natural bacteria that inhabit the gut are overly sensitive to dietary changes, diseases, environmental factors such as pathogen load and stress. The microbiota present in the gut derives their nutrients from the diet consumed by the bird, and in return increases the value of nutrients by synthesizing essential nutrients and converting them to complimentary enzymes (Kogut *et al.*, 2017). When the gut is infected by bacteria such as coccidiosis or necrotic enteritis, a decrease in digestibility and nutrient transportation will follow (Choct, 2009).

2.6.1 Iron on gut health

Foods that contain environmentally oxidized Fe have low solubility levels and therefore must develop mechanisms to compete for available sources. Iron binding proteins aid in the transport of Fe in the digestive system. Iron binds to mucins in the stomach due to the stomach's acidic conditions, and therefore it remains soluble for periods of alkalinity further in the digestive tract (Rusu *et al.*, 2020). Dietary products, O₂, and pH affect the solubility and valency of Fe (Yilmax & Li, 2018). Iron deficiency or Fe overload in the gastrointestinal tract occurs due to the malabsorption of Fe and subsequently alters the host's mucosal immune response. An Fe deficiency arises due to a lack of Fe-rich sources in the diet or because the body cannot absorb Fe due to genetically related problems (Rusu *et al.*, 2020). Although the inflammatory response between Fe deficiency and excess is similar, it appeared that inflammation did not alter the morphology. Dietary Fe influences the susceptibility to other diseases related to the dysfunction of the intestinal barrier in pigs (Li *et al.*, 2016). In contrast, heme-rich diets decrease the gut microbial diversity and abundance. Iron availability in the colon can promote the virulence and replication of gut enteric pathogens such as *Salmonella*, *Shigella*, and *Campylobacter* due to it being a critical signal for their expression (Yilmaz & Li, 2018).

An overload of Fe can result from Fe toxicity or a chronic accumulation of Fe. Majority of Fe is stored in hepatocytes in the liver and other tissues as ferritin when there is a high level of transferrin saturation. However, some of this Fe is catabolized to ROS (Weiss, 2010). Supplementing excess levels of Fe into the diet can cause acute Fe toxicity, and in this case resulted in oxidative stress that caused structural damage to the jejunal enterocytes and a reduction in the number of villi. There was also a reduction in nitric oxide synthase (NOSi) from 27.2% to 17.5% in medium to high supplemented Fe diets (Gou *et al.*, 2018).

Iron toxicity can result in hemosiderosis or hemochromatosis. Hemosiderosis is the accumulation of Fe in an organ without causing organ injury or dysfunction, whereas hemochromatosis refers to organ damage and dysfunction due to an overload of Fe. Hemosiderosis normally takes place where a hemorrhage had occurred in a tissue (Weiss, 2010). The duodenum is responsible for most of the Fe absorption and is, therefore, more sensitive to dietary Fe changes. Significant increases in haemoglobin concentration along with increases in the intestinal and hepatic Fe concentration occurred in pigs fed diets high in Fe. This excess absorption of Fe binds with hydrogen peroxide produced by neutrophils and results in hydroxyl radicals that cause mucosal damage and lipid peroxidation. Conclusively, high Fe diets in pigs negatively affected the morphology of the small intestine by reducing the villus height by 25% (Li *et al.*, 2016).

2.6.2 Necrotic enteritis

Necrotic enteritis is caused by a gram-positive, spore-forming anaerobe called *Clostridium perfringens* that causes concern to poultry producers since it increases mortality and reduces production performance. As the pathogenic strain proliferates into the gut it increases the NetB toxin that damages the epithelium (Moore, 2016). Subsequently, this results in the formation of intestinal lesions in the small intestine, liver, and kidneys followed by thin duodenum, ileum, and jejunum walls. (Timbermont *et al.*, 2011). Feed components such as indigestible and non-starch polysaccharides are predisposing factors to necrotic enteritis. Diets that are high in poorly digestible proteins result in high levels of the protein that are retained in the gastrointestinal tract and serve as substrates for bacteria. Any disease that weakens the immune system of the bird that decreases the gut's resistance to pathogens, such as Gumboro disease, can increase the incidence of ascites (Timbermont *et al.*, 2011; Moore, 2016).

Studies have shown that cold stress increases the birds' susceptibility to necrotic enteritis due to the immunosuppression of birds that occurs during low environmental temperatures. Changes in environmental temperature alter the serum thyroid hormone levels, therefore a subsequent alteration in thyroid hormones can ultimately affect the immune status of the bird, predisposing them to other diseases. Cold stress not only increased the caecal content pH but increased the population of *C. perfringens* in the ceca (Tsiouris *et al.*, 2015).

2.7 Strategies to alleviate ascites

2.7.1 Growth manipulation

Past research has stated that by restricting feed intake you control the growth rate of the broiler and subsequently reducing the incidence of ascites (Aftab & Khan, 2005). Restricting feed intake in rapidly growing broilers prior to breeding resulted in a decreased respiratory frequency, an increased tidal volume accompanied by an improved arterial O₂ saturation, and decreased haematocrit (Reeves *et al.*, 1991). By implementing feed restriction, you can potentially improve the blood flow through the lungs and subsequently improve future growth rates (Julian, 2000). The purpose of feed restriction programs is to limit the quantity of feed per day or to shorten the length of feeding time per day. Restricting a broiler's fast body growth could potentially prevent the right ventricular hypertrophy induced by hypoxia (Peacock *et al.*, 1989; Kahjali & Wideman, 2016). This reduction of rapid growth prevents the bird from depositing large amounts of muscle and fats and thereby reducing the O₂ requirement (Balog, 2003). By implementing the feed restriction program earlier in the broiler's life, you reduce the negative effect on the body weight at market age (Julian, 1993).

Table 1 The effects of feed restriction and cold environmental temperatures on ascites mortality, hematocrit value, heart weight relative to body weight, and RV/TV ratio in broilers (Bolukbasi *et al.*, 2004).

Groups	Heart (%)	RV/TV ratio	Hematocrit value (%)	Ascites mortality (%)	Total mortality (%)
Ad libitum control	0.567 ± 0.01 ^c	0.26 ± 0.007 ^c	35.0 ± 2.6	6 ^{ab}	10 ^a
Ad libitum cold	0.611 ± 0.008 ^a	0.32 ± 0.008 ^a	35.8 ± 2.6	13.3 ^a	13.3 ^a
*FR control	0.544 ± 0.008 ^c	0.25 ± 0.008 ^c	32.6 ± 1.9	1.6 ^b	3.2 ^b
*FR cold	0.580 ± 0.008 ^b	0.29 ± 0.006 ^b	33.5 ± 1.7	1.6 ^b	1.6 ^b

^{abc} Having different superscripts in a column differ significantly (P<0.01); *FR: Feed restricted

Baghbanzadeh and Decuyper (2008), determined that reducing the growth of the bird during day 3 to 14 of age improves bird health later in life compared to birds that experienced slow growth. Restricting feed intake has been proved to be successful to reduce metabolic disease but the severity of restriction must be balanced with the period required to attain market weight. Implementing feed restriction to reduce the growth rate improved ventilation rates concerning metabolic rate or body size Peacock *et al.*, (1989), and Reeves *et al.* (1991) discussed a further improvement of oxygenation in broilers. Özkan *et al.*, (2006) concluded that low levels of O₂ consumption per metabolic body weight at low ambient temperatures indicate insufficient metabolic action and could potentially lead to the development of ascites. Reduced levels of O₂ consumption during the last week of age could be due to the overall deterioration of the condition of the bird.

2.7.2 Nutrition

Manipulation of the diet form can be used as a strategy to decrease the growth rates of broilers to prevent ascites syndrome (Bolukbasi *et al.*, 2004). Any complication related to hypoxaemia and heart

disease can be eradicated completely by controlling the growth rate of the bird (Olkowski *et al.*, 2005). High nutrient densities and high feed intake along with feed form are factors contributing to the incidence of ascites in birds (Balog *et al.*, 2000; Aftab & Khan, 2005; Ozkan *et al.*, 2006). This explains why broilers on pelleted diets exhibit higher incidences of ascites compared to mash diets, because pelleted diets are more digestible and nutrient-dense which allows for a higher intake (Bagbanzadeh & Decuypere, 2008). The diet can be manipulated by changing from pelleted feed to mashed feed as it reduces the feed intake of the broiler (Julian, 2000).

2.7.2.1 Fatty acids

Including omega-3 fatty acids into the broiler diet will provide protection against lipid peroxidation and reactive O₂ to reduce the progression of ascites (Das & Deka., 2019). Increasing the flexibility of the red blood cell membrane to ensure deformability of erythrocytes by increasing the unsaturated fatty acid concentration in the diet can aid in the reduction of ascites incidence among broilers (Singh *et al.*, 2011).

2.7.2.2 Bicarbonate

Including potassium carbonate (KHCO₃) into the drinking water of broilers at 1000mg/L of water, it can improve blood oxygenation and be a potential prophylactic to reduce ascites-related mortalities. To alkalize the diet by adding KHCO₃ slightly decreased the incidence of pulmonary hypertension as well as increasing blood oxygenation in broilers that were exposed to temperatures below the comfort zone (Shlosberg *et al.*, 1998). Although Squires and Julian, (2001), as well as Wideman *et al.*, (2003), concluded that HCO₃ did not alter the cardiac output or arterial pressure by alkalizing the diet. By alkalizing a diet with sodium bicarbonate (NaHCO₃) can potentially function as a pharmacological method to reduce ascites mortality without causing concern for residues (Owen *et al.*, 1994). However, the addition of other sodium sources should be done carefully as it would require a decrease in other sodium sources to prevent over-supplementation. Supplementation of NaHCO₃ in the diet results in blood alkalization which increases the affinity for O₂ in the lung (Julian, 2000; Squires & Julian, 2001).

2.7.2.3 Arginine

Arginine (Arg) is an essential amino acid that cannot be synthesized by the broiler itself due to broilers having incomplete urea cycles (Kidd *et al.*, 2001). It is required by the bird to support protein synthesis, feathering, and growth. This amino acid functions as a substrate to generate nitric oxide (NO) that serves in the maintenance of the immune system as well as the regulation of cardio-pulmonary blood flow and pressure (Khajali & Wideman, 2016). During heat stress, Brake *et al.* (1998) advised supplementing Arg to meet the increased requirement for Arg in higher ambient temperatures. NO is an effective vasodilator that aids in the reduction of pulmonary vascular resistance by stimulating the

smooth muscles to relax. NO also prevents the production of vasoconstrictors such as serotonin (Wideman *et al.*, 2001). Studies have shown that NO production reduces plasma Fe and liver serum Fe due to the disruption of the mechanisms of Fe storage in rats (Xiao *et al.*, 2003). Supplementing higher levels of Arg than the requirement can potentially improve broiler performance such as FCR and body weight (BW) (Kidd *et al.*, 2001; Castro *et al.*, 2019). The NRC recommends supplementing 1.25% Arginine from day 0 to 21, and 1.1% from day 22 onwards (NRC, 1994).

2.7.2.4 Iron

Iron in broiler diets is supplemented to prevent any clinical deficiencies such as anaemia. Hemoglobin, Hct, and growth are used to determine the iron requirements on a molecular and metabolic basis (Cork, 2000; Ma *et al.*, 2016). Iron and copper (Cu) function mainly in the formation of the heme complex found in erythrocytes to be able to transport O₂ to and from tissues. Iron can be provided to cells by absorption from the gastrointestinal tract, recycled Fe from destroyed red blood cells or from the Fe storage in the liver (Olver, *et al.*, 2010). Dietary Fe is absorbed and transported across the intestinal mucosa; therefore, it is highly dependent on the Fe status of the bird. Along with other microminerals, Fe is supplemented in a premix usually as a salt or as ferrous sulphate. Fe supplementation greatly depends on the ingredient composition of the commercial diet; therefore, it is recommended to perform a mineral analysis on feed prior to formulation to avoid any deficiencies or supplementing in excess (Conrad *et al.*, 2000, Taschetto *et al.*, 2017).

Iron participates in enzymatic reactions as either non-heme weak complexes (catalase and succinate dehydrogenase) or stainable heme Fe (cytochrome c oxidase) (Hagler *et al.*, 1981; Theil, 2004). The enzymes succinate dehydrogenase (SDH), catalase, and cytochrome-c oxidase (COX) function as antioxidant enzymes to aid in the elimination of ROS (Rao & Jagadeesan, 1996). Ma *et al.*, (2016) and Liao *et al.*, (2017) both concluded that SDH, COX, and catalase activity in the heart and liver are more sensitive measurements to determine the Fe requirements in broilers. Food components such as tannins and phytate catch nonheme Fe (in weak complexes) during digestion and absorption and ultimately altering the bioavailability of Fe in food. Fe³⁺ is pH-dependent in comparison to Fe²⁺ ions and much like other minerals will be surrounded by water unless they are in a chelated form (Theil, 2004).

Iron is traditionally supplemented in the form of inorganic FeS, and recently have been supplemented in the organic form as inorganic minerals have many disadvantages. Organic Fe whether in the form of a proteinate or amino acid chelate, had a much higher bioavailability (Ma *et al.*, 2014). Minerals that are bound to amino acids present with no electrical charge, implying that they are unresponsive to fluctuations in pH while traveling through the digestive tract (Milanovic *et al.*, 2008).

In animals, Fe forms part of Hb, myoglobin, cytochromes, and all other Fe-containing enzymes including ferritin and hemosiderin. Ferritin is mainly a cytoplasmic protein that functions in transporting iron (Theil, 2004). The intestinal lumen lacks transferrin receptors and is capable of transporting non-transferrin-bound Fe, whereas plasma Fe requires transferrin to be transported. Ferric iron must be chelated in order to remain soluble at a pH of three and above. The number of chelators must be controlled because they compete for Fe and compromise the integrity of the cells. Biochemically it was shown that Mn inhibits the uptake of ferrous Fe but not ferric Fe, therefore it can be hypothesized that ferrous Fe can be catalytically oxidized to ferric Fe before being transported (Conrad *et al.*, 2000).

Studies have shown that organic sources of Fe not only improved chick performance but also increased Hb concentration (McNaughton *et al.*, 1974). The NRC recommendation for Fe in broiler diets from day 1 to 21 is 80 mg Fe/kg (NRC, 1994). These contrasts the findings of Ma *et al.*, (2016) and Liao *et al.*, (2017) who determined that amount of Fe required to support the full performance and expression of enzymes in broilers was 97-136 mg Fe/kg for day 1-to-21-day old broilers and 104-110 mg Fe/kg for day 22–42-day-old broilers respectively when they are fed maize soybean meal diets.

2.7.3 Environmental

Low house temperatures especially during brooding have been shown to intensify ascites incidence around the age of 6 weeks when compared to higher house temperatures (Deaton *et al.*, 1996). Scheele *et al.*, (2003) concluded that in juvenile chickens the low pCO₂ tension in venous return is an appropriate measurement for genetic selection compared to a high pCO₂ which indicates the requirement for improved management. Monitoring house temperatures together with an adequate ventilation schedule can eradicate practically all cases of ascites on farms (Aviagen, 2009). Being proactive by increasing the ventilation program as the house biomass increases can reduce ascites almost completely in their flocks. Managing fan cycle timers are especially important as the bird's age or as the biomass increase to ensure a constant flow of clean air (Aviagen, 2009).

Studies have shown that birds that are reared on intermittent lighting programs improved broiler health as well as reduce the incidence of metabolic disorders by indirectly controlling their growth. Manipulating broiler lighting schedules at the starter period reduces ascites mortality (Hassanzadeh, 2009). To limit the number of hours of light the juvenile broiler receives reduces the amount of O₂ required by temporarily reducing the growth rate (Julian, 1993). At high altitudes, increasing the dark period for another 1 to 2 hours can help in reducing the ascites incidence (Aviagen, 2009).

2.7.4 Medicinal

Ascites syndrome increases blood viscosity and causes pulmonary vasoconstriction. Therefore, by supplementing a drug that can improve vascular lung capacity or reduce vascular resistance you can theoretically reduce ascites (Aftab & Khan, 2005). Diaoxinxuekang is Chinese medicine, and the inclusion of it at 3500mg/kg in the diet reduces ascites mortality by decreasing the O₂ consumption by the cardiac muscle cells and improving the hydraulic pressure of blood. Diaoxinxuekang improves blood circulation and can be used without any negative effects on broiler performance (Wang & Hacker, 1993).

2.8 Conclusion

Higher metabolic rates require higher O₂ levels and if it cannot be satisfied, it results in hypoxemia and ultimately ascites. When the blood flow reaches its limit, there is a resultant increase in cardiac output to supply enough blood through the capillaries, and therefore there is an increase in pulmonary pressure. By decreasing the O₂ requirements, it allows for the compensation of the physiological limits caused by this selection for increased growth. In previous studies, supplementing organic minerals and essential amino acids will have a positive effect on broiler performance due to their greater bioavailability. Yu *et al.*, (2000) concluded that the supplementation of Availa Fe (organic Fe) increased the liver serum Fe concentration in weanling pigs more efficiently in contrast to inorganic Fe. Numerous studies have reported the beneficial impacts on performance with organic minerals however, the precise recommendation is unknown.

Chapter 3: Material and methods

3.1 Introduction

The objective of this trial was to evaluate broiler performance in terms of feed conversion ratio (FCR), feed intake (FI), body weight (BW), average daily gain (ADG), and ascites mortality when fed different sources of iron (Fe) and levels of dietary Arginine to dietary Lysine ratio (dArg/dLys) for the duration of the trial (0 to 35 days). Approval for the use of this data ethics was obtained from the NAS Ethics Committee (NAS171/2022) at the University of Pretoria (NAS171/2022), South Africa. This broiler trial took place on a private commercial broiler farm in Grainfield, and all performance and blood data were collected and analysed on the farm. These parameters were taken weekly (on days 0, 7, 14, 21, 28, and 35) as well as on dietary phase changes. Ascites mortality and other related mortalities were recorded daily.

3.1.1 Facilities and husbandry

The commercial broiler house was environmentally controlled and was controlled by SKOV temperature systems. Prior to placement the facility was disinfected and left to stand for 14 days. Strict biosecurity measures were implemented throughout the trial such as footbaths and controlled access to the facility. The broilers were exposed to a standard lighting program recommended by the breed standard Aviagen (Aviagen, 2018). This lighting program shown in Table 2 stimulates the development of vital organs and ensures healthy eating and drinking habits.

Table 2 Lighting program used in trial

Age (Days)	Daylength (Hours)
0-7	23 light, 1 darkness
7-35	18 light, 6 darkness

A total of 4800-day-old Ross 308 male chicks were placed in the commercial broiler house with 96 pens. Only male chicks were used for the purpose of this trial because it reduces statistical variance and improves statistical significance as female broilers have lower metabolic and growth rates in contrast to male broilers. Pens were covered with clean pine shavings to ensure maximal comfort. Each pen had its own tube feeder and nipple drinkers to allow *ad libitum* consumption of feed and water. The nipple lines were flushed with water and tested before the arrival of the chicks. For the first 7 days, fountain drinkers, feeder pans, and chick paper were placed in each pen for supplemental feeding and drinking to maximize feed intake. Fountain drinkers and feeder pans were cleaned and replaced with fresh water and feed every morning. Nipple and feeder heights were adjusted daily to facilitate correct drinking and eating behaviour.

3.2 Pen and house design

The commercial broiler house used for this trial had treatment pens located in the middle of the house, allowing commercial birds to roam around the outside of the pens. A diagram of the pen layout can be seen in Appendix 1. The treatments were set up in a complete randomized blocking design where 6 treatments were divided and blocked into 96 pens (16 replicates per treatment), to provide adequate statistical power to prove significance $P < 0.05$. Each pen consisted of 2.0m x 1.0m in size with 50 birds placed within each pen creating a stocking density of 25 birds/m².

3.3 Dietary treatments and formulations:

This trial consisted of a five-phase feeding program, a pre-starter (days 1-7), starter (days 8-14), grower (days 15-22), finisher (days 23-28) and post-finisher treatment (days 29-35). All feeding phases were lined up with the correlating weigh days for the purpose of convenience. All diets were standard maize-soybean diets with dietary compositions shown in Table 3 to 5. All dietary treatments were formulated to commercial specifications.

Treatment diets were mixed in a 1.5-ton fountain blender at Simple Grow Agricultural services feed mill (M26 Knoppieslaagte 385-Jr, 0157). All base diet components were obtained from Simple Grow Agricultural services. The basal diets were mixed and thereafter divided into aliquots of six dietary treatments. A carrier-premix was made beforehand using one kilogram of ground yellow maize and the different sources of Fe and levels of dArg/dLys correlating to each treatment. This carrier-premix was added slowly into the fountain blender while blending took place. This was done for each dietary phase to ensure the correct premix was added to the correct treatment. The organic Fe source is a metal amino acid complex consisting of one metal ion bound to one amino acid ion called *Availa*®Fe (Zinpro Performance minerals, Zinpro Corporation).

3.3.1 Chemical analyses:

A sample of each dietary treatment from pre-starter to post-finisher was collected for dry matter, ash, moisture, crude fiber, crude protein, apparent metabolizable energy (AME), and Fe analyses at ChemNutri Analytical (Cedar Lake Industrial Park, Olifantsfontein, 1665). All analyses were determined by the official methods of the Association of Official Analytical Chemists (AOAC) and Coreset Variational Bayes (CVB) method of ileal digestible amino acids (AA) and retainable phosphorus (CVB, 2021). The Fe in the feed was measured by the Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES) (method 985.01; AOAC, 2000). One base feed sample from each phase were analysed for ash (method 942.05; AOAC, 2000), calcium and phosphorus (method 985.01; AOAC, 2000), moisture content (method 943.01; AOAC, 2000), crude fiber (method Ba 6a-05; AOCS, 2000), crude protein (method 990.03; AOAC, 2000), and crude fat (method 920.39; AOAC, 2000).

Table 3 Ingredient composition (%) of the different dietary phases

Ingredient	Pre-starter	Starter	Grower	Finisher	Post-finisher
Yellow Maize	49.80	49.80	59.14	58.66	60.84
Extruded FF Soya	8.00	8.00	12.00	12.00	12.00
Soya O/C	36.01	36.01	25.28	25.41	23.05
L-Threonine	0.17	0.17	0.16	0.13	0.13
L-Valine	0.04	0.04	0.05	0.05	0.05
L-Methionine	0.37	0.37	0.31	0.31	0.30
L-Isoleucine	0.00	0.00	0.00	0.01	0.02
Lysine (HCL)	0.20	0.20	0.23	0.22	0.23
L-Arginine	0.10	0.04	0.04	0.04	0.035
Soya Oil (Degummed)	1.75	1.75	0.42	1.16	1.39
Limestone	1.26	1.26	1.48	1.17	1.10
MDCP	1.29	1.29	0.00	0.00	0.00
Salt	0.52	0.52	0.30	0.25	0.25
Sodium Bicarbonate	0.00	0.00	0.09	0.09	0.09
Enzyme pack*	0.05	0.05	0.05	0.05	0.05
Mineral premix	0.15	0.15	0.12	0.12	0.12
Vitamin premix	0.15	0.15	0.12	0.12	0.12
Cycostat	0.02	0.02	0.20	0.02	0.02
Zinc Bacitracin 15%	0.02	0.02	0.02	0.02	0.02

*Phytase 2000 FTU + Syncra (500g/t)

Table 4 Ingredient composition of the broiler vitamin premix per kg of premix

Ingredient	Unit	Pre-starter/Starter	Grower/Finisher/Post-finisher
Vitamin A	I.U	12 0000	10 000
Vitamin D3	I.U	5 000	4166.67
Vitamin E	mg	60	50
Vitamin K3	mg	2	1.67
Vitamin B1	mg	2	1.67
Vitamin B2	mg	5	4.167
Vitamin B3 (Niacin)	mg	50	41.67
Vitamin B5	mg	12	10
Vitamin B6	mg	3	2.5
Vitamin B9 (Folic acid)	mg	2	1.67
Vitamin B12	mg	0.01	0.0083
Biotin	mg	0.1	0.083
Antioxidant	mg	125	104.17
Manganese	mg	110	91.67
Zinc	mg	100	83.33
Copper	mg	10	8.33
Cobalt	mg	0.5	0.42
Iodine	mg	2	1.67
Selenium	mg	0.3	0.25
Choline chloride	g	0.35	0.29
Limestone	g	1.478	1.232

Table 5 Treatment description including Fe source and level of dArg/dLys

Treatment	Description		Arginine (dArg/dLys) ³
	Organic ¹	Inorganic ²	
1	No supplemented Fe (NC)		1.03
2	70 ppm FeS		1.03
3	30ppm Availa Fe		1.03
4	No supplemented Fe (NC)		1.10
5	70ppm FeS		1.10
6	30ppm Availa Fe		1.10

¹Organic Fe supplemented on top of the basal diet in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

²Inorganic Fe supplemented on top of the basal diet in the form of iron sulphate obtained from Nutroteq

³Synthetic L-Arginine was supplemented in ratio to Lysine

Table 6 Analysed iron content in feed (mg/kg)

Phase	Treatment	Description	Supplemented Fe	Fe analyses	Actual Fe ²
Pre-Starter	1 & 4	NC*	0	186.11	186.11
	2 & 5	FeS	70	301.54	231.54
	3 & 6	Availa Fe	30	206.69	176.69
Starter	1 & 4	NC	0	164.16	164.16
	2 & 5	FeS	70	257.19	187.19
	3 & 6	Availa Fe	30	236.21	206.21
Grower	1 & 4	NC	0	104.72	104.72
	2 & 5	FeS	70	204.91	134.91
	3 & 6	Availa Fe	30	176.15	146.15
Finisher	1 & 4	NC	0	101.95	101.95
	2 & 5	FeS	70	179.22	109.22
	3 & 6	Availa Fe	30	158.05	128.05
Post-finisher	1 & 4	NC	0	86.45	56.45
	2 & 5	FeS	70	170.04	100.04
	3 & 6	Availa Fe	30	163.04	133.04

¹Negative control containing no additional supplemented iron.

²Analysed iron – supplemented iron = Actual iron

Table 7 Analysed proximate composition for each dietary phase (as is)

Phase	dArg/dLys	Ash	Fat (EE)	Fibre (CF)	Moisture	Protein (Dumas)
Pre-Starter	High ¹	5.87	6.63	3.68	10.45	24.83
	Low ²	4.44	5.88	3.61	10.19	22.67
Starter	High	5.4	5.54	3.58	9.52	25.75
	Low	5.4	5.77	3.46	10.03	23.76
Grower	High	5.82	5.65	3.84	9.91	22.14
	Low	4.74	6.1	3.61	10.1	22.27
Finisher	High	4.16	6.44	3.55	9.72	20.18
	Low	4.27	6.53	3.59	10.52	21.29
Post-Finisher	High	4.47	6.36	3.36	10.9	21.61
	Low	4.57	6.65	4.21	9.5	20.28

¹1.10 dArg/dLys

²1.03 dArg/dLys

3.4 Measurements

3.4.1 Production performance

On weigh days, birds were placed into portable crates of known weights, by which this weight was subtracted from the total weight. This final total weight was then divided by the number of birds in the pen to determine the average individual BW of the birds. Statistical analyses were run after each sampling day to look for outliers and those pens was then reweighed.

$$BW = \frac{(Weight\ of\ crate + birds) - Weight\ of\ crate}{Number\ of\ birds\ in\ crate}$$

$$ADG = \frac{Amount\ weight\ gained\ since\ last\ weigh\ day}{number\ days\ since\ last\ weigh\ day}$$

The average FI per bird was determined by subtracting the residual feed in the feeder and bins from each pen from the initial total amount of feed supplied and dividing it by the number of birds in the pen. ADG and FCR were then calculated from the abovementioned parameters and the FCR was corrected for mortalities:

$$FI/bird = \frac{(Residual\ feed\ from\ feeder\ and\ bin) - Initial\ feed\ supplied}{Amount\ of\ birds/pen}$$

$$FCR_{mc} = \frac{Feed\ intake\ per\ pen}{(total\ weight\ gain\ of\ the\ pen\ over\ the\ period + weight\ of\ mortalities\ per\ pen)}$$

3.4.2 Blood sampling

Only male broilers were used in this study for all parameters to decrease statistical variation, in particular for blood physiology, since males are more prone to ascites than females due to their higher metabolic rate. On days 7 and 30 one bird per pen was subjected to a 1mL blood sample from their brachial wing vein. These blood samples were analysed immediately on-farm for the partial pressure of carbon dioxide (pCO₂), partial pressure of oxygen (pO₂), blood pH, hemoglobin (Hb), hematocrit (Hct), O₂ saturation, and total carbon dioxide (TCO₂) by the i-STAT device with EG7 cartridges from Abbott point of care systems. All broilers were humanely handled by one student restraining the bird while a trained person performed the blood withdrawals.

3.5 Statistical analyses

All data was analysed in JMP statistical software, version 15.0.0 with a 2-way Analyses of Variance (ANOVA) with Fe source, dArg/dLys level and Fe x dArg/dLys. Block was included as a random effect. Where main or interaction effects were significant, means were separated using Tukey's HSD test at $P < 0.05$ in accordance with a completely randomized block design. JMP, a subsidiary of SAS, statistical version 15.0.0 was used for analyses to determine the means and averages, and an overall significant difference ($P < 0.05$) was determined between means using Tukey's HSD test (Tukey, 1949).

The linear mixed model used in this trial is described by the following equation:

$$Y_{ijkl} = \mu + T_i + W_j + H_k + B(H)_l + TW_{ij} + e_{ijkl}$$

where Y_{ijkl} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i th treatment

W_j = effect of the j th week or phase

H_k = effect of the k th house

$B(H)_l$ = effect of the l th block in the house

TW_{ij} = effect of the ij th interaction between treatment and house

e_{ijkl} = error associated with each Y

Chapter 4: Results

4.1 Performance parameters

4.1.1 Average daily gain (g/bird)

The cumulative and weekly average daily gain for interaction and main effects are presented in Table 8 and Table 9. Significant interaction between treatments ($P<0.05$) for cumulative average daily gain between the periods of 0-7 and 0-14 days are shown in Table 8, where in both periods the organic iron (Fe) and negative control (NC) with low supplemented dArg/dLys presented higher daily gains compared to the same treatments with a higher supplemented dArg/dLys. No further significant differences ($P>0.05$) in body weight gain were seen after the period of 0-14 days and were comparable to the NC diets. Broilers supplemented with the low level of dArg/dLys presented significantly higher ($P<0.05$) body weight gains in contrast to the high level of dArg/dLys up and till the period of 0-28 days.

Table 8 Interaction and main effects on cumulative average daily gain (grams) on broilers from 0-35 days of age¹

Iron Source	dArg/dLys	Cumulative average daily gain (g) ¹				
		0-7	0-14	0-21	0-28	0-35
NC ²	1.03 (low)	17.47 ^{ab}	29.48 ^a	41.68	51.64	54.43
Availa Fe ³	1.03	17.97 ^a	29.72 ^a	41.63	51.61	54.69
FeS ⁴	1.03	17.02 ^b	29.03 ^{ab}	41.27	51.24	54.93
NC	1.10 (high)	17.41 ^{ab}	28.85 ^{ab}	40.97	50.37	53.79
Availa Fe	1.10	16.95 ^b	28.23 ^b	40.74	51.19	54.65
FeS	1.10	17.25 ^{ab}	28.84 ^{ab}	41.11	51.03	54.31
SEM		0.22	0.26	0.27	0.41	0.56
Main effect probability						
Iron source						
NC		17.44	29.16	41.32	51.00	53.90
Availa Fe		17.46	28.97	41.19	51.40	54.67
FeS		17.13	28.93	41.19	51.13	54.62
SEM		0.16	0.19	0.21	0.32	0.50
dArg/dLys						
1.03 (low)		17.49	29.41 ^a	41.53 ^a	51.50 ^a	54.68
1.10 (high)		14.20	28.64 ^b	40.94 ^b	50.86 ^b	54.11
SEM		0.22	0.17	0.18	0.30	0.48
P-Value						
Iron source		0.2673	0.6038	0.8271	0.5378	0.0631
dArg/dLys		0.1201	0.0003*	0.0046*	0.0332*	0.0552
Interactions		0.0171*	0.0303*	0.3162	0.3039	0.3758

¹Two-way ANOVA to determine effects between dietary treatments

²Negative control (No supplemented iron)

³Organic in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P<0.05$)

Similar results can be seen with the weekly average daily gain in Table 9. Broilers that were fed diets of organic Fe and a low level of dArg/dLys presented with a significantly higher weekly daily gain ($P<0.05$) compared to organic Fe and high level of dArg/dLys. For the main effect of dArg/dLys level, the lower level of dArg/dLys had significantly higher daily gains ($P<0.05$) than the high level of dArg/dLys. Supplementing different Fe sources with different levels of dArg/dLys during the last 4 weeks of the trial had no significant effect ($P>0.05$) on broiler average daily gain.

Table 9 Interaction and main effects on average daily gain (grams) per week (phase) on broilers from 0-35 days of age¹

Iron Source	dArg/dLys	Average daily gain (g) ¹				
		0-7	7-14	14-21	21-28	28-35
NC ²	1.03 (low)	17.37 ^{ab}	41.36	65.38	79.90	61.99
Availa Fe ³	1.03	17.95 ^a	41.09	65.02	80.83	63.46
FeS ⁴	1.03	16.98 ^b	40.63	64.64	79.70	65.45
NC	1.10 (high)	17.38 ^{ab}	39.96	64.58	76.59	63.06
Availa Fe	1.10	16.89 ^b	39.23	64.91	80.33	64.60
FeS	1.10	17.20 ^{ab}	40.24	64.59	79.36	62.93
SEM		0.23	0.42	0.63	1.45	1.74
Main effect probability						
Iron source						
NC		17.37	40.66	64.98	78.25	62.53
Availa Fe		17.42	40.16	64.97	80.58	64.03
FeS		17.09	40.44	64.62	79.53	64.19
SEM		0.16	0.32	0.46	1.13	1.56
dArg/dLys						
1.03 (low)		17.43	41.03 ^a	65.01	80.14	63.64
1.10 (high)		17.16	39.81 ^b	64.70	78.76	63.53
SEM		0.13	0.27	0.39	0.99	1.50
P-Value						
Iron source		0.2974	0.4378	0.7929	0.1975	0.2541
dArg/dLys		0.1422	0.0002*	0.5254	0.1916	0.9049
Interactions		0.0136*	0.1513	0.7900	0.4315	0.1693

¹Two-way ANOVA to determine effects between dietary treatments

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P<0.05$)

4.1.2 Body weight

The broiler body weights are presented in Table 10. Significant ($P<0.05$) interaction between treatments for Fe source and dArg/dLys level on broiler body weight are presented for the period 0-14 days of age, with Fe source and low level of dArg/dLys having higher body weights in contrast to Fe source and high levels of dArg/dLys. Supplementing lower levels of dArg/dLys resulted in significantly higher ($P<0.05$) body weights from day 14-28, with day 14 having an incredibly significant effect ($P=0.0002$) on body weight between dArg/dLys levels. On day 35, there was a tendency ($P=0.0632$ and $P=0.0543$) towards heavier body weights when supplementing organic Fe compared to the NC, as well as a tendency towards heavier body weights when supplementing lower levels of dArg/dLys.

Table 10 Interaction and main effects on weekly (phase) body weights in broilers from 0-35 days of age ¹

Iron Source	dArg/dLys	Body weights (g) ¹				
		7	14	21	28	35
NC ²	1.03 (low)	161.74 ^{ab}	452.09 ^a	914.61	1485.31	1944.33
Availa Fe ³	1.03	165.33 ^a	455.65 ^a	913.86	1484.56	1953.56
FeS ⁴	1.03	158.53 ^b	445.77 ^{ab}	906.01	1474.11	1962.02
NC	1.10 (high)	161.30 ^{ab}	443.39 ^{ab}	899.70	1449.91	1907.69
Availa Fe	1.10	158.09 ^b	434.63 ^b	894.99	1472.70	1952.04
FeS	1.10	159.98 ^{ab}	442.95 ^{ab}	902.47	1467.98	1940.16
SEM		1.54	3.62	5.66	11.50	19.51
Main effect probability						
Iron source						
NC		161.52	447.74	907.16	1467.61	1926.01 ^y
Availa Fe		161.71	445.14	904.43	1478.63	1952.80 ^x
FeS		159.25	444.36	904.24	1471.05	1951.09 ^x
SEM		1.08	2.71	4.31	9.05	17.38
dArg/dLys						
1.03 (low)		161.86	451.17 ^a	911.50 ^a	1481.33 ^a	1953.30 ^x
1.10 (high)		159.79	440.32 ^b	899.06 ^b	1463.53 ^b	1933.29 ^y
SEM		0.88	2.32	3.75	8.08	16.61
P-Value						
Iron source		0.2146	0.5841	0.8197	0.5314	0.0632*
dArg/dLys		0.1042	0.0002*	0.0044*	0.0322*	0.0543*
Interactions		0.0158*	0.0285*	0.3134	0.3051	0.3764

¹Two-way ANOVA to determine effects between dietary treatments, mortality corrected

²Negative control (No supplemented iron)

³Organic Fe diet in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

^{XYZ}Columns with different superscripts indicate a tendency for treatments to differ ($P < 0.1$)

4.1.3 Feed intake

Cumulative and average feed intake per bird can be seen in Table 11 and 12, respectively. No significant differences ($P > 0.05$) were found neither for interaction effects nor Fe sources on feed intake of broilers. However, significant differences ($P < 0.05$) in feed intake were seen with different levels of dArg/dLys on from day 21 onward, where broilers fed lower levels of dArg/dLys had significantly higher feed intakes in contrast to the higher level of dArg/dLys. This was observed for both cumulative and average feed intake.

The different levels of dArg/dLys had a significant difference ($P < 0.05$) on average feed intake for the periods of 7-14 (starter) and 14-21 (grower) days. Birds fed the lower levels of dArg/dLys had significantly higher feed intakes compared to birds fed the higher level of dArg/dLys.

Table 11 Interaction and main effects on cumulative feed intake on broilers from 0-35 days of age (in grams)¹

Iron Source	dArg/dLys	Cumulative FI (g/bird) ¹				
		0-7	0-14	0-21	0-28	0-35
NC ²	1.03 (low)	129.31	467.41	1099.88	1984.55	2698.77
Availa Fe ³	1.03	128.83	463.50	1090.75	1975.21	2695.34
FeS ⁴	1.03	127.50	458.18	1079.98	1971.14	2692.65
NC	1.10 (high)	128.00	458.14	1074.74	1944.81	2651.66
Availa Fe	1.10	129.77	458.62	1072.32	1959.48	2680.48
FeS	1.10	129.30	459.77	1079.74	1955.82	2656.73
SEM		1.42	3.47	6.40	10.09	15.97
Main effect probability						
Iron source						
NC		128.66	462.78	1087.31	1964.68	2675.22
Availa Fe		129.30	461.06	1081.53	1967.34	2687.92
FeS		128.40	458.98	1079.86	1963.48	2674.69
SEM		1.10	2.59	4.71	7.82	13.2
dArg/dLys						
1.03 (low)		128.55	463.03	1090.20 ^a	1976.97 ^a	2695.59 ^a
1.10 (high)		129.02	458.85	1075.60 ^b	1953.37 ^b	2662.96 ^b
SEM		0.97	2.22	3.99	6.90	12.13
P-Value						
Iron source		0.7705	0.5099	0.4447	0.9087	0.5036
dArg/dLys		0.6519	0.1209	0.0045*	0.0020*	0.0024*
Interactions		0.4607	0.2536	0.1151	0.3070	0.4408

¹Two-way ANOVA to determine effects between dietary treatments, mortality corrected

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

Table 12 Interaction and main effects on average feed intake on broilers from 0-35 days of age (in grams)¹

Iron Source	dArg/dLys	Average FI (g/bird) ¹				
		0-7	7-14	14-21	21-28	28-35
NC ²	1.03 (low)	129.31	338.10	632.47	884.67	714.25
Availa Fe ³	1.03	128.83	334.67	627.25	884.47	720.13
FeS ⁴	1.03	127.50	330.68	621.80	891.16	721.51
NC	1.10 (high)	128.00	330.14	616.60	870.07	706.85
Availa Fe	1.10	129.77	328.85	613.70	887.16	721.01
FeS	1.10	129.30	330.47	619.97	876.08	700.90
SEM		1.42	2.86	3.97	8.50	11.34
Main effect probability						
Iron source						
NC		128.66	334.12	624.53	877.37	710.54
Availa Fe		129.30	331.76	620.47	885.81	720.57
FeS		128.40	330.58	620.89	883.62	711.20
SEM		1.10	2.11	2.84	6.40	9.98
dArg/dLys						
1.03 (low)		128.55	334.48 ^a	627.17 ^a	886.76	718.62
1.10 (high)		129.02	329.82 ^b	616.76 ^b	877.77	709.89
SEM		0.97	1.79	2.34	5.51	9.50
P-Value						
Iron source		0.7705	0.4200	0.5283	0.5462	0.3433
dArg/dLys		0.6519	0.0396*	0.0018*	0.1692	0.1505
Interactions		0.4607	0.3449	0.1676	0.4472	0.3682

¹Two-way ANOVA to determine effects between dietary treatments, mortality corrected

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

4.1.4 Feed conversion ratio (mortality corrected)

The interaction and main effects on the cumulative and weekly FCR of broilers are shown in Table 13 and 14, respectively. Significant interaction between treatments ($P < 0.05$) are observed for the periods of 0-7 and 0-14 days of age for cumulative FCR. Broilers fed organic Fe with low levels of dArg/dLys had a significantly lower cumulative FCR in contrast to broilers fed organic Fe and higher levels of dArg/dLys during days 0-7. During day 0-14, broilers fed low levels of dArg/dLys independent of the Fe source had significantly lower ($P < 0.05$) cumulative FCR compared to the higher level of dArg/dLys. After day 14, no significant interaction effects ($P > 0.05$) were observed.

The effect of dArg/dLys level on cumulative FCR was significant ($P < 0.05$), where broilers fed a lower level of dArg/dLys presented with lower cumulative FCR in contrast to the higher level of dArg/dLys between 0-14 days. A tendency towards a lower cumulative FCR ($P = 0.0607$) was observed between 0-7 days, with this tendency indicating that broilers fed lower levels of dArg/dLys have lower cumulative FCR. This tendency was confirmed to be true because significant differences ($P < 0.05$) in cumulative FCR between the low and high level of dArg/dLys was observed between 0-14 days.

Table 13 Interaction and main effects on cumulative feed conversion ratio, mortality corrected in broilers from 0-35 days of age¹

Iron Source	dArg/dLys	Cumulative FCR _{mc} (g) ¹				
		0-7	0-14	0-21	0-28	0-35
NC ²	1.03 (low)	1.060 ^{ab}	1.133 ^b	1.257	1.373	1.418
Availa Fe ³	1.03	1.026 ^b	1.115 ^b	1.248	1.367	1.409
FeS ⁴	1.03	1.072 ^{ab}	1.128 ^b	1.247	1.375	1.402
NC	1.10 (high)	1.051 ^{ab}	1.135 ^{ab}	1.249	1.380	1.421
Availa Fe	1.10	1.096 ^a	1.161 ^a	1.254	1.368	1.404
FeS	1.10	1.073 ^{ab}	1.140 ^{ab}	1.251	1.370	1.399
SEM		0.014	0.008	0.005	0.010	0.012
Main effect probability						
Iron source						
NC		1.055	1.134	1.253	1.377	1.420 ^a
Availa Fe		1.061	1.138	1.251	1.368	1.406 ^{ab}
FeS		1.072	1.134	1.249	1.373	1.400 ^b
SEM		0.011	0.006	0.004	0.008	0.010
dArg/dLys						
1.03 (low)		1.052 ^y	1.125 ^b	1.250	1.372	1.409
1.10 (high)		1.073 ^x	1.145 ^a	1.251	1.373	1.408
SEM		0.009	0.006	0.004	0.007	0.010
P-Value						
Iron source		0.4495	0.7731	0.6360	0.5886	0.0272*
dArg/dLys		0.0607*	0.0004*	0.8097	0.8941	0.8202
Interactions		0.0072*	0.0025*	0.2899	0.7813	0.8182

¹Two-way ANOVA to determine effects between dietary treatments, mortality corrected

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

^{XYZ}Columns with different superscripts indicate a tendency for treatments to differ ($P < 0.1$)

There was no significant interaction between treatments ($P>0.05$) in weekly FCR between treatments over the trial period except for the starter period (0-7 days), in which broilers that were fed organic Fe with a lower level of dArg/dLys had a significantly lower ($P<0.05$) FCR compared to organic Fe with high levels of dArg/dLys. The other treatments presented all with similar feed conversions.

As seen in Table 14 below, there were no significant differences ($P>0.05$) in FCR for Fe source throughout the entire trial period. During the pre-starter period (0-7 days) there was a tendency ($P=0.0684$) towards a lower weekly FCR when birds were fed lower levels of dArg/dLys. Significant differences ($P<0.05$) are observed between 7-14 and 14-21 days, where birds fed diets with lower levels of dArg/dLys had lower weekly FCR.

Table 14 Interaction and main effects on weekly (phase) feed conversion ratio in broilers from 0-35 days of age¹

Iron Source	dArg/dLys	Weekly FCR _{mc} (g/bird) ¹				
		0-7	7-14	14-21	21-28	28-35
NC ²	1.03 (low)	1.067 ^{ab}	1.138	1.383	1.585	1.654
Availa Fe ³	1.03	1.027 ^b	1.164	1.379	1.565	1.628
FeS ⁴	1.03	1.073 ^{ab}	1.163	1.375	1.603	1.582
NC	1.10 (high)	1.053 ^{ab}	1.183	1.366	1.639	1.607
Availa Fe	1.10	1.099 ^a	1.198	1.351	1.581	1.625
FeS	1.10	1.076 ^{ab}	1.174	1.373	1.584	1.598
SEM		0.014	0.010	0.009	0.025	0.030
Main effect probability						
Iron source						
NC		1.060	1.175	1.374	1.612	1.630
Availa Fe		1.063	1.181	1.364	1.573	1.627
FeS		1.075	1.169	1.374	1.594	1.590
SEM		0.01	0.008	0.007	0.020	0.027
dArg/dLys						
1.03 (low)		1.056 ^y	1.165 ^b	1.378 ^a	1.584	1.621
1.10 (high)		1.076 ^x	1.185 ^a	1.363 ^b	1.601	1.610
SEM		0.009	0.007	0.006	0.017	0.026
P-Value						
Iron source		0.4982	0.3582	0.5006	0.2391	0.0985*
dArg/dLys		0.0684*	0.0086*	0.0395*	0.3651	0.4964
Interactions		0.0037*	0.3676	0.3537	0.2719	0.2847

¹Two-way ANOVA to determine effects between dietary treatments, mortality corrected

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P<0.05$)

^{XYZ}Columns with different superscripts indicate a tendency for treatments to differ ($P<0.1$)

4.1.5 Ascites mortalities

Ascites mortality results are presented in Table 15. Data showed that there were no significant differences ($P>0.05$) for ascites mortality throughout this trial for neither interactions nor any main effects.

Table 15 Interaction and main effect on ascites mortality in broilers from day 0-35¹

Iron Source	dArg/dLys	Cumulative mortalities (%) ¹				
		0-7	0-14	0-21	0-28	0-35
NC ²	1.03	1.70	2.21	3.25	4.43	5.99
Availa Fe ³	1.03	0.40	1.95	2.60	3.13	4.69
FeS ⁴	1.03	0.65	2.34	3.90	4.95	6.77
NC	1.10	0.52	1.95	3.86	4.30	5.34
Availa Fe	1.10	0.91	2.08	3.40	4.95	6.64
FeS	1.10	0.78	1.56	3.13	4.20	6.12
SEM		0.42	0.629	0.803	0.93	1.00
Main effect probability						
Iron source						
NC		1.11	2.10	3.06	4.36	5.66
Availa Fe		0.65	2.02	2.99	4.04	5.66
FeS		0.72	1.95	3.52	4.56	6.44
SEM		0.298	0.428	0.603	0.680	0.775
dArg/dLys						
1.03		0.91	2.17	3.25	4.17	5.82
1.10		0.74	1.86	3.13	4.47	6.03
SEM		0.244	0.336	0.520	0.574	0.682
P-Value						
Iron source		0.5072	0.9803	0.7517	0.8412	0.6097
dArg/dLys		0.6151	0.5698	0.8322	0.6784	0.7695
Interactions		0.1163	0.7825	0.5580	0.3223	0.2571

¹Two-way ANOVA to determine effects between dietary treatments, mortality corrected

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

4.2 Blood parameters

Interaction and main effects on day 7 on blood parameters such as pH, pCO₂, pO₂, HCO₃ are presented in Table 16 and TCO₂, SO₂, HCT, HB are presented in Table 17, respectively. No significant interaction between treatments ($P > 0.05$) were observed for blood parameters on day 7, except for TCO₂. Broilers that were fed diets with zero supplemented Fe and high levels of dArg/dLys had a significantly higher ($P < 0.05$) total blood carbon dioxide in contrast to the treatments with supplemented Fe and high level of dArg/dLys.

Evaluating the main effect Fe source specifically, significant effects ($P < 0.05$) were observed for pH, PCO₂, HCO₃ and TCO₂. Broilers fed diets supplemented with organic Fe had significantly higher ($P < 0.05$) blood pH compared to broilers fed inorganic Fe. Significant differences ($P < 0.05$) are also presented for pCO₂ where treatments with organic Fe had lower pCO₂. This could potentially be due to organic Fe higher bioavailability for O₂ transport, indirectly reducing CO₂ concentration. HCO₃ present results in which the inorganically supplemented treatments had significantly lower ($P < 0.05$) HCO₃ levels confirming this statement with the respective lower pH for inorganic Fe treatments. The NC with zero supplemented Fe had a significantly higher TCO₂ compared to supplemented organic and inorganic Fe. Supplementing different levels of dArg/dLys had no significant effect ($P > 0.05$) on any blood parameters on day 7.

Table 16 Interaction and main effects on blood parameters in broilers on day 7¹

Iron Source	dArg/dLys	Blood parameters			
		pH	pCO ₂	pO ₂	HCO ₃
NC ²	1.03 (low)	7.36	37.96	33.88	22.09
Availa Fe ³	1.03	7.40	36.55	34.25	22.77
FeS ⁴	1.03	7.37	37.09	35.44	21.38
NC	1.10 (high)	7.39	38.21	33.84	23.18
Availa Fe	1.10	7.40	33.20	36.23	20.65
FeS	1.10	7.34	38.67	33.89	20.59
SEM		0.018	1.28	1.38	0.671
Main effect probability					
Iron source					
NC		7.38 ^{ab}	38.08 ^a	33.86	22.63 ^a
Availa Fe		7.40 ^a	34.87 ^b	35.24	21.71 ^{ab}
FeS		7.35 ^b	37.88 ^{ab}	34.66	20.98 ^b
SEM		0.0125	0.920	1.01	0.506
dArg/dLys ratio					
1.03 (low)		7.38	37.20	34.52	22.08
1.10 (high)		7.38	36.69	34.65	21.47
SEM		0.01	0.764	0.862	0.439
P-Value					
Iron source		0.0198*	0.0236*	0.5763	0.0301*
dArg/dLys		0.8750	0.620	0.9017	0.2385
Interactions		0.2307	0.143	0.4168	0.0407*

¹Two-way ANOVA to determine effects between dietary treatments

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

pH, concentration of hydrogen ions; pCO₂, partial pressure of carbon dioxide; pO₂, partial pressure of oxygen; HCO₃, bicarbonate concentration

Although there were no significant differences seen with the other blood parameters whether interaction or main effect, numerical differences ($P > 0.05$) in pO₂, SO₂, and Hb were observed. Broilers fed any supplemented source of Fe independent of the dArg/dLys level had HCO₃ levels compared to treatments with zero supplemented Fe. Similar findings can be seen with SO₂ and Hb.

Table 18 and Table 19 displayed that there was no significant ($P > 0.05$) interaction between treatments recorded for day 30 on blood parameters. There was also no significant effect ($P > 0.05$) observed for dArg/dLys level on day 30 blood parameters. Similarly, no significant effects ($P > 0.05$) were found on Fe source on day 30 with the exception of PCO₂. Iron source had a significant effect ($P < 0.05$) on PCO₂ in which broilers fed organic Fe presented with significantly lower blood PCO₂ in contrast to inorganic Fe and NC treatments. This corresponds to the numerically higher ($P > 0.05$) value of SO₂ of broilers fed organic Fe confirming that organic Fe promotes O₂ transport.

Table 17 Interaction and main effects on blood parameters in broilers on day 7 continued¹

Iron Source	dArg/dLys	Blood parameters			
		TCO ₂	SO ₂	Hct	Hb
NC ²	1.03	23.19 ^{ab}	62.63	20.56	6.89
Availa Fe ³	1.03	24.12 ^{ab}	68.24	20.23	6.97
FeS ⁴	1.03	22.56 ^{ab}	65.38	20.75	7.06
NC	1.10	24.52 ^a	63.54	20.27	6.83
Availa Fe	1.10	21.68 ^b	68.78	20.07	7.02
FeS	1.10	21.68 ^b	61.32	20.07	6.82
SEM		0.698	2.567	0.599	0.192
Main effect probability					
Iron source					
NC		23.85 ^a	63.08	20.41	6.86
Availa Fe		22.90 ^{ab}	68.51	20.15	6.99
FeS		22.12 ^b	63.35	20.41	6.94
SEM		0.523	1.908	0.424	0.133
dArg/dLys					
1.03		23.29	65.41	20.51	6.98
1.10		22.63	65.55	20.16	6.89
SEM		0.451	1.632	0.347	0.107
P-Value					
Iron source		0.0312*	0.0529	0.8815	0.7874
dArg/dLys		0.2188	0.6644	0.4422	0.5940
Interactions		0.0193*	0.5193	0.9060	0.7539

¹Two-way ANOVA to determine effects between dietary treatments

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

TCO₂, Total carbon dioxide; SO₂, Saturated oxygen; Hct, hematocrit; Hb, Hemoglobin.

Table 18 Interaction and main effects on blood parameters in broilers on day 30¹

Iron Source	dArg/dLys	Blood parameters			
		pH	pCO ₂	pO ₂	HCO ₃
NC ²	1.03	7.43	37.67	36.38	24.64
Availa Fe ³	1.03	7.46	35.20	34.94	24.78
FeS ⁴	1.03	7.43	36.58	36.25	24.33
NC	1.10	7.41	41.21	34.19	25.68
Availa Fe	1.10	7.43	35.83	36.00	23.54
FeS	1.10	7.41	37.67	35.00	23.82
SEM		0.0173	1.428	1.188	0.641
Main effect probability					
Iron source					
NC		7.42	39.44 ^a	35.28	25.16
Availa Fe		7.44	35.52 ^b	35.47	24.16
FeS		7.42	37.12 ^{ab}	35.62	24.08
SEM		0.0124	1.10	0.841	0.455
dArg/dLys					
1.03		7.44	36.48	35.86	24.58
1.10		7.41	38.24	35.06	24.35
SEM		0.0104	0.965	0.688	0.374
P-Value					
Iron source		0.2562	0.0115*	0.9590	0.1765
dArg/dLys		0.0648	0.1001	0.4148	0.6537
Interactions		0.9406	0.4783	0.3725	0.1940

¹Two-way ANOVA to determine effects between dietary treatments

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

^{AB}Columns with different superscripts indicate significant differences ($P < 0.05$)

pH, concentration of hydrogen ions; pCO₂, partial pressure of carbon dioxide; pO₂, partial pressure of oxygen; HCO₃, bicarbonate concentration

Table 19 Interaction and main effects on blood parameters in broilers on day 30¹

Iron Source	dArg/dLys	Blood parameters			
		TCO ₂	SO ₂	HCT	HB
NC ²	1.03	25.69	70.13	26.00	8.94
Availa Fe ³	1.03	25.83	71.03	27.18	9.30
FeS ⁴	1.03	25.34	67.20	26.38	8.94
NC	1.10	26.81	65.54	27.50	9.19
Availa Fe	1.10	24.56	70.94	26.75	9.11
FeS	1.10	24.92	67.16	27.00	9.15
SEM		0.694	2.488	0.620	0.217
Main effect probability					
Iron source					
NC		26.25	67.83	26.75	9.07
Availa Fe		25.20	70.98	26.97	9.20
FeS		25.13	67.18	26.68	9.05
SEM		0.497	1.740	0.45	0.158
dArg/dLys					
1.03		25.62	69.45	26.52	9.06
1.10		25.43	67.88	27.08	9.15
SEM		0.410	1.41	0.375	0.132
P-Value					
Iron source		0.1897	0.2736	0.8870	0.7273
dArg/dLys		0.7366	0.4460	0.2578	0.5892
Interactions		0.2083	0.5883	0.2771	0.5071

¹Two-way ANOVA to determine effects between dietary treatments

²Negative control (No supplemented iron)

³Organic Fe in the form of Availa®Fe (Zinpro Performance Minerals, Zinpro Corporation)

⁴Inorganic ferrous sulphate obtained from Nutroteq

TCO₂, Total carbon dioxide; SO₂, Saturated oxygen; Hct, hematocrit; Hb, Hemoglobin.

Chapter 5: Discussion

5.1 Experimental diets

Feeding programmes greatly influence the success of poultry operations as broiler performance can easily be manipulated by nutrition. Growth rates of broilers have been heavily influenced by genetic alteration and therefore the diets should be altered to meet their new requirements. The chemical characteristics of minerals are of great importance when determining bio-availabilities. Several studies have demonstrated that organic Fe is more bioavailable to broilers compared to inorganic Fe (Ma *et al.*, 2014; Kwiecien *et al.*, 2015; Zhang *et al.*, 2016).

In the present study, performance parameters such as BW, ADG, FCR, ascites mortality and blood parameters such as pCO₂, pO₂, blood pH, SO₂, HCT and Hb were used to evaluate and compare ascites development between organic AvailaFe and inorganic FeSO₄. The abovementioned blood parameters have been proved to be a sensitive index towards the indication of ascites development and are known to be affected by dietary Fe concentration (Bounous & Stedman, 2010; Ma *et al.*, 2014). Similar results were also found in this study.

The Fe analyses of the treatments are presented in Table 6. Hygroscopicity, along with background Fe in the water, feedstuff, and metallic Fe from mixing machinery could account for the varying levels of Fe within treatment diets, although these amounts were not measured. In a study done by Vahl and Koolster. (1986) they estimated that approximately 20mg Fe/kg can originate from mixing machinery.

As stated previously, broilers with ascites syndrome have higher O₂ demands than what can be supplied by heart and lungs. Reports have been made that by reducing the protein content of the feed, you can indirectly reduce the O₂ demand therefore reducing PHS. The statement was based off the assumption that in order to catabolise 1g of protein, you need 1L of O₂ (Khajali & Wideman, 2016). Conflicting results were shown by Behrooj *et al.* (2012) where broilers that were fed higher levels of protein had less mortalities caused by PHS in contrast to broilers fed low-protein diets. The possible reasons as to why mortality increased in broilers fed low protein diets were summarized by Khajali and Wideman. (2016) as a decrease in uric acid production, decrease in Arginine intake, and increased lipogenesis. The key protein sources used for these experimental diets were soybean meal and full fat soya, and both are known for their high protein contribution and amino acid profile (Dei, 2011). The results from this study established that higher levels of protein and Arginine presented with numerically lower ascites mortality during the first production stage (0-21d).

5.2 Performance parameters

Previously, little research has been done on the supplementation of dArg/dLys level and Fe source in combination, however both have been individually researched and beneficial results from both have been observed. The supplementation of Fe and dArg/dLys at the levels such as in this study was enough to maximize broiler performance, however it was not enough to increase performance above our desired level. Growth performance parameters are mostly influenced by the type of diet and is not generally a sensitive index to assess Fe requirements in broilers (Ma *et al.*, 2016; Han *et al.*, 2022). The Fe analyses results from this present study, both in the basal diets and Fe supplemented diets present with higher Fe levels than the NRC recommendation for broilers (NRC, 1994), yet no additional benefit was observed with Fe source on broiler performance for the total duration of the trial; only a beneficial FCR and BW was observed at the end of the trial period when administering supplemental Fe in contrast to no additional Fe. Subsequently, supplementing organic Fe at lower than the recommendation such as in this study did not decline the production performance of broilers compared to inorganic Fe treatments and NC, and this is in line with the study of Bao *et al.* (2007) and Nollet *et al.* (2007). Supplementing organic minerals have been shown to increase broiler performance due to their higher bio-availabilities and because they have less antagonistic reactions in the intestinal tract (Nollet *et al.*, 2007; Milanovic *et al.*, 2008; Zhang *et al.*, 2016).

In the treatments where iron was supplemented, a decrease in broiler mortality was observed. Despite the lack of significance, there was a trend towards a lower mortality when supplementing Fe regardless of source; and this is in accordance with the study done by Kwiecien *et al.* (2015). In the present study, broilers fed organic Fe had better ADG than broilers fed inorganic Fe which is similar to the study of Sun *et al.* (2015) who too found that broilers had performed better in regard to FCR when supplemented organic Fe. Therefore, both the lower antagonistic effects and greater absorption of organic Fe may have contributed to the numerically greater performance in ADG, BW and FCR which compared to the study done by Han *et al.* (2022) who also found an increase in BW and ADG with supplementing 80 mg Fe /kg. However, Ma *et al.* (2016), Gou *et al.* (2018) and many other studies found no significant improvements in broiler performance with additional Fe supplementation. Perhaps, the reason why no further significant results in broiler performance were found in this study was because the Fe content was sufficient for the growth of broilers.

In a study done by Miroshnikov *et al.* (2017) they concluded that supplementing nano-Fe in combination with Arginine, Lysine and methionine provided the most efficient use of Fe. This is because methionine aids in the increase of migration of iron particles. A possible reason why we did not see the desired results in this present study could have been because we did not supply enough methionine in contrast to the supplied Arginine and Lysine level, which could have resulted in a decrease in their amino acid complex.

The dArg/dLys supplementation in this study was sufficient to maintain a steady body weight gain and was in accordance with the recommendation of the Arginine requirements by the NRC (1994). Broilers raised on feed with mainly maize and soybean such as in this study, contain enough crude protein levels as recommended by the NRC (1994) and would not be deficient in Arginine unless Lysine is supplemented above the requirement. In this present study the higher level of dArg/dLys did not increase broilers performance, and had lower BWG, BW and FI in contrast to treatments fed the low level of dArg/dLys. Throughout this study, no significant effects were seen in broiler performance with the supplementation of the higher level of dArg/dLys compared to other studies. This could have been due to interactions with other minerals and antagonisms with amino acids.

Xiao *et al.* (2003) and his previous studies found a significant decrease in plasma Fe and liver serum Fe when additional Arginine was supplemented, and higher levels of NO was produced which decreased the level of Fe further. In this study, the high level of dArg/dLys supplemented with organic Fe had numerically the lowest performance. This could have been due to the higher production of NO, which subsequently decreased the Fe storage in the liver which is the main site of Fe metabolism (Ma *et al.*, 2014). Decreasing the Fe storage, indirectly reduces plasma Fe resulting in less Fe available for uptake. The NRC recommends a dArg/dLys of between 1.10 to 1.04 which is similar to the ratios used in this study. Brake *et al.* (1998) determined that the optimum dArg/dLys will decrease when broiler diets contain higher concentrations of sodium chloride (salt). He concluded that salt and dArg/dLys have an inverse relationship as sodium reduces the antagonism between Arginine and Lysine. In this study, relatively high levels of salt were supplied, and this serves as a plausible reason why broilers fed the higher ratio of dArg/dLys had lower performance in contrast to broilers fed the low dArg/dLys.

In this study, significant interaction effects were found between organic Fe and the dArg/dLys level in early broiler performance of 0-14 days of age. Broilers that were supplemented with organic Fe and the low level of dArg/dLys had significantly ($P<0.05$) greater ADG, BW, and FCR in contrast to organic Fe and the high level of dArg/dLys. Similarly, the supplementation of inorganic Fe showed beneficial effects, however the supplementation of organic Fe with the low level of dArg/dLys outperformed the other treatments. This occurrence could possibly be explained by the increased availability of organic Fe, resulting in the greater absorption and storage of available Fe, and a reduction of the antagonisms with Arginine. In this study, it can be seen that the benefit of supplementing organic Fe lowers when supplemented with a higher dArg/dLys in the early phases of broiler performance (0-14 days).

5.3 Blood parameters

Standard Hct and Hb levels for broilers can range anything from 22-35% and 7-13 g/dL respectively and are sensitive to changes in dietary Fe levels and source (Bounous & Stedman, 2010). In the present study, hematological indicators were significantly affected ($P < 0.05$) by Fe source and is reflected in Tables 16-19 however, Hct and Hb were not affected which is contradicting to the results found in Bounous & Stedman, (2010), Kwiecien *et al.* (2015) and Ma *et al.* (2016).

Even though there were no significant differences found in Hct and Hb, they were still within the reference range for broilers. The present study suggests the possibility that Fe only has a significant effect on Hct and Hb when the broiler has an Fe deficiency or because the supplemented levels of Fe were not high enough to affect them and this is an accordance with the study of Han *et al.* (2022) who found no significant effect on blood Fe levels with 40-80 mg Fe/kg supplemented. Despite the non-significant differences in Hb and Hct in this study, Hb was numerically higher ($P > 0.05$) in contrast to the inorganically supplemented treatments and could be a consequence of the higher bioavailability of the organic Fe.

Various studies have shown the correlation between blood gas parameters in the prediction of ascites syndrome however, no previous research has been done on the effects of Fe nor dArg/dLys on blood gas. Blood pH has a significant effect on O₂ transport and binding to Hb and the lower the blood pH, the less O₂ will be bound and transported (Bagbanzadeh & Decuyper, 2008). In this study, Fe source had a significant effect on broiler blood pH on day 7 with broilers fed inorganic Fe had significantly lower blood pH. Subsequent to their low pH, these treatments also had numerically lower HCO₃ and pO₂ levels in contrast to broilers fed organic Fe. This is in accordance with the literature of Bagbanzadeh & Decuyper, (2008) in that a broiler with a low blood pH (metabolic acidosis), will have signs of panting which results in excessive loss of HCO₃ and a build-up of pCO₂. Broilers that were fed no supplemental Fe or inorganic Fe had significantly higher ($P < 0.05$) pCO₂, and lower pO₂ in contrast to broilers fed organic Fe. Therefore, the low levels of pO₂ could be a result of their respective higher level of pCO₂. The supplementation of Fe had significant improvement effects ($P < 0.05$) on pCO₂ and TCO₂. This can be connected by Fe participating in the uptake and transport of O₂ along with Hb. Therefore, supplementing Fe could have aided in the uptake of O₂, and subsequently decreasing the pCO₂ and TCO₂ by means of the better removal of them from the cardiovascular system.

The results in this study presented no significant effects of dArg/dLys supplementation on any blood parameters that were measured. However, due to Arginine influencing Fe absorption and storage, there is a possibility that it could indirectly affect blood gasses by influencing the Fe availability for binding with Hb.

Chapter 6: General Conclusion

Rapid selection of faster-growing broilers has resulted in the impairment of some mechanisms to sustain this increased metabolic rate. Like other metabolic syndromes, ascites is a multifactorial and non-infectious disorder caused by interactions amongst environmental, genetic, and physiological effects. Many factors play a role in the onset of ascites, but the main mechanism is the increase in metabolic rate as the growth rate of broilers increase. Higher metabolic rates have higher O₂ demands and heart rates and if these cannot be satisfied, it results in hypoxemia and ultimately ascites. When the blood flow reaches its limit, there is a resultant increase in cardiac output to supply enough blood through the capillaries, and therefore there is an increase in pulmonary pressure. Nutritional and management factors such as feed restriction, feed density, and diet form can reduce the incidence of ascites by reducing the growth rate of the broiler. By decreasing the O₂ requirements, it allows for the compensation of the physiological limits caused by this selection for increased growth. Understanding the conditions and predisposing factors that cause ascites is the key factor for the elimination of ascites prevalence. As in this and previous studies, supplementing organic minerals and essential amino acids will have a positive effect on broiler performance due to their greater bioavailability. Numerous studies have reported the beneficial impacts on performance with organic minerals however, the precise recommendation is unknown and changes throughout lifespan of the broiler.

The results from this study showed significant interactions between Fe and dArg/dLys on early broiler performance (0-14 days of age) for BW and FCR. Iron source had significant effects on broilers blood pCO₂, pO₂ and HCO₃; while dArg/dLys had significant effects on ADG, BW and FCR, especially when supplemented in higher levels. It was noteworthy that the mortality rate remained unaffected throughout the trial. This is contrary to the null hypothesis proving that the alternative hypothesis is true. In conclusion, broiler performance was affected when supplementing organic Fe and higher levels of dArg/dLys regardless of supplementing below or on the NRC recommendation. During early performance it was evident that early broiler performance was positively influenced when using organic Availa Fe and the low/industry standard level of dArg/dLys, but the opposite effect was seen when increasing the level of dArg/dLys as the organic iron loses its benefits. Considering the outcome of this trial, supplementation of organic Fe and different levels of dArg/dLys could be implemented to aid in the relief of ascites development however, more research is needed when supplementing these together as the results from this trial showed some possible antagonisms. More research is needed to assess the interaction between these two.

Chapter 7: Critical review and recommendations

Despite the lack of extensive research available on the interactive effects of Fe and dArg/dLys in broilers, there are various recommendations and starting points to clearly evaluate the interaction and inter-relationship between Arginine and Fe. However, from previous research performed in rats it has been shown that supplementing high levels of Arg decreases plasma Fe and Fe in the liver (Xiao et al., 2003). A great possibility in this trial was that dArg/dLys and Fe had significant interaction effects, but this was not measured. One remarkable challenge is the Fe contribution by various other sources, such as water and machinery, other than the diet itself. Another likely limitation and cause of data variation in this study was that blood collections were done at different times and by a different person on both days. Therefore, human error should be considered during sampling days.

Although both Arg and Fe have been separately proven to alleviate ascites mortality, in this trial some significant effects were observed in performance, however no alleviation of ascites mortality was observed. A detailed nutrient analysis of the ingredients and finished diets may be warranted to avoid any antagonistic or negative interaction effects that may limit the expression of the experimental nutrients or additives in broiler cells and/or tissues. A recommendation for future research would be to intensively study the interaction effects of Arg and Fe in terms of NO production, plasma Fe and liver Fe content and avoiding all outside contributing factors. This could allow for a better understanding of the mode of action and interactions between minerals and amino acids in broilers to help alleviate ascites development and mortality.

Chapter 8: References

- Abbott Group. (2014). Procedure Manual for the i-STAT® System. 24–27. Accessed on 8 June 2021
<http://www.abbottpointofcare.com/Customer-Info-Center/User-Documentation.aspx>
- Aftab, U., & Khan, A., 2005. Strategies to alleviate the incidence of ascites in broilers: a review. *Braz. J. Vet. Res. Anim. Sci.* 7, 199–204. <https://doi.org/10.1590/s1516-635x2005000400001>
- Arvedson, T. L., & Sasu, B. J., 2009. Role and regulation of iron metabolism in erythropoiesis and disease. Erythropoietins. *Erythropoietic Factors, and Erythropoiesis.* 1, 279–298. https://doi.org/10.1007/978-3-7643-8698-6_15
- Aviagen., 2009. Effective Management Practices to Reduce the Incidence of Ascites in Broilers. www.aviagen.com
- Aviagen., 2018. Broiler Management Handbook. 1–148.
- Bao, Y. M., Choct, M., Iji, P. A., & Bruerton, K., 2007. Effect of Organically Complexed Copper, Iron, Manganese, and Zinc on Broiler Performance, Mineral Excretion, and Accumulation in Tissues. *J. Appl. Poult. Res.* 16, 448–455. <https://doi.org/10.1093/japr/16.3.448>
- Balog, M.J., Anthony, N.B., Cooper, M.A., Kidd, B.D., Huff, G.R., Huff, W.E. & Rath, N.C., 2000. Ascites syndrome and related pathologies in feed restricted broilers raised in a hypobaric chamber. *Poult. Sci.* 79, 318–323.
- Balog, J.M., 2003. Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: are we seeing the light at the end of the tunnel? *Avian. Biol. Res.* 14, 99–126.
- Baghbanzadeh, A. and Decuypere, E., 2008. Ascites syndrome in broilers: physiological and nutritional perspectives. *Avian. Pathol.* 37, 117–126.
- Behrooj, N., Khajali, F., & Hassanpour, H., 2012. Feeding reduced-protein diets to broilers subjected to hypobaric hypoxia is associated with the development of pulmonary hypertension syndrome. *Br. Poult. Sci.* 53, 658–664. <https://doi.org/10.1080/00071668.2012.727082>
- Beker, A., Vanhooser, S. L., Swartzlander, J. H., & Teeter, R. G., 2003. Graded atmospheric oxygen level effects on performance and ascites incidence in broilers. *Poult. Sci.* 82, 1550–1553. <https://doi.org/10.1093/ps/82.10.1550>
- Bölükbası, S. C., Güzel, M., & Aktas, M. S., 2004. Effect of early feed restriction on ascites induced by cold temperatures and growth performance in broilers. *J. Appl. Anim. Res.* 26, 89–92. <https://doi.org/10.1080/09712119.2004.9706514>

- Bottje, W. G., Wang, S., Kelly, F. J., Dunster, C., Williams, A., & Mudway, I., 1998. Antioxidant Defenses in Lung Lining Fluid of Broilers: Impact of Poor Ventilation Conditions. *Poult. Sci.* 77, 516–522. <https://doi.org/10.1093/ps/77.4.516>
- Bottje, W. G., 2016. Oxidative metabolism and efficiency: the delicate balancing act of mitochondria. *Poult. Sci.* 98, 4223–4230. <https://doi.org/10.3382/ps/pey405>
- Bounous, D. I. & Stedman, N. L., 2010. Normal avian hematology: chicken and turkey. In: D. J. Weiss & K. J. Wardrop, eds. *Schalm's Veterinary Hematology*. 6th ed. Ames(Iowa): Wiley-Blackwell, 958-967.
- Brake, J., Balnave, D., & Dibner, J. J., 1998. Optimum dietary arginine:lysine ratio for broiler chickens is altered during heat stress in association with changes in intestinal uptake and dietary sodium chloride. *Br. Poult. Sci.* 39, 639–647. <https://doi.org/10.1080/00071669888511>
- Castro, F. L. S., Su, S., Choi, H., Koo, E., & Kim, W. K., 2019. L-Arginine supplementation enhances growth performance, lean muscle, and bone density but not fat in broiler chickens. *Poult. Sci.* 98, 1716–1722. <https://doi.org/10.3382/ps/pey504>
- Choct, M., 2009. Managing gut health through nutrition. *Br. Poult. Sci.* 50, 9–15.
- Chau-Berlinck, J. G., & Monteiro, L. H. A., 2017. Frank-Starling mechanism and short-term adjustment of cardiac flow. *J. Exp. Biol.* 220, 4391–4398. <https://doi.org/10.1242/jeb.167106>
- Chapman, M. E., & Wideman, R. F., 2000. Pulmonary Wedge Pressures Confirm Pulmonary Hypertension in Broilers Is Initiated by an Excessive Pulmonary Arterial (Precapillary) Resistance. *Poult. Sci.* 80, 468–473. <https://doi.org/10.1093/ps/80.4.468>
- Closter, A. M., van As, P., Elferink, M. G., Crooijmanns, R. P. M. A., Groenen, M. A. M., Vereijken, A. L. J., van Arendonk, J. A. M., & Bovenhuis, H., 2012. Genetic correlation between heart ratio and body weight as a function of ascites frequency in broilers split up into sex and health status. *Poult. Sci.* 91, 556–564. <https://doi.org/10.3382/ps.2011-01794>
- Conrad, M.E., Umbreit, J.N., Moore, E.G., Hainsworth, L.N., Porubcin, M., Simovich, M.J., Nakada, M.T., Dolan, K. and Garrick, M.D., 2000. Separate pathways for cellular uptake of ferric and ferrous iron. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 279, G767-G774.
- Cork, S. C., 2000. Iron storage diseases in birds. *Avian Pathol.* 29, 7–12. <https://doi.org/10.1080/03079450094216>

- Das, S., and Deka, P., 2019. Ascites syndrome (Water belly) in broilers and its management. *J. Entomol. Zool. Stud.* 7, 388-390. <https://doi.org/10.13140/RG.2.2.10353.02408>
- Decuypere, E., Buyse, J. & Buys, N., 2000. Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. *Worlds. Poult. Sci. J.* 56, 367-376.
- Decuypere, E., Buyse, J., Hassanzadeh, M., & Buys, N., 2005. Further insights into the susceptibility of broilers to ascites. *Vet. J.* 169, 319–320. <https://doi.org/10.1016/j.tvjl.2004.07.005>
- Dei, H.K., 2011. Soybean as a feed ingredient for livestock and poultry. In: Recent Trends for Enhancing the Diversity and Quality of Soybean Products. Ed. Prof. Krezhova, D., Rijeka, Croatia. IntechOpen.
- Druyan, S., and Moschandreou T.E., 2012. Ascites Syndrome in Broiler Chickens - A Physiological Syndrome Affected by Red Blood Cells. *Blood Cell - An Overview of Studies in Haematology* 1, 243-270.
- Fathi, M., 2016. Effects of zinc oxide nanoparticles supplementation on mortality due to ascites and performance growth in broiler chickens. *Iran. J. Appl. Anim. Sci.* 6, 389–394.
- Gou, Z. Y., Jiang, S. Q., Fan, L. L. Q. L., Jiang, X. J. L. Z. Y., & Ding, C. T. Z. F. Y., 2018. Effects of oxidative stress induced by high dosage of dietary iron ingested on intestinal damage and caecal microbiota in Chinese Yellow broilers. *J. Anim. Physiol. Anim. Nutr.* 102, 924-932.
- Han, M., Fu, X., Xin, X., Dong, Y., Miao, Z., & Li, J., 2022. High Dietary Organic Iron Supplementation Decreases Growth Performance and Induces Oxidative Stress in Broilers. *Anim.* 12, 1604. <https://doi.org/10.3390/ani12131604>
- Hassanzadeh, M., 2009. New approach for the incidence of ascites syndrome in broiler chickens and management control the metabolic disorders. *Poult. Sci.* 8, 90-98.
- Hassanzadeh, M., Fard, M.B., Buyse, J., Bruggeman, V. and Decuypere, E., 2004. Effect of chronic hypoxia during embryonic development on physiological functioning and on hatching and post-hatching parameters related to ascites syndrome in broiler chickens. *Avian Pathol.* 33, 558-564.
- Hassanzadeh, M., Buyse, J., Toloei, T., & Decuypere, E., 2014. Ascites syndrome in broiler chickens: A review on the aspect of endogenous and exogenous factors interactions. *J. Poult. Sci.* 51(3), 229–241.

- Havenstein, G. B., Ferket, P. R., Scheideler, S. E., & Larson, B. T., 1994. Growth, liveability, and feed conversion of 1957 vs 1991 broilers when fed “typical” 1957 and 1991 broiler diets. *Poult. Sci.* 73, 1785–1794.
- Hilpert, P., Fleischmann, R.G., Kempe, D. and Bartels, H., 1963. The Bohr effect related to blood and erythrocyte pH. *Am. J. Physiol.* 205, 337-340.
- Hulan, H. W., Proudfoot, F. G., & McRae, K. B., 1980. Effect of vitamins on the incidence of mortality and acute death syndrome (“flip-over”) in broiler chickens. *Poult. Sci.* 59, 927–931.
- Isaacks, R., Goldman, P., and Kim, C., 1986. Studies on avian erythrocyte metabolism. XIV. Effect of CO₂ and pH on P50 in the chicken. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 250, 260–266.
- Jensen, F. B., 2004. Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O₂ and CO₂ transport. *Acta. Physiol. (Oxf)*. 182, 215–227.
- Julian, R. J., and Goryo, M., 1990. Pulmonary Aspergillosis Causing Right Ventricular Failure and Ascites in Meat-Type Chickens. *Avian Pathol.* 19, 643–654.
- Julian, R. J., and Mirsalimi, S. M., 1992. The blood oxygen concentration of fast-growing and slow-growing broiler chickens, and chickens with ascites from right ventricular failure. *Avian Dis.* 36, 730–732.
- Julian, R.J., 1993. Ascites in poultry. *Avian Pathology*, 22, 419-454.
- Julian, R.J., 1998. Rapid growth problems: ascites and skeletal deformities in broilers. *Poult. Sci.* 77, 1773-1780.
- Julian, R. J., 2000. Physiological management and environmental triggers of the ascites syndrome: A review. *Avian Pathol.* 29, 519–527.
- Kenyon, B., 2019. Why the first week’s feed can make or break broiler performance. Available at: [Why the first week’s feed can make or break broiler performance | The Poultry Site](#) (Accessed: 6 October 2022).
- Khajali, F., & Wideman, R. F., 2016. Nutritional approaches to ameliorate pulmonary hypertension in broiler chickens. *J. Anim. Physiol. Anim. Nutr.* 100, 3–14 <https://doi.org/10.1111/jpn.12315>
- Kidd, M. T., Peebles, E. D., Whitmarsh, S. K., Yeatman, J. B., & Wideman, R. F., 2001. Growth and Immunity of Broiler Chicks as Affected by Dietary Arginine. *Poult. Sci.* 80, 1535–1542.

- Kim, J. C., Wilcock, P., & Bedford, M. R., 2018. Iron status of piglets and impact of phytase superdosing on iron physiology: A review. *Anim. Feed. Sci. Technol.* 235, 8–14.
- Kogut, M. H., Yin, X., Yuan, J., & Broom, L., 2017. Gut health in poultry. *CAB. Rev.* 12, 0–7.
- Kwiecień, M., Samolińska, W., & Bujanowicz-Haraś, B., 2015. Effects of iron-glycine chelate on growth, carcass characteristic, liver mineral concentrations and haematological and biochemical blood parameters in broilers. *J. Anim. Physiol. Anim. Nutr.* 99, 1184–1196. <https://doi.org/10.1111/jpn.12322>
- Laurell, C. B., 1951. What is the function of transferrin in plasma? *Blood*, 6, 183–187.
- Li, Y., Hansen, S. L., Borst, L. B., Spears, J. W., & Moeser, A. J., 2016. Dietary iron deficiency and over-supplementation increase intestinal permeability, ion transport, and inflammation in pigs. *J. Nutr.* 146, 1499–1505.
- Liao, X., Ma, C., Lu, L., Zhang, L., & Luo, X., 2017. Determination of dietary iron requirements by full expression of iron-containing cytochrome c oxidase in the heart of broilers from 22 to 42 d of age. *Br. J. Nutr.* 118, 493–499.
- Lin, H. Y., 2009, Erythropoiesis: The Roles of Erythropoietin and Iron. In: Textbook of Nephro-Endocrinology. Ed: Ajay K. Singh, Gordon H. Williams., Academic Press, 19-26. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-373870-7.00002-8>
- Luger, D., Shinder, D., Rzepakovsky, V., Rusal, M., & Yahav, S., 2001. Association Between Weight Gain, Blood Parameters, and Thyroid Hormones and the Development of Ascites Syndrome in Broiler Chickens 1. *Poult. Sci.* 80, 965–971.
- Luger, D., Shinder, D., & Yahav, S., 2002. Hyper- or hypothyroidism: Its association with the development of ascites syndrome in fast-growing chickens. *Gen. Comp. Endocrinol.* 127, 293–299.
- Luger, D., Shinder, D., Wolfenson, D., & Yahav, S., 2003. Erythropoiesis regulation during the development of ascites syndrome in broiler chickens: A possible role of corticosterone. *J. Anim. Sci.* 81, 784–790.
- Ma, X., Liao, X., Lu, L., Li, S., Zhang, L., & Luo, X., 2016. Determination of dietary iron requirements by full expression of iron-containing enzymes in various tissues of broilers. *J. Nutr.* 146, 2267–2273. <https://doi.org/10.3945/jn.116.237750>

- Ma, X. Y., Liu, S. B., Lu, L., Li, S. F., Xie, J. J., Zhang, L. Y., Zhang, J. H., & Luo, X. G., 2014. Relative bioavailability of iron proteinate for broilers fed a casein-dextrose diet. *Poult. Sci.* 93, 556–563. <https://doi.org/10.3382/ps.2013-03296>
- Malte, H., & Lykkeboe, G., 2018. The Bohr/Haldane effect: a model-based uncovering of the full extent of its impact on O₂ delivery to and CO₂ removal from tissues. *J. Appl. Physiol.* 125, 916–922.
- Malte, H., Lykkeboe, G., & Wang, T., 2021. The magnitude of the Bohr effect profoundly influences the shape and position of the blood oxygen equilibrium curve. *Comparative Biochemistry and Physiology Part A. J. Mol. Integr. Physiol.* 254, 110880.
- Martin, C. L., 2010. i-STAT - Combining Chemistry and Haematology in PoCT. *Clin. Biochem. Rev.* 31, 81–84.
- Maxwell, M. H., S. Spence, W. G. Robertson, and M. A. Mitchel., 1990. Hematological and morphological responses of broiler chickens to hypoxia. *Avian Pathol.* 19, 23–40.
- McNaughton, J. L., Day, E. J., Dilworth, B. C., & Lott, B. D., 1974. Iron and copper availability from various sources. *Poult Sci.* 53, 1325–1330.
- Milanović, S., Lazarević, M., Jokić, Ž., Jovanović, I., Pešut, O., Kirovski, D., & Marinković, D., 2008. The influence of organic and inorganic Fe supplementation on red blood picture, immune response, and quantity of iron in organs of broiler chickens. *Acta. Vet.* 58, 179–189. <https://doi.org/10.2298/AVB0803179M>
- Miroshnikov, S. A., Yausheva, E. v., Sizova, E. A., Kosyan, D. B., & Donnik, I. M., 2017. Research of opportunities for using iron nanoparticles and amino acids in poultry nutrition. *International J. Geom.* 13, 124–131. <https://doi.org/10.21660/2017.40.99216>
- Monge, C., & Leon-Velarde, F., 1991. Physiological Adaptation to High Altitude: Oxygen Transport in Mammals and Birds. *Physiol. Rev.* 71, 1135–1172. www.physiology.org/journal/physrev
- Moore, R. J., 2016. Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathol.* 45, 275–281.
- Moghadam, H. K., Mcmillan, I., Chambers, J. R., Julian, R. J., & Tranchant, C. C., 2005. Heritability of sudden death syndrome and its associated correlations to ascites and body weight in broilers. *Br. Poult. Sci.* 46, 54–57.

- Morita, V. S., Boleli, I. C., & Cargnelutti Filho, A., 2009. Hematological values and body, heart, and liver weights of male and female broiler embryos of young and old breeder eggs. *Braz. J. Vet. Res. Anim. Sci.* 11, 7–15.
- Mushtaq, M. M. H., Pasha, T. N., Mushtaq, T., & Parvin, R., 2013. Electrolytes, dietary electrolyte balance and salts in broilers: An updated review on growth performance, water intake, and litter quality. *Worlds. Poult. Sci. J.* 69, 789–802.
- Nollet, L., Van Der Klis, J.D., Lensing, M. & Spring, P., 2007. The effect of replacing inorganic with organic trace minerals in broiler diets on productive performance and mineral excretion. *J. Appl. Poult. Res.* 16, 592–597. <https://doi.org/10.3382/japr.2006-00115>.
- NRC, 1994. Nutrient requirements of poultry. (Washington, National Academic Press, 9th revised eds.).
- Olanrewaju, H. A., Thaxton, J. P., Iii, W. A. D., & Branton, S. L., 2007. Electrolyte Diets, Stress, and Acid-Base Balance in Broiler Chickens. *Poult. Sci.* 86, 1363-1371.
- Olanrewaju, H. A., Purswell, J. L., Collier, S. D., & Branton, S. L., 2013. Interactive effects of photoperiod and light intensity on blood physiological and biochemical reactions of broilers grown to heavyweights. *Poult. Sci.* 92, 1029–1039.
- Olkowski, A. A., Duke, T., & Wojnarowicz, C., 2005. The aetiology of hypoxaemia in chickens selected for rapid growth. *Comparative Biochemistry and Physiology. J. Mol. Integr. Physiol.* 141, 122–131.
- Olkowski, A. A., Wojnarowicz, C., Nain, S., Ling, B., Alcorn, J. M., & Laarveld, B., 2008. A study on the pathogenesis of sudden death syndrome in broiler chickens. *J. Vet. Sci.* 85, 131–140.
- Olver, C. S., Andrews, G. A., Smith, J. E. & Kaneko, J. J., 2010. Erythrocyte Structure and Function. In: Schalm's Veterinary Hematology. Ed: D. J. Weiss & K. J. Wardrop (6th edition), Iowa, Wiley-Blackwell, pp. 123-130.
- Owen, R. L., Wideman, R. F., Dunn, P. A., & Ford, B. C., 1994. Effect of age of exposure and dietary acidification or alkalization on broiler pulmonary hypertension. *Poult. Sci.* 3, 244–252.
- Ozkan, S., Plavnik, I. & Yahav, S., 2006. Effects of early feed restriction on performance and ascites development in broiler chickens subsequently raised at low ambient temperature. *J. Appl. Poult. Res.* 15, 9-19.
- Pakdel, A., Rabie, T., Veenendaal, T., Crooijmans, R.P.M.A., Groenen, M.A.M., Vereijken, A.L.J., Van Arendonk, J.A.M. & Bovenhuis, H., 2004. Genetic analysis of ascites-related traits in broilers.

- Doctoral thesis, Animal Breeding and Genetics Group, Department of Animal Sciences, Wageningen University, The Netherlands, p. 144.
- Powell, F. L., 2000. Respiration. In: *Avian Physiology*. Ed: G. C. Witthow, Sturkie's (Fifth edition), United States, Academic Press, pp 233-246.
- Peacock, A. J., Pickett, C., Morris, K., & Reeves, J. T., 1989. The relationship between rapid growth and pulmonary hemodynamics in the fast-growing broiler chicken. *Am. Rev. Respir. Dis.* 139, 1524–1530.
- Peacock, A. J., Pickett, C., Morris, K., & Reeves, J. T., 1990. Spontaneous hypoxaemia and right ventricular hypertrophy in fast-growing broiler chickens reared at sea level. *Comparative Biochemistry and Physiology -- Part A. Physiol.* 97, 537–541.
- Rao, J., & Jagadeesan, V., 1996. Lipid peroxidation and activities of antioxidant enzymes in iron deficiency and effect of carcinogen feeding. *Adv. Redox. Res.* 21, 103–108.
- Reeves, J. T., Ballam, G., Hofmeister, S., Pickett, C., Morris, K., & Peacock, A., 1991. Improved arterial oxygenation with feed restriction in rapidly growing broiler chickens. *Comp. Biochem. Physiol.* 99, 481–485
- Richards, M. P., 1996. Trace Mineral Metabolism in The Avian Embryo. *Poult. Sci.* 76, 152–164.
- Richards, M. P., Stock, M. K., & Metcalfe, J., 1991. Effects of brief hypoxia and hyperoxia on tissue trace element levels in the developing chick embryo. *Magnes. Trace. Elem.* 10, 305–320.
- Rouwet, E.V., Tintu, A.N., Schellings, M.W.M., Van Bilsen, M., Lutgens, E., Hofstra, L., Slaaf, D.W., Ramsay, G. & Le Noble, F.A.C., 2002. Hypoxia induces aortic hypertrophic growth, left ventricular dysfunction, and sympathetic hyperinnervation of peripheral arteries in the chick embryo. *J. Circ.* 105, pp.2791-2796.
- Rusu, I. G., Suharoschi, R., Vodnar, D. C., Pop, C. R., Vulturar, R., Istrati, M., Socaci, S. A., Morosan, I., Farcas, A. C., Kerezsi, A. D., Muresan, C. I., Pop, O. L., 2020. Iron Supplementation Influence on the Gut Microbiota and Probiotic Intake Effect in Iron Deficiency - A Literature-Based Review. *Nutrients.* 12, 1993.
- Scheele, C. W., Van Der Klis, J. D., Kwakernaak, C., Buys, N., & Decuypere, E., 2003. Haematological characteristics predicting susceptibility for ascites. 1. High carbon dioxide tensions in juvenile chickens. *Br. Poult. Sci.* 44, 476–483.

- Shlosberg, A., Bellaiche, M., Berman, E., Perk, S., Deeb, N., Neumark, E., & Cahaner, A., 1998. Relationship between broiler chicken haematocrit-selected parents and their progeny, with regard to haematocrit, mortality from ascites, and bodyweight. *Res. Vet. Sci.* 64, 105–109.
- Shlosberg, A., Bellaiche, M., Berman, E., David, A. B. E. N., Deeb, N., & Cahaner, A., 1998. Comparative Effects of Added Sodium Chloride, Ammonium Chloride, or Potassium Bicarbonate in the Drinking Water of Broilers, and Feed Restriction, on the Development of the Ascites Syndrome. *Poult. Sci.* 77, 1287–1296.
- Shinde, P. L., Ingale, S. L., Choi, J. Y., Kim, J. S., Pak, S. I., & Chae, B. J., 2011. Efficiency of inorganic and organic iron sources under iron depleted conditions in broilers. *Br. Poult. Sci.* 52, 578–583. <https://doi.org/10.1080/00071668.2011.607430>
- Singh, P. K., Shekhar, P., & Kumar, K., 2011. Nutritional and managemental control of ascites syndrome in poultry. *Int. J. Livest. Prod.* 2, 117–123.
- Smith, F. M., West, N. H. & Jones, D. R., 2000. Chapter 9- The Cardiovascular system. In: Sturkie's Avian Physiology. Ed: G. C. Whittow (Fifth edition), Canada, Academic Press, pp. 141-231.
- Stolz, J.L., Rosenbaum, L.M., Jeong, D. & Odom, T.W., 1992. Ascites syndrome, mortality, and cardiological responses of broiler chickens subjected to cold exposure. *Poult. Sci.* 71, 4.
- Sun, J., Liu, D., & Shi, R., 2015. Supplemental dietary iron glycine modifies growth, immune function, and antioxidant enzyme activities in broiler chickens. *Livest. Sci.* 176, 129–134. <https://doi.org/10.1016/j.livsci.2015.03.004>
- Squires, E. J., & Julian, R. J., 2001. The effect of dietary chloride and bicarbonate on blood pH, haematological variables, pulmonary hypertension, and ascites in broiler chickens. *Br. Poult. Sci.* 42, 207-212
- Taschetto, D., Vieira, S. L., Angel, C. R., Stefanello, C., Kindlein, L., Ebbing, M. A., & Sim, C. T., 2017. Iron requirements of broiler breeder hens. *Poult. Sci.* 96, 3920–3927.
- Tekeli, A., 2014. Effects of ascites (Pulmonary Hypertension Syndrome) on blood gas, blood oximetry parameters, and heart sections of broilers grown at high altitude. *J. Anim. Plant. Sci.* 24, 998–1002.
- Theil, E. C., 2004. Iron, ferritin, and nutrition. *Annu. Rev. Nutr.* 24, 327–343. <https://doi.org/10.1146/annurev.nutr.24.012003.132212>

- Timbermont, L., Haesebrouck, F., Ducatelle, R., Immerseel, F. Van, Timbermont, L., Haesebrouck, F., Ducatelle, R., & Immerseel, F. Van., 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis Necrotic enteritis in broilers: an updated review on the pathogenesis. *Avian Pathol.* 40, 341-347.
- Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., & Fortomaris, P., 2015. The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. *Avian Pathol.* 44, 430–435.
- Tukey, J. W., 1949. Comparing Individual Means in the Analysis of Variance. *Biometrics*, 5, 99-114.
- Van As, P., Elferink, M. G., Closter, A. M., Vereijken, A., Bovenhuis, H., Crooijmans, R. P. M. A., Decuypere, E., & Groenen, M. A. M., 2010. The use of blood gas parameters to predict ascites susceptibility in juvenile broilers. *Poult. Sci.* 89, 1684–1691. <https://doi.org/10.3382/ps.2009-00430>
- Weiss, D. J., 2010. Iron and Copper Deficiencies and Disorders of Iron Metabolism. In: Schalm's Veterinary Hematology. Ed: D. J. Weiss & K. J. Wardrop (6th edition), Iowa, Wiley-Blackwell, pp. 167-171.
- Wideman, R.F., 1997. Understanding pulmonary hypertension syndrome (ascites). Hubbard Farms Technical Report (pp. 1-6), Walpole, NH, USA.
- Wideman, R.F., 1998. Causes and control of ascites in broilers. 6- National Meeting on Poultry Proceeding, 33, 56- 85.
- Wideman, R. F., 2000. Cardio-Pulmonary Hemodynamic and Ascites in broiler chickens. *Avian. Poult. Biol.* 11, 21-43.
- Wideman, R. F., Fedde, M. R., Tackett, C. D., & Weigle, G. E., 2000. Cardiopulmonary function in preascitic (hypoxemic) or normal broilers inhaling ambient air or 100% oxygen. *Poult. Sci.* 79, 415–425.
- Wideman, R. F., Erf, G. F., & Chapman, M. E., 2001. Intravenous Endotoxin Triggers Pulmonary Vasoconstriction and Pulmonary Hypertension in Broiler Chickens. *Poult. Sci.* 80, 647–655.
- Wideman, R. F., Hooge, D. M., & Cummings, K. R., 2003. Dietary Sodium Bicarbonate, Cool Temperatures, and Feed Withdrawal: Impact on Arterial and Venous Blood-gas Values in Broilers. *Poult. Sci.* 82, 560–570.

- Wideman, R. F., Rhoads, D. D., Erf, G. F., & Anthony, N. B., 2013. Pulmonary arterial hypertension (ascites syndrome) in broilers: A review. *Poult. Sci.* 92, 64–83.
- Xiao, D.-S., Jiang, L., Che, L.-L., & Lu, L., 2003. Nitric oxide and iron metabolism in exercised rat with L-arginine supplementation. *Mol. Cel. Biochem.* 252, 65-72.
- Yilmaz-Dikmen, B., Sahan, U., Ipek, A., Aydin, C., & Kederli, E., 2014. Effect of oxygen supplementation in a hatchery at high altitude and growth performance of broilers reared at low altitude. *S. Afr. J. Anim. Sci.* 44, 350–359.
- Yilmaz, B. & Li, H., 2018. Gut Microbiota and Iron: The Crucial Actors in Health and Disease. *Int. J. Pharm.* 11, 1–20.
- Yu, B., Huang, W.J., & Chiou, W.S., 2000. Bioavailability of iron from amino acid complex in weanling pigs. *Anim. Feed Sci. Technol.* 86, 39–52.
- Zafra, R., Pérez, J., Pérez-Écija, R. A., Borge, C., Bustamante, R., Carbonero, A., & Tarradas, C., 2008. Concurrent aspergillosis and ascites with high mortality in a farm of growing broiler chickens. *Avian Dis.* 52, 711–713.
- Zhang, L. Y., Lu, L., Zhang, L. Y., & Luo, X. G., 2016. The chemical characteristics of organic iron sources and their relative bioavailabilities for broilers fed a conventional corn–soybean meal diet. *J. Anim. Sci.* 94, 2378–2396. <https://doi.org/10.2527/jas.2016-0297>

Appendix 1

		Pen	Treatment	MIDDLE ISLE	Pen	Treatment		
Block 1		1	2		96	5	Block 16	
		2	5		95	6		
		3	1		94	2		
		4	3		93	1		
		5	6		92	3		
		6	4		91	4		
Block 2		7	2		90	5	Block 15	
		8	4		89	6		
		9	3		88	4		
		10	1		87	3		
		11	5		86	2		
		12	6		85	1		
Block 3		13	3		84	3	Block 14	
		14	4		83	2		
		15	6		82	5		
		16	2		81	1		
		17	1		80	6		
		18	5		79	4		
Block 4		19	6		78	1	Block 13	
		20	3		77	2		
		21	4		76	3		
		22	5		75	5		
		23	1		74	6		
		24	2		73	4		
Block 5		25	6		72	5	Block 12	
		26	5		71	1		
		27	3		70	4		
		28	2		69	3		
		29	1		68	2		
		30	4		67	6		
Block 6		31	6		66	4	Block 11	
		32	4		65	2		
		33	5		64	5		
		34	1		63	1		
		35	2		62	3		
		36	3		61	6		
Block 7		37	3		60	5	Block 10	
		38	5		59	1		
		39	1		58	4		
		40	4		57	6		
		41	2		56	3		
		42	6		55	2		
Block 8		43	6		54	2	Block 9	
		44	3		53	5		
		45	4		52	3		
		46	1		51	1		
		47	2		50	4		
		48	5		49	6		