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Dietary supplementation of di- and trimethylglycine to attenuate the effects of pulmonary hypertension syndrome (ascites) in broilers

by

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DECLARATION

I, Bonita Nortjé, declare that the dissertation, which I hereby submit for the MSc. (Agric) Animal Nutrition degree 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: . . . . . .

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DEDICATION

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LIST OF ABBREVIATIONS

4-HNE	4-Hydroxy-2-nonenal
AA	Amino Acid
AS	Ascites/Ascites Syndrome
ADG	Average Daily Gain
ADP	Adenosine Diphosphate
AHI	Ascites Heart Index
AHI_{DM}	Ascites Heart Index on a Freeze-Dried Matter Basis
AHI_{FM}	Ascites Heart Index on a Fresh Matter Basis
AdjFCR	Adjusted Feecd Conversion Ratio
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate Protein Kinase
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BHMT	Betaine Homocysteine Methyltransferase
BMW	Breast Meat Weight
BMY	Breast Meat Yield
BW	Bodyweight
BWG	Body Weight Gain
Ca^{2+}	Calcium
CH_3	Methyl group
CHF	Congestive Heart Failure
ChoCl	Choline Chloride
CF	Crude Fiber
Cl	Chloride
CP	Crude Protein
CTNNT-2	Cardiac Troponin Type 2
CumAdjFcr	Cumulative Adjusted Feed Conversion Ratio
CumBWG	Cumulative Body Weight Gian
CumFI	Cumulative Feed Intake
CumFCR	Cumulative Feed Convertion Ratio
CYS	Cysteine

Da	Dalton
DL-MET	DL-Methionine
DM	Dry Matter
DMG	N, N – Dimethylglycine
DNA	Deoxyribonucleic Acid
DP	Dressing Percentage
e^-	Electron
ED	Embryonic Day
EE	Ether Extract
EST	Eggshell Temperature
EWL	Egg Weight Loss
FA	Fatty Acid
FCR	Feed Conversion Ratio
FI	Feed Intake
GE	Gross Energy
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
GSSG	Oxidised Glutathione/Glutathione Disulfide
H^+	Proton
H_2O_2	Hydrogen Peroxide
HC	Homocysteine
IL	Intermittent Lighting
K^+	Potassium
KOH	Potassium Hydroxide
LVS	Left Ventricle plus Septum
LVS/BW	Left Ventricle plus Septum Ratio on a fresh matter basis
LYS	Lysine
MCC	Medincine Control Council
MDA	Malondialdehyde
MI	Myocardial Infarction
Min	Minimum
MS	Methionine Synthase
mtDNA	Mitochondrial DNA

mtRNA	Mitochondrial RNA
MW	Molecular Weight
MT	Machine Air Temperature
Na^+	Sodium
Na/K-ATPase	Sodium-Potassium Adenosine Triphosphatase
NADPH	Nicotinamide Dinucleotide Phosphate
NAFLD	Non-alcoholic Fatty Liver Syndrome
NC	Negative Control
NFE	Nitrogen Free Extract
NO	Nitric Oxide
$O_2^{\cdot-}$	Superoxide Anion Radical
OH	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
P	Phosphorus
PAH	Pulmonary Arterial Hypertension
PC	Positive Control
Pcr/Cr ratio	Phosphocreatine-to-Creatine ratio
PEF	Performance Efficiency Factor
PH	Pulmonary Hypertension
PHS	Pulmonary Hypertension Syndrome
PFK-2	Phospho-fructo-kinase-2
PKCζ	Protein Kinase C zeta
PS	Processing Stage
RH	Relative Humidity
RHF	Right Heart Failure
RBC	Red Blood Cells
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RV	Right Ventricle
RV/BW	Righ Ventricle to Body Weight Ratio on a fresh matter basis
RVF	Right Ventricular Failure
RV/TV ratio	Right Ventricle to Total Ventricle Ratio on a fresh matter basis

SAA	Sulphur Amino Acids
SAM	S-adenosylmethionine
SH	Thiol Group
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
THFMT	Methyl-tetrahydrofolate-homocysteine-methyltransferase
THFT	Tetrahydrofolate Cycle
TNF-α	Tumour Necrosis Factor Alpha
TMG	N, N, N – Trimethylglycine (betaine)
TSAA	Total Sulfur Amino Acids
TV	Total Ventricle
TV/BW	Total Ventricle to Body Weight Ratio on a freshmatter basis
VSMC	Vascular Smooth Muscle Cells
WW	Wing Weight
w/w %	Weight per Weight Percentage
WY	Wing Yield
YFBM	Yolk Free Body Mass

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ABSTRACT

Both N, N-dimethylglycine (DMG) and trimethylglycine (Betaine; TMG) are intermediary metabolites in the pathway of choline-to-glycine metabolism. DMG possess non-enzymatic anti-oxidant properties and have been implicated in enhanced oxygen utilisation while TMG posses osmolytic, methyl-donating, lipolytic and also potentially anti-oxidant-like properties. Recently, DMG has been supplemented to broiler chicken diets as a means to alleviate pulmonary hypertension syndrome (PHS or ascites) in rapidly growing broiler chickens. The present trial aimed to evaluate the effects of these different methylamines on production and slaughter performance and ascites-relates traits in broilers, and to assess oxidative stress in broilers with ascites.

A challenge study was conducted where broilers were subjected to ascites-inducing conditions (AIC) from embryonic stage until 40 days of age. These AIC included high (>38.8°C) eggshell temperatures during mid-late incubation (Embryonic Day 11 (ED_{11}) to Embryonic Day 18 (ED_{18})) and cold temperatures during brooding which increases the bird's relative oxygen requirements and basal metabolic rate, as a means to incite pulmonary hypertension syndrome (PHS). A total of 1,632-day-old separate sex (males vs. females) broiler chicks were distributed equally amongst 96 pens. Birds were allocated to treatments based on a factorial arrangement comprising of two sexes and four dietary treatment groups of which each group had 12 replicate pens with 17 birds each. The treatment diets comprised of the following: a negative control group (NC; basal diet which was low in supplemental methionine (0.16 % methionine) and no additional choline chloride; a betaine group (NC + Betaine, where 1.042 g/kg of pure betaine (100 %) was added to the basal diet); a DMG group (NC + DMG, where equal molar equivalents of DMG (1.380 g/kg) was added to the basal diet); a positive control (PC) group (basal diet which contained additional levels of DL-methionine (0.21 % methionine) and additional choline chloride (250mg/kg)). Both DMG and TMG were fed at 100 % methyl group donors, i.e. concentrations higher than recommended levels. The experiment was divided into three phases: a starter phase (1-14 d), a grower phase (15-28 d) and a finisher phase (29-40 d). All birds were fed basal diet consistinf of maize-soybean meal. Animal performance was determined at weekly intervals. At termination of the study (d40 & 41), slaughter performance traits were determined on two birds per replicate. All birds were monitored twice daily for overt signs of PHS and mortality were recorded. During both processing stage 1 (d 20 & 21) and 2 (d 40 & 41) of the experiment, the risk of heart failure associated with the

abovementioned dietary treatments was tested followed by gross and biochemical investigation from randomly selected individuals to determine the progression of PHS.

Overall, body weight (BW), body weight gain (BWG) and cumulative BWG (CumBWG) were consistently higher in birds fed dietary betaine in comparison with birds fed the negative control (basal) or DMG-supplemented diets, whereas dietary treatment appeared to have little to no effect on feed intake. Weekly and cumulative feed conversion ratios (cumFCR) were consistently lower (P <0.05) in the betaine group compared with both the NC and DMG-supplemented groups, but only during the starter period (up to 14 d of age). It was also demonstrated that the use of betaine significantly (P <0.05) improved carcass parameters such as dressing percentage, breast meat weight and breast meat yield in comparison with both the NC (basal) and DMG-supplemented diet. Dietary treatment had no effect on any of the abdominal fat parameters. Furthermore, there was a highly significant (P <0.05) response following dietary betaine supplementation in pre-slaughter live BW, carcass weight, leg portion- and wing weight but not yield, in contrast to birds fed DMG. CumBWG, cumulative feed intake (CumFI) and CumFCR were significantly better (P <0.0001) for the commercial Ross male broiler chickens in contrast to the females. The heavier male broilers had significantly higher pre-slaughter live body- and carcass weights (P <0.0001), lower abdominal fat content (P <0.0001), and higher carcass cut (leg portion and wing) yields (P <0.05) than the females at slaughter.

The incidence of PHS was not significantly different in broilers fed either of the control diets as compared to the betaine-supplemented group although a 19 % lower mortality rate was observed (P >0.05) in the betaine-supplemented group compared to the NC treatment group. Broilers fed dietary betaine was less likely to succumb to heart failure due to PHS compared to birds fed dietary DMG (i.e. 33% lower mortality rate) (P <0.05). Male broilers showed a ~4 times higher (P <0.001) ascites mortality rate compared to their female counterparts, confirming male broiler chickens to be more prone to develop ascites due to their inherently faster growth rate.

The beneficial effects of betaine supplementation were also evident by a lower concentration of total serum homocysteine concentration (an independent risk factor for cardiovascular disease) compared to the control and DMG-supplemented groups during both processing stages. The level of 4-hydroxy-2-nonenal (4-HNE), an indicator of lipid peroxidation, was significantly lower for both the betaine-supplemented and PC treatment groups compared to either the NC or DMG treatment groups during processing stage 1 (d 20 & 21) (P <0.05). No significance was observed between the different treatment diets fed at 40 and 41 days of age (P >0.05). TBARS, an additional indicator of lipid peroxidative damage, was significantly lower for the DMG-supplemented group at

40 and 41 days of age compared to birds that received either the betaine-supplemented or Control diets. A slightly elevated AMPK concentration following oxidative-induced stress was obtained in birds fed dietary betaine at 40 and 41 days of age compared to birds fed either the DMG-supplemented or either of the control diets; however this difference was not significant ($P>0.05$).

In conclusion, current data from the present study demonstrates the beneficial effects of supplementing diets with betaine in contrast to its methyl derivative, DMG, and methyl-inadequate diets on production and slaughter performance and attenuating PHS in broilers. Oxidative stress contributed to the pathogenesis of PHS in broilers in this study and supplementation of betaine can, to some extent, prevent oxidative damage and alleviate cumulative mortalities in broilers that develop PHS.

CHAPTER 1: INTRODUCTION AND AIMS

1.1 Introduction

The commercial broiler industry has expanded significantly over the past several decades and has evolved into an efficient mass food production system. Successful genetic breeding programmes in conjunction with improvements in nutrition and management have resulted in increased bird growth and production efficiency that has greatly driven the poultry industry's progress. The primary focus of the broiler industry continues to be maximising profits through improvements in feed conversion ratio, and carcass yield while maintaining overall bird health and welfare. Any interference with the bird's health, such as the development of metabolic diseases, will decrease profitability because of the deterioration in production value due to an increase in mortality rate, impaired nutrient utilisation, or a rise in condemned carcasses at slaughter. Owing to enhanced bird growth, modern broilers are capable of reaching their market weights much earlier than their 1950's predecessors (Havenstein *et al.*, 2003a, b). However, these advances have not been adequately balanced with similar improvements in organ development needed to support such dramatic fast growth, resulting in an uneven dichotomy of supply and demand where growth and body weight often exceed heart and lung capacity (Decuypere *et al.*, 2000). Consequently, the birds are able to grow at an unsustainable rate and suffer increased incidences of metabolic diseases such as pulmonary hypertension syndrome (PHS or ascites), which has become prevalent within the poultry industry worldwide.

Even though the highest incidence of ascites is mostly evident when the birds are five to six weeks of age due to increased metabolic stress as a result of bird growth exceeding the rate at which oxygen can be supplied to the organs and tissues, the aetiology of this syndrome is believed to be initiated as early as the embryonic stage (Decuypere *et al.*, 2000; De Smit *et al.*, 2005; Baghbanzadeh & Decuypere, 2008). Although broiler grow-out time has decreased dramatically over the past 50 years, the incubation time for eggs during which the embryo develops has remained the same (Havenstein *et al.*, 2003a, b) although the requirements of the chicks may have changed, with the temperature for optimal incubation being between 37 to 38°C. High eggshell temperature (EST) during incubation in conjunction with cold brooding temperatures have been indicated with decreased post-hatch chick performance, leading to diminished cardiovascular and pulmonary development and function (Balog, 2003; Leksrisompong, 2005; Leksrisompong *et al.*, 2009, Molenaar *et al.*, 2011). Therefore, failure to maintain the embryos within the required temperature range can result in reduced organ development, specifically decreased heart weights that can lead to

a smaller heart-to-body weight-ratio and, not surprisingly, increased incidence of ascites as the bird grows (Leksrisompong *et al.*, 2007; Molenaar *et al.*, 2011). The smaller the heart, the harder it will have to work in order to supply the high amount of oxygen needed to meet the high oxygen demand of the fast-growing tissues during the rearing period.

The primary occurrence of PHS in broilers is mostly attributed to a hypoxemic condition (Julian, 1993). Modern broilers are particularly susceptible to hypoxia because of a combination of rapid growth and their proportionately underdeveloped cardio-pulmonary system (Havenstein *et al.*, 2003a, b). Furthermore, male meat-type poultry lines are considered more susceptible to ascites because of their higher growth rates compared to females of the same type (Balog, 2003). Next to rapid growth, any external factor that increases the bird's need for oxygen to the tissues may contribute in the cascade of organ/heart failure, ascites, and death. High altitudes have been noted to increase the incidence of ascites (Julian, 1993). A strong association has also been demonstrated between cold environmental temperatures and heart failure (Julian *et al.*, 1989b) and ascites is well known to be more prevalent during winter compared to summer. When a chick is placed outside its temperature comfort zone, its demand for oxygen by the organs increases (Julian *et al.*, 1989b). This high oxygen requirement is exasperated by limited ventilation during winter (in an attempt to retain heat within the broiler house), which lowers the oxygen concentration and increases the potential for the development of ascites.

Recent work has revealed that a high level of reactive oxygen species (ROS) is a common feature shared by many individuals succumbing to ascites (Olkowski, 2007a; Xi *et al.*, 2012). Elevated levels of ROS have also coincided with a marginal antioxidant capacity in ascitic broiler chickens (Sibrian-Vazquez *et al.*, 2010; Xi *et al.*, 2012) as well as the negatively influence of cellular ion pumps and subsequent homeostasis (Cronje, 2005). These birds become more susceptible to ascites due to oxidative damage to the cardio-pulmonary system by lipid peroxidative end-products (Bayés *et al.*, 2001; Sibrian-Vazquez *et al.*, 2010), which may further aggravate the birds existing hypoxemic status even more (Bottje & Wideman, 1995). Normally, these chemical radicals are metabolised by the enzymatic antioxidants (i.e. superoxide dismutase and glutathione peroxidase) and non-enzymatic antioxidants (glutathione and α-tocopherol).

Glutathione peroxidase in particular plays an important role in mitochondrial antioxidant protection following oxidative stress caused by PHS (Cawthon *et al.*, 1999). In cases of severe oxidative stress, the body has evolved several compensatory mechanisms in order to protect cells from the consequences of limited oxygen supply and metabolic substrate deprivation (Mungai *et al.*, 2011). One such important system is the adenosine monophosphate protein kinase (AMPK)

system. AMPK may have a potential cardio-protective role in the ascitic broiler due to its function in restoring the energy imbalance brought about during oxidative stress (Young *et al*., 2005; Fisslthaler & Fleming, 2009; Shirwany & Zou, 2010). In addition, AMPK has also been shown to trigger pathways that inhibit the production of ROS (Young *et al*., 2005).

In the past, ascites has been addressed mainly by means of feed restriction (qualitative vs. quantitative) or through changes in lighting regimes (Olkowski, 2007a). However, these methods do not appear to be very effective since they occur at the expense of optimal bird growth and performance. Although breeding for ascites resistant broiler lines would appear to be a more permanent solution (Balog, 2003), it may take years of genetic selection to achieve, during which billions of rands may be lost to the poultry industry. In this regard, optimisation of the diet appears to be a more feasible and contemporary solution to the persistent problem of ascites.

1.2 Motivation of the study

As a methyl group donor, trimethylglycine (TMG; betaine) has been demonstrated to be more efficient than methionine, choline, and possibly dimethylglycine (DMG). Through sparing these methyl group donors, betaine may indirectly make them more available for other important physiological functions in the body, such as protein synthesis of muscle tissues and/or the breakdown of lipid (Eklund *et al*., 2005; Ratriyanto *et al*., 2009). Furthermore, due to its methyl donating properties, betaine may also be involved in immediate and long-term lowering of total plasma homocysteine (HC) (Bidulescu *et al.*, 2007), which is well known to be an independent risk factor for cardiovascular related disease such as PHS (Bayés *et al.*, 2001; Sibrian-Vazquez *et al.*, 2010). Elevated levels of HC may be coupled with ROS production (Bayés *et al.*, 2001; Kumar *et al.*, 2012), facilitating oxidative damage to the pulmonary and circulatory system (Bayés *et al.*, 2001; Sibrian-Vazquez *et al.*, 2010). By lowering HC and resulting in reduced ROS, betaine may be able to reduce oxidative induced stress.

Betaine may also exert protective mechanisms during PHS through its action as an organic osmolyte during periods of hyperosmotic stress, thereby maintaining cellular water balance by regulating membrane ATPase's (Craig, 2004). During osmotic stress, cells lose water and the subsequent increase in intracellular ions disrupts protein and enzyme structure and function as well as ATP production and ultimately cell death can occur if not corrected (Craig, 2004). Through accumulation of osmotically stressed cells, betaine saves the energy necessary to pump ions across the cell membrane via activating the Na^+/K^+ pump (Eklund *et al*., 2005) and reducing the energy

requirements for maintenance, thereby allowing more energy for other important functions such as growth and production. Furthermore, the energy saved may stimulate cell proliferation that in turn may promote digestion and absorption within the intestinal tract, thus indirectly supporting intestinal growth and development. Betaine may serve a similar function during ascites since poor osmoregulation following oxidative stress has been shown to be linked with PHS in broiler chickens. Finally, it has also been suggested that betaine may be considered an anti-stress agent (Saunderson & MacKinlay, 1990), thereby reducing the deleterious effects following oxidative induced stress by making the cells more resilient to osmotic stress during PHS.

1.3 Aims and hypotheses

The main aim of the current trial was to evaluate different methyl derivatives of the amino acid glycine, dimethylglycine (DMG) and trimethylglycine (TMG, betaine), in alleviating pulmonary hypertension syndrome (PHS) in broiler chickens. It was postulated that both DMG and betaine (TMG) will be equally effective in attenuating progression towards PHS in rapidly growing broilers under ascites-inducing conditions (AIC). This study was also conducted to compare the difference in efficacy between these methyl derivatives on growth and carcass performance. It was postulated that dietary betaine would improve growth and carcass performance to a greater extent than its methyl derivative DMG due to its methyl donating and osmolytic properties. Furthermore, it was hypothesised that male broiler chickens will be more prone to develop PHS compared to their female counterparts. This study was also conducted to compare the differences in growth and carcass performance between the different sexes under AIC such as high incubation and cold temperatures at brooding.

To summarise, the following null hypotheses, each with its respective alternative hypothesis, have been tested during this study:

H0: Supplementation of trimethylglycine (betaine) or dimethylglycine to a diet deficient in methyl groups will not improve growth and carcass performance and reduce the incidence of pulmonary hypertension syndrome (PHS) in broilers.

H1: Supplementation of trimethylglycine (betaine) or dimethylglycine to a diet deficient in methyl groups will improve growth and carcass performance and reduce the incidence of pulmonary hypertension syndrome (PHS) in broilers.
H0: Supplementation of trimethylglycine (betaine) to a diet deficient in methyl groups will not be more effective than supplementation of dimethylglycine in improving growth and carcass performance and reducing the incidence of pulmonary hypertension syndrome in broilers.

H1: Supplementation of trimethylglycine (betaine) to a diet deficient in methyl groups will be more effective than supplementation of dimethylglycine in improving growth and carcass performance and reducing the incidence of pulmonary hypertension syndrome in broilers.

H0: There will be no difference in the incidence of pulmonary hypertension syndrome (PHS) between male and female broilers.

H1: The incidence of pulmonary hypertension syndrome (PHS) will differ between male and female broilers.

H0: There will be no difference in growth and carcass performance between male and female broilers reared under ascites-inducing conditions such as high incubation and cold temperatures at brooding.

H1: There will be differences in growth and carcass performance between male and female broilers reared under ascites-inducing conditions such as high incubation and cold temperatures at brooding.

CHAPTER 2: LITERATURE REVIEW

2. Pulmonary hypertension syndrome (PHS)

2.1 The economic impact of pulmonary hypertension syndrome (PHS)

The modern chicken (*Gallus gallus domesticus)* has been vigorously selected for certain desirable characteristics in order to produce birds that meet modern food production needs (Wheeler & Campion, 1993; Kalmar, 2011). Fast growth allowing market weight to be achieved quicker and a favourable feed conversion ratio are considered the most important factors contributing to economically efficient broiler production (Druyan *et al*., 2008). Further advances in nutritional physiology and general management have enhanced production efficiency and allowed for maximal expression of improved genetic traits (Lorenzoni & Ruiz-Feria, 2006; Baghbanzadeh & Decuypere, 2008). Additionally, a comprehensive understanding regarding the response of chickens to their environment has also led to optimal housing conditions to promote bird growth (Wheeler & Campion, 1993; Rauw *et al.*, 1998; Kalmar, 2011). These factors have all contributed greatly towards meat-type poultry lines exhibiting superior genetic potential with regard to growth rate, conformation, and breast yield (Balog, 2003) and has allowed the modern broiler to reach their market weight in approximately 60% less time than 40 years ago (Lorenzoni & Ruiz-Feria 2006; Bagbanzadeh & Decuypere, 2008) while consuming 20% less feed (Havenstein *et al.*, 2003b). Along with increases in growth and production, the metabolic rate of the modern broiler has also more than doubled over the past few decades (Havenstein *et al*., 1993; Lubritz & McPherson, 1994).

However, the outstanding progress made in genetic selection has not been without its negative consequences, such as an increase in the occurrence of several metabolic related conditions including heart failure and leg problems due to rapid growth (Rauw *et al.*, 1998; Nian, 2008; Kalmar, 2011). Enhanced growth has also been accompanied by a comparable increase in broiler mortality rates (Havenstein *et al*., 2003a) which may have resulted due to higher incidences of specific physiological insufficiencies (Nian, 2008). It is thus clear that the emphasis of breeding programmes has focussed mainly on traits for improving efficiency of production (i.e. economic traits such as growth rate, feed efficiency, and age of slaughter) (Havenstein *et al.*, 1993a, b), while overlooking traits critical for supporting broiler welfare (Nian, 2008). Consequently, this has led to the prevalence of one of the most widespread and costly metabolic diseases amongst commercial poultry producers, known as pulmonary hypertension syndrome (PHS) (Balog, 2003). In the

scientific literature, PHS, pulmonary arterial hypertension (PAH), and ascites syndrome/ascites (AS) are commonly used synonymously (Wideman *et al.*, 2013) due to their overlapping causes and symptoms.

The tremendous increase in the incidence of ascites worldwide over the past few years is well known to be the primary cause of death in meat-type poultry (Luger *et al.*, 2001; Daneshyar *et al.*, 2009; Hassanzadeh, 2009). Generally, death due to ascites is greatest after 4 weeks of bird age, with the peak incidence occurring during the fifth or sixth week of the growing period just before slaughter (Gupta, 2011), or even during transport to the slaughterhouse (Nijdam *et al.*, 2006; Kalmar, 2011). According to the 1996 World Broiler Ascites Survey, ascites was determined to affect 4.7% of the broiler population worldwide (Maxwell & Robertson, 1997) and more recent investigations indicated that ascites could account for over 25% of total mortalities (Balog, 2003; De Smit et al., 2005; Guo *et al.*, 2007; Daneshyar *et al.*, 2009). Apart from the high mortality rates observed in broiler production systems, ascites has also been associated with diminished weight gain (Daneshyar *et al.*, 2009), as well as condemned carcasses accounting for up to 5 to 7% at slaughter (Han *et al.*, 2005; Gupta *et al.*, 2011). Considering that an estimated amount of 106 million tonnes of broiler carcasses are expected to be produced worldwide (The Poultry Site, 2013) it is relatively evident that as little as 1% of mortality due to ascites can have a significant impact on economic loss (Lubritz & McPherson, 1994; Maxwell & Robertson, 1997; Druyan *et al.*, 2009).

Being the most prevalent cardiovascular syndrome in broilers, PHS has a marked detrimental impact on production performance and economic efficiency of broiler production systems, making it of global importance to the commercial industry. It may further be expected that the incidence of ascites will persist due to the continuous on-going genetic and nutritional improvements of modern broilers in the areas of growth rate and feed efficiency (Julian *et al.*, 1986). Next to financial cost, broiler ascites syndrome will also remain an important welfare concern (Lubritz & McPherson, 1994; Maxwell & Robertson, 1997; Balog, 2003; Baghbanzadeh & Decuypere, 2008; Hassanzadeh, 2009) because of breathing difficulties that are distressing to the animal (Beker *et al.*, 1995; Aksit *et al.*, 2008; Kalmar, 2011). Furthermore, with poultry meat having the highest per capita consumption owing to its affordability and reputation as a healthy food option (Du Toit, 2005), it can be expected that on a global scale poultry consumption will increase at a rate that will exceed its production (Makube & Janovsky, 2005). Owing equally to its nature and its incidence, ascites will therefore remain a serious concern to poultry producers worldwide (Kalmar, 2011) because of the considerable losses of heavy, fast-growing broilers (Balog, 2003; Baghbanzadeh & Decuypere, 2008; Hassanzadeh, 2009) and the economic consequences of losing these birds (Kalmar, 2011).

2.2 Pathogenesis of pulmonary hypertension syndrome (PHS)

Undoubtedly, the most prevalent cause of ascites in broilers has been attributed to PHS (Julian; 1993; Odom, 1993; Tankson *et al.*, 2001; Geng *et al*., 2004; Julian, 2005). Two different categories of PHS can be distinguished, with both ultimately resulting in ascites. First, primary or spontaneous PHS can arise in fast-growing broilers as a result of insufficient oxygen and blood capillary capacity in their lungs, which is unrelated to any known organ dysfunction (i.e. heart or lung pathologies) (Julian *et al.*, 1987; Julian *et al.*, 1989b). Conversely, secondary PHS may occur in situations where hypoxemic broilers experience an increase in blood volume (hypervolemia) or if a lung pathology or dysfunction exists (Julian, 1987; Julian *et al.*, 1989a, b).

The modern broiler chicken seemingly has more genetic potential for growth than providing a sufficient amount of oxygen in order to sustain this rapid growth rate (Druyan, 2012). Numerous exhaustive investigations have revealed that the central aetiology of this metabolic disorder is believed to be the result of a general hypoxemic condition (i.e. systemic hypoxia) (Julian, 1998; Currie, 1999; Huchzermeyer; 2012). Hypoxia, which is characterised by abnormally low oxygen content in a tissue or organ, normally results due to a drop in the partial pressure of air oxygen, for example at high altitudes and reduced oxygen percentage in the air capillaries of the lungs. Because of the bird's inherently fast growth rate, which requires a high resting metabolic rate and an increase demand for oxygen (Druyan, 2012), it would be reasonable to think that this would be the primary cause leading to ascites. However, it is believed that the most critical trigger of ascites is brought about due to the imbalance between the bird's oxygen requirement and supply in order to sustain their fast rate of growth (Wideman, 2000; Balog, 2003; Decuypere *et al.*, 2005; Druyan *et al*., 2007; Khajali *et al*., 2007; Baghbanzadeh & Decuypere, 2008).

Various reports have documented the general pathogenesis of PHS very well (**Figure 2.1**) (Julian, 1993; Shlosberg *et al.*, 1998; Currie, 1999; Wideman, 2001; Balog, 2003; Bagbanzadeh & Decuypere, 2008). Although PHS accounts for most cases of ascites in commercial broilers, the aetiology of this syndrome appears to be rather complex (Balog, 2003; Wideman *et al.*, 2013). The

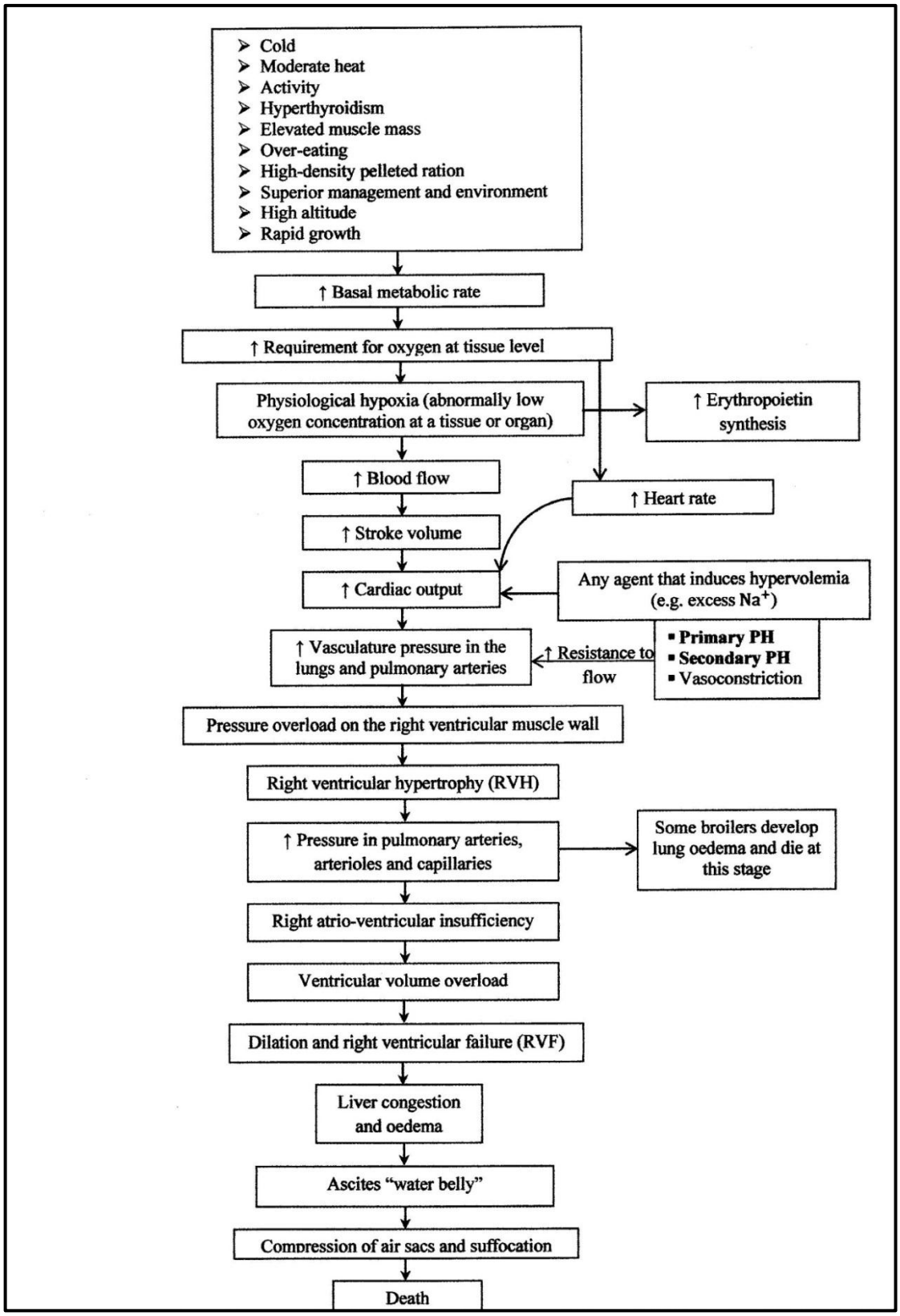

Figure 2.1: Physiological and pathophysiological factors affecting ascites syndrome in chickens [Adapted from Julian (1993), Bagbanzadeh & Decuypere (2008), and Huchezermeyer (2012)]

lack of oxygen at tissue level required for general metabolism ultimately triggers a cascade of compensatory mechanisms in order to rectify this imbalance (Julian, 1998; Currie, 1999). However, this interaction of an often-fatal cascade of events ultimately leads towards PHS, which in turn culminates in ascites incidence (Mirsalimi *et al*., 1992, 1993; Julian & Squires, 1994; Julian, 1998; Currie, 1999).

The pulmonary circulation of the clinically healthy broiler normally operates at relatively low hydrostatic gradients (<22 mmHg pulmonary arterial pressures (PAP)) to minimise the threat of fluid filtration into the gas exchange spaces by maintaining a low resistance to blood (Wideman *et al.,* 2013). Following an oxygen deficit, such as during hypoxia, the arteries of the lungs are progressively stimulated to contract (pulmonary vasoconstriction) (Huchzermeyer, 2012) while the heart responds by increasing its output of deoxygenated blood to the lungs for oxygenation (Olkowski *et al.*, 1998; Druyan, 2012). This brings about an increase in heart rate because of the insufficient supply of oxygen, which eventually results in an abnormally high blood pressure that is required to push the blood through the capillaries in the lungs (Silversides *et al*., 1997; Wideman *et al*., 1999; Druyan *et al*., 2007). Because of the rapid flow of red blood cells (RBC) through the pulmonary vasculature, the amount of time available for gas exchange is decreased and the cells are unable to achieve full blood-gas equilibration (Wideman *et al.,* 2013). Consequently, the blood exiting the lungs into the systemic circulation also has a lower than normal oxygen partial pressure (hypoxemia) and a higher than normal carbon dioxide (CO_2) partial pressure (hypercapnia), which causes the apparently healthy broilers to develop PAH/PH (Fracoisini *et al.*, 2012; Wideman *et al.*, 2013). With prolonged hypoxia, the body will also attempt to increase its oxygen carrying capacity of the blood via stimulating red blood cell (erythrocyte) production; however, this results in a higher haematocrit and an increase in blood viscosity that ultimately exacerbates the problem (Silversides *et al*., 1997; Aftab & Khan, 2005).

Persistent engorgement of the pulmonary vascular channels with blood to an extent where they become essentially nondistendable and nonrecruitable or excessive vascular tone within the primary resistant vessels (pulmonary arterioles) (Wideman *et al.,* 2013) subsequently forces the heart (specifically the right ventricle) to contract more vigorously. In order to overcome an oxygen deficiency and to cope with the high venous return (Aftab & Khan, 2005), the resultant cardiac output to the lungs is subsequently increased, however the modern bird is incapable of handling such a rapid increase in cardiac output (Julian; 1993). This may be greatly attributed to the broiler's pulmonary vasculature lacking functional elasticity that is generally required for maintaining normal cardiac output (Wideman & Kirby, 1995b, Wideman, 2001; Franciosini *et al.*, 2012) so that

broilers with the most restrictive pulmonary vascular capacity are more susceptible to pulmonary hypertension (Wideman & Bottje, 1993; Wideman, 2000; Wideman *et al.*, 2013). Under hypoxic conditions, the resultant increase in workload causes an increased pressure load on the right ventricular wall that causes it to distend (Olkowski *et al.*, 1998a; Currie, 1999). The ratio of the right ventricle to total ventricle mass can therefore be used as a measure of increased workload on the right heart ventricle due to this enlargement or damage (Julian *et al.*, 1989a, b). Owing to ventricular wall stress, parallel sarcomeres are added to the cardiac muscle cells resulting in thickening (hypertrophy) of the right ventricular wall, which in turn elevates the pressure in the pulmonary arteries, arterioles, and capillaries of the lungs, causing additional hypertrophy (Olkowski *et al.*, 1998a; Druyan, 2012). Meanwhile the right atrio-ventricular valve thickens and starts to leak, aggravating the excess pressure problem even more by admitting excess volume. This may be partly attributed to the thickened valve being less effective and partly because of an increase backpressure from the pulmonary arteries and right ventricle (Druyan, 2012). Consequently, the right side of the heart dilates (Julian, 1998; Currie, 1999; Balog, 2003; Cisar *et al*., 2004) and the wall-muscle cells lengthen by producing longitudinally arranged sarcomeres. The succeeding rise in venal blood pressure continues to raise the pressure overload in the heart until valvular insufficiency (Julian, 2000) and congestive or right ventricular failure result, causing a drop in cardiac output and pulmonary hypertension. This is followed by a marked pressure increase in the right atrium, sinus venoses, vena cava, and portal veins that leads to liver congestion and liquid seepage from the liver into the abdominal cavity (oedema), as well as fluid leakage from the veins and into the pericardium (pericardial effusion) (Arce *et al.*, 1992; Olkowski *et al*., 2003). This pathology of the accumulation of transudate fluid within the abdominal cavity has led to the common name of “water belly” and hence ascites for describing the syndrome (Balog, 2003). Finally, pressure exerted on the air sacs in conjunction with congestive heart failure leads to unimproved cardiac function and finally death (Silversides *et al*., 1997; Bottje *et al*., 1998; Julian, 1998). Consistent available evidence supports the hypothesis that terminal ascites is therefore the end-result of a pathophysiological progression initiated by the elevated blood pressure exerted on the pulmonary vascular system (pulmonary hypertension) (Wideman *et al.*, 2013). Therefore, it is important to point out that a large number of organs (i.e. the heart, liver, lungs, etc.) are affected by PHS, of which the heart is the most important (Bagbanzadeh & Decuypere, 2008).

In acute cases of PHS, clinical signs may not be obvious. Under these conditions, the birds may succumb to a sudden death and may frequently be found on their backs (Franciosini *et al.*, 2012). This results due to lung oedema secondary to PH (Julian, 2005). The symptoms are visible

when right heart failure (RHF) occurs, as can be distinguished by measuring the RV/TV ratio of the bird as well as other visible signs (including fluid accumulation in the abdominal cavity and pericardial sac). If these individuals survive for several days they are likely to develop ascites, which is characterised by the distinct yellowish clear liquid observed in the peritoneal cavity with the presence of fibrin clots (Franciosini *et al.*, 2012). When alive, these birds are of normal size but may show signs of cyanosis (especially around the comb and wattles) and they may be in a state of respiratory distress (Julian, 2005) due to physical restriction of the large abdominal air sacs (Franciosini *et al.*, 2012).

2.3 Aetiology

2.3.1 Predisposing factors to heart failure

Despite the modern meat-type chicken being inherently predisposed to ascites, the main factor contributing to ascites susceptibility still appears to be the result of an altered physiology because of their superior rate of growth (Nian, 2008). Furthermore, interactions between genetics and other secondary factors (such as nutrition, environment, management, and bird anatomy and physiology) can promote the development of ascites (Julian, 1998), since their actions converge in causing an increase of oxygen requirement, thereby exacerbating the bird's hypoxic status (Khajali *et al*., 2007; Franciosini *et al.*, 2012).

2.3.1.1 Genetics

It is evident that there is a genetic component to ascites syndrome. Genetic selection accompanied by rapid growth has greatly reduced the time of broilers to reach a desired market weight (2.5-3.0 kg). In the 1940's, birds took roughly 16 weeks to reach a market weight of 2.0-2.5 kg and the feed consumed was mostly used to support their maintenance requirements (Balog, 2003). After intense genetic selection, broiler strains in the 1990's achieved the same weight within 43-47 days with a smaller proportion of feed required for maintenance (Balog, 2003). Modern broiler strains have improved growth rates of up to 4 to 5% a year (Julian, 1990a, b, 2000; Druyan *et al.*, 2007), with commercial broilers growing up to 4 times more than egg-type Leghorns (Olkowski & Classen, 1998b; Balog, 2003). However, these on-going genetic improvements have also favoured the occurrence of ascites in broiler chickens (Julian *et al*., 1986). Previous reports confirmed that 85-90% of the changes observed in commercial broilers may be directly associated

with these genetic advancements (Havenstein *et al*., 2003 a, b), including an enhanced rate of growth. However, these changes may also be associated with PHS due to a lack of a comparable development of the cardiovascular and respiratory systems (Decuypere *et al.*, 2000; Havenstein *et al.*, 2003).

Many studies have revealed a lower incidence of ascites in slow-growing chickens such as egg-type Leghorns (Olkowski & Classen, 1998b; Olkowski *et al.*, 2005) and slow-growing broilers (Buys *et al.*, 1998). Therefore, the high growth rate of modern meat-type poultry lines likely plays a significant role in ascites prevalence, which is also driven by a higher feed intake per unit time and higher metabolic rate. Consequently, a higher demand of oxygen is required by tissues and organs of the birds from embryonic stage onward (Druyan, 2010; Druyan, 2012). This has gradually shifted the focus of the breeder industry towards the production of several ascites resistant lines of broilers (Balog, 2003) and stock that are adaptable to different environments in order to limit the metabolic disorders such as PHS. However, differences in susceptibility between broiler lines might be expected since each breeding company follows a different selection program (Silversides *et al*., 1997).

2.3.1.2 Anatomical and physiological considerations of PHS or ascites

2.3.1.2.1 Marginal pulmonary vascular capacity

Over the years domestication introduced numerous anatomical and physiological alterations to the modern bird (Balog, 2003), which may have unintentionally contributed to a marginal cardiopulmonary capacity in poultry (Wideman, 1988). Therefore, it can be suggested that various pre-existing pathophysiological disturbances predispose the modern commercial broiler to the risk of pulmonary hypertension, RVF and ultimately ascites (Wideman *et al.*, 2013).

It is well known that the average weight of a broiler chick is about 40 g at hatch and it has the capacity to reach 4,000 g in about 8 weeks (Wideman *et al.*, 2013). The consequence of this ongoing doubling and redoubling of its body mass by almost 7 times in 8 weeks cannot be sustained without a proportional increase in body organ size (i.e. the heart and the lungs) (Decuypere *et al*., 2000; Julian; 2000; Havenstein *et al*., 2003; Druyan *et al.*, 2007; Wideman *et al*., 2013). The left ventricle of the heart of the chicken is responsible for pumping 200 mL of oxygenated blood per kg of BW every minute (defined as cardiac output) (Wideman, 1999). Wideman *et al.* (2013) extrapolated that the absolute cardiac output during the 8 week period of the bird's life needs to be increased by 100-fold, which in turn must equal the rate at which venous blood is returned to the

heart in order to sustain normal basic functions. In order to support the heart, the bird's lungs must therefore rapidly mature within a relatively short period (2 months) in order to achieve a pulmonary vasculature capacity to receive and oxygenate a 100-fold increase in venous return (Wideman *et al.*, 2013). However, it has been clearly demonstrated that even under ideal conditions, the pulmonary vascular system of the modern bird only possesses a marginal reserve capacity (Wideman & Kirby, 1995b; Wideman, 2000, 2001; Odom *et al.,* 2004; Wideman *et al.,* 2013) and is unable to sustain optimal cardiac output.

Several unique anatomical factors may further have contributed to an enhanced ascites susceptibility in broilers (Julian, 1998; Balog, 2003; Bagbanzadeh & Decuypere, 2008). Unlike mammals, birds have small rigid lungs that are fixed within the body cavity, which limits respiratory expansion and contraction. In addition, the lungs of birds (as a percentage body weight) are generally smaller than that of mammals, with broiler lungs also being smaller compared to that of Leghorn chickens (Julian, 2000). The limitations of the bird's small stature, the abdominal pressure exerted on the air sacs from increased feed intake, growth or ascitic fluid, and their relative small lung volume (Julian, 1998; Balog, 2003; Bagbanzadeh & Decuypere, 2008) have limited the modern broiler chicken to employ key compensatory mechanisms that would have enabled them to readily accommodate for the increase in cardiac output (Wideman & Kirby, 1995b; Wideman, 2000, 2001; Odom *et al.,* 2004; Wideman *et al.,* 2013). During an event of increased blood viscosity and blood flow, the lack of space for blood flow through the lungs due to the limited expansion ability of the bird's small pulmonary capillaries (Wideman *et al.*, 2000; Julian, 2000) may aggravate the oxygen deficit occurring in these tissues. This, together with the little emphasis placed on selection for increasing lung area to match increasing BW and breast yield (Julian, 1989; Owen *et al*., 1995a, b; Wideman, 1999, 2000), has resulted in many individuals succumbing to ascites by means of outgrowing their pulmonary vascular capacity (Balog, 2003). Due to the modern bird's cardiac and pulmonary capacity still being very similar to that of historic broiler strains, it has ultimately forced the bird's cardiopulmonary system to work very close to its physiological limit (Lorenzoni & Ruiz-Feria, 2006; Baghbanzadeh & Decuypere, 2008; Singh *et al*., 2011), precipitating the cascade of organ/heart failure, ascites syndrome, and finally death (Balog, 2003).

Modern commercial broilers also possess compromised heart function, which is inadequate to meet the demand for its rapid rate of growth (Julian; 2007; Nian, 2008). This may greatly be attributed to the avian heart being different from that of mammals, as a result of several anatomical differences, including: (1) the right ventricle acting as a volume pump as opposed to functioning as

a pressure pump (Balog, 2003), (2) the right atrio-ventricular valve being composed of muscle fibres from the right ventricular wall, and (3) the left ventricle wall being thicker compared to mammals. All these may interfere with the effectiveness of the heart valve of the bird, thus making them very susceptible to valvular insufficiency causing the right ventricle and valve to become hypertrophic (Julian *et al.*, 1987; Julian, 1990a; 1993; Balog, 2003). It is important to take note however that the heart should not be seen as a physiological trigger. The heart simply responds to the work overload imposed by the bird's high metabolic rate or due to the increase in resistance of blood flowing through the lungs (Julian, 2000).

Furthermore, current meat-type poultry lines have been developed to promote high rates of metabolism (Druyan *et al.*, 2007), which seems to be directly correlated with ascites incidence (Julian, 2000; Decuypere *et al.*, 2005). Together with the selection for fast growth and efficient feed conversion, modern broilers have also been selected for large, heavy breast mass and hence, high rates of protein synthesis (Decuypere *et al.*, 2005). However, these advancements were made without dramatic increases in the functional capacities of the heart and lungs (Wideman *et al.*, 2013), leading to an aggravated mismatch between the cardiopulmonary system and increased muscle mass and raising the bird's demand for oxygen even more while further augmenting ascites susceptibility (Decuypere *et al.*, 2000; Balog; 2003). In addition, muscle and bone growth are at their peak during the early growth period (starting period), which is well known to be one of the most stressful periods during the bird's life, placing the cardiopulmonary system under severe pressure. These birds will subsequently enter the grower and finisher period having a weaker cardiopulmonary system and may be more prone to develop ascites (Arce *et al.*, 1992) due to the heart and lungs being compromised and incapable of providing enough oxygen to sustain the body (Julian, 1990a, b; Julian, 2000; Luger *et al.*, 2003; Aftab & Khan, 2005; Baghbanzadeh & Decuypere, 2008). Males generally have greater oxygen requirements compared to females due to their rapid growth and are therefore at greater risk to develop ascites as a result (Peacock *et al.*, 1990; Olkowski and Classen, 1998a; Balog, 2003; Nian, 2008).

2.3.1.2.2 Blood

Unlike mammals, the red blood cells of birds are characterised as having a large nucleus (Julian, 2000), which provides rigidity to the erythrocytes of the bird (Mirsalimi & Julian, 1991; Franciosini *et al.*, 2012) and can reduce blood flow in both the peripheral and lung blood vessels (Franciosini *et al.*, 2012). During hypoxic hypoxemia, an increase in erythropoietin production leads to RBC hyperplasia, resulting in a condition known as polycythaemia (Julian *et al.*, 1986;

Maxwell *et al.*, 1987a, b; Julian, 2000; Balog, 2003) and is responsible for causing an increase in resistance to blood flow throughout the lungs due to the bird's anatomical structure (Julian, 2000; Balog, 2003). Any contributing factor towards high blood viscosity may therefore be an important factor in contributing towards ascites (Mirsalimi & Julian, 1991; Maxwell *et al.*, 1992; Julian, 2000; Balog, 2003).

Domestication has placed a limit on the gas exchange capacity of the modern chicken and turkey (Mirsalimi *et al.*, 1993; Wideman, 1998; Balog, 2003). Hocking *et al.* (1994) reported that blood viscosity was elevated in genetically fat chickens compared to their leaner broiler breeder controls. Blood viscosity is closely correlated to both the percentage of erythrocytes and the amount of haemoglobin (Hb) in the blood (Julian, 2000). In addition, a high growth rate in these birds suggests an increase in blood flow through the lungs that may further lower the oxygen diffusion capacity of the red blood cells (Wideman & Kirby, 1995a, b; Kalmar, 2011). The pH of blood is yet another factor that may also influence the affinity of Hb for oxygen in the lungs and the ability of oxygen to be released to the tissues; a reduction of the blood pH lowers the Hb oxygen affinity and it is believed that modern broilers may be in a state of metabolic acidosis (Franciosini *et al.*, 2012). This was also confirmed by other authors that observed rapidly growing chickens having a lower blood oxygen concentration compared to that of their slower growing counterparts (Reeves *et al.*, 1991; Julian & Mirsalimi, 1992; Franciosini *et al.*, 2012). Additionally, chickens have a relatively thicker respiratory membrane compared to other birds, with broilers having a thicker respiratory membrane compared to Leghorn-type fowl, thus further limiting their capacity to fully oxygenate haemoglobin (Julian, 2000; Bagbanzadeh & Decuypere, 2008).

2.3.1.3 Environmental/management causes of PHS

Along with the previously mentioned genetic factor, altitude, incubation, house management and dietary nutrient content are closely associated with the development of PHS.

2.3.1.3.1 Altitude

The most evidential environmental factor triggering broiler ascites syndrome in many countries is high altitude (1300 m above sea level) (Julian, 1993; Julian, 2000; Bagbanzadeh & Decuypere, 2008; Singh *et al.*, 2011). Mortality rates between 15 to 25% have been reported in commercial broiler production systems where birds were raised at high altitudes (Lopez-Coello *et al.*, 1985; Beker *et al.*, 1995). The effects of high altitude on ascites and heart disorders were

already being reported as early as the 1950's and 1960's (Balog, 2003; Druyan, 2012). Mortalities observed in these birds were due to RVH, congested and oedematous lungs, and fluid in the abdominal cavity or "water-belly" (Druyan, 2012). Apart from documented incidences of ascites detected in commercial flocks maintained at high altitudes in Bolivia, Peru, and Columbia, broiler flocks in South Africa also suffer from this major metabolic disorder (Huchzermeyer, 1984; Maxwell *et al.*, 1986a). Higher incidences of ascites observed were mostly restricted to altitudes greater than 1300 m above sea level, which generally entails lower atmospheric partial pressure that increased blood viscosity and diminished oxygen supply (Julian, 1993; Julian, 2000), thus increasing the workload on the heart (Owen *et al.*, 1995; Wideman *et al.*, 1998; Luger *et al.*, 2003; Druyan, 2012; Franciosini *et al.*, 2012; Tekeli, 2014). However, over the last three decades, similar signs of ascites have also been observed at sea level and poultry producers have been struggling with this metabolic disease almost everywhere where broilers are raised (Julian, 1993, 2000; Hassanzadeh *et al*., 2001; Balog, 2003; Hassanzadeh, 2009). Koç (2007) reported that 13%, 27%, and 80% of broilers had ascites at altitudes of 1980 m, 2438 m, and 2896 m, respectively. Thus, a high ascites incidence no longer seems to be limited to high altitude conditions (Albers & Frankenhuis, 1990; Hassanzadeh, 2009). Nevertheless, ascites at moderate (above 800 m) (Julian, 1998) and high altitude (above 1200 m) (Tekeli, 2014) appears to be a more serious concern due to polycythaemia induced by hypoxia at these altitudes (Julian, 1998).

2.3.1.3.2 Incubation parameters

a) The potential role of incubation on ascites development

Although the peak incidence of ascites is mostly evident towards the end of the growing period, the aetiology of this syndrome is considered to be initiated as early as during the embryonic stage (Decuypere *et al.*, 2000; De Smit *et al*., 2005; Baghbanzadeh & Decuypere, 2008). This is mainly due to incubation comprising a significant portion of the total lifespan of a broiler chicken (Molenaar *et al.*, 2011). When conditions are optimal, it takes about 21d for a chicken embryo to develop and hatch (Yalcin & Siegel, 2003). Numerous factors are of fundamental importance for correct embryonic development to occur during the period of incubation, such as temperature and humidity control (Romanoff, 1960; Lourens, 2008), egg turning, gas exchange, moisture loss, egg quality factors (i.e. eggshell porosity), and the age of the breeder hen (Leksrisompong, 2005). All of these seem to affect subsequent embryonic metabolism, growth and development (Romanoff, 1960; Lourens, 2008), as well as hatchability (Decuypere *et al.*, 2001; Tona *et al.*, 2005; Bahadoran *et al.*, 2010). Temperature has been deemed as one of the most important physical factors

influencing embryo development and growth (Leksrisompong, 2005; Willemsen *et al.*; 2010; Molenaar *et al.*, 2011), and as such will partially be the focus of this study in terms of initiating ascites. Achieving the correct environment during incubation is crucial to optimal embryo development and will have a marked influence on bird performance during the grow-out period (Tona *et al*., 2005; Decuypere & Bruggeman, 2007; Hulet, 2007; Lourens, 2008; Molenaar *et al.*, 2011). Failure to do so may increase the chick's susceptibility to and the incidence of cardiovascular-related metabolic disorders (especially later in life), such as ascites (Leksrisompong *et al.*, 2007), since it will have a significant effect on later development and functioning (Decuypere *et al.*, 2001; Tona *et al.*, 2005; Bahadoran *et al.*, 2010). Molenaar and his colleagues (2011) were among the first to investigate the effect of high eggshell temperature (EST) on broiler mortality due to ascites, therefore research is still limited.

b) The role of high incubation temperature on organ growth and development and chick quality parameters

Three important factors greatly contribute towards the temperature experienced by the developing embryo. These include: (1) the incubator/machine temperature (MT), (2) the ability to transfer heat between the incubator and embryo, and (3) the metabolic heat produced by the embryo itself because of growth and metabolism (French, 1997). By measuring EST compared to MT, one can get a true estimate of the temperature experienced by the embryo, since EST will not deviate more than 0.1-0.2˚C from internal embryonic temperature (Lourens, 2008). It is well known that the optimum operating temperature for poultry species during incubation ranges between 37˚C and 38ºC (Tona *et al.*, 2005; Decuypere & Bruggeman, 2007), and it should not vary more than 0.3ºC (Yalçin & Siegel, 2003). It is therefore critical for temperature to be controlled within a very narrow range.

Fluctuating incubation temperatures have a marked influence on the growth efficiency of the embryo (Leksrisompong, 2005), with lower incubation temperatures depressing growth and development (Yalçin & Siegel, 2003), and higher incubation temperatures accelerating it (Romanoff, 1960; Christensen *et al.*, 1999). Therefore, embryo growth is rendered most efficient at a temperature optimum for attaining maximum hatchability (Romanoff, 1936; Leksrisompong, 2005). Both acutely high and low incubation temperatures have been shown to adversely affect embryo development and growth (Romanoff, 1960), leading to progressive weakening of the chicken embryo. These adverse temperatures negatively affect embryogenesis, organ development,

mitotic rates, metabolic adaptations, and disturb metabolic transitions, leading to reduced embryo and chick viability (Romanoff, 1960).

Embryos incubated at high EST may be at an increased risk for developing ascites (Balog, 2003; Molenaar *et al*., 2011). Several investigators have documented the adverse effects of high incubation temperatures on major organ development (Leksirompong *et al*., 2007; Leksirompong *et al.,* 2009; Molenaar *et al*., 2011). Generally, embryonic development of the avian heart starts during a very early stage and continues up to 10d post-hatch, which provides the basis for the development of the circulatory system (Leksrisompong, 2005). Olivo (1931) reported that eggs incubated at three different temperatures 34.5˚C, 36.5˚C, and 39.5˚C had significant differences in the number of mitotically active myocytes of the heart. Research therefore demonstrates that a clear relationship exists between incubation temperature and cardiac cell division, with embryos incubated at high EST exhibiting smaller heart weights. Similar results have been obtained by various other researchers, showing both a reduction in heart size, heart weight, and heart weight relative to chick weight when embryos were subjected to elevated EST's (Wineland *et al*., 2000; Leksirompong *et al.,* 2007; Lourens *et al.,* 2007; Molenaar *et* al., 2011). Molenaar *et al.* (2011) reported reduced heart weights of up to 31% and mortality rates as high as almost 4% in the high incubation temperature treatment group compared to chicks incubated at normal EST. High temperature during incubation showed a comparable decline in growth of the liver as it did in the heart (Leksrisompong, 2005), suggesting that the mitotic cell division of both the liver and heart is very similar due to the reduced rate of growth in these organs.

During the last 7 days of embryonic development (which is recognised as preparation for the commencement of pulmonary breathing and hatch) growth is at its fastest, resulting in a 60% higher oxygen consumption than compared to earlier stages (Decuypere *et al.*, 2000; Balog, 2003; Singh *et al.,* 2011). An oxygen shortage (hypoxia) may arise in the chicken embryo during the interval between internal pipping and hatching (Villamor *et al.,* 2004; Singh *et al.,* 2011), resulting in reduced embryonic growth rate and causing insufficient development of the pulmonary vasculature while increasing the embryo's metabolic demands for oxygen (Lubritz & McPherson, 1994; Balog, 2003). In addition, chicks subjected to incubator hypoxia had congested lungs that appear to remain this way until they are about 5 weeks of age (Maxwell *et al.*, 1987; Balog, 2003). In an attempt to supply more oxygen to the higher oxygen demanding tissues and organs, RBC ovoduction is increased resulting in an elevated viscosity of blood (Decuypere *et al.*, 2000; Molenaar *et al.,* 2011). The subsequent rise in cardiac output may lead to pulmonary hypertension, RVH, and finally fluid accumulation in the abdominal cavity and pericardium, which are well-known signs for the

development of ascites (Julian, 1993; Decuypere *et al.*, 2000; Molenaar *et al.*, 2011). Therefore, developing embryos exposed to high temperatures at incubation may be at increased risk of being susceptible to ascites. It can further be suggested that a long-term compromise of the young chick's cardiopulmonary system post hatch may increase their susceptibility to ascites development (Balog, 2003).

It is well recognised that any deviation from the optimum temperature may also influence metabolic and physiologic regulation within the embryo (French, 1997), altering prenatal embryonic development. This may be attributed to its subsequent effect on the metabolic rate, oxygen consumption, and nutrient utilisation (Brake, 1997). The physiological changes brought about by high EST may also have an effect on the bird's postnatal neuronal hypothalamic thermo-sensitivity making the chick more sensitive to suboptimal temperatures during the grow-out period (Yalçin & Siegel, 2003). Through changing the hormone concentrations related to growth and metabolism, high EST may subsequently alter the growth rate of the embryo and 1-day old chick quality (Christensen *et al.* 2001*a*; Decuypere & Bruggeman, 2007), the birds' thermoregulatory ability, and also post-hatch performance (French, 1997).

Beyond embryonic day (ED) 9-10, the gradual increase in EST and heat produced by the embryo (Lourens, 2008) together with insufficient cooling and air velocity in the incubators (French, 1997; Elibol & Brake, 2006) may result in incorrect conditions for proper embryonic development. During the second half of incubation, constant exposure to high EST (≥38.9˚C) results in a higher rate of embryonic mortality (Willemsen *et al.*, 2010; Molenaar *et al*., 2011) due to a higher rate of embryonic malpositions (Gladys *et al*., 2000; Decuypere & Bruggeman, 2007, Hulet, 2007; Barri *et al.*, 2011), reduced hatchability, and poor hatching quality compared to chicks experiencing normal EST (37.8˚C) (Lourens *et al.*, 2005, 2007; Hulet *et al.*, 2007; Leksrisompong *et al.*, 2007; Molenaar *et al.*, 2011). Van der Hel *et al.* (1991) also showed that chicks incubated at high EST during the last stages of incubation might hatch with as much as a 12% lower body weight because of inadequate development. Overheated developing embryos have also been characterised by enlarged retained yolk sacs and therefore a reduced yolk-free body mass (YFBM), resulting in a stunted chick due to reduced nutrient absorption from the yolk (Lourens *et al*., 2005, 2007; Hulet *et al.*, 2007; Leksrisompong *et al*., 2007; Molenaar *et* al., 2011). Poor chick viability with high incubation temperatures have also been recognised by poor navel conditions (i.e. unclosed or unhealed navels) (Lourens *et al*., 2005, 2007; Hulet *et al.*, 2007; Leksrisompong *et al*., 2007; Molenaar *et al*., 2011), extended stomachs (Hulet, 2007; Barri *et al*., 2011), and red or bruised hocks (Decuypere & Bruggeman, 2007). These issues can have detrimental effects during

the grow-out period, altering post-hatch chick performance (Leksirompong, 2005; Decuypere & Bruggeman, 2007; Lourens, 2008; Molenaar *et al*., 2011) and resulting in depressed body weight gain (BWG) and increased feed conversion (Leksrisompong *et al*., 2007, 2009). Poor growth associated with high incubation temperatures may be due to the chicks being less alert, having compromised nutrient supply to organs, and an increased sensitivity to poor brooding conditions (Leksrisompong *et al*., 2007, 2009).

2.3.1.3.3 Cold Temperature

A strong correlation has been recognised in poultry between cold temperature and heart failure for several decades (Julian *et al*., 1989; Bendheim *et al*., 1992; Shlosberg *et al.*, 1992a, b; Sato *et al*., 2002). Low temperature is regarded as the most important secondary environmental factor to cause PHS/AS in commercial broiler production systems (Julian, 2000; Balog; 2003; Bagbanzadeh & Decuypere, 2008; Singh *et al*., 2011) and will be the second focus in the scope of this study in terms of enhancing ascites susceptibility. More than a two-fold increase in the rate of ascites-associated mortalities have occurred in birds grown under cold temperatures compared to birds subjected to normal temperatures (Molenaar *et al.*, 2011), with deaths accounting for up to 25% as a result of ascites in flocks reared under moderately cold conditions (Balog, 2003; Druyan *et al*., 2009). However, under more severe conditions mortality rates can be anticipated to be even higher, with previous reports reaching mortalities of up to 50% in extreme cold environments (18-20˚C) (Druyan *et al*., 2007, 2009).

The timing of cold exposure to the juvenile chick during brooding appears to have a long-lasting effect on ascites susceptibility (Shlosberg *et al.*, 1992b; Julian & Squires, 1995; Julian, 2000; Groves, 2002; Singh *et al*., 2011; Druyan, 2012). Young chicks are most vulnerable to develop ascites during the first two weeks of rapid growth due to the effect that cold stress has on their metabolic rate (Decuypere *et al*., 2000; Bagbanzadeh & Decuypere, 2008; Singh *et al*., 2011). Although chicks may initially survive the cold stress, their growth is impaired and mortality increases two weeks later (Groves, 2002; Balog, 2003). Julian (2000) reported that chicks subjected to low temperatures before day six are predisposed to develop ascites, with temperature stress becoming less critical after three weeks of age.

The importance of temperature on chick performance cannot be stressed enough, since any deviation from the chick's optimum temperature requirements may result in impaired chick performance. Young growing chicks have a very narrow thermoneutral zone, therefore a drastic drop below the optimum may subsequently increase their requirements for oxygen (Julian *et al*.,

1989b) in order to maintain a normal body temperature (Julian, 2000; Balog, 2003). During the first week of life, the proposed thermoneutral temperature for brooding broiler chicks should range between 33-35˚C (Leksrisompong *et al*., 2009). Therefore, the thermoregulatory system of the growing chick operates within very narrow limits in order to maintain body temperature and cannot easily withstand moderate to extreme changes in environmental conditions (Druyan, 2012).

Upon exposure to cold or moderate heat, the young chick is placed outside its thermoneutral zone, subsequently inducing stress (Moraes *et al.*, 2002; Leksrisompong *et al.*, 2009) and resulting in an increase in their metabolic rate (Decuypere *et al*., 2000; Bagbanzadeh & Decuypere, 2008; Singh *et al*., 2011; Druyan, 2012) as well as increased demand for oxygen as they attempt to stabilise their body temperature (Beker *et al.*, 1995; Julian, 2000; Balog, 2003; Geng *et al*., 2004). Additionally, cold temperatures outside the broiler house due to the ventilation system decreasing to conserve heat within the house can lead to reduced O_2 availability within the broiler house at a time when O_2 demand is higher (Buys *et al*., 1999; Daneshyar *et al*., 2009). Research has demonstrated that with a rapid drop in environmental temperature (from 20˚C to 2˚C), the oxygen requirement for White Leghorn hens was almost doubled, while another study detected a 32.7% increase in oxygen requirement in response to low temperatures (Huchzermeyer *et al.*, 1989). Birds grown under low environmental conditions exhibited similar pathological symptoms than those that developed ascites under low oxygen partial pressure (Druyan, 2012). Geng *et al*. (2004) revealed that low ambient temperatures increased the growing chick's requirement for the oxygen-carrying erythrocytes to facilitate their high metabolic needs, causing a proliferation in RBC (haemo-concentration), more viscous blood and higher blood pressure (Julian, 2000): all symptoms associated with PHS.

Temperature during brooding has also been shown to affect the efficiency of broiler growth and organ development (Leksrisompong, 2005). Moraes *et al.* (2002) stated that the development during the first week of the chick's life may influence their subsequent performance, with the first two days of the brooding period being the "critical time window" that birds must be active and eating (Leksrisompong, 2005). During the first week of life, physiological processes such as cellular hypertrophy and hyperplasia, maturation of thermoregulation and the immune system, gastrointestinal development and growth may subsequently influence BWG and feed conversion until slaughter age (Moraes *et al.*, 2002), which may be unfavourably affected by low environmental temperatures. This is supported by reduced chick performance under cold brooding temperatures that results in depressed BW, reduced feed efficiency, and increased mortality rate during the first week of life because of the chicks being less active (Moraes *et al.*, 2002; Leksrisompong, 2005). Most importantly, the effects of low brooding temperature have also been

shown to affect the heart, which is regarded as the most sensitive and responsive organ to temperature (Leksrisompong, 2005). Reports indicated that chicks reared at a lower brooding temperature (32.2˚C) had significantly lower liver and heart weights relative to body weight (BW) compared to chicks reared at higher temperatures (37.2˚C) (Leksrisompong, 2005; Leksrisompong *et al.*, 2009). In addition, birds grown under low temperatures exhibited an increase in the right ventricle: total ventricular (RV:TV) ratios (Julian *et al.*, 1989; Scheele *et al.*, 1991; Shlosberg *et al.*, 1991, 1992; Lubritz *et al.*, 1995; Druyan *et al.*, 2007, 2008; Druyan 2012).

It is thus evident that environmental temperature (i.e. cold brooding or cold grow-out) together with the chick's fast growth is one of the most important triggers of PHS (Scheele *et al.*, 1991; Acar *et al.*, 2001; Wideman, 2001; Molenaar *et al*., 2011). Therefore, it can be expected that ascites development would be intensified at below optimum brooding temperatures and when paired with high temperatures at incubation may further increase the burden placed on the bird's cardiovascular system.

2.3.1.3.4 Lighting programmes

Domestic poultry consume feed almost continuously throughout the entire daylight period (Hassanzadeh, 2009) and as such, they are usually grown on a near continuous lighting schedule for the purpose of maximising growth and feed consumption (Balog, 2003; Bagbanzadeh & Decuypere, 2008; Hassanzadeh, 2009; Singh *et al.*, 2011). However, constant lighting may be disadvantageous to the bird, resulting in increased bird activity, feed intake, and hence growth that will require additional oxygen (Julian, 2000). Thus, broilers on a long day period are at a higher risk of developing metabolic disorders, including heart related disorders (Hassanzadeh *et al*., 2003; Leeson & Summers, 2005; Nian, 2008) such as sudden death syndrome and ascites (Hassanzadeh, 2009; Franciosini *et al.*, 2012). Alternatively, intermittent lighting programmes (IL) consisting out of shorter dark-light cycles may result in reduced feed intake and growth rate since birds are reluctant to eat during the dark period (Hassanzadeh *et al*., 2003). Although ascites incidence have been reduced by increasing the duration of the dark period (Albers & Frankenhuis, 1990; Hassanzadeh *et al.*, 2000; Julian, 2000), it can be suggested that this may not solely be responsible for reducing the rate of mortality, since many interacting factors culminates in PHS development.

2.3.1.3.5 Air quality and ventilation

The oxygen content of air brought into poultry houses can have a significant impact on the housing environment and bird performance (Singh *et al.*, 2011). Moreover, any challenges

compromising the bird's ability to exchange oxygen with its environment will predispose it to develop ascites (Singh *et al.*, 2011). For example, poor ventilation and incorrect litter management can lead to low environmental oxygen and accumulative toxic gases (*e.g.* carbon dioxide or ammonia) (Bottje *et al.*, 1998; Wideman, 1998; Baghbanzadeh & Decuypere, 2008; Singh *et al.*, 2011; Tekeli, 2014). These gases negatively affect the air quality and can have marked detrimental effects on the respiratory or cardiovascular system of the birds (Wideman, 1998; Balog, 2003; Baghbanzadeh & Decuypere, 2008), causing decreased oxygen transfer between the bird and the environment (Singh *et al.*, 2011). For example, high levels of ammonia may reduce cilia motility in the lungs favouring the appearance of respiratory diseases. High levels of environmental dust may also contribute to the onset of ascites due to the damage it can inflict in the lungs (Franciosini *et al.*, 2012). In addition, inhalation of spores from disease-causing microorganisms originating from mouldy litter or from the hatchery can attach themselves to dust particles and be respired. Consequently, this may lead to irritation of the respiratory tract as well as infection, causing damage to the bird's lungs and precipitating ascites (Julian, 1993; Julian, 2000; Singh *et al*., 2011). Ascites occurrence may also increase on commercial farms during winter periods because of carbon monoxide emission from brooders, which lowers the oxygen concentration within the poultry house (Franciosini *et al.*, 2012). Maintaining optimal air quality via correct litter management together with adequate ventilation is therefore crucial in order help reducing ascites prevalence.

2.3.1.4 Dietary risk factors precipitating PHS or ascites

With the main aim of poultry nutrition being maximised production efficiency at minimum input cost, manipulation of feed supply or composition in broilers can affect the severity of metabolic disorders (Nian, 2008). Therefore, diet also plays a part in influencing ascites development in meat-type chickens (Julian, 2000).

2.3.1.4.1 Diet form and nutrient density

Diet has also been found to influence ascites development in meat-type chickens (Julian, 2000). Modern commercial broilers are mostly fed crumbled or pelleted diets as it is easier for them to consume and more digestible than mash feed (Julian & Squires, 1995; Balog, 2003; Singh *et al*., 2011), which can contribute toward an increase in ascites prevalence (Wideman, 1988; Julian, 2000; Balog, 2003). In addition, pelleted feed being more nutrient dense may also enhance bird growth due to a higher nutrient intake thereby further increasing the bird's subsequent metabolic rate and

oxygen consumption (Julian & Squires, 1995; Balog, 2003). This is especially true during the initial stages of bird growth (0 to 21 days of age), which can predispose the bird to ascites (Baghbanzadeh & Decuypere, 2008). It is also generally accepted that broilers fed exceptionally high levels of protein and amino acids are more prone to develop ascites due to the rapid growth that occur on these diets (Mirsialimi *et al.*, 1993; Julian, 2000; Balog, 2003). However, several researchers have found no specific significant effect of dietary protein, lysine, or energy: protein ratios on ascites incidence (Julian *et al.*, 1989b; Buys *et al.*, 1998; Balog, 2003). It can be hypothesised that diets containing high protein concentrations are subjected to increased deamination of excess nitrogen as uric acid, which is an energetically costly process for the bird (Julian, 2000). Research has shown that broilers consuming diets high in metabolisable energy and dietary fat are also more vulnerable in developing ascites (Julian *et al.*, 1989b; Scheele *et al.*, 1991; Julian, 1993; Balog, 2003). This may be attributed to the resulting increase in growth rate (Julian *et al.*, 1989b; Scheele *et al.*, 1991; Julian, 1993; Balog, 2003) and the elevated oxygen requirements of the birds to meet the high oxygen demand in order to complete oxidation (Balog, 2003).

2.3.1.4.2 Miscellaneous dietary factors

Various studies have indicated the effect of specific nutrients or other dietary compounds to broiler ascites syndrome. Meat meal, poultry by-product meal (Julian, 1993, 2000), and excessive dietary levels of rapeseed oil (Balog, 2003) have been associated with a higher broiler ascites incidence. Excessive levels of vitamin A, D, E, C, and niacin in the feed may be associated with metabolic lesions that negatively affect heart performance, leading to an increased risk of heart failure (Nian, 2008). High dietary levels of chloride (Wideman *et al.*, 1998a; Balog, 2003) can be a trigger for PHS because of acidosis (Franciosini *et al.*, 2012), which reduces oxygen affinity of blood (Julian, 2000). Excessive sodium in the diet has also been coupled with PHS due to hypervolaemia (Mirsalimi *et al.*, 1992; Franciosini *et al.*, 2012). Additional factors also influencing ascites development include feed containing excess salt (Julian, 1987, 1990b; Arce *et al.*, 1992; Julian *et al.*, 1992; Mirsalimi *et al.*, 1992) or cobalt (Wideman *et al.*, 1998a; Balog, 2003), low phosphorus levels (Julian *et al.*, 1986), and several non-nutritional compounds (i.e. mycotoxins, coal tar disinfectants, carbon, solvents, drugs and other chemicals) (Balog, 2003). These agents impose an increase in the bird's metabolic rate (Julian, 1993; Julian, 2000) that may elevate the risk of ascites. Most importantly, the presence of these cardio-toxic compounds in the diet may further raise the risk of heart failure through either directly influencing the cardiomyocytes or indirectly via remodelling of the extracellular matrix of the heart (Nian, 2008). Furthermore, it has been

suggested that dietary deficiency of vitamin D or Ca (rickets) may also be a predisposing factor of AS. The reasoning behind this is that the resultant impaired calcification of the ribs interferes with breathing and the bird's potential to oxygenate the blood (Julian, 1998). Reduced mortality due to PHS has been observed following supplementation of vitamin E, which may be due to its antioxidant capacity (Bottje *et al.,* 1995; Franciosini *et al.,* 2012).

2.4 The potential role of oxidative stress and lipid peroxidation during metabolic disease

Over the past few decades, substantial evidence has revealed a significant involvement of oxidative stress in the pathophysiology of congestive heart failure (CHF) (Griendling & FitzGerald, 2003; Giordano, 2005; Seddon *et al.*, 2006) and its predisposing conditions such as ischaemic and non-ischaemic cardiomyopathy, pressure and volume overload, cardiac hypertrophy and adverse remodelling after myocardial infarction (MI) (Seddon *et al.,* 2006). The principle pathological effects of oxidative stress have been well thought to result from free radical induced oxidation and damage that disrupt cell function and lead to necrosis and/or apoptosis (cell death) (Seddon *et al.*, 2006; Mee, 2009; Tahara *et al.,* 2009; Kolamunne, 2010). A well-defined relationship has also been established between the production of mitochondrial reactive oxygen species (ROS), an oxygen-derived radical, and susceptibility of broiler chickens to PHS (Bottje & Wideman, 1995; Currie, 1999; Cawthon *et al.,* 1999, 2001; Iqbal *et al*., 2001b, 2002; Tang *et al.*, 2002; Cisar *et al*., 2004; Geng *et al*., 2004; Han *et al.,* 2005). Furthermore, ROS may contribute to many of the abnormalities associated with vascular disease, possibly through its individual or combined action within the vessel wall (Griendling & FitzGerald, 2003). Damage to the pulmonary endothelium and supportive structures along with hypertension induced by ROS may be the main two contributing factors that ultimately culminate in ascites development (Han *et al.,* 2005). It is thus inevitable that the effects of ROS are implicated not only in CHF but also in its predisposing conditions, including PHS, since mitochondrial radical generation has been associated with metabolic disease (Cawthon *et al.,* 1999).

2.4.1 Generation of reactive oxygen species (ROS) in the cardiovascular system

Reactive oxygen species are free radicals derived from molecular oxygen and encompasses both oxygen radicals and specific non-radicals that are also capable of generating free radicals (i.e. H_2O_2) (Turrens, 2003; Seddon *et al.*, 2006; Mackenzie, 2010). These unstable and chemically

reactive atoms or groups of atoms are generally characterised by containing an unpaired number of electrons (Balog, 2003; Turrens, 2003; Seppanen, 2005; Song, 2013) and can be formed in the heart as well as other tissues through several mechanisms (Giordano, 2005). Free radicals are well known to cause lipid peroxidation of fatty acids in the presence of oxygen (Seppanen, 2005; Song, 2013). The main reactive oxygen species that plays a central role in vascular physiology and pathophysiology include superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) (Griendling & FitzGerald, 2003). A newly formed radical may become stable or unreactive through either donating its unpaired electron(s) to other molecules, or through reacting with a free radical scavenger (i.e. primary antioxidant or chain breaking species) (Kolamunne, 2010). Upon reactivity (oxidation) with another molecule, excess free radicals (i.e. ROS) become highly reactive, perturbing cellular antioxidant defense systems, causing damage to cells, tissues, and macromolecules including protein, carbohydrates, lipids, and nucleic acids (Han *et al.*, 2005; Seddon *et al.*, 2006; Tahara *et al.*, 2009; Kolamunne, 2010). By exerting cytotoxic effects, these oxygen free radicals cause peroxidation of membrane phospholipids, which in turn results in membrane fluidity, increased permeability, and loss of membrane integrity (Han *et al.*, 2005). ROS have also been reported to directly damage tissues, including that of the pulmonary endothelium and related supportive structures (Barnes, 1990; Han *et al.*, 2005). ROS may therefore play a significant role in pulmonary arterial hypertension in chicks through directly damaging the pulmonary endothelium, degrading and/or collapsing alveoli, and increasing resistance in the pulmonary vasculature (Han *et al.*, 2005).

Many metabolic reactions within a living system are responsible for generating low levels of radicals (Kolamunne, 2010), with each of the reactive oxygen species being derived from specific or enzymatic chemical reactions (Griendling & FitzGerald, 2003). The mitochondrial respiratory chain has been established to be the principal contributor to intracellular levels of ROS in most tissues (Turrens, 2003; Giordano, 2005; Hamanaka & Chandel, 2010) (**Figure 2.2**), including the heart (Suematsu *et al.*, 2003), with as much as 2% of the oxygen utilised by the respiratory chain being incompletely reduced to ROS (Cawthon *et al.*, 1999). High levels of ROS may result due to "electron leakage" from several redox centres of the mitochondrial electron transport chain (ETC) (Turrens, 2003; Giardano, 2005). During oxidative processes such as oxidative phosphorylation, subsequent transfer of one electron at a time within the mitochondria results in the production of ROS such as superoxide ($O_2^{\cdot -}$) (Turrens, 2003). During oxidative metabolism, complexes I, II, and III of the mitochondrial ETC contain specific sites that will ultimately result in the formation of $O_2^{\cdot -}$ (Turrens, 2003; Murphy, 2009; Hamanaka & Chandel, 2010).

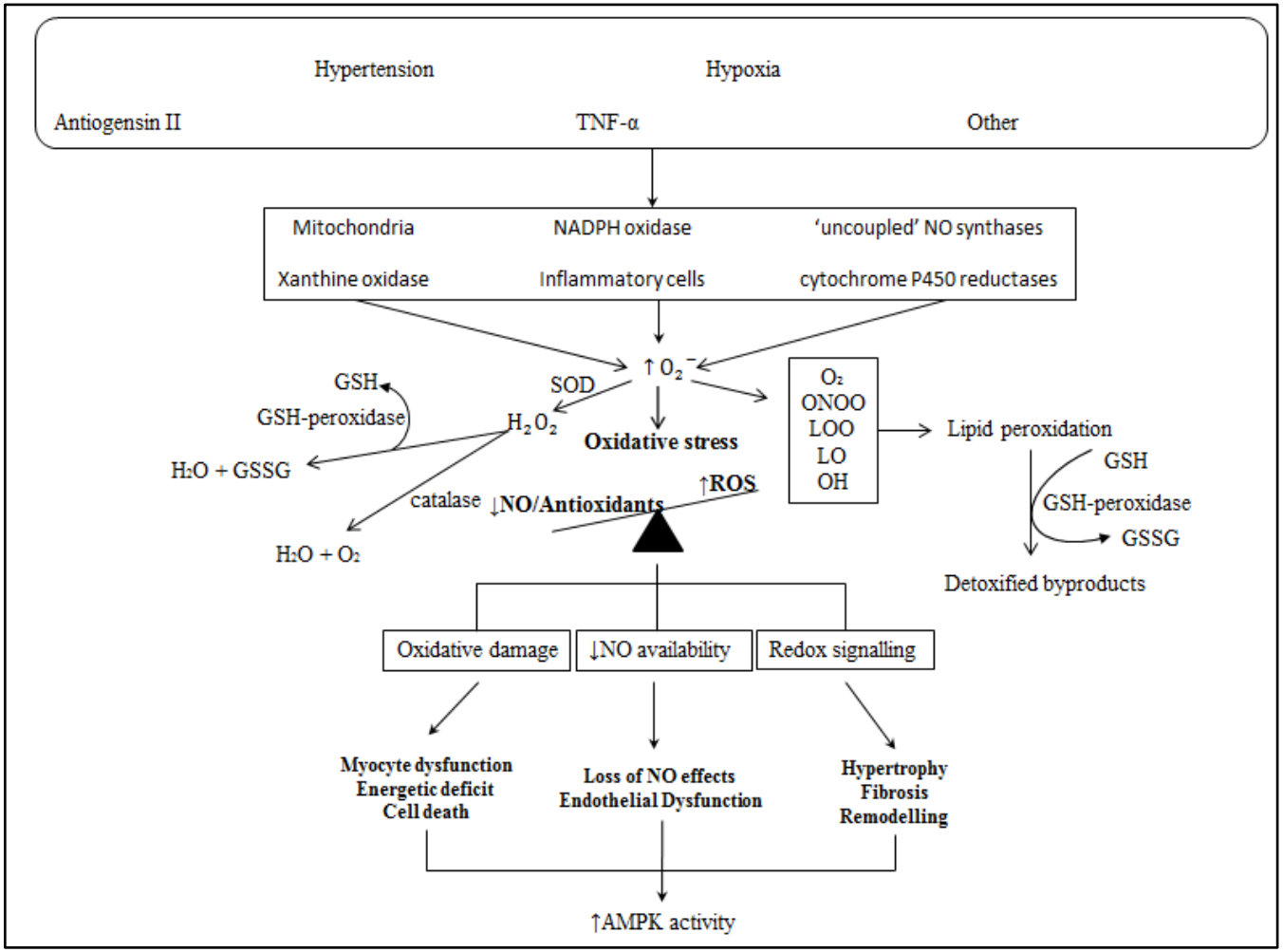

Figure 2.2: Oxidative Stress in Cardiovascular Disease. Environmental and physiological factors leading to the generation of superoxide (O2 -) in the vasculature. O2- is enzymatically converted to hydrogen peroxide (H2O2) by superoxide dismutases (SODs) and further processed by catalase and glutathione peroxidase (GSH-Px). However, excess O2- can generate a number of other reactive oxygen species (ROS), disrupting the balance of nitric oxide (NO) and ROS. Increased oxidative stress leads to reduced NO bioavailability which is associated with endothelial dysfunction and cardiovascular disease (CVD) [Adapted from Seddon et al. (2006), Hamanaka & Chandel (2010), and Mackenzie (2010)]

Superoxide anion ($O_2^{\cdot -}$) is the precursor of most reactive oxygen species as well as a mediator in oxidative chain reactions and is formed upon donation of a single electron to molecular oxygen (Turrens, 2003; Giordano, 2005; Murphy, 2009; Hamanaka & Chandel, 2010). A family of superoxide dismutase enzymes rapidly converts $O_2^{\cdot -}$ to H_2O_2, which in a second reaction gets broken down by glutathione peroxidase or catalase to water (Griendling & FitzGerald, 2003; Turrens, 2003; Giordano, 2005; Seddon *et al.*, 2006). Virtually all types of vascular cells produce O_2^- and H_2O_2 (Griendling *et al.*, 2000) and high amounts of these radicals will negatively influence vascular function (Griendling & FitzGerald, 2003). Under pathological conditions, a single-electron reduction of H_2O_2 may direct towards the formation of the highly reactive hydroxyl (OH^-) radicals (Griendling & FitzGerald, 2003; Turrens, 2003; Giordano, 2005; Seddon *et al.*, 2006). Furthermore, $O_2^{\cdot -}$ may react with other radicals, yielding reactive nitrogen species such as nitric oxide (NO) (Turrens, 2003) which can lead to the formation of peroxynitirtite ($ONOO^-$). Peroxoxynitrite is known to play an important role in vascular homeostasis and modulation of cardiac function (Li & Shah, 2004; Seddon *et al.*, 2006) and is regarded an important mediator of lipid peroxidation (Griendling & FitzGerald, 2003).

Apart from mitochondrial sources, there are several other potential sources of ROS contributing towards cardiac hypertrophy and CHF (Seddon et al., 2006) (**Figure 2.2**). The enzyme xanthine oxidase has been implicated in contributing to oxidative stress in CHF as a result of $O_2^{\cdot -}$ production (Seddon *et al.*, 2006). Other enzymes that are also considered to be important in the generation of $O_2^{\cdot -}$ and/or H_2O_2 include cytochrome P450 (Griendling & FitzGerald, 2003) and the membrane-associated NADPH oxidase(s). The complex enzyme, NADPH oxidase, catalyses the electron transfer from NADPH to molecular oxygen, which results in the formation of $O_2^{\cdot -}$ (Verhaar *et al.*, 2004; Seddon *et al.*, 2006). In experimental models of left ventricular hypertrophy, CHF, and end-stage heart failure in humans, elevated NADPH activity was observed (Seddon *et al.*, 2006), suggesting an increase in ROS production. Furthermore, Verhaar *et al.* (2004) observed that $O_2^{\cdot -}$ from NADPH oxidase resulted into nitric oxide synthase (NOS) uncoupling, which has been supported in experimental models in diabetes and hypertension. Subsequent uncoupling of NOS will yield $O_2^{\cdot -}$ instead of nitric oxide (NO), which has also been incriminated in the development of vascular endothelial dysfunction in patients with heart failure (Seddon *et al.*, 2006). Additionally, ROS produced by NADPH oxidases also promotes the production of ROS by other sources, thereby augmenting the total levels of ROS (Verhaar *et al.*, 2004; Seddon *et al.*, 2006).

Enzymes similar to NADPH oxidases found within various cell types, including endothelial cells, fibroblasts, vascular smooth muscle cells (VSMCs), and cardiac myocytes, have also been

associated with a diverse range of stimuli important for cardiovascular pathology (Seddon *et al.*, 2006). For example, antiogensin II, tumour necrosis factor-α (TNF-α), thrombin, and platelet-derived growth factor have all been implicated in an increase of oxidase activity that raises intracellular levels of $O_2^{\cdot -}$ and H_2O_2 in VSMCs (Griendling & FitzGerald, 2003). In cultured cardiac myocytes, TNF-α has been shown to elevate mitochondrial ROS production thereby impairing mitochondrial electron transport activity (Suematsu *et al.*, 2003). It has also been demonstrated that TNF-α mitochondrial ROS-induced production results in mitochondrial DNA (mtDNA) damage and cardiac mitochondrial dysfunction because of its contribution to enhanced intracellular oxidative stress. Therefore, high levels of ROS may also act as signalling molecules for TNF-α (Suematsu *et al.*, 2003) and thus play an important role in the pathophysiology of various cardiovascular diseases, including heart failure due to oxidative stress (Li *et al*., 2001; Suematsu *et al.*, 2003).

2.4.2 Pathophysiological actions of Reactive Oxygen Species (ROS)

The pathophysiological effects of ROS depend on different factors, such as the type of ROS produced, its concentration, and the specific site of production (Seddon *et al.*, 2006). A steady state of ROS will be maintained as long as the various antioxidant defense and repair mechanisms keep the ROS concentration at relatively low, non-toxic levels (Turrens, 2003). When the generation of these pathogenic species exceeds the antioxidant protection, they may trigger cellular dysfunctions and irreversible cell damage or death (Seddon *et al.*, 2006; Mee, 2009). This may be attributed to their role in altering the proportions of several critical membrane associated or intracellular macromolecules, including nucleic acids, proteins, and phospholipids of the membranes (Cawthon *et al*., 1999; Nian, 2008), as well as the generation of other more reactive radicals (Li & Shah, 2004; Seddon *et al.*, 2006).

2.4.2.1 The role of ROS in the pathophysiology during cardiovascular disease

Overwhelming evidence confirms the role of the pathogenic species on tissue injury and/or impaired repair mechanisms, which is a common feature shared by many cardiovascular disease states (i.e. hypertension, heart failure) (Suematsu *et al.*, 2003; Sugamura & Keany, 2011; Song & Zou, 2012). The imbalance in ROS brought about by oxidative stimuli may lead to the generation of several lipid peroxidation end products, such as 4-hydroxy-2-trans-nonenal (4-HNE) (Ma *et al.*, 2011; Wang *et al.*, 2012) and thiobarbituric acid reactive substances (TBARS) (Kalmar *et al.*, 2010)

as a result of cellular damage. Since both 4-HNE and TBARS have been associated with various negative biological effects, they have been widely used as biomarkers for oxidative damage in tissues (Seppanen, 2005; Song, 2013). The 4-hydroxykenals, including 4-HNE, are extremely toxic aldehydes and have been related to many diseases in humans, such as Parkinson's and Alzheimer's disease (Seppanen, 2005; Song, 2013). Diaz-Cruz *et al.* (1996) revealed elevated TBARS concentrations, a measure of aldehyde content (*i.e.* malondialdehyde (MDA), in the liver and cardiac tissue of ascitic broilers, which is indicative of lipid peroxidative damage initiated by high levels of reactive oxygen species. This was substantiated by Han *et al.* (2005) that reported a high relationship between MDA content and ascites incidence in broiler chickens. Furthermore, other characteristics associated with myocardial cellular damage due to oxidative stress have also been observed in ascitic broilers; such as the breakdown and subsequent release of contractile proteins from the myocardium (i.e. myosin and troponin T) into general circulation (Maxwell *et al*., 1994, 1995; Currie, 1999). Elevated serum cardiac troponin-T levels have also been observed in 30-day-old broilers as a result of hypoxia (Maxwell *et al*., 1994, 1995; Currie, 1999). Similarly, Undhad *et al.* (2012) reported an increase in serum cardiac troponin-T following myocardial injury within 3 to 8 hours, with peak levels occurring at 12-24 hours that persisted for approximately 1-2 weeks after the incident. Studies in humans have indicated that measuring serum cardiac troponin T levels is a highly specific, non-invasive technique to determine the extent of cardiac damage since this specific protein is related to the contractile tissues of the striated heart muscle (Maxwell *et al.*, 1995) and may be useful in birds to help identify PHS.

Previous reports in mammals showed that ROS, because of normal metabolism, were not adequately removed under normal metabolic conditions due to the antioxidant defense systems being overwhelmed by oxidative stress (Alirezaei *et al.,* 2012b). Correspondingly, commercial broilers with well-advanced symptoms of PHS exhibited reduced levels of nonenzymatic antioxidants (Vitamin C and E), lower levels of reduced glutathione (GSH) in liver and lung tissues, as well as elevated levels of lipid peroxides in the plasma compared to their control counterparts (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995; Cawthon *et al*., 1999). Since birds with PHS had lower levels of GSH compared to healthy birds, and mitochondrial GSH levels depend on the movement of GSH into the mitochondria from the cytosol, it is clear that antioxidant defense is compromised in birds with PHS (Cawthon *et al*., 1999). The resultant damage to liver and lung tissue due to high ROS production (Tang *et al*., 2002) causes the birds to be less efficient in using oxygen compared to broilers without PHS (Cawthon *et al*., 1999, 2001; Iqbal *et al.,* 2001a, b; Cisar *et al.,* 2004). In addition, generation of ROS within broiler lung mitochondria have been implicated

in the elevated release of pulmonary vaso- and broncho-constrictors (i.e., thromboxines and leukotrines) that further aggravate the already existing cardiopulmonary insufficiency during PHS (Iqbal *et al*., 2001a).

Other researchers have also implicated ROS to be involved in heart failure supported by histological evidence that demonstrates increased ROS activity in the mitochondria of damaged broiler hearts (Maxwell *et al.*, 1996; Cawthon *et al*., 1999; Iqbal *et al*., 2001a). In addition, the generation of high levels of intracellular ROS has also been involved in impaired myocardial contractility and increased tone in the peripheral vasculature causing cardiac-induced hypertrophy, which is a well-recognised component regarding the phenotype of heart failure (Cave *et al.*, 2005; Han *et al.*, 2005; Seddon *et al.*, 2006). Several researchers have also established a relationship between the generation of mitochondrial ROS and myocardial contractile dysfunction during CHF (Ide *et al.*, 2001; Seddon *et al.*, 2006). For example, *in vivo* studies have revealed impaired myocardial contractile function as a result of enhanced ROS activity, ultimately causing disruption of calcium cycling, altered myofilament responsiveness to calcium, along with impaired cellular and energy metabolism (Ide *et al.*, 2001; Cave *et al.*, 2005; Seddon *et al.*, 2006), all of which are crucial to sustain normal cardiac function. Furthermore, the inactivation of NO may be another contributing factor towards myocardial dysfunction, as NO plays an important role in reducing myocardial oxygen consumption and thus helps improve cardiac efficiency (Seddon *et al.*, 2006). Finally, ROS have also been implicated in the development of interstitial cardiac fibrosis, which is considered an important detrimental aspect of left ventricular hypertrophy and CHF (Seddon *et al.*, 2006). It is relatively evident that elevated production of ROS contribute a great deal with regards to the failing heart and can therefore contribute towards tissue and/or organ damage and failure (such as the lungs, liver, and heart) considering the pathophysiology of PHS.

2.4.3 Mechanisms of defense against oxidative stress and impaired energy status

2.4.3.1 Antioxidant System - Glutathione Peroxidase (GSH-Px)

Cells express various antioxidant enzymes to minimise potential damage to macromolecules and the cellular environment (Kolamunne, 2010). As previously mentioned, inadequate antioxidant protection or elevated levels of free radicals may induce oxidative stress, promoting PHS development (Bottje & Wideman, 1995; Cawthon *et al.*, 1999; Balog, 2003). Several endogenous cellular mechanisms operate to counterbalance the production of high levels of ROS during normal

metabolism, including enzymatic antioxidants (i.e. superoxide dismutase, glutathione peroxidase (GSH-Px) and nonenzymatic antioxidants (i.e. GSH and α-tocopherol)) (Yu, 1994; Cawthon *et al.*, 1999; Giordano, 2005; Song, 2013). However, the glutathione antioxidant system is paramount in its role of cellular defense against ROS (Alirezaei *et al.*, 2012b), with GSH-Px being particularly important in mitochondrial antioxidant protection (Cawthon *et al.*, 1999) and will therefore be discussed further for the purpose of this study.

The GSH peroxidases consist of a family of tetrameric intrinsic antioxidant enzymes that contribute to both a first and second line oxidant defense system (Mee, 2009; Mackenzie, 2010). GSH-Px inhabits a particularly important role in antioxidant protection in the mitochondria (Cawthon *et al.*, 1999) by directly scavenging ROS and converting them into less reactive species (Cawthon *et al.*, 1999; Giordano, 2005; Mackenzie, 2010). On the whole, GSH-Px operates to convert hydrogen peroxide (H_2O_2), the product of $O_2^{\cdot -}$ dismutation and the main precursor of OH^- in the presence of reduced transition metals, to water and lipid peroxides and finally to less reactive alcohols. Failure to metabolise these lipid peroxides will result in a reaction with metal ions and thus the formation of the highly reactive hydroxyl radical as well as conversion of GSH to oxidised glutathione (GSSG) (Cawthon *et al.*, 1999; Turrens, 2003; Mackenzie, 2010). It is known that toxic levels of GSSG may accumulate following oxidative stress within the mitochondria (Cawthon *et al.*, 1999). A phospho-lipid-hydroperoxide GSH-Px associated with the mitochondrial membrane have shown to be specifically involved in reducing lipid hydroperoxides (Turrens, 2003) to their corresponding alcohols via the conjugation and oxidation of GSH (Mee, 2009; Mackenzie, 2010). Therefore, GSH-Px also plays a role in detoxification of secondary oxidation products (Mackenzie, 2010).

There are several studies showing that antioxidants may ameliorate the development of heart failure or hypertrophy in different animal models (Mee, 2009). Although mitochondrial antioxidant capacity in birds with PHS may be low (Cawthon *et al.*, 1999), recent investigations have revealed that supplemental betaine, a methyl group donor, may exert antioxidant-like properties in experimental animal models (Alirezaei *et al.*, 2010; Alirezaei *et al.*, 2011; Alirezaei *et al.*, 2012b). Alirezaei and his colleagues (2012b) suggested that supplementation of betaine to broiler diets at 1 g/kg of the final feed may act as an antioxidant following oxidative stress. The GSH-Px levels were significantly higher in broilers in both the betaine (B) and methionine-low + betaine (ML+ B) groups (240-260 mU/mg protein) compared to broilers that received the control (C) (159 mU/mg protein) and methionine-low (ML) (140 mU/mg protein) diets, indicating that GSH-Px may be promoted. In addition, diets that contained betaine also had a significant effect in reducing lipid

peroxidation mediated damage of breast muscle meat, which implies a reduced rate in the generation of ROS (Alirezaei *et al*., 2012b). Therefore, it is relatively evident that due to the increased GSH-Px enzyme activity found in birds fed betaine-containing diets, betaine may indirectly possess antioxidant effects through preserving cellular antioxidant stores and by this means, can reduce subsequent oxidative stress (Alirezaei *et al*., 2012b). However, another potential mechanism of betaine in its protective role against oxidative stress may also be its ability to re-establish S-adenosylmethionine (SAM) levels; SAM is needed for the synthesis of glutathione, which is well-known to protect the cell from reactive metabolites and ROS (Ganesan *et al.*, 2007a; Alirezaei *et al*., 2011; Alirezaei *et al*., 2012b). It can therefore be hypothesised that betaine may alleviate the pathophysiological development of RVH that would occur as a result of PHS due to its possible enhancement of the antioxidant GSH-Px.

2.4.3.2 Energy Systems - AMP-activated protein kinase (AMPK), a core signalling pathway in the heart

2.4.3.2.1 Control of energy metabolism in the heart

Under normal physiological circumstances, all cellular processes are highly dependent upon the dynamic regulation of cellular oxygen supply and energy metabolism (Huss & Kelly, 2005; Mungai *et al.*, 2011; Sanli, 2012). The continual high-energy demands of the heart are related to the maintenance of specialised processes, including ion transport, sarcomere function, and intracellular Ca^{2+} homeostasis (Huss & Kelly, 2005). Therefore, normal, healthy hearts require a constant supply of fuels and oxygen (Marsin *et al*., 2000) in order to maintain proper contractile function and basal metabolic functions, which is derived primarily from mitochondrial oxidative metabolism (with a small amount derived from glycolysis) (Giordano, 2005; Dolinsky & Dyck, 2006; Lopaschuck, 2008; Mungai *et al.*, 2011).

Various literature supports that cardiac energy metabolism may be severly altered during various physiological and pathophysiological conditions that places strain on the heart (Dolinsky & Dyck, 2006; Schimmack *et al.*, 2006); such as exercise, volume overload (Tian *et al*., 2001; Kim *et al.*, 2009), cardiac hypertrophy (Tian *et al*., 2001; Schimmack *et al.*, 2006; Kim *et al.*, 2009), myocardial ischemia (Kudo *et al.*, 1996; Hardie & Hawley, 2001; Xing *et al.*, 2003; Musi, 2006; Schimmack *et al.*, 2006) and hypoxia (Hardie & Hawley, 2001; Musi, 2006; Hutchinson *et al*., 2008; Shirwany & Zou, 2010). During oxidative stress (i.e. hypoxia), cellular oxygen supply is limited to such an extent that oxidative metabolism suffers, threatening cell survival (Mungai *et al*.,

2011). This is probably the result of a progressive decline in the activity of the respiratory pathways (Huss & Kelly, 2005), which undermines the essential ATP-dependent processes within the cell (Mungai *et al*., 2011). Hypoxia therefore necessitates the cells to expend less energy in order to minimise oxygen usage (Hamanaka & Chandel, 2010). In addition, given the close relationship between workload and energy demand of the heart, any form of cardiac pathology (i.e. cardiac hypertrophy) will also impact the energy generation of the mitochondria, which are the key organelles for cellular ATP production (Abel & Doenst, 2011). Maintaining intracellular ATP reserves is therefore critical since an energy deficit can result in cardiac heart failure due to diminished cardiac pump function (Huss & Kelly, 2005). For this reason, multiple systems have evolved to protect the cells from the consequences of oxygen supply limitation and metabolic substrate deprivation (Mungai *et al*., 2011); one such an important system is the adenosine monophosphate protein kinase (AMPK) system.

2.4.3.2.2 AMPK structure and function

AMP-activated protein kinase is a phylogenetically heterotrimeric serine/threonine protein kinase that is found across all eukaryotic species (Evans *et al.*, 2009). Structurally, this enzyme complex system consists out of multiple subunit isoforms (Woods *et al.*, 1996; Cheung *et al*., 2000; Marsin *et al*., 2000; Stein *et al.*, 2000), which include two α-subunits (α1-2), two β-subunits (β1-2), and three γ-subunits (γ1-3) (Hardie & Carling, 1997; Hardie, 2003; Schimmack *et al.*, 2006; Hardie, 2011). The α subunits are known to contain the important Thr-172 regulatory residue, which is vital for subsequent AMPK activation through its phosphorylation **(**Stein *et al*., 2000; Towler & Hardie, 2007; Hutchinson *et al*., 2008) and have catalytic activity, whereas the remainder of the subunits play a regulatory role (Woods *et al.*, 1996; Cheung *et al*., 2000; Towler & Hardie, 2007; Kim *et al.*, 2009; Shirwany & Zou, 2010).

A very high homology in amino acid sequence has been demonstrated between chickens and mammals regarding AMPK subunits, suggesting that chicken AMPK may perform a similar function to that observed in mammals. High levels of phosphorylated (active) AMPK (pAMPK) have been observed in the liver, spleen, and most importantly the heart of the chicken (Proszkowiec-Weglarz *et al*., 2006b). Most of the isoforms of each subunit are expressed in the heart (Kim *et al.*, 2009), with the exception of the γ3 isoform, which is exclusively expressed only in skeletal muscle (Hardie, 2003; Young *et al*., 2005; Kim & Tian, 2011). However, complexes containing the α2 isoform are primarily responsible for cardiac AMPK activity under resting conditions, exercise, and ischeamia (Russell *et al*., 2004; Kim *et al.*, 2009).

2.4.3.2.3 Pathophysiological actions of AMPK in the cardio-vascular system

a) Activation of AMPK

Several researchers support AMPK being strongly activated under hypoxic conditions as a result of endogenous mitochondrial ROS production (Choi *et al.*, 2001; Kim *et al.*, 2009; Hamanaka & Chandel, 2010; Li & Keany, Jr., 2010; Mungai *et al.*, 2011); therefore, severe hypoxia would represent an important condition where enhanced AMPK activity would be expected (Mungai *et al*., 2011). However, the exact mechanism underlying this ROS-dependent activation of AMPK still remains to be elucidated (Mungai *et al.*, 2011). Upon exposure of cells to hypoxia, activation of AMPK has been shown to phosphorylate and activate protein kinase C zeta (PKCζ) (Gusarova *et al.*, 2009; Hamanaka & Chandel, 2010), which ultimately triggers endocytosis of the Na/K-ATPase protein complex (Gusarova *et al.*, 2009; Hamanaka & Chandel, 2010; Gusarova *et al.*, 2011). The Na/K-ATPase pump is responsible for pumping Na^+ and K^+ across the cell in order to maintain ionic gradients and is considered a major consumer of cellular ATP, accounting for 20-80% of oxygen expenditure in mammals (Hamanaka & Chandel, 2010).

Apart from hypoxia, AMPK is also extremely sensitive to a broad spectrum of other stress stimuli and enhanced activity is signalled in response to various physiological or pathological metabolic stresses (**Figure 2.3**) (Hardie, 2004; Hardie, 2008; Fogarty & Hardie, 2010; Li & Keany, Jr., 2010). During a severe oxygen deprivation, limitations in cellular processes (including mitochondrial respiration) result in the depletion of cellular energy levels, causing a drop in ATP levels together with a rise in intracellular AMP levels (Hardie & Carling, 1997; Young *et al*., 2005; Fogarty & Hardie, 2010; Li & Keany, Jr., 2010; Shirwany & Zou, 2010; Mungai *et al.*, 2011). Similarly, an increase in ATP consumption together with an unaltered ATP production will also contribute to an increased AMP/ATP ratio (Shirwany & Zou, 2010). This is considered the primary trigger of AMPK activation in an AMP-dependent manner (Dolinsky & Dyck, 2006) and is regarded as an indicator of metabolic stress (Dolinksy & Dyck, 2006; Evans *et al.*, 2009). Small changes in the AMP concentration indicate larger changes in the cellular energy balance, which is closely monitored by AMPK in order to maintain ATP concentrations within a very narrow range (Dolinksy & Dyck, 2006).

AMPK is allosterically activated upon binding of AMP to the γ-subunit which, in turn promotes the phosphorylation of the α-subunit by an upstream kinase at the critical threonine residue (Thr172) (Hardie & Carling, 1997; Mackenzie, 2010). Nevertheless, AMPK may confer greater protection if the kinase were to be activated before the cell reaches the point where the

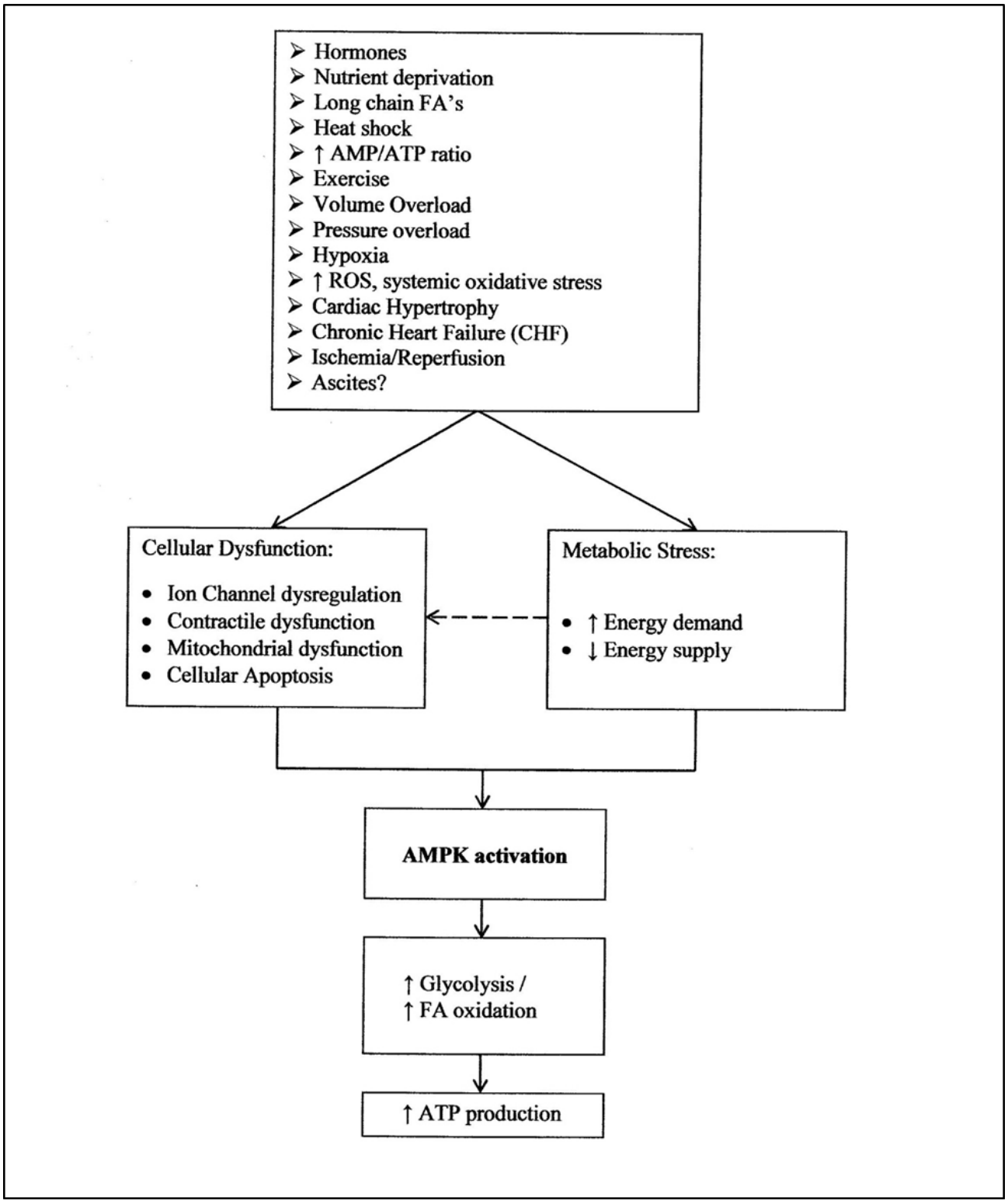

Figure 2.3: AMPK activation following various stress stimuli [Adapted from Dolinsky & Dyck (2006), Li & Keany, Jr. (2010), and Shirwany & Zou (2010)]

AMP/ATP ratio has increased (Mungai *et al.*, 2011). Some beneficial effects of AMPK signalling are not instantaneous, including gene expression and mitochondrial biogenesis (Mungai *et al.*, 2011); therefore enhanced signalling of AMPK in anticipation of a lethal bioenergetic crisis could enhance AMPK-mediated protection (Mungai *et al.*, 2011). During an energy shortage, phosphocreatine is also depleted to replenish ATP and the resultant fall in the phosphocreatine-to-creatine (Pcr/Cr) ratio in skeletal muscle has also been shown to activate AMPK (Dolinsky & Dyck, 2006). Although Pcr/Cr may not directly be involved in the heart, it is clear that AMP is involved in controlling cardiac AMPK activity and thus contributes to re-establishing the ATP content, which is important for cellular function (Dolinsky & Dyck, 2006).

More recently, mechanisms independent of bioenergetic changes have also been implicated to activate AMPK in various cells (Mackenzie, 2010). Stimuli causing an increase in intracellular Ca^{2+} activate a second AMPK kinase (AMPKK) called Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) in mammalian cells (Mackenzie, 2010; Mungai *et al.*, 2011). Activation of CaMKK, particularly the β-isoform (CaMKKβ) sequentially results in the activation of AMPK by also phosphorylating Thr-172, but in an AMP-independent manner (Mackenzie, 2010; Mungai *et al.*, 2011). Furthermore, it has been proposed that this enzyme is also activated upon ROS signalling (Mackenzie, 2010). High cytoplasmic Ca^{2+} concentrations may increase the demand for ATP due to its involvement in triggering ATP-demanding processes, such as muscle contraction or membrane trafficking (Mungai *et al.*, 2011).

Once activated, AMPK acts as a "fuel gauge" (Hardie & Carling, 1997; Marsin *et al*., 2000; Shirwany & Zou, 2010) or master metabolic switch turning on catabolic pathways to generate ATP (i.e. carbohydrate and fatty acid metabolism), while concomitantly terminating anabolic and nonessential processes that consume ATP (i.e. protein synthesis) (Hardie & Hawley, 2001; Hardie & Pan, 2002; Hardie *et al.*, 2003; Hardie, 2003, 2004; Young *et al.*, 2005; Allard *et al.*, 2007; Shirwany & Zou, 2010). In this regard, AMPK has been referred to as the "guardian of cellular energy" (Evans *et al.*, 2009). AMPK therefore functions as an energy sensor of the cell by closely regulating and balancing cellular energy resources (Hardie, 2008; Kim *et al*., 2012), which may restore the energy imbalance that occurs during oxidative stress (Choi *et al*., 2001; Young *et al*., 2005; Fisslthaler & Fleming, 2009; Shirwany & Zou, 2010). Along with AMPK's involvement in energy metabolism, AMPK may also phosphorylate targets such as ion channels and transporters, thereby contributing to other aspects of cellular function (Evans *et al.*, 2009). Moreover, AMPK has also been shown to activate pathways that inhibit the production of ROS (Young *et al*., 2005), modulate endogenous antioxidant gene expression, and/or suppress the production of oxidants (Shirwany & Zou, 2010).

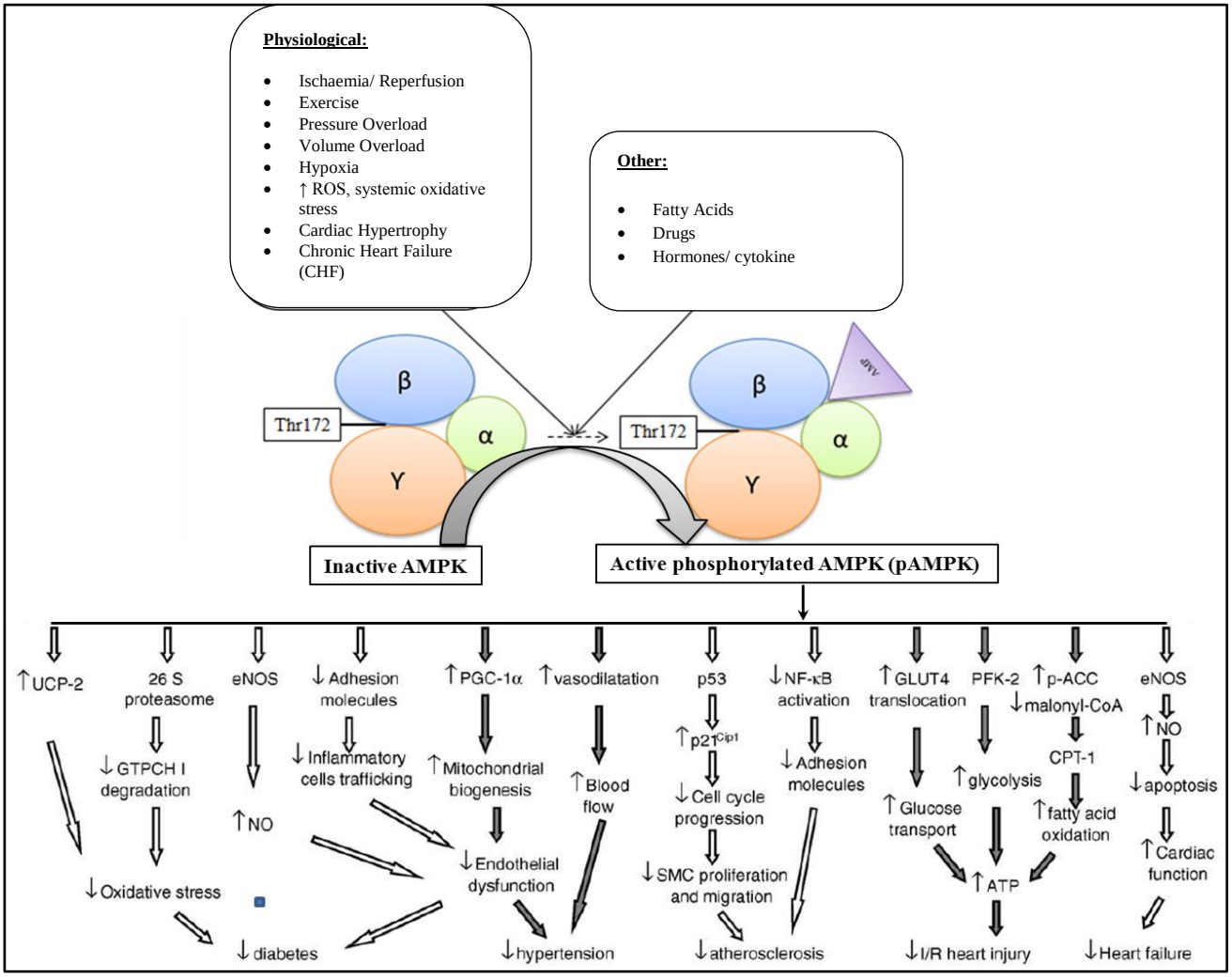

Figure 2.4: AMPK activation in response to various stimuli and its implication in cardiovascular disease [Adapted from Dolinsky & Dyck (2006), and Li & Keany, Jr. (2010)]

b) Regulation of energy metabolism by AMPK in response to various physiological and pathophysiological metabolic-related conditions

Growing evidence is revealing that AMPK may play a potential protective role in the pathological settings of the cardiomyocyte (Dolinsky & Dyck, 2006; Li & Keany, Jr., 2010; Shirwany & Zhou, 2010; Kim & Tian, 2011) (**Figure 2.4**). This may be attributed to the fact that AMP-activated protein kinase is a energy conserving enzyme signalling system that monitors systemic and cellular energy status (Hardie & Carling, 1997; Kemp *et al*., 1999; Dolinsky & Dyck, 2006; Musi, 2006; Viollet *et al*., 2009). Cardiac AMPK activation is thus essential to the heart through coordinating a cellular program that limits any further intracellular ATP depletion (Young *et al*., 2005; Allard *et al*., 2007; Shirwany & Zou, 2010). It may be suggested that AMPK plays a modulatory role during cardiac stress since many cardiovascular-related pathologies (such as hypertension, ischemia, left ventricular hypertrophy, heart attack and stroke) represent an environmental stress that induces "injury" signals to the vascular tissues and the heart (Hutchinson

et al., 2008; Li & Keany, Jr., 2010). In this way, the AMPK system may sustain life when faced with environmental challenges through continually adjusting biological processes of the organism (Kim *et al*., 2011).

Under normal conditions, AMPK activity in the unstressed heart is considered to be very low (Tian *et al*., 2001; Kim & Tian, 2011); however when the heart is subjected to various stresses, activation of AMPK is rapid and vigorous (Kudo *et al*.**,** 1996; Sambandam & Lopaschuck, 2003; Xing *et al.*, 2003; Russell *et al.*, 2004; Young *et al.*, 2005; Kim & Tian, 2011). Following a diminished oxygen or substrate supply, AMPK results in subsequent changes of myocardial energetics (Dolinsky & Dyck, 2006; Kim *et al.*, 2009) through regulating key steps in both fatty acid and glucose metabolism (Xing *et al.*, 2003; Sambandam & Lopaschuck, 2003; Russell *et al*., 2004; Dolinsky & Dyck, 2006; Kim *et al.*, 2009), thereby conserving intracellular energy stores (Marsin *et al.*, 2000). For this reason, AMPK has been dubbed the "guardian of energy status" in the heart (Hardie, 2004; Young *et al*., 2005).

AMPK has also been regarded as a central mediator of fatty acid utilisation in the heart (Dolinsky & Dyck, 2006). This may be attributed to its role in promoting fatty acid availability, fatty acid transport and also the oxidation of fatty acids within the cardiac myocytes (Dolinsky & Dyck, 2006; Steinberg & Kemp, 2009; Mungai *et al.*, 2011). Enhanced fatty acid transport into the cardiac myocyte may be the result of either an increase in the fatty acid concentration within the cell or a greater ATP generation as a result of enhanced fatty acid β-oxidation within the mitochondria (Kudo *et al.*, 1996; Dolinsky & Dyck, 2006). Kim e*t al.* (2009) suggested that AMPK may also be involved in accelerated fatty acid uptake from the triglyceride-containing lipoprotein by inactivating lipoprotein lipase, thus further liberating energy.

AMPK has also been shown to enhance glucose utilisation in the heart (Lopaschuck, 2008). Activation of AMPK may elevate the levels of the glucose transporter GLUT 4 to the muscle plasma membrane, leading to improved glucose uptake into the cell (Russell *et al.*, 1999; Steinberg & Kemp, 2009). AMP-activated protein kinase may also stimulate glycolysis through directly phosphorylating glycolytic enzymes such as 6-phosphofructo-kinase-2 (PFK-2) (Marsin *et al.*, 2000; Baron *et al.*, 2005; Dolinsky & Dyck, 2006; Viollett *et al*., 2009; Mungai *et al.*, 2011). These and other responses triggered by AMPK activation confer defense against hypoxic injury in tissues such as the heart by preserving energy supply, mitochondrial metabolism, and glycolytic flux (Russell *et al.*, 2004; Mungai *et al.*, 2011).

Another potential mechanism by which AMPK can compensate for the reduced ATP levels in the cardiac myocyte is the repression of non-essential energy-consuming pathways (*e.g.*, protein synthesis) that are not required for immediate cell survival. In an energetically compromised heart, the suppression of energy-consuming metabolic processes will direct ATP towards essential cellular

processes such as contractile function and homeostasis. During an oxygen deficit, the resulting intracellular ATP levels decrease, resulting in the suppression of protein synthesis. This mechanism has now been linked to the activation of AMPK. The concept of AMPK activation via protein inhibition may be one mechanism by which cellular ATP is conserved for more essential cellular processes. However, in the healthy heart inhibition of protein synthesis appears to play a very small role in maintaining mechanical function, but AMPK-mediated control of protein synthesis may be more relevant in severely stressed hearts (Dolinsky & Dyck, 2006).

Several researchers have proposed that AMPK regulation is essential in the heart since it is a critical mediator permitting myocardial adaptation in response to cardiac stress associated with ischemia/reperfusion, pressure overload, heart failure (Russell *et al.*, 2004; Li & Keany, Jr., 2010), and also pathological cardiac hypertrophy (Tian *et al.*, 2001; Russell *et al.*, 2004; Kim *et al.*, 2009; Kim *et al.*, 2012). Owing to the increase in AMPK concentration found in hypertrophied (Young *et al.*, 2005; Li & Keany, Jr., 2010; Kim & Tian, 2011) and ischemic hearts (Russell *et al.*, 2004; Baron *et al.*, 2005; Young *et al.*, 2005; Dolinsky & Dyck, 2006), it is clear that AMPK are associated with regulating metabolism during cardiac stress (Li & Keany, Jr., 2010). Evidence has also shown AMPK to reduce the incidence of cardiac hypertrophy in the heart (Dyck *et al.*, 2004; Kim *et al.*, 2009; Song and Zou, 2012). Most importantly, Beauloye *et al.* (2011) have revealed that AMPK activity may impede the transition of cardiac hypertrophy to heart failure. Investigations have uncovered that both α1- and α2-subunits of AMPK are activated in the heart during cardiac hypertrophy (Tian *et al.*, 2001; Kim *et al.*, 2009) and ischemia, though α2 appears to be activated to a greater extent as a result of myocardial ischemia (Russell *et al.*, 2004; Dolinsky & Dyck, 2006). Furthermore AMPK-deficient hearts in rats demonstrated a poor recovery of left ventricular function, increased necrosis, and enhanced myocyte apoptosis and dysfunction after ischemia and reperfusion **(**Russell *et al.*, 2004; Baron *et al.*, 2005; Li & Keany, Jr., 2010). In addition, canine studies have also revealed that *in vivo* pacing-induced heart failure was significantly improved by AMPK activation (possibly via eNOS stimulation), resulting in decreased apoptosis and improved cardiac function (Li & Keany, Jr., 2010). A deficiency in eNOS may be associated with pulmonary hypertension incidence (Han *et al.*, 2005).

Apart from its protective role in the heart, AMPK has also been regarded as a vital homeostatic mechanism that is capable of motoring O_2 supply and can adjust respiratory and circulatory function in order to meet demands (Evans *et al.*, 2009). Evans *et al.* (2009) demonstrated that during hypoxia, AMPK are activated leading to the phosphorylation of target proteins, such as ion channels, which initiate pulmonary artery constriction and carotid body activation. Through this, ventilation-perfusion is matched within the lungs, to divert blood flow from areas with an oxygen deficit to oxygen-rich environments, together with corrective changes in

breathing patterns. Furthermore, investigations based on exposing pulmonary arterial smooth muscle to physiological hypoxia confirmed a precipitated drop in cellular energy status (i.e. ↑ AMP/ATP ratio) as would be expected, which was accompanied with two-fold increase in AMPK activity (Evans *et al.*, 2009). These results clearly demonstrated that AMPK is also necessary for hypoxia-response coupling and may therefore regulate O_2 and energy supply at both whole body and cellular level (Evans *et al.*, 2009).

2.5 The effect of dietary betaine and related compounds in attenuating PHS

The concept that dietary betaine (TMG) supplementation and its intermediary metabolite dimethylglycine (DMG) may attenuate PHS is just starting to emerge. To our knowledge, we are the first to investigate the effect of betaine in ameliorating pulmonary hypertension syndrome (ascites) in broiler chickens.

2.5.1 Dietary sources of betaine

Betaine, also known by other names including trimethylglycine (TMG), glycine betaine, lycine or oxyneurine (Apicella, 2011), is a chemically derived N-trimethylated derivative of the amino acid glycine (Kidd *et al.*, 1997; Craig, 2004; Kalmar *et al.*, 2011). It exists in zwitterionic form at neutral pH (Craig, 2004; Ueland *et al.*, 2005; Apicella, 2011) with a chemical formula of $(CH_3)_3N^+CH_2COO^-$ (Craig, 2004) and molecular weight of 117.15 Da (Junnila, 2000; Lipiński *et al.*, 2012). TMG has been characterised as a methylamine due to the presence of its 3 chemical reactive methyl groups (Craig, 2004), whereas its intermediary metabolite, dimethylglycine (DMG), is characterised by containing only 2 methyl groups (**Figure 2.5**).

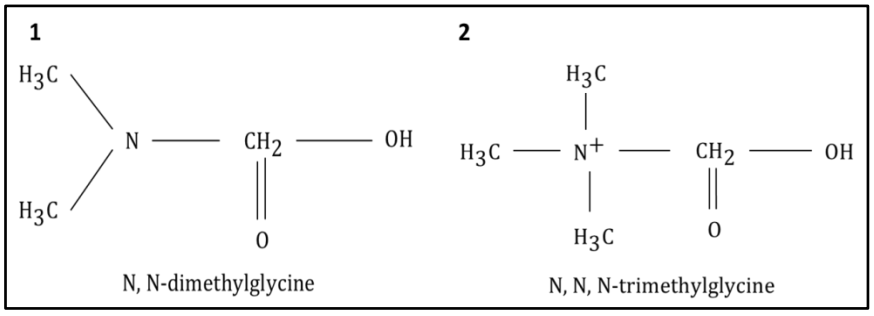

Figure 2.5: Chemical structure of (1) dimethylglycine and (2) trimethylglycine (betaine) (Bashir *et al.*, 2014)

Betaine was first discovered in the 19th century in the juice of sugar beets (*Beta vulgaris*) and then later found in several other organisms, such as shrimp and spinach (Zeisel *et al.*, 2003; Craig, 2004). It has been well documented that certain other food/feed components also contain considerable amounts of betaine (Craig, 2004) (**Table 2.1**). However sugar beet still remain the principal dietary source of betaine (Kidd *et al.*, 1997; Craig, 2004; Eklund *et al.*, 2005) with the highest level occuring in condensed molasses solubles, a by-product of sugar beet processing.

Betaine has been made commercially available as a feed additive to livestock diets in its purified form. Supplementary forms of feed-grade betaine include anhydrous betaine, betaine monophosphate, and betaine hydrochloride (Eklund *et al*., 2005). Apart from being obtained from dietary sources, betaine can also be acquired endogenously via the oxidation of free, non-lipid bound choline (Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009; Slow *et al*., 2009; Apicella, 2011).

However, most intracellular betaine is probably attained by uptake from the extracellular medium instead of from its subsequent synthesis (Kempson & Montrose, 2004; Ueland *et al*., 2005). In humans, the bioavailability of betaine is assumed to be close to a 100% (Schwahn *et al.*, 2003; Ueland *et al.*, 2005). Once ingested, betaine is rapidly absorbed in the ileum (Ueland *et al.*, 2005) or duodenum of the small intestine, with peak serum betaine concentrations occurring approximately 1-2 hours postprandial (Apicella, 2011).

In living systems, betaine is known to have two important functions. As a methyl group donor, betaine serves to convert homocysteine into methionine (Finkelstein, 1990; Simon, 1999; Junnila, 2000; Ueland *et al*., 2005; Apicella, 2011), and as an organic osmolyte, it aids in maintaining cellular water homeostasis (Metzelr-Zebeli *et al.*, 2009). It has also been reported that betaine may assist in stabilising protein structure under denaturing (Kempson & Montrose, 2004; Ueland *et al*., 2005); however, the focus here will be primarily on betaine's role as a methyl group donor and organic osmolyte.

2.5.2 Betaine – Nature's most efficient methyl source

2.5.2.1 The importance of methyl group metabolism

In order to understand the role of betaine as a methyl group donor one needs to have a clear understanding as to why methyl group metabolism is important in an organism's body. Methyl groups (CH_3) are essential molecules consisting out of one carbon atom and three hydrogen atoms, which are provided through the transmethylation cycle (Kim *et al.*, 2010). All biological systems including the nervous system, the immune system, the kidneys, the liver, the blood vessels and the heart, are dependent on these methyl groups for normal functioning to occur (James *et al.*, 2004). In addition, numerous substances require the synthesis of these methyl groups such as

Table 2.1: Betaine content of selected feed/food ingredients [Adapted from Kidd *et al.* (1997), and Craig *et al.* (2004)]

Feed ingredient	Betaine content (mg/kg)
Barley	730
Beets	1140-2970
Canola meal	Below detection limit*
Condensed molasses solubles	116000
Crackers	490-1990
Fish meal	400-1180
Groundnut meal	2520
Lucerne meal	3175-3850
Maize	Below detection limit*
Maize gluten meal	Below detection limit*
Oats	590
Peas	160
Pretzels	2370
Rice	590
Shrimp	2190
Soyabean meal	Below detection limit*
Spinach	6000-6450
Rapeseed meal	Below detection limit*
Wheat	1400 – 3960
Wheat bran	2675
Wheat bread	2010

*Average detection limit for betaine in feedstuff is about 150 mg/kg

creatine, phosphatidylcholine, carnitine, adrenaline, methyl purines, and the methylated amino acids (Kidd *et al.*, 1997). The methylation cycle is thus responsible for catalysing several methyltransferase reactions, which involves passing on methyl groups to the rest of the body facilitating in the production of various essential compounds including DNA, RNA, proteins, phospholipids (essential molecules involved in cell membrane and nerve health), stress hormones (eg., epinephrine and nor-epiniphrine), and the neurotransmitters (James *et al.*, 2004).

The body cannot synthesise sufficient methyl groups, thus they form an essential part of the vertebrate's dietary requirements (Kidd *et al.*, 1997; Metzler-Zebeli *et al.*, 2009). This demand for methyl groups is affected by numerous factors, including, age, sex (Mudd & Poole, 1975; Mudd *et al.*, 1980), dietary protein level (Pesti *et al.*, 1981), amino acid balance (Pesti *et al.*, 1981; Finkelstein & Martin, 1986), coccidian challenge (Tiihonen *et al.*, 1997), and chemical toxicity (Junnilla *et al.*, 1998). Inadequate supply of methyl groups will result in extreme damage to the afore-mentioned biological systems and this can be exacerbated during periods of stress. Therefore, following oxidative stress because of PHS, it would be expected that the requirements for methyl groups might be increased due to their subsequent depletion.

With regards to poultry and various other livestock production systems, the most important carriers of preformed, transferable methyl groups are the nutrients betaine, methionine, and choline (Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al.*, 2009; Selvakumar *et al.*, 2011). In addition, the intermediary metabolite of betaine, dimethylglycine (DMG), may also indirectly act as a methyl group donor (Eklund *et al.*, 2005) through the tetrahydrofolate cycle that requires structural changes to S-adenosylmethionine (SAM). After oxidation, the two methyl groups contained by DMG may split off to yield one-carbon fragments, which can be used to synthesise methyl groups *de novo* via the tetrahydrofolate pathway (Eklund *et al.*, 2005). Similarly, choline and methionine also necessitate structural changes before they can play their part as methyl group donors. Choline requires conversion to betaine in the liver to act indirectly as a methyl group donor, while methionine accepts an adenosyl group from ATP to form SAM before it is able to donate a methyl group (Eklund *et al.*, 2005). Other methyl group donors considered to be of less importance are the B vitamins (B_6, B_{12}) and folic acid, however, little thought is given to these due to their cost of dietary inclusion (Selvakumar *et al.*, 2011).

2.5.3 Betaine metabolism

Biochemical literature reveals that the metabolism of choline, betaine, methionine, and folate are all closely interrelated by transmethylation metabolic pathways (Kidd *et al.*, 1997; Bidulescu *et al.*, 2007). Perturbation in any one of these pathways will result in compensatory changes in the other (Bidulescu *et al.*, 2007). A clear discussion of betaine is therefore incomplete without referring to the metabolism of the other methyl groups (Kidd *et al.*, 1997). However, this literature review will mainly focus on the role of betaine in the liver since this is the main site for metabolism in poultry (personal communication, Dr. Janet Remus). Choline and methionine will be discussed when necessary to provide a better understanding of the role of betaine in the methylation cycle.

As previously mentioned, betaine (TMG) serves as a methyl group donor (Simon, 1999; Junnila, 2000; Apicella, 2011). Plasma concentration of betaine (TMG) is under homeostatic control (Slow *et al*., 2009) and it is released from the cell mitochondria of the liver and kidney into the cytosol (Kidd *et al.*, 1997; Simon, 1999; Junnila, 2000; Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009). Betaine requires no activation once in the cytosol and regardless of its origin, can then be directly used as a methyl group donor (Rafeeq *et al.*, 2011). As a natural intermediary metabolite in the conversion of choline to glycine (Kidd *et al.*, 1997; Kalmar, 2011), it is then catabolised through a series of transmethylation reactions (**Figure 2.6;** Kidd *et al.*, 1997; Junnila, 2000, Apicella, 2011). In short, choline is converted through a two-step enzyme-dependent reaction to betaine in the liver and kidney (Kidd *et al.*, 1997; Ueland *et al.*, 2005), which donates one of its 3 methyl groups to homocysteine. Choline, the precursor of betaine, is first oxidised to betaine aldehyde, which is catalysed by mitochondrial choline dehydrogenase (EC 1.1.99.1) (Junnila, 2000; Ueland *et al*., 2005). The second reaction encompasses the oxidation of betaine aldehyde through a NAD^+-dependant reaction (Junnila, 2000) to betaine in the mitochondria or cytoplasm by betaine aldehyde dehydrogenase (EC 1.1.1.8) (Ueland *et al.*, 2005). Once betaine is formed, it cannot further be metabolised within the mitochondria and it is transported out to the cytosol, possibly via passive diffusion (Ueland *et al.*, 2005), although the exact mechanism of transport still needs to be identified. Other metabolic routes of choline metabolism include the formation of acetylcholine (Simon, 1999; Siljander-Rasi *et al.*, 2003; Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al.*, 2009) and phospholipids, including phosphatidylcholine (Junnila, 2000; Ueland *et al.*, 2005). A high efficiency in the conversion of choline to betaine has been reported (Junnila, 2000), with this conversion being at least as great as the phosphorylation of choline to phophatidylcholine in the rat liver. In addition, choline has been involved in the methylation of homocysteine (HC) to methionine through the betaine dependant pathway; therefore, it indirectly contributes to the donation of labile methyl groups (Junnila, 2000; Bidulescu *et al.*, 2007). In this way, the oxidation of choline may serve as a spill over pathway during high levels of dietary choline (Ueland *et al.*, 2005).

Next to methionine, de-methylation of betaine in the cytosol results in the formation of DMG, which still contains two methyl groups (Eklund *et al.*, 2005; Ueland *et al.*, 2005; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al*., 2009). This reaction is catalysed by the enzyme betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5) (Apicella, 2011), which is regarded as one of the essential pathways necessary to re-methylate HC to methionine (Slow *et al.*, 2004; Kalmar, 2011). DMG is a feedback inhibitor of BHMT and is normally excreted in urine (McGregor *et al.*, 2001) or degraded to sarcosine (N-methylglycine) and finally to glycine (McGregor *et al.*, 2001; Craig, 2004; Slow *et* al., 2004; Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009; Kalmar *et al.*, 2011). During oxidation,

DMG's two methyl groups can split off forming two one-carbon fragments that can be used together with other sources, such as formic acid or carboxyl groups of other organic acids, to synthesise methyl groups *de novo* (Finkelstein, 1998; Eklund *et al.*, 2005; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al*., 2009). This occurs via the tetrahydrofolate pathway in order to yield 5-methyltetrahydrofolate, which are then transferred via the enzyme THFMT to homocysteine to form methionine (Eklund *et al.*, 2005; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al*., 2009). Thus, both TMG (Kidd *et al.*, 1997) and DMG can also donate methyl groups to HC via the folate pool in order to form methionine (Bidulescu *et al.*, 2007). However, it is evident that DMG has to cycle through a complex series of reactions (**Figure 2.6**) before that carbon group, as a part of 5-methyltetrahydrofolate, can be used by the transmethylation cycle for the ultimate production of SAM (Devlin, 1982). Therefore, when folate's availability is reduced, the subsequent demand for betaine as a methyl group donor is then especially increased (Bidulescu *et al.*, 2007).

2.5.3.1 The role of betaine in homocysteine re-methylation

Betaine in its role as a methyl group donor plays a crucial role in methyl group metabolism and the methylation cycles necessary to maintain normal body functions. Most importantly, betaine conserves methionine by re-methylating HC to methionine, detoxifies HC, and produces S-adenosylmethionine (SAM) (Craig, 2004; Apicella, 2011), which is considered the principle methyl group donor in transmethylation reactions including the synthesis and metabolism of phosphatidylcholine (a phospholipid that is an essential molecule, involved in cell membrane and nerve health), carnitine, and creatine (Metzler-Zebeli *et al*., 2009). In this way, methionine is conserved under conditions of dietary deficiency (Finkelstein *et al.*, 1983) and both SAM and HC are maintained at relatively constant levels (Kettunen *et al.*, 2001c). This suggests that poultry has a specific requirement for preformed labile methyl groups when these are deficient in the diet (El-Husseiny *et al.*, 2007; Ratriyanto *et al*., 2009).

Two mechanisms are involved in the redistribution of HC during the methylation cycle. The first reaction involves the removal of HC during the transulphuration pathway through the irreversible transformation of HC to cystathionine through cystathionine β-synthase and afterwards to cysteine, which can be utilised for protein synthesis (Finkelstein, 1990; Junnila, 2000). The second reaction involves the re-methylation of HC by means of other sources to form methionine (Finkelstein & Martin, 1984; Kidd *et al.*, 1997; James *et al.*, 2004; Kumar *et al.*, 2012). This can be achieved via either (i) the supplementation of methyl groups to the diet, (ii) or via the previously mentioned betaine pathway involving BHMT or (iii) by means of the tetrahydrofolate pathway (involving folates and vitamin B_{12}) through methyl-tetrahydrofolate-homocysteine-

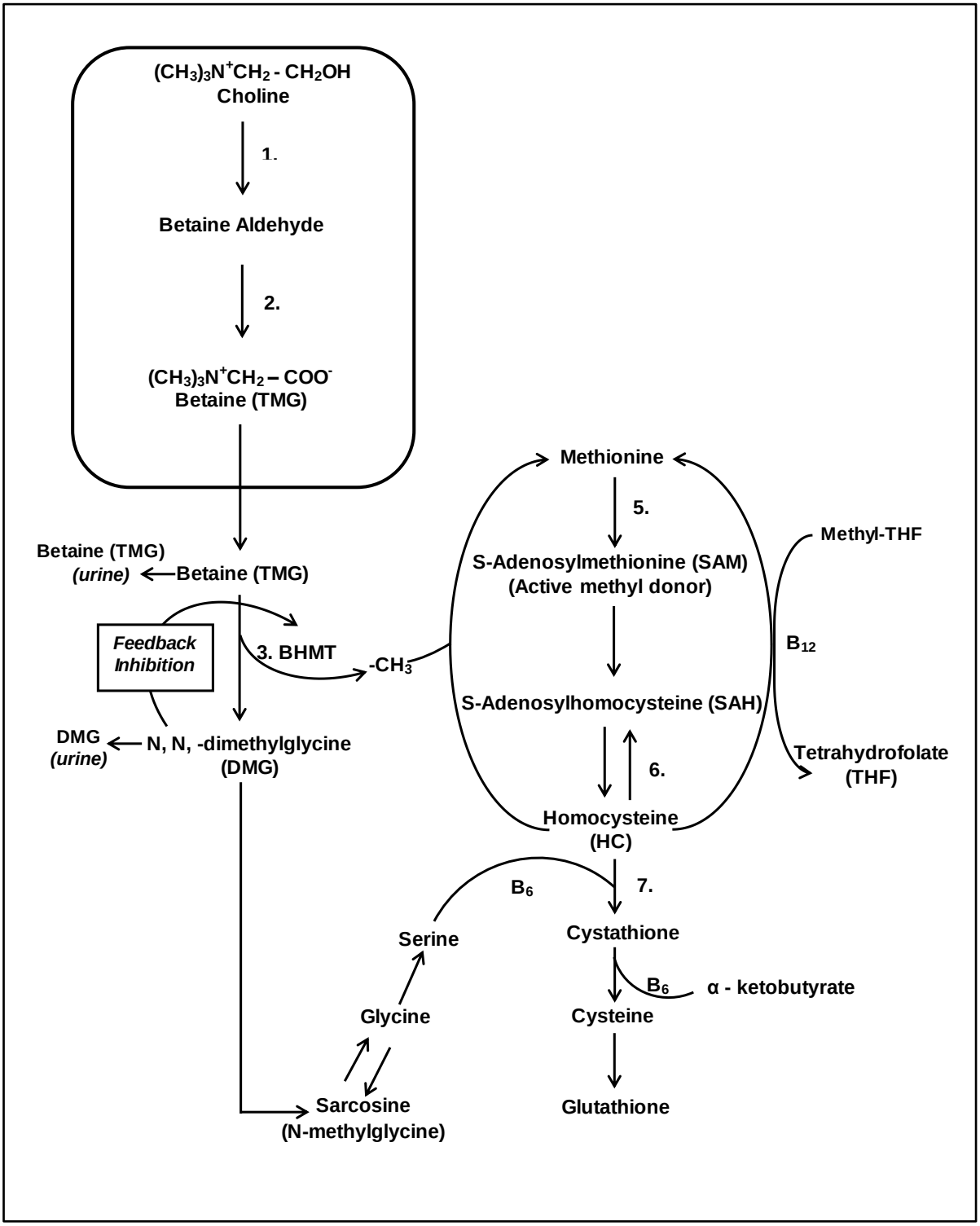

Figure 2.6: Betaine and choline metabolism in the poultry liver and kidney. Upon entry into the mitochondrion, choline is metabolised to betaine by two sequential and physiologically irreversible oxidative reactions, first by the action of choline oxidase, followed by betaine aldehyde dehydrogenase. Betaine is transported out of the mitochondria into the cytosol where it is further metabolised yielding N, N, -dimethylgycine and re-methylating homocysteine (HC) to methionine via betaine-homocysteine methyltransferase (EC 2.1.1.5). Numbered enzymes include (1) choline oxidase (EC 3.1.1.27); (2) betaine aldehyde dehydrogenase (EC 1.2.1.8); (3) betaine homocysteine methyltransferase (EC 2.1.1.5); (4) methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.3); (5) methionine adenosyl transferase (EC 2.5.1.6); (6) adenosylhomocyteinase (EC 3.3.1.1); (7) cystathione- beta-synthase (EC 4.1.22) [Adapted from Kidd *et al.* (1997), Junnila (2000), McGregor *et al.* (2001), and Clow *et al*. (2008)]

methyltransferase (THFMT; methionine synthase (MS); EC 2.1.1.3) (Finkelstein & Martin, 1984; Finkelstein, 1990; Kidd *et al.*, 1997; Junnila, 2000). Both the BHMT and MS pathways are equally important due to their dual role in transulphuration. Firstly, these enzymes are crucial for maintaining methylation capacity, and secondly, they are involved in the reduction of HC levels (Eklund *et al.*, 2005; Ratriyanto *et al.*, 2009; Lever & Slow, 2010; Kalmar, 2011), which has been put forward as an independent risk factor for cardiovascular disease (Bayés *et al.*, 2001; Lever & Slow, 2010; Sibrian-Vazquez *et al.*, 2010; Kalmar, 2011).

Several researchers observed enhanced re-cycling of total HC through both BHMT and tetrahydrofolate (THFT) pathways when betaine was supplemented to poultry diets (Kettunen *et al.*, 2001c; El-Husseiny *et al.*, 2007). Hepatic BHMT activity was increased in broilers upon dietary changes in betaine, choline, or sulfur containing amino acid (SAA) concentrations in poultry diets (Saunderson & Mackinlay, 1990; Emmert *et al.*, 1996). Saunderson & Mackinlay (1990) reported that BHMT activity was higher than MS activity in the avian liver following dietary betaine supplementation. Wang *et al*. (2004) found that betaine is an effective methyl group donor when added to methionine-deficient diets during insufficient homocysteine re-methylation. However, limited betaine response can be expected when cysteine is deficient in the basal diet because homocysteine would be completely used in the transsulfuration pathway (Rostagno & Pack, 1996; Schutte *et al.*, 1997; Esteve-Garcia & Mack, 2000). It is important to note that the underlying mechanism of the MS pathway on hepatic homocysteine re-methylation containing excessive dietary choline or betaine levels in poultry have not been clearly explained yet (Metzler-Zebeli *et al.*, 2009). Pillai *et al.* (2006a, b) suggested that MS-dependant remethylation of HC clearly predominates and seems to be influenced by dietary changes. They observed an increased level of MS-dependant re-methylation rather than BHMT-dependant re-methylation and an absence of re-methylation response to SAA level. Therefore, HC appears to be partitioned equally between the re-methylation pathways in broiler diets containing adequate levels of SAA (Pillai *et al.*, 2006a, b).

Failure to re-methylate HC to methionine will result in inadequate removal of HC and consequently reduced transmethylation and transsulfuration. Inadequate HC re-methylation may be the result of either (i) an inadequate intake of sufficient methyl groups resulting in a deficiency of methyl group donors and/or acceptors involving methionine-conserving and methionine–catabolising enzymes (Finkelstein, 1998); (ii) high SAM levels inadequately regenerating methionine back to HC (Bidulescu *et al.*, 2007); (iii) toxic accumulation of methionine and HC in the blood under non-physiological conditions (Kidd *et al.*, 1997); or (iv) abnormal methylation due to low plasma choline or plasma betaine (Bidulescu *et al.*, 2007). The resultant high HC

concentrations may all contribute to the potential hazard of cardiovascular diseases (Bidulescu *et al.*, 2007; Kim *et al.*, 2010). Furthermore, the resultant reduced flux of transsulfuration has also been coupled with low levels of SAM, which is required for normal methylation activity as well as reduced synthesis of cystathionine, cysteine (the rate-limiting amino acid required for certain antioxidants), and total glutathione (James *et al.*, 2004). The decrease associated with the rate-limiting amino acids, cysteine and glutathione, creates an increase in vulnerability to oxidative stress (James *et al.*, 2004). Thus, it is clear that the regulation of the transmethylation cycle is of crucial importance in order to maintain physiological levels of HC and methionine (Finkelstein, 1998).

Several researchers have described the role of HC levels with several major diseases, most importantly cardiovascular disease (Bayés *et al.*, 2001; Sibrian-Vazquez *et al.*, 2010). As a free amino acid, HC can occur either in the reduced form containing a thiol group (SH) or it can be present in its oxidised form. Its redox chemistry is dominated by its thiol group, which is readily oxidised. Oxidation of two homocysteine molecules yields the disulphide, two protons (H^+) and two electrons (e-) (Kumar *et al.*, 2012). Rapid oxidation upon its subsequent release in the plasma (Bayés *et al.*, 2001) has been reported to encourage oxidative stress due to the generation of reactive oxygen species (ROS) (Sibrian-Vazquez *et al.*, 2010). The underlying mechanism imposing a cardiovascular risk is thought to act through a mechanism involving oxidative damage (Bayés *et al.*, 2001). During the course of oxidation of the sulfhydryl group (in the presence of oxygen and metal ions) of HC, highly reactive partially reduced oxygen species are generated, including the superoxide anion radical (O^{2-}) and hydrogen peroxide (H_2O_2) (Bayés *et al.*, 2001; Kumar *et al.*, 2012). These potent reactive molecules facilitate oxidative injury through instigating cytotoxicity and lipid peroxidation (Bayés *et al.*, 2001) and oxidative stress is also linked with reduced pulmonary and circulatory function (Sibrian-Vazquez *et al.*, 2010). Regulating the oxidation state of sulphur-containing amino acids is therefore important in reducing cellular-mediated damage due to the potential role of thiols initiating lipid peroxidation, producing hydroxyl radicals, and oxidatively cleaving proteins (Kumar *et al.*, 2012).

Several researchers have described the potential of dietary supplemented methyl groups on lowering HC levels, of which betaine appears to have the greatest impact (Clarke *et al.*, 2002; Olthof & Verhoef, 2005; Bidulescu *et al.*, 2007). Stekol *et al.* (1953) found that dietary betaine methylated HC to methionine three times more efficiently than dietary choline in chickens. Furthermore, supplementation of the methyl group donors TMG and DMG have been reported to

have an immediate as well as a long-term effect in lowering HC concentrations in the plasma (Clarke *et al.*, 2002; Olthof & Verhoef, 2005; Bidulescu *et al.*, 2007).

2.5.4 The economic benefit of supplementing diets with betaine

There is considerable interest about the efficacy of betaine as a feed additive in poultry and pig diets due to its potential role to replace part of the dietary choline and methionine (Metzler-Zebeli *et al*., 2009). Commercial broiler production systems have difficulty in incorporating labile methyl groups into poultry feed (Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009) and have combined choline and methionine as feed additives in commercial poultry rations to overome these constraints (Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009). Methionine and choline are both essential in that they cannot be produced by the bird itself in sufficient amounts to support maximum growth (Moritz *et al*., 2005) and broilers particularly have a high methionine requirement that cannot be obtained from the maize and soybean fraction of diets. In addition, choline may be unavailable, likely due to it being used at a faster rate than being synthesised. For these reasons, broilers require ingredient sources of methionine and/or choline to meet their requirements. In order to have an adequate supply of available methyl groups, additional betaine may therefore be required in the diet (Metzler-Zebeli *et al.*, 2009). The use of betaine as a methyl group donor can be attributed to its methionine (Finkelstein *et al.*, 1983; Kidd *et al.*, 1997; Overland *et al.*, 1999; Lipiński *et al*., 2012) or choline sparing effect, thus making more methionine and choline available for other important metabolic functions (Kidd *et al*., 1997; Simon, 1999; Craig, 2004; Lipiński *et al*., 2012). On a more practical basis, Kidd *et al*. (1997) estimated that 1 kg of anhydrous betaine (97%) supplies equivalent amounts of methyl groups as 1.25 kg DL-methionine or 1.65 kg of choline chloride (70%). Therefore, betaine contains 0.90 and 3.75 times the methyl groups than contained by choline and methionine, respectively (Kidd *et al*., 1997). By sparing the total amount of synthetic choline and methionine, betaine may thereby reduce the overall feed costs of broiler rations while still meeting the bird's nutritional requirements (Lipiński *et al*., 2012).

Many investigators have studied the interrelationship between choline, and methionine paired with betaine to determine if these compounds can spare the needs of chickens for both choline and methionine (Rostagno & Pack, 1996; Esteve-Garcia & Mack, 2000; Pillai *et al*., 2006b; Zhan *et al*., 2006). Research results have found betaine, as a methyl donor, to be far more efficient compared to the other methyl group donors when routinely added to poultry and pig diets (Horne & Remus, 2012). To support this, Saarinen *et al*. (2001) reported that dietary betaine is almost twice as

efficient for increasing betaine levels in broiler chicks compared to an equal molar of choline. It is thus clear that betaine is potent in providing adequate amounts of methyl groups for various metabolic functions.

2.5.4.1 Methionine sparing

It is well known that methionine lies at the crossroads of both protein synthesis and the formation of the universal methyl group donor, SAM (Finkelstein, 1998; Ratriyanto *et al.*, 2009). Therefore, any alternative methyl group donors that can replace methionine as a donor or provide methyl groups from HC to methionine to act as a methyl group donor should promote methionine's use for protein synthesis (McDevitt *et al*., 2000). In this regard, betaine has been proven to spare methionine's methyl donor activity, and positively influenced traits like breast yield and abdominal fat in broiler diets containing suboptimal concentrations of methionine and supplemental betaine (Rama-Rao *et al*., 2011).

There have been many controversies in the literature on the ability of betaine to spare some added synthetic methionine to the diet. However, much of the conflicting results can perhaps be ascribed to the specific diet's formulation and whether it allows methionine sparing or not (personal communication, Dr. Janet Remus). The amount of betaine required to spare a part of the added dietary methionine also depends on the supply of dietary cysteine (Firman & Remus, 1999; Eklund *et al*., 2005). When the animal's cysteine requirements are met, methionine is exclusively used for protein synthesis, but when cysteine is deficient, methionine will be irreversibly degraded to cysteine (Metzler-Zebeli *et al.*, 2009). Therefore, it is evident that dietary formulations must suffice in the animals' requirements for cysteine in order for betaine to effectively spare added methionine.

2.5.4.2 Sparing of choline

Betaine has been shown to be a more efficient methyl donor than choline, possibly due to betaine being directly used as a methyl source whereas choline must first go through a two-step enzymatic reaction to be converted to betaine before being used (Kidd *et al*., 1997). Furthermore, the methylating efficiency of choline can be highly variable due to inefficiencies in the metabolic conversion of choline to betaine (personnel communication, Dr. Janet Remus) thus confirming betaine to be more bio-available as a methyl source.

Apart from acting as a methyl group donor, choline also has other essential functions within the animal's body, including the synthesis of the neurotransmitter acetylcholine, fat utilisation and transport by the liver, as well as cellular integrity through its involvement in the synthesis of

membrane phospholipids (i.e. phosphatidylcholine) (Eklund *et al.*, 2005). Dilger *et al.* (2007) showed that 50% of the chick's choline requirements have to be met by choline itself, but the remaining 50% can be replaced by dietary betaine. Therefore, it appears that there is a specific minimum dietary requirement for choline (1200ppm in the starter diets, 1000ppm in grower and finisher diets) to support its non-methyl roles (personnel communication, Dr. Janet Remus). Several studies that have investigated the interchange between dietary synthetic choline and betaine have shown that in most instances the bird's endogenous choline requirements for non-methyl needs can invariably be satisfied from the raw materials added to the diet (Horne & Remus, 2012). Betaine may therefore adequately substitute for supplemental choline in the diet in many cases and more effectively satisfy the needs of the methyl donors.

2.5.5 Role of betaine as an organic osmolyte

2.5.5.1 Osmoregulation

In order to understand the importance of betaine as an organic osmolyte, it must first be understood that water is the key factor determining the physiological state of the cell (Häussinger, 1996), which is governed by osmoregulation. Osmoregulation is simply the ability of cells to maintain its structure and function by regulating both intra- and extracellular cell water volume (Kidd *et al.*, 1997). Most compounds present in the animal body are comprised of water, which clearly demonstrates its fundamental role in cell survival and function; for example chick heart fibroblast cells have been shown to contain about 87% water (Kidd *et al.*, 1997). It is therefore evident that the main goal of osmotic regulation is to maintain cellular homeostasis by keeping the cell volume as constant as possible, since cell activity is highly influenced upon exposure to different osmotic pressures (Häussinger, 1996).

It is well known that hyperosmotic stress results in dehydration of cells. Loss of intracellular water leads to cell shrinkage, negatively affecting nutrient absorption and cell membrane transport as well as impairing processes important for cell function, such as the metabolism of amino acids, ammonia, carbohydrates, and fatty acids (Häussinger, 1996; Cronje, 2007). In response to intracellular water loss, several inorganic ions, including potassium, interact with cellular organelles and enzymes in order to increase the electrolyte concentration within the cell. However, these inorganic ions can bind to active sites of the enzyme perturbing the activity of the affected enzymes, which eventually overwhelms the cell's ability to handle the high levels of intracellular potassium as water balance stress continues (Burg, 1994; Petronini *et al.*, 1992, 1994; Kidd *et al.*, 1997; Craig,

2004). In extreme cases of water loss, cells may shrink to such an extent that it can disrupt the tight junctions that hold them together and the resultant cellular damage allows unknown pathogens and toxins to enter the cells (Cronje, 2007). The overall outcome of this is cell death as a result of its inability to potentially hold water.

Maintaining stable intracellular and extracellular ion concentration gradients and a stable water level requires a considerable amount of energy. This energy is essential to drive ion pumps, in particular the Na/K-ATPase pump, in order to preserve the water cellular balance (Cronje, 2005; Lipiński *et al*., 2012) (**Figure 2.7**). The Na/K-ATPase pumps function to minimise water movement out of the cell through pumping electrolytes (mainly K^+) into the cell thereby increasing the concentration within the cell. While these pumps maintain water balance mostly during short-term stresses, they consume a lot of energy (ATP) making them energetically costly. Research conducted by Kettenun *et al.* (2001a, b) demonstrated that during periods of osmotic stress, broilers were able to reduce cellular osmotic pressure by transferring organic osmolytes into and out of their cells thereby maintaining a stable osmotic gradient within the body without altering nutrient absorption or metabolism.

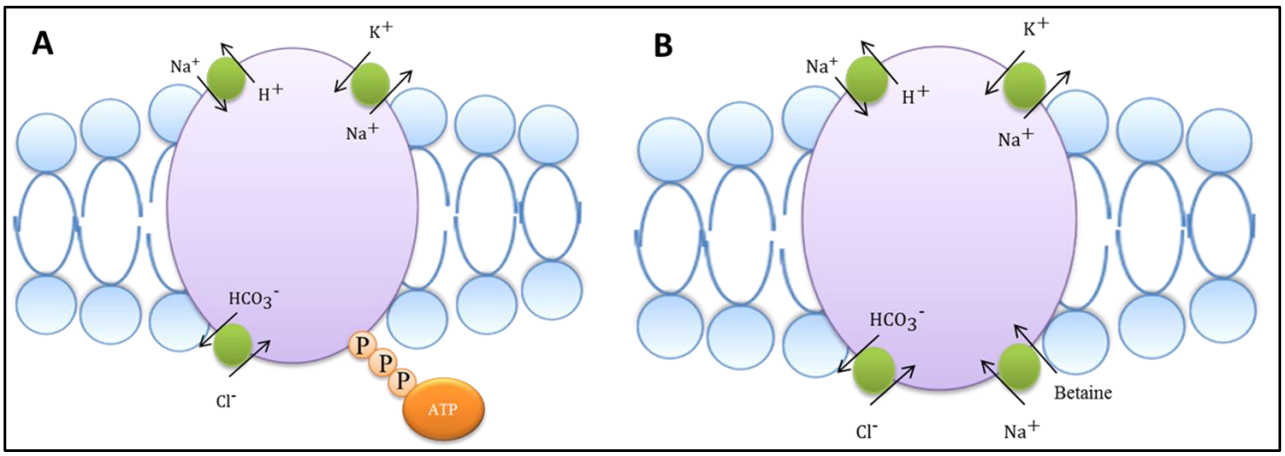

Figure 2.7: Compensation of ion pumps during hyperosmotic stress via (A) elevated electrolyte concentrations and (B) via betaine accumulation within cells. A - In order to maintain water balance, electrolytes are pumped into the cell. One unit of energy (ATP) is required for every one electrolyte pumped into the cell. B - Betaine (mostly from the diet) is taken up via passive diffusion, which does not require ATP to pump it into the cells. Although a carrier for betaine has been identified in humans, this still yet remains to be determined for farm animals [Adapted from Horne & Remus (2012)]

a) Betaine as an osmoprotectant during osmotic stress

Osmotic stress may be caused by numerous factors including disease, malnutrition and/ or inadequate environmental conditions (Lipiński *et al*., 2012). Most cells must adapt to these external osmotic pressures or stresses in order to maintain homeostasis and thus avoid osmotic stress. This may be achieved through accumulating organic osmolytes (i.e. methylated amines, certain amino acids, and sugar alcohols) and low molecular weight inorganic ions (i.e. sodium, potassium, and chloride). As previously mentioned, high concentrations of inorganic ions alter protein structure and enzyme function, and are thus limited as osmotic effectors (Burg, 1994; Petronini *et al.*, 1992, 1994; Kidd *et al.*, 1997; Craig, 2004). On the other hand, alterations of organic osmolyte concentrations have been shown to be highly compatible with the former functions and therefore do not disrupt normal metabolic processes (Kidd *et al.*, 1997; Wehner *et al.*, 2003). These compatible osmolytes therefore play an important role in minimising water loss against a prevailing osmotic gradient (Kettunen *et al.*, 2001b*;* Klasing *et al*., 2002) and in doing so maintain cell water volume (Metzler-Zebeli *et al*., 2009; Ratriyanto *et al.*, 2009).

Betaine has been viewed as a widespread compatible osmolyte for various organisms including prokaryotes, animals, algae, and salt-tolerant plants (Metzler-Zebeli *et al.*, 2009). Interestingly, the activity of BHMT is also osmoregulated, thus dividing betaine between its two separate paths as a methyl group donor and as an organic osmolyte (Apicella, 2011; Ueland, 2011). Therefore, apart from betaine being catabolised, it also plays a crucial role in regulating cell volume (Kidd *et al*., 1997; Craig, 2004; Apicella, 2011; Ueland, 2011; Lipiński *et al*., 2012). Betaine's osmotic activities have been attributed to both its zwitterion character and its exceptionally high water solubility (Eklund *et al*., 2005; Metzler-Zebeli *et al.*, 2009).

Accumulation of betaine may take place either through its synthesis or by the uptake of dietary betaine. Due to betaine metabolism being limited only to the liver and kidney, as well as it being utilised by these tissues, dietary uptake of betaine seems to be the primary mechanism of obtaining betaine for osmoregulation in vertebrates (Burg, 1994), since it is not available for metabolism. Dietary betaine can be taken up by diffusion, which is coupled to the transport of Na^+ and K^+ across the cell wall (Cronje, 2007) and can increase the betaine concentration within the cell so that it is higher than outside the cell (**Figure 2.7**). The resultant concentration difference across the cell attracts water and allows the cell to "hold" water during adverse situations. In this way betaine may act as an osmoprotectant, thereby replacing inorganic ions as well as protecting enzymes and cell membranes from inactivation by inorganic ions since it does not interfere with enzyme activity (Petronini *et al.*, 1992). In addition, accumulation of betaine may be advantageous

as it has no harmful effect on energy production and is thus less energetically costly compared to other electrolytes. This makes betaine an ideal supplement for improving resilience of gut cells during periods of osmotic stress (Cronje, 2007) as it has been demonstrated to be involved in the osmoregulation of the small intestine of poultry (Kettenun *et al.*, 2001a).

Many studies have shown the positive effects of betaine as an organic osmolyte in withstanding osmotic stress. This has been demonstrated by Petronini *et al*. (1992) who found that betaine replaced inorganic ions and also protected enzymes and cell membranes from inactivation by these ions; for this reason, it has been termed an 'osmoprotectant' (Petronini *et al*., 1992). Further studies showed that betaine significantly reduced the transport of water out of intestinal cells when osmotic pressure was increased (Kettenun *et al.*, 2001b) and improved gut morphology (Kettenun *et al.*, 2001c) when added to poultry diets. Moeckel *et al.* (2002) reported a 42% increase in the resilience of gut cells upon osmotic stress following betaine supplementation. Moreover, betaine has been found to exert anti-apoptotic effects (Alfieri *et al.*, 2002), thus enhancing the proliferation of cells subjected to hyperosmotic shock and counteracting the inhibition of cell protein synthesis (Petronini *et al*., 1992). Further work has demonstrated that betaine may increase nutrient absorption in poultry by overcoming these osmotic stresses (Eklund *et al.*, 2005) and may also modulate immunity in the osmotically stressed liver macrophages and phagocytes (Craig, 2004), making it an extremely comprehensive supplement.

It is also known that the osmotic challenge imposed by modern production diets results in the limited ability of the ion pumps to maintain normal function; however supplemental betaine has shown to spare energy expenditure for ion pumping thereby making more energy available for cell proliferation (Moeckel *et al.*, 2002). Most significantly, betaine has also been shown to aid in the neutralisation of cell volume in erythrocytes through regulating membrane ATPases (Moeckel *et al.*, 2002; Craig, 2004), thereby reducing some of the energy expended during ion pumping in cells exposed to hyperosmotic media (Moeckel *et al*., 2002; Eklund *et al*., 2005). Moeckel *et al.* (2002) found that both the activity of the Na^{+}/K^{+} pump and that of the Ca^{++} pump were significantly reduced by 64% and 73%, respectively, following betaine supplementation. As a consequence, betaine may thus also reduce the energy requirements needed for maintenance when added to the feed due to lower energy expenditures (Ratriyanto *et al.*, 2009; Lipiński *et al.*, 2012). This would allow more energy to be spared and become available for intestinal cell proliferation, subsequently increasing the absorptive capacity of the bird (Eklund *et al.*, 2005). By supplementing betaine to poultry diets, it will therefore enable them to retain water allowing more energy for growth (Jahanian and Rahmani, 2008). Finally, Saunderson and Mackinlay (1990) have suggested that

betaine may also be regarded as an anti-stress agent. Betaine can therefore be regarded an important osmoprotectant that assits in maintaining normal cell volume since metabolic processes (i.e. protein turnover, membrane transport, endocytosis, as well as the metabolism of amino acids, ammonia, carbohydrates, and fatty acids) in the cell are deeply influenced by small changes in cell volume (Häussinger, 1996; Metzler-Zebeli *et al.*, 2009).

Due to dysfunctional osmoregulation as a result of PHS (Kidd *et al.*, 1997), the beneficial effects of supplementing betaine, whose main effect is to protect against osmotic stress, may therefore offer compelling support to help ameliorate this metabolic condition. Furthermore, cellular destruction by ROS as a result of oxygen and energy deprivation can disrupt cell structure and membrane integrity, as well as impair the ability of ion pumps to maintain cellular ion homeostasis (Cronje, 2007); by activating AMPK, betaine may also help diminish the susceptibility to the adverse consequences of PHS, thus providing a causal link between PHS, AMPK, and betaine.

2.5.6 Practical application of betaine

A significant amount of research has been carried out over the last few decades concerning the efficacy of betaine in poultry with regards to growth performance, carcass composition, and intestinal health (Metzler-Zebeli *et al.*, 2009). Some of the investigated effects of betaine and its intermediary metabolite dimethylglycine on poultry performance are presented in **Tables 2.3** and **2.4.** Although not a topic of this literature review, other potential benefits of betaine have also been considerably studied (**Table 2.2**).

Table 2.2: Benefits of adding betaine to poultry diets

Benefits of betaine	References
Betaine and coccidiosis	*E.g.* Augustine *et al.*, 1997; Augustine & Danforth, 1999; Teeter *et al.*, 1999; Kettunen *et al.*, 2001b,c; Klasing *et* al., 2002; Metzler-Zebeli *et al*., 2009.
Betaine and heat stress	*E.g.* Moeckel *et al.*, 2002; Cronje, 2005; Cronje 2007; Mahhoudnia & Madani, 2012.
Betaine and nutrient digestibility	*E.g.* Remus *et al.*, 1995; Remus & Virtanen, 1996; Augustine & Danforth, 1999; Overland *et al.*, 1999; Xu & Yu, 2000; Kettunen *et al.*, 2001c; Eklund *et al.*, 2005; El-Husseiny *et al*., 2007; Honorbakhsh *et al*., 2007; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al.*, 2009

2.5.6.1 The effects of betaine on performance and carcass characteristics

a) Role of betaine in lipid metabolism

In birds, fat synthesis (lipogenesis) mainly occurs in the liver rather than the adipose tissue, making fat deposition of adipose tissue highly dependent on the availability of lipoproteins either originating from the diet or from the liver (Kalmar *et al.*, 2011). Diets containing oil have shown to increase the chick's response to supplemental betaine, thereby indicating that betaine supplementation may be beneficial with important fat additions in broiler diets, especially during the summer months in an attempt to reduce heat stress (Mahmoudnia & Madani, 2012).

Numerous studies suggest that betaine may have an effect on lipid metabolism (Wang *et al.*, 2004; Attia *et al.*, 2005; Zhan *et al.*, 2006; Baghaei *et al.*, 2009) and therefore is it considered as a lipotropic compound. It is well understood that dietary betaine supplementation can positively affect liver fat metabolism by improving choline availability (Eklund *et al.*, 2005). By sparing choline, betaine may increase choline's accessibility for lecithin synthesis which is required for fat transport throughout the body (Saunderson & Mackinlay, 1990). Sparing choline may increase the synthesis of very-low-density-lipoprotein, thereby preventing fat deposition and accelerating fat removal from the liver (Metzler-Zebeli *et al*., 2009; Ratriyanto *et al*., 2009). Additionally, betaine may also interact with lipid metabolism through indirectly increasing creatine and carnitine synthesis in the liver and muscle (Remus *et al.*, 1995; Xu and Zhan, 1998; McDevitt *et al*., 2000; Zhan *et al.*, 2006; Baghaei *et al.*, 2009; Maghoul *et al.*, 2009; Metzler-Zebeli *et al*., 2009;

Ratriyanto *et al.*, 2009; Mahmoudnia & Madani, 2012). In the mitochondria, carnitine plays an important role in the transport of long-chain fatty acids across the inner membrane, where fatty oxidation will take place (Wang *et al.*, 2004; Eklund *et al.*, 2005; Ratriyanto *et al.*, 2009; Lipiński *et al.*, 2012; Mahmoudnia & Madani, 2012), while creatine promotes ATP availability.

Since betaine is involved in lipid metabolism, it offers an interesting prospective in meat production to satisfy consumer's demand for lean meat (Attia *et al.*, 2005; Hassan *et al.*, 2005; Eklund *et al.*, 2005; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al.*, 2009). According to some authors, betaine may result in a reduced carcass fat content, increased carcass lean content, and improved feed conversion (Attia *et al.*, 2005; Eklund *et al.*, 2005; Hassan *et al.*, 2005; Metzler-Zebeli *et al.*, 2009; Ratriyanto *et al.*, 2009). The improvements observed in the reduced fat percentage and lean meat yield (i.e. increase in breast weight) may be due to its role in methyl group function as previously described. By sparing methionine and cysteine (McDevitt *et al.*, 2000; Attia *et al.*, 2005) and improving lysine and methionine absorption (Maghoul *et al.*, 2009), it makes these essential amino acids more available for protein synthesis and hence protein deposition, resulting in leaner carcasses (McDevitt *et al.*, 2000). This also means that the dietary nutrients will be used more efficiently and thus fewer amino acids are left for deamination and eventual synthesis into adipose tissue (McDevitt *et al.*, 1999; Ratriyanto *et al.*, 2009). Kalmar *et al.* (2011) also suggested that DMG's (the intermediary metabolite of betaine) might have an effect on depot fat storage because of the fatty acids being used as a direct source of energy instead of being stored in the abdominal fat pad as a result of enhanced fatty acid metabolism. It can therefore be expected that betaine may exert the same effect due to its lipotropic properties, thereby increasing the lean: fat ratio of poultry carcasses. In addition, the observed changes in hormone levels and growth factors that are involved for regulating fat synthesis, as well as lower lipogenic enzyme activity following betaine supplementation to poultry diets, may further contribute to improved carcass fat and lean percentage (Huang *et al.*, 2006). For these reasons, betaine has been deemed as an important 'carcass modifier' (McDevitt *et al.*, 2000; Hassan *et al.*, 2005).

b) Effect of betaine on carcass composition

Different studies have revealed considerable changes in carcass composition upon betaine supplementation to poultry diets (**Table 2.3**) (Saunderson & Mackinlay, 1990; Virtanen & Rosi, 1995; Rostagno & Pack, 1996; Schutte *et al.*, 1997; McDevitt *et al.*, 2000; Wang *et al.*, 2004; Waldroup *et al.*, 2006). Konca *et al.* (2008) and others indicated that betaine was effective in improving carcass quality parameters (Hassan *et al.*, 2005; Zhan et al., 2006). Following betaine

supplementation to poultry diets, betaine effectively improved lean meat deposition through an increase in the amount of breast meat along with a reduction in abdominal fat. Breast meat yield was improved in broiler chickens (McDevitt et al., 2000; Sun *et al*., 2008), turkeys (Noll *et al*., 2002) and meat ducks (Wang *et al*., 2004). Other researchers have also found that betaine was as effective as choline in improving breast meat yield in male broilers, independent of dietary methionine (Waldroup *et al*., 2006; Dilger *et al*., 2007).

Conversely, several other studies did not reveal any improvements on carcass traits when betaine (Esteve-Garcia & Mack, 2000; Kermashahi, 2001; Waldroup & Fritts, 2005; Baghaei *et al*., 2009) or its intermediary metabolite dimethylglycine (Kalmar *et al*., 2011) were added to poultry diets. Moreover, supplemental betaine seemed to have no major influence on carcass weight, carcass yield (Türker *et al*., 2004), and abdominal fat content in broilers (Rostagno & Pack, 1996; Schutte *et al*., 1997). However, Honorbakhsh *et al*. (2007) demonstrated that betaine increased both breast yield and abdominal fat percentage. This is in agreement with other researchers that also found an increase in abdominal fat deposition upon dietary betaine supplementation (Attia *et al*., 2005) but relatively lighter breast muscles (McDevitt *et al*., 2000). Clearly more work is needed to elucidate what effect dietary betaine supplementation has on broiler carcass composition.

c) Effect of betaine on growth performance

Certain studies have found that betaine supplementation was effective in improving weight gain and feed conversion in broilers (Virtanen & Rosi, 1995; Augustine *et al*., 1997; Waldenstedt *et al*., 1999; Attia *et al*., 2005; Hassan *et al*., 2005; Zhan *et al*., 2006; Honorbakhsh *et al*., 2007; Sayed & Downing, 2011), while others have indicated minimal or no effect of supplemental betaine on subsequent broiler performance (Schutte *et al*., 1997; Matthews *et al*., 1998, 2001a, b; Esteve-Garcia & Mack, 2000; Kermanshahi, 2001; Fernández-Fígares *et al*., 2002; Feng *et al*., 2006; Waldroup *et al*., 2006; Dilger *et al*., 2007; El-Husseiny *et al*., 2007; Sun *et al*., 2008). Additionally, despite improvements observed in weight gain and feed efficiency, betaine did not seem to affect feed intake (McDevitt *et al*., 2000; Zhan *et al*., 2006; Konca *et al*., 2008).

Hassan *et al.* (2005) compared the effect of different levels of betaine in the presence of its precursor choline on performance traits of broilers. These animals were offered diets supplemented with different levels of betaine (0, 72, and 144 mg/kg) and choline chloride (0, 300, and 600mg/kg). Betaine treatments resulted in an increase in BWG between 3.9 and 5.1% and an improved FCR between 4.1 and 4.8%, compared to the control groups. Furthermore, betaine addition at 0.072 or 0.144% to the basal diet resulted in similar BWG and FCR. These results are in agreement with

that of Virtanen & Rosi (1995), Augustine *et al.* (1997), and Waldenstedt *et al.* (1999) who also found improvements in performance with dietary betaine supplementation.

Researchers also investigated the effects of supplemental betaine under heat stress conditions in broiler chickens and found that it increased their body weight and feed intake and as a result, improved their feed conversion (Attia *et al.*, 2009). Likewise, Farooqi *et al*. (2005) and Konca *et al*. (2008) also noted significant improvements in weight gain under heat stress, whereas Zulkifli *et al.* (2004) reported no beneficial effects of betaine on weight gain and feed conversion in broilers reared under similar conditions when fed betaine supplemented diets. Saunderson & Mackinlay (1990) also observed no difference in body weight gain when betaine was added to the diet, which is in agreement with other authors who found little or no effect in terms of body weight gain and feed conversion upon betaine addition to broiler diets (Maghoul *et al*., 2009). Although Baghaei *et al*. (2009) also observed no change in body weight following betaine supplementation, an increase in feed conversion efficiency was detected. Similar results were obtained by Esteve-Garcia & Mack (2000) who also found a small improvement in FCR following dietary betaine supplementation, however it was non-significant.

Studies were also conducted to examine the effect of betaine in broilers infected with coccidia. They found that betaine supplementation enhanced the performance of chickens challenged with *Eimeria* (coccidia), through indirectly supporting intestinal structure and function, but also directly via partially inhibiting further coccidial invasion and/or development (Augustine *et al.*, 1997; Matthews *et al*., 1997; Schutte *et al.*, 1997; Matthews & Southern, 2000). During periods of osmotic disturbance due to water salinity stress, betaine also resulted in improved growth and feed efficiency, which may be attributed to the role of betaine in protection of intestinal epithelia (Honorbakhsh *et al.*, 2007a, b). The increase observed in total body and carcass weight when betaine is added to the diet may be the result of an altered water-retention capacity of the muscle tissue (Eklund *et* al., 2005; Honorbakhsh *et al*., 2007). This may be attributed the osmolytic properties of the accumulated betaine that lead towards a favourable growth performance (Augustine *et al.*, 1997; Allen *et al.*, 1998; Kettunen *et al*., 2001*b*; Remus *et al*., 2004).

The findings of Rama-Rao *et al.* (2011) also support that betaine was effective in improving body weight gain and feed efficiency at 21 days of age and body weight gain at 42 days of age in broilers that received maize-soybean meal based diets containing sub-optimal or lower concentrations of methionine. These improvements in broiler performance may be attributed to betaine's methionine-sparing effect (Virtanen & Rosi, 1995; Kidd *et al.*, 1997), thus making more of this essential amino acid available for protein synthesis and immune modulation (Rama-Rao *et*

al., 2011). Improved weight gain and breast yield with betaine supplementation at 21d but not 42d of age, suggests that broiler chickens have a relatively higher requirement for methyl group donors during the starter phase when compared to the finisher phase (Rama-Rao *et al.*, 2011). Florou-Panerri *et al*. (1997) found comparable improvements in broiler performance following betaine supplementation with lower concentrations of methionine.

Numerous authors have also reported a linear relationship between growth performance traits (i.e. body weight and feed efficiency) in broiler chicks in response to dietary methionine and betaine supplementation, with betaine being twice as efficient as methionine (Virtanen and Rosi, 1995; Virtanen and Rumsey, 1996; Garcia-Neto *et al.*, 2000; Ratriyanto *et al.*, 2009). Weight gain, feed intake, and feed efficiency were also improved when betaine was added to broiler diets marginally deficient in methionine and choline (Rostagno & Pack, 1996; Türker *et al*., 2004; Pillai *et al*., 2006). The intermediary metabolite of betaine (TMG) also seems to improve broiler performance as Kalmar *et al.* (2011) reported a significant improvement in FCR when 1 g Na-DMG/kg feed was added to the diet in contrast to birds fed the control diet or diets supplemented with 10 g Na-DMG/kg of feed.

d) The effect of betaine on energy utilisation

Some authors have suggested that betaine could play an energy-sparing role under certain conditions (Fernández-Fígares *et al*., 2002) due to the energy requirements for maintenance being reduced with betaine supplementation. Schrama *et al*. (2003) investigated the effect of betaine on energy metabolism in growing pigs and found that when betaine was added to the diet the amount of heat produced was reduced, which corresponded with lower energy being required for maintenance and increased energy retention. Dietary betaine supplementation could thus have affected energy metabolism by firstly reducing the amount of heat produced and secondly by subsequently changing the lipid/ protein deposition ratio to the advantage of protein. Additionally, some of the improvements in energy utilisation may also be attributed to betaine's osmolytic properties, as well as reducing the amount of energy expended for the ion pump, particularly in the cells of the gastrointestinal tract (Schrama *et al.*, 2003). This outlook was also shared by Simon (1999). However, the exact mechanism underlying the reduction in energy requirements after betaine supplementation remains to be determined and therefore necessitates some detailed investigation. It can be proposed that the effect of betaine can be attributed to its improvements in energy utilisation under conditions of disease, metabolic and/ or nutritional stress to limit their effects on growth, performance, and carcass parameters.

2.6 Association between AMPK, betaine, and metabolic disease such as PHS

As previously explained, it is evident that oxidative stress is involved in the pathophysiology of PHS and its antecedent conditions, such as cardiac hypertrophy and congestive heart failure. The premise that betaine might attenuate PHS through subsequent antioxidant protection and AMPK activation is novel and intriguing. These approaches were primarily obtained from Song *et al.* (2007) and from Alirezaei *et al.* (2012).

Song *et al.* (2007) studied the effect of betaine on fat accumulation in the liver using a mouse-model where they induced hepatic steatosis, an initiator of non-alcoholic fatty liver syndrome (NAFLD) (a metabolic syndrome commonly found in humans). While attempting to identify possible mechanisms for preventing this metabolic disease, it was discovered that AMPK activity increased following dietary betaine supplementation. Although the underlying causes of NAFLD and PHS are clearly different, overlapping responses are shared in normal metabolism, including the depletion of SAM in conjunction with SAH and HC elevation (Bayés *et al.*, 2001; Song *et al.*, 2007; Lever & Slow, 2010; Sibrian-Vazquez *et al.*, 2010). Some histological similarities also seem to exist between NAFLD and PHS. For example, NAFLD is associated with a broad spectrum of liver abnormalities, including necrosis and inflammation, with more severe cases progressing to fibrosis, cirrhosis, and liver failure (Song *et al.*, 2007). Betaine significantly reduced hepatic steatosis caused by NAFLD in an animal model, with this change being associated with an increase in AMPK activation (Song *et al.*, 2007). In an animal model, betaine supplementation significantly improved hepatic seatosis caused by NAFLD, which was mainly contributed to its subsequent increase in activating AMPK. With betaine being effective in improving NAFLD through increasing AMPK activity it may therefore be suggested that betaine might also have a potential role in alleviating PHS through enhancing AMPK activity due to their similar aetiologies. This may introduce a new paradigm, establishing a link between betaine, AMPK, and metabolic disease such as PHS. Another approach through which PHS may be alleviated stems from its cardio-protective role. Studies have indicated that AMPK is activated during chronic stresses in the heart (i.e. cardiac hypertrophy, ischaemia) due to an energy imbalance brought about during oxidative stress. Thus, AMPK activation in the cardiomyocyte may mediate cellular adaptation in response to environmental and/or nutritional stress factors that can lead to various cardiac pathologies, some of which are also shared by PHS.

Recently, Alirezaei *et al.* (2012) found that betaine enhanced antioxidant activity (particularly GSH-Px) in broiler meat following ROS-mediated damage. Because of ROS-mediated damage to the cardiopulmonary vasculature following oxidative stress, it might be suggested that betaine may play a similar functional role, thereby improving antioxidant defenses during PHS.

The concept that betaine can alleviate PHS may also be attributed to its role as an osmolyte, with its methyl donating properties also playing a minor but significant role. Various research has also indicated that as an osmolyte betaine may have an energy sparing effect in the body, which may be another approach to alleviate ascites.

Given the substantial evidence, attempts to find novel, safe, and efficacious therapies for PHS via targeting AMPK and antioxidant (particularly GSH-Px) systems are therefore definitely worthy of pursuit. However, a lack of research necessitates this to be further investigated.

Table 2.3: Effect of dietary betaine and its intermediary metabolite, dimethylglycine, supplementation on carcass characteristics of poultry

Strain/Sex	Betaine level (%)	Betaine effects	Reference
Broilers; male	0.06	↓ Carcass fat ↓ Liver weight	Saunderson & Mackinlay (1990)
Broilers	0.05-0.15	↓ Percentage fat ↑ Breast yield	Virtanen & Rosi (1995)
Broilers	0.08	↑ Breast yield	Virtanen & Rosi (1995)
Broilers; male	0.04	↑ Breast yield (trend)	Schutte *et al.* (1997)
Broilers; female	0.05	↑ Carcass yield	Esteve-Garcia & Mack (2000)
Broilers; male	0.05	↑ Breast yield ↓ Abdominal fat	McDevitt *et al.* (2000)
Broilers; unsexed/mixed	0.035-0.07	↑ Carcass yield ↑Feather weight ↑ Protein muscle yield	Attia *et al.* (2005)
Broilers; unsexed/mixed	0.072-0.144	↓ Abdominal fat ↑ Serum total protein	Hassan *et al.* (2005)
Broilers; male			Waldroup & Fritts (2005)
0-14 d	0.10	-	
0-35 d	0.10	-	
0-42 d	0.10	↑ Dressing percentage	
0-49 d	0.10	-	
Broilers; male			Waldroup *et al.* (2006)
0-14 d	0.10	-	
0-35 d	0.10	-	
0-42 d	0.10	↑ Breast yield	
0-56 d	0.10	↑ Breast yield	
Broilers, unsexed	0.05-0.10	- dressing percentage	El-Husseiny *et al.* (2007)
Broilers; male	0.08-0.23	↑ Breast yield ↑ Abdominal fat	Honorbakhsh *et al.* (2007)
Broilers; male	0.05	↑ Breast yield ↓ Abdominal fat	Zhan *et al.* (2006)

Broilers; male	0.1-0.2	-	Konca *et al.* (2008)
Broilers; mixed (Arbor Acres)	0.3-0.6		Sun *et al.* (2008)
21d		- Breast yield	
		↓ Abdominal fat	
42d		↑ Breast yield	
		↓ Abdominal fat	
Broilers	10-30%	- Carcass characteristics	Baghaei *et al.* (2009)
		↑ thigh meat percentage	
Broilers; mixed (Cobb 500)	0.1	-	Kalmar *et al.* (2011)
	1	-	
Broilers; mixed (Cobb 500)	0.1-1	↑ Breast yield	Kalmar *et al.* (2011)
		↓ Abdominal fat	
Broilers; male (Cobb 400)	0.8	↑ Breast yield	Rama-Rao *et al.* (2011)

-No effect (*P* <0.05); ↑, increase (*P* <0.05); ↓, decrease (*P* <0.05)
ADFI=Average daily feed intake, ADG=average daily gain, FCR=feed conversion ratio, PV=production value

Table 2.4: Effect of dietary betaine and its intermediary metabolite, dimethylglycine, supplementation on performance traits of poultry

Strain/Sex	Betaine level (%)	Betaine effects	Reference
Broilers	0.05 – 0.15	↑ ADG ↓ FCR	Vitanen & Rosi (1995)
Broilers	0.08	↑ ADG ↓ FCR	Vitanen & Rosi (1995)
Broilers, mixed	0.15	↑ ADG ↓ FCR	Augustine *et al.* (1997)
Broilers	0.10-0.50	↑ ADG ↓ FCR	Matthews *et al.* (1997)
Broilers	0.10	↓ FCR	Matthews *et al.* (1997)
Broilers; male	0.04	↓ FCR (Met deficient) - ADG	Schutte *et al.* (1997)
Broilers	0.15	↓ FCR (0-14 d)	Teeter *et al.* (1999)
Broilers	0.10	↑ ADG (21-35 d)	Teeter *et al.* (1999)
Broilers; female	0.10	↑ ADG ↓ FCR	Waldenstedt *et al.* (1999)
Broilers; female	0.05	-	Esteve-Garcia & Mack (2000)
Broilers	0.65	↑ ADG	Garcia-Neto *et al.* (2000)
Broilers	0.08	↑ ADG ↑Total plasma protein	Matthews & Southern (2000)
Broilers; male	0.05	- Feed intake - FCR	McDevitt *et al.* (2000)
Broilers; male	0.15		Fetterer *et al.* (2003)
E. acervulina		↑ ADG	
E. maxima		↑ADG	
E.tenella		-	
Broilers; male	5-10	-	Zulkifli *et al.* (2004)

Broilers; unsexed	0.035-0.07	↑ ADG ↓ FCR ↑ Feather weight	Attia *et al.* (2005)
Broilers; unsexed	0.10	↑ ADG (Under heat stress)	Farooqi *et al.* (2005)
Broilers; unsexed	0.072-0.144	↑ ADG ↓ FCR	Hassan *et al.* (2005)
Broilers; male			Waldroup & Fritts (2005)
0-14 d	0.10	-	
0-35 d	0.10	↓ FCR	
0-42 d	0.10	↓ FCR	
0-49 d	0.10	-	
Broilers; unsexed	0.28	-	Pillai *et al.* (2006)
Broilers; male			Waldroup *et al.* (2006)
0-14 d	0.10	-	
0-35 d	0.10	↓ FCR	
0-42 d	0.10	↓ FCR	
0-56 d	0.10	-	
Broilers; male (Arbor Acres)	0.05	↑ ADG ↓ FCR	Zhan *et al.* (2006)
Broilers; mixed	0.05-0.10	↑ ADG ↓ FCR	El-Husseiny *et al.* (2007)
Broilers; male	0.08-0.23	↑ ADG ↓ FCR	Honorbakhsh *et al.* (2007a,b)
Broilers; male	0.1-0.2	-	Konca *et al.* (2008)
Broilers; mixed (Arbor Acres)	0.3-0.6	-	Sun *et al.* (2008)
Broilers; unsexed (El-Salem strain)	0.05-0.10	↑ ADG (under heat stress) ↑ Feed intake ↓ FCR	Attia *et al.* (2009)

Broilers	10-30		Baghaei *et al.* (2009)
1-21 d		- ADG	
		- FCR	
22-42 d		- ADG	
		↑ FCR	
1-42 d		↑ ADG	
		↑ FCR	
Broilers, mixed			Hamidi *et al.* (2010)
7-21 d	0.6-1.2	-	
21-42 d	0.6-1.2	↑ ADG	
		↓ FCR	
7-42 d	0.6-1.2	↑ ADG	
		↓ FCR	
Broilers; females (Ross 308)	0.0167	-	Kalmar *et al.* (2010)
Broilers; mixed	0.01	↓ FCR	Kalmar *et al.* (2011)
	1	-	
Broilers; mixed (Cobb 500)	0.01-1	-	Kalmar *et al.* (2011)
Broilers; male (Cobb 400)	0.8	↑ ADG	Rama-Rao *et al.* (2011)
21d		↑FCR	
42 d		↑ ADG	
Broilers; males (Cobb)	0.05-0.10		Sayed *et al.* (2011)
32-35 d		↑ ADG	
36-41 d		-ADG, FCR	

- No effect (*P* <0.05); ↑, increase (*P* <0.05); ↓, decrease (*P* <0.05); *N, N-dimethylglycine; *N,N,N-trimethylglycine
ADG=Average daily gain; FCR=Feed conversion rate

CHAPTER 3: MATERIALS AND METHODS

The Animal Ethics Committee of the University of Pretoria (EC034-13) approved all animal care and use procedures applied in these experiments (Phase A and Phase B). The use of dimethylglycine (DMG), which is not a registered product in South Africa, was approved by the Medicine Control Council (MCC).

The study was conducted in two different phases, as follows **(Table 3.1):**

- Phase A – Incubation Trial
- Phase B – Broiler Grow-out Trial

Table 3.1: Experimental Design and Sampling Protocol

PHASE A	
Number of incubator machines	2
Number of trays per incubator	10
Number of eggs per tray	132
Number of eggs per incubator	1320
Number of chicks selected per incubation tray	1
Number of male chicks selected per incubator and pooled hatching trays	408
Total number of male chicks selected for Phase B	816
Number of female chicks selected per incubator and pooled hatching trays	408
Total number of female chicks selected for Phase B	816
PHASE B	
Number of Dietary Treatments	4
Number of Sexes	2
Total number of treatments	8
Number of replicates per dietary x sex treatment	12
Number of birds per dietary x sex treatment	204
Number of Chicks per pen	17
Total birds per treatment	
Number of Processing Stages (Processing Stage 1 and 2)	2
Number of Birds Processed during Processing Stage 1 (PS1)	96
Sampling Protocol:	
▪ Total number of birds processed per dietary x sex treatment	192
▪ AHI determination per dietary x sex treatment (2 birds/replicates per pen)	24

Table 3.1 (Continues): Experimental Design and Sampling Protocol

PHASE B	
▪ Number of birds sampled per dietary x sex treatment for blood plasma homocysteine analysis (2 birds/ replicates per pen)	24
▪ Number of birds sampled per dietary x sex treatment for blood plasma 4-HNE analysis ▪ (2 birds/ replicates per pen)	24
Number of Birds Processed during Processing Stage 2 (PS2)	96
Sampling Protocol:	
▪ Total number of birds processed per dietary x sex treatment	192
▪ AHI determination per dietary x sex treatment (2 birds/replicates per pen)	24
▪ Carcass Composition Determination per dietary x sex treatment	24
▪ Number of birds sampled per dietary x sex treatment for blood plasma homocysteine analysis (2 birds/ replicates per pen)	24
▪ Number of birds sampled per dietary x sex treatment for blood plasma 4-HNE analysis (2 birds/ replicates per pen)	24
▪ Number of birds sampled per dietary x sex treatment for blood plasma TBARS analysis (2 birds/ replicates per pen)	24
▪ Number of birds sampled per dietary x sex treatment for blood plasma AMPK analysis (2 birds/ replicates per pen)	24

PHASE A: Incubation Trial

3.1 Hatching eggs

Astral Operations Ltd. (Pretoria, South Africa) generously donated 2700 Commercial Ross 308 broiler eggs upon 3-4 days of storage at 20ºC for the study. These eggs were selected from the slow-feathering broiler breeder parent flock of 42 weeks of age. A total of 2640 hatching eggs within a 0.4-gram range of the average total egg weight were selected to minimise variation in egg weight and size (73.48-73.86 g). Selected eggs were evenly distributed at random across 20 egg trays immediately after arrival at the hatchery facilities at the Research Farm of the University of Pretoria (Hatfield, Pretoria). All egg trays were labelled in order to prevent errors during the

transfer process, and weighed for determination of egg weight loss (EWL) for the duration of incubation.

Three reference eggs per tray (30 eggs in total per incubator machine), positioned in the front, middle, and back (**Figure 3.1**) of each egg tray were numbered and marked (X) on the equator of the egg for later determination of eggshell temperatures.

Eggs were kept at 20ºC until placed into incubators. Eggs were not pre-warmed prior to placement into the different incubator machines in order to incite PHS development from the embryonic stage.

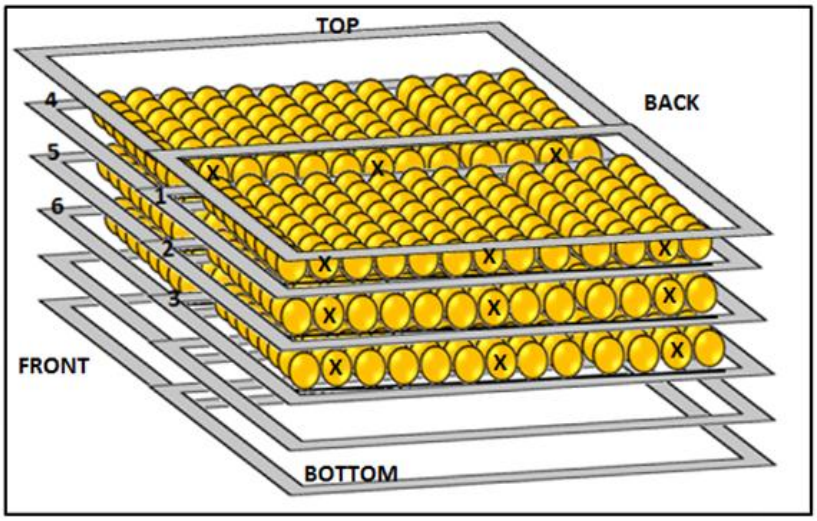

Figure 3.1: Schematic representation of tray placement within the incubator

3.2 Experimental design

The 20 incubator trays were equally distributed between two identical incubators (Chick Master; Model 1056: HQ), that were specifically modified to be single stage. Ten trays each containing 132 eggs were placed into each machine for incubation (i.e. 1320 eggs per incubator) (**Figure 3.2**). Egg rotation occurred every hour through an angle of 90º from setting until day of transfer (ED_{18}) of the incubation study.

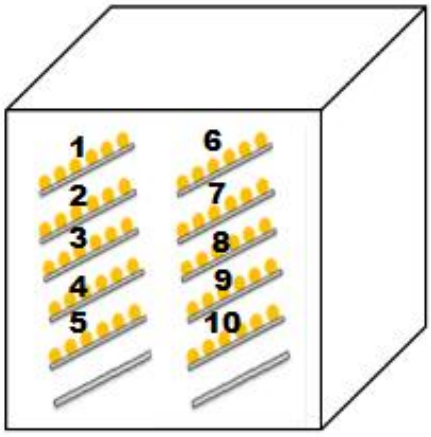

Figure 3.2: Schematic representation of tray placement within the incubator

Two days prior to the start of incubation, both incubators were switched on and allowed to reach equal air temperatures of 37.5°C to 37.8°C (99.5°F to 100.0°F). Ventilation and RH in the incubators were maintained more or less similar at all times. RH between 45 and 55% was maintained during the incubation. For eggshell temperature monitoring, a plastic tent was constructed over all incubators. The tent was heated with two small electric heaters for approximately 30-60 minutes in order to stabilise the temperature within the tent to match the incubator so that the internal and external incubator environment would be equalised for egg temperature measurements. This was necessary to prevent any major heat loss from the eggs during the measurement of eggshell temperatures. Egg temperatures were taken daily from embryonic day 11 (ED_{11}) until day of transfer (ED_{18}) at the same position each time (on the equator of each reference egg, marked (X)) with a Braun Thermoscan infrared thermometer. Eggs were removed if believed to be infertile and replaced by a new egg that was marked. The Braun Thermoscan infrared thermometer was placed inside one of the incubators for 30-60 minutes before use in order to equilibrate. The machine controls for air temperature of both incubator machines were adjusted daily based on the average temperature trends observed in the 30 reference eggs in each machine during the period of eggshell temperature measurements (ED_{11} to ED_{18}). Machine controls were adjusted in order to reach eggshell temperatures for the respective incubation treatments within the desired temperature ranges as follow:

Table 3.2: Desired egg temperatures during incubation

Embryonic Day (ED)	High temperature treatment (ºC)
ED_0 to ED_{10}	37.5-37.8
ED_{11} to ED_{15}	38.8-39.4
ED_{16} to hatch	> 38.8

At ED_{18}, all egg trays from each incubator were taken out and weighed in order to determine egg weight loss (EWL). Eggs were then candled and those with evidence of living embryos were transferred to the same numbered plastic hatching basket to prevent any error during the transfer process. Therefore, each hatching basket contained the eggs of the respective numbered incubator tray. Each hatching basket was returned in the same order within the same machine immediately after transfer and the temperature treatments continued until the day of hatch (21. 5 d).

At 21.5 d of incubation, the chicks that have completed the hatching process were removed from the hatching baskets, counted, scored, and sexed using the feather-sexing method. Chicks were seperated by hatching basket number and sex. Chicks were then graded, and first and second grade chicks of each hatching basket were seperated. An almost equal number of first-grade male and female chicks were selected from each hatching basket and then equally mixed before placement of the chicks into their specific pens.

3.3 Measurements and sampling

3.3.1 Egg weight loss (EWL):

Egg weights at setting and egg weights at ED_{18} of incubation were used to calculate relative egg weight loss (EWL) up to ED_{18} as follows:

$$EWL = \frac{W0-W18}{W0}\ x\ 100\%$$

Where:

W0= full tray weight of eggs at setting, and W18= full tray weight of eggs at ED_{18}.

3.3.2 Hatchability:

On both the day of transfer (ED_{18}) and day of hatch (21.5 d), infertile eggs and eggs with dead embryos (i.e. eggs that failed to hatch) were removed and counted per incubator tray. These eggs were opened and evaluated visually in order to determine the stage of embryonic mortality and to determine hatchability. Embryonic mortality were categorised as infertile, early dead (week 1), mid dead (week 2), late dead (week 3) and malpositioned.

The hatchability for each incubation temperature treatment was determined as follow:

$$\text{Hatchability \%} = \frac{number\ of\ eggs\ hatched}{number\ of\ eggs\ set}\ x\ 100\% \quad \text{and,}$$

$$\text{Hatch of Fertile \%} = \frac{number\ of\ eggs\ hatched}{number\ of\ fertile\ eggs}\ x\ 100\%$$

3.3.3 Chick quality scoring:

On the day of hatch, all of the hatched chicks were visually evaluated in order to score for quality based on the following subjective criteria, as decsribed by Tona *et al.* (2003a):

Chicks were evaluated on feather colour and quality, navel quality, leg colour, bruising, chick behaviour and overall appearance and behaviour. According to their visual score, the chicks' were then divided into the different grades as follow:

- First grade: Active and alert chicks, good feather cover, clean beak and feathers, open eyes (good quality chicks)
- Second grade: Chicks that showed visible signs of sub-optimal incubation conditions such as red hocks, navel infections (black buttons), water bellies, and chicks covered in yolk/egg content.
- Third grade: The most severe of the second-grade chicks were re-graded and classed as third-grade chicks. These chicks were weak and less active than the 2nd grade chicks.
- Culls: Chicks with severe abnormalities such as deformed chicks (i.e. sprayed legs, not able to stand up, one eye, ectopic viscera, etc.) or chicks with severe navel infections and water bellies (i.e. unlikely to survive post hatch).

PHASE B: Broiler grow-out trial

3.4 Birds

A total of 816 male and 816 female chicks were specifically selected at hatch from each incubation machine and hatching basket. The chicks were transferred to the 96-pen environmentally controlled broiler trial facility on the Research Farm of the University of Pretoria (Hatfield, Pretoria) where Phase B of the experiment was conducted. The 1632 (816 males, 816 females) chicks were equally distributed amongst 96 floor pens with 17 birds per pen so that each pen contained the same number of chicks from each hatching basket within the same incubation machine. Before placement, all chicks were identified with a neck-tag that contained a unique number in order to trace them back to their respective pen and sex/dietary treatment during bird processing.

3.5 Experimental design

During Phase B, birds were reared in groups for a period of 40 days. A 2x4 factorial design was used that consisted of two sexes and four dietary treatments, hence there were eight treatment groups that were each replicated 12 times within the 96-pen broiler facility (**Table 3.3**).

Table 3.3: Summary of treatments in grow-out trial

Sex/Group	Dietary Treatment		Replicates/pen	Birds/pen
Male	Treatment 1	Negative Control Group (NC-Group)	12	17
Male	Treatment 2	Betaine-treated Group (BT-Group)	12	17
Male	Treatment 3	DMG-treated Group (DMG-Group)	12	17
Male	Treatment 4	Positive Control-Group (PC-Group)	12	17
Female	Treatment 1	Negative Control Group (NC-Group)	12	17
Female	Treatment 2	Betaine-treated Group (BT-Group)	12	17
Female	Treatment 3	DMG-treated Group (DMG-Group)	12	17
Female	Treatment 4	Positive Control-Group (PC-Group)	12	17
			96 pens	**1 632**

3.6 Animal housing and care

The experiment was conducted in an environmentally controlled broiler house that consisted out of 2 smaller houses of 48 pens each fitted with concrete floors. The chicks from each replicate (pen) were housed in a pen of 2.25 m² covered with clean pine wood shavings as bedding material. The climate was automatically regulated with a Skov computer (Model DOL 539) in order to facilitate an optimum environment in the 96-pen broiler trial facility. The temperature and ventilation of the trial facility was closely monitored and regulated through the combined usage of fogging coolers, air master fans, and electrical heaters.

The brooding facilities were preheated for approximately 12 hours prior to chick placement. The experimental house was operated in order to maintain suboptimal brooding temperatures as a means to further induce ascites (**Table 3.4**). Litter temperatures were gradually decreased by 1-2˚C every week from d_0 until d_{21} and then maintained according to Ross' temperature guidelines until d_{40}. Litter temperatures were determined daily using a Major Tech Infrared Thermometer (Model MT 694) to randomly check 3-5 spots in each pen throughout the house every morning and afternoon during the brooding and grow-out period. Temperatures were monitored approximately every 4 hours during d_1-d_{10} from 08:00 to 23:00. From day 11 and onwards, litter temperatures were recorded 3 times a day until the end of the study whenever possible. Inside air temperatures were determined via temperature sensors at bird level in the middle on each side of the broiler house, whereas outside temperatures were determined via temperature sensors hanged outside the broiler house under the roof; information from both sensors was recorded by the Skov DOL 539.

Table 3.4: Desired temperatures during broiler grow-out for chicks

Age of Chick	Desired brooding temperature at chick level (°C)	Ross Temperature Guidelines (°C) @ 50% RH
D_0-D_7	25-26	29-34
D_7-D_{14}	23-24	27-29
D_{14}-D_{21}	21	24-27
D_{21}-D_{28}	21	22-24
D_{28}-D_{35}	19-21	
D_{35}-D_{40}	17-19	

Chicks were provided 24 hours of light on d_0 to stimulate feed and water intake. Thereafter, a lighting programme consisting out of 23 hours light and 1-hour darkness was employed during the first week of the chick's life. During the second week, daylight length was reduced to 12 hours and thereafter increased by 2 hours each week until 16 hours of light was reached at d_{21}, at which point it was then kept constant until d_{40} (**Table 3.5**).

Each pen was equipped with one tube feeder and 6 nipple drinkers to provide feed and water for *ad libitum* consumption. During the first week, one chick supplemental drinking font was also used, as well as one pan feeder and brown paper for supplemental feed were placed in each pen; these were removed at d_7.

Table 3.5: Lighting schedule employed during the brooding and broiler grow-out period

Age (in days)	Hours dark	Hours light
D_0	0	24
D_{1-7}	1	23
D_{7-14}	12	12
D_{14-21}	10	14
D_{21-28}	8	16
D_{28-35}	8	16
D_{35-40}	8	16

3.7 Diets

3.7.1 Phase feeding, diet composition and feed supplementation

A three-phase feeding programme was followed that consisted of a starter (d_0-d_{14}), grower (d_{14}-d_{28}), and finisher (d_{28}-d_{40}) phase. The starter was fed as crumbles while the grower and finisher diets were fed as pellets.

The total amount of feed required by the birds for the 40-day trial period was roughly calculated from the average intakes according to the Ross 308 guidelines (**Table 3.6)**. All birds received an industry standard commercial maize-soya based broiler diet (basal diet); each supplemented according to the 4 experimental dietary treatments (**Table 3.7**).

Betaine (96%, Betafins S1) was provided by Chemunique International (PTY) Ltd, Pretoria, South Africa. Na-DMG (97%) was provided by Taminco (Global Chemical Corporation), Gent,

Belgium. The different feed supplements were each added to the premix individually and mixed homogenously into the respective basal diets for each treatment to be mixed. All feeds and mineral premixes were mixed and manufactured by Pennville Animal Nutrient Solutions (Pretoria, South Africa). All diets were formulated using Format Software (Format International, UK). The compositions of the different treatment diets for the different phases are given in **Table 3.8** to **Table 3.13**, respectively.

Feed samples were obtained for each dietary phase and treatment during the course of the experimental trial. Feed samples were acquired by taking a few small random feed samples from each feedbag from the respective dietary treatments, which was then labelled. This procedure was repeated at the beginning of each dietary phase.

Table 3.6: Calculated feed intake per treatment per phase during the grow-out period

Final amount of feed required (rough estimate)								
Feed needed	**Days**	**Estimated average Intake/bird (kg**	**Number of animals 20/pen**	**Total Feed consumed 20/pen (kg)**	**Feed samples (kg)**	**Total Feed required (kg)**	**Rounded Estimate (kg)**	**Feed required per dietary treatment (kg)**
Starter	0-14	0.6	1920	1152	8	1160	~1200	300
Grower	14-28	1.7	1920	3264	8	3272	~3300	825
Finisher	28-42	2.9	1920	5568	8	5576	~5600	1400
TOTAL AMOUNT OF FEED REQUIRED							**10100**	**2525**

Table 3.7: Different dietary treatments and levels of dimethylglycine (DMG) and trimethylglycine (TMG; betaine) supplementation, as formulated with Betacheck[1]

Sex/Group	Description		Dimethylglycine (DMG) Supplementation (g/kg feed)	Trimethylglycine (betaine) Supplementation (g/kg feed)
Male (Group 1)	Negative Control (NC)	Basal diet low in methionine with no added choline chloride.	0	0
Male (Group 2)	NC + Betaine (BET)	Negative Control diet with added trimethylglycine (betaine).	0	1.042
Male (Group 3)	NC + DMG (DMG)	Negative Control diet with added dimethylglycine.	1.38	0
Male (Group 4)	Positive Control (PC)	Basal diet with added methionine and choline chloride levels	0	0
Female (Group 5)	Negative Control (NC)	Basal diet low in methionine with no added Choline Chloride.	0	0
Female (Group 6)	NC + Betaine (BET)	Negative Control diet with added trimethylglycine (betaine).	0	1.042
Female (Group 7)	NC + DMG (DMG)	Negative Control diet with added dimethylglycine.	1.38	0
Female (Group 8)	Positive Control (PC)	Basal diet with added methionine and choline chloride levels	0	0

[1] Betacheck is software developed by DuPont Danisco that is a useful tool in determining the amount of dietary methionine and/or choline that can be replaced by betaine in poultry diets, relative to the bird's requirements

Table 3.8: Composition of feed ingredient and calculated nutrient levels of broiler starter diets in treatments 1, 2, 3 and 4

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Ingredient Composition (%)				
Yellow Maize (fine)	59.09	59.09	59.09	59.09
Soya oilcake meal	26.50	26.50	26.50	26.50
Full fat soya	4.66	4.66	4.66	4.66
Sunflower oilcake meal	3.0	3.0	3.0	3.0
Gluten 60	3.0	3.0	3.0	3.0
Limestone ($CaCO_3$)	1.42	1.42	1.42	1.42
Bicarbonate of soda ($NaHCO_3$)	0.073	0.073	0.073	0.073
Salt (fine)	0.429	0.429	0.429	0.429
MCP	0.949	0.949	0.949	0.949
Lysine HCl	0.314	0.314	0.314	0.314
DL-Methionine	**0.167**	**0.167**	**0.167**	**0.231**
L-Threonine	0.063	0.063	0.063	0.063
1000 Starter Phytase XP10K	0.01	0.01	0.01	0.01
Broiler Starter Premix	0.25	0.25	0.25	0.25
Calculated Nutrient Composition				
Dry matter (%)	89.15	89.15	89.15	89.15
Moisture (%)	10.85	10.85	10.85	10.85
AME (MJ/kg)	11.50	11.50	11.50	11.50
Crude protein (%)	22.22	22.22	22.22	22.22
Crude fibre (%)	3.73	3.73	3.73	3.73
Fat (%)	3.56	3.56	3.56	3.56
Ash (%)	4.79	4.79	4.79	4.79
Nitrogen-free extract (%)	54.33	54.33	54.33	54.33
Ca (%)	0.94	0.94	0.94	0.94
P (%)	0.79	0.79	0.79	0.79
Available P (%)	0.45	0.45	0.45	0.45
Na (mg/kg)	0.20	0.20	0.20	0.20
Cl (mg/kg)	0.30	0.30	0.30	0.30

K (mg/kg)	0.92	0.92	0.92	0.92
Total LYS (%)	1.32	1.32	1.32	1.32
TD[1] LYS (%)	1.20	1.20	1.20	1.20
Total MET (%)	0.593	0.593	0.593	0.593
TD MET (%)	0.563	0.563	0.563	0.563
Total CYS (%)	0.372	0.372	0.372	0.372
TD CYS (%)	0.314	0.314	0.314	0.314
Total M + S (%)	0.964	0.964	0.964	0.964
TD M + S (%)	0.876	0.876	0.876	0.876
Total THR (%)	0.883	0.883	0.883	0.883
TD THR (%)	0.78	0.78	0.78	0.78
Total TRP (%)	0.245	0.245	0.245	0.245
TD TRP (%)	0.206	0.206	0.206	0.206
Total ILE (%)	0.919	0.919	0.919	0.919
TD ILE (%)	0.840	0.840	0.840	0.840
Total ARG (%)	1.378	1.378	1.378	1.378
TD ARG (%)	1.260	1.260	1.260	1.260

[1] **TD: Total digestible amino acid**

Table 3.9: Micro-mineral composition of broiler starter premix pack used in treatments in 1, 2, 3 and 4

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Micro-mineral Composition per unit)				
Vitamin A (I.U.)	11,000,000	11,000,000	11,000,000	11,000,000
Vitamin D3 (I.U.)	5,000,000	5,000,000	5,000,000	5,000,000
Vitamin E (mg)	60,000	60,000	60,000	60,000
Vitamin K_3 (mg)	2,000	2,000	2,000	2,000
Vitamin B_1 (mg)	2,000	2,000	2,000	2,000
Vitamin B_2 (mg)	5,000	5,000	5,000	5,000
Niacin (Vitamin B_3) (mg)	50,000	50,000	50,000	50,000
Calcium Panthonate (Vitamin B_5) (mg)	12,000	12,000	12,000	12,000
Vitamin B6 (Pyridoxine) (mg)	3,000	3,000	3,000	3,000
Folic Acid (Vitamin B_9) (mg)	2,000	2,000	2,000	2,000
Vitamin B_{12} (mg)	10	10	10	10
Biotin (mg)	100	100	100	100
Antioxidant (mg)	125,000	125,000	125,000	125,000
Manganese (mg)	110,000	110,000	110,000	110,000
Iron (mg)	40,000	40,000	40,000	40,000
Zinc (mg)	100,000	100,000	100,000	100,000
Copper (mg)	10,000	10,000	10,000	10,000
Cobalt (mg)	500	500	500	500
Iodine (mg)	2,000	2,000	2,000	2,000
Selenium (mg)	300	300	300	300
Choline (mg)	**0**	**0**	**0**	**300,0000**
N, N, N – Trimethylglycine (betaine)	**0**	**1.042**	**0**	**0**
N, N – Dimethylglycine (DMG) (g/kg)	**0**	**0**	**1.38**	**0**

Table 3.10: Composition of feed ingredient and calculated nutrient levels of broiler grower diets in treatments 1, 2, 3 and 4

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Ingredient Composition (%)				
Yellow maize (fine)	62.532	62.532	62.532	62.532
Soya oilcake meal	18.980	18.980	18.980	18.980
Full fat soya	9.251	9.251	9.251	9.251
Sunflower oilcake meal	3.0	3.0	3.0	3.0
Gluten 60	3.0	3.0	3.0	3.0
Limestone ($CaCO_3$)	1.336	1.336	1.336	1.336
Bicarbonate of soda ($NaHCO_3$)	0.038	0.038	0.038	0.038
Salt (fine)	0.430	0.430	0.430	0.430
MCP	0.560	0.560	0.560	0.560
Lysine HCl	0.299	0.299	0.299	0.299
DL-Methionine	**0.163**	**0.163**	**0.163**	**0.212**
L-Threonine	0.051	0.051	0.051	0.051
1000 Grower Phytase XP10K	0.01	0.01	0.01	0.01
Broiler Grower Premix	0.30	0.30	0.30	0.30
Calculated Nutrient Composition				
Dry mattter (%)	89.429	89.429	89.429	89.429
Moisture (%)	10.571	10.571	10.571	10.571
AME (MJ/kg)	11.95	11.95	11.95	11.95
Crude protein (%)	20.738	20.738	20.738	20.738
Crude fibre (%)	3.858	3.858	3.858	3.858
Fat(%)	4.428	4.428	4.428	4.428
Ash (%)	4.442	4.442	4.442	4.442
Nitrogen-free extract (%)	55.668	55.668	55.668	55.668
Ca (%)	0.825	0.825	0.825	0.825
P (%)	0.694	0.694	0.694	0.694
Available P (%)	0.375	0.375	0.375	0.375
Na (mg/kg)	0.19	0.19	0.19	0.19
Cl (mg/kg)	0.3	0.3	0.3	0.3

K (mg/kg)	0.842	0.842	0.842	0.842
Total LYS (%)	1.214	1.214	1.214	1.214
TD[1] LYS (%)	1.1	1.1	1.1	1.1
Total MET (%)	0.557	0.557	0.557	0.557
TD MET (%)	0.527	0.527	0.527	0.527
Total CYS (%)	0.353	0.353	0.353	0.353
TD CYS (%)	0.298	0.298	0.298	0.298
Total M + S (%)	0.910	0.910	0.910	0.910
TD M + S (%)	0.825	0.825	0.825	0.825
Total THR (%)	0.815	0.815	0.815	0.815
TD THR (%)	0.715	0.715	0.715	0.715
Total TRP (%)	0.225	0.225	0.225	0.225
TD TRP (%)	0.186	0.186	0.186	0.186
Total ILE (%)	0.855	0.855	0.855	0.855
TD ILE (%)	0.776	0.776	0.776	0.776
Total ARG (%)	1.271	1.271	1.271	1.271
TD ARG (%)	1.155	1.155	1.155	1.155

[1] **TD: Total digestible amino acid**

Table 3.11: Micro-mineral composition of broiler grower premix pack used in treatments 1, 2, 3 and 4

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Micro-mineral Composition (per unit)				
Vitamin A (I.U.)	9,000,000	9,000,000	9,000,000	9,000,000
Vitamin D_3 (I.U.)	4,000,000	4,000,000	4,000,000	4,000,000
Vitamin E (mg)	50,000	50,000	50,000	50,000
Vitamin K_3 (mg)	1,500	1,500	1,500	1,500
Vitamin B_1 (mg)	1,700	1,700	1,700	1,700
Vitamin B_2 (mg)	4,000	4,000	4,000	4,000
Niacin (Vitamin B_3) (mg)	42,000	42,000	42,000	42,000
Calcium Panthonate (Vitamin B_5) (mg)	10,000	10,000	10,000	10,000
Vitamin B_6 (Pyridoxine) (mg)	2,500	2,500	2,500	2,500
Folic Acid (Vitamin B_9) (mg)	1,700	1,700	1,700	1,700
Vitamin B_{12} (mg)	8	8	8	8
Biotin (mg)	80	80	80	80
Antioxidant (mg)	125,000	125,000	125,000	125,000
Manganese (mg)	90,000	90,000	90,000	90,000
Iron (mg)	35,000	35,000	35,000	35,000
Zinc (mg)	80,000	80,000	80,000	80,000
Copper (mg)	8,000	8,000	8,000	8,000
Cobalt (mg)	400	400	400	400
Iodine (mg)	1,600	1,600	1,600	1,600
Selenium (mg)	250	250	250	250
Choline (mg)	**0**	**0**	**0**	**250,000**
N, N, N – Trimethylglycine (betaine)	**0**	**1.042**	**0**	**0**
N, N – Dimethylglycine (DMG) (g/kg)	**0**	**0**	**1.38**	**0**

Table 3.12: Composition of feed ingredient and calculated nutrient levels of broiler finisher diets in treatments 1, 2, 3 and 4

	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Ingredient Composition (%)				
White maize (fine)	66.744	66.744	66.744	66.744
Soya oilcake meal	14.388	14.388	14.388	14.388
Full fat soya	10.110	10.110	10.110	10.110
Sunflower oilcake meal	3.0	3.0	3.0	3.0
Gluten 60	3.0	3.0	3.0	3.0
Limestone ($CaCO_3$)	1.181	1.181	1.181	1.181
Bicarbonate of soda ($NaHCO_3$)	0	0	0	0
Salt (fine)	0.432	0.432	0.432	0.432
MCP	0.313	0.313	0.313	0.313
Lysine HCl	0.286	0.286	0.286	0.286
DL-Methionine	**0.157**	**0.157**	**0.157**	**0.197**
L-Threonine	0.038	0.038	0.038	0.038
1000 Finisher Phytase XP10K	0.01	0.01	0.01	0.01
Broiler Finisher Premix	0.3	0.3	0.3	0.3
Calculated Nutrient Composition				
Dry matter (%)	89.036	89.036	89.036	89.036
Moisture (%)	10.964	10.964	10.964	10.964
AME (MJ/kg)	12.25	12.25	12.25	12.25
Crude protein (%)	19.265	19.265	19.265	19.265
Crude fibre (%)	3.832	3.832	3.832	3.832
Fat (%)	4.662	4.662	4.662	4.662
Ash (%)	4.036	4.036	4.036	4.036
Nitrogrn-free extract (%)	57.523	57.523	57.523	57.523
Ca (%)	0.715	0.715	0.715	0.715
P (%)	0.626	0.626	0.626	0.626
Available P (%)	0.325	0.325	0.325	0.325
Na (mg/kg)	0.18	0.18	0.18	0.18
Cl (mg/kg)	0.301	0.301	0.301	0.301
K (mg/kg)	0.771	0.771	0.771	0.771

Total LYS (%)	1.105	1.105	1.105	1.105
TD[1] LYS (%)	1.0	1.0	1.0	1.0
Total MET (%)	0.526	0.526	0.526	0.526
TD MET (%)	0.497	0.497	0.497	0.497
Total CYS (%)	0.334	0.334	0.334	0.334
TD CYS (%)	0.283	0.283	0.283	0.283
Total M + S (%)	0.861	0.861	0.861	0.861
TD M + S (%)	0.78	0.78	0.78	0.78
Total THR (%)	0.744	0.744	0.744	0.744
TD THR (%)	0.65	0.65	0.65	0.65
Total TRP (%)	0.205	0.205	0.205	0.205
TD TRP (%)	0.167	0.167	0.167	0.167
Total ILE (%)	0.787	0.787	0.787	0.787
TD ILE (%)	0.712	0.712	0.712	0.712
Total ARG (%)	1.159	1.159	1.159	1.159
TD ARG (%)	1.05	1.05	1.05	1.05

[1] **TD: Total digestible amino acid**

Table 3.13: Micro-mineral composition of broiler finisher premix pack used in treatments 1, 2, 3 and 4

	Treatment1	Treatment 2	Treatment 3	Treatment 4
Micro-mineral Composition (per unit)				
Vitamin A (I.U.)	7,500,000	7,500,000	7,500,000	7,500,000
Vitamin D3 (I.U.)	3,300,000	3,300,000	3,300,000	3,300,000
Vitamin E (mg)	40,000	40,000	40,000	40,000
Vitamin K_3 (mg)	1,300	1,300	1,300	1,300
Vitamin B_1 (mg)	1,300	1,300	1,300	1,300
Vitamin B_2 (mg)	3,300	3,300	3,300	3,300
Niacin (Vitamin B_3) (mg)	33,000	33,000	33,000	33,000
Cal Panthonate (Vitamin B_5) (mg)	8,000	8,000	8,000	8,000
Vitamin B_6 (Pyridoxine) (mg)	2,000	2,000	2,000	2,000
Folic Acid (Vitamin B_9) (mg)	1,300	1,300	1,300	1,300
Vitamin B_{12} (mg)	6.5	6.5	6.5	6.5
Biotin (mg)	70	70	70	70
Antioxidant (mg)	125,000	125,000	125,000	125,000
Manganese (mg)	70,000	70,000	70,000	70,000
Iron (mg)	25,000	25,000	25,000	25,000
Zinc (mg)	70,000	70,000	70,000	70,000
Copper (mg)	6,500	6,500	6,500	6,500
Cobalt (mg)	350	350	350	350
Iodine (mg)	1,300	1,300	1,300	1,300
Selenium (mg)	200	200	200	200
Choline (mg)	**0**	**0**	**0**	**250,000**
N, N, N – Trimethylglycine (betaine)	**0**	**1.042**	**0**	**0**
N, N – Dimethylglycine (DMG) (g/kg)	**0**	**0**	**1.38**	**0**

3.7.2 Dietary calculations

Research over the last few years has shown that betaine supplementation may spare added dietary methionine and choline under practical trial conditions when fed to animals receiving typical maize-soybean meal diets. Therefore, treatment diets were adjusted to offset the potential methionine and choline surpluses to allow betaine alone to be the primary dietary influence.

3.7.2.1 Betafin bio-equivalency calculations with choline, methionine and Na-dimethylglycine

It is possible to calculate an equivalency for methionine, choline, and betaine as methyl donors, which allows nutritionists to decide on the amount of betaine required when substituting for choline or sparing methionine and ensures a sufficient source of methyl group donors to the animal. In this trail, the methyl equivalents of betaine (trimethylglycine) were used to determine the amount of dimethylglycine to be added to the animal's diet. For calculating the amount of betaine to be used, the molecular weight of 100% pure trimethylglycine (TMG) was corrected for the bioavailability of betaine in the specific product.

The recommended level(s) for including Betafin S1 (crystalline product containing 96% trimethylglycine) into poultry diets is 1-1.5kg per ton of finished feed (DuPont Danisco), whereas the recommended levels for including Na-DMG (Taminizer D) is 1kg per ton of finished poultry feed (Taminco Global Amine Company).

For the purpose of this study, we wanted to add 100% trimethylglycine and therefore all calculations were based on the addition of 100% trimethylglycine to the broiler diets.

A) Methionine to betaine unit conversion

MW of methionine = 149.2, MW of betaine/TMG = 117.15

Ratio of 100% methionine to 100% betaine = 149.2/117.15 = 1.2736

Thus, 1.2736 units methionine (100%) = 1 unit betaine (100%)

Correction for bio-efficacy (assuming 90% bio-efficacy of methionine compared to betaine):

1.2736/0.90 = 1.4151

Thus, 1.4151 unit methionine (100%) = 1 unit betaine (100%)

Correction for purity of product:

Methionine = 99% pure, betaine = 96% pure

0.96/0.99 x 1.4151 = 1.3722

So, 1.37 units DL-MET = 1 unit betaine (96% TMG)

So, 1.4293 units DL-Methionine = 1.042 betaine (100% TMG)

B) Choline to betaine unit conversion

MW of choline chloride = 139.63, MW of betaine/TMG = 117.15,

Ratio of 100% choline chloride to 100% betaine = 139.63/117.15 = 1.1916

Thus, 1.1916 units ChoCl (100%) = 1 unit betaine

Correction for bio-efficacy (assuming a 55% bio-efficacy of choline compared to vs. betaine):

1.1916/0.55 = 2.1671

Thus, 2.1671 unit choline chloride (100%) = 1 unit betaine (100%)

Correction for purity of product:

Choline Chloride = 60% pure, betaine = 96% pure

0.96/0.60 x 2.1671 = 3.4673

So, 3.47 units choline chloride (60%) = 1 unit betaine (96% TMG)

So, 3.6118 units choline chloride (60%) = 1.042 betaine (100% TMG)

C) Methionine to DMG unit conversion

MW of methionine = 149.2, MW of sodium N, N-DMG (Taminizer D) = 125.1,

Ratio of 100% MET to 100% sodium N, N-DMG = 149.2/125.1 = 1.1926

Thus, 1.1926 units MET (100%) = 1 unit sodium N, N-DMG (100%)

Correction for bio-efficacy (assuming 90% bio-efficacy of methionine compared to dimethylglycine):

1.1926/0.90 = 1.3252

Thus, 1.3252 unit methionine (100%) = 1 unit Na- DMG (100%)

Correction for purity of product (i.e. dimethylglycine is :

Methionine 99% pure, Na-DMG = 97% pure

0.99/0.97 x 1.0786 = 1.3525

So, 1.0353 units methionine = 1 unit sodium N, N-DMG (97%)

D) Betaine to DMG unit conversion

1.4293 units DL-methionine = 1.042 betaine (100% TMG)

So, 1 unit DL-methionine = 1.0423/1.4293 = 0.7290 unit betaine (100%TMG)

And, 1.03523 units DL-methionine = 1 unit sodium N, N-DMG

So, 1 unit DL-methionine = 1/1.0353 = 0.9659 sodium N, N-DMG

Therefore, 0.7290 units of betaine = 0.9659 units of sodium N, N-DMG

So <u>1.042 units of betaine</u> = (1.042/0.7290) x 0.9659 = 1.3806 <u>(~1.381) units of sodium N, N-DMG</u>

From the inclusion level of TMG (Betafin, betaine) and DMG, the inclusion level of methionine and choline chloride was then determined using Betacheck (**Figure 3.3** to **Figure 3.5**) or via manual calculations (**Table. 3.13**).

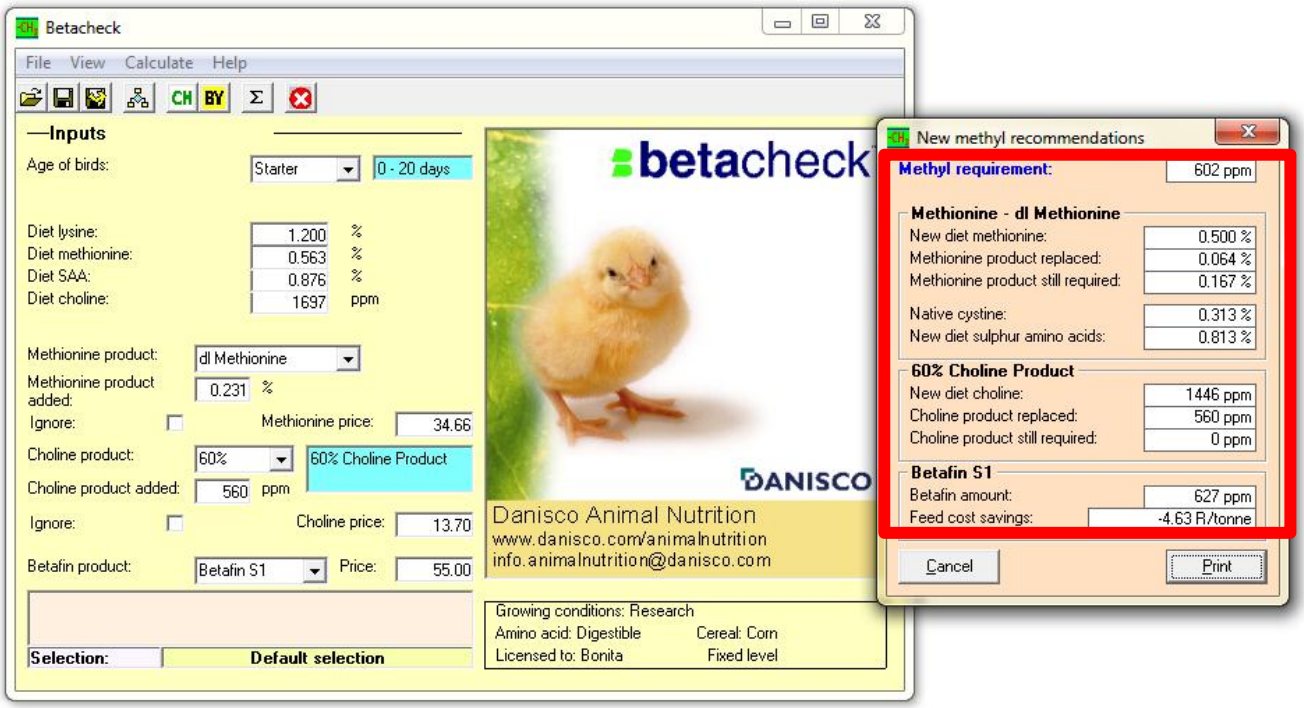

Figure 3.3: New dietary choline and methionine content for the broiler starter diet as formulated containing 1.042g Betafin S1/kg feed (based on digestible AA ratios and bird age)

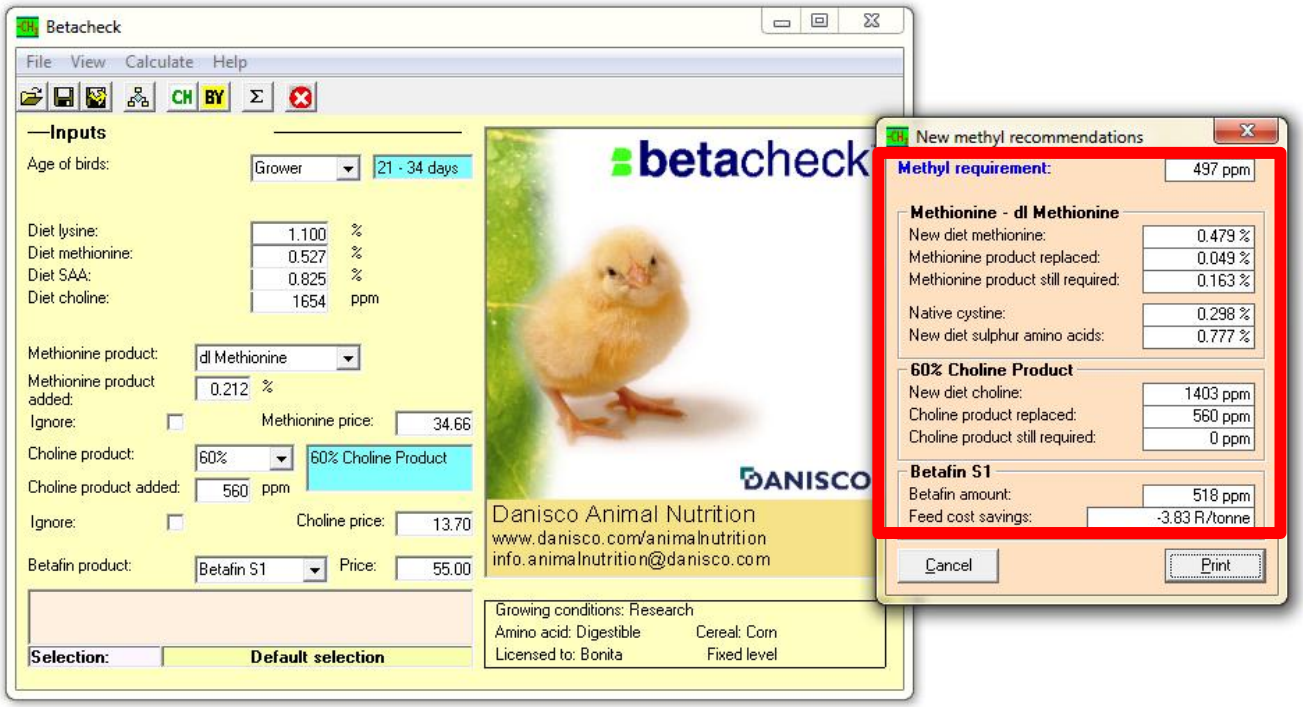

Figure 3.4: New dietary choline and methionine content for the broiler grower diet as formulated containing 1.042g Betafin S1/kg feed (based on digestible AA ratios and bird age)

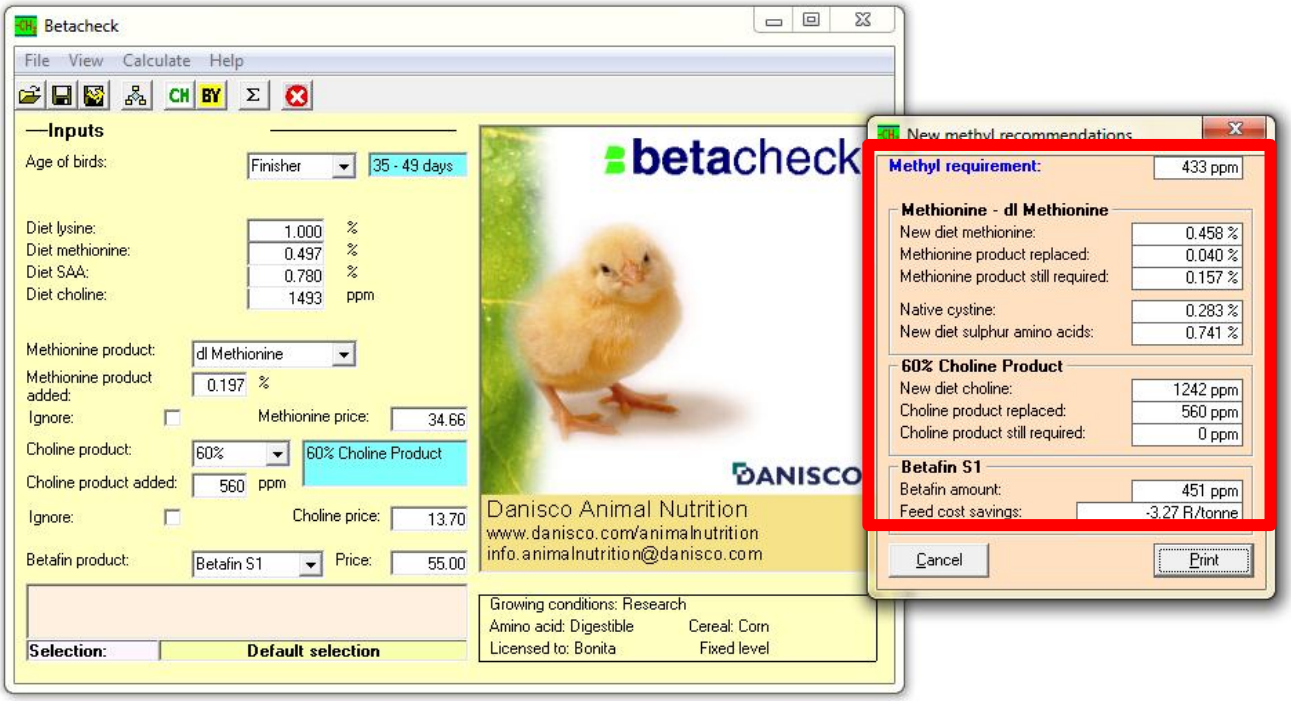

Figure 3.5: New dietary choline and methionine content for the broiler finisher diet as formulated containing 1.042g Betafin S1/kg feed (based on digestible AA ratios and bird age)

Table 3.14 shows the calculate specifications for choline and methionine and shown in **Figure 3.3, 3.4** and **3.5** as formulated using Betacheck by illustrating the differences between the basal diets where no betaine is added to the diet (A), compared to when betaine is added to the broiler diet (B).

Table 3.14: Methyl optimisation of the broiler diet

	Starter	**Grower**	**Finisher**
(A) Original diet with added choline and methionine (Positive Control):			
1. Endogenous choline from raw materials	1347	1354	1193
2. Added dietary choline	350	300	300
3. Added choline chloride (60%)	560	560	560
1 + 2 Total dietary choline	1697	1654	1493
4. Dietary choline specific requirement	1200	1000	800
5. Dietary lysine (based on digestible AA ratios)	1.20	1.10	1.00
6. Dietary methionine (based on digestible AA ratios)	0.56	0.53	0.50
7. Dietary TSAA (based on digestible AA ratios)	0.88	0.83	0.78
8. Synthetic methionine	0.23	0.21	0.20
(B) Original diet with new choline and methionine levels (basal diet and/or Negative Control):			
1. Endogenous choline from raw materials	1347	1354	1193
2. Added dietary choline	0	0	0
3. Added choline chloride (60%)	0	0	0
1 + 2 Total dietary choline	1347	1354	1193
4. Dietary choline specific requirement	1200	1000	800
5. Dietary lysine (based on digestible AA ratios)	1.20	1.10	1.00
6. Dietary methionine (based on digestible AA ratios)	0.50	0.48	0.46

7. Dietary TSAA (based on digestible AA ratios)	0.81	0.78	0.74
8. Synthetic methionine	0.17	0.16	0.16
9. Methyl requirement	602	497	433
10. Betaine amount	627	518	451
11. Betaine added (100% TMG)	1000	1000	1000
11- 10 "Extra" betaine	373	482	549

3.8 Measurements and sampling

3.8.1 Broiler performance

Chicks were weighed in groups at placement and at 7, 14, 21, 28, 35, and 40 d of age. Weekly bodyweight gain (BWG), cumulative BWG (CumBWG), weekly feed intake (FI), Cumulative FI (CumFI), weekly feed conversion ratio adjusted for mortality (AdjFCR) and Cumulative AdjFCR (CumAdjFCR) for each pen were also determined. Adjusted and Cumulative FCR was corrected for the mortalities in each pen and was calculated as follow:

$$\text{Adjusted FCR (AdjFCR)} = \frac{total\ feed\ consumed}{total\ BW\ of\ surviving\ chick + total\ BW\ of\ birds\ that\ died}$$

Weekly mortality percentage, cumulative total mortality and ascites-related mortality percentages were also calculated during the experimental period.

Performance efficiency factor (PEF) for each pen were also calculated as follow:

$$\text{Performance efficiency factor (PEF)} = \frac{[(100 - total\ cumulative\ mortality\ \%)\ X\ CumBWG\ X\ 100)]}{(CumAdjFCR * \#\ of\ days\ grown)/1000}$$

3.8.2 Ascites (AS) or pulmonary hypertension syndrome (PHS) diagnosis

Throughout the experiment, all dead birds were removed on a daily basis. Mortalities were recorded and all dead chickens were necropsied (see necropsy procedure) and visually inspected to determine the incidence of ascites-related heart failure. Birds that died from other causes were

recorded as such. Birds were examined for macroscopic lesions related to pulmonary hypertension syndrome or ascites, such as (Scheele *et al*., 2003; Kalmar *et al*., 2010)**:**

- Peritoneal effusion (abdominal fluid accumulation, "water belly").
- Pericardial effusion (fluid accumulation around the heart).
- Ratio of right ventricle to total ventricle weight (RV/TV ratio).

Bird necropsy was performed as follow (Adapted from Davis & Morishita, 2012):

1. *The dead bird was weighed and the individual's weight was recorded*
2. *The bird was examined internally:*

- The dead bird was placed on its back with its feet facing towards the researcher.
- The bird was then grasped at both legs and legs were pushed down and away from the pelvis, thus disarticulating the hips to loosen the joints.
- With sharp scissors, a small cut was made into the body cavity just below the breast bone. Next, the skin overlying the abdomen and breast from the neck to cloaca were removed (**Figure 3.6**).
- The abdominal muscle were incised and cut through the ribs on both sides of the breast muscle.
- The abdominal muscle was pulled caudally (upwards) to expose the internal organs and chest cavity (**Figure 3.7**). Any fluid accumulation in the abdominal cavity was noted.

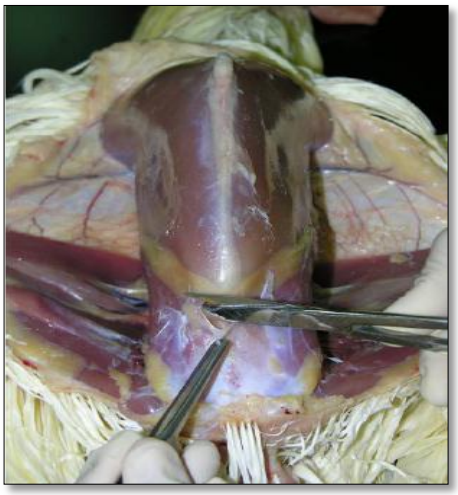

Figure: 3.6: Cutting into the body cavity of a chicken

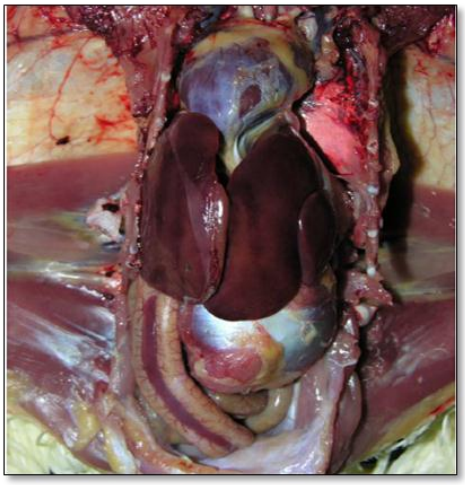

Figure 3.7: Exposure of internal organs and chest cavity

- Next, the heart muscle as well as the valves were examined for hypertrophy (**Figure 3.9** and **3.10**). Any excessive fluid located between the heart and the pericardium were noted, as this is an indication of right heart failure, hence ascites. The RV/TV ratios were also determined.

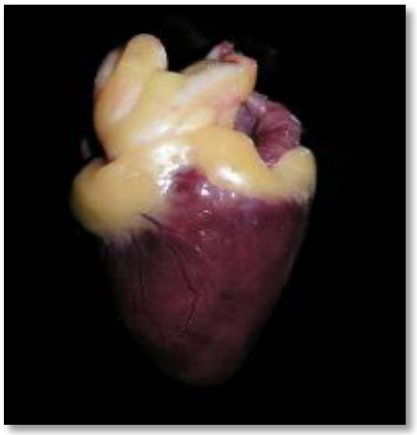

Figure 3.8: Chicken Heart

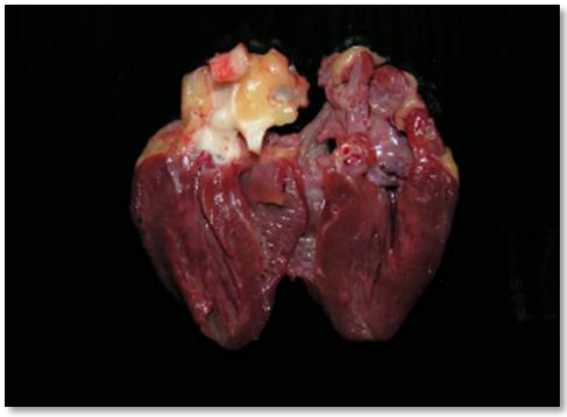

Figure 3.9: The interior of the heart's valves

3.8.3 Bird processing

Birds were transported to the slaughtering facilities of the Research Farm at the University of Pretoria (Hatfield, Pretoria). The trial consisted out of two processing stages, with two consecutive sampling days each. Two birds per pen were randomly sampled during each processing stage. The processing stages were as follow:

- **Processing stage 1**:
  - Sampling day 1(d20) = 96 birds
  - Sampling day 2 (d21) = 96 birds

- **Processing stage 2:**
  - Sampling day 3 (d40) = 96 birds
  - Sampling day 4 (d41) = 96 birds

During processing, neck tags remained attached to each bird in order to trace them back to their respective pens and dietary/sex treatments.

3.8.3.1 Carcass yield and abdominal fat determination:

At the end of the experiment (bird processing stage 2), a yield study was conducted using a total of 2 birds per pen (replicate). Water and feed was not removed from the birds until approximately 30-60 minutes prior to processing. At the University of Pretoria abattoir, the birds were individually weighed prior to processing. Birds were then electrically stunned, followed by immediate bleeding via severing of the jugular vein and scalded for approximately 30-45 seconds at approximately 60-80°C. Carcass weight of the individual birds was then determined after removal of the head, neck, feet, feathers, abdominal fat pad, and manual evisceration. The abdominal fat pad was removed and weighed separately for determination of abdominal fat, which was expressed as a percentage of the individual bird's live weight. The breast fillets were skinned and deboned, while the hind quarters were dissected to remove the drums and thigh as a whole (leg portion). All individual cuts were weighed separately and the yields were expressed relative to the final live bodyweight. Any signs of ascites during processing were noted and recorded.

3.8.3.2 Ascites heart index (AHI) determination:

The ascites heart index (AHI) was determined from all birds that died during the trial, however this was only done on a fresh matter basis. The AHI was also measured from two birds per pen during each processing stage, in order to quantify the progression towards pulmonary hypertension-induced heart failure. This was performed by cutting away the atria, major vessels, and gross fat from the left ventricle. The AHI was then calculated by dividing the weight of the right heart ventricle by the combined weight of both heart ventricles (right ventricle + left ventricle + septum; RV/TV ratio). This was performed on both fresh matter basis (AHI_{FM}) during both bird processing stages (1 and 2) and on freeze-dried matter basis (AHI_{DM}) (Kalmar *et al.*, 2010) during bird processing stage 2. After AHI_{FM} determination, hearts were placed in sealable plastic Ziploc bags and frozen at -20˚C until AHI_{DM} could be determined the following week. A value of AHI_{FM} above 0.27 (Huchzermeyer & De Ruyck, 1986; Paecock *et al.*, 1988) or AHI_{DM} above 0.30 (Kalmar *et al.*, 2010) were considered to be an accurate measure of right ventricular hypertrophy and therefore pulmonary hypertension, which is regarded as the onset of ascites, as described by Huchzermeyer & De Ruyck (1986) and Paecock *et al*. (1988). Furthermore, the RV/BW ratio was also calculated for each bird and recorded (Julian *et al*., 1989a; Kalmar *et al.*, 2010).

3.8.3.3 Blood collection

At the processing plant, whole blood samples were collected from the same birds that were subjected for AHI quantification during each bird processing stage. Blood was collected from the jugular vein following decapitation after electrical stunning of the chicken. All blood samples were collected in heparinised micro-capillary tubes and immediately centrifuged with a portable centrifuge at 1,000g for 15 minutes. After centrifugation, plasma/serum samples were allocated to different cryotubes. Serum and plasma samples for lipid peroxidation and AMPK analyses were directly placed on ice and stored at -80ºC for further analysis. Plasma samples for homocysteine analysis were stored at 20-25°C. These samples were shipped to Ampath Pathologists (Pretoria, South Africa) for total homocysteine (tHCY) analysis the day after collection.

3.9 Laboratory analyses

3.9.1 Nutrient composition analyses

All feed samples were analysed in duplicate for the various nutrient levels. All nutritional analyses were performed at the Nutrilab, University of Pretoria.

3.9.1.1 Dry Matter (DM), moisture, and ash

The DM and ash analyses were performed according to the methods of AOAC (2000). DM analyses were performed by weighing ~2g of the representative feed samples into their separate crucibles. Thereafter, crucibles were placed into an oven at 105°C for approximately 24 hours and weighed back soon after cooling in a desiccator (±30 minutes) for determination of water loss, thus DM content.

$$\%DM = \frac{[\,weight\ of\ crucible\ +\ feed\ sample\ weight\ (wet)(g)] - [weight\ of\ crucible\ +\ feed\ sample\ weight\ (dry)(g)]}{sample\ weight\ (g)}\ x\ 100\%$$

The moisture content was determined as follow:

$$Moisture\ \% = 100 - \%\ DM$$

The ash content was determined by incinerating the feed samples that were used for DM determination in a muffle furnace for 4 hours at 550°C and then weigh back the sample soon after cooling in a desiccator (±30 minutes).

$$\% \, Ash = \frac{weight \; of \; crucible \; \& \; ash \; (g) - weight \; of \; crucible \; (g)}{weight \; of \; crucible \; \& \; feed \; sample \; (g) - weight \; of \; crucible \; (g)} \; x \; 100$$

3.9.1.2 Crude protein (CP)

CP was determined by using the Dumas combustion method on the Leco TruMac N determinator (Leco Corporation), which measures the total nitrogen content in the feed (AOAC, 2000). Nitrogen content was measured by placing ~0.2g of the feed sample into their respective sample holders, referred to as boats. The boats were placed in order in the purge region of a combustion tube and eventually pushed into the "hot zone" of the furnace. This caused the sample to combust in the high temperature chamber in the presence of oxygen and leads to the release of CO_2, water and nitrogen. The gases were then passed over special columns that absorb the CO_2 and water. The samples were then passed through a column containing a thermal conductivity detector, called the TC cell, that reduces NOX gases to N_2. The remaining content was then quantified from the signal of the TC cell and corrected for ballast temperature, pressure and sample mass. Finally, the nitrogen content was converted to crude protein content by multiplying the percentage nitrogen with a conversion factor (x6.25 for protein) (AOAC, 2000).

3.9.1.3 Crude fibre (CF)

CF was determined by using the Foss Fibertec^{TM} 2010 machine according to the methods of AOAC, 1984. The analysis was performed by weighing approximately ~1g of the individual feed samples into their respective crucibles. Crucibles were placed tightly onto a pre-heated hot plate and filled with approximately 150mL sulphuric acid solution (H_2SO_4, 1.25%). Three drops of n-Octanol was added to each sample as antifoaming agent and samples were brought to a boil, after which the heat was reduced and the samples were allowed to boil for a further 30 minutes. After 30 minutes, the sulphuric acid solution was vacuumed out and samples were washed for three washes with hot distilled water. After draining the last wash, 150mL of preheated potassium hydroxide solution (KOH, 1.25%) and 3 drops of n-Octanol were added to the separate samples and brought to

a boil again. After 30 minutes, the washing process was repeated. Crucibles were removed and the dry weight was determined after drying the samples overnight in an oven at 105°C. These weights represent the crude fibre plus the ash content. Samples were ashed for 3 hours at 550°C and CF was determined as follow:

$$\%\ Crude\ fiber = \frac{[CF + Ash] - [initial\ dry\ weight\ (g)]}{initial\ dry\ weight\ (g)}$$

3.9.1.4 Ether extract (EE)

EE analysis was performed by using the Foss Soxtec™ 2043 according to the methods of AOAC (2000). Feed samples (1.995-2.005g) were weighed onto filter paper, folded, and placed into their respective thimble holders. Fat beakers were filled three quarter full with ether (±30-40mL). Fat beakers were lined up in front of the fat extraction apparatus and tightly clamped onto the extractor. Next, the thimbles were matched and slipped into their corresponding fat beakers containing the ether and boiled for 4 hours, followed by a 2-hour rinsing period. Afterwards, fat beakers were dried in an oven at 100°C until all the ether has evaporated. Fat beakers containing the fat or lipid content of the feed was then placed into a desiccator (±20 minutes) and weighed back soon after cooling.

$$\%\ Crude\ Fat\ (DM\ basis)\ =\ (W3\ -\ W2)\ X\ 100\ /\ W1\ X\ Lab\ DM\ /\ 100$$

- W1 = initial sample weight in grams
- W2 = tare weight of beaker in grams
- W3 = weight of beaker and fat residue in grams

3.9.1.5 Nitrogen-Free extract (NFE)

The NFE content of the feed samples, which represents the non-structural carbohydrates such as starches and sugars, was calculated as follow:

$$\%\ \mathrm{NFE} = 100 - (\%\mathrm{Water} + \%\ \mathrm{CP} + \%\mathrm{CF} + \%\mathrm{Ash} + \%\mathrm{EE})$$

3.9.1.6 Gross energy (GE)

The GE of the samples was measured with a MC-1000 Modulator Calorimeter according to the operator's manual. The GE content was determined by heat of combustion of the individual feed samples (~0.5g) in a water bomb calorimeter in the presence of oxygen.

3.9.1.7 3.9.1.7 Amino acid (AA) analysis

Representative samples of each feed were shipped to Evonik Industries AG Analytical Lab (Germany, UK) for amino acid content by means of high performance liquid chromatography (HPLC).

3.9.1.8 Mineral analysis

Before mineral analyses, all feed samples (0.5g) were digested with 25mL nitric acid (HNO_3) and 10 mL perchloric acid ($HCLO_4$) on a heating block at 240°C until they appeared orange-yellow. Samples were transferred to 50mL volumetric flasks and filled up with deionised water. All samples were prepared according to AOAC (2000).

3.9.1.8.1 Calcium (Ca), sodium (Na), and potassium (K)

Calcium (Ca), sodium (Na), and potassium (K) concentrations were obtained from the standard curve read at 432nm on a Perkin Elmer Atomic Spectrophotometer.

3.9.1.8.2 Phosphorus (P)

Phosphorus (P) readings were performed in accordance with AOAC (2000) on a Technicon Autoanalyser II Continuous-flow Analytical instrument (Spekol).

3.9.1.8.3 Chloride (Cl)

Chloride (CL) readings were performed in accordance with AOAC (2000) on a titrating plate.

3.9.1.9 Dietary choline, methionine, betaine and dimethylglycine analysis

Representative samples of each feed were shipped to Eurofins Scientific Inc. Nutrition Analysis Center (St. Louis, USA) from Danisco Animal Nutrition for analyses of choline chloride

(free) in accordance with AOAC (2008) and betaine and dimethylglycine HCL via internal methods.

3.9.2 Freeze drying of chicken hearts

All hearts collected during both sampling stages were freeze-dried. Firstly, all right ventricles and left ventricle + septum samples were weighed into separate containers and frozen overnight at -20°C. Next, the freeze-drying apparatus was pre-cooled until it had reached -50 to -60°C and the Temperature Control and Condenser were switched on. Hearts were packed into the machine and the vacuum and shelve temperature controller was set to -20°C. After approximately 4-5 hours, when the vacuum has reached 100 millitors, the shelve temperature controller was set to minus 10°C for a further 4-5 hours, followed by 0°C overnight. The next morning the shelve temperature was set to 10°C for ~ 4-5 hours, followed by 20°C for a further 4-5 hours. After freeze-drying, all heart samples was weighed back and placed in an oven of 105°C for approximately 5 hours. Once completely dried, hearts were immediately weighed pack to prevent any moisture accumulation and the RV: TV ratio was expressed on a dry matter basis.

3.9.3 Biochemical analysis

All sample analyses were performed in duplicate.

3.9.3.1 Homocysteine analysis

200µL of blood plasma from each bird per pen collected during both processing stage 1 and 2 for Total homocysteine was measured the day after collection by Ampath Pathologists (Pretoria, South Africa).

3.9.3.2 Lipid peroxidation assays

a) Thiobarbituric reactive substances (TBARS) assay

Plasma thiobarbituric acid reactive substances (TBARS) content was determined by using a colorimetric MDA Quantification assay Kit (Oxiselect™ TBARS Assay Kit; Cell Biolabs, INC., South Africa) according to the manufacturer's instructions. All plasma samples were stored at -80˚C for up to 1-2 months until assayed.

Calculation of results

The standard curve was used to determine the concentration of samples corresponding to the mean absorbance from the standard curve. The standard curve was constructed by plotting the average optical density (O.D.) for each standard on the vertical axis (Y) against the concentration on the horizontal (X) axis using statistical software (MyAssays) to generate a Linear Regression (LN) curve.

b) 4-Hydroxynonenal antibody (4-HNE) assay

Plasma 4-hydroxynonenal (4-HNE) content was determined as a second index of oxidative stress by using an enzyme Immunoassay Kit (Chicken 4 Hydroxynonenal (4-HNE) ELISA kit, MBS 739943; MyBioSource; California) according to the manufacturer's instructions.

Calculation of results

The standard curve was used to determine the concentration of samples corresponding to the mean absorbance from the standard curve. This was performed for both TBARS and 4-HNE Assays. However, with 4-HNE x^2 weighting was applied in order to take account for heteroscedasticity (unequal variability amongst a range of values) which results in less biased parameter estimates.

The standard curve was constructed by plotting the average O.D. for each standard on the vertical axis (Y) against the concentration on the horizontal (X) axis using statistical software (MyAssays) to generate a four-parameter logistic (4-PL) curve-fit used specifically for bioassays or immunoassays such as ELISAs. The concentrations were determined for samples which fell within the range of the upper and lower asymptotes of the fit (thus the A and D parameters).

$$Y = D + \frac{A-D}{1+(\frac{x}{B})^B}$$

- A = Minimum asymptote
- B = Slope/ steepness of the curve
- C = Inflection point (point where curve concaves upwards/downwards)
- D = Maximum asymptote

3.9.3.3 Chicken Adenosine Monophophate Activated Protein (AMPK) ELISA Assay

Plasma AMPK concentration was quantified using a solid phase enzyme immunoassay technique (Chicken Adenosine Monophosphate Activated Protein (AMPK) ELISA kit, MBS 742103; MyBioSource; California) according to the manufacturer's instructions.

Sample preparation

All plasma samples were diluted by adding 100 µL of PBS (pH 7.0-7.2) diluting buffer to 50 µL of plasma before the start of the assay and end-values were multiplied by the appropriate dilution factor for determination of final AMPK concentration.

Calculation of results

The standard curve was used to determine the concentration of samples corresponding to the mean absorbance from the standard curve.

The standard curve was constructed by plotting the average O.D. for each standard on the vertical axis (Y) against the concentration on the horizontal (X) axis on a spreadsheet (Microsoft Excel (2010)) to perform linear regression analysis.

3.10 Statistical analysis

A 2 x 4 factorial design was utilised with dietary treatment and sex as the main factors. There were four dietary treaments and two sexes, with six replicates (12 replicates in total) per treatment in each side of the 96-pen trail house. Each side of the house was devided into six blocks containing eight pens per block. One replicate per diet x sex treatment was randomly allocated to each block. The variation due to block and house effects was accounted for by including house and block as fixed effects in the model. Upon initial analysis of performance data a significant difference was found for initial (d0) body weight (P <0.05). To account for this difference initial (d0) body weight was included as a covariant in the subsequent statistical analysis to assess for treatment effetcs on body weight for this trial.

Statistical analyses were performed on all pooled data with pen as the experimental unit. Data were analysed statistically as a randomised block design using the General Linear Model (Statistical Analysis Systems, 2014) for the average effects over time. Repeated Measures Analyses Of Variance with the GLM model was used for grow out performance (except for mortality), carcass traits, indices of pulmonary hypertension syndrome (heart characteristics), and blood biochemical

parameters. Means and standard deviations were calculated and significance of difference (P <0.05) between means was determined by Fischers test (Samuels, 1989).

Total mortality and ascites-related mortality data were not normally distributed, hence these data were analysed with the General Linear Model as proportions of mortality out of 17 birds per pen that are binomially distributed. The data were transformed by the arcsin transformation prior to analysis to normalise the distributions for all mortality data. The effects of treatment, sex, and their interactions was tested for at a 5% significance interval. Fisher's protected least significant difference test was used to seperate predictions of proportions at the 5% confidence level.

Linear Regression analysis using unpooled data was carried out to estimate AHI_{DM} from AHI_{FM} (Draper & Smith, 1966). The adjusted R^2 value was determined as it is more reliable than the ordinary R^2 value ($R^2_{adjusted} < R^2$). This was performed using GenStat® for Windows™ (VSN, International, UK).

CHAPTER 4: RESULTS

I PHASE A: Incubation trial

4.1 Eggshell temperature (EST) and egg weight loss (EWL)

In order to obtain the desired high EST in each incubator, the individual machine temperatures had to be adjusted daily. **Table 4.1** and **Figures 4.1** and **4.2** depict the difference between the internal machine air temperature (MT) and EST for each machine. EST did not vary more than 1.1°C from MT for incubator machine 1 and 1.5°C for incubator machine 2 during the time that high temperatures were employed. EST for machine 1 was slightly higher compared to EST in machine 2 during ED_{11} to ED_{18}, where the eggs were subjected to higher than normal (>38.8°C) temperatures. This was most probably due to the higher percentage of infertile eggs in machine 2 (8.86%) compared to machine 1 (7.58%), hence more heat was produced by the embryos in machine 1. During the incubation period at high temperatures, the average EST of the two incubators ranged between 38.09°C to 39.40°C from ED_{11} to ED_{18} (**Figure 4.3**). Molenaar *et al.* (2010) suggested that an egg weight loss between 6.5 and 14.0% of the initial egg weight is satisfactory for the embryo to create a large enough air cell and to stimulate internal pipping. As illustrated in **Table 4.2**, average EWL between d0 and d18 was 10.62%, which is considered adequate.

Table 4.1: Comparison between the machine temperature (MT) and eggshell temperature (EST) of the two incubators

Embryonic Day (ED)	MT – Machine 1	EST - Machine 1	MT – Machine 2	EST – Machine 2	AVG MT	AVG EST
0	37.3	NA[1]	37.6	NA	37.5	NA
1	37.5	NA	37.4	NA	37.4	NA
2	37.6	NA	37.4	NA	37.5	NA
3	37.4	NA	37.5	NA	37.4	NA
4	37.5	NA	37.5	NA	37.5	NA
5	37.5	NA	37.6	NA	37.5	NA
6	37.5	NA	37.5	NA	37.5	NA
7	37.5	NA	37.6	NA	37.5	NA
8	37.5	NA	37.6	NA	37.5	NA
9	37.5	NA	37.7	NA	37.6	NA
10	37.5	NA	37.6	NA	37.6	NA
11	37.6	38.1	37.5	38.2	37.5	38.2
12	37.9	38.4	37.8	38.6	37.8	38.5
13	38.0	38.8	38.0	39.0	38.0	38.9
14	38.1	39.1	38.0	39.0	38.1	39.0
15	38.3	39.1	38.1	39.4	38.2	39.2
16	38.1	39.3	38.0	39.4	38.0	39.3
17	38.1	38.8	37.7	39.1	37.9	38.9
18	38.1	39.2	37.9	39.0	38.0	39.1
19	37.7	NA	37.9	NA	37.8	NA
20	37.4	NA	37.3	NA	37.3	NA
21	37.3	NA	36.9	NA	37.1	NA

[1] NA: Not Applicable. EST was measured only between embryonic day 11 (ED_{11}) and embryonic day 18 (ED_{18})

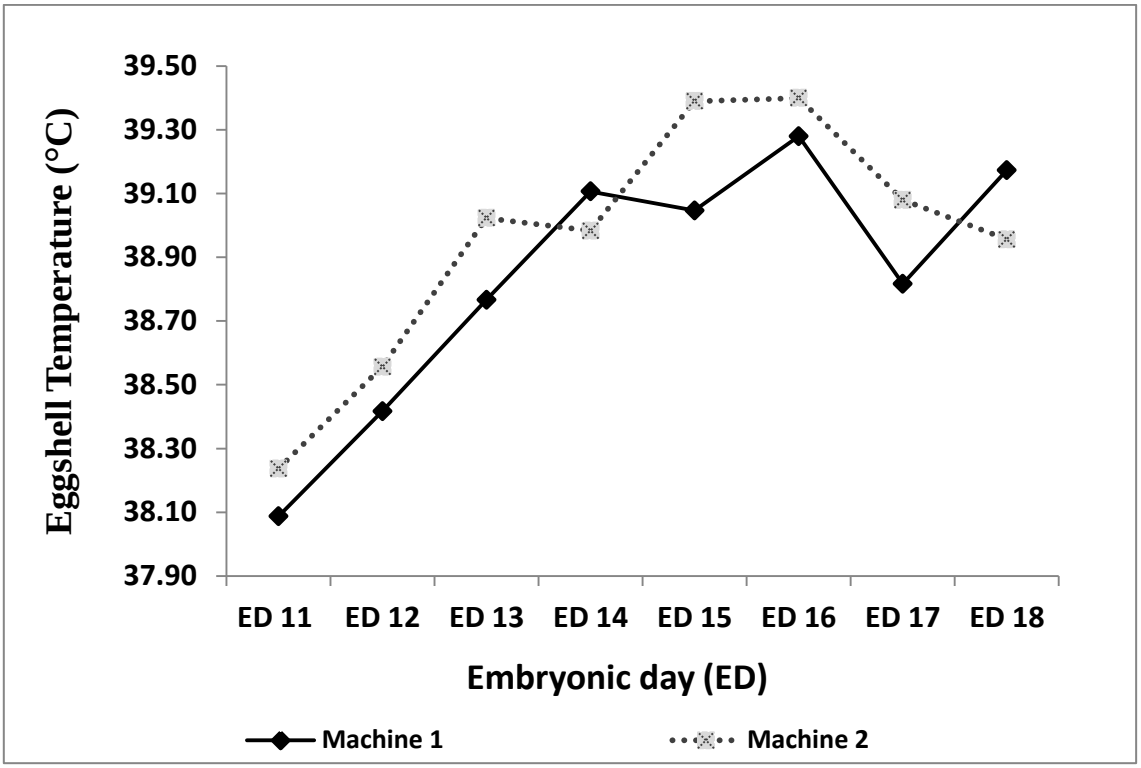

Figure 4.1: Eggshell temperature (EST) reached in machine 1 & 2 because of induced high incubation temperatures during ED_{11} to ED_{18}

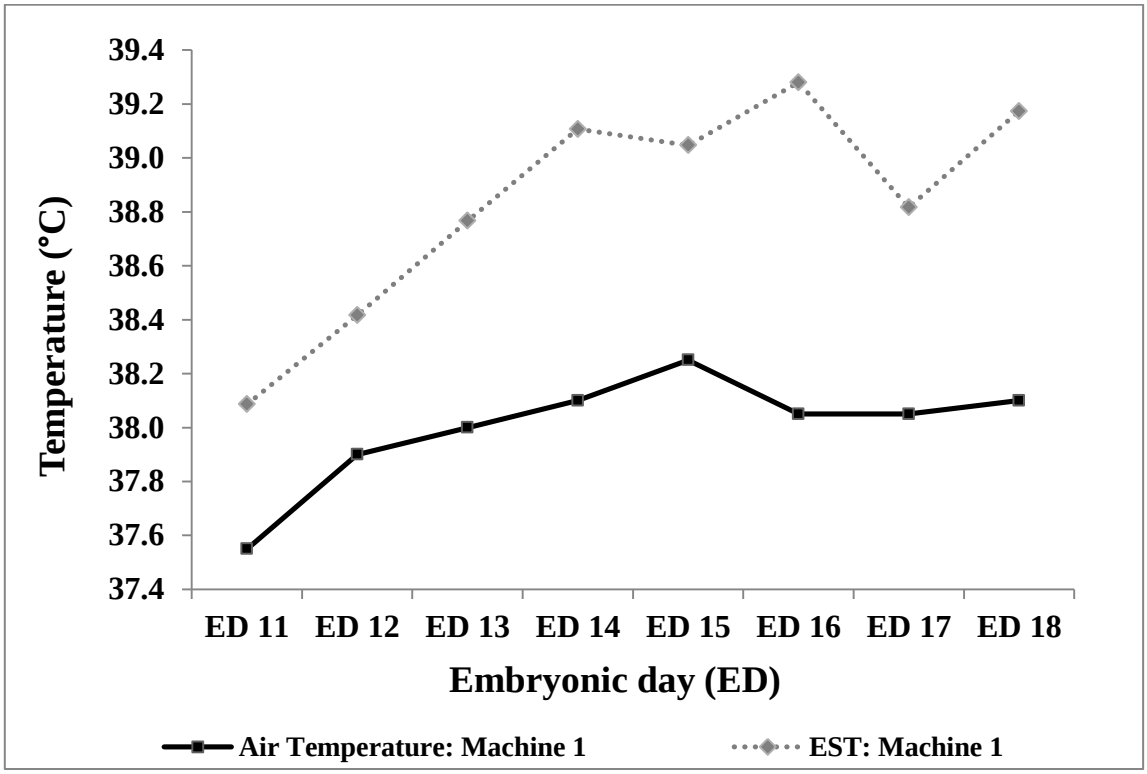

Figure 4.2: Difference between machine temperature (MT) and eggshell temperature (EST) of Machine 1 during ED_{11} to ED_{18}

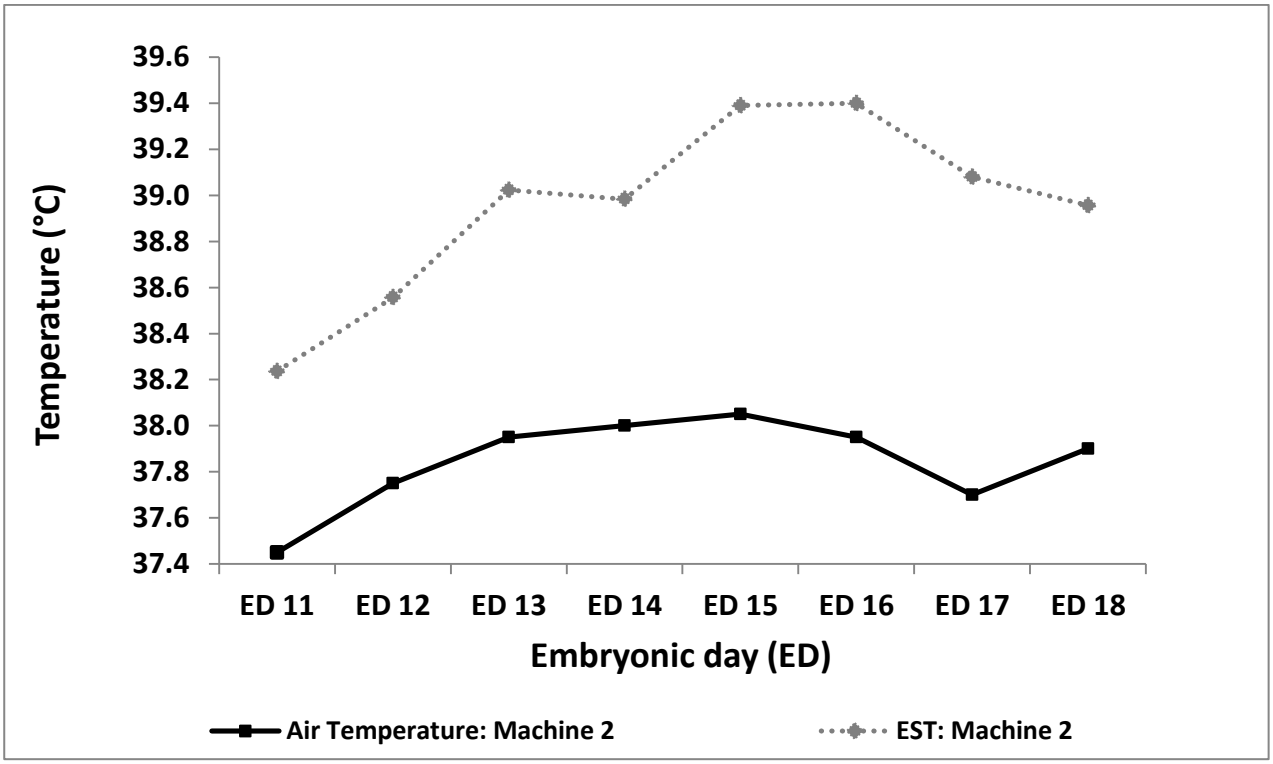

Figure 4.3: Difference between machine temperature and eggshell temperature of Machine 2 during ED_{11} to ED_{18}

4.1.1 Hatching performance and chick quality

Table 4.2: Percentage of fertile eggs, hatchability of fertile eggs, first grade chicks, second grade chicks, third grade chicks, embryonic mortality, and malpositioned embryos of eggs incubated at high (>38.8°C) eggshell temperatures (EST)

	Machine 1	Machine 2	Average for Machine 1 & 2
Incubation:			
Egg weight loss (EWL) percentage (%)	10.36	10.88	10.62
% Infertile eggs[1]	7.58	8.86	8.22
Hatchability of fertile eggs[2] (%)	84.93	79.77	82.35
Hatchability[3] (%)	77.88	72.65	75.27
% First-grade chicks[4]	84.96	77.65	81.31
% Second-grade chicks[5]	6.31	10.72	8.28
% Third-grade chicks[6]	6.61	9.95	8.67
% Culls[7]	1.57	0.98	1.28
% Dead[8]	0.54	0.71	0.63
Embryo mortality[9]:			
Early (1week)	3.41	4.55	3.98
Mid (2 week)	8.54	4.85	6.70
Late (3 week)	5.75	7.84	6.80
% Malpositioned embryos	31.91	41.44	36.68
% Abnormal	2.31	2.24	2.28
Hatchling[10]:			
% Males	0.48	0.48	0.48
% Females	0.52	0.52	0.52

[1] Expressed as a percentage of the total number of eggs

[2] Expressed as a percentage of fertile eggs

[3] Expressed as a percentage of eggs set

[4,5,6,7,8] Expressed as a percentage of hatched eggs

[9] Expressed as a percentage of unhatched eggs

[10] Expressed as a percentage of the first- and second- grade chicks

Hatching performance and chick quality of the chicks incubated at high eggshell temperatures is presented in **Table 4.2**. Our results showed an increase in embryonic mortality from the first week until the last week of incubation that can be attributed to the high temperatures employed from the second half of incubation onwards. The relatively high third week embryonic mortality (6.80%) observed can possibly be related to the increased number of malpositioned embryos (36.68%). From our personal observations, the chicks incubated at the high eggshell temperatures showed signs of chick quality problems (**Figure 4.4, 4.5** and **4.6**). These included: blood inside the eggshell, blood on the down and feathers, short feathers, red hocks, unhealed navels, "black buttons", externalised yolk sac remnants, ectopic viscera as well as some conformational deformities (e.g. no eyes, cross-beak, extra limbs and ectopic viscera). Generally, chicks were also pale in colour. In line with the judged poor quality chicks was a relatively high second- (17.03%) and third-grade (16.52%) chick percentage of the hatch. However, due to a lack of adequate sampling, no statistical analysis could be performed relating the effect of high incubation temperatures on organ development and chick quality in male and female broiler chicks. The percentage of male chicks comprised of 0.48% of the total number of chicks that have hatched whereas the percentage of females constitued of 0.52% of the total number of chicks that have hatched under high incubation temperatures.

Figure 4.4: Chick exhibiting white colour, poor feathering around face and beak, and splayed legs due to high incubation temperatures (>38.8°C)

Figure 4.5: Chick exhibiting white colour and conformational deformities (absence of left eye, cross-beaked) possibly due to high incubation temperatures (>38.8°C)

Figure 4.6: Chick exhibiting red hocks possibly due to high incubation temperatures (>38.8°C)

II PHASE B: Broiler grow-out trial

4.2 Nutrient composition of diets

4.2.1 Proximate composition of the different test diets

The treatment diets were closely formulated to avoid extensive variations in raw ingredient compositions between the different dietary treatments as well as between the different dietary phases. After completion of the study, proximate analysis was performed on all treatment diets (**Tables 4.3, 4.4,** and **4.5**).

Table 4.3: Formulated (expected) values versus analysed (actual) values for the broiler starter, grower, and finisher diets, respectively

	Starter		**Grower**		**Finisher**	
	Formulated (expected) Values	*Analysed Values*	*Formulated (expected) Values*	*Analysed Values*	*Formulated (expected) Values*	*Analysed Values*
Proximate Composition:						
Moisture (%)	10.85	10.84	10.57	11.59	10.96	12.61
Dry matter (%)	89.15	89.16	89.43	88.41	89.04	87.39
Ash (%)	4.79	4.60	4.44	4.08	4.04	3.56
Crude protein (%)	22.22	22.78	20.74	20.91	19.27	18.34
Crude fibre (%)	3.73	3.11	3.86	3.48	3.83	3.34
Ether extract (%)	3.56	3.40	4.43	4.23	4.66	4.44
Nitrogen-free extract (%)	54.33	55.28	55.67	55.72	57.52	57.71
Gross energy (MJ/kg)	NA	15.99	NA	16.18	NA	15.97
Mineral Analysis:						
Ca (%)	0.940	0.971	0.825	0.702	0.715	0.601
Total P (%)	0.790	0.608	0.694	0.491	0.626	0.419
Ca: P	1.190	1.596	1.189	1.429	1.142	1.433
Na (%)	0.200	0.176	0.190	0.175	0.180	0.166
K (%)	0.920	0.836	0.842	0.759	0.771	0.694
Cl (%)	0.300	0.331	0.300	0.342	0.301	0.352

4.2.2 Feed additives and amino acid concentrations of the test diets

The nutrient concentrations of the intermediate metabolites in the choline-to-glycine pathway of the broiler test diets are summarised in the following section. Nutrient analysis showed slightly higher betaine concentrations relative to the basal (Negative Control) diet for the various phases. Dietary betaine content for the individual test diets varied from 0.68 to 0.80 w/w % for the broiler starter, 0.56 to 0.61 w/w % for the broiler grower, and 0.59 to 0.64 w/w % for the broiler finisher diets (**Table 4.4**). When expressed on a percentage weight-to-weight basis, betaine was added at the amount of 0.1042 w/w % to test diet 2.

Results from **Table 4.5** showed that the highest dietary DMG concentration was obtained with Na-DMG addition, as intended. Overall, the DMG content for Treatments 3 (DMG-groups) were 0.15 w/w % for the broiler starter, 0.13 w/w % for the broiler grower, and 0.15 w/w % for the broiler finisher diets compared to the other test diets that contained minimal DMG levels (<0.09 w/w %). These analysed values were in good agreement with the formulated values of 0.1380 when expressed on a weight per weight percentage basis.

The feed was formulated to contain no additional choline in the basal diet, hence the choline present in the different test diets are mainly of dietary origin. The calculated and analysed choline content of the different test diets for the respective age categories is shown in **Table 4.6**. As expected, the analysed choline content of the NC (basal) diet was lower for the broiler starter and grower but not for the finisher diets in contrast to the choline content of Treatment 4 (PC diet), where choline was added.

The formulated and analysed total sulphur containing amino acids (TSAA) and total cysteine contents for the different bird age categories is shown in **Table 4.7** and **Table 4.9**, respectively. Overall, the analysed values for both TSAA and cysteine for the different test diets were in good agreement with the expected values across all bird age categories. The total TSAA content ranged from roughly 94-97%, 97-98%, and 97-104% for the broiler starter, grower, and finisher diets, respectively.

Table 4.4: The analysed levels of betaine in the different treatment diets

	Treatment diets	Added betaine w/w %	Total measured betaine w/w %
Starter:			
1	Negative Control (NC)	0	0.68
2	NC + Betaine	0.1042	0.71
3	NC + DMG	0	0.72
4	Positive Control (PC)	0	0.80
Grower:			
1	Negative Control (NC)	0	0.56
2	NC + Betaine	0.1042	0.61
3	NC + DMG	0	0.61
4	Positive Control (PC)	0	0.56
Finisher:			
1	Negative Control (NC)	0	0.59
2	NC + Betaine	0.1042	0.61
3	NC + DMG	0	0.59
4	Positive Control (PC)	0	0.64

The formulated and analysed total and free methionine content for the different bird age categories is shown in **Table 4.8**. The feed was formulated to contain 26.09%, 19.05%, and 20.0% less synthetic methionine in the starter, grower, and finisher diets of Treatments 1 (basal or NC diets), respectively. As expected, higher methionine values (both total and free) were obtained following dietary methionine supplementation (Treatment 4, PC diet) in contrast to the basal diet where methionine was removed from the diet for the different bird age categories. Furthermore, actual methionine (total and free) contents of the different treatment diets were relatively similar to what was anticipated (formulated). Further analysed contents of the remaining amino acids for the different broiler starter, grower, and finisher diets are shown in **Tables 4.10, 4.11** and **4.12**, respectively.

4.3 Brooding and grow-out temperatures

Figure 4.7 summarises the temperature profile experienced by the chicks during the brooding and grow-out period. As illustrated by **Figure 4.8**, a gradually colder brooding and rearing temperature was employed from day of chick placement (d0) until the end of the growing period (d40) in relation to commercial broiler brooding and grow-out temperatures. This gradual decrease in litter temperature might have contributed to a possible increase in the basal metabolic rate and oxygen consumption of the growing chickens and as a result, the development of PHS was increased.

Table 4.5: Analysis of dimethylglycine (DMG) concentrations in the different treatment diets

	Treatment diets	Added DMG w/w %	Total measured DMG w/w %	Analysed DMG as % of expected DMG
Starter:				
1	Negative Control (NC)	0	<0.09	NA
2	NC + Betaine	0	<0.09	NA
3	NC + DMG	0.1380	0.15	108.70
4	Positive Control (PC)	0	<0.09	NA
Grower:				
1	Negative Control (NC)	0	<0.09	NA
2	NC + Betaine	0	<0.09	NA
3	NC + DMG	0.1380	0.13	94.20
4	Positive Control (PC)	0	<0.09	NA
Finisher:				
1	Negative Control (NC)	0	<0.09	NA
2	NC + Betaine	0	<0.09	NA
3	NC + DMG	0.1380	0.15	108.70
4	Positive Control (PC)	0	<0.09	NA

Table 4.6: The expected and analysed content of added choline in the different treatment diets

Treatment diets		Added choline g/100g	Expected total choline	Total measured choline g/100g	Measured choline as % predicted choline
Starter:					
1	Negative Control (NC)	0	1347	1320	98.00
2	NC + Betaine	0	1347	1360	100.97
3	NC + DMG	0	1347	1510	112.10
4	Positive Control (PC)	300	1647	1470	89.25
Grower:					
1	Negative Control (NC)	0	1354	1260	93.06
2	NC + Betaine	0	1354	1210	89.36
3	NC + DMG	0	1354	1460	107.83
4	Positive Control (PC)	250	1604	1410	87.91
Finisher:					
1	Negative Control (NC)	0	1193	1070	89.69
2	NC + Betaine	0	1193	1040	87.18
3	NC + DMG	0	1193	1250	104.78
4	Positive Control (PC)	200	1393	995	71.43

Table 4.7: The expected and analysed content of total sulphur amino acids (TSAA) in the different treatment diets

	Treatment diets	Expected total TSAA	Total actual TSAA	Expected total TSAA as % of analysed
Starter:				
1	Negative Control (NC)	0.920[1]	0.890	96.74
2	NC + Betaine	0.920[1]	0.895	97.28
3	NC + DMG	0.920[1]	0.886	96.30
4	Positive Control (PC)	0.964	0.914	94.84
Grower:				
1	Negative Control (NC)	0.880[1]	0.858	97.50
2	NC + Betaine	0.880[1]	0.854	97.05
3	NC + DMG	0.880[1]	0.865	98.30
4	Positive Control (PC)	0.910	0.896	98.46
Finisher:				
1	Negative Control (NC)	0.820[1]	0.852	103.90
2	NC + Betaine	0.820[1]	0.822	100.24
3	NC + DMG	0.820[1]	0.799	97.44
4	Positive Control (PC)	0.861	0.838	97.33

[1] The new TSAA for the different age categories that the diet will allow after formulating with Betacheck

Table 4.8: The expected and analysed content of total and free methionine content in the different treatment diets

Treatment diets	Expected total methionine (%)	Analysed total methionine (%)	Analysed total methionine as % of expected total methionine	Expected free methionine (%)	Analysed free methionine (%)	Analysed free methionine as % of expected free methionine
Starter:						
1 Negative Control (NC)	0.580[1]	0.512	87.93	0.167[1]	0.158	94.61
2 NC + Betaine	0.580[1]	0.513	88.45	0.167[1]	0.160	95.81
3 NC + DMG	0.580[1]	0.502	86.55	0.167[1]	0.140	83.83
4 Positive Control (PC)	0.593	0.537	90.56	0.231	0.199	86.15
Grower:						
1 Negative Control (NC)	0.560[1]	0.487	86.96	0.163[1]	0.148	90.80
2 NC + Betaine	0.560[1]	0.485	86.61	0.163[1]	0.146	89.57
3 NC + DMG	0.560[1]	0.494	88.21	0.163[1]	0.153	93.87
4 Positive Control (PC)	0.557	0.518	93.00	0.212	0.159	75.00
Finisher:						
1 Negative Control (NC)	0.520[1]	0.484	93.08	0.157[1]	0.145	92.36
2 NC + Betaine	0.520[1]	0.469	90.19	0.157[1]	0.148	94.27
3 NC + DMG	0.520[1]	0.475	91.35	0.157[1]	0.171	108.92
4 Positive Control (PC)	0.526	0.509	96.77	0.197	0.188	95.43

[1] The new minimum methionine levels (total and free) for the different age categories that the diet will allow after formulating with Betacheck

Table 4.9: The expected and analysed content of total cysteine content in the different treatment diets

Treatment diets		Expected total cysteine	Analysed total cysteine	Analysed total cysteine as % expected total cysteine
Starter:				
1	Negative Control (NC)	0.370[1]	0.378	102.16
2	NC + Betaine	0.370[1]	0.382	103.24
3	NC + DMG	0.370[1]	0.383	103.51
4	Positive Control (PC)	0.372	0.377	101.34
Grower:				
1	Negative Control (NC)	0.350[1]	0.371	106.00
2	NC + Betaine	0.350[1]	0.369	105.43
3	NC + DMG	0.350[1]	0.370	105.71
4	Positive Control (PC)	0.353	0.379	107.37
Finisher:				
1	Negative Control (NC)	0.330[1]	0.368	111.52
2	NC + Betaine	0.330[1]	0.354	107.27
3	NC + DMG	0.330[1]	0.323	97.88
4	Positive Control (PC)	0.334	0.329	101.52

[1] The new minimum cysteine level for the different age categories that the diet will allow after formulating with Betacheck

Table 4.10: Analysed amino acid composition of the different broiler starter diets (expressed as percentage standardise DM)

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**
	Neg. Control (NC)	**Neg. Control + Betaine**	**Neg. Control + DMG**	**Pos. Control (PC)**
Content (%):				
Methionine (total)	**0.512**	**0.513**	**0.502**	**0.537**
Methionine (free)	**0.158**	**0.160**	**0.140**	**0.199**
Cystine	0.378	0.382	0.383	0.377
TSAA (Methionine + Cystine)	0.890	0.895	0.886	0.914
Lysine (total)	1.330	1.335	1.317	1.275
Lysine (free)	0.249	0.248	0.226	0.228
Threonine (total)	0.863	0.845	0.858	0.830
Threonine (free)	0.062	0.064	0.057	0.057
Tryptophan	0.261	0.253	0.252	0.251
Argenine	1.482	1.460	1.461	1.429
Isoleucine	0.965	0.965	0.935	0.922
Leucine	2.009	1.994	1.987	1.958
Valine	1.092	1.094	1.063	1.048
Histidine	0.591	0.585	0.582	0.573
Phenylalanine	1.131	1.119	1.119	1.097
Glycine	0.943	0.936	0.931	0.913
Serine	1.057	1.018	1.068	1.042
Proline	1.379	1.341	1.359	1.351
Alanine	1.158	1.150	1.145	1.130
Aspartic acid	2.179	2.161	2.166	2.214
Glutamic acid	4.112	4.057	4.061	3.985
Total (without NH_3)	21.440	21.209	21.189	20.846
Ammonia	0.462	0.466	0.451	0.444
Total	21.903	21.676	21.641	21.290

Table 4.11: Analysed amino acid composition of the different broiler grower diets (expressed as percentage standardise DM)

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**
	Neg. Control (NC)	**Neg. Control + Betaine**	**Neg. Control + DMG**	**Pos. Control (PC)**
Content (%):				
Methionine (total)	**0.487**	**0.485**	**0.494**	**0.518**
Methionine (free)	**0.148**	**0.146**	**0.153**	**0.159**
Cystine	0.371	0.369	0.370	0.379
TSAA (Methionine + Cystine)	0.858	0.854	0.865	0.896
Lysine (total)	1.182	1.176	1.220	1.292
Lysine (free)	0.223	0.218	0.222	0.251
Threonine (total)	0.781	0.762	0.804	0.810
Threonine (free)	0.050	0.047	0.053	0.053
Tryptophan	0.225	0.217	0.236	0.245
Argenine	1.311	1.309	1.348	1.395
Isoleucine	0.876	0.881	0.863	0.908
Leucine	1.928	1.899	1.890	1.924
Valine	1.008	1.015	0.989	1.046
Histidine	0.551	0.549	0.549	0.568
Phenylalanine	1.032	1.029	1.037	1.065
Glycine	0.861	0.858	0.870	0.903
Serine	0.956	0.915	10.014	0.999
Proline	1.316	1.303	1.301	1.323
Alanine	1.104	1.093	1.097	1.116
Aspartic acid	1.930	1.926	1.991	2.051
Glutamic acid	3.808	3.776	3.816	3.909
Total (without NH_3)	19.726	19.562	19.889	20.450
Ammonia	0.439	0.446	0.417	0.441
Total	20.165	20.008	20.306	20.891

Table 4.12: Analysed amino acid composition of the different broiler finisher diets (expressed as percentage standardise DM)

	Treatment 1	**Treatment 2**	**Treatment 3**	**Treatment 4**
	Neg. Control (NC)	**Neg. Control + Betaine**	**Neg. Control + Taminizer D**	**Pos. Control (PC)**
Content (%):				
Methionine (total)	**0.484**	**0.469**	**0.475**	**0.509**
Methionine (free)	**0.145**	**0.148**	**0.171**	**0.188**
Cystine	0.368	0.354	0.323	0.329
TSAA (Methionine + Cystine)	0.852	0.822	0.799	0.838
Lysine (total)	1.164	1.062	0.935	0.997
Lysine (free)	0.232	0.220	0.210	0..221
Threonine (total)	0.756	0.712	0.640	0.663
Threonine (free)	0.039	0.037	0.036	0.038
Tryptophan	0.222	0.212	0.184	0.192
Argenine	1.275	1.187	1.066	1.123
Isoleucine	0.835	0.788	0.724	0.738
Leucine	1.850	1.802	1.766	1.749
Valine	0.964	0.917	0.857	0.872
Histidine	0.539	0.512	0.464	0.478
Phenylalanine	1.002	0.953	0.896	0.906
Glycine	0.843	0.790	0.725	0.755
Serine	0.959	0.900	0.821	0.843
Proline	1.292	1.284	1.200	1.198
Alanine	1.077	1.042	1.020	1.018
Aspartic acid	1.886	1.765	1.570	1.638
Glutamic acid	3.693	3.528	3.281	3.320
Total (without NH_3)	19.209	18.276	16.946	17.328
Ammonia	0.414	0.401	0.389	0.390
Total	19.624	18.677	17.335	17.718

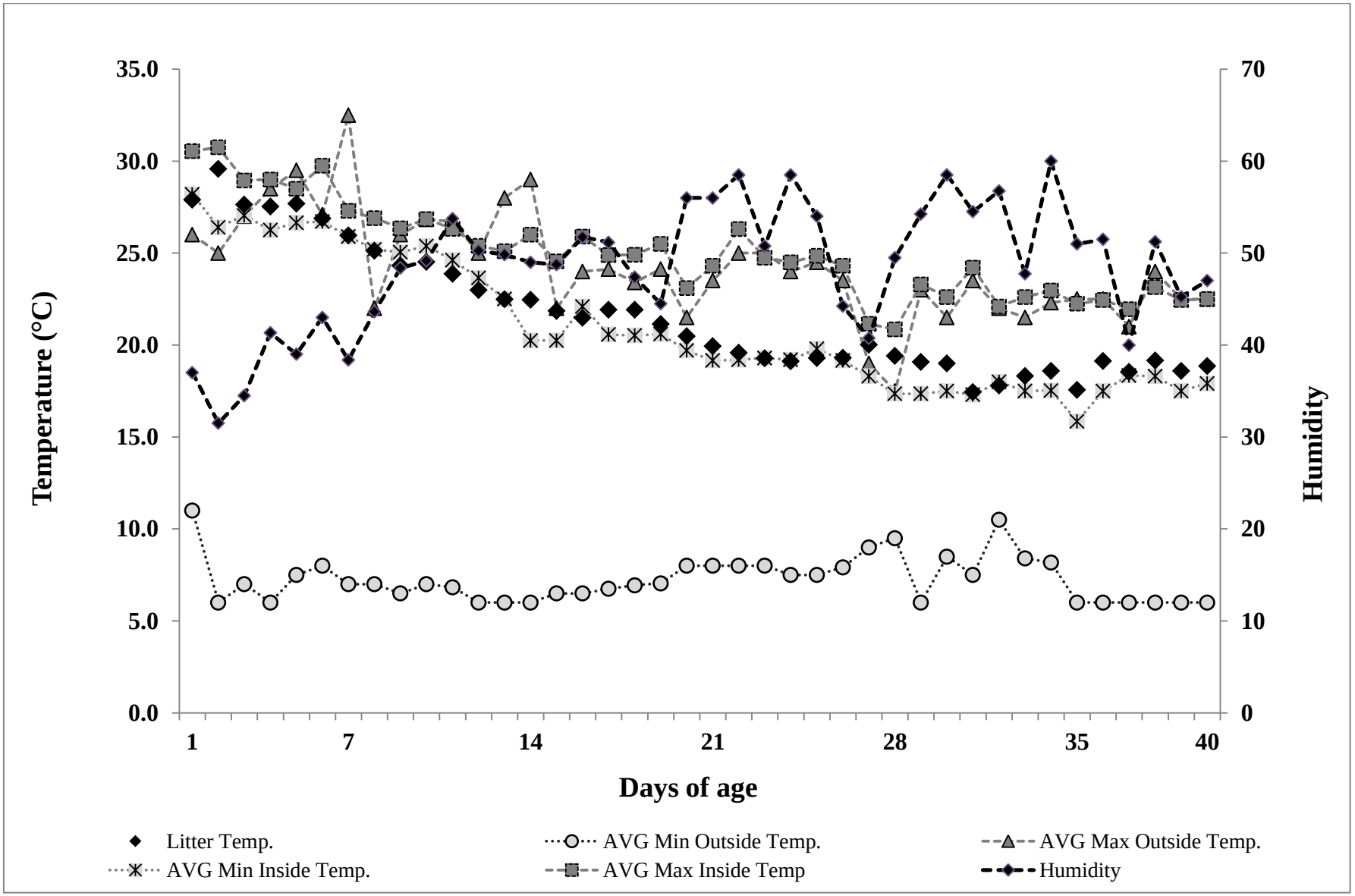

Figure 4.7: Temperature profile during the brooding and grow-out period of the broiler chicken for the duration of the trial

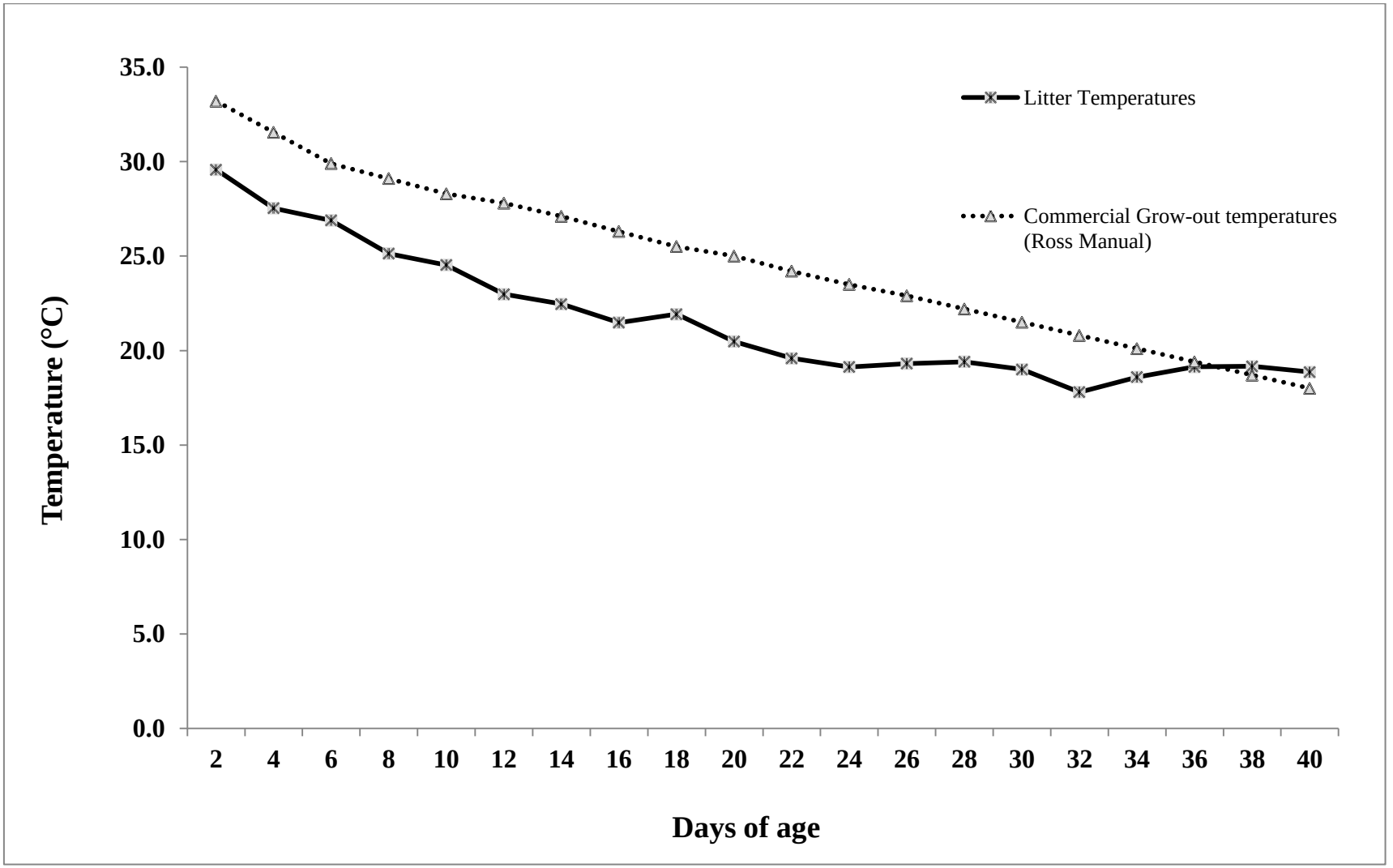

Figure 4.8: A comparison of cold temperatures employed during our study vs. temperatures employed during commercial grow-out of broiler chickens

4.4 Broiler performance and carcass characteristics

Data concerning chick growth, feed intake, feed conversion, and carcass composition are summarised in the following section.

4.4.1 Effect of dietary treatment, sex, and dietary treatment by sex interaction on growth and efficiency

The effects of dietary treatment, sex, and dietary treatment by sex interaction on body weight (BW), body weight gain (BWG), and cumulative body weight gain (CumBWG) of male and female broilers for all ages is shown in **Tables 4.13, 4.14,** and **415** and **Figure 4.9**, respectively.

Initial (day 0) BWs were similar for birds between treatments except for the PC-group which exhibited a significantly lower (P <0.05) BW. BW was significantly increased (P <0.05) following dietary betaine supplementation, especially at the beginning (d7 and d14 of age) and towards the end (d35 and d40 of age) of the growth phase, while BW tended to be higher (P <0.10) at 21d and 28d of age compared to birds fed either of the remaining treatment diets. Chicks fed dietary betaine also demonstrated significantly heavier (P <0.05) weekly BWG and CumBWGs up to 14d of age (hence during the starter period) in contrast to birds fed either the control diets or the DMG-supplemented diets. Overall, 14 day old chick weight was improved (P <0.05) by 7.33 g, 12.03 g and 12.72 g compared to chicks fed the NC-, DMG-supplemented, or PC treatment diets, respectively, during the first two weeks, which is considered the most critical period for bird growth and development. Furthermore, supplementation of betaine to the basal diet caused an appreciable (P <0.05) increase in CumBWG at 0-35d and 0-40d of age (the finisher period), and 21-40d of age compared to supplementation of DMG to the basal diet. Despite the lack of any further significant growth response to the treatment diet fed beyond d14 (P >0.05), it is important to note that the betaine-supplemented group consistantly yielded numerically higher weekly BWGs and CumBWGs compared to the PC-group and NC-group for almost all periods of this experiment. Bird growth was depressed for the DMG-supplemented group in comparison to the NC-group during the last two weeks (finisher period) of this study (P <0.05).

Table 4.13: Average body weight (g) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		Body Weight						
Age (days)		**0**	**7**	**14**	**21**	**28**	**35**	**40**
Dietary treatment group		----------(g)----------						
Negative Control (NC)		43.92^{A}	183.18^{AB}	463.79^{B}	1015.02^{B}	1664.99^{B}	2370.44^{A}	2940.82^{A}
Betaine		43.75^{A}	185.82^{A}	471.68^{A}	1028.58^{A}	1692.28^{A}	2399.67^{A}	2966.99^{A}
DMG		43.92^{A}	181.63^{B}	459.08^{B}	1013.86^{B}	1669.32^{AB}	2302.65^{B}	2849.68^{B}
Positive Control (PC)		43.21^{B}	182.31^{B}	460.73^{B}	1021.28^{AB}	1688.47^{AB}	2354.51^{AB}	2893.94^{AB}
Sex								
Male		43.97^{1}	188.11^{1}	482.87^{1}	1081.57^{1}	1780.05^{1}	2470.30^{1}	3080.99^{1}
Female		43.43^{2}	178.36^{2}	444.77^{2}	957.80^{2}	1577.48^{2}	2243.34^{2}	2744.72^{2}
Diet*Sex Interaction								
NC	Male	44.02^{ab}	188.73^{ab}	485.39^{b}	1078.72^{ab}	1762.92^{b}	2501.60^{ab}	3114.22^{ab}
NC	Female	43.82^{abc}	177.63^{c}	442.20^{de}	951.33^{de}	1567.06^{d}	2239.28^{cd}	2767.42^{d}
Betaine	Male	44.31^{a}	191.45^{a}	494.01^{a}	1092.15^{a}	1801.15^{a}	2539.55^{a}	3181.55^{a}
Betaine	Female	43.19^{cd}	180.18^{c}	449.34^{d}	965.01^{cd}	1583.40^{cd}	2259.80^{cd}	2752.43^{d}
DMG	Male	44.21^{ab}	186.66^{b}	479.16^{bc}	1084.29^{ab}	1786.01^{ab}	2417.51^{b}	2996.04^{c}
DMG	Female	43.63^{bcd}	176.60^{c}	439.01^{e}	943.43^{e}	1552.64^{d}	2187.79^{d}	2703.31^{d}
PC	Male	43.33^{cd}	185.59^{b}	472.93^{c}	1071.12^{b}	1770.13^{ab}	2422.53^{b}	3032.16^{bc}
PC	Female	43.09^{d}	179.04^{c}	448.53^{d}	971.43^{c}	1606.81^{c}	2286.49^{c}	2755.71^{d}
----------		----------Probability----------						
Diet		0.0129	0.0440	0.0001	0.0970	0.0976	0.0293	0.0138
Sex		0.0024	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Diet*Sex		0.2065	0.3970	0.0022	0.0172	0.0449	0.1315	0.1932
Pooled SEM		0.8236	1.1567	1.6467	4.8333	9.5200	24.2967	28.3433

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.14: Average body weight gain (BWG) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		Body Weight Gain					
Age (days)		**0-7 (week 1)**	**7-14 (week 2)**	**14-21 (week 3)**	**21-28 (week 4)**	**28-35 (week 5)**	**35-40 (week 6)**
Dietary treatment group		----------(g)----------					
Negative Control (NC)		139.38^{AB}	280.60^{B}	550.96^{B}	649.59	705.85^{A}	568.85
Betaine		142.31^{A}	285.99^{A}	558.08^{AB}	665.32	719.34^{A}	557.36
DMG		137.89^{B}	277.03^{B}	554.63^{AB}	657.79	646.34^{B}	546.49
Positive Control (PC)		138.84^{B}	278.38^{B}	561.52^{A}	668.38	649.13^{B}	559.69
Sex							
Male		144.46^{1}	294.63^{1}	599.12^{1}	700.26^{1}	703.01^{1}	604.50^{1}
Female		134.75^{2}	266.37^{2}	513.48^{2}	620.28^{2}	657.32^{2}	511.70^{2}
Diet*Sex Interaction							
NC	Male	144.91^{ab}	296.66^{b}	593.00^{a}	683.67^{a}	739.29^{ab}	610.40^{a}
NC	Female	133.85^{c}	264.55^{de}	508.93^{c}	615.50^{b}	672.42^{c}	527.30^{ab}
Betaine	Male	148.11^{a}	302.89^{a}	600.25^{a}	711.56^{a}	763.44^{a}	618.43^{a}
Betaine	Female	136.51^{c}	269.09^{d}	515.92^{bc}	619.08^{b}	675.25^{bc}	496.29^{b}
DMG	Male	142.93^{b}	291.68^{bc}	604.90^{a}	706.32^{a}	657.76^{c}	576.91^{ab}
DMG	Female	132.85^{c}	262.38^{e}	504.36^{c}	609.25^{b}	634.93^{c}	516.07^{b}
PC	Male	141.89^{b}	287.29^{c}	598.34^{a}	699.48^{a}	651.56^{c}	612.26^{a}
PC	Female	135.79^{c}	269.47^{d}	524.70^{b}	637.27^{b}	646.71^{c}	507.12^{b}
----------		----------Probability----------					
Diet		0.0427	<0.0001	0.1789	0.3103	0.0017	0.9291
Sex		<0.0001	<0.0001	<0.0001	<0.0001	0.0081	0.0005
Diet*Sex		0.3108	0.0004	0.0471	0.2578	0.2330	0.8221
Pooled SEM		1.1933	1.4467	3.5233	7.7767	16.8033	25.3633

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.15: Average cumulative body weight gain (CumBWG) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

	Cumulative Body Weight Gain						
Age (days)	**0-7**	**0-14**	**0-21**	**0-28**	**0-35**	**0-40**	**21-40**
Dietary treatment group							
Negative Control (NC)	139.38^{AB}	420.81^{B}	972.84^{AB}	1623.00^{B}	2329.46^{A}	2898.02^{AB}	1925.18^{A}
Betaine	142.31^{A}	428.14^{A}	985.21^{A}	1648.96^{A}	2356.58^{A}	2923.49^{A}	1938.27^{A}
DMG	137.89^{B}	416.11^{B}	971.68^{B}	1627.34^{AB}	2261.67^{B}	2806.88^{C}	1835.20^{B}
Positive Control (PC)	138.84^{B}	415.42^{B}	974.20^{AB}	1640.96^{AB}	2304.76^{AB}	2848.22^{BC}	1874.02^{AB}
Sex							
Male	144.46^{1}	440.05^{1}	1039.72^{1}	1738.44^{1}	2429.92^{1}	3038.40^{1}	1998.67^{1}
Female	134.75^{2}	400.19^{2}	912.24^{2}	1531.68^{2}	2196.31^{2}	2699.91^{2}	1787.67^{2}
Diet*Sex Interaction							
NC Male	144.91^{ab}	442.73^{de}	1037.22^{b}	1721.69^{b}	2461.83^{ab}	3071.82^{ab}	2034.61^{ab}
NC Female	133.85^{c}	398.90^{b}	908.47^{cd}	1524.31^{cd}	2197.10^{cd}	2724.23^{d}	1815.76^{de}
Betaine Male	148.11^{a}	452.30^{a}	1052.67^{a}	1762.21^{a}	2503.41^{a}	3140.36^{a}	2087.70^{a}
Betaine Female	136.51^{c}	403.96^{d}	917.76^{cd}	1535.70^{cd}	2209.74^{cd}	2706.61^{d}	1788.85^{e}
DMG Male	142.93^{b}	437.14^{b}	1044.13^{ab}	1746.30^{ab}	2380.17^{b}	2954.46^{c}	1910.33^{cd}
DMG Female	132.85^{c}	395.07^{e}	899.23^{d}	1508.37^{d}	2143.18^{d}	2659.30^{d}	1760.08^{e}
PC Male	141.89^{b}	428.02^{c}	1024.8^{bd}	1723.57^{b}	2374.29^{b}	2986.95^{bc}	1962.07^{bc}
PC Female	135.79^{c}	402.82^{de}	923.51^{c}	1558.35^{c}	2235.23^{c}	2709.50^{d}	1785.98^{e}
----------Probability----------							
Diet	0.0276	0.0001	0.1551	0.1569	0.0305	0.0127	0.0235
Sex	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Diet*Sex	0.2296	0.0010	0.0105	0.0255	0.0983	0.1630	0.2053
Pooled SEM	1.1933	2.1567	4.8467	9.3533	23.7400	27.5533	27.0676

$^{A-C}$ **Column means with different superscripts differ significantly at *P* <0.05**
^{1,2} **Column means with different superscripts differ significantly at *P* <0.05**
$^{a-e}$ **Column means with different superscripts differ significantly at *P* <0.05**

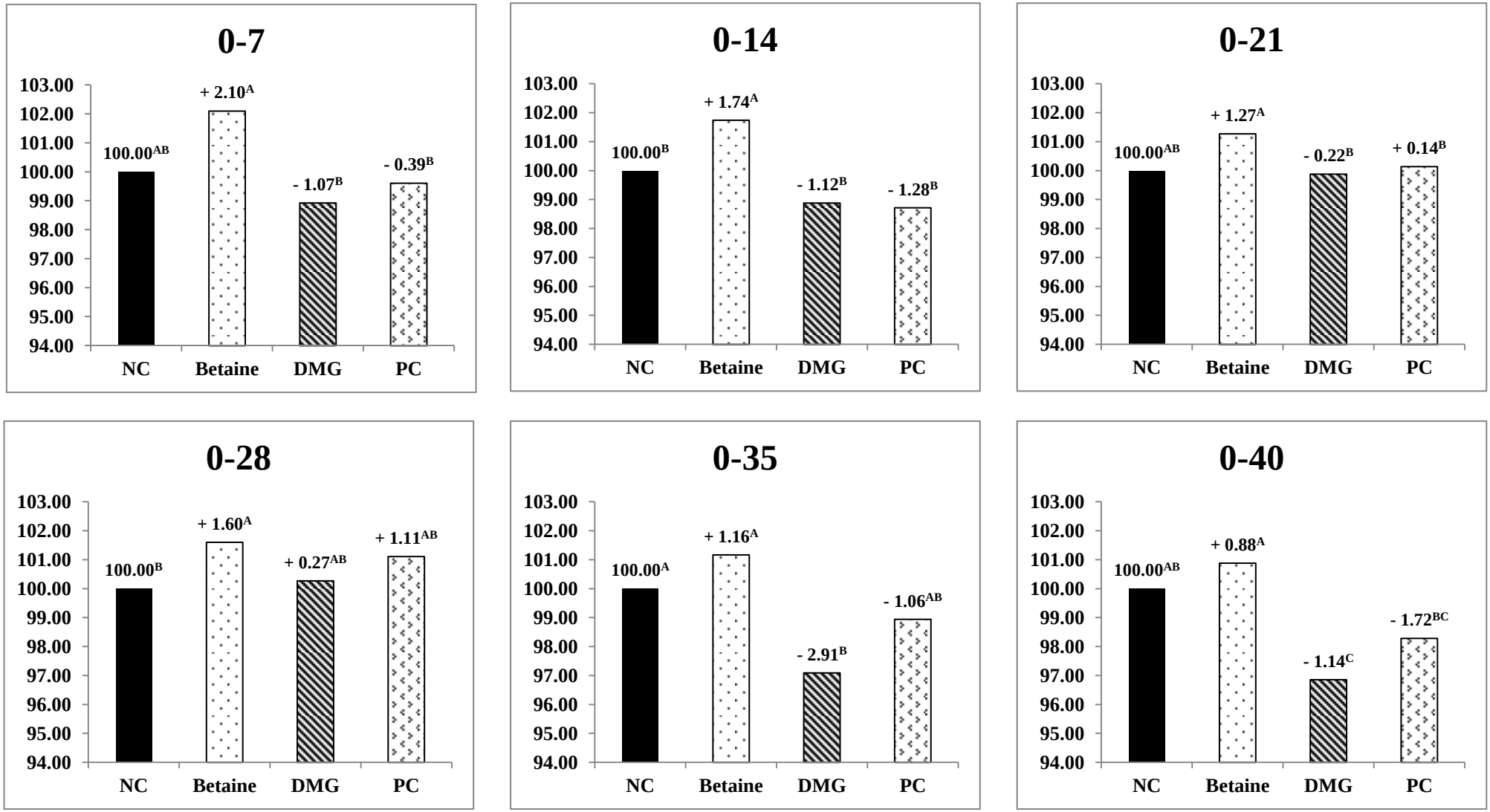

Figure 4.9: Effect of dietary di- and trimethylglycine (betaine) on cumulative body weight gain in broiler chickens exposed to ascites-inducing conditions (high EST and cold temperature) during rearing and grow-out. All values expressed relative to the performance of the broilers in the negative control (NC). $^{A-C}$ Different superscripts are significantly different at $P < 0.05$

Average live BW was increased (P <0.05) to a greater extent in male broiler chickens compared to their female counterparts, which became more conspicuous from day 21 and onwards. In addition, significant effects (P <0.05) were also obtained between males and females concerning BWG and CumBWG, with males exhibiting heavier (P <0.05) weight gains than females from week 1 through to week 6.

There were interactions between dietary treatment and sex for live BW at 14, 21, and 28 days of age (P <0.05) where male birds, irrespective of the treatment diet fed, displayed heavier weights than their female counterparts. Likewise, an interaction effect for weekly BWG and CumBWG was also found at week 2 (7-14d) and week 3 (14-21d), as well as 0-14d, 0-21d, and 0-28d, respectively. These results therefore clearly indicate an overall strong sex influence on growth performance from day 7 up to 28 days of age.

4.4.2 Effect of dietary treatment, sex, and dietary treatment by sex interaction on feed consumption

The effects of dietary treatment, sex, and dietary treatment by sex interaction on feed intake (FI) and cumulative feed intake (CumFI) of male and female broilers for all ages are shown in **Table 4.16** and **4.17**, respectively.

Dietary treatment had little to no significant effect (P >0.05) on weekly FI and CumFI for almost all ages shown.

Significant effects were observed between sexes with males having higher weekly FI and CumFI compared to females (P <0.05).

A significant diet by sex interaction was detected only for CumFI at 0-35d, 0-40d, and 21-40d with males consuming more feed than their female counterparts, irrespective of the treatment diet that was fed.

Table 4.16: Average feed intake (FI) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		Feed Intake					
Age (days)		**0-7 (week 1)**	**7-14 (week 2)**	**14-21 (week 3)**	**21-28 (week 4)**	**28-35 (week 5)**	**35-40 (week 6)**
Dietary treatment group		----------(g)----------					
Negative control (NC)		189.39	355.02	905.36^{B}	1062.65^{AB}	1366.76	1077.08
Betaine		186.57	355.30	939.00^{A}	1094.34^{A}	1391.09	1079.26
DMG		192.50	355.89	919.34^{AB}	1076.31^{AB}	1421.82	1138.20
Positive control (PC)		192.91	350.88	905.77^{B}	1055.72^{B}	1362.59	1107.94
Sex							
Male		194.61^{1}	365.09^{1}	977.65^{1}	1132.20^{1}	1415.49^{1}	1075.97^{2}
Female		186.07^{2}	343.46^{2}	857.09^{2}	1012.30^{2}	1355.64^{2}	1125.97^{1}
Diet*Sex Interaction							
NC	Male	192.59^{ab}	370.03^{a}	967.09^{b}	1111.85^{bc}	1383.29^{abc}	1057.55^{a}
NC	Female	186.18^{bc}	340.01^{c}	843.63^{c}	1013.45^{d}	1350.24^{bc}	1096.61^{a}
Betaine	Male	194.70^{ab}	368.90^{a}	1005.92^{a}	1169.82^{a}	1486.49^{a}	1094.80^{a}
Betaine	Female	178.44^{c}	341.70^{c}	872.08^{c}	1018.85^{d}	1295.69^{c}	1063.72^{a}
DMG	Male	196.37^{a}	363.15^{a}	976.59^{ab}	1152.62^{ab}	1431.59^{ab}	1070.62^{a}
DMG	Female	188.63^{ab}	348.63^{bc}	862.10^{c}	999.99^{d}	1412.06^{ab}	1205.77^{b}
PC	Male	194.77^{ab}	358.28^{ab}	961.00^{b}	1094.52^{c}	1360.61^{bc}	1080.92^{a}
PC	Female	191.04^{ab}	343.49^{c}	850.54^{c}	1016.93^{d}	1364.57^{bc}	1134.96^{ab}
----------				Probability			
Diet		0.2373	0.6577	0.0462	0.1335	0.40566	0.2315
Sex		0.0009	<0.0001	<0.0001	<0.0001	0.0322	0.0402
Diet*Sex		0.3149	0.1772	0.8247	0.0764	0.0601	0.1156
Pooled SEM		2.5567	3.2533	9.8133	12.7567	28.5167	24.5533

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-d}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.17: Average cumulative feed intake (CumFI) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		Cumulative Feed Intake						
Age (days)		**0-7**	**0-14**	**0-21**	**0-28**	**0-35**	**0-40**	**21-40**
Dietary treatment group		---------- (g)----------						
Negative control (NC)		189.39	544.41	1449.77^{B}	2512.42^{B}	3879.18	4956.27^{B}	3506.50
Betaine		186.57	541.87	1480.87^{A}	2575.20^{A}	3966.29	5045.55^{AB}	3564.68
DMG		192.50	548.39	1467.73^{AB}	2544.04^{AB}	3965.86	5104.06^{A}	3636.33
Positive control (PC)		192.91	543.79	1449.56^{B}	2505.29^{B}	3867.87	4975.81^{AB}	3526.4
Sex								
Male		194.61^{1}	559.70^{1}	1537.35^{1}	2669.55^{1}	4085.05^{1}	5161.01^{1}	3623.67^{1}
Female		186.07^{2}	529.53^{2}	1386.62^{2}	2398.55^{2}	3754.05^{2}	4879.82^{2}	3493.21^{2}
Diet*Sex Interaction								
NC	Male	192.59^{ab}	562.62^{c}	1529.71^{ab}	2641.57^{bd}	4024.85^{bc}	5082.41^{bc}	3552.69^{bcd}
NC	Female	186.18^{bc}	526.19^{a}	1369.82^{c}	2383.28^{a}	3733.52^{d}	4830.13^{de}	3460.30^{cd}
Betaine	Male	194.70^{ab}	563.60^{a}	1569.52^{a}	2739.34^{c}	4225.83^{a}	5320.63^{a}	3751.11^{a}
Betaine	Female	178.44^{c}	520.14^{c}	1392.22^{c}	2411.07^{a}	3706.75^{d}	4770.47^{e}	3378.25^{d}
DMG	Male	196.37^{a}	559.52^{a}	1536.10^{ab}	2688.72^{bc}	4120.32^{ab}	5190.94^{ab}	3654.84^{ab}
DMG	Female	188.63^{ab}	537.26^{bc}	1399.36^{c}	2399.35^{a}	3811.41^{d}	5017.18^{bcd}	3617.82^{abc}
PC	Male	194.77^{ab}	553.05^{ab}	1514.06^{b}	2608.58^{d}	3969.19^{c}	5050.10^{bc}	3536.05^{bcd}
PC	Female	191.04^{ab}	534.53^{c}	1385.07^{c}	2401.99^{a}	3766.56^{d}	4901.52^{cde}	3516.45^{bcd}
----------				Probability				
Diet		0.2373	0.7505	0.1080	0.0199	0.0970	0.1256	0.2156
Sex		0.0009	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0062
Diet*Sex		0.3149	0.1371	0.3548	0.0896	0.0225	0.0168	0.0313
Pooled SEM		2.5567	4.4277	6.5733	14.1633	37.7567	50.0833	41.4257

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05

4.4.3 Effect of dietary treatment, sex, and dietary treatment by sex interaction on feed efficiency

The effects of dietary treatment, sex, and dietary treatment by sex interaction on FCR of male and female broilers for all ages are shown in **Tables 4.18** and **4.19** and **Figure 4.10**, respectively. All FCR values are adjusted for mortality.

Weekly FCR was significantly improved (P <0.05) following dietary betaine supplementation at 0-7d of age compared to birds fed either of the remaining treatment diets. In addition, birds fed betaine had a considerably better FCR at 7-14d of age, 28-35d, and 35-40d of age compared to birds fed dietary DMG (P <0.05). This corresponded to the significantly improved cumulative FCR (CumFCR) (P <0.05) observed for the betaine-supplemented group during the starter (0-7d and 0-14d of age) period compared to birds fed either of the remaining treatment diets. Birds fed dietary DMG demonstrated the poorest CumFCR at 0-35d and 0-40d of age (P <0.05) (the finisher period) and 21-40d of age (P <0.05) compared to birds fed either of the remaining treatment diets. These results therefore clearly indicate an overall improvement in feed efficiency following dietary betaine supplementation, but only during the starter and finisher periods of this study. These results also correspond to the improvement in growth performance observed during these periods, in contrast to birds fed dietary DMG. No further significant effects were detected for dietary treatment and weekly FCR or CumFCR during the remainder of this study.

Although inconsistent effects have been observed for weekly AdjFCR for sex, male broilers had a significant better (P <0.05) CumFCR compared to females throughout the entire study.

The significant interactions for CumFCR over the 0-35 d and 0-40 d periods observed are mainly due to better feed conversion efficiency observed in male broilers in contrast to their female counterparts, regardless of the dietary treatment fed, while no diet by sex interaction was observed for weekly FCR.

Table 4.18: Feed Conversion Ratio (FCR) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		Feed Conversion Ratio					
Age (days)		**0-7 (week 1)**	**7-14 (week 2)**	**14-21 (week 3)**	**21-28 (week 4)**	**28-35 (week 5)**	**35-40 (week 6)**
Diet		----------(g: g)----------					
Negative control (NC)		1.35^{B}	1.26^{AB}	1.48^{AB}	1.62^{A}	2.02^{AB}	2.16^{B}
Betaine		1.30^{C}	1.24^{B}	1.50^{A}	1.63^{A}	1.93^{B}	2.20^{B}
DMG		1.38^{AB}	1.28^{A}	1.49^{AB}	1.59^{B}	2.24^{A}	2.37^{A}
Positive control (PC)		1.41^{A}	1.26^{AB}	1.45^{B}	1.56^{B}	2.12^{A}	2.24^{AB}
Sex							
Male		1.39^{1}	1.23^{2}	1.47	1.56^{2}	2.08	2.17^{2}
Female		1.34^{2}	1.29^{1}	1.50	1.63^{1}	2.07	2.32^{1}
Diet*Sex Interaction							
NC	Male	1.32^{d}	1.23^{d}	1.48^{ab}	1.58^{de}	2.02^{bc}	2.13^{c}
NC	Female	1.38^{bc}	1.29^{ab}	1.48^{ab}	1.67^{a}	2.02^{bc}	2.19^{bc}
Betaine	Male	1.29^{d}	1.21^{d}	1.50^{ab}	1.61^{bcd}	1.96^{bc}	2.16^{bc}
Betaine	Female	1.32^{d}	1.27^{bc}	1.51^{ab}	1.64^{ab}	1.91^{c}	2.24^{bc}
DMG	Male	1.35^{cd}	1.23^{d}	1.44^{b}	1.54^{ef}	2.24^{a}	2.26^{bc}
DMG	Female	1.42^{ab}	1.32^{a}	1.54^{a}	1.63^{abc}	2.24^{a}	2.48^{a}
PC	Male	1.39^{abc}	1.24^{cd}	1.45^{b}	1.53^{f}	2.11^{ab}	2.11^{c}
PC	Female	1.42^{ab}	1.28^{bc}	1.46^{b}	1.59^{cde}	2.12^{ab}	2.36^{ab}
----------		----------Probability----------					
Diet		0.0002	0.1018	0.2358	0.0002	<0.0001	0.0245
Sex		0.0029	<0.0001	0.0790	<0.0001	0.8554	0.0028
Diet*Sex		0.7390	0.2658	0.1630	0.3534	0.9659	0.4272
Pooled SEM		0.0171	0.0104	0.0183	0.0127	0.0444	0.0514

$^{A-C}$ **Column means with different superscripts differ significantly at *P* <0.05**
^{1,2} **Column means with different superscripts differ significantly at *P* <0.05**
$^{a-f}$ **Column means with different superscripts differ significantly at *P* <0.05**

Table 4.19: Cumulative Feed Conversion Ratio (CumFCR) of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		Cumulative Feed Conversion Ratio						
Age (days)		**0-7**	**0-14**	**0-21**	**0-28**	**0-35**	**0-40**	**21-40**
Dietary treatment group		(g:g)						
Negative control (NC)		1.35^{B}	1.29^{A}	1.40	1.48^{A}	1.60^{B}	1.79^{B}	1.88^{BC}
Betaine		1.30^{C}	1.26^{B}	1.39	1.48^{A}	1.59^{B}	1.78^{B}	1.86^{C}
DMG		1.38^{AB}	1.31^{A}	1.41	1.47^{A}	1.65^{A}	1.86^{A}	1.98^{A}
Positive control (PC)		1.41^{A}	1.31^{A}	1.39	1.45^{B}	1.60^{B}	1.79^{B}	1.91^{B}
Sex								
Male		1.34^{2}	1.26^{2}	1.38^{2}	1.44^{2}	1.57^{2}	1.74^{2}	1.84^{2}
Female		1.39^{1}	1.32^{1}	1.42^{1}	1.50^{1}	1.65^{1}	1.87^{1}	1.97^{1}
Diet*Sex Interaction								
NC	Male	1.32^{d}	1.26^{de}	1.38^{bc}	1.45^{cd}	1.56^{e}	1.74^{d}	1.82^{e}
NC	Female	1.38^{bc}	1.32^{bc}	1.41^{b}	1.50^{a}	1.65^{b}	1.84^{bc}	1.93^{bc}
Betaine	Male	1.29^{d}	1.23^{e}	1.38^{bc}	1.46^{cd}	1.57^{e}	1.75^{d}	1.82^{e}
Betaine	Female	1.32^{d}	1.29^{cd}	1.41^{b}	1.50^{ab}	1.61^{cd}	1.81^{c}	1.90^{cd}
DMG	Male	1.35^{cd}	1.27^{de}	1.37^{c}	1.43^{d}	1.58^{de}	1.77^{d}	1.88^{cde}
DMG	Female	1.42^{ab}	1.36^{a}	1.46^{a}	1.52^{a}	1.71^{a}	1.96^{a}	2.08^{a}
PC	Male	1.39^{abc}	1.29^{cd}	1.38^{bc}	1.43^{d}	1.56^{e}	1.73^{d}	1.84^{de}
PC	Female	1.42^{ab}	1.33^{ab}	1.40^{bc}	1.48^{bc}	1.64^{bc}	1.86^{b}	1.98^{b}
		Probability						
Diet		0.0002	0.0003	0.4664	0.0459	0.0005	<0.0001	<0.0001
Sex		0.0029	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Diet*Sex		0.7390	0.3077	0.0526	0.0645	0.0074	0.0003	0.0950
Pooled SEM		0.0171	0.0189	0.0103	0.0083	0.0097	0.0099	0.0207

[A-C] Column means with different superscripts differ significantly at *P* <0.05
[1,2] Column means with different superscripts differ significantly at *P* <0.05
[a-e] Column means with different superscripts differ significantly at *P* <0.05

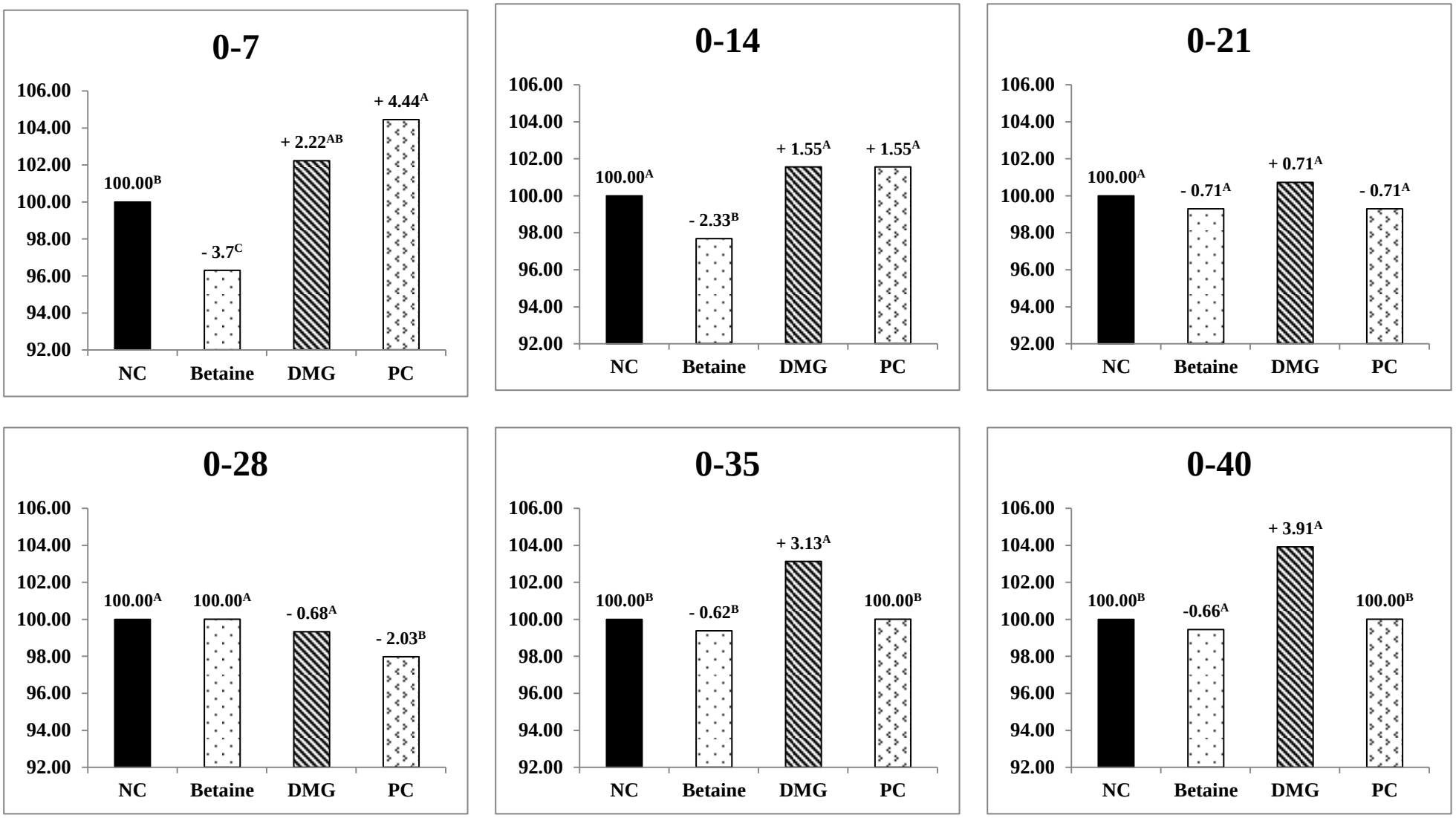

Figure 4.10: Effect of dietary di- and trimethylglycine (betaine) on cumulative feed to gain in broiler chickens exposed to ascites-inducing conditions (high EST and cold temperature) during rearing and grow-out. All values expressed relative to the performance of the broilers in the negative control (NC). [A-C] Different superscripts are significantly different at $P < 0.05$

4.4.4 Effect of dietary treatment, sex, and dietary treatment by sex interaction on carcass characteristics

The effect of dietary treatment, sex, and dietary treatment by sex interaction on carcass characteristics at slaughter age (Processing stage 2; d 40 and 41) are summarised in **Table 4.20** and **Figure 4.11**.

At the end of the experiment, there was a marked and highly significant response for the carcass traits following dietary treatment. The results presented in **Table 4.20** indicated that the betaine supplemented birds had a significanty higher (P <0.05) pre-slaughter live BW, carcass weight, dressing percentage, breast meat weight and yield, and wing weight compared to birds fed dietary DMG. Furthermore, leg portion weight also tended to be higher for the betaine-supplemented group compared to the DMG-supplemented group, although not significantly (P >0.05). Non-significant effects (P >0.05) were observed for all treatment diets concerning any of the abdominal fat parameters. Dietary betaine also showed an improvement (P <0.05) in dressing percentage, breast meat weight and yield, and wing yield compared to the NC, but yielded similar results for all carcass parameters when compared to birds fed the PC treatment diet. In contrast, similar results (P >0.05) were obtained for dressing percentage and breast meat yield; however lower breast meat, leg portion and wing weights were observed for the DMG-supplemented group in comparison to birds fed the NC treatment diet. Average live pre-slaughter body weight, carcass weight, breast meat weight, leg portion yield, and wing weight were similar (P >0.05) between the two control diets. However, the PC-group exhibited a significantly higher (P <0.05) dressing percentage, breast meat yield and wing yield than did the NC-group.

The effects of sex on carcass parameters were highly significant (P <0.01), with males exhibiting higher weights in all variables except breast meat and wing yield when compared to their female counterparts. However, the effects of sex on dressing percentage were relatively small and non-significant (P >0.05).

An interaction effect between diet and sex was only shown for dressing percentage, wing weight, and wing yield (P <0.05). The interaction observed for dressing percentage is mainly due to male birds fed the DMG-supplemented diets exhibiting the lowest dressing percentage compared to male birds fed any of the other treatment diets. Females fed the NC-diet exhibited the lowest dressing percentage of all female birds.

Table 4.20: Carcass characteristics of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

	Live body weight	Carcass weight	Dressing	Abdominal Fat	
				Weight	Yield
Dietary treatment group	**(g)**	**(g)**	**(%)**	**(g)**	**(%)**
Negative control (NC)	3269.22^{A}	2352.67^{A}	72.01^{B}	40.64	1.25
Betaine	3252.74^{A}	2383.98^{A}	73.32^{A}	42.48	1.30
DMG	3110.16^{B}	2230.21^{B}	71.70^{B}	39.90	1.28
Positive control (PC)	3236.10^{A}	2349.71^{A}	73.20^{A}	44.09	1.36
Sex					
Male	3417.22^{1}	2478.48^{1}	72.48	39.61^{2}	1.15^{2}
Female	3016.89^{2}	2188.80^{2}	72.63	43.95^{1}	1.45^{1}
Diet*Sex Interaction					
NC Male	3439.28^{a}	2500.40^{a}	72.66^{ab}	36.57^{b}	1.06^{e}
NC Female	3099.15^{c}	2204.93^{cd}	71.37^{bc}	44.72^{a}	1.44^{ab}
Betaine Male	3502.15^{a}	2565.94^{a}	73.26^{a}	44.41^{a}	1.26^{bcd}
Betaine Female	3003.32^{cd}	2202.01^{cd}	73.38^{a}	40.55^{ab}	1.35^{abc}
DMG Male	3298.28^{b}	2342.66^{b}	70.94^{c}	36.91^{b}	1.10^{de}
DMG Female	2922.04^{d}	2117.77^{d}	72.45^{ab}	42.89^{ab}	1.47^{ab}
PC Male	3429.15^{a}	2504.93^{a}	73.06^{a}	40.55^{ab}	1.17^{cde}
PC Female	3043.05^{c}	2230.49^{c}	73.33^{a}	47.62^{a}	1.56^{a}
----------Probability----------					
Diet	0.0006	<0.0001	0.0002	0.3335	0.4738
Sex	<0.0001	<0.0001	0.6453	0.0197	<0.0001
Diet*Sex	0.2627	0.2032	0.0281	0.0759	0.1316
Pooled SEM	48.2045	36.0454	0.5149	2.8999	0.0846

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.20(Continue): Carcass characteristics of broiler chickens as affected by dietary treatment, sex and dietary treatment by sex interaction

		Breast Meat		Leg Portion[x]		Wing	
Dietary treatment group		**Weight (g)**	**Yield (%)**	**Weight (g)**	**Yield (%)**	**Weight (g)**	**Yield (%)**
Negative control (NC)		666.30^{B}	20.46^{C}	666.011^{A}	20.40	228.53^{AB}	6.97^{B}
Betaine		695.42^{A}	21.46^{A}	659.71^{AB}	20.30	233.21^{A}	7.16^{A}
DMG		639.51^{C}	20.60^{BC}	636.66^{B}	20.43	222.55^{B}	7.16^{A}
Positive control (PC)		684.43^{AB}	21.16^{AB}	670.32^{A}	20.68	235.09^{A}	7.27^{A}
Sex							
Male		701.65^{1}	20.52^{2}	708.18^{1}	20.71^{1}	241.77^{1}	7.05^{2}
Female		641.17^{2}	21.32^{1}	608.17^{2}	20.19^{2}	217.92^{2}	7.23^{1}
Diet*Sex Interaction							
NC	Male	685.00^{c}	19.91^{c}	717.70^{a}	20.89^{a}	243.24^{a}	7.04^{cd}
NC	Female	647.60^{b}	21.00^{b}	614.33^{c}	19.90^{b}	213.82^{d}	6.91^{d}
Betaine	Male	723.65^{a}	20.65^{bc}	718.00^{a}	20.52^{ab}	250.94^{a}	7.14^{bcd}
Betaine	Female	667.19^{bc}	22.27^{a}	601.41^{c}	20.52^{b}	215.49^{d}	7.18^{bc}
DMG	Male	674.12^{bc}	20.42^{bc}	677.26^{b}	20.48^{ab}	229.10^{b}	6.94^{cd}
DMG	Female	604.91^{e}	20.78^{bc}	596.05^{c}	20.40^{ab}	216.01^{cd}	7.38^{ab}
PC	Male	723.85^{a}	21.10^{b}	719.74^{a}	20.95^{a}	243.82^{a}	7.10^{cd}
PC	Female	645.01^{c}	21.22^{b}	620.90^{c}	20.41^{ab}	226.35^{bc}	7.44^{a}
----------Probability----------							
Diet		<0.0001	0.0031	0.0212	0.4603	0.0040	0.0127
Sex		<0.0001	0.0005	<0.0001	0.0055	<0.0001	0.0096
Diet*Sex		0.4635	0.1172	0.5421	0.3565	0.0235	0.0159
Pooled SEM		14.1941	0.3549	13.5337	0.2884	4.2920	0.1043

[A-C] Column means with different superscripts differ significantly at *P* <0.05
[1,2] Column means with different superscripts differ significantly at *P* <0.05
[a-e] Column means with different superscripts differ significantly at *P* <0.05
[x] Leg Portion = thigh + drumstick

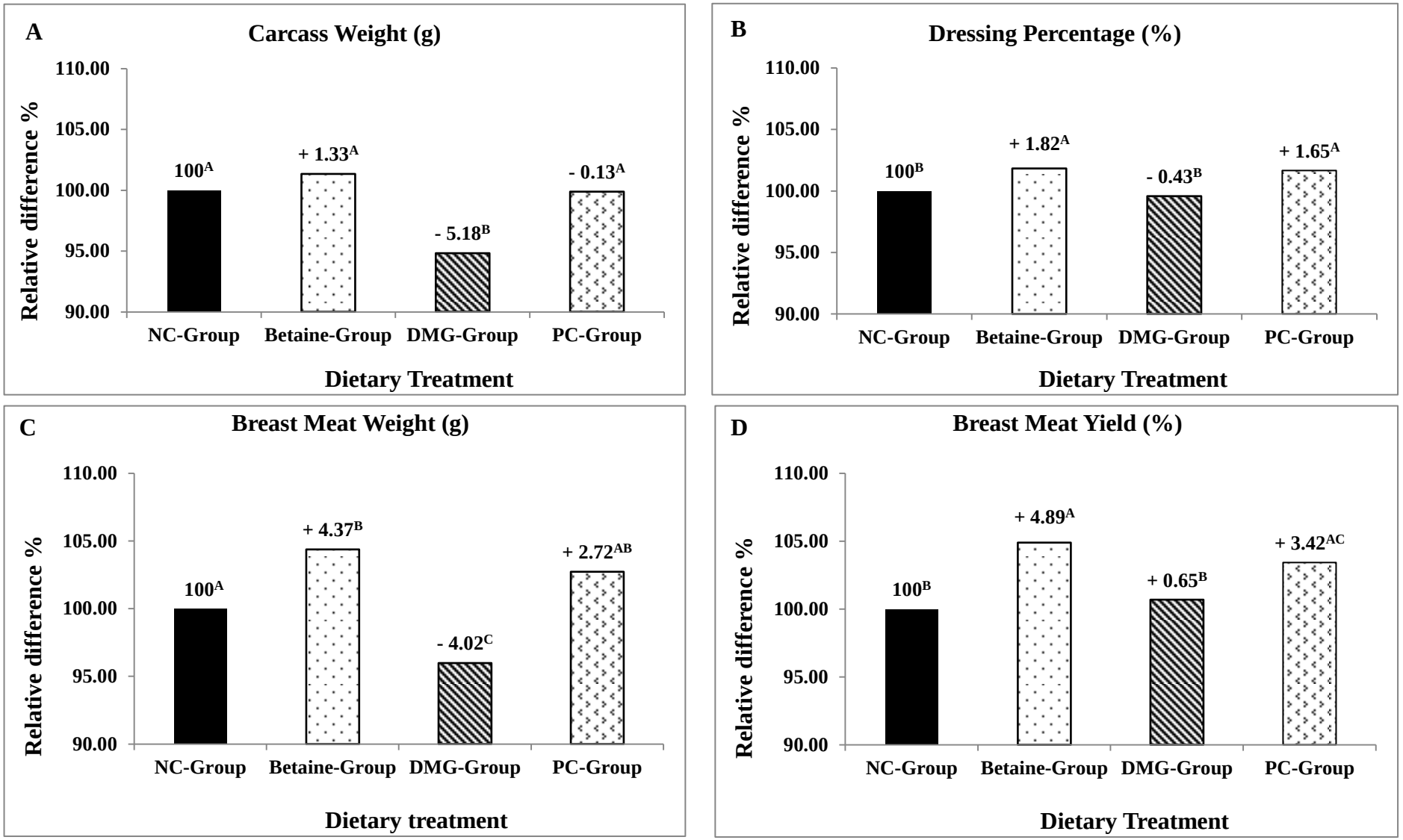

Figure 4.11:: Effect of dietary di- and trimethylglycine (betaine) on A) Carcass weight (CW), B) Dressing Percentage (DP), C) Breast Meat Weight (BMW) and D) Breast Meat Yields (BMY) exposed to ascites-inducing conditions (high EST and cold temperature) during rearing and grow-out. All values expressed relative to the performance of the broilers in the negative control (NC). $^{A\text{-}C}$ Different superscripts are significantly different at P <0.05

Following DMG supplementation, males obtained the lowest values for wing weight compared to males fed any of the other treatment diets. Females from the PC group demonstrated the highest values for both wing weights and yield compared to females fed any of the remaining treatment diets.

4.5 Ascites performance

4.5.1 Ascites and ascites characteristics

Post mortem examination of broilers with PHS revealed gross dilation of the right ventricle (an indication of right heart damage) (**Figure 4.12)**, excessive fluid in the pericardial sac **(Figure 4.13 B)** and/or abdominal cavity (**Figure 4.13 A**) as well as an extended abdomen; ocassionally, subcutaneous oedema was also observed. In severe cases, a swollen liver as well as cysts were present **(Figure 4.13 C)**. From our personal observations, most ascitic birds were generally smaller compared to their pen mates, although this was not always the case. Some birds also displayed signs of cyanosis, which is an indication of oxygen deprivation (i.e. hypoxia) and was characterised by their bluish comb. Difficulty in breathing was also observed in some birds that might be mainly attributed to physical restriction of the large abdominal air sacs.

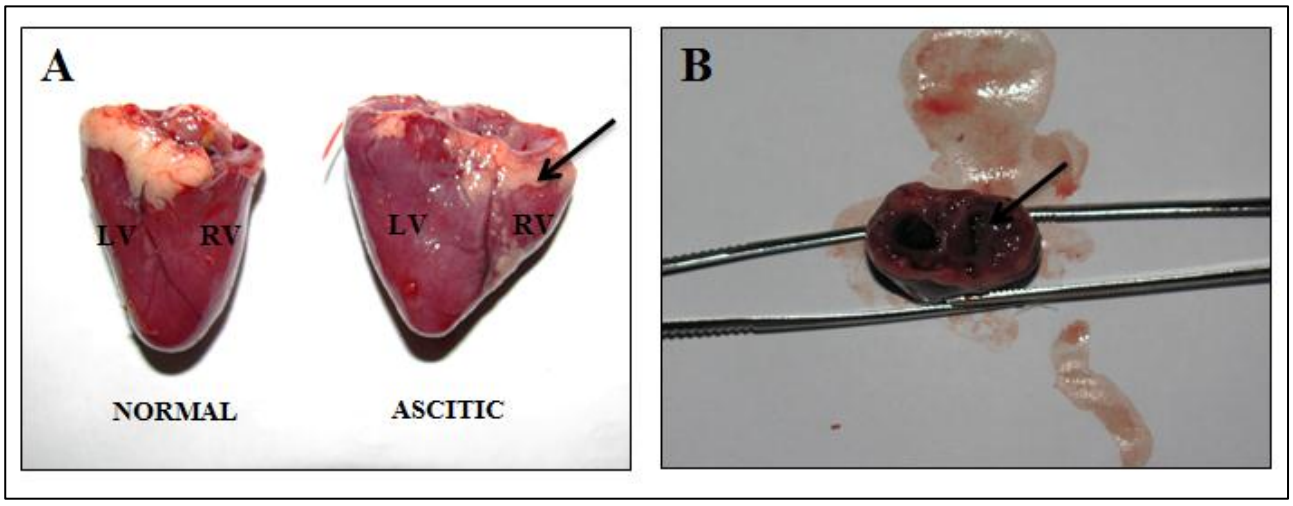

Figure 4.12: (A) A comparison between a normal and an ascitic heart. (B) An illustration of the ascitic heart, as taken from above. A clear dilation and hypertrophy of the right ventricle of the ascitic heart, as indicated by the arrows (A & B), can be seen

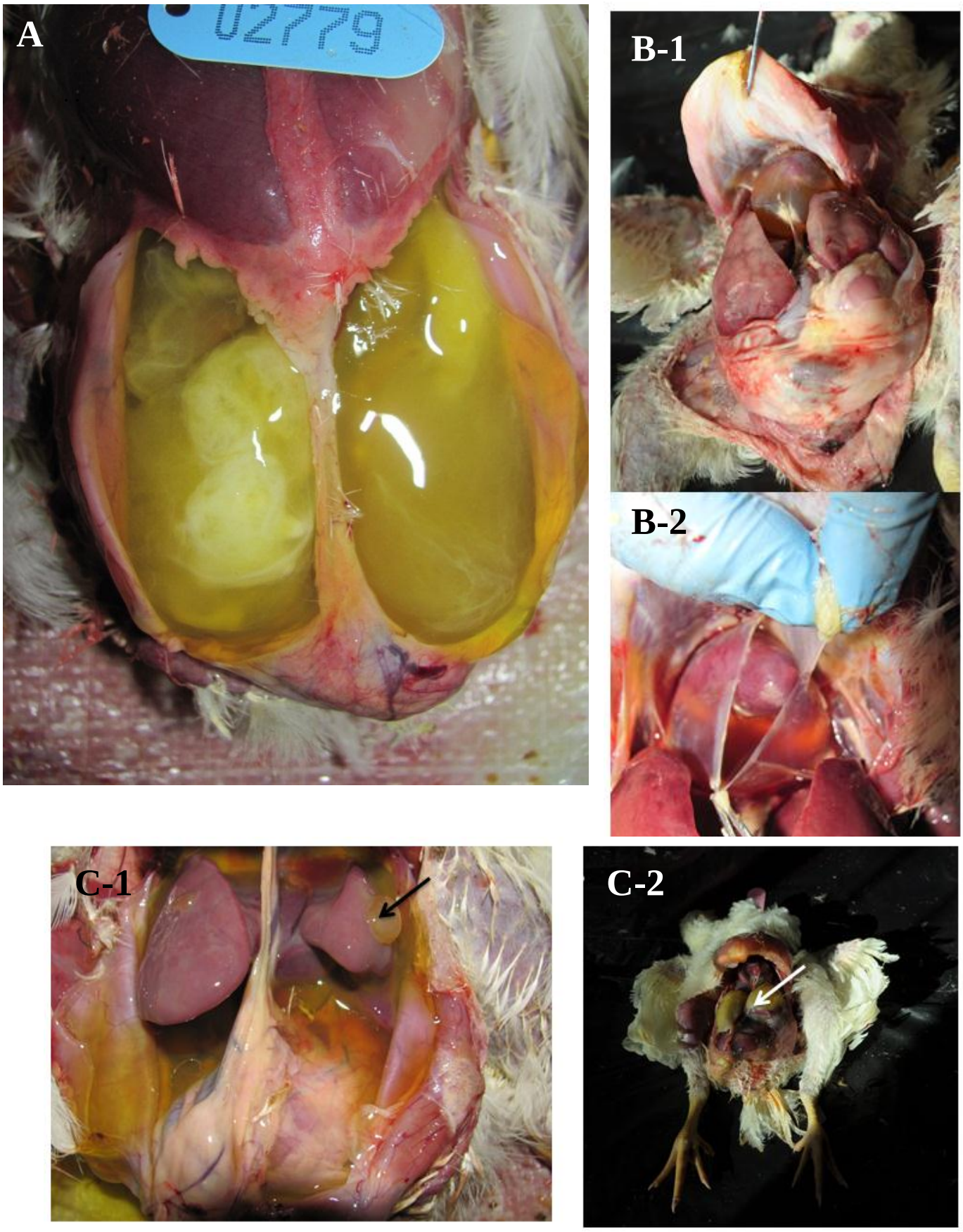

Figure 4.13: Some common characteristics exhibited by ascites individuals such as **(A)** water belly, **(B1&2)** pericardial fluid, as well as **(C1&2)** cysts on the liver in extreme cases

The effects of diet, sex, and diet by sex interaction on ascites characterstics during processing stage 1 (d20 & d21) and processing stage 2 (d40 & d41) are shown in **Tables 4.21** and **4.22**.

Post mortem examination was performed on apparently healthy individuals at both days of slaughter. Among apparently normal birds selected at each processing day, the number of individuals from the NC-group, betaine-supplemented, and DMG-supplemented groups showed progressive increases of 2.08, 2.10, and 6.25%, respectively, in the presence of fluid in the abdominal cavity. In contrast, fewer birds from the PC-group showed signs of waterbelly towards the end of the study.

Over the course of this study, the proportion of birds with pericardial effusion (water around the heart) decreased numerically from 10.42% to 8.33% for the betaine-group whereas the proportion of birds with pericardial effusion increased numerically by 8.33% and 4.16% for the DMG-supplemented and PC-group, respectively. However, birds fed the NC diet revealed similar pericardial fluid percentages at both 20 & 21d and 40 & 41d of age.

No obvious signs of liver damage were observed for the betaine-supplemented or PC groups during any of the processing stages. However, 1/24 birds (4.17%) in the NC-group had severe liver damage at day 20 & 21, whereas 2/24 birds (6.25%) in the DMG-group suffered from severe liver damage at day 40 & 41.

A 4.17%, 5.21% and 2.09% increase in abdominal fluid, pericardial fluid, and liver damage (important indicators of ascites) in male broilers was observed from d20 & 21 towards the end of the growing period (d40 & 41), respectively, in contrast to their female counterparts.

In both processing stages, almost twice as many male birds fed DMG had fluid present in the abdominal cavity compared to males fed either the betaine-supplemented or NC diet, whereas the DMG supplemented male group had a 8.3% higher incidence of waterbelly compared to the PC group. Pericardial fluid percentage was numerically higher for the male group fed the NC diet throughout this study in contrast to males fed either of the remaining three treatment diets. A progressively higher percentage (12 times more) of male individuals of the DMG- and PC-group had fluid present in the pericardium compared to male betaine-supplemented group. Males fed the NC or DMG diet had a 4.2 or 12.5% higher incidence of liver damage during processing stages 1 and 2, respectively, compared to males fed the remaining treatment diets. Similar results were obtained between females fed the DMG and betaine supplemented diets, while no individuals showed signs of waterbelly in the NC group during both processing stages. Fewer females had fluid present in their abdominal cavity for the PC treatment group at both d20 & 21 and d40 & 41.

Table 4.21: Percentage of broilers that showed signs of ascites (abdominal fluid, pericardial fluid, liver damage) at Processing Stage 1 (d20 & 21) that had been incubated at high (>38.8°C) egg shell temperature and grown under cold brooding and grow-out temperatures

		n	Abdominal Fluid (%)	Pericardial Fluid (%)	Liver Damage (%)
Dietary treatment group					
Negative control (NC)		48	0	22.92	4.17
Betaine		48	2.08	10.42	0
DMG		48	2.08	2.08	0
Positive control (PC)		48	4.17	4.17	0
Sex					
Male		96	2.08	17.71	1.04
Female		96	2.08	2.08	1.04
Diet*Sex Interaction					
NC	Male	24	0	45.8	4.2
NC	Female	24	0	0	4.2
Betaine	Male	24	0	16.7	0
Betaine	Female	24	4.2	4.2	0
DMG	Male	24	4.2	4.2	0
DMG	Female	24	4.2	0	0
PC	Male	24	4.2	4.2	0
PC	Female	24	4.2	4.2	0

Table 4.222: Percentage of broilers that showed signs of ascites (abdominal fluid, pericardial fluid, liver damage) at Processing Stage 2 (d40&41) that had been incubated at high (>38.8°C) egg shell temperature and grown under cold brooding and grow-out temperatures

		n	Abdominal Fluid (%)	Pericardial Fluid (%)	Liver Damage (%)
Dietary treatment group					
Negative control (NC)		48	2.08	22.92	0
Betaine		48	4.17	8.33	0
DMG		48	8.33	10.42	6.25
Positive control (PC)		48	2.08	8.33	0
Sex					
Male		96	6.25	22.92	3.13
Female		96	2.08	2.08	0
Diet*Sex Interaction					
NC	Male	24	4.2	41.7	0
NC	Female	24	0	4.2	0
Betaine	Male	24	4.2	16.7	0
Betaine	Female	24	4.2	0	0
DMG	Male	24	12.5	16.7	12.5
DMG	Female	24	4.2	4.2	0
PC	Male	24	4.2	16.7	0
PC	Female	24	0	0	0

The number of females suffering from pericardial effusion numerically decreased for both the betaine-supplemented and PC groups from d20 & 21 to d40 & 41, whereas the opposite was observed for the DMG-supplemented and NC groups. No liver damage was detected among any of the females, irrespective of the dietary treatment fed, except for the NC group at 20 and 21 days of age.

4.5.2 Mortality rate

4.5.2.1 Total and ascites-related mortality rates

The effects of diet, sex, and diet by sex interaction on total weekly and cumulative mortalities during the course of the study are shown in **Table 4.23** and **Table 4.24**, respectively. The total cumulative mortality rate and cumulative ascites mortality rate during the experiment over the course of this study given per dietary treatment group is summarised in **Figure 4.14.**

Based on mortality data, no significant differences were obtained for total weekly mortality percentage between individuals fed different dietary treatments. Nevertheless, birds fed either the betaine-supplemented or PC treatment diets revealed a 23.14% and 18.30 % lower total cumulative mortality rate than birds fed the DMG-supplemented diet (P <0.05). However, no further significant differences were obtained concerning total weekly or total cumulative mortality percentage for the different treatment diets fed.

Males exhibited a significantly higher (P <0.001) total weekly mortality percentage during week 4, 5 and 6 in this study than their female counterparts. Likewise, our study revealed a significantly higher total cumulative mortality rate in male broiler chickens during the 0-21d, 0-28d, 0-35d, and 0-40d periods in contrast to female broiler chickens **(Figure 4.16).**

No significant interaction effects between dietary treatment and sex were observed for both total weekly and total cumulative mortality rate were observed during the course of this study.

Table 4.23: Percentage total weekly mortality as influenced by dietary treatment, sex, and dietary treatment by sex interaction in male and female broilers subjected to high incubation (>38.8ºC) and cold brooding temperatures

		Total weekly mortality percentage					
Age (days)		**0-7 (week 1)**	**7-14 (week 2)**	**14-21 (week 3)**	**21-28 (week 4)**	**28-35 (week 5)**	**35-40 (week 6)**
Dietary treatment group							
Negative control (NC)		0.98	1.96^{AB}	0.74	5.91	10.63	7.15^{AB}
Betaine		0.98	1.23^{AB}	1.48	4.43	10.85	4.92^{B}
DMG		1.72	2.70^{A}	0.74	5.91	11.41	8.37^{A}
Positive control (PC)		0.74	0.49^{B}	1.48	5.41	10.10	7.14^{AB}
Sex							
Male		1.47	1.96	1.35	8.86^{1}	17.80^{1}	10.96^{1}
Female		0.74	1.23	0.86	1.97^{2}	3.69^{2}	2.83^{2}
Diet*Sex Interaction							
NC	Male	0.49^{b}	2.94	0.49	9.35^{a}	18.31^{a}	11.83^{a}
NC	Female	1.47^{ab}	0.98	0.98	2.46^{b}	2.95^{b}	2.46^{c}
Betaine	Male	1.48^{ab}	1.96	1.48	8.34^{a}	17.27^{a}	8.36^{ab}
Betaine	Female	0.49^{b}	0.49	1.48	0.49^{b}	4.43^{b}	1.48^{c}
DMG	Male	2.96^{a}	2.45	1.48	10.34^{a}	19.37^{a}	11.82^{a}
DMG	Female	0.49^{b}	2.94	0.00	1.48^{b}	3.45^{b}	4.92^{bc}
PC	Male	0.99^{ab}	0.49	1.97	7.38^{a}	16.26^{a}	11.83^{a}
PC	Female	0.49^{b}	0.49	0.98	3.45^{b}	3.94^{b}	2.46^{c}
----------Probability----------							
Diet		0.6642	0.1038	0.5531	0.6337	0.9382	0.2082
Sex		0.2110	0.2632	0.3357	<0.0001	<0.0001	<0.0001
Diet*Sex		0.2292	0.4972	0.5088	0.2671	0.7736	0.7662
Pooled SEM		0.0288	0.0321	0.0249	0.0451	0.072	0.0566

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-c}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.24: Percentage total cumulative total mortality as influenced by dietary treatment, sex, and dietary treatment by sex interaction in male and female broilers subjected to high incubation (>38.8ºC) and cold brooding temperatures

		Total cumulative mortality percentage					
Age (days)		**0-7**	**0-14**	**0-21**	**0-28**	**0-35**	**0-40**
Dietary treatment group							
Negative control (NC)		0.98	2.96^{AB}	3.69	9.61	20.50	28.09^{AB}
Betaine		0.98	2.21^{A}	3.69	8.13	19.28	24.45^{B}
DMG		1.72	4.43^{A}	5.17	11.11	22.82	31.81^{A}
Positive control (PC)		0.74	1.23^{B}	2.71	8.13	18.37	25.99^{B}
Sex							
Male		1.47	3.45	4.80^{1}	13.70^{1}	31.97^{1}	43.84^{1}
Female		0.74	1.97	2.83^{2}	4.80^{2}	8.50^{2}	11.33^{2}
Diet*Sex Interaction							
NC	Male	0.49^{b}	3.45^{ab}	3.94^{ab}	13.32^{ab}	32.14^{ab}	44.87^{ab}
NC	Female	1.47^{ab}	2.46^{ab}	3.45^{ab}	5.91^{cd}	8.86^{c}	11.32^{c}
Betaine	Male	1.48^{ab}	3.45^{ab}	4.92^{ab}	13.32^{ab}	31.07^{ab}	40.05^{b}
Betaine	Female	0.49^{b}	0.98^{b}	2.46^{b}	2.95^{d}	7.38^{c}	8.86^{c}
DMG	Male	2.96^{a}	5.41^{a}	6.89^{a}	17.30^{a}	37.26^{a}	50.33^{a}
DMG	Female	0.49^{b}	3.45^{ab}	3.45^{ab}	4.92^{d}	8.38^{c}	13.30^{c}
PC	Male	0.99^{ab}	1.48^{b}	3.45^{ab}	10.85^{bc}	27.40^{b}	40.13^{b}
PC	Female	0.49^{b}	0.98^{b}	1.97^{b}	5.41^{cd}	9.35^{c}	11.84^{c}
----------				Probability			----------
Diet		0.6642	0.0656	0.3517	0.3796	0.3255	0.0469
Sex		0.2110	0.0874	0.0443	<0.0001	<0.0001	<0.0001
Diet*Sex		0.2292	0.8428	0.7304	0.3077	0.2122	0.4666
Pooled SEM		0.0288	0.0419	0.0473	0.0683	0.0876	0.0941

[A-B] Column means with different superscripts differ significantly at *P* <0.05
[1,2] Column means with different superscripts differ significantly at *P* <0.05
[a-d] Column means with different superscripts differ significantly at *P* <0.05

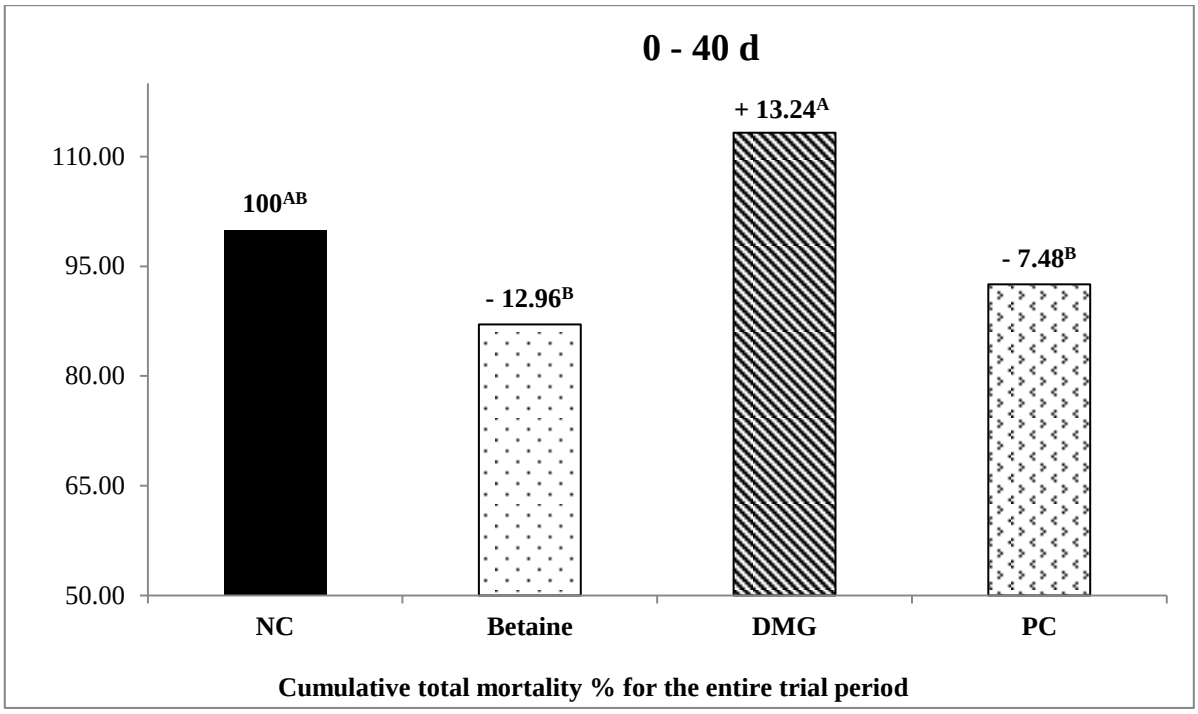

Figure 4.14: The effect of diet on total cumulative mortality percentage over the course of this study. Relative difference as expressed as a percentage of the negative control (NC). [A,B] Mean values with different superscripts were statistically different from each other at P <0.05

4.5.2.2 Mortality due to ascites

The effects of diet, sex, and diet by sex interaction on weekly and cumulative ascites-related mortalities during the course of the study are shown in **Table 4.25** and **Table 4.26** and **Figure 4.15**, respectively. The effect of sex on total cumulative and ascites-related mortality percentage over the course of this study is summarised in **Figure 4.16.**

The first cases of PHS occurred during the second week (day 10), and was observed onwards until the end of the study (day 40). Over the course of the study, PHS was observed in 358 out of 433 birds that died in total. Therefore, out of a total mortality rate of 26.53%, 21.94% (thus ~83%) of these mortalities were due to heart failure, hence PHS.

Birds supplemented with DMG had a significantly higher weekly ascites mortality rate during the 2nd week (7-14d) of this study compared to birds fed any of the other three treatment diets. However, no further significant differences were obtained concerning weekly ascites mortality rate and the different treatment diets fed for the remainder of the study. In contrast, significant differences were obtained for almost all periods concerning cumulative ascites mortality rate and dietary treatment fed.

Table 4.25: Percentage weekly ascites-related mortality as influenced by dietary treatment, sex, and dietary treatment by sex interaction in male and female broilers subjected to high incubation (>38.8ºC) and cold brooding temperatures

		Weekly ascites mortality percentage				
Age (days)		**7-14 (week 2)**	**14-21 (week 3)**	**21-28 (week 4)**	**28-35 (week 5)**	**35-40 (week 6)**
Dietary treatment group						
Negative control (NC)		0.49^{B}	0.49	5.41	10.12	6.90
Betaine		0.25^{B}	0.74	3.69	10.11	4.18
DMG		2.46^{A}	0.49	5.42	10.16	8.12
Positive control (PC)		0.25^{B}	0.49	3.94	8.34	6.89
Sex						
Male		0.86	0.74	7.75^{1}	16.43^{1}	10.35^{1}
Female		0.86	0.37	1.48^{2}	2.95^{2}	2.71^{2}
Diet*Sex Interaction						
NC	Male	0.99^{ab}	0.49	8.86^{ab}	17.79^{a}	11.34^{a}
NC	Female	0.00^{b}	0.49	1.97^{c}	2.46^{b}	2.46^{c}
Betaine	Male	0.49^{b}	0.98	6.89^{ab}	16.28^{a}	7.38^{ab}
Betaine	Female	0.00^{b}	0.49	0.49^{c}	3.94^{b}	0.98^{c}
DMG	Male	1.76^{ab}	0.98	9.36^{a}	17.36^{a}	11.32^{a}
DMG	Female	2.96^{a}	0.00	1.48^{c}	2.95^{b}	4.92^{bc}
PC	Male	0.00^{b}	0.49	5.91^{b}	14.27^{a}	11.32^{a}
PC	Female	0.49^{b}	0.49	1.97^{c}	2.46^{b}	2.46^{c}
----------Probability----------						
Diet		0.0095	0.9506	0.2346	0.7334	0.0874
Sex		1.0000	0.3101	<0.0001	<0.0001	<0.0001
Diet*Sex		0.5473	0.7359	0.3227	0.7653	0.7431
Pooled SEM		0.0261	0.0177	0.0378	0.0660	0.0543

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-c}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.26: Percentage cumulative ascites-related mortality as influenced by dietary treatment, sex, and dietary treatment by sex interaction in male and female broilers subjected to high incubation (>38.8ºC) and cold brooding temperatures

		Cumulative ascites mortality percentage				
Age (days)		**0-14**	**0-21**	**0-28**	**0-35**	**0-40**
Dietary treatment group						
Negative control (NC)		0.49^{B}	0.98^{B}	6.40^{AB}	16.68^{AB}	23.98^{AB}
Betaine		0.25^{B}	0.98^{B}	4.67^{B}	14.91^{AB}	19.28^{B}
DMG		2.46^{A}	2.96^{A}	8.38^{A}	18.68^{A}	27.21^{A}
Positive control (PC)		0.25^{B}	0.74^{B}	4.68^{B}	13.09^{B}	20.24^{B}
Sex						
Male		0.86	1.23	9.36^{1}	26.02^{1}	36.99^{1}
Female		0.86	1.60	2.71^{2}	5.66^{2}	8.37^{2}
Diet*Sex Interaction						
NC	Male	0.99^{ab}	1.48	10.35^{ab}	28.45^{a}	40.57^{a}
NC	Female	0.00^{b}	0.49	2.46^{e}	4.92^{c}	7.38^{c}
Betaine	Male	0.49^{b}	1.48	8.36^{bc}	24.90^{ab}	32.66^{b}
Betaine	Female	0.00^{b}	0.49	0.98^{e}	4.92^{c}	5.91^{c}
DMG	Male	1.76^{ab}	2.96	12.33^{a}	29.97^{a}	42.12^{a}
DMG	Female	2.96^{a}	2.96	4.43^{de}	7.39^{c}	12.31^{c}
PC	Male	0.00^{b}	0.49	6.40^{ce}	20.76^{b}	32.61^{b}
PC	Female	0.49^{b}	0.98	2.95^{de}	5.42^{c}	7.88^{c}
----------Probability----------						
Diet		0.0095	0.0579	0.0275	0.0753	0.0085
Sex		1.0000	0.5664	<0.0001	<0.0001	<0.0001
Diet*Sex		0.5473	0.8031	0.3196	0.2513	0.3669
Pooled SEM		0.0261	0.0314	0.0484	0.0761	0.0875

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05

In general, birds fed the betaine-supplemented or either of the control diets revealed consistently lower cumulative ascites mortality rates than birds fed the DMG-supplemented diet ($P \leq 0.05$). It is also important to note that the betaine-supplemented and PC groups revealed numerically lower deaths due to ascites compared to the NC-group, although no significance was obtained ($P > 0.05$).

Overall, there was a significant difference between males and females in both weekly and cumulative ascites-related mortalities during the last 3 weeks of this study, with males having a higher ($P < 0.0001$) incidence of ascites than did females.

Similar to earlier reports, no significant differences were observed between sex and diet with regards to weekly- and cumulative ascites mortalities ($P > 0.05$).

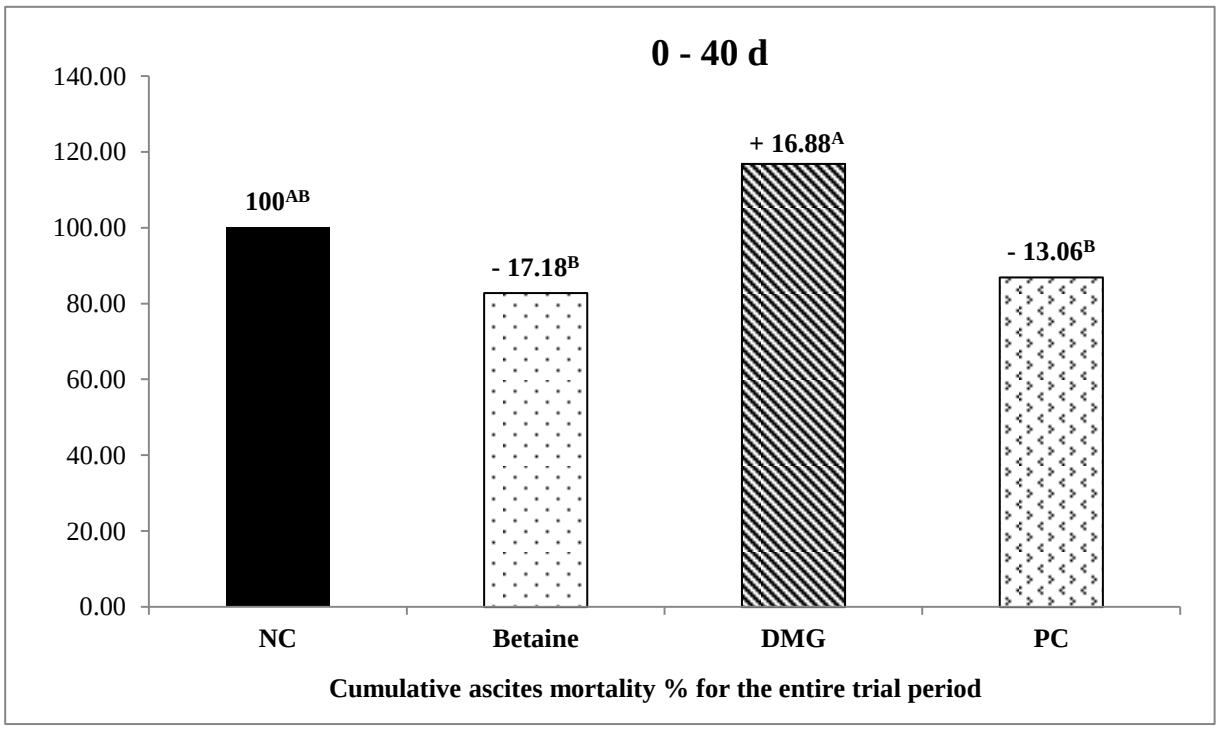

Figure 4.15: The effect of diet on total cumulative ascites mortality percentage over the course of this study. Relative difference as expressed as a percentage of the negative control (NC). ^{A,B} Mean values with different superscripts were statistically different from each other at $P < 0.05$

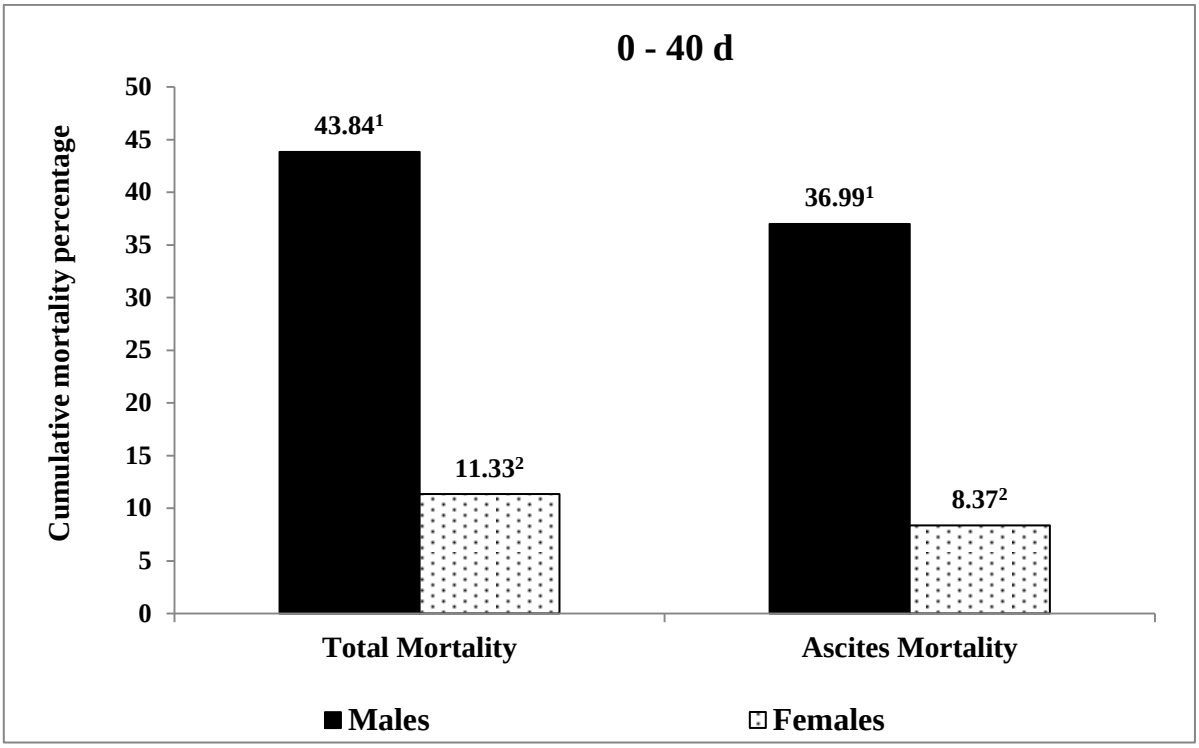

Figure 4.16: The effect of sex on total cumulative and ascites-related mortality percentage over the course of this study. [1,2] Mean values with different superscripts were statistically different from each other at P <0.001

4.5.3 Ascites incidence and heart characteristics

The effects of dietary treatment, sex, and dietary treatment by sex interaction on the different heart characteristics for processing stage 1 (d20 & 21) and 2 (d40 &41) are shown in **Tables 4.28** and **Table 4.29**, respectively.

Bird weight was significantly higher (P <0.05) for the betaine-supplemented group at 20 & 21 days of age compared to birds fed either of the remaining treatment diets, while birds fed DMG demonstrated the lowest weights at 40 & 41 days of age compared to birds fed either of the remaining treatment diets (P <0.05). Heart characteristics during processing stage 1 (d20 & 21) revealed no significant differences among the dietary treatment groups for any of the heart parameters measured except for the LVS/BW and TV/BW ratios (P <0.05). Betaine supplementation had a significant reducing effect (P <0.05) on LVS/BW ratio of 6.61%, 3.90%, and 0.90%, and TV/BW ration of 6.67%, 4.44%, and 4.44% compared to birds fed the NC, DMG supplemented, and PC diets, respectively. The PC treatment group also revealed a significantly lower (P <0.05) LVS/BW ratio than did the NC dietary treatment group. It is therefore noteworthy

that birds fed diets containing adequate methyl groups (i.e. betaine-, DMG and PC groups) revealed numerically lower LVS/BW and TV/BW ratios than did birds fed diets containing inadequate methyl groups (i.e. NC group). A significant difference was found only for TV weight during processing stage 2, while insignificant effects were found for all other heart parameters at 40 & 41 days of age for the dietary treatment fed. Data presented in **Table 4.28** revealed a 6.34% and 6.51% higher TV weight following dietary betaine supplementation at 40 & 41 days of age compared to birds fed either the DMG-supplemented or PC treatment diets. Furthermore, although not significant (P <0.05), the betaine-supplemented group had a 0.90% higher TV weight compared to birds from the NC group.

Bird weight was significantly heavier (P <0.001) for males during both processing stage 1 (20 & 21d of age) and 2 (40 & 41d of age) when compared to their female counterparts. The RV weight, TV weight, RV/TV_{FM} ratio and RV/BW ratio were constantly higher for the males in comparison to the females during slaughter stage 1 (d20 & 21). In contrast, a lower LVS/BW ratio was observed in males than in females. A significant difference was found for all heart parameters during processing stage 2 (40 & 41d of age), where males revealed consistently heavier weights for all measured heart parameters than did their female counterparts (P <0.05).

No interactions were observed for dietary treatment by sex during processing stage 1 (20 & 21d of age), while a significant interaction effect was only observed for RV and TV weight during processing stage 2 (40 & 41d of age). Irrespective of the treatment diet fed, males yielded significantly higher values for RV and TV weight than females. In addition, males fed either betaine or the NC treatment diet had significantly higher RV and TV weights than males fed the DMG supplemented or PC treatment diets (P <0.05).

During each processing stage, the hearts collected were also used to express linear regression between ascites heart index calculated on a fresh matter basis (AHI_{FM}) and on dry matter basis (AHI_{DM}). Results indicated an increase in the number of data points situated above the horizontal line or to the right of the vertical line from processing stage 1 (d20 & 21) (data not shown) to processing stage 2 (d40 & 41). As illustrated by **Figure 4.17**, the cut-off value (0.270) during processing stage 2 as measured by AHI_{FM} for the onset of PHS corresponded to a similar value of 0.272 as measured by AHI_{DM} ($R^2_{adjusted}$ = 0.955). The AHI_{FM} ranged from 0.152 to 0.431 while the AHI_{DM} ranged from 0.146 to 0.448 from all hearts measured.

Table 4.27: Heart characteristics of birds at Processing Stage 1 (d20 & 21) of broiler chickens that had been incubated at high (>38.8°C) eggshell temperature and reared under cold brooding and grow-out temperatures

	Chick Weight (g/bird)	RV (g/bird)	LVS (g/bird)	TV (g/bird)
Dietary treatment group				
Negative control (NC)	861.80^{B}	0.955	2.898	3.854
Betaine	910.58^{A}	0.939	2.867	3.806
DMG	893.60^{B}	0.915	2.881	3.796
Positive control (PC)	898.45^{B}	0.897	2.997	3.894
Sex				
Male	922.91^{1}	1.046^{1}	2.918^{1}	3.964^{1}
Female	859.30^{2}	0.808^{2}	2.904^{2}	3.711^{2}
Diet*Sex				
NC Male	883.28^{bc}	1.060^{a}	2.917	3.978^{a}
NC Female	840.33^{ce}	0.851^{bc}	2.879	3.730^{ab}
Betaine Male	953.34^{a}	1.087^{a}	2.884	3.971^{a}
Betaine Female	867.83^{cde}	0.791^{c}	2.850	3.641^{b}
DMG Male	940.95^{a}	1.053^{a}	2.875	3.928
DMG Female	846.25^{cde}	0.777^{c}	2.887	3.665^{b}
PC Male	941.08^{ab}	0.983^{ab}	2.996	3.979^{a}
PC Female	882.82^{bd}	0.811^{c}	2.998	3.809^{ab}
----------Probability----------				
Diet	0.0078	0.6927	0.2970	0.7263
Sex	<0.0001	<0.0001	0.7998	0.0005
Diet*Sex	0.1037	0.6437	0.9878	0.8974
Pooled SEM	16.2223	0.0575	0.0762	0.1058

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
$^{1-2}$ Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05
Abbreviations:
RV = right ventricle weight on a fresh matter basis;
LVS = left ventricle & septum weight on a fresh matter basis;
TV = total ventricle weight on a fresh matter basis

Table 4.27 (Continue): Heart characteristics of birds at Processing Stage 1 (d20 & 21) of broiler chickens that had been incubated at high (>38.8°C) eggshell temperature and reared under cold brooding and grow-out temperatures

		RV/TV_{FM} Ratio	RV/BW Ratio	LVS/BW Ratio	TV/BW Ratio
Dietary treatment group					
Negative control (NC)		0.244	0.00109	0.00333^{A}	0.0045^{A}
Betaine		0.246	0.00102	0.00311^{C}	0.0042^{B}
DMG		0.239	0.00102	0.00320^{AB}	0.0043^{A}
Positive control (PC)		0.229	0.00100	0.00330^{B}	0.0043^{A}
Sex					
Male		0.264^{1}	0.00113^{1}	0.00314^{2}	0.0043
Female		0.215^{2}	0.00093^{2}	0.00332^{1}	0.0042
Diet*Sex					
NC	Male	0.265^{a}	0.00119^{a}	0.00328^{a}	0.0045^{a}
NC	Female	0223^{bc}	0.00100^{d}	0.00337^{cd}	0.0044^{ab}
Betaine	Male	0.275^{a}	0.00113^{a}	0.00300^{c}	0.0042^{b}
Betaine	Female	0.218^{c}	0.00091^{d}	0.00323^{d}	0.0042^{b}
DMG	Male	0.268^{a}	0.00113^{ab}	0.00304^{ab}	0.0042^{ab}
DMG	Female	0.209^{c}	0.00091^{cd}	0.00336^{cd}	0.0043^{ab}
PC	Male	0.247^{ab}	0.00108^{bc}	0.00325^{b}	0.0044^{ab}
PC	Female	0.211^{c}	0.00091^{d}	0.00335^{cd}	0.0043^{ab}
----------Probability----------					
Diet		0.3894	0.3436	0.0382	0.0368
Sex		<0.0001	<0.0001	0.0028	0.8729
Diet*Sex		0.6733	0.9694	0.4913	0.7966
Pooled SEM		0.0116	0.00006289	0.001542	0.0001119

$^{A-C}$ Column means with different superscripts differ significantly at P <0.05
$^{1-2}$ Column means with different superscripts differ significantly at P <0.05
$^{a-d}$ Column means with different superscripts differ significantly at P <0.05
Abbreviations:
RV/TV_{FM} = right ventricle to total ventricle weight on a fresh matter basis;
RV/BW = right ventricle to body weight ratio on a fresh matter basis;
TV/BW = total ventricle to body weight ratio on a fresh matter basis

Table 4.28: Heart characteristics of birds at Processing Stage 2 (d40 & 41) of broiler chickens that had been incubated at high (>38.8°C) eggshell temperature and reared under cold brooding and grow-out temperatures

		Bird Weight (g/bird)	RV (g/bird)	LVS (g/bird)	TV (g/bird)
Dietary treatment group					
Negative control (NC)		3293.43^{A}	2.709^{A}	7.601^{AB}	10.310^{AB}
Betaine		3298.17^{A}	2.548^{AB}	7.780^{A}	10.328^{A}
DMG		3119.47^{B}	2.441^{AB}	7.233^{B}	9.674^{B}
Positive control (PC)		3278.66^{A}	2.327^{B}	7.330^{AB}	9.657^{B}
Sex					
Male		3447.25^{1}	3.132^{1}	8.478^{1}	11.610^{1}
Female		3047.62^{2}	1.881^{2}	6.494^{2}	8.375^{2}
Diet*Sex					
NC	Male	3493.34^{a}	3.465^{a}	8.904^{a}	12.372^{a}
NC	Female	3093.52^{c}	1.953^{c}	6.295^{d}	8.248^{c}
Betaine	Male	3556.99^{a}	3.415^{a}	8.838^{ab}	12.254^{a}
Betaine	Female	3039.36^{cd}	1.680^{c}	6.722^{d}	8.402^{c}
DMG	Male	3273.47^{b}	2.921^{b}	7.969^{c}	10.889^{b}
DMG	Female	2965.47^{d}	1.962^{c}	6.497^{d}	8.458^{c}
PC	Male	3465.19^{a}	2.725^{b}	8.199^{bc}	10.923^{b}
PC	Female	3092.14^{c}	1.930^{c}	6.462^{d}	8.392^{c}
----------			**Probability**	----------	
Diet		<0.0001	0.0673	0.0948	0.0397
Sex		<0.0001	<0.0001	<0.0001	<0.0001
Diet*Sex		0.1141	0.0175	0.1478	0.0312
Pooled SEM		44.5890	0.1621	0.2585	0.3419

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
$^{1-2}$ Column means with different superscripts differ significantly at *P* <0.05
$^{a-d}$ Column means with different superscripts differ significantly at *P* <0.05
Abbreviations:
RV = right ventricle weight on a fresh matter basis;
LVS = left ventricle & septum weight on a fresh matter basis;
TV = total ventricle weight on a fresh matter basis

Table 4.28 (Continue): Heart characteristics of birds at Processing Stage 2 (d40 & 41) of broiler chickens that had been incubated at high (>38.8°C) eggshell temperature and reared under cold brooding and grow-out temperatures

	RV/TV_{FM} Ratio	RV/BW Ratio	LVS/BW Ratio	TV/BW Ratio
Dietary treatment group				
Negative control (NC)	0.258	0.00081	0.00233	0.0031
Betaine	0.243	0.00075	0.00238	0.0031
DMG	0.253	0.00079	0.00236	0.0031
Positive control (PC)	0.242	0.00070	0.00226	0.0029
Sex				
Male	0.272^{1}	0.00091^{1}	0.00250^{1}	0.0034^{1}
Female	0.227^{2}	0.00061^{2}	0.00217^{2}	0.0027^{2}
Diet*Sex				
NC Male	0.282^{ab}	0.00099^{a}	0.00259^{a}	0.0035^{a}
NC Female	0.234^{cd}	0.00062^{d}	0.00206^{d}	0.0026^{c}
Betaine Male	0.283^{a}	0.00096^{a}	0.00253^{ab}	0.0034^{a}
Betaine Female	0.204^{e}	0.00054^{d}	0.00224^{bcd}	0.0027^{c}
DMG Male	0.271^{ab}	0.00091^{ab}	0.00248^{abc}	0.0033^{ab}
DMG Female	0.236^{cd}	0.00067^{cd}	0.00223^{cd}	0.0029^{c}
PC Male	0.251^{bcd}	0.00078^{bc}	0.00240^{abc}	0.0031^{b}
PC Female	0.233^{d}	0.00062^{d}	0.00212^{d}	0.0027^{c}
----------Probability----------				
Diet	0.3379	0.1286	0.3949	0.2209
Sex	<0.0001	<0.0001	<0.0001	<0.0001
Diet*Sex	0.0513	0.0863	0.2511	0.1823
Pooled SEM	0.0112	0.00005308	0.00008591	0.0001119

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
$^{1-2}$ Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05
Abbreviations:
RV/TV_{FM} = right ventricle to total ventricle weight on a fresh matter basis;
RV/BW = right ventricle to body weight ratio on a fresh matter basis;
TV/BW = total ventricle to body weight ratio on a fresh matter basis

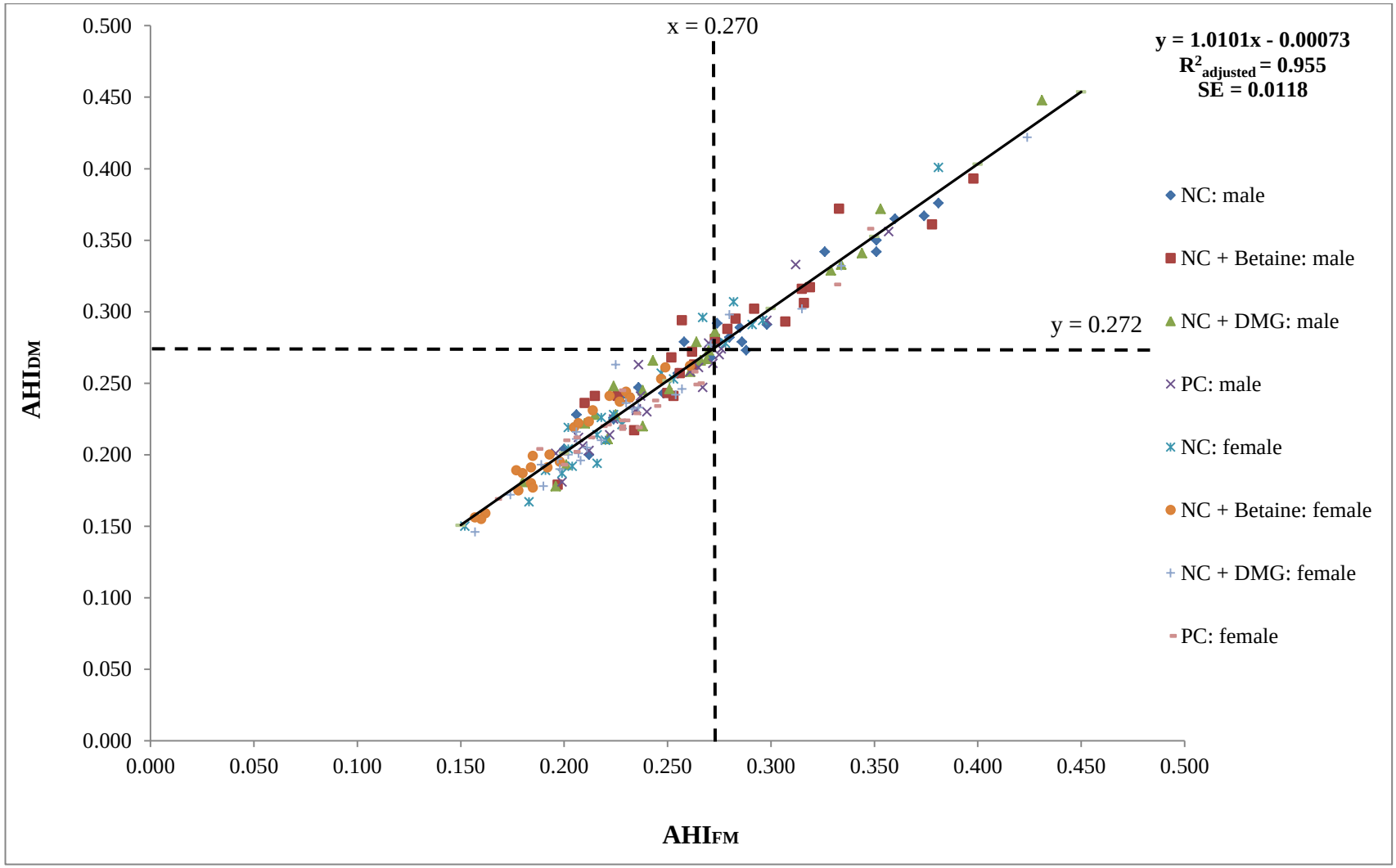

Figure 4.17: Linear regression between ascites heart index calculated on a fresh matter basis (**AHI_{FM}**) and on dry matter basis (**AHI_{DM}**) for the different treatment diets as calculated during Processing Stage 2. All data points situated above the horizontal line or to the right of the ventricle line indicate individuals with pulmonary hypertension syndrome

4.6 Performance Efficiency Factor (PEF)

The effect of dietary treatment, sex, and dietary treatment by sex interaction on performance efficiency factor (PEF) is summarised in **Table 4.30** and **Figures 4.18** and **4.19**, respectively.

Table 4.29: Peformance efficiency factor (PEF) at 40 & 41 days of age of broiler chickens as affected by dietary treatment, sex, and dietary treatment by sex interaction

		PEF
Dietary treatment group		
Negative control (NC)		282.77^{A}
Betaine		304.62^{A}
DMG		255.50^{B}
Positive control (PC)		288.29^{A}
Sex		
Male		251.80^{2}
Female		313.79^{1}
Diet*Sex		
NC	Male	244.74
NC	Female	320.81
Betaine	Male	277.06
Betaine	Female	332.18
DMG	Male	223.73
DMG	Female	287.27
PC	Male	261.66
PC	Female	314.92
----------Probability----------		
Diet		0.0007
Sex		<0.0001
Diet*Sex		0.7283
Pooled SEM		38.04

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
$^{1-2}$ Column means with different superscripts differ significantly at *P* <0.05
$^{a-e}$ Column means with different superscripts differ significantly at *P* <0.05

As shown in **Table 4.29** and **Figure 4.18**, performance efficiency factor (PEF) was significantly lower (*P* <0.05) for the DMG supplemented treatment group in contrast to birds receiving either the NC, betaine supplemented, or PC treatment diets. Female birds showed the highest PEF (*P* <0.0001) which can mainly be attributed to their lower cumulative total mortality rate, hence a higher total cumulative body weight at the end of this study in comparison to the

rapidly growing male broiler birds **(Figure 4.19).** No significant interaction was observed for PEF for dietary treatment and sex (P >0.05).

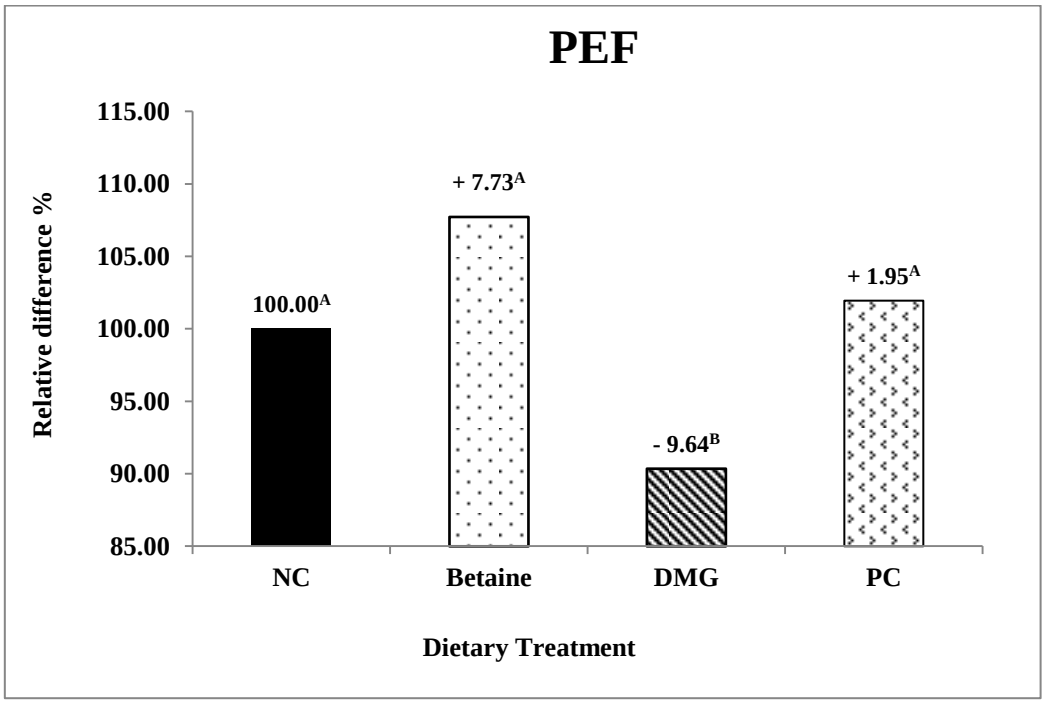

Figure 4.18: Effect of dietary di- and trimethylglycine (betaine) on performance efficiency factor (PEF) in broiler chickens under ascites inducing conditions (high eggshell temperature and cold temperature) during rearing and grow-out. [A, B] Different superscripts are significantly different at P < 0.05

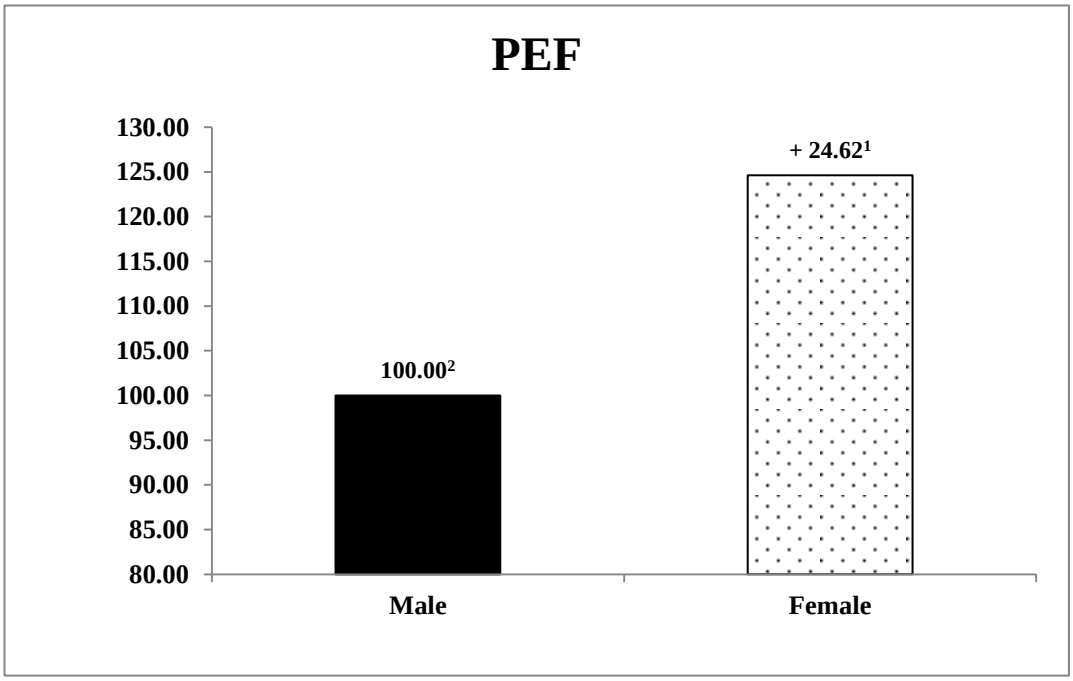

Figure 4.19: Effect of sex on performance efficiency factor (PEF) in broiler chickens under ascites-inducing conditions (high eggshell temperature and cold temperature) during rearing and grow-out. [1,2] Different superscripts are significantly different at P <0.001

4.7 Biochemical parameters

The effect of diet, sex, and diet by sex interaction on the measured biochemical parameters associated with the risk of heart failure, hence PHS, are shown in **Table 4.30**.

Plasma homocysteine (HCY) levels were significantly lower in broilers consuming the betaine supplemented diet in contrast to broilers consuming either of the control diets at approximately 3 weeks of age (d20 & 21) (P <0.05) **(Figure 4.20).** It is also important to note that the betaine group had a 5.46 µmol/L lower HCY concentration at 20 & 21 days of age than the DMG group, however, this difference was not significant. At termination of the study (d40 & 41), birds reared on betaine had a significantly lower (P <0.05) HCY concentration than any of the other three treatment diets (**Figure 4.21**). Male birds had a higher (P <0.05) level of HCY in their plasma than did females during week 3 (d20 & 21), but no significance was obtained in HCY concentration between the sexes at termination of the study (d40 &41). Little to no significant interactions were observed between diet and sex concerning HCY concentration at any of the ages (P >0.05), except for the NC treatment group during week 3 (P <0.05).

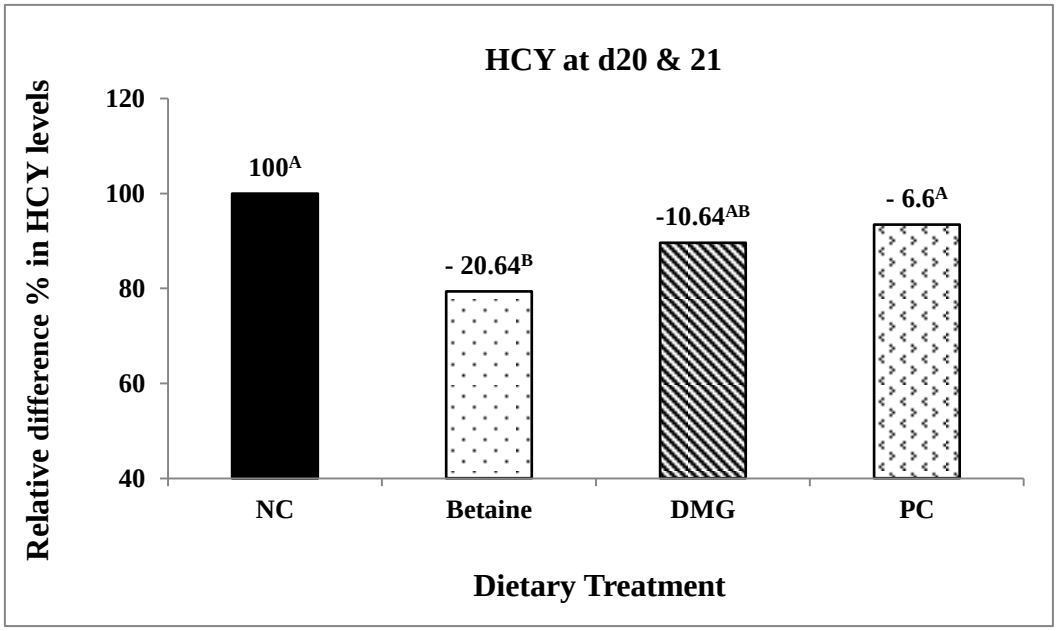

Figure 4.20: Relative levels of homocysteine (HCY) in blood plasma of broilers at 20 & 21 days of age fed the different treatment diets expressed as a percentage basis of birds fed the negative control (NC) diet representing a graph reference value of 100. ^{A,B} Mean values with different superscripts were statistically different from each other at P <0.05

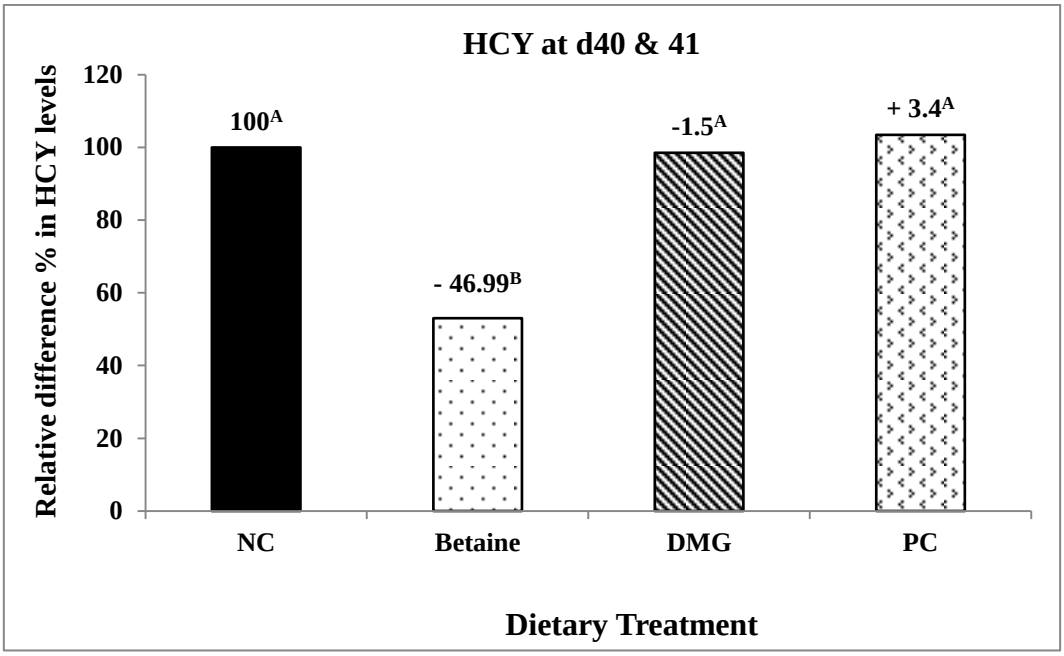

Figure 4.21: Relative levels of homocysteine (HCY) in blood plasma of broilers at 40 & 41 days of age fed the different treatment diets expressed as a percentage basis of birds fed the negative control (NC) diet representing a graph reference value of 100. ^{A,B} Mean values with different superscripts were statistically different from each other at P <0.05

At 20 & 21 days of age, 4-hydroxynonenal (4-HNE) concentration (an indicator of lipid peroxidation) was lowest (P <0.05) in broilers consuming the betaine-supplemented or PC diets in contrast to birds consuming the NC or DMG supplemented diets (**Figure 4.22**). In contrast, no difference was obtained in plasma 4-HNE concentration at the end of the study (d40 & 41) (**Figure 4.23)**. Significant differences were obtained between males and females for 4-HNE throughout the study, with males having lower lipid peroxidation (4-HNE) values at d20 & 21 but higher lipid peroxidation values at d40 & 41 (P <0.05). A significant diet by sex interaction was obtained during week 3 for the betaine supplemented, DMG supplemented, and PC groups, where males generally had lower levels of 4-HNE than females. However, little to no positive interaction effects were observed for 4-HNE concentration at 40 & 41d of age (P >0.05). Although no significant differences were obtained at 40 & 41 days of age, both males and females in the betaine group tended to have lower levels of 4-HNE than males and females fed the other treatment diets.

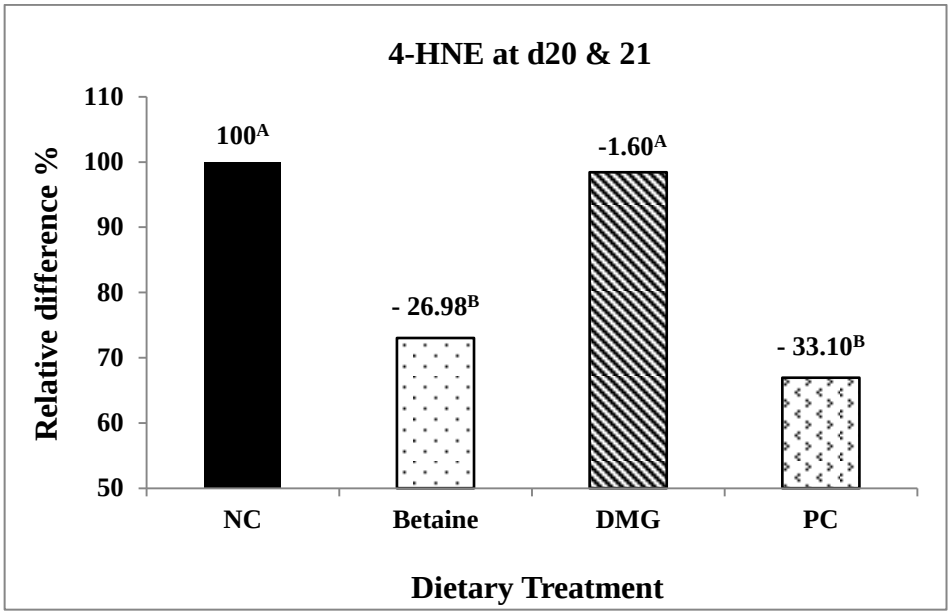

Figure 4.22: Relative levels of lipid peroxidative end-products (4-HNE) in blood plasma at 20 & 21 days of age in broilers fed the different treatment diets expressed as a percentage basis of birds fed the negative control (NC) diet representing a graph reference value of 100. [A,B] Mean values with different superscripts were statistically different from each other at P <0.05

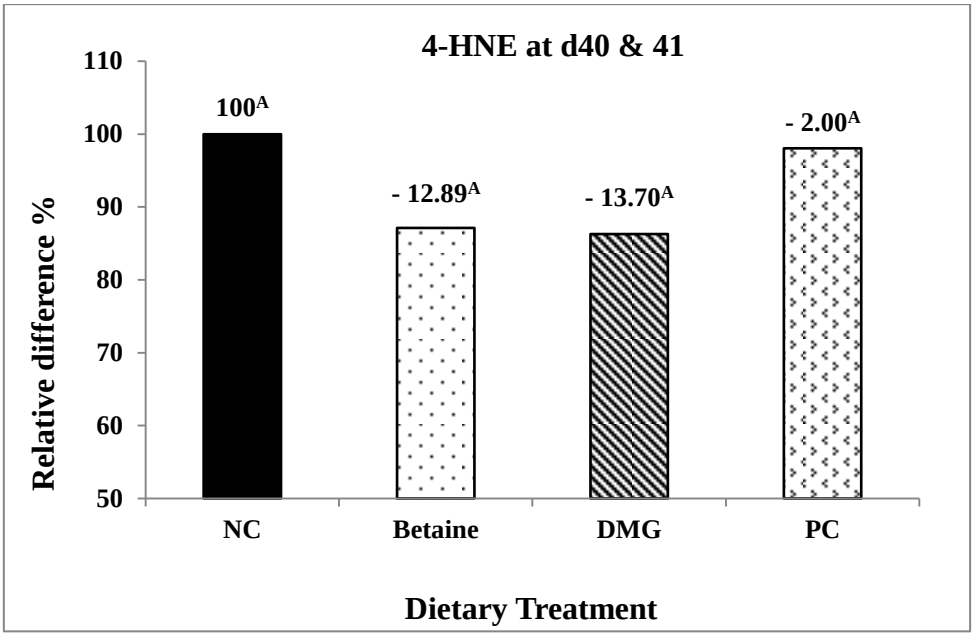

Figure 4.23: Relative levels of lipid peroxidative end-products (4-HNE) in blood plasma at 40 & 41 days of age in broilers fed the different treatment diets expressed as a percentage basis of birds fed the negative control (NC) diet representing a graph reference value of 100. [A, B] Mean values with different superscripts were statistically different from each other at P <0.05

Figure 4.24 summarises the difference in thiobarbituric acid (TBARS) levels in plasma between the different treatment diets fed to broilers chickens at 40 & 41 days of age. Findings from the TBARS assay showed that lipid peroxidation was significantly lower in the DMG supplemented group compared to birds fed the NC treatment diet ($P \leq 0.05$), however no diference was observed between the DMG-supplemented and PC control groups ($P > 0.05$). It is also noteworthy that birds fed dietary betaine revealed numerically lower lipid peroxidation values than birds fed either of the control diets, although little to no significance was observed. In addition, similar lipid peroxidation values were obtained for the betaine-supplemented group in comparison to birds fed dietary DMG ($P > 0.05$). Male broilers tended to have a higher TBARS concentration than females, however this difference was not significant. There were no significant diet by sex interactions concerning the TBARS assay ($P > 0.05$).

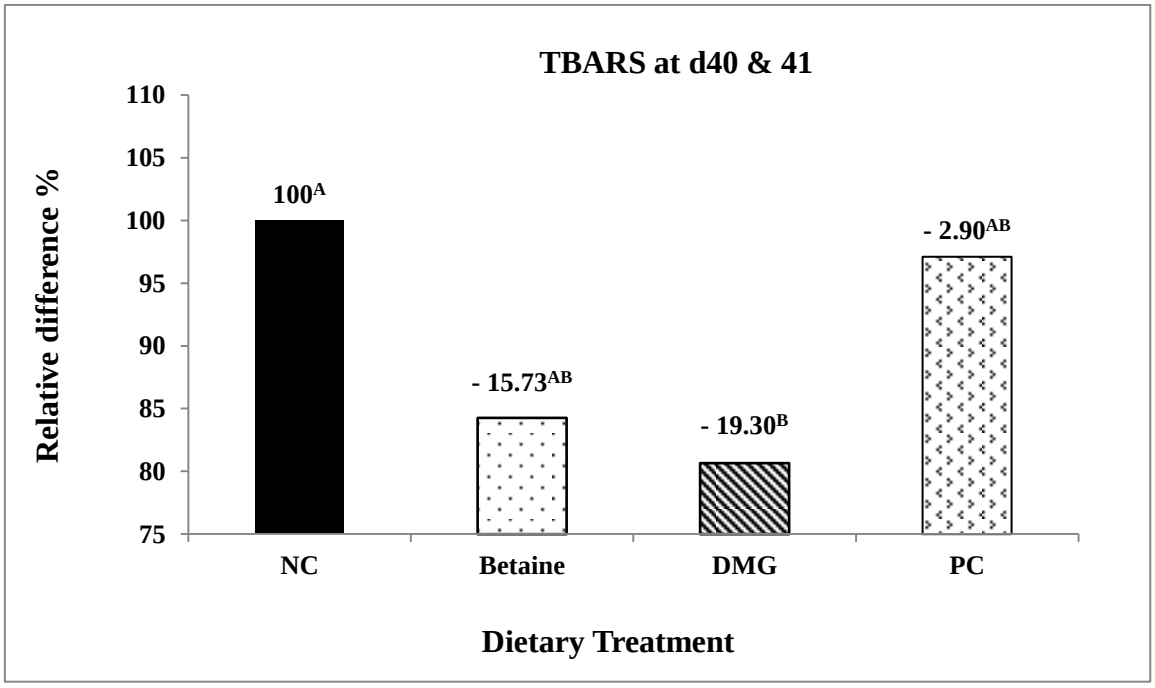

Figure 4.24: Relative levels of lipid peroxidative end-products (TBARS) in the blood plasma at 40 & 41 days of age in broilers fed the different treatment diets expressed as a percentage basis of birds fed the negative control (NC) diet representing a graph reference value of 100. ^{A,B} Mean values of different superscripts were statistically different from each other at $P < 0.05$

Figure 4.25 summarises the difference in plasma adenosine monophosphate proteinkinase (AMPK) levels between the different treatment diets fed to broilers chickens at 40 & 41 days of age. Plasma levels of AMPK tended to be higher in the Betaine-group compared to the remaining treatment diets, although no significant differences were obtained between these diets. Similarly, no significant differences were observed among males and females and there were no significant interactions observed between diet and sex at the termination of the study. However, it is important to note that both the male and female group supplemented with dietary betaine had numerically higher AMPK activity compared to male and female birds fed the remaining treatment diets.

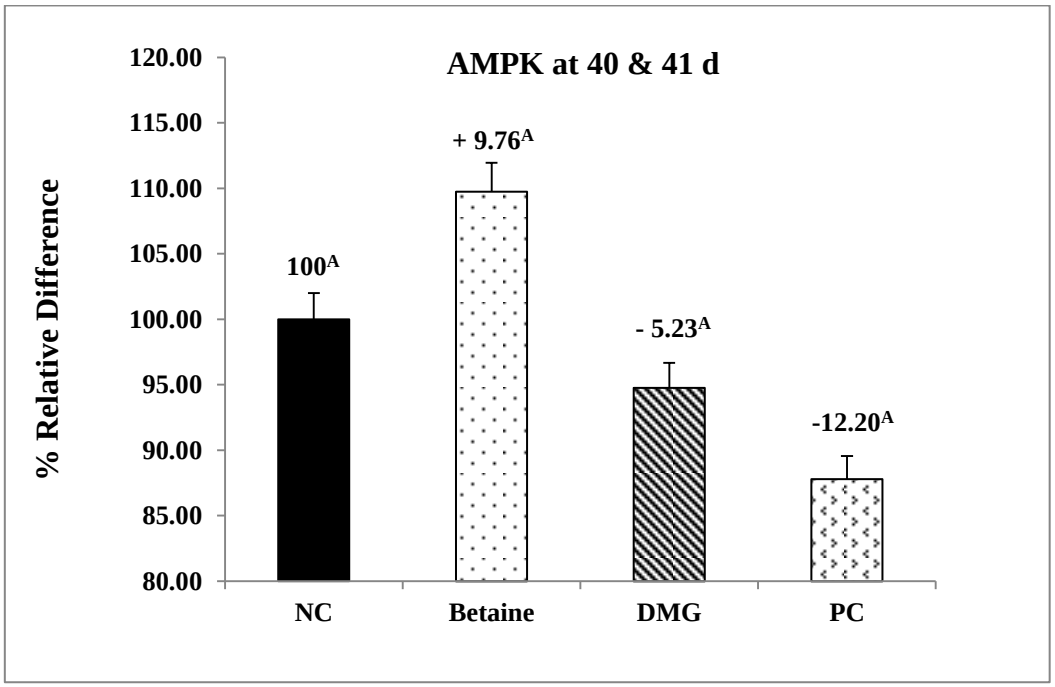

Figure 4.25: A comparison of AMPK activity between different treatment diets under ascites-inducing conditions (high EST and cold brooding). [A,B] Mean values with different superscripts were statistically different from each other at $P < 0.05$

Table 4.30: Biochemical parameters related to ascites (mean ± standard error)

		Homocysteine concentration (µmol/L)		4-HNE concentration (µg/mL)		TBARS concentration (µmol/L)	AMPK concentration (ng/mL)
	n	**d20 & 21**	**d40 &41**	**d20 & 21**	**d40 &41**	**d40&41**	**d40&41**
Dietary treatment group							
Negative control (NC)	**24**	53.29^{A} (± 2.03)	38.92^{A} (± 1.27)	0.378^{A} ± (0.020)	0.357 ± (0.024)	4.602^{A} (± 0.28)	0.287 (± 0.25)
Betaine	**24**	42.29^{B} (± 2.03)	20.63^{B} (± 1.27)	0.276^{B} ± (0.020)	0.311 ± (0.024)	3.878^{AB} (± 0.28)	0.315 (± 0.25)
DMG	**24**	47.75^{AB} (± 2.03)	38.33^{A} (± 1.27)	0.372^{A} ± (0.020)	0.308 ± (0.024)	3.712^{B} (± 0.28)	0.272 (± 0.25)
Positive control (PC)	**24**	49.79^{A} (± 2.03)	40.25^{A} (± 1.27)	0.253^{B} ± (0.020)	0.350 ± (0.024)	4.469^{AB} (± 0.28)	0.252 (± 0.25)
Sex							
Male	**48**	50.40^{1} (± 1.43)	34.04 (± 0.90)	0.253^{2} (± 0.014)	0.371^{1} (± 0.017)	4.325 (± 0.20)	0.276 (± 0.02)
Female	**48**	46.17^{2} (± 1.43)	35.02 (± 0.90)	0.386^{1} (± 0.014)	0.291^{2} (± 0.017)	4.006 (± 0.20)	0.286 (± 0.02)

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-d}$ Column means with different superscripts differ significantly at *P* <0.05

Table 4.30 (Continue): Biochemical parameters related to ascites

		n	Homocysteine concentration (µmol/L) d20 & 21	Homocysteine concentration (µmol/L) d40 &41	4-HNE concentration (µg/mL) d20 & 21	4-HNE concentration (µg/mL) d40 &41	TBARS concentration (µmol/L) d20 & 21	AMPK concentration (ng/mL) d40 &41
Diet*sex								
Negative control (NC)	Male	**12**	45.08^{bc} (± 2.87)	39.00^{a} (± 1.80)	0.343^{ad} (± 0.03)	0.405^{a} (± 0.04)	4.839^{ab} (± 0.40)	0.279 (± 0.04)
Negative control (NC)	Female	**12**	61.50^{a} (± 2.87)	38.83^{a} (± 1.80)	0.413^{a} (± 0.03)	0.309^{ab} (± 0.04)	4.366^{a} (± 0.40)	0.295 (± 0.04)
Betaine	Male	**12**	39.33^{b} (± 2.87)	20.58^{b} (± 1.80)	0.216^{b} (± 0.03)	0.346^{ab} (± 0.04)	4.104^{b} (± 0.40)	0.293 (± 0.04)
Betaine	Female	**12**	45.25^{bc} (± 2.87)	20.67^{b} (± 1.80)	0.336^{ad} (± 0.03)	0.275^{b} (± 0.04)	3.652^{ab} (± 0.40)	0.336 (± 0.04)
DMG	Male	**12**	51.42^{c} (± 2.87)	38.25^{a} (± 1.80)	0.248^{bd} (± 0.03)	0.354^{ab} (± 0.04)	3.980^{b} (± 0.40)	0.278 (± 0.04)
DMG	Female	**12**	44.08^{bc} (± 2.87)	38.41^{a} (± 1.80)	0.496^{c} (± 0.03)	0.262^{b} (± 0.04)	3.444^{ab} (± 0.40)	0.266 (± 0.04)
Positive control (PC)	Male	**12**	50.75^{c} (± 2.87)	42.25^{a} (± 1.80)	0.207^{b} (± 0.03)	0.381^{a} (± 0.04)	4.375^{a} (± 0.40)	0.256 (± 0.04)
Positive control (PC)	Female	**12**	48.83^{c} (± 2.87)	38.25^{a} (± 1.80)	0.299^{d} (± 0.03)	0.320^{ab} (± 0.04)	4.563^{ab} (± 0.40)	0.248 (± 0.04)
----------Probability----------								
Diet			0.0027	<0.0001	<0.0001	0.3548	0.0774	0.3510
Sex			00402	0.4446	<0.0001	0.0016	0.2656	0.6918
Diet*Sex			0.0011	0.5994	0.0128	0.9531	0.7834	0.8567
Pooled SEM			2.1088	1.3257	0.0212	0.0254	0.2952	0.0260

$^{A-B}$ Column means with different superscripts differ significantly at *P* <0.05
^{1,2} Column means with different superscripts differ significantly at *P* <0.05
$^{a-d}$ Column means with different superscripts differ significantly at *P* <0.05

CHAPTER 5: DISCUSSION

5.1 Nutrient values for the different dietary treatment groups

All analysed proximate contents of the different test diets were in good agreement with the expected values for the different phases, however some discrepencies were noted for mineral content between the test diets of the different phases (**Table 4.3**). The small deviations in the analysed mineral content may be attributed to either error in methodology of sampling or possible analytical error. In addition, the actual nutrient composition of raw ingredients was unknown and therefore formulations were based on estimated values which may have been a contributing factor to the differences obtained between the formulated (expected) and the actual (analysed) values.

The dietary betaine content of the diet was unknown, which might explain the differences between the analysed versus the expected betaine levels. The method of analysis could also have been another contributing factor for the discrepencies in the dietary betaine concentration. Interestingly, variations in dietary betaine content were discovered across all of the dietary phases and test diets. It remains uncertain as to why diets not supplemented with additional betaine yielded values similar to or higher than the betaine supplemented diet, since poultry diets composed primarily of maize and soybean meal have very low natural betaine content (<150 mg/kg) (Kidd *et al.*, 1997).

The broiler starter and grower choline concentrations were relatively close to the formulated values, so the small deviation obtained in the broiler finisher diets may have occurred due to incorrect sampling or possible error in the analytical procedure. Unfortunately, when using a small amount of sample for different analytical procedures, the chance that an error may occur can be high. This also makes it difficult to analyse a relatively representative sample of what is expected in the actual diet. Interestingly, dietary treatment 3 revealed the highest choline content across all bird age categories, although no choline chloride (60%) was supplemented. It remains uncertain as to why the diets supplemented with DMG yielded the highest choline concentration, however it appears that dietary DMG supplementation may have an effect on dietary choline content.

The total cysteine content was above 100% of expected values for almost all phases. The latter deviations are most likely due to estimated values of raw ingredients from previous batches being used, resulting in an error in the percent of the expected amount. It can be concluded that almost all formulated total TSAA and total cysteine values were present in the diets at or above what was anticipated.

In agreement with previous results, the small variations in the analysed methionine contents were most likely due to an error in sampling or an error in the analytical method. The fact that the formulations were based on predicted values and that an error in the expected amount might have occurred must also not be disregarded.

Despite the discrepencies in analysed versus expected dietary analyses, it is noteworthy to mention that the dietary treatment groups performed as expected with the betaine and positive control groups showing an overall improvement in growth and carcass performance as well as amelioration in ascites related mortality in contrast to the negative control group. Unexpectedly, the DMG supplemented groups performed poorly in comparison to its methyl derivative betaine and the positive control. For this reason, it is important to note that all discussions in this dissertation concerning the feed supplement levels refer to the concentrations as initially formulated, hence, the intended levels.

5.2 The effect of dietary treatment and sex on PHS development

The main aim of the present study was to investigate the effect of different methyl-derivatives of the amino acid glycine, di- (DMG) and trimethylglycine (betaine), on reducing the severity and incidence of PHS when broilers were subjected to ascites inducing conditions (i.e. high EST and cold environmental temperatures during rearing).

5.2.1 The influence of diet and sex on morphological changes during PHS

The present study indicated that most of the morphological changes of PHS (ascites), such as fluid in the abdominal cavity (i.e. waterbelly), fluid build-up around the heart (i.e. pericardial effusion), and liver damage, were represented by a numerically higher portion of birds fed either the NC (methyl-inadequate) or DMG-supplemented treatment diets. It is well known that the morphological changes mentioned above have been associated with an increased risk of heart failure or ascites in rapidly growing broiler chickens. Interestingly, betaine was the only dietary treatment to reduce pericardial effusion related to PHS. Additionally, supplementation of either betaine or adequate methyl groups (i.e. PC diet) resulted in reduced liver damage and liver failure compared to the NC (methyl-inadequate) and DMG-supplemented treatment groups.

During this study, the average ventricular weight data for all heart parameters were normalised as described by Wideman *et al.* (1998b). This was achieved by expressing the ventricle

weights relative to body weight during both slaughter stages in order to enable a better comparison of results for the different treatment groups. The present study revealed few differences in the different heart parameters as measured between the dietary treatment groups at both 3 and 6 weeks of age. In contrast, significant differences were observed among males and females for almost all heart parameters during both processing stages. This might be largely attributed to the difference in growth rate between males and females, with males growing more rapidly than their female counterparts. It isapparent that sex had a larger overall effect than diet on the measured heart parameters in this study.

The Ascites Heart Index (AHI), also known as RV/TV ratio, is a useful indicator of PHS in birds (Huchzermeyer & De Ruyck, 1986; Peacock *et al.*, 1989; Julian, 1993; Odom, 1993; Wideman & Bottje, 1993; Lubritz *et al.*, 1995; Olkowski *et al.*, 1995; Wideman *et al.*, 1998). For this reason, the AHI or RV/TV ratio has extensively been used to correlate an elevated blood pressure in the right ventricle to PHS (Wideman & French, 1999). Broilers are considered to have normal pulmonary arterial pressures when RV/TV values range from 0.15 to 0.27, which is the range considered for clinically healthy broilers. In contrast, RV/TV values ≥0.28 are indicative of right ventricular hypertrophy or pulmonary hypertension syndrome (Julian, 1987; Julian, 1988; Lubritz *et al.*, 1995; Owen *et al.*, 1995b; Wideman & French, 1999; Wideman, 2001; Olanrewaju *et al.*, 2008). However, Julian *et al.* (1989) have reported RV/TV ratios above 0.25 to be an indicator for the development of ascites. Principally, the weight of the right ventricle (RV) signifies the work performed in order to maintain the existing elevation in pulmonary arterial vasculature (Huchzermeyer & DeRuyck, 1986; Peacock *et al.*, 1989; Julian, 1993; Odom, 1993; Wideman & Bottje, 1993; Lubritz *et al.*, 1995; Olkowski *et al.*, 1995; Wideman *et al.*, 1998) and therefore a higher right ventricular weight would indicate that the right ventricle was under excessive pressure. This may in turn indicate a higher RV/TV ratio at slaughter age and thus a higher incidence of PHS. No significant ($P>0.05$) differences were recorded at nearly 6 weeks of age for RV/TV_{FM} ratio between the different treatment diets. However, it is important to note that supplementing diets with either dietary betaine or adequate methyl groups, such as choline and methionine (i.e. PC diet), demonstrated the lowest RV/TV ratios (0.243 and 0.242, repsectively) compared to birds fed dietary DMG or the NC treatment diets (0.253 and 0.258, respectively) (**Table 4.29**). These values observed for RV/TV_{FM} ratio for the betaine-supplemented and PC control groups were in line with the values as described by Julian *et al.* (1989) (slightly below 0.25), which also coincided with lower ascites related mortalities. Futhermore, the higher RV/TV ratios (slightly above 0.25) for the DMG or NC diets corresponded with a higher ascites incidence, although non-significant at 6 weeks

of age. The latter improvements in the related heart parameters might therefore suggest that betaine was more successful in attenuating PHS in comparison to DMG and both the control diets by altering cardiovascular related traits in proportion to body mass.

Another important heart parameter to measure during PHS is the LV/BW ratio, since the proportional increase of left ventricle weight to body weight (LV/BW) reflects the amount of work performed to deliver a cardiac output sufficient to supply oxygen and nutrients for sustaining the whole body's requirements (Wideman *et al.*, 1998). The current study revealed a lower LVS/BW and TV/BW ratio for the betaine-supplemented group compared to that of the other treatment diets (P <0.05). The lower LVS/BW and TV/BW ratios observed might be attributed to the significantly (P <0.05) heavier pre-slaughter weights obtained for the betaine-suppplemented birds compared to birds fed any of the remaining treatment diets. Beyond these results, dietary treatment had little to no further beneficial effect on the remaining heart parameters at nearly 6 weeks of age.

As shown in **Table 4.28** and **4.29**, the RV/TV ratios (on a fresh matter basis) were higher (P <0.001) for male birds, which also exhibited a higher mortality rate due to PHS compared to their female counterparts, irrespective of age at sampling. Furthermore, almost all heart parameters were more severely affected in males compared to their female counterparts throughout this study (P <0.05). The higher RV/TV ratio observed in males clearly explains the earlier morphological findings as well as the higher ascites related deaths observed in male broiler chickens. Indeed, the consistently elevated RV/TV ratio among male broilers strongly supports the idea that the fast growth rate of male birds may be a predisposing factor for PHS. Several other researchers have also investigated the effects of ascites in male and female broiler chickens (Mignon-Grasteau *et al.* 1999; Wideman & French, 2000; Pakdel *et al.*, 2002; Druyan *et al.*, 2007; Molenaar *et al.*, 2011) and in agreement with our findings, these authors concluded that total mortalities and RV/TV ratios were higher in male broiler chickens compared to females. In contrast, Wideman *et al.* (1997) did not find an apparent sex difference in ascites development when pulmonary hypertension was initiated by surgical occlusion of the pulmonary artery or an extra-pulmonary primary bronchus. More recent research by Wideman and co-workers (2010) confirmed their previous findings, with no significant difference noted in RV/TV ratio between males and females. However, it is important to note that birds in the present study were subjected to a combination of high incubation and cold brooding temperatures at high altitudes whereas birds from Wideman *et al.* (2010) were reared under standard commercial conditions.

The current study was unable to support an advancement in methodology to study PHS as reported by Kalmar *et al.*, (2010). Kalmar and her colleagues (2010) reported an improvement in

ventricle weights when expressed on a dry matter basis (AHI_{DM}). However, limited research is available supporting this concept and for this reason, hearts collected during the final processing stage were also expressed on a dry matter basis. In contrast to Kalmar *et al.* (2010), the AHI in fresh material (AHI_{FM}) was not numerically improved when ventricle weights were expressed on a dry matter basis (AHI_{DM}). The predicted AHI_{DM} from the AHI_{FM} was highly significant ($R^2_{adjusted}$ = 0.955) (**Figure 4.17**), therefore it appears that AHI_{FM} and AHI_{DM} may be highly correlated, collaborating with reports of Kalmar *et al.*, (2010). However, regression analysis did not reveal an improvement in methodology by freeze-drying the heart ventricles, since similar values were obtained for both AHI_{FM} and AHI_{DM} (0.270 and 0.272, respectively).

5.2.1.1 The influence of diet and sex on mortality due to PHS

In addition to our morphological findings, supportive evidence was provided by the consistently higher total mortalities (**Figure 4.14**) and mortalities due to PHS (**Figure 4.15**) found in the treatments where methyl groups were limited (DMG and NC diets), as these tended to be higher throughout the study. Our data showed that birds that received dietary betaine had a 26.2% and 34.06% decrease in total mortalities and ascites related deaths, while birds fed the PC treatment diet had a 20.72% and 29.94% decrease in total mortalities and ascites mortalities, respectively, compared to the DMG supplemented or NC (P <0.05). In addition, birds fed adequate methyl groups (betaine and PC groups) also revealed numerically lower total and ascites related mortalities in contrast to birds fed the NC (methyl-inadequate) dietary treatment group. The latter data therefore clearly suggests that supplementing betaine to the broiler chicken's diet under ascites conditions may have exerted positive effects in attenuating both the incidence and progression towards PHS as revealed by the morphological and mortality data. To our knowledge, our results are therefore the first to report the favourable effect of supplementing betaine to the bird's diet in an attempt to reduce PHS in rapidly growing broilers. Our findings, however, did not support that of Kalmar *et al.* (2010) who reported a lower incidence of broiler ascites syndrome following dietary DMG supplementation, an intermediary metabolite of the amino acid glycine, in comparison to both the control diets. Despite the improvements observed in mortality rates indicated in this trial following adequate methyl group supplementation (betaine and PC groups), it was apparent that management ultimately played a much larger role in the induction of PHS under such severe incidences of ascites (i.e. high EST, low rearing temperatures and high altitude). However, supplementation of betaine to the broiler chicken's diet ameliorated PHS under current trial conditions.

The current study also corroborated with reports from other authors (Decuypere *et al.*, 2000; De Smit *et al*., 2005; Baghbanzadeh & Decuypere, 2008) that indicated a higher incidence of PHS towards the end of the growing period with deaths being more prevalent from week 4 to week 6 (P <0.0001).

Our study also confirmed that male broiler chickens are more susceptible to develop ascites compared to their female counterparts (P <0.05). Furthermore, the progressive increase in gross morphological lesions due to PHS, as mentioned above, in males throughout the study clearly demonstrated males to be more severely influenced by ascites inducing conditions in contrast to females. Similar to the results concerning dietary treatment and ascites incidence, the morphological changes in male broilers were in parallel with their significantly higher incidence of mortalities (both total and ascites related). Most importantly, male broiler chickens had an approximately 4.0 fold higher total and ascites related mortality percentages when compared to females (**Figure 4.16**).

Mignon-Grasteau *et al.* (1999) revealed that female broiler chickens have a slower initial growth rate but a higher maturation rate than male broilers, which may explain their reduced incidence of PHS. It has also been proposed that the incidence of metabolic related disorders later in life might be reduced through restricting growth at an early stage in the broiler chicken's life (Baghbanzadeh & Decuypere, 2008). However, this might not be advantageous to the commercial broiler farmer since early growth restriction will have a subsequent effect on performance throughout the bird's life, resulting in higher input costs and later age at slaughter. The higher growth rate of male broiler chickens observed in this study may explain why males were at greater risk of succumbing to PHS. Mignon-Grasteau *et al.* (1999) also reported a higher ascites incidence in the inherently faster growing male birds in contrast to the females, concluding with our findings.

The modern broiler that has been selected for improved rate of growth, feed efficiency, and higher breast meat yield has difficulty in dealing with the high oxygen demand of the fast growing tissues, making the bird more prone to develop ascites. As PHS and growth rate are positively interrelated (Kalmar, 2010), it is therefore important to evaluate growth rate in conjunction with any alleged treatment for PHS (Wideman, 1988; Reeves *et al.*, 1991; Shlosberg *et al.*, 1991, 1992; Arce *et al.*, 1992; Wideman *et al.*, 1995a, b; Wideman & French, 1999). It has been proposed that the reduction in relative growth rate of birds that succumb to PHS is thought to result due to the bird's restricted cardiopulmonary capacity, which is inadequate to support the body's oxygen and nutrient requirements (Wideman & Kirby, 1996; Wideman & French, 1999). By reducing the rate of growth, oxygen demand for the rapidly growing tissues is also subsequently reduced, which can

lead to a lower incidence of PHS (Julian, 1993). Interestingly, the significant decrease in PHS in birds fed supplemental betaine or the PC (methyl adequate) diets did not result from a slower rate of growth. It is apparent from the BW data that the reduced incidence of PHS when 100% of betaine was added to the diet was not due to a reduction in growth rate of the birds, despite the birds increased oxygen requirements during cold exposure. In contrast, birds fed dietary DMG showed significantly (P <0.05) lower growth rates, however this depressed rate of growth was not accompanied by a reduced incidence of PHS.

5.2.2 The role of different methyl derivatives and sex during oxidative stress in rapidly growing broiler chickens

Interest in various biochemical risk factors related to the heart was investigated due to their potential protective roles in cardiovascular disease development such as PHS. Due to limited biochemical information following ascites induction in literature, these results therefore deserves further consideration. In order to understand the trends in biochemical parameters following ascites induction in birds, all comparisons presented here were made against broilers fed the NC treatment diet, as it was hypothesised that birds fed a diet inadequate in methyl groups would exhibit the highest incidence of PHS. Although some of the biochemical results were not statistically significant, the changes observed generally supported the overall hypotheses of this trial.

Currently, there is little published information available concerning the effects of dietary betaine and its methyl derivative dimethylglycine (DMG) on circulating plasma total homomcysteine (tHCY) concentrations in broiler chickens. This study was therefore the first to compare the effects of different methyl derivatives, di- (DMG) and trimethylglycine (betaine), on plasma tHCY concentration in ascitic broiler chickens. In recent years, there has been increasing evidence indicating that elevated tHCY is a mediating factor for the development of cardiovascular disease, however its role in broiler heart failure still remains to be elucidated. Past human and animal studies have shown a positive association between high tHCY concentrations and cardiovascular (Boushey *et al.*, 1995; Selhub, 1999; Samueals, 2003; Steenage *et al.*, 2003; Trinidad, 2005; Nasir *et al.*, 2007; Alirezaei *et al.*, 2012) or artheroschlerotic disease (Boushey *et al.*, 1997; McGregor *et al.*, 2001). Homocysteine is a sulphur containing amino acid product of methionine metabolism that undergoes auto-oxidation in the plasma, through which free radicals are produced that can cause damage to endothelial cells and promotes low density lipoprotein oxidation (McGregor *et al.*, 2001). Long-term elevation of HCY has been recognised as a mediating factor in

acute myocardial infarction in humans (Nasir *et al.,* 2007; Alirezaei *et al.,* 2012). On this basis, it was hypothesised that elevated HCY levels might be a risk factor for metabolic diseases such as PHS in broilers and that dietary supplementation with betaine and DMG may reduce total plasma HCY levels. Although the exact mechanism through which elevated tHCY concentrations increases the risk of cardiovascular disease remains unknown in poultry, it may be postulated that the high circulating tHCY levels may alter vascular endothelial function (Jacobsen, 1998; Selhub, 1999; Samuels, 2003) which leads to heart failure. Consequently, this has generated interest in finding dietary means to lower HCY, which can be toxic (Trinidad, 2005).

The general finding has been that dietary betaine supplementation to broilers reared under ascites conditions resulted in appreciable effects on plasma tHCY levels throughout this study. A significant elevation (P <0.05) in the level of plasma tHCY was noted in the NC (methyl inadequate), DMG supplemented and PC (methyl adequate) groups in contrast to the betaine group at both 3 and 6 weeks of age (**Figure 4.20** and **Figure 4.21**). Furthermore, a progressive decrease of 26.35% compared to the NC diet for plasma tHCY concentration was observed following betaine supplementation. Broiler diets supplemented with either DMG or adequate methyl group donors (i.e. the PC diet) had no significant influence on plasma tHCY response when compared to the NC diet throughout this study. Based on these findings, it appears that the elevation of homocysteine levels in both the PC and DMG supplemented groups resulted in increased catabolism of methionine to tHCY in the transsulfuration pathway. The assocation between plasma tHCY and PHS in broilers in this study seems to be independent of the dietary choline and methionine concentration, suggesting that other protective effects of methionine and choline alone or in combination might have exerted a protective effect against PHS in broilers. Several possible mechanisms may possibly explain the protective effect of methionine and/or choline against heart failure in broiler chickens. One potential mechanism might be indepently increasing the levels of TSAA which can result in a high level of HDL and lower levels of VLDL and LDL, and has been associated with atherosclerotic disease (Andi, 2012). Furthermore, methionine can have a free radical scavenging effect by being oxidised to methionine sulfoxide in many animal species (Atmaca, 2004; Song, 2013), thereby possibly reducing the ascites induced mortalities observed. However, more research is necessary in broilers to substantiate the latter interpretations.

The important role of betaine compared to DMG and methyl adequate/inadequate diets in lowering plasma tHCY levels was established in this study. Through progressively lowering tHCY, dietary betaine supplementation might have slowed down or reduced the incidence and/or severity of PHS experienced by the rapidly growing broilers under ascites inducing conditions.

Samuels (2003) reported that plasma tHCY concentrations only need to be slightly above normal in order to impose a risk for developing cardiovascular disease. Overall, chicken plasma tHCY concentrations (42.3-53.3 µmol/L at 3 weeks of age; 20.6-40.25 µmol/L at 6 weeks of age) were considerably higher compared to humans (plasma tHCY between 5-15 µmol/L is considered normal for humans) (Jacobsen, 1998). These results provide a valuable reference for the levels of tHCY in broilers reared under ascites inducing conditions. It was demonstrated that birds with tHCY levels lower than 30 µmol/L had a reduced risk of cardiovascular associated disease such as PHS. This data also suggests that the re-methylation pathway is considerably slower in broilers when compared to humans; however, supplementation of dietary betaine successfully enhanced re-methylation and thus lowered tHCY concentration in plasma.

In the present study, differences in tHCY concentrations were only significant at 3 weeks of age between males and females, with numerical differences occuring at 6 weeks of age. This might suggest that differences exist concerning HCY metabolism between males and females, but that it is age dependant. Non-significant effects between tHCY and sex has also been previously reported in rabbits (Alirezaei *et al.*, 2012a), which has been related to specific sex steroids (Giltay *et al.*, 1998).

Re-methylation of HCY can occur either via betaine, through increasing betaine homocysteine methyl transferase (BHMT) activity, or via CH_3-THF, or through increasing Methionine Synthase (MS) activity (Craig, 2004; Alirezaei *et al.*, 2012a, b). Various studies in both animals and humans have reported re-methylation of HCY to be equally partitioned between both these two pathways and that betaine is a vital methylating agent (Barak & Tuma, 1983; Finkelstein & Martin, 1984, 1986; Pillai *et al.*, 2006). However, little evidence is available concerning HCY re-methylation in poultry. Ethanol feeding in animals as a means to induce oxidative stress has been shown to affect several hepatic enzymes, including decreasing the activity of MS (Alirezaei *et al.*, 2011). Consequently, betaine dependant BHMT activity is increased in order to maintain hepatic SAM at normal concentrations (Craig, 2004; Alirezaei *et al.*, 2011). In addition, previous investigations in broilers and rats showed that dietary changes in TSAA, choline, or betaine levels did affect hepatic BHMT activity (Barak *et al.*, 1996; Finkelstein *et al.*, 1983; Saunderson & Mackinlay, 1990; Emmert *et al.*, 1996; Park & Garrow, 1999; Saarinen *et al.*, 2001; Pillai *et al.*, 2006b). Although measuring the BHMT and MS activity was beyond the scope of this study, previous studies with rats (Barak *et al.*, 1996) and chickens (Saarinen *et al.*, 2001) revealed elevated hepatic betaine pools following dietary betaine supplementation. A possible mechanism through which betaine demonstrated a HCY reducing effect could be attributed to an increase in betaine dependent remethylation due to a higher betaine availability and increased BHMT enzyme activity

in the liver and kidney. In light of this research, it may also be suggested that betaine supplementation to broiler chicken diets may have reduced HCY concentrations through conserving methionine levels, which, based on previous research, would be expected to increase hepatic enzymatic activity in the liver or kidney. However, it remains uncertain whether the betaine associated HCY reduction was due to a higher BHMT or MS activity and this warrants further investigations. In contrast, N, N-dimethylglycine (DMG) (a by-product of BHMT re-methylation) is known to be a potent inhibitor of BHMT (McGregor *et al.*, 2001; Stipanuk, 2004) and is normally excreted in the urine or metabolised to sarcosine (McGregor *et al.*, 2001). Due to lack of SAM remethylation, this might possibly explain why diets containing additional DMG did not progressively reduce plasma tHCY concentrations, since the increase in BHMT activity that one might have expected could have been inhibited by the negative feedback from DMG. It may also be proposed that DMG metabolism might be disturbed in individuals with PHS; however more research is needed to investigate this theory. Furthermore, dietary supplementation with betaine might have overcome the competitive inhibition of DMG on BHMT and in this way progressively reduced total plasma HCY concentrations to a greater extent than did dietary supplementation of DMG alone. Optimal tHCY lowering through supplementing betaine might present a new opportunity for research in order to overcome DMG feedback inhibition of BHMT and thereby reduce PHS in broiler chickens. One proposed method includes shifting the equilibrium from DMG to betaine in order to continue remethylation of homocysteine to methionine (McGregor *et al.*, 2001). However, plasma betaine and DMG concentrations were not measured in this study. Further research is needed to elucidate the relationship between betaine, elevated plasma DMG, and BHMT activity and their roles in PHS in broilers.

High tHCY concentrations have been found to have a negative influence on the expression of antioxidant enzymes (Alirezaei *et al.*, 2011), which may escalate the damaging effects of ROS produced under ascites inducing conditions. Furthermore, it has been well established that auto-oxidation of homocysteine generates reactive oxygen species (ROS) or reactive nitrogen species (RNS), which in turn can lead to oxidative stress and injury (Alirezaei *et al.*, 2011). Although the body has several natural defenses against ROS, such as dietary free radical scavengers, the endogenous tripeptide glutathione, and the enzymatic antioxidants (Alirezaei *et al.*, 2011), there is considerable evidence that oxidative damage may directly or indirectly lead to heart damage due to the generation of the free radicals (Orrenius *et al.*, 2007; Nian, 2008). These end products of lipid peroxidation (i.e. ROS and RNS) can alter the structure, and thus properties, of several biological macromolecules (i.e. lipids, proteins, and nucleic acids), ultimately leading to myocardial

dysfunction (Orrenius *et al.*, 2007; Nian, 2008). Most importantly, lipid peroxidative damage produced by ROS was previously reported to play an important role in the pathogenesis of PHS in rapidly growing broiler chickens (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995; Bottje & Wideman, 1995; Geng *et al.*, 2004). In fast growing broilers, lipid peroxidation damage is almost universally associated with the excessive generation of H_2O_2 (Maxwell *et al.* 1996; Nian *et al.*, 2008) and therefore, lipid peroxide levels may also be involved in the degeneration of heart tissue or development of right ventricular hypertrophy (Aksit *et al.*, 2008).

The present study was also designed to examine whether the dietary supplementation of betaine or DMG could prevent oxidative heart damage in broilers via its indirect antioxidant-like properties (Alirezaei *et al.*, 2010, 2011, 2012). Currently, one of the most common and well-recognised approaches to measure the effects of free radicals, including ROS, is by measuring the oxidative damage resulting from lipid peroxidation (Nian, 2008). In the present study, two indicators of lipid peroxidative damage, 4-HNE and TBARS, were measured in order to evaluate the effect of both diet and sex on ascites development. Due to its negative biological effects, both HNE and TBARS have been used widely as biomarkers for lipid peroxidative damage in tissues (Seppanen, 2005).

The NC group demonstrated consistently higher lipid peroxidative (i.e. 4-HNE and TBARS) values compared to the remaining treatments diets and it may therefore be suggested that oxidative stress is involved in the pathogenesis of PHS in rapidly growing broilers. Furthermore, high levels of lipid peroxidation end-products throughout the study indicated that broilers reared at cold temperatures suffered from elevated lipid peroxidation. In the present study, significant differences were found for both 4-HNE and TBARS at 3 and 6 weeks, respectively. Birds fed either dietary betaine or the PC treatment diets revealed a 26.98% and a 33.10% lower (P <0.05) 4-HNE concentration, respectively, than did birds fed the NC treatment diet**.** These results corresponded well with the lower concentrations of lipid peroxidation end-products observed for the betaine supplemented group suggesting that these factors are associated with a reduced risk of PHS in rapidly growing broilers as also indicated by the morphological changes in the liver and heart and reduced ascites mortality rate. The current findings therefore revealed that supplementation with dietary betaine may have some beneficial effect in preventing heart failure in broilers due to the constantly lower concentrations of lipid peroxidation end-products. Although no significant differences were obtained at nearly 6 weeks of age concerning 4-HNE concentration, it is important to note that birds fed adequate methyl groups (i.e. betaine, DMG, choline, and methionine) had numerically lower lipid peroxidation (4-HNE) values than did birds fed inadequate methyl groups

(i.e. NC-diet). DMG supplementation to broilers reared at low temperatures caused a significant decrease in TBARS concentration at 6 weeks of age in contrast to broilers fed the NC treatment diet ($P \leq 0.05$). Furthermore, it is also noteworthy that birds fed either dietary betaine or the PC treatment diet displayed numerically lower TBARS values than did broilers fed the NC treatment diet.

Despite the divergent findings regarding the different lipid peroxidation assays (i.e. 4-HNE and TBARS), overall the results of the present study showed that betaine as well as DMG were able to lower the concentration of potential indicators of lipid peroxidative damage that may have been brought about by PHS. Both betaine and DMG have previously been reported to exert antioxidant like properties (Alirezaei *et al.*, 2010, 2011, 2012; Kalmar, 2011; Kalmar *et al.*, 2010). Ganesan *et al.* (2010) and Alirezaei *et al.* (2011) have reported increased antioxidant enzyme activity in the cerebellum of rats fed dietary betaine following ethanol induced oxidative stress. Betaine might have therefore stabilised both cellular and subcellular membranes in the liver and myocardium cells by equally restoring non-enzymatic and enzymatic antioxidants (Ganesan *et al.*, 2010; Alirezaei *et al.*, 2011).

Although antioxidant enzymes were not measured in the current study, the decreased plasma lipid peroxidative contents, together with the lower ascites related mortality rate following dietary betaine supplementation, suggest that betaine might have reinforced the antioxidant pool through possibly enhancing the activity of the various antioxidant enzymes such as SOD, CAT, and GSH-Px; however, more research is needed to confirm this. Furthermore, betaine might have made the cardiac myocytes and other lipid membranes more resistant to peroxidative damage from free radicals such as ROS; as suggested by the decrease observed in tHCY. The results of the present investigation therefore clearly indicated a protective effect of betaine that might possibly be related to both its methyl group donor properties as well as its antioxidant properties, such as by scavenging free radicals. In contrast, although DMG has been reported to exert antioxidant properties (Kalmar *et al.*, 2010; Kalmar, 2011), it remains uncertain why the DMG supplemented birds in this study exhibited lower lipid peroxidation values, since a higher incidence of PHS and elevated tHCY concentrations were also observed for these birds.

The conflicting responses of the different methyl group derivatives in the present study may possibly be attributed to differences in sensitivity of the different biochemical assays. Since TBARS measures saturated aldehydes (Palmquist & Jenkins, 2003; Song, 2013), a low TBARS value could have resulted either due to aldehydes that had not yet been produced or due to volatile aldehydes that had already been lost during processing and storage of the lipid (Song, 2013).

Compared to the TBARS assay used in the current study, using a HPLC method might be a more direct and accurate measure for quantifying specific aldehydes; however, these methods are very complex and expensive and may not be economically feasible or practical for use on a large number of samples (Song, 2013). Additionally, due to a lack of biochemical knowledge of TBARS assay, the TBA acid diluent in the assay was diluted at 3 times the concentration instead of 2 times, thus suggesting human analytical error. Although the manufacturer suggested these analysed values to be only slightly lower than the expected values, it may be proposed that under the conditions of this study the 4-HNE concentrations may be a more precise indicator of lipid peroxidation and oxidative stress. It is also noteworthy that indicators of lipid peroxidation products (i.e. ROS) were only measured in the blood plasma and not in the heart and lung tissue during this trial, which are tissues well known to be effected during PHS by end products of lipid peroxidation. Furthermore, during normal metabolism, free radicals such as ROS that have a very short lifespan are also being generated in tissues, which might briefly cause localised lipid peroxidation (Nain *et al.*, 2008) and thus result in false positive results.

The current study also demonstrated a significantly increased (P <0.05) 4-HNE content that was 21.56% higher in males compared to females, but only a 7.38% increase in TBARS content was seen in the male broilers at roughly 6 weeks of age. It is therefore suggested that the male broiler chicken's inherently faster growth rate contributed to an increased risk of deaths due to heart failure or PHS as a result of more oxidative induced stress. Moreover, the observed oxidative damage in males can be readily associated with the noticeable changes in morphology observed due to ascites.

It has been well established that impaired heart function has been coupled with insufficiency of energy substrate (Olkowski *et al.*, 2007a). AMPK has been extensively viewed as the energy gauge of the cell. Furthermore, it has been documented that AMPK is phosphorylated (activated) in response to hypoxic stress (Mackenzie, 2010; Mungai *et al.*, 2011). Although the results in this trial failed to achieve significance between the different dietary treatment groups, plasma AMPK concentrations were 9.76% higher in the betaine supplemented group but lower by 5.23% and 12.20% in the DMG supplemented and PC (methyl-adequate) groups, respectively, in contrast to the NC (methyl-inadequate) group at roughly 6 weeks of age. Here a trend towards increased AMPK activity following betaine supplementation in ascitic broilers was observed, linking elevated AMPK activity to betaine supplementation in birds exposed to oxidative induced stress during PHS. These findings may suggest that enhanced AMPK activity might have been part of an adaptive mechanism in broilers fed dietary betaine, with betaine playing a possible role in defense against

oxidative induced stress during the ascites challenge. Through activating AMPK, betaine might have indirectly overcome the cardiac energy dysfunction that was brought about during oxidative stress because of PHS. These results substantiated those of Song *et al.* (2007), who found a remarkable improvement in hepatic steatosis that was associated with a significantly higher hepatic AMPK activation following betaine-supplementation to drinking water in rats. In the present study however, the effects of betaine on the intracellular AMP-to-ATP ratio as well as upstream enzymes modulating AMPK activation, were not investigated.

5.3 The effect of dietary treatment and sex on growth performance and carcass characteristics

5.3.1 Growth and slaughter performance and overall performance efficiency

5.3.1.1 Betaine and DMG as functional substitutes for methionine and choline

PHS remains an important issue challenging the poultry industry, especially during winter periods as a result of the bird's inherently fast rate of growth which can exert deleterious effects on productive performance in poultry under adverse rearing conditions. Supplementation of betaine to the broiler's diet is gaining interest due to its possible economic and bird health benefits. Both choline and methionine, important methyl group donors, are added to commercial broiler diets as essential nutritional feed additives as vertebrates are unable to synthesise these methyl groups endogenously (Kidd *et al.*, 1997). Therefore, the livestock industry is continually being pressured to develop new strategies, such as the use of alternative ingredients (i.e. feed additives), in order to further improve growth and feed efficiency despite the increases and unpredictability in feed prices. However, these additives can be expensive and there is great interest in reducing the need for supplementing broiler diets with methionine and choline through addition of betaine, and possibly DMG, to meet the bird's methyl group requirements in a more economically sound way. Hence, the second goal of this study was to compare the efficacy of the different methyl group derivatives, di- and trimethylglycine (betaine), as possible functional substitutes for dietary methionine and choline.

Performance traits of broilers were influenced by betaine supplementation. It should be noted at this point that the entire discussion concerning cumulative performance was divided into the following periods: 1) 0-14d which is referred to as the starter period; 2) 0-28d which is referred to as the grower period; however, also includes the data of the starter period and, 3) 0-40d which is referred to as the finisher periods; however, includes the data of both the starter and grower periods.

In general, broiler chicks fed diets with added betaine had consistently higher CumBWG while broilers fed DMG had similar or poorer CumBWG compared to both control groups (**Figure 4.9**). Supplementing betaine to the broiler chicken's diet resulted in a 1.74% and 1.60% significantly (P <0.05) higher CumBWG compared to birds fed the NC (methyl-inadequate) diet during the starter and grower periods, respectively; although not significant it should also be noted that the betaine supplemented group exhibited a 0.88% numerically higher CumBWG during the finisher period. Likewise, dietary betaine supplementation showed a significantly higher (P <0.05) CumBWG of 2.86% and 4.02% than did birds fed DMG and showed significantly higher (P <0.05) CumBWG of 3.02% and 2.60% than did birds fed the PC diets, but only during the starter and finisher periods respectively. It is also noteworthy that the betaine supplemented birds displayed a numerically (but not significant) higher CumBWG compared to both the DMG supplemented and PC birds during the grower period. Dietary DMG supplementation however did not demonstrate significant improvements concerning CumBWG compared to both the control diets and in fact resulted in a significantly depressed rate of growth in comparison to the NC group during the entire growth (0-40d) period. Therefore, this study is, to our knowledge,are the first to demonstrate an overall improvement in growth rate in broilers supplemented with betaine compared to broilers that received similar amounts of methyl groups in other forms, either as methionine and choline and/or DMG.

It would be expected that birds with a greater BWG should consume more feed, firstly because these birds would be expected to have a greater maintenance requirement and secondly because of the need to sustain their superior rate of growth (Dono, 2012). However, no significant effects were observed for feed consumption during this experiment, except during the grower phase, where birds fed betaine-supplemented feed consumed considerably more than birds fed either of the control diets, and during the finishing phase, where birds fed DMG-supplemented feed consumed considerably more than birds fed the NC diet. Nevertheless, feed to gain ratio was significantly improved (P <0.05) by betaine, but only during the starter period when compared to birds from either of the control diets. Additionally, a slightly lower (0.66%) CumFCR was also observed for birds fed dietary betaine during the finisher period compared to birds fed either of the control diets, however this difference was insignificant. Similarly dietary betaine supplementation improved feed to gain ratio (P <0.0.5) by 3.88% and 4.57%, respectively, during the starter and finisher periods in comparison to birds fed dietary DMG. These results therefore suggests an overall improvement in feed efficiency following dietary betaine supplementation in contrast to the DMG-supplemented diets and either of the control diets, but only during the starter and finisher periods,.

Despite the effect of dietary betaine supplementation on broiler growth performance, it was also thought that there might have been an improvement in production performance in birds fed the diets containing adequate amounts of methyl group donors (methionine and choline, PC diet) compared to birds fed the diets marginally deficient in these methyl groups (i.e. NC diet). In the case of the NC diet, practically all supplemental choline and some supplemental methionine were removed, thus the only source of TSAA was strictly from the raw materials in the diet whereas the PC diet contained additional methionine and choline that were initially removed. Unexpectedly, similar values were obtained between the two control diets for almost all growth performance parameters (i.e. body weight, body weight gain, feed intake, and feed conversion), except during the grower period when the PC birds exhibited a significantly lower FCR than the birds fed the NC diet. Due to a methionine and choline deficiency, it is possible that a lack of difference in growth performance was due to a slightly higher 0-35d cumulative feed intake in order to overcome this deficiency, which in turn resulted in a similar growth rate and feed efficiency exhibited by birds fed the PC (methyl adequate) diet. Furthermore, it may also be suggested that although methionine and choline were marginally lower in the NC (methyl inadequate) diet, the raw materials from this diet contained sufficient amounts of nutrients, as was the case for choline, to meet the chicken's minimum requirements to sustain growth. Nevertheless, the nutrients supplied by the raw materials of the NC diet were insufficient to support optimal carcass performance in contrast to the birds fed diets containing adequate amounts of methyl groups (PC diet).

The results of this study also demonstrated important benefits for adding betaine to broiler diets in terms of improvements in carcass traits. Similar responses were obtained when feeding dietary betaine in terms of slaughter weight, carcass weight and yield, and carcass portion weights and yields in relation to birds fed the PC (methyl adequate) diet. Such finding support the hypothesis presented here that dietary betaine may be able to substitute the need for supplemental methyl group donors such as methionine and choline without adversely affecting growth and slaughter performance. More importantly, dietary betaine supplementation resulted in significant improvements in carcass yield, breast meat weight and yield, and wing yield in contrast to the NC birds, suggesting an improvement in slaughter performance following dietary betaine supplementation. However, no effects were observed between dietary treatments for carcass fat content. Apart from the improvements in growth performance and feed efficiency, this study also was the first to demonstrate significant improvements (P <0.05) in slaughter performance traits (i.e. preslaughter live body weight, carcass weight, dressing percentage, breast meat weight and yield, leg portion weight, and wing weight) following dietary betaine supplementation in contrast to birds

fed its methyl derivative, DMG. Most importantly, birds fed dietary betaine had a 2.25% and a 4.24% higher (P <0.05) dressing percentage and breast meat yield, respectively, in contrast to birds fed DMG. Overall, these results suggests more efficient nutrient utilisation in birds fed dietary betaine under ascites inducing conditions in contrast to birds fed either the NC (methyl inadequate) and DMG supplemented diets. The mode of action of betaine as a so-called 'carcass modifier' is highly likely to be attributed to its methyl donating capacity (McDevitt *et al*., 2000; Hassan *et al.*, 2005), which may explain its superior performance compared to dietary treatments with fewer methyl groups.

Currently, literature related to the effects of supplemental betaine on broiler chickens under ascites inducing conditions is very limited. The current work clearly illustrated that dietary betaine resulted in improved growth and feed efficiency, but only during the starter and finisher periods in relation to the remaining treatment diets. In this context, betaine may have partially reduced the dietary need for supplemental methionine and choline, especially during a period of an osmotic challenge (such as during PHS) in contrast to DMG, presumably by supplying the portion of methionine and/or choline required as a methyl group donor (Emmert *et al.*, 1998). Consequently, dietary methionine levels could be lowered on average by approximately 23.05% and all supplemental choline could be completely removed from the starter and finisher diets, respectively, provided these diets were supplemented with betaine under ascites inducing conditions. In contrast, a sparing effect of dietary DMG on methionine and choline, as well as betaine, during this study was not observed, contradicting earlier reports of Kalmar *et al.* (2010) who suggested a sparing effect of DMG on choline and betaine.

The ability of betaine to spare supplemental methionine and/or choline has been extensively investigated in poultry, however, it has also been a subject of controversy. Although results have been variable, there is some evidences in the literature indicating that dietary betaine can partially spare methionine (Virtanen, 1995; Rostagno & Pack, 1996; Virtanen & Rosi, 1995; Matthews *et al.*, 1997; Wang *et al.*, 2004; Attia *et al.*, 2005; Zhan *et al.*, 2006) or choline (Waldroup & Frits, 2005), both important methyl group donors in the broiler chicken's diet. The results presented here supported those of other authors suggesting that betaine can partially reduce the dietary need for supplemental methionine (Türker *et al.*, 2004; Wang *et al.*, 2004; Attia *et al.*, 2005) and choline (Emmert *et al.*, 1998; Waldroup & Frits, 2005) under ascites inducing conditions, but only during the starter and finisher periods. Interestingly, the current results partially agreed with reports of other researchers who concluded betaine to be more effictive in improving body weight gain and feed efficiency compared to the supplementation of methionine and/or choline (Virtanen & Rosi,

1995; Virtanen & Rumsey, 1996; Hassan *et al*., 2005). Furthermore, Florou-Paneri *et al.* (1997) recommended that between 30% and 80% of supplemental methionine could be substituted by betaine, while between 20% and 26% of supplemental methionine and 100% of supplemental choline could be replaced by betaine under the conditions of this study without having any adverse effects on bird performance. However, the results of the current study are in contrast to experiments conducted by Lowry *et al.* (1987) who reported that only 25% of total dietary choline could be replaced by dietary betaine in broiler chicken diets. Furthermore, certain authors did not report any improvements in growth performance following betaine supplementation in broilers when reared under heat stress conditions (Zulkifli *et al.*, 2004) or betaine supplementation to a choline free diet (Dilger *et al.*, 2007). Although the current study's findings support betaine as an acceptable alternative for methionine and choline, more research is needed to elucidate under what conditions these substitution may be optimised.

Contemporary consumers prefer lean meat and higher breast meat yield as these are considered to be healthier options. Therefore, the involvement of betaine and methionine in lipid metabolism may be appealing to poultry producers with regards to meat production as a tool to satisfy the consumer preference while also maintaining feed efficiency (Attia *et al.*, 2005). The present results indicated that the betaine, methionine and choline (methyl adequate) diets were effective in providing lean meat via increasing breast meat yield. This was in agreement with results of other researchers (Wang *et al.*, 2004; Hassan *et al.*, 2005; Zhan *et al.*, 2006; Konca *et al.*, 2008), however betaine did not have an effect on carcass fat content, which is in contrast with results from these same authors. The improvement in carcass yield of the betaine supplemented group may be attributed to its role as an organic osmolyte and its ability to increase water retention (Attia *et al.*, 2005). McDevitt *et al.* (2000) suggested that betaine caused relatively lighter breast meat yield, but relatively higher abdominal fat pad, while Esteve-Garcia & Mack (2000) reported that betaine significantly improved carcass yield, but not abdominal fat weight and percentage, corroborating with results of this study.

The reason for the variation in published literature may be contributed to a variety of factors such as the different trial conditions, the varying modes of actions tested, the differences in animal's health and stress status, and concentration of other methyl donors (choline and methionine) in the broiler's diet (Schrama *et al.*, 2003; Lukić *et al.*, 2012). Previous studies by researchers contained adequate levels of methionine and choline (Rostagno & Pack, 1996; Matthews *et al.*, 1997; Schutte *et al.*, 1997; Waldenstedt *et al.*, 1999; McDevitt *et al.*, 2000; Wang *et al.*, 2004; Zhan *et al.*, 2006; Konca *et al.*, 2008), which may possibly shadow the positive effects of dietary betaine

supplementation. The extent to which methionine can be substituted by betaine may also depend on the dietary supply of cysteine (Metzler-Zebeli *et al.*, 2009). When the animal's requirement for cysteine is met, methionine is exclusively used for protein synthesis; otherwise, part of the methionine will be irreversible degraded to cysteine (Metzler-Zebeli *et al.*, 2009). The presence of ionophores in the diet may also help to further explain some of these differences in experiments, which could impair the conversion of choline to betaine (Esteve-Garcia & Mack, 2000). In this experiment, an anticoccidial was included in the diet and there was no coccidial infection evident during the course of this study; it may therefore be suggested that the positive effects of betaine under a coccidiosis challenge did not play a role in the current results.

A number of mechanisms might be attributed to the improved bird performance following dietary betaine supplementation in contrast to DMG or the control diets, which may be ascribed to its diverse physiological roles within the animal's body. First, the improvements in performance and carcass characteristics in the betaine groups compared to the remaining treatment diets might have been attributed to its methyl donating function. As a methyl group donor, betaine might have spared these methyl group donors making them more available for their other important physiological functions in the body. Btaine might have improved broiler performance under ascites inducing conditions by either partially replacing methionine as a methyl group donor or by providing adequate amounts of methyl groups that are necessary to remethylate homocysteine into methionine. By assuming some of methionine's important functions, betaine might have indirectly made more methionine available for other fundamental biological processes such as protein synthesis and the formation of SAM, which both compete for methionine (Finkelstein, 1998; Ratriyanto *et al.*, 2007).

Another mechanism involved in overall improved performance following dietary betaine supplementation might strongly be related to its function as an osmolyte within the animal's body. It is well known that nutrient digestion and absorption is strongly influenced by osmolytic protective mechanisms (Eklund *et al.*, 2005). Due to betaine being one of nature's most effective organic osmolytes (Simon, 1999; Craig, 2004), it is therefore usually added to pigs and poultry diets for this purpose (Kidd *et al.*, 1999), since betaine may substantially provide more benefits than its role as methyl group donor (Horne & Remus, 2012). During osmotic stress, cells loose water and the subsequent intracellular increase of ions interferes with protein and enzyme structure and function and ATP production, eventually leading to cell death if allowed to continue unchecked (Horne & Remus, 2012). In response, cells try to rectify the ionic imbalance through activating the Na/K pump in order to pump inorganic ions (i.e. K^+) across the cell membrane as a means to

maintain cellular homeostasis, however this is an energetically costly process (Horne & Remus, 2012). In addition, these inorganic ions can alter protein structure and can bind to the active sites of several enzymes, thus diminishing their activity and water imbalance will continue until the cell ultimately shrinks due to its inability to hold water (Kidd *et al.*, 1997; Craig, 2004). In contrast, organic osmolytes such as betaine have been shown to be highly compatible with the former functions and therefore do not disrupt normal metabolic processes (Kidd *et al.*, 1997; Wehner *et al.*, 2003). In addition, the proposed uptake of betaine is by diffusion, which has no harmful effect on energy production and is therefore less energetically costly than other electrolytes (Cronje, 2007). Betaine has been shown to result in improved growth and feed efficiency during periods of osmotic disturbance caused by water salinity stress through protection of intestinal epithelium (Honorbakhsh *et al.*, 2007a, b). Since osmotic disturbance is considered to be related to PHS, it could be expected that betaine would have accumulated more in the hypososmotic cells of the osmotically challenged chicks. Through accumulating in the cells of the ascitic chicks, betaine might have improved the intracellular water imbalance and disruption in intestinal volume, in turn facilitating digestive and absorptive processes (Petronini *et al.*, 1992; Eklund *et al.*, 2005; Metzler-Zebeli *et al.*, 2009). Therefore, by indirectly supporting intestinal structure and function (Attia *et al.*, 2005) betaine might have contributed to improved bird growth and carcass performance. Furthermore, being an anti-stress agent (Saunderson & MacKinlay, 1990), betaine might have improved the response of broiler chickens under ascites inducing conditions through rendering the cells more resilient to stress during a PHS occurrence, especially during the first two weeks of life (when birds were cold stressed) and during the last two weeks of life (where there is an increase in the prevalence of ascites), thus, the activity of betaine was likely to be enhanced during these periods in the osmotically stressed chicks.

Additionally, the improvements in performance and carcass characteristics in the betaine groups compared to the remaining treatment diets might have also been indirectly attributed to its energy sparing role. High intracellular betaine concentrations might have ultimately reduced the need for cells to pump ions across the cell membranes in order to maintain an osmotic equilibrium, thus sparing energy for ion pumping (Moeckel *et al.*, 2002; Cronje, 2007; Mahmoudnia & Madani, 2012). This spared energy may stimulate cell proliferation (Eklund *et al.*, 2005) which in turn may promote digestion and absorption within the gastrointestinal tract. In essence, birds consuming more betaine are therefore better able to retain water, allowing more energy for important functions such as growth and production (Jahanian & Rahmani, 2008; Mahmoudnia & Madani, 2012).

Finally, improved bird performance following dietary betaine addition to the broiler chicken's diet might also be explained by a negative feedback mechanism of DMG, a by-product of BHMT re-methylation, which strongly inhibits BHMT activity and controls methyl group transfer (Stipanuk, 2004). Indirectly, DMG might have thus spared betaine rendering it more available for its other important functions such as its osmolytic activity which is considered particularly important during stressors such an ascites challenge. Although desirable effects have been demonstrated following betaine supplementation in contrast to DMG supplementation, future studies are certainly warranted to assess the repeatability of these results when using different broiler strains, basal diets, and different ascites inducing conditions. These studies also need to elucidate the different mechanisms likely to be involved in improving growth and carcass performance during an ascites challenge.

5.3.1.2 Males versus females

Many researchers have reported a difference in growth rate between male and female broilers (Rodenlli, 2005; Sam *et al.,* 2010). The results in our study showed significant differences (P <0.05) between males and females in body weight gain, feed intake, and feed to gain ratio from placement until the end of the growing period (d40). In agreement with other researchers (Rondelli *et al.* 2003; Sam *et al.,* 2010), a significant variation in feed intake was observed, with males consuming more feed than did their female counterparts. Fischer (1985) acknowledged that males are behaviourally more active than females and this may explain their higher feed intakes and faster weight gains compared to females. Similar to the findings of Sam *et al.* (2010), males exhibited superior performance, as these individuals displayed heavier body weights and had higher weight gains than the female birds. Our results also confirmed that of Leeson (2000), who noted that the feed to gain ratio is usually lower in males than in females at a corresponding weight after about 30 days of age. Thus, the growth rate of females plateaus at an earlier age than males and fat deposition also starts at a relatively earlier age at the expense of muscle growth, hence the lower weight gains and decrease feed efficiency in females. On the other hand, Samarakoon (2012) only showed a lower FCR in male broilers at 3 weeks of age, with little other significance during the first and last two weeks of rearing. In conclusion, growth performance was significantly improved in male broiler chicken's above that of females as depicted by their higher BW's, higher weight gains, and lower feed conversion ratio regardless of dietary treatment.

A comparison was made for the commercial Ross broiler strain between the different sexes and significant effects due to sex were observed in the slaughter parameters. Generally, poultry

companies select the broiler strain and sex that will result in the most economic performance (Shim *et al.*, 2012). As expected, males demonstrated higher final body weights (P <0.05) and carcass weights (P <0.0001) compared to the females, agreeing with results from López *et al.* (2011). These heavier weights are most likely related to the distinct differences observed in growth rate between the male and female individuals. Likewise, Shim *et al.* (2012) reported a significantly higher preslaughter live body weight for males compared to females, whereas other authors (Ojedapo *et al.*, 2008) reported no significant effects in final live body weight between the different sexes. Rondelli *et al.* (2003) and Ojedapo *et al.* (2008) revealed heavier carcass weights in Ross females compared to their male counterparts, contradicting the outputs found in this study. The results presented here showed no significant affect on dressing percentage between male broilers and their female counterparts, which is in agreement with those obtained from Rahayu *et al.* (2008). However, reports from Kidd *et al.* (2005) and López *et al.* (2011) contradict these results, instead observing the opposite for males compared to their female counterparts. It is well known that sex has a significant influence on carcass and abdominal fat deposition (Ojedapo *et al.*, 2008), with females in general having a higher carcass fat content in comparison to males. This phenomenon can primarily be ascribed to differences in hormone function between sexes, with female hormones being responsible for stimulating fat production (Rondelli *et al.*, 2003). Current results revealed that sex, irrespective of diet, significantly affected abdominal fat characteristics, with males having lower carcass fat content compared to female birds, as expected. However, Rondelli *et al.* (2003) differs with these results as no significant differences for either abdominal fat weight or abdominal fat yield between males and females. The higher breast meat weight (P <0.05) obtained in males compared to female Ross broilers agreed with reports from that of Shim and co-workers (2012) and differences observed in breast meat weight (López *et al.*, 2011) and breast meat yield between sexes were in accordance with those previously documented (Young *et al.*, 2001; Rondelli *et al.*, 2003; Kidd *et al.*, 2005; López *et al.*, 2011; Shim *et al.*, 2012). Similar to difference in response between dietary treatment groups, the difference in response to carcass fat content between sexes might also be accredited to different ages at slaughter, with values expected to be more pronounced when birds have sexually matured (Rahayu *et al.*, 2008). The weights of the whole thighs and drumstick (leg portion) were significantly affected by sex (P <0.0001), with males having heavier weights than females. Although Shim *et al.* (2012) did not find any significant effects on leg portion weight between males and their female counterparts, Rondelli *et al.* (2003) have indicated that females generally display a lower leg and thigh proportion (leg portion yield) in comparison to males. The results of the current study are consistent with Rondelli *et al.* (2003), together with previous

observations from others (Rahayu *et al.*, 2008, Shim *et al.*, 2012) that showed a proportionally smaller leg portion (LP) in females than males. The meat of the broiler wings also marginally contributes to the total carcass meat compared to the other cuts of the carcass, such as the breast, drumstick, and thigh meat. Males showed heavier wing weights (P <0.0001), but lower wing yields (P <0.05) compared to females. Shim *et al.* (2012) reported significantly heavier wing weights for males in comparison to female, however, did not observe a significant effect for wing yield between sexes. The heavier weights and yields for the individual cuts for most of the carcass traits in males is likely to be attributed to the heavier live body weight and carcass weights (400.33g and 289.68g, respectively) compared to that of the females. The differences observed concerning these and the breast meat parameters might be explained by the difference in anatomical structure between males and females. According to Mountney (1976) (as cited by Rahayu *et al.*, 2008) females encompass a finer bone structure and a more rounded body and shorter keel. Generally, the thighs and drumsticks (leg portion) are also comparatively shorter in males.

Commercial farmers generally place broilers as-hatch, thus disregarding the influence of sex on performance parameters. However, using separate sexes for the purpose of grow-out enables better uniformity of the flock, in turn allowing better management as well as reaching market weight at an earlier age. Furthermore, by rearing males and females separately, males can be slaughtered earlier than females, therefore saving on feed cost (Veerapen & Driver, 1999). In order to meet modern consumer demands for value-added poultry choice cuts, males can be grown for longer to achieve the require target slaughter live weight, heavier carcass weight and higher meat yields of the different choice cuts (Veerapen & Driver, 1999). In the case of the smaller female carcasses, they can be marketed to meet the whole carcass market (Veerapen & Driver, 1999).

5.3.2 Peformance efficiency factor (PEF)

5.3.2.1 Effect of dietary betaine and DMG on overall production performance efficiency in broiler chickens

Overall, PEF gives an excellent indication of the performance efficiency of broiler production systems that includes feed efficiency, mortality rates, and bird body weight (Samarakoon, 2012); a high PEF value depicts a higher overall return for the operation. PEF was significantly affected by the dietary treatments used in this study. In accordance with improved growth and carcass performance, dietary betaine supplementation also resulted in a 17.37% higher PEF value than did its methyl derivative, DMG (P <0.05). Although PEF did not differ significantly between the

betaine supplemented, NC, and PC dietary treatment groups (P >0.05), it is noteworthy that betaine supplementation yielded a 7.73% higher and a 5.78% higher PEF than did birds fed the NC or PC diets, respectively. Overall, these results suggest that dietary betaine supplementation resulted in the best performance and therefore may offer optimal return when fed to broilers under ascites inducing conditions in comparison to DMG or choline and methionine deficient/supplemented (control) diets.

5.3.2.2 Effect of males versus females on overall Production Performance Efficiency in broiler chickens

Interestingly, the current data demonstrated female birds to have a 24.62% higher PEF value in contrast to male birds. This is most likely attributed due to their higher survival rate (i.e. low ascites related mortalities) and therefore a higher overall CumBWG at the end of this study. Under ascites challenged conditions such as high altitudes and cold temperatures it would therefore be more feasible to grow female birds when opting for a higher return on investment.

5.4 The Ascites Model

One main objective of this study was to create an ascites model in order to explore dietary means to reduce the severity and incidence of ascites. This was achieved through a combination of high incubation temperatures followed by colder temperatures during brooding and grow-out. However, the following should be noted regarding temperature in this dissertation. Similar to recent authors (Leksrisompong, 2005), the temperature concerning high incubation strictly refers to the temperature as experienced by the embryo, hence eggshell temperature (EST), which was measured daily during mid to late incubation (ED_{11} to ED_{18}). However, most other authors based their observations and conclusions solely on machine air temperatures. The two are not the same and therefore cannot be compared in a consistent and logical manner (Leksrisompong, 2005). Furthermore, the temperature referred to during brooding and broiler grow-out indicated the temperature as experienced by the juvenile chick, hence litter temperature, and not that of air temperature.

The main purpose of the chosen temperatures during incubation and brooding to induce ascites development was twofold. The primary aim was to increase the workload on the heart from incubation onwards by reducing the relative size of the organs (especially that of the heart) in proportion to the body. Chickens incubated at high EST may have increased risk to develop ascites

due to an inadequate development of the pulmonary vasculature, which in turn increases their metabolic demands for oxygen (Lubritz & McPherson, 1994; Leksrisompong *et al.*, 2007). The next step was to expose the chicks to exogenous hypoxemic conditions that have been well associated with ascites development. This was achieved by exposing the chicks to cold stress conditions during brooding. The cold temperatures employed during brooding theoretically should have induced a higher metabolic rate, which would have increased the oxygen need of the broiler chicken. While trying to maintain a constant body temperature, their heart rate would also subsequently be increased. Furthermore, these birds were hatched and reared at relatively high altitudes (1350m or 4500ft. above sea level) that could have placed an even greater burden on the chick's hypoxemic status. In order to fulfil the high oxygen requirement, general circulation of red blood cells would be enhanced to increase the oxygen supply to the body tissues, and also causing an increase in viscosity of the blood (Decuypere *et al.*, 2000). In broilers raised under these circumstances, cardiac output (or heart contraction rate) must increase to cope with the oxygen deficiency in the animal, ultimately causing pulmonary hypertension, right ventricular hypertrophy, and heart failure. The resultant increase in the intravascular pressure would cause the fluid to leak from the cells into the abdominal cavity and pericardium, until the birds finally die from these lesions (Julian, 1993; Decuypere *et al.*, 2000; Tekeli, 2014). The effects of high incubation and cold brooding and rearing temperatures at high altitudes on bird performance and ascites development are therefore clearly in line with the literature and are discussed below.

5.4.1 Did the Ascites Model lead to changes in ascites susceptibility?

Over the past few years incubation has become a critical component of the broiler's life since modern broilers are marketed at 35 days, which means that approximately 34% of its total life span occurs as an egg. For this particular reason, incubation has been deemed as one of the most challenging phases of broiler management (Leksrisompong, 2005; Leksrisompong *et al.*, 2007). Previous researchers have reported chicken embryos to respond to high incubation temperatures through accelerated growth and development (Romanoff, 1960; Christensen *et al.*, 1999; Leksrisompong, 2005; Leksrisompong *et al.*, 2007). However, beyond a certain point, high incubation temperatures interfere with proper embryonic development (Leksrisompong, 2005; Leksrisompong *et al.*, 2007, 2009) and have been reported to negatively affect hatchability and general post-hatch broiler performance (i.e. BW, BWG, and feed conversion efficiency) (Gladys *et al.*, 2000; Leksrisompong *et al.*, 2007, 2009).

In the present study, average eggshell temperature reached 39.1°C (102.38°F) on ED_{18} and was kept at constant machine temperatures thereafter. Therefore, it can be expected that the chicken embryos might have experienced temperatures even greater than 39.1°C (102.38°F) from ED_{18} until day of hatch (21.5 days). This explains our general finding of the high amount of second and third grade chicks; hence, chicks were of poor quality. The "white" coloured chicks observed may have resulted due to poor yolk sac absorption, and consequently diminished absorption of nutrients required by the developing embryo to develop correctly. Furthermore, other chick quality problems associated with high incubation temperatures were also seen in this study, such as excessive blood inside the eggshell, blood on the feathers, short feathers, red hocks, unhealed navels, cross beaks, and various other deformities that are consistent with the observations of other authors (Leksrisompong, 2005; Lourens *et al.*, 2005; Leksrisompong *et al.*, 2007, 2009).

It has previously been demonstrated that the heart is the organ most critically affected by high temperatures during incubation (Wineland *et al*., 2000; Leksrisompong, 2005; Leksrisompong *et al.*, 2007). Leksrisompong *et al.* (2007) reported that chicks incubated at high (39.7-39.9°C, 103.0-103.8°F) temperatures had a significantly smaller heart than embryos incubated at normal (37.5°C - 37.7ºC) temperatures. Likewise, Wineland *et al.* (2000) also noted a smaller heart in chicks subjected to high setter and hatcher temperatures. Due to time constraints, as well as a limited amount of chicks, this study was unable to quantify the effect of high incubation temperatures on organ development. Nevertheless, due to the proven effect of elevated incubation temperatures on organ development (particularly the heart), it can be surmised that the chicks in this study might have had smaller hearts and therefore marginally developed or underdeveloped cadio-pulmonary systems after hatching. Furthermore, given that the chicks in this study displayed classic external symptoms of excessive incubation temperatures (such as poor chick quality and abnormalities), it may be inferred that organ development was likely also negatively influenced, thus the chicks were already limited by their cardiopulmonary system at hatch and already susceptible to ascites.

5.4.2 Severity of the challenge

It is well understood that hypoxia remains the primary cause for ascites development, since any condition that would necessitate a greater metabolic demand or decreased oxygen availability would increase the incidence of PHS (Buys *et al.*, 1999; Fathi *et al.*, 2011). High EST during embryonic development in combination with cold environmental temperatures during rearing successfully induced high incidences of PHS from d_{10} until the end of the study (d_{40}). Ascites was

only expected from 3 weeks of age and upwards due to its incidence being more prevalent towards the end of the growing period (week 4-6), however, some birds displayed signs of ascites development as early as 10 days of age. The total mortality rate, irrespective of dietary treatment or sex, in this study was 25.06% with 21.94% of total mortality being due to PHS; thus approximately 88% of all mortalities were ascites related. Similar results were obtained in previous studies (Balog, 2003; Druyan *et al*., 2009) where chicks were grown under naturally cold conditions. More recently, Tekeli (2014) observed a 17.3% ascites mortality rate in broilers grown under high altitudes (1727 m). In contrast, other researchers have failed to induce such high incidences of PHS under ascites inducing conditions such as cold environmental temperatures (de Greef *et al.,* 2001; Pakdel *et al.,* 2002; Closter *et al.,* 2009; Closter, 2014). The high incidence of ascites might have been related to a reduced cardiovascular development at hatch, thus reducing the chick's ability to supply the rapidly growing tissues with sufficient oxygen during the growing period (Dewil *et al.,* 1996; Leksrisompong *et al.,* 2007; Willemsen *et al.,* 2011). By subjecting an already physiological compromised chick to cold temperatures (Buys *et al*, 1999; Daneshyar *et al.,* 2009; Fathi *et al.,* 2011), this might have further contributed to a greater PHS incidence. The high altitude (1339m; 4393ft) of the Research Farm (Pretoria, South Africa) might also have contributed towards the incidence of ascites, although ascites symptoms can also be displayed at relatively low altitudes, the effects are intensified at altitudes of 1200 m or more, especially during winter (Tekeli, 2014).

The right ventricle to total ventricle ratio (RV/TV ratio) can be used to indicate the development of PHS in the ascitic broiler chicken (Tekeli, 2014). The average RV/TV ratio for all birds that died due to PHS in this study was 0.33, irrespective of diet and sex, which was also higher than values reported in previous studies on broilers (de Greef *et al.,* 2001; Pakdel *et al.,* 2002; Closter *et al.,* 2009; Closter, 2014). These results have been substantiated by other studies where a high RV/TV ratio was associated with cold temperatures during rearing (Shlosberg *et al.,* 1992; Lubritz & McPherson, 1994; Aksit *et al.,* 2008). In addition, other authors (Tafti & Karima, 2000; Ocak, 2006; Dryan, 2012) reported distension of the right ventricle (ventricular hypertrophy) in ascitic broilers, matching the results of this study. This clearly emphasises the severity of the combining high incubation and cold brooding temperatures in causing right ventricular failure, and ultimately ascites. The findings of the current study are also supported by Olkowski *et al.* (2001), who observed similar morphological and pathological changes in ascitic broilers raised at low altitudes (350m).

In conclusion, the conditions used in the present study appeared to have successfully triggered high cumulative incidences of PHS throughout this experiment.

CHAPTER 6: SUMMARY AND CONCLUSIONS

The main aim of this study was to investigate different dietary strategies that would safely reduce the severity and incidence of ascites, especially during winter and at high altitudes, without adversely influencing growth and carcass performance. The primary objective of this study was to create an "Ascites Model" to induce high cumulative incidences of PHS throughout the rearing period. The second aim was to examine if dietary betaine and its methyl derivative DMG could alleviate PHS. The third aim was to evaluate the effect of the methyl derivatives, di- (DMG) and trimethylglycine (betaine), on growth and slaughter performance in broilers. The final aim was to compare male and female broiler chickens in terms of growth and slaughter performance, as well as PHS susceptibility.

The first aim was addressed by subjecting young chicks to a combination of high EST during mid to late induction (ED_{11} to ED_{18}) in combination with cold environmental temperatures during grow out in order to induce high cumulative incidences of PHS. Furthermore, broilers were reared at a relatively high altitude that might have contributed to a greater PHS incidence. From the results presented here, it is clear that an interaction of these various factors, from incubation until the end of the growth period, all may have initiated a pathophysiological progression towards PHS.

Results showed an important protective effect of dietary betaine in contrast to both DMG and the NC groups in attenuating the incidence of PHS. Herein, PHS was significantly (P <0.05) reduced by 34.06% and 17.18% in the betaine supplemented groups compared to birds fed either dietary DMG or methyl inadequate diets (i.e. the NC), respectively. Overall, this data also supports the hypothesis that betaine has the potential to reduce ascites-induced oxidative stress and improve the energy status of cells in osmotically challenged situations. The decreases observed for total homocystine (tHCY) concentration, the different lipid peroxides (4-HNE and TBARS), and increase in AMPK in the betaine treatment groups provide evidence that betaine may prevent oxidative damage in PHS susceptible broiler chickens. The important protective effect of betaine during PHS is most likely attributed to a number of factors, such as its osmolytic and methyl donating properties.

The third aim intended to assess the efficacy of the different methyl derivatives (di- versus trimethylglycine (betaine)) on growth and carcass performance when added to a broiler diet under challenging conditions. In the present study, it was evident that the significant reduction in PHS following betaine supplementation did not result from a slower growth rate and did not negatively influence the carcass performance traits. When birds were placed in a physiologically challenging

situation, the advantages of supplemental betaine became apparent. Results presented here demonstrated a consistent improvement in growth and feed efficiency following dietary betaine supplementation in contrast to birds fed either the PC, NC, or DMG supplemented diets, especially during the starter period (d0 to d14) (P <0.05). Furthermore, the inclusion of betaine resulted in a significant improvement (P <0.05) in carcass traits, including a higher DP, BMW and BMY, and WW in contrast to birds fed dietary DMG or methyl insufficient diets (i.e. the NC diet).

The final aim adressed the difference between male and female broilers in terms of PHS susceptibility and the difference in growth and slaughter performance. The main conclusions were that males were more prone to develop PHS, having a 4-fold or higher total and ascites related mortality rate compared to females (P <0.0001). Necropsies conducted during both week 3 (d 20 & 21) and week 6 (d 40 & 41) revealed significantly higher values in male birds for almost all heart parameters measured in contrast to females (P <0.05). Most importantly, the RV weights were increased (P <0.0001) and became dilated to a greater extent in male birds in comparison to females during both slaughtering stages. Indeed, the consistently elevated RV/TV ratios observed in male broiler chickens strongly indicates that PH due to the rapid growth rate of males was the initiating trigger for PHS (ascites) in this experiment. At final autopsy (d40 &41), the weights of the TV and LVS were also significantly increased in male birds (P <0.0001). Despite the higher RV/TV ratios obtained in males, the latter results also indicated a heavier RV/BW, LVS/BW, and TV/BW ratio in male birds (P <0.0001). Furthermore, the characteristics of PHS were clear and in line with the literature as evidenced by a dilated RV, as well as effusion in both the pericardial and abdominal sacs. The latter morphological changes observed in this experiment clearly indicated male birds to be more severely influenced by PHS in contrast to their female counterparts and that their inherently faster rate of growth was believed to be the primary cause for PHS in the pathogenesis of ascites. Male birds exhibited superior growth rates, achieved heavier body weights, had higher feed intakes, and were more efficient in converting feed to live weight in contrast to their female counterparts (P <0.05). Male broiler chickens also yielded heavier carcass weights, lower abdominal fat depots, and higher weights and yields of the breast and leg portions compared to females (P <0.05).

As a general conclusion, our research demonstrated that betaine is a valuable feed additive in contrast to its methyl derivative DMG in broiler diets, especially under environmentally challenging situations. Dietary betaine reduced tHCY concentration, a well-known indicator of cardiovascular related disorders and was able to increase the AMPK concentration in the bird's plasma. This feed additive was also effective at reducing oxidative stress and the progression towards PHS and

therefore may reduce the severe financial losses incurred by such a metabolic disease. Betaine improved the rate of growth, feed conversion, and carcass performance even when birds were exposed to cold temperatures during the brooding and grow out period. The research presented here confirms males to be more prone to develop PHS due to their inherently faster growth rate. However, despite their higher mortality rates, male broiler chickens had better growth rates, feed conversion efficiencies, and carcass performance traits than did their female counterparts.

CHAPTER 7: CRITICAL EVALUATION AND RECOMMENDATIONS

7.1 Limitations of the study

The effectiveness, reliability and validity of scientific research, no matter how well controlled, are subjected to several limitations that mostly arise from circumstances beyond our control.

The initial aim of this study was to compare the difference in ascites susceptibility between chicks incubated at high vs. normal eggshell temperatures. Unforeseen circumstances on the last day of incubation changed the direction of the initial study and it was decided to use sex as a treatment instead of eggshell temperature (EST), although it was previously shown that sex has an influence on ascites development (Molenaar *et al.,* 2011) with male chicks being more susceptible. Nonetheless, this change did not affect the primary objectives and the statistical outline of the study remained the same. Despite this challenge, the ascites model developed was proven successful and the chicks subjected to the betaine diets exhibited lower ascites related mortalities.

It is essential to prevent heat loss from the embryonic egg when handling eggs with the incubator door open for an extended period of time in order to measure eggshell temperatures. For that reason, we constructed a plastic tent around all of the incubators in order to heat the outside atmospheric temperature to match the machine temperatures. One potential problem undergone when measuring eggshell temperatures was loss of heat from the incubators, especially from the bottom two trays in all of the incubators. In addition, due to the cold winter temperatures, it was noted that the tent took longer to heat up to the desired temperature before the incubator door could be open for recording egg temperatures. Unfortunately, chick samples were not taken on the day of hatch, so that the effect of the high incubation temperature treatment on embryonic organ development, especially of that of the heart, was not ascertained. Furthermore, the "machine effect" was noted during the course of this study and it is recommended that this is to be included in the MANOVA analyses in future studies.

Technical difficulties with equipment a few days before chick placement led to limited pre-heating of the 96-pen trial facility on the day of chick placement. Fortunately, the chicks were to be brooded at temperatures below the standard as part of the ascites inducing model. The mild chilling experienced by the chicks could have influenced the birds' normal feeding behaviour, growth and development, and ascites susceptibility. Nevertheless, all chicks were subjected to the same environment and for that reason it can be presumed that the cooler temperatures experienced by the chicks for the 2 day period did not have a significant impact on the subsequent performance of any particular treatment.

One goal of this research was to achieve new insights for changes in biochemical parameters as indicators of oxidative damage, a common feature shared by individuals with ascites. Although this study aimed to investigate several biochemical mechanisms of defenses used to alleviate ascites incidence, not all the parameters examined were successfully analysed. The GSH-Px activities were examined, but unfortunately misguided kit information and lack of technical support resulted in poor repeatability, inconsistent and inaccurate results. For these reasons, it was decided to discontinue with the analysis. Better GSH-Px results might have been obtained using red blood cell homogenate washed with physiological saline and expressing GSH-Px activity/mg protein. Determining results upon unit/mL plasma is possible, but less accurate (personnal communication, Dr. Masoud Alirezaei). Although all samples should have been analysed for both slaughter stages, limitations in laboratory experience performing assays resulted in some of the assays being unsuccessful. For this reason, some parameters had to be repeated in order to improve the reproducibility and reliability of the assays and therefore some parameters could only be analysed for the second stage of slaughter.

7.2 Recommendations and future research opportunities

The relationship between different EST and ascites susceptibility has not been fully investigated and therefore provides great opportunities for future research.

Despite the improvement in mortality rate, the data here did not reveal any significant improvement with the different methyl derivatives fed on any of the heart parameters, especially that of the RV/TV ratio which is associated with PHS. Nevertheless, birds fed either betaine or methyl-adequate diets (i.e. choline and methionine sufficient diets, PC group) had numerically lower RV/TV ratios than did birds fed DMG and the NC diets. More research is required in terms of assessing the repeatability of these results using different broiler strains, different basal diets, and different management and housing conditions. In addition, further research is needed into the different mechanisms of both betaine and its methyl derivative, DMG, in terms of PHS susceptibility.

The results of the present study indicated that the protective effect of betaine is most likely related to its membrane stabilising action, its methyl group donor properties, role in tHCY remethylation, its prevention of lipid peroxidation through its antioxidant properties and finally its role in conserving energy in osmotically stressed cells. Through relating some of these biomarkers to PHS incidence, methyl group donors such as betaine can possibly be used as a useful

preventative tool for metabolic related disorders, especially during winter periods, at high altitudes, or in stressful environments. Due to limited research however, the potential benefits of supplementing betaine against cardiovascular related diseases such as PHS in broilers still requires further investigation. More trials are required to evaluate the different biomarkers, including extra biomarkers such as GSH-Px, CTNNT-2, and TNF-α, and to evaluate supplementation of different methyl group donors as preventative therapy for PHS in broilers. A strong link between TNF-α and metabolic syndrome such as NAFLD has been observed in humans and therefore a potential benefit may also be found for broilers.

The results of this study failed to show an alleviation of the metabolic syndrome PHS following DMG supplementation in contrast to reports from Kalmar and collegues (Kamar *et al.*, 2011). In this regard, more research is required to assess the repeatability of these results under various conditions. In addition, further research is needed on the different mechanisms of both betaine and its methyl derivative DMG and PHS susceptibility. The degree of the effect of the different methyl derivatives in attenuating metabolic disease and improving growth and carcass performance in broiler chickens may be modulated by its chemical nature, difference in metabolism, difference in rate of absorption, and/or difference in mode of action.

A number of questions remain unresolved in this study and therefore more work on this topic is required. In most instances, the DMG supplemented group performed similar or poorer than birds fed the NC treatment diet**.** Furthermore, the lower lipid peroxidation values (4-HNE and TBARS) at termination of this study for the DMG supplemented group was not explained by a lower total or ascites related mortality rate as one would expect. In this study, DMG was supplemented at 100% methyl group donors, which is above the recommended dosage (1138 ppm instead of 167 ppm). One possible theory might be that this over-supplementation of dietary DMG in the broiler chicken's diet may have increased the risk and/or incidence of PHS. However, further studies under similar conditions are needed to establish safe levels of dietary DMG in broiler chickens. Lastly, knowledge about betaine that can induce AMPK activation in the broiler chicken is very limited and provides great opportunities for future research.

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Appendix

Table 8. 1: Proximate composition of the broiler starter diet as expressed on a percentage of dry matter

	Neg. Control (NC)	Neg. Control + Betaine	Neg. Control + Taminizer D	Pos. Control (PC)	Average
Proximate Analyses:					
Moisture (%)	10.478	10.303	11.431	11.133	10.836
DM (%)	89.522	89.697	88.569	88.867	89.164
Ash (%)	4.026	5.229	4.652	4.503	4.603
CP (%)	23.024	22.983	22.291	22.803	22.776
CF (%)	3.197	3.299	2.800	3.155	3.112
EE (%)	3.620	3.348	3.387	3.235	3.397
NFE (%)	55.655	54.838	55.439	55.172	55.276
GE (MJ/kg)	16.095	16.118	15.838	15.915	15.992
GE (kCal/g)	3.844	3.850	3.783	3.801	3.820
Mineral Analyses:					
Ca (%)	1.013	1.060	0.940	0.873	0.971
P (%)	0.636	0.619	0.593	0.584	0.608
Ca:P	1.591	1.714	1.584	1.495	1.596
Na (%)	0.195	0.175	0.180	0.155	0.176
K (%)	0.850	0.840	0.830	0.825	0.836
Cl (%)	0.353	0.334	0.313	0.324	0.331

Table 8. 2: Proximate composition of the broiler grower diet as expressed on a percentage of dry matter

	Neg. Control (NC)	Neg. Control + Betaine	Neg. Control + Taminizer D	Pos. Control (PC)	Average
Proximate Analyses:					
Moisture (%)	11.903	12.56	11.031	11.256	11.586
DM (%)	88.097	87.844	88.969	88.744	88.414
Ash (%)	4.428	4.403	3.076	4.402	4.078
CP (%)	21.114	20.653	21.324	20.561	20.913
CF (%)	3.600	3.252	3.455	3.601	3.477
EE (%)	4.444	4.304	4.025	4.149	4.231
NFE (%)	54.510	55.232	57.089	56.031	55.716
GE (MJ/kg)	16.105	16.006	16.306	16.293	16.178
GE (kCal/g)	3.847	3.823	3.895	3.892	3.864
Mineral Analyses:					
Ca (%)	0.698	0.677	0.776	0.658	0.702
P (%)	0.481	0.477	0.511	0.496	0.491
Ca: P	1.453	1.418	1.518	1.326	1.429
Na (%)	0.165	0.160	0.205	0.170	0.175
K (%)	0.735	0.740	0.791	0.771	0.759
Cl (%)	0.330	0.327	0.350	0.360	0.342

Table 8. 3: Proximate composition of the broiler finisher diet as expressed on a percentage of dry matter

	Neg. Control (NC)	Neg. Control + Betaine	Neg. Control + Taminizer D	Pos. Control (PC)	Average
Proximate Analyses:					
Moisture (%)	12.986	12.281	12.713	12.478	12.607
DM (%)	87.044	87.719	87.287	87.522	87.393
Ash (%)	3.377	4.002	3.604	3.252	3.558
CP (%)	18.536	19.079	17.847	17.912	18.343
CF (%)	3.550	3.000	3.398	3.402	3.337
EE (%)	4.497	4.748	4.374	4.151	4.442
NFE (%)	57.085	56.890	58.064	58.806	57.711
GE (MJ/kg)	15.960	16.062	16.020	15.827	15.967
GE (kCal/g)	3.812	3.836	3.826	3.780	3.814
Mineral Analyses:					
Ca (%)	0.606	0.614	0.607	0.575	0.601
P (%)	0.409	0.424	0.432	0.411	0.419
Ca: P	1.481	1.447	1.406	1.399	1.433
Na (%)	0.160	0.170	0.180	0.155	0.166
K (%)	0.705	0.725	0.680	0.666	0.694
Cl (%)	0.352	0.362	0.337	0.358	0.352

Table 8.4: Analysed content of selected intermediate metabolites in the choline-to-glycine pathway of the broiler test diets (expressed on an as-fed basis)

		Final Feed Supplement Concentration		
Dietary Treatment		Choline Chloride (Free) (mg/100g)	Betaine (w/w %)	Dimethylglycine HCL (w/w %)
Starter:				
Starter 1	Neg. Control (NC)	132	0.68	<0.09
Starter 2	NC + Betaine	136	0.71	<0.09
Starter 3	NC + DMG	151	0.72	0.15
Starter 4	Positive Control (PC)	147	0.80	<0.09
Grower:				
Grower 1	Neg. Control (NC)	126	0.56	<0.09
Grower 2	NC + Betaine	121	0.61	<0.09
Grower 3	NC + DMG	146	0.61	0.13
Grower 4	Positive Control (PC)	141	0.56	<0.09
Finisher:				
Finisher 1	Neg. Control (NC)	107	0.59	<0.09
Finisher 2	NC + Betaine	104	0.61	<0.09
Finisher 3	NC + DMG	125	0.59	0.15
Finisher 4	Positive Control (PC)	99.5	0.64	<0.09