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**Amelioration of the Effects of Cyclic Heat Stress in
Broiler Chickens with Dietary Betaine and
Antioxidants**

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Submitted in total fulfilment of the requirements of the degree of Doctor of
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Faculty of Veterinary and Agricultural Sciences

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Declaration

I declare that this thesis comprises only my original work towards the degree of Doctor of Philosophy. Due acknowledgement has been made in the text to all other material used. This thesis does not exceed 100,000 words, and complies with the stipulations set out for the degree of Doctor of Philosophy by the University of Melbourne.



Majid Shakeri

November 2019

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Abstract

Controlling and managing environmental conditions is crucial to avoid hyperthermia and heat stress, and hence to successful poultry production and welfare. Heat stress (HS) is one of the most important environmental factors challenging poultry production globally. The detrimental effects of HS on broilers range from reduced growth rate to decreased poultry meat quality. Increasingly, there has been a focus on the use of nutritional supplements as a cost effective HS amelioration strategy. The use of antioxidants such as selenium, vitamin E and polyphenols to improve productivity via reduced levels of oxidative stress in tissues when broiler chickens were exposed to cyclic HS. Another molecule of interest is the osmolyte betaine, which is accumulated in cells under osmotic stress, but also has properties as a methyl donor and antioxidant. The principal findings were that cyclic HS impaired growth performance and physiological responses as increased respiratory rate and rectal temperature caused alteration in blood haematology as pCO₂ reduced and blood pH increased which could lead to impaired product quality. While supplementation of betaine improved growth performance, including improved final body weight and this effect was observed irrespective of HS or additional antioxidants supplementation where it had no impact on blood haematology. The effects of HS and betaine were investigated on meat quality and it was found that HS reduced moisture content, increased lipid oxidation and reduced myofibril degradation, indicating reduced post-mortem proteolysis. This indicates worsening of meat quality as increased lipid oxidation reduces shelf life and myofibrillar degradation is important for the meat tenderisation process. When supplemented betaine was distributed to the breast muscle, and corresponded to improvements in meat quality. This was evidenced by reduced drip loss, lipid oxidation and reduction of Evans Blue dye (EBD) concentration in the breast muscle. Most importantly, supplementation of betaine reduced EBD concentration in vital organs such as kidney and ileum, indicating that betaine could have protective effects under HS. Furthermore, supplementation of betaine improved intestinal and breast muscle structure, including improved villous length and increased muscle fibre diameters which contribute to better growth performance and meat quality. Betaine supplementation was also performed against or with selenium, vitamin E or polyphenols. In general, selenium and vitamin E did not have additive effects on growth, whereas some benefits were

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List of abbreviations

ADFI	Average daily feed intake
ADG	Average daily weight gain
ADJ	Adherent
AOX	Antioxidants
BCCA	Branch chain amino acid
BET	Betaine
BHMT	Betaine-homocysteine methyltransferase
bmp	Breath per minute
Ca	Calcium
CK	Creatine kinase
CON	Control
CP	Crude protein
DS	Desmosomes
DNPH	Dinitrophenylhydrazine
EBD	Evans blue dye
FB	Final breast weight
FBW	Final body weight
FCR	Feed conversion ratio
FD4	fluorescein isothiocyanate-dextran
GPx	Glutathione peroxidase
HPLC	High performance liquid chromatography
h	Hour
HS	Heat stress

KCL	Potassium chloride
Kg	Kilogram
MAPK	Mitogen activated protein kinases
min	Minute
MFI	Myofibril fragmentation index
Mgcl ₂	Magnesium dichloride
MJ ME	Metabolic energy
MLCK	Myosin light kinase
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaN ₃	Sodium azide
PC	Protein carbonyl
PES	Pale soft and exudative
PKC	Protein kinase
POL	Polygain or polyphenol
ROS	Reactive oxygen species
RR	Respiration rate
Se	Selenium
sec	Second
T ₃	Triiodothyronine hormone
T ₄	Thyroxine hormone
TBARS	Thiobarbituric acid reactive substances
TJ	Tight junctions
TN	Thermoneutral
VitE	Vitamin E

Preface

Majid Shakeri wrote the original manuscripts in the thesis, contributing 60% to the final product of the thesis. Majid Shakeri has received contributions from Frank R. Dunshea (15% contribution) and Jeremy J. Cottrell (15% contribution), who kindly revised and edited the thesis. Majid Shakeri also received help from Stuart Wilkinson, Hieu H. Le, Weicheng Zhao, Hafiz A. R. Suleria, Mitchell Ringuet, Rachel M^cQuade, John B. Furness and Robyn D. Warner who contributed to the thesis (10% contribution) by helping him with research methods and the execution of experiments.

There is no work towards the thesis that has been submitted for other qualifications. The current thesis does not include any works carried out prior to the enrolment in the degree. No third party editorial assistance was provided in preparation of the thesis.

For all multi-authored publications or under-review articles in the thesis: Majid Shakeri, Jeremy J. Cottrell, Stuart Wilkinson and Frank R. Dunshea designed the experiments; Majid Shakeri and Frank R. Dunshea conducted the data analysis; Majid Shakeri, Hieu H. Le (chapters 4, 5 and 6) and Weicheng Zhao (chapter 6) conducted the experimental works; Majid Shakeri, Hafiz A. R. Suleria (chapters 4 and 5), Mitchell Ringuet (chapter 3) and Rachel M^cQuade (chapter 6) processed the samples; Majid Shakeri, John B. Furness (chapters 3 and 6) and Robyn D. Warner (chapters 4 and 5) developed methods for the analysis.

The candidate contributed 60% to the following papers and conference proceedings, and co-authors contributed 40%.

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2. Shakeri, M., J. J. Cottrell, S. Wilkinson, R. D. Warner, and F. R. Dunshea. 2018. Betaine ameliorates the impact of heat stress in broilers, improving breast muscle growth and reducing drip loss. 64th International Congress of Meat Science and Technology – Australia Melbourne.
3. Shakeri, M., J. J. Cottrell, S. Wilkinson, J. B. Furness, and F. R. Dunshea. 2017. Betaine improves performance and reduces rectal temperature in broiler chickens during heat stress. The Annual Meeting of the European Federation of Animal Science – Estonia.
4. Shakeri, M., J. J. Cottrell, S. Wilkinson, J. B. Furness, and F. R. Dunshea. 2017. Rearing in hot environments reduces the transepithelial resistance of Ross 308 broilers. 5th International Conference on Food Digestion. Rennes, France.

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CHAPTER 1

Introduction

1.1 The importance of poultry industry

Global production of poultry meat production has increased rapidly over the past few decades, in response to the rise in global poultry meat consumption per capita from 2.88 kg to 14.13 kg (Mottet and Tempio, 2017). Demand for poultry meat production is expected to increase because of the higher feed efficiency leading to lower meat production costs. Poultry meat is more economical than other meats, makes it a globally significant animal protein source. Furthermore, poultry meat does not experience cultural and religious taboos. Poultry is important for small local and large commercial meat production, making it one of the fastest growing animal products (Mottet and Tempio, 2017). Declining poultry final prices results in increased demand for the poultry products (Taha, 2003). The significant increase in demand for chicken meat production in most countries around the world has important implications for the global trading of poultry meat products. Among the broiler strains used for meat production, Cobb and Ross are the most widely produced worldwide (Tona et al., 2010) with almost the same growth performance results. However, it has been indicated that Ross strain is more heat tolerant than Cobb (Zaglool et al., 2019). With the impacts of climate change increasing affecting the production of poultry, the Ross genotype may become the favoured genotype in the coming years. Furthermore, most of commercial farms are private investments, it increases public concerns about the impacts of the environmental temperature on final products which is related to human health, that it is pushing governments to respond to public policies and concerns.

1.2. Climate change and production

Global warming is a result of the atmosphere getting warmer due to greenhouse gas emissions (Stocker et al., 2013). Greenhouse gas emission level sharply increases yearly as a results of human activities. The intergovernmental panel on climate change (IPCC) reports that global temperatures have increased considerably. This organisation predicts

an increases in global temperature at about 1.8-3.9 °C by 2100 (Koneswaran and Nierenberg, 2008) which are much greater than the increase in the last century, where average temperatures grew 0.06 °C per decade (NOAA, 2009). The IPCC's latest report also indicated that climate change "could have severe impacts on different aspects of human life (Gregory et al., 2007), one of the areas is livestock production, an important source of human food. Global warming can have adverse effects on livestock production through different aspects such as competition for natural resources and environmental challenges including heat stress, while the demand for livestock products is expected to increase by 100% by 2050 (Garnett, 2009). Therefore, the challenge is to maintain the balance between productivity, household food security, and environmental preservation (Wright et al., 2012).

In response to market demand, commercial broiler chickens having undergone intensive genetic selection for a better growth rate, which makes them the fastest-growing farmed species. However, selections for rapid growth and heavy musculature have been associated with risks of behavioural, physiological and immunological problems caused by global warming (Elijah and Adedapo, 2006). Among farm animals, poultry compared to other farm animals is more susceptible to environmental temperature changes. There is a range of thermal conditions within which animals are able to cope with and maintain their body temperature stable which could reduce required behavioural and physiological activities that happens during stress conditions. In this regard, chickens are able to only tolerate narrow temperature ranges to sustain the peak of their production, and any unpredictable environmental temperature changes could cost them a series of adjustment and readjustments which may have adverse consequences on poultry production. Therefore, it is important to understand and develop heat-related strategies to reduce the harmful effects of high environmental temperature on chicken's performance.

1.3. Implications of heat stress

Heat stress (HS) results from a negative balance between the amount of energy the body emits to the environment and the amount of heat produced by their body. This adverse impact can be caused by various environmental factors, such as thermal irradiation, humidity and movement; and the scale of said negative balance can be subject to

characteristics of the animal itself, such as its species, metabolism rate, and thermoregulatory mechanisms (Renaudeau et al., 2012).

Heat stress can be devastating to commercial broiler chicken production; and the effects could be worsened with high humidity (Butcher and Miles, 2009). Heat stress interferes with the broilers' comfort and suppresses productive efficiency. During HS, broiler chickens require more energy to make critical life sustaining physiological adjustments in order to reduce their body temperature. Depressed feed intake and minimized activity lessen the heat burden. Reduced amount and quality of final productions are consequences of these responses. Therefore, introducing strategies to reduce the negative effects of HS is important, and the most common strategies are nutritional strategies. Dietary adjustments help reduce metabolic heat production and maintain nutrient intake. Energy intake and amino acid balance is of extreme importance in HS.

1.4. Solutions to cope with high environmental temperature

Genetically modified broiler chickens have higher growth rate in a shorter period of time as they consume more feed, their body produce more heat due to their greater metabolic activity, making them more susceptible to HS than natural breeds. The increasing demand for poultry production around the world calls for re-evaluation of long-term strategies for modern genetically-modifying commercial breeding programmes.

Many strategies have been introduced to the industry, such as improving the housing system, and adding supplementations to the diet to help broiler chickens cope with HS. However, many of these strategies have limited effects on broiler chicken health, while many supplementations such as osmolytes and antioxidants could help them to improve their health and the body's heat tolerance. One of the most important function of the additives is improving gut health. A healthy gut is critically important for the efficient conversion of feed into absorbable form for optimal nutrient utilization, thereby better growth performance of poultry. A healthy gut improves nutrient utilization, stability of the microbiota, and the status of the immune system (Jha et al., 2019). When gut health is compromised, digestion, and nutrient absorption are affected, which in turn, may have a detrimental effect on feed efficiency and greater susceptibility to diseases leading to

economic loss. Therefore, nutritional strategies could be a promising method to cope with HS. This motivates researchers to look for affordable and effective additives to ameliorate the effects of HS. Betaine as an osmolyte can protect tissues, and antioxidants such as selenium, vitamin E and polyphenols improve enzyme activities and reduce oxidative damages. Therefore, they have been introduced to improve poultry growth performance and product quality. However, limited researches have been evaluated the effects of mentioned additives alone or in combination with each other on Ross-308 breed which is a common breed in Australia under HS conditions.

Selenium (Se) is shown to be an essential element for poultry nutrition, having several important roles in biological processes. Organic forms of Se are more efficient than inorganic forms, and can be produced biologically via Se microbial reduction. Selenium deficiency led to several negative effects, including impaired growth, poor feathering, reduced hatchability, and reduced immune function, which resulted in large economic losses for the industry (Brandt-Kjelsen et al., 2017). The National Research Council recommends a level of 0.1 to 0.15 mg/kg of selenium in various poultry diets (NRC, 1997). However, a review by (Habibian et al., 2015) showed that several studies suggested that higher doses of Se could have better effects under HS conditions. Furthermore, as there is a relation between Se and vitamin E in the sense that vitamin E “spares” the need of Se, it may be deduced that a positive effect on chicken yield indicators may be manifested even in the case of an insufficient supply of both these substances. A combination of Se with VitE up to 300 IU/kg has been recommended to improve the humoral immunity and provide better immunity response of broiler chickens under HS (Sahin and Kucul, 2001).

Furthermore, polyphenol compounds have considered additives in broiler diets because of their antioxidant activities in various in vitro systems. However, low level of them in a diet may not be effective, as these compounds are poorly absorbed in the gut and their concentrations in target tissues are too low to provide effective antioxidant defences (Surai, 2014). Many studies observed positive effects of polyphenols when supplemented in broiler diets up to 2.5 % under TN and HS conditions (Aengwanich and Suttajit, 2010; Tufarelli et al., 2016).

1.5. Importance of this project

High environmental temperature decrease meat industry production resulting in significant economic losses globally. The United States livestock industry alone has an annual economic loss between 1.69 and 2.36 billion dollars due to this issue (Rojas-Downing et al., 2017)) when the environmental temperature is higher than 30 °C (Esminger et al., 1990). Due to the common occurrence of environmental stressors across the globe, many studies have investigated the detrimental effects of HS on poultry production (Lin et al., 2006; Habibian et al., 2016) and suggested solutions, one of the promising strategies is the nutritional strategy, such as using betaine and antioxidants to reduce the adverse impacts of HS and improve the quality of products. It has been shown that HS negatively affects the welfare and productivity of broilers chickens. However, further research is still needed to improve the knowledge of the basic mechanisms associated to the negative effects of HS in poultry, as well as to develop effective interventions. . Therefore, this thesis attempted to investigate the effects of an osmolyte as betaine and different antioxidants on productivity, and provide better understanding of the mechanisms involved in this matter.

1.6. Research overview

This thesis is divided in 7 chapters, of which two have been published and two papers under review. Chapter 2 presents the current literature and researches related to this thesis, in which the importance of poultry industry and poultry meat is discussed. Then, the effects of HS on production and meat quality and strategies to reduce the effects are introduced. Chapters 3, 4 and 5 explores the effects of betaine and antioxidants (selenium, vitamin E and polyphenols) alone or in combination on performance and meat quality parameters, antioxidants activities and gut physiology when broiler chickens are exposed to cyclic HS. In chapter 6, betaine distribution, gut and muscle morphology were examined when chickens were exposed to short-term HS and injected with a marker named Evans Blue Dye to determine the amount of damages in different tissues. Chapter 7 contains general discussion, conclusions and recommendations of this thesis. The main findings are discussed and appropriate recommendations are provided. Finally, at the end of this written work is the Reference List.

CHAPTER 2

Review of the literature

2.1. The poultry industry

Chicken meat was once a by-product of egg production, being derived from end of life laying hens or surplus cockerels. However, from approximately the 1960's there has been the development of dedicated meat chickens, or "broilers". Since then chicken meat consumption per capita has increased from approximately 5 kg per annum to ~49 kg, to become the most popular meat in Australia (Lindgreen et al., 2008), with a similar trend internationally. In Australia, over 800 functional commercial poultry farms produce 80 % of Australia's meat chicken, some of which having over 4 sheds with the capacity of 50,000 chickens each. Commercial farms produce nutritious and high quality products that become more affordable for customers every year (Australian chicken meat federation, <http://www.chicken.org.au/facts-and-figures/>). The success of the industry is mostly attributed to a more efficient structural organization, improved production and processing technologies, and a continuing responsiveness to consumer demands.

2.2. Benefits of poultry production

Poultry production is highly efficient, with the short breeding interval of the birds lending themselves to more rapid rates of genetic improvement. This is evidenced by the rapid improvements in feed conversion ratio (FCR) in Australian broilers, with efficiency gains equating to a reduction of ~1kg feed per kg of growth (Figure 1). To produce 1 kg of chicken's meat requires about 2-2.5 kg feed, whereas for other meats this increases to about 7 kg (Anonymus, 2017). This makes poultry meat comparatively inexpensive, costing approximately 40 % the price of beef and 70 % the price of pork. Poultry production has high economic return due to its short production cycle, where the cycle normally takes 7-8 weeks, which means the cycle can be repeated 6 times a year (Wahyono and Utami, 2018). Furthermore, poultry production needs a smaller area in comparison with other animals, making it more achievable for small investors. Despite

the advantages of the poultry production, chickens seems to be sensitive to environmental temperature changes compared to other farm animals (Lara et al., 2013).

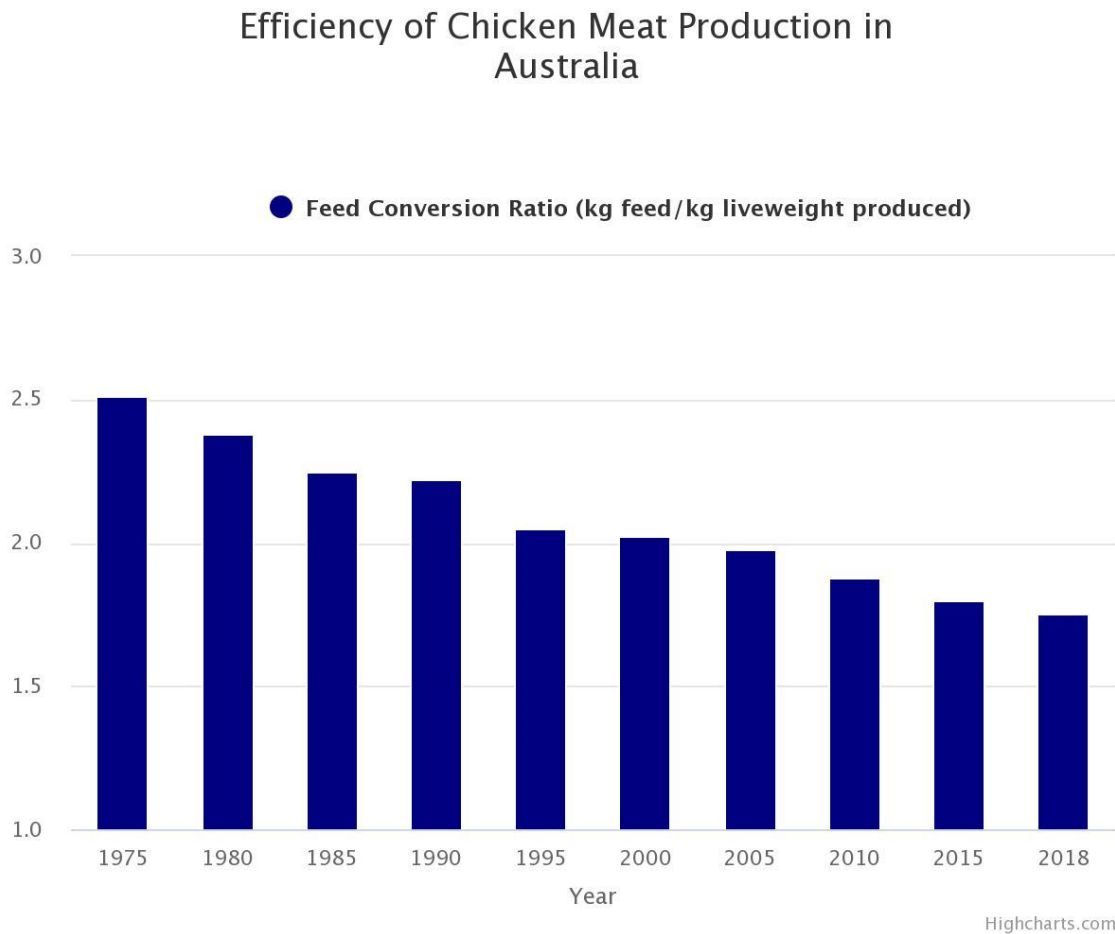


Figure 1. Improvements in feed conversion ratio of Australian broilers since 1975 (Federation, 2006).

2.3. Shifts in animal production and the challenges presented by a changing climate

One of the most significant structural shifts in global meat production is the emergence of large, vertically integrated broiler production facilities in South Asia (Bruinsma, 2017), meaning that there is a far greater proportion of animal production in tropical and subtropical areas. Additionally the impacts of climate change are exerting themselves on all animal production systems. The Australian climate has warmed by 1°C since 1909, which is increasing the frequency, duration and severity of heat waves (Figure 2). With existing projections that CO₂ emissions are above those required to meet the 2 °C targets of the COP21 “Paris agreement” the issues of producing animal protein in hotter climates is one that will only gain in significance in the coming decades. Poultry and pigs are the

most abundant protein sources internationally, and both are poorly adapted to hot environments in that they lack active sweating responses. Where animals cannot maintain their core body temperature within their biological set points they can enter what is referred to as a “heat stressed” state. This can range in effects from reduced feed intake and poorer growth to more overt tissue damage and in extreme incidences even heat stroke (Tang et al., 2018). This negatively affects efficient production of animal protein and in the USA alone has been evaluated to cost animal production industries billions of dollars a year in lost production (St-Pierre et al., 2003). The aim of the following section is to discuss how broilers respond to environmental heat and how this influences their productivity.

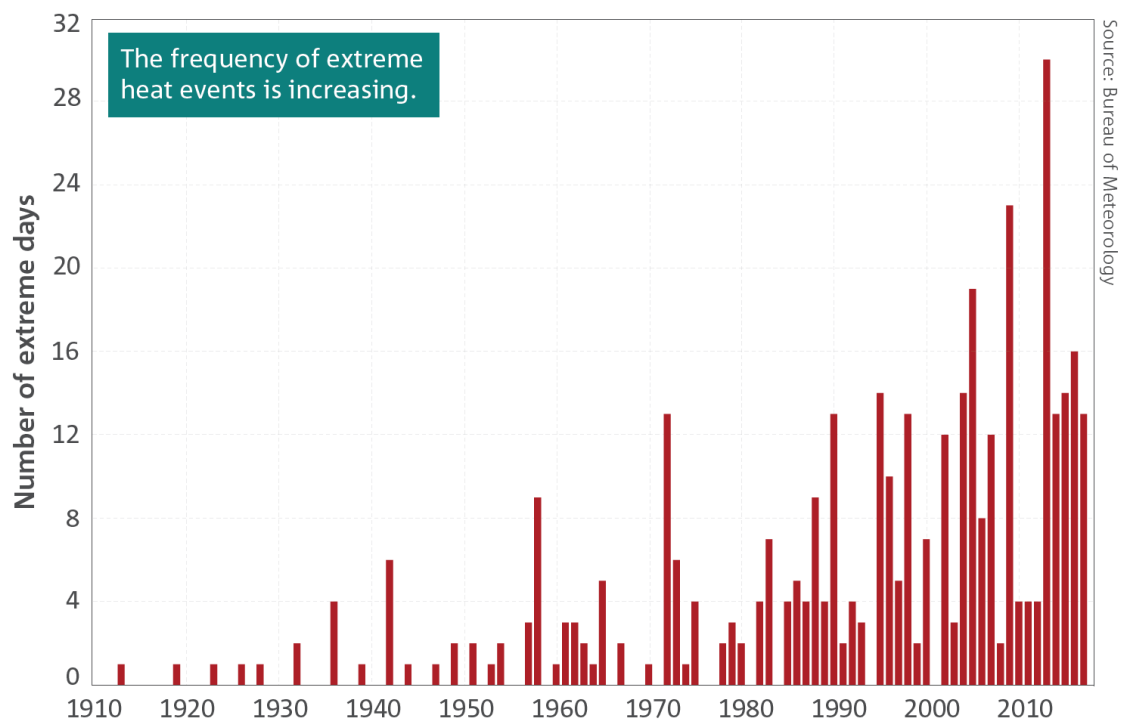


Figure 2: Proportion of days above the 99th percentile for the dairy mean between 1909 and 2017, representing the number of “extreme” hot days per year (Phipps et al., 2011).

2.4. Temperature and poultry production

High environmental temperature and high humidity are the main cause of HS among chickens and it negatively affects poultry production and final product quality (Meremikwu et al., 2013) (Figure 3). Broiler chickens try to lower their body temperature by panting, but with high environmental humidity, panting decreases poultry heat loss from the lungs, which makes them more prone to HS. Therefore, chickens could have

more welfare problems when high temperature couples with high humidity (Butcher and Miles, 2009) which may lead to heat stroke and death. Usually, the optimum humidity should not be over 50 % when environmental temperature is over 35 °C, or the chickens may have difficulty in breathing, impaired ability to deliver adequate amounts of oxygen to the body, resulting in over loading of the respiratory system (<http://www.poultryworld.net/Health/Article>). Usually, the optimum temperature for growing broilers is 18-22 °C (Sahin et al., 2006) and any temperature higher than that could cause HS (Daghir, 2009). Heat stress normally happens in tropical areas, during the summer season in non-tropical areas or when there is a negative balance between environmental temperature and body heat production. In fact, chickens confront with three different ranges of temperature zones as comfort zone, critical zone and upper critical zone. In the comfort zone (18-25 °C), chickens can maintain their body temperature with minimum effort while in the critical zone (26-35 °C), maintaining body temperature requires the help of physical heat regulation. Furthermore, in the upper critical zone (higher than 35 °C) chickens cannot dissipate their body's temperature, and physiological disorders appear following multi organs dysfunction resulting in death (Sohail et al., 2012). In warm tropical and subtropical regions, meat type chickens are kept in semi-open house systems that are more in contact with the environmental temperature changes leading to adverse production efficiency.

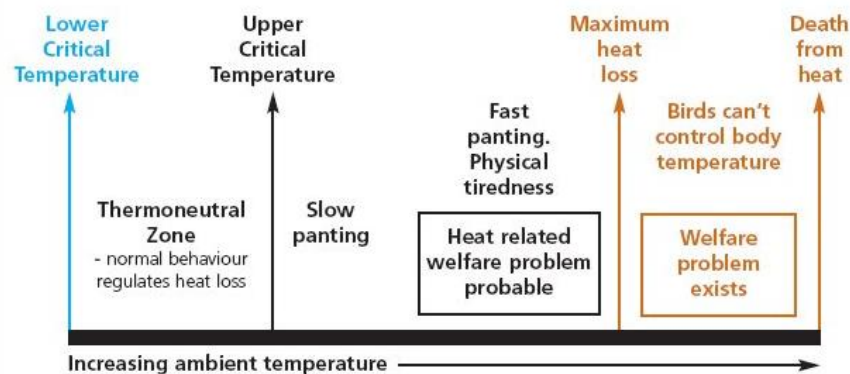


Figure 3. Diagram of thermoneutral zone for broiler chicken (Youssef et al., 2015). In the thermoneutral zone, chickens are in a comfort condition. In the upper critical temperature, chickens attempt to cope with environmental temperature by increasing

energy expenditure on defensive mechanisms to reduce their body temperature. Higher temperatures cause serious welfare problem and lead to death.

2.5. The housing systems and impacts of environmental temperature

The housing systems vary in form due to environmental temperature or other management factors, from ventilated sheds to free-range farms where chickens roam outside the sheds in daytime. Different systems entail distinct requisites, among which stocking density, the use of medication, and the use of purpose developed feed. Chicken production in Australia predominantly features large-scale establishments of intensive production. The majority of broiler farm comprises modern tunnel ventilated sheds equipped with automated mechanical ventilation systems, or tunnel ventilation converted from naturally ventilated sheds. Tunnel ventilated sheds are usually long, each keeping up to 50,000 chickens. Nevertheless, both systems are affected by the environment at higher temperatures. In these systems, sheds must be open during the day to provide a chance for chickens to walk freely, or poor ventilation may result in broilers expressing obvious signs of HS (heavy breathing). Therefore, HS may cause a decline in growth performance, final meat product and increase mortality even in fully automatic housing systems. Therefore, broiler chickens under HS must regulate their body's temperature through different thermoregulation mechanisms to reduce the negative effects of HS.

2.6. Thermoregulatory responses

2.6.1. Overview

Chickens are homeothermic and capable to regulate their body heat balance and save most of obtained energy from feed for growth (Yahav et al., 2004). Broiler chickens are able to control and maintain their body's temperature when temperature is not upper than critical temperature zone by thermoregulation mechanisms. Thermoregulation is the ability to keep the body temperature within certain boundaries to ensure the function of all vital organs, even when the surrounding temperature is very different. However, when the environmental temperature reach to upper than critical zones, to minimize the energy cost of thermoregulation, chickens use a variety of morphological and behavioural responses to adjust their body's temperature by balancing the ratio of heat loss and heat gain (Yahav et al., 2005). Thermoregulation in broilers is mediated by the hypothalamus

in conjunction with central and peripheral thermoreceptors. Thermoreceptors are specialized nerve cells located in the preoptic anterior hypothalamus that are able to detect environmental temperature and are found throughout the skin, allowing sensory reception throughout the body to evoke adequate thermoregulatory responses via the control of physiological, endocrinological, and behavioural responses to adjust the rates of heat produced by metabolism, dissipated to and absorbed from the environment (Yahav et al., 2006).

2.6.2. Behavioural responses

Physiological response is a reaction that causes a physical response to a stimulus. In broiler chickens, physiological responses are initial defensive actions against HS. Broiler chickens attempt to reduce their body temperature through different actions. Featherless parts of body appear to act as “thermal windows” where heat dissipation is most efficient, a means of thermoregulation at high temperatures in broilers (Yalcin et al., 1997). Plumage related adaptive behaviours in the context of thermoregulation include: keeping the wings away from the body, ruffling of feathers and dustbathing. Furthermore, they limit feed intake, avoid walking and standing while increase, gular fluttering, drinking and resting (Suganya et al., 2015). They splash water to increase evaporative cooling from skin areas (Dawson and Whittow, 2000), use sand baths or bath in bedding such as wood shaving (commercial), or move to a shaded area to avoid absorbing more heat from the environment. Chickens under HS tend to distance themselves from each other to maximize heat loss from their bodies (Etches et al., 2008). Despite of the benefits of these initial responses that help chickens to keep their body’s temperature in the safe area, they might not be sufficient when their body’s temperature goes higher than tolerance area, when other biological mechanisms are required to reduce their body temperature. Increasing peripheral blood flow to reduce excessive produced heat and increasing panting to facilitate evaporative cooling (Daghir, 2008; Mack et al., 2013a) are biological responses that can help chickens to cope with the excessive body temperature.

2.6.3. Biological responses

Since chickens cannot cool their bodies by evaporation of moisture from their skin as they lack sweat glands, they are equipped with air sacs located in the lung that help them to

increase heat loss to the environment from their body. Air sacs improve air circulation on surfaces contributing to increase gas exchanges with the air, in order to lose excessive heat from the body. Furthermore, the air sacs can maintain normal body temperature and fluid levels. When the environmental temperature increases, their principal metabolic option for cooling is to evaporate moisture from the lining of the throat and the air-sacs. The increase in air movement over the air-sacs, leads to a significant increase in moisture evaporation, leads to loss of body heat and cooling. To further aid with radiant heat loss to the environment chickens redistribute blood flow to the skin. This requires a commensurate reduction of blood flow from elsewhere and is principally derived from the body core. Both mechanisms help chickens to reduce their body's temperature when the environmental temperature is above the tolerance area. However, there are consequences for these responses against HS.

2.7. Effects of heat stress and responses

2.7.1. Heat stress and performance

Heat stress happens when chicken's body temperature reaches to critical levels and mechanisms for heat dissipation cannot cope with environmental and metabolic heat load. Such failure would cause heat stroke results the body begins shutting down. Heat stress generates behavioural, physiological and immunological responses which is a known to impaired growth performance and reduced meat quality of commercial broilers (Cahaner and Leenstra, 1992; Shakeri et al., 2018). Moreover, genetically modified breeds of meat-type chickens has higher rate of growth and metabolic rate, which is accompanied by a higher heat production level due to increased feed intake (Havenstein et al., 2003). In fact, less selected broiler breeds have higher feed wastage compared to modern breeds (Zuidhof et al., 2014). Apart from a high mortality rate (Meremikwu et al., 2013), decreased feed intake and growth performance, HS could negatively affect intestinal development (Garriga et al., 2006; Mitchell and Carlisle, 1992a). Heat stress reduces feed consumption resulting in reduced the amount of meat production, this is more significant when temperatures increases from 21.1-32.2 °C or 32.2-37.8 °C that cause a reduction in feed consumption of about 9.5-9.9 % chicken/day (North and Bell, 1990). As a consequence, body weight decreases by about 34 % when environmental temperature

increases from ~ 21 to 32 °C, and this reduction is about 14.3 % to 21.2 % when the environmental temperature is at 32.2 °C to 37.8 °C, respectively (North and Bell, 1990).

2.7.2. Endocrine and biochemical responses to heat stress

During HS conditions, chickens pant faster due to the increase in blood pH increases which is a reason for respiratory alkalosis and hyperthermia (Sahin et al., 2003). Moreover, decreasing in serum vitamin and mineral concentrations happen (Anderson, 1994). Increased panting rate is an physiological response to maintain body temperature, as it entails evaporative cooling and hence the respiratory dissipation of heat, contributing to keep thermal homeostasis (Calder Jr and Schmidt-Nielsen, 1966; Yahav et al., 2004). Higher panting rate causes respiratory alkalosis by increasing the exhalation of CO₂ that reduces blood bicarbonate concentration, which leads to a lower concentration of hydrogen ions and a boost in plasma pH (Mongin, 1968). Respiratory alkalosis is a condition that occurs when there is an acid/base imbalance that triggers metabolic reactions in muscle tissue, ATP depletion, and increases osmotic activity in the cell membrane (Sandercock et al., 2001) and gives rise to body water imbalance (Sandercock et al., 2001). All of the abovementioned processes accelerate the rate of metabolism, energy expenditure, and the depletion of ATP (Yahav et al., 2004). There are two pathways that generate required ATP for muscle contraction: glycolysis which converts glucose into pyruvate, and increasing creatine kinase (CK) through the tricarboxylic acid (TCA) cycle (Zhang et al., 2012b). Pyruvate oxidized by the TCA cycle in order to produce abundant ATP when sufficient amount of oxygen is provided. If the rate of pyruvate production surpasses the rate of its oxidation through the TCA cycle, redundant pyruvate will be converted into lactic acid, which dissociates into lactate and H⁺ at physiological pH resulting in lower muscle pH and decreasing Ca²⁺ (Stackhouse et al., 2001). The metabolic acidosis converts pyruvate to lactate and induces higher dependency to anaerobic metabolism in order to produce more energy in the muscle, which remains so during the early stage of post-mortem. The aerobic metabolism in muscle switches to anaerobic metabolism once the oxygen supply stops, which raise lactic acid production through the conversion of pyruvate to lactate under anaerobic conditions by the relevant rate limiting enzyme, lactate dehydrogenase (Zhang et al., 2012b). Thus, under HS, the rate of anaerobic glycolysis to generate energy by breaking down muscle

glycogen is faster than under the normal temperature condition. The escalated lactic acid production creates a rapid drop in pH when the body temperature is elevated (Wang et al., 2017). Actually, the increased activity of CK is an indicator of muscle damage and the malfunction of sarcolemma in muscle cells (Ostrowski-Meissner, 1981), which corresponds the disturbance of intracellular calcium ions (Ca^{2+}) homeostasis in muscle. Calcium ions are involved in a variety of eukaryotic cell signalling pathways such as muscle contraction, cellular proliferation and secretion of hormones. Furthermore, it mediates alterations in the permeability of muscle membrane could be caused by hyperthermia or acid/base imbalance (Sandercock et al., 2001). The swift fall in muscle pH before and after slaughter accelerates protein denaturation and decreases water holding capacity in muscle (Channon et al., 2000; Owens et al., 2000). However, pH is not the only factor that influences the meat quality, and other metabolic pathways could be involved to identify the effects of HS.

It has been indicated that HS could alter the functions of hypothalamo-pituitary axis and orthosympathic nervous system. Under chronic HS, the changes in the blood pH impair the immune system function and alter body's hormonal activity (Mack et al., 2013a). As a results, the concentration of thyroid hormones such as triiodothyronine (T_3) and thyroxine (T_4) were altered (Mack et al., 2013a) that are involved in metabolic activity. Thyroid hormones are essential in metabolic pathways which are necessary for skeletal development, growth and maintain body temperature (Darras et al., 2000) that help chickens to adapt their body's temperature with the environment. Due to the involvement of the thyroid hormones for reproductive function in chickens, a disruption of thyroid activity could impairs performance (Lara and Rostagno, 2013). The main hormone produced with thyroid gland is T_4 , but it converts to a more active form which is T_3 . Both hormones determine the rate of metabolic and have a positive correlation with growth rate. There are two main factors that determine the thyroid activity are feed intake and environmental temperature. The thyroid gland activity regulates by thyroid stimulating hormone (TSH) which it is depends on thyrotropin releasing hormones (TRH) and environmental temperature. It has been indicated that thyroid hormones activity decrease during summer or hot seasons (Aleena et al., 2016). These changes are harmful for lean tissue accretion and for growth (Figure 4).

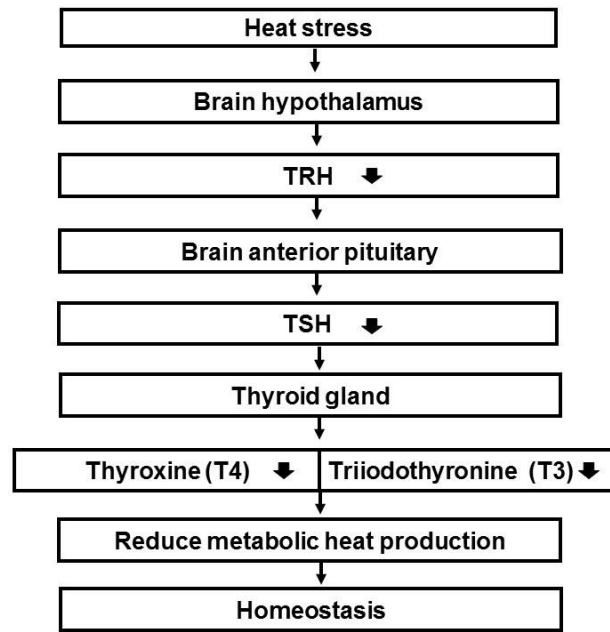


Figure 4. The depressed activity of the thyroid gland reduces concentration of the thyroid hormones during the heat stress. Heat stress impacts the hypothalamic pituitary axis and reduce thyroid stimulating hormone (TSH) secretion which it is depends on thyrotropin releasing hormones (TRH) due to less thyroid gland function and reduced T₃ and T₄ level (Aleena et al., 2016).

2.7.3. Heat stress and the digestive system

The consequence of increase of blood flow to skin is a reduction in gastrointestinal, hepatic and renal blood flow, making these organs particularly sensitive to HS. Reduction of blood flow reduces oxygen supply to these organs with high metabolic activities leading to oxidative damages (Li et al., 2015). Epithelial cells in the intestine provides a barrier isolating the external environment from the internal body, providing tolerance to water and digested nutrients. Intestinal epithelial cells adhere to each other through three distinct intercellular junctional complex as desmosomes (DS), adheren junctions (ADJ), and tight junctions (TJ). Desmosomes are localized dense plaques that are linked to keratin filaments, while ADJ and TJ are composed of transcellular proteins (Figure 5). These proteins are connected intracellularly through adaptor proteins to the actin cytoskeleton. In contrast to transcellular transport, the transfer of molecules through the space between the cells across an epithelium is passive down a concentration gradient, and this transport is regulated by the TJ. As multi-protein complexes, TJ not only hold

cells together, but they form channels allowing the transport of substances across the epithelium.

The molecular composition, ultrastructure, and function of TJ is regulated by intracellular proteins through a series of intracellular signalling pathways that includes myosin light kinase (MLCK), mitogen-activated protein kinases (MAPK), protein kinase C (PKC) among others. Any external or environmental factors that alter the balance between protein kinases and protein phosphatases that affect gut permeability due to disruption of TJ. Tight junctions regulate epithelial permeability and paracellular diffusion via two pathways, leak and pore. The leak pathway allows transport of large noncharged solutes while the pore pathway allows the transfer of large charged molecules. Tight junctions also function as a fence between the lumen and host. Any changes and alteration in gut cells and disruption of tight junctions could increase permeability by elevated serum levels of fluorescein isothiocyanate dextran (FITC). Fluorescein isothiocyanate dextran is used as a fluorescent probe to study cell permeability such as intestinal and renal cells that supports the measurement of processes such as blood flow and membrane damage. It is a large molecule which cannot pass through gastrointestinal barrier except when these barriers disrupted.

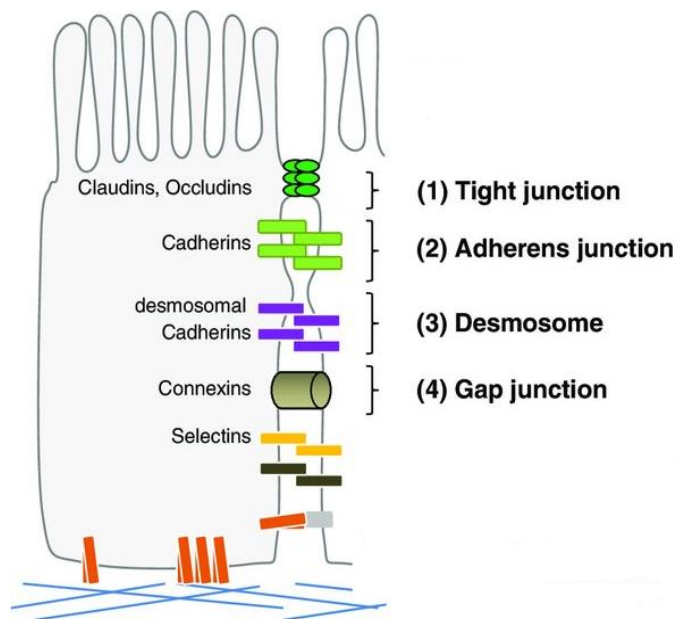


Figure 5. Cell adhesion molecules and junctional are available in large quantities in epithelial intestinal tissues. Tight junctions are linked to actin filaments (1). Adherens junctions are linked to the actin cytoskeleton (2). Desmosomes are connected to intermediate filaments (3). Gap junctions connect the cytoplasm of two adjacent cells and are linked to microfilaments (4) (Schnell et al., 2013).

Heat stress increases intestinal permeability to various external microorganisms such as pathogens that could cause infection and inflammation. Under thermal neutral conditions, paracellular junction are rigorously regulated. However, under HS conditions, the TJ barrier becomes compromised and luminal substances leak into the blood stream. . Indeed, HS dysregulates intestinal barrier function and induces leaky gut via alteration of tight junction proteins (Tellez et al., 2017). Leaky gut is a condition that induces chronic systemic inflammation, as it makes the immune system more active which requires high resources of energy and causes malabsorption that prevents the absorbing of the vitamins and nutrients that are necessary to make energy which that impact negatively the performance of the chickens. The leaky gut problem can be attributed to osmotic stress. Heat stress increases water losses, consequently, it leads to deregulated osmotic pressures and reduces intestinal integrity results of such osmotic stress and oxidative stress. Osmotic stress leads to large changes of Ca^{2+} , H^{+} and K^{+} , and strongly influences the accumulation of H_2O_2 in the external medium, indicative of ROS production (Beffagna et al., 2005). Therefore, alteration in gut structure is accountable for stimulating free radicals production such as reactive oxygen species (ROS) by allowing unwanted pathogens enter into the blood. High levels of ROS production increases damages to biological molecules such as proteins, lipids or carbohydrates. The final outcome is impaired performances.

2.7.4. Heat Stress and meat quality characterises

The effects of HS on organs functions directly or indirectly affects meat quality by causing mitochondria to malfunction and affecting energy-substance aerobic metabolism, resulting in increased glycolysis and intramuscular fat deposition (Lu et al., 2017), in which leads to less customer acceptability (Lara and Rostagno, 2013) due to paler meat colour (Wang et al., 2017; Zhang et al., 2012b) with low water holding capacity (Feng et

al., 2008) and increased cook and drip losses (Wang et al., 2017). The loss of water holding capacity in pale, soft and exudative meat is especially deleterious, as a considerable part of broiler meat is used for further processing that involves marination, tumbling, and cooking. Heat stress can contribute to the development of pale, soft and exudative meat (Wang et al., 2017; Wilhelm et al., 2010).

2.7.4.1. pH decline

pH is among the most promising indicators that could impair meat quality (El Rammouz et al., 2004). Consequences of post-mortem muscle energy metabolism are the drop in pH through glycolysis pathway (Figure 6), due to the accumulation of H⁺ ions in muscle from adenosine triphosphate hydrolysis (Sandercock et al., 2001; Wang et al., 2009) by pyruvate and lactate dehydrogenase enzymes in anaerobic conditions that pyruvate converts to lactate. As the energy metabolism declines post-mortem, the muscles enter rigor mortis and the muscle temperature lowers because of the reduction of blood supply and the application of chilling. The rates of pH and the drop of temperature during rigor development are arguably two of the most important post-mortem factors affecting meat quality known as a protein-denaturing condition, associated to pale colour and significant water loss (Wang et al., 2009), protein denaturation due to poor texture as the results of the rapid post-mortem glycolysis (Alvarado and Sams, 2002; Wilhelm et al., 2010).

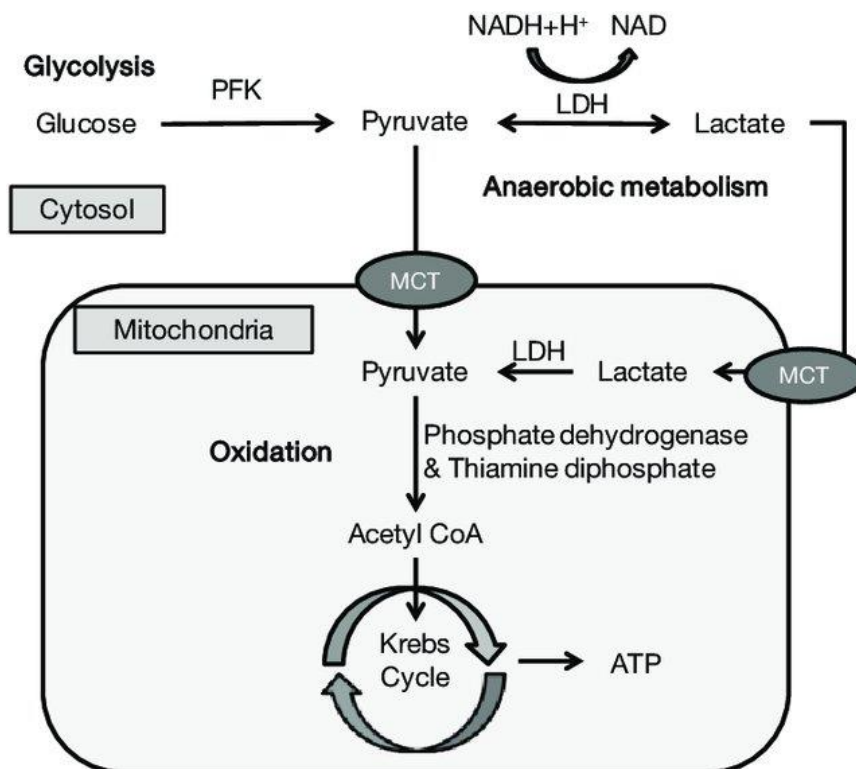


Figure 6. Glycolysis produces pyruvate and lactate in aerobic or anaerobic conditions. In aerobic conditions, pyruvate is transported into the mitochondria through MCT. In anaerobic conditions, LDH enhances lactate formation in the cytosol. Excess lactate is transported into the mitochondria again through MCTs and is oxidized to pyruvate. Monocarboxylate transporter (MCT); adenosine triphosphate (ATP); lactate dehydrogenase (LDH); tricarboxylic acid (TCA); phosphofructokinase (PFK) (Ryoo and Kim, 2018).

2.7.4.2. Increasing reactive oxygen species (ROS)

Broiler chickens are genetically modified to produce more meat leading to higher metabolic rate and body temperature than normal breeds that makes them more vulnerable to HS and oxidative damages than their ancestors (Altan et al., 2003; Tan et al., 2010). Oxidative stress happens when production of ROS is increased, resulting in altering vitamins and mineral concentration by increasing excretion from the body (Smith et al., 2003; Şahin and Gümüşlü, 2007; Song and King, 2015) that are important to enhance the defence system. Reactive oxygen species as a major reason for oxidation produces by cellular metabolism and external sources, including feed that contain oxidized from fats and lipids in mitochondria (Cadenas and Davies, 2000) by stimulating metabolic

oxidation and increasing the activity of enzymes related to β -oxidation and fatty acid transport (Mujahid et al., 2007a). Fatty acid β -oxidation is a process during which fatty acids are broken down resulting in cleavage of two-carbon units from the carboxyl end of fatty acyl-CoA with the formation of acetyl CoA. The acetyl groups generated from β -oxidation of the fatty acid participate in the Krebs's cycle resulting in the formation of NADH and FADH₂. The reduced coenzymes are oxidized by donating the protons and electrons to oxygen in the mitochondria to synthesize ATP.

When HS is more severe for mitochondrial homeostasis, this leads to decreased ATP synthesis, cellular calcium dyshomeostasis, and induction of the mitochondrial permeability transition. Instances of mitochondrial damage following HS have demonstrated that HS increased mitochondrial malondialdehyde (Mujahid et al., 2009; Mujahid et al., 2007b) that resulted from increasing substrate oxidation and membrane potential of mitochondria. Mitochondria are especially susceptible to oxidative attack as their membranes have plenty of polyunsaturated fatty acids and proteins. Additionally, ROS impacts on the cellular redox environment and interfere with the oxidation potential of reactive thiols in proteins. Overproduction of ROS in mitochondria increases when animals are under HS conditions (Mujahid et al., 2007a) that damage proteins and lipids further reducing energy generation efficiency enhanced the activity of superoxide dismutase and glutathione reductase (Altan et al., 2003). It also been indicated that HS induces oxidative stress of the breast muscle, *pectoralis major* (Feng et al., 2006) leads to oxidation of sarcoplasmic and myofibrillar proteins (Wang et al., 2009) (Figure 6). Oxidation impairs muscle protein functions by impairing cell membrane and mitochondrial integrity and cell damages through lipid peroxidation (Meng et al., 2008) that these changes can be determine by malondialdehyde and protein carbonyl changes (Figure 7).

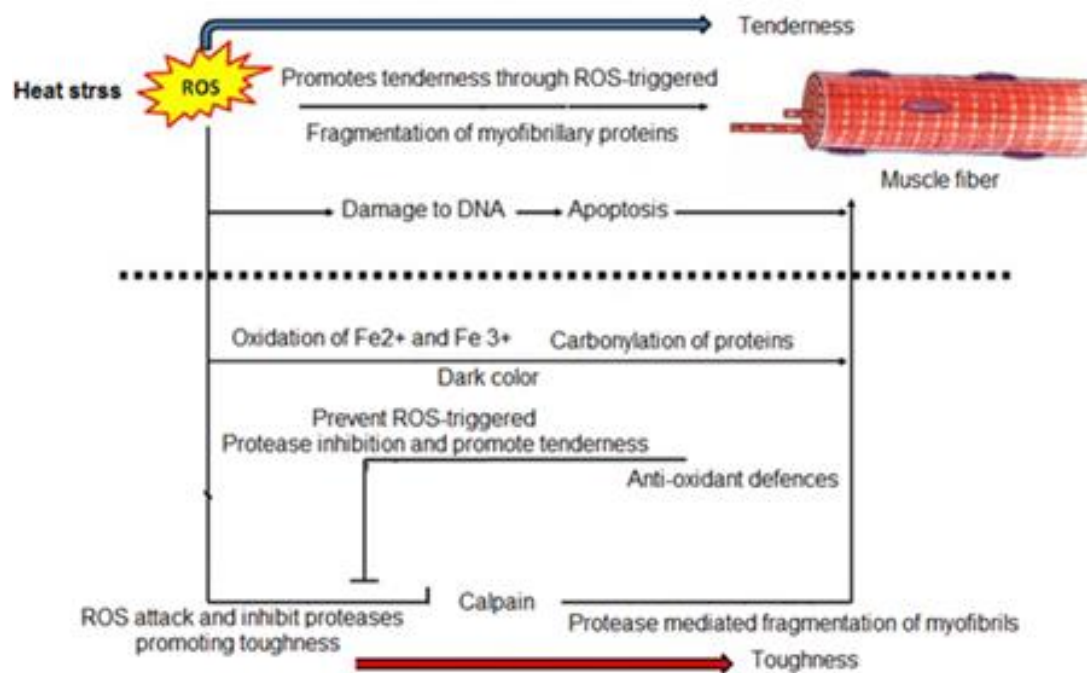


Figure 7. The role of oxidative stress in damaging tissues. Reactive oxygen species (ROS) produced during stress conditions promotes toughness by attacking and inhibiting μ -calpain and thus impeding its pro-tenderization activity. They may improve tenderization through direct triggering of ROS-mediated fragmentation of myofibrillary proteins. Thus, ROS-mediated damage to DNA favours apoptosis and thus tenderization processes. Antioxidant defences improve tenderness by preventing ROS-triggered calpain inhibition. It has adverse influence on the appearance of the meat by contributing to the oxidation of haemoglobin and myoglobin iron from Fe^{2+} to Fe^{3+} , due to undesirable color (D'Alessandro and Zolla, 2013).

.When the environmental temperature is below the critical zone, chickens attempt to reduce their body's temperature through different metabolic and physiological mechanisms (Etches et al., 2008). However, these defensive mechanisms alone are insufficient when the temperature reaches to intolerance area. As a consequence, poultry farmers in hot regions must apply management strategies to facilitate the chickens to endure HS. However, there are not enough academic studies on broiler chickens under HS in extensively managed systems, for instance in tropical regions. Solutions for the prevention of HS calls for a multifactorial approach that may include disciplines including genetics (Gowe and Fairfull, 2008), housing system (Yahav et al., 2004), thermal

conditioning (Yahav and McMurtry, 2001), and feeding and nutrition (Syafwan et al., 2011). Among these strategies, nutritional strategies have shown to be promising, easy to apply and cheap to help chickens to cope with high environmental temperatures. Furthermore, the investigation into the effects of HS on different tissues and organs which may cause organs dysfunction could provide valuable information on how to ameliorated the adverse effects of HS. Therefore, markers such as Evans Blue Dye has been considered for this purpose, as it is a non-toxic substance that binds to damaged tissue.

2.8. Quantification of tissue damages with Evans Blue Dye

Previously the tissue staining method with a coloured dye (Nidavani et al., 2014) has been applied to measure cell and tissue damages. As most plasma proteins are albumin, Evans Blue Dye (EBD) can be useful in detecting the extravasation of plasma proteins in interstitial or muscle tissues (Radu and Chernoff, 2013) (Figure 8). Under normal conditions, plasma albumin is highly restricted within blood vessels via regulation of the semi- 27 permeable vascular barriers. However, pathologies can result in increased vascular permeability, leading to accumulation of serum proteins in interstitial space (Radu and Chernoff, 2013). Generally, there are two methods to identify damages to tissues by using EBD: identifying the stained blue colour areas macroscopically after intravenously infusion investigating the damaged tissues microscopically by using EBD fluorescence (Tidball et al., 1999), or extracting the dye from target tissues by using solvents such as formamide, and the concentration of extracted dye is then quantitatively measured by using a spectrophotometer at its optical absorbance of 610 nm (Nagy et al., 2008; Radu and Chernoff, 2013). The second method is a common and trustable method to determine damages to tissues as described by (Miles and Miles, 1952). As EBD is a reliable and highly sensitive tracer, previous literature showing the feasibility of using EBD as a marker of vascular leakage are well-documented. It has been suggested that the endothelial cells with blue stains appeared to be more irregular and damaged. In addition, more breaks and gaps of endothelial boundary lines were observed in blue-stained areas compared to non-stained areas, indicating endothelial cell damage (Caplan and Schwartz, 1973).



Figure 8. Evans Blue Dye extravasation in breast muscle tissue when chickens exposed to heat stress that causes oxidative damages.

2.9. Nutritional additives to ameliorate heat stress

2.9.1. Overview

Nutritional solutions is one of the cheapest ways to protect chickens against HS by selecting nutrients having a low heat increment, or provide bioactive nutrients that improve the physiological dysfunctions associated with HS such as gut leakage and oxidative stress. During HS, maintenance energy increases as more energy is required for reducing body's temperature with panting. Furthermore, feed intake is reduced in order to reduce metabolic heat. Therefore, it requires to provide suitable nutrients to correct body's requirement and improve the performance when chickens are exposed to HS. Nutrients provided for broiler chickens can be divided into two categories: macronutrient and micronutrients.

2.9.2. Macronutrients and micronutrients

Macronutrients are called energy providing nutrients that are required in larger quantities such as carbohydrates, fat and protein. Carbohydrates provides energy, they break down into smaller sugars such as glucose to be used as a source of energy. Any unused glucose will be converted into glycogen and stored in the muscles and liver for future use.. Fats are important to make steroids and hormones, and burn to provide sufficient amount of

energy when the body has run out of carbohydrates. Proteins are source of amino acids and responsible for cell structures including the cell membrane. Under stress conditions such as HS, the muscles use their proteins to provide sufficient energy for the body. Therefore, macronutrient as an energy provider is essential for growing, repairing and developing tissues that might help the body to cope with HS effects by improving thermoregulations pathways without using the stored proteins in tissues. However, to maintain muscle, blood circulation and immune system body requires a supply of different material such as vitamins and other micronutrients.

Micronutrients' main function is to participate in metabolic pathways, they do not provide energy for the body. They are required in small quantities and include minerals and vitamins. Vitamins are essential for the body to have a normal metabolism, growth, and regulation of cell functions. Vitamins are fat soluble and available in the fatty tissues and included vitamins E and K. Minerals are co-factors and body needs them in minor quantities, they can be found in ionized such as selenium and essential for many pathways including the function of enzymes.

It is poorly characterised but the NRC arbitrarily states that maintenance requirements increases 7-25 % above 30 °C (Cottrell et al., 2015a). This means that animals under HS or grown in tropical environments require higher levels of micronutrients, and this may be exacerbated by reduced levels of feed intake. Reduced levels of micronutrients will compromise growth performance (Swennen et al., 2007), and therefore the replacement of micronutrients with increased utilisation during HS is one micronutrient amelioration strategy. One example is increasing supplementation of antioxidants such as selenium, vitamin E and phytochemicals to combat oxidative stress in HS livestock. Other strategies include organic osmolytes such as betaine. Heat stress can increase the cellular osmotic stress and the initial discovery of betaine was that it was accumulated in plants experiencing osmotic stress. Betaine has since has been demonstrated to fulfil multiple roles, including as a methyl donor and chaperone (Lever and Slow, 2010).

2.9.3. Betaine

Previous studies unveil the critical roles of amino acids in prevention of oxidative stress, intestinal barrier against pathogens, immune system function and growth performance

(Geraert and Mercier, 2010; Gous, 2010). Among amino acids, betaine is suggested as an important amino acid in alleviating stress symptoms in different animals (Figure 9).

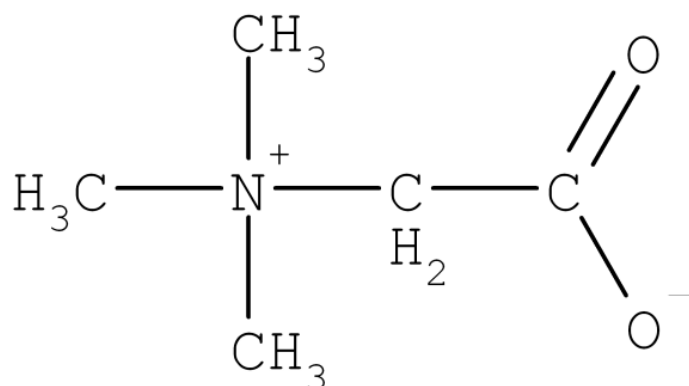


Figure 9. Chemical structure of betaine.

Betaine (trimethylglycine) is composed of the amino acid glycine which is attached to three methyl groups with a formula of $(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$. It can be extracted from different natural sources including sugar beets, which is where the name betaine is derived from. There are two major functions that betaine undertakes, as osmolyte to protect cells against osmotic stress or “methyl donor” as a catabolic source of methyl groups via transmethylation (Craig, 2004a; Zhao et al., 2018a) to reduce homocysteine, which is harmful in high level, into L-methionine. A methyl group is a hydrogen and carbon molecule that can be transferred via a process known as methylation. The methylation process taking place in all cells to generate and donate methyl groups (CH_3) for regulation of other process such as synthesis of melatonin and many other biological important molecules (Figure 10).

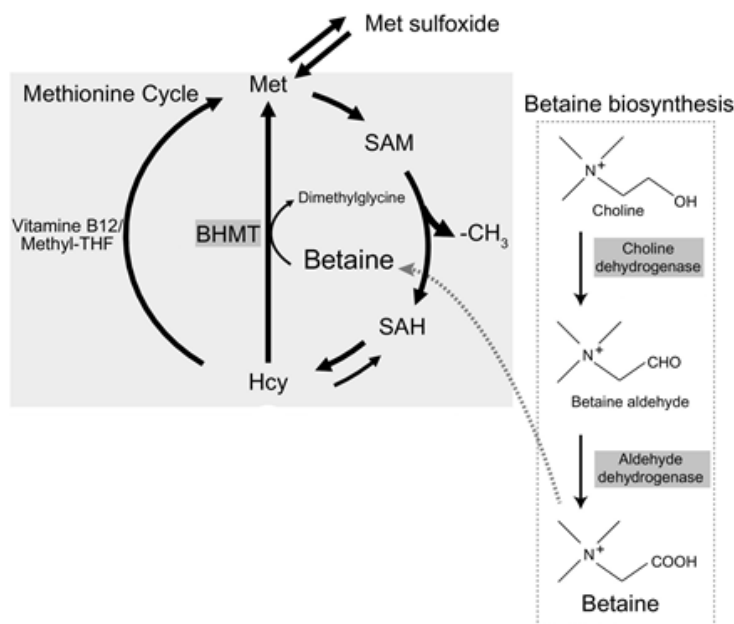


Figure 10. Methionine–homocysteine cycle and betaine biosynthesis pathways. Metabolism of Met interact when Hcy converts to Met. Betaine homocysteine methyltransferase catalyzes the methylation of homocysteine using betaine as the methyl donor. Abbreviations are: betaine homocysteine methyltransferase (BHMT);methyl group ($-CH_3$); 5-methyltetrahydrofolate (methyl-THF); methionine (Met); S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH) (Ohnishi et al., 2019).

Betaine acts as an osmolyte and is a highly soluble small organic compound that accumulate in cells without disrupting their function; it affects the process of osmosis by creating fluid level balance outside and inside of cells that protect cells against osmotic inactivation (Virtanen, 1995), alleviates negative impacts of osmotic stress in the intestine (Kettunen et al., 2001a), and maintains water and metabolic balance in cells (Ferket, 1995). The results of imbalanced fluid levels can increase cellular shrinkage, dependent upon whether there's excess fluid on the inside or the outside of the cell. Hyperosmosis causes water efflux and a concomitant reduction in cell volume; which is important for cells survival (Neuhofer and Beck, 2005). In contrast to other osmolytes and inorganic salts, such as Na^+ , betaine reduces the ability of water molecules to solvate proteins, thus stabilizing the native protein structures (Adamczak et al., 2016). Additionally, betaine increases the cytoplasmic volume and free water content of cells to prevent shrinkage in hyperosmotic conditions and to inhibit various hyperosmotic-induced apoptosis-related proteins (Ratriyanto et al., 2009). Therefore, based on the positive effects of betaine, using

it as supplementation during osmotic stress conditions as HS can help cells and tissues to counter the pressure when tissues are hypertonic and alleviating stress symptoms.

Osmolarity effects of betaine has been discussed that could help the gut tissues during stress conditions by continuing their normal metabolic activities. Damages to the intestine cells during HS cause reduction in villus height and crypt, and also increases osmolarity in the intestine lead to impaired cell metabolism and its enzyme activity. Any alteration in cell structure could reduce nutrients absorption, and provide a better conditions for external pathogens or toxics to enter into the blood. During HS, intestinal villus membrane absorb less Na^+ and Cl^- lead to hyperosmolar in the intestine. Betaine could enhances the intestinal integrity that helps the intestine works normally resulting in optimise nutrient digestibility and reduce excretion (Panda et al., 2010). Betaine could prevent nitric oxide synthase expression which could be initiated by inflammation by which it facilitated vascular function (Iqbal et al., 2006; Panda et al., 2010). Nitric oxide is responsible for critical illness in the gut include: intrinsic intestinal tissue (epithelium and smooth muscle), resident and infiltrating leukocytes (neutrophils and monocytes), reduction of luminal gastric nitrate, and denitrification by commensal anaerobes (Salzman, 1995). Furthermore, betaine is effective during early ages as chick's gut is immature. During this period, gut structure is not developed fully and not functional to absorb or digest nutrients well due to creates osmotic pressure and moves water to the lumen. Thus, betaine moves into cells when osmolarity increases resulting the accumulation of betaine into gut cells and protecting epithelial cell morphology and stabilized gut mucosa (dos Santos et al., 2019a) as well as reducing movement of water from cells (Kettunen et al., 2001a). Thus, supplementing a diet with betaine from early age could help to improve the intestinal integrity health leading to better growth performance.

Another pathway that betaine is involved is methylation. Methylation is an essential biochemical process in animals. Betaine is an important methyl group donors present in diets. Methionine metabolise takes place in liver and it requires for protein synthesis and regulating cellular functions (Pacana et al., 2015). The transmethylation reaction of betaine, which is part of a one-carbon metabolism via the methionine cycle, occurs

principally in the mitochondria of liver and kidney cells. One of the important cellular methylation processes that betaine is involved in is remethylation of homocysteine and eliminates toxic metabolites (Craig, 2004b). Homocysteine is generated by deamination of methionine and is a toxic sulphur-containing intermediate product, and has various consequences for cells as oxidative stress, mitochondrial dysfunction, increase of cytosolic calcium and protein damages (Borowczyk et al., 2018) which contribute to apoptosis (Alirezai et al., 2015). It generates ROS and inhibits the expression of antioxidant enzymes which might potentiate the toxic effects of ROS (Alirezai et al., 2015). The high level of homocysteine appears to be toxic to the cells by slowing the growth rate, therefore, it requires re-conversion to methionine through remethylation process (Orth et al., 1992; Xie et al., 2014). There are two major remethylation pathways that employ the methionine synthase enzyme and cofactor such as B₁₂ and folic acid while the minor pathway involves betaine-homocysteine methyltransferase and betaine as cofactor (Craig, 2004b). Under stressful thermal conditions, the ROS may impair the major pathway (folate-dependent methionine synthesis) hence the minor pathway betaine-homocysteine methyltransferase (BHMT) regulates the homocysteine homeostasis (Weisberg et al., 2003). In addition, betaine enhances the activity of BHMT and 5,10-methylenetetrahydrofolate reductase (MTHFR) (Purohit et al., 2007). Betaine enhances methionine-homocysteine cycle and increases production of methionine in the cell which may result in reduction of methionine requirement in animals. Thus, this reduces the required amount of methionine for a diet results lower feed cost. Furthermore, betaine could improve carcass quality through methionine pathway by synthesis of carnitine. Carnitine is concentrated in skeletal muscle and functioning as a source of energy by transporting fatty acids into the mitochondria, and oxidized them. It boosts protein metabolism and alters carcass composition by reducing protein turnover, results in higher nitrogen retention resulting in a positive effect on accretion of protein in muscle. Moreover, it through carnitine pathway improves lipid catabolism (Hanje et al., 2006) due to low carcass fat disposition (Panda et al., 2010).

In addition, betaine being demonstrated to be an antioxidant agent for prevention of oxidative stress by enhancing glutathione peroxidase activity (GPx), activity of superoxide dismutase (SOD) and catalase activity (CAT) subsequently decreasing in the

lipid peroxidation process (Alirezaei et al., 2015). The protective effects of betaine against oxidative damages is associated with the restoration of S-adenosyl methionine by supplying required substrate for synthesis of the enzymes (Alirezaei, 2015). Glutathione peroxidase activity has peroxidase activity that protect the organism from oxidative damage by reducing lipid hydroperoxides and free hydrogen peroxide (Muthukumar et al., 2011). The activity of superoxide dismutase is an enzyme that converts the superoxide radicals to ordinary molecular oxygen or hydrogen peroxide. As hydrogen peroxide itself is harmful to cells, it converts to nontoxic compounds with CAT (Hayyan et al., 2016). The catalase activity is an enzyme that protect cells against oxidative damages caused with ROS by converting hydrogen peroxide to water and oxygen (Goodsell, 2004).

2.9.4. Selenium

Selenium is an essential micronutrient and can be grouped into inorganic and organic categories. Inorganic selenium exists as minerals in the form of selenite, selenate and selenide. The primary organic forms of selenium include selenomethionine and selenocysteine. The selenium is incorporated into enzymes such as glutathione peroxidase, superoxide dismutase and thioredoxin reductase and contributes to antioxidant pathway. Moreover, selenium is considered as a cofactor for iodothyronine deiodinases enzymes which are involved in activation or inactivation of the initially released hormone precursor thyroxine (T_4) to triiodothyronine (T_3). Thus, selenium by indirect regulation of T_3 and T_4 production affects basal metabolic rate, protein synthesis, and metabolisms of fat, carbohydrate, protein and vitamins. It is known that under physiological, pathological or environmental stimuli the synthesis of thyroid hormones are impaired and supplementing selenium affectively regulates the synthesis of thyroid hormones and recovering the body homeostasis.

The cellular metabolism pathway of the selenium is shown in Figure 11. Initially, the organic or inorganic forms of selenium compound is metabolised to hydrogen selenide. Selenomethionine could act as methionine function or transforming to selenocysteine via a trans-sulfuration pathway. Then, selenocysteine may transform to hydrogen selenide. Selenate reduces to selenite by glutathione and then hydrogen selenite. The selenophosphate synthetase converts the hydrogen selenite to selenophosphate. Then, it

reacts with L-seryl-tRNA^{Sec} to form L-selenocysteinyl-tRNA^{Sec} which then further forms the selenoproteins. Selenoprotein is any protein containing selenocysteine and the common selenoproteins are glutathione peroxidase and thioredoxin reductase which are involved in oxidative damages and thyroid hormones metabolism, respectively. The function of immune system has also been enhanced at the presence of these two selenoproteins (Hoffmann, 2007).

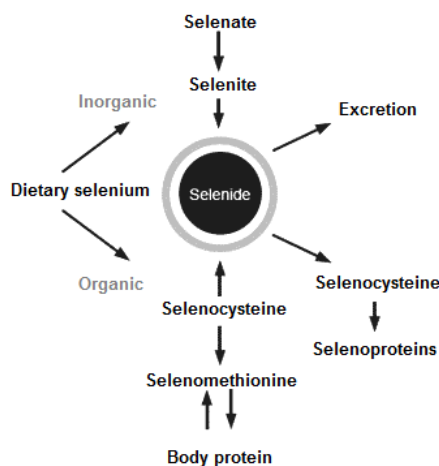


Figure 11. Basic selenium metabolism pathway. Inorganic forms of selenium convert to selenite which is further convert to selenide. Organic forms of selenium convert to selenocysteine which in turn degrade to selenide and used to form selenocysteine, which then incorporate into the active site of a selenoprotein.

Early studies indicated that dietary supplementation of selenium with the doses ranged from 0.4-1 mg/kg of the broiler diet could significantly enhance growth performance of chickens challenged by HS (Ibrahim et al., 2011; Krstić et al., 2012). These results were further attributed to the effects of selenium on thyroid hormone metabolism, cellular antioxidant level and immune system response (Habibian et al., 2016; Niu et al., 2009; T Tayeb and Qader, 2012).

2.9.5. Vitamin E

Vitamin E is a group of compounds containing both tocopherols and tocotrienols which are fat-soluble with antioxidant properties (Packer et al., 2001). The fat-soluble nature of vitamin E, render it as a suitable cell membranes antioxidant against oxidative damage. The alpha-tocopherol is the most biologically active form of vitamin E which is involved

in glutathione peroxidase pathway and protects organisms from oxidative damages by reacting with lipid radicals produced in the lipid peroxidation reaction (Gao et al., 2010; Traber and Stevens, 2011) and acts as a cellular enzymatic activity regulator (Schneider, 2005). Moreover, it activates dephosphorylation enzyme called protein phosphatase 2 which cuts phosphate groups from Protein kinase C (PKC) and resulted in inactivation of PKC, therefore, controls the smooth muscle growth and proliferation (Azzi et al., 1997). Protein kinase C is involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. Furthermore, it protects the lymphocytes, macrophages and plasma cells against oxidative damage and improves their survival, proliferation and function. Thus, it enhances the immune response under stress conditions such as HS (Attia et al., 2016), and reduces synthesis of malonyldialdehyde in the liver by acting against lipid peroxidation and cell damages (McDowell, 2012) and resulted in improvement of the chicken's performance (Sahin and Kucuk, 2001b; Sahin et al., 2001).

The required vitamin E for chicken's body should be provided through dietary supplement as they are not capable of vitamin E synthesis. Under stress condition, particularly HS, the levels of hormones like corticosterone and catecholamines increases and lipid peroxidation in cells membranes is initiated. Therefore, under HS conditions, more vitamin E is required to balance the level of hormones to cope with HS. Emerging literature revealed that addition of vitamin E at the average concentration of 250 IU/kg is feasible protective practice to alleviate the symptoms of HS and may result in optimum performance and improved meat quality in broiler chickens (Gao et al., 2010; Hashizawa et al., 2013; Hosseini-Mansoub et al., 2010).

2.9.6. Polyphenols

Polyphenols are recognised by their phenol structure, and polyhydroxy substitution of the benzene ring (Figure 12). They can be classified in many classes, whereas the main classes are phenolic acids and alcohols, stilbenes, and flavonoids (Hu et al., 2019; Hussain et al., 2016). Polyphenols can be found in high amount in fruits and vegetable with antioxidant activity such as spinach (Fotina et al., 2013; Lipiński et al., 2017; Surai, 2014)

and immunostimulatory properties (Chamorro et al., 2015; Gerasopoulos et al., 2015). The biological effects of polyphenols are briefly presented in Figure 13.

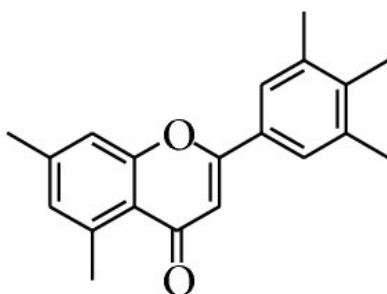


Figure 12. Chemical structure of polyphenols (Kaur and Kaur, 2014).

Polyphenols have a hydrogen group in their chemical structure that eliminate ROS. Phenolic groups accept unpaired electrons which have short half-life to form stable phenoxyl radicals (Brenes et al., 2016; Landete, 2013), which in turn highlights the short reaction distance and susceptibility to neighbouring molecules (Sies, 1993). Indeed, free radicals are capable of damaging every major category of biomacromolecule, have oxidative modification of lipids as well as damaging proteins by carbonyl modification (Smith, 1995). Furthermore, they have protein sparing effects that can stimulate the activity of antioxidants and increase the concentrations of some antioxidants such as α -tocopherol (Masella et al., 2005). Fluorophenols decrease the catalytic activity of enzymes involved in ROS generation due to reduce oxidative damages (Kumar et al., 2012). Reactive oxygen species increases free metal ions by reduction of hydrogen peroxidase with generation of the highly reactive hydroxyl radical. Lower redox potentials of the polyphenols are thermodynamically able to reduce highly oxidizing free radicals because of their capacity to chelate metal ions and free radical (Mishra et al., 2013). Therefore, the functions of polyphenols make them a promising additive in broiler chickens diets (Surai, 2014) especially under HS (Xue et al., 2017) where there is need to reduce ROS production.

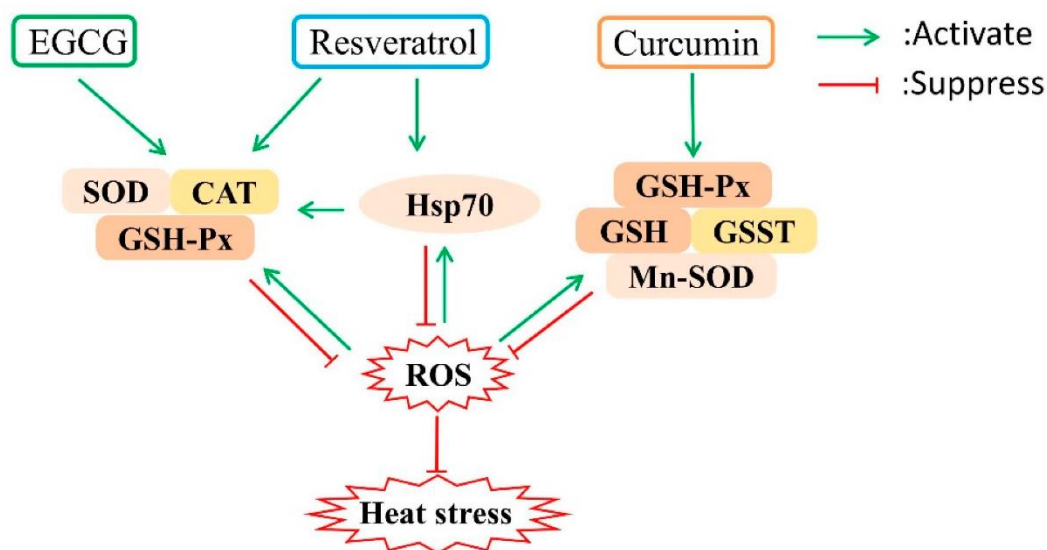


Figure 13. Effects of polyphenols (Resveratrol and Curcumin) against heat stress. Polyphenols suppress reactive oxygen species by upregulating the expression of stress response proteins. Abbreviations are: CAT: catalase; EGCG: epigallocatechin gallate; GSH: glutathione; GSH-Px: glutathione peroxidase; GSST: glutathione S-transferase; Hsp70: heat shock proteins 70; Mn-SOD: manganese superoxide dismutase; SOD: superoxide dismutase; (Hu et al., 2019).

2.10. Conclusion

Average global temperatures have risen considerably, and this increase has direct impact on livestock production by increasing incident of HS. The environment of a chicken is a composite of interacting stressors and a chicken's success in coping with its environment therefore depends on the severity and its physiological ability to respond properly. When the biological cost of coping with the stressors diverts resources away from other biological functions, such as maintaining reproduction or growth. The animal's welfare is compromised before the resources are taken away from growth and reproduction, and the economic impaction happens at this point.. Heat stress is an unpleasant challenge with the major welfare issues in broiler chickens production in tropical area or hot seasons that could cause a huge economic loss. For several years, different strategies have been introduced such as improving housing system, but these strategies cannot be promising when the environmental temperature is higher than the tolerance area. Therefore, it's required to find a better solution to ameliorate adverse effects of HS. It has been established that the supplementations of osmolytes and antioxidants individually could

ameliorate the adverse effects of HS. However, limited studies have examined the combination of these additives and the involved mechanisms in this matter. Micronutrients such as betaine, selenium, vitamin E and polyphenols are low cost and potential supplementations to ameliorate the adverse effects of HS. Previous studies indicated that betaine or antioxidants individually could ameliorate negative impacts of HS on performance and product quality. Most studies have examined individual effects of the above additives on other strains of broiler chickens such as Cobb. Therefore, this thesis attempts to provide further information on the effects of HS on Ross-308 that is vastly used in Australia commercial broiler farms, as well as the effects of supplementation of betaine and antioxidants when chickens are subjected to cyclic HS.

2.11. Aims of the thesis

Betaine contributes to multiple biological pathways that may ameliorate the impacts of HS in the Ross-308 broiler. Therefore, the overall aim of this thesis was to improve understanding into how betaine affects physiology, growth and product quality in the HS growing Ross-308 broiler. This was to be done alone and in conjunction with other additives that may have complimentary actions. The specific aims of these were:

- 1 – To investigate the effects of cyclic HS on growth performance, enzymes activities and gut physiology of Ross-308 broiler chickens supplemented with betaine, selenium and vitamin E.
- 2 – To investigate the effects of cyclic HS on growth performance and meat quality of Ross-308 broilers chickens supplemented with betaine, selenium, vitamin E and polyphenols.
- 3 – To investigate the effects of cyclic HS on different organs, betaine distribution in tissues by using a marker named Evans Blue Dye of Ross-308 broilers chickens supplemented with betaine. Furthermore, the effects of the diets and HS were examined on gut and muscle morphology.

The outcomes from this thesis will improve our understanding the underlying effects of HS on Ross-308 performance and meat quality. These results will be of benefit to the

Australian domestic production systems. This will particularly be the case for Ross-308 and other poultry species reared outdoors or indoors and therefore has ramifications for international poultry production, particularly when chickens are exposed to the critical environmental temperature such as HS.

The survey on recent progresses in HS management in broilers revealed the potential applications of bioactive compounds (betaine, selenium, vitamin E and polyphenols) as a feasible approach to combat HS symptoms. Therefore, due to the importance of these functional bioactive compounds in HS management, the thesis aims to highlight the detailed information on how these bioactive compounds may contribute to alleviate HS symptoms in the poultry industry.

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CHAPTER 3

Betaine and Antioxidants Improve Growth Performance, Breast Muscle Development and Ameliorate Thermoregulatory Responses to Cyclic Heat Exposure in Broiler Chickens

3.1. Introduction, aims and hypotheses

The physiological and metabolic responses of broiler chickens to control thermoregulation during HS divert energy from efficient production in addition to increasing morbidity and mortality. Therefore, HS amelioration strategies may improve the productivity of poultry meat production over the summer months and in tropical regions. Increasingly, affordable feed additives are being investigated as potential amelioration strategies against HS.

The aim of this chapter was to investigate the effects of the osmolyte betaine, selenium and vitamin E supplementations on growth performance, physiological responses, and gut physiology in broiler chickens when chickens were exposed to cyclic HS.

Section 3.2 presents the published paper that showed effects of betaine and antioxidants on broiler growth performance and gut physiology.

3.2. Published paper

Betaine and antioxidants improve growth performance, breast muscle development and ameliorate thermoregulatory responses to cyclic heat exposure in broiler chickens

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Simple Summary: The physiological and metabolic responses of broiler chickens to control thermoregulation during heat stress divert energy from efficient production in addition to increasing morbidity and mortality. Therefore, heat stress amelioration strategies may improve the productivity of poultry meat production over the summer months and in tropical regions. Increasingly, low-cost feed additives are being investigated as potential amelioration strategies against heat stress. Scholars have investigated the effects of betaine alone on growth performance and gut physiology, while a small number of studies have been made regarding the impacts of the combination of betaine and anti-oxidants. Therefore, this study was conducted to investigate the effects of the osmolyte betaine and selenium and vitamin E supplementation on growth performance, physiological responses and gut physiology in broiler chickens exposed to cyclic heat stress.

Abstract: Heat stress (HS) is an environmental stressor challenging poultry production and requires a strategy to cope with it. A total of 288-day-old male broiler chicks were fed with one of the following diets: basal diet, basal with betaine (BET), or with selenium and vitamin E (AOX), or with a combination of BET and AOX, under thermoneutral and cyclic HS. Results showed that HS reduced average daily feed intake

(ADFI) ($P = 0.01$) and average daily gain (ADG) ($P < 0.001$), and impaired feed conversion ratio (FCR) ($P = 0.03$) during rearing period (0-42 d). BET increased ADG ($P = 0.001$) and decreased FCR ($P = 0.02$), whereas AOX had no effects. Breast muscle weight was decreased by HS ($P < 0.001$) and increased by BET ($P < 0.001$). Rectal temperature was increased by HS ($P < 0.001$), and improved by BET overall. Respiration rate was increased by HS ($P < 0.001$), but BET decreased it during HS ($P = 0.04$). Jejunum transepithelial resistance was reduced by HS and had no effect on permeability whereas BET increased jejunum permeability ($P = 0.013$). Overall, the reductions in ADG of broiler chickens during HS were ameliorated by supplementation with BET, with much of the increase in ADG being breast muscle.

Keywords: betaine; antioxidant; broiler; growth; heat stress

1. Introduction

Heat stress (HS) causes reductions in feed intake and body weight gain (Syafwan et al., 2011), thus posing a major concern for poultry industry. Furthermore, HS increases mortality rate, possibly by impairing intestinal development (Garriga et al., 2006) or disrupting barrier function (Quinteiro-Filho et al., 2010). It increases radiant heat loss, through the redistribution of blood flow from the body core to the periphery, where it can more readily radiate to the environment. This redistribution of blood flow during HS arguably underpins hypoxia and tissue damage within the gastrointestinal tract (GIT) (Lambert, 2009a). The GIT forms the digestive and absorptive apparatus of the body, and damages to it is likely to compromise feed efficiency. In addition, reductions in barrier function are likely to inflict a portal of entry for pathogens, increasing disease risk (Dunshea et al., 2013; Keita and Söderholm, 2010) and is of particular concern for intensive production systems with high stocking densities due to the relative ease of transmission of enteric infection.

Betaine (BET) is a natural compound that accumulates within the intestine of broilers when included in diets. The protective effects of dietary BET during HS are threefold. To begin with, BET serves as an extracellular osmolyte, lowering the activity of Na^+/K^+ ATPases and therefore reducing overall energy expenditure (Moeckel et al., 2002; Tsai et al., 2015a). Because the GIT accounts for a disproportionately large amount of whole-body energy expenditure, the GIT is particularly sensitive to perturbations in blood flow

and it has been proposed the reducing the GIT energy demand with BET may serve as a protective mechanism against HS (Cronjé, 2005). Furthermore, BET acts as a methyl donor. This has wide-ranging effects, including DNA methylation and increasing methionine remethylation from homocysteine to increase protein synthesis. Finally, osmolytes act as chaperones, stabilising protein folding. In broilers, BET supplementation improves villus morphology, including following coccidia infection (Kettunen et al., 2001b). While one study has been inconsistent (Matthews and Southern, 2000), it has been proposed that BET protects against the effects of HS by ameliorating damage to the GIT (Saeed et al., 2017). As studies sought to explore the ideal dosage of BET added in broiler chickens' diets, it has been established that growth performance improved with 0.5-2 g/kg added BET, and within the range of 0.5-1 g/kg, the increase in the improvement is proportional to the increase in the dosage of BET in the diets, with 1 g/kg being the most ideal dosage for such improvement; while BET 1-2 g/kg, albeit effective, did not improve growth performance any further than BET 1 g/kg did (Hassan et al., 2005; Zhan et al., 2006; Attia et al., 2009).

Selenium (Se) is a trace element that improves growth performance by improving the efficiency of feed utilization (Krstić et al., 2012), by protecting and preserving of cells and cell membrane and is required for expression of the anti-oxidant enzyme glutathione peroxidase (GP_x). Under thermoneutral (TN) conditions, the dietary Se requirement for poultry is approximately 0.10 to 0.15 mg/kg (Dale, 1994). However, higher levels of dietary Se, of the order of 0.4 to 1.0 mg/kg, have been recommended to enhance cellular antioxidant levels, improve immunity and ameliorate the negative impacts of HS (Habibian et al., 2015; Niu et al., 2009; Yang et al., 2012). Furthermore, organic forms of Se, such as selenised yeast or protein-bound Se, may be more available and bioavailable than inorganic forms of Se in broiler chickens (Uglietta et al., 2008) and pigs (Packer et al., 2001).

Vitamin E (VitE) is a fat-soluble vitamin with antioxidant properties (Villacorta et al., 2003) that regenerates damaged tissues (Gao et al., 2010; Sahin and Kucuk, 2001a) during oxidative stress by participating in the GP_x pathway resulting in improved chicken performance (Hosseini-Mansoub et al., 2010). For example, dietary supplementation with up to 250 mg/kg VitE improved the growth performance of broiler chickens reared under both TN and HS conditions (Hashizawa et al., 2013; Hosseini-Mansoub et al., 2010).

Most of the studies investigating the effects of dietary strategies on alleviating HS have been performed on chickens with only a moderate growth potential, rather than genetically improved chickens. Moreover, based on our best knowledge, a small number of studies have investigated effects of the combination of BET with selenium and vitamin E in broiler chickens of Ross-308 under HS. Therefore, we hypothesize that BET, Se, and VitE might ameliorate some of the effects of HS and therefore the aims of this experiment were to determine the effects of supplemental BET and antioxidants (AOX), either alone or in combination on growth performance, breast muscle development and the physiological responses to HS in contemporary broilers chickens.

2. Materials and Methods

2.1. Ethics Approval and Consent to Participate

The study was performed in Animal facility in The University of Melbourne, Parkville, Victoria in Australia. All procedures of this experiment were approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia (Protocol no. 1613936.1). The use of animals for research is directed by federal and state legislation according to the Prevention of Cruelty to Animals Act 1986 and the Prevention of Cruelty to Animals Regulations 2008.

2.2. Birds and Husbandry Housing

A total of 288-day-old-male Ross-308 chicks were obtained from a local commercial hatchery (Tri Foods Pty. Ltd, Bannockburn Victoria, Australia) located within less than 2 hours driving distance from The University of Melbourne. Chicks were transported in 3 boxes (100 chicks per box) in an environmentally controlled vehicle (where the temperature inside the vehicle was ~30 °C) to the experimental site. Chicks were randomly selected and then weighed as a group of 6 chicks and allocated to equally-sized pens (1m × 0.5m) with wire flooring and walls and with the floors covered with wood shavings (10cm deep approximately) in two environmentally controlled rooms (24 pens in each room). Temperature rooms were operated independently and there were temperature and humidity sensors located within each room. For the first 7 days of the experiment, the temperature of both rooms was a constant 33 °C for the full 24 h in each day. From day 8, the temperature in each room was either TN where the temperature was decreased

gradually from 33 °C to 25 °C on 21d (1 degree every 2 days starting from day 7) and then maintained at 25 °C from 21-42d, which is in the range of the optimum temperature for Ross-308 broiler chickens (Kamely et al., 2015) or HS conditions. The HS conditions were characterized as cyclic 33 °C from 1-42d where the chickens were kept at 33 °C between 08:00 to 17:00 h and then the prevailing temperature in the TN room for the rest of the day. The relative humidity was between 45-65% during the experiment. Light (fluorescent, 10 lux) was provided for 24 h for the first 3 days after placement and gradually decreased (1 h per day) to 20 h at day 7 for both rooms. Each pen was equipped with a nipple drinker and a ball type feeder. To minimize feed wastage during the experiment, each feeder was placed on a box with a net on the top to collect spilled feed. Each room was equipped with two fans to exchange air (~0.2 m³/min.).

2.3. Experimental Treatments

The experiment was a 2 × 2 × 2 factorial design the respective factors being environmental temperature (TEMP) regime (TN versus HS), added dietary BET (0 versus 1 g/kg BET (Betafin S1, DuPont, UK) and dietary AOX (Basal (50 IU/kg VitE and 0.3 mg Se/kg) and 300 IU/kg VitE and 0.8 mg Se/kg). The basal AOX diet was formulated to satisfy NRC requirements (Dale, 1994) with Se provided as selenised yeast (SelenoSource 3,000, Diamond V Mills, USA) and the Vit E as natural Vit E (VitE 250, dl- α -tocopheryl acetate, ADM, USA). Feed (Feedworks_BESTMIX, The University of Sydney) was formulated as a commercial starter was crumbled from 1-14d (CP 22.19% 2950 MJ ME/kg), grower was pelleted from 15-28d (CP 20.54% 3100 MJ ME/kg) and finisher was pelleted from 29-42d (CP 19.16% 3.200 MJ ME/kg) (Table 1). It has been recommended (Aviagen, 2014) that the finisher diet should be provided for broiler chickens after approximately 25 days. Feed and water were provided ad-libitum and live weight and feed disappearance were recorded weekly with a digital scale (Teraoka Seiko, S-YBS, Tokyo, Japan) to calculate weekly average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). Mortality rate was recorded daily. All chicks were vaccinated against coccidiosis and Newcastle in the hatchery to avoid coccidiosis during the rearing period.

Table 1. Nutrient composition of commercial experimental diets.

Ingredients %	Starter	Grower	Finisher
Wheat	57.86	60.82	63.61
Soybean meal 48%	31.13	25.28	19.69
Canola meal 38%	4.00	6.00	8.00
Soya oil	3.21	4.55	5.68
Dicalcium phosphate	1.27	0.99	0.81
Limestone	1.12	1.04	0.96
Sodium bicarbonate	0.25	0.25	0.31
Lysine	0.25	0.23	0.20
Celite	0.20	0.20	0.20
DL-methionine	0.28	0.23	0.19
Salt	0.20	0.20	0.16
Starter premix ^{#, *}	0.10	0.10	0.10
Threonine	0.11	0.09	0.06
Axtra XB 201 [†]	0.01	0.01	0.01
Axtra Phy TPT 10,000 [‡]	0.01	0.01	0.01
Total	100	100	100
Calculate analysis			
AME ¹ (kcal/kg)	2950.00	3100.00	3200.00
Crude protein (%)	22.19	20.54	19.16
Sodium (%)	0.16	0.16	0.16
Fat (%)	5.04	6.46	7.35
Salt (%)	0.27	0.28	0.24
Calcium (%)	0.96	0.87	0.79
Chloride (%)	0.23	0.23	0.20
Lysine (%)	1.37	1.23	1.10
Methionine (%)	0.61	0.54	0.48
Starch (%)	35.54	37.18	38.90
P available	0.48	0.44	2.00

^{#2} Supplied per kg of diet: retinol, 3600 µg; cholecalciferol, 125 µg; menadione, 3 mg; thiamine, 3 mg; riboflavin, 9 mg; pyridoxine, 5 mg; cobalamin 25 µg; niacin, 50 mg; pantothenic acid, 18 mg; folic acid, 2 mg; biotin, 200 µg; Cu, 20 mg; Fe, 40 mg; Mn, 110 mg; Co, 250 µg; I, 1 mg; Mo, 2 mg; Zn, 90 mg; ethoxyquin, 125 mg.

^{*}Total betaine, selenium and vitamin E were measured in the feed. Only the starter premix was used (formulated to provide the required multi-minerals and multi-vitamins). The premix was free of vitamin E, selenium and betaine. Betaine was only added to the betaine groups.

[†]Contained: endo-1,4-β-xylanase; endo- 1,3 (4)-β-glucanase.

[‡]Contained: dried *Trichoderma reesei*; sodium sulphate; starch; hydrate magnesium silicate; sucrose; poly- vinyl alcohol; vegetable oil; phytic acid.

¹Apparent metabolisable energy.

2.4. Physiological Responses

Physiological responses to HS were measured by quantifying rectal temperature and respiration rate. Forty-eight chickens (1 per pen) were randomly selected to measure

rectal temperature weekly from 7d at 7:00, 12:00, and 16:00 by using a digital thermometer (Comark PDT 300, UK) inserted about 3-4 cm into the cloaca for 35 sec. until the temperature stabilized. Forty-eight chickens were randomly selected to measure respiration rate at 12:00 on 41d. Chickens were filmed using an android mobile phone (Samsung Electronics Co., Ltd. Korea) and then the number of breaths taken over a 10 second period was quantified and then expressed as breaths per minute. The same researcher measured rectal temperature, and filmed and measured respiration rate to keep consistency.

2.5. Tissue Sampling and Euthanasia

At 21d of age half of the chickens (3 per pen) were randomly chosen to be slaughtered to obtain liver samples for subsequent GPx analysis with the remaining chickens slaughtered at 42d to measure gut integrity, permeability, breast muscle weight and abdominal fat pat. Abdominal fat pat was collected and weighed after slaughtering. Chickens were killed by injecting 3 ml lethobarb (pentobarbitone sodium, Virbac Animal Health, Milperra, NSW, Australia) into the wing vein; death was confirmed by the absence of heartbeat and respiratory movement, by observation and lack of response to a toe pinch.

2.6. Intestinal Barrier Integrity and Permeability

At day 42, jejunum samples were collected from 80 randomly pre-selected chickens. The mucosal integrity and permeability were measured as previously described (Liu et al., 2016) using chambers by trans-epithelial resistance (TER) and FITC-Dextran 4kDa (FD4) permeability. In brief, fresh jejunum samples were inserted into chambers (EasyMount Diffusion Chambers; Physiologic Instruments, San Diego, CA, USA). Each chamber had a set of four electrodes and contained 5 ml of the Krebs bicarbonate buffer. Tissues were allowed to equilibrate for about 25 min. in the chambers before measurements were made. Transepithelial electrical resistance was calculated by Ohm's law and multiplied by the exposed area. Macromolecular permeability was quantified by passage of 4kDa fluorescein isothiocyanate dextran from the mucosal side (1 mg.ml^{-1}) to the basolateral side. The FD4 fluorescence was measured using a fluorescence reader (FlexStation II; Molecular Devices, Sunnyvale, CA, USA). The apparent permeability coefficient of FD4 was calculated by equation given by (Pearce et al., 2013).

2.6. Glutathione Peroxidase Assay

Glutathione peroxidase was measured kinetically in liver homogenates by the oxidation of NADPH and measured at 340 nm as per the manufacturer's instructions (Cayman Chemical Company, USA). Briefly, non-enzymatic and positive control wells were prepared by diluting 120 µl of assay buffer and 50 µl of co-substrate mixture, and diluting 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of diluted GPx, respectively. Samples wells were prepared by adding 100 µl of assay buffer, 50 µl co-substrate mixture and 20 µl samples. Then, 20 µl cumene hydroperoxide was added to all wells and gently shook for 10 sec. Absorbance was read at 340 nm for 5 times. Then, actual values were calculated by a provided formula based on obtained absorbance results.

2.7. Statistical Analysis

All growth performance, breast muscle development, abdominal fat pad, carcass, and respiration rate data were analysed by ANOVA for the main and interactive effects of TEMP, dietary BET or dietary AOX with pen as the experimental unit using the GLM procedure (Institute, 2004). Mortality data were analysed using the χ^2 maximum likelihood contingency test to determine the main effects of TEMP, BET and AOX. Rectal temperature was analysed for main and interactive effects of day of study (DAY), time of day (TM), TEMP, BET and AOX with pen as the random effect. Means were separated using Duncan's multiple range test. Significance was considered as $P < 0.05$.

3. Results

3.1. Performance

Between days 21 and 42 there was a reduction in ADFI ($P = 0.001$) and ADG ($P < 0.001$) and an increase in FCR ($P = 0.04$) in response to HS (Table 2). Dietary BET increased ADG ($P = 0.001$) and decreased FCR ($P = 0.04$) between days 21 and 42. Although there were no main effects of AOX on either ADFI, ADG or FCR between days 21 and 42 of the experiment.. Over the entire experiment, there was a reduction in ADFI ($P = 0.01$) and ADG ($P < 0.001$) and an increase in FCR ($P = 0.03$) in response to HS (Table 2). Dietary BET increased ADG ($P = 0.001$) and decreased FCR ($P = 0.02$) between days 0 and 42 but had no significant effect on ADFI. Although there were no significant main effects of AOX on either ADFI, ADG or FCR over the entire experiment, there tended ($P = 0.09$) to be an effect of AOX and an interaction between TEMP and

AOX ($P = 0.02$) such that AOX decreased ADFI under TN conditions but not during HS (Table 2). As a result of the effects on ADG, final live weight was decreased by HS ($P < 0.001$) and increased by dietary BET ($P = 0.003$) whereas there was no effect of AOX. A large part of the changes in live weight appeared to be as breast muscle which was decreased by HS ($P < 0.001$) and increased by dietary BET ($P < 0.001$) with no main effect of AOX. However, there were significant TEMP $\times$ AOX ($P = 0.046$), AOX $\times$ BET ($P = 0.007$) and TEMP $\times$ AOX $\times$ BET ($P = 0.015$) interactions such that the combined BET and AOX treatment increased ADG during HS but not under TN conditions (Table 2). There were no main effects of HS, BET or AOX on fat pad weight, although there was a significant AOX $\times$ BET ($P = 0.019$) interaction indicating that AOX alone increased fat pad weight, but not when combined with BET. The mortality rate was low but was increased by HS (0.7 versus 4.2%, $\chi^2=4.05$, 1 df, $P = 0.044$) whereas there was no effect of dietary BET (1.4 versus 3.5%, $\chi^2=1.36$, 1 df, $P = 0.24$) or AOX (2.1 versus 2.8%, $\chi^2=0.15$, 1 df, $P = 0.70$). However, these data must be treated with caution because of the low mortality rates and hence low frequency in some cells.

3.2. Physiological Responses

Heat stress increased rectal temperature ($P < 0.001$) with the response being greatest ($P < 0.001$) during the afternoon when the ambient temperature was highest (Figure 1b) and no effects under TN (Figure 1a). Although there were no main effects of BET, there was a reduction in rectal temperature in response to dietary BET during the initial stages of the experiment in chickens exposed to HS, particularly late in the afternoon, as indicated by DAY $\times$ BET ($P < 0.001$), TM $\times$ BET ($P = 0.015$) and TM $\times$ DAY $\times$ BET ($P = 0.04$) interactions (Figure 1b). Also, the positive effects of BET on rectal temperature were reduced when combined with dietary AOX as indicated by DAY $\times$ BET $\times$ AOX ($P < 0.001$) and TM $\times$ DAY $\times$ TEMP $\times$ BET $\times$ AOX ($P < 0.014$) interactive effects (Figures 1b and 1d). Interestingly, rectal temperature increased at the end of the experiment in chickens fed the AOX diet and housed under TN conditions as indicated by the DAY $\times$ TEMP $\times$ AOX ($P < 0.001$) interaction (Figure 1c).

Table 2. Effect of heat stress and dietary betaine and antioxidants on growth performance ¹.

Temperature(TEMP)	Thermoneutral				Heat Stress				SE ³	P-values		
Antioxidants ² (AOX)	Control		Supplemental		Control		Supplemental					
Betaine (g/kg)	0	1.0	0	1.0	0	1.0	0	1.0		TEMP	AOX	BET
<u>0 to 21 days</u>												
ADFI (g/d)	59.2	56.7	54.4	60.1	62.0	58.4	57.4	55.3	2.82	0.64	0.12	0.66
ADG (g/d)	36.6	36.3	35.2	35.2	34.1	36.4	35.5	36.3	1.58	0.87	0.57	0.49
FCR	1.62	1.56	1.55	1.71	1.82	1.61	1.62	1.43	0.10	0.52	0.35	0.34
<u>21 to 42 days</u>												
ADFI (g/d)	162	173	167	169	161	155	153	159	6.30	0.001	0.82	0.30
ADG ⁴ (g/d)	93.5	102	91.8	91.4	80.5	86.9	79.6	89.3	3.59	< 0.001	0.13	0.001
FCR ⁵	1.74	1.69	1.83	1.86	1.99	1.80	1.93	1.78	0.09	0.04	0.36	0.04
<u>0 to 42 days</u>												
ADFI (g/d)	111	115	111	115	111	109	105	107	3.92	0.01	0.45	0.49
ADG ⁶ (g/d)	65.0	69.3	63.5	63.3	57.6	61.6	57.5	62.8	1.90	< 0.001	0.09	0.001
FCR ⁷	1.71	1.66	1.75	1.81	1.93	1.74	1.83	1.70	0.06	0.03	0.60	0.02
Final weight (g)	2769	2949	2704	2699	2457	2627	2454	2675	161.9	< 0.001	0.20	0.003
Breast weight ⁸ (g)	598	746	612	584	500	587	498	575	66.2	< 0.001	0.17	< 0.001
Fat weight ⁹ (g)	31.9	38.3	42.7	35.2	28.9	34.0	37.2	33.7	9.31	0.13	0.10	0.95

*ADFI: average daily feed intake; ADG: average daily weight gain; FCR: feed conversion ratio.

¹There were no interactions ($P > 0.10$) except where indicated.

²Control diet contained 50 IU/kg Vit E and 0.3 mg Se/kg and 250 IU/kg Vit E and 0.5 mg Se/kg added to control diet for supplemented diets.

³Standard error of the difference for effect of TEMP × AOX × BET.

⁴Trend for TEMP × AOX ($P = 0.057$) and TEMP × AOX × BET ($P = 0.088$) interactions.

⁵Trend for TEMP × AOX ($P = 0.080$) and TEMP × BET ($P = 0.082$) interactions.

⁶Significant TEMP × AOX ($P = 0.029$) interaction.

⁷Significant TEMP × AOX ($P = 0.016$) and TEMP × BET ($P = 0.013$) interactions.

⁸Breast weight (m. pectoralis major plus m. pectoralis minor) in broiler chickens aged 42d. Significant TEMP × AOX ($P = 0.046$), AOX × B ($P = 0.007$) and TEMP × AOX × BET ($P = 0.015$) interactions.

⁹Abdominal fat pad in broiler chickens aged 42 d. Significant AOX × BET ($P = 0.019$) interaction.

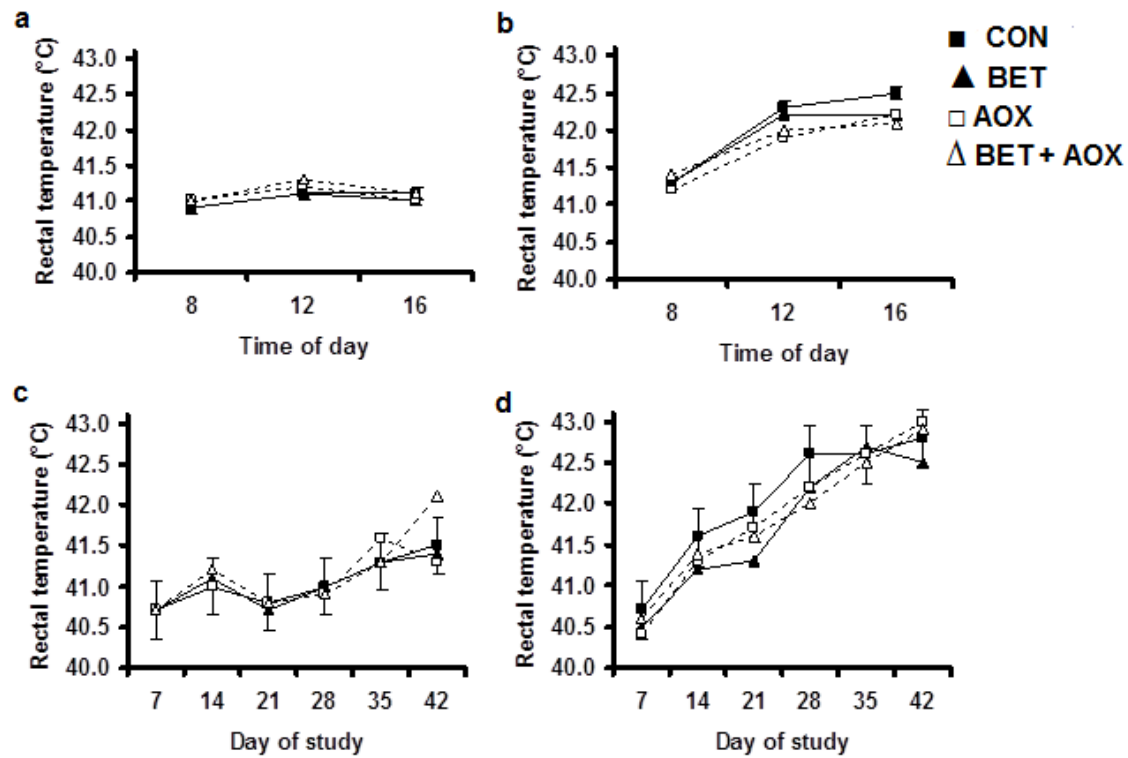


Figure 1. Rectal temperature in chickens fed either a control diet (CON, filled square), the control diet plus betaine (BET, filled triangle), the control diet plus supplemental antioxidants (AOX, open square) or the control diet plus both BET and AOX (open triangle) under either thermoneutral (a, c) or cyclic heat stress (b, d). Panels (a) and (b) indicate the effect of time of day (TM) pooled across days of the experiment with the standard error of the difference for the interaction between TM, BET and AOX displayed on the data from the chicken receiving the control diet. Panels (c) and (d) indicate the effect of day of experiment pooled across time of day with the standard error of the difference for the interaction between day of experiment (DAY), BET and AOX displayed on the data from the chicken receiving the control diet. There were significant main effects of temperature (TEMP) ($P < 0.001$), DAY ($P < 0.001$) and TM ($P < 0.001$) and TEMP $\times$ AOX ($P = 0.048$), TM $\times$ DAY ($P < 0.001$), TEMP $\times$ TM ($P < 0.001$), DAY $\times$ TM ($P < 0.001$), TM $\times$ BET ($P = 0.015$), DAY $\times$ BET ($P < 0.001$), TM $\times$ DAY $\times$ BET ($P = 0.040$), DAY $\times$ TEMP $\times$ AOX ($P < 0.001$), DAY $\times$ BET $\times$ AOX ($P < 0.001$) and TM $\times$ DAY $\times$ TEMP $\times$ BET $\times$ AOX ($P < 0.014$) interactive effects. There were no other main or interactive effects ($P < 0.05$).

Respiration rate was increased by HS ($P < 0.001$) and decreased by BET ($P = 0.017$) particularly during HS conditions as indicated by the TEMP $\times$ BET ($P = 0.048$) interaction (Figure 2). There were no main or interactive effects of AOX on respiration rate (Figure 2).

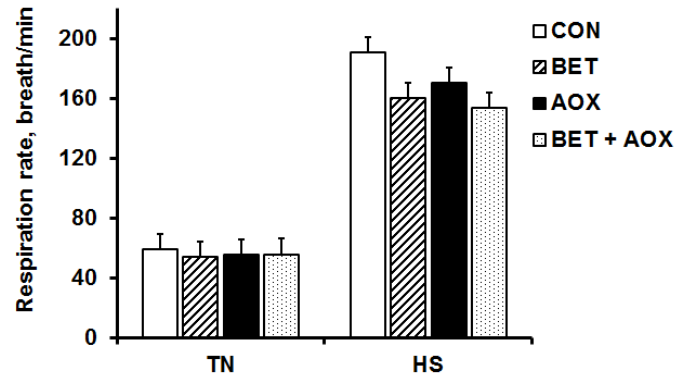


Figure 2. Respiration rate in chickens fed either a control diet (CON), CON plus betaine (BET), CON plus supplemental antioxidants (AOX) or CON plus both BET and AOX under either thermoneutral (TN) or cyclic heat stress (HS) at 12:00 h on day 41 of the experiment. Data are means with the standard error of the difference for the interaction between temperature (TEMP), BET and AOX. The P -values for the effects of TEMP, BET, AOX, TEMP $\times$ BET, TEMP $\times$ AOX, BET $\times$ AOX and TEMP $\times$ BET $\times$ AOX were < 0.001 , 0.017 , 0.16 , 0.048 , 0.24 , 0.37 and 0.69 , respectively.

3.3. Intestinal Epithelial Barrier Integrity

Heat stress reduced jejunum TER by approximately 30% compared to their TN counterparts (149 vs $104 \Omega \cdot \text{cm}^2$) but there were no main or interactive dietary effects (Figure 3). Heat stress had no effect on jejunum FD4 permeability ($P = 0.24$) whereas dietary BET increased jejunum FD4 permeability ($P = 0.013$) compared to control chickens (Figure 4).

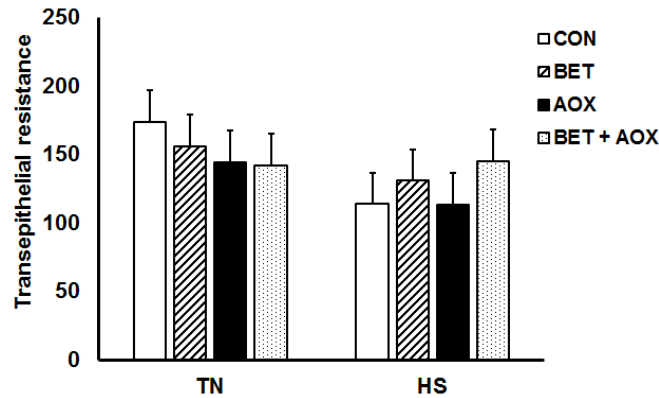


Figure 3. Jejenum transepithelial electrical resistance (TER) in chickens under either thermoneutral (TN) or cyclic heat stress (HS) and fed either a control diet (CON), CON plus betaine (BET), CON plus supplemental antioxidants (AOX) or CON plus both BET and AOX.

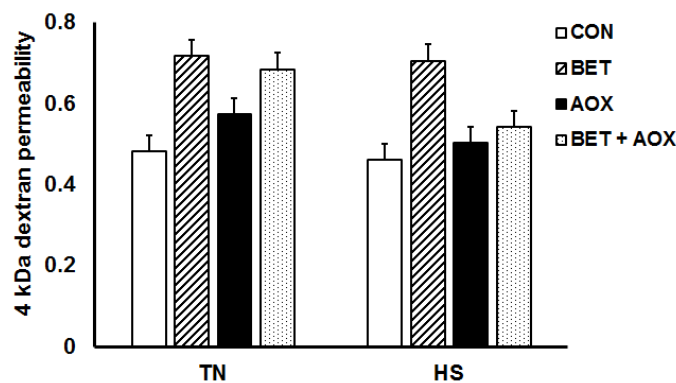


Figure 4. Jejenum fluorescein isothiocyanate (FITC)-dextran (4k Da) permeability in chickens under either thermoneutral (TN) or cyclic heat stress (HS) and fed either a control diet (CON), CON plus betaine (BET), CON plus supplemental antioxidants (AOX) or CON plus both BET and AOX.

3.4. Glutathione Peroxidase Activity

All of the main and interactive effects of HS, day, BET and AOX on liver GPx activity were significant ($P < 0.001$) (Figure 5) which requires a comprehensive interrogation of the data to obtain a clear picture. There was no effect of HS on GPx in the chickens consuming the control diet on day 21 (12.5 vs 11.9 nmol/(mg protein.min)) whereas GPx on day 42 decreased in those chickens maintained under TN conditions but was unchanged in those exposed to HS (6.33 vs 13.9 nmol/(mg protein.min)). Dietary

BET and AOX increased GPx on day 21 but the effects were not additive and there was no main effect of HS at this time. While there was a decrease in GPx activity between day 21 and day 42 for all dietary groups under TN conditions, nevertheless supplemental BET and AOX increased GPx over that of the controls. On day 42 the effects of dietary BET and AOX were exacerbated by HS as indicated by highly significant 3- and 4-way interactions (Figure 5).

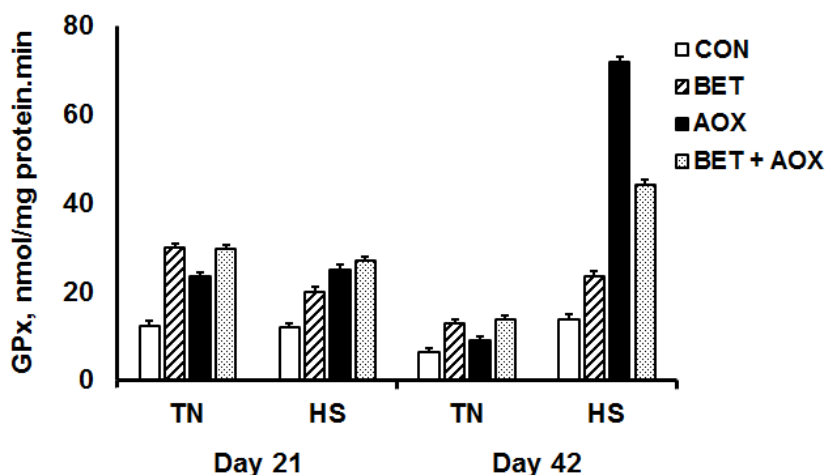


Figure 5. Glutathione peroxidase activity (GPx) in chickens under either thermoneutral (TN) or cyclic heat stress (HS) and fed either a control diet (CON), CON plus betaine (BET), CON plus supplemental antioxidants (AOX) or CON plus both BET and AOX at day 21 and 42.

4. Discussion

4.1. Effects of Betaine, Selenium and Vitamin E on Growth Performance and Physiological Responses

Under some rearing conditions HS can be a major cause of impaired performance, reduced muscle development and increased mortality rate in chickens. For example, HS impairs growth performance (Farooqi et al., 2005; Zhang et al., 2012a) by increasing body temperature and panting rate due to respiratory alkalosis with subsequent growth depression in chickens (Teeter et al., 1985a). During HS, the chicken diverts energy towards reducing their body temperature and away from growth and breast muscle development through different metabolic mechanisms (Teeter et al., 1985a). This experiment confirmed that HS increased rectal temperature (+1.3°C) and respiration rate (+223%), and reduced ADG (-11%), ADFI (-4%) and breast weight (-16%) in broiler

chickens fed a conventional commercial ration. One major finding from the present experiment was that supplementation with 1g/kg BET partially ameliorated rectal temperature, respiration rate, ADFI and ADG and fully ameliorated breast muscle weight in chickens exposed to chronic cyclic HS, which is in line with (Alirezai et al., 2012). The effects of AOX were not as pronounced although AOX reduced rectal temperature and respiration rate during the hottest part of the day in chickens exposed to HS conditions. The results can be explained by improvements obtained from AOX which are an important part of many metabolic pathways, and show that AOX can enhance the immunity, which decreases body temperature and reduces oxidative damages in cell (Swan and Boles, 2002). Importantly, these improvements were observed in rapidly growing chickens of an improved genotype. Furthermore, dietary BET but not AOX, improved ADG (+7%), FCR (-3%), final live weight (+7%) and breast muscle (+25%) in chickens housed under TN conditions. While others have found that dietary BET can increase ADG and final live weight (Alirezai et al., 2012; Craig, 2004b; He et al., 2015; Nofal et al., 2015) and reduce rectal temperature and respiration rate (Craig, 2004b) during HS, the present experiment is the first with an improved genotype capable of reaching 2.8 kg by 42 days of age under TN conditions. By comparing the production results of different genotypes of broiler chickens, it can be inferred that breed selection, genetic progress and nutritional strategies are among the most cost-effective approaches to increasing poultry production. Among different broiler breeds, Ross and Cobb breeds have achieved better final growth performance, and a recent study (Attia et al., 2018) showed even better performance for Ross-308 under HS. Previous studies (Garcia et al., 2000; Nofal et al., 2015) observed growth improvement in other genotypes of broiler chickens when different levels of betaine up to 1g/kg were supplemented to the diet, while Ross-308 supplemented with 1 g/kg betaine in the current study showed greater growth performance compared to the previously published studies under HS conditions.. Dietary BET can assist the performance during HS by playing an important role in the methionine-homocysteine cycle by acting as a methyl group donor (Rao et al., 2011). Dietary BET may spare methionine from methyl donation for other metabolic mechanisms such as protein synthesis and immune modulation (Kettunen et al., 2001a). Moreover, BET alleviates the negative effects of osmotic stress in the intestine and other

tissues (Egbuniwe et al., 2016), eliminates toxic metabolites (Rao et al., 2011) and enhances antioxidant status (Zhan et al., 2006).

4.2. Effects of Betaine, Selenium and Vitamin E on Gut Permeability and Integrity

Heat stress may impair growth performance and health by damaging intestinal barrier function in the gut (Gabler et al., 2013) and the findings from this experiment confirm that HS reduces TER in broilers (Ibrahim et al., 2011; Liu et al., 2015; Tellez Jr et al., 2017). The reason for this phenomenon is likely due to a reduction in blood flow to intestinal and other visceral tissue which can cause hypoxia and resultant oxidative damage. Despite the decrease in TER, HS did not increase permeability compared to TN which is in contrast to (Dunshea et al., 2008). The reason may be related to the positive impacts of BET and AOX under HS conditions. Betaine has been shown to improve growth performance in pigs (Dunshea et al., 2008), poultry (Attia et al., 2009b; Zulkifli et al., 2004) and sheep (DiGiacomo et al., 2016). These positive effects can be explained by improved intracellular water retention, protection of intracellular enzymes against osmotically induced inactivation (Craig, 2004b) and a reduction in energy expenditure. In the present study, dietary BET increased permeability of jejunum compared to other diets. Dietary AOX and BET can reduce gut cell damage and improve (reduce) intestinal permeability in broilers (Ibrahim et al., 2011; Tellez Jr et al., 2017). The reason for differences between our results with other studies may be due to the difference in site of measurement. Betaine is mostly absorbed in the duodenum and has a greater effect on the duodenum because osmotic pressure in this section is higher (Chauhan et al., 2014; Egbuniwe et al., 2016).

4.3. Effects of Betaine, Selenium and Vitamin E on Glutathione Peroxidase Activity

Heat stress disturbs the balance between the production of reactive oxygen species (ROS) and GPx activity in broiler chickens (Lin et al., 2008; Lin et al., 2006a; Williams et al., 2004). Increased ROS concentrations lead to oxidative damage and decreased GPx activity (de Vega et al., 2016). Glutathione peroxidase production and activation is an adoptive mechanism in the cellular anti-oxidant defence system which is increased when free radical production increases to deal with oxidative stress to protect against tissue damage (Altan et al., 2003). These results are in concordance with those of (Azad et al., 2010b; Gu et al., 2012; Tan et al., 2010) where cyclic HS increased liver GPx activity in

chickens. Moreover, Se, Vit E and BET supplementation increased GPx activity in broiler chickens (Alirezai et al., 2012; Cai et al., 2012; Harsini et al., 2012; Zhou and Wang, 2011) and pigs (Liu et al., 2016). The present data indicate that the decrease in GPx levels over time in chickens that were not supplemented with BET or AOX is possibly related to a reduction in GPx synthesis, and that this mechanism can be enhanced by Se that helps regulate the synthesis of GPx enzyme (Baker et al., 1993). The reduction in GPx over time may also indicate that as fractional growth rate decreases, so too does the production of ROS and hence the need for GPx. Moreover, BET enhances anti-oxidant defence (Egbuniwe et al., 2016) by acting as a free radical scavenger to neutralize free radicals, and promotes the main antioxidant enzyme activities including GPx (Alirezai et al., 2012; Carocho and Ferreira, 2013; Kheradmand et al., 2013).

4.4. Betaine, Selenium and Vitamin E Functions During Heat Stress

The ability of BET to stimulate ADG and breast muscle under TN conditions has also been observed previously (Kettunen et al., 2001a), although the magnitude of the effects here were impressive given the high basal performance of the control chickens. The breast muscle is the most valuable muscle in the chicken carcass (Suster et al., 2004a), so an increase in muscle weight of the magnitude observed here is profound. When protein deposition is limited by energy intake, the energy spared from the reduction in ion-pumping during BET supplementation can be used to stimulate skeletal muscle deposition (Sakomura et al., 2005). It is likely that the protein deposition potential of rapidly growing chickens of an improved genotype is limited by energy intake (Nunes et al., 2005), therefore dietary BET would be expected to stimulate skeletal muscle deposition.

Selenium protects tissues against cell damage and excessive ROS generation via the glutathione peroxidase pathway, thereby protecting tissues against oxidation of lipids and proteins (McDowell, 2012). Vitamin E regenerates damaged cells by reducing synthesis of malonyldialdehyde and protecting them against lipid peroxidation and cell damage (Perić et al., 2009), thereby improving chicken growth performance (Hosseini-Mansoub et al., 2010). In the present experiment AOX decreased growth performance under TN conditions but not under HS conditions. In addition, the rectal temperature in chickens fed supplemental AOX, particularly when combined with BET, was increased towards the end of the experiment in chickens housed under TN conditions. These findings are

consistent with findings of (Rao et al., 2011) and (Zduńczyk et al., 2011a) who found that supplementation with Se above 0.3 mg/kg impaired growth performance in chickens in tropical climatic conditions. Moreover, (Habibian et al., 2016) and (Yoon et al., 2007) indicated that the combination of 0.5 mg/kg Se with 250 IU/kg VitE or 0.5 mg/kg Se with 200 IU/kg VitE had no positive impacts on chicken performance. However, in other studies supplementation of up to 0.5 mg/kg Se alone (Zhou and Wang, 2011) or 250 IU/kg Vit E alone (Hosseini-Mansoub et al., 2010) improved performance. Interestingly, (Ramnath et al., 2008) found an interaction between AOX and thermal treatment such that AOX reduced the HS-induced increase in reactive oxygen species whereas under TN conditions AOX tended to increase reactive oxygen species and other indices of oxidative damage. These findings indicate that when there is little oxidative stress, such as under TN conditions, then excess AOX may create pro-oxidant conditions. Despite there not being any growth promoting effects, supplementation with AOX did reduce rectal temperature and respiration rate during HS in the present experiment indicating some partial mitigation against HS.

5. Conclusions

Betaine has an important role as an osmolyte and methyl donor in poultry and therefore may be useful under dysfunctional osmoregulatory conditions such as HS. The results of this experiment demonstrate that supplementation of 1g/kg BET individually, or in combination with AOX, could alleviate the effects of HS and improve ADG, ADFI, FCR and breast development of Ross-308 broiler chicken for up to 6 weeks. However, AOX did not improve growth performance under TN condition which may be related to creation of pro-oxidant conditions. Moreover, the beneficial effects of BET might be related to lower rectal temperature and respiration rate during HS which helps chickens convert more consumed energy and nutrients into meat rather than mechanisms to reduce body temperature.

Author Contributions: F.R.D, J.J.C and M.S designed the experiment; M.S performed the experiment; M.S, M.R and J.J.C analysed the samples; F.R.D, M.S analysed the data; F.R.D, J.J.C and M.S wrote the manuscript; All authors read and approved the final manuscript.

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CHAPTER 4

Growth Performance and Characterization of Meat Quality of Broiler Chickens Supplemented with Betaine and Anti-oxidants Under Cyclic Heat Stress

4.1. Introduction, aims and hypotheses

Heat stress occurs in broilers when the temperature is higher than optimum (Lin et al., 2006b), and results in adverse effects on performance (Al-Fataftah and Abu-Dieyeh, 2007) and meat quality (Zhan et al., 2006). During HS, chickens attempt to dissipate excess body heat by increasing panting rate and spend a greater proportion of dietary energy on reducing core temperature than on metabolic pathways, resulting in impaired performance as well as reduced meat quality. Consequently, there is a growing interest in affordable dietary strategies to reduce the adverse effects of HS on meat quality and performance in domestic animals (Cottrell et al., 2015b; DiGiacomo et al., 2014a).

The aim of this chapter was to investigate the effects of the osmolyte betaine alone or in combination with selenium and vitamin E supplementations on growth performance, physiological responses, and meat quality when chicken were exposed to cyclic HS.

Section 4.2 presents the published paper that showed the effects of betaine and antioxidants on broiler growth performance and meat quality.

4.2. Published paper

Growth performance and characterization of meat quality of broiler chickens supplemented with betaine and antioxidants under cyclic heat stress

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Abstract: Heat stress (HS) causes oxidative stress, which compromises broiler performance and meat quality. The aim of this study was to determine if dietary antioxidants could be used as an amelioration strategy. Seventy-two day-old-male Ross-308 chicks were exposed to either thermoneutral or cyclical heat stress conditions. Diets were either control commercial diet (CON), CON plus betaine (BET), or with a combination of betaine, selenized yeast and vitamin E (BET+AOX). Heat stress increased rectal temperature ($p < 0.001$), respiration rate ($p < 0.001$), decreased blood $p\text{CO}_2$ ($p = 0.002$) and increased blood pH ($p = 0.02$) indicating the HS broilers had respiratory alkalosis. Final body weight was decreased by HS ($p < 0.001$) whereas it was improved with BET ($p = 0.05$). Heat stress reduced cooking loss ($p = 0.007$) and no effect on drip loss, while BET decreased drip loss ($p = 0.01$). Heat stress reduced myofibril fragmentation index ($p < 0.001$) and increased thiobarbituric acid reactive substances ($p < 0.001$) while these were improved with the combination of BET+AOX ($p = 0.003$). In conclusion, BET overall improved growth rates and product quality in this small university study whereas some additional benefits were provided by AOX on product quality in both TN and HS broilers.

Keywords: heat stress; betaine; antioxidants; growth performance; meat quality; broiler chickens

1. Introduction

With increasing global temperatures and incidence of sub-tropical and tropical broiler production (Henry et al., 2012), the management of heat stress (HS) is a subject of increasing concern for industry. Broilers are susceptible to increased environmental heat loads for a variety of reasons, including that they lack sweat glands, feather coverage and that increased selection for muscling means they are comparatively less resilient to heat than other production animals (Renaudeau et al., 2012). Broilers reared under hot conditions have a reduced growth rate, in part due reduced feed intake. However only half of the consequential reduction in growth rates is due to feed intake alone (Geraert et al., 1996), and skeletal muscle from thermally challenged broilers has reduced rates of protein synthesis and to a lesser extent proteolysis (Temim et al., 2000). This is in part due to adaptive endocrine alterations to HS, for example thyroid hormones are permissive for growth and negatively correlated with elevated temperature. Furthermore, insulin is an important regulator of muscle metabolism and protein synthesis, and downstream anabolic and metabolic pathways of insulin signaling are suppressed in skeletal muscle of the HS broiler (Ezzine et al., 2010). Other implications for product quality include reduced muscle glycogen and muscle pH, paler colour (Akşit et al., 2006), increased lipid oxidation (Love and Pearson, 1971) and changes muscle fiber structure (Huang et al., 2015; Rajagopal and Oommen, 2015). Consequently, there is a growing interest in affordable dietary strategies to reduce the adverse effects of HS on meat quality and performance in domestic animals (Cottrell et al., 2015b; DiGiacomo et al., 2014a). Some of the most promising strategies include betaine (BET), selenium (Se) and vitamin E (VitE) which individually have shown positive effects on chicken performance and meat quality during HS.

Betaine is a natural compound which can be found in many organisms and is commercially derived from sugar beets. It protects cells against osmotic inactivation, increases water retention of cells (Craig, 2004b), improves protein and energy metabolism (Alirezai et al., 2012) by acting as a methyl donor which is important for synthesis of methionine and carnitine. Betaine is a potent antioxidant (Alirezai et al., 2015) and dietary BET can increase plasma glutathione peroxidase in chickens under both thermoneutral (TN) and HS conditions (Shakeri et al., 2018). It also reduces core body temperature by reducing the activity of the ion pumps required for osmoregulation,

allowing more energy for growth (Dunshea et al., 2013; Suster et al., 2004b; Tsai et al., 2015b). In this regard, BET may improve body weight and meat quality in chickens (Attia et al., 2009b; Sun et al., 2008). Selenium is an antioxidant that may affect growth performance by the maintenance of tissue cellular integrity (Oliveira et al., 2014; Zhou and Wang, 2011). The activity of Se is often augmented through interactions with VitE, maximizing the efficiency of the vitamin as an antioxidant. There are indications that Se deficiency has deleterious effects on growth performance and meat quality (Choct et al., 2004; Perić et al., 2009). Moreover, VitE improves performance and meat quality by protecting and regenerating damaged tissues during oxidative stress (Gao et al., 2010; Sahin and Kucuk, 2001b) and reducing lipid peroxidation and drip loss (Hashizawa et al., 2013).

In a previous investigating the individual and combined effects of BET and the antioxidants Se and VitE (AOX) on growth performance and physiological response to HS we observed improved performance in response to BET alone, and no positive effects of AOX (Shakeri et al., 2018). However, we did see some additional benefits of AOX when combined with BET. Therefore, the aims of the present study were to investigate the effects of BET alone and in combination with Se and VitE on growth performance and meat quality characterize and the physiological responses to HS in broiler chickens.

2. Materials and Methods

2.1. Ethics Statement

The study was performed in Animal facility approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia (Protocol no. 1714224.1). The use of animals for research is regulated by federal and state legislation according to the Prevention of Cruelty to Animals Act 1986 and the Prevention of Cruelty to Animals Regulations 2008.

2.2. Animals, Diet and Experimental Design

Seventy-two one day-old male Ross-308 chicks were obtained from a commercial hatchery and transported at ~30 °C within 2 h distance to our facility (Tri Foods Pty. Ltd, Bannockburn Victoria, Australia). Chicks were randomly selected, wing-tagged, and individually weighed and housed in pens of 3 birds in one of two environmentally controlled rooms (12 pens /room).The temperature for both rooms was held constant at

33 °C for the first 7 days of the study before being gradually decreased to 25 °C from days 7 to 21 for the TN room (1 °C decrease every 2 days), and then remained at 25 °C from days 21 to 42 (Aviagen, 2009); or cyclic HS conditions where the temperature was kept at 33 °C from days 7 to 42 between 9 am and 5 pm and at the prevailing temperature in the TN room from 5 pm until 9 am. The relative humidity was between 45-60 % during the study. Light was provided 24 h for the first 3 days after placement and was gradually reduced (1 h/day) to 20 h at day 7 for both rooms. The pens measured 1 m × 1 m and had ~10 cm deep wood shavings on the floor.

Chickens were fed either a commercial control diet (50 IU/kg VitE and 0.3 mg/kg Se) (CON), or the control diet supplemented with 1 g/kg betaine (Betafin S1, DuPont, UK) (BET) or the control diet plus a combination of 1 g/kg betaine with 0.3 mg/kg Se (selenised yeast, selenoSource 3,000, Diamond V Mills, Cedar Rapids, USA) and 200 IU/kg natural yeast VitE (VE 200, dl- α -tocopheryl acetate, ADM, USA) (BET+AOX). The control diet was formulated to satisfy NRC (NRC, 1994) requirements with Se provided as selenised yeast and the VitE as natural VitE as outlined above. Feed (Feedworks BESTMIX, The University of Sydney, Australia) was formulated as a commercial starter from days 1 to 14 (CP 24.7 % and 12.6 MJ ME/kg), grower from days 15-28 (CP 22.8 % and 13.0 MJ ME/kg) and finisher from days 29-42 (CP 21.24 % and 13.4 MJ ME/kg). Feed and water were provided *ad-libitum*. Live weight and consumed feed were recorded weekly to calculate average daily body weight gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR).

Starting from day 14, two chickens per pen were randomly selected weekly to measure rectal temperature at 8 am, 12 pm, and 15 pm with a digital thermometer (Comark PDT 300, UK) inserted about 3-4 cm into the rectum for 30 sec. Respiration rate was measured from two randomly selected chickens per pen at 12 pm on days 35 and 41. Chickens were filmed with a cellphone (iPhone 7, Apple Inc., USA) and then the number of breaths taken over a 20 sec. period was quantified and then expressed as breaths per minute.

Twenty-four chickens were randomly selected to collect blood samples (4 from each treatment) on day 41. Approximately 3 mL blood was collected from the wing vein and centrifuged at 10,000 x g for 15 min. to obtain plasma. A portion (~0.3 mL) of the fresh

blood sample was loaded into an automatic blood gas analysis (EPOC®; Alere, Waltham, Massachusetts, USA) to measure blood gas and chemistry parameters.

2.3. Slaughter and Meat Quality Measures

All chickens were killed with a dry electrical stunner (standard 15 amp 220-volt, Mitchell Engineering Food Equipment, Pty Ltd, Australia). Then, chickens were immersed in hot water of 60-62 °C for 80 sec. before they were mechanically stripped of the feathers (1.5 Kw, 660 mm, Bellsouth Poultry Equipment, Pty Ltd, Australia). All carcasses were then placed in sealable plastic bags and sealed before being suspended in ice water (~4 °C) for 40 min. and the carcasses were then transferred to a cool room (4 °C). These slaughter procedures follow standard industry practice. The *m. Pectoralis major and minor*, hereon referred to as the breast muscle, were removed from the carcass at 24 h after slaughter.

Muscle pH was measured with a polypropylene spear type electrode (Ionode IJ44A, Pty Ltd, Queensland, Australia) calibrated at pH 4 and 7 and portable pH meter (WP-80M, TPS, Pty Ltd, Queensland, Australia) by inserting ~2 cm deep for 30 sec. Immediately after the removal of the feathers, the breast muscles were partially skinned and pH measured 3 times at >10 min, 1 h and 24 h after slaughter before deboning. Drip loss was quantified by weighing 10 g of breast meat and then suspending in a bottle with a sealed cap for 48 h. All samples had freshly trimmed surfaces and were devoid of covering fat or connective tissue. After 48 h, all samples were re-weighed to calculate drip loss. Warner Bratzler shear force (WBSF) was quantified on blocks of cooked meat 1 cm wide, 1 cm high and 3 cm lengthwise (Instron, canton, Massachusetts, USA). Prior to cooking, samples (80g) were weighed and were then placed in a plastic bag and then cooked in an 80 °C water bath for 40 min. After cooking, samples were cooled in running tap water for 10 min, then gently dried with a piece of paper towel without being squeezed to remove all moisture lost on the meat surface before they were re-weighed to measure cooking loss. Cooked samples were chilled overnight at 4 °C to measure WBSF after 24 h. Cooked samples were cut into blocks and WBSF measured using an inverted V-blade and cross head speed 200 mm/min perpendicular to muscle fiber orientation and Lloyd (Lloyd Instruments LS5S/C Materials testing instrument, Lloyd Instruments Ltd., Hampshire, UK) texture analyser fitted with a 500 N load cell. Warner Bratzler shear force was performed at least 6 times for each sample. A Minolta chromameter CR-400

with 8 mm aperture (Minolta Pty Ltd., Japan, light source D65, observer angle 2°) was used to determine colour at days 1 and 4 post-slaughter after a 20 min. bloom at 4 °C with measurements in the CIE L*, a*, b* system where L* indicates relative lightness, a* redness and b* yellowness. Breast muscle was placed on retail trays, over-wrapped, then placed in simulated retail display, with illumination, at 4 °C for three days before measuring colour. All readings were taken in triplicate and averaged.

The method of (Culler et al., 1978) modified by (Koohmaraie et al., 1987) was used with some modifications to measure myofibrillar fragmentation index (MFI). Briefly, 2 g minced breast meat was added to 20 mL MFI buffer (100 mM KCl, 20 mM potassium phosphate (pH 7), 1 mM EGTA, 1 mM MgCl₂, and 1 mM NaN₃) and homogenized for 30 sec at 10,000 x g (IKA Ultra Turrax[®] T 25 digital, Selangor, Malaysia). All samples were kept in crushed ice during homogenizing. Samples were centrifuged at 100 x g for 15 min. at 2 °C. After centrifuging, supernatants were discarded and 20 mL MFI buffer was added to each tube. Once more, the samples were centrifuged for another 15 min, at 100 x g at 2 °C. Then, supernatant and fat cap were discarded. 10 mL MFI was added to each tube and vortexed until well mixed. Samples were filtered through a polyethylene cell strainer (100 µm). Protein assay was done by adding 0.25 mL suspension with 0.75 mL MFI buffer and 4 mL biuret reagent (1.5 g CuSO₄×5H₂O, 6g NaKC₄H₄O₆.4H₂O, 300 mL 10 % NaOH adding to 700 mL dH₂O). The mixture was kept in a dark place at room temperature for 30 min. Simultaneously, bovin serum albumin was prepared as 0, 2.5, 5, 7.5 and 10 mg/mL. The absorbance of prepared standards were read at 540 nM with a spectrophotometer (UV-1800, Shimadzu, Japan). The myofibril protein concentration of the samples was adjusted to 0.5 mg/ml and then all the prepared samples were vortexed and mixed well prior to reading absorbance with spectrophotometer at 540 nM (UV-1800, Shimadzu, Japan).

Thiobarbituric acid reactive substances (TBARS) were measured as per (Ohkawa et al., 1979) with minor modifications. To determine TBARS, 2 g of breast muscles were homogenized in 8 mL KCL with homogenizer (IKA Ultra Turrax[®] T 25 digital, Selangor, Malaysia) for 30 sec. at 10,000 x g. Then, 200 µL of homogenized samples was mixed with 35 µL butylated hydroxy toluene 7.0 mM, 165 µL 8.1 % sodium dodecyl sulphate, 2 mL thiobarbituric acid 0.8 %, topped up with 300 µL distilled water. Then, the mixture was heated at 95 °C for 60 min. The mixture was cooled under running water for 5 min,

and 3 mL n-butyl alcohol was added into the mixture, and vortexed for 60 sec. The mixture was centrifuged at 3000 x *g* for 10 min. and the supernatant A₅₃₂ quantified vs a malondialdehyde (MDA) (mg/kg) standard curve (UV-1800, Shimadzu, Japan).

2.4. HPLC Analysis of Betaine and Derivatives

Betaine concentrations were determined in liver, muscle and plasma samples. Standards and samples were prepared using the method of (Laryea et al., 1998) with some modifications. Frozen tissue samples were first pulverized in a dry mill pre-cooled in liquid nitrogen (The Cellcrusher, Cork Ireland). Pulverized tissue (0.5 g) was homogenized with homogenizer (IKA Ultra Turrax[®] T 25 digital, Selangor, Malaysia) for 1 min at 10,000 x *g* in 5 mL of tris buffer (1 M, pH 7), then centrifuged at 24,000 x *g* for 20 min and the supernatant collected. The supernatant and plasma samples (50 µL) was added to 50 µL of 100 mmol/L KH₂PO₄ and 900 µL derivatization solution containing 50 mmol/L 4-bromophenacyl bromide and 2.5 mmol/L 18-crown-6 in acetonitrile and vortex mixed. The samples were heated to 80 °C in a block heater for 1 h, then cooled to room temperature before filtering through a 0.22 µm filter into a glass HPLC vial and placed on a wisp model 712 autosampler attached to a HPLC. The HPLC (Waters 2998, Massachusetts, USA), comprising a quaternary pump, vacuum degasser, an autosampler, and a column compartment with thermostat and a photodiode array (PDA) detector, with data acquisition from Empower software (Water, Massachusetts, USA). A XBridge columns (HILIC 3.5 µm, 4.6×150 mm, Waters, Massachusetts, USA) was used, with the temperature maintained at 25 °C. An isocratic mobile phase solution contained 22 mmol/L choline chloride (Sigma-Aldrich, St. Louis Missouri, USA) in 900 mL/L acetonitrile and 100 mL/L distilled water were used at a flow rate of 1.0 mL/min. The mobile phases were filtered by using a 0.45 µm filter and degassed by sonication for 30 min before use. The injection volume for the analysis of all samples was 10 µL. The UV detection was set to monitor the analytes at 254 nm.

2.5. Plasma Thyroid Hormone Analysis

The thyroid hormones were measure and validated by using free triiodothyronine (T₃) and Thyroxine (T₄) radioimmunoassay kit in plasma (MP Biomedicals, LLC Diagnostics Division, Fountain Parkway, Solon, Ohio, USA). For both T₃ and T₄, plasma samples were kept at room temperature before using in order to minimise deterioration.

For T₃, 100 µL of standards and samples were added to each tube. 1 mL free T₃ tracer was added to each tube and vortexed for 3-5 sec. before incubated in a water bath for 150 min. at 37 °C. After incubating, aspirated all tubes. Added 1 mL distilled water to each tube and aspirated again. All tubes were read with a gamma counter (PerkinElmer Life Science, Wallac 1470 Wizard gamma counter, Waltham, Massachusetts, USA). The obtained results were calculated based on the structure provided by the company and results were presented as pg/mL for T₃. All procedures were the same for T₄, except 50 µL of standards and samples were used, and incubation time was 90 min, and the results were presented as ng/dl.

2.6. Statistical Analyses

All data were analysed using ANOVA for the main and interactive effects of temperature, pooled BET (Control vs BET and BET+AOX) and within BET (BET vs BET+AOX) using GenStat version 16th (VSNi Ltd, Hemel Hempstead, UK). These planned contrasts allows the separation of the response to BET as well as any additional responses to AOX (Dunshea et al., 2002). For physiological parameters and meat pH and color data the effects of main and interactive effects of time were also included as repeated measures effects in the residual maximum likelihood model. Due to the large increase in the magnitude and variation in TBARS between 0.5 h and 24 h post slaughter these data were analysed separately. Differences were considered a trend when $0.10 > p > 0.05$ and significant when $p < 0.05$.

3. Results

3.1. Growth Performance

From days 1-21 there were no effects of temperature or dietary treatments on average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) (Table 1). Between days 21 and 42 there was a reduction in ADG (96.7 vs 85.9 g/d, $p < 0.001$) and ADFI (188 vs 172 g/d, $p = 0.017$) but no effect on FCR in response to HS. BET tended to increase ADG between days 21 and 42 (88.3 vs 92.9 g/d, $p = 0.10$) but had no effect on ADFI or FCR. Over the duration of the entire study there was a reduction in ADG (72.0 vs 65.4 g/d, $p < 0.001$) and ADFI (128 vs 118 g/d, $p = 0.033$) but no effect on FCR in response to HS. BET tended to increase ADG over the entire study (66.4 vs 69.9 g/d, $p = 0.056$) but had no effect on ADFI or FCR. As expected from the growth

performance data, HS reduced final weight (3065 vs 2783 g, $p < 0.001$) and breast weight (737 vs 633 g, $p < 0.001$). Dietary BET increased final weight (2825 vs 2974 g, $p = 0.052$) and breast weight (634 vs 710 g, $p < 0.002$). There were no within BET effects of AOX of growth performance or final and breast weight indicating that there were no additional or negative effects of additional supplementation with AOX nor were there any significant interactions.

Table 1. Effect of dietary betaine (BET) and antioxidants (AOX) on average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) and carcass characteristics during thermoneutral (TN) and heat stress (HS) conditions.

Temperature (T)	Thermoneutral			Heat Stress			s.e.d. ²	<i>p</i> – value ³		
Diet	CON ¹	BET	BET+ AOX	CON ¹	BET	BET+ AOX		T	BET	Within BET
Day 0 -21										
ADG, g/d	45.5	47.0	49.5	43.4	44.1	46.8	1.85	0.11	0.15	0.17
ADFI, g/d	70.8	64.3	67.3	59.7	67.9	66.3	4.35	0.50	0.88	0.87
FCR, g/g	1.56	1.37	1.36	1.38	1.55	1.42	0.098	0.70	0.46	0.49
Day 21-42										
ADG, g/d	93.3	98.9	98.0	83.3	85.2	89.3	3.09	<0.001	0.10	0.61
ADFI, g/d	182	184	197	164	171	180	7.4	0.017	0.13	0.15
FCR, g/g	1.96	1.86	2.01	1.97	2.02	2.02	0.075	0.32	0.86	0.34
Day 0-42										
ADG, g/d	69.4	72.9	73.8	63.3	64.7	68.1	1.99	<0.001	0.056	0.30
ADFI, g/d	126	124	132	112	120	123	4.8	0.033	0.21	0.24
FCR, g/g	1.82	1.70	1.79	1.76	1.85	1.81	0.055	0.35	0.82	0.69
Final weight, g	2953	3103	3138	2697	2755	2898	83.6	<0.001	0.052	0.30
Breast weight, g	672	753	785	596	640	662	24.2	<0.001	0.002	0.24

¹ Control diet (CON) contained 50 IU/kg VitE and 0.3 mg/kg Se. ² Standard error of the difference for T × CON vs pooled BET. ³ T- TN vs HS; BET – CON vs pooled BET treatments; Within BET – BET vs BET+AOX.

3.2. Physiological Responses

Heat stress increased rectal temperature ($p < 0.001$) with the response being greatest ($p < 0.001$) during the afternoon when the ambient temperature was highest (Figure 1b) and no effects under TN (Figure 1a). Dietary BET tended to reduce rectal temperature in response to dietary BET particularly towards the end of the experiment as indicated by

the DAY $\times$ BET ($p = 0.063$) interaction. The response appeared to be greater for those chickens receiving dietary BET alone as indicated by DAY $\times$ BET $\times$ within BET interaction ($p = 0.004$) interactions (Figure 1c,d).

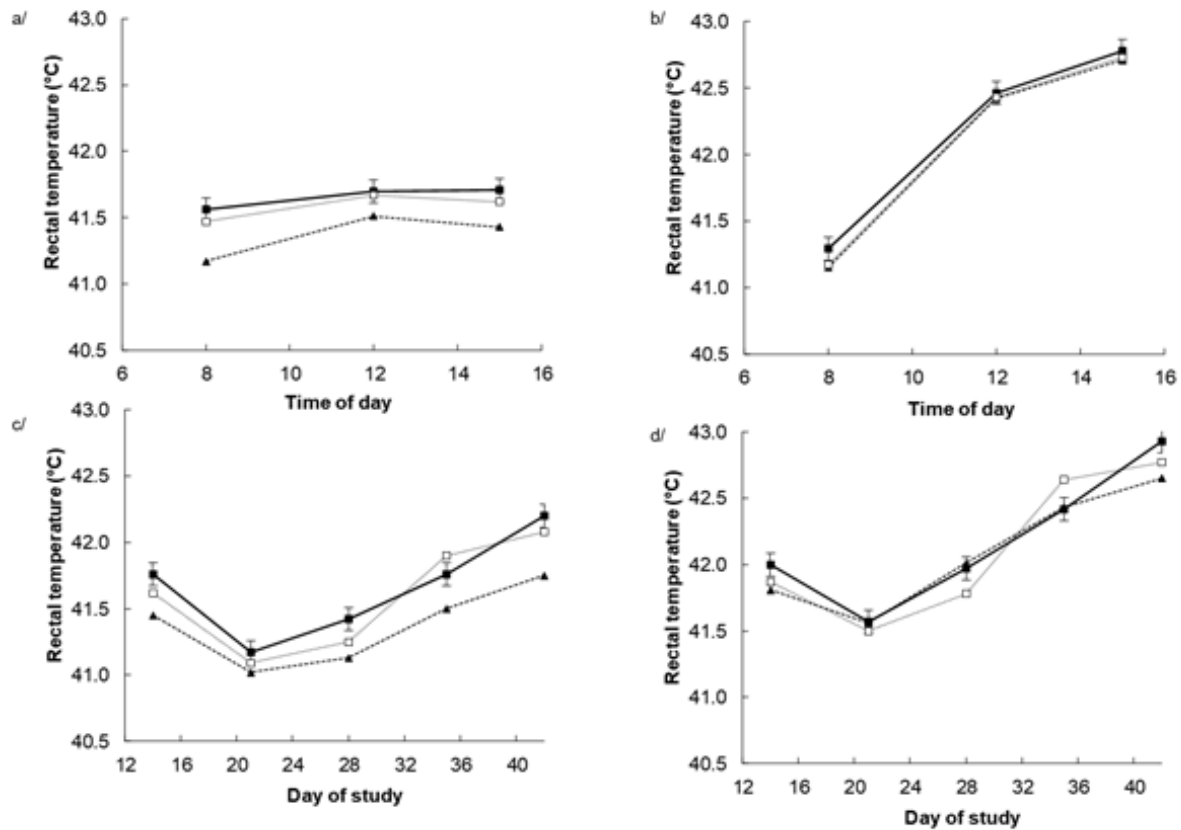


Figure 1. Rectal temperature in chickens fed either a control diet (CON, filled square), the control diet plus betaine (BET, filled triangle) or the control diet plus both BET supplemental antioxidants (BET+AOX, open square) under either thermoneutral (a,c) or cyclic heat stress (b,d). Panels (a,b) indicate the effect of time of day (TM) pooled across days of the experiment with the standard error of the difference for the interaction between TM, BET and within BET displayed on the data from the chicken receiving the control diet. Panels (c,d) indicate the effect of day of experiment pooled across time of day with the standard error of the difference for the interaction between day of experiment (DAY), BET and AOX displayed on the data from the chicken receiving the control diet. There were main effects ($p < 0.10$) of temperature (TEMP) ($p < 0.001$), DAY ($p < 0.001$) and TM ($p < 0.001$), BET ($p = 0.059$) and TEMP $\times$ Day ($p < 0.001$). TEMP $\times$ TM ($p < 0.001$), DAY $\times$ TM ($p = 0.004$), DAYS $\times$ BET ($p = 0.063$) TEMP $\times$

DAY $\times$ TM ($p < 0.001$) and (DAY $\times$ within BET) interactive effects. There were no other main or interactive effects ($p < 0.10$).

Respiration rate was increased with HS (62.4 vs 141 breaths/min, $p < 0.001$) and was reduced with BET (118 vs 99 breaths/min, $p < 0.001$) (Figure 2). However, there was a significant interaction ($p < 0.001$) between HS and BET such that dietary BET reduced respiration rate during HS (171 vs 126 breaths/min) but not under thermoneutral conditions (62.4 vs 62.0 breaths/min) (Figure 2). There were no within BET main or interactive effects indicating that there were no effects of additional AOX over that of BET alone.

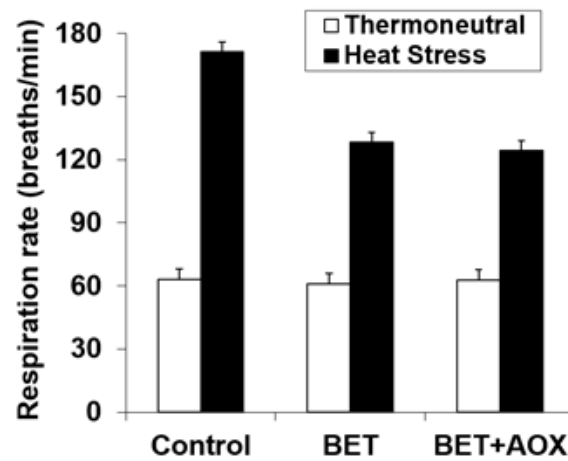


Figure 2. Effect of heat stress and dietary BET or BET+AOX on respiration rate. Since there were no main or interactive effects ($p > 0.10$) of day of study the data are pooled across days 35 and 41 with the standard error of the difference for the interaction between temperature, BET and within BET displayed. There were main effects of temperature (TEMP) ($p < 0.001$) and BET ($p < 0.001$) and a TEMP $\times$ BET ($p < 0.001$) interaction. There were no other main or interactive effects ($p < 0.10$).

Table 2. Effect of dietary betaine (BET) and antioxidants (AOX) on acid base balance and blood gas during thermoneutral (TN) and heat stress (HS) conditions.

Temperature (T)	Thermoneutral			Heat Stress			s.e.d. ²	<i>p</i> – value ³		
Diet	CON ¹	BET	BET+ AOX	CON ¹	BET	BET+ AOX		T	BET	Within BET
pH	7.31	7.31	7.32	7.41	7.37	7.38	0.037	0.021	0.64	0.84
pCO ₂ , mm Hg	54.1	51.9	54.1	40.5	42.7	41.4	4.13	0.002	0.96	0.92
Total CO ₂ , mM	27.1	27.7	32.1	26.0	27.2	28.4	1.39	0.14	0.067	0.058
pO ₂ , mm Hg	31.3	34.0	29.6	35.1	31.9	33.9	1.61	0.15	0.54	0.47
O ₂ saturation, %	49.2	58.8	56.8	62.0	64.0	67.1	4.88	0.029	0.17	0.91
HCO ₃ , mM	27.3	26.5	30.4	24.7	25.9	26.8	1.14	0.024	0.17	0.050
Anion gap, mM	19.3	16.0	15.8	16.3	15.5	13.3	1.36	0.089	0.039	0.37
Base excess, mM	-1.38	-0.23	1.90	-0.10	0.93	1.78	0.994	0.36	0.047	0.15

¹ Control diet (CON) contained 50 IU/kg VitE and 0.3 mg/kg Se. ² Standard error of the difference for T × CON vs pooled BET. ³ T- TN vs HS; BET – CON vs pooled BET treatments; Within BET – BET vs BET+AOX.

Blood pH increased during HS (7.31 vs 7.39, $p = 0.021$) but was unchanged by BET (Table 2). Blood partial pressure of CO₂ was decreased during HS (53.4 vs 41.5 Pa, $p = 0.002$) and was unchanged by BET whereas there was no effect of HS or BET on the partial pressure of O₂. Blood O₂ saturation was increased during HS (54.9 vs 64.4 %, $p = 0.029$) but was unchanged by BET (Table 2). There was no effect of HS on total CO₂ but there were trends towards an effect of BET ($p = 0.067$) and a within BET effect ($p = 0.058$) that showed that the combination of BET and AOX increased total CO₂ compared to both control and BET (26.6 vs 27.4 and 30.3 mM for CON, BET and BET+AOX, respectively). Blood HCO₃ was decreased during HS (28.1 vs 25.8 mM, $p = 0.024$) but was unchanged by BET. However, there was a within BET effect ($p = 0.050$) such that the combination of BET and AOX increased blood HCO₃ compared to both control and BET (26.0 vs 26.2 and 28.6 mM). The anion gap tended to be decreased by HS (17.0 vs 15.0 mM, $p = 0.089$) and was decreased by BET (17.8 vs 15.1 mM, $p = 0.039$) (Table 2). There was no effect of HS on base excess whereas BET increased base excess (-0.74 vs 1.09 mM, $p = 0.047$).

Table 3. Effect of dietary betaine (BET) and antioxidants (AOX) on haematology, electrolytes, metabolites and plasma thyroid hormones during thermoneutral (TN) and heat stress (HS) conditions.

Temperature (T)	Thermoneutral			Heat Stress			s.e.d. ²	<i>p</i> – value ³		
Diet	CON ¹	BET	BET+ AOX	CON ¹	BET	BET+ AOX		T	BET	Within BET
Haematocrit, %	21.5	21.8	22.0	18.8	18.0	19.8	1.04	0.003	0.78	0.35
Hgb ⁴ , g/dL	6.93	7.38	7.45	6.38	6.20	6.83	0.312	0.006	0.26	0.28
Potassium, mM	6.38	5.80	5.80	5.73	5.83	5.30	0.239	0.071	0.092	0.29
Sodium, mM	150	149	150	149	150	149	1.3	0.82	0.96	0.93
Chloride, mM	112	112	110	114	115	114	1.3	0.014	0.82	0.26
Calcium, mM	1.48	1.46	1.49	1.36	1.43	1.42	0.037	0.034	0.42	0.82
Lactate, mM	9.17	7.89	5.24	5.53	5.17	3.73	0.74	<0.001	0.010	0.012
Glucose, mM	15.6	14.5	14.8	15.2	15.7	15.5	0.61	0.30	0.56	0.92
T ₃ ^{4,5} , pg/mL	4.76	6.47	6.08	5.16	3.76	3.43	0.977	0.008	0.97	0.61
T ₄ ⁴ , pg/mL	4.57	5.54	5.74	3.23	3.16	4.49	1.366	0.049	0.33	0.78

¹ Control diet (CON) contained 50 IU/kg VitE and 0.3 mg/kg Se. ² Standard error of the difference for T × CON vs pooled BET. ³ T- TN vs HS; BET – CON vs pooled BET treatments; Within BET – BET vs BET+AOX. ⁴ Haemoglobin (Hgb); triiodothyronine (T₃); Thyroxine (T₄). ⁵ T × BET interaction (*p* = 0.018).

Blood haematocrit (21.8 vs 18.3 %, *p* = 0.003) and haemoglobin (7.25 vs 6.47, *p* = 0.006) decreased during HS but were unchanged by BET (Table 3). Blood potassium tended to be decreased by HS (5.99 vs 5.62 mM, *p* = 0.071) and decreased by BET (6.05 vs 5.68 mM, *p* = 0.092) whereas there was no effect of either HS or BET on sodium. Blood chloride was increased during HS (111 vs 114 mM, *p* = 0.014) but was unchanged by BET. Blood calcium was decreased during HS (1.47 vs 1.40 mM, *p* = 0.034) but was unchanged by BET. Plasma lactate was decreased during HS (7.43 vs 4.81 mM, *p* < 0.001) and was decreased (*p* = 0.010) by BET. However, there was also a strong within BET effect (*p* = 0.012) such that the combination of BET and AOX decreased blood lactate to an even greater extent than BET alone (7.35 vs 6.53 and 4.48 mM for CON, BET and BET+AOX, respectively). There was no effect of either HS or BET on blood glucose concentrations. Plasma T₃ was decreased by HS (*p* = 0.008) while there was no main effect of BET. However, there was a significant interaction between temperature regime and BET (*p* = 0.018) such that plasma T₃ was increased by BET under TN conditions (4.76 vs 6.28 pg/mL) and decreased under HS conditions (5.16 vs 3.60 pg/mL). Plasma

T₄ concentrations were decreased during HS (4.57 vs 3.23 pg/mL, $p = 0.049$) but was unchanged by BET.

There were no main or interactive effects of temperature regime on meat colour or pH the data were pooled for presentation (Table 4). Breast muscle L* increased between 24 and 96 h post slaughter (54.6 vs 55.2, $p = 0.011$) although there was an interaction between time and BET ($p = 0.067$) such that the L* value increased over time in the breast of those chickens fed BET (54.7 vs 55.7) but not in those consuming the CON diet (54.3 vs 54.2) (Table 4). There were no effects of diet or time on a* while b* tended to increase between 24 and 96 h post slaughter (1.56 vs 1.81, $p = 0.062$). There was the expected decline ($p < 0.001$) in muscle pH over the first 24 h post slaughter. However, there were indications of a within BET ($p = 0.094$) and time $\times$ within BET ($p = 0.10$) effects such that the initial pH was higher in breast muscle from those chickens supplemented with both BET and AOX (Table 4).

Table 4. Effect of dietary betaine (BET) and antioxidants (AOX) and time on meat colour and pH during thermoneutral (TN) and heat stress (HS) conditions. Since there were no main or interactive effects of HS the data have been pooled across temperature regimes.

	Hours	Diet				p – value ³				
		CON ¹	BET	BET +AOX	s.e.d ²	Time	BET	Within BET	Time $\times$ BET	Time $\times$ within BET
L*	24	54.3	55.1	54.3	0.967	0.011	0.31	0.52	0.06	0.82
	96	54.2	56.0	55.4						
a*	24	2.20	2.50	2.30	0.283	0.91	0.68	0.57	0.26	0.82
	96	2.22	2.31	2.40						
b*	24	1.43	1.59	1.65	0.268	0.062	0.97	0.49	0.77	0.82
	96	1.74	1.82	1.88						
pH	0.25	6.68	6.68	6.78	0.053	<0.001	0.27	0.094	0.67	0.10
	1	6.48	6.49	6.58						
	24	5.92	5.93	5.93						

¹ Control diet (CON) contained 50 IU/kg VitE and 0.3 mg/kg Se. ² Standard error of the difference for Time $\times$ CON vs pooled BET. ³ Time – 24 vs 96 or 0.25 vs 1 vs 24 hours; BET – CON vs pooled BET treatments; Within BET – BET vs BET+AOX.

Cooking loss was decreased in breast meat from chickens exposed to HS (27.3 vs 24.9%, $p = 0.007$) while there was no main effect of BET. However, there was an

indication of an interaction between temperature regime and BET such this response was most pronounced in the breast from chickens fed the CON diet (27.5 vs 23.0 %) rather than those consuming BET (27.2 vs 25.9 %) (Table 5). While there was no main effect of HS on drip loss there were main ($p = 0.007$) and interactive ($p = 0.052$) effects of BET such that HS increased drip loss in meat from chickens consuming the CON diet (2.11 vs 3.43%) but not in those consuming BET (1.76 vs 1.69 %). Total water content tended to be lower after HS (75.7 vs 75.0 %, $p = 0.079$) but was not significantly altered by BET. There were no main or interactive effects of HS or BET on shear force although there were indications of a HS $\times$ BET ($p = 0.10$) and HS $\times$ within BET ($p = 0.087$) interactions that may be attributed to the meat from the chickens consuming the CON diet and housed under TN conditions having the lowest shear force. The MFI was decreased by HS (84.6 vs 67.5) but unchanged by BET. However, there was highly significant within BET effect ($p = 0.003$) which when coupled with the HS $\times$ BET interaction ($p = 0.031$) clearly showed that the combination of BET and AOX, but not BET alone, protected against the negative effects of HS on MFI (Table 5). There were no effects of HS or BET on TBARS at 0.5 h post- slaughter. However, there were many main and interactive effects at 24 h post-slaughter. Heat stress increased TBARS post slaughter (3.60 vs 5.65, $p < 0.001$) whereas TBARS were decreased by BET (5.72 vs 4.08, $p < 0.001$). However, there was a within BET effect ($p = 0.029$) and HS $\times$ BET ($p = 0.008$) and Hs $\times$ within BET ($p = 0.10$) interactions such that supplemental AOX provided addition antioxidative effects above BET alone under TN conditions but to a lesser extent under HS conditions.

Table 5. Effect of dietary betaine (BET) and antioxidants (AOX) on chicken breast objective eating quality measures during thermoneutral (TN) and heat stress (HS) conditions.

Temperature (T)	Thermoneutral			Heat Stress			s.e.d. ²	<i>p</i> – value ³		
Diet	CON ¹	BET	BET+ AOX	CON ¹	BET	BET+ AOX		T	BET	Within BET
Cooking loss ⁴ , %	27.5	27.4	26.9	23.0	25.8	26.0	1.27	0.007	0.21	0.84
Drip loss ⁵ , %	2.11	1.74	1.78	3.43	1.15	2.22	0.51	0.33	0.007	0.20
Water content, %	74.8	76.5	75.9	74.6	75.2	75.3	0.79	0.079	0.13	0.56
Shear force ⁶ , N	18.4	20.4	23.9	23.0	22.8	21.3	1.95	0.30	0.24	0.34
Breast yield, %	22.8	24.4	24.9	22.7	22.9	23.6	0.92	0.057	0.066	0.37
MFI ⁷	86.3	79.7	87.7	58.1	62.8	81.6	6.23	<0.001	0.14	0.003
0.5 h TBARS ⁸	0.21	0.20	0.17	0.21	0.21	0.17	0.040	0.88	0.37	0.20
24 h TBARS ^{8,9}	5.32	3.48	2.00	6.12	5.51	5.32	0.545	<0.001	<0.001	0.029

¹ Control diet (CON) contained 50 IU/kg VitE and 0.3 mg/kg Se. ² Standard error of the difference for T × CON vs pooled BET. ³ T- TN vs HS; BET – CON vs pooled BET treatments; Within BET – BET vs BET+AOX. ⁴ T × BET interaction (*p* = 0.075). ⁵ T × BET interaction (*p* = 0.052). ⁶ T × BET (*p* = 0.10) and T × within BET (*p* = 0.087) interactions. ⁷ Myofibrillar fragmentation index (MFI); T × BET interaction (*p* = 0.031). ⁸ Thiobarbituric acid reactive substances (TBARS) in mg/kg of malondialdehyde (MDA). ⁹ T × BET (*p* = 0.008) and T × within BET (*p* = 0.10) interactions.

Table 6. Effect of dietary betaine (BET) and antioxidants (AOX) on plasma and tissue betaine concentrations during thermoneutral (TN) and heat stress (HS) conditions.

Temperature (T)	Thermoneutral			Heat Stress			s.e.d. ²	<i>p</i> – value ³		
Diet	CON ¹	BET	BET+ AOX	CON ¹	BET	BET+ AOX		T	BET	Within BET
Plasma ⁴ , µmol/L	85.0	140	145	102	109	125	10.50	0.043	<0.001	0.14
Muscle ⁵ , µmol/g	321	635	413	425	518	567	9.38	0.43	0.009	0.16
Liver ⁶ , µmol/g	403	191	187	208	143	145	45.1	<0.001	<0.001	0.98

¹ Control diet (CON) contained 50 IU/kg VitE and 0.3 mg/kg Se. ² Standard error of the difference for T × CON vs pooled BET. ³ T- TN vs HS; BET – CON vs pooled BET treatments; Within BET – BET vs BET+AOX. ⁴ T × BET interaction (*p* = 0.002). ⁵ T × within BET interaction (*p* = 0.050). ⁶ T × BET interaction (*p* = 0.002).

Plasma BET concentrations were decreased by HS (123 vs 112 µmol/L, *p* = 0.043) and increased by BET (94.0 vs 130 µmol/L, *p* < 0.001) (Table 6). However, there was a

HS $\times$ BET interaction ($p = 0.002$) such that HS increased plasma betaine concentrations in chickens consuming the CON diet (85.0 vs 102 $\mu\text{mol/L}$) whereas it was decreased in those consuming BET (143 vs 117 $\mu\text{mol/L}$). There was no main effect of HS on muscle betaine concentrations whereas it was increased by dietary BET (373 vs 456 $\mu\text{mol/g}$, $p = 0.009$) (Table 6). However, there was a HS $\times$ within BET interaction ($p = 0.050$) such that HS decreased muscle betaine concentrations in chickens supplemented with BET alone (635 vs 518 $\mu\text{mol/g}$) whereas it was increased in those consuming AOX as well as BET (413 vs 567 $\mu\text{mol/g}$). Liver betaine concentrations were decreased by HS (260 vs 165 $\mu\text{mol/g}$, $p < 0.001$) and decreased by BET (306 vs 166 $\mu\text{mol/g}$, $p < 0.001$) (Table 6). However, there was a HS $\times$ BET interaction such that HS decreased liver betaine concentrations to a greater extent in chickens on the CON diet (403 vs 189 $\mu\text{mol/g}$) whereas it was decreased in those consuming BET (208 vs 144 $\mu\text{mol/g}$). Indeed, chickens consuming the CON diet and housed under TN conditions had the highest liver betaine concentrations. Although statistical comparisons were made, there appeared to be a >1000 fold difference between plasma and tissue betaine concentrations suggesting accumulation against a strong concentration gradient.

4. Discussion

The principal findings of this study were that supplementation of BET alone or in combination with AOX ameliorated symptoms of HS in broilers, as evidenced by reduced respiration rate and rectal temperature. This corresponded to improvements in productive performance, with BET improving final body and breast weights. Irrespective of HS or additional antioxidant supplementation. Betaine supplementation resulted in accumulation within the breast muscle and this corresponded to improved meat quality, namely improved drip loss and lipid stability post-mortem. The temperature profile used in this study markedly increased respiration rate and blood pH, indicating that the broilers were experiencing both HS and respiratory alkalosis. In agreement with other studies this resulted in an adaptive endocrine state, reducing thyroid hormone concentrations and growth performance (Alirezai et al., 2012; He et al., 2015; Mello et al., 2015; Nofal et al., 2015; Shakeri et al., 2018). Heat stress reduced meat quality, tending to reduce moisture content, increasing lipid oxidation and reducing MFI, a marker of post-mortem myofibrillar breakdown and proteolytic activity. In general, the positive effects of betaine on HS amelioration were not augmented with antioxidant co-supplementation. However,

when BET was combined with AOX there were positive effects on meat quality, reducing postmortem myofibrillar degradation and lipid peroxidation.

Betaine is accumulated in plant and animal tissue when under osmotic stress, and has previously been reported to accumulate in the hepatic tissue of chicks (Kettunen et al., 2001b). Surprisingly in this study hepatic BET concentrations were lower with BET and BET+AOX supplementation. The opposite pattern was observed in skeletal muscle, which had higher overall concentrations than the liver. Betaine distribution changed during HS, with concentrations falling in the liver and increasing by ~30% in HS CON chickens. The BET and BET+AOX supplemented chickens further increased their BET concentrations by another 30%, under TN or HS conditions. As an osmolyte, BET reduces the osmotic gradients, and therefore the activity of cellular ATPases required to maintain them (Moeckel et al., 2002). The energy saving from reduced ATPase activity could be substantial, and approximated to be as much as 8% (Cronje, 2005). Although no interactions were observed with circulating thyroid hormones, reduction of basal metabolic rate has parallels to adaptive endocrine regulation of by thyroid hormones during HS. Triiodothyroxine and T₄ were lower in this study and others (Raghebian et al., 2016), and amongst their biological roles thyroid hormones serve to set basal metabolic rate though regulating the rate of futile cycling of cellular ATPases. Perhaps part of the reason for the accumulation of betaine in skeletal muscle during HS is thermoregulatory, and a reflection on the physical size of the organ and its contribution to basal metabolic rate. Alternatively, it may reflect accumulation as a protective chaperone.

It has been reported elsewhere that elimination of betaine is primarily by metabolism and not excretion (Schwahn et al., 2003). Furthermore, the liver is a major site of BET utilization through methyl donor reactions to the methionine cycle via betaine homocysteine methyltransferase (BHMT) and other metabolic pathways. It requires further investigation, however, it is possible that the reductions in hepatic betaine observed with supplementation may reflect an increase in utilization. Outside of DNA methylation reactions, fates of betaine could be increased remethylation of homocysteine to methionine as part of the methionine cycle. Methionine is an essential amino acid, utilized in protein synthesis, as a precursor to cysteine, taurine and glutathione. If so this apparent increase in hepatic betaine utilization may explain some of the protective effect of betaine during HS and enhanced growth performance seen in this study and elsewhere

(Eklund et al., 2005; Sayed and Downing, 2011; Zhang et al., 2016). It has been indicated that the antioxidant properties of betaine provide protection by relieving oxidative pressure and increasing productivity, and the enhancement of meat quality in poultry under stress conditions (Akhavan-Salamat and Ghasemi, 2016). During HS, betaine adjusts osmotic pressure, preserves water in cells (Attia et al., 2005; Eklund et al., 2005) and reduces energy expenditure used in the sodium-potassium pump which reduces production of heat from metabolic pathways (Remus, 2001). By reducing heat production, deep body temperature and respiration rate will decrease as the chicken's body does not produce extra heat. Moreover, Se and Vit E are an important part of many metabolic pathways and they enhance immunity which can improve body temperature management and reduce oxidative damages in cells (Habibian et al., 2016; Swain et al., 2000). However, the present results showed no positive effects of Se and VitE on growth performance which is in line with (Habibian et al., 2016; Zduńczyk et al., 2011b). Indeed, the establishment of Se and VitE requirements are difficult, mainly because of their interactions with diverse factors that may affect the necessity of diet supplementation and the physiological status of the broiler chickens (Kuttappan et al., 2012).

The present results of blood analysis are in line with a study that showed sodium was unchanged and potassium was decreased with HS (Borges et al., 2004) while a study reported reduction of both sodium and potassium (Ait-Boulahsen et al., 1995). Apparently, the time of blood sampling could be a reason for differences of the results. During HS, hemodilution occurs as chicken's body temperature increased due to a reduction of sodium concentration, and some of the tissue potassium exits into the bloodstream apparently due to altered membrane permeability (Smith and Teeter, 1987). The results are agree with studies that showed HS increased blood pH (Borges et al., 2004; Teeter et al., 1985b) and reduced HCO_3 and chloride, pCO_2 and haematocrit (Altan et al., 2000; Borges et al., 2004). The increased body temperature that occurs during HS causes broiler chickens to reduce their body temperature through different mechanisms, such as increasing respiration rate. Respiration rate is increased to dissipate heat as well as being a response to the increase in blood pH owing to respiratory alkalosis. Then, more calcium is needed to exert an acidic action to balance blood pH resulting in reduction of HCO_3 . Increased HCO_3 level in BET+AOX could be related to supplementation of Se that increases calcium release from the sarcoplasmic reticulum (Bodnár et al., 2016)

which is responsible to store calcium ions. Respiratory alkalosis causes a primary deficit of CO₂ and hence causes the ratio of bicarbonate to pCO₂ to increase, resulting in increased pH. Subsequently, the kidneys attempt to normalize blood pH by increasing the excretion of HCO₃, by reclaiming H⁺ and by decreasing HCO₃. Total CO₂ was higher for BET+AOX that could be attributed to Se level, as it was indicated that higher dosages of Se caused pulmonary congestion and haemorrhages in lungs (Kumar et al., 2018) which could reduce the ability of lungs to reduce CO₂ in blood. However, this cannot be attribute to Se only, as total CO₂ increased for BET as well. This could be related to heavier chickens, there is a negative relationship between surface area available for exchange in lungs and body size (Maina et al., 1989). The decreased haematocrit could be related to increased circulating blood volume (Zhou et al., 1999) due to increase in plasma volume and lesser increase in total red blood cells. In broilers, it was observed that the haematocrit decreased when the environmental temperature is higher than 30 °C (Zhou, 1998). Therefore, as hemoglobin is a protein in red blood cells that is responsible for delivery of oxygen, haematocrit reduction results in reduction of hemoglobin (Billett, 1990). Unexpectedly, lactate in blood was reduced with HS, BET and BET+AOX, while the duration of recovery time after exposing to HS could be a reason for this reduction (Zhang et al., 2009). Furthermore, BET and BET+AOX could provide sufficient energy for the body by reducing body temperature to avoid anaerobic pathways due to lower lactic acid. The thyroid hormones have major function in the control of metabolic rate and thermogenesis in broiler (Tao et al., 2005). The results of the present study confirmed with (Ayo et al., 2014; Hosseini et al., 2013; Tao et al., 2005) showed that HS reduces both T₃ and T₄. Reduction in thyroid hormones production during HS is one of the pathways for maintenance of homeostasis (Tao et al., 2005). While T₃ results under HS are consistent in most studies and have shown reduction (Elnagar et al., 2010; Mack et al., 2013b; Star et al., 2008), T₄ results are inconsistent among studies that showed reduction (Bobek et al., 1980), increase (Elnagar et al., 2010) or no alteration (Mitchell and Carlisle, 1992b). While there is a correlation between ultimate muscle pH and colour, pH is not the only factor impacting meat color (Priolo et al., 2001). In the present study there were no effects of HS on pH or colour which disagrees with studies that argued that both cyclic and consistent HS could reduce initial and ultimate pH (Cramer et al., 2018; Sandercock et al., 2001). However, other studies support the present results, which

showed that consistent HS (Hashizawa et al., 2013) and short term HS have no effects on initial and ultimate pH (Lu et al., 2017). Furthermore, pH level reduction over time agrees with (Ismail and Joo, 2017; Warner et al., 2007). These studies could provide the insight that the duration of HS is an important factor which could affect pH level. From the colour aspect, the results are in line with (Cramer et al., 2018) indicated that HS has no effect on colour. Lighter breast muscles over time in BET is related to BET functioning as an osmolyte that can improve and regulate cells osmotic balance (DiGiacomo et al., 2014b). The amount of water available in meat is important, as losing water could affect the weight and correspondingly the economic value of the final product. Additionally, it may affect the appearance, the juiciness and the tenderness of the meat. The results of the present study agree with (Feng et al., 2008; Fouad et al., 2016; Wang et al., 2017; Zhang et al., 2012b), which indicated that HS increases water loss, which is evident in the reduced total water content, resulting in reduced cooking loss and reduced breast yield, whereas BET could improve it. It is likely that HS causes leaky membranes, resulting in loss of water from muscle cells and would explain the reduced muscle total water content and reduced breast yield. Betaine, as an osmolyte, maintains the osmotic pressure to prevent dehydration and preserves water in cells (Park and Kim, 2016). The present results are in disagreement with (Feng et al., 2008; Zhu et al., 2017) that showed HS increased shear force, however, the results agree with (Gu et al., 2008) that HS has no impact on shear force. The duration of HS could be a reason for inconsistency results among studies.

Although no overall differences in WBSF were observed in this study, there was a reduction in the MFI in HS broilers. Texture measurements such as WBSF measure the force required to shear through the myofibrils and connective tissue in a standard block of meat. and are a product of both the skeletal muscle fibres and connective tissue. Alternatively, MFI is a measure of the myofibrillar filament fragmentation only. The meat tenderization process is initiated by calpain mediated proteolysis of the skeletal muscle fibre, forming smaller fragments and an increase in the MFI (McDonagh et al., 2001). The results of this study agree with (Fouad et al., 2016; Santos et al., 2008) that HS reduces MFI. The mechanism is that because HS generates high amounts of reactive oxygen species, this either (i) leads to the oxidation of myofibrillar proteins which can cause protein cross-linking and toughness or (ii) leads to direct oxidation of calpain,

preventing proteolysis from occurring, or a combination of both (Frank et al., 2017). Elsewhere, HS has been observed to increase Atrogin-1 gene expression, associated with muscle wasting which would likely have consequences for meat tenderisation. Notably, BET+AOX improved MFI under HS, which may indicate that BET in combination with AOX provides a number of benefits to both product yield and quality.

Lipid oxidation, a process during which meat lipids oxidize and interact with other meat constituents, causes deterioration in the quality of meat and causes undesirable effects in nutritive value. The TBARS in biological pathways, which include lipid hydroperoxides, increases in response to oxidative stress. The results are consistent with (Azad et al., 2010a; Fouad et al., 2016; Mujahid et al., 2007b) whose studies indicated that HS increases oxidative damages to muscle due to increased TBARS. Heat stress generates a high amount of reactive oxygen species due to denaturation of proteins and cell death, resulting in higher oxidative damages in tissues (Fouad et al., 2016). Moreover, it has been indicated that additives such as BET can improve TBARS concentration and preserve the cellular antioxidant storage under stress conditions (Alirezai et al., 2012; Fouad et al., 2016), and there are additional effects of antioxidants (Habibian et al., 2016; Skrivan et al., 2008) through removing ROS. In this context, the combination of dietary BET and AOX reduced meat TBARS to a greater extent than BET alone.

5. Conclusions

Supplemented BET conferred overall benefits as well as improved resilience against HS in this small university study. Some additional benefits such as increased MFI and decreased plasma lactate and muscle TBARS were provided by supplemental BET+AOX. This study appears to show that supplemented BET is utilized by the liver, potentially to benefit important metabolic pathways such as the methionine cycle and antioxidant production. However, this requires further investigation. Likewise, accumulation in skeletal muscle may also have a protective effect, and contribute to improved growth and meat quality. The additional effects of AOX warrant further investigation particularly with respect to shelf life and oxidation.

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CHAPTER 5

Growth Performance and Characterization of Meat Quality of Broiler Chickens Supplemented with Betaine and polyphenols under Cyclic Heat stress

5.1. Introduction, aims and hypotheses

Heat stress has been demonstrated to have undesired effects on the growth performance (Al-Fataftah and Abu-Dieyeh, 2007; Shakeri et al., 2018) and meat quality (Zhang et al., 2012b) of broiler chickens. Heat stress among broilers ensues from an elevated temperature (Lin et al., 2006b), detrimental to performance and meat quality. Under HS, chickens seek to deal with the unwanted and exceed body heat by increasing panting which in turn gives rise to respiratory alkalosis (Davies et al., 1986) and disruptions in muscle membrane integrity (Zhang et al., 2012b). Additionally, maintaining body temperature consumes more energy and drives away energy from the metabolic pathways. As a result, deteriorate performance, and so do meat quality by impairing drip loss and tenderness (Akşit et al., 2006). Moreover, HS enhances the production of lipid oxidation, the latter being detrimental to meat colour and flavour (Love and Pearson, 1971), while modifying the conditions of muscle fibre structure (Huang et al., 2015; Rajagopal and Oommen, 2015). To mitigate the unfavourable impact of HS on meat quality and performance, solutions have been developed. Affordable feed additives are among the solutions that have the most potential, as betaine (BET) and polyphenols (POL) have been known to encourage chicken performance and meat quality under HS.

The aim of this chapter was to investigate the effects of the osmolyte BET and POL supplementation on growth performance, physiological responses, and meat quality in broiler chickens exposed to cyclic heat stress.

Sections 5.2 and 5.3 present the papers (under review and prepared) that showed effects of betaine or polyphenols on broiler growth performance and meat quality.

5.2. Under review, Agriculture journal

Dietary Betaine Reduces the Negative Effects of Cyclic Heat Exposure on Growth Performance, Blood Gas Status and Meat Quality in Broiler Chickens

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Abstract: Heat stress (HS) impairs growth performance and has a severe impact on lipid and protein metabolism leading to serious adverse effects on meat quality. Forty-eight day-old-male Ross-308 chicks were assigned to two temperature conditions, thermoneutral or cyclical HS, and fed with either a control diet (CON) or the CON plus betaine (BET). Heat stress increased rectal temperature ($p < 0.001$), respiration rate ($p < 0.001$) and increased blood pH ($p = 0.017$), indicating that HS caused respiratory alkalosis. Heat stress reduced body weight during the final stage of growing period ($p = 0.005$), while BET improved it ($p = 0.023$). Heat stress tended to reduce breast muscle water content and drip loss ($p = 0.089$ and $p = 0.082$), while both were improved with BET ($p = 0.008$ and $p = 0.001$). Heat stress tended to reduce the myofibril fragmentation index ($p = 0.081$) whereas it increased with BET ($p = 0.017$). Heat stress increased thiobarbituric acid reactive substances ($p = 0.017$), while BET improved it ($p = 0.008$). Meat tenderness was not effected by HS, but was improved with BET ($p < 0.001$). Overall, growth performance was improved with BET at the last stage of growing period, and improved product quality of broiler chickens when chickens exposed to HS.

Keywords: cyclic heat stress; growth performance; betaine; product quality; blood gas status; hormone activity; broilers

1. Introduction

With the severity and incidence of cyclic heat events grow along with a rise in poultry production in sub-tropical and tropical areas (Henry et al., 2012), it is necessary to improve the management regarding heat stress (HS). Broiler chickens reared under HS exhibit a decreased growth performance and increased respiration rate and body temperature. There is also some limited evidence that skeletal muscle from broilers exposed to HS has reduced protein synthesis and proteolysis (Temim et al., 2000) which may impact on subsequent meat quality. Other possible impacts on chicken meat quality include reduced pH, resulting in paler meat (Akşit et al., 2006), increased level of lipid oxidation (Love and Pearson, 1971) and altered muscle fiber structure (Huang et al., 2015; Rajagopal and Oommen, 2015). As a result, there is a high demand for affordable nutritional solutions to ameliorate the impacts of HS on growth performance in domestic animals (Cottrell et al., 2015b; DiGiacomo et al., 2014a). In this regard, dietary betaine (BET) is considered as an additive which has been shown to improve growth performance in HS chickens and possibly meat quality.

Betaine (trimethylglycine) is a ubiquitous amino acid derivative which can be extracted from sugar beets. Betaine protects tissues against osmotic stress thereby increasing cellular water retention (Craig, 2004b), acts as a methyl donor which improves synthesis of methionine and carnitine (Alirezai et al., 2012). Betaine has antioxidant function that can reduce oxidative stress in broiler chickens under HS (Alirezai et al., 2015; Shakeri et al., 2018). Furthermore, it reduces maintenance requirements and body heat production providing more energy for growth (Shakeri et al., 2018). Therefore, that purpose of the present experiment was to investigate whether dietary BET could ameliorate the negative effects of HS on growth performance, physiological responses and meat quality in contemporary Ross-308 broiler chickens.

2. Materials and Methods

2.1. Animal Ethics Statement

The experiment was approved by the Animal Ethics Committee, The University of Melbourne, Australia (Protocol no. 181442601.1).

2.2. Animals, Experimental Design and Diets

Forty-eight day-old male Ross-308 chicks were purchased from a local hatchery (Tri Foods Pty Ltd, Bannockburn Victoria, Australia) within 2 h distance from the research facility. Upon arrival the chicks were wing-tagged, individually weighed and randomly assigned to 16 pens of 3 chicks, and allocated to two climate controlled rooms (8 pens/room). There were an additional 16 pens of chicks from the same hatching in each room that were utilized in a concurrent experiment to be reported separately. The temperature in both rooms was 33 °C for the first week and dropped to 25 °C during the first 3 weeks of the experiment for the TN (1 °C/2 days), before being maintained at 25 °C for the last 2 weeks of the experiment (Aviagen, 2009); or 33 °C for cyclic HS group, 8 h at 33 °C (9 am-5 pm) and dropped to TN temperature for 16 h (5 pm- 9 am). The environmental humidity was approximately 40-55 % throughout the experiment. Lights were on for both rooms continuously for the first 3 days was decreased (1 h/day) to 20 h. The pen dimensions were 1 m × 0.5 m and the floor each pen was covered with a depth of ~10 cm with wood shavings. Feed was either a control (CON) or the CON + 1 g/kg betaine (Betafin, Dupont, UK) (BET). Feed (Feedworks Bestmix, Sydney, Australia) was provided as a starter (1-14 days, crude protein 24.9 % and 12.0 metabolisable energy per kilogram), grower (15-28 days, crude protein 23.1 % and 12.4 metabolisable energy per kilogram) and finisher (29-53 days, crude protein 21.3 % and 12.7 metabolisable energy per kilogram). Feed and water were available at all times. Body weight gain and feed intake were recorded weekly to determine daily body weight gain (ADG), daily feed intake (ADFI) and feed conversion ratio (FCR).

Commencing on day 14, rectal temperature was measured weekly at 08:00, 12:00 and 15:00 h with a thermometer (Comark PDT 300, Norwich, UK). Number of panting was measured (2 chickens/pen) at 12 pm at the end of the experiment with a digital cell phone (iPhone 7, Apple Inc., California, USA) to measure number of breaths per minutes (bpm).

Approximately 3 mL Blood sample (16 chickens, 1/pen) was collected from the wing vein (4 from each treatment) at the end of experiment. Furthermore, some of the blood sample (~0.3 mL) was added into an blood gas analyzer (EPOC®; Waltham, Massachusetts, USA) to analysis blood chemistry. The rest of blood sample was centrifuged at 15,000 x *g* for 15 min. to obtain plasma for subsequent betaine and thyroid hormone determination.

2.3. Killing Procedure and Objective Meat Quality

Chicken were slaughtered and preceded as explained in details in our previous publication (Shakeri et al., 2019). After feather removal, the breast muscles (*m. Pectoralis major and minor*) were prepared to measure pH with a pH meter (WP-80M, Pty Ltd, Queensland, Australia) approximately within 10 min., at 60 min. and 24 h. pH meter was calibrated at pH 4.00 and 7.00 before measuring. Carcasses were then rapidly chilled by being placed in sealed plastic bags, cooled for 40 min. under running water, then the samples were transferred to a fridge (4 °C) to simulate commercial conditions. At 24 h after slaughter, the whole breast muscles (both sides) were removed for subsequent objective meat quality and betaine determination. Other meat quality parameters such as drip loss, cooking loss, shear force and colour were measured as explained in detail previously (Shakeri et al., 2019).

2.4. Biochemical and Endocrinological Analyses

Muscle myofibrillar fragmentation index (MFI) and lipid peroxidation (TBARS) were determined as described in detail previously (Shakeri et al., 2019). Plasma and muscle betaine concentrations were determined using High-Performance Liquid Chromatography (Waters 2998, Massachusetts, USA) technique as described by (Shakeri et al., 2019). Thyroid hormones, triiodothyronine (T₃) and thyroxine (T₄), were measured using free radioimmunoassay kits in plasma (MP Biomedicals, LLC Diagnostics Division, Ohio, USA) and explained previously (Shakeri et al., 2019).

Muscle protein carbonyl concentrations were determined using a previously published method with some modifications (Fagan et al., 1999). Muscle (1 ± 0.01 g) was minced and homogenized (Ultra Turrax[®] T25 digital, Selangor, Malaysia) in 5 mL pyrophosphate buffer (2 mM sodium pyrophosphate, 10 mM tris maleate, 100 mM potassium chloride and 2 mM ethylene tetra acetic acid at pH = 7.4) at 10,000 x g for 30 sec. After splitting into 2 tubes, 9 mL hydrogen chloride-acetone (3:100 v/v) was added in each tube, vortexed and centrifuged at 5000 x g at 4 °C for 5 min. The mixture was washed twice with 1 mL trichloroacetic acid 10% and centrifuged at 5000 x g for 5 min. at 4 °C. Tube 1 was then incubated with 0.5 mL 10 mM 2,4 dinitrophenylhydrazine (DNPH) dissolved in 2 mM hydrogen chloride whereas tube 2 was incubated with only 0.5 mL 2 mM hydrogen chloride to calculate protein concentration. Both tubes were kept in darkness for 30 min. (vortexed for 10 sec. every 10 min.). Next, 0.5 mL 20%

trichloroacetic acid was added to both tubes which were vortexed and placed on crushed ice for 10 min. before being centrifuged at 5000 x *g* for 5 min. at 4 °C. After washing with 1 mL trichloroacetic acid 20% the centrifuged pellets were washed 3 times with 1 mL ethanol ethyl acetate and then centrifuged at 5000 x *g* for 5 min. at 4 °C. Then, 1 mL of a guanidine solution (6 M guanidine hydrochloride dissolved in 20 mM potassium dihydrogen phosphate at pH 2.3) was added and the mixture shaken overnight at 4 °C. Absorbance was measured at 370 nm to determine DNPH concentration in tube 1 and at 280 nm to determine protein concentration in tube 2.

2.5. Branch Chain Amino Acid

Branch chain amino acid (BCAA) was measured based on a protocol provided by the manufacturer (Abcam, Discovery Drive Cambridge Biomedical Campus, Cambridge, UK). In brief, 10 µL of plasma samples or different level of standards were mixed with assay buffer (provided by the manufacturer), in a 96 wells plate, to bring the total volume to 50 µL. Then, 50 µL of reaction mix reagent was added to the mixture, incubated under room temperature for 30 min. in a dark place before the plate was read at 450 nm using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). The results were calculated based on a formula provided by the manufacturer and presented as nmol/ µL.

2.6. Statistical Analyses

Data were subjected to ANOVA using GenStat version 16th (VSNi Ltd, Hemel Hempstead, UK). All data analysed for the main and interactive effects of temperature (T) and dietary betaine (D) with pen as a random effect for all data except growth performance where pen was the unit of observation. For physiological parameters and muscle TBARS the effects of time (TM) were included as repeated measures effects in the residual maximum likelihood model. Due to heterogeneity of variances in MFI these data were log₁₀ transformed before analyses. Data differences were indicated a trend when $0.10 > p > 0.05$ and significant when $p < 0.05$.

3. Results

3.1. Body Weight, Feed Intake and Feed Conversion Ratio

There was no effect of T or dietary D on either ADFI, ADG or FCR between days 0 and 21 of the experiment (Table 1). However, from day 21 to 35 ADG was reduced (90.2 vs 80.2 g/day, $p = 0.005$) with no change in ADFI or FCR in response to HS (Table 1). Dietary D increased ADG (81.1 vs 89.3 g/day, $p = 0.023$) but did not effect ADFI or FCR between days 21 and 35. Over the whole experiment, ADG was reduced (56.4 vs 51.6 g/day, $p = 0.009$) with no change in ADFI or FCR in to the HS group (Table 1). Dietary D tended to increase ADG (52.5 vs 55.5 g/day, $p = 0.094$) between days 0 and 35 but had no significant effect on ADFI or FCR. As a result of the effects on ADG, final live weight was decreased by HS (2011 vs 1845 g, $p = 0.009$) and tended to be increased by BET (1876 vs 1980 g, $p = 0.092$).

Table 1. Impact of supplementation of betaine (BET) on daily weight gain, daily feed consumption, feed conversion and final slaughter weight when chickens were under thermoneutral (TN) or heat stress (HS) conditions.

Temperature (T)	TN		HS		SED ¹	<i>p</i> -values		
Diet (D)	CON	BET	CON	BET		T	D	T × D
Daily weight gain, g/day								
0 to 21 days	33.6	34.0	33.4	31.8	1.52	0.27	0.57	0.32
21 to 35 days	84.4	96.0	77.7	82.7	4.58	0.005	0.023	0.14
0 to 35 days	53.9	58.8	51.1	52.1	2.42	0.009	0.094	0.26
Daily feed consumption, g/day								
0 to 21 days	48.7	50.4	45.4	45.1	5.93	0.33	0.86	0.81
21 to 35 days	147	150	128	148	11.0	0.22	0.17	0.31
0 to 35 days	87.9	90.2	78.5	86.2	7.48	0.23	0.36	0.62
Feed conversion ratio								
0 to 21 days	1.46	1.48	1.45	1.43	0.162	0.78	0.98	0.85
21 to 35 days	1.76	1.58	1.60	1.80	0.159	0.78	0.96	0.12
0 to 35 days	1.65	1.53	1.54	1.66	0.131	0.93	0.95	0.23
Slaughter weight, g	1925	2097	1827	1862	85.1	0.009	0.092	0.26

¹ Standard error of the difference, T × D.

3.2. Respiration Rate and Rectal Temperature

Heat stress increased rectal temperature ($p < 0.001$) with the response being greatest ($p < 0.001$) during the afternoon when ambient temperature was highest (Figure 1a) and towards the end of the experiment (Figure 1b). Dietary BET decreased rectal temperature

($p < 0.001$) with the response being greatest in those chickens exposed to HS, during the afternoon when the temperature was highest (Figure 1a) and towards the end of the experiment (Figure 1b) as indicated by $TM \times D$ ($p < 0.001$), $T \times D$ ($p < 0.001$) and $T \times TM \times D$ ($p < 0.001$) interactions. Consequently, on the afternoon of day 34 of age HS increased rectal temperature by 1.1 °C in the CON (41.6 vs 42.7 °C), whereas chickens consuming the BET diet had a rectal temperature of only 41.0 °C during HS (Table 2). Respiration rate on day 34 was increased by HS (64.0 vs 160 bpm, $p < 0.001$), was reduced by dietary BET (122 vs 102 bpm, $p = 0.004$) (Table 2). However, there was a $T \times D$ ($p = 0.017$) where the increase in respiration rate in response to HS was greater in the CON (66.0 vs 177 bpm) than in the BET (62.0 vs 142 bpm) (Table 3).

3.3. Blood and Plasma Parameters and Muscle Betaine

pH in blood was increased by HS (7.34 vs 7.40, $p = 0.017$) and tended to be increased by dietary BET (7.36 vs 7.40, $p = 0.096$) (Table 2). However, interaction was indicated ($p = 0.003$) when HS increased blood pH in the CON (7.27 vs 7.45) whereas it was elevated under both thermal environments in those fed BET (7.41 vs 7.38). Blood pCO₂ was reduced by HS (51.6 vs 43.1 mm Hg) whereas and no main effect of D was indicated. However, there was an interaction ($p = 0.050$) such that HS decreased blood pCO₂ in the CON (55.6 vs 40.1 mm Hg) whereas it was low under both thermal environments in those fed BET (47.6 vs 46.1 mm Hg). There was no main effect of T on blood total CO₂, pO₂, O₂ saturation, HCO₃, anion gap and base excess (Table 2). There were also no main effects of D on blood total CO₂, pO₂, O₂ saturation and HCO₃ while dietary BET decreased the anion gap (17.9 vs 16.5 mM, $p = 0.050$) and tended to increase base excess (1.53 vs 3.34 mM, $p = 0.080$). However, there were interactions such that the CON under TN conditions had lower total CO₂ ($p = 0.054$), HCO₃ ($p = 0.033$), and base excess ($p = 0.006$) and higher anion gap ($p < 0.001$) than the other groups (Table 2).

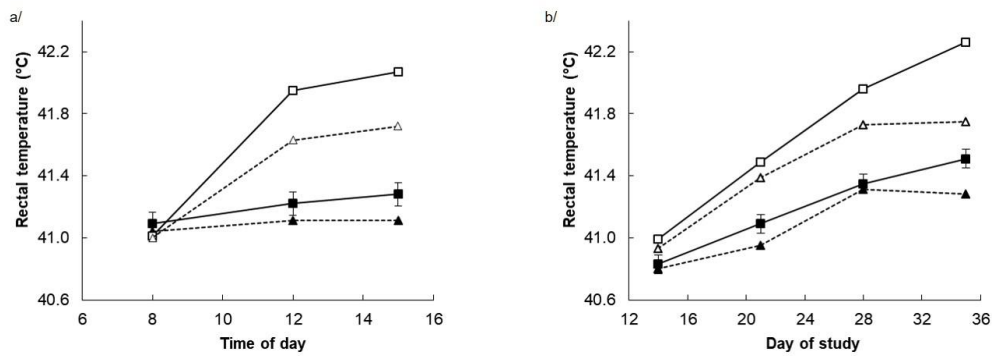


Figure 1. Rectal temperature in chickens fed either a control diet (CON, squares) or the CON diet with betaine (BET, triangles) when chickens were under either thermoneutral (filled symbols) or cyclic heat stress (open symbols). (a) Indicates the impact of time of day (TM) pooled across days of the experiment (DAY) with the standard error of the difference (SED) for the interaction between TM, environmental temperature (T) and diet (D) displayed on the data from the chickens receiving the CON diet. (b) Indicates the impact of DAY pooled across TM with the SED for the interaction between DAY, T and D displayed on the data from the chickens receiving the CON diet. There were main effects ($p < 0.10$) of T ($p < 0.001$), DAY ($p < 0.001$), TM ($p < 0.001$) and D ($p < 0.001$) and interactive of T $\times$ DAY ($p < 0.001$), T $\times$ TM ($p < 0.001$), DAY $\times$ TM ($p < 0.001$), TM $\times$ D ($p = 0.005$), DAY $\times$ D ($p < 0.001$), T $\times$ D ($p = 0.053$) and T $\times$ DAY $\times$ TM ($p = 0.042$). There were no indication of other main or interactive effects ($p < 0.10$).

Table 2. Impact of supplementation of betaine (BET) on rectal temperature, respiration rate and blood gas analysis when chickens were under thermoneutral (TN) or heat stress (HS) conditions.

Temperature (T) Diet (D)	TN		HS		SED ¹	<i>p</i> -value		
	CON	BET	CON	BET		T	D	T $\times$ D
Rectal temperature, °C	41.6	41.3	42.7	42.0	0.11	<0.001	<0.001	0.005
Respiration, bpm	66.0	62.0	177	142	8.30	<.001	0.004	0.017
pH	7.27	7.41	7.45	7.38	0.034	0.017	0.09	0.003
pCO ₂ , mm Hg	55.6	47.6	40.1	46.1	4.47	0.023	0.53	0.050
Total CO ₂ , mM	27.4	31.5	29.8	28.5	1.76	0.79	0.17	0.054
pO ₂ , mm Hg	30.6	35.4	32.8	34.2	4.38	0.87	0.29	0.59
O ₂ saturation, %	48.6	66.6	64.8	63.7	9.11	0.32	0.15	0.17
HCO ₃ , mM	25.7	30.1	28.5	27.1	1.68	0.95	0.13	0.033
Anion gap, mM	20.8	14.8	15.0	18.3	1.28	0.26	0.050	<.001
Base excess, mM	-1.18	4.95	4.23	1.73	1.78	0.40	0.080	0.006

¹ Standard error of the difference, T $\times$ D.

There was no effect of T on blood haematocrit while it was decreased by the BET (19.3 vs 18.0 %, $p = 0.050$) (Table 3). Similarly, there was no effect of T on blood haemoglobin concentrations whereas it tended to be decreased by dietary BET (6.53 vs 6.14 %, $p = 0.058$). However, there was an indication of an interaction ($p = 0.093$) such that haemoglobin concentrations were only decreased by dietary BET under TN conditions. Blood potassium (6.45 vs 5.63 mM, $p = 0.029$) and sodium (150 vs 148 mM, $p = 0.056$) concentrations were decreased during HS but there was no effect on blood chloride or calcium concentrations (Table 3). While there was no main effect of D on any of these blood mineral concentrations there was an interaction such that blood potassium concentrations were decreased by HS in the CON (6.78 vs 5.20 mM) but not when consuming the BET diet (6.13 vs 6.07 mM). Blood lactate concentrations were reduced by HS (7.97 vs 5.23 mM, $p = 0.017$) where tended to be reduced by dietary BET (7.26 vs 5.95 mM, $p = 0.069$). However, there was an interaction such that blood lactate concentrations were markedly decreased by HS the CON (10.80 vs 3.76 mM) whereas this was not the BET diet (5.19 vs 6.70 mM). Blood glucose concentrations was increased by HS (14.1 vs 14.8 mM, $p = 0.093$) whereas there was no effect of D. Plasma T₃ (4.69 vs 5.79 pg/mL, $p = 0.009$) and T₄ (6.62 vs 9.66 pg/mL, $p = 0.016$) concentrations were increased by HS. Plasma T₃ concentrations tended to be increased by dietary BET (4.87 vs 5.62 pg/mL, $p = 0.069$) whereas plasma T₄ concentrations remained unchanged.

Betaine concentrations were reduced in plasma by HS (149 vs 121 $\mu\text{mol/L}$, $p < 0.001$) and were increased by the BET (118 vs 152 $\mu\text{mol/L}$, $p < 0.001$). Muscle betaine concentrations were higher by HS (210 vs 163 $\mu\text{mol/g}$, $p = 0.047$). There was no main effect of D on muscle betaine concentrations there was an interaction ($p = 0.008$) such that muscle betaine concentrations were highest in the CON under TN conditions and lowest in the CON under HS conditions (246 vs 131 $\mu\text{mol/g}$). The betaine concentration in muscle obtained from chickens consuming the BET supplemented diet were intermediate and not different (Table 3).

Branch chain amino acid was decreased by HS (2.67 vs 1.90 nmol/uL, $p < 0.001$) and dietary BET (2.62 vs 1.95 nmol/uL, $p < 0.001$). There was an interaction ($p = 0.002$) such that BCAA was higher in the CON diet under TN conditions compare to other groups (Table 3).

3.4. Objective Meat Quality

The water content of breast muscle was decreased after HS (77.70 vs 76.10 %, $p = 0.089$) and was increased by dietary BET (75.50 vs 78.30 %, $p = 0.008$) (Table 4). Drip loss of the breast muscle was decreased by HS (2.29 vs 1.82 %, $p = 0.082$) and was reduced by dietary BET (2.54 vs 1.57 %, $p = 0.001$). There was no effect of either T or D on cooking loss of the breast muscle. There was no effect of T on shear force whereas shear force was decreased by dietary BET (24.40 vs 18.90 N, $p < 0.001$). Carbonyl units were increased by HS (2.62 vs 3.33 nmol/mg, $p = 0.053$) and decreased by dietary BET (3.50 vs 2.46 nmol/mg, $p = 0.008$). The myofibrillar fragmentation index was decreased by HS (92.30 vs 68.61, $p = 0.081$) and was increased by dietary BET (63.61 vs 97.30, $p = 0.017$) (Table 4). There was little effect of either T or D on meat color with the exception that dietary BET tended to increase a^* (1.44 vs 1.93, $p = 0.10$) (Table 5). There were significant changes in meat color between 24 h and 72 h post-slaughter with an decrease in L^* (56.22 vs 50.91, $p < 0.001$), and a decrease in a^* (1.97 vs 1.27, $p = 0.006$) and b^* (5.70 vs 4.75, $p < 0.001$). There was no main effect of HS on the pH of breast muscle ($p = 0.36$) while pH decreased over time post-slaughter (6.80 vs 6.53 and 5.86 at 10 min., 60 min. and 24 h after slaughter, $p < 0.001$) and was increased by dietary BET (6.33 vs 6.47, $p = 0.020$) (Table 5). However, there were interactions such that the effects of were BET were most pronounced at 24 h post-slaughter, particularly under TN conditions.

Lipid peroxidation was increased between 24 h and 72 h (1.64 vs 3.06 mg/kg, $p < 0.001$), was increased by HS (2.12 vs 3.03 mg/kg, $p = 0.017$) and was decreased by dietary BET (3.06 vs 2.10 mg/kg, $p = 0.008$) (Figure 2). There was an interaction between time post-slaughter $\times$ dietary BET ($p = 0.036$) such that dietary BET decreased TBARS to a greater extent after 72 h (4.37 vs 2.66 mg/kg) than at 24 h (1.74 vs 1.54 mg/kg) post-slaughter (Figure 2).

Table 3. Impact of supplementation of betaine (BET) on blood electrolytes, , plasma thyroid hormones, betaine distribution and branched chain amino acid when chickens were under thermoneutral (TN) or heat stress (HS) conditions.

Temperature (T) Diet (D)	TN		HS		SED ¹	p-value		
	CON	BET	CON	BET		T	D	T × D
Haematocrit, %	19.5	17.3	19.0	18.7	0.917	0.50	0.050	0.17
Hgb ² , g/dL	6.65	5.88	6.40	6.40	0.296	0.53	0.058	0.09
Potassium, mM	6.78	6.13	5.20	6.07	0.453	0.029	1.00	0.040
Sodium, mM	151	149	148	148	1.1	0.056	0.18	0.13
Chloride, mM	112	110	110	109	1.7	0.35	0.43	0.82
Calcium, mM	1.51	1.50	1.48	1.47	0.042	0.25	0.78	0.88
Lactate, mM	10.8	5.19	3.76	6.70	1.348	0.017	0.069	0.001
Glucose, mM	14.2	14.0	15.1	14.4	0.53	0.09	0.33	0.58
T ₃ , pg/mL	4.11	5.27	5.62	5.96	0.547	0.009	0.069	0.30
T ₄ , pg/mL	6.95	6.29	9.56	9.75	1.64	0.016	0.85	0.72
Plasma betaine, µmol/L	133	164	102	140	10.9	<0.001	<0.001	0.66
Muscle betaine, µmol/g	246	174	131	194	30.2	0.047	0.83	0.008
BCAA, nmol/µL	3.25	2.09	1.98	1.82	0.204	<0.001	<0.001	0.002

¹ Standard error of the difference, T × D. ² Haemoglobin (Hgb); Triiodothyronine (T₃); Thyroxine (T₄); Branched chain amino acid (BCAA).

Table 4. Impact of supplementation of betaine (BET) on breast muscle (*pectoralis major*) objective eating quality measures after being housed when chickens were under thermoneutral (TN) or heat stress (HS) conditions.

Temperature (T) Diet (D)	TN		HS		SED ₁	p-value		
	CON	BET	CON	BET		T	D	T × D
Water content, %	76.2	79.1	74.7	77.5	1.42	0.08	0.008	0.96
Drip loss, %	2.95	1.62	2.13	1.52	0.39	0.08	0.001	0.22
Cooking loss, %	22.8	21.2	21.5	20.3	1.69	0.36	0.26	0.88
Peak shear force, N	24.1	18.6	24.8	19.1	1.87	0.49	<0.001	0.92
Carbonyl, nmol/mg	3.22	2.02	3.77	2.89	0.48	0.05	0.008	0.64
MFI ²	1.88	2.04	1.72	1.93	0.10	0.08	0.017	0.74
	(75.2)	(109)	(52.0)	(85.1)				

¹ Standard error of the difference for T × D. ² Myofibrillar fragmentation index (MFI).

Data were skewed so were log₁₀-transformed before analysis. Back-transformed means are in parentheses.

Table 5. Impact of supplementation of betaine (BET) and on breast muscle (*pectoralis major*) colour and pH when chickens were under thermoneutral (TN) or heat stress (HS) conditions.

Temperature (T)		TN		HS		SED ¹	<i>p</i> -value		
Diet (D)	Time (TM)	CON	BET	CON	BET		T	D	TM ²
L*	24 h	55.5	58.6	55.7	55.2	2.22	0.48	0.20	<0.001
	72 h	50.1	52.5	50.6	51.0				
a*	24 h	1.99	2.22	1.54	2.40	0.60	0.27	0.10	0.022
	72 h	1.55	1.47	0.69	1.63				
b*	24 h	6.07	6.09	5.11	5.69	0.78	0.34	0.21	0.015
	72 h	4.46	5.18	4.50	5.11				
pH	10 min	6.77	6.78	6.83	6.82	0.14	0.36	0.02	<0.001
	1 h	6.44	6.57	6.60	6.50				
	24 h	5.72	6.30	5.60	5.82				

¹ Standard error of the difference, T × D. ² TM × T *p* = 0.023; TM × D *p* = 0.009; T × D *p* = 0.008.

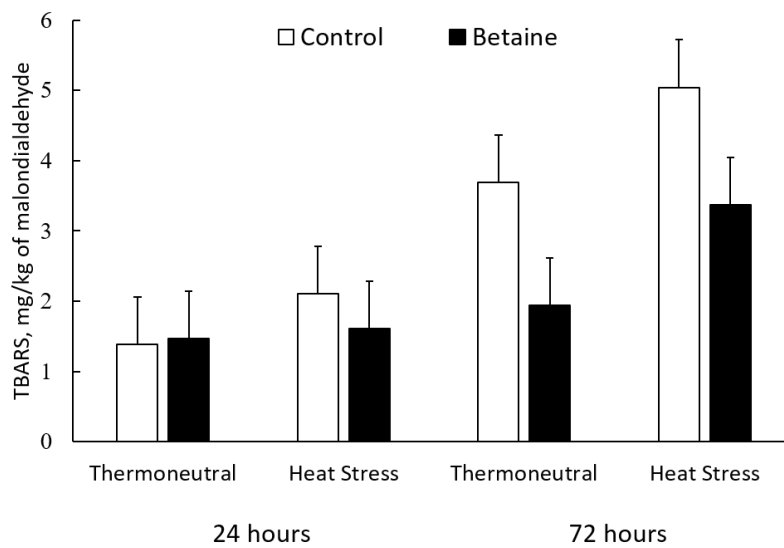


Figure 2. Effect of heat stress, dietary betaine, and time post-slaughter on breast muscle lipid peroxidation (TBARS) in mg/kg. Data are mean and standard error of the difference for the interaction between environmental temperature (T), diet (D) and time post-slaughter (TM). There were main effects (*p* < 0.10) of T (*p* = 0.017), TM (*p* < 0.001) and D (*p* = 0.008) and an interactive of TM × D (*p* = 0.036).

4. Discussion

The major finding from this experiment was that dietary BET could reverse some of the negative effects of HS on growth performance, physiology, acid base balance and

meat quality in contemporary broiler chickens. The effects of HS and dietary BET on ADG and final live weight confirm our previous findings (Shakeri et al., 2019; Shakeri et al., 2018) and of others (He et al., 2015; Nofal et al., 2015). The effects were such that there was no significant difference between the final live weight of the chickens consuming the CON diet under TN conditions and those consuming the BET diet during HS. The ability of dietary BET to reduce rectal temperature during peak HS towards the end of the experiment was quite profound, particularly given the rapid growth rate. The same was true for respiration rate. These data are again consistent with our previous observations (Shakeri et al., 2019; Shakeri et al., 2018) and (Attia et al., 2009a). The protective effects of dietary BET are most likely multifaceted but at least part of the effect on rectal temperature and respiration rate is thought to be due to its osmoprotective effects. Betaine, or tri-methyl glycine, is a naturally occurring amino acid derivative found in many plant and invertebrate species. Physiologically, BET has a crucial osmoregulatory action and has been reported to improve growth performance by reducing the maintenance energy requirements by reducing the need for sodium/potassium pumping to maintain cellular osmolarity (Schrama et al., 2003; Suster et al., 2004b). As a result, heat production is decreased with a resultant reduction in the amount of heat dissipated, rectal temperature and respiration rate. Furthermore, reduction of BCAA in the BET groups could lead to better growth performance as BCAA might use to synthesis protein and reduced protein breakdown (Chen et al., 2016; Wolfe, 2017), and stimulates glucose uptake in skeletal muscle that provides energy sources for tissues (Nie et al., 2018). However, literature on this topic in broiler chickens are still limited and further studies are required to delineate the role of BCAA.

There has been relatively little research conducted on the effects of HS on chicken meat quality. Genetic improvement has meant that modern poultry with their rapid lean growth are more susceptible to HS than non-selected or indigenous breeds because of their higher heat production. In this context, there is a negative relationship between rectal temperature, susceptibility to HS and production traits in chickens (Cooper and Washburn, 1998; Lu et al., 2007; Settar et al., 1999). Thus, modern genotypes have lower survival rates, growth rate, body weights, and feed efficiency, and higher rectal temperature than slow-growing breeds when exposed to HS conditions (Deeb et al., 2002; Yalcin et al., 2001). Genetic selection for rapid muscle growth in poultry has altered the ability of animals to respond and adapt to environmental stressors such as HS. For

example, in the present experiment there was an increase in plasma T₃ and T₄ in response to HS which is counter to the normally observed natural thermoregulatory reduction in plasma thyroid hormone concentrations observed in birds and mammals during exposure to elevated temperatures (Collin et al., 2005). Although this is in contrast to our previous experiment (Shakeri et al., 2019), others have seen that modern avian genotypes exhibit increased plasma thyroid concentrations during HS. For example, several Japanese quail and chicken genotypes that have been selected for lean rapid growth display elevated plasma thyroid hormone concentrations during HS, which is not the case for non-selected lines (Bowen and Washburn, 1985). Similarly, turkeys from non-selected lines produced stable thyroid responses to HS compared with commercial lines (Chiang et al., 2008).

Increased thyroid response during HS could influence meat quality through increasing the incidence of pale soft and exudative (PSE) meat. Although, there was no change in plasma Ca²⁺ concentrations during HS in the current experiment, the Ca in muscle is regulated by T₃ and T₄. Pale soft and exudative meat may occur as the result of muscle hyper-metabolism mediated by increased Ca²⁺ release from the sarcoplasmic reticulum or by direct action on the ryanodine receptor increasing the open state of the channel (Chiang et al., 2008; Strasburg and Chiang, 2009). In this context, breast muscle from broiler chickens exposed to chronic HS exhibited increased muscle glycolysis as evidenced by increased muscle lactate and lower pHu resulting in paler meat with a greater cooking loss than meat from chickens reared under TN conditions (Zhang et al., 2012a). Similarly, the breast muscle from turkeys exposed to HS had lower pH and was paler and had greater drip and cooking loss than muscles from chickens maintained under TN conditions (Sams, 1997). It was found that cyclic HS resulted in paler meat and lower ultimate pH than that observed in chickens housed under TN conditions (Akter et al., 2019). Despite the increase in plasma T₃ and T₄, there was no evidence of PSE during HS in the present experiment since there was no change in meat color and pH while drip loss and muscle water content were actually lower during HS. However, there was an increase in MFI indicating an increase in post-mortem myofibrillar breakdown in response to HS. Previously we have observed an increase in MFI and a decrease in cooking loss and muscle water content but no change in drip loss after HS (Shakeri et al., 2019). Others have also observed a reduction in cooking loss during HS although in that experiment the meat was paler and had a high pHu after the chickens were exposed to HS (Dai et al., 2012). Therefore, it appears that there is no consistent effect of HS on the incidence of

PSE meat in chickens with perhaps the potential negative effects being offset by reduced water content due to dehydration of the meat. Interestingly, dietary BET reversed the effects of HS on muscle MFI and water content while reducing drip loss indicating improvements in eating quality under both TN and HS conditions. Also of interest, BET resulted in elevated 24 h pH, particularly in the TN chickens, which would explain the higher total water content of BET muscles. As high ultimate pH is well-known to be associated with higher water-holding capacity due to reduced charge between myofilaments (Hamm, 1986).

There was no effect of HS on WBSF which is consistent with our previous findings (Shakeri et al., 2019). In contrast, others have found that HS increased WBSF in chickens compared to their counterparts held under TN conditions (Zhang et al., 2012b). Interestingly, dietary BET reduced WBSF regardless of thermal environment.

Cyclic HS resulted in oxidation of chicken meat as evidenced by the increase in TBARS and carbonyl concentrations, with the increase in TBARS being more pronounced with time post-slaughter. These findings indicate that HS is a major cause of oxidative stress in chickens as it is in other species (Chauhan et al., 2014; Liu et al., 2016). Increased lipid and protein oxidation characterized by increased TBARS and microbiological growth, color deterioration, and shorter shelf life of the breast have been reported in broiler chickens housed under HS conditions (Imik et al., 2012; Mujahid et al., 2007b; Wang et al., 2009). Importantly, dietary BET was able to counteract the effects of HS on oxidation and even reduce oxidation under TN conditions.

5. Conclusions

This experiment clearly demonstrated that HS decreased growth and increased respiration rate and rectal temperature in broilers with resultant deleterious effects on meat quality. Supplemental BET was able to ameliorate the effects of HS on growth and physiological parameters. Importantly, dietary BET was able to reduce oxidation, myofibrillar protein fragmentation and WBSF under both temperature conditions.

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M.S.; Resources, S.W., F.R.D., and J.J.C.; Supervision, F.R.D., J.J.C., and S.W.; Writing—original draft, M.S., and F.R.D.; Writing—review and editing, M.S., J.J.C., and F.R.D.

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5.3. Reformatting for submission, Animal Feed and Science Technology Journal

A Dietary Sugarcane-Derived Polyphenol Mix Reduces the Negative Effects of Cyclic Heat Exposure on Growth Performance, Blood Gas Status and Meat Quality in Broiler Chickens

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Abstract: Heat stress (HS) compromises growth performance and meat quality of broiler chickens by interrupting lipid and protein metabolism, resulting in increased oxidative damages. The experiment attempted to investigate whether dietary polyphenols (Polygain (POL)) could ameliorate the aforementioned adverse effects of HS on performance and meat quality. One hundred twenty one day-old-male chicks were allocated to two temperature conditions, thermoneutral (TN) or HS, and fed with either a control diet (CON) or the CON plus 4 different doses of POL (2, 4, 6 and 10 g/kg). Heat stress increased rectal temperature ($p < 0.001$) and respiration rate ($p < 0.001$) due to increased blood pH ($p < 0.001$) leading to respiratory alkalosis.. Heat stress decreased final body weight ($p = 0.061$) and breast muscle water content ($p = 0.013$) while POL improved both ($p = 0.002$ and $p = 0.003$, respectively). Heat stress amplified muscle damages, indicated by increased thiobarbituric acid reactive substances ($p < 0.001$) and reduced myofibril fragmentation index ($p = 0.006$) whereas POL improved both ($p = 0.037$ and $p = 0.092$, respectively). Heat stress impaired meat tenderness ($p < 0.001$) while POL improved it ($p = 0.003$). In conclusion, HS impaired growth performance and meat quality whereas POL ameliorated these responses in a dose-dependent manner, and effects of POL were evident under both temperature conditions.

Keywords: heat stress; polyphenols; growth rate; meat quality; hormones; broiler chickens

1. Introduction

Heat stress (HS) has been demonstrated to have undesired effects on the growth performance (Al-Fataftah and Abu-Dieyeh, 2007; Shakeri et al., 2018) and meat quality (Zhang et al., 2012b) of broiler chickens. Heat stress among broilers ensues from an elevated temperature (Lin et al., 2006b), detrimental to performance and meat quality. Under HS, chickens seek to deal with the unwanted and exceed body heat by increasing panting, resulting in alteration of blood acid/base status, which in turn gives rise to respiratory alkalosis (Davies et al., 1986) and disruptions in muscle membrane integrity (Zhang et al., 2012b). Additionally, maintaining body temperature consumes more energy and drives away energy from the metabolic pathways. As a result, HS reduces growth performance and impairs meat quality by lowering tenderness (Akşit et al., 2006). Furthermore, HS enhances the production of lipid oxidation, the latter being detrimental to meat colour and flavour (Love and Pearson, 1971), while modifying the conditions of muscle fibre structure (Huang et al., 2015; Rajagopal and Oommen, 2015). To mitigate the unfavourable impact of HS on meat quality and performance, solutions have been developed.

Polyphenols are a group of compounds with antioxidant properties and have anti-inflammatory, immunomodulatory effects that prevent oxidative stress (Paszkievicz et al., 2012; Petti and Scully, 2009) by reducing production of reactive oxygen species. Polyphenols can be found in various part of vegetables and fruits including roots, leaves and flowers (Landete, 2013; Paszkievicz et al., 2012). Polyphenols also acts as hormones and growth regulators (Majewska and Czczot, 2009) and enzymes regulators (D Archivio et al., 2007). Therefore, polyphenols as supplementation could ameliorate the negative effects of HS on performance and meat quality.

Nonetheless, a limited amount of studies has explored the effects of polyphenols on alleviating HS on broilers' growth performance and meat quality of Ross-308, albeit various studies on other breeds (Aengwanich and Suttajit, 2010; Tufarelli et al., 2016). The present experiment attempts to examine the performance, meat quality characteristics and the physiological responses to cyclic HS in broiler chickens fed various doses of polyphenols as named polygain in this experiment.

2. Materials and Methods

2.1. Ethics Statement

The experiment was approved by The University of Melbourne (FAVS, Protocol no. 18144261.1).

2.2. Animals, Experimental Design and Diets

One hundred and twenty one day-old male Ross-308 chicks obtained from a local hatchery (Tri Foods Pty. Ltd, Bannockburn Victoria, Australia) were transported for 2 h while being maintained at ~30 °C to the research site located in The University of Melbourne. Upon arrival the chicks were wing-tagged, individually weighed and randomly allocated to 16 pens (3 chicks/pen) and in one of two climate controlled rooms (8 pens/room). There were an additional 4 pens of chicks from the same hatching in each room that were utilized in a concurrent experiment with both experiments utilizing the same control diet (unpublished results). The temperature for both rooms was maintained at 33 °C (continuously for the first 7 days) and decreased to 25 °C for TN (days 7-35) (Aviagen, 2009); or HS where the temperature was at 33 °C (8 h/day) and 25 °C (16 h/day) from days 7-35. The environmental humidity was maintained at 50 ± 5 % throughout the experiment. Light was provided continuously for the first 3 days and was reduced (1 h/day) to 20 h on day 7 for both rooms. Each pen (100 cm × 50 cm) was equipped with a nipple drinker and a feeder and the floor was covered with wood shaving (~10 cm deep).

Chickens were fed either a commercial control diet (CON) or the CON diet supplemented with 2, 4, 6 or 10 g/kg polygain (The Product Makers, Dandenong, Australia) (POL). Commercial feed was provided as a starter (for 2 weeks, 24.9 % crude protein and 12.0 metabolic energy/kilogram), grower (for 2 weeks, crude protein 23.1 % and 12.4 metabolic energy/kilogram) and finisher (one week, 21.3 % crude protein and 12.7 metabolic energy/kilogram). Chickens had unlimited access to feed and water. Body weight and consumed feed were recorded weekly.

Rectal temperature was measured weekly at three time points (morning before increasing the room's temperature, mid-day, afternoon before dropping the room's temperature) with a thermometer (Comark PDT 300, UK). Respiration rate was measured (2 chickens/pen) at 12 pm on day 34 with a smartphone (iPhone 7, Apple Inc., USA) to measure respiration rate as breaths per minute (bpm).

Forty eight chickens (1/pen) were randomly selected for blood analysis (4/treatment) on day 35. About 3 mL blood was collected and ~0.2 mL was used to analysis blood gas with an automatic analyser (EPOC®; Alere, Waltham, Massachusetts, USA. The remaining blood was centrifuged at 15,000 x *g* for 15 min. to obtain plasma for further analysis.

2.3. Slaughter and Objective Meat Quality Measures

Slaughter procedure was performed in details previously (Shakeri et al., 2019). After feather removal, the breast muscles (*m. Pectoralis major and minor*) were partially skinned to measure pH at less than 10 min., 1 h and 24 h after killing by using a digital pH meter (WP-80M, TPS, Pty Ltd, Queensland, Australia) and was calibrated at pH 4.00 and 7.00. Carcasses were then rapidly chilled by placing in sealed plastic bags suspended in cold water (~4 °C for 40 min.) before transfer to a refrigerated room (4 °C) to simulate commercial conditions. Other meat quality analysis such as drip loss, cooking loss, shear force and colour were performed as explained in details previously (Shakeri et al., 2019).

2.4. Biochemical and Endocrinological Analyses

Muscle myofibrillar fragmentation index (MFI) and thiobarbituric acid reactive substances (TBARS) were determined as described in detail (Shakeri et al., 2019). Plasma and muscle betaine concentrations were determined using the High-Performance Liquid Chromatography (Waters 2998, Massachusetts, USA) technique as described previously (Shakeri et al., 2019). Thyroid hormones activity in plasma were measured using free triiodothyronine (T₃) and thyroxine (T₄) radioimmunoassay kits in plasma (MP Biomedicals, LLC Diagnostics Division, Ohio, USA) based on a protocol provided by the manufacturer and described previously (Shakeri et al., 2019). Muscle protein carbonyl concentrations were determined using the method previously published with some modifications (Fagan et al., 1999). Muscle (1 g ± 0.01) was minced and homogenized (IKA Ultra Turrax® T 25 digital, Selangor, Malaysia) in 5 mL pyrophosphate buffer (2 mM Sodium pyrophosphate, 10 mM tris maleate, 100 mM potassium chloride and 2 mM ethylene tetra acetic acid at pH = 7.4) at 5000 x *g* for 30 sec. After splitting into 2 tubes, 9 mL hydrogen chloride-acetone (3:100 v/v) was added in each tube, vortexed and centrifuged at 5000 x *g* for 5 min. at 4 °C. The mixture was washed twice with 1 mL trichloroacetic acid 10 % and centrifuged at 5000 x *g* at 4 °C for 5 min. Tube 1 was then

incubated with 0.5 mL 10 mM 2,4 dinitrophenylhydrazine (DNPH) dissolved in 2 mM hydrogen chloride whereas tube 2 was incubated with only 0.5 mL 2 mM hydrogen chloride to calculate protein concentration. Both tubes were kept in darkness for 30 min. (vortexed for 10 sec. every 10min.). Next, 0.5 mL 20 % trichloroacetic acid was added to both tubes which were vortexed and placed on ice for 10 min. before being centrifuged at 5000 x g for 5 min. at 4 °C. After washing with 1mL trichloroacetic acid 20 % the centrifuged pellets were washed 3 times with 1 mL ethanol ethyl acetate and then centrifuged at 5000 x g for 5 min. at 4 °C. Next, 1mL of a guanidine solution (6 M guanidine hydrochloride dissolved in 20 mM potassium dihydrogen phosphate at pH 2.3) was added and the mixture shaken overnight at 4 °C. Absorbance was measured at 370 nm to determine DNPH concentration in tube 1 and at 280 nm to determine protein concentration in tube 2.

2.5. Statistical Analyses

All results were analysed with ANOVA for the main and interactive effects of temperature (T) and the linear (L) and quadratic (Q) effects of POL using GenStat V.16th (VSNi Ltd, Hemel Hempstead, UK). For physiological parameters and muscle TBARS the main and interactive effects of time (TM) were also included as repeated measures effects in the residual maximum likelihood model. Differences were considered a trend when $0.10 > p > 0.05$ and significant when $p < 0.05$.

3. Results

3.1. Body Weight and Feed Consumption

There was no effect of T on either daily feed intake (ADFI), daily weight gain (ADG) or feed conversion ratio (FCR) between days 0 - 21 of the experiment (Table 1). However, between days 21 - 35 there was a reduction in ADG (90.3 vs 85.2 g/d, $p = 0.029$) and ADFI (143 vs 135 g/d, $p = 0.024$) with no change in FCR in response to HS (Table 1). Dietary POL increased ADG (82.1 vs 85.6, 87.6, 91.5 and 92.0 g/d, $p = 0.004$) and decreased FCR (1.68 vs 1.65, 1.60, 1.47 and 1.55 g/d, $p = 0.015$) in a linear manner with increasing dose of POL but did not effect ADFI between days 21 and 35. Over the entire experiment, there tended to be a reduction in ADG (55.9 vs 53.8 g/d, $p = 0.060$) and ADFI (87.8 vs 83.3 g/d, $p = 0.062$) with no change in FCR in response to HS (Table 1). Dietary POL increased ADG (52.5 vs 53.6, 55.3, 54.8 and 57.9 g/d, $p = 0.002$) and decreased

FCR (1.60 vs 1.64, 1.61, 1.52 and 1.50 g/d, $p = 0.050$) in a linear manner with increasing dose of POL but did not affect ADFI over the entire experiment. As a result of the effects on ADG, final live weight tended to be decreased by HS (1994 vs 1920 g, $p = 0.061$) and increased linearly with increasing dose of POL (1877 vs 1915, 1972, 1957 and 2065 g, $p = 0.002$).

Table 1. Effect of dose (D) of dietary polygain on performance parameters when chickens exposed to thermoneutral (TN) and heat stress (HS) temperature (T) conditions.

		Dose of polygain, g/kg					SED ¹	Significance ^{2,3}
		0	2	4	6	10		
Daily body gain, g/d								
0 to 21 days	TN	33.3	30.6	33.9	29.8	37.0	2.49	
	HS	31.8	33.8	31.4	31.4	32.8		
21 to 35 days	TN	84.0	88.4	88.8	94.0	96.4	5.04	T*, L**
	HS	80.1	82.8	86.3	89.0	87.6		
0 to 35 days	TN	53.9	53.7	55.9	55.5	60.4	2.46	T+, L**
	HS	51.1	53.5	54.7	54.2	55.4		
Daily feed intake, g/d								
0 to 21 days	TN	48.7	52.6	52.1	52.2	48.0	5.2	
	HS	45.4	52.7	51.8	47.2	48.3		
21 to 35 days	TN	147	135	145	140	149	8.04	T*
	HS	128	147	135	128	136		
0 to 35 days	TN	87.9	85.5	89.3	87.5	88.4	5.05	T+
	HS	78.5	90.2	85.0	79.7	83.3		
Feed conversion ratio								
0 to 21 days	TN	1.46	1.72	1.54	1.77	1.30	0.186	
	HS	1.45	1.56	1.68	1.51	1.50		
21 to 35 days	TN	1.76	1.53	1.64	1.50	1.55	0.098	L*
	HS	1.60	1.78	1.57	1.44	1.55		
0 to 35 days	TN	1.65	1.59	1.60	1.58	1.46	0.101	L*
	HS	1.54	1.69	1.61	1.46	1.53		
Slaughter weight, g	TN	1925	1917	1994	1978	2154	86.4	T+, L**
	HS	1828	1912	1950	1936	1976		

¹ Standard error of the difference for T × D. ² Level of significance for main and interactive effects of T and linear (L) and quadratic (Q) effects of polygain. ³ + - $p < 0.10$; *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$.

3.2. Rectal Temperature and Respiration Rate

Heat stress significantly increased rectal temperature ($p < 0.001$) with more significant response ($p < 0.001$) during the afternoon (Figure 1b) and towards the end of the experiment (Figure 1d). Dietary POL decreased ($p < 0.001$) rectal temperature in a

linear fashion ($p < 0.001$) with the response being greatest in those chickens exposed to HS, during the afternoon (Figure 1b) and towards the end of the experiment (Figure 1d) as indicated by $T \times \text{DAY}$ ($p < 0.001$), $T \times \text{TM}$ ($p < 0.001$), $\text{DAY} \times \text{TM}$ ($p < 0.001$), $\text{TM} \times \text{L}$ ($p < 0.001$), $\text{DAY} \times \text{L}$ ($p < 0.001$), $\text{DAY} \times \text{T} \times \text{L}$ ($p = 0.008$) and $T \times \text{DAY} \times \text{TM}$ ($p < 0.001$) interactions. Consequently, on the afternoon of day 35 of age HS increased rectal temperature by 1.1 °C in chickens consuming the CON diet (41.55 vs 42.71 °C) whereas chickens consuming the highest POL diet had a rectal temperature of only 42.01 °C during HS (Table 2). Respiration rate at 11:00 am on day 35 was increased by HS (63.8 vs 160 bpm, $p < 0.001$) and was decreased linearly by dietary POL (122 vs 114, 111, 109 and 106 bpm, $p = 0.012$) (Table 2). However, there was a $T \times \text{L}$ interaction ($p = 0.022$) such that the increase in respiration rate in response to HS was greater in chickens consuming the CON diet (66.0 vs 177 bpm) than in those consuming the highest POL diet (64.0 vs 148 bpm) (Table 2).

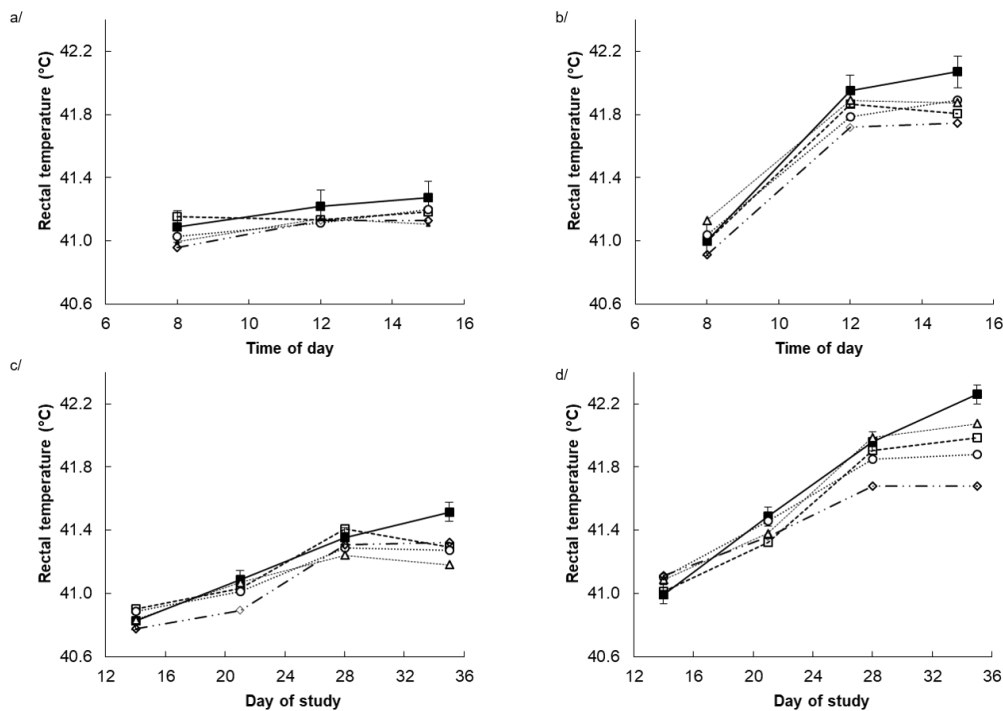


Figure 1. Rectal temperature for chickens fed with a control diet (CON, ■) or the CON plus various doses of polygain (POL, 2 g/kg Δ, 4 g/kg □, 6 g/kg ○ and 10 g/kg ◇) when chickens exposed to thermoneutral (a/ and c/) or cyclic heat stress (b/ and d/) conditions. Panels a/ and b/ show the effect of time of day (TM) pooled across days of the experiment

(DAY) with the standard error of the difference (SED) for the interaction between TM, environmental temperature (T) and diet (D) displayed on the data from the chickens receiving the CON diet. Panels c/ and d/ show the effect of DAY pooled across TM with the SED for the interaction between DAY, T and D displayed on the data from the chickens receiving the CON diet. There were main effects ($p < 0.10$) of T ($p < 0.001$), DAY ($p < 0.001$), TM ($p < 0.001$) and linear effects of POL (L) ($p < 0.001$) and interactive effects of T $\times$ DAY ($p < 0.001$), T $\times$ TM ($p < 0.001$), DAY $\times$ TM ($p < 0.001$), TM $\times$ L ($p < 0.001$), DAY $\times$ D ($p < 0.001$), DAY $\times$ T $\times$ L ($p = 0.008$) and T $\times$ DAY $\times$ TM ($p < 0.001$). There were no other main or interactive effects ($p < 0.10$).

3.3. Blood and Plasma Parameters and Muscle Betaine

Heat stress increased blood pH (7.35 vs 7.44, $p < 0.001$) and tended to increase linearly with increasing dose of POL (7.36 vs 7.39, 7.39, 7.40 and 7.42, $p = 0.068$) (Table 2). Blood pCO₂ was decreased by HS (51.6 vs 41.1 mm Hg, $p < 0.001$) whereas there was no effect of POL. There was no main effect of T on blood total CO₂, pO₂, O₂ saturation, HCO₃ and base excess (Table 2). However, there was an interaction ($p = 0.014$) between temperature and a linear effect of POL such that blood total CO₂ increased with increasing dose of POL under TN conditions but not during HS. There were quadratic effects of POL on blood pO₂ and O₂ saturation ($p < 0.001$) in that both were highest at intermediate doses of POL. There was a main linear POL effect ($p = 0.015$) and an interaction ($p = 0.003$) between temperature and a linear effect of POL such that blood HCO₃ increased with increasing dose of POL under TN conditions but not during HS. Similarly, there was a main linear POL effect ($p = 0.006$) and an interaction ($p = 0.002$) between temperature and a linear effect of POL such that blood base excess increased with increasing dose of POL under TN conditions but not during HS. The blood anion gap was decreased by HS (17.5 vs 15.5 mM, $p < 0.001$) and decreased linearly with increasing dose of POL (17.9 vs 17.2, 15.3, 17.2 and 15.2 mM, $p < 0.001$). However, there was an interaction with temperature such that this decrease with increasing dose of POL only occurred under TN conditions and not during HS.

Table 2. Effect of dose (D) of dietary polygain on physiological responses and blood gas changes under thermoneutral (TN) and heat stress (HS) temperature (T) conditions.

		Dose of polygain, g/kg					SED ¹	Significance ^{2,3}
		0	2	4	6	10		
Rectal temperature, °C	TN	41.55	41.32	41.29	41.25	41.41	0.129	T***, L***, Q*, T.L**
	HS	42.71	42.29	42.24	42.39	42.01		
Respiration rate, bpm	TN	66.0	64.0	62.0	63.0	64.0	8.20	T***, L*, T.L*
	HS	177	163	160	154	148		
pH	TN	7.27	7.40	7.35	7.32	7.40	0.037	T***, L+
	HS	7.45	7.39	7.44	7.48	7.43		
pCO ₂ , mm Hg	TN	55.6	41.5	54.6	55.4	50.7	4.73	T***
	HS	40.1	45.3	42.5	35.0	42.4		
Total CO ₂ , mM	TN	27.4	26.9	31.3	30.1	31.8	1.56	L+, T.L*
	HS	29.8	29.0	29.8	26.9	29.5		
pO ₂ , mm Hg	TN	30.6	45.6	36.7	42.0	29.3	4.11	Q***
	HS	32.8	36.9	36.4	40.2	31.9		
O ₂ saturation, %	TN	48.6	81.2	65.6	70.7	54.1	7.59	Q***
	HS	64.8	68.9	71.1	78.7	62.8		
HCO ₃ , mM	TN	25.6	25.6	29.6	28.4	31.5	1.54	L*, T.L**
	HS	28.5	27.6	28.5	25.8	28.2		
Anion gap, mM	TN	20.8	19.0	16.0	17.7	14.3	0.92	T***, L***, T.L***
	HS	15.0	15.3	14.7	16.7	16.0		
Base excess, mM	TN	-1.17	0.72	3.40	2.03	6.23	1.55	L**, T.L**
	HS	4.23	2.43	3.93	2.10	3.50		

¹ Standard error of the difference for T × D. ² Level of significance for main and interactive effects of T and linear (L) and quadratic (Q) effects of polygain. ³ + - $p < 0.10$; *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$.

Blood haematocrit tended to be decreased by HS (19.7 vs 18.9 %, $p = 0.087$) and increased quadratically ($p = 0.042$) with dose of POL peaking at 4 g/kg POL (Table 3). There was no effect of T on blood haemoglobin concentrations whereas it tended to increase quadratically ($p = 0.082$) with increasing dose of POL peaking at 4 g/kg POL. Blood potassium (6.40 vs 5.50 mM, $p < 0.001$) and sodium (150 vs 148 mM, $p = 0.001$) concentrations were decreased during HS but there was no effect on blood chloride or calcium concentrations (Table 3). While there was no main effects of POL on blood potassium concentrations there was an interaction ($p = 0.028$) such that blood potassium concentrations decreased linearly with increasing dose of POL under TN conditions but not in those exposed to HS conditions. There were linear ($p = 0.042$) and interactive ($p = 0.056$) effects of POL such that blood chloride decreased linearly with increasing dose of POL particularly under TN conditions (Table 3). There were quadratic ($p = 0.062$) and

interactive ($p = 0.035$) effects of POL such that blood calcium decreased quadratically before increasing again with increasing dose of POL with the nadir occurring at different doses of POL under TN (0.2 g/kg) and HS (0.6 g/kg) conditions (Table 3). Blood lactate concentrations were decreased by HS (7.51 vs 4.77 mM, $p < 0.001$) and decrease linearly ($p = 0.011$) with increasing dose of POL. However, there was an interaction such that blood lactate concentrations decreased with increasing dose of POL under TN conditions whereas they increased with increasing dose under HS conditions. Blood glucose concentrations tended to be increased by HS (14.4 vs 15.0 mM, $p = 0.058$) whereas there was no effect of POL. Although there were no main effects of temperature or POL on plasma T_3 concentrations there tended to be a $T \times L$ interaction ($p = 0.083$) such that plasma T_3 concentrations were increased with increasing dose of POL under TN conditions not under HS conditions (Table 3). Plasma T_4 (6.64 vs 9.27 pg/mL, $p < 0.001$) concentrations were increased by HS but were unchanged by dietary POL. Plasma betaine concentrations were decreased by HS (139 vs 108 $\mu\text{mol/L}$, $p < 0.001$) and responded quadratically to increasing dose of POL (118 vs 132, 129, 121 and 116 $\mu\text{mol/L}$, $p = 0.023$). Muscle betaine concentrations were decreased by HS (155 vs 126 $\mu\text{mol/g}$, $p = 0.002$). There were main and interactive linear ($p < 0.001$, both) and quadratic ($p = 0.006$, both) effects of POL such that muscle betaine concentrations were only lower during HS in the chickens fed the control diet (Table 3).

3.2. Objective Meat Quality

There was no effect of temperature or dietary POL on breast muscle cooking loss (Table 4). No main effect of HS on drip loss there were main ($p = 0.007$) and interactive ($p = 0.018$) linear effects of POL such that drip loss decreased with increasing dietary POL content especially under TN conditions. Water content was decreased by HS (77.2 vs 76.0 %, $p = 0.013$) and increased linearly with dietary POL content (75.4 vs 75.9, 77.8, 76.3 and 77.7 %, $p = 0.003$). Shear force increased after HS (20.9 vs 24.1 N, $p < 0.001$) and decreased linearly in response to dietary POL (24.4 vs 23.2, 22.1, 21.5 and 21.3 N, $p = 0.003$). The myofibrillar fragmentation index was decreased by HS (93.8 vs 69.7, $p = 0.006$) and tended to increase linearly with increasing dose of dietary POL (73.5 vs 64.9, 96.1, 83.5 and 91.0, $p = 0.092$) (Table 4). There was no effect of temperature on carbonyl units which tended to exhibit a quadratic decline with increasing dose of dietary POL (3.50 vs 3.22, 3.01, 2.87 and 3.12 nmol/mg protein, $p = 0.078$).

There were significant changes in breast muscle color between 24 h and 72 h post-slaughter with decreases in L* (56.0 vs 50.8, $p < 0.001$), a* (1.69 vs 1.34, $p = 0.009$) and b* (6.10 vs 4.75, $p < 0.001$) (Table 5). Breast muscle L* (52.7 vs 54.1, $p = 0.025$) was increased and a* (1.85 vs 1.18, $p < 0.001$) was decreased by HS but were unchanged by dietary POL. While there was no main effect of temperature on b* there were main and interactive effects of dietary POL such that b* increased with increasing dose of POL before declining with the peak value occurring at a lower dose of POL under TN (4 g/kg) conditions that during HS (6 g/kg) (Table 5). Breast muscle pH decreased over time post-slaughter (6.86 vs 6.61 and 5.87 at 10 min., 1 h and 24 h post-slaughter, $p < 0.001$), was decreased by HS (6.50 vs 6.39, $p < 0.001$) and increased in a linear manner with dose of POL (6.33 vs 6.40, 6.48, 6.51 and 6.54, $p < 0.001$) (Table 5). However, there were interactions such that the linear effects of were POL were most pronounced at 24 h post-slaughter, particularly under TN conditions. In this context, the pH of the breast muscle from chickens consuming a diet containing 10 g/kg POL and maintained under TN conditions was markedly higher than in muscle from those consuming the control diet (5.72 vs 6.33).

Table 3. Effect of dose (D) of dietary polygain on haematology, electrolytes, metabolites, plasma thyroid hormones and plasma and muscle betaine (*Pectoralis major*) during thermoneutral (TN) and heat stress (HS) temperature (T) conditions.

		Dose of polygain, g/kg					SED ¹	Significance ^{2,3}
		0	2	4	6	10		
Haematocrit, %	TN	19.5	19.0	21.7	19.7	18.7	1.10	T+, Q*,
	HS	19.0	19.0	19.3	19.0	18.0		
Hgb ⁴ , g/dL	TN	6.65	6.30	7.37	6.60	6.30	0.383	Q+
	HS	6.40	6.37	6.60	6.47	6.10		
Potassium, mM	TN	6.78	6.49	6.43	6.33	5.97	0.46	T***, T.L*
	HS	5.20	5.33	5.50	5.60	5.87		
Sodium, mM	TN	151	148	151	149	148	1.0	T***
	HS	148	147	148	148	148		
Chloride, mM	TN	112	111	112	110	108	1.2	L*, T.L+
	HS	110	109	110	111	109		
Calcium, mM	TN	1.52	1.40	1.50	1.52	1.46	0.043	Q+, T.Q*
	HS	1.48	1.44	1.47	1.34	1.51		
Lactate, mM	TN	10.8	7.65	7.09	7.39	4.67	1.25	T***, L**, T.L**
	HS	3.76	6.24	4.26	4.30	5.31		
Glucose, mM	TN	14.2	14.0	14.7	14.3	15.0	0.59	T+,

T ₃ ⁴ , pg/mL	HS	15.1	15.1	15.1	14.3	15.2	0.754	T.L+
	TN	4.11	6.68	4.31	4.88	6.16		
T ₄ ⁴ , pg/mL	HS	5.61	6.07	4.76	4.27	5.44	1.261	T***
	TN	6.95	6.56	6.21	7.63	5.85		
Plasma betaine, µmol/L	HS	9.56	9.34	9.16	8.89	9.39	7.76	T****, Q*
	TN	133	149	142	136	133		
Muscle betaine, µmol/g	HS	102	115	116	107	101	18.3	T**, L****, Q**, T.L**** T.Q**
	TN	246	152	144	117	115		
	HS	131	148	122	118	113		

¹ Standard error of the difference for T × D. ² Level of significance for main and interactive effects of T and linear (L) and quadratic (Q) effects of polygain. ³ + - $p < 0.10$; *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$. ⁴ Haemoglobin (Hgb); Triiodothyronine (T₃); Thyroxine (T₄).

Table 4. Effect of dose (D) of dietary polygain on breast muscle quality (*Pectoralis major*) during thermoneutral (TN) and heat stress (HS) temperature (T) conditions.

		Dose of polygain, g/kg					SED ¹	Significance ^{2,3}
		0	2	4	6	10		
Cooking loss, %	TN	22.8	21.6	23.0	20.9	22.4	1.28	
	HS	21.5	22.2	21.2	22.5	21.4		
Drip loss, %	TN	2.95	2.11	2.21	1.92	1.58	0.356	L**, T.L*
	HS	2.13	1.71	2.10	2.49	1.76		
Water content, %	TN	76.2	76.6	77.3	77.1	78.7	1.02	T*, L**
	HS	74.7	75.1	78.3	75.4	76.7		
Shear force, N	TN	24.1	20.1	20.9	19.9	19.4	1.61	T****, L**
	HS	24.8	26.3	23.3	23.1	23.1		
MFI ⁴	TN	82.0	69.8	106	97.8	113	19.13	T**, L+
	HS	64.9	60.0	86.0	69.1	68.7		
Carbonyl, nmol/mg protein	TN	3.22	3.00	3.08	2.84	2.89	0.403	Q+
	HS	3.77	3.45	2.94	2.90	3.35		

¹ Standard error of the difference for T × D. ² Level of significance for main and interactive effects of T and linear (L) and quadratic (Q) effects of polygain. ³ + - $p < 0.10$; *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$. ⁴ Myofibrillar fragmentation index.

Table 5. Effect of dose (D) of dietary polygain and time post-slaughter (TM) on chicken breast (*Pectoralis major*) colour and pH after being housed thermoneutral (TN) and heat stress (HS) temperature (T) conditions.

	Time (TM)		Dose of Polygain, g/kg					SED ¹	Significance ^{2,3}
			0	2	4	6	10		
L*	24h	TN	55.5	53.0	56.3	56.3	55.2	1.95	TM****, T*
		HS	55.7	55.3	60.3	56.2	56.3		
	72h	TN	50.1	47.5	49.8	51.4	51.8		
		HS	50.6	50.9	53.1	50.8	51.7		
a*	24h	TN	1.99	1.99	1.82	2.06	2.38	0.420	TM**, T***
		HS	1.54	1.26	1.42	1.19	1.29		
	72h	TN	1.55	2.21	1.49	1.26	1.79		
		HS	0.69	1.29	1.29	1.21	0.62		
b*	24h	TN	6.07	5.60	6.25	6.79	6.26	0.606	TM***, L*, Q*, T.L*
		HS	5.11	6.01	6.50	6.44	6.00		
	72h	TN	4.46	3.97	4.43	5.21	5.53		
		HS	4.50	4.28	6.09	4.74	4.31		
pH	10min	TN	6.77	6.88	6.88	6.90	6.90	0.112	T***, TM***, L***, T.TM*** TM.L*, T.TM.L*
		HS	6.83	6.78	6.86	6.84	6.99		
	1h	TN	6.44	6.72	6.64	6.75	6.60		
		HS	6.60	6.49	6.64	6.58	6.67		
	24h	TN	5.72	5.91	5.98	6.15	6.33		
		HS	5.61	5.60	5.86	5.81	5.73		

¹ Standard error of the difference for T × D. ² Level of significance for main and interactive effects of T and linear (L) and quadratic (Q) effects of polygain. ³ + - $p < 0.10$; *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$.

Breast muscle TBARS increased between 24 h and 72 h post-slaughter (2.15 vs 3.01 mg/kg of *malondialdehyde* (MDA), $p < 0.001$), was increased by HS (2.01 vs 3.15 mg MDA/kg, $p < 0.001$) and decreased linearly with increasing dose of dietary POL (3.06 vs 2.42, 3.06, 2.09 and 2.27 mg MDA/kg, $p = 0.037$) (Figure 2). However, there were significant interactions between time and dose of POL that were largely driven by possibly an anomalous high value at 24 h for samples from chickens fed 4 g POL/kg and housed under HS conditions (Figure 2). This is particularly so given that there was no further increase between 24 h and 72 h for this group.

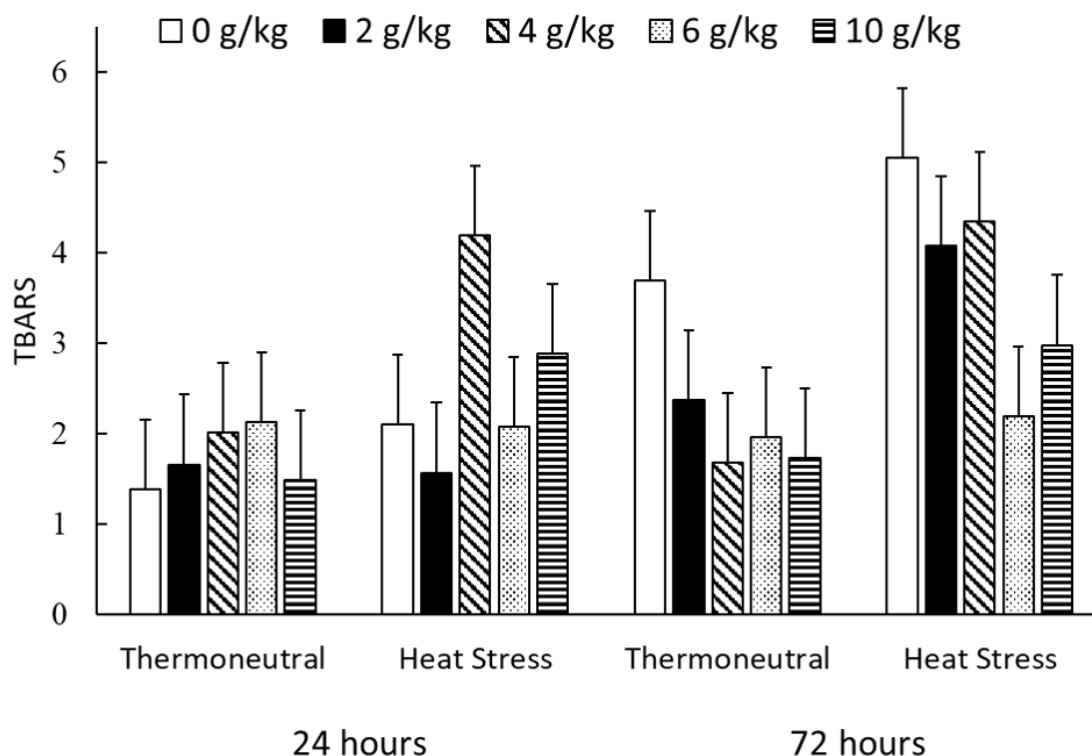


Figure 2. Effect of temperature condition, dose of dietary polygain (POL), and time post-slaughter on breast muscle lipid peroxidase (TBARS) in mg/kg of malondialdehyde triangles. Data are mean and standard error of the difference for the interaction between environmental temperature (T), diet (D) and time post-slaughter (TM). There were main effects ($p < 0.10$) of T ($p = 0.001$), TM ($p < 0.001$) and linear (L) effects of POL ($p = 0.037$) and interactive TM $\times$ L ($p < 0.001$) and TM $\times$ quadratic ($p = 0.008$) effects. There were no other main or interactive effects ($p < 0.10$).

4. Discussion

The major finding from this experiment was that dietary POL could partially reduce the negative effects of HS on growth performance and meat quality in broiler chickens. The adverse effects of HS on weight gain and meat quality confirm our previous findings (Shakeri et al., 2019; Shakeri et al., 2018) and others (He et al., 2015; Nofal et al., 2015). In this regard, potential additives such as POL could play an important role to reduce the negative effects of HS. The effects were such that POL improved body weight gain and FCR under both TN and HS conditions with these effects being most pronounced over

the latter stage of the growing period. The POL diet was not effective at the beginning of growing time, indicating that POL is more effective when chickens were exposed to HS (after day 21). Dietary POL reduced rectal temperature and respiration rate under HS and again these effects were more evident at the end of experiment (Abu Hafsa and Ibrahim, 2018; Starčević et al., 2015). The responses were also dose-dependent with the highest dose investigated being most efficacious. The positive effects of POL on regulating the expression of heat shock proteins (Hu et al., 2019) could help chickens to reduce their body temperature. Polyphenols are micronutrients with strong antioxidants properties and potential health benefits such as improving digestion issues and weight gain (Manach et al., 2004). Furthermore, POL has protective effects that can be related to inhibition of glucoses absorption (Abu Hafsa and Ibrahim, 2018), hormones and growth regulators function (Majewska and Cieczot, 2009), and they can act as an anti-inflammatory agents that prevent oxidative stress (Paszkiwicz et al., 2012; Petti and Scully, 2009). As a result, POL could reduce heat production leading to lower rectal temperature and respiration rate. The positive effects of POL on lowering body temperature could allow chickens to utilize more ingested energy on growth performance rather than other metabolic pathways during HS.

Current genetically improved commercial broilers, such as the Ross-308, can gain weight faster than unimproved breeds, and in a shorter period of time, but this is not without consequences. These improved broiler chickens are more susceptible to HS because of higher endogenous heat production. In this regard, there is a negative relationship between susceptibility to HS and production traits in chickens (Cooper and Washburn, 1998; Lu et al., 2007). Therefore, the body of fast growing genotypes produces more heat, leading to higher mortality rate than slow-growing genotypes under thermal conditions (Deeb et al., 2002; Yalcin et al., 2001). In fact, fast growing genotypes have a reduced ability to respond and adapt to environmental temperature changes such as HS.

Thyroid hormones play a vital role in growth, metabolic rate and oxygen consumption; any alteration in the amount of thyroid hormones could cause metabolic disorders in various organs such as the kidney (Dev et al., 2016; Mansourian, 2012). Furthermore, altered thyroid hormones could modify kidney structure due to reduction of sodium, potassium and other electrolyte (Mansourian, 2012). The present results indicated an increase in thyroid hormones in response to HS which is in contrast with our previously published results (Shakeri et al., 2019), but consistent with others studies

(Bowen and Washburn, 1985; Chiang et al., 2008). However, the differences between the present results and other studies or our previous experiment could be related to differences in age, sex or breed of broiler chickens. This suggests that genetic selection for growth and age of chickens may affect how thyroid hormones respond to environmental temperature. The change of thyroid response in growth-selected birds could affect Ca^{2+} regulation in muscle and thus influence the postmortem meat quality. Although there was no change in plasma Ca^{2+} concentrations during HS in the current experiment, the Ca content in skeletal muscle is regulated by thyroid hormones. Also, HS reduced potassium which participates in acid-base balance, maintenance of osmotic pressure, amino acid absorption and development of membrane potentials of cells (Leeson and Summers, 2001). Potassium does not function independently, and it is in relation with sodium concentrations that is necessary for optimal growth performance and amino acid metabolism (NRC, 1994). Therefore, the reduced plasma potassium and sodium concentrations during HS could impair growth performance and water content as indicated in the present results. In this regard, higher level of potassium during HS could increase thermotolerance in broiler chickens (Ait-Boulahsen et al., 1995), leading to better performance. Interestingly, the level of potassium increased linearly with doses of POL under HS, suggesting that POL could help chickens to cope with HS.

Pale soft and exudative (PSE) meat may occur in chickens exposed to HS as a result of increased muscle glycolysis as demonstrated by that the lower pHu in their muscle which may have led to paler meat with a lower water content than meat from chickens reared under TN conditions (Qiao et al., 2001). Similarly, meat from chickens exposed to HS had lower pH, was paler, and had greater drip loss than muscles from chickens maintained under TN conditions (Sams, 1997). Also, in another study cyclic HS resulted in paler meat with a lower ultimate pH than that observed in chickens housed under TN conditions (Akter et al., 2019).

There was an increase in MFI regardless of the additives using in the present experiment indicating that HS generated large amounts of reactive oxygen species, thus leading to the oxidation of myofibrillar proteins. In our previous study, HS increased MFI and reduced cooking loss and water content (Shakeri et al., 2019). In another study, cooking loss was reduced after HS although the meat was paler with higher pHu (Dai et al., 2012). As there is inconsistency between different studies regarding the effects of HS on the incidence of PSE meat in broiler chickens, other factors may have been involved.

Despite the adverse effects of HS on muscle MFI and water content, dietary POL ameliorated the negative effects, thereby improving meat quality under both temperature conditions. Furthermore, POL resulted in elevated ultimate pH which would explain the higher total water content of POL muscles. High ultimate pH is well-known to be associated with higher water-holding capacity due to reduced charge between myofilaments (Hamm, 1986). Heat stress increased WBSF which is inconsistent with our previous findings (Shakeri et al., 2019). In contrast, others have found that HS increased WBSF in chickens compared to their counterparts held under TN conditions (Zhang et al., 2012a), while dietary POL reduced WBSF. The age of chickens could be a reason for differences between current results and previous studies.

Heat stress increased oxidative damages to muscle tissues as determined by increased TBARS. Heat stress produces reactive oxygen species due to denaturation of proteins, resulting in more significant oxidative damages in tissues (Fouad et al., 2016). These results suggest that regardless of supplementation, HS is one of the major causes of oxidative stress in chickens and other farm animals muscle (Chauhan et al., 2014; Liu et al., 2016). Increased lipid and protein oxidation can be determined by increased TBARS and microbiological growth, and color deterioration for broiler chickens exposed to HS (Imik et al., 2012; Wang et al., 2009). Importantly, higher doses of dietary POL were able to counteract the effects of HS on oxidation and even reduce oxidation under both temperature conditions.

5. Conclusions

The present data indicate that HS decreased growth performance and increased respiration rate and rectal temperature in broilers with resultant deleterious effects on meat quality. Supplemental POL was able to partially ameliorate the negative effects of HS on performance and meat quality as indicated by the reduction in TBARS, myofibrillar protein fragmentation and WBSF under both TN and HS conditions. The responses to POL were linear in nature with the greatest responses occurring at the highest dose investigated.

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and S.W.; Supervision, S.W., J.J.C., and F.R.D.; Writing—original draft, M.S., and J.J.C.; Writing—review and editing, M.S., J.J.C., and F.R.D.

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Appendix A

Details of the nutrition composition of the diets in these experiments are provided in the material method of the chapter 3. For this chapter, polygain was added to the final mixture as 0.2, 0.4, 0.6 and 1% equal to 2, 4, 6 and 10g/kg feed, respectively.

CHAPTER 6

Dietary Betaine Improves Intestinal Barrier Function and Ameliorates the Impact of Heat Stress in Multiple Vital Organs in Broiler Chickens

6.1. Introduction aims and hypotheses

Heat stress (HS) causes multiple organs dysfunction, by increasing blood flow to the skin areas to accelerate the heat dissipation, resulting in the reduction of blood supply to internal organs, leading to hypoxia. Therefore, affordable nutritional supplements such as betaine have been considered to improve organs conditions as it is accumulated in tissues under osmotic stress. Studies indicated that osmolyte effects of betaine could improve tissue conditions under HS conditions, while limited studies investigated the distribution of betaine in tissues and its effects on tissues structure. Therefore, in order to understand the effects of HS and betaine on tissues, it's required to use indicators such as Evans Blue Dye to achieve this purpose. Evans Blue Dye (EBD) is widely used to study blood vessel and cellular membrane permeability as it is non-toxic, it can be administered as an intravital dye and it binds to serum albumin – using this as its transporter molecule. Using markers such as EBD is a common method to identify amount of damages to different tissues under stress conditions such as HS.

Therefore, the aim of this chapter was to investigate the effects of short term cyclic heat stress when chickens were supplemented with the osmolyte betaine on different tissues, physiological responses, and tissues morphology.

Section 6.2 presents the paper (under review) that showed the effects of short term cyclic heat stress on tissues when chickens were injected with EBD and supplemented with betaine.

6.2. Published paper

Dietary Betaine Improves Intestinal Barrier Function and Ameliorates the Impact of Heat Stress in Multiple Vital Organs as Measured by Evans Blue Dye in Broiler Chickens

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Simple Summary: Heat stress alters the normal physiological status, compromising the function of organs such as the small intestine. However, evidence exists of a wider distribution of organ dysfunction, stemming through factors such as a reduction in blood flow due to redistribution to the skin for increased radiant heat loss to the environment. Simultaneously assessing organ dysfunction at multiple locations presents technical difficulties and hence studies are lacking. Therefore, the aim of this experiment was to determine the pattern of Evans Blue Dye distribution as a cost-effective indicator of organ dysfunction in heat-stressed chickens supplemented with betaine, which is protective against heat stress. The results showed that Evans Blue Dye accumulated in the kidney and muscle during heat stress and that this was ameliorated by betaine. Therefore, Evans Blue Dye may be a useful tool to investigate the effects of heat stress on broiler organ dysfunction.

Abstract: In a 2 × 2 factorial design, 60 male Ross-308 broilers were fed either a control vs. 1 g/kg betaine diet and housed under thermoneutral (TN) vs. heat stress (HS)

conditions. Broilers were acclimated to diets for 1 week under TN (25 °C), then either kept at TN or the temperature increased 8 h/day at 33 °C and 16 h/day at 25 °C (HS) for up to 10 days. Respiration rate (RR) was measured at four time points, and on each of 1, 2, 3, 7 and 10 days of HS, 12 broilers were injected with 0.5 mg/kg of Evans Blue Dye (EBD) solution to quantify regional changes in tissue damage. Betaine was quantified in tissues and ileal damage was assessed via morphometry and transepithelial resistance (TER). Heat stress elevated RR ($p < 0.001$) and resulted in reduced villous height ($p = 0.009$) and TER ($p < 0.001$) while dietary betaine lowered RR during HS ($p < 0.001$), increased betaine distribution into tissues and improved ileal villous height ($p < 0.001$) and TER ($p = 0.006$). Heat stress increased EBD in muscle and kidney of chickens fed the control diet but not in those receiving betaine. Overall, these data indicate that supplemented betaine distributes to vital organs and the gastrointestinal tract where it is associated with improved tolerance of HS.

Keywords: heat stress; betaine; Evans blue dye; histology; intestinal barrier; broiler chickens

1. Introduction

Due to the impacts of a changing climate, heat stress (HS) is of increasing concern for animal production. Broilers are sensitive to HS due to the presence of feather coverage, increased selection for muscling and because they lack sweat glands. Heat stress compromises efficient broiler production in part by reducing voluntary feed intake. However, some studies have shown that the reduction in feed intake does not fully explain the reductions in growth rate (Renaudeau et al., 2012). The reasons for this include factors such as altered endocrine status (Shakeri et al., 2019; Tao et al., 2005). Furthermore, increased evaporative heat loss by panting and radiant heat loss by blood flow redistribution also compromise efficient growth. As the distribution of blood flow to the skin to facilitate radiant heat loss relies on a commensurate reduction in blood flow elsewhere, continued HS can precipitate dysfunction in affected organs due to reduced nutrient delivery and removal of metabolic by-products. Heat stress compromises intestinal barrier integrity in broiler chickens and other species (Goo et al., 2019; Liu et al., 2016; Shakeri et al., 2018), presumably by splanchnic blood restriction, and that can

lead to bacterial translocation and a systemic inflammatory response (Alhenaky et al., 2017; Pearce et al., 2013).

While investigations into the etiology of heat stroke have confirmed the central role of gastrointestinal tract (GIT) damage (Eshel et al., 2001), it is apparent that heat stroke has a wider pattern of organ damage than the GIT alone, with increased incidences of renal and liver injury (Davis et al., 2017; Hifumi et al., 2018). It is likely that compromised hepatic and renal function also contribute to reduced growth efficiency in broilers and other production species during heat exposure. While quantifying systemic changes in organ damage is technically difficult, this may in part be overcome by using blood-borne markers such as Evans Blue Dye (EBD). Within the blood, EBD binds tightly to plasma albumin and is used as an exogenous marker of plasma volume (Crooke and Morris, 1942). Following inflammation or tissue injury, EBD extravasates into the surrounding tissue where it may be quantified as a marker of tissue damage (Caplan and Schwartz, 1973; Radu and Chernoff, 2013). Identifying localized sites of stress is a useful strategy for the development of amelioration strategies, as has been demonstrated with the supplementation of antioxidants in mitigating the impact of HS on intestinal permeability (Liu et al., 2016). Therefore, the aims of this experiment were to investigate changes in EBD extravasation in the broiler during HS. Furthermore, the organic osmolyte betaine has been demonstrated to be an effective supplement for ameliorating the effects of HS. It protects cells against osmotic inactivation, increases water retention of cells (Craig, 2004b), reduces core body temperature by reducing the activity of the ion pumps required for osmoregulation, allowing more energy for growth (Dunshea et al., 2013; Suster et al., 2004b; Tsai et al., 2015b) and acts as a methyl donor for homocysteine remethylation (Alirezai et al., 2012). Therefore, the secondary aim of the experiment was to determine if supplementation of betaine ameliorated the effects of HS and altered the pattern of EBD extravasation.

2. Materials and Methods

2.1. Ethics Statement

The experiment was performed in an animal facility approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia (Protocol no. 1814704.1). The use of animals for research is

regulated by federal and state legislation according to the Prevention of Cruelty to Animals Act 1986 and the Prevention of Cruelty to Animals Regulations 2008.

2.2. *Animals, Diets and Experimental Design*

Four week-old-male Ross-308 chickens ($n = 60$) were obtained from a local commercial farm (Turosi, Bannockburn Victoria, Australia) located within 2 h driving distance from The University of Melbourne. Chickens were randomly allocated to 4 equally sized pens (1.9 m $\times$ 3.4 m) in two environmentally controlled rooms. All pens were covered with wood shavings (8-10 cm deep) with 4 drinkers and 4 feeders for each pen. The chickens were allowed to acclimate to the pens and facility for 7 days at a constant 25 °C (thermoneutral, TN). From the arrival in the facility chickens were given either a standard finisher control diet (CON, $n = 30$) which was formulated as a commercial finisher diet (Feedworks BESTMIX CP 21.3 % and 12.65 MJ ME/kg) and exceeded nutrient requirements (NRC, 1994), or a CON plus 1 g/kg betaine (Betafin S1, DuPont, UK) diet (BET, $n = 30$). After 7 days acclimation the temperature in one room was increased to 33 °C for 8 h/day (9 am-5 pm, 16 h/day 25 °C) to induce heat-stress (HS) for 10 days while the alternate room was maintained under TN conditions. The relative humidity for both rooms was between 40-55 % during the experiment. Light was provided 20 h/day, and chickens had *ad-libitum* access to feed and water during the period of experiment. On each of days 1, 2, 3, 7 and 10 of environmental treatment, 3 chickens from each pen (each diet $\times$ temperature group) were assessed for EBD extravasation.

2.3. *Physiological Responses*

Respiration rate (RR) was measured at 11:00 am after chickens were exposed to 1, 3, 7 and 10 days HS (corresponding to 8, 10, 14 and 17 days in the facility and consuming the experimental diets). Chickens were filmed with a cell phone (iPhone 7, Apple Inc., Cupertino, California, USA) and then the number of breaths taken over a 20 sec. period was quantified and then expressed as breaths per min.

2.4. *Evans Blue Dye Injection, Slaughter and Tissue Collection*

Chickens were injected with 0.5 mg/kg of an EBD solution (1.5% w/w EBD, Sigma, Aldrich, Missouri, USA in 0.9 % saline solution) into the brachial vein on days 1, 2, 3, 7 and 10 of the environmental treatment. Three chickens were briefly removed from each pen for the injection, then returned to their designated rooms for 2 h. Chickens were then

removed from the rooms, electrically stunned (15 amp 220-volt, Mitchell Engineering Food Equipment, Pty Ltd, Clontarf, Queensland, Australia), placed in an inverted restraining funnel, slaughtered by severing the major blood vessels in the neck and then exsanguinated. Tissue samples were collected from the ileum, jejunum, muscle, liver, spleen and kidney for measuring EBD concentration. Furthermore, about 5 cm of ileum tissue and a piece of *psoas major* (breast muscle) were collected for morphometric analysis.

2.5. Evans Blue Dye Extraction and Qualification

Evans Blue Dye concentration was measured in the collected tissues according to a published method (Radu and Chernoff, 2013). Tissue samples were dried in an oven at 70 °C for 48 h, then EBD extracted from 100 mg of dried and pulverised tissue with 500 µL formamide (Sigma-Aldrich, St Louis, Missouri, USA) and incubated at 55 °C for 24 h. Samples were then centrifuged for 15 min. at 14,000 x *g* and 4 °C, and the A₆₁₀ of 200 µL of supernatant quantified in duplicate against standards using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). The obtained results were expressed as ng EBD per mg tissue dry weight.

2.6. Intestinal Transepithelial Electrical Resistance

Intestinal transepithelial electrical resistance (TER) was measured according to a previously published method (Liu et al., 2016). Sections of ileum were (from Meckel's diverticulum to the cecum junction) collected immediately after euthanasia on days 3, 7 and 10 of the environmental challenge. The samples were collected (~3 cm length) from the ileum, without squeezing them which could damage the section. Subsequently, the samples were washed with saline buffer to remove all contents before placing them in chilled phosphate buffered saline, then transferred to Krebs solution (pH 7.4). The ileal sample was then opened along the mesenteric border and the external muscle was removed. The remaining layers were mounted onto a round slider (0.71 cm²) and placed into a two-part Ussing chamber (EasyMount Diffusion Chambers, Physiologic Instruments) and 5 mL Krebs' solution added to each side. On the mucosal side the 11.1 mM glucose was replaced with mannitol. Voltage and I_{sc} readings were acquired using a PowerLab amplifier and recorded using LabChart®5 (AdInstruments Pty Ltd., Lexington, New South Wales, Australia). Tissue was left to equilibrate for 20 min. before clamping

the voltage to 0 V and epithelial resistance determined by administering five 2 sec. pulses of 2 mV. The TER was calculated by Ohm's law and multiplied by the exposed area.

2.7. High-Performance Liquid Chromatography Analysis of Betaine and Derivatives

Betaine was quantified in ileum, kidney and spleen following derivatisation with bromophenacyl bromide catalysed with 18-crown-6 and the bromophenacyl esters quantified by High-Performance Liquid Chromatography (Laryea et al., 1998). Briefly, 100 mg of pulverised tissue samples were homogenized with a bead beater (AnytimeLabTrader LLC, Fallbrook, California, USA) in 1 mL tris buffer (1 M, pH 7) then centrifuged at 10,000 x *g* for 15 min. to obtain supernatant. The obtained supernatant was added to monopotassium phosphate (100 mmol/L) and derivatisation solution containing 4-bromophenacyl bromide (50 mmol/L) and 18-crown-6 (2.5 mmol/L) in acetonitrile and vortex mixed. The samples were heated at 80 °C in a block heater for 1 h, cooled to room temperature before filtering into a glass High-Performance Liquid Chromatography. The A_{254} of the bromophenacyl esters of betaine were then quantified versus standards using a High-Performance Liquid Chromatography following a 10 μ L injection (Model 2998, Waters, Milford, Massachusetts, USA).

2.8. Morphometric Analysis

Tissue samples were collected on days 1, 2, 3, 7 and 10 of the environmental challenge. The midpoint of the ileal section, and *psoas major* were excised and transferred in 10 % formalin (Sigma-Aldrich, St Louis, Missouri, USA) and fixed in paraffin wax (Baddeley et al., 1986). Slides were prepared using 8 μ m sections, stained by hematoxylin and eosin, and the villous height, crypt depth, ileum seromuscular layer and *psoas major* fibre diameter (width) were quantified using a light microscope equipped with a camera (Leica, ICC50 W, Wetzlar, Germany), and analysed with ImageJ software (Collins, 2007). The distance from the tip of the villous to the villous crypt junction represents the villous height, crypt depth was defined as the depth of the invagination between adjacent villous, and seromuscular layer was the smooth muscular layer located under the crypt. A total of 10 samples per section were quantified.

2.9. Statistics Analysis

All data were analysed using ANOVA for the main and interactive effects of temperature and diet (CON vs BET) and time (1, 2, 3, 7 and 10 days) using Genstat

version 18 (VSNi Ltd., Hemel Hempstead, UK). Statistical significance was considered at $p \leq 0.05$ and when achieved a Duncan's multiple range post-hoc test was performed to differentiate between treatment groups, which were then labelled with differing alphabetic superscripts. Where skewed data occurred normality was restored following a Log_{10} transformation and analysed as above. The predicted means were then back-transformed (10^x) and presented in tables in parentheses. The replication for the main effects of temperature and diet were 30 chickens respectively. The replication for the interaction between temperature and diet was 20 chickens per group and for temperature $\times$ diet $\times$ time was 3 chickens per treatment/time.

3. Results

3.1. Respiration Rate

Heat stress increased RR at each time point measured ($p < 0.001$, Figure 1). Dietary BET supplementation reduced RR during HS ($p < 0.001$) at each time point (Figure 1), but there was no effect of dietary BET under TN conditions. No main or interactive effects of time on RR were observed.

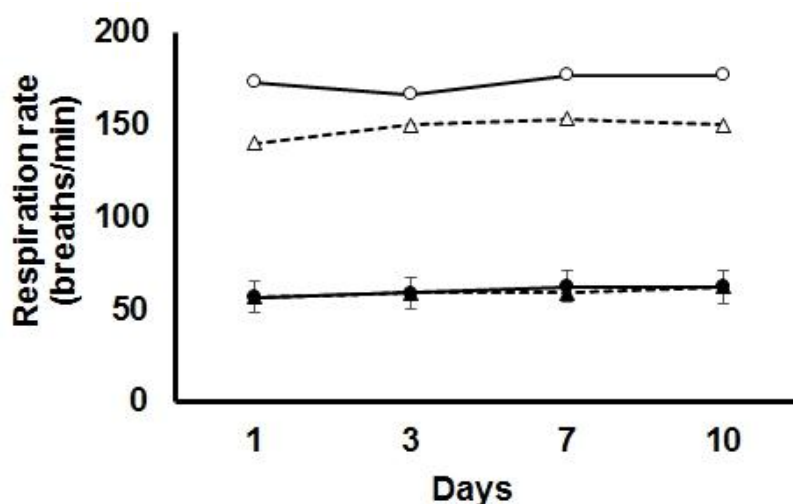


Figure 1. Respiration rate in broilers fed either a control diet (CON, round symbols) or betaine supplemented diet (BET, triangle symbols) after 1, 3, 7 and 10 days being exposed to either thermoneutral (TN, filled symbols) or heat stress (HS, open symbols) conditions. The standard error of the difference for Temperature $\times$ Diet $\times$ Day is displayed on the data for the chickens fed the CON diet under TN conditions.

3.2. Evans Blue Dye Distribution

Evans Blue Dye concentration was quantified in muscle, liver, ileum, jejunum, spleen and kidney (Table 1). Heat stress increased EBD concentrations in the kidney (74 vs 99 ng/mg, $p = 0.007$) but reduced concentrations in the spleen (213 vs 162 ng/mg, $p = 0.024$). Dietary BET decreased EBD concentrations in the jejunum (94 vs 76 ng/mg, $p = 0.043$), ileum (100 vs 76 ng/mg, $p = 0.028$) and kidney (113 vs 61 ng/mg, $p < 0.001$). There were significant interactions between diet and temperature in muscle and kidney such that HS increased EBD concentrations in chickens consuming the CON diet but not in those on the BET supplemented diet (Figures 2A and 2B). There was also an interaction between temperature and diet in the spleen where BET increased EBD concentrations under TN conditions, but not during HS (Figure 2C). There were no main or interactive effects of temperature, diet or time for EBD concentration in the liver, while interactions were observed for diet, temperature and time in the kidney, jejunum and ileum (Table 1). These interactions typically reflected that BET under HS conditions reduced tissue EBD concentrations. In the kidney, EBD concentration was higher under HS CON than all other groups after 1 day of HS. In the jejunum, this was observed after 7 days and in the ileum at day 10.

3.3. Transepithelial Electrical Resistance

Ileal TER was quantified after 3, 7 and 10 days of the environmental challenge (Table 2). Overall, TER was increased by BET (182 vs 235 $\Omega\cdot\text{cm}^2$, $p = 0.006$) and reduced by HS (256 vs 161 $\Omega\cdot\text{cm}^2$, $p < 0.001$). Ileal TER declined with time over the course of the experiment ($p < 0.001$). An interaction between HS and time occurred such that the TER of TN chickens at day 3 was almost double that of HS chickens (346 vs 186 $\Omega\cdot\text{cm}^2$, $p = 0.049$). An interaction between diet, temperature and day was observed such that a reduction in ileal TER by day 10. Alternatively, the TER from HS BET chickens was nearly double than HS CON at this time (93 vs 161 $\Omega\cdot\text{cm}^2$) (Table 2).

Table 1. Effects of a control diet (CON) or dietary betaine (BET) on Evans Blue Dye distribution in broilers housed under thermoneutral (TN) or heat stress (HS) conditions for 1, 2, 3, 7 and 10 days.

	Diet	Temp (T)	Day of Thermal Challenge					SED	Significance ¹
			1	2	3	7	10		
Psoas Major (ng/mg)	CON	TN	8.3	16.0	8.0	14.3	29.4	11.3	D × T*
		HS	8.3	42.1	24.5	29.1	39.1		
	BET	TN	6.7	30.8	16.4	18.8	25.2		
		HS	9.5	6.2	16.7	14.3	31.8		
Liver (ng/mg)	CON	TN	37	91	96	110	94	32.8	
		HS	65	107	111	83	80		
	BET	TN	128	111	106	90	80		
		HS	58	141	65	60	87		
Kidney (ng/mg)	CON	TN	96 ^a	93 ^{ab}	62	61	119	28.2	T**, D***, D × D × T × Day+
		HS	158 ^b	117 ^b	98	94	161		
	BET	TN	90 ^a	31 ^a	41	53	93		
		HS	62 ^a	61 ^{ab}	36	45	91		
Spleen ² (ng/mg)	CON	TN	2.05	2.08	2.20	2.30	2.36	0.15	T*, D × T*
			(112)	(120)	(157)	(199)	(229)		
		HS	2.04	1.89	2.31	2.31	2.41		
			(110)	(78)	(206)	(206)	(258)		
	BET	TN	2.28	2.19	2.51	2.43	2.49		
			(191)	(153)	(321)	(267)	(309)		
		HS	2.04	2.03	2.36	2.14	2.22		
			(110)	(108)	(231)	(175)	(167)		
Jejunum ² (ng/mg)	CON	TN	2.02	2.29 ^a	1.90	1.86 ^{ab}	2.08	0.14	D*, D × T ×
			(104)	(194)	(79)	(73)	(121)		
		HS	1.85	1.84 ^b	1.95	2.08 ^b	1.90		
			(71)	(69)	(89)	(119)	(79)		
	BET	TN	1.84	1.76 ^b	1.86	1.90 ^{ab}	1.98		
			(69)	(58)	(73)	(80)	(100)		
		HS	1.99	2.10 ^{ab}	1.87	1.69 ^a	1.85		
			(97)	(125)	(75)	(49)	(70)		
Ileum (ng/mg)	CON	TN	151	100	77	75	54 ^a	34.2	D*, D × T ×
		HS	115	68	69	85	209 ^b		
	BET	TN	70	146	49	32	108 ^a		
		HS	83	106	55	34	75 ^a		

¹ + $p < 0.10$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$. Differing superscripts within a column denotes significant ($p < 0.05$) differences for D × T × Day on a single day of the experiment.

² Due to skewed data the values were Log₁₀ transformed before statistical analysis. Back transformed means are presented in parentheses.

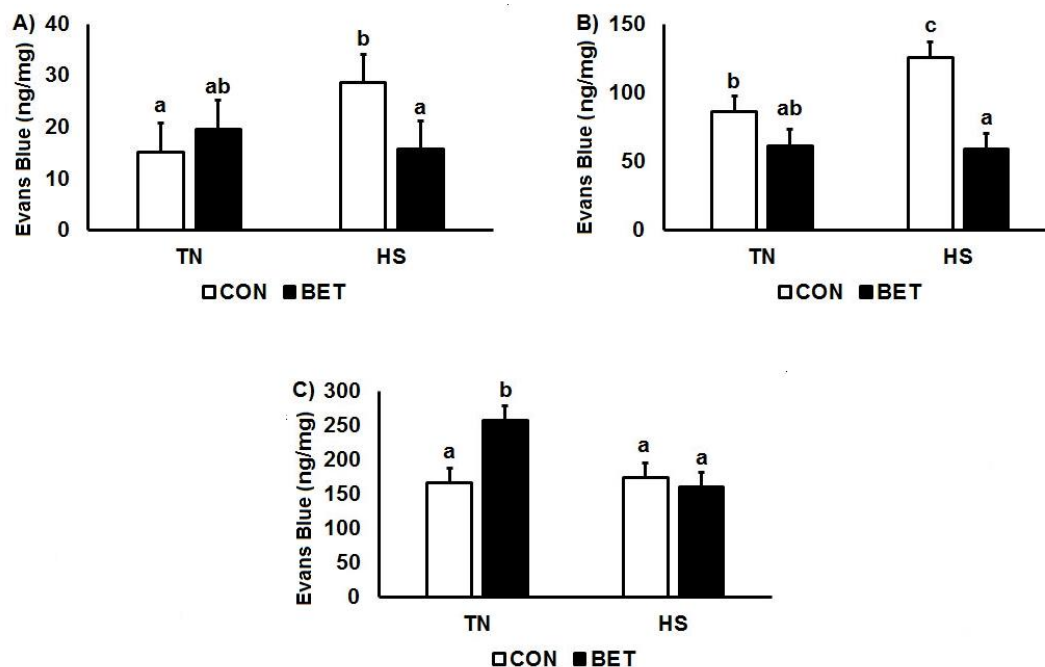


Figure 2. Changes in the distribution of Evans Blue Dye in A) psoas major, B) kidney and C) spleen in broilers during a thermoneutral (TN) vs heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET) and the mean represents the main effect of 5 time points (1, 2, 3, 7 and 10 days challenge). Means with differing superscripts denote $p < 0.05$. Refer to Table 1 for full interactive effects.

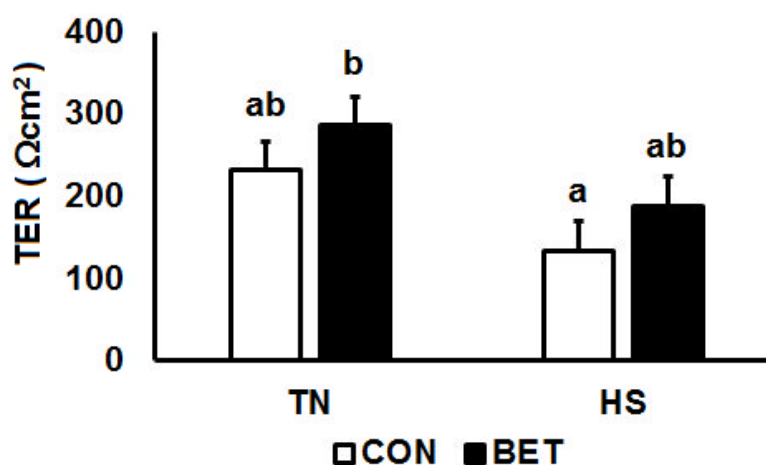


Figure 3. Transepithelial electrical resistance (TER) changes in broilers during a thermoneutral (TN) vs heat stress (HS) environmental challenge. Broilers were fed either

a control diet (CON) or betaine diet (BET) and the mean represents the main effect of 3 time points (3, 7 and 10 days challenge).

3.4. Morphometric Analysis

Ileal villous height was decreased by HS (826 vs 779 μm , $p = 0.009$) and increased by dietary BET (741 vs 864 μm , $p < 0.001$) (Table 2; Figure 4). There was no main effect of HS on villous area whereas dietary BET increased villous surface area (97 vs 136 μm^2 , $p < 0.001$) (Table 2; Figure 5A). However, there were interactions such that villous surface area reduced over time particularly in those chickens exposed to HS. There was no main effect of HS on crypt depth whereas it was increased by dietary BET (152 vs 192 μm , $p < 0.001$) (Table 2). However, there were interactions such that crypt depth decreased over time particularly in those chickens exposed to HS. The seromuscular layer depth was decreased by HS (227 vs 174 μm , $p < 0.001$) and increased by dietary BET (161 vs 240 μm , $p < 0.001$) (Table 2; Figure 5B). However, there were interactions such that seromuscular layer depth increased over time in those chickens consuming the BET diet and housed under TN conditions whereas it declined in those chickens exposed to HS (Table 2). Heat stress reduced *psoas major* fibre diameter (227 vs 174 μm , $p < 0.001$) whereas it was increased by dietary BET (161 vs 240 μm , $p < 0.001$) (Figure 6). However, there were interactions such that *psoas major* fibre diameter was initially higher in chickens fed dietary BET and then increased over time in those chickens consuming the BET diet and housed under TN conditions whereas it declined in those chickens exposed to HS. *Psoas major* fibre diameter remained constant in those chickens consuming the CON diet.

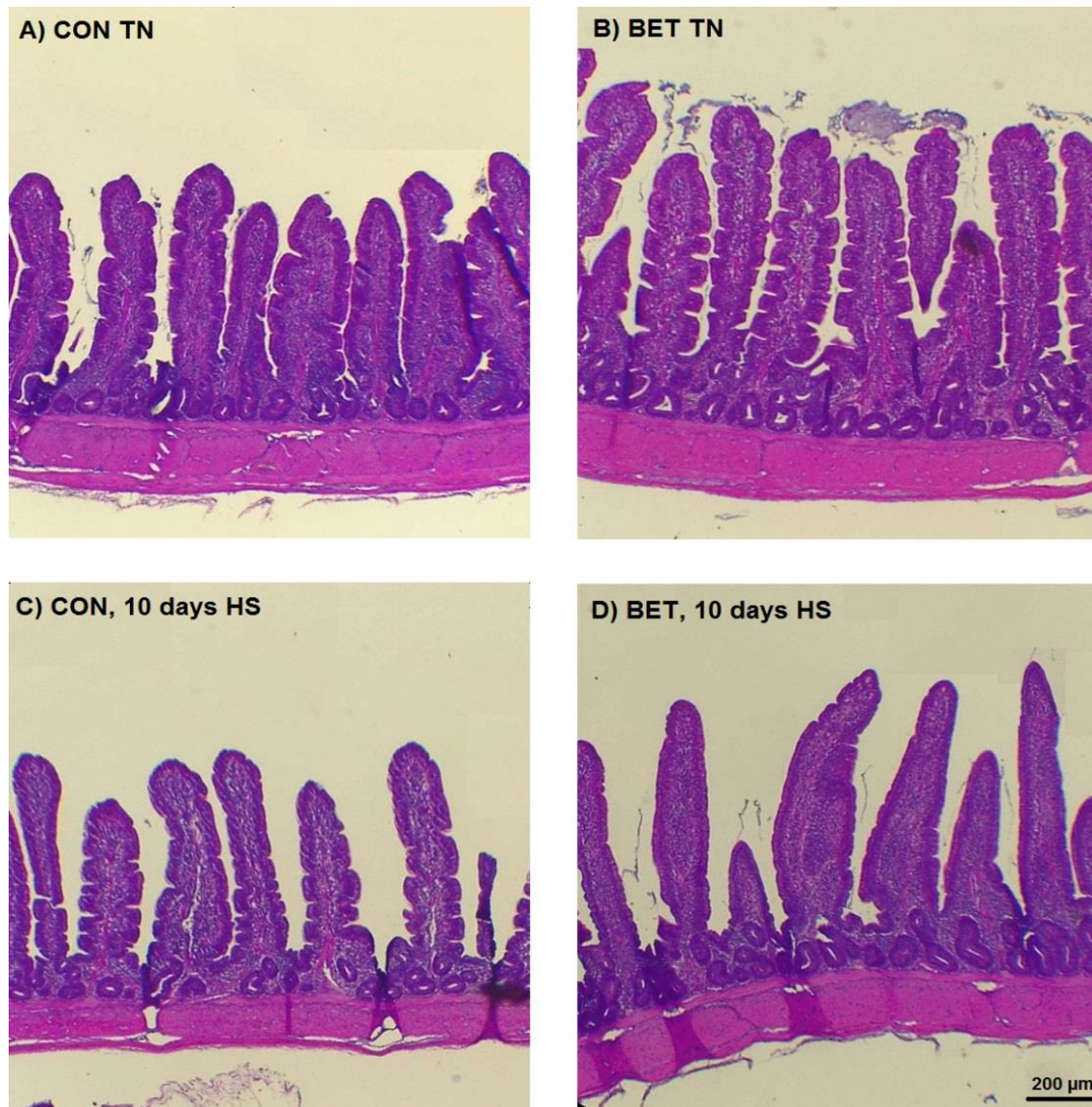


Figure 4. Representative photomicrographs of the ileum after 10 days of the experiment from broilers fed a control diet (CON, A and C) and betaine (BET, B and D) on villous height under thermoneutral (TN, A and B) or after 10 days being exposed to heat stress (HS, C and D).

Table 2. Effects of a control diet (CON) or dietary betaine (BET) on ileal transepithelial electrical resistance and ileal morphology in broilers under thermoneutral (TN) or heat stress (HS) conditions for 1, 2, 3, 7 and 10 days.

	Diet (D)	Temp	Day of Thermal Challenge					SED	Significance ¹
			1	2	3	7	10		
Transepithelia resistance ² ($\Omega \cdot \text{cm}^2$)	CON	TN	-	-	268 ^a	211	215 ^a	35.7	T***, D**, Day***, D $\times$ T $\times$ Day+
		HS	-	-	159 ^a	148	93.0 ^b		
	BET	TN	-	-	424 ^b	246	175 ^{ab}		
		HS	-	-	212 ^a	192	161 ^{ab}		
Villous height (μm)	CON	TN	754 ^a	763 ^a	727 ^a	741 ^a	775 ^a	55.5	T***, D***, D $\times$ D $\times$ T $\times$ D***
		HS	749 ^a	722 ^a	718 ^a	763 ^a	696 ^b		
	BET	TN	885 ^b	827 ^b	894 ^b	947 ^b	939 ^c		
		HS	921 ^b	852 ^b	775 ^{ab}	796 ^a	797 ^a		
Villous area ³ (μm^2)	CON	TN	2.10 ^a (130)	1.90 ^a (82)	2.00 ^b (104)	2.06 ^a (118)	2.14 ^c (140)	0.22	D***, Day**, D $\times$ T $\times$ Day*, D $\times$ T $\times$
		HS	1.98 ^a (100)	1.96 ^a (95)	1.87 ^a (76)	1.96 ^a (92)	1.92 ^a (85)		
	BET	TN	2.08 ^a (134)	2.14 ^b (142)	2.04 ^b (112)	2.19 ^b (165)	2.14 ^{bc} (141)		
		HS	2.35 ^b (228)	2.09 ^b (127)	2.07 ^b (126)	2.17 ^b (156)	2.08 ^b (123)		
Crypt depth (μm)	CON	TN	172 ^a	183 ^b	152	132 ^a	144 ^b	8.9	D***, Day***, D $\times$ Day***, T $\times$
		HS	141 ^a	197 ^{bc}	162	126 ^a	112 ^a		
	BET	TN	250 ^b	153 ^a	170	211 ^c	186 ^c		
		HS	253 ^c	217 ^c	158	179 ^b	151 ^b		
Seromuscular layer (μm)	CON	TN	160 ^a	175 ^a	159 ^a	160 ^a	149 ^a	11.4	T***, D***, D $\times$ D $\times$ Day**, T $\times$ D $\times$ T $\times$ Day**
		HS	161 ^a	185 ^a	167 ^a	145 ^a	150 ^a		
	BET	TN	282 ^b	235 ^b	259 ^b	315 ^b	375 ^c		
		HS	233 ^c	209 ^a	151 ^a	172 ^a	171 ^b		

¹ *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$.

Differing superscripts within a column denotes significant ($p < 0.05$) differences for D $\times$ T $\times$ Day on a single day of the experiment.

² Due to logistical constraints ileal transepithelial electrical resistance was only measured on days 3, 7 and 10.

³ Due to skewed data the values were Log₁₀ transformed before statistical analysis. Back transformed means are presented in parentheses.

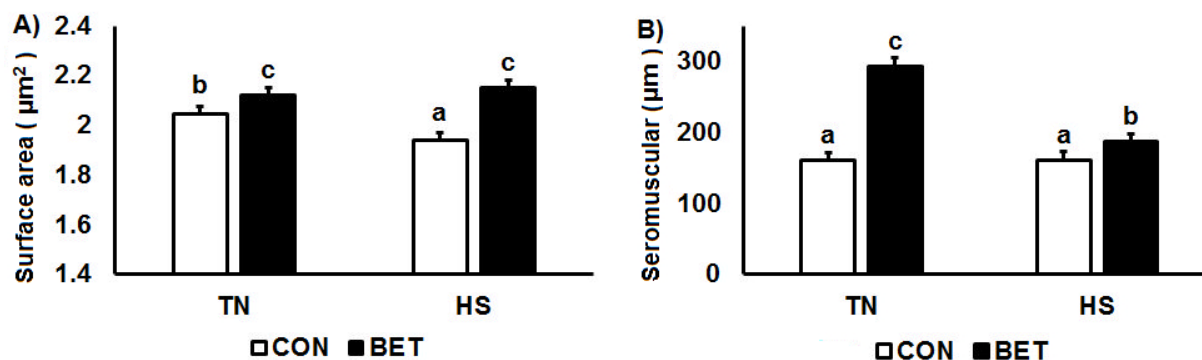


Figure 5. Ileal morphology A) villous surface area and B) seromuscular layer in broilers during a thermoneutral (TN) vs heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET) and the mean represents the main effect of 5 time points (1, 2, 3, 7 and 10 days challenge). Means with differing superscripts denote $p < 0.05$, refer to Table 2 for full interactive effects.

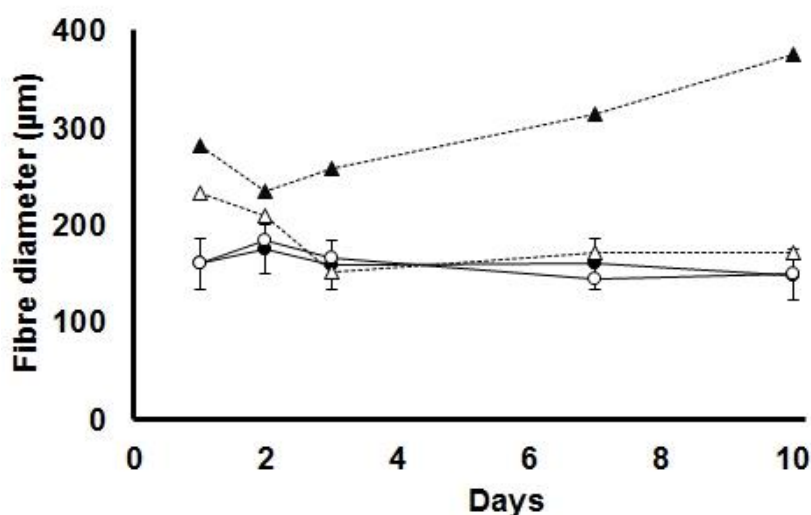


Figure 6. Psoas major fibre diameter in broilers fed either a control diet (CON, round symbols) or betaine supplemented diet (BET, triangle symbols) after 1, 3, 7 and 10 days being exposed to either thermoneutral (TN, filled symbols) or heat stress (HS, open symbols) conditions. Psoas major fibre diameter increased over the course of experiment in TN BET ($p < 0.001$) while HS groups reduced HS CON. The standard error of the difference for Temperature × Diet × Day is displayed on the data for the chickens fed the CON diet under TN conditions.

3.5. Betaine Distribution

Heat stress decreased betaine concentrations in the ileum (117.1 vs 84.7 $\mu\text{mol/g}$, $p < 0.001$) whereas they were increased in the kidney (59.6 vs 74.2 $\mu\text{mol/g}$, $p < 0.001$) and spleen (59.9 vs 64.2 $\mu\text{mol/g}$, $p = 0.02$) (Table 3; Figure 7). Overall, chickens supplemented with BET had higher betaine concentrations in the ileum (83.2 vs 118.6 $\mu\text{mol/g}$, $p < 0.001$), kidney (60.5 vs 73.4 $\mu\text{mol/g}$, $p < 0.001$) and spleen (57.7 vs 66.4 $\mu\text{mol/g}$, $p < 0.001$) than their control counterparts. However, there were interactions such that ileal betaine concentrations increased over time under TN conditions particularly in those chickens consuming the BET diet. Conversely, for the kidney and spleen the increase in tissue betaine concentrations in response to dietary BET were greater during HS than under TN conditions (Figure 7).

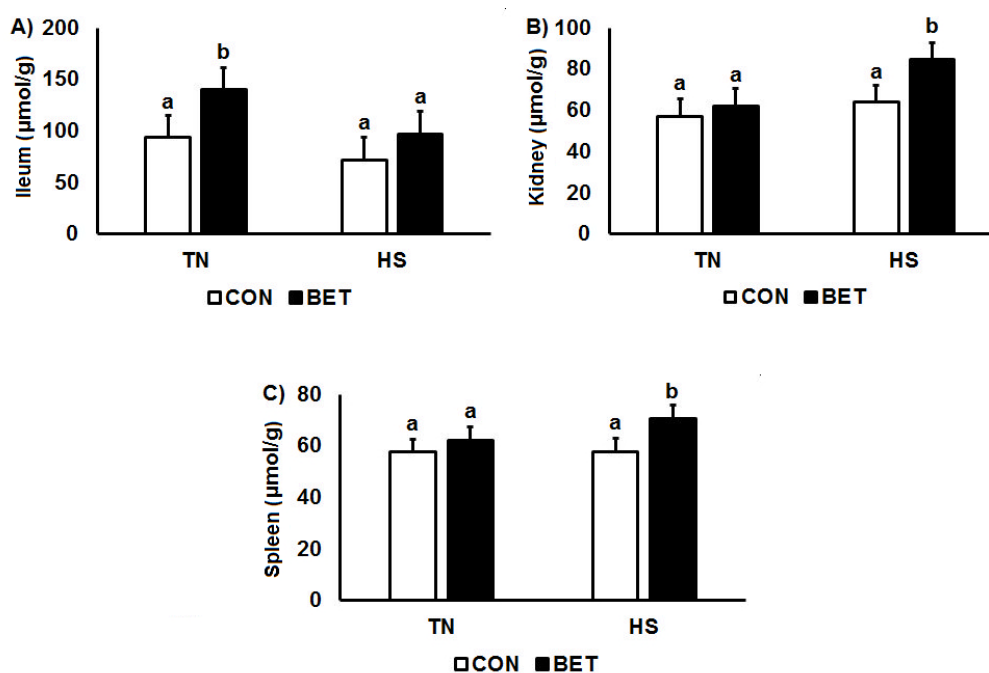


Figure 7. Changes in the distribution of betaine A) ileum, B) kidney and C) spleen in broilers during a thermoneutral (TN) vs heat stress (HS) environmental challenge. Broilers were fed either a control diet (CON) or betaine diet (BET) and the mean represents the main effect of 5 time points (1, 2, 3, 7 and 10 days challenge). Means with differing superscripts denote $p < 0.05$, refer to Table 3 for full interactive effects.

Table 3. Effects of a control diet (CON) or dietary betaine (BET) on tissue betaine concentration in broilers housed under thermoneutral (TN) or heat stress (HS) conditions for 1, 3, 7 and 10 days.

			Day of Thermal Challenge						
	Diet (D)	Temp (T)	1	2	3	7	10	SED	Significance ¹
Ileum (μmol/g)	CON	TN	77.7 ^a	90.5 ^b	95.7 ^a	113 ^{ab}	77.7 ^a	21.3	T***, D***, T × D × T × Day*
		HS	68.1 ^a	48.7 ^a	106 ^a	66 ^a	68.1 ^a		
	BET	TN	91 ^{ab}	153 ^c	152 ^b	165 ^b	91 ^{ab}		
		HS	126 ^b	90.1 ^b	84.1 ^a	89.4 ^{ab}	126 ^b		
Kidney (μmol/g)	CON	TN	41.2 ^a	85.1 ^b	54.1	47.8 ^a	41.2 ^a	8.3	T***, D***, T × Day***, D ×
		HS	48.1 ^a	87 ^b	64.1	56.7 ^{ab}	48.1 ^a		
	BET	TN	55.9 ^b	53.9 ^a	75.6	63.6 ^b	55.9 ^b		
		HS	90.4 ^c	81.2 ^b	77.6	88.8 ^c	90.4 ^c		
Spleen (μmol/g)	CON	TN	57.1	50.5 ^{ab}	62.7 ^a	60.1 ^a	57.1	5.1	T*, D***, D × T*, D × T × Day*, D × T ×
		HS	62.5	42.5 ^a	75.1 ^b	50.9 ^a	62.5		
	BET	TN	63.5	59.3 ^{bc}	69.6 ^{ab}	56.5 ^a	63.5		
		HS	52.5	68.5 ^c	72.1 ^{ab}	89.2 ^b	52.5		
Main effect of 5 time points ²									
Ileum (μmol/g)	CON	TN			93.6 ^a			36.8	D*, T*
		HS			71.5 ^a				
	BET	TN			139.3 ^b				
		HS			96.6 ^a				
Kidney (μmol/g)	CON	TN			56.4 ^a			14.3	D* , T* , D × Day *
		HS			63.3 ^a				
	BET	TN			61.6 ^a				
		HS			83.8 ^b				
Spleen (μmol/g)	CON	TN			56.9 ^a			8.5	D*, Day* , D × T × Day *
		HS			57.1 ^a				
	BET	TN			61.6 ^a				
		HS			69.6 ^b				

¹ *- $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$. Other main and interactive effects $p > 0.10$.

Differing superscripts within a column denotes significant ($p < 0.05$) differences for D $\times$ T $\times$ Day on a single day of the experiment. ² Refer to figure 7.

4. Discussion

Consistent with other experiments in the area (Marchini et al., 2011), HS compromised the small intestinal mucosa, as evidenced by reduced villous height and crypt depth. Furthermore, as in other experiments HS reduced the TER of the small

intestinal mucosa (Goo et al., 2019). The current results indicated that HS did not influence EBD concentration in the jejunum or ileum suggesting there was no discernible extravasation in the small intestine during HS. Taken together, these data support that the reduction in villous height observed was not due to damages of the villous, as observed in some other investigations into the effect of HS on the intestinal mucosa of pigs (Pearce et al., 2014). Alternatively, the reduction in crypt depth and absence of increases in EBD indicates that the reduction in villous height was likely due to reduced crypt cell proliferation and would be consistent with reductions in splanchnic blood flow observed in HS layers and other species.

Blood flow redistribution between organs in HS animals have been quantified using radioactive microspheres to determine localised changes in capillary blood flow (CBF). In layer hens it was found that HS increased CBF to the skin, comb, wattles and upper respiratory tract while CBF in the digestive and reproductive tracts reduced by approximately one half (Wolfenson et al., 1981). In baboons, HS increased skin CBF by approximately 10 % and was compensated by reductions in splanchnic and renal CBF of 35 % and 27 % respectively (Hales et al., 1979). The splanchnic bed and kidneys receive approximately 25 % and 30 % of cardiac output in resting animals. And it has been postulated that this makes them sensitive to disruptions in reductions in blood flow, which in turn can precipitate oxidative stress and hypoxic damage (Hall et al., 1999), and is a prelude to loss of intestinal barrier function, inflammatory damage and bacterial translocation (Lambert, 2009b; Lambert et al., 2002). Although, there are exceptions and under milder forms of HS the consequences are likely reduced protein synthesis and crypt cell proliferation, resulting in a gradual decline in intestinal barrier function (Goo et al., 2019), which appears to be supported by the present findings.

The organs where differences in EBD distribution due to HS were observed were the kidney and spleen. The results from these organs was quite different, with HS increasing kidney EBD concentration, but alternatively reducing EBD concentration content in the spleen. That the kidney is a site of impairment during HS has been indicated in clinical studies (Davis et al., 2017; Hifumi et al., 2018). Compared to the GIT, less is known about the etiology of HS mediated kidney damage, parallels may exist as the kidneys also receive a large proportion of cardiac output, that can be disrupted by HS (Hales et al., 1979; Sils et al., 2001). The interruption in blood flow may be more closely linked to

decompensation and heat stroke and may not be applicable under HS (Kregel et al., 1988). Elsewhere, increased incidences of nephropathy have been recorded in rural communities in rural tropical communities and this has been attributed to a warming climate, but it has also been postulated to be in part due to dehydration (Glaser et al., 2016). Although not quantified in this experiment, previously we have quantified reductions in haematocrit in broilers (Shakeri et al., 2019; Shakeri et al., 2018), and in pigs and this has been accompanied by an apparent reduction in plasma volume, even with *ad-libitum* water intake in all experiments (Zhao et al., 2018b). There are fewer reports into the effects of HS on the spleen, and the result from this experiment was primarily driven by an increase in EBD concentration in the TN BET group, and TN and HS CON groups were not significantly different. Recently it was observed in ducks that HS reduced spleen size, complement production (He et al., 2019). Additionally, a study observed that HS altered spleen lymphocyte populations (Trout and Mashaly, 1994), collectively these results may indicate that HS may compromise immune function.

Consistent with earlier experiments by our research group and others (Egbuniwe et al., 2018; Shakeri et al., 2019; Shakeri et al., 2018), BET supplementation reduced respiration rate and rectal temperature, indicating partial amelioration of the effects of HS. Furthermore, the BET improved ileal villous height, area, crypt depth and seromuscular thickness. This result is in agreement with the experiments in young broilers up to 3 weeks of age (dos Santos et al., 2019b; Kettunen et al., 2001b), but differs to the results of a study who also investigated the effects of betaine on finisher broilers (Sakomura et al., 2013). The experiment observed that the improved morphology was associated with improved resistance to coccidiosis infection, while morphology was not quantified (Kettunen et al., 2001b). A study showed that dietary betaine reduced coccidiosis intestinal damage scores (Hamidi et al., 2009). As per our earlier work, betaine has been shown to improve growth digestive function in HS broilers (Shakeri et al., 2018). Still, it should be noted in our earlier experiment no improvement in ileal TER was observed.

Although the fractional oral bioavailability of betaine has not been quantified, it has been reported to be readily available (Schhwahn et al., 2003), and in broilers has been reported to be absorbed in the jejunum (Kettunen et al., 2001b). In this experiment, betaine concentrations were increased with supplementation in the ileum, kidney and

spleen, and previously we have observed that supplementation increases plasma, liver and muscle concentrations (Shakeri et al., 2019). Elevated, ileal betaine concentrations may be an indication of betaine absorption across a wider area of the GIT than previously thought, while the contribution of arterial second-pass betaine metabolism cannot be excluded. Regardless, localised increases in betaine support a direct role for betaine in the ileum, as the ileum is not a site of betaine homocysteine methyltransferase expression (Bauchart-Thevret et al., 2009), it is unlikely that the ileum is utilising betaine. Despite lower concentrations of betaine being quantified in the HS ileum, as the ileum is not recognized as a site of betaine utilisation the most likely role for betaine in the HS broiler ileum is as an osmolyte.

The effects of betaine on EBD distribution were that increases in EBD concentration in the HS CON group were not evident with BET, possibly indicating reduced extravasation and muscle damage. This may support the role of betaine in improving growth rates and feed conversion ratio in HS broilers (Shakeri et al., 2018), improved meat tenderness and reduced drip loss (Shakeri et al., 2019). Likewise, increased EBD concentration observed in the kidney of the HS CON were not evident in the HS BET group, also indicating amelioration of HS mediated damage. As the kidney is a site of betaine homocysteine methyltransferase expression, it is possible that betaine protects the kidney by roles as a methyl donor or as an osmolyte. However, the effect of betaine in the spleen was perplexing, increasing under TN but not HS conditions. Furthermore, overall reductions (independent of HS) in EBD were observed in the jejunum and ileum, which is consistent with the improvements in intestinal morphometry. Perhaps surprisingly no effect of HS or BET was observed on liver EBD concentration. Elsewhere, HS has been observed to induce oxidative stress in the liver (Hall et al., 1999) and has been reported to be a major site of betaine distribution (Kettunen et al., 2001b).

5. Conclusions

Supplementation of betaine partially ameliorated physical symptoms of HS in finishing Ross-308 broilers and when supplemented betaine was widely distributed in the kidney, spleen and ileum. In particular, betaine benefitted the small intestine, improving ileal resistance and villous height while reducing EBD concentrations, indicating an improvement in intestinal barrier function and gut health. The results of the current study

showed that HS compromised intestinal barrier function and intestinal structure.. However, by using EBD as a marker of organ dysfunction it was apparent that a wider pattern of compromised function exists. This was evidenced in the muscle, kidney, jejunum, ileum and spleen having elevated EBD concentrations, likely reflecting underlying inflammation or damage. Importantly beneficial interactive effects with betaine were observed in muscle, jejunum, ileum and the kidneys, where elevated EBD concentrations were ameliorated by betaine. This indicates that the wide tissue distribution of betaine enables it to have multiple protective effects against HS, contributing to improved productivity and meat quality.

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CHAPTER 7

General discussion, conclusion and recommendations

7.1. General discussion

Global warming is a result of climate change, and it refers to the long-term rise of temperature on earth. It happens following a rise in the level of greenhouse gases in the atmosphere. The increased global temperature is a severe challenge (causing HS) for the poultry industry to sustain the level of production, especially for genetically modified broilers which are more sensitive to the environmental temperature changes. To sustain poultry production, research has focussed on means to improve many factors that have negative effects on poultry production including global warming.

The contemporary commercial broiler chicken, through intensive genetic selection, is among the fastest growing farmed species but are capable of tolerating a narrow range of the environmental temperature variations. In order to realize their potential the broiler chickens require adequate nutrients and need to be raised in a suitable environment. However, the environment of a broiler chicken is often a composite of interacting stressors that, in the broadest sense, can include all conditions in which the chickens lives. A chicken's success in coping with its environment therefore depends on the severity and its physiological ability to respond appropriately. The nutritional status of a chicken is crucial in determining its ability to respond to environmental insults such as HS. Betaine and antioxidants as selenium, vitamin E and polyphenols are relatively low cost additives that can play a profound role in the response to osmotic stress, boosting metabolic pathways and organs functions, and in the maintenance of the intestinal epithelium. Hence, betaine and antioxidants supplementation are beneficial in improving growth performance and well-being of farm animals (Liu et al., 2016; Shakeri et al., 2018) particularly under stressful conditions such as HS. The present thesis determined the effects cyclic HS on growth performance, gut physiology and morphology and meat quality (physiology and biochemistry) when Ross-308 broiler chickens were

supplemented with dietary betaine, selenium, vitamin E and polyphenols or combination of them with betaine.

All the experiments in the present thesis were the effects of cyclic HS when chickens were supplemented with betaine, selenium, vitamin E and polyphenols. In chapter 2, the effects of betaine alone or in combination with selenium and vitamin E were investigated on growth performance, physiological responses and gut physiology. In chapters 3 and 4, the effects of betaine, selenium, vitamin E and different doses of polyphenols on growth performance and meat quality characteristics were investigated. In chapter 5, the distribution of betaine and EBD were investigated during TN and HS conditions. The EBD was used as a marker to determine the amount of damages to tissues, and also the effects on gut morphology. The benefits of the above supplementations are confirmed in improving growth performance, immune response, health and survivability of other livestock (Liu et al., 2016). However, little information is available on the effect of betaine and antioxidants supplementations in Ross-308 broiler chickens which is a common strain in commercial farms in Australia.

7.2. Effects of betaine and antioxidants on physiological responses and blood chemicals

Ambient temperature and diets have important role to balance the acid-base in poultry. High environmental temperatures increases body temperature with a resultant increase in rectal temperature and respiration rate to dissipate heat as observed in all experiments (chapters 2, 3, 4 and 5). Betaine as osmolyte, helps maintain cellular water balance and reduces energy required for this mechanism resulting lower heat production in the body as evidenced by lowering rectal temperature and respiratory rate in chapters 2, 3, 4 and 5. Furthermore, additional effects of antioxidants (chapters 3, 4 and 5) to regulate body temperature can be related to their positive effects on the glutathione peroxidase enzyme as observed in chapter 2. These enzymes are essential for thyroid health which plays an important role in regulating and maintaining body temperature.

The acid-base physiology of broiler chickens is altered as a consequence of HS, because of the respiratory alkalosis resulting from the sharp decrease in CO₂ and the increase in pH via panting during HS, as observed in chapters 4 and 5. Respiratory alkalosis increases respiration, and elevates blood pH beyond the normal range with a concurrent reduction in arterial levels of carbon dioxide (Singh et al., 2013). There was a reduction in potassium concentration in the blood whereas sodium and chloride concentrations were unchanged (chapters 4 and 5). When the intake and excretion of other minerals is assumed constant, the sum of the acid/base altering effects of sodium and potassium intake equals the difference in excreted cations and anions plus the production of endogenous acid, plus a reduction in plasma levels of potassium and sodium due to HS (Borges et al., 2004) as observed in chapter 5 for potassium. However, potassium and sodium levels were unchanged for the betaine group as it was expected to be increased by reduced need for ion pumps in cells. Further investigations are required about potassium and sodium changes, as there are inconsistencies in the obtained results of studies that previously mentioned in the previous chapters, which could be related to the time of blood sampling. Heat stress reduces bicarbonate level as observed in chapters 4 but not 5, more calcium is needed to exert an acidic action to balance blood pH, resulting in a reduction of bicarbonate. This differences might be related to the age of chickens (5 versus 6 weeks old). While betaine individually had no effects on bicarbonate level (chapters 4 and 5), the bicarbonate level increased when chickens were supplemented with Se and VitE. It was indicated that VitE could prevent the reduction of bicarbonate in response to respiratory alkalosis (Liu et al., 2014) or could be related to the supplementation of Se that increases calcium release from the sarcoplasmic reticulum which is responsible for storing calcium ions. Haematocrit was reduced with HS (chapter 4) and betaine (chapter 5). The difference between the two experiments could be related to the age of chickens, increased circulating blood volume due to an increase in plasma volume and a lesser increase in the total red blood cells. Indeed, younger chickens (chapter 5) were less affected by HS. Also, betaine by regulating body temperature and lowering circulating of blood volume reduced haematocrit. Reduction in lactate in blood with HS and betaine (chapters 4 and 5) could be related to the duration of recovery time after exposure to HS. Furthermore, Betaine could provide sufficient energy for the body by reducing body temperature to avoid anaerobic pathways, due to lower lactic acid.

7.3. Effects of betaine and antioxidants on gut health

Heat stress reduces feed intake and the associated decreased blood flow to the digestive tract leads to a reduced supply of oxygen and nutrients to the intestine due to impaired intestinal barrier function, nutrient absorption and impaired production performance (Elphick and Mahida, 2005). The negative impacts of HS on the gut epithelium is evidenced by a reduction of villous height and changes in the membrane function of the enterocytes as it was observed in chapter 5. Heat stress alters the number and function of the enterocytes and reduces the digestive and absorptive capacity of the intestine. Besides the reduction of the intestine absorption capacity, the damage to the intestinal mucosa causes intestinal inflammation resulting in oxidative stress, which can disrupt the tight junction proteins and increase intestinal permeability, compromising the integrity of the intestinal barrier as it was indicated in chapters 2 and 5 by reduced transepithelial electrical resistance. Betaine acting as an osmolyte, an antioxidant and a methyl donor improves epithelial cells morphology and stabilizes the structure of the intestine cells as it was observed in chapter 5. However, some surprising findings reported in chapter 2 where betaine appeared to increase jejunal permeability could be related to the difference in the site of measurement or differences in the chicken's body weight in betaine and control diet groups. It has been indicated that betaine is mostly absorbed in the duodenum and has a greater effect on the duodenum because osmotic pressure in this section is highest (Chauhan et al., 2014). Furthermore, improving gut morphology can be related to glutathione peroxidase production that increased under HS, as was indicated in chapter 2, when reactive oxygen species production increases (Altan et al., 2003). The results reported in chapter 2 indicated that the decrease in glutathione peroxidase levels for chickens fed the control diet is possibly related to a reduction in glutathione peroxidase synthesis, and that this mechanism can be enhanced by dietary selenium which helps regulate the synthesis of glutathione peroxidase (Baker et al., 1993). Moreover, betaine enhances antioxidant defence by acting as a free radical scavenger to neutralize free radicals, and promotes the main antioxidant enzyme activities including glutathione peroxidase (Alirezai et al., 2012; Egbuniwe et al., 2016).

7.4. Betaine distribution in tissues and thyroid hormones

Betaine is actively accumulated in tissues where it plays a vital role as an intracellular osmolyte, regulating cell volume by countering changes in extracellular tonicity and stabilizing macromolecules against a variety of physiological perturbations (Slow et al., 2009). In addition, betaine is an important methyl donor in one-carbon metabolism, where betaine-homocysteine methyltransferase, the only known enzyme which uses betaine as a substrate, mediates the transfer of a methyl group from betaine to homocysteine, forming methionine and N,N-dimethylglycine. In this regard, increasing concentration of betaine in tissues and plasma could be a positive sign as it may indicate an improvement in animal's health and tissues. In chapters 3 and 4 and 5, tissues with higher concentration of betaine showed better health condition (ie. Less damage during HS) such as improved meat quality parameters (chapters 3 and 4) by improving lipid preoxidation and fibre structures, and reduced damage to the gut and other visceral tissues (chapter 6) as indicated by reduced EBD extravasation during HS. Furthermore, gut morphology was improved as determined by improving villous height (chapter 6). However, the reduction of EBD concentration (chapter 6) during HS in the spleen could be related to a reduction in blood flow to spleen during HS. During HS, blood flow increases to the periphery to dissipate heat resulting in a reduction in blood flow to internal organs such as spleen which works like a filter to clean and replenish blood cells, while betaine appears to work otherwise leading to a higher concentration of EBD in spleen. Furthermore, the reduction of blood flow caused by HS in the gut is one of the major reasons for oxidative damage to gut cells resulting in the reduced growth performance that was observed in the first three experiments. Also, the reduction in EBD concentration in muscle tissues of chickens consuming betaine during HS is consistent with the meat quality improvements observed in the experiments reported in chapters 3 and 4 when broiler chickens were supplemented with betaine or antioxidants.

The thyroid hormones increase basal metabolic rate, affect protein synthesis, regulate growth and regulate body temperature. Heat stress causes a reduction in plasma thyroid hormones concentrations which is possibly related to the reduction feed intake caused by exposure to a high environmental temperature observed in the study reported in chapter 4, but not the study reported in chapter 5. The reason is unknown but it could be related

to age of chickens used in the experiments (5 versus 6 weeks old). Also, the thyroid hormones responses to HS in the literature are in consistent with some studies showed increased (Elnagar et al., 2010), decreased (Bobek et al., 1980) or no alteration in concentrations (Mitchell et al., 1992). During TN, thyroid hormones are involved in metabolic activities, leading to an increase in basal metabolic rate. One consequence of this activity is to increase body heat production, which seems to result, at least in part, from increased oxygen consumption and rates of ATP hydrolysis. Therefore, reduction of thyroid hormones during HS can help the body to regulate body's temperature. As it mentioned earlier, antioxidants such as selenium play important roles in thyroid health by increasing glutathione peroxidase activity as it was found in the chapter 4.

7.5. Effects of betaine and antioxidants on performance and meat quality

As previously explained, exposure of chickens to HS generates behavioural, physiological and immunological responses, which impose detrimental consequences to their productivity by reducing feed intake, daily gain, and feed utilization. (Hu et al., 2019). When chickens are exposed to HS, their body temperature, blood circulation, and peripheral blood flow increases sharply, whereas their visceral blood flow decreases. These changes lead to limited nutrients utilization and thus reduce the poultry's production performance and feed conversion efficiency as was observed in the experiments reported in chapters 3, 4 and 5. Heat stress decreases the gastrointestinal motility and prolongs gastric emptying, which in turn results in lowered feed intake (Datta, 2001), and increase water intake under HS due to reduction the concentration of digestive enzymes in the intestinal tract, which can affect food digestion and absorption. The effects of HS are not limited to performance only and could impact product quality as well. Heat stress adversely impairs the meat quality by changing the aerobic metabolism, increasing oxidative damages as determine by increasing lipid peroxidation and fibre degradation. Oxidative damages occurs when the oxidants exceed the antioxidants, including glutathione peroxidase as it was indicated in the chapter 3. Oxidative damages initiated by reactive oxygen species which are generated by cellular metabolism as well as external sources, including feed that contain oxidized fats and lipids (Cadenas and Davies, 2000). Therefore, the addition of antioxidants such as

betaine, selenium, vitamin E and polyphenols in a diet might be a good tool to ameliorate the adverse effect of HS on the performance and the meat quality.

Betaine as a methyl donor protects cells against osmotic inactivation, and improves protein and energy metabolism. Betaine increases plasma glutathione peroxidase in chickens as it was indicated in chapter 3. It also reduces body's temperature by reducing the activity of the ion pumps that are required for osmoregulation, allowing more energy for growth as the body's temperature was determined by reduction in rectal temperature in chapters 3, 4 and 5. In this regard, betaine improves body weight and meat quality. Moreover, betaine supplementation resulted in betaine accumulation within the breast muscle and this corresponded to improved meat quality as reported in chapters 4 and 5. The results presented in chapters 3, 4 and 5 indicate that betaine at 1g/kg for 1-21 days of age had negligible effect on growth performance of broiler chickens raised under cyclic HS, although these conditions were possibly before any signs of HS. However, beyond 21 days the effects were profound. It has been suggested that the benefits of betaine supplementation in poultry is more obvious during late life and under "challenged" conditions such as infection and HS. In the present thesis, the stressfulness of the HS was apparent as the chickens increased in size and heat production during the finisher stage. During the starter phase HS may be considered less "challenging", and thus betaine supplementation had negligible benefits in the first three weeks.

The fact that no there were additional effects of selenium and vitamin E on performance may be related to a reduction in selenium availability in muscle tissues which could have been the outcome of other environmental factors. Selenium improves tissue cellular integrity through interactions with vitamin E and improves meat quality by protecting and regenerating damaged tissues during oxidative stress (Gao et al., 2010) and reducing lipid peroxidation and drip loss (Hashizawa et al., 2013) as was indicated in chapters 4 and 5. A review of the literature revealed that the effects of selenium and vitamin E on growth performance of chickens are inconsistent. The inconsistencies could be attributed to variations in dosage and duration of supplementation, form of selenium (organic versus inorganic), age and breed, number of chickens used and the rearing environment. Moreover, even in the present series of studies there were some differences between

experiments (chapters 4 and 5) with respect to effects on meat quality aspects such as drip loss and perhaps these differences were related to the different ages of chickens. In this context, the age of chickens could make a huge differences when chickens are subjected to the same temperature condition and supplementation since the responses to HS become greater as the chickens get older and larger. Despite of absence of the effects of selenium and vitamin E on growth performance of chickens, supplementation with polyphenols had positive effects on the performance and the meat quality possibly by acting as hormones and growth regulators (Majewska and Czczot, 2009), enzyme regulator (D Archivio et al., 2007) that make them a promising additive to improve the final products. Additionally, they have anti-inflammatory, immunomodulatory, and a most powerful antioxidant that prevent oxidative stress (Paszkievicz et al., 2012; Petti and Scully, 2009).

7.6. General conclusion

The present thesis findings suggest that rectal temperature and respiration rate are potential measurements to evaluate the amount of physiological stress in Ross-308 broiler chickens whereas thiobarbituric acid reactive substances, myofibril fragmentation index are measurements to evaluate the products quality as well as drip loss. Furthermore, these studies indicated that glutathione peroxidase activity has a defensive function that increases when ROS level increases under HS to protect tissues against oxidative damages due to improve growth performance and reduction in undesired effects on tissues such as muscle (Altan et al., 2003). Therefore, supplementing a diet with betaine, selenium, vitamin E or polyphenols could partially reduce oxidative damages resulting in improvement of the meat quality. Therefore, the additives used in these experiments can be important tools to be utilised in commercial practices to increase the final amount and quality of products where exposing chickens to a short-term HS as is very common practice in free range farms and tropical areas.

7.7. General recommendation

The results of experiments recommended that 1 g/kg supplement betaine from an early age is an effective strategy to improve growth performance and meat quality when broiler chickens are being exposed to cyclic HS. Betaine distribution was increased in tissues such as ileum, kidney, spleen and muscle when chickens were supplemented by 1 g/kg

betaine and this attributed to lowering damages to tissues as measured by Evans Blue Dye. Furthermore, it improved breast muscle fibre diameter and villous structure of ileum which can attribute to healthier gut and better muscle developments. However, the positive effects were more obvious under TN condition. There were additional effects of supplementations of selenium at dose of 0.6 mg/kg and vitamin E at dose of 250 IU/kg on the product quality as reduced lipid peroxidation and increased myofibril fragment index which are indicators of lower oxidation in the muscles leading to better product quality. Polyphenols at dose of up to 10 g/kg had better final performance and reduced oxidation in meat as lowered lipid peroxidation. However, at an early age, it seemed polyphenols are ineffective while effects were more obvious at the final stage. Therefore, supplementing chickens with higher doses of betaine and introducing polyphenols midway through the growth cycle merited as a strategy, although this requires further investigations.

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