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Amelioration of the effects of heat stress with antioxidant enzymes superoxide dismutase and catalase in broiler chickens

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Abstract:

Heat stress has become a growing concern in the livestock industry. Elevated temperatures, coupled with rising global animal production is severely impacting animal health, welfare and overall production capacity. Heat stress is associated with a myriad of physiological, metabolic and gastrointestinal changes and has been shown to induce oxidative stress, a condition in which harmful free radicals are produced in excess. Oxidative stress is particularly damaging to macromolecules and causes widespread cell and tissue damage. Emerging research suggests supplementation with antioxidants, such as Vitamin C and Vitamin E, can partially ameliorate the damaging effects of heat stress. Despite considerable research on antioxidant supplementation in livestock, there has been little investigation into the therapeutic effects of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Therefore, the aims were to determine the effect of both antioxidants and antioxidant enzymes on growth parameters, meat quality, gastrointestinal health and markers of oxidative stress in heat stressed broiler chickens.

Declaration

This is to certify that

i. The thesis comprises only my original work towards the master's except where indicated in the preface; and

ii. Due acknowledgement has been made in the text to all other material used; and

iii. The thesis is fewer than 50000 words in length, exclusive of tables, maps, bibliographies and appendices.

_____

Olivia Artaiz

August 2022

Preface

The work towards this thesis was carried out in collaboration with DSM Nutritional Products. DSM contributed to the work as they were involved in the project planning, development of the feed supplements and the plasma analysis for Vitamin C levels. No portion of this thesis was carried out prior to enrolment in the degree and there is no work in this thesis that has been submitted for other qualifications. Chapter 2 and 3 contain unpublished material that has not yet been submitted for publication. Contributors for these publications in preparation include Jeremy Cottrell, Carrie Walk, Frank Dunshea, Linda Fothergill, Weicheng Zhao, Huu Hieu Le, Yasir Iqbal and Katherine Papastratos. Financial support for this degree includes funding from DSM, Melbourne Research Scholarship (fee offset) and Studentship (stipend) from the University of Melbourne.

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Abbreviations

ADFI: average daily feed intake

ADG: average daily gain

CLD: claudin

CON: control CAT: catalase

d: day

FCR: feed conversion ratio

FI: feed intake

GPx: glutathione peroxidase

h: hour

HSP: heat shock protein

FITC: fluorescein isothiocyanate

g: gram

GIT: gastrointestinal tract

GSH: glutathione

GSSG: glutathione disulfide

HS: heat stress

Kg: kilogram

MDA: malondialdehyde

MFI: myofibrillar fragmentation index

NN: naked neck

OX: Oxicare

OCLN: occludin

PPLA: post pellet liquid application

SOD: superoxide dismutase

TBARS: thiobarbituric acid

TER: transepithelial resistance

TJP: tight junction protein

TN: thermoneutral

w: week WB: wooden breast

WBSF: Warner-Bratzler shear force

WS: white stripping

ZO: zonula occludin

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1 Chapter 1: Literature Review

1.1 Introduction

Global meat consumption increased 58% between 1998 to 2018. This change is largely explained by the worldwide increase in white meat production and consumption such as Brazil, India, the European Union and the United States (Whitnall and Pitts, 2019; USDA, 2021). Overall trends in global meat production reflect this, with South America and Asia representing regions of prominent growth, and pigs and poultry serving as the leading production animals worldwide (Ritchie and Roser, 2017). By 2030, it is expected poultry will make up 41% of meat production, followed by pork (34%), beef (20%) and lamb (5%) (OECD et al., 2021). The trend of increased poultry meat consumption is largely due to its lower cost and perception as a convenient, versatile and healthy protein compared to other meat types (Kennedy et al., 2004; OECD et al., 2021).

While meat production in tropical and subtropical regions is increasing, there has also been an increase in global temperatures and in the frequency and severity of heat waves. Perkins-Kirkpatrick and Gibson (2017) speculate regional heat waves will change in intensity, frequency and duration as a function of degree of global warming. Furthermore, these changes are expected to be most damaging in tropical regions. This combination of increased meat production and rising temperatures has triggered growing concern regarding the impact of high ambient temperatures on livestock health and production yield. In fact, Baumgard et al. (2012) identifies heat stress as the primary factor impeding access by people to high-quality protein and achieving global food security. In addition to issues of food security, decreases in production capacity in the livestock industry cause severe economic losses. In 2003, industry loses due to heat stress were estimated to be $2.4 billion (St-Pierre et al., 2003). Adjusting for inflation, this would equate to approximately $3.6 billion in 2022.

1.2 Heat Stress

Heat stress (HS) is a physiological state when the body can no longer maintain thermal homeostasis, meaning that the amount of heat produced exceeds the amount of heat dissipated (Belhadj Slimen et al., 2016). Heat stress can be acute, involving a short and rapid increase in temperature, or chronic,

involving increased temperatures over days or weeks. Chronic HS can be further classified as constant or cyclic. Constant HS (referred to as “chronic”) refers to sustained elevated heat exposure over a period of time whereas cyclic HS (referred to as “cyclic”) refers to a period of heat exposure followed by thermoneutral (TN) conditions (Akbarian et al., 2016). Although the exact metabolic effects of HS can vary depending on the intensity and duration of HS , it is clear HS negatively impacts animal health and performance and may result in increased mortality rates (St-Pierre et al., 2003; Quinteiro-Filho et al., 2010).

1.3 Thermoregulation

Thermoregulation is the balance between the mechanisms of heat production and heat loss, which helps maintain homeostasis. Animals maintain homeostasis through thermoregulatory processes. There are two main thermoregulatory process that are involved in managing high ambient temperatures - radiant and evaporative heat loss (Renaudeau et al., 2012). Radiant heat loss occurs as blood is redirected from the core to the periphery, allowing excess heat to be released to the external environment, while evaporative heat loss encourages cooling through panting and sweating.

In hyperthermic laying hens, Wolfenson et al. (1981) found increased capillary blood flow to the back, breast, comb, wattle, tongue, larynx and trachea. Simultaneously, there was a decrease in blood flow to internal organs, such as the gastrointestinal tract and reproductive organs. This diversion of blood to the extremities promotes heat loss from the blood through the skin. As chickens maintain a core body temperature between 41°C and 42°C, heat is released due to the temperature gradient between the bird and the environment. If the ambient temperatures exceeded that of the core temperature of the bird, radiant heat loss would not be possible, leading to thermodynamic dysregulation and cell injury (Sato et al., 2019) This once again highlights the dangers of increasingly hotter environments to animal health.

Pigs and poultry are particularly susceptible to heat stress as they lack functional sweat glands and thus rely heavily on panting, drooling (pigs) and behavioral changes that encourage heat loss (Weaver, 2002; Renaudeau et al., 2006; Cottrell et al., 2015; Kerr, 2015; Gourdine et al., 2021). In response to acute or chronic HS, animals will increase respiration and begin to pant to encourage

evaporative heat loss. In broilers (types of chickens used for meat production) exposed to cyclic HS, 33°C for 8 hours (h) followed by 25°C for 16 h for 42 days (33°C 8h/25°C 16h; 42d), respiration rates more than doubled when compared to those exposed to TN conditions (Shakeri et al., 2018b). This pattern is also reflected in pigs (46 ± 6 kg body weight) with respiration rates increasing over 50% in pigs exposed to 24h of HS (35°C) compared to TN controls (Pearce et al., 2012).

While radiant and evaporative heat loss assist in heat dissipation, they may also have negative consequences if overused. Redistribution of blood flow to the periphery can create hypoxic conditions in splanchnic tissue, which can ultimately lead to cell injury and oxidative damage (Hall et al., 1999). During evaporative heat loss, increased respiration triggers changes in the respiratory exchange rate and acid-base balance. In particular, there is an initial decrease in acid (carbon dioxide) causing blood pH to rise, followed by a decrease in base (bicarbonate) in an attempt to maintain homeostasis (Hopkins et al., 2022). However, when the internal buffering system is inadequate in returning pH to normal physiological levels, respiratory alkalosis can occur. Respiratory alkalosis will be described in depth in Section 1.5. Despite these negative consequences associated with thermoregulation, animals prioritize heat dissipation at the expense of efficient production (Cottrell et al., 2015).

1.4 Susceptibility to heat stress

There are many factors that contribute to an animals' susceptibility to HS including genetics, production stage, presence and function of sweat glands and hair, skin or feather covering (Bernabucci et al., 2010). Livestock are often selectively bred for high-production efficiency, such as increased growth rates, improved meat quality and high product yield. Between 2001 and 2017, the portion of breast muscle to carcass weight of Ross-308 broilers increased from 15.8% to 22% (Soglia et al., 2019). This increase in breast proportion meant birds reached their kill weight (approx. 2.2 kg) 9 days quicker in 2017 than 2001. However, selecting for performance may result in undesirable side effects that leave livestock more vulnerable to stressors. As livestock weight increases, so does metabolic heat production (Nascimento et al., 2017a). Therefore, rapid growth rates are more detrimental during periods of high ambient temperature as birds bred for rapid growth will have a harder time dissipating heat and maintaining homeostasis than their slower-growing counterparts.

Numerous studies have investigated the correlation between ambient temperature and thermal homeostasis in livestock. The difficulty in maintaining a homeothermic state in high ambient temperatures may explain why Settar et al. (1999) found a depression in broiler growth rates during hotter months compared to during cooler months. Likewise, Lu et al. (2007b) found a similar disruptions to growth rates during chronic HS (34°C; 3 weeks (w)), but these effects were breed specific. Commercial fast-growing broiler (Arbor Acres) had significantly higher feed intake, body weight gain and final body weight compared to slow growing breeds (Beijing You chicken), reflecting their respective growth phenotypes. However, when exposed to chronic heat stress (34°C; 3w), AA chickens had decreased body weight and lower breast proportion, while slow-growing birds were not affected. Collectively, these data suggest that while increased growth rates are advantageous for production output, the rise in metabolic heat production increases susceptibility to HS.

While some birds are selected for their growth rates and meat yield, others are selected for their thermotolerance. For example, birds with the frizzle gene have curled feathers that reduce insulation while birds with naked neck (NN) gene display reduced feather cover by 20-40 %. Both genotypes lead to improved radiant heat loss (Wasti et al., 2020). Interestingly, birds carrying both NN genes (NN +/+) also displayed improved heat loss performance compared to NN -/- birds under TN conditions (23°C). Growth performance was also improved under HS conditions (32°C), indicating that the NN gene has beneficial properties across at both normal and high temperature environments (Cahaner et al., 1993). Heterozygous NN birds also present a heat tolerant phenotype, with NN +/- birds having lower body temperature, increased feed efficiency and superior performance in hot climates. However, in moderate climates only NN +/- males showed improved performance and in cool climates NN +/- actually had lower feed efficiency (Yalcin et al., 1997). These differences suggest the interplay of genetics, temperature and sex on thermotolerance.

Livestock appear to be most susceptible to HS during late-stage production compared to initial-stage production. A meta-analysis by Andretta et al. (2021) found HS to reduce only average daily gain (ADG) in broiler chickens from hatch to 21 days of age, whereas both ADG and average daily feed intake (ADFI) was reduced from 21 to 42 days of age. Although a reduction in ADG will lead to lighter birds, the additional reduction in ADFI will lead to less nutrient availability and utilization and further

impact production outputs. Similarly, finishing pigs (54-79 kg) reared under tropical conditions (22-32°C; 3w) experienced the largest decline in performance compared to young (8- 25 kg) and growing (29-50 kg) pigs reared under similar conditions (Christon, 1988). Furthermore, sows exposed to cyclic heat stress (25°C 16h/ 32°C 8h) during lactation saw greater changes in rectal temperature (RT), respiration rate (RR), feed intake and metabolic hormone levels compared to sows exposed to heat stress during gestation or breeding (Williams et al., 2013). While these studies highlight the dangers of HS during vulnerable stages of production, they also highlight times when animals are most susceptible to HS and could benefit from targeted interventions.

1.5 Effect of heat stress on physiology

Thermoregulation efficiency is reflected in body temperature, as RT rises when the heat produced exceeds the heat dissipated (Serviento et al., 2020). Pearce et al. (2013a) reported significantly higher RT in pigs exposed to 1, 3 or 7 days of HS (35°C) compared to TN controls. A similar response is documented in poultry across acute and chronic HS models. Altan et al. (2003) saw a significant rise in rectal temperature in birds 36-37 days old after acute heat exposure (38°C; 3h), with rectal temperatures rising over 1°C. Lin et al. (2006) also saw a dramatic increase in RT after 3h of heat exposure (32°C), which remained elevated over 6h of heat exposure. Likewise, Azad et al. (2010) found cyclic HS (32°C 8h/24°C 16h; 2w) significantly increased RT compared with TN controls, while chronic HS (32°C; 2w) significantly increased RT compared to TN controls and cyclic HS groups. Collectively, these data support the idea that RT represents an early and lasting indicator of HS.

As mentioned previously in Section 1.4, HS often increases respiration rate (RR) to encourage evaporative cooling. Alterations of respiratory rate significantly impact metabolic and respiratory pH. Increased respiration leads to increased carbon dioxide (CO_2) exhalation and a subsequent fall in partial pressure CO_2 (pCO_2) in the blood. With less CO_2 available for carbonic acid formation, pH increases, ultimately leading to respiratory alkalosis (Fausnacht et al., 2021). However, most animals employ an internal buffering system to help maintain acid- base homeostasis which relies on the following reaction (Hamilton et al., 2017):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

In response to the rise in pH, the renal system increases bicarbonate (HCO_3^-) excretion. This in turn reduces the amount of base available and reduces pH (Berend et al., 2014). Respiratory alkalosis is one of four acid-base disorders, and often occurs in conjunction with metabolic acidosis due to an increase acid or a reduction in base, such as HCO_3^- (Robertson, 1989).

Teeter et al. (1985) found chronic HS (32°C; 3w) increases blood pH while simultaneously reducing CO_2 and HCO_3^- levels in broilers compared to TN controls. Interestingly, HS birds that were actively panting had significantly higher pH than both HS non-panting birds and TN controls, while there was no difference in CO_2 and HCO_3^- between HS panting and non-panting birds. This study is significant as it highlights the variability in acid-base balance depending on respiration rate and the speed at which pH can fluctuate. Another study investigated the differences in blood chemistry parameters across acute HS (35°C; 4-6h), or cyclic HS (35°C 7h/30°C 17h; 2w or 4w) (Barrett et al., 2019). As expected, acute HS increased pH and reduced CO_2 and HCO_3^-. This pattern was also seen after 2 weeks of cyclic HS. However, after 4 weeks of cyclic HS, levels were approaching near pre-HS values indicating an adaptive response as HS persists. Studying pigs, Cottrell et al. (2020) reported reduced pCO_2 and HCO_3^- and a near significant ($p = 0.087$) increase in pH in response to cyclic HS (35°C 8h/28°C 16h; 7d), indicating respiratory alkalosis. This was further supported by the reduction of base excess in the blood, indicating lower circulating HCO_3^-. However, not all studies have reported respiratory alkalosis in response to HS. Lin et al. (2006) found blood pH, pCO_2 and partial pressure oxygen (pO_2) remained unchanged during acute HS, (32°C; 6h) and Shakeri et al. (2020b) reported similar findings in response to cyclic HS (33°C 8h/25°C 16h; 4w). Together, these findings indicate that pH is a highly labile variable with respect to HS. The variability in response of blood gas and chemical parameters is likely due to the constant compensatory mechanisms to maintain homeostasis (Ahmad and Sarwar, 2006).

1.6 Effect of Heat Stress on Growth Performance

Heat stress reduces production capacity by reducing feed intake and body weight gain, altering metabolism and negatively impacting meat quality. Several studies report reduced feed intake in response to acute and chronic heat stress. Pearce et al. (2012) reported a 53% decrease in feed intake and reduced body weight in growing pigs subject to acute HS (35°C; 24h) compared to TN controls. Shakeri et al. (2018b) also found cyclic HS (33°C 8h/25°C 16h; 42d) to reduce ADFI, ADG and increased feed conversion ratio (FCR- ratio of feed consumed to weight gained) in broilers between 21 and 42 days of age. However, changes in performance are not always consistent across studies. In a similar study, Shakeri et al. (2019a) found cyclic HS (33°C 8h/25°C 16h; 5w) to only significantly reduce ADFI and ADG in broilers but had no effect on FCR, which is calculated by the weight of feed consumed divided by the weight gained by the animal. Feed conversion ratio can be helpful in determining the effect of HS on animal efficiency, as it gives insight into whether animals are merely eating and gaining less or if the amount consumed is processed differently. Taken together, these data indicate that the effects of HS on animal performance produce lighter and less efficient animals (Wasti et al., 2021).

Pair-fed studies are critical in determining the effects of HS in isolation from reduced feed intake. Several studies show HS increases adiposity and alters lipid mobilization, regardless of feed intake, suggesting HS alters metabolism. In support of this observation, pigs exposed to heat stress had increased back fat thickness, proportion of flare fat (visceral fat) and lipid content of back fat compared to pair fed TN (PFTN) controls (Kouba et al., 2001; Serviento et al., 2020). Pigs reared in tropical (21°C-32°C) compared to TN (17°C-21°C) had higher levels of plasma free fatty acids, triglycerides and cholesterol and decreased thyroid hormones, all suggesting changed metabolic function (Christon, 1988). A similar pattern is observed in poultry, with heat stressed (34°C; 3w) birds having increased abdominal and intramuscular fat compared to PFTN controls (Lu et al., 2007b). These studies are significant as they support the position that the HS alters postabsorptive metabolism, with HS animals favoring glucose and protein metabolism over lipid metabolism. Altered metabolism may, in turn, lead to decreased carcass value and challenges for the livestock industry.

1.7 Effect of Heat Stress on Meat Quality

Chronic heat stress has also been shown to negatively affect meat quality, including pH, tenderness, color, drip and cook loss (Gonzalez-Rivas et al., 2020). Santos et al. (1997) found acute HS (35°C; 2-3h) pre-slaughter led to greater instances of pale, soft exudative (PSE) meat compared to pigs held at normal ambient temperatures. The breast muscle of broilers exposed to cyclic HS (36°C/6h, 23°C/18h; 2w) showed decreased initial and final pH as well as increased shear force values compared to the breast muscle of TN controls (Awad et al., 2020). Reduced pH levels indicate an increase rate of glycolysis and subsequent accumulation of lactic acid, while higher shear force indicates increased toughness. Myofibrillar fragmentation is also an indicator of meat toughness and has also been shown to be reduced in response to cyclic HS (33°C 8h/25°C 16h; 3w) (Shakeri et al., 2019a). Lower myofibrillar fragmentation may be due to a lack of proteolysis or increased protein crosslinking in the meat. However, both causes indicate a reduction of the naturally occurring meat tenderization process which ultimately leads to tougher meat. pH and tenderness are key indicators of customer satisfaction, thus changes here may lead to unfavorable meat quality. Moeller et al. (2010) investigated consumer perceptions of pork eating quality against pH and Warner-Bratzler Shear Force (WBSF), which measures the amount of force needed to cut through a sample of meat. They found that higher pH and lower WBSF values were associated with more positive consumer responses, indicating that changes in these two parameters can negatively impact eating quality and therefore are directly related to profitability.

The changes in meat quality are often dictated by the intensity and duration of HS. Zhang et al. (2012) investigated the differing effects of cyclic (36°C 6h/23°C 18h; 2w) and chronic HS (34°C; 2w) on meat quality in broilers. Broilers exposed to cyclic HS had higher fat deposition and lower protein content in breast muscle, compared to broilers exposed to cyclic heat stress. There was also increased lightness (L*) and shear force and decreased initial pH (pH_i) and final pH (pH_u). Broilers exposed to chronic HS expressed similar changes in breast muscle color, pH and shear force as chickens exposed to cyclic heat stress. However, they also had increased moisture content and increased cook loss. This observation that the intensity and duration of HS plays a role in meat quality was reinforced by Goo et al. (2019b), who found little to no negative effect of heat stress on meat quality measures in broilers

kept at a constant 27.8°C for 2 weeks. While 27.8°C is outside of a broilers TN zone, it appears to be insufficient to cause changes in meat quality.

Broiler myopathies, such as wooden breast (WB) and white stripping (WS), are becoming increasingly common and are suspected to be related to the fast growth rates of modern-day broilers (Kuttappan et al., 2012). In fact, both Xing et al. (2020) and Estevez and Petracci (2019) found size and weight of the breast fillet to be positively correlated with the occurrence of myopathies. Wooden breast is characterized by a pale, hardened breast while white stripping is characterized by white striations along the muscle fibers and are likely a result of rapid growth and increasingly large breast muscles of broilers (Petracci et al., 2019). WB and WS are associated with hypoxia, increased ROS production, oxidative stress and increased lipid peroxidation, all of which are indicators of HS (Kuttappan et al., 2016). Thus, HS may exacerbate the severity of broiler myopathies. The first few days post hatch are critical for the proliferation of satellite cells (SC) which ultimately determines the muscle structure of grown birds (Halevy, 2020). HS here can alter SC proliferation and number, often causing a reduction of SC but an increase in lipogenesis. For example, broiler chicks raised under HS conditions during the first 13 days of life (39°C; d0-4, 37°C; d4-6, 34°C; d6-8, 33; d8-11) saw an increase in fat and collogen accumulation at day 35 compared to chicks raised under TN condition (Patael et al., 2019). As a buildup of lipid and collogen is often a feature of myopathies, including WS, this study suggests a link between early exposure to HS and muscle damage later in life (Kuttappan et al., 2012; Halevy, 2020; Velleman, 2020)

Despite these correlations, some studies have actually reported a reduction in WB and WS in response to HS applied during the grower and finisher phases. Malila et al. (2021) saw a decrease in occurrence of WS and WB (91% vs. 63%, 62% vs. 59% respectively) in broilers exposed to cyclic HS (35 °C 6h, 26°C 18h; 3w) compared to TN controls. This may be due to the depressed growth rates seen during HS, as the development of the breast muscle may not be as rapid and therefore a lower chance to develop myopathies. Aslam et al. (2021) also reported a decrease in WS in broilers exposed to chronic HS (32°C; 3w). Interestingly, despite being a decrease in the number of cases of WS, there was an increase in *severity* of WS in HS broilers when compared to TN controls. Collectively, these studies suggest ambient temperatures do play a role in the development of broiler myopathies. However,

chronology matters: when the birds are exposed to HS determines how it can change muscle structure. HS applied immediately post hatch tends to increase the occurrence of myopathies, while HS applied during the growing and finishing stages may be more related to the severity of myopathies. Regardless of WS and WB development, they severely downgrade meat quality and because the breast muscle is the most profitable portion of the chicken, myopathies here result in severe economic losses (Zanetti et al., 2018; Soglia et al., 2019).

Collectively, the studies in the above sections indicate the widespread effects of heat stress on feed intake, metabolism and meat quality. Reducing feed intake is a compensatory mechanism to reduce metabolic heat load but can lead to reduced feed efficiency and reduced weight gain. Meat becomes tougher, changes color and tends to display other undesirable characteristics such as PSE. The resulting carcass is lighter and fattier with reduced meat proportions, leading to decreased production value and economic losses for the meat industry (Baumgard et al., 2012; Pearce et al., 2013a; Shakeri et al., 2019a).

1.8 Effects of Heat Stress on Gastrointestinal Health and Integrity

As mentioned above, HS often leads to visible changes to livestock such as reduced body weight, lower meat yield and poor meat quality. However, HS can also lead to less apparent, internal changes including impaired gut health and function. The gastrointestinal tract (GIT) is responsible for selective nutrient digestion and absorption and plays a key role in the host immune response. The intestinal barrier in particular controls the passage of ions, nutrients, pathogens and harmful molecules between the lumen and blood stream. It is comprised of a single layer of epithelial cells connected by tight junction proteins, which control paracellular transport.

Intestinal integrity is sensitive to external stressors such as HS. As previously mentioned, The reduction in splanchnic blood flow brought on by HS may lead to intestinal hypoxia and reduced nutrient availability. This can then trigger gastrointestinal dysfunction, whether through modulation of gut morphology, altered gene expression or changes in gut permeability and resistance (Lian et al., 2020). The following sections will discuss the effect of HS on these three components of the GIT.

Changes in villus and crypt architecture are indicative of gut health. Using an in vitro model of HS and using tissues from rat small intestine, Lambert et al. (2002) showed sloughing of epithelial cells and damage to microvillus structure across the jejunum and ileum after 30 minutes of heat exposure (41.5°C-42.5°C). After 60 minutes, there was a complete lifting of the epithelial layer off the villus which was accompanied by increased permeability. This study is significant as it demonstrates that even short and severe exposure to heat stress can destroy intestinal epithelial cells and directly impact intestinal permeability. In vivo studies in pigs and poultry also demonstrated HS can impact gut morphology by altering villus height (Garriga et al., 2006; Pearce et al., 2013b; Shakeri et al., 2020d; Wasti et al., 2021), villus area (Wasti et al., 2021), crypt depth (Shakeri et al., 2020d) and villus height: crypt depth ratio (Pearce et al., 2013b). However, in some studies, no morphological changes were detected, indicating the variability in response to differing levels of HS (Quinteiro-Filho et al., 2010).

It should be noted that changes in gut morphology may in part be due to decreased feed intake. Thermoneutral pigs fed a restricted diet expressed altered gut morphology when compared to pigs fed *ad libitum* (Pearce et al., 2015). Interestingly, these changes mirrored those seen during acute HS (37°C; 12h), which suggests the importance of considering feed intake when investigating changes in gut structure. Regardless of the causes of morphological changes, these changes are often indicative of villus atrophy and epithelial cell damage. Structural alterations to the GIT have serious effects on digestion and absorption capacity; intact villi allow for maximized absorption through increased epithelial surface area while reduced surface area will hinder absorption (Aviello and Knaus, 2017; Celi et al., 2017).

Another way HS causes gastrointestinal damage is through changes in expression, abundance or distribution of key proteins that manage paracellular or intercellular transport (Aviello and Knaus, 2018). These include tight junction proteins (TJPs) and mucins (MUC), both of which contribute to GI barrier integrity. Tight junction proteins, including occludens (OCLN), zonula occludens (ZO), and claudins (CLDN), control paracellular passage of molecules via tight junctions. Changes here can lead to altered gut permeability and changes in absorption, section and immunological functions of this tissue. Growing pigs exposed to acute HS (35°C; 12h) saw an upregulation of CLDN-3 and OCLN in the ileum but no change in CLDN-1 (Pearce et al., 2013b). Broilers exposed to cyclic HS (33°C 8h/22°C

16h; 3w) had a different response and saw a downregulation in mRNA expression of OCLN, ZO-1, CLDN-1 and CLDN-4 (Alhotan et al., 2021). Taken together, these studies highlight the differences in genetic response to acute or chronic HS. The immediate upregulation of TJPs in response to acute HS suggests compensatory and protective response to gastrointestinal damage. However, we see a reduction in TJP expression over time, indicating a breakdown of barrier integrity. A similar effect is seen in mucin expression. Mucin plays a protective role in the gut and forms the mucus layer that protects the body from luminal pathogens and toxins (Aviello and Knaus, 2018). Acute HS (35°C; 12h) tends to increase MUC2 protein abundance (Pearce et al., 2015) while cyclic HS (33°C 10h/22°C 12h; 3w) tends to decrease it (Zhang et al., 2017). In some cases, no changes in TJP or MUC are reported in response to HS, which shoes the variation across studies (Goo et al., 2019b; Wasti et al., 2021).

Intestinal permeability and transepithelial resistance (TER) are also markers of intestinal integrity and are typically measured ex vivo in isolated gut tissue. Permeability is determined by the passage of a fluorescein isothiocyanate (FITC) labelled dextran marker across isolated tissue, while TER is determined by measuring electrical resistance across the tissue. Permeability can also be determined in vivo by orally administering FITC-dextran and measuring the subsequent serum levels. Several studies have utilized these methods to demonstrate the negative effects of acute and chronic HS by decreasing intestinal integrity in pigs and poultry.

Pearce et al. (2015) found acute HS (35°C; 12h) to increase ileal permeability and decrease ileal TER in growing pigs. Similarly, Pearce et al. (2012) and Pearce et al. (2013b) reported reduced TER in the ileum and colon in heat stressed growing pigs (35°C; 24h). In a slightly longer study, Liu et al. (2016b) found 48h of cyclic HS (35°C 8h/28°C 16h) increases FITC-dextran flux and reduced TER in the small intestine (ileum and jejunum samples pooled). In poultry, chronic and cyclic HS (HS (33°C; 4w, 33°C 10h/22°C 12h; 3w respectively) have been shown to increase gut permeability and increase FITC-dextran serum levels after being given an oral gavage of the marker (Zhang et al., 2017; Kikusato et al., 2021). Shakeri et al. (2018b) reported decreased jejunum TER in broilers exposed to cyclic HS (33°C 8h/25°C 16h; 3w) but no effect of HS on jejunum permeability. In a related study, looking at the effects of cyclic HS (33°C 8h/25°C 16h) over 3, 7 or 10 days Shakeri et al. (2020d) found HS to

significantly reduce ileum TER but only at day 10. The lack of effect of HS on days 3 and 7 suggests the duration of HS can impact the severity of intestinal injury.

Increased permeability and reduced TER compromise overall intestinal integrity and cause the GIT to become "leaky." A leaky gut may subsequently lead to the passage of microbial products and toxins into the blood stream. In fact, reduced integrity in the ileum and colon due to acute HS was associated with increased serum endotoxin concentrations (Pearce et al., 2012; Pearce et al., 2013b). Endotoxins, often referred to as lipopolysaccharides (LPS), trigger inflammation and encourage reactive oxygen species formation, which ultimately leads to compromised performance as energy and nutrients are diverted to immune requirements instead of growth (Mani et al., 2012; Aviello and Knaus, 2017).

It is clear HS decreases gastrointestinal function and integrity, whether it is through physically damaging the structure, altering gene expression, increasing permeability or decreasing transepithelial resistance. These widespread changes suggest the GIT is highly susceptible to HS and should be considered when developing heat management strategies. However, it is important to consider other factors, such as the severity and duration of HS and reduced feed intake, when determining these effects.

1.9 Effect of Heat Stress on the Immune System

Several studies investigated the effects of HS on the immune response, often reporting immunosuppressing effects (Lara and Rostagno, 2013). In poultry, HS decreases the relative weight of lymphatic organs including the bursa of Fabricius, spleen, and thymus (Bartlett and Smith, 2003; Liu et al., 2014; Hirakawa et al., 2020). In pigs, chronic HS (30°C; 3w) reduced liver weight and upregulated several proteins involved in stress and immune response including heat shock proteins (HSP) (Cui et al., 2016). HSPs are stress-induced proteins that act as molecular chaperones and aid in protein folding and repair and can induce cell death. Rising levels of HSPs often signal an increased stress response, such as during HS (Sonna et al., 2002). In broilers, cyclic HS (36°C 6h/24°C 18h; 3w, 32-33°C 8h/22°C 16h; 3w) was shown to upregulate HSP70 in the liver (Roushdy et al., 2018) and increase mRNA abundance of HSP70 and HSP90 in breast muscle (Chen et al., 2021). Pigs exposed to acute HS (35°C; 24h) also had upregulated HSPs in the ileum and colon compared to TN controls (52% and 24% increase

respectively) (Pearce et al., 2012). Regardless of intensity and duration of heat stress and tissue, these studies highlight the immediate and long-lasting effects of HS on HSP expression.

Not only does HS alter lymphatic organ weight and gene expression, but it can also trigger extensive immunomodulatory effects, leading to weakened adaptive and innate immune responses. Cyclic HS has been shown to decrease antibodies immunoglobulin Y (IgY), IgM (Bartlett and Smith, 2003; Awad et al., 2020), anti-inflammatory cytokines (Alhotan et al., 2021), and increase acute phase proteins (Awad et al., 2020) and proinflammatory cytokines (Alhotan et al., 2021). However, changes in immune markers are inconsistent across studies. In pigs, Montilla et al. (2014) saw inconsistent changes in inflammatory signaling molecules across glycolytic and oxidative skeletal muscles (IκB-α, NF-κβ, p-NF- κβ, Il-6, TNFα), with the majority remaining unchanged over 1 and 3 days of HS. Liu et al. (2016b) also reported no changes in inflammatory marker abundance in pigs exposed to 2 days of cyclic HS (38°C 8h /28°C 16h). Pearce et al. (2013a) even saw a downregulation of proinflammatory cytokines IL-8, IL-1β and TNF-α in pigs after acute heat exposure (35°C; 12h). The difference in immune response across acute and chronic HS suggests the longer the HS, the more likely it is to trigger inflammation and suppress the immune system. This will negatively impact animal health and welfare and even increase risk to secondary infections. In fact, Tang et al. (2021) explored how acute HS (41°C; 12h) alters the intestinal immune response to *Escherichia coli.* Chickens subjected to heat stress and then exposed to *E. coli* infection saw increased bacterial load in the cecum and decreased IgA antibody levels compared to chickens exposed solely to *E. coli.* Production of proinflammatory cytokines (IL-6, TNF-a, and IL- 1b) also increased in the small intestine, but these results varied across the jejunum, ileum and duodenum. In a longer study, Quinteiro-Filho et al. (2012) found broilers infected with *Salmonella* Enteritidis and exposed to chronic HS (31°C; 6 days) to have more higher *Salmonella* counts and more severe enteritis than *Salmonella*-infected broilers kept in TN conditions. These studies highlight how HS not only alters intestinal immune parameters but can increase susceptibility to infection and cause a myriad of negative effects. This is of particular concern as production animals are often kept in proximity which increases the likelihood of transmission of pathogens and disease.

1.10 Heat Stress and Oxidative Stress

As discussed in the previous sections, HS causes a myriad of negative effects across performance, physiological, gastrointestinal and immune parameters. However, there is yet another way HS negatively impacts livestock: oxidative stress. Briefly, oxidative stress occurs when there is an imbalance between oxidants and antioxidants in the body. Oxidants induce oxidative stress through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive oxygen and reactive nitrogen species come in radical or non-radical forms. The main difference between them is that radical species contain one or more unpaired electrons in their valence shell and non-radicals do not. While ROS and RNS are critical to cellular processes at low concentrations, they are highly unstable and can trigger a cascade of reactions leading to lipid, protein, and DNA damage. During periods of oxidative stress, ROS and RNS are increase, thus leading to widespread injury (Aviello and Knaus, 2017)

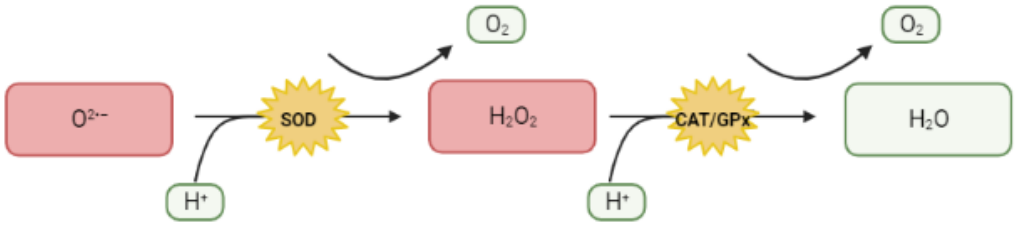
1.10.1 Reactive Oxygen Species and the Antioxidant Defense System

ROS and RNS are derived from endogenous and exogenous sources. Endogenous ROS are produced by the electron transport chain (ETC), NADPH oxidase, and xanthine oxidase while RNS are produced through nitric oxide synthase (Shaji et al., 2015; Aviello and Knaus, 2017). During cellular respiration, electrons are shuttled through the four respiratory enzyme complexes embedded in the inner mitochondrial membrane, creating a proton gradient that ultimately drives adenosine triphosphate (ATP) production. Nearly all oxygen is reduced to water in Complex IV. However, a small percentage is converted to superoxide ($^{\bullet}O^{-}_{2}$) due to electron leakage from Complex I (NADH dehydrogenase) and Complex III (cytochrome reductase) (Phaniendra et al., 2015). While $^{\bullet}O^{-}_{2}$ is not a potent oxidant, its derivative H_2O_2 can propagate further ROS formation. Through the Fenton reaction, hydroxide (OH^{-}) and hydroxyl ($OH^{\bullet}$) are formed by the reaction of Iron (II) (Fe^{2+}) and H_2O_2. Through the Haber-Weiss reaction, OH^{-} and $OH^{\bullet}$ are formed by the reaction of H_2O_2 and $^{\bullet}O^{-}_{2}$ (Phaniendra et al., 2015; Gunn et al., 2019).

NADPH oxidases (NOX/DUOX enzymes) are the only mammalian enzymes solely dedicated to ROS generation and are recruited in response to invading pathogens. When activated, NADPH

oxidases trigger an oxidative burst, generating $^{\bullet}O^{-}_{2}$ or H_2O_2 through the conversion of NADPH to NADP+. The ROS attack the invading pathogens and aid in the immune response (Panday et al., 2015). ROS generated by xanthine oxidase are produced as the byproduct of two subsequent reactions: conversion of hypoxanthine into xanthine and xanthine into uric acid (Dröge, 2002). And lastly, NOS generates nitric oxide radical ($^{\bullet}NO$) through the conversion of L arginine to L citrulline. NO is a prominent signaling molecule involved in blood pressure regulation, smooth muscle relaxation, neurotransmission and defense and immune function. However, in the presence of $^{\bullet}O^{-}_{2}$, it can form peroxynitrite ($ONOO^{-}$) which is highly toxic and can lead to lipid peroxidation, protein damage, nitrosylation and further disruption of NOS signaling (Fukai et al., 2011; Candas and Li, 2014). In addition to these endogenous sources of ROS, exogenous sources include bacteria, such as H_2O_2-producing lactobacilli, diet, climate pollution, toxins, drugs and pathogens (Rahal et al., 2014).

ROS/RNS concentrations are maintained through redox reactions and by the antioxidant defense system. The antioxidant defense system is responsible for clearing excess free radicals and involves both enzymatic and nonenzymatic antioxidants to assist in neutralizing harmful molecules. Antioxidant enzymes include superoxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GPx), with each playing a specialized role. SOD converts $^{\bullet}O^{-}_{2}$ into H_2O_2. H_2O_2 can then be neutralized by CAT or GPx and converted to molecular oxygen (O_2) and water (H_2O) (**Error! Reference source not found.**). While CAT and GPx target H_2O_2 they do so in slightly different ways. CAT does not require another substrate for action, while GPx requires glutathione (GSH) to act upon H_2O_2. In this reaction, GPx simultaneously coverts reduced glutathione to oxidized glutathione (GSSG) and H_2O_2 to



O_2 and H_2O (Bacou et al., 2021).

Figure 1. Antioxidant enzymatic reaction. Created with BioRender.com

While SOD, CAT and GPx are the primary endogenous antioxidants, there are other antioxidants that assist in converting ROS into more stable molecules. These include glutathione,

selenium, vitamins (A, C, E, B_2), alpha-lipoic acid, ubiquinol and flavonoids (Rahal et al., 2014; Bacou et al., 2021). Other compounds, such as magnesium sulfate or some plant extracts, may not be conventional antioxidants but have antioxidant properties to help mitigate oxidative stress. Vitamin E (α -tocopherol) is a lipid soluble antioxidant largely found in the cell membrane (Lauridsen, 2019). It acts as a chain-breaking antioxidant and prevents free radical propagation, especially lipid peroxidation (Khan et al., 2019). Vitamin C (ascorbic acid) is a water-soluble antioxidant synthesized by most species, including poultry (McDowell, 2000). Due to its strong reducing capabilities, vitamin C acts as a scavenger and helps neutralize free radicals such as H_2O_2, hydroxyl radical and singlet oxygen (Padayatty et al., 2003; Hacişevkđ, 2009). It also supports the formation and function of other antioxidants, including vitamin E, as it returns oxidized vitamin E back to its reduced form (Sato et al., 1990).

Free radicals tend to be difficult to measure as they are highly reactive and depending on the stability, can have a very short half-life. Therefore, one way to measure oxidative stress through byproducts of DNA, protein and lipid damage. Thiobarbituric acid reactive substances (TBARS), including malondialdehyde (MDA), are the main products of lipid peroxidation while protein carbonyls serve as biomarkers of protein damage. These byproducts of oxidation can lead to further free radical formation and propagation, thus perpetuating oxidative stress. Shakeri et al. (2020b) and Chen et al. (2021) both found cyclic HS (35°C 8h/25°C 16h; 3w, 32-33°C 8h/22°C 16h; 3w), to increase lipid peroxidation in the breast muscles of broilers, as indicated by increased levels of TBARS and MDA in the breast tissue, respectively. Similarly, Tan et al. (2010) found acute HS (32-38°C; 3h) to increase liver and serum MDA as well as increase formation of liver protein carbonyls. Liu et al. (2018) reported reduced biological antioxidant potential and increased advanced oxidized protein product in broilers exposed to cyclic HS (35°C 8h/20°C 16h; 7d), which suggest antioxidant capacity and higher protein damage, respectively. These studies suggest that high ambient temperatures result in lipid and protein damage, consistent with oxidative stress.

Another way to measure oxidative stress is through antioxidant activity. However, depending on the duration and intensity of heat stress and when the samples are taken, the resulting antioxidant

activity may increase, decrease or remain unchanged. . In poultry, Altan et al. (2003) found acute HS (38°C; 3h) to increase MDA concentrations and SOD, CAT and GPx in the blood. Similarly, Harsini et al. (2012) saw increased MDA concentrations and SOD activity and in muscle but no change in GPx activity in response to cyclic HS (37°C/23.9°C; 49d). Increased antioxidant activity may suggest an upregulation in antioxidant defense systems in response to HS and subsequent oxidative stress. On the other hand, decreased antioxidant activity may suggest a downregulation, weakening or even overuse of the antioxidant defense system. Liu et al. (2014) found chronic HS (37°C; 15d) inhibited SOD, CAT and GPx activity and decreased reduced glutathione (Gao et al., 2020). Liu et al. (2016b) also reported reduced in GPx activity and an increased ratio of GSSG to reduced glutathione, both of which suggest depressed antioxidant capacity because of oxidative stress.

1.10.2 Superoxide Dismutase

SOD is a metalloenzyme present as different isoforms in mammalian, bacterial, and plant cells. It was first isolated in 1938 but it wasn't until 1969 that its function was finally characterized. As its name indicates, SOD, catalyzes the dismutation of $^{\bullet}O^{-}_{2}$ to molecular oxygen and hydrogen peroxide (McCord and Fridovich, 1969; Perry et al., 2010). SOD activity relies on the presence of catalytic metal ions at the active site, such as copper (Cu) and manganese (Mn), which are alternatively reduced and re-oxidized during the dismutation process (Perry et al., 2010).

The three mammalian isoforms include CuZnSOD (SOD1), MnSOD (SOD2) and CuZnSOD (SOD3/ecSOD), all of which are differentially localized. SOD1 is predominately expressed in the cytosol but can also be found in the intermembrane space of the mitochondria, nuclei, lysosomes and peroxisomes. Both Cu and Zn are required for SOD1 to function properly, with Zn playing a key role in protein folding and stability and Cu taking a more mechanistic role (Perry et al., 2010; Fukai et al., 2011). SOD2 is found in the mitochondrial matrix and is responsible for scavenging superoxide radicals produced by the ETC (Candas and Li, 2014) SOD3 is located in the extracellular matrix and cell surfaces and is homologous to SOD1 in its active sites and recruitment of Cu and Zn (Fukai et al., 2011).

Knockout studies have revealed the importance of SOD in the antioxidant defense system and redox signaling. Mice lacking CuZnSOD, both SOD1 and SOD3, display an increase in protein, lipid

and DNA damage in skeletal muscles. These changes are associated with a decrease in motor function and an increase in age-related loss of muscular mass (Muller et al., 2006). Erythrocytes from SOD1 (-/-) mice also showed increased levels of ROS and decreased viability, despite not displaying signs of being under oxidative stress (Grzelak et al., 2009). In turn, SOD3 (-/-) mice also show changes in their redox status, as they experience an increase in renal mRNA expression of NADPH oxidase and markers of oxidative stress under hypoxic conditions (Suliman et al., 2004). These changes suggest the importance of SOD3 in the oxidant/antioxidant balance and bodily response to hypoxia. This is especially important during HS, as the thermoregulatory processes often create hypoxic conditions in key organs such as the liver, gastrointestinal tract, and kidneys.

Nevertheless, SOD2 may be the most critical SOD isoform, as knockout mice commonly suffer perinatal mortality or have a life expectancy of 3 weeks or less (Li et al., 1995). SOD2 (-/-) mice also display diminished growth rates, oxidative mitochondrial injury, motor deficiencies, neuron damage, anemia and cardiac myopathy (Li et al., 1995; Lebovitz et al., 1996). In contrast, over expression of SOD2 in mice have a phenotypic reduced superoxide formation in hippocampal neurons and increased longevity. Heterozygous SOD2 (-/+) mice display increased survival rates and no external abnormalities. Furthermore, they show significantly decreased MnSOD activity in liver, kidney, heart, lung, brain, muscle, spleen and stomach tissue. Interestingly, there was no change in expression of other major antioxidant enzymes in these tissues, suggesting there was no compensation for the reduction in SOD2 (Van Remmen et al., 1999; Van Remmen et al., 2001).

SOD1, SOD2 and SOD3 all play key biological roles and a lack of any of these isoforms results in increased oxidative damage. Therefore, targeting SOD and perhaps increasing abundance or activity may assist in protecting again oxidative stress. Utilizing SOD as a therapeutic intervention for heat stress will be discussed in the next section.

1.11 Heat Stress Mitigation Strategies

There are number of heat abatement strategies that aim to help minimize the effects of HS in livestock. These may include housing improvements, genetic selection and nutritional strategies. One of the first steps to mitigate HS is through housing design and function such as providing shade,

adopting an open barn layout, using forced ventilation or installing an air conditioning or misting system (Gunn et al., 2019). By reducing the ambient temperature through these methods, the heat load on the animals is reduced and potential for heat stress is diminished. However, when these methods are not sufficient to protect livestock again rising temperatures, other strategies must be employed. This can be done through altering feed formulation to meet the changing metabolic needs of livestock under HS or through feed additives, such as vitamins, minerals or plant extracts (Renaudeau et al., 2012). Effective feed additives often act directly as antioxidants or possess antioxidant capabilities.

1.11.1 Antioxidant Supplementation

Several studies have explored the use of feed additives such as betaine, selenium, vitamin E, vitamin C and polyphenols to help ameliorate the effects of HS across performance, gastrointestinal and immune parameters. Le et al. (2020b) demonstrated betaine supplementation lowered respiration rate and rectal temperatures in pigs exposed to cyclic HS (33°C 8h/28°C 16h; 3d). Betaine has also been shown to improve growth rates and meat quality (Shakeri et al., 2018b; Shakeri et al., 2020a), decrease TBARS (Shakeri et al., 2019a) and reduce tissue damage in vital organs in broilers exposed to cyclic HS (33°C 8h/25°C 16h; 42d). Supplementing vitamin E or vitamin C independently or in conjunction yields beneficial results in livestock exposed to HS. Sahin et al. (2003) reported higher live weight, feed intake and egg production in Japanese quail exposed to seasonal HS (average 41°C; ~3 mo) in birds supplemented with either vitamin E or vitamin C. In a similar study, Sahin and Kucuk (2001) investigated the combined effects of different doses of vitamin C and vitamin E supplementation on performance, digestion and carcass characteristics in Japanese quails exposed to chronic HS (34°C; 4w). Results showed higher levels of vitamin C (200 mg/kg compared to 100 mg/kg) increased feed intake, and body weight and improved feed efficiency and nutrient digestibly. These changes were enhanced when combined with higher vitamin E levels, suggesting a synergistic effect.

The effects of vitamin E and C go beyond performance, with Panda et al. (2008) finding vitamin E (125 IU/kg) and vitamin C (200 mg/kg diet) independently improve antioxidant enzyme activity (GPx) in White Leghorn layers during season HS. When combined, vitamin E and C improved activity of antioxidant enzyme glutathione reductase and reduced activity of lipid peroxidase, signifying a

protective effect against oxidative stress and lipid peroxidation. While not a heat stress study, Gao et al. (2010) found high levels of vitamin E (200 mg) reduced lipid peroxidation in plasma and skeletal muscles of broilers subject experiencing dexamethasone oxidative stress.

Kuttappan et al. (2016) investigated the effects of antioxidant ethoxyquin (ETX), mineral methionine hydroxy analog chelates (MMHAC; combination of Zn, Cu and Mn) and organic selenium (OrgSe) on breast muscle quality heat stressed broilers (30°C; 2w). They found MMHCA supplementation increased the number of normal fillets and reduced the severity scores of WB when they did occur. Furthermore, MMHCA increased tibial strength improved the appearance of the breast muscle by reducing breast blisters and skin scratches. Combining MMHCA with ETX and OrgSe significantly improved upon these changes suggesting a key role in antioxidants in improving meat quality and appearance.

As outlined above, it is clear antioxidants can work synergistically to strengthen their protective effects against HS (Sahin and Kucuk, 2001; Sahin et al., 2003; Panda et al., 2008; Sahin et al., 2009). While vitamin E and C are two common antioxidants to be used in conjunction, vitamin E and selenium have also been shown to ameliorate the effects of HS. Pigs supplemented with supra-nutritional levels of selenium (1.0 p.p.m.) and vitamin E (200 IU kg^{-1})and exposed to 2 days of cyclic HS (38°C 8h /28°C 16h) reported improved intestinal barrier function when compared to heat stressed controls (Liu et al., 2016b). In poultry, vitamin E and selenium have also been shown to increase feed intake and reduce breast meat MDA concentrations in broilers exposed to cyclic HS (24°C 8h/24-37°C 4h/37°C 8h/37-24°C 4h; 4w) (Harsini et al., 2012).

While uncommon in the livestock industry, magnesium sulfate ($MgSO_4$) has also been shown to improve performance in HS broilers and protect against oxidative stress. Broilers exposed to cyclic HS and supplemented with magnesium sulfate exhibited increased body weight gain, improved feed conversion ratio and lower mortality rates compared to HS controls (Yang et al., 2012). In addition, SOD, CAT and GPx activity increased, and byproducts of oxidative damage decreased, suggesting $MgSO_4$ can improve antioxidant defense system and protect against oxidative stress. Other studies investigating the therapeutic effects of $MgSO_4$ against oxidative stress found it to reduce lipid

peroxidation (Yavuz et al., 2013; Abad et al., 2015; Mohammadi et al., 2020), reduce protein carbonyls (Mohammadi et al., 2020), increase antioxidant enzyme activity (Yavuz et al., 2013), and improve mitochondria function (Mohammadi et al., 2020).

While the mechanisms of $MgSO_4$ are still largely unknown, Mg itself plays a key role in cellular functions and acts as a cofactor to several enzymes throughout the body (Touyz, 2004). Magnesium deprived or magnesium deficient cells increase mitochondrial malfunction and ROS production (Liu and Dudley, 2020). Furthermore, acute and cyclic HS can reduce Mg retention increase Mg excretion and low magnesium levels have also been linked to increased ROS production (Belay and Teeter, 1996). $MgSO_4$ may therefore maintain the mineral balance under high ambient temperatures and improve the antioxidant defense system.

Plant-derived polyphenols and plant extracts are also interesting feed additives due to their potential antioxidant capacities and therapeutic application in livestock production. The botanical phenolic compound resveratrol has been shown to increase feed intake and body weight gain as well as serum SOD and CAT activity in chronic heat stressed chickens (37°C; 15d) (Liu et al., 2014). *Macleaya cordata* (Plume Poppy) extract has been shown to increase total antioxidant capacity (T-AOC) and activity of GPx and SOD in weaned piglets, highlighting its antioxidant effect. Furthermore, it alters gut microbiota, decreases diarrhea severity and increases tight junction protein expression, suggesting beneficial effect on gut health (Liu et al., 2016c; Chen et al., 2019). A recent study found dried plum increases expression of HSPs (HSP70, HSP90), antioxidant enzymes (SOD1, SOD2, GPx1, GPx3), TJPs (CLDN1, OCLN) and immune related genes (IL, MUC2) in the ileum of broilers exposed to cyclic HS (33°C 8h/22-24°C 16h; 3w). Once again, these data suggest the potent effects of plant derived, antioxidant rich, compounds when administered during a heat challenge.

Collectively, these studies suggest the protective effects of antioxidants and compounds with antioxidant capabilities against HS and highlight their role in reducing oxidative damage. However, there is limited information addressing the effects of antioxidant enzymes, such as SOD in heat stressed livestock. Due to its role in the antioxidant defense system, it is possible SOD may ameliorate the effects of HS, particularly regarding gastrointestinal health and oxidative stress status.

1.11.2 SOD Supplementation

The current literature suggests beneficial effects of SOD supplementation in cell survival, ischemia/reperfusion, inflammatory and infectious disease in mice, rats and horses. Notin et al. (2010b) have shown orally administered melon derived SOD has protective benefits and reduced blood hemolysis in horses during training. A reduction in hemolysis, as well as an enhanced resistance to apoptosis, was also reported by Vouldoukis et al. (2004b) following melon SOD supplementation in mice. The protective effects of SOD are also documented in ischemia-reperfusion models. Rats administered with SOD prior to reperfusion, maintained villus height and were protected from villus damage after intestinal ischemia (Stone et al., 1992). Postischemic isolated rabbit hearts were found to be protected by human recombinant MnSOD and had a higher percentage of developed muscle tension compared to untreated hearts. However, the response was dose-dependent and a high dose of MnSOD elicited toxic effects, indicating SOD operates in a dose-dependent manner.

Further studies have demonstrated the beneficial effects of SOD during inflammatory and infectious diseases. Intravenous injection of lecithinized SOD have displayed therapeutic potential in the treatment of ulcerative colitis, as severity of symptoms and progression of disease were suppressed in rodent and clinical models (Hori et al., 1997; Suzuki et al., 2008). Inhalation of lecithinized SOD has also been shown to ameliorate the inflammatory effects of pulmonary inflammation and emphysema. Although not related to disease model *per se*, Vouldoukis et al. (2004b) found oral melon SOD to reduce TNF-α production and enhance IL-10 synthesis in response to IFN-γ proinflammatory cytokines. In cats infected with feline immunodeficiency virus (FIV), SOD was found to increase erythrocyte SOD and CD4+/CD8+ ratio compared to un-supplemented FIV-infected cats. As CD4+ are lost in FIV, the elevated CD4+/CD8+ ratio may indicate T-cell survival and suppression of disease progression by SOD supplementation.

The use of SOD in agricultural settings is less common, with few studies investigating the potential benefits in livestock. However, melon pulp extract (MPC) has been used as a feed additive as it is rich in SOD. Carillon et al. (2016) found MPC to increase egg weight, shell and yolk weight, and SOD, CAT and GPx expression in laying hens. Lallès et al. (2011) also found MPC to decrease stress proteins in the gastrointestinal tract of piglets during weaning stress. Similarly, Ahasan et al. (2019)

reported an increase in ADG, ADFI and antioxidant capacity in weaning piglets. However, studies with unprotected SOD, such as recombinant SOD, found little biological effect after supplementation, indicating that SOD must be protected when administered for maximum efficiency (Le et al., 2019).

The various routes of SOD administration and whether the enzyme is protected, can have varying effects on efficacy. The primary concern is that orally administered exogenous SOD will be denatured and digested in the gastrointestinal tract before reaching the target tissue. Indeed, several studies have looked to enhance the therapeutic effects of SOD though encapsulation and protection methods, including encapsulation of SOD with vegetable oil, wheat gliadin, liposomes, dietary fibers or though binding to lectin. Encapsulated or protected SOD tend to elicit a stronger biological effect, and in some cases are critical for SOD to be effective (Regnault et al., 1995; Hori et al., 1997; Vouldoukis et al., 2004b; Carillon et al., 2013; Stephenie et al., 2020). For example, subcutaneous administration of SOD demonstrated higher absorption rates than oral administration and SOD encapsulated in liposome, with or without ceramide, displays increased bioavailability when compared to free SOD (Regnault et al., 1995). Therefore, the type of SOD and route of administration should be carefully considered when using SOD as a feed additive.

1.11.3 CAT Supplementation

Catalase supplementation is rare, when compared to SOD supplementation. The published studies are from recent years and tend to investigate the effect of catalase on animal performance in the absence of a challenge (i.e. HS). Guo et al. (2022) investigated maternal CAT supplementation on reproductive performance, antioxidant capacity, mineral transport and mRNA expression of Cu/Zn SOD, GPX and CAT on sows and piglets. CAT (280 U/kg) improved T-AOC in sow serum and umbilical cords of piglets, reduced intrauterine growth restriction and increased CAT activity in neonatal piglet serum and umbilical cords. It also modulated mineral elements and increased GPx and CAT expression in the piglet ileum. In another study looking at reproductive performance in sow, CAT (75 U/kg) was shown to reduce birth time, number of still births, mortality and increased ADG and litter weight of piglets at day 21 (Sun et al., 2022). Weaned piglets supplemented with CAT (120 U/kg) for 14 days showed higher CAT and SOD activity as well as lowered MDA concentrations, suggesting

higher antioxidant capabilities and lower lipid peroxidation. When these piglets were challenged with LPS, those supplemented with CAT showed lower levels of liver injury and maintained GPx and CAT activity compared to those fed the control diet (Li et al., 2020). Turning to poultry, Tang et al. (2022) also found altered performance, antioxidant activity and even changes in gut integrity in response to CAT supplementation over 60 days. Male Lingnan yellow broilers fed CAT (200 U/kg) showed improved weight gain and feed to gain ration from days 1 to 60 compared to birds fed the control diet. By day 30, supplemented birds also had increased T-AOC along with increased CAT, SOD and GPx activity in the liver and reduced MDA in the serum and jejunum. Villus height and ratio of villus height to crypt depth was increased and crypt depth was decreased in the duodenum, jejunum and ileum. These changes in antioxidant activity and gut morphology were maintained through day 60. However, there was no significant change in MDA and the ileum saw a reduction in crypt depth and no change in villus height. mRNA expression of tight junction proteins was also improved across the jejunum and ileum. Collectively, these studies highlight the potential of CAT to improve growth parameters, reproduction performance, antioxidant capacity and gut integrity. Although there were no studies investigating the use of CAT during heat stress, one can speculate that it would have protective effects, particularly in mitigating HS-induced oxidative stress.

1.12 Study Aims

This work aims to support the growing body of research investigating feed additives to ameliorate the effects of HS. Specifically, it aims to help fill a gap on the effects of antioxidant enzyme supplementation in heat stressed production animals. . Poultry, particularly Ross-308 broilers, were selected as a model for this study due to their rapid growth rates and high prevalence in animal production systems worldwide. Additionally, poultry provide the ability to support high *n* amidst time and space constraints. This study is meant to mimic commercial conditions, and therefore birds will be reared from hatch to 42 days of age, during which growth performance, meat quality and multiple physiological parameters will be measured. A portion of the birds will be reared to 21 days of age, to document the onset of the effects of HS and/or diet.

Therefore, the aims of this study are:

1. Document the effects of HS across production and physiological parameters.
2. Investigate whether antioxidants, antioxidants, antioxidant enzymes or a combination of the two are effective in ameliorating the effects of HS across production and physiological parameters. We will focus our attention on the antioxidant supplement Oxicare (OX) which is a combination of vitamin E, vitamin C and $MgSO_4$, and the antioxidant enzymes SOD and CAT.
3. Determine temporal changes due to temperature or diet.

I anticipate HS to negatively impact growth performance, meat quality, antioxidant status and TER and increase markers of HS such as RR and RT. Furthermore, it is expected that HS will alter blood chemistry and oximetry in a way that suggests the birds are experiencing respiratory alkalosis. The addition of feed additives, such as Oxicare, SOD or CAT, will ameliorate these effects to some degree and I believe the combination of two or more of the additives will yield the best results. Looking at the onset of these changes, I anticipate growth performance to be affected earlier on than the other measures, as the divergence of temperatures between TN and HS groups begins at day 7.

The relevance of this work is two-fold. Primarily it will help improve animal health and welfare as it will give insight into the detrimental effects of HS and investigate potential therapeutic strategies. Secondly, it will help strengthen animal production amidst a changing climate by improving meat quality and output. With global meat production and consumption increasing, particularly in tropical and subtropical regions, HS is an increasing problem.

2 Chapter 2: Effects of antioxidants and antioxidant enzymes on growth performance and meat quality in heat stressed broilers.

2.1 Introduction

This work was conducted as a single study carried out at the University of Melbourne from April 2021 – May 2021. The experiment was approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia (Protocol no. 20277). Due to the variety of parameters taken during this study, the methods, results and discussion will be divided across Chapters 2 and 3. Chapter 2 will focus on the effects of HS and antioxidant and antioxidant enzymes supplementation on growth performance and meat quality. Chapter 3 will focus on the effects of HS and antioxidant and antioxidant enzymes on physiology, blood chemistry and oximetry, gut integrity and plasma antioxidant status. The animals used, diet and experimental design were the same for both chapters and will be reported in Chapter 2 only. Several tissue samples were taken during this study but were not analyzed. They will be reported as being collected during the study but will not be included in the results section.

2.2 Methods

2.2.1 Animals, Diet and Experimental Design

Two hundred-and sixteen, day-old male Ross-308 chicks were obtained from a commercial hatchery (Turosi Food Solutions Group, Devon Meadows, Victoria, Australia). Upon arrival at the facility, birds were wing tagged, weighed and randomly allocated to floor pens in one of two climate-controlled rooms,TN (TN) or heat stress (HS), and to one of six diets, control (CON) or CON supplemented with SOD (SOD), CAT (CAT), SOD and CAT (O+S), Oxicare premix (OX) or OX and SOD and CAT (O+S+C), creating a 2 x 6 factorial arrangement. Both rooms were maintained at 33°C for the first 7 days of the study, after which there was a divergence in temperature. TN conditions gradually decreased 1°C every 2 days until reaching 25°C. Heat stress conditions remained at 33°C from 9:00-17:00 and reflected TN conditions from 17:00-9:00.While the temperature change was not instantaneous, it took approximately 1h for climate rooms to adjust to the desired temperature. Humidity was maintained between 40-60 % for the entirety of the study. Light was provided for 24h for the first

three days after entering the facility then reduced 1h/day to 20h by day 7. Birds experienced darkness 1h after the decrease in temperature and darkness lasted for 4h: 18:00-22:00. Each pen (0.9m x 0.45m) was covered in ~10cm deep wood shavings and equipped with a nipple drinker, feeder and enrichment in the form of lattice balls. Food and water were provided *ad libitum*.

Feed (University of Sydney, Sydney Australia) was formulated as a commercial starter crumble from days 0-14 (CP 24.28%, ME 2950 kcal/kg), grower pellet from days 14-28 (CP 22.36%, ME 3070 kcal/kg) and finisher pellet from days 28-42 (CP 20.20%, ME 3170 kcal/kg) (Table 1). All phase diets contained 5% titanium dioxide as marker to measure ileal digestibility. Chickens were fed with the commercial CON or a diet supplemented with OX containing vitamin E (125 g/t), vitamin C (100 g/t) and magnesium sulfate (2 kg/t) (DSM Nutritional Products, Moorebank, New South Wales, Australia) at an inclusion rate of 250 mg/kg, SOD with 3064 U/kg superoxide dismutase (Novozymes, Bagsværd, Denmark) , CAT with 100 U/kg catalase (Novozymes, Bagsværd, Denmark), O+C with 3064 U/kg SOD and 100 U/kg CAT (Novozymes, Bagsværd, Denamark)), or O+S+Cwith Oxicare, 3064 U/kg SOD, and 100 U/kg CAT) (Table 1)**.**

From day 0-21, there were 18 birds/treatment. Birds were divided into 3 replicate pens with 6 birds/pen. On day 21, 6 birds/treatment were sacrificed, and samples collected. From day 21-42, there were up to 12 birds/treatment (variation due to mortalities). Birds were divided into 4 replicate pens with 3 birds/pen. Pen densities were adjusted to accommodate the growing birds.

Enzymes were administered to the chickens via post pellet liquid application (PPLA). Briefly, feed was placed into a 65L cement mixer (Westmix 65L ½ HP Cement Mixer, Ames True Temper Australia Pty Ltd, Doncaster, Victoria, Australia). Enzymes, either SOD, CAT or the combination of S+C, were gently mixed with distilled water at room temperature to complete a total of 1,000 ml/MT (20 ml/20 kg) immediately before application. Enzymes were sprayed uniformly into the mixer, directly onto the feed with a micro fluid nozzle (Nylex Trigger Garden Sprayer, Nylex, Doncaster, Victoria, Australia). The mixer was allowed to run for 15 seconds prior to and after applying the enzymes. Feed was mixed in batches of 20 kg, stored at 4°C and used within 7 days. Oxicare was added to the feed during pelleting

and for O+S+C feed, SOD and CAT were added via PPLA, as described above, onto the pelleted OX feed.

Table 1.Nutritional composition of control and Oxicare starter, grower and finisher diets.

Ingredient Name	Starter Control	Starter Oxicare	Grower Control	Grower Oxicare	Finisher Control	Finisher Oxicare
Wheat 13 %	61.18	60.53	63.11	62.71	67.33	66.63
Soybean Meal	26.25	26.50	21.00	21.00	14.50	14.75
Meat Meal 55%	4.75	4.75	4.00	4.00	3.25	3.25
Canola Expellers Meal [1]	4.00	4.00	6.00	6.00	8.00	8.00
Canola Oil	1.65	1.80	3.30	3.45	4.40	4.60
Lime Fine 38%	0.750	0.745	0.765	0.765	0.780	0.780
Lysine-HCl	0.3150	0.3150	0.3140	0.3140	0.3160	0.3160
DL-Methionine	0.2750	0.2750	0.2200	0.2200	0.1600	0.1600
Vitamin and Mineral Premix [2]	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
Oxicare Premix	0.0000	0.2500	0.0000	0.2500	0.0000	0.2500
Salt (NaCl)	0.1950	0.1950	0.1500	0.1500	0.1400	0.1400
Sodium Bicarbonate	0.1800	0.1800	0.2150	0.2100	0.2350	0.2350
L-Threonine	0.1450	0.1450	0.1300	0.1300	0.1100	0.1100
Choline Chloride, 75%	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
Phystase [3]	0.0250	0.0250	0.0250	0.0250	0.0250	0.0250
L-Valine	0.0250	0.0275	0.0125	0.0125	0.0000	0.0000
Xylanase [4]	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100
Titanium Dioxide	0.0000	0.0000	0.5000	0.5000	0.5000	0.5000
Ingredient Total:	100.00	100.00	100.00	100.00	100.00	100.00

Nutrient Name	Starter Control	Starter Oxicare	Grower Control	Grower Oxicare	Finisher Control	Finisher Oxicare
Broiler ME. Enz (Kcal/Kg)	2950	2950	3070	3070	3170	3170
Broiler NE (Kcal/Kg)	2316	2317	2433	2435	2532	2534
Moisture (%)	9.31	9.26	9.19	9.15	9.13	9.09
Crude Protein (%)	24.28	24.27	22.36	22.35	20.20	20.19
T. Dig.Lys. Poult (%)	1.271	1.271	1.157	1.157	1.027	1.027
T. Dig.Met. Poult (%)	0.592	0.593	0.523	0.523	0.442	0.443
T. Dig.M+C. Poult (%)	0.943	0.943	0.859	0.859	0.762	0.762
T. Dig.Thr. Poult (%)	0.854	0.854	0.778	0.778	0.69	0.69
Dry Matter (%)	90.69	90.73	90.80	90.84	90.86	90.90
Crude Fibre (%)	3.088	3.081	3.090	3.083	3.083	3.076
Phytate Phosphorous. (%)	0.250	0.249	0.244	0.243	0.237	0.236
Calcium (%)	0.950	0.950	0.900	0.900	0.850	0.850
Av. Phosphorous (%)	0.475	0.475	0.450	0.450	0.425	0.425
Total Phosphorous (%)	0.548	0.547	0.512	0.511	0.475	0.474
Sodium (%)	0.190	0.190	0.180	0.180	0.180	0.180
Chloride (%)	0.270	0.270	0.240	0.240	0.230	0.230
Na+K-Cl (Meq)	230	231	213	213	192	192

[1]contains 36% crude protein, [2] vitamin and mineral premix include (expressed as unit/2kg of premix): vitamin D3, 5.0 MIU, Vitamin A 12.0 MIU, vitamin E 75.0 g, vitamin K3 3.5 g, vitamin V1 3.0 g, vitamin B2 9.0 g, vitamin B6 5 g, vitamin B12 0.02 5g, biotin 0.025 g, panthothenic acid 18.0 g, folic acid 2.0 g, niacin 55.0 g, copper 16.0 g, iodine 1.250g , selenium 0.3 g, iron 40.0 g, zinc 100.0 g, manganese 80.0 g, ethoxyquin 75.0 g, [3] HiPhos, DSM, Switzerland, [4] Ronozyme, WX, DSM, Switzerland

2.2.2 Growth performance

Live individual weights were measured upon arrival and then recorded weekly from day 14. Feed consumption was recorded and used to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). Average daily gain, ADFI and FCR were corrected for mortalities.

2.2.3 Slaughter and Sample Collection

On day 21, seventy-two birds *(n*= 6 birds/treatment) were randomly selected to be slaughtered to obtain tissue samples. Of the three replicate pens, 3 birds were taken from two of the replicate pens. The 6 birds in the remaining pen were then divided, resulting in 4 replicate pens with 3 birds/treatment for the second half of the study. Birds were first stunned with a dry electrical stunner (Standard 15amp 220-volt, Mitchell Engineering Food Equipment, Pty Ltd., Clontarf, Queensland, Australia) then exsanguinated. The liver and spleen were removed and weighed. A 1cm^2 portion of the liver and spleen were taken and snap frozen in liquid nitrogen. A 1 cm^2 section of the left breast muscle (*pectoralis major)* was also taken and snap frozen in liquid nitrogen. The gastrointestinal tract was removed and a 0.5cm section of the middle of the jejunum was taken and preserved in RNAlater (RNAlater Stabilizing Solution, Invitrogen). A 1 cm sample of the same section of the jejunum was taken and snap frozen in liquid nitrogen. A 0.5 cm section from the middle of the distal two thirds of the ileum was taken and preserved in RNAlater for gene expression analysis. A 1 cm sample from the same section of the ileum was taken and snap frozen in liquid nitrogen and another 2 cm section was taken to measure transepithelial resistance (TER) (see section 3.2.4 for TER methods). Digesta content was collected from the jejunum, ileum and one lobe of the ceca and snap frozen in liquid nitrogen to measure ROS, ileal digestibility and microbiome abundance and diversity respectively. Ileum, jejunum and digesta samples were stored at -80°C until being shipped to Village-Neuf, France for further analysis by DSM.

On day 42, the remaining birds were slaughtered (*n*= 8-12 birds/trt) and tissue was collected using the same procedure as per day 21. Additionally, a 3cm section from the middle of the jejunum and from the distal two thirds of the ileum were collected and placed in 5mm tissue processing cassettes

and placed into 10% neutral buffered formalin. After 48 h, samples were transferred to 70% ethanol and kept there until further analysis.

2.2.4 Meat Quality

Meat quality was also determined on day 42 and included measuring pH, drip loss, cook loss, shear force and color (*n*= 8-12 birds/trt). After general dissection and tissue collection, the carcasses were transferred to a cool room (4°C) overnight. After 24 h, pH was measured using a polypropylene spear type electrode (Ionode IJ44A, Pty Ltd., Tennyson Queensland, Australia) inserted 2cm into the right breast muscle for 30 s. Measurements were taken in duplicate and averaged. The right breast muscle (*pectoralis major and minor)* was then removed, stripped of connective tissue and weighed. A 10g sample of the *pectoralis major* was taken and stored at -80°C for further analysis to determine myofibrillar fragmentation. Drip loss was quantified by suspending a 10 g (W1) piece of breast muscle in a sealed bottle for 48 h. After 48 h, samples were weighed again (W2), and drip loss calculated as a percentage using the following equation:

$$\text{Drip loss (\%)} = [(W1 - W2)/W1] \times 100$$

The tissue was then stored at -80°C for further analysis of lipid peroxidation. Cook loss was quantified by cooking samples (80g) for 40 min in an 80°C water bath. After 40 min, samples were cooled under running water, gently dried and re-weighed. The cooked samples were then cut into blocks 1 cm high, 1 cm wide and 3 cm lengthwise and used to quantify Warner Bratzler shear force (WBSF). Briefly, WBSF was measured using an inverted V-blade and cross head (200mm/min) perpendicular to the muscle fibers and Lloyd texture analyzer (Lloyd Instruments LS5S/C Materials testing instrument, Lloyd Instruments Ltd., Hampshire, UK) with a 500 N load cell. Color was measured 24 h post slaughter using a Minolta chromameter CR-400 with an 8mm aperture (Minolta Pty Ltd., Tokyo Japan, light source D65, observer angle 2°). Measurements were made in accordance with the CIE color space model to determine lightness (L*), redness (a*) and yellowness (b*).

2.2.5 Statistical Analysis

Results were analyzed by residual maximum likelihood model (REML) for main and interactive effects of temperature (T; TN vs. HS) and diet (D; CON vs. OX vs. SOD vs. CAT vs. S+C vs. O+S+C) using Genstat 21st Edition (VSN International Ltd., Hemel Hempstead, UK). Fishers unprotected LSD test was used to separate group means. Pen was the experimental unit for growth performance, while bird was the experiment unit for all other parameters. Standard errors were pooled and represented as the mean standard error across all treatment groups. Differences were considered a trend when $0.1 > p > 0.05$ and significant when $p < 0.05$.

2.3 Results

Results are reported by the effects of temperature first, followed by the effects of diet, and lastly by the temperature by diet interaction.

2.3.1 Adverse Events, Euthanasia, Mortality

It should be note that on day 3 of the experiment, both climate control rooms malfunctioned, causing the temperature to drop from 33°C to 25°C between 8:00 and 13:30. Due to the rapid temperature decrease, there was also a spike in humidity, reaching > 50 % humidity. The error was noticed at 13:30 and steps were taken to re-establish the appropriate temperature and humidity. The temperature returned to 33°C, 45 % humidity by 16:00. A trained veterinarian was called to inspect the birds on day 4 of the study and noted some raspy lungs in a few of the birds. However, no lasting effects were noted regarding this adverse event.

The temperature profiles were adjusted on day 36 of the study as broilers in the TN room were showing mild signs of HS. The temperature of the TN room was changed to 23°C while the HS room was changed to 32°C during the day and 24°C overnight for the remainder of the study. Both rooms were adjusted due to concern over animal welfare.

Heat stress reduced overall survivability by nearly 10 % (95.9 vs. 86.8 %)

Figure 2a). The HS CAT treatment experienced the highest mortality at 4 deaths, followed by HS SOD with 3 deaths, TN OX, HS OX, TN SOD, TN S+C, HS SOD + CAT and HS OX + SOD +CAT with 1 death

Figure 2b). Both CON treatments, TN CAT and TN OX + SOD + CAT with zero mortalities. Four birds were euthanized over the course of the study. Three were due to improperly placed wing tags that could not safely be removed and one due to poor health and lameness. Six birds were noted as being lame over the course of the study, although no official gait/configuration scoring was determined. Lameness was distributed across treatment groups and did not appear to be related to treatment or diet.

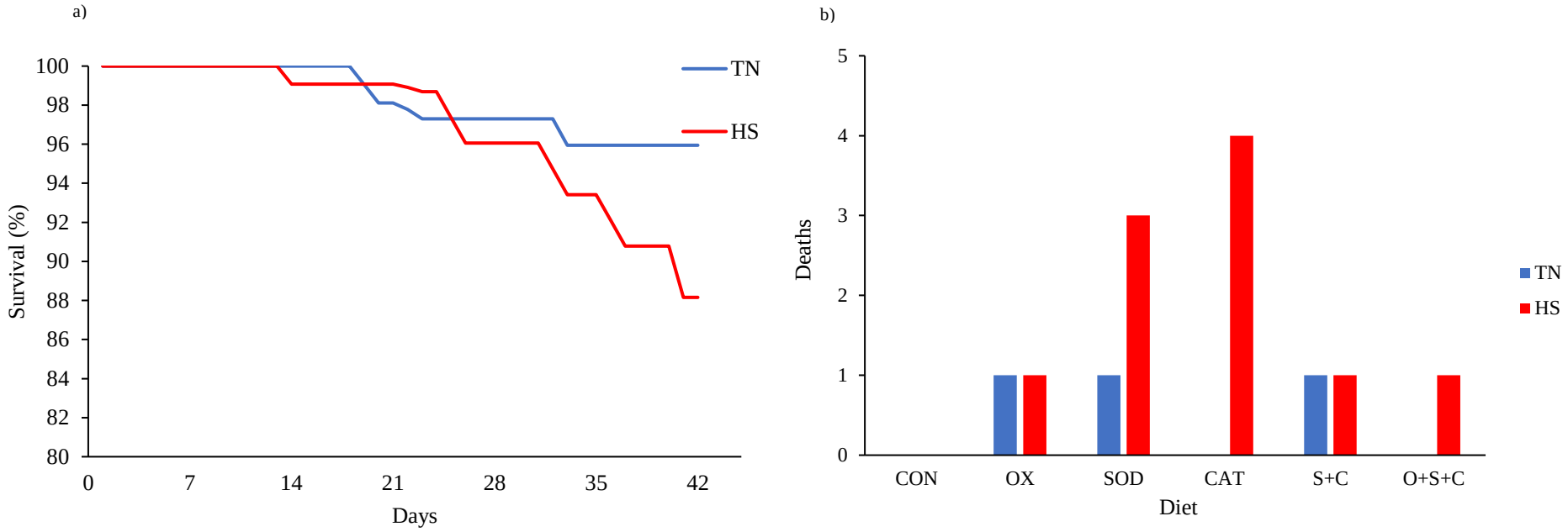


Figure 2. Survivability and mortality. Panel a) provides the survivability percentage of broilers across thermoneutral and heat stress conditions over the course of the study. Panel b) indicates the number of mortalities per treatment group over the course of the study.

2.3.2 Performance

Body weight (BW) and feed intake (FI) were measured weekly starting on day 14 and used to calculate ADFI, ADG and FCR. Heat stress significantly reduced mean BW on day 20 (989 vs. 911 g, $p < 0.001$) (Table 2). There was also a significant effect of diet ($p = 0.008$) such that birds fed OX were significantly heavier than birds fed SOD, CON and OX+S+C (985 vs. 912, vs. 933, vs. 941 g respectively), when pooled across TN and HS groups. However, BW of birds fed SOD, CAT, S+C or O+S+C were not significantly different from BW of CON birds. There was a near significant interactive effect of temperature and diet on BW at day 20. Under TN conditions, supplementing CAT and S+C increased BW compared to CON (951 vs. 1025 g, 1015 g respectively, both $p < 0.05$). Alternatively, under HS conditions, OX increased BW compared to CON (915 vs 975 g, $p < 0.05$). Heat stress significantly reduced BW on day 41 (2842 vs. 3339 g, $p < 0.001$). There was a near significant effect of diet on BW ($p = 0.072$), such that S+C, CAT and OX all significantly increased BW compared to SOD (1737 vs. 1827 g, 1861 g and 1870 g respectively, all $p < 0.05$). However, there was no significant difference in BW between CON and any of the experimental diets.

From day 0 to 20, HS tended to reduce ADFI (64.1 vs. 61.3 g/d, $p = 0.086$) (Table 2). There was no main or interactive effect of diet on ADFI during this time. HS significantly reduced ADG (46.7 vs. 43.3 g/d, $p < 0.001$). There was a significant effect of diet on ADG ($p = 0.033$), with OX increasing ADG when compared to O+S+C and SOD groups (47.2 g vs. 43.4 and 42.8 g/d respectively, $p < 0.05$). However, there was no significant difference in BW between CON and any of the experimental diets. There was no interactive effect of temperature and diet on ADG from day 0 to 20. There was no main or interactive effect of temperature and diet on FCR from day 0 to 20.

From day 21 to 42, HS significantly reduced ADFI (176 vs. 143 g/d, $p < 0.001$) and ADG (113 vs. 85.4 g/d, $p < 0.001$) (Table 2). There was no main or interactive effect of diet on either ADFI or ADG. Furthermore, there was no main or interactive effect of temperature and diet on FCR from day 21 to 42. Over the course of the entire study (day 0 to 42) HS reduced ADFI (120 vs. 102 g/day, $p <0.001$) and ADG (79.7 vs. 64.5 g/d, $p < 0.001$). HS also tended to increase FCR (1.47 vs 1.57, $p =$

0.052). There was no main or interactive effect of diet on ADFI, ADG or FCR from day 0 to 42.

Table 2. Growth performance in broilers exposed to thermoneutral (TN) or heat stress (HS) conditions and fed a control diet (CON) or a diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C).

	Temp	Diet							Significance		
		CON	OX	SOD	CAT	S+C	O+S+C	SED1	T	D	T x D
Day 0-20											
ADFI*(g/d)	TN	72.1	65.7	63.6	62.1	61.4	59.6	4.21	0.086	0.15	0.14
	HS	61.8	63.9	55.2	59.7	61.5	65.5				
ADG* (g/d)	TN	45.7	47.7	45.7	48.9	47.5	44.5	1.96	<0.001	0.03	0.32
	HS	43.9	46.8	39.8	42.9	44.3	42.4				
FCR* (g/d)	TN	1.58	1.38	1.40	1.27	1.29	1.35	0.11	0.47	0.30	0.23
	HS	1.41	1.36	1.39	1.39	1.40	1.54				
Day 21-41											
ADFI (g/d)	TN	183	171	177	175	170	178	13.1	<0.001	0.95	0.48
	HS	134	150	131	146	149	148				
ADG (g/d)	TN	108	110	116	114	117	112	10.2	<0.001	0.48	0.70
	HS	78.1	89.7	77.5	79.9	97.8	89.3				
FCR (g/d)	TN	1.72	1.56	1.53	1.55	1.46	1.59	0.212	0.076	0.73	0.86
	HS	1.73	1.70	1.85	1.85	1.55	1.66				
Day 0-41											
ADFI (g/d)	TN	76.7	79.1	80.8	81.1	82.0	78.6	6.50	<0.001	0.75	0.104
	HS	60.8	68.4	58.8	61.4	71.4	66.0				
ADG (g/d)	TN	128	119	121	118	116	119	5.24	<0.001	0.33	0.53
	HS	97.5	108	92.9	103	106	107				
FCR (g/d)	TN	1.66	1.47	1.48	1.40	1.38	1.46	0.12	0.052	0.34	0.56
	HS	1.57	1.55	1.61	1.63	1.46	1.60				
Final Weight Day 20 (g)	TN	951	995	954	1025	1015	996	29.8	<0.001	0.008	0.076
	HS	915	975	870	896	925	886				
Final Weight Day 41 (g)	TN	3287	3296	3322	3379	3426	3324	144	<0.001	0.072	0.13
	HS	2778	2994	2878	2572	3141	2763				

[1]SED pooled standard error of the difference across treatment groups. T- main effect of temperature; D- main effect of diet; T x D – temperature diet interaction effect. ADFI: average daily fed intake, ADG: average daily gain, FCR: feed conversion ratio. *n* = 8-12 birds/trt.

2.3.3 Meat Quality

Breast weight, as measured by the right breast muscle, was reduced by 20% in response to HS (334 vs. 266 g, $p < 0.001$) (Table 3). However, there was no main or interactive effect of diet on breast weight. There were mixed temperature, diet and interactive effects on meat colour. HS birds tended to have lighter breast muscle compared to their TN counterparts, as reflected by higher L* values (59.1 vs. 60.2, $p = 0.094$). Diet significantly influenced L* ($p < 0.001$), with birds supplemented with SOD having darker meat than birds fed the CON diet (60.2 vs 56.8, $p < 0.001$). There was also a significant temperature and diet interactive effect on L*($p = 0.038$), whereby SOD decreased lightness across both TN and HS treatments. Furthermore, CAT significantly increased lightness, but only under TN conditions. There was no effect of temperature or diet on a*, although there was a near significant temperature and diet interaction ($p = 0.071$). There was no effect of temperature on b* but there was a main effect of diet on b* ($p = 0.027$). Meat from broilers fed CAT was significantly more yellow than meat from broilers fed SOD and S+C (5.53 vs. 4.38, 4.80 respectively, $p < 0.05$). However, there was no difference between CON and experimental groups when it came to redness. There was a temperature and diet interaction effect on b* ($p = 0.047$), whereby CAT significantly increased yellowness, but only under TN conditions (4.82 vs. 6.39, $p < 0.05$).

Heat stress had no significant effect on pH_u, but there was a significant effect of diet ($p = 0.01$), with SOD increasing pH_u compared to CON (5.89 vs 6.00, $p < 0.05$). There was no temperature and diet interaction effect on pH_u. There was no main or interactive effect of temperature and diet on drip loss. Conversely, HS significantly reduced cook loss by 2.2% (23.3 vs. 21.1 %, $p < 0.001$). There was no main or interactive effect of diet on cook loss. Shear force was not influenced by temperature. However, it was influenced by diet ($p = 0.014$), with SOD reducing shear force compared to controls (18.2 vs. 21.0 N, $p < 0.05$). This was particularly apparent under TN conditions, as reflected by the near significant temperature and diet interaction effect ($p = 0.062$).

A summary of the effects of temperature and diet on growth performance and meat quality can be found in Table 4. Summary of effects of temperature and diet on growth performance and meat

quality. This provides a visual for both performance and MQ data together, with indicators whether measures increased or decreased or remained unchanged.

Table 3. Meat quality of broilers exposed to thermoneutral (TN) or heat stress (HS) conditions and fed a control diet (CON) or a diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C).

Temp		Diet							Significance		
		CON	**OX**	**SOD**	**CAT**	**S+C**	**O+S+C**	**SED[1]**	**T**	**D**	**T x D**
Lightness (L*)	TN	60.1^{a}	60.4^{a}	56.8^{b}	64.4^{c}	58.6^{ab}	60.9^{b}	1.37	0.094	<0.001	0.038
	HS	60.4^{a}	58.8^{a}	56.8^{b}	59.3^{ab}	59.7^{a}	59.7^{a}				
Redness (a*)	TN	2.13	2.85	2.54	1.75	2.73	2.28	0.39	0.42	0.16	0.071
	HS	2.31	2.21	3.04	2.47	2.29	2.88				
Yellowness (b*)	TN	4.82^{ab}	5.35^{b}	4.30^{a}	6.39^{b}	4.55^{ab}	5.15^{ab}	0.51	0.21	0.027	0.047
	HS	4.90	4.72	4.46	4.67	5.05	5.05				
Ultimate pH	TN	5.89	5.91	6.00	5.98	5.92	5.88	0.05	0.35	0.01	0.88
	HS	5.89	5.93	6.01	5.92	5.88	5.86				
Cooking loss (%)	TN	22.2	22.2	23.3	25.7	22.3	24.1	1.46	<0.001	0.25	0.20
	HS	19.9	21.9	21.5	21.3	22.0	20.1				
Drip Loss (%)	TN	3.24	3.70	3.29	3.61	3.10	3.86	0.55	0.26	0.60	0.78
	HS	3.44	3.06	2.99	2.77	3.10	3.64				
Shear Force (N)	TN	20.2	20.1	17.2	18.8	22.6	20.9	1.43	0.17	0.014	0.062
	HS	21.8	20.6	19.2	22.5	21.0	19.1				
Breast Weight (g)	TN	324	337	333	335	346	326	22.6	<0.001	0.26	0.79
	HS	258	274	270	241	300	252				

[1]SED pooled standard error of the difference across treatment groups. T- main effect of temperature; D- main effect of diet; T x D – temperature diet interaction effect. Means denoted with a different letter signify significant differences between treatments in the same temperature conditions ($p < 0.05$). Significant T x D interactions without differences between CON and experimental groups were not denoted with a letter. *n* = 8-12 birds/trt.

Table 4. Summary of effects of temperature and diet on growth performance and meat quality.

Parameter	Temperature Effect	Diet Effect				
	HS	OX	SOD	CAT	S+C	O+S+C
Growth Performance						
Day 0-21	Trend ↓ ADFI ↓ ADG	-	-	-	-	-
Day 21-42	↓ ADFI ↓ ADG	-	-	-	-	-
Day 0-42	↓ ADFI ↓ ADG Trend ↑ FCR	-	-	-	-	-
Body Weight, g	↓ d21 ↓ d42	↑ HS d21	-	↑ TN d21	-	↑ TN d21
Meat Quality						
L*	Trend ↑	-	↓	↑ TN	-	-
a*	-	-	-	-	-	-
b*	-	-	-	↑	-	-
pH_u	-	-	↑	-	-	-
Cook Loss, %	↓	-	-	-	-	-
Drip Loss, %	-	-	-	-	-	-
Shear Force, N	-	-	↓; *TN	-	-	-
Breast Weight, g	↓	-	-	-	-	-

HS: heat stress, OX: Oxicare, SOD: superoxide dismutase, CAT: catalase, S+C: SOD + CAT, O+S+C: Oxicare + SOD + CAT, ADFI: average daily feed intake, ADG: average daily gain, FCR: feed conversion ratio, L*: lightness, a*: redness, b* yellowness, pH_u : ultimate pH.. Effects are characterized as significantly ($p < 0.05$) increasing (↑), decreasing (↓) or no effect (-). Trend denotes the effect as being near significant ($0.05 < p < 0.1$). d21 or d42 indicate whether this effect was seen around the day 21 or day 42 timepoint respectively. HS or TN indicates if the effects were seen under heat stress or TN conditions respectively. HS or TN accompanied by * indicates a temperature and diet interaction in addition to a diet effect

3 Chapter 3: Effects of antioxidants and antioxidant enzymes across physiological parameters

3.1 Introduction

The previous chapter outlines the effects of temperature and diet on animal performance, including growth performance and meat quality. These two measures are critical in determining industry output, as light carcasses and poor meat quality can directly impact profitability. Looking beyond direct output, other effects of heat stress can alter physiology and trigger a cascade of negative biological effects. This chapter investigates the effect of temperature and diet on physiological parameters including respiration rate, rectal temperature, blood chemistry and oximetry, antioxidant status and gut physiology in broilers. Changes in these parameters in response to heat stress will determine the robustness of the heat model, while changes in response to diet will highlight potential intervention strategies.

3.2 Methods

As mentioned at the start of Chapter 2, this study was conducted as a single project, but methods and results divided across two chapters. Thus, the experimental design and animal husbandry methods (Section 2.2.1) for both chapters are the same. Methods specific to this stage of the study are described in the following sections.

3.2.1 Physiological Measures

Rectal temperature (RT) was recorded on days 14, 20, 28, 35 and 41. Three chickens per pen were randomly selected each week to measure rectal temperature (RT) at 8:00, 12:00 and 15:00. A digital thermometer (Ri-gital® Digital Thermometer, Riester, Junjingen, Germany) was inserted 3-4cm into the rectum and recorded for 30 sec. In the event fewer than three chickens remained in the pen, only the afternoon time points were recorded. If RT exceeded 44°C, the bird was removed from the room and returned once RT dropped below 44°C. While these birds were temporarily removed from the heat room, they were not removed from the analysis and were analyzed as have a RT of 44°C.

Respiration rate (RR) was recorded weekly at a single time point (11:00) on days 15, 19, 29, 35, 40. Each pen was filmed using a cellphone (iPhone 7, Apple Inc., Cupertino, CA, USA). A minimum of two birds per pen were randomly selected and breaths were counted over a 10 second period and quantified as breaths/minute. To minimize researcher interference and handling of the birds, RT and RR were recorded on different days of the week.

3.2.2 Blood Gas Analysis

Seventy-two chickens were randomly selected to collect venous blood samples (*n* = 6/trt; 2 birds/replicate pen) on day 17. Bleeds were conducted over a two-day period, with each day balanced across treatments. This timepoint will be referred to as day 17 going forward. Approximately 3-5mL of blood was collected from the wing vein. ~0.2mL of the fresh blood was loaded into an automatic blood gas analyser (EPOC, Alere, Waltham, MA, USA) for biochemical and oximetry analysis. The remaining fresh blood was decanted into a heparin coated vacutainer (BD Vacutainer, BD Australia, North Ryde, NSW, Australia) and centrifuged for 10 minutes at 1100 x g and 4°C. Plasma was then collect and aliquoted into separate tubes for antioxidant status, fat soluble vitamins (A and E) and vitamin C analysis. For Vitamin C determination, exactly 100 µL of plasma was added to 900 µL of 5% meta-phosphoric acid solution. Venous blood samples were taken again on day 38 (*n* = 8-11 birds/trt) using the same method described above. This timepoint will be referred to as day 38. All plasma samples were stored at -20°C until being shipped to Village-Neuf, France for further analysis by DSM.

3.2.3 Plasma Antioxidant Quantification

Plasma was analyzed for vitamin A, E and C levels by DSM in Village-Neuf, France. Vitamin A (as retinol) and E (as alpha-tocopherol) concentrations were determined by reversed-phase high performance liquid chromatography (HPLC) as per Aebischer et al. (1999). Briefly, alpha-tocopherol (E. Merck, Darmstadt, Germany) and retinol (E. Merck, Darmstadt, Germany) were diluted, to a concentration of with 0.5 µg/l. with absolute ethanol (Lichrosolv, E. Merck, Darmstadt, Germany) and *n*-hexane (Uvasol, E. Merck, Darmstadt, Germany) respectively. Standards were made up containing 400 µL nanopure grade water (>18 M~, Millipore, Bedford, MA), 200 µg ethanol (400 µg for retinol), 200 µl standard solution of either alpha-tocopherol or retinol, and 800 µl n-hexane/2,6-di-tert-butyl-4-

methylphenol (E. Merck, Darmstadt, Germany) (600 µl for retinol) and used to calibrate the HPLC system. The HPLC system was assembled using a high-pressure pump (Kontron, T 414), an autosampler (Kontron, 360), a column oven (Kontron, Oven Controller 480), a photometric (GAT LCD 501, Stagroma, Wallisellen, Switzerland), and a fluorometric (Perkin Elmer LS40, England) detector. Data were acquired via a chromatography server (Fissions Instruments, England) and analyzed by a VAX based multichannel data acquisition system (Multichrom, VG Data Systems Ltd., England). The autosampler was kept at 25°C to avoid disintegration of the redissolved plasma extract. The separation was done on a reversed-phase column (Primesphere C~8-HC 5 txm 110 A, 250 by 4.6 ram, Phenomenex, Torrance, CA) at 30°C with a mixture of acetonitrile (684), tetrahydrofuran (220), methanol (68), and a 1% (w/v) ammonium acetate solution (28) at a flow of 1.6 (ml/min). The resulting backpressure on the system was between 40 and 70 bar.

Plasma samples were thawed slowly (approx. 30 min) and vortexed (Bender Hobein, Zürich, Switzerland). Two hundred microliters of plasma was then transferred to a 4-ml glass tube (inner diameter 8 mm), diluted with 200 µL water, and deproteinized with 400 µL absolute ethanol. The suspension was vortexed again for 30sec before eight hundred microliters of *n*-hexane was added and the solution was mixed on a mechanical shaker (Vetter AG, St. Leon, Switzerland) for 7 minutes. It was then spun down (4°C at 2000g/10 min) and 400/ µL of the supernatant was dried on a Speed-Vac (Savant Instruments, Farmingdale, NY) at room temperature and 40 mbar. The residue was then redissolved in 150 µL of a mixture of methanol (1) and 1,4-dioxane (1) and further diluted with 100 µl acetonitrile (HPLC grade S, Rathburn Chemicals, Walkerburn, Scotland). One hundred microliters of the resulting solution were injected into the HPLC system and vitamin A and E concentrations were determined using liquid chromatography separation with a fluorescent detection.

Vitamin C, analyzed as sum of ascorbic acid and dehydroascorbic acid, was determined through enzymatic oxidation followed by a derivatization reaction with a fluorescent reagent as per Esteban and Ho (1997) Briefly, plasma samples were thawed slowly (approx. 30 min) and vortexed (Bender Hobein, Zürich, Switzerland). One hundred microliters of plasma were then transferred to a 10mL volumetric flask containing 1000uL 1,2 Phenylenediamine (OPDA) (Thermo Fischer Scientific, Waltham, Massachusetts, USA), 1000uL phosphate buffer and 100uL ascorbate oxidase (Sigma Alrdich, St. Louis

Missouri, USA). Deaerated dionized water was then added to the flask to make up a total of 10mL of solution. The solution was left to react at room temperature overnight. The next day samples were loaded into spectrophotometer (Hewlett Packard Model 8451 diode array spectrophotometer, Helwett Packard, Palo Alto, CA) and read at 358nm. Samples were compared against a standard curve of known ascorbic acid concentration, prepared similarly to the sample solutions, with 100uL ascorbic acid (Thermo Fischer Scientific, Waltham, Massachusetts, USA) replacing the 100uL of plasma.

3.2.4 Transepithelial Resistance

On day 21 (n= 6 birds/trt) and 42 (n= 6-11 birds/trt), gut samples ($2cm^2$) were collected from the middle of the distal two thirds of the ileum to measure transepithelial resistance (TER). Briefly, tissue was placed in chilled phosphate-buffered saline (PBS). Tissue was then opened along the mesentery, flushed with PBS and pinned onto a round aperture slide ($0.3cm^2$). The slide was then mounted in a two-part Ussing chambers apparatus (EasyMount Diffusion Chambers, Physiologic Instruments, San Diego, CA, USA). Krebs bicarbonate buffer was added to both chambers, with the serosal chamber containing 11.1mM added glucose and the mucosal chamber containing 11.1mM added mannitol. Krebs bicarbonate buffer was continually gassed with carbogen (95% O_2- 5% CO_2) and maintained at 37°C. A multichannel voltage–current clamp (Physiologic Instruments, model VCC MC6) was connected to the chambers by four electrodes (two voltage-sensing and two current passing electrodes) inserted on opposite sides of the tissue. Tissue was allowed to equilibrate for 25 min before tissue was clamped to 0 V and 2 s pulses of 2 mV were administered for 5 minutes. Change in voltage(V) and current (I) were determined and averaged across three pules. Transepithelial resistance (R) calculated using Ohm's law (R= V/I) and multiplied by the exposed surface area of the aperture.

3.2.5 Statistical Analysis

Results were analyzed by residual maximum likelihood model (REML) for main and interactive effects of temperature (T; TN vs. HS) and diet (D; CON vs. OX vs. SOD vs. CAT vs. S+C vs. O+S+C) using Genstat 21st Edition (VSN International Ltd., Hemel Hempstead, UK). Rectal temperature was analyzed for main and interactive effects of T, D, day of study (DAY) and time of day (TM). Fishers unprotected LSD test was used to separate group means. Pen was the experimental unit for rectal

temperature, while bird was the experiment unit for all other parameters. Standard errors were pooled and represented as the mean standard error across all treatment groups. Differences were considered a trend when $0.1 > p > 0.05$ and significant when $p < 0.05$.

3.3 Results

Results are reported by the effects of temperature first, followed by the effects of diet, and lastly by

the temperature by diet interaction.

3.3.1 Physiology

Rectal Temperature (RT):

Rectal temperature was taken weekly, starting on day 14 at three time points during the day: 8:00, 12:00 and 15:00. Pooling data across each day (14, 20, 28, 35 and 41) and time point (8:00, 12:00 and 15:00), HS significantly increased RT (41.7 vs. 42.5°C, $p < 0.001$) (**Error! Reference source not found.**a-d). There was also a main effect of diet, such that CAT significantly increased RT (42.1 vs. 42.3°C, $p < 0.001$) compared to CON, whereas S+C reduced RT compared to CON (42.1 vs. 41.9°C, $p < 0.05$). There was a significant effect of day of study ($p < 0.001$). Rectal temperature was highest on day 35 (42.6°C), followed by day 41 (42.4°C) then day 20 (42.0°C) and 28 (41.89°C). Rectal temperature was lowest on day 14 (41.4°C) (all $p < 0.005$). Time also had a significant impact on RT ($p < 0.001$), with the highest RTs recorder at 15:00, followed by 12:00 and lastly 8:00 (all $p < 0.05$).

There was a temperature and diet interaction on RT ($p < 0.008$). Under TN conditions, both S+C and OX diets reduced RT compared to CON (41.7 vs. 41.5°C, 41.5°C respectively, both $p < 0.05$). Under HS conditions, CAT significantly increased RT compared to CON (42.4 vs. 42.7°C, $p < 0.05$). The significant temperature and day interaction ($p < 0.001$) revealed an increase in RT, under both TN and HS conditions, as the study progressed. Lastly, there was an interactive effect of temperature and time on RT ($p < 0.001$). Under HS conditions, RT at 12:00 was significantly higher than RT at 8:00, and RT at 15:00 was significantly higher than RT at 12:00 (all $p < 0.05$). There were no changes in RT across the three timepoints under TN conditions. There were no other interactive effects across temperature, diet, day of study and time of day on RT.

To isolate the effects of temperature and diet on RT at day 20 and 40, we pooled data from both 12:00 and 15:00 on the respective days. On day 20, there was a main effect of temperature, with HS increasing RT by 1.5°C (41.5 vs. 43.0°C, $p < 0.001$). There was also a main effect of diet ($p = 0.05$), with CAT and SOD having the highest RT (42.5°C and 42.4°C respectively) and O+S+C with the lowest (42°C). However, there was no significant difference between CON and the experimental diets. The significant temperature and diet interaction ($p = 0.012$) revealed that under HS conditions, broilers fed SOD or CAT diets had higher RT compared to broilers fed CON diet (42.8 vs. 43.5°C, 42.8 vs. 43.4°C respectively, all $p < 0.05$). There were no differences in RT between diets under TN conditions. On day 41, the main effect of HS persisted, with HS increasing RT compared to TN controls (42.0 vs. 43.3°C, $p < 0.001$). However, there was no main or interactive effect of diet on RT on day 41.

Respiration Rate (RR):

Respiration rate was recorded weekly starting on day 15 but the two time points of interest are day 19 and 40, as they align with the other midpoint and end of study measures. On day 19, HS increased RR from 86 to 133 breath/min ($p < 0.001$) (**Error! Reference source not found.Error! Reference source not found.**). There was a significant effect of diet ($p = 0.013$), with birds fed OX and O+S+C having a significantly lower RR than birds fed CAT ($p < 0.05$). However, there was no difference in RR between CON and experimental groups. There was a near significantly temperature and diet interaction effect ($p = 0.058$), such that under TN conditions, SOD and OX significantly reduced RR when compared to CON (92 vs. 79 breaths/min, 80 breaths/min, both $p < 0.05$). Under HS conditions, S+C, CAT and SOD significantly increased RR when compared to CON (125 vs. 141 breaths/min, 140 breaths/min, 138 breaths/min respectively, all $p < 0.05$).

The main effect of temperature on RR continued to day 40, with HS increased RR from 72 to 115 breaths/min ($p < 0.001$) (**Error! Reference source not found.**b). There was a near significant effect of diet ($p = 0.066$), with birds fed S+C experiencing high RR when compared to birds fed CON (88 vs. 101 breath/min, $p < 0.05$). There was no temperature and diet interactive effect on RR on day 40.

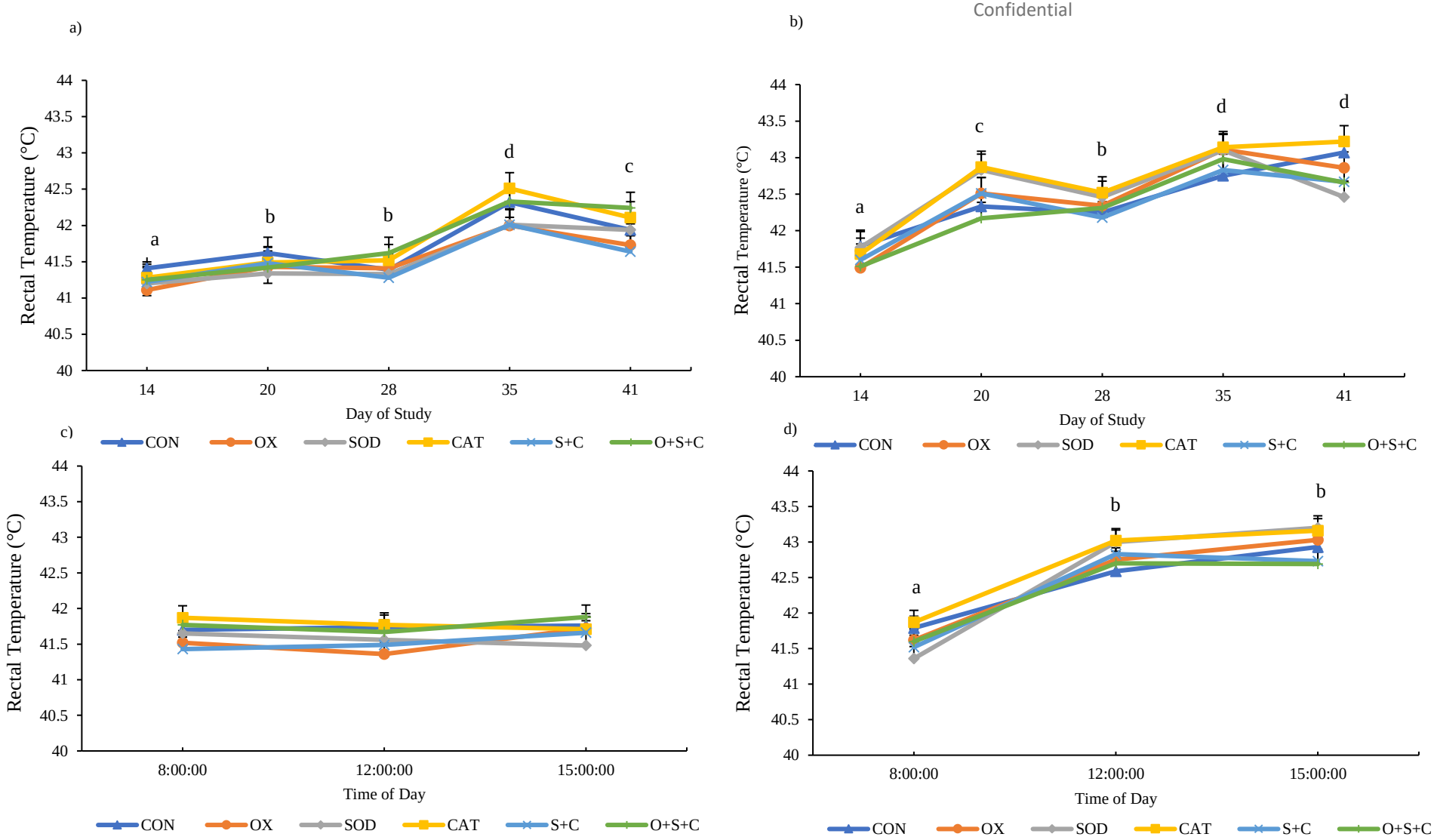


Figure 3. Rectal temperature (RT) of broilers exposed to TN (TN) or heat stress (HS) conditions and fed a control diet (CON) or a diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C). RT was taken weekly starting on day 14 and measured at 8:00, 12:00 and 15:00. Panels a and b represent the effect of temperature (T), diet (D) and day of study (DAY under TN (a) and HS (b) conditions. with the standard error of the difference for the interaction between T, D and DAY. Panels c and d represent the effect of T, D and time of day (TM) on RT of broilers under TN (c) and HS (d) conditions with the standard error of the difference for the interaction between T, D and TM. There were main effects of T ($p < 0.001$), D ($p < 0.001$), TM ($p < 0.001$) and DAY ($p < 0.001$) and interactive effects of T x D ($p = 0.008$), T x DAY ($p < 0.001$) and T x TM ($p < 0.001$). There was no interactive effect of D x DAY ($p = 0.809$), D x TM ($p = 0.733$), DAY x TM ($p = 0.085$), T x D x DAY ($p = 0.789$), T x D x TM ($p = 0.141$), T x DAY x TM ($p = 0.275$), D x DAY x TM ($p = 0.503$) and T x D x DAY x TM ($p = 0.975$). Means denoted with a different letter signify significant differences between treatments in the same temperature conditions ($p < 0.05$). Significant differences between groups are denoted by a different letter. *n* = 3-4 birds/trt.

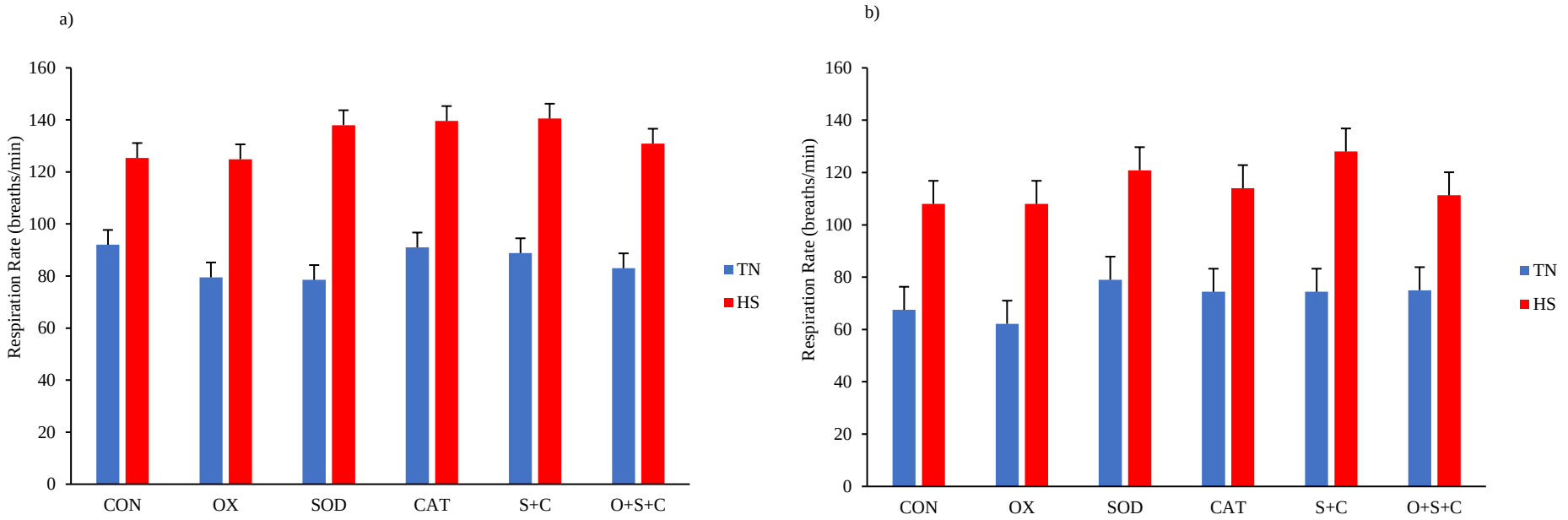


Figure 4. Respiration rate of broilers exposed to TN (TN) or heat stress (HS) conditions and fed either a control diet (CON) or diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C). RR was taken at 11:00 on day 19 and 40. Panel a represents the effect of temperature (T) and diet (D) on RR on day 19 with standard error of the difference for the interaction of T x D. There was a main effect of T ($p < 0.001$) and D ($p = 0.013$) on RR and a near significant T x D interaction ($p = 0.058$). Panel b represents the effect of T and D on day 40 with standard error of the difference for the interaction of T x D. There was a main effect of T ($p < 0.001$) and a near significant effect of D ($p = 0.066$). There was no T x D interaction ($p = 0.80$). Day 19 *n* = 10-12/trt. Day 40 *n* = 7-12 birds/trt

3.3.2 Blood Chemistry and Oximetry

Day 17:

Blood haematocrit percentage was reduced in HS broilers compared to TN controls (18.0 vs. 16.7 %, $p = 0.005$) on day 17 (

There was no main or interactive effect of diet on haematocrit percentage. There was no main effect of temperature or diet on haemaglobin levels but there was a slight interactive effect ($p = 0.071$). Turning to electrolytes, HS had no effect on potassium concentration in the blood. However, there was a main effect of diet ($p = 0.02$), whereby birds fed CAT showed significantly higher levels of potassium than birds fed the control diet (5.38 vs. 6.45 mM). There was no temperature and diet interaction on blood potassium. Chloride concentrations remained unaffected by temperature, diet or a temperature and diet interaction. Calcium concentrations were reduced by HS (1.58 vs, 1.52 mM, $p = .04$) but there was no main or interactive effect of diet. Blood lactate tended to decrease in response to HS (6.56 vs. 5.59 mM, $p = 0.061$), while blood glucose tended to increase (14.4 vs. 15.0 mM, $p = 0.08$). There was no main or interactive effect of diet on lactate or glucose. Blood sodium remained unchanged in response to temperature, diet or temperature and diet interaction.

Blood pH was increased by HS (7.38 vs. 7.43, $p = 0.031$) but remained unchanged by diet or a combination of temperature and diet on day 17. Partial pressure CO_2 was also affected by HS, with HS decreasing pCO_2 by 12.6% (45.8 vs. 40.0 mm Hg, $p < 0.001$). There was no diet or temperature and diet interactive effect on pCO_2. HS tended to reduce total CO_2 in the blood (29.2 vs. 28.2 mM, $p = 0.072$). There was also a significant effect of diet ($p = 0.042$) on total CO_2, although no treatment group was significantly different than controls. There was no temperature and diet interaction on total CO_2. Partial pressure O_2 and O_2 saturation were unchanged by temperature, diet or temperature and diet interaction. Similarly, no significant change was detected in blood HCO_3^- levels, base excess in the extracellular fluid (BE^{ECF}) and anion gap across treatments. There was no main effect of temperature on base excess in the blood (BE^b) but there was a near significant effect of diet ($p =$

0.064). However, no treatment group differed significantly from CON. There was no temperature and diet interaction on BE^b.

Day 38:

On day 38, haematocrit was reduced by HS (18.2 vs, 16.5 %, $p < 0.001$) (**Error! Reference source not found.**). Haematocrit was also significantly affected by diet ($p = 0.036$), with both OX and SOD increasing the amount of red blood cells (RBC) in the blood compared to controls (16.4 vs. 18.2%, 16.4 vs. 17.9%, both $p < 0.05$). There was a near significant temperature and diet interaction ($p = 0.098$). Under TN conditions, birds fed SOD or S+C had higher haematocrit levels than birds fed CON diets (16 vs. 19.3%, 16 vs. 18.7% respectively, both $p < 0.05$). Under HS conditions, birds fed OX saw higher levels than birds fed CON (16.0 vs. 18.0%, $p < 0.05$). Haemaglobin was reduced by HS (6.19 vs. 5.60 g/dL, $p < 0.001$). There was also a main effect of diet ($p < 0.039$), with OX and SOD both increasing haemaglobin (5.60 vs. 6.20 g/dL, 5.60 vs. 6.08g/dL, both $p < 0.05$) compared to CON. The interactive effect of temperature and diet ($p = 0.095$) showed SOD, S+C and OX to significantly increase haematocrit compared to CON under TN conditions (5.74 vs. 6.56 g/dL, vs. 6.37 g/dL, vs. 6.3 g/dL, all $p < 0.05$). Under HS conditions, only OX increased haematocrit levels compared to controls (5.46 vs. 6.09 g/dL, $p < 0.05$). HS tended to reduce blood potassium concentrations (5.70 vs. 5.88 mM, $p = 0.091$) but there was no main or interactive effect of diet. HS increased calcium concentrations (109.4 vs. 110.9 mM, $p = 0.003$) and reduced chloride (1.56 vs. 1.52 mM, $p < 0.001$). There was no main or interactive effect of diet on either chloride or calcium. Lactate, glucose and sodium concentrations remained unchanged in response to temperature, diet or an interaction between the two.

There was no main effect of temperature, diet or an interactive effect on blood pH on day 38 (**Error! Reference source not found.**). There was a near significant effect of temperature on pCO_2, as HS tended to reduce pCO_2 (48.6 vs. 45.6 mm Hg, $p = 0.079$). There was no main or interactive effect of diet. Turning to total CO_2, HS significantly reduced total CO_2 (28.0 vs. 27.0 mM, $p = 0.023$). There was no main effect of diet but there was a near significant temperature and diet interaction ($p = 0.074$). For pO_2 and O_2 saturation, there was no effect of temperature, but there was

an effect of diet ($p = 0.002$ and $p < 0.001$ respectively). OX and CAT reduced pO_2 (40.4 vs. 30.7, 40.4 vs. 32.8 mm Hg), as well as O_2 saturation (72.2 vs. 56.0 %, 72.2 vs. 55.6 % respectively, both $p < 0.05$), when compared to CON groups. There was no temperature, diet or temperature and diet interaction on either parameter. HCO_3^- levels were reduced during HS (26.4 vs. 25.6 mM, $p = 0.042$) but there was no significant effect of diet. There was a temperature and diet interaction ($p = 0.044$), but there were no significant differences between CON and experimental groups under TN or HS conditions. Anion gap remained unchanged in response to a temperature, diet or an interaction between the two. Base excess of the extracellular fluid was decreased by HS (1.04 vs. -0.005 mM, $p = 0.013$). There was a significant effect of diet ($p = 0.08$), with CAT reducing BE^{ECF} compared to CON (0.833 vs. -0.826 mM, $p < 0.05$). There was also a significant temperature and diet interaction ($p = 0.006$), such that SOD reduced BE^{ECF} under TN conditions (1.38 vs. 0.613 mM, $p < 0.05$), while CAT reduced BE^{ECF} under HS conditions (0.289 vs. -2.69 mM, $p < 0.05$). Base excess tended to be reduced by HS (0.917 vs. 0.199 mM, $p = 0.063$) but there was no effect of diet. There was a near significant temperature and diet interaction on BE^b ($p = 0.07$), although no treatment group differed significantly from CON in either temperature conditions.

Table 5. Blood gas analysis of broilers exposed to thermoneutral (TN) or heat stress (HS) conditions and fed either a control diet (CON) or a diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C) on day 17.

	Temp	Diet							Significance		
		CON	OX	SOD	CAT	S+C	O+S+C	SED[1]	T	D	T x D
Day 17											
Haematocrit, %	TN	18.0	15.8	17.3	21.0	17.0	19.0	1.35	0.05	0.13	0.16
	HS	17.0	17.0	16.0	16.8	16.3	17.3				
Haemaglobin, g/dL	TN	6.13	5.35	5.90	7.13	5.77	6.48	0.60	0.29	0.58	0.071
	HS	5.76	6.47	5.38	5.58	5.55	5.77				
Potassium, mM	TN	5.23	5.53	5.63	6.53	5.73	5.18	0.52	0.33	0.02	0.84
	HS	5.52	5.40	5.04	6.38	5.18	5.00				
Chloride, mM	TN	105	107	107	111	106	107	1.58	0.54	0.32	0.25
	HS	107	107	108	108	108	107				
Calcium, mM	TN	1.57	1.54	1.55	1.59	1.58	1.63	0.08	0.04	0.49	0.69
	HS	1.52	1.50	1.52	1.57	1.41	1.58				
Lactate, mM	TN	5.78	5.88	6.22	7.21	7.12	7.13	1.31	0.061	0.86	0.69
	HS	6.57	5.40	4.88	6.06	5.28	5.33				
Glucose, mM	TN	14.7	13.8	14.7	14.7	14.3	14.4	0.90	0.08	0.32	0.55
	HS	14.3	15.0	16.4	15.1	14.1	15.3				
Sodium, mM	TN	143	144	144	143	145	145	1.66	0.105	0.31	0.49
	HS	143	143	142	143	142	146				
pH	TN	7.39	7.42	7.40	7.35	7.37	7.39	0.06	0.031	0.77	0.78
	HS	7.42	7.42	7.48	7.41	7.46	7.41				
pCO_2, mm Hh	TN	45.8	45.4	46.3	46.9	49.3	40.9	3.95	<0.001	0.90	0.15
	HS	41.4	41.8	37.1	41.7	36.0	42.3				
Total CO2, mM	TN	28.9	30.8	29.8	27.3	29.5	28.8	1.31	0.072	0.042	0.49
	HS	29.2	29.9	28.3	27.4	26.6	27.9				
pO_2, mm Hg	TN	44.3	42.7	42.0	41.2	34.1	44.7	5.20	0.14	0.26	0.16
	HS	42.0	36.2	34.7	30.4	41.5	40.1				
O_2 saturation, %	TN	76.8	76.4	74.8	72.6	61.3	76.5	8.05	0.84	0.26	0.102
	HS	77.5	70.8	74.1	57.4	79.2	74.9				
HCO_3^-, mM	TN	27.5	29.5	28.4	25.8	28.0	27.3	1.44	0.13	0.12	0.73
	HS	26.7	28.6	27.1	26.7	25.5	26.6				
Anion Gap, mM	TN	16.0	13.0	16.5	12.7	16.0	16.3	1.94	0.27	0.30	0.15
	HS	13.8	13.7	12.4	16.0	13.8	17.0				
Base excess extracellular fluid	TN	2.52	4.95	3.57	0.17	2.62	2.38	2.06	0.86	0.24	0.98
	HS	2.28	4.02	3.60	1.45	1.68	2.00				
Base excess	TN	2.10	4.58	3.30	0.10	2.37	2.10	1.68	0.87	0.064	0.66
	HS	3.53	3.63	3.14	1.30	1.56	-0.23				

[1]SED pooled standard error of the difference across treatment groups. T- main effect of temperature; D- main effect of diet; T x D – temperature diet interaction effect. *n* = 3- 6 birds/trt.

Table 6.Blood gas analysis of broilers exposed to thermoneutral (TN) or heat stress (HS) conditions and fed either a control diet (CON) or diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C) on day 38.

	Temp	Diet							Significance		
		CON	OX	SOD	CAT	S+C	O+S+C	SED[1]	T	D	T x D
Day 38											
Haematocrit, %	TN	16.9	18.4	19.3	18.4	18.7	17.4	0.84	<0.001	0.036	0.098
	HS	16.0	18.0	16.4	15.9	15.8	16.6				
Haemaglobin, g/dL	TN	5.74	6.30	6.56	6.28	6.37	5.91	0.28	<0.001	0.039	0.095
	HS	5.46	6.09	5.60	5.36	5.40	5.69				
Potassium, mM	TN	5.60	5.47	5.49	6.26	5.69	5.67	0.28	0.091	0.17	0.42
	HS	5.96	5.99	5.70	5.98	5.98	5.69				
Chloride, mM	TN	110	108	109	109	111	110	1.23	0.003	0.29	0.89
	HS	111	110	111	112	111	112				
Calcium, mM	TN	1.55	1.58	1.56	1.56	1.55	1.56	0.03	<0.001	0.47	0.85
	HS	1.51	1.53	1.51	1.55	1.48	1.51				
Lactate, mM	TN	5.62	5.28	6.47	6.72	5.70	6.01	1.01	0.87	0.60	0.90
	HS	6.19	5.93	5.66	6.68	5.25	5.83				
Glucose, mM	TN	15.4	15.4	15.0	15.3	15.0	14.7	0.58	0.35	0.21	0.47
	HS	15.5	15.2	14.6	16.5	15.1	15.4				
Sodium, mM	TN	144	145	146	145	146	146	1.03	0.47	0.51	0.75
	HS	145	145	146	145	145	146				
pH	TN	7.35	7.36	7.35	7.34	7.35	7.35	0.03	0.29	0.88	0.97
	HS	7.35	7.36	7.38	7.35	7.38	7.36				
pCO_2, mm Hh	TN	46.8	47.7	46.9	50.5	50.0	49.4	3.94	0.079	0.97	0.81
	HS	46.8	45.4	46.1	44.6	43.5	47.5				
Total CO2, mM	TN	27.7	27.9	26.3	28.5	29.0	28.6	1.05	0.023	0.66	0.074
	HS	27.3	26.5	28.1	25.8	27.0	27.6				
pO_2, mm Hg	TN	40.8	26.5	39.3	33.7	43.2	35.7	4.27	0.64	0.002	0.44
	HS	40.1	34.9	37.4	32.0	40.8	38.2				
O_2 saturation, %	TN	74.6	49.4	69.0	58.1	73.6	62.1	6.52	0.62	<0.001	0.36
	HS	69.8	62.6	73.1	53.1	73.4	65.9				
HCO_3^-, mM	TN	26.3	25.8	24.9	26.9	27.5	27.1	0.94	0.042	0.35	0.044
	HS	25.9	25.1	26.7	24.3	25.6	26.2				
Anion Gap, mM	TN	14.3	15.8	15.2	15.0	14.6	14.7	0.87	0.65	0.29	0.94
	HS	14.8	15.5	14.7	15.1	14.8	14.0				
Base excess extracellular fluid	TN	1.38[a]	-0.38[a]	1.51[b]	1.03[a]	1.86[a]	0.72[a]	0.97	0.013	0.08	0.006
	HS	0.29[a]	1.07[a]	-0.61[a]	-2.69[b]	0.51[a]	1.53[a]				
Base excess	TN	1.20	0.97	-0.60	0.92	1.64	1.37	0.93	0.06	0.43	0.07
	HS	0.31	-0.41	1.36	-1.21	0.50	0.65				

[1]SED pooled standard error of the difference across treatment groups. T- main effect of temperature; D- main effect of diet; T x D – temperature diet interaction effect. Means denoted with a different letter signify significant differences between treatments in the same temperature conditions ($p < 0.05$). Significant T x D interactions without differences between CON and experimental groups were not denoted with a letter. n = 7-10 birds/trt

3.3.3 Plasma Antioxidant Quantification

Vitamin A:

Plasma antioxidant levels, including vitamin A, E and C, were quantified on day 17 and day 38. On day 17, there was no main effect of temperature or diet on vitamin A concentrations. However, there was an interactive effect of temperature and diet ($p < 0.001$) (a). Under TN conditions, S+C increased vitamin A levels compared to CON (558 vs. 677 mg/mL, $P < 0.05$). Under HS conditions, supplementing CAT or SOD independently increased vitamin A levels when compared to CON (538 vs. 641 mg/l, 714 mg/mL respectively, both $p < 0.05$). By day 38, HS tended to increase plasma vitamin A levels (644 vs. 677 mg/mL, $p = 0.051$) (b). There was no main or interactive effect of diet on plasma vitamin A.

Vitamin E:

HS increased vitamin E levels on day 17 (29308 vs. 33854 mg/mL, $p < 0.001$) (

Figure *6*a). Diet also had a significant effect on plasma vitamin E levels ($p < 0.001$), with both OX and O+S+C increasing vitamin E compared to CON (23075 vs. 44684 mg/mL, 52612 µg/l respectively, both $p < 0.05$). The combination of O+S+C increased vitamin E to a greater degree than OX alone (94% vs. 128% increase). Interestingly, the opposite effect of temperature was seen on day 38: HS reduced plasma vitamin E levels (32103 vs. 26994 mg/mL, $p = 0.002$) (

Figure *6*b). However, the effect of diet was sustained, with both OX and the combination of O+S+C increasing vitamin E under TN and HS conditions (2223 vs. 43997 mg/mL, 49664 mg/mL, $p < 0.005$). There was no temperature and diet interactive effect on vitamin E concentration at either timepoint.

Vitamin C:

On day 17, there was no effect of temperature, diet or an interactive effect of temperature and diet on plasma vitamin C levels (Figure *7*a). However, broilers fed O+S+C had the highest levels of vitamin C (9.77 µg/ml) followed by OX, CAT, CON, S+C and lastly SOD (8.38, 8.26, 6.06 and 5.74 µg/ml respectively). On day 38, there was a main effect of temperature, with HS significantly reducing plasma vitamin C by nearly 15% (12.7 vs. 10.8 µg/ml, $p < 0.001$) (Figure *7*b). There was also a main

effect of diet (p = 0.03), with birds supplemented with OX having significantly higher plasma vitamin C levels than birds fed the CON diet.

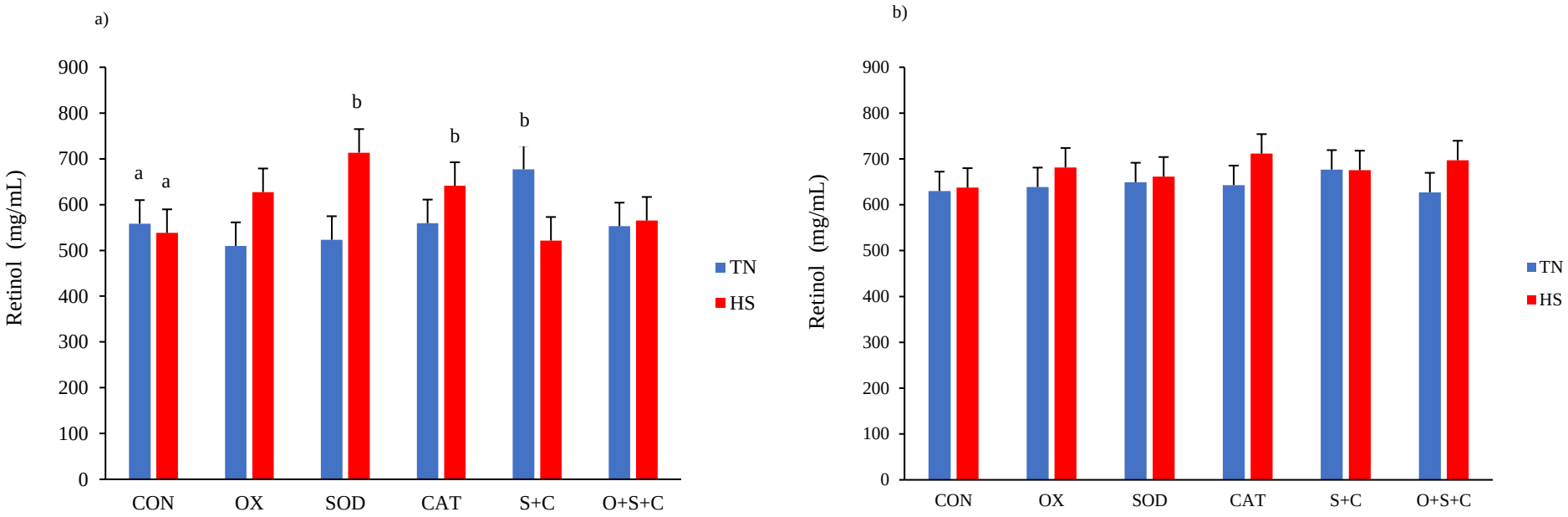


Figure 5.Plasma vitamin A concentrations in broilers exposed to TN (TN) or heat stress (HS) conditions and fed either a control diet (CON) or diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C). Panel a represents vitamin A levels on day 17 with standard error of the difference for the interaction of T x D. There was no main effect of T (p = 0.107) or D (p = 0.33) but there was a significant T x D interaction (p < 0.001). Panel b represents vitamin A levels on day 38 with standard error of the difference for the interaction of T x D. There was a slight main effect of T (p = 0.051) and no main effect of D (p = 0.739). There was no T x D interaction effect (p = 0.742). Means denoted with a different letter signify significant differences between treatments in the same temperature conditions (p < 0.05). Significant T x D interactions without differences between CON and experimental groups were not denoted with a letter. Day 17 n = 3-6 birds/trt. Day 38 n = 8-12 birds/trt.

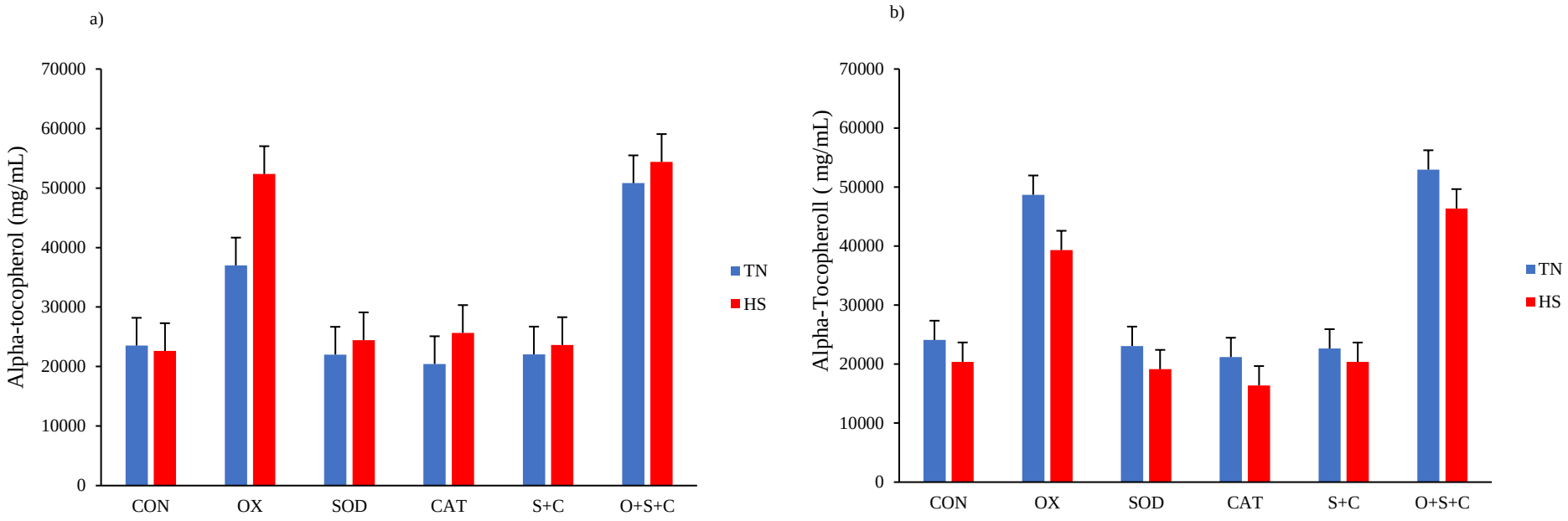


Figure 6. Plasma vitamin E concentrations in broilers exposed to TN (TN) or heat stress (HS) conditions and fed either a control diet (CON) or diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C). Panel a represents vitamin E levels on day 17 with standard error of the difference for the interaction of T x D. There was a main effect of T ($p < 0.001$) and D ($p < 0.001$) but there was no significant T x D interaction ($p = 0.112$). Panel b represents vitamin E levels on day 38 with standard error of the difference for the interaction of T x D. There was a main effect of T ($p = 0.002$) and D ($p < 0.001$). There was no significant T x D interaction ($p = 0.65$). Day 17 n = 4-6 birds/trt. Day 38 n = 8-12 birds/trt.

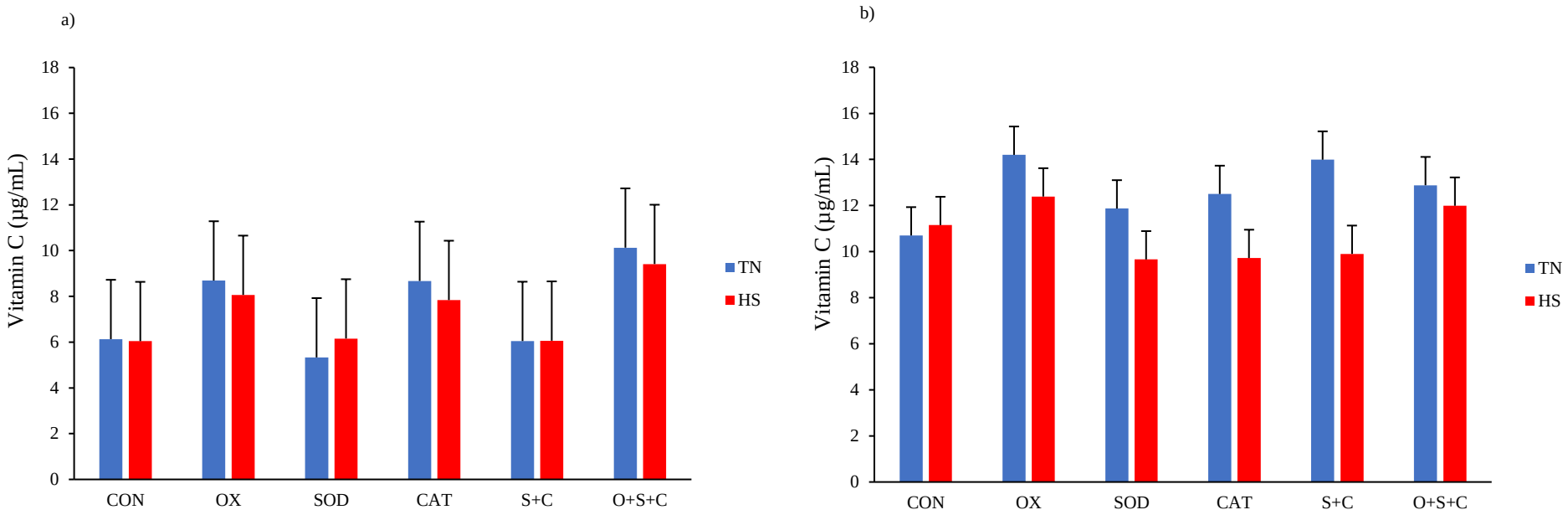


Figure 7. Plasma vitamin C concentrations in broilers exposed to TN (TN) or heat stress (HS) conditions and fed either a control diet (CON) or diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C). Panel a represents vitamin C levels on day 17 with standard error of the difference for the interaction of T x D. There was no main effect of T ($p = 0.835$), D ($p = 0.161$) or T x D interaction ($p = 0.998$). Panel b represents vitamin C levels on day 38 with standard error of the difference for the interaction of T x D. There was a main effect of T ($p < 0.001$) and a main effect of D ($p = 0.01$). There was no interactive effect of T x D ($p = 0.145$). Day 17 *n* = 5-6 birds/trt. Day 38 *n* = 8-12 birds/trt.

3.3.4 Transepithelial Resistance

Ileal TER was determined on day 21 and day 42 of the study. On day 21, there was no effect of temperature, diet or interactive effect of temperature and diet on TER (Figure 8a). However, TER was lower in HS broilers (151 vs. 140 $\Omega*cm^2$) compared to TN broilers and birds supplemented with SOD showed significantly reduced TER values (153 vs. 111 $\Omega*cm^2$, $p < 0.05$) compared to birds supplemented with CON. Birds fed O+S+C and CON diets had the highest TER at 175 $\Omega*cm^2$ and 153 $\Omega*cm^2$ respectively. SOD had the lowest TER at 111 $\Omega*cm^2$.

On day 42, HS reduced TER by nearly 15% (163 vs. 138 $\Omega*cm^2$, $p = 0.043$) (Figure 8b). There was no main or interactive effect of diet on TER. However, birds fed S+C and SOD had the highest TER at 174 $\Omega*cm^2$ and 169 $\Omega*cm^2$respectively, and birds fed CAT had the lowest TER at 126 $\Omega*cm^2$.

A summary of the effects of temperature and diet on physiology, blood gas status, antioxidant status and TER is reported in Table 7. This provides a visual for both performance and MQ data together, with indicators whether measures increased or decreased or remained unchanged.

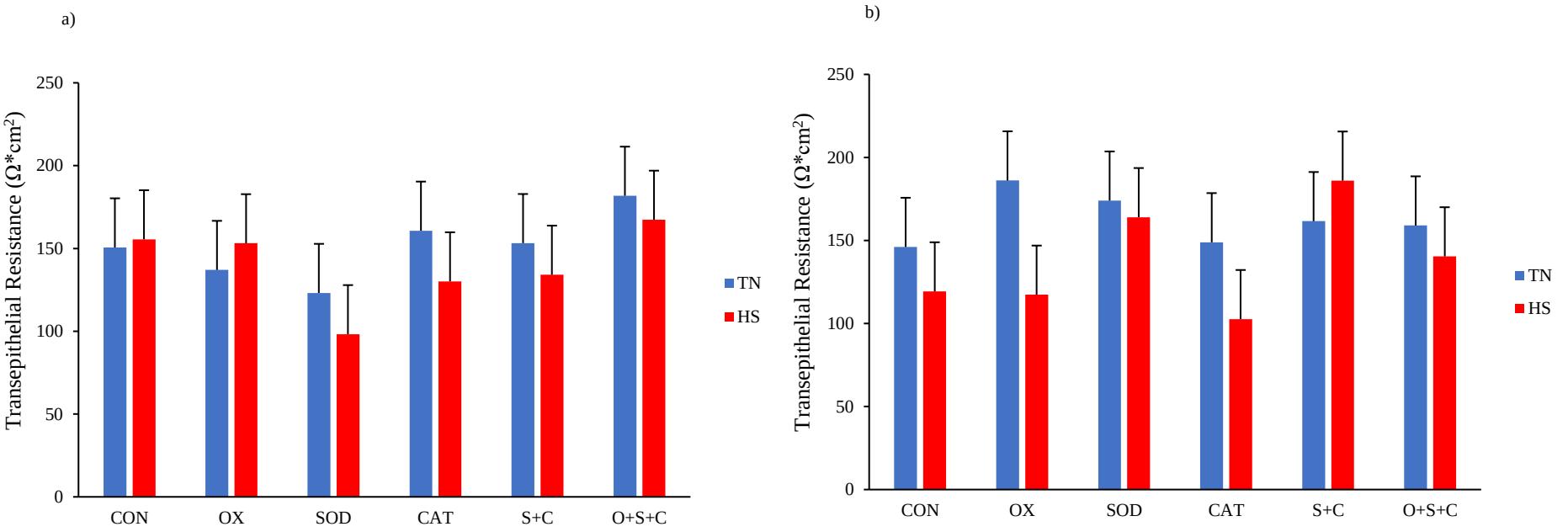


Figure 8. Transepithelial resistance in broilers exposed to TN (TN) or heat stress (HS) conditions and fed either a control diet (CON) or diet supplemented with Oxicare (OX), superoxide dismutase (SOD), catalase (CAT), SOD and CAT (S+C) or Oxicare, SOD and CAT (O+S+C). Panel a represents TER levels on day 21 with standard error of the difference for the interaction of T x D. There was no main effect of T (p = 0.353), D (p = 0.103) or Tx D interaction (p = 0.864) on TER at day 2. Panel b represents TER levels on day 42 with standard error of the difference for the interaction of T x D. There was a main effect of T (p = 0.043) on TER, but no main effect of D (p = 0.216) or T x D interaction (p = 0.339) on TER. Day 21 n = 6 birds/trt. Day 42 n = 6-11 birds/trt.

Table 7. Summary of the effects of temperature and diet on physiology, blood gas status, antioxidant status and gut physiology.

Parameter	Temperature Effect	Diet Effect				
	HS	OX	SOD	CAT	S+C	O+S+C
Physiology						
RT, °C	↑ all pooled ↑ d21 ↑ d42	↓ TN all pooled	↑ HS d21	↑ all pooled ↑ HS all pooled ↑ HS d21	↓ all pooled *TN all pooled	-
RR, breath/min	↑	↓ TN d21	↑ HS d21 ↓ TN d21	↑ HS d21	↑ HS d21 ↑d42	-
Blood Analysis						
pH	↑ d21	-	-	-	-	-
pCO_2, mm Hg	↓ d21 Trend ↓ d42					
Total CO_2, mM	Trend ↓ d21 ↓ d42					
pO_2, mm Hg		↓ d42		↓ d42		
O_2 Saturation, %		↓ d42		↓ d42		
HCO_3^-, mM	↓ d42					
Anion Gap, mM	-	-	-	-	-	-
BE^{ECF}	↓ d42	↓ TN d42		↓ d42; *HS		

BE	Trend ↓ d42					
Haematocrit, %	↓d21 ↓d42	↑ d42; *HS	↑ d42; *TN	-	↑ TN d42	-
Haemaglobin, g/dL	↓ d42	↑ d42; *TN; *HS	↑ d42; * TN	-	↑ TN d21	-
Potassium, mM	Trend ↓ d42	-	-	↑ d21	-	-
Chloride, mM	↓ d42	-	-	-	-	-
Calcium, mM	↓ d21 ↑ d42	-	-	-	-	-
Lactate, mM	Trend ↓d21	-	-	-	-	-
Glucose, mM	Trend ↑ d21					
Sodium, mM						
Antioxidant Status						
Vitamin A, mg/mL	Trend ↑ d42	↑ HS d21	↑ HS d21		↑ TN d21	
Vitamin E, mg/mL	↑ d21 ↓ d42	↑ d21, ↑ d42	-	-	-	↑ d21 ↑ d42
Vitamin C, µg/mL	↓ d42	↑ d42	-	-	-	
Gut Physiology						
TER (Ω*cm^2)	↓	-	-	-	-	-

HS: heat stress, OX: Oxicare, SOD: superoxide dismutase, CAT: catalase, S+C: SOD + CAT, O+S+C: Oxicare+SOD+CAT RT: rectal temperature, RR: respiration. Effects are characterized as significantly ($p < 0.05$) increasing (↑), decreasing (↓) or no effect (-). Trend denotes the effect as being near significant ($0.05 < p < 0.1$). D21 or d42 indicate whether this effect was seen around the day 21 or day 42 timepoint respectively. For RT, data from d21 and d42 were pooled across 12:00 and 15:00 time points. HS or TN indicates if the effects were seen under heat stress or TN conditions respectively. HS or TN accompanied by * indicates a temperature and diet interaction in addition to a diet effect. Under RT, all pooled indicates day was pooled across days and time points.

4 Chapter 4: Discussion

This study set out to confirm the effects of cyclic HS on broiler chickens as well as investigate the potential therapeutic effects of antioxidants, antioxidant enzymes or a combination of the two. We tested this by creating an environment to mimic that of a heat wave, with high daytime temperatures followed by cooler overnight temperature. Birds were fed either the control diet or one of five experimental diets and reared to day 42. Growth performance, meat quality, physiological measures, blood parameters, antioxidant status and gut integrity were measured across the study to determine the effects of temperature and diet across different time points. A portion of the birds were also slaughtered on day 21 to investigate the onset of the effects of temperature and/or diet. Ross-308 broilers were selected due to their prevalence in commercial farming and reared to an age that reflects common industry practices, in which broilers are usually slaughtered between day 35 and 42 days of age. It should be noted that measures taken on or around day 21 (i.e., day 17, 18, 19 or 20) will be referred to as representing the day 21 timepoint. Likewise, measures taken on or around day 42 (i.e., 38, 39, 40, 41) will be referred to as representing the day 42 time point.

The functionality of the HS model was confirmed by strong temperature effects across both production and physiological parameters. The depressed growth performance over the entirety of the study is consistent with previous studies from our lab (Shakeri et al., 2018a; Shakeri et al., 2019b) as well as others (Cooper and Washburn, 1998; Gu et al., 2008). Yet, when isolating the effects of HS from day 0 to 21, HS tended to reduce ADFI and significantly reduced ADG. These results contrast with past work, which reported no effect of HS on ADFI, ADG and FCR during this period (Shakeri et al., 2018a; Shakeri et al., 2019b, 2020c). This may be due to the differences in body weight across studies. For example, when comparing final BW from this study to others (Shakeri et al., 2018a; Shakeri et al., 2019b, 2020c), TN control birds we approx. 400-500g heavier and HS control birds were approx. 100-300g heavier perhaps indicating a smaller weight difference between the TN and HS groups. While these weights were from day 42, it is likely day 21 weights were also greater than previous studies. Nascimento et al. (2017a) found heat production to be positively correlated with age and body weight

in broilers. Therefore, older and heavier are more susceptible to HS as they struggle to maintain homeothermy and cannot dissipate heat effectively.

The lack of effect of diet on growth performance contradicts our hypothesis and juxtaposes previous work. Sahin and Kucuk (2001) reported improved growth performance after vitamin E and C supplementation while Yang et al. (2012) reported a similar effect after $MgSO_4$ supplementation. Oxicare consists of vitamin E, C and $MgSO_4$ so it was expected diets containing Oxicare would improve growth performance to some degree. Furthermore, SOD supplementation also improved growth performance in weaning piglets (Ahasan et al., 2019).

The effects of HS were apparent during the first half of the study, as reflected by the reductions in ADFI and ADG from days 0 to 21 and intensified as the study progressed. These results indicate changes in growth performance can be detected as early as day 21 and may serve as an early indicator of heat stress and timepoint for intervention. Overall, these findings support the theory that HS negatively impacts performance, and these effects are felt to a greater extent as the birds approach slaughter.

In contrast with our hypothesis that HS would negatively influence meat quality, there was no effect of HS on L*, a*, b*, tenderness (as measured by WBSF) or drip loss. Meat quality is often affected by stress, including environmental stress. Meat from heat stressed livestock is often lighter in weight (Zhang et al., 2012; Shakeri et al., 2019a), altered pH (Zhang et al., 2012; Shakeri et al., 2020b) lighter in colour (Lu et al., 2007a; Shao et al., 2019; Shakeri et al., 2020b), less tender (Shakeri et al., 2020b) and have higher drip (Sandercock et al., 2001b; Lu et al., 2007b; Lu et al., 2017) and cook loss (McKee and Sams, 1997). While the results from this study are in contrast to the expectation, they are in line with other studies which also found a lack of response to HS across a*, b*, tenderness and drip loss (Lu et al., 2007a; Baghban Kanani et al., 2017; Shakeri et al., 2019b).

The two parameters that did change in response to HS, were breast muscle weight and cooking loss. This can be linked to the reduced overall BW and reduced feed intake and in birds exposed to HS. The lack of available nutrients due to reduced FI can lead to reduced protein content, synthesis, turnover

and retention (Geraert et al., 1996; Yunianto et al., 1997; Temim et al., 2000; Gu et al., 2008). As muscle is made up of protein, altered protein availability and function can directly result in a reduction of muscle mass.

Cook loss refers to the amount of water loss during cooking and is expressed as a percentage of the precooked weight. In this study, there was a reduction in cook loss in response to heat stress. These results coincide with other studies which also reported a decrease in cook loss in poultry in response to cyclic HS (Shakeri et al., 2019a)(ADD). However, other studies draw contradictory conclusions regarding cook loss and heat stress. Lu et al. (2017) reported no change in cook loss in response to chronic HS (32°C; 7d) while McKee and Sams (1997) even reported a decrease in cook loss in response to chronic heat stress (32-38°C; 4w).

One factor that contributes to cook loss is water holding capacity (WHC). WHC refers to the amount of water bound to protein and is inversely proportional to the water released during protein denaturation or meat transport, storage or cooking (Warner, 2017). There are many ways HS can contribute to protein denaturation and thus impact WHC and cook loss. As mentioned in Section 1.5, HS can trigger respiratory alkalosis which is often coupled with metabolic acidosis. The rise in acidity can lead to protein denaturation and thus a lower WHC (Robertson, 1989; Zaboli et al., 2019) . Protein denaturation can also occur in response to oxidative stress. HS has been shown to stimulate mitochondrial superoxide production (Mujahid et al., 2006; Del Vesco and Gasparino, 2013), reduce T-AOC and antioxidant enzyme concentrations, and increase MDA formation in breast muscle (Lu et al., 2017). These compounds all promote protein degradation and may affect WHC to some degree. A lower WHC may suggest increase water loss prior to cooking, thus decrease the amount of water lost due to cooking alone, as we have reported in this study.

It should be noted that changes in ROS levels, T-AOC, antioxidant enzymes and byproducts of oxidative stress are not always consisted across studies and often depend on the variation and intensity of HS. For instance, Lu et al. (2017) reported increased markers of oxidative stress after 7 days of chronic HS (32°C), while these effects dissipated after 14 days of chronic HS. Similarly, Kikusato and Toyomizu (2019) found an increase in H_2O_2 but no change in SOD, CAT or GPx expression in the

breast muscles after acute HS (34°C; 12h). Therefore, the difference in cook loss across studies in response to HS may be attributed to the molecular changes occurring within the meat.

While cook loss is just one measure of meat quality, it is closely related to juiciness which also is reduced in response to HS (Sandercock et al., 2001a). Juiciness is a prominent factor contributing to customer satisfaction of meat consumption, with higher perceived juiciness contributing to higher customer satisfaction Felderhoff et al. (2020). Therefore, a reduction in cook loss would also lead to reduced satisfaction and negatively impact industry sales and profitability.

Muscle pH is closely linked to other meat characteristics, including colour, water holding capacity, cook loss, drip loss and tenderness (Petracci et al., 2004; Jankowiak et al., 2021). There was no observed change in pH_u in this study, which may contribute to the lack of changes in colour, drip loss and tenderness. However, pH_u did increase in response to SOD. SOD supplementation was also linked to lower L*scores, indicating the meat was darker than control birds. This negative correlation between pH_u and L* is reported elsewhere (Fletcher, 1999; Qiao et al., 2001; Petracci et al., 2004).

Ultimate pH is also correlated meat tenderness, although there is opposing research whether or not it is a positive or negative correlation, and this may be due to the degree of glycolysis and lactic acid production post mortem (Ouali et al., 2006). In this study, broilers fed SOD had the highest pH_u, lowest WBSF suggesting more tender meat. This is in line with Shakeri et al. (2020b) and (Li et al., 2014)but in contrast with Jankowiak et al. (2021). While WBSF provides one indicator of meat tenderness, the myofibrillar fragmentation index (MFI) provides another. Determining MFI for the breast samples of this study will give further insight into whether there are ultrastructure changes to the meat due to diet.

There are many underlying components that contribute to meat quality. Therefore, it would be beneficial to look at MFI, antioxidant enzyme abundance and expression and the level of peroxidation in the breast tissue from this study. These results will determine the association between the previously measured meat quality parameters and the molecular changes occurring in response to temperature or diet.

Rectal temperatures and RR were taken weekly to monitor physiological changes in response to HS and diet. As expected, HS increased RT and these effects were apparent as early as day 14 and persisted to the end of the experiment. Under both TN and HS conditions, RT continued to rise as the study progressed. However, the increase in RT was greater under HS conditions. Shakeri et al. (2019a) reported a similar response in RT over time, with both TN and HS birds experiencing rising RT over the 42-experiment. The steeper rise in RT during HS may be a function of the compounding effect of high ambient temperatures making it more difficult to dissipate heat.

Interestingly, RT was highest at day 35 of the study under both TN and HS conditions. The decrease in RT at day 41 may reflect the decrease in house temperatures for the last week of the study: the TN room was changed to 23°C while the HS rooms was adjusted to 32°C during the day and 24°C overnight. The temperature adjustment would have created a less severe thermal environment for both TN and HS birds, thus reducing RT. However, the decrease over time may be a result of HS acclimatization. Sykes and Fataftah (1986) found the rate of increase in RT in response to daily heat exposure (38°C/4h) continued to decrease over 21 days of HS. This suggests the birds become less susceptible to HS over time. However, this acclimatization was seen over multiple days rather than hours. This could explain why the RT of birds under HS was highest at 15:00, as this time point was taken after 7h of HS exposure.

These results correspond with other studies from this lab (Shakeri et al., 2018a; Shakeri et al., 2019c; Shakeri et al., 2020c), which also reported an increase in RT in response to cyclic HS. Furthermore, the rise in RT was also accompanied by depressed growth performance as reported Section 2.3.2. This is in line with Cooper and Washburn (1998), who also reported a negative correlation between RT and ADFI, ADG and feed efficiency under HS conditions.

Diet played an interesting role in RT, with some diets increases or decreasing RT. When administered separately, both SOD and CAT increased RT. However, when supplemented together, SOD+CAT reduced RT. expected to dampen the progression of oxidative stress to some degree, either by targeting O^{-}_{2} or H_2O_2 respectively. In contrast, the combination of S+C reduced RT. This may be

due to the fact when SOD or CAT are supplemented together, they work in conjunction to neutralize both O^{-}_{2} or H_2O_2 and prevent further oxidizing chain reactions.

When an animal is exposed to high ambient temperatures, they employ thermoregulatory processes to maintain homeostasis, which include evaporative cooling (i.e., panting, sweating) and radiant heat loss (raised wings, shunting of blood to periphery) (Cottrell et al., 2015). However, when these mechanisms fail or if the heat produced exceeds the heat dissipated, core body temperature rises. High core temperature can result in cell damage and apoptosis (Tang et al., 2016; Sato et al., 2019), particularly in dividing cell populations (Khan and Brown, 2002). Cell damage can lead to mitochondrial malfunction and trigger oxidative stress (Sato et al., 2019). Therefore, high RT can directly damage cells through apoptosis or trigger a cascade of negative effects through oxidative stress.

As mentioned in Section 1.3, when exposed to high ambient temperatures, animal try to dissipate heat through evaporative cooling, in this case, panting. Increased RR allows for more air movement through the respiratory tract which promotes moisture evaporation, thus cooling down the animal (Robertshaw, 2006). Therefore, it was expected that those birds reared under HS conditions had higher RR than birds reared under TN conditions. These results are in line with previous studies from this lab (Shakeri et al., 2018a; Shakeri et al., 2019a; Shakeri et al., 2020c) and others (Shao et al., 2019)). While RR serves as a visual clue that may indicate HS, determining the acid-base balance will give further insight into the effect of HS on the respiratory system. The link between RR and respiratory alkalosis will be discussed shortly.

Respiration rate can serve as an early sign of HS, as supported by the higher RR recorded on day 21, which was sustained for the remainder of the study. In another study looking at even younger birds (7-days old), increased RR was recorded after just 1 day of cyclic HS (33°C/8h, 25°C/16h) (Shakeri et al., 2019c). The same is seen in pigs, with higher RR in those exposed to acute (Pearce et al., 2012), chronic (Liu et al., 2018) or cyclic (Le et al., 2020a) HS compared to TN controls. Interestingly, RR was higher on day 19 than day 40. This can be explained through the relationship between BW, RR and tidal volume. As a bird grows, tidal volume of the lungs increases (Nascimento et al., 2017b). The higher tidal volume allows for a reduction in RR because the volume per breath is

higher, thus fewer breaths are needed to achieve the same amount of gas exchange. Therefore, as birds grow, RR naturally declines which was confirmed with this study.

Rectal temperature and RR are clear and early indicator of HS. The marked changes in both measurements suggest the birds were heat stressed but do not indicate whether these physiological changed trigger biochemical changes and oxidative changes in the body. To investigate this, we measured blood gas and electrolyte parameters, plasma antioxidant levels (vitamin A, E and C) and TER.

Understanding interplay of pCO_2, pH and HCO_3^- is useful in determining an animal's acid-base status. The simultaneous reduction in blood pCO_2 and increase in pH in HS broilers at day 21 suggests the birds were experiencing acute respiratory alkalosis. Acute respiratory alkalosis is characterized by an initial reduction in pCO_2, followed by an increase in pH and steady HCO_3^- levels (Brinkman and Sharma, 2022). Acute respiratory alkalosis turned to chronic respiratory alkalosis by day 42, which was characterized by reduced pCO_2, reduced HCO_3^- but normal pH levels. This change suggests a compensatory response as cyclic HS progressed. However, prolonged exposure to HS is not always characterized by a return of pH to normal physiological levels. In fact, some studies investigating the effects of chronic (Teeter et al., 1985) and cyclic HS (Shakeri et al., 2019a) for three weeks or more reported a reduction in blood pCO_2 and HCO_3^- but a significantly higher pH. The high pH represents a different snapshot in time, where the compensatory mechanisms (i.e., reduction in HCO_3^-) have not yet effectively brought down the high pH levels. Understanding the interplay of these parameters suggests a) whether the animal is experiencing respiratory alkalosis and b) the strength of the internal buffering system to maintain homeostasis.

Haematocrit is representative of the percentage of blood that is made of up red blood cells (RBC) (Billett, 1990). High haematocrit may indicate an increase in RBCs or a reduction in blood volume, such as during dehydration, while low haematocrit indicates reduced RBCs. The reduction of haematocrit may suggest RBC damage, as dehydration would have caused the opposite effect. Diets containing OX, SOD or S+C all increased haematocrit, highlighting the protective effect of these antioxidants and antioxidant enzymes. This is in line with our hypothesis, as the components of OX all

have all been show to increase antioxidant enzyme activity and reduce by products of oxidative stress (Panda et al., 2008; Gao et al., 2010; Yang et al., 2012; Yavuz et al., 2013; Abad et al., 2015; Mohammadi et al., 2020). Likewise, SOD is a key antioxidant enzyme in neutralizing $\bullet O^{-}_{2}$, while the combination of SOD and CAT neutralizes both $\bullet O^{-}_{2}$ and H_2O_2, offering further protection against oxidative stress. SOD has also been shown to protect cells from hemolysis, highlighting its role in cell survival (Vouldoukis et al., 2004a; Notin et al., 2010a). Haemaglobin levels were similarly affected to haematocrit, with HS reducing it and OX and SOD increasing it. As hemoglobin is the oxygen carrying protein in RBCs, it positively correlates with haematocrit (Billett, 1990). Therefore, it is expected hemoglobin will fluctuate in a similar manner as haematocrit. This is in line with Shakeri et al. (2019c), who also saw reductions in haematocrit and hemoglobin at day 42. Interestingly, Shakeri et al. (2020c) reported no change in either parameters but this was only after 10 day of cyclic HS. While inconsistent with our results, it is indicative of the temporal changes as cyclic heat stress progresses.

Other changes in blood chemistry included changes in the electrolyte balance. However, most of these changes were only apparent at day 21 and dissipated by day 42. The inconsistency may be indicative of the lability of these measures and testing blood at two time points rather than over times provides a small snapshot of these changes.

As expected, HS decreased plasma vitamin E and C levels. A reduction in antioxidants in response to HS is reported by Kutlu and Forbes (1993) and Padilla et al. (2006). Both vitamins are prominent antioxidants, with vitamin E acting as a chain breaking antioxidant, and vitamin C neutralizing free radicals and regenerating oxidized vitamin E. The reduction in these antioxidants suggest the heat stressed broilers are experiencing some degree of oxidative stress and utilizing the available antioxidants.

Supplementing birds with OX increased vitamin E and C levels, likely due to the inclusion of both vitamins in the diet. This is in accordance with Lohakare et al. (2005) which also reported an increase in vitamin E and C levels after supplementation. Interestingly, the combination of OX with SOD and CAT (O+S+C) only increased vitamin E and not vitamin C levels. These results may be due to the indirect interaction between the antioxidant enzymes and vitamin E. If left unchecked $\bullet O^{-}_{2}$ can

be converted to H_2O_2, and subsequently $OH^{\bullet}$. Hydroxyl radicals initiate lipid peroxidation can a cascade of oxidative events. Vitamin E is a lipid soluble vitamin thus can protect polyunsaturated fats from oxidative damage. It is likely SOD and CAT reduced lipid peroxidation, thus reducing the substrate for which vitamin E acts upon. Vitamin C was likely unchanged as it was still utilized to neutralize non-specific free radicals.

There was an unexpected increase in vitamin A concentrations in response to heat stress. Vitamin A plays is a lipid soluble vitamin and plays a prominent role in reproduction, gut barrier, immune function, and growth performance (Clagett-Dame and DeLuca, 2002; Kucuk et al., 2003 McCullough et al., 1999;). At low levels can lead to compromised gut barrier function, altered TJPs and reduced immune response (McCullough et al., 1999; Quadro et al., 2000; Clagett-Dame and DeLuca, 2002; Kucuk et al., 2003) . Vitamin A can also acts as an antioxidant and can stabilize singlet oxygen, thiyl radical and peroxyl radicals (Palace et al., 1999).

Data on vitamin A levels after a heat challenge or other stressor is scarce, although vitamin A supplementation can improve growth performance (Lin et al., 2002; Kucuk et al., 2003), gut health (Pang et al., 2021) and immunity (Lin et al., 2002) . However, two possible theories are: 1) HS triggered epithelial cell injury leading to the emptying of cellular contents, such as vitamin A, into the blood stream or 2) vitamin A was recruited to reduce oxidative damage, boost immunity and pacify the increased circulating toxins due to increased gut permeability. Although the study was designed to mimic industry standards, it would have been helpful to see if vitamin A levels remained high or decreased past the 42-day mark. This would give insight into vitamin A utilization after sustained stress. Furthermore, measuring LPS in the blood stream or vitamin A and MDA concentrations in tissue to determine oxidative damage would be beneficial in determining vitamin A's protective effects against both endotoxins and oxidative stress. Lastly, measuring vitamin A levels in the liver will indicate stored vitamin A, which can be toxic at high amounts (Olson, 1984; Tang et al., 1985; Yuan et al., 2014).

The reduction in TER in response to HS agrees with the hypothesis that HS will negatively impact gut barrier function. It is also supported by past work from the lab which found cyclic HS to reduce TER in ileum (Shakeri et al., 2018a; Shakeri et al., 2020d). Other studies have reported a

reduction in jejunum TER, indicating damage is not isolated to one section of the GIT (Song et al., 2014; Goo et al., 2019a; Shakeri et al., 2019c). The negative effects of HS on TER have also been reported in pigs (Pearce et al., 2013a; Pearce et al., 2013b; Sanz Fernandez et al., 2014; Liu et al., 2016a) and in vitro cell culture models (Varasteh et al., 2015; Sandner et al., 2020).

This reduction in barrier function in response to HS may be linked to several possible factors including hypoxia and changes in TJPs. During heat stress, blood is shunted away from the splanchnic bed and to the periphery to encourage heat dissipation. The lack of blood flow to the gut and other organs reduces nutrient and oxygen availability creating hypoxic conditions (Hall et al., 1999). Hypoxia can increase ROS formation and reduce antioxidant defense, thus triggering oxidative stress (Bottje et al., 1995; Ramanathan et al., 2005; Coimbra-Costa et al., 2017). Alterations in TJPs have also been associated with depressed barrier function and TER (Pearce et al., 2013b; Pearce et al., 2015).

It is postulated the animals in this study were undergoing heat stress and therefore were shunting blood away from the splanchnic bed. This may have induced oxidative stress in the GIT, altered expression of proteins involved in barrier function, damaged the intestinal epithelium and/or encouraged the translocation of harmful toxins into the blood stream. The absence of a diet effect is interesting, as it was expected antioxidant enzymes would reduce oxidative stress and thus protect gut barrier integrity.

Further work is required to investigate the extent of GIT damage and provide insight into specific areas of intervention. For example, morphometric analysis will help determine possible structural damage associated with HS, as previous studies have reported damaged epithelium (Mitchell et al., 1992; Al-Fataftah and Abdelqader, 2014; Shakeri et al., 2020d) and sloughing of endothelial cells in response to HS (Lambert et al., 2002). Looking at abundance and gene expression of TJPs will also help give insight into gut barrier integrity, as TJPs control cells polarity and the selective passage of ions and molecules in between cells (Bhat et al., 2019). Measuring endotoxin levels in the blood is yet another marker of gut permeability, as acute, chronic and cyclic HS have all been shown to increase circulating LPS (Pearce et al., 2013b; Alhenaky et al., 2017). Reduced gut barrier function can leave

birds vulnerable to infection, such as *Salmonella*, which not only poses a risk to the birds themselves, but to consumers as well (Quinteiro-Filho et al., 2012).

5 Chapter 5: Concluding remarks

5.1 Summary of findings

We set out to determine if the HS model used in this study was adequate to induce heat stress in the broilers, and if so, under which parameters were these changes most prominent? We also sought to determine if the antioxidants and/or antioxidant enzymes added to feed were effective in ameliorating the effects of HS. And finally, we attempted to show when the effects of HS, antioxidants and/or antioxidant feed on broiler production and physiology could be detected.

The effects of HS were most apparent in heat stress and physiological measures including RT and RR and confirm that our climate model provoked a heat stress response.. HS reduced growth performance and increase RR and RT, suggesting the birds were suffering from the raised temperatures. There was also strong negative effect on TER, antioxidant levels and respiratory alkalosis which indicates a biochemical change in response to HS. However, the effect was weaker in areas such a meat quality and some blood parameters. It may be possible the decrease in temperature in the climate rooms during the final days of the study may have dampened the effects of heat stress. Bringing down the temperature from 33°C during the day and 25°C overnight to 32°C and 24°C respectively, was a joint decision between myself, my supervisor and the Animal Ethics Committee. Although it may have added a confounding variable to the study making the effects of HS less intense, it was necessary to maintain the health and welfare of the birds. If the temperatures were maintained at the proposed 33°C and 25°C, here may have been a stronger effect of HS across all parameters but may have results in increased mortality and excessive stress. Further lab analysis is required to determine the expression of antioxidant enzymes and HSPs, extent of lipid peroxidation, gut morphology, ileal digestibility and ROS formation in the gut. These tests will provide further insight into if the extent of oxidative stress and the effects of temperature at the molecular level.

The data indicate that the feed additives can elicit a detectable response in some parameters. However, it is hard to conclude whether they would be beneficial, as some additives elicited positive, negative or conflicting effects across parameters. For example, SOD produced darker and more tender meat, but at times increased RR and RT compared to controls. Thus we cannot conclude that a particular diet is more favorable over the other or the CON diet itself. The inconsistent interaction effects across the study also support this, with clear conclusions not being able to be drawn at the present time.

Some of the measured variables are labile and only provide a snapshot in time, such as with the blood gas analysis. Further research would need to be conducted regarding temporal changes in these biomarkers. Due to the mild effects of diet across parameters in the study, it would also be beneficial to analyze composition and activity of antioxidants of the diet. Determining SOD and CAT activity in the respective diets would give us insight into the potential activity of the enzymes in vivo. Looking at the undigested antioxidant enzymes in the digesta, prior to exposure to air, would also provide insight into its digestibility and whether it was or can be digested.

Looking at temporal changes across the study, there were some parameters that were detected as early as day 21, including growth performance, RT, RR respiratory alkalosis and haematocrit levels. However, there were also effects that were only detected at day 21 and dissipated by day 42 including changes in blood electrolytes due to heat stress or the effects of diet on growth performance. Knowing the time of onset of these effects can be beneficial when designing intervention strategies. For example, implementing a heat abatement strategy by day 21 may help ameliorate the changes in growth performance, RT and RR which can prevent further damage due to reduced nutrient availability, hypoxia, oxidative stress and respiratory alkalosis.

The rising animal production, meat consumption and global temperatures has created a risky environment for both producers and animals. The economic loss and deceased animal welfare associated with such an environment makes heat stress mitigation a central focus for the livestock industry over the subsequent years. While there is still considerable research to be done in the field regarding the use of antioxidant enzymes as feed additives, this study highlights the potential indicators of HS and when a suitable intervention may be necessary.

5.2 COVID-19 Impact

Due to the complexity of COVID-19, loss of research time and changing from a PhD to MPhil in April 2022, some outstanding analysis remains. The tissue samples, including ileum, jejunum and cecal digesta, plasma, breast muscle, liver, spleen, ileum and jejunum, were shipped to Village Neuf, France to be analyzed by DSM. The outstanding analysis includes ROS levels (jejunum digesta), ileal digestibility (ileum digesta) microbiome analysis (cecal digesta), antioxidant enzyme abundance (plasma, jejunum, ileum, breast muscle, liver), MDA (plasma, breast muscle, liver), TBARS/MFI (breast muscle), TJPs and MUC2 (jejunum, ileum), HSP70/90 (jejunum, ileum, breast muscle, liver, spleen), vitamin E, A (plasma) and oxidative stress index (breast muscle, liver, spleen).

As mentioned previously, this project was intended to be the first installment of a four-experiment PhD project. The following projects intended to focus on the biomarkers of oxidative stress and the mechanisms of how antioxidant enzyme supplementation may protect against heat stress. Prior to deciding on changing to MPhil, the second project was outlined and granted AEC approval. While this project was and will not be completed under the MPhil program, it intended to be completed by the end of 2022.

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