Amelioration of gastrointestinal damage with Superoxide Dismutase (SOD) in the heat stressed pig

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Declaration

This is to certify that:

The work contained in this thesis is my original work, performed and written to satisfy the criteria outlined in the Honours Guidelines for the Faculty of Veterinary and Agricultural Sciences.

Animal husbandry and physiological observations were performed by a team under the guidance of animal technician Miss Shannon Holbrook BSci (Hons) and Mrs Maree Cox (Animal Facility Manager). However I was the primary contact for this work and assumed primary responsibility. Furthermore I assisted the laboratory results presented under the guidance of Mr Huu Hieu Le (PhD candidate).

The Ussing chambers were performed by Mr Weicheng Zhao (MSci) and Rachel McQuade (PhD) and raw data analysis and interpretation performed by myself.

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Abstract

Heat Stress (HS) causes high morbidity rates in pigs. Pigs' health is negatively affected after a HS event. Pigs raised in tropical and subtropical areas of the world are increasingly effected by overall higher rises in temperature across the globe. Many pigs in intensive farming are lost due to HS which in turn causes substantial economic losses. Due to the actions of climate change increasing global temperature and a structural shift towards tropical agriculture, future animal production systems will require increased management of HS. Pigs that have been exposed to HS experience reduced growth rates, have poorer morbidity and mortality and have poorer welfare outcomes.

Pigs are susceptible to heat stress as they lack active sweat glands and rely overtly on panting and redistributing blood to the skin to radiate heat to the environment. Utilising evaporative cooling can be beneficial to a degree, but when respiration rate (RR) becomes fivefold higher than Thermoneutral (TN) pigs, alterations in the blood, urine and interstitial fluids begin to occur, changing the pH balance and leading to respiratory alkalosis and blood alkalosis.

It has been shown in previous studies that the use of antioxidants are of extreme benefit in correcting and alleviating oxidative stress. Therefore in this study the use of two of the biggest antioxidant pathways in the body will be investigated, namely Superoxide Dismutase (SOD) by using two forms of the enzymes, the raw recombinant form (rSOD) and the protected, plant-based form (Melofeed). The second antioxidant pathway would be Glutathione Peroxidase (GPx), a selonoprotein, and therefore using Selenium (Se) as the ingredient.

96 female, ~35 kg grower, Large White x Landrace pigs, were subjected to varying combinations of diets containing feeds of control diets (fed at piggeries), Se enhanced,

and SOD (rSOD and Melofeed) diets. The pigs were divided evenly between Thermoneutral (TN) and HS conditions and data obtained for physiological parameters, such as respiration rate (RR), rectal temperature (RT) and skin temperature (ST). Terminal blood and urine collections were analysed for blood gas analyses (BGA) and urinalyses, respectively. These data gave valuable information of the amount of HS pigs were under in comparison to TN pigs.

Overall the data presented significant temperature effects seen between HS vs TN groups. Pigs in HS conditions showed increased levels for RR, RT, ST. Reductions in blood bicarbonate, CO_2 and an increase in base excess indicate that the HS pigs were experiencing respiratory alkalosis, a symptom of HS. Furthermore increased urinary creatinine concentrations may reflect compromised renal function through extended buffering against respiratory alkalosis. A reduction in plasma SOD activity was observed in HS pigs, indicating that they were experiencing oxidative stress. This result demonstrates the importance of the anti-oxidant pathway in ameliorating the impact of HS, however the addition of levels of the anti-oxidant Selenium commonly used in industry did not ameliorate the impact of HS on the pigs. Furthermore using SOD enzyme additives either alone or in combination with Selenium did not ameliorate the impact of heat stress or improve anti-oxidant status. In conclusion augmentation of SOD activity appears to be a sound strategy to ameliorate the impact of HS in the pig, but further work remains to identify micronutrient additives and concentrations that may ameliorate the effects of HS on anti-oxidant activity.

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List of Abbreviations

AGapK	Anion Gap, K+
ANOVA	Analysis Of Variance
BE (b)	Base Excess of blood
BE (ecf)	Base Excess of extra cellular fluid
BGA	Blood Gas Analysis
Ca2+	Ionised Calcium
$cHCO_3$-	Bicarbonate
cHgB	Concentration of Haemoglobin
Hgb	Haemoglobin
Cl-	Chloride
Crea	Creatinine
cSO_2	Oxygen saturation
FD4	Fluorescein Isothiocyanate Dextran 4kDa
GIT	Gastrointestinal Tract
Glu	Glucose
GPx	Glutathione Peroxidase
Hct	Haematocrit
HS	Heat Stress
HSPs	Heat Stress Proteins
IU	International Units
K+	Potassium
Lac	Lactate
Na+	Sodium

pCO_2	partial pressure Carbon Dioxide
pO_2	partial pressure of Oxygen
RH	Relative Humidity
RR	Respiration Rate
rSOD	recombinant Superoxide Dismutase
RT	Rectal Temperature
Se	Selenium
SI	Small Intestines
SOD	Superoxide Dismutase
ST	Skin Temperature
TCO_2	total Carbon Dioxide
TER	Transepithelial Electrical Resistance
TN	Thermoneutral
WB	Whole Blood

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Chapter 1

Introduction – Aim, Hypothesis & Literature Review

1. 1 Aim

In previous studies it has been established that in HS pigs the intestinal barriers are damaged due to a heat stressing event or overall hot climates that pigs are exposed to (Pearce et al. 2013b; Liu et al. 2016). HS causes oxidative stress to occur in the gut tissues and therefore an improper balance of free radicals and antioxidants occurs (Liu et al. 2016). Therefore, the aim of this study is to determine the efficacy of supplementing SOD and ameliorating HS in the pig.

1. 2 Purpose and Context

Previous studies of antioxidants (Liu et al. 2015), demonstrate that antioxidants play and important role and reduce oxidative stress in the gastrointestinal tract (GIT) of HS pigs significantly. The use of Selenium and Vitamin E in varying degrees of concentration demonstrated how higher doses significantly reduced and removed oxidative stress to the gut tissues.

For this study the use of two major enzymatic pathways were chosen, SOD and GPx. SOD is able to convert superoxide into less harmful molecules, oxygen and hydrogen peroxide. Oxygen is utilized by the body while hydrogen peroxide is either exhaled or further broken down by catalase enzymes, oxygen and water.

GPx is the second biggest pathway and utilizes Selenium to become activated, and works by continuously recycling reduced glutathione for further scavenging of free radicals.

1. 3 Hypothesis

Therefore the hypothesis for this study were:

1. HS will increase oxidative stress and barrier function of the GIT.
2. Supplementing SOD will increase endogenous SOD levels.
3. Feeding protected SOD (Melofeed) will have larger effects than raw enzyme SOD (rSOD).

1. 4 Literature Review

1. 4. 1 Overview

HS can be a seasonal element that compromises efficient production, such as in the spring and summer seasons in Australia. Around the world in tropical and subtropical countries, this may be problematic for longer periods of time throughout the year due to the nature of the climates. These HS occurrences are likely to increase in time due to drivers such as climate change increasing overall temperatures around the world and increase participation in tropical agricultural leading to more intensive farming practices. Further to this the Food and Agricultural Organization (FAO) has put out a statement saying that:

"The strongest structural trend in livestock production has been the growth of intensive, vertically integrated, intensive establishments close to large urban centers, particularly for pig and poultry meat production in East Asia and Latin America, and broiler production in South Asia."

Bruinsma et al. (2003) World agriculture: Towards 2015/2030 a FAO perspective.

These points must be considered in the approach taken towards intensive farming practices if animal welfare. Therefore predicted impacts of climate change are that there will be an increased average temperature, which translates to extended duration and severity of summer heat waves (Simonetti, Perna, Giudice, Cappuccio, & Gambacorta,

2018). Because of intensive practices this will further pose a challenge for feeding growing populations with safe and affordable meat (Aksit, Yalcin, Ozkan, Metin, & Ozdemir, 2006), dairy and eggs (Hristov et al., 2018).

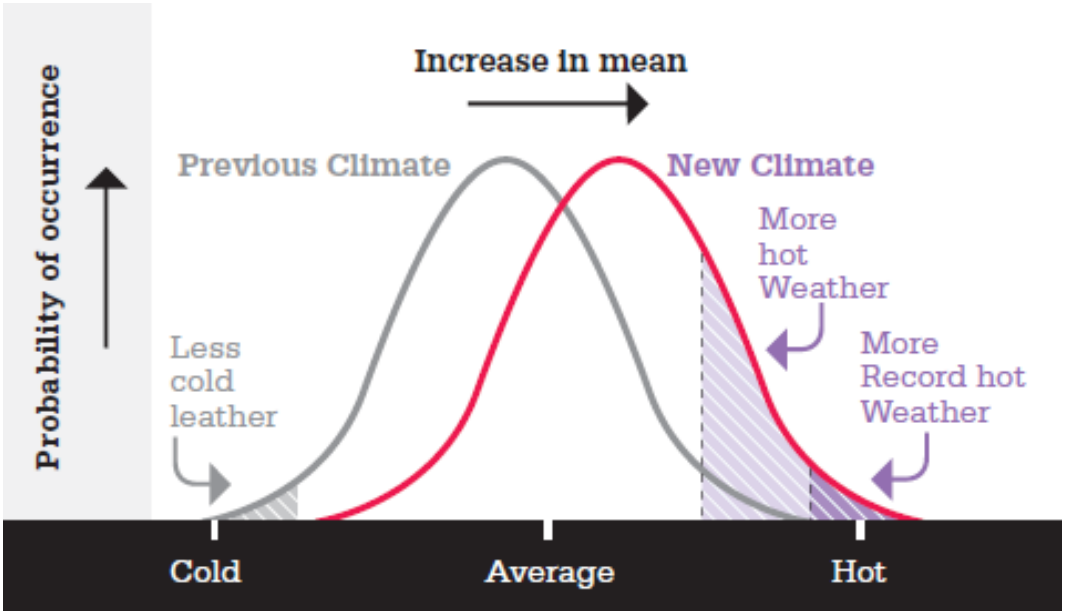


Figure 1. *The two bell curves demonstrates the amount of time livestock now spend in hot weather due to the bell curve extremities set by the new climate temperatures. The extremities from previous climate remain, and thus overall animals are exposed to hotter temperatures. (Bell curve chart courtesy of Lesley Hughes, Climate Council).*

Pig farming is an excellent case in point for how important it is to find apt alternative ways to care for livestock during a heat wave, or for pigs that are farmed in tropical or hot climates (Cottrell et al., 2015). In pig farming, the decrease in sow productivity, as a result of HS in one year amounted to $118 million dollars in the USA (St-Pierre et al. 2003) and highlights the need to find cost effective interventions to alleviate HS in farmed animals. As pigs lack sweat glands, it is challenging to raise them in hot climates (Cottrell et al., 2015; Liu et al., 2016). Many are lost to HS events due to their inability to thermoregulate efficiently. Pigs rely on conductive, evaporative and radiative heat loss to cool their core body temperature (Huynh et al., 2005a; Liu et al., 2016). In its natural

environment, or given the opportunity a pig would wallow in mud to help aid in cooling off (Bracke, 2011). Using mud allows for conductive heat loss and why some farms like to provide hogs with wallowing opportunities, this practice is not common on factory or organic farms, but it is not unheard of (Bracke, 2011). Wallowing gives pigs an efficient way to keep cool and manage thermoregulation and the added benefit of removing ectoparasites (Bracke, 2011). In factory farms, or open organic farms, it is difficult to offer pigs mud to wallow in (Bracke, 2011). Other methods have been put in place, such as shade shelters, air-conditioning and misters. However, in very hot climates and areas prone to heat waves, like Australia, this is not sufficient. Pigs will suffer from HS as their internal organs, and in particular the GIT, are often irreversibly damaged from oxidation that occurs during a HS event (Cottrell et al., 2015; Liu et al., 2016).

HS causes an imbalance between free radicals and antioxidants. Free radicals are increased whilst antioxidants are utilised and decreased. This upregulation of free radicals negatively affects cells and tissues, causing irreversible damage to lipid, protein and nucleotide molecules (F. Liu et al., 2018).

To correct oxidative stress from HS, the use of antioxidants proves to be of key importance. In a previous study the use of antioxidants was shown to be of significant benefit in correcting and alleviating oxidative stress (Liu et al., 2016). Liu showed significant improvement to oxidative stress by the use of antioxidants. The use of Vitamin E and Selenium together in varying degrees of concentrations was able to demonstrate that higher doses significantly reduced and removed oxidative stress to the gut tissues. Therefore the effects of HS were ameliorated in those pigs.

The experiment showed that HS reduced intestinal resistance (TER) and increased permeability, indicating reduced intestinal barrier function. Furthermore it highlighted that the use of anti-oxidants (Selenium and Vitamin E) ameliorated the impact of HS.

The use of the antioxidant enzyme Superoxide Dismutase (SOD) has been shown to alleviate oxidative stress in weanling pigs (Lalles, Lacan, & David, 2011). Where the study used weaning piglets to measure the amount of heat shock proteins (HSP) in the stomach, small and large intestinal tissues as a result of HS.

1. 4. 2 Controversies

The controversies surrounding livestock production is the fact that it itself contributes significantly to greenhouse gas emissions, global warming and environmental pollution (Devun 2016, Mourao 2017 and Boontiam 2016). It's a hard topic to bring to farmers, as this is not a beneficial statement to make about their farming future (Mourao 2017 and Boontiam 2016). Major problems of large scale animal farming is not limited to just greenhouse gas emissions, but also manure management, saturation of land from waste excrement and the waste runoff from abattoirs into the oceans (Dedekea 2016).

1. 4. 3 Environmental Impacts

Along with more animals comes additional land required for farming and in many parts of the world deforestation has taken place to account for this (Bonaudo et al., 2014). The effects of deforestation is extensive and comes at the cost of, but not limited to, the destruction of forests, loss of wildlife, the addition of newer endangered species, and passes an unwelcome imbalance to otherwise healthy ecosystems (Bonaudo et al., 2014; Zhang et al., 2015). It is of course understandable that livestock are being raised in more

intensive farming systems. Often, animals inside intensive farming facilities are burdened with this demand, but it is not isolated to just this group. Animals raised in paddocks can also be dealt extreme weather conditions to bear.

1. 4. 4 Impacts of Positive and Negative Stressors on Livestock

Amidst the many stressors that livestock endure is HS and it is one of the biggest contributors to livestock loss (St-Pierre, Cobanov, & Schnitkey, 2003). Livestock animals namely in tropical or warm climates, as well as in open fields are subjected to this impact. These animals in hot climates are exposed to heat waves and long periods of hot weather, along with periods of drought. With so many animals to farm and care for it is becoming difficult to offer them adequate amounts of shade, cooling off areas or adequate air-conditioning especially when they are out in open fields. Add to this climate change, and this has become a serious problem to abate (Cottrell et al., 2015). Piggeries have existing heat reduction technologies in place, such as enhanced shade areas, spray misters and improved ventilation. These will always be a part of the heat management systems, however with climate change as a back drop to increasing intensive farming practices these will not suffice overall management of HS events in pigs.

Pigs subjected to intense HS show the effects of damaged intestinal tissues by losing weight. These are not immediate effects and are more evident approximately a week post the HS event. Animals in this situation are essentially starving. In an ideal situation pigs losing weight and starving should be euthanised on the farm, however at times pigs are found dead before human intervention to euthanise. These animals do not make it to market. An undesirable outcome for the farmer and a suffering way to go for any animal. This phenomenon is common and is sadly a reality of the industry (Hristov et al., 2018; Liu et al., 2016). Animal welfare is interlinked with productivity and profit. Stressed

animals don't grow and aren't profitable. It is therefore important to manage animal welfare standards and minimize possible stressors to animals. Demands from different communities are wanting better living conditions and standards for livestock animals (Bracke, 2011; Bray & Ankeny, 2017) but it is also shown that content animals, that are not stressed, yield better quality and quantity of food products (Bray & Ankeny, 2017; Carroll et al., 2018). It is imperative that this is further researched and novel useful practices be adopted, to reduce where possible and to prevent further loss of animal life from HS on farms.

1. 4. 5 Thermoregulation in the Pig

Metabolic Heat Production

Pigs generate metabolic heat which is a normal process of all animals. There is a constant baseline of metabolic heat production and is increased during periods of activity such as eating, digestion, lactation, pregnancy and all physical activity and growing. During these periods metabolic activity is increased and therefore body temperature is also increased. For a pig to aid its thermoregulation to reduce core body temperature, food intake and activity levels will cease (Cottrell et al., 2015). This is a problem for production animals as no feed intake will affect growth rates.

Other physiological systems that are influenced by a hot climate is fertility of the animals, oestrus cycles in females, bringing pregnancy to full term, litter sizes, offspring sizes and general ability of animals to have adequate nutrient uptake due to metabolic stressors (Hristov 2018). However the metabolic activity for a pregnant and lactating sow would be much higher than for a grower pig. Thus higher metabolic activity is generating increased heat and therefore would be placed under additional HS.

Evaporative Heat Loss

Evaporative cooling involves loosing heat via panting and drooling (Cottrell et al., 2015). High respiration rate (RR) is when the panting increases to high levels of breaths per minute (BPM) and is such a high rate that blood gases in the blood and respiratory system turn to an alkaline environment (Cottrell et al., 2015). To reduce an alkaline state the body would naturally lower the breathing rate however this is not possible in a HS pig. Due to the acid-base upset, the kidneys become involved more intensely to filter out the excess ions and help balance the pH of the blood and this results in low urine pH (Patience, Umboh, Chaplin, & Nyachoti, 2005).

In extreme cases of hyperthermia, very high RR accompanied by open-mouthed panting is observed. RR has been seen to be increased by minimum 2-fold in a HS pigs (Pearce et al., 2013). The respiratory system is effected by the elevated RR due to the hyperventilation implemented by the pig to cool down via evaporative heat loss. The affect is alkalosis of respiratory system (F. Liu et al., 2018). Alkalosis of the respiratory system causes further negative downstream effects on other blood gas parameters in the HS pig (F. Liu et al., 2018). In very hot environments the thermoregulatory system will redistribute blood flow to the periphery of the body to aid in thermoregulation and lose heat via radiative heat loss.

Radiative and Conductive Heat Loss

Most mammals are able to thermoregulate in hotter temperatures to a certain degree, however due the pig's physiology and thermoregulation processes, it is a bit more challenging for pigs to keep cool in hot temperatures, particularly in piggeries and intensive farms (Cottrell et al., 2015). Pigs do not have sweat glands and therefore will manage thermoregulation in HS temperatures by radiant and evaporative heat loss. Radiant heat loss involves the redistribution of blood to the surface of the skin to aid in exchange of metabolic heat energy generated by the pig with the external environment. This would also be used as conductive heat loss if the pig were able to lie down in cool mud or water to draw away excess heat energy and cool the surface blood. Radiative heat loss can only be useful to a certain degree while the temperature is cooler in the environment than that of the pig. This is true in piggeries as there is no opportunity for pigs to wallow and dissipate heat via conductive heat. Once the atmospheric temperature equals or is higher than that of the pig's core temperature, this method will not no longer

be of much aid, as heat is no longer being exchanged. This is when evaporative cooling via panting will occur.

During hyperthermia a blood surge is sent to the periphery away from the splanchnic tissues and visceral organs, to aid in dissipating maximum heat out and away from the body, conversely causing hypoxia to the these very same internal organs (Collin et al., 2001). The villi lining the walls of the GIT tract become oxygen starved and causes cells to die in these tissues.

Hypoxia causes a breakdown in the integrity of the GIT. This is a grave problem and here in lies the major effects of HS (Liu et al., 2016). A compromised intestinal wall lining will interfere with proper transportation and absorption of nutrients (Pearce et al., 2013). HS causes oxidation to occur and degenerate the integrity of the mucosal barrier in the small intestines (SI) and colon (Liu et al., 2016).

Tight junctions are connections and serve to create a free movement of water and electrolytes between the intestinal lumen, passing through the apical membrane, through to the basolateral membrane to the blood (Cunningham, 1997).

After a HS event, such as in cases of extremely hot weather or a heat wave, animals often lose the ability to uptake nutrients from their feed, due to a disarray of physiological and metabolic factors, leading to weight loss and often death (Liu 2016 and Hristov 2018). Furthermore the way in which nutrients are utilised also changes and therefore regardless of how much the animals are fed, they will not be able to uptake nutrients efficiently (Hristov 2018 and Liu 2016).

1. 4. 6 Oxidative Stress

In addition to hypoxia is oxidative stress, where there is an upregulation of free radicals vs antioxidant enzymes (F. Liu et al., 2018). Oxidative stress occurs during HS at the cellular level by the increase in Superoxide free radicals. Superoxide damages lipid, protein and nucleotides thus negatively affecting cells and organ and oxidised proteins are associated with metabolic disorders in animals (F. Liu et al., 2018).

In some instances inflammation may also be present as a result of this environment (Lambert et al., 2002). The length and time an animal is exposed to a heat stressing environment also plays a key role in the amount of oxidative stress that will develop to the gut barrier. This has been shown by an increase intestinal oxidative stress markers in HS pigs. (Pearce et al., 2013).

Oxidative stress affects tight junctions (Rao, 2008) in the HS pig (Liu et al., 2016). Tight junctions play a role in absorption of water and electrolytes but do not allow large organic molecules to pass through. However in the HS pig, where the oxidative stress has caused damage to intestinal barrier integrity (Liu et al., 2016). Since the permeability condition has changed and is now compromised, more water, nutrients and molecules are able to pass incorrectly through the tight junctions into the abdominal cavity, causing excess fluid build-up in the pig, known as ‘leaky gut’ (Cottrell et al., 2015).

HS in animals can upset many physiological systems and declining food intake and reduced activity is one such effect that results when animals are HS (Liu 2016, Cottrell 2015 and Kamal 2018). The process of digestion increases metabolism and further raises core body temperature, thus reducing feed intake is a mechanism adaptation to control to

control thermoregulation (page 637 Veterinary Physiology Text Book 2nd Edition Cunningham).

Reduced feed intake is a direct result of HS in pigs and the main concern in pig farming as it directly affects growth performance of livestock animals (Collin et al., 2001).

1. 4. 7 Antioxidants to Combat Oxidative Stress

In two separate previous HS studies, using sheep (Chauhan, Celi, Leury, Liu, & Dunshea, 2016) and using pigs (Liu et al., 2016) it was presented that using Vitamin E and Selenium together, was beneficial in ameliorating gut barrier damage from oxidative stress in these animals.

Superoxide Dismutase (SOD)

The largest anti-oxidant pathway is Superoxide Dismutase (SOD) and one of the most important to all life that utilises oxygen. SOD breaks down superoxide (O_2-) a free radical, into either oxygen or hydrogen peroxide:

$2\ HO_2 \rightarrow O_2 + H_2O_2$

Superoxide is a by-product of oxygen metabolism. Oxygen is utilised by the body or simply excreted via exhalation, hydrogen peroxide is further degraded by other catalase enzymes (Hayyan, Hashim, & AlNashef, 2016).

Recently a plant base additive that is highly enriched in SOD has been developed. Melofeed, a derivative from melons, contains adequate amounts of SOD that can be harvested and added to feed and diets to enrich SOD levels (Lalles et al., 2011). Recombinant SOD (rSOD) has also be developed and is a laboratory product by-product made by bacteria, and harvested for the sole purpose of collecting SOD.

When this SOD was fed to weanling pigs it was observed to reduce the expressions of heat shock proteins (HSPs) along the gastrointestinal tract, indicating tolerance to weaning stress (Lalles et al., 2011).

HSPs are a stress proteins and their role is to stabilise other proteins during a heat stress event. Their presence or absence in tissue when being analysed allows us to understand if the tissues at the time of collection were under significant heat stress.

Glutathione Peroxidase (GPx) and Selenium (Se)

Selenium itself is not an antioxidant, but forms part of the antioxidant enzyme glutathione peroxidase (GPx), which is one of the body's largest enzymatic anti-oxidant pathways (Liu et al., 2016).

Promising results from initial experiments has spiked interest in these antioxidants and by providing pigs with these antioxidants, or combinations of these antioxidants it is hoped to achieve positive results in maintaining gut integrity in HS pigs (Liu et al., 2016). By using Se as an additive in diets, it spares the requirements of other antioxidants such as Vitamin E and Vitamin C, and conserves their use for other needs.

1. 4. 8 Managing HS

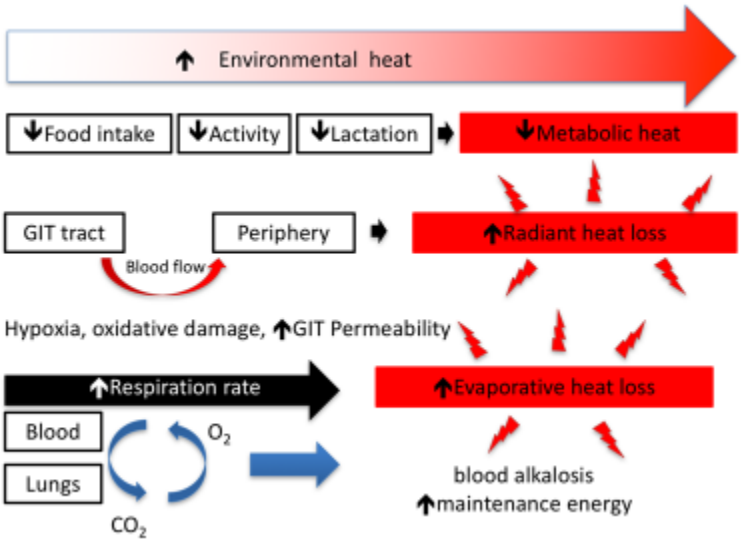


Figure 2. *Showing the overview of heat management during HS in the pigs. As HS increases, metabolic activity decreases. Blood is sent to the peripheries away from the GIT tract, causing hypoxia, oxidative damage and increased GIT permeability. Respiration increases and induces blood alkalosis and respiratory alkalosis.*

Developments into managing HS have been made by the addition of supplementing a pigs' diet with antioxidant feed. In a weather forecast that predicts high temperatures, or a sudden heat wave on the horizon, farmers are able to switch their pigs' feed to an antioxidant diet, whereby the antioxidants can combat or keep at bay the damaging effects of oxidation and allow the gut lining to maintain its integrity. This has proven in past studies to be a very useful and cost effective way to manage the HS (Liu et al., 2016).

It is clear from much work of (Liu 2016, Cottrell 2015, Pearce 2013 and Hristov 2018) that physiological barriers and parameters of metabolic enzymes and hormones are upset by HS. These works have shown the disorder that is thrown upon an individual animal's biological systems and it is very clear that these animals need intervening strategies to

abate the destruction that results from HS. One such method is by diet. By providing antioxidant supplements to the diets of animals, such as adding Selenium and Vitamin E to pig feed prior to an onset of a HS event (Liu 2016), it can have a positive effect on the animal. This has been shown to keep gut integrity throughout and post a HS event (Liu 2016).

1. 4. 9 Future Directions

A previous study (Lalles et al., 2011) was able to demonstrate in a previous study that weanling piglets fed SOD had reduced damaged to the GIT and lowered levels of HSPs. This is an interesting and affordable approach should it prove efficient at reducing or ameliorating GIT damage in the heat stressed pig, and would be worth investigating further.

Developing and implementing new practices into farms is not easy. Added costs and applying new systems takes time, money and energy. Hence, this diet supplementation is the kind of practice and new technology that farmers are on the lookout for. They can be made available and integrated into practice easily, with minimal costs involved. Meat, dairy and egg production needs to be significantly reduced by the public demand if mass changes in the amount of animals reared is going to have any hopes of shifting. It is a hot topic as the growing concerns of our environment are biting at our heels. Meat consumption is not dramatically decreasing, and for greenhouse gas emissions to have a notable drop from livestock gas emissions, our meat consumption must significantly dip before we will notice any positive differences in our global warming problem. Hence, animal farming will continue, but the future of animal farming must beyond doubt use practices in which HS is alleviated or significantly reduced to abate animal loss of

life. Without doing so, more and more animals will be subjected to needless suffering, especially when this particular stressor can be addressed using our technological advances. By using new practices that will give better farming standards it is going to not only help the livestock industry lose fewer animals to HS, but it will also raise happier animals as they will be subjected to one less chief stressor.

Livestock animals at the best of times are subject to many stressors, it would be a beneficial outcome for both the animal and industry aspects to keep further research going in this field, and bring about new and improved practices where animals, farmers and the environment can benefit from.

Chapter 2

Methodology – Materials, Methods & Statistics

2. 1 Ethics Approval

The Animal Ethics Committee (AEC) of the University of Melbourne, approved the experimental design and protocols of this study, including the procedures performed on the experimental pigs. The ethics approved number for this study is: AEC #1714291. The experiment described in this thesis adhered to the code of practice outlined in the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013).

2. 2 Animals and Animal Housing Conditions and Acclimatisation

96 female *Large White x Landrace* grower pigs were sourced from a commercial piggery farm, Berrybank Farm, Hendersons Road, Windermere, 3352 Victoria, Australia. They were transported via a small truck in groups of ten, directly to the Animal Facility, Building 147, Faculty of Veterinary and Agricultural Sciences (FVAS), Parkville Campus, The University of Melbourne, Parkville, 3052 Victoria, Australia.

Upon arrival at the Animal Facility, pigs were weighed (28.1 kg ± 2.3 kg) and then randomly assigned to an individual pen and acclimated for 14 days to their experimental diets. Throughout the acclimation period pigs maintained audial, visual and snout to snout contact with their cohort (Figure 2-1).

Figure 3. *Neighbouring pigs in floor pens, during acclimatisation period. Floors have soft plastic flooring to allow for both comfort and ease of cleaning. These pens have gaps between the walls to allow for socialising and snout touching, an important behaviour for pigs to be able to express in housing conditions.*

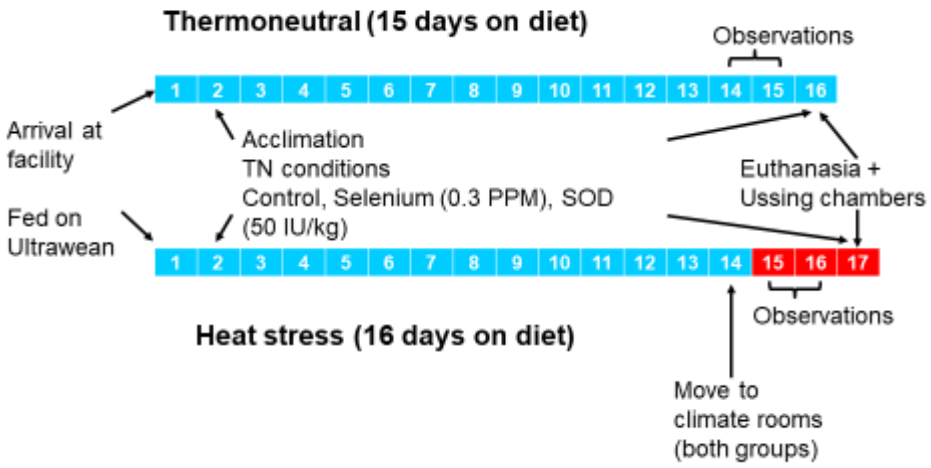


Figure 4. *Time line showing the layout of the experimental design. The top and bottom row highlighting the time line for the TN and HS pigs respectively. Note that the HS pigs were allowed an extra 24 hours of acclimatisation before having the heat turned on to begin the HS.*

For the duration of the acclimatisation period both the temperature and humidity remained a constant 20 ºC / 35 - 45 % RH in the acclimatisation room. Toys such as balls and chew toys were provided to each pig to offer them enrichment to their environment. The radio was left on quietly to provide soft background music in the room.

Figure 5. *Pig in acclimatisation room in a floor pen with enrichment toy.*

At the end of the acclimatisation period the pigs reached a final weight of (33.2 kg ± 2.5 kg) before being moved into climate controlled rooms.

As the pigs were commercially sourced the health status reflected a commercial herd. There were instances of dermatitis and, elevated temperature that required treatment and one pig was euthanised soon after arrival due to a rectal prolapse. Treatment of pigs with ill thrift was managed by the Animal Welfare Officer (AWO) and none of the medication was expected to interfere with the experiment

2. 3 Experimental Diets

Water is always readily available *ad libitum* to all the pigs, all the time and is supplied from a nipple drinking system.

Pigs are fed twice daily, (0900 h and 1500 h) *ad libitum* with formulated diets in pellet form with additional top coat dressing, where required – depending on the type of diet assigned. On Day 1, pigs arrive at the facility at noon and at 1500 h fed e Ultrawean diet, of 500 g and dosed with 1.5 mL of Ulcerguard (Renvet) oral paste to reduce the incidence of ileitis and gastric ulcers after transport. On Day 2, the morning feed at 0900 h consists of each pig receiving 250 g of Ultrawean diet and 250 g of the experimental diet (Table 1) and a final dose of 1.5 mL of Ulcerguard. At 1500 h the complete afternoon feed was given as the specialised assigned diet to all pigs. All pigs were pair-fed at 2.5 x maintenance energy (Huynh et al., 2005b) to ensure the same level of additives were consumed. Pigs were weighed weekly and feed intake adjusted accordingly.

Table 1. *List of all diets used in the experiment with their abbreviations and active ingredients.*

Diet abbreviated name	**Diet Composition**	**Antioxidant / Active Ingredients**
Con	Control diet	Standard grower feed
Se	Control diet with added Selenium 0.3 PPM	Selenium 0.3 PPM
rSOD	Control diet with rSOD	recombinant derived SOD 50 IU top dress
MF	Control diet with Melofeed	melon derived SOD 50 IU top dress
Se*rSOD	Selenium diet with rSOD	Selenium 0.3 PPM + recombinant derived SOD 50 IU top dress
Se*MF	Selenium diet with Melofeed	Selenium 0.3 PPM + melon derived SOD 50 IU top dress

2. 4 Climate Controlled Rooms and Metabolic Cages

After the acclimatisation period, the pigs were then moved into one of the two climate controlled rooms. Thermoneutral (TN) room and the heat stressed (HS) room. Rooms B103 and B104 respectively, in the Parkville Large Animal House. Due to constraints for the speed of processing tissue samples with the Ussing chambers the thermoneutral pigs began the climate challenge one day earlier than the heat stress pigs. The size and capacity of the animal facility and the limited amount of personnel to help on tissue collection days, the 96 pigs were divided up into much smaller replicates of ten pigs per replicate / per week.

There were five pigs in each climate controlled room. The TN room had a constant temperature of 20 ºC and humidity of 35 – 45 % RH. The HS room was set to TN conditions for an extra 24 hours, for those pigs that were assigned the HS room, to give additional time to adjust to the new room and metabolic cages. On Day 2 of the pigs in the HS room the heat stressing began and the temperature was set to rise to 35 ºC / 35 – 45 % RH from 0900 h – 1700 h (during daytime hours) and 28 ºC / 35 – 45 % RH from 1700 h – 0900 h (evening and overnight hours).

The pigs moved from individual floor pens in the acclimatisation room to individual metabolic cages in the climate controlled rooms. Each of these metabolic cages was approximately 1 m x 0.6 m in dimension and allowed for the pig to stand, sit and lie down, but not turn around. Whereas in the floor pens were more spacious and allowed space for a pig to turn around. Therefore as a result of the restrictive nature of metabolic cages, pigs assigned to a HS room were given extra time (an additional 24 hours) to acclimatise to this new condition and environment before the heat stressing experimental period began.

The purpose of the metabolic cages was to allow researchers to perform rectal temperatures five times daily and to ensure that each pig received and ingested the correct amount and type of specialised diet.

Figure 6. *Overview of placement of the metabolic cages in the TN room are seen. Each metabolic cage has an individual water hose attached to supply water to the nipple drinkers. The food receptacles are assembled on the front end, which is also the opening to the cage. Pigs are pushed backwards into the cage and walk forwards when required to exit the cage.*

2. 5 Physiological Monitoring

For the first 48 hours the pigs were in metabolic cages and under experimental conditions, the respiration rate (RR), rectal temperature (RT) and skin temperature (ST) was checked and recorded every two hours during the day. A minimum of five observations were carried out each day at 0900 h, 1100 h, 1300 h, 1500 and 1700 h. In the HS room and pigs that needed closer monitoring, it would have been more than five checks performed, to ensure their safety during the experiment and following instructions of an AWO when a pig was noticed to be very hot.

As part of the intervention criteria for the experiment if a pig reached a rectal temperature of 42^{o}C pigs were cooled for 5 minutes with wet cloths soaked in cold water. If the cooldown was insufficient to aid relief then it was applied a second time. If the pig was not responding to the second cool down treatment, it would then be removed from the HS room and allowed to fully recover from HS. Such measures were never required, but the occasional cool down was necessary. A pig with a rectal temperature of 42 ^{o}C and showing any of the following signs or a combination of them (but not limited to) would be subject for a 10 minute cool down. These symptoms were very high RR (above 143.25 / min), open mouthed panting, drooling while panting, difficulty getting comfortable, bright pink skin, not eating feed and general observation of a distressed pig. Alone any of these signs did not warrant immediate response but were flagged and checked at the next observational time point.

A cool down on a pig consisted of a 10 minute interval using cloths saturated in icy cool water from a bucket and draped over the neck, back and belly of the HS pig. Every few minutes the cloths would be dipped in the icy cool water and reapplied. After 10 minutes

this procedure proved affective enough to relieve the pig of extreme distress and allow for some comfort. The pig receiving the cool off treatments would then be monitored further monitored at 15 minute intervals to observe any changes.

A secondary cool down was sometimes required to a few of pigs, but often the initial cool down allowed the pig to settle down and complete the rest of the day in the HS conditions. No pigs ever warranted complete removal from the HS room.

RR was measured by counting the rise and fall of the thoracic cavity movements of each breath for 20 seconds and then multiplying by three to calculate overall breaths / minute. An electronic stop watch was used to flag when the 20 seconds were up for counting the breaths. Grunts interfered with RR counting and were, recounted a few minutes later if there was an excitable pig.

RT was checked by inserting a thermometer into the rectum every two hours. An S+M (Surgical & Medical Products) Digital Thermometer (South Australia, Australia) was used while also applying lubricant to the device to allow for comfort and minimise distress for the pigs. Pigs were also comforted by belly rubs, pats and ear scratches to allow for some positive association with the unpleasant procedure.

ST was assessed by using a hand held InfraRed Thermometer (DIGITECH). It was held at a distance of about 30 cm from the flank of the pig and the infrared lasers read the surface temperature.

All physiological observational data was recorded on a monitoring sheet, including any flagged pigs or unusual observations made.

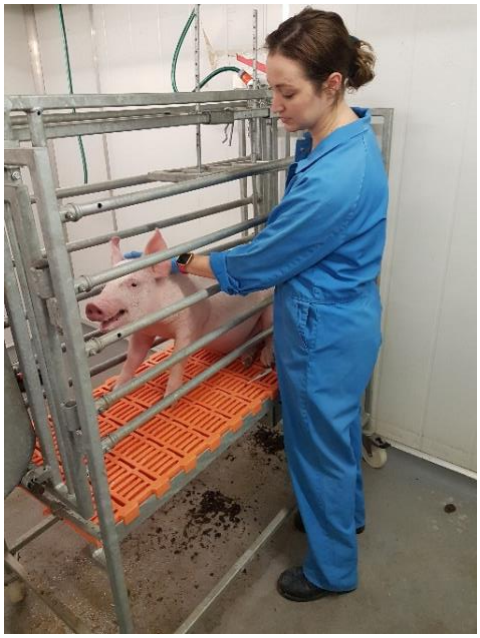


Figure 7. *Observations for RT and ST being carried out on a pig in a metabolic cage in the HS room. Here a pig is shown positive reinforcement by scratching the ears. As pigs are intelligent, when they learn to associate the procedure as a pleasant experience, subsequent observations become easier for both operator and the pig.*

Figure 8. *Cool down procedure, towels saturated in icy cool water is applied to the body of a HS pig. This procedure is very easy and has quick affects.*

2. 6 Anaesthesia

Pigs were first anaesthetised while still in the climate rooms with a mixture of ketamine and xylazine and injected via intramuscular (IM) injection using a butterfly needle with extension tubing. The pigs were monitored until they lost palpebral reflexes and blood collections from the ear vein puncture and jugular were performed. The entire duration of anaesthesia was closely monitored and the depth of anaesthesia was maintained. Periodically a pig would need a top up of ketamine and xylazine to continue the depth of anaesthesia. This was necessary to safely and accurately obtain blood collections for blood gas analysis (BGA) and for whole blood (WB) and plasma collections.

Dose of injectable anaesthesia: 7 mL ketamine (Ketamine 100 mg / mL) and 6 mL xylazine (Xylazil 20 mg / mL). Dosage based on approx. 35 kg body weight of pig.

Administration: using 20 mL syringe and 18 Gauge x 38 mm needles used for IM injection of anaesthetic with extension tubing (BRAUN Heidelberg Extension Tubing 22 cm Luer Lock) for administration.

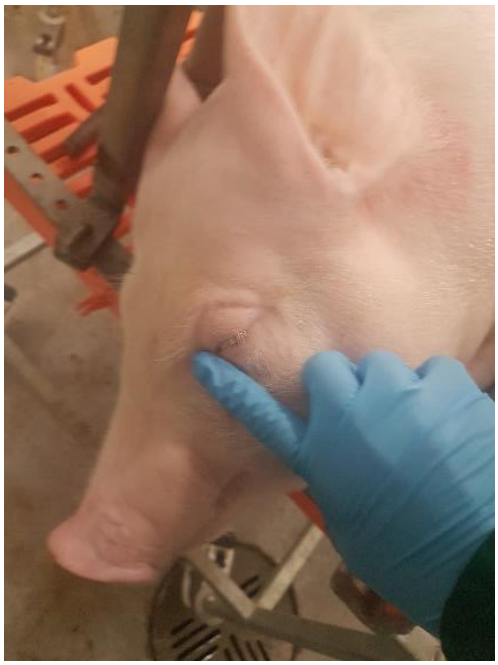


Figure 9. *Testing for absence of a palpebral reflex to indicate depth of anaesthesia level is achieved. Lack of blinking when a finger is gently placed near the corner of the eye indicates the pig is anaesthetised correctly and is ready to have the bloods taken.*

2. 7 Ear Venepuncture Blood Collection and Blood Gas Analysis

Analysis of blood gas concentrations required a small sample of venous blood from the subject. Whilst under anaesthesia, using a 1 mL syringe and a 21 G x 32 mm needle, approximately 1.0 mL venous blood was collected from a superficial ear vein from the pig's ear. For HS condition pigs this procedure was performed in the HS room to maintain the temperature of the blood collected and conserve blood gas concentrations in the sample retrieved. Once the blood was collected, a swab was placed over the puncture site on the vein and pressure applied to cease the bleeding.

The freshly collected blood sample was checked for air bubbles (to minimise error reads and unnecessary aeration to sample) and was immediately introduced into the waiting sample card in the Blood Gas Analyser machine (EPOC®, MA, USA) to promptly analyse and deliver data from the sample.

The panels that the BGA machine tests for in the blood are:

Oximetry levels: pH, pCO_2, pO_2, TCO_2 and cSO_2 ;

Ionic levels: $cHCO_3$-, Na+, K+, Ca2+ and Cl- ;

Metabolic levels: Glu, Lac, Crea, Hct and cHgB and

Base excess: BE[ecf], BE[b] and AGapK.

Figure 10. *Ear venepuncture performed on anaesthetised pig. This blood sample is immediately introduced to the BGA machine to analyse the blood with minimal delay. (A) Demonstrating position of pig (B) Close up of procedure.*

2. 8 Jugular Venepuncture Blood Collection for Whole Blood and Plasma

Approximately 40 mL of venous blood was taken from each pig via venepuncture from the external jugular vein. This was required to sample whole blood and serum. The procedure was undertaken whilst the pig was under anaesthesia and in a supine position. The neck was extended towards the operator collecting the blood. The forelegs were retracted caudally and held in place by an assistant. This position allows the jugular furrow and groove to be visible and guide the placement of the needle to the skin. Using a BD Vacutainer needle 18 G x 38 mm and attached to a Vacutainer holder with a 10 mL LH BD Vacutainer collection tube that is heparinised ready to be clicked on. The needle must be inserted perpendicular to the site and once inserted the Vacutainer may be clicked on. Four collection tubes were collected from each pig. Once the blood was collected it was rocked back and forth to allow the blood to mix with the heparin and avoid any clot formations to occur. Once blood was collected the needle was gently removed and

pressure applied with a finger and gauze pad to the venepuncture site for approximately one minute.

Once blood was collected, WB and serum were pipetted out from the Vacutainer tubes into Eppendorf tubes. To achieve the serum component, the blood was centrifuged for 10 minutes at 10, 000 RPM and the serum (top, clear supernatant layer) pipetted off and aliquoted out into several Eppendorf tubes. WB and serum was then stored at -20 ºC until further analysis in the laboratory. All blood collections were performed under aseptic conditions.

Figure 11. *Jugular venepuncture performed on anaesthetised pig, showing the location of where the collection point is on the neck. The forelegs are drawn back caudally to help obtain the sample promptly.*

2. 9 Euthanasia

After receiving immediate BGA results and collecting enough WB without any clots, the pig was euthanised. Euthanasia was performed by an overdose of Lethabarb (Virbac, 325 mg / mL Pentabarbitone sodium). Each pig received a dose of ~ 20 mL Lethabarb via intracardiac injection using an angiocatheter specialty needle (BD Angiocath 14 G x 83 mm) and a 30 mL syringe. Under anaesthetic conditions, with the pig in supine position, the needle was injected at an approximately 45 degree angle, aimed cranially on the left side, and injected through the 5^{th} and 6^{th} rib. Drawing back on the syringe allowed the operator to see when the needle reached the correct depth and was positioned inside the

heart. This was seen due to a positive surge of bright red blood in the syringe. At this point the full 20 mL of Lethabarb was injected.

Death was confirmed by absence of heartbeat and breath by checking with a stethoscope.

Figure 12. *Intracardiac injection of Lethabarb. Showing the position and angle to attain accurate dosing of the drug and ensure death is achieved quickly.*

2. 10 Tissue Harvest Post Mortem and Urine Collection

Rapid harvesting of tissues and dissection began once death was confirmed. The carcass was placed in a supine position on a dissecting table. Two rolled up surgical drape cloths were placed underneath the shoulder blades to support the carcass during dissection proceedings.

The initial incision was made from the sternum to the navel on the abdomen using a clean sharp scalpel blade. A cut was made until the intestinal cavity was exposed and sharp dissecting scissors were used to cut down and open up the abdominal cavity.
All tissue samples collected from organs and stored in cryovials were immediately snap frozen with liquid nitrogen and stored at -80 ºC until further analysis in the laboratory.

2. 10. 1 Urine Collection Sample

First the bladder was exposed after a mid-line incision and then 10 mL Urine was collected using an uncoated 10 mL Vacutainer and 21 g needle. The urine pH was then immediately quantified using a pH meter (EUTECH Instruments).

2. 10. 2 Small and Large Intestine Tissue Collection

Ileum, jejunum and colon were collected using surgical clamps to section off approximately 30 cm in length of tract and cut using dissection scissors. Ileum and jejunum were flushed with physiological saline using a 50 mL syringe to remove bowel contents. A section of this of about 4 cm in length was cut and put into cryovials and snap frozen in liquid nitrogen. The remaining 25 cm of tract was put into sample pots containing physiological saline and placed on ice. The colon was cut length wise and

washed with physiological saline using the 50 mL syringe to wash out thoroughly faecal debris from between folds.

2. 11 Ussing Chamber Tissue Analysis

Assessment of the gut permeability by Transepithelial Electrical Resistance (TER) and Fluorescein Isothiocyanate-Dextran [molecular weight 4 kDa] (FD4) was analysed by the use of Ussing Chambers (Physiologic Instruments, San Diego, CA, USA). The ileum, jejunum and colon tissue segments were prepared in the laboratory for this analysis. To prepare, each tissue type was cut down to an appropriate size of ~ 8 cm x 5 cm. This was achieved by cutting the gut tubing lengthwise and pinning the tissue with the villi-exposed side, facing down in the Petri dish. A large glass Petri dish with a silicon base was used to allow for tissue pinning. The Petri dish is filled with Krebs' mannitol and physiological saline solutions with additional gasses carbogen (95% O2 and 5% CO2) bubbled into the buffer to keep the tissues oxygenated and carbonated. This step was to allow the tissues to remain in near as possible conditions that were present in the body, and to slow down the tissue degradation process during preparation and analysis. Once the tissue was pinned taught the submucosal and muscularis mucosae layers were removed by gently peeling the tissues back with forceps and exposing the fine mucosal gut lining with the villi. This fine tissue layer is then cut into stamp-sized segments and positioned villi-side down onto a slide with an opening. The tissue is mounted over the opening and fastened into place by the surrounding eight prongs. The prongs allow the tissue to be fastened into position. When positioned correctly the tissue will sit taught and form the area that will be analysed. This section must have no creases or tears to cause false readings. A cover slide to push down and secure the tissue and prongs sits over the slide. At this stage

the slide is ready to be placed in the Ussing Chambers and begin analysis for TER and FD4 but is allowed to sit and equilibrate in the new setting.

Multiple chambers are set up in the Ussing Chambers and each is linked to a multichannel voltage clamp that is attached to four electrodes and agar bridges. Each chamber consists of two parts to assess this study.

The TER analysis is performed by administering five x 2 second pulses of 2 mV. Transepithelial voltage was determined by administering five x 2 sec pulses of 2 mV. The TER was calculated by using Ohm's Law.

FD4 analysis was performed by adding 200 μL 50 mg / mL of Fluorescein isothiocyanate-dextran [molecular mass 4 kDa] to the chamber #1 that contained the mucosal side of tissue (i.e. replicating the inside of the lumen). The readout here was analysing the permeability of the FD4 leaking into the neighbouring chamber #2 (i.e. replicating the abdominal cavity). Permeability was expressed as a linear rate. Therefore any excessive amounts of FD4 detected in chamber #2, would indicate gut leakage and therefore measuring the amount of damage to the gut integrity.

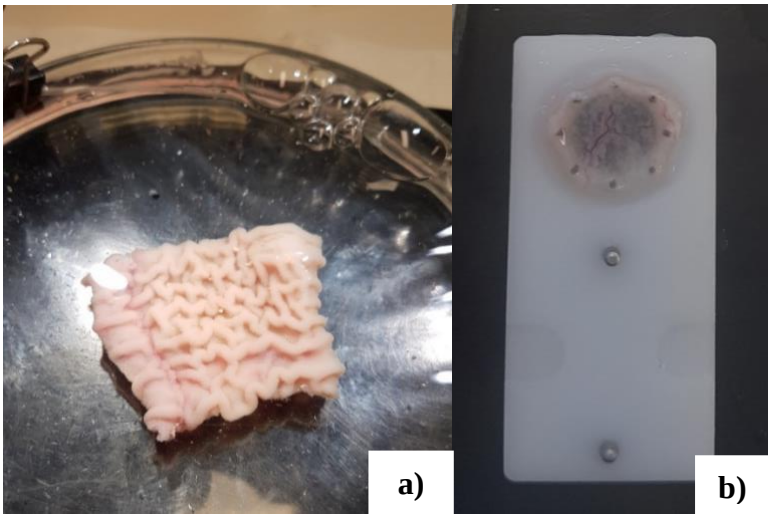


Figure 13. *a) Cleaned tissue segment of the jejunum placed in a Petri dish of oxygenated and carbonated buffering solution. Here the mucosal layer is removed and mounted onto a slide. b) Ussing chamber slide. There is no coverslip and the 8 prongs surrounding the tissue retain the tissue in place and allow the tissue to be mounted and kept taught.*

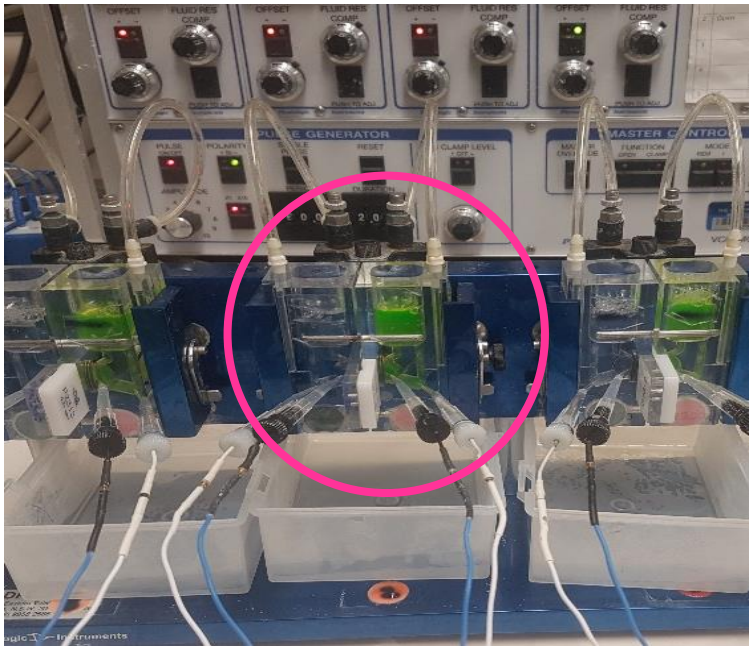


Figure 14. *Ussing chambers, showing the two separate chambers and a slide positioned in the centre of the two, containing the mounted mucosa. The chamber on the right contains the FD4. Within the pink circle, highlights a set of Ussing chambers, with two chambers situated on either side of the mounted tissue slide. The FD4 concentrated solution can be visibly seen here, shown by the bright green colour.*

2. 12 Experimental Design and Statistics

This study design is 6 x 2 factorial design, using six diets (Table 1) across two climates (TN vs HS). Due to fact of a maximum of five pigs allowed per climate controlled room, and a study design using six different diets, this experiment had an unbalanced design where one diet per replicate was excluded, however all diets within a replicate were given to both climate rooms..

All data were analysed by ANOVA using GenStat V18 (VSNi Ltd, Hemel Hempstead, UK) for the main effects of diet, temperature and their interaction. The data collected

from physiological observations (RR, RT and SK) and BGA were analysed by ANOVA using GenStat 18th version (VSNi Ltd, Hemel Hempstead, UK). A pig stands as the experimental unit in this study, whereas the treatment factors are considered as the treatment (diet) and temperature effects.

Block analysis was performed first in the ANOVA, however Unblock analysis worked significantly better and showed cleaner results from the raw data.

Statistical significance was considered when $P \leq 0.05$.

Chapter 3

Results

3. 1 Physiological Observations

3. 1. 1 Respiration Rate

Markers of increased thermoregulation were observed in the HS room. Namely the respiration rate increased nearly 6-fold (25.6 vs 148 breaths/min for TN vs HS, P<0.001 Figure). This increase was observed from 2 h of HS onwards. A significant effect of diet was observed such that all diets excepting the rSOD were higher (P<0.001, data not shown). This effect was centred in the HS cohort and no differences in RR were observed in the TN cohort (Trt*Temp P<0.001, Figure). However under HS conditions only the MF, Se*rSOD and se*MF diets had increased RR than control pigs.

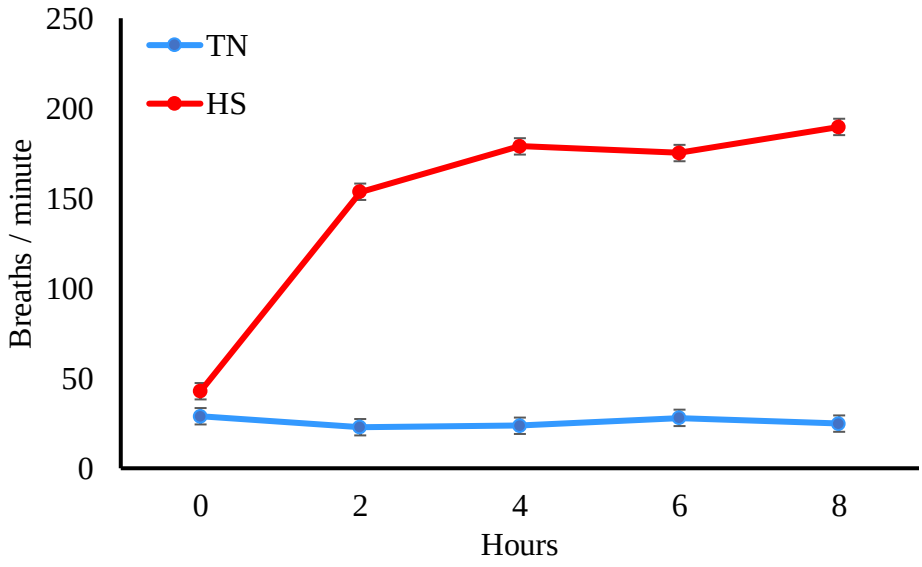


Figure 15. *Conditions in the HS room increased respiration rate nearly 6-fold (P<0.001).*

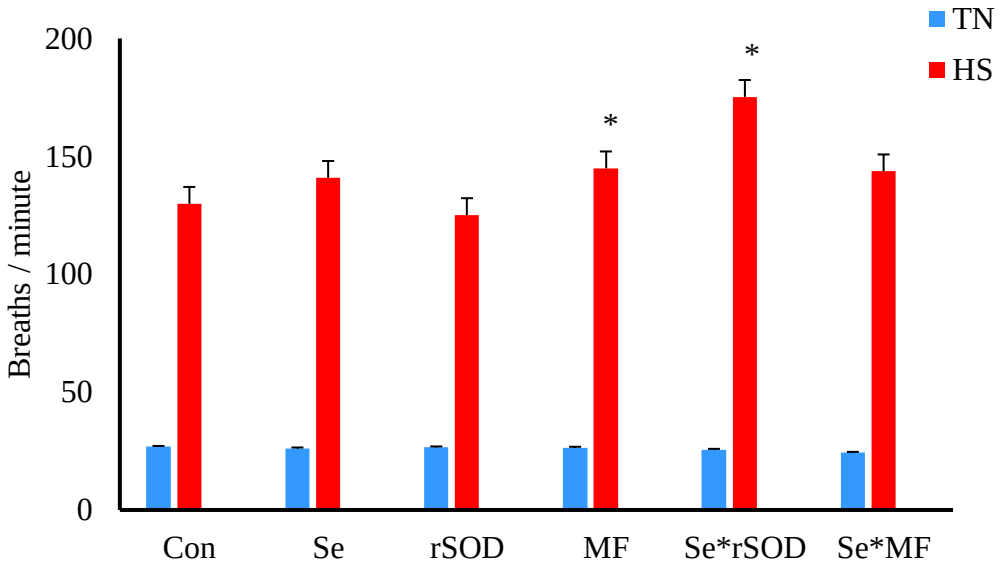


Figure 16. *The effects of experimental diet on the respiration rate of TN and HS pigs. * Denotes P<0.05 from HS control, the effects of diet, temperature and D*T were all P<0.001.*

3. 1. 2 Rectal Temperature

Conditions in the HS room increased RT overall (38.2 ºC vs 40.0 ºC P<0.001, Figure). This increase was evident from approximately 2 h of HS and was greater than 2ºC hotter than TN between 4 and 8 h (Temp*Time P<0.001). All diets other than Se*MF were higher than control overall (P<0.001, data not shown). No differences between diets were observed under TN conditions, however MF and Se*SOD diets had higher RTs than control HS pigs (D*Temp P<0.001, Figure).

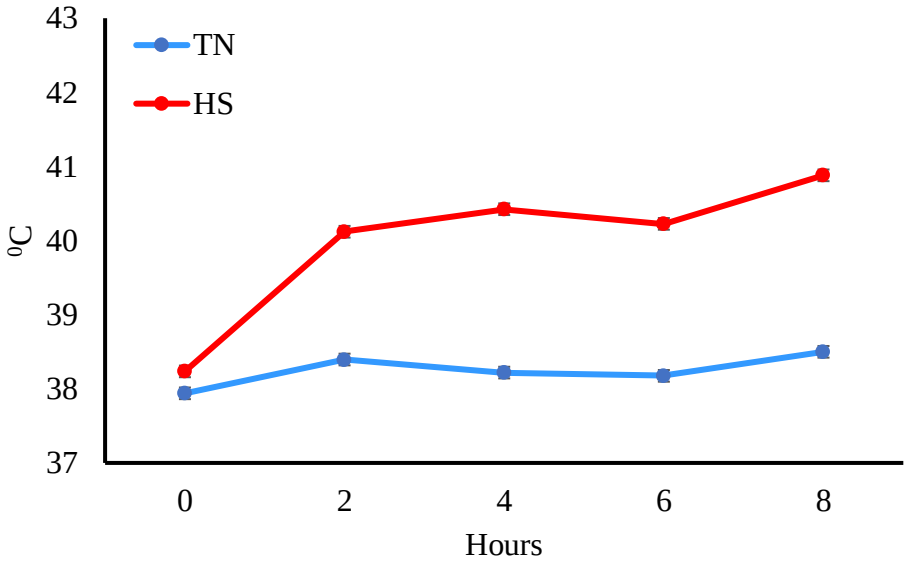


Figure 17. *Conditions in the HS room increased rectal temperature by approximately 2^0C overall ($P<0.001$).*

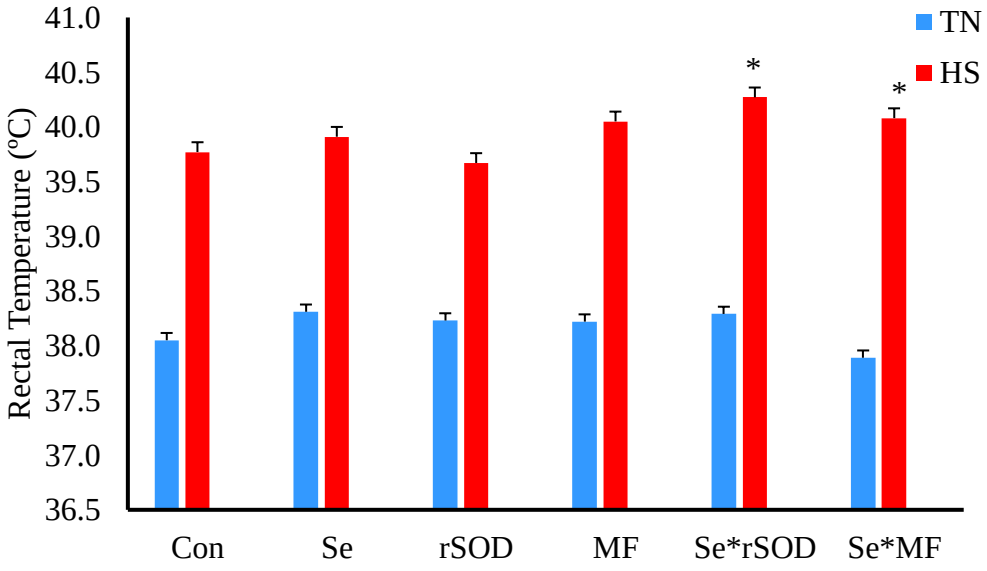


Figure 18. *The effects of experimental diet on the RT of TN and HS pigs. * Denotes $P<0.05$ from HS control. The effects of diet, temperature and D*T were all $P<0.001$.*

3. 1. 3 Skin Temperature

Skin temperature increased by approximately 6ºC in the HS group (33.9 vs 40.1 P<0.001, Figure). The increase in skin temperature occurred by approximately 2h and remained elevated for the rest of the observation period (Temp*Time P<0.001) and the pigs were noticeably redder in colour, indicating flushed skin (

Figure). Significant differences were observed between some of the diets, however none of the diets were different to control (P<0.014). Furthermore no significant interactions with HS were observed (P=0.092, Figure .

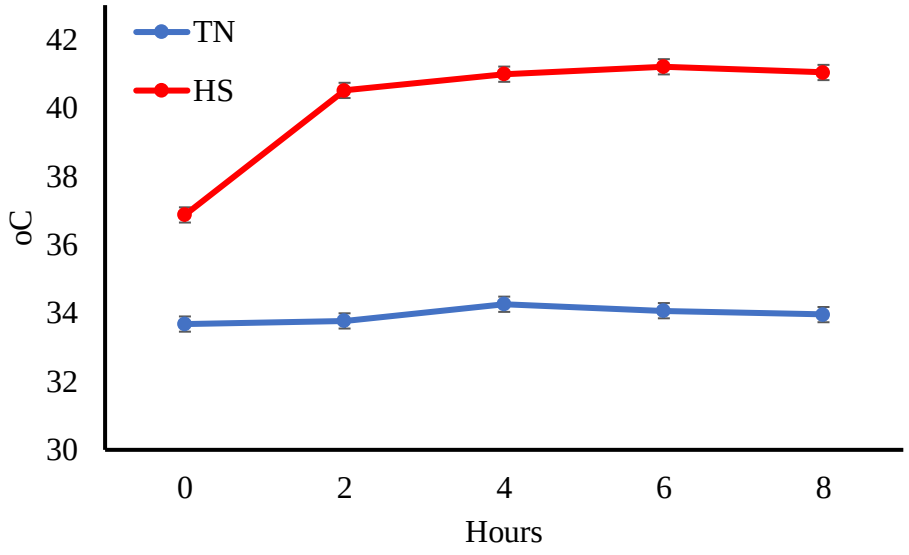


Figure 19. *Skin temperature increased by approximately 6ºC for pigs in the HS room (P<0.001).*

Figure 20. *Pigs under HS conditions had red flushed skin (b) compared to pigs housed under TN conditions (a), consistent with the increase in skin temperature.*

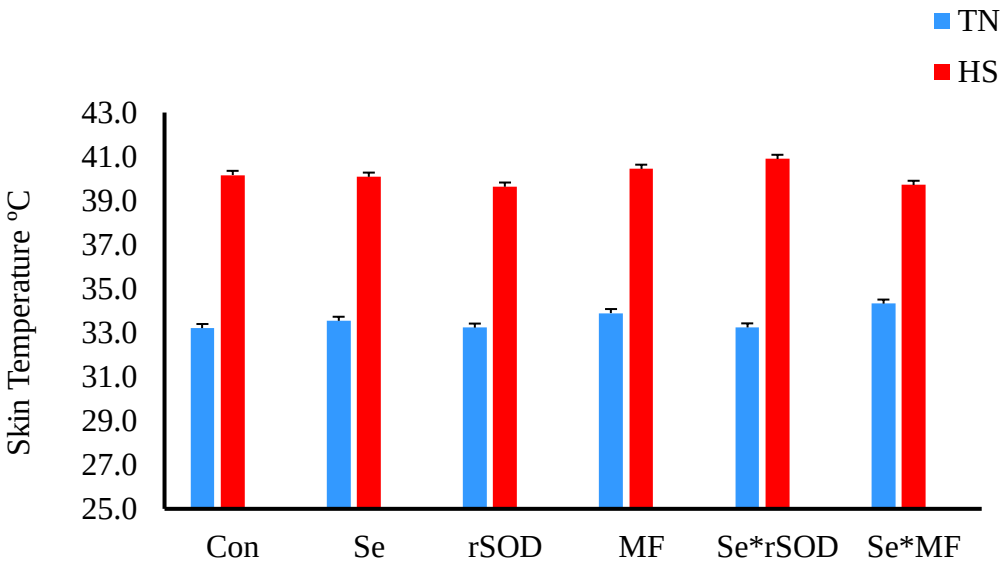


Figure 21. *The effects of experimental diet on skin temperature. The effects of diet, temperature and D*T were P=0.014, <0.001 and 0.092.*

3. 2 Blood Gas Analysis

Blood gas parameters were collected at the end of the experiment and are presented in Table . Heat stress increased oxygen concentrations (pO_2, sO_2) and reduced CO_2 concentrations ($_TCO_2$). Furthermore HCO_3^- concentrations reduced by over 2 mmol/L in HS pigs compared to TN (33.5 vs 31.1 mmol/L, P=0.002). The base excess in whole blood ($BE_{[b]}$) and extracellular fluid ($BE_{[ecf]}$) and was lower in HS pigs than TN (8.10 vs 6.03 and 9.35 vs 6.87 mmol/L for [b] and [ecf], P<0.001), however pH was not significantly affected. Blood potassium was elevated in HS pigs (4.03 vs 4.29 mmol/L, P<0.001), however no other electrolytes were influenced by temperature and no difference in the anion gap was observed. Both the haematocrit (34.2 vs 31.2 %, P<0.001) and haemoglobin concentration (11.6 vs 10.6 g/dL, P<0.001) were lower in HS pigs than TN. No differences in glucose concentrations were observed, however blood lactate (2.16 vs 1.18 mmol/L, P<0.001) and creatinine (2.09 vs 1.74 mmol/L, P<0.001) were lower in HS pigs. No significant main effects of diet or interactions with temperature were observed.

Table 2. *Results of blood gas analysis from pigs housed under TN and HS conditions.*

	Thermoneutral						Heat Stress						SEM	P-Value		
	Con	Se	rSOD	MF	Se*rSOD	Se*MF	Con	Se	rSOD	MF	Se*rSOD	Se*MF		Diet	Temp	D*T
pH	7.45	7.44	7.43	7.44	7.45	7.44	7.44	7.43	7.43	7.42	7.44	7.43	0.01	0.25	0.88	0.32
pO_2 (mmHg)	65.1	63.1	59.3	58.8	67.3	71.6	56.1	47.8	55.2	47.6	50.1	48.1	4.78	0.36	<0.001	0.64
pCO_2 (mmHg)	48.3	49.7	51.6	48.8	48.8	46.1	46.2	47.4	46.5	47.6	44.8	49.3	2.25	0.36	0.19	0.91
$_cSO_2$ (%)	90.6	91.6	89.0	88.9	93.1	94.3	88.7	81.9	87.6	80.9	85.1	82.5	3.37	0.49	0.003	0.33
$_TCO_2$ (mmol/L)	35.1	35.3	35.7	34.7	35.3	32.5	32.9	32.8	32.6	32.4	31.4	33.3	1.27	0.87	0.002	0.64
$_cHCO^{3-}$ (mmol/L)	33.6	33.7	34.1	33.2	33.9	31.1	31.5	31.4	31.2	301.0	30.1	31.9	1.21	0.92	0.002	0.57
Na^+ (mmol/L)	143	144	145	144	144	143	141	141	143	143	144	142	2.57	0.57	0.40	0.91
K^+ (mmol/L)	4.01	4.03	4.10	4.06	3.99	3.94	4.21	4.16	4.43	4.25	4.59	4.11	0.16	0.35	0.002	0.25
Ca^{2+} (mmol/L)	1.39	1.40	1.39	1.35	1.40	1.39	1.34	1.41	1.37	1.37	1.37	1.39	0.04	0.53	0.25	0.78
Cl^- (mmol/L)	99.6	100.1	100.8	99.0	100.5	100.6	99.6	100.4	101.9	102.3	103.3	99.9	1.76	0.62	0.50	0.97
$BE_{[b]}$	8.44	8.30	8.25	7.86	8.51	5.99	6.51	6.14	6.10	5.69	5.16	6.56	1.10	0.88	<0.001	0.27
$BE_{[ecf]}$	9.66	9.59	9.78	9.03	9.74	6.86	7.36	7.03	6.91	6.51	5.86	7.54	1.27	0.92	<0.001	0.48
AGapK	13.4	13.9	14.0	15.3	13.9	15.6	14.0	13.3	13.9	13.8	15.6	14.5	1.06	0.48	0.96	0.37
Hct (%)	34.4	34.6	35.7	33.0	32.9	34.6	30.1	31.0	31.3	30.4	31.8	32.4	1.32	0.63	<0.001	0.56
cHgB (g/dL)	11.7	11.7	12.2	11.3	11.2	11.8	10.3	10.5	10.6	10.3	10.7	11.0	0.43	0.60	<0.001	0.56
Glucose (mmol/L)	6.21	6.93	6.40	6.89	6.24	7.31	6.47	6.55	6.70	6.63	6.95	6.53	0.40	0.65	0.69	0.59
Lactate (mmol/L)	1.58	2.25	2.26	2.84	1.56	3.86	0.99	1.23	1.15	1.07	1.34	1.33	0.60	0.38	0.010	0.18
Creatinine (mg/dL)	2.02	2.07	2.27	2.03	1.95	2.08	1.87	1.65	2.00	1.69	1.74	1.79	0.19	0.098	0.036	0.80

3. 3 SOD activity

Plasma SOD activity was reduced by ~14% in HS pigs compared to TN (0.053 vs 0.045 IU/mg, P=0.030, Figure). No overall effect of diet was observed (P=0.91), however lower SOD activity was seen in HS pigs on the Se and MF diets compared to pigs on the equivalent diet under TN conditions (P=0.004).

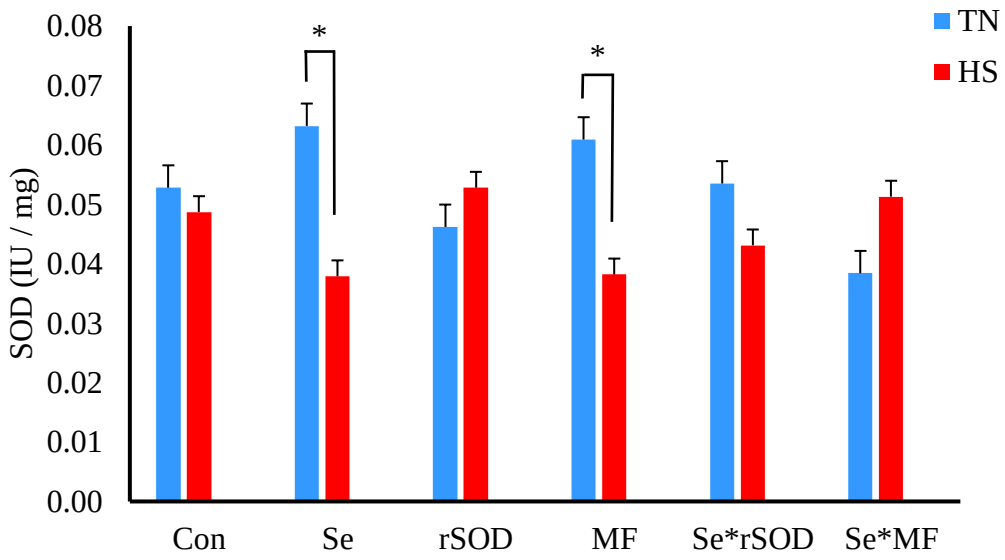


Figure 22. *The effect of experimental diet on plasma SOD activity under TN and HS conditions. * denotes significantly lower (P<0.05) under HS conditions than TN. The effects of diet, temperature and time were 0.91, 0.030 and 0.004 respectively.*

3. 4 Urinalysis

Urine pH was lower in HS pigs than TN (6.12 vs 5.82 P=0.002). However no effect of diet or interaction with temperature was observed (Figure 1). Urine osmolality was increased in HS pigs in excess of 30% overall (298 vs 455 mOsm P=0.004, Figure), however no effect of diet or interaction with temperature was observed. Urine albumin was not influenced by temperature or diet (Figure) and bilirubin increased in HS pigs (1.04 vs 1.58 g/L, P=0.023) however no effects of diet or interactions were observed.

Urinary creatinine was also elevated in HS pigs (93 vs 152 mg/dL, P=0.004), with no effects of diet or interactions with temperature observed.

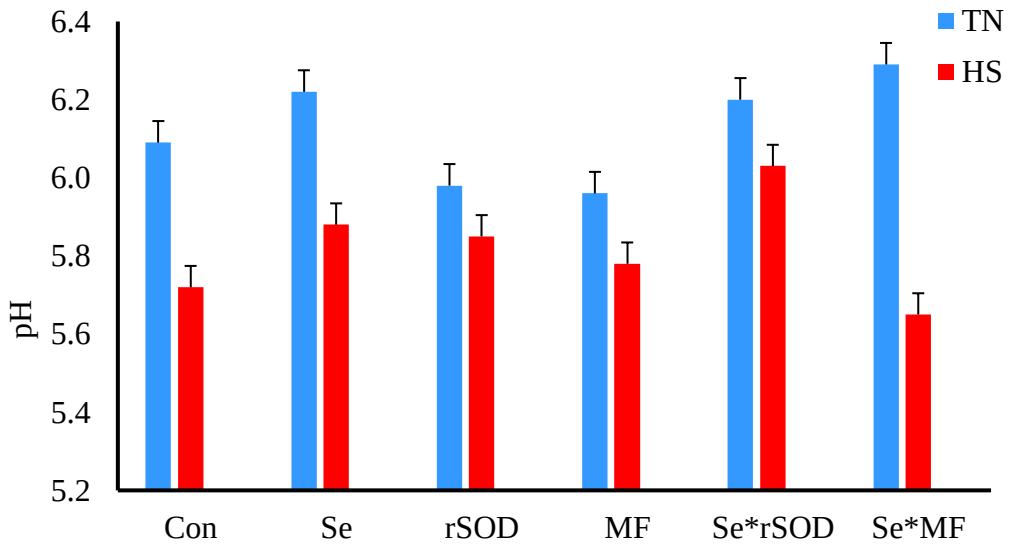


Figure 1. *The effect of experimental diet on urine pH in TN and HS pigs. The effects of diet, temperature and D*T were P=0.66, 0.002 and 0.65 respectively.*

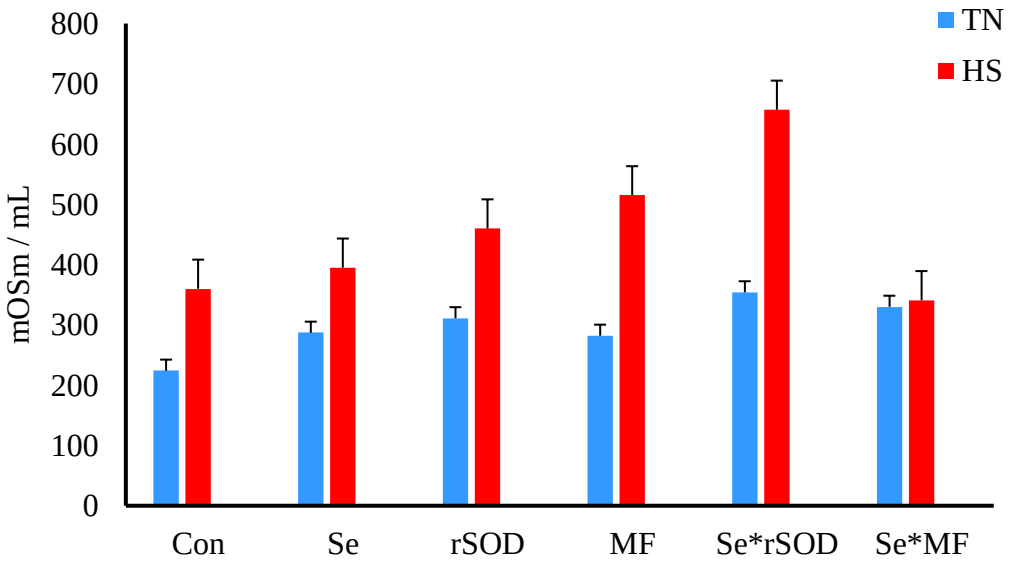


Figure 24. *The effect of experimental diet on urine osmolality in TN and HS pigs. The effects of diet, temperature and D*T were 0.28, 0.004 and 0.70 respectively.*

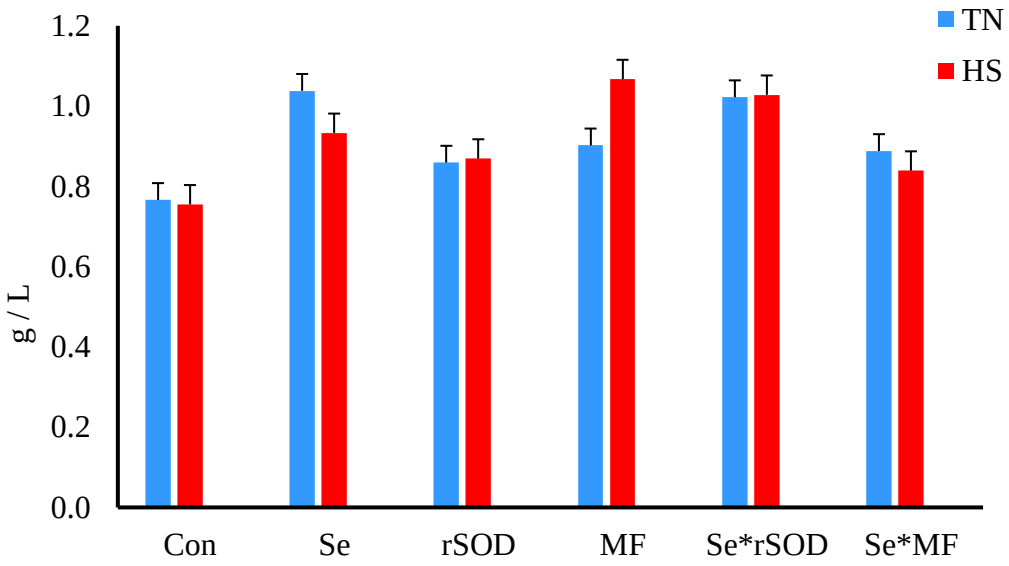


Figure 25. *The effect of experimental diet on urine Albumin in TN and HS pigs. The effects of diet, temperature and D*T were 0.37, 0.29 and 0.36 respectively.*

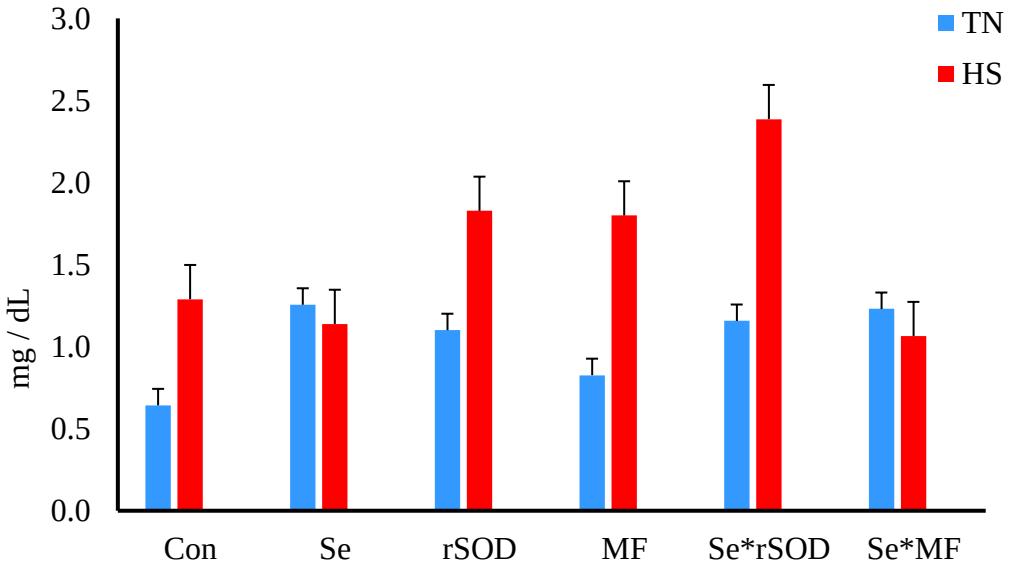


Figure 26. *The effect of experimental diet on urine Bilirubin in TN and HS pigs. The effects of diet, temperature and D*T were 0.46, 0.023 and 0.44 respectively.*

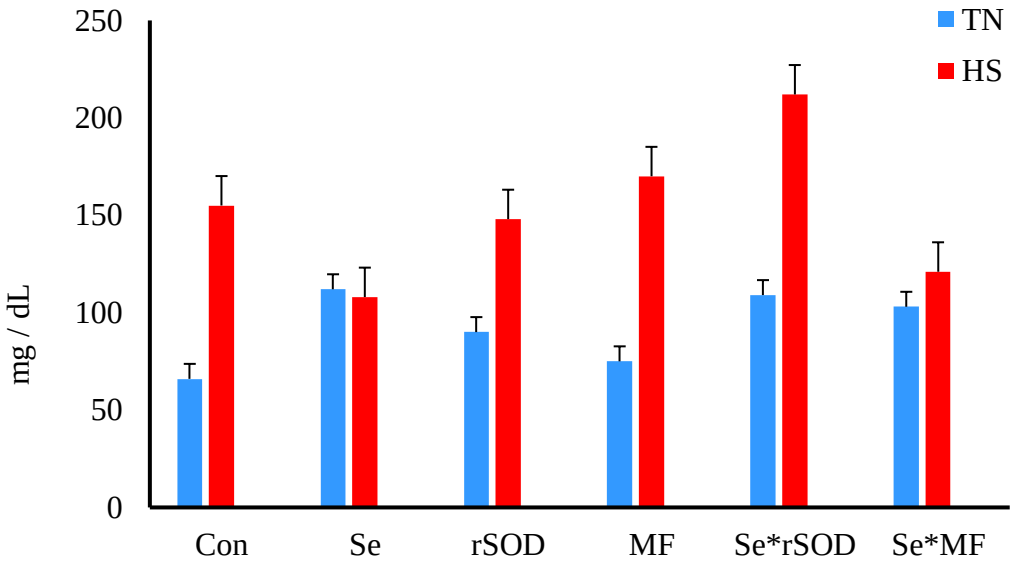


Figure 27. *The effect of experimental diet on urine Creatinine in TN and HS pigs. The effects of diet, temperature and D*T were 0.68, 0.004 and 0.54 respectively.*

3. 5 Ussing Chamber Analysis

No effects of diet or temperature were observed on the jejunum (TER) or ileum (TER and FITC, Table 3. However the colon TER was significantly higher in HS pigs than TN (34.2 vs 43.7 Ω/cm^2). Furthermore a trend existed for MF pigs to have an increased TER under HS conditions than TN, and against all other diets under HS with the exception of Se*rSOD (Diet*Temp P=0.072). Corresponding to increased colon TER in HS pigs, the FD4 permeability was lower in HS pigs than TN (0.153 vs 0.024 mg.min/cm, P=0.049).

Table 3. *The effects of experimental diet and TN vs HS conditions on transepithelial resistance (TER) and FD4 permeability in different regions of the gastrointestinal tract.*

	Thermoneutral						Heat Stress							Significance		
	Con	**Selenium**	**SOD**	**MF**	**Se*SOD**	**Se*MF**	**Con**	**Selenium**	**SOD**	**MF**	**Se*SOD**	**Se*MF**	**SED**	**Diet**	**Temp**	**D*T**
TER ($m\Omega/cm^2$)																
Jejunum	40.2	36.7	38.9	30.8	34.9	35.9	36.9	33.2	33.2	46.8	33.8	34.6	6.77	0.90	0.94	0.25
Ileum	41.8	33.8	34.6	32.3	38.7	34.8	39.1	34.4	34.2	35.0	33.9	33.9	4.67	0.31	0.63	0.91
Colon	39.4	26.1	38.4	28.4	40.5	32.4	37.9	40.4	37.2	60.1	48.0	36.1	8.59	0.31	0.012	0.072
Permeability (FD4, ug.min.cm)																
Ileum (log_{10})	-0.191	-0.111	-0.182	-0.373	-0.206	-0.146	-0.034	-0.164	-0.112	-0.198	-0.055	-0.023	0.150	0.50	0.095	0.90
(10^x)	(0.644)	(0.774)	(0.658)	(0.424)	(0.622)	(0.714)	(0.925)	(0.685)	(0.773)	(0.634)	(0.881)	(0.948)				
Colon (log10)	0.180	0.397	0.069	0.145	0.133	0.005	0.063	0.092	0.146	-0.230	0.012	0.078	0.156	0.20	0.047	0.22
(10^x)	(1.51)	(2.49)	(1.17)	(1.40)	(1.36)	(1.01)	(1.16)	(1.24)	(1.40)	(0.59)	(1.03)	(1.20)				

* As the results were not normally distributed a Log_{10} transformation was performed

+ Values in parenthesis represent the back-transformed means. NB the SEM was not back-transformed.

Chapter 4
Discussion

4. 1 Overview

Overall the model clearly induced heat stress in the pigs. This was evidenced by greatly elevated physiological parameters of heat stress (RR, RT and ST). The onset was within 2h of the beginning of the heat period, meaning that the pigs had at least 6 h of heat stress each day for the first two days of the experiment. The heat stress was also reflected by marked changes in blood gas, electrolytes and metabolites, with an increase in base excess indicating that the pigs were experiencing respiratory alkalosis. Furthermore blood SOD activity was reduced by HS, indicating that the pigs were experiencing oxidative stress. Unlike in previous experiments the GIT was not negatively affected by HS, and parameters for the colon were actually improved by HS. However increased urinary bilirubin may indicate that the pigs had an upregulated acute phase response and increased creatinine excretion may indicate impaired renal function.

While the effects of heat stress on pig physiology the effects of the experimental diets were few. The MF, Se*rSOD and Se*MF diets resulted in increased parameters of heat stress, whoever this did not translate to any shift in biochemical parameters. Therefore it may be concluded that the diets did not ameliorate the impact of HS on the pig.

4. 2 Physiological Parameters

The effects of HS on the RR, RT and ST were as expected, with all the HS groups showing elevated RR and elevated RT and ST. The RR in the HS pigs was increased by up to fivefold, compared to the TN groups. Proving how intensely a HS pig must

thermoregulate. In the case of high RR the pigs were desperately aiding thermoregulation by using evaporative heat loss, as their responses to the HS via radiative heat loss was inadequate by this stage. Panting in pigs is recognized as a severe heat stress sign. However the elevated RR in turn caused acid-base disturbances in the blood gas parameters (as discussed in section 5.3 BGA panels). HS caused serious stress on the pigs that were effected and it was apparent not just in the physiological parameters but in their overall state of being. Figure 12 highlights the crucial difference in their stressed conditions. TN pig is relaxed, curious and attentive to human handler, whilst HS pig is showing little interest, open-mouthed panting with drool, and is focused on conserving energy levels. Pigs are overall curious and inquisitive animals, therefore such disinterest behaviour also reflects their heightened level of stress.

A pig's physiology will send a surge of blood to the peripheries to aid thermoregulation via radiant heat loss. This is a simple yet affective way of cooling down where pigs have access to water, mud or cool earth to wallow in. In factory farming pigs do not have this available therefore radiant heat loss is only effective whilst the surrounding air temperature is lower than their body temperature, to allow transfer of energy. When the environmental temperature equals that of the pig's surface body temperature, radiant heat loss is no longer effective. A pig will begin to pant to further aid thermoregulation and attempt to lose heat via evaporative heat loss.

RT is used as the measurement of core body temperature. Healthy adult pigs have a rectal temperature of around 37.7 – 38.8 ºC. RT does increase normally during digestion / feeding times as digestion requires energy and metabolic activities produce heat. However, outside of these times, high RT is an excellent indicator of infection or HS in the pig.

RT and ST was lowest at the 0 hours of observations, and gradually increased to maximum levels 2 hours post heat being turned up (data not shown here).

In the HS groups, RT was elevated for all the HS groups compared to TN, however their corresponding ST results gave much higher readings. Indicating that blood is surging to the peripheries to aid in thermoregulation via radiant heat loss. RT is not as high as ST, (in the HS pigs) further explaining the movement of blood away from core internal organs. Visually HS pigs had more pink flushed skin colour compared to that of TN pigs, as seen in Figure 12.

4. 3 SOD Activity

In the plasma there was a downregulation of SOD activity in the HS groups and a significant effect seen for Temperature. The suppression of SOD in the plasma of HS pigs is a strong indicator of antioxidants being required to neutralise oxidative effects of HS at the site of the intestinal mucosal walls. The blood is a proxy of things that are occurring within organs and tissues, however venous blood cannot give us answers to which tissues in particular. Therefore this downregulation of SOD in the plasma is an indicator that oxidative stress is occurring as the reduction of SOD signifies that a portion of the antioxidants has been spent to neutralise the effects of free radicals and prevent intestinal wall damage.

As TN are not effected by heat, their internal tissues are not exposed to oxidative stress. Therefore the SOD activity levels in their plasma remains typically unchanged. Any effects that were seen may be due to slight errors in data collection or variations in personnel interpretations of data read out.

4. 4 BGA Panel Screen

In this experiment blood gas analysis panel screen, assessed 18 different parameters from the terminal venous blood collected, which included oximetry, ionic, metabolic and base excess levels.

4. 4. 1 Carbon Dioxide and Oxygen Saturation Levels (TCO_2 and cSO_2)

Respiratory alkalosis is caused by respiratory hyperventilation (Cunningham, 1997). In HS pigs CO_2 is expelled more quickly than tissues can produce it, and therefore will cause a drop in overall CO_2 levels in the blood. This leads to respiratory alkalosis and blood alkalosis (Liu et al., 2016). In this study oxygen saturation (cSO_2) was seen to overall be reduced in the HS groups compared with TN. Total carbon dioxide (TCO_2) levels were also reduced in the HS groups compared to TN. The lower TCO_2 results seen in the HS groups is expected due to the high RR in pigs under HS conditions. The high RR is expelling more CO_2 than in a neutral state of respiration. However the low cSO_2 values were not expected, as it was thought to have rather increased than decreased, due to the high RR. Further analysis of the BGA shows that haemoglobin (Hgb) levels are significantly lower in the HS pigs and thus the lowered cSO_2 levels is understandable. Lower levels of cHgB would naturally mean that less oxygen is able to be carried by the blood and the decreased levels of cHgB is caused by the acid-base disturbance and metabolic upset that is created by HS environment. Urine analysis of bilirubin as seen in Figure 4-25, has shown significant increased levels of bilirubin in the urine of HS pigs compared to TN pigs. This is an indicator of Hgb breakdown in the blood and correlates the low levels of cHgB and Hct seen in the blood with the high levels of bilirubin seen in the urine. Urinalysis is further discussed in Section 5.5.(Voiculeț, Zară, Bogeanu, Văcăroiu, & Aron, 2016)

4. 4. 2 Bicarbonate Levels ($cHCO_3$-)

Bicarbonate ($cHCO_3$-) is a form of carbon dioxide (CO_2) and is a by-product of cell metabolism in the body. $cHCO_3$- is also an electrolyte and very important in regulating pH in the blood (Voiculeț et al., 2016).

In this study $cHCO_3$- levels were significantly lowered for temperature effects in the HS groups. Low $cHCO_3$- indicates metabolic acidosis in the blood and is directly the effect of the high RR in HS pigs. During the high RR, CO_2 (an acid) is lost through respiration, leaving $cHCO_3$- (a weak base) circulating in the blood, causing the blood to become more alkaline. The kidneys intervene by filtering out excess amounts of $cHCO_3$- to stabilise the pH of the blood. Hence the low $cHCO_3$- seen in the BGA screen.

4. 4. 3 Plasma Creatinine Levels (Crea)

Creatinine (Crea) in the plasma lowered in the HS pigs, with a significant temperature effect seen between TN and HS pigs. Lowered Crea in the blood indicates that ion filtrations is incorrectly managed by the kidneys and could possibly be a sign of renal damage. This correlates with the high levels of Crea found in the urinalysis of HS pigs, see Urinalysis section 5.5.

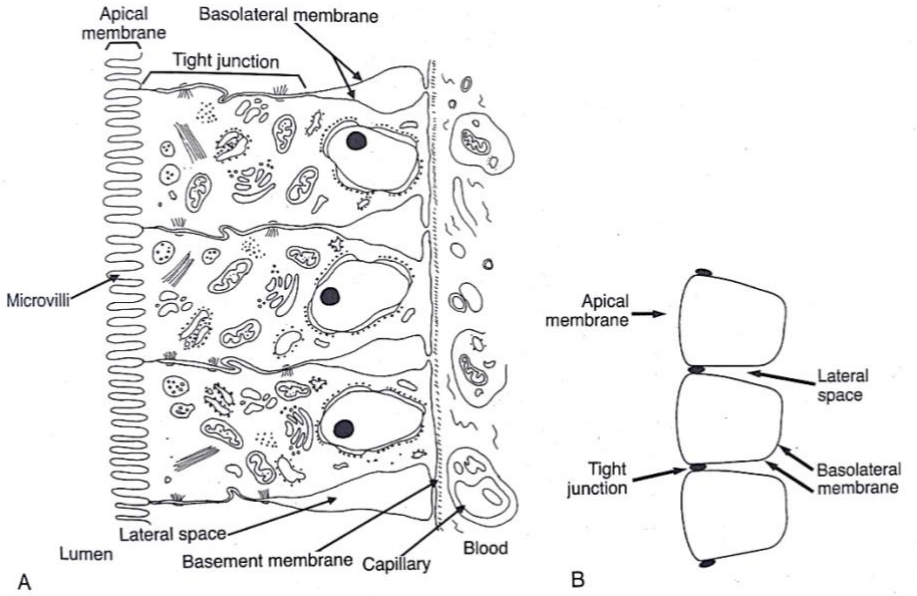
4. 4. 4 Base Excess of Blood and Extra Cellular Fluid (BE (b) and BE (ecf))

Results obtained from the Base Excess of blood and Base Excess of extra cellular fluid (BE (b) and BE (ecf) respectively) these values are both lowered significantly for temperature, indicating acidic levels. When blood is oxygen saturated it will turn BE (b) slightly acidic. As cSO_2 increases, pH will decrease in a poorly buffered extra cellular fluid (ecf), than in a well buffered ecf. This is due to blood H^+ ions tending to diffuse from the ecf to the blood, and therefore cause a drop in BE. This would be accurate for

the HS pigs, as their metabolic acid-base buffering is disturbed. Overall the BE (b) and BE (ecf) are an indicator of buffering upset in the HS pigs.

The overall results from the BGA show acid-base disturbances in the blood. The various panels indicate that at various stages the blood would be quite alkaline due to overcompensation for buffering, and at other times more acidic. These are all strong indicators of the buffering system driving to maintain blood pH levels that are severely affected by the high RR from the pigs. Overall blood pH is maintained demonstrating the buffering regulation is in operation. However, this comes at a cost of energy, that is put into buffering and thermoregulation that could otherwise be spent in growth and production by the pig. Furthermore it is observed that renal damage may be at present in HS pigs, due to the improper filtration and leakage of Crea by the kidneys. This creates further concern in HS pigs.

4. 5 Ussing Chambers TER and FD4



(Cunningham, 1997).

Figure 28. *Diagram illustrating the tight junctions and their positioning in the body in regards to the intestinal tissues and blood vessels. (A) Illustrating the epithelium tissues in the intestines. (B) Epithelium containing a capillary containing formed elements of blood. Understanding intestinal absorption is crucial to this experiment and the use of the Ussing Chambers.*

In this experiment damage to the intestinal mucosal layer lining the GIT was measured by TER and FD4 to determine tissue integrity and permeability. The purpose of this test was to measure ion channel activity that is occurring between the lumen of the GIT and the capillary blood vessels. Situated between these are the tight junctions located between the apical tissues and the basolateral membrane that sits closely over a capillary (Cunningham, 1997). Damaged GIT walls will have "leaky gut" and allow the passage of FD4 molecules to pass more freely into the blood, and will be picked up by detecting the

FD4 in the chamber representing the blood. TER values are higher for GIT tissues that have integrity intact whilst the lower values are signs of GIT tissues that have lost their integrity and show gut permeability increased.

Overall effects of HS on both the TER and FD4 results showed significance for temperature in the Colon tissues. High value results for TER indicated that tissues integrity was maintained, or improved, and Melofeed treatments across the three tissues sampled, jejunum, ileum and colon all seemed to have a trend showing a positive effect of this diet.

FD4 results followed the pattern seen in the TER results, where there was a significance seen in the Colon tissues for temperature effect.

This experiment therefore shows that high TER values and low FD4 values represents tissue integrity of the mucosal layer maintained, whilst low TER values and high FD4 values indicate that the mucosal layer has been compromised and is no longer functioning correctly. Therefore improper absorption of nutrients will be absorbed at incorrect stages in the GIT, causing leaky gut and no nutritional value from the food ingested will result in being properly metabolised and used.

4. 6 Urinalysis

4. 6. 1 Urine pH

The final urine pH is measured by the collecting duct in the kidney before it is excreted, and the collecting duct is responsible for the pH of the urine. It is within the nephron where acids and bases are either reabsorbed or excreted depending on the blood pH. (Cunningham, 1997). Therefore it's understandable that the urine excreted can have a significantly different pH to the blood.

In the terminally collected urine of the pigs, it was evident that HS pigs had significantly lower pH of urine across all the different treatment groups compared to that of the TN groups. This effect shows us that HS pigs will have more acidic urine compared to that of TN pigs and is a result of the kidneys performing maintenance buffering of the blood, and filtering out excess acids, to elevate the blood pH. This further highlights the strain on the kidneys to maintain blood pH at such heightened times of stress.

4. 5. 2 Urine Osmolality

Urine osmolality was shown to be significantly increased for temperature in the HS groups compared with TN groups. Higher urine osmolarity indicates that urine volume was significantly less compared to TN groups. This was found to be particularly interesting as it was noted clearly that HS pigs were seen to be drinking more water throughout the duration of the HS events compared to that of the TN pigs. Regardless of the higher intake of water, their urine volume was still significantly less. This shows that much of the water was lost through evaporative panting and how hard the pig is working to reduce core body temperature.

4. 5. 3 Urine Albumin

Urine albumin is a protein that is synthesized by the liver and excreted into the blood via hepatic capillaries. Therefore it is a plasma protein and it is important during immune responses (Cunningham, 1997). Urine albumin is a measurement of proteins that have leaked into the urine. A healthy functioning renal system will produce urine with none or very little protein leakage. Large amounts of Albumin present in the urine are a solid indicator of early kidney damage. This occurs when the glomeruli tubules within the kidneys are damaged and therefore routinely leak out proteins into the urine. The

glomeruli tubules can become damaged from inflammation or from some type of scarring that occurred previously.

In this study HS pigs showed a slight trend of Albumin protein leakage in the urine. Although not a significant effect was seen in this test, it is a noteworthy result as other urinalysis results significantly reported possibly kidney damage.

4. 5. 4 Bilirubin

The foundation of bile pigment is bilirubin and used during the normal process of blood turnover (Cunningham, 1997). Therefore old and damaged red blood cells when broken down produce bilirubin as a waste product. The liver is responsible for breaking down bilirubin in order for it to be excreted by the body via feces or urine (Cunningham, 1997). Large amounts of bilirubin in the urine tend to indicate liver disease, but high levels of bilirubin in the urine may also potentially indicate renal damage (Y. Liu et al., 2018).

The results of this study showed that HS pigs compared to TN pigs contained large amounts of bilirubin in their urine, see Figure 4-25. High amounts of bilirubin are anticipated as the Hgb levels have also significantly dropped in HS pigs. However bilirubin is understood to be broken down further by the liver before being excreted by the body and therefore large amounts in the urine indicate the possibility of liver or kidney disease (Cunningham, 1997).

It is not an unlikely result that it points to kidney disease, as other tests also have indicated the possibility of this. See section 5. 5. 3 Albumin and 5. 5. 5 Creatine. The incorrect filtering out of bilirubin, before it has been correctly broken down by the liver, could indicate kidney damage.

4. 5. 5. Urine Creatinine (Crea)

Creatinine is a protein and is a byproduct of muscle metabolism. When incorrectly filtered through kidneys, in large amounts, it becomes a marker of kidney damage and improper globular filtration rates (Hyun et al., 2016; Nayak et al., 2013). This was a significant occurrence in the study and apparent for temperature effect.

Analysis of urine collected terminally from the pigs, showed that HS groups were excreting higher amounts of creatinine compared to TN groups. This implies renal damage as there is improper filtration rate of proteins (Cunningham, 1997).

(Aksit et al., 2006)

4. 5. 6 Creatinine normalised to Osmolality (Creatinine : Osmolality)

Creatinine ratio to osmolality was performed to demonstrate that regardless of urine volume, that the creatine levels were still high in the HS pigs. The creatine levels are significantly higher in the HS groups compared to TN. Further highlighting a possibility of renal damage in HS pigs.

Chapter 5

Conclusion

5. 1 Conclusion

In conclusion, HS pigs showed downregulation of SOD levels in the plasma, indicating that due to oxidative stress occurring in tissues, endogenous levels of SOD was then suppressed to combat the effects of oxidation. In essence, balancing out the free radicals with the antioxidant capacity. Furthermore this indicates that SOD is an important pathway during HS.

Unlike previous studies heat stress did not compromise GIT barrier function, and therefore no benefits of SOD were observed. Furthermore SOD, either as rSOD or Melofeed did not ameliorate parameters of heat stress and in some instances were marginally worse, such as the RR (Figure 4-13) and RT (Figure 4-14). However this study highlighted that rSOD and Melofeed are the right pathways and worth further investigation as two of the biggest antioxidant pathways, SOD and GPx, were found to be downregulated.

Finally homeostasis involvement in the buffering of blood pH, tightly involves the respiratory system and the renal system and the blood, and while the focus was on the high RR, it is possible that kidney damage at the globular level has occurred and might be a contributor to acid-base disturbances.

5. 2 Future directions

Further investigation of rSOD and Melofeed could be of interest as both SOD and GPx has been found to be an important antioxidant pathway during HS.

This experiment provided basic evidence of impaired renal function during HS and may be a target for future amelioration strategies. Of importance was albumin and creatine excretion and could possibly be worth further investigation.

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Eva Vidacs (Honours student)

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