The Effect of Supplemental Grape Seed Extract on Pig Growth Performance and Body Composition during Heat Stress

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Life Science
In
Food Science and Technology

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April 14, 2016
Blacksburg, VA

Keywords:
Grape Seed Extract, Pigs, Heat Stress, Body Composition

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Academic Abstract

Prolonged exposure to high ambient temperature without cooling causes heat stress (HS) resulting in altered growth, body composition and metabolic dysfunction in pigs. Grape seed extract (GSE) has been shown to reduce inflammation, and improve glucose transport and metabolism. Thus, GSE may be an effective supplement to combat the consequences of heat stress; however this possibility has not been evaluated in a large animal model. The objective of the current study was to examine the effect of GSE supplementation on pig performance and body composition during HS. Twenty-four female pigs (62.3± 8 kg BW) were randomly assigned to a 2X2 factorial experiment; thermal neutral (TN; 21-22°C) or heat stress conditions (HS; 33-34°C) for 7 days and fed either a control or a GSE supplemented diet (12mg/kg body weight). Body temperature (T_B), respiration rate (RR) and feed intake (FI) were measured daily. Body composition was measured by dual-energy X-ray absorptiometry (DXA). Respiration rate and T_B increased in the HS control group compared to the TN control group ($p<0.05$), however GSE did not alter these parameters compared to control for the duration of the 7 day period. HS decreased FI ($P < 0.05$). Fasting blood glucose concentrations were approximately 1.5-fold greater in the control diet compared to their GSE supplemented counterpart ($p=0.067$) on day 6 of the HS period, but did not differ between groups at the end of day 7 of HS. Body composition analysis indicated bone mineral density, bone mineral content, and percent change of fat remain unchanged between treatment groups. Percent change in weight was significantly reduced in HS. Lean tissue accretion was 45% greater in TN compared to HS groups ($p<0.05$). Endotoxin concentrations were approximately 2-fold lower in the HS-GSE group compared to the control ($P=0.083$). Grape seed extract supplementation does not appear to alter pig growth performance or body composition, but does appear to delay the onset of reduced feed intake by 1 day, reduce intestinal permeability, and improve insulin sensitivity during additional stress.

The Effect of Supplemental Grape Seed Extract on Pig Growth Performance and Body Composition during Heat Stress

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General Abstract

Prolonged exposure to high temperatures without cooling causes heat stress (HS) resulting in stunted growth as well as altered body composition (bone mineral content, bone mineral density, fat accumulation, and lean tissue gain) and nutrient (glucose, lipid and protein) metabolism in pigs. Grape seed extract (GSE) has been shown to reduce inflammation and improve glucose and lipid metabolism showing that GSE, if added to the diet, can alleviate the symptoms of heat stress; however, this has never been tested in a pig. The objective of the current study was to examine GSE supplementation on pig growth and body composition during HS. Twenty-four female pigs (137.35 ± 17.64 lbs BW) were randomly assigned to one of four treatment groups; thermal neutral (TN; 69.8 to 71.6°F) or heat stress conditions (HS; 91.4 to 93.2°F) for 7 days and fed either a control (typical swine diet) or a GSE supplemented diet (12mg/kg body weight). Body temperature (T_B), respiration rate (RR) and feed intake (FI) were measured daily. Body composition was measured by a dual-energy X-ray absorptiometry (DXA) scanner. Respiration rate and T_B significantly increased in the HS control group compared to the TN control group, however GSE did not alter RR and T_B compared to the control for the duration of the 7 day period. HS decreased FI. Fasting blood glucose concentrations were approximately 1.5-times greater in the control diet compared to their GSE supplemented counterpart on day 6 of the HS period, but did not differ between the four treatment groups at the end of day 7 of HS. Body composition indicated bone mineral density, bone mineral content, and percent change of fat remain unchanged between treatment groups. Percent change in weight was significantly reduced due in HS. Lean tissue gain was 45% higher in TN compared to HS groups. Endotoxin concentrations were approximately 2-times higher in the HS-GSE group compared to the HS-control. Grape seed extract addition to the diet does not appear to improve pig growth or body composition, but does appear to delay the onset of reduced feed intake by 1 day, reduce intestinal permeability and improved glucose regulation during additional stress.

Acknowledgements

To Dr. Andrew Neilson: I can't thank you enough for the opportunity to come to graduate school and work in your lab! I have learned so much in every possible way and have greatly improved as a scientist because of you. Thank you for giving me the opportunity to be a part of so many other research projects not only at this university, but also around the country. It definitely kept me busy, but I would not have wanted it any other way or would have gotten this experience anywhere else.

To my committee (Dr. Rob Rhoads, Dr. Shelly Rhoads, Dr. Monica Ponder, and Dr. Amanda Stewart): Thank you Rob and Shelly for all of your help and expertise with completing the pig study. Those were some long and hard days, but you always had smile on your faces, so it made it a bit easier. Thank you Dr. Ponder and Dr. Stewart for always being there to support me and answering any needed questions.

To the Neilson lab group (Andrew Gilley, Chris Winslow, Tommy Haufe, Caroline Ryan, Karen Strat, and Reem Ajlan): I am forever in your debt for helping me wrangle hogs at all hours of the day and night. I can't thank you all enough for sticking it out and helping me out whenever I asked. You guys are the best lab group anyone could ask for. There is never a dull moment around any of you. Thanks for always making it fun and keeping it interesting. Special shout-out to Dr. Katie Goodrich: I wouldn't be where I am today without out you. Most importantly you were an incredible friend, but also a great mentor.

To Pat Williams, Kevin Young, and the Rhoads lab groups: Pat, thank you for all of your help with the DXA scans. Kevin, I can't thank you enough for all of the help with the initial phases of this project and bringing me continuous deliveries of feed even though some of them were at the last minute. Thank you for being patient with me. To all the members of the Rob and Shelly's lab groups (Morgan Biggs, Lidan Zhao, Zhenhe Zhang, Rebecca Poole, and Audra Harl), thank you to each and every one of you for your help, support, and advice during the completion of this study.

To the faculty and staff of the food science department: Over the last six years as an undergraduate and now a graduate student I've noticed that this department has hands down the best faculty and staff members. You all are always incredibly friendly and talkative which makes it easy for students to feel welcome, ask questions, and excel in their careers. Thank you all for being supportive of me in career and being available whenever I have questions or need help.

To the FST graduate students: Thank you to each and every one of you for great friendship. It has definitely been an incredible past two years with you all and we've all accomplished so much.

Saving the best for last, to my mom and dad, Jean and Todd Smithson: I can never begin to thank you enough for all of your love and support you've given me through my college career. You've always encouraged me to set goals and strive for them. Not only that, but be the best person I can be. Thank you for being the best role models.

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GSE, grape seed extract; HS, heat stress; TN, thermal neutral; TNZ, thermal neutral zone; VFI, voluntary feed intake; ATP, adenosine triphosphate; TAGs, triacylglycerol; GLUT4, glucose transporter type-4; SGLT, sodium-glucose transporter; GI, gastrointestinal; ROS, reactive oxygen species; RNS, reactive nitrogen species; TJP, tight junction protein; LPS, lipopolysaccharide; IL, interleukin; NOS II, nitric oxide synthase II; NEFA, non-esterified fatty acid; AGP, antibiotic growth promoter; DP, degree of polymerization; DXA, dual x-ray absorptiometry; BW, body weight; RR, respiration rate; RT, rectal temperature; T_B, body temperature; BMD, bone mineral density; BMC, bone mineral content; EU, endotoxin unit; NS, nonsignificant.

Introduction

During this century, North American temperatures are projected to increase 1.8°C to 4°C and regions within the United States which experience summer "heat waves" are expected to have more frequent and prolonged seasons. As increasing temperatures rise above and beyond thermal neutrality, there has been a subsequent increase in mortality rates due to heat related illnesses that has economically hampered the U.S. and Global livestock industry [1, 2]. Heat distress in animal agriculture have shown to impact milk yields and composition, animal growth, reproduction, and carcass traits [1]. More specifically, the projection of rising temperatures and prolonged heat wave seasons poses a considerable threat to the swine industry that loses approximately $300 million in the United States and over billions globally due to heat stress [3].. Heat stress, as described by St-Pierre et al [2], is the result of increased thermal energy in the animal as a result of warm environmental temperatures, heat produced by nutrient metabolism, and increased metabolic rates from thermoregulatory mechanisms without the ability to efficiently dissipate the heat load to maintain homeostasis.

This heat stress in growing pigs compromises gut integrity by causing intestinal inflammation, metabolic imbalance [4], and reduced feed intake [5]. The impacts of heat stress are found in increased monetary losses associated with growth retardation and the cost of finishing swine. In addition, current strategies to ameliorate heat stress such as cooling, shading using insulated roofs, or the extensive use of water and electricity are not cost effective. The result is a need for an alternative method for alleviating the negative effects of heat stress. Grape seed extract, a byproduct of wine production, has shown promising results in improving growth

performance, nutrient metabolism [6-8], and colonic barrier integrity and reducing circulating endotoxin levels, therefore providing the potential to improve overall animal health [9].

The **long-term** goal of this study is to develop dietary strategies through supplemental feed additives that will aid in combating the deleterious effects of physiological stress in animal health. The **overall objective** for this project is to determine the efficacy of grape seed extract to ameliorate the detrimental effects of heat stress in pigs. The **central hypothesis** is that grape seed extract will improve intestinal integrity, body composition, and nutrient metabolism thus preventing deterioration of the animal's health under heat stressed conditions. In order to achieve the overall objective and test the central hypothesis, the following specific aims were proposed:

Aim 1: To evaluate the effects of grape seed extract supplementation on intestinal integrity in heat stressed pigs.

Working Hypothesis: Grape seed extract supplementation will reduce key inflammatory mediators while improving formation of tight junction proteins leading to increased intestinal integrity in heat stressed pigs.

Aim 2: To evaluate the effects of grape seed extract supplementation on body composition such as lean mass accretion, total body fat mass, total body bone mineral content, and bone mineral density in heat stressed pigs.

Working Hypothesis: Grape seed extract supplementation will alleviate the deleterious effects of heat stress by reducing total body fat mass production and increasing lean mass accretion.

Total body bone mineral content and bone mineral density will return to normal levels prior to heat stress.

Aim 3: To evaluate the effect of grape seed extract supplementation on nutrient metabolism in heat stressed pigs

Working Hypothesis: Animals fed a grape seed extract supplemented diet under heat stressed conditions will have improved glucose tolerance along with increased lipid and protein metabolism compared to those pigs fed a control diet under heat stressed conditions.

Literature Review

Overview

United States Pork Industry – At a Glance

The United States pork industry is the world's third largest pork industry attributing nearly 10% of all pork produced throughout the world, only behind China (46%) and the European Union (26%) [10, 11]. In 2013, it was estimated that the U.S. produced nearly 23.1 billion pounds of fresh, chilled, or frozen pork for consumption or processing. The U.S. swine production is currently valued, as of 2013, at $21.4 billion which is increased from $12.8 billion in 2009 and currently accounts for 6% of the total U.S. agriculture sales [10, 12]. In the U.S., large scale hog production (>2,000 hogs/producer) accounts for only 13% of total hog production with nearly 66% coming from small scale operations (<100 hogs); however, the large scale production accounts for more than 85% of the total number of hogs in the country [10]. Most large scale swine production relies on the availability of feed and is found where it is grown. Being said, the top swine producing states are those in the Midwest, known as the Corn Belt, or in the southeast. By sales, the top pork producing states are Iowa ($6.8 billion), North Carolina ($2.9 billion), Minnesota ($2.8 billion), Illinois ($1.5 billion), Indiana ($1.3 billion), and Nebraska ($1.1 billion) [12]. While the U.S. pork industry is only third largest pork producer, it is also the world's largest exporter. The majority of pork from the U.S. is exported to Japan, Mexico, and mainland China [11].

Heat Stress Impact on Animal Agriculture and the Swine Industry

During this century, average temperatures in the United States are estimated to elevate approximately 1.67°C to 6.67°C (3°F to 12°F) while average global temperatures are projected to rise 0.28°C to 4.8°C (0.5°F to 8.6°F) [13-15]. This rise in ambient temperatures throughout the world is commonly attributed to an increase in human population activity stimulating the "greenhouse effect" and solar radiation emission[16]. As temperatures are predicted to rise above and beyond thermal neutrality, which is defined as the temperature in which metabolic heat production is not increased due to cold stress or heat stress, there has been a subsequent increase in complications in animal agriculture related to heat stress that has formerly and still currently hampers animal production in the U.S. and Global livestock industry. [2, 17, 18].

Heat stress has greatly impacted animal agriculture in the United States. In a review in 2003, it was estimated that the U.S. livestock industry lost approximately $1.7 billion – $2.4 billion annually in livestock due to heat stress [2]. Heat related illnesses in animal agriculture have shown to impact animal growth, carcass traits, reproduction, and milk yields [1]. Nearly half (43%) of the annual losses were accounted from states in the west (California), Midwest (Iowa, Minnesota, Nebraska), and Southeast (North Carolina) where those regions regularly exceed thermal neutral conditions [2]. For example, in 2006, a heat wave in the United States accounted for the losses of over 30,000 dairy cows in California and 4,000 beef cattle in Iowa [19].

More specifically, the projection for rising temperatures and prolonged heat wave seasons poses a considerable threat to the swine industry. It is estimated that this industry loses >$300

million per year due to heat stress [2]. Approximately two-thirds is lost on the animal and the remaining third on capital costs for ameliorating the effects of heat stress which are characterized by the hallmark symptoms of compromised intestinal integrity [4, 20, 21], metabolic imbalance [4], and reduced feed intake [5]. The impacts of heat stress are found in growth retardation and the cost of finishing swine leading to monetary losses. In addition, current strategies for ameliorating heat stress such as cooling by means of extensive use of water and electricity or shading using insulated roofs are not cost effective; calling for alternative methods in alleviating the negative effects of heat stress.

Swine Temperature Homeostasis

Homeostasis in the animal can be affected by three zones of temperature: the lower critical temperature, the thermal neutral zone (TNZ), and the upper critical temperature [22, 23]. The most significant of the three zones is in the TNZ. The TNZ provides the animal with the most comfort (i.e. no sweating) and highest rate of performance (i.e. metabolism and feed conversion) [22, 24]. Dependent on a decrease in ambient temperature, an animal may reach the lower critical temperature in which metabolism is significantly increased and heat loss is minimized in order to raise core body temperature to regain homeostasis. In the event of an increase in ambient temperature, the opposite occurs. Metabolism is significantly reduced while heat loss is maximal to offset the elevation in temperature [23].

Generally, for most animals to maintain temperature homeostasis, they are able to regulate heat loss or heat gain through radiation (giving off heat to the surrounding environment), convection (air or water flow coming into contact with the skin and dissipating

heat), conduction (body conducts heat to skin/surface contact such as lying down on the cool ground), and most commonly through evaporation (heat loss through the change of water from a liquid to a vapor) whether that be evaporative loss from the skin in sweating or from the respiratory system [25, 26]. When homeostasis is not achieved a decline in animal performance and health is observed. This is most noticeable in growing-finishing swine when the temperature is greater than the 18-24°C optimal thermal neutral zone. Temperatures that exceed the TNZ may induce heat stress or hyperthermia in the animal. Heat stress, as defined by St. Pierre et al. [2], "results from a negative balance between the net amount of energy flowing from the animal to its surrounding environment and the amount of heat energy produced by the animal" amounting from a variety of factors which include metabolism, environmental factors, and thermoregularity. As temperatures continue to rise, the most commonly used mechanism to manage heat stress and dissipate heat in the pig, evaporation, is not very effective because as observed by Ingram et al. [27], the sweat glands in the pig are not responsive to temperatures above the thermal neutral zone as they are in other mammals such as cats, dogs, and humans [5, 28].

As temperatures exceed 25°C, pigs begin to show hallmark signs of distress. While these symptoms will develop at various points depending on the different characteristics of the animal (i.e. body weight, breed, sex, and physiological status), they are visibly noticeable when the animal has been stressed [29]. For example, heavier pigs are more easily stressed than younger, lighter weight pigs [30]. The hallmark symptoms of stress include various physiological parameters such as escalated respiration rates (RR) and body temperature (T_B), usually measured using rectal temperature (RT) as an indicator. Normal RR and RT for pigs are 60-80 breaths per minute (BPM) and 39.1°C±0.5°C (102.5°F±1°F), respectively, while distressed pigs have heart

rates well above 80 BPM and rectal temperatures >39.1°C±0.5°C [31]. Other noticeable signs of distress in the animal include diminished voluntary feed intake (VFI) to minimize the thermic effect of feeding [3, 5, 29]. Heitman and Hughes [32] and Heitman et al. [33] estimate that pigs reduced feed intake roughly 60-100g/day per °C of heat stress. Along with the previous estimation, Collin et al [5] reports that in young pigs, around 20kg, VFI is at the highest level in the thermal neutral zone, decreased between 25-33°C, and significantly depressed at more than 35°C. Level of activity by the animal also becomes decreased to reduce heat production in favor of maximizing the amount of time lying down to dissipate heat through methods such as evaporation through the respiratory system or conduction through lying on a cooler surface [23].

Metabolism

Carbohydrate Metabolism

During the fed state, the majority of glucose is obtained from the diet and is in the form of complex carbohydrates [34]. These complex carbohydrates can be broken down to single glucose units and further degraded to produce energy for the cell via cellular respiration. According to Scheepers et al [35], glucose homeostasis is monitored by glucose absorption in the small intestines, glucose production in the liver, and consumption of glucose by peripheral tissue. The basal glucose levels for a healthy pig can range from 80-120 mg/dl [36, 37].

In the body, adenosine triphosphate (ATP) can be produced through the cellular respiration of glucose that is broken down to carbon dioxide. Respiration begins in the cytosol of the cell with glycolysis. In glycolysis, glucose, a 6-carbon molecule, is oxidized through a series of reactions to produce two 3-carbon molecules of pyruvate and 2 ATP. Next, the 2 molecules of

pyruvate enter the mitochondria matrix where they are individually stripped of a carbon molecule. The stripped carbon is expelled as carbon dioxide and the remaining 2 carbon molecule attaches to coenzyme-A to form acetyl-CoA which subsequently enters the Kreb's cycle. Briefly, in the Kreb's cycle, each molecule of pyruvate is broken down into carbon dioxide and ATP. Simultaneously, the key electron carriers, NAD^+ and FAD are reduced to NADH and $FADH_2$. Furthermore in the mitochondria, in the final step of cellular respiration, oxidative phosphorylation, maximizes the amount of ATP produced by one molecule of glucose. In oxidative phosphorylation, electrons released from NADH and $FADH_2$ go through the electron transport chain in order to reduce oxygen to water. As the electron goes through the phases of the electron transport chain, it releases energy which in turn pumps protons across the membrane of the crista into the intermembrane space creating an electrical gradient and a highly acidic environment. With the increase in protons in the intermembrane space, the protons now have a want to travel from the area of high concentration to the area of low concentration back inside the matrix. This occurs through the ATP synthase in which protons are pumped through this molecule causing it to turn which assists in the combination of a phosphate group to adenosine diphosphate to produce ATP [38]. One molecule of glucose can create upwards of 38 ATP molecules [39].

Lipid Metabolism

Another form of fuel in the diet is in the forms of lipids. Upon consumption and reaching of the intestinal lumen, triacylglycerols (TAGs) mix with bile salts from the liver to form micelles which are broken down by pancreatic lipases to free fatty acids and monoacylglycerols where they can be readily absorbed by the intestinal mucosa. Once inside the intestinal mucosa,

TAGs are reformed from free fatty acids and monoacylglycerols and packed into transport particles called chylomicrons that can be shipped throughout the body to the target tissue [38]. Chylomicrons bind to membrane-bound lipoprotein lipases where the TAGs are once again catabolized for transport across the membrane into the cell and then resynthesized for storage, typically in the cytoplasm of adipose cells [40, 41].

During excess fasting, fatty acids undergo mobilization in order to be oxidized to maintain energy homeostasis of the cell while reserving the remaining glucose for obligate glucose using central nervous system (brain), red blood cells and immune system. When the cell is in need of energy, glucagon stimulates hormone sensitive lipase which is used to hydrolyze TAGs to free fatty acids and their glycerol backbone [41]. In the muscle and heart tissue, free fatty acids are oxidized to produce energy and the remaining glycerol portion is used in gluconeogenesis to synthesize new glucose molecules. In the liver, free fatty acids are absorbed and converted to ketone bodies which are released in the blood and used to be converted elsewhere in the tissue to be converted to acetyl CoA [42]. Acetyl CoA can then be used in the TCA which further produces ATP saving all available glucose for the glucose dependent tissue [38].

Protein Metabolism

Amino acids, twenty of which are considered as the primary building blocks to life, are used to construct proteins and peptide chains as well as other nucleotide bases that use nitrogen such as adenine, uracil, guanine, cytosine, and thymine, which make up the genetic code of DNA or RNA [43]. Consumed protein can either be broken down into fuel or used for synthesis of new proteins or peptides. As protein is digested, it rapidly begins to denature when mixed with the

high acid (pH 2) juices and proteolytic enzyme, pepsin, of the stomach. Further down in the intestines, proteins and peptides are further catabolized into individual amino acid units where they can be then absorbed by the gut mucosal cells in intestinal wall [38, 44]. Certain amino acids and their metabolites such as glutamine, glutamate, and aspartate are used as fuel at this location while the remaining amino acids reach the portal veins and travel to the liver for further degradation [38]. Amino acids consist of a carbon skeleton and α-amino group. In the liver, amino acids are split into these two constituents. The carbon skeleton is transformed acetyl CoA, pyruvate, or other major intermediates of the Kreb's cycle so it can be further converted to fuel. The remaining α-amino group is converted to urea via the urea cycle where it is eventually excreted from the body in the urine [38, 44].

The second fate of protein occurs with protein synthesis in the body. Protein synthesis takes place on the ribosome of the cell where messenger RNA (mRNA) acts as a template of replicate protein and transfer RNA (tRNA) compliments this code to work together along with others to form new proteins and peptides [45]. mRNA contains a template of a series of 3 base pairs, otherwise known as codons, that integrate a specific amino acid to each pair[43]. tRNA reads the template and brings with it the specific amino acid that binds to that spot. There are a multitude of tRNA proteins that complete this process until a protein or peptide is formed [38]. Typically, the amino acids used in this process consist of essential amino acids that are used for skeletal muscle synthesis whereas nonessential amino acids are primarily used as a fuel source [44].

Insulin

Insulin, a hormone secreted by β-cells in the pancreas, plays a multifaceted anabolic role in carbohydrate, lipid, and protein metabolism [46]. In carbohydrate metabolism, insulin's primary role is to maintain glucose homeostasis. Insulin stimulates glucose uptake via facilitated diffusion by glucose transporter type 4 (GLUT 4). The surfacing of this transporter at the cell surface, expressed in insulin sensitive tissue such as the heart, skeletal muscle, and adipose tissue, dramatically increases the uptake of glucose up to nearly twenty times the normal rate [35]. Insulin also plays a role in glucose storage. Insulin upregulates the conversion of glucose to glycogen that, to store in the liver for fuel, while reducing the breakdown of glycogen in the muscle. In conjunction to insulin, when glucose levels begin to lower, glucagon, produced by α-cells in the pancreas, promotes the catabolism of glycogen to glucose in order to fulfil energy needs of the cell [47]. Both insulin and glucagon work simultaneously to maintain glucose homeostasis.

In lipid metabolism, insulin has the opposite effect of the lypolytic stimulating hormones epinephrine, norepinephrine, and glucagon, in that it inhibits lipolysis in the muscle and liver [38]. To do this, glucagon, for example, inhibits hormone sensitive lipase which in turn blocks off the pathways in which TAGs are broken down for fuel in the muscle, heart tissue, or liver as previously explained [48]. Being that insulin inhibits lipolysis, this enzyme stimulates the synthesis of TAGs by increasing lipoprotein lipase activity which increases the fatty acids available for esterification [49]. Insulin also stimulates the transport and metabolism of glucose in adipocytes which provides the substrate, glyceraldehyde-3-phosphate, for TAG synthesis [50].

Metabolic Adaptation to Heat Stress

Carbohydrate

Heat stress markedly alters carbohydrate metabolism. Typically during heat stress, there is an increase in circulating insulin concentration which has been shown to cause hypoglycemia in many animals such as the pig due to increased glucose uptake [51]. The rise in circulating insulin levels stimulates an increase in hepatic glucose output as a response for diminished levels [51]. Interestingly, due to the constant rise in insulin levels, there is also a possibility for increased risk of cellular insulin resistance. With this increased hepatic glucose output and insulin resistance, over production of glucose could lead to hyperglycemia that could negatively impact the animal's health [52]. Independently of insulin, there is an *in vitro* (cell culture) increase in the glucose transporters sodium-glucose transporter 1 (SGLT1) and glucose transporter type 1 (GLUT1) during heat stress [51].

Lipid

Along with carbohydrate metabolism, lipid metabolism is also altered to promote the health of the animal. One of the hallmark symptoms of heat stress is a reduction in voluntary feed intake (VFI) [3, 53]. Generally, in times of inadequate nutrient intake, fatty acids stored in the adipose tissue are mobilized and oxidized to conserve glucose and compensate for the lack of energy available to the body from the diet; however, during heat stress, this is not the case [54]. Despite reduced VFI, multiple studies [3, 51, 52] show that pigs have reduced plasma non-esterified fatty acid concentrations while showing increased lipid retention on the carcass at slaughter due to the increased capacity for storage of triglycerides in the adipose tissue [55].

Ramifications of Hyperthermia on the Gastrointestinal System

The gastrointestinal tract (GI tract) is a long pathway of organs, leading from the esophagus to the colon, that is ultimately responsible for the breakdown of food products and the absorption of nutrients found in those food products [56]. The GI tract, specifically the regions containing the stomach, small and large intestines, and colon, are comprised of a multitude of organisms that make this pathway efficient and create a barrier between unwanted food antigens, bile, and endotoxin, otherwise known as lipopolysaccharide (LPS), from entering the abdominal cavity. These organisms include enterocyte membranes and tight junction proteins (TJPs) in between the enterocytes which makeup the wall-like structure of the intestinal epithelium. The intestinal epithelium also contains tissue macrophages as well as a mucus layer that aid in the prevention of unwanted substances leaving or entering the GI tract [57, 58]. Finally, one of the largest components in the GI tract is the gut microflora. The GI tract is home to millions, up to trillions, of obligate anaerobic microorganisms throughout the pathway. Particularly, in viable counts/g of material, the stomach, distal small intestines, and the colon in pigs, can contain up to 10^7-10^9, 10^9, and 10^{10}-10^{11} counts of bacteria, respectively, that aid in the metabolism of essential nutrients for the body [59-61].

However, during periods of heat waves or prolonged environmental exposure to heat, a chain of detrimental reactions occur in the gastrointestinal tract that can greatly affect the overall well-being of an animal. Generally, under heat stress, animals redistribute their internal blood to peripheral regions in order to maximize heat dissipation inducing a cellular, tissue, and systemic

response in body [57, 62]. Redistribution of the blood to the peripheral causes vasoconstriction of the blood vessels in the splanchnic region. Following vasoconstriction, the lack of blood flow leads to hypoxia of the GI tract and the cells that comprise this region. In turn, cellular hypoxia induces mitochondrial stress, reducing ATP production and increasing the production of transition metals (Ca^{2+}). Specifically, the increase in transition metals stimulates free radical production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the mitochondria [62]. ROS, such as superoxide (O_2^-), and RNS, such as nitric oxide (NO), produced by the enzymatic activities of NADPH oxidase and nitric oxide synthase II (NOS II), respectively, have shown, in overproduction, can cause severe damage to cells [62, 63].

Consequently, ROS and RNS overproduction causes overall cell damage to the enterocyte membrane and TJP integrity compromising the intestinal epithelium [20, 57, 62]. With damage to the enterocytes and TJPs, increased intestinal permeability is imminent. According to G.P. Lambert [57], "intestinal permeability is defined by the nonmediated diffusion of large (i.e., molecular weight > 150 Da), normally restricted molecules from the intestinal lumen to the blood." Permeability is measured in two ways: First, through the concentration of FITC-dextran in plasma over time [64]; second, through the concentration of blood LPS. In animals, intestinal permeability is measured by a fluorescently labelled polysaccharide, fluorescein isothiocyanate-dextran, which is ingested and measured for fluorescence in plasma. The concentration of this dextran is proportional to the permeability of the cells to this macromolecule [57]. Along with measuring permeability using FITC-dextran, gut permeability can also be measured by the serum concentration of LPS. LPS is a component of the walls of the anaerobic, Gram-negative bacteria that are found in the gastrointestinal tract [65]. Increased

circulating endotoxin concentration shows the ability for LPS to pass through the lumen into circulating blood potentially leading to sepsis and death of the animal. Furthermore, the rise in blood endotoxin triggers a local cytokine response at the site of infection through binding to toll-like receptor 4 (TLR4) found on cells that respond to LPS such as macrophages, monocytes, and leukocytes [66, 67]. The immune system's response to LPS is found in the release of cytokines, specifically, Interleukin-1alpha (IL-1 α), IL-6, IL-1β, Interferon-γ (IFN-γ), and tumor necrosis factor alpha (TNFα) that induce inflammation causing damage and injury throughout the site of the response [57, 65].

Moreover, the increase of gut permeability leads to the leakage of LPS into blood circulation, stimulating a cytokine response, and local inflammation, also increases the enzymatic activity of NOS II. Escalation of NOS II activity promotes the further production of ROS and RNS, specifically, nitric oxide. Nitric oxide has been found to promote the relaxation of the blood vessels, which in turn is a precursor to the vasodilation of vessels in the splanchnic region. Dilation of the splanchnic region brings about hypotension which leads to cardiac shock, heat stroke, and ultimately a potential death of the animal [62, 68].

Overview of the Detrimental Effects of Heat Stress in Swine

Table 1, below, is an overview of the major detrimental effects of heat stress in swine. The most noticeable symptoms of physiological distress in the animal can be seen through elevated RR and RT along with depressed VFI. Further investigation confirms the effect of heat stress on altered nutrient metabolism. Studies by Pearce et al (2013) [3] and Sanz Fernandez et al [52] showed that there are reduced blood concentration levels of non-esterified fatty acids

(NEFA) showing altered lipid metabolism along with showing a reduction of blood glucose levels displaying altered carbohydrate metabolism during heat stress. Yu et al [21] and Pearce et al (2012) [4] show the effects of heat stress on the gastrointestinal tract. Yu et al [21] found reduced microvilli height in the jejunum and Pearce et al [4] found a 200% increase in LPS compared to TN controls which shows the effects of heat stress on TJPs in the gastrointestinal tract.

Overall, not only does heat stress reduce feed intake [3], alter nutrient metabolism [52], increase intestinal permeability [20], but it also has a much larger impact. The altered nutrient metabolism leads to increased lipid carcass values which leads to a lower quality of meat. The lower quality of meat impacts not only the farmer in that he/she is getting less value for their product, but also the consumer who will have to pay higher costs due to the demand for higher quality product [2, 69]. Heat stress also financially impacts farmers due to the increased cost of finding methods to effectively cool the animals [70].

Table 1. Studies showing the detriments of HS in the pig

Paper	Animal Species	HS Conditions	Major Findings
Pearce *et al*. (2013) [3]	Cross-bred gilts	35°C; 20%-35% humidity for 1,3, or 7 days	- Increase in rectal temp (40.8 vs. 39.3°C) vs. TN[1] - 7.1% reduction in blood glucose vs. TN - Immediate increase in NEFA[2] concentration, but similar to TN control past day 1 - PFTN[1a] had 137% increase in NEFA
Pearce *et al*. (2012) [4]	Cross-bred gilts and barrows	35°C; 24-43% humidity for 24 hours	- 53% reduction in VFI[3] - 200% increase in serum endotoxin levels in HS[4] group - Pigs were hyperglycemic after 24hr - 283% increase in activity of ileum glucose transport activity
Sanz Fernandez et al. (2015) [52]	Cross-bred female pig	32°C, ~23% humidity for 8 days	- 1.5°C and 4.5 fold increase in RR[5] and RT[6], respectively - 39% decrease in VFI - HS pigs were more hypoglycemic - HS pigs had an overall 20% reduction in NEFA
Patience *et al*. (2005) [71]	Cross-bred gilts (Yorkshire x Landrace) 25kg in weight	Diurnal HS fluctuation between 20°C and 38°C in 24hrs for 11 days	- DHS pigs consumed more feed in morning than night - DHS decreased VFI and increased water intake vs. TN - HS had 7-fold increase in respiration rate - 1.5°C increase in core body temp
Marple *et al*. (1974) [72]	Chester White gilts (65 to 70kg in weight)	27°C (increase 5°C per hour until HR ceased); 70-80% humidity	- Increase in RR and RT (60 min antemordem showed a rapid increase in body temp) - Death occurred at RT of approx. 43°C - Variable plasma glucose levels, but were 2-fold increase compared to rested
Yu et al., (2010) [21]	Chinese Mini Pig (2 months old) 7.6±0.5kg weight	40°C for 5 hr/day for 1,3,6, or 10 days	- Significant increase in body temp (37.4 vs. 35.4°C) compared to TN - Microvilli height (2232 vs. 2013nm) was shorter in jejunum vs. TN group - Increase in HSP70[7], HSP90 and HSP27 mRNA expression in HS groups (jejunum)

[1]TN – Thermal Neutral; [1a]PFTN – Pair fed thermal neutral
[2]NEFA – Non-esterified fatty acids
[3]VFI – Voluntary feed intake
[4]HS – Heat Stress; [4a]DHS – Diurnal Heat Stress
[5]RR – Respiration rate
[6]RT – Rectal Temperature
[7]HSP – Heat Shock Protein

Analysis of Alternative Methods for Alleviating Heat Stress

As the deleterious effects of heat stress have been studied in the pig for more than 50 years, there has been a push to find methods to alleviate these symptoms in the animal. These methods include a wide range of treatments such as environmental cooling, changing the base diet composition, and supplementing the diet with antibiotics, other nutrients or chemicals/drugs that contain properties that assist in surviving a heat load; however, due to the varying results and cost effectiveness of these treatments, a more in depth analysis is needed.

Cooling

There are a multitude of cooling methods that are applied in the swine industry to reduce heat stress in the animal. These methods are through convective, evaporative, and conductive measures [73]. Convective cooling relies on natural or increased air flow such as air conditioners or snout coolers. In Kentucky, most swine production facilities have little to no ventilation and rely on natural air flow [74]. During summer months this could easily result in increased environmental heat loads that could be detrimental to the animal. Air conditioning, that removes heat from inside the building to the outside environment, is an exceptional way to lower ambient temperature around the animal, but is terribly inefficient in terms of cost [75]. The last convective measure used to cool pigs is via the snout cooler. This is a tool that is used to increase (blow) air over the animal in the lying, standing, or sitting position [73]. Evaporative cooling methods used in the industry include misting or direct sprinkling of the water onto the animal. Misting involves the spraying of tiny water droplets into the air to reduce ambient temperature around the animal [73, 74, 76-78]. Direct spraying of the animal involves placing water on the skin and the direct contact by larger water droplets leads to increased evaporative cooling [78].

Finally, conductive cooling methods used are primarily water cooled pads in which the animal can orient itself in multiple different positions on the pad in order to reduce the animal's heat load [73, 79].

To go more in depth, various studies have attempted to compare and contrast these methods used for cooling in terms of benefits to minimize the symptoms of heat stress (i.e. reduced growth performance and intestinal integrity, and altered metabolism). For example, Bull *et al* [73] performed a preference test a preference test among heat stressed gilts to determine if the pigs preferred snout coolers, water dripper, or a cooling pad along. Physiological parameters, respiration rate and rectal temperatures, were also taken following the use of the cooling methods to determine if there was a correlation between reduction of heat stress in the animal and preference of cooling method. It was concluded that heat stressed gilts chose the cooling pad nearly half (50%) of the time that cooling methods were desired by the animal. Cooling pads also reduced rectal temperatures more so than the other methods tested (water drip and snout cooler) and caused a vast reduction in respiration rate (72.7 bpm [cooling pad] vs. 114.7 [snout cooler], 102.7 [drip cooler], and 103.2 [no cooling]) [73].

Another examination of the use of cooling methods came from the University of Kentucky cooperative extension service in which researchers examined the use of evaporative (misting system) cooling systems through a computer simulated 22 year model based on weather and pig placement date (April to July). From this model, it showed that the misting cooling method does show an increased growth performance (i.e. increased growth rate, feed intake, and earlier market date) at most of the facilities throughout the state of Kentucky. In terms of

profitability, this correlated to a profit margin ranging from $4.16/pig to as little as $0.41/pig depending on placement date, weather, and location of facility. Bridges *et al* [74], reported that this method was generally profitable throughout the 22 year simulation, but the profit margins were higher with placement dates in mid-spring compared to those in the heat of the summer in July. Due to the low initial investment cost into this system, the authors believe it be more cost effective with earlier placement dates. Despite showing the ability to have profitable margins, this analysis did not take into account operating costs associated (i.e. electricity and water) with providing these benefits to the animal so it is hard to determine the overall capital worth of this investment [74].

Antibiotics

In the pork industry, the use of antibiotics has a multitude of roles from its normal use in protecting herds from deadly diseases (which cause diarrhea to potentially death) to being used to stimulate growth in pigs [80-82]. In the protection of the pigs, antibiotics may be used therapeutically, to treat groups of pigs when some pigs begin to show clinical symptoms of disease, or prophylactically and given to groups of pigs in advance of showing clinical signs of disease [80]. As well as disease prevention, antibiotics have also been given to pigs to promote growth. In a study by Mordenti and Zahini [83], antibiotic growth promoters (AGP) were shown to stimulate weight gain by 6-7% and feed conversion efficiency in 3.7-4.5% in pigs, therefore, combating some of the symptoms associated with heat stress. To stimulate weight gain, it is hypothesized that antibiotic growth promoters work through the following methods, as reviewed by Bach Knudsen [59]: thinning the epithelium leading to greater nutrient absorption, reducing

microflora leading to higher nutrient availability, and the reduction of growth suppression toxins and microbial deconjugation of bile acids.

However, while antibiotics have the ability to protect the animal from disease and stimulate growth in pigs, the side effects associated with the usage of these chemicals has become controversial and alarming topic amongst the industry and consumers following a 1994 bacterial outbreak in European countries. In 1994, a bacterial outbreak of vancomycin-resistant enterococci (VRE) was found in food-animals in the United Kingdom, Germany, and Denmark. The effects of this outbreak rippled throughout the world's hospitals as individuals could not be cured due to the microorganism's resistance to nearly all antibiotics [80]. With this outbreak, scientists traced chemical residues on meat products back to a Danish farm that used the glycopeptide antibiotic avoparcin. There, significant evidence linked between the usage of avoparcin as antibiotic growth promoter and glycopeptide resistant enterococci [84].

While this product (avoparcin) was eventually banned by the EU in 1997 and was never used in the U.S., the use of antibiotics throughout the world is still prevalent. For example, Tetracycline has been used for many decades and is still currently used today, in the U.S., for protection against Gram-negative bacteria and as a supplemental AGP. Although there has never been a correlation between the use of AGP and resistant bacteria, there is still reason for a call for the use of different methods for treatment of animals [80].

Changes in Diet Composition

Heat increment, according to the National Research Council, is the increase in heat following consumption of food [85]. During heat stress one of the largest factors affecting pig growth is reduction in voluntary feed intake to reduce the thermal effect of feeding [86]. In order to reduce the heat increment following feeding, researchers have investigated altering the composition of the swine's diet to reduce the heat load upon consumption. Methods for altering the diet that have been investigated include reduction of protein or fiber with an increase in fat or starch content of the diet [26]. Heat increments as a percentage of energy converted to heat for protein and carbohydrates are 36% and 22%, respectively, which are higher than that of fat 4 to 10% [87, 88]. Heat production from proteins and fiber come from the catabolism of excess amino acids for urea synthesis and the fermentation production of ATP from short-chained fatty acids, respectively [26]. Researchers at the University of Kentucky and Georgia have looked into increasing the fat content of the diet by 5%, but did not report any benefits for growth performance in weaning and growing pigs; however, they did notice improvement in growing-finishing pigs at ≥ 29°C. Unfortunately, this increase in growth performance was associated with increased fatty carcass at slaughter [89]. A reduction in crude protein itself was found to stunt growth performance and be deemed unfavorable because of the lack of necessary amino acids for growth [26].

Supplements to the diet

Supplements to the original diet have been found in many forms. Some of these may be in the form of amino acids or other nutrients, chemicals, or byproducts of processes used in the body. In the first instance, the addition of L-lysine to the diet showed the benefits of increased

growth performance with no additional carcass fat traits as with additional fat in the diet [89]. Sanz Fernedez et al [90] supplemented zinc (normal levels in diet and 100/200ppm increase) to heat stressed crossed gilts, but failed to see any improvements in growth performance. Rhoads et al reviewed a series dietary supplements, chromium, thiazolidinediones, and lipoic acid that have varying effects to combat heat stress. Chromium, a micronutrient used in the body to regulate glucose, lipid, and protein metabolism shows reduced weight loss in lactating cows and an increase in feed consumption in broilers, but it did not stimulate feed intake in swine. Thiazolidinediones, a drug used to treat diabetes, was able to improve heat tolerance in diabetic patients and improve insulin responsiveness in dairy cows, but has never been tested in swine. Finally, a third supplement that has been tested is lipoic acid which is synthesized in the mitochondria and plays a role in enzymes that perform oxidative decarboxylation. Lipoic acid has the ability to be a potent reactive oxygen and nitrogen species scavenger as well as enhance glucose uptake in chickens and horses, but has never been tested in swine [1].

Polyphenols

Polyphenols are a class of chemical that are biosynthesized via the shikimate or acetate pathways as a result of secondary metabolism. Secondary metabolites are advantageous to a plant's longevity and are produced as a natural phytoalexins to give resistance against pathogens, protect from ultraviolet damage from the sun, and aid in the determent of predators due to the highly astringent nature of the chemical when consumed [91, 92]. To this date, more than 8000 different phenolic structures have been discovered [91, 93]. Being that polyphenols are ubiquitously found in plants, they are frequently consumed as a part of an everyday diet. Polyphenolics can be found in an extreme variety of fruits, some vegetables, and even beverages

[91, 94, 95]. Examples of these foods and their phenolic content range can be found, below, in **Table 2**.

Table 2. Phenolic content in various foods and beverages

Fruits	mg/100g fresh matter	Vegetables	mg/100g fresh matter
Apples	27-298	Onion	100-2025
Blueberries	135-280	Celery	94
Grapes	50-490	Potato	14-28.5
Tomato	85-130	**Beverages**	
Orange	50-100	Tea, cup (mg/200mL)	150-210
Cranberry	77-247	Coffee, cup (mg/150mL)	200-550
Cherry	60-90	Wine (mg/L)	
Plum	4-225	Red	1000-4000
Strawberry	38-218	White	200-300

While polyphenols are a wide class of chemical, they can be further broken down into approximately 10 classes with varying base structures, according to Bravo *et al* [91]. For example, some of the most commonly found polyphenols are those in the phenolic acid, stilbene, lignin, and flavonoid classes [94]. Furthermore, flavonoids, the most commonly found class of polyphenols found in plants, can be broken down into 13 more subclasses consisting of more than 5000 different chemical structures [91, 94]. The basic structure of flavonoids, as seen in articles by Bravo *et al* [91], Motohashi *et al* [96], and Aron and Kennedy [97] can be seen in **Figure 1Error! Not a valid bookmark self-reference.**

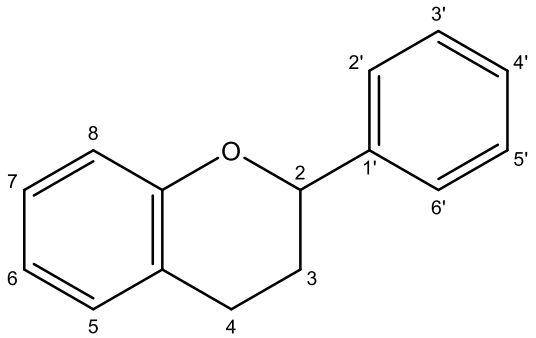
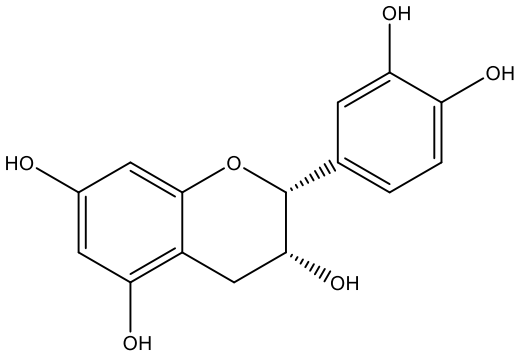
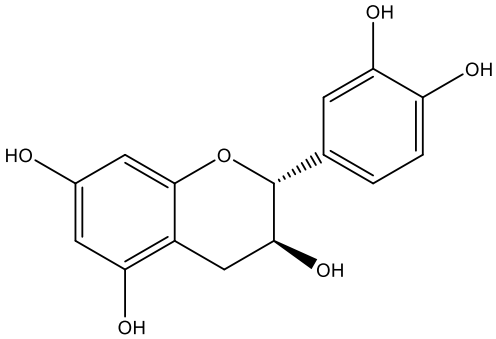


Figure 1. Basic flavonoid structure

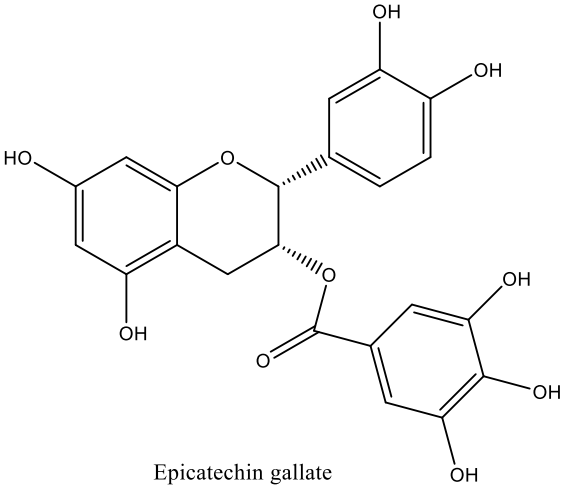
The flavonoid structure is characterized as containing two aromatic rings bound together by three carbons that form an oxygenated heterocycle [97] . Flavonoids are of interest to researchers due to their numerous therapeutic properties, which include antidiarrheal [82], antibiotic [98] , anti-inflammatory [9, 99], and extreme antioxidant capacities [93, 100] as well as improved vision [101], cardioprotection [102], among others [103]. A group known as procyanidins, consisting of flavanols or flavan-3-ols, a further subdivision of the flavonoid, needs attention due to the predominance of this chemical found in fruits. Flavan-3-ols, are identified by the hydroxyl (-OH) group located at the 3 position of the basic flavonoid structure **Figure 1**. It is to note at this time, in **Table 2**that grapes (50-490 mg/100g fresh matter) and a product of its fermentation, wine (200-4000 mg/L), have one of the highest phenolic contents compared to other fruits, vegetables, and beverages. Grapes (seeds and skins) are rich in procyanidins, which are predominately found as the monomeric units, (+)-catechin and (-)-epicatechin. In grapes, flavan-3-ols can also exist as oligomeric procyanidins (OPCs) that are 2 to upwards of 16 monomeric units covalently bonded between the C4 → C8 position [97]. The predominant OPCs are those in the dimeric and trimeric form, including procyanidin B2 and procyanidin C1 [9, 104]. These oligomeric procyanidins are generally recognized by the deep red or purple color of grapes and/or the astringent flavor they give to the fruit. Structures of the most common procyanidins found in grapes as described earlier can be seen in **Figure 2**, below.



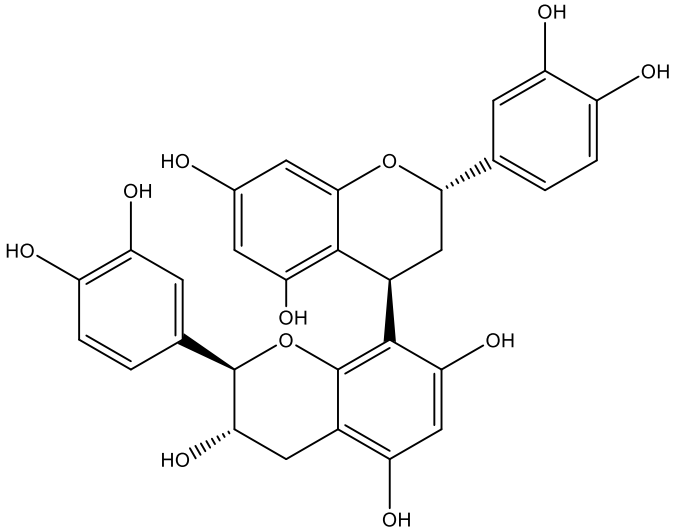
(-)-Epicatechin



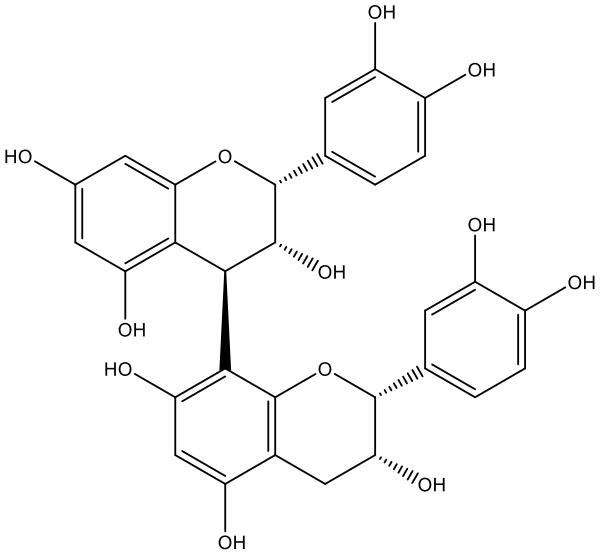
(+)-Catechin



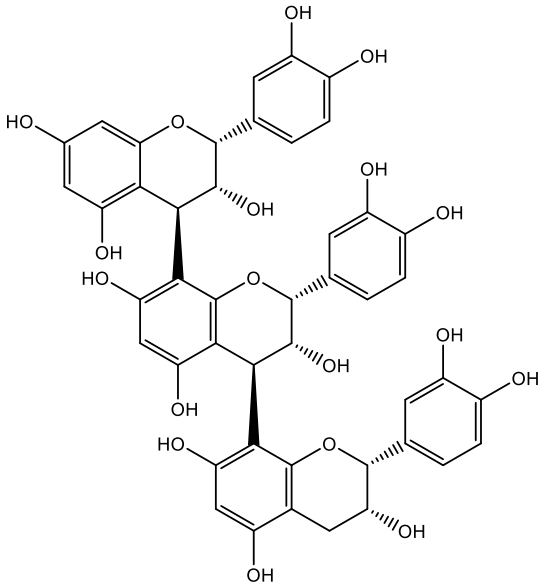
Epicatechin gallate



Procyanidin B1



Procyanidin B2



Procyanidin C1

Figure 2. Select predominant flavan-3-ols found in Vitaflavan® GSE

Grape Seed Extract Composition

Table 3. Vitaflavan® GSE chemical composition

Compound	%(wt/wt)	Compound	%(wt/wt)
Monomers	22.0	Dimers + Trimers	32-35
Catechin	15.3-15.9	Procyanidin B1	7.7-7.8
Epicatechin	4.1-11	Procyanidin B2	5.7
Epicatechin gallate	2-5.7	Procyanidin B3	3.8-4.0
		Procyanidin B4	1.8
Total Procyanidins	>75	Total Polyphenols	>96

Grape seed extract composition that was used in this study can be seen above **Table 3**. Vitaflavan® grape seed extract is characterized by having 22% (wt/wt) monomers, 32-35% dimers and trimers as well as characterized by having more than 75% oligomeric procyanidins and being composed of more than 96% total polyphenols [9]. Other grape seed extracts that are commercially available are Gravinol Super™, Activin®, and Mega Natural™ Gold, all having very similar flavan-3-ol composition. Gravinol Super™ contains 6.6% monomers, 11.6% dimers + trimers, 77.7% oligomers + polymers and is characterized has having 95.9% total polyphenols [101]. Activin® contains 67% dimers + trimers, 7% oligomers + polymers and is characterized as having 79.1% total polyphenols[105, 106]. MegaNatural™ Gold contains 7.41% monomers, 5.8% dimers + trimers, 74.63% oligomers + polymers and is characterized by containing 95.17% total polyphenols [106]. As seen, four different grape seed extracts have similar compositions, but differ due to type of grapes and extraction procedure used.

Grape/ Grape Seed Extract History

Archaeological data suggests that viticulture has been around since 6500 B.C. during the Neolithic era. Following that time, grapes were not only used as a fruit for food consumption, but were also transformed into a fermentation product, wine, that was used for consumption by royalty and widely used for religious purposes during the fall of the Roman Empire. Subsequent

to the fall of the Roman Empire, viticulture and the use of wine began to grow beyond royalty and religious purposes to more social activity. The growth and use of grapes took off during the 16th century and continues in today's world [107, 108] . During 2013, more than 76 million tonnes of grapes were produces, approximately a third of which was fermented into 26 million tonnes of wine, leaving the remains of more than 3 million tonnes of grape marc or pomace (skins, seeds, and stems) [109, 110]. Until recently, grape marc has generally been designated as industrial waste and left to decompose in fields of nearby wineries or in massive landfills [111]. It was in the 1950's when Jacques Masquelier, a French scientist, patented a technique used for the extraction of oligomeric procyanidins (OPCs), finding a use for this industrial waste. Dr. Masquelier discovered these OPCs as potentially having therapeutic benefits after reading in the journal of a French explorer, Jacques Cartier, that he and his crew miraculously recovered from a bout of scurvy after drinking tea made from pine bark in the 1590's. Due to the destruction of the tree from the patented extraction method, Dr. Masquelier discovered that grapes contained a high source of oligomeric procyanidins while maintaining the health of the plant. From that time, he discovered the potential for procyanidins to have a high capacity for scavenging radical oxygen species leading the way to new potential health benefits [112, 113]. Today, grape seed extract has been associated with numerous therapeutic benefits, especially those pertaining to ones needed to alleviate the deleterious effects of heat stress in pigs.

In **Table 4**, below, is a summary of the major health benefits that have been as a result of the intake of varying levels of grape seed extract. GSE has shown to affect both growth and cellular parameters. GSE provided a weight gain of approximately 1 g/day more than control groups [82]. The majority of the benefits of GSE can be found at the cellular level. As stated

earlier, some symptoms of heat stress include reduced intestinal integrity leading to a leaky gut and altered metabolism. Many studies have shown to promote the formation of the tight junction proteins occluding and zonula occluden-1 and also a reduction of claudin 2, a TJP that forms pores and is found at high levels in crohn's patients [9, 82, 99]. Along with the increased TJP formation, there is a reduction in formation and circulating levels of ROS [102]. In terms of metabolism, GSE increases GLUT4 protein expression and a reduced serum TAG concentration [114]. No studies have been performed using GSE to alleviate the symptoms of heat stress in a large animal model making it a viable alternative to traditional methods.

Table 4. Summary of GSE health benefits as related to this study

Paper	Animal Species	Dose	Duration	Major Findings
Song et al (2011) [82]	Sprague Dawley rats (recently weaned; 21 days old)	250 mg/kg BW Grape Seed Procyanidins	29 days	- Increased weight gain (4.12 vs. 3.14 g/day) vs. control - Significantly reduced intestinal permeability (lactulose to mannitol ratio) - Significant increase in expression of Occudin and zonula occluden-1
Wang et al (2013) [99]	Mice (IL-10[1] deficient)	1% GSE[2a] (g GSE/g dry feed weight) GSE	16 weeks	- Reduction of key inflammatory mediators IL1β[3a], IL6[3b], and IFN-γ[3c] - Reduction of claudin 2 TJP[4]
Goodrich et al (2012) [9]	Male Wistar Furth rats (6-7 weeks old)	0.1% (~100mg/kg/d) GSE	Treated for 21; 7 day acclimate	- GSE has potential to inhibit circulating endotoxin after high fat diet - Increased expression of Occludin in proximal/distal colon - Drastically reduced fecal calprotectin in a non-diseased model
Meeprom et al (2011) [114]	Male Wistar rats	0.5 or 1% in food GSE	8 weeks	- Reduction in plasma fructosamine - 40% reduction in plasma adiponectin concentration - Reduced serum triacylglycerols - Increased GLUT4[5] protein content
Pinent et al (2004) [115]	Male Wistar rats	250mg/kg BW GSPE[2b]	Acute	- Significantly reduced blood glucose levels in PE[2c] groups vs. Control (acute dose) - PE dose with low insulin dose boosted effects similar to that of other antihyperglycemic drugs
Sato et al (2001) [102]	Male Sprague Dawley rats	50 or 100mg/kg/d GSPE	3 weeks	- 100mg/kg dose significantly reduced apoptotic cell death - Reduced number of ROS[6] - 50mg/kg reduced ROS formation by 50% - 100mg/kg reduced ROS by 75%

[1]IL-10 defiction – Model used that is translatable to human chronic inflammatory bowel disease
[2a]GSE=Grape seed extract; [2b] GSPE=Grape seed procyanidins extract; [2c] procyanidins extract
[3a]IL-1β=Interluekin 1 Beta; [3b]IL-6=Interleukin-6; [3c]IFNγ=Interferon gamma
[4]TJP= Tight junction protein
[5]GLUT4=Glucose transporter type 4
[6]ROS = reactive oxygen species

Effect of Grape Seed Extract on Carbohydrate and Lipid Metabolism

As previously stated, carbohydrate and lipid metabolism are drastically altered during periods of heat stress in swine. Of the many health benefits of grape seed extract, it has also shown to improve carbohydrate and lipid metabolism. In carbohydrate metabolism, grape seed extracts have shown to have properties similar to insulin, otherwise known as insulinomimetic properties, in which glucose metabolism is improved. *In vitro*, in 3T3-L1 cell cultures, GSE stimulated uptake in the insulin-sensitive cell lines [6]. This insulinomimetic property has also been shown in a high fructose diet induced insulin resistant model done by Suwannaphet et al [116]. In this rat model that faced an eight week meal challenge supplemented with either 0.5 or 1% GSE, the glucose tolerance test showed significantly reduced incremental plasma glucose levels at 15 and 30 minutes compared to the high fructose control. This study also shows the glucose area under the curve concentration was significantly reduced for the 1% GSE supplemented diet [7]. Another study, in which participants were challenged with a high carbohydrate meal and supplemented with 100 or 300mg GSE, also showed significantly reduced blood glucose levels at 15 and 30 minutes compared to the control. The glucose area under the curve concentrations were reduced by 46% and 76% for 100 and 300mg GSE supplementation, respectively, compared to the control diet [116]. All of these studies display GSE's ability to enhance glucose uptake, but the methods in which they do so remain poorly understood.

There are a few mechanisms which GSE's insulin-like properties can be explained. The first is through the PI3K and p38 MAPK insulin signaling pathways. In insulin resistant cells, GSE has the ability to activate insulin receptors and acting through these two pathways to

increase glucose uptake [6]. While the uptake of glucose is less efficient than that of insulin, likely due to the compounds in GSE, their structure and non-ideal binding to the insulin receptor, GSE still can significantly improve glucose uptake. In insulin resistant cells, GSE also promotes the translocation of GLUT-4 to the cell membrane, encouraging glucose uptake [6, 114]. Along with signaling pathways, GSE has been shown to be a potent inhibitor the enzymes α-glucosidase and pancreatic α-amylase which play an important role in glucose metabolism in the gastrointestinal tract [7, 117]. By inhibiting these enzymes, the breakdown of complex carbohydrates to single glucose units and their absorption is slowed, therefore, lowering blood glucose levels [115].

GSE's improvement in lipid metabolism can be attributed to those results found primarily high fat feeding/obesity related models. In three studies, weight gain associated with a consumption of GSE was significantly reduced compared to the high fat control [8, 118, 119]. In addition, the study by Park et al [8], showed a decrease in back fat mass which is a sign of carcass traits in heat stressed reared swine [2]. More importantly, GSE significantly reduced circulating triglycerides [8, 119, 120] as well as liver triglycerides [8], total cholesterol [8, 119], and total phospholipid levels [119].

Two mechanisms through which GSE can improve lipid metabolism are through the increase in fatty acid oxidation and the inhibition of lipases. The first, which appears to be the primary mechanism, is through increased levels of β-oxidation [8, 118]. The expression of many key β-oxidation related genes (ACADVL, CPT1B, and PPAR-α) were increased in GSE diets compared to their controls. In addition, total carnitine concentrations, a compound used in the

transfer of fatty acid chains to the mitochondria for β-oxidation, was also significantly increased with GSE supplementation, therefore, showing an increased level in lipid oxidation [8]. The second mechanism in which GSE can improved lipid metabolism is shown in a cell culture study by Moreno et al [121]. It is reported that GSE, at levels of 1mg/mL, can partially inhibit pancreatic lipase and lipoprotein lipase, therefore, slowing down metabolism of triglycerides into free fatty acids and their uptake and improving overall lipid metabolism.

Flavan-3-ol rich matrices can reduce circulating endotoxin

During periods of reduced intestinal integrity, there is an accompaniment of increased circulating endotoxin due to the leakage of gram-negative bacteria through the intestinal wall into circulation [65, 66]. Along with promoting the formation of TJP's to rebuild the intestinal wall, GSE or other flavan-3-ol rich matrixes also reduce circulating endotoxin levels. A study by Gu et al [122], in which patients were fed 80mg/g unsweetened cocoa powder in addition to a high fat diet, the cocoa supplemented diet reduced endotoxin levels by 40.8% compared to that of the control high fat diet. In another study, Dorenkott et al [123] interestingly found an increase in endotoxin levels when the model was given cocoa extract; however, when the extract was broken down into fractions (monomers, oligomers, and polymers), the monomer and oligomer fractions significantly reduced circulating endotoxin compared to the high fat control. In addition to reducing circulating endotoxin, GSE and other flavan-3-ol rich matrixes can reduce the effects that are induced by an increase in endotoxin. Pallerès et al [124] found that the increase in nitric oxide formation and anti-inflammatory molecule, IL-10, can be significantly reduced at varying levels of grape seed extract, therefore, reducing potential harm to the animal.

Bioavailability of Phytochemicals

Generally, substances used for treatment are administered though intravenous injection or orally ingested. Upon oral administration, the substance immediately begins to breakdown. In addition, due to barriers of absorption and detoxification mechanism, only a portion of the original dose reaches its intended target [125]. This concept, otherwise known as bioavailability, is defined through the Food and Drug Administration as "the rate and extent to which an active ingredient or active moiety is absorbed from a drug product and becomes available to the site of action" [126]. Absolute bioavailability can be determined by measuring the area under the pharmacokinetic curve (AUC) compared to that of the intravenous injection AUC, the maximum plasma concentration of the drug (C_{max}), and also the time required for that drug to reach maximum concentration (T_{max}) [127]. Another term, bioaccessibility, aids in determining the bioavailability of a substance. Bioaccessibility is the fraction of a compound that is available in the gastrointestinal lumen for absorption by the intestinal epithelium upon release from a food matrix after withstanding exposure to saliva, gastric juice, intestinal secretions and varying pH [127-129].

Being that phytochemicals are xenobiotic in nature (foreign to the human body), it remains unclear whether the therapeutic benefits of those substances reside in the intact compounds or are a result of their metabolites [130, 131]. Therefore, it essential to understand their metabolism, how they become systemically bioavailable, and factors that affect bioavailability. The process in which this occurs in the body is digestion and release, absorption, distribution, xenobiotic metabolism, and finally excretion [125, 132].

Digestion, Absorption and Metabolism

Digestion of polyphenols begins in the mouth with enzymatic breakdown via the saliva present. Once swallowed, polyphenols are then subjected to very acidic gastric juices in the stomach and later in the small intestines to secretions of bile from the gallbladder and digestive juices from the pancreas [133]. Fortunately, flavan-3-ols and procyanidins are relatively stable when subjected to saliva and gastric juices, therefore, increasing bioavailability [127].

Once in the small intestine, most polyphenols, 90-95%, will not be absorbed into the small intestines because they exist as glycosides, esters, large oligomers (DP>3) or polymers and therefore pass along to the colon for subsequent metabolism by the gut microbiota [131, 134]. Of those polyphenols remaining are monomeric flavan-3-ol units, some of which are absorbed by enterocytes in the small intestines, but those that are not continue into the colon for microbial degredation [131]. After passing through the epithelium, flavan3-ols, such as (+)-catechin and (-)-catechin, are metabolized by phase II enzymes (UGTs; uridine 5'-diphosphate glucuronosyltransferases, and methylated; methyltransferases) and converted into glucuronide or methyl derivatives. Once metabolized in the enterocytes, these units can be further metabolized in by hepatocytes in the liver into methyl derivatives or sulfate conjugates by catechol-O-methyltransferase (COMT) and sulfotransferases (SULT), respectively, or excreted by bile into feces or though urine. Furthermore, after reacting the liver, these metabolites, if not excreted, can then be transported into systemic circulation [131, 135].

Larger polyphenols and those procyanidins, oligomers (DP>3) and polymers, that are not metabolized in the small intestines reach the colon intact where they encounter approximately 10^{10} – 10^{11} bacteria cell counts per gram of intestinal lumen and are primarily broken down by the resident Fermicutes and Bacteriodetes bacteria [60, 136]. These procyanidins are broken down into smaller procyanidins (monomeric flavan-3-ol units), phenolic acids, and γ-valerolactones [134, 136-139]. As in the small intestines, metabolites present in the intestinal lumen of the colon are absorbed into the colonocytes where they can be transported into the portal vein to the liver. Once in the liver, these metabolites can be sulfonated, methylated, or glucoronated as mentioned earlier and transported to into systemic circulation or excreted in urine or feces [131, 135].

Rationale for using grape seed extract

Over the years, heat stress has negatively impacted animal agriculture and more specifically, the swine industry. Heat stress has been shown to be detrimental to swine health due to the animal's inability to efficiently dissipate heat [27]. This leads to an increase in body temperature, respiration rate, and reduced feed intake [3]. In addition, heat stress markedly alters carbohydrate and lipid metabolism [51, 52], and reduces intestinal integrity resulting in increased oxidative stress leading to increased circulating endotoxin [4]. Current methods for alleviating heat stress rely on various cooling methods [70], antibiotics [140], or changes in dietary composition [1], all of which do not effectively alleviate the symptoms of heat stress. Some of these methods, in fact, create new problems, such as antibiotic resistant bacteria. These new problems have led consumers to demand change of currently used methods in favor of

alternatives that will promote the animal's health and be effective under these environmental conditions.

Grape seed extract has been shown to have many therapeutic health benefits many of which are essential to this study. Those health benefits include improved carbohydrate [7] and lipid metabolism [8], formation of TJP's promoting intestinal integrity [82], reduction of circulating endotoxin, as well as having potent antioxidant properties that promote cell health [124]. All of these health benefits are vital in alleviating the majority of the symptoms of heat stress and provide warrant for its use in a larger heat stressed model.

Unknowns

Being that this is the first time that GSE has been evaluated to ameliorate the symptoms of HS in the pig, there are a few unknowns that need to be addressed. The first unknown is if GSE provides the same health benefits under HS conditions as it has shown under TN conditions in the literature. The health benefits provided by GSE under TN conditions are extensive, but does it provide the same results under different conditions? The second unknown is if these health benefits shown primarily in small rodents will translate to a larger animal with a different metabolism. The third unknown is at what concentration or dose is GSE the most beneficial to the animal and will provide the most health benefits. There are a wide variety of doses in the literature ranging from acute 100mg doses in humans that have provided health benefits to chronic 50mg/kg BW to upwards of 2000 mg/kg BW doses in rats that have also shown health benefits vital to this study. Finally, the final unknown is with treatment delivery. What is the best method for treatment delivery? Pigs are a bit different compared to other animals and humans in that they excessively root or play through their food making it extremely difficult to accurately

measure feed or dose intake. Pigs also have a more sensitive palate comparable to the human palate, and how would this delivery method affect dose consumption [141, 142]?

GSE Dose Justification

To calculate approximate dose of grape seed extract for each pig per day, a dose translation from rats to pigs was used. Starting with the formula (**Table 5**) used in an animal to human equivalent dose study, a modification of this equation to an animal to animal (rat to pig) translation was used.

Table 5. Dose translation equations.

Species to Species	Formula
Original: Animal to Human Equivalent Dose (HED)	$HED\left(\frac{mg}{kg}\right) = Animal\ dose\left(\frac{mg}{kg}\right) x \frac{Animal\ K_m}{Human\ K_m}$
Modified: Animal to Animal (Rat to Pig)	$Eq_{rat}Km_{rat} = Eq_{pig}Km_{pig}$

Note: The original formula was originally published in the study a "Dose translation from animal to human studies revisited" by Reagan-Shaw *et al.*

Unknowns: Eq_{pig} and Km_{pig}

To find Km_{pig}: From a study of dose translations from animal studies to human studies by Reagan-Shaw *et al* [143], one can calculate the Km factor by the following equation as derived from **Table 6** (below).

$$Equation\ to\ find\ Km: Km = \frac{Weight\ (kg)}{BSA\ (m^2)}$$

Table 6. Body surface area based animal to human dose conversion factors

Category	BSA (m^2)	K_m Factor	Weight (kg)
Human - Adult	1.6	37	60
Farm Pig – 15-16 wks	**?**[1]	**?** [1]	50
Human – Child	0.8	25	20
Baboon	0.6	20	12
Dog	0.5	20	10
Monkey	0.24	12	3
Rabbit	0.15	12	1.8
Guinea Pig	0.05	8	0.4
Rat	0.025	6	0.15
Hamster	0.02	5	0.08
Mouse	0.007	3	0.02

[1] Represents an unknown value. This data was originally published in the study a "Dose translation from animal to human studies revisited" by Reagan-Shaw *et al* [143].

Although BSA(m^2) in this equation is unknown, a study "Swine as Models in Biomedical Research and Toxicology Testing" performed by Swindle *et al* [144], has defined farm pigs to have a BSA(m^2) = $0.0734BW_{kg}$. Upon arrival, the pigs will weighed approximately 50kg. At this weight, the BSA value was $3.67m^2$.

$$BSA(m^2) = 0.0734\ x\ BW_{kg}$$
$$At\ 50kg: BSA(m^2) = 0.0734\ x\ 50kg = 3.67$$

The BSA value was taken and plugged into the Km equation (above) to find Km value for pigs.

$$Km_{pig} = \frac{50\ kg}{3.67\ m^2} = 13.62$$

As outlined by Song et al [82], a 250mg/kg Grape seed extract diet was used to evaluate growth performance in weaning rats. If a similar dose is used to evaluate effectiveness of GSE in heat stressed pigs, the Km value for pigs is used to find the equivalent dose (Eq_{pigs}) in pigs as described by the modified formula (see **Table 5** for modified formula).

$$Eq_{rat}Km_{rat} = Eq_{pig}Km_{pig}$$
$$Eq_{pig} = \frac{Eq_{rat}Km_{rat}}{Km_{pig}}$$
$$Eq_{pig} = \frac{(250mg/kg)(6)}{13.62} = 110.1321mg/kg/day$$

Each pig will need to receive a dose of 110.1321 mg/kg/day GSE in order to match this equivalence of 250mg/kg BW. However, it is important to **note** that only approximately **one-tenth (12 mg/kg BW)** of this dose was given to the animals due to the extremely astringent nature of the compound. It has been shown in sensory studies that pigs have a sensitive palate like that of the human and tend to dislike or refuse samples that are bitter in a dose dependent nature in favor of more sweet tasting compounds [142, 145]. Therefore, it was necessary to cut the dose and encase the desired treatment dose in a more palatable vehicle. Reducing the dose to this amount was also the most feasible amount that could be given and consumed by the animal.

Materials and Methods

Animal Care and Experimental Design

Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University (Virginia Tech). Twenty-four female, cross-bred pigs, 15-16 weeks old, were obtained from the Virginia Tech Swine Center and transported to the Litton Reaves animal facility. Pigs were immediately weighed upon arrival using a Way Pig 300 (Raytec LLC, Ephrata, PA, USA) market hog scale (dimensions: 31" H x 49"L x 15" W). Following weighing, all pigs were individually scanned using a dual-energy x-ray absorptiometry (DXA) scanner (General Electric, Fairfield, CT, USA) for a baseline body composition analysis and then assigned into one of four treatment groups. Treatment group assignment was based on the weight of the animal in order to make the average weight the same across groups. The experimental design for this study was of a 2x2 factorial design (**Error! Reference source not found.**). A description of all treatments groups can be seen below in **Table 7**. Following DXA scan and treatment selection, pigs were housed in four separate rooms for the duration of the study. Two of the four rooms were designated as TN rooms and the remaining two rooms were designated as HS rooms. In each room, a total of six pigs were housed; 3 consuming the control diet and three consuming the GSE supplemented diet. Each room in the Litton Reaves facility was equipped with a discrete, 100% clean air ventilation system under constant negative pressure. Pigs were individually housed in either a 4x4ft) or a 3x5ft pen. In each room, pens were aligned adjacent to one another and each pig was given a rubber "chew" toy in order to maximize animal stimulation and interaction with the other animals.

		Heat Stress	
		-	+
GSE	-	-/-	-/+
	+	+/-	+/+

Figure 3. Pig study 2x2 factorial design

Table 7. Description of pig study treatment groups.

Treatment Number	Environment	Diet	Abbreviation	N
1	Heat Stress	Control	HS-control	6
2	Heat Stress	Grape Seed Extract Supplement	HS-GSE	6
3	Thermal Neutral	Grape Seed Extract Supplement	TN-GSE	6
4	Thermal Neutral	Control (Normal Swine Diet)	TN-control	6

Initially, all pigs were placed under thermal neutral conditions, 21°C-22°C (69.8°F-71.6°F), for a 10 day acclimatization period to adjust the animal to its new environment and respective diet. Twelve pigs (n=12; 6 pigs x 2 groups) were fed a control diet (**Table 8**) while the remaining twelve pigs (n=12; 6 pigs x 2 groups) were fed a grape seed extract supplemented diet. Pigs were given food and water *ad libitum*, but were fed on a twice a day schedule (6AM and 6PM). Beginning on day 4 of the experiment, feed refusals were measured each afternoon at 6PM and feed given was appropriately adjusted for the following day to ensure a target of 0.5kg refusals. Feed intake and refusal measurements were recorded to ensure each animal was consuming an adequate amount food and to study the effects of the treatments on feed intake. Following acclimatization, two rooms (n=12), were subjected to a 7 day heat stress period in which environmental heat conditions were increased to 33°C-34°C. Physiological parameters were taken twice a day in thermal neutral groups (6AM and 6PM) and 4 times a day (6AM, 12PM, 6PM, and 12AM) in the heat-stressed groups. At the beginning of each session,

respiration rates were visibly measured and recorded in the health log by counting the number of breaths for 15 seconds and converting to breaths per minute (BPM). Immediately following, to measure core body temperatures, rectal temperatures were measured using a digital thermometer. Body weights were measured three times during the study, upon arrival, prior to heat stress, and prior to euthanasia. A timeline of events can be seen in **Figure *4***.

Table 8. Formulation of control diet (standard diet for pigs).

Component	Percent (%)	g/kg
Corn	78.09	780.9
Soy Bean Meal (SBM)	19	190
Soy Oil	1	10
L-Lysine-HCL	0.1	1
Monocalcium Phosphate (MCP)	0.23	2.3
Limestone	1.18	11.18
Vitamin Mix	0.08	0.8
Mineral Mix	0.05	0.5
Salt	0.25	2.5
Phytase	0.02	0.2
Total	100	1000g

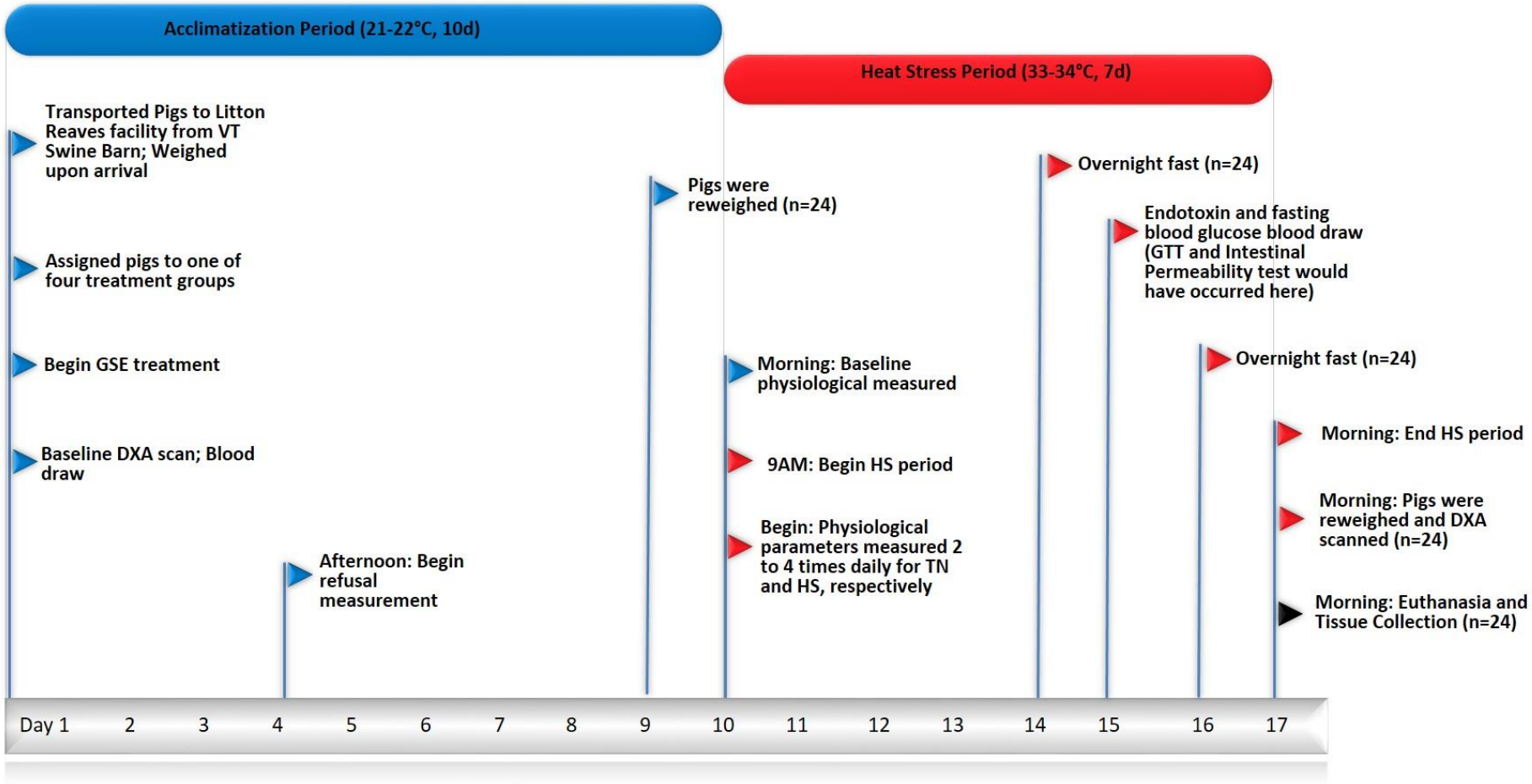


Figure 4. Timeline of events

Grape Seed Extract and Treatment Delivery

Vitaflavan® GSE, purchased from DRT Neutraceutics (Dax, France), was supplemented into the diet of twelve (n=12) pigs to study its effects during heat stress. This extract, extracted from the seeds of white grapes, is comprised of <25% monomers, >30% Dimers + Trimers, and is characterized as containing >96% total polyphenols and >75% oligomeric procyanidins (w/w). The GSE was supplemented into the diet at a dose of 12mg/kg BW via a sugar cookie dough vehicle. Cookie dough was used in order to increase palatability of the bitter and astringent GSE substance and to ensure complete consumption of the supplemental dose. Half of the GSE dose was given with morning feeding (6AM) while the remaining half dose was given with the afternoon feeding (6PM). To construct this food vehicle, each half dose was mixed thoroughly into 10g of sugar cookie dough and layered thinly with another 10g of cookie dough for an approximate vehicle size of 20g. Pigs who did not receive grape seed extract supplementation were given a blank 20g cookie dough vehicle.

Sample Collection

Blood

Blood samples were collected through venipuncture from the cephalic, external jugular, internal jugular or common jugular. Pigs were restrained by one assistant using a snout snare to limit head and upper body movement while the second assistant performed the venipuncture. During restraint, the puncture area was cleaned using a 5% iodine solution (betadine). A sterile needle (18-22Ga by 1-1/2 inch) was used to draw blood into a sterile BD Vacutainer serum separation tube (SST). Once the sample was obtained, pressure was applied, using gauze, to the puncture site until bleeding stopped, sprayed with 70% ethanol, and then the pig was returned to

its cage. Blood collections occurred at the following events: DXA scan (baseline and prior to euthanasia), endotoxin/intestinal permeability (baseline and 3 hours), and a blood draw prior to heat stress.

Serum

Using the method for blood collection, mentioned above, blood was collected into a 4mL BD Vacutainer serum separation tubes (SST). Following collection, blood was allowed to clot for 30 minutes before being centrifuged at 3500xg for 10 minutes at room temperature. Serum was collected in a series of three 2mL microtubes. One aliquot of serum was stored with 0.25µL 2% acetic acid/1% ascorbic acid (w/v) per 100µL of serum for polyphenol preservation and the remaining serum was split in half and stored in separate tubes. Serum was immediately snap frozen and stored at -80°C until further analysis.

Euthanasia Method

Pigs were euthanized by penetrative captive bolt gun followed by exsanguination. Captive bolt stunning was performed with a Supercash Mark 2 captive bolt pistol using a two grain charge (Accles & Shelvoke Ltd., Sutton Coldfield, UK). Animals were restrained to ensure proper placement of the captive bolt. The captive bolt was directed to the brain from a central point slightly above the line between the eyes to induce sudden loss of consciousness and subsequent death. Exsanguination followed the captive bolt to ensure rapid death of the animal.

Tissue collection and storage

Immediately following sacrifice, sections of intestinal tissue (duodenum, jejunum, ileum, cecum, spiral colon, and distal colon), skeletal muscle (longissimus dorsi), and liver were collected. Approximately 5 inch sections from the top of each section of intestines were incised as follows: For the duodenum, 12 inches following the pyloric sphincter. For the jejunum, 36 inches post the pyloric sphincter. For the ileum, 12 inches inward towards the ileum starting at the ileal-cecal valve. For the colon, 12 inches inward toward the rectum. For the cecum and spiral colon, identical sections were cut from animal. Tissue collected was promptly flushed with chilled phosphate buffered saline (PBS) to remove any residual fecal content (for intestinal tissue only). Following flushing, a 1-inch ring of intestine was incised and placed into a tissue cassette for Histology/Immunohistochemistry, placed in a 10% neutral buffered formalin solution and allowed to fixate, until storage in 70% ethanol. A second 1-inch ring of intestine was incised then cut into thirds and placed into a series of 3 cryogenic 5mL tubes for microbial, polyphenol, and protein analysis. For microbial analysis, approximately 0.5 to 1g pieces of tissue were collected, stored in 1mL RNALater (Qiagen, Valencia, CA, USA), snap frozen in liquid N_2, and then stored at -80°C until further analysis. For protein analysis of tissue samples, pieces of tissue (approximately 0.5g to 1g of tissue) were collected, stored in 0.42mL Lysis Buffer/HALT protease inhibitor solution, snap frozen in liquid N_2, and stored at -80°C until further analysis. For polyphenol analysis, sections of tissue (approximately 0.5g to 1g) were collected, stored in 1mL 2% acetic acid/1% ascorbic acid (w/v) (for polyphenol preservation), snap frozen in liquid N_2, and stored at -80°C until further analysis. Fecal contents from the lumen of the distal colon were collected in a series of three tubes with the same reagents and volume of reagents as during the tissue collection. Approximately 0.5g of fecal contents were collected. Tubes used for the

collection of tissue and feces were individually weighed prior to the collection of tissue and immediately following tissue collection to ensure an accurate dilution calculation.

Procedures

Body Composition Analysis

A dual-energy x-ray absorptiometry (DXA) body scanner was used to measure total body bone mineral content (BMC), bone mineral density (BMD), lean tissue, and total body fat. To perform this procedure, the pigs were fasted for 12 hours overnight (to prevent regurgitation of feed stuff) before anesthetization with isoflurane in oxygen via the snout-covering face mask. The DXA scan took 7 minutes to complete and up to 15 minutes in its entirety from induction to recovery for each pig. Body composition analysis were performed on day 0 for a baseline body composition analysis upon arrival and immediately prior to euthanasia. Blood samples (4mL) were collected in BD Vacutainer serum separation tubes during these two time points via the venipuncture method. Blood samples were centrifuged to separate serum (see "serum" under "sample collection" for method), collected, snap frozen in liquid N_2, and stored at -80°C until further analysis.

Glucose Tolerance/Intestinal Permeability/Endotoxin

On day 6 of the 7 day heat stress period, pigs were initially supposed to be subjected to a glucose tolerance test and intestinal permeability test; however, the glucose tolerance test aspect of this procedure could not be completed due to the following circumstance. Two pigs that were anesthetized and outfitted with a butterfly ear vein catheter could not keep the rubber tubing for the blood draw attached to the ear vein catheter due to the animal's movement. Nevertheless,

baseline blood draws were still completed via the venipuncture method (see – blood collection for method). Immediately following the blood draw, each pig was given a 1mg/kg BW dextrose along with 3.5mg/kg BW 10,000 Da Fluorescein isothiocyanate-dextran (FITC-Dextran) in a cookie dough vehicle. Following the complete consumption of the food vehicle, the current time was recorded and another blood draw was subsequently taken after 3 hours via the venipuncture method.

Endotoxin Assay

The Limulus Amebocyte Lysate (LAL) Pyrogent™-5000 kinetic turbidimetric assay (Lonza; Walkersville, MD, USA) was used to measure LPS in the circulating system of each pig. Serum was initially diluted 10-fold and heat treated in a 70°C for 15 minutes then further diluted 4-fold for a final 1/40 dilution concentration. A 100 EU/mL to 0.01 EU/mL standard curve was constructed using *E. coli* 055:B5 to measure the concentration of each analyte. 100µL of each sample were added a pyrogen free 96-well plate and allowed to incubate at 37°C for ≥ 10 minutes. Finally, LAL Pyrogent 5000 reagent was reconstituted with LAL Pyrogent 5000 buffer and 100µL aliquots were added to each well. Samples were immediately analyzed on a Biotek Synergy plate reader using Gen 5 software. Absorbance readings were taken every minute for 100 minutes. Positive product controls were run with each sample to ensure there was no assay inhibition in the sample.

Preparation of Fecal Homogenates

Luminal contents, or feces, were subjected to 3 super-sonifications. Next, an additional 0.5mL lysis buffer was added to the feces and fecal contents were homogenized by bead beating (4°C for 10 minutes). Homogenized fecal contents were centrifuged (23°C; 10,000xg for 10 min)

and supernatants collected and used immediately for calprotectin analysis or snap frozen in liquid N_2 and stored at -80°C for further analysis.

Calprotectin Assay

Calprotectin, a protein released by neutrophils into the intestinal lumen in response to inflammation, was measured using a Bluegene porcine calprotectin assay kit (Shanghai, China). Fecal homogenates were diluted 6-fold and analyzed according to manufacturer's instructions. 100µL of standards or samples were added to the appropriate wells in the antibody pre-coated Microtiter Plate. 100µL of PBS was added in the blank control well. Next, 10µL of balance solution was added to the 100µL specimen wells and 50µL of conjugate was added to each well. This was thoroughly mixed, covered and incubated for 1 hour at 37°C. Following the incubation period, the microtiter plate was manually washed 5 times. To perform this washing, each well was completely filled with 1x washing solution, aspired into a waste container, blotted until dry, and repeated. Subsequently 50µL of substrate A and 50µL of substrate B was added to each well, including the blank, covered and incubated for 10 to 15 min at 37°C. Finally, 50µL of the stop solution was added to each well and the optical density (O.D.) was immediately determined at 450nm using a BioTek Synergy plate reader and Gen 5 software (Winooski, VT, USA).

Statistical Analysis

Being that this study is a 2x2 factorial design (**Error! Reference source not found.**), ifferences between various treatments will be analyzed using a two-way analysis of variance (ANOVA). The significance level will be set at $P < 0.05$. All data will be analyzed using the statistical software GraphPad Prism v.6 (La Jolla, CA, USA).

The main effects of the factors in this study are described below as “main effect of factor 1” and “main effect of factor 2”. These groups are classified as the “within group” measurements. There are also “between groups” interactions between factor 1 and factor 2, listed below, that will be used in the analysis of the data for this study.

Main effect of Factor 1

Diet effect; Grape Seed Extract (GSE supplementation vs. control diet): Is there a difference between intestinal permeability, endotoxin concentration, blood glucose levels, etc. of GSE supplemented pigs vs. permeability, endotoxin concentration, blood glucose, etc. of control diet pigs?

Main effect of Factor 2

Environment effect (Thermal neutral conditions, 21-22°C, vs. Heat stressed conditions, 33-34°C): Is there a difference between, endotoxin concentration, blood glucose levels, calprotectin, etc. of pigs housed in TN conditions (21-22°C) vs. endotoxin concentration, glucose tolerance, calprotectin, etc. of pigs housed in heat stressed (33-34°C) conditions?

Interactions

Interaction between diet (Control vs. GSE) and the environment (TN vs. HS). For example, would the supplementation of grape seed extract or control diet have an effect on the animal during HS or TN conditions?

Tukey's HSD

Tukey's honest significant difference (HSD) test will be used in conjunction to the two-way ANOVA for the statistical analysis of this study. Tukey's test will be used to compare the means of every treatment and determine if they are significantly different than the means of every other treatment (multiple comparison). Tukey's HSD test is a moderately conservative approach to determine if differences between treatments are significantly different compared to the most conservative test, Bonferroni, and the least conservative test, Fisher's least significant difference test. Tukey's test will have an overall experimental error (α_E; type I error) lower than Fisher's and a narrower confidence interval than Bonferroni's method.

Dixon's Q Test

All data was reviewed an analyzed for statistical outliers. All outliers were statistically eliminated by Dixon's Q test using the following formula and compared to $Q_{Critical}$ values at 95% confidence. The gap is the difference between the potential outlier and the next closest number. This is divided by the range of values, the largest value in the set of data minus the smallest. If $Q_{exp} > Q_{Crit}$, then the value was eliminated.

$$Q_{exp} = \frac{Gap}{Range} = \frac{X_2 - X_1}{X_n - X_1}$$

Equation 1. Equation for Dixon's Q test

Results

Baseline DXA scan (Figure 5)

At the baseline (arrival) DXA scan, BMD, BMC, percent fat, total fat, lean tissue, and weight before HS period were measured for each pig. Pigs assigned to groups, HS-control, HS-GSE, TN-GSE, and TN-Control had baseline BMD (**Figure 5- A**) values of 0.902±0.04, 0.924±0.34, 0.933±0.38, and 0.945±0.38 g/cm^2, respectively. Mean BMC (**Figure 5 – B**) was 933±100.18, 915±80.30, 938.3±109.25, and 983±83.79 grams, respectively. Tissue (%fat) (**Figure 5– C**) was 13.63±0.85, 13.42±0.89, 15.03±1.13, and 14.33±0.765%, respectively for treatment groups. Total fat (**Figure 5 - D**) was 7212.17±3586.13, 7108.83±833.47, 7940.50±3590.32, and 7522±750.4 grams, respectively. Lean tissue (**Figure 5– E**) was 44697.67 ± 2701.66, 45184.17±2324.67, 43620.67±2542.16, and 44368.5±2250 grams, respectively. Pig weight before HS (**Figure 5– F**) was 61.5±3.81, 63.12±2.88, 61.8±4.46, and 62.6±2.88 kg, respectively, for each treatment. All values are expressed as mean±SEM. There were no significant differences between treatment groups (P>0.05) at the baseline (arrival) for each DXA scan parameter and body weight before the HS period.

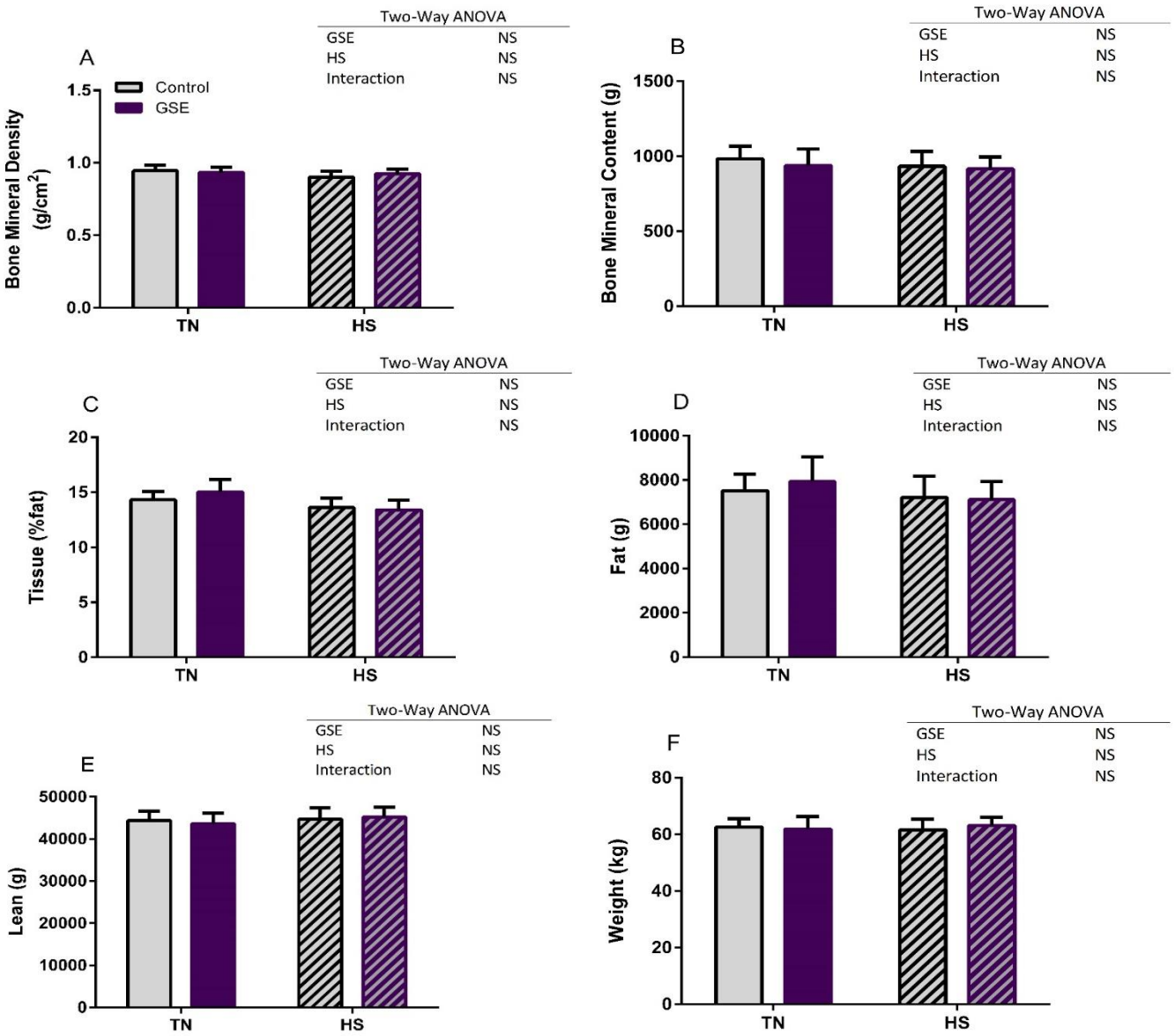


Figure 5. Baseline dual x-ray absorptiometry scan results. This scan was upon arrival at the Litton Reaves animal facility for the TN-Control (grey; solid), TN-GSE (12mg/kg BW GSE; purple; solid), HS-Control (grey; striped), and HS-GSE (12mg/kg BW GSE; purple; striped) groups (n=6 per group). Results include: A) bone mineral density, B) bone mineral content, C) percent fat, D) fat, and E) lean tissue. Weight (F), in kilograms, was measured a day prior to the HS period. All values are expressed as mean±SEM. Data analyzed via two-way ANOVA with Tukey's HSD test

Body Temperature (Figure 6-Left Panel)

At the baseline (day 0), all treatment groups, HS-Control (red; diamond), HS-GSE (12mg/kg BW GSE; purple; square), TN-GSE (12mg/kg BW GSE; green; triangle), and TN-Control (blue; circle) had similar rectal temperatures, 38.72±0.09, 39.02±0.09, 39.04±0.09, 38.99±0.14°C, respectively. There were no significant differences between treatment groups at the baseline (P>0.05). Following the initiation of HS, represented by the vertical dotted line on day 1, rectal temperatures for both the control and GSE groups immediately increased >40°C on day 1 and maintained this average on day 2 until progressively declining as the HS period continued. On day 1, this increase was characterized by a rectal temperature increase of 3.89% (1.51°C), where it reached its maximum of 40.2±0.11°C in the HS-control, and 2.77% (1.08°C) in the HS-GSE group compared to their respective baselines. From the initial rise in temperatures on day 1, RTs declined by 0.21% (0.086°C) in the HS-control group and raised in the HS-GSE group by 0.05% (0.021°C) where it reached its maximum temperature of 40.1±0.12°C on day 2. On day 3, RTs continued to decline by 0.71% (0.28°C) for the HS-control and 0.53% (0.21°C) for the HS-GSE from day 2. Furthermore, RTs decreased by 0.34% (0.14°C), 0.063% (0.025°C), 0.51% (0.2°C), and 0.21% (0.08°C) from day 3 to 4, day 4 to 5, day 5 to 6, and day 6 to 7, respectively for the HS-control until it reached a minimum of 39.4±0.10°C (remaining approximately 0.7°C above its baseline). RTs for the HS-GSE group fluctuated over the remaining days as it decreased by 0.39% (0.15°C), increased by 0.19% (0.075°C), decreased by 0.80% (0.32°C), and finally increased by 0.13% (0.05°C) from day 3 to 4, day 4 to 5, day 5 to 6, and day 6 to 7, respectively, and on the final day was recorded at 39.56±0.13°C (0.54°C above its baseline value).

RTs for TN groups fluctuated within a small range for the entirety of the heat stress period. For the TN-GSE group, fluctuated between a minimum temperature of 38.76±0.08°C and a maximum temperature of 39.07±0.09°C during the HS period. RTs for the TN-control fluctuated between a minimum of 38.87±0.09°C to a maximum of 39.03±0.06°C during the HS period. Values are expressed as mean (in parenthesis) or mean±SEM. During the entirety of the HS period, the environmental effect (HS) was significant ($P<0.0001$) compared to the TN groups, but at no time was the treatment significantly different within HS or TN groups ($P>0.05$).

Respiration rates (Figure 6– Right Panel)

At the baseline, respiration rates for each group, HS-Control (red; diamond), HS-GSE (12mg/kg BW GSE; purple; square), TN-GSE (12mg/kg BW GSE; green; triangle), and TN-Control (blue; circle) were 39.3±2.36, 40.4±1.99, 51±2.66, and 57.8±3.56 BPM. At the baseline, TN groups were significantly higher ($P<0.0001$) compared to HS groups. Following the initiation of the HS period, represented by the vertical line on **Figure 6-Right**, respiration rates were immediately increased compared to the TN groups. The HS-control and HS-GSE groups had a 3.09-fold and 3.35-fold respective increase compared to their baseline values. Day 1 had the highest RR for both HS groups. Max RR were 121.5±7.66 BPM for HS-control and 135.5±8.39 BPM for the HS-GSE group. From the initial day, RR rates dropped 10.5% (12.7 BPM) for the HS-Control and 11.4% (15.4 BPM) for the HS-GSE group on day 2. Furthermore, RR progressively declined throughout the rest of the HS period with mild increases found on a few days. From day 2, RR decreased 14.9% (16.1 BPM), increased 10.8% (10 BPM), decreased 14.5% (14.9 BPM), increased 3.91% (3.43 BPM), and increased 1.09% (1 BPM) from day 2 to 3, day 3 to 4, day 4 to 5, day 5 to 6, and day 6 to 7, respectively, for the HS-control group. Overall,

there was a 24.13% decrease in RR from the day 1 RR reading to the day 7 RR reading for this HS-control group. RR for HS-GSE group decreased 11.4% (15.4 BPM), decreased 11.1% (13.3 BPM), decreased 5.77% (6.2 BPM), decreased 7.61% (7.6 BPM), increased 11.5% (10.7 BPM), and decreased 9.00% (9.3 BPM) from day 2 to 3, day 3 to 4, day 4 to 5, day 5 to 6, and day 6 to 7, respectively. Overall, there was a 30.4% reduction in RR from the day 1 RR reading to day 7 RR reading for this HS-GSE group.

RR for TN groups fluctuated within a small range for the entirety of the heat stress period and no dramatic changes were seen as in the HS groups. For the TN-GSE group, RR fluctuated between a minimum mean rate of 37.6±1.84 BPM and a maximum rate of 51.0±2.66 BPM. For the TN-control, RR fluctuated between a minimum mean rate of 40.6±2.14 BPM and a maximum of 57.8±3.56 BPM. Values are expressed as mean (in parenthesis) or mean±SEM. During the entirety of the HS period (day 1 to day 7), the environmental effect (HS) was significant ($P<0.0001$) compared to the TN groups, but at no time was the treatment significantly different within the HS or TN groups ($P>0.05$).

Two-Way ANOVA - RT

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
GSE	NS	NS	NS	NS	NS	NS	NS	NS
HS	NS	****	****	****	****	****	***	****
Interaction	NS	NS	NS	NS	NS	NS	NS	NS

Two-Way ANOVA - RR

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
GSE	NS	NS	NS	NS	NS	NS	NS	NS
HS	****	****	****	****	****	****	****	****
Interaction	NS	NS	NS	NS	NS	NS	NS	NS

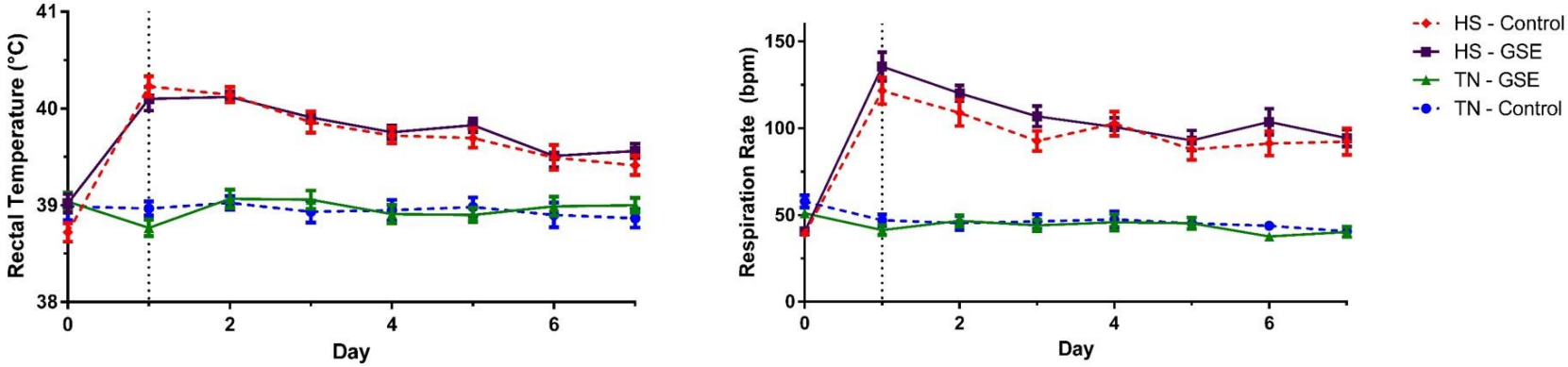


Figure 6. Pig rectal temperatures (left) and respiration rates (right) at baseline (day 0) and during 7 day HS period. Treatment groups are defined as HS-Control (red; diamond), HS-GSE (12mg/kg BW GSE; purple; square), TN-GSE (12mg/kg BW GSE; green; triangle), and TN-Control (blue; circle) with n=6 per group. RTs are expressed in degrees Celsius and RRs are expressed in beats per minute. Vertical dashed line on day 1 represents transition from acclimatization to HS period. All values are expressed as mean ± SEM. ***Environment effect, P<0.001 or ****P<0.0001. Data analyzed via two-way ANOVA with Tukey's HSD test.

Feed Intake (Figure 7)

All treatment groups, HS-control (2.85±0.19kg), HS-GSE (2.8±0.14kg), TN-GSE (2.79±0.19kg), and TN-control (3.05±0.057kg) had approximately 2.9kg feed intake at the baseline (day 0). Baseline feed intake did not differ between treatment groups (P>0.05). Following the initiation of the HS period (represented by the dotted line on **Figure 7** at day 0), the HS-control and HS-GSE groups had an immediate 27.7% (0.8kg) and 13.7% (0.38kg) reduction in feed intake, respectively, compared to their baseline values. This reduction of feed intake trend continued as feed intake for the HS-control group was further depressed by 21.4% (0.44kg) on day 2 and by 37.9% (0.62kg) on day 3 where it reached its minimum intake of 1.0±0.14kg. The HS-GSE group's feed intake was decreased further by 38.6% (0.93kg) on day 2, but remained similar on day 3 where it reached a minimum feed intake of 1.48±0.11kg. From day 3, feed intake began to gradually increase for each HS treatment group. HS-control feed intake increased by 33.9% (0.34kg) from day 3 to day 4 and by 21.6% (0.29kg) from day 4 to 5 where feed intake was approximately 1.64±0.22kg. HS-GSE feed intake increased by 21.5% (0.32kg) from day 3 to 4 and remained similar from day 4 to 5 where feed intake was approximately 1.76±0.14kg before the fasting period.

TN group's feed intake fluctuated slightly during the baseline and 7 day HS period. For the TN-GSE group, feed intake decreased by 3.28% (0.09kg), 0.62% (0.01kg), and 14.9% (0.4kg) from baseline to day 1, day 1 to 2, and day 2 to 3, respectively, where minimum feed intake for this period was reached on day 3 at approximately 2.3±2.28kg. Following these three days, feed intake slightly increased by 5.11% (0.12kg) and 5.90% (0.14kg) from day 3 to 4 and day 4 to 5, respectively, where feed intake was approximately 2.54±0.24kg before the fasting

period on day 6. For the TN-control, feed intake decreased by 11.4% (0.35kg) from the baseline to day 1, slightly increased 6.46% (0.18kg) from day 1 to day 2, decreased 15.3% (0.44kg) from day 2 to day 3, remained unchanged from day 3 to 4, and increased 9.09% (0.23kg) at which feed intake was at approximately 2.7±0.18kg before the overnight fast. Values are expressed as mean (in parenthesis) or mean±SEM.

During the entirety of the HS period (day 1 to day 7), the environmental effect was significant as feed intake was lower in the HS groups compared to the TN groups. Feed intake was significant ($P<0.01$) on day 1 and 4, significant ($P<0.0001$) on days 2, 3, and 7, and significant ($P<0.001$) on day 5. Feed take did not differ between TN diets ($P>0.05$) at any time during the HS period. The interaction between HS-control vs. HS-GSE and TN-control vs. TN-GSE was only significantly different during day 3 ($P<0.05$) in that the difference between HS-GSE FI and HS-control FI was greater than the difference between the TN-GSE and TN-control groups. The most interesting result of the FI resulted on day 1 and 4. The FI for the HS-GSE group was not statistically different compared the TN groups ($P>0.05$) whereas the HS-Control was statistically different ($P<0.05$). FI on day 1 for the HS-GSE group was not statistically significant from the baseline (day 0) ($P>0.05$) whereas the HS-Control was statistically different from its baseline ($P<0.05$).

Two-Way ANOVA – Feed Intake

	Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
GSE	NS	NS	NS	NS	NS	NS	NS	NS
HS	NS	NS	**	****	****	**	***	****
Interaction	NS	NS	NS	NS	*	NS	NS	NS

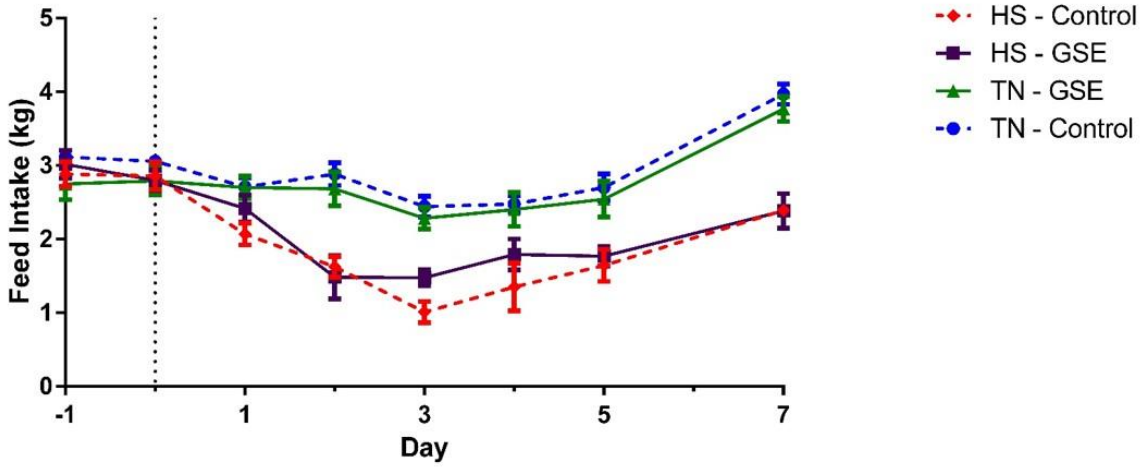


Figure 7. *Ad libitum* feed intake at baseline and during HS period. Groups for HS-Control (red; diamond), HS-GSE (12mg/kg BW GSE; purple; square), TN-GSE (12mg/kg BW GSE; green; triangle), and TN-Control (blue; circle) groups (n=6 per group). Feed intake was measured in kilograms. Vertical line at day 0 represents transition between baseline acclimatization (day -1 and 0) and heat stress period (day 1). No feed intake was recorded on day 6 due to an overnight fast. Feed intake was measured once daily at 6PM and is reported as feed given (AM and PM) minus refusals. All values are expressed as mean ± SEM. **Environment effect, P<0.01 or *** P<0.001 or **** P<0.0001, and *Interaction P<0.05. Data analyzed via two-way ANOVA with Tukey's HSD test.

DXA scan – End of Study (Figure 8)

At the conclusion of the HS period, pigs in all treatment groups were again DXA scanned and measured for BMD, BMC, percent fat, fat accumulation, and lean tissue. Weight was also taken at the time. The change in these parameters and percent increase in weight can be seen in **Figure 8**. BMD (**Figure 8– A**) increased by 0.0625±0.12, 0.0603±0.01, 0.0683±0.16, and 0.0667±0.013 g/cm^2 for the HS-control, HS-GSE, TN-GSE, and TN-control, respectively. There were no significant differences between groups (P>0.05). BMC (**Figure 8– B**) increased by 238.6±16.33, 276.1±25.65, 276.2±41.62, and 296.1±34.11 grams for the HS-control, HS-GSE, TN-GSE, and TN-control groups, respectively. The HS-GSE group had a 15% higher BMC than the HS-control. There were no significant differences between groups (P>0.05). Tissue (%fat) (**Figure 8– C**) increased by 2.7±0.31, 3.83±0.47, 2.68±0.53, and 3.43±0.50% for the HS-control, HS-GSE, TN-GSE, and TN-control groups, respectively. HS-GSE's change in percent fat was 1.1% higher than the HS-control while TN-control was 0.75% higher than the TN-GSE supplemented diet. TN-control had a 0.7% higher fat accumulation than HS-control while the HS-GSE group had a 1% higher percent fat than the TN-GSE group. There were no significant differences between groups (P>0.05). Fat (**Figure 8– D**) had similar trends to **Figure 8-C**. Mean fat was increased by 3062.17±251.90, 3966.33±367.70, 4011.17±527.19, and 4489.5±352.7 grams for the HS-control, HS-GSE, TN-GSE, and TN-control groups, respectively. TN-control had a significantly higher (P<0.05) fat gain than the HS-control. There was also a significant difference in treatment interaction between the TN and HS groups (P<0.05). Mean lean tissue gains (**Figure 8– E**) for the entirety of the study were 7,333.167±495.13, 7,524.833±527.23, 10,658±748.15, and 10,925.17±960.91 grams for the HS-control, HS-GSE, TN-GSE, and TN-control groups, respectively. Comparing treatments, the TN-control had a very significant

increase (P<0.01) of 48.98% lean tissue accretion compared to the HS-control group while the TN-GSE had significant increase (P<0.05) of a 41.64% gain in lean tissue accretion compared to the HS-GSE group. Being said, the TN groups had an average significant increase (P<0.001) in lean tissue accretion by 45% compared to the HS group. Mean percent weight (**Figure 8– F**) increases were 4.21, 2.13, 8.46, and 10.59% for the HS-control, HS-GSE, TN-GSE, and TN-control groups, respectively. TN groups had an average 3-fold increase in percent weight gain compared to the HS groups. TN-control and TN-GSE had a significant increase (P<0.01) compared to their HS counterparts. Values are expressed as mean±SEM. Overall, the percent weight increase in the TN groups was significantly higher than the HS groups (P<0.0001).

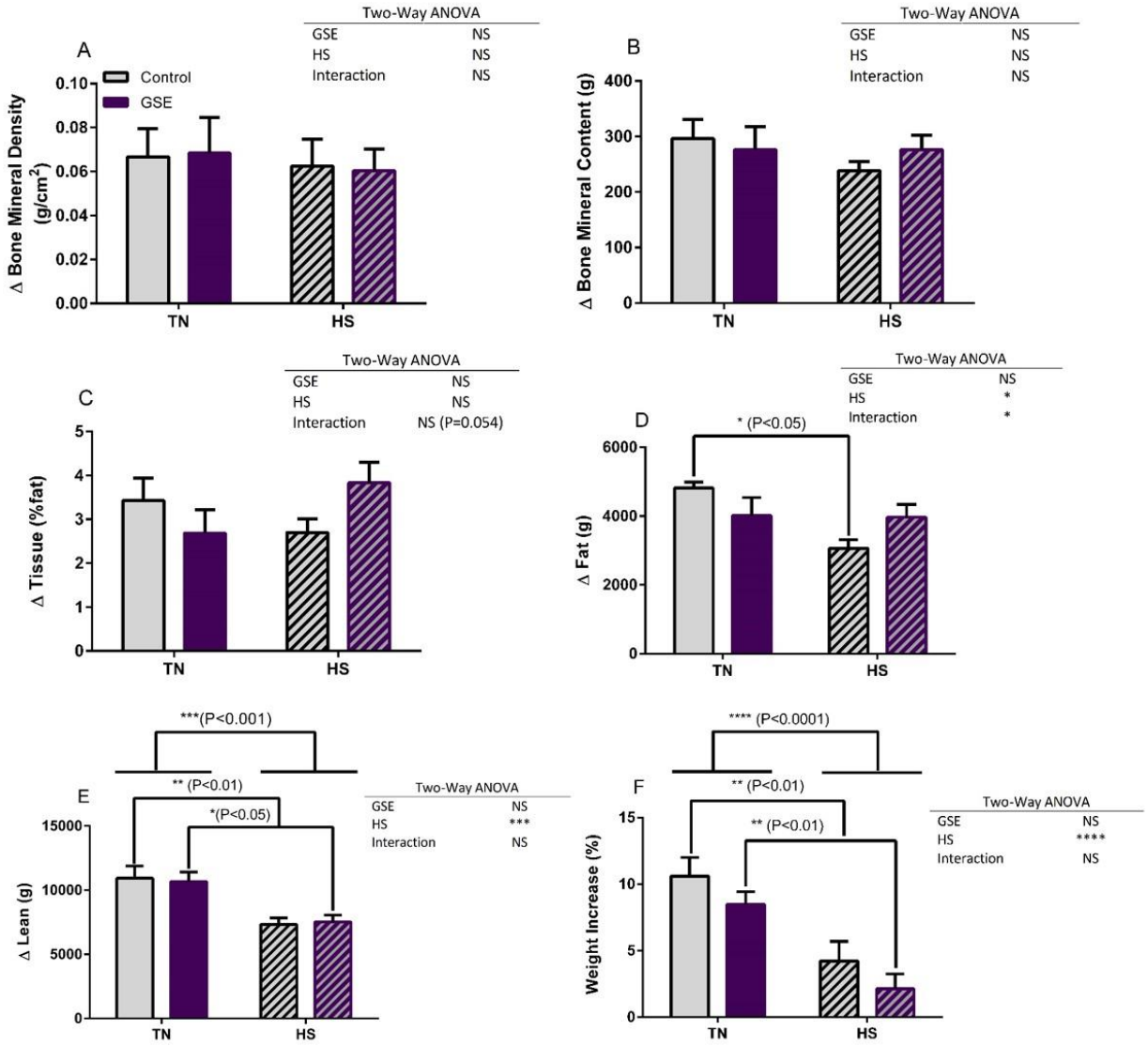


Figure 8. Dual x-ray absorptiometry scan results at the conclusion of the HS period. Results were for the TN-Control (grey; solid), TN-GSE (12mg/kg BW GSE; purple; solid), HS-Control (grey; striped), and HS-GSE (12mg/kg BW GSE; purple; striped) groups (n=6 per group). Results include: A) change in bone mineral density, B) change in bone mineral content, C) change in percent fat, D) change in fat accumulation, and E) lean tissue accretion. Weight (F) was also taken immediately following the conclusion of the 7 day HS period and is expressed as a percent increase. All values are expressed as mean±SEM. *Environment effect, P<0.05 or *** P<0.001 or ****P<0.0001; *Interaction P<0.05. Data analyzed via two-way ANOVA with Tukey's HSD test.

Fasting Blood Glucose (**Figure 9**)

Fasting blood glucose levels were measured on day 6 and at the conclusion of the HS period. On day 6 (**Figure 9** – A), mean blood glucose levels were 90.83±14.09, 64.83±9.73, 41.17±11.12, and 65.80±16.43 mg/dl for the HS-Control, HS-GSE, TN-GSE, and TN-Control groups, respectively. TN-control had 1.4-fold higher blood glucose levels compared to the TN-GSE. HS-Control had 1.6-fold higher blood glucose compared to its GSE counterpart. Together, GSE groups had approximately 1.5-fold lower blood glucose levels compared to the thermal neutral groups. Comparing control groups, the HS-control had a 38% higher blood glucose compared to the TN-control. Comparing GSE groups, the HS-GSE supplemented diet had a 57.47% higher blood glucose levels compared to TN. During the day, blood glucose levels were extremely variable with a ≥ 9-16mg/dl SEM. The environmental effect was borderline significant (P=0.067); however, there were no statistical differences between treatments (P>0.05).

Fasting blood glucose levels measured at the conclusion of the HS period had mean levels of 90.5±6.55, 75.5±7.36, 87.8±2.50, and 90.6±5.60 mg/dl. There were no differences between TN blood glucose levels (P>0.05). HS-control fasting blood glucose levels were 19.87% higher than GSE supplemented diet. On this day, fasting blood glucose were much less variable with a 2-7mg/dl SEM. Values are expressed as mean±SEM. There were no statistical differences between the two treatments (P>0.05). Environmental effect did not have any significant differences between groups (P>0.05).

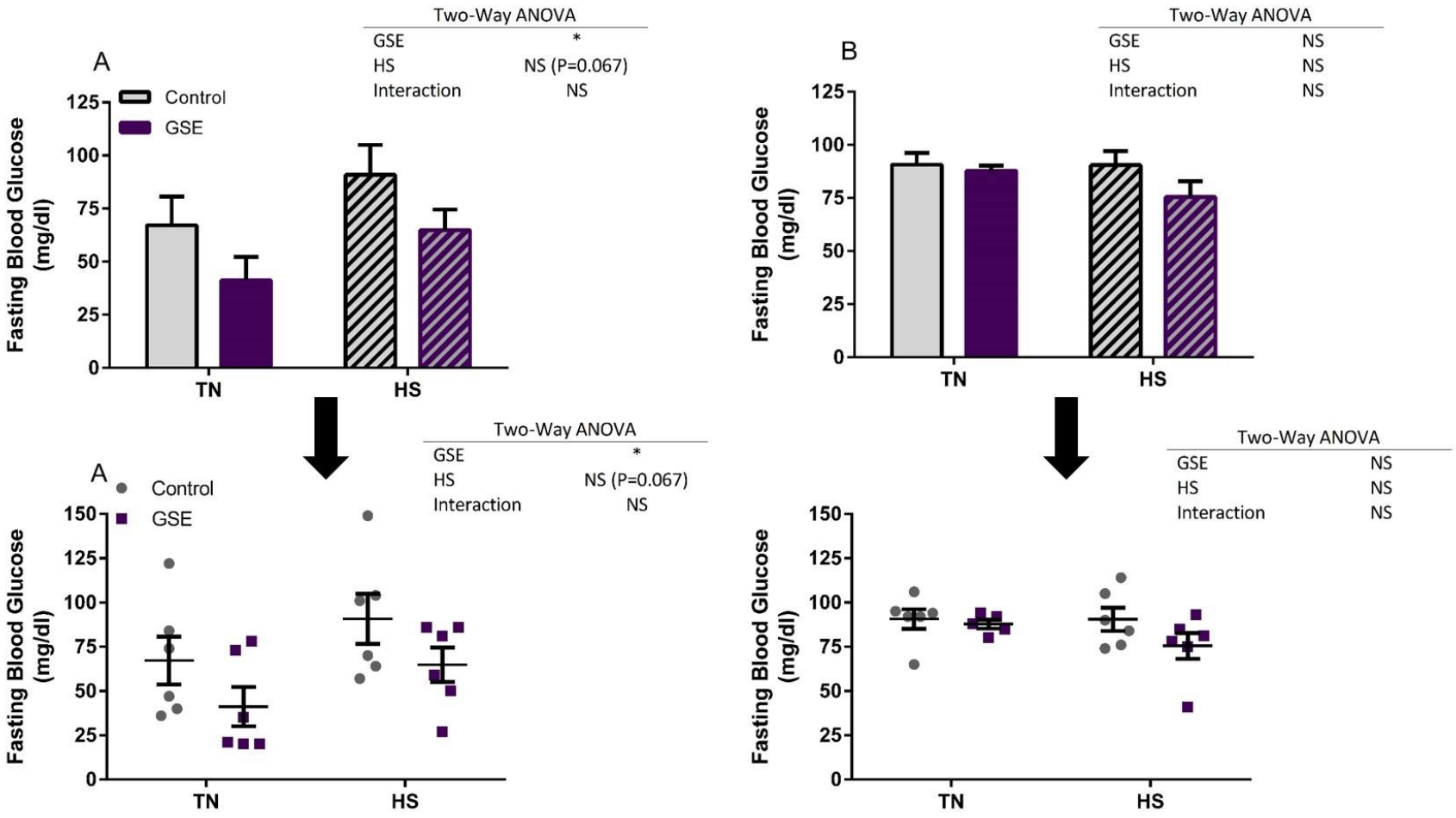


Figure 9. Fasting blood glucose levels taken on A) day 6 and B) at the conclusion of the 7 day HS period following an overnight (>12hr) fast. Results for TN-Control (grey; solid), TN-GSE (12mg/kg BW GSE; purple; solid), HS-Control (grey; striped), and HS-GSE (12mg/kg BW GSE; purple; striped) groups. Blood glucose was measured on a standard glucometer using One Touch Ultra® test strips. Values which read as "low" were recorded as 20mg/dl which is the minimum glucose concentration for a reading. All values are expressed as mean±SEM. *Diet effect, P<0.05. Data analyzed via two-way ANOVA with Tukey's HSD test.

Serum Endotoxin (**Figure 10**)

On day 6 of the HS period, fasting serum endotoxin concentrations were measured for all treatments. Mean endotoxin levels were 0.817±0.26, 0.386±0.03, 0.424±0.038, and 0.479±0.05 endotoxin units per milliliter for the HS-Control, HS-GSE, TN-GSE, and TN-Control groups, respectively. There were no significant differences (P>0.05) between TN-control and TN-GSE groups as mean endotoxin levels varied only by 0.05 EU/ml. HS-control samples were highly variable with a SEM of 0.26 EU/mL while the HS-GSE group had an SEM of 0.03 EU/ml. Endotoxin levels were approximately 2.1 fold higher in the HS-control compared to the HS-GSE supplement. HS-GSE also had the lowest endotoxin concentrations of all the groups. Diet treatment tended toward significance (P=0.083); however, there were no significant differences in the environmental effect (P>0.05). All statistical outliers were eliminated by Dixon's Q test.

Serum endotoxin concentrations were also measured 3 hours following the ingestion of a high carbohydrate load. There was an approximate 1.5 fold increase in the mean endotoxin concentration of the HS-GSE compared to the HS-control. There was no significant differences between groups or treatments (P>0.05)

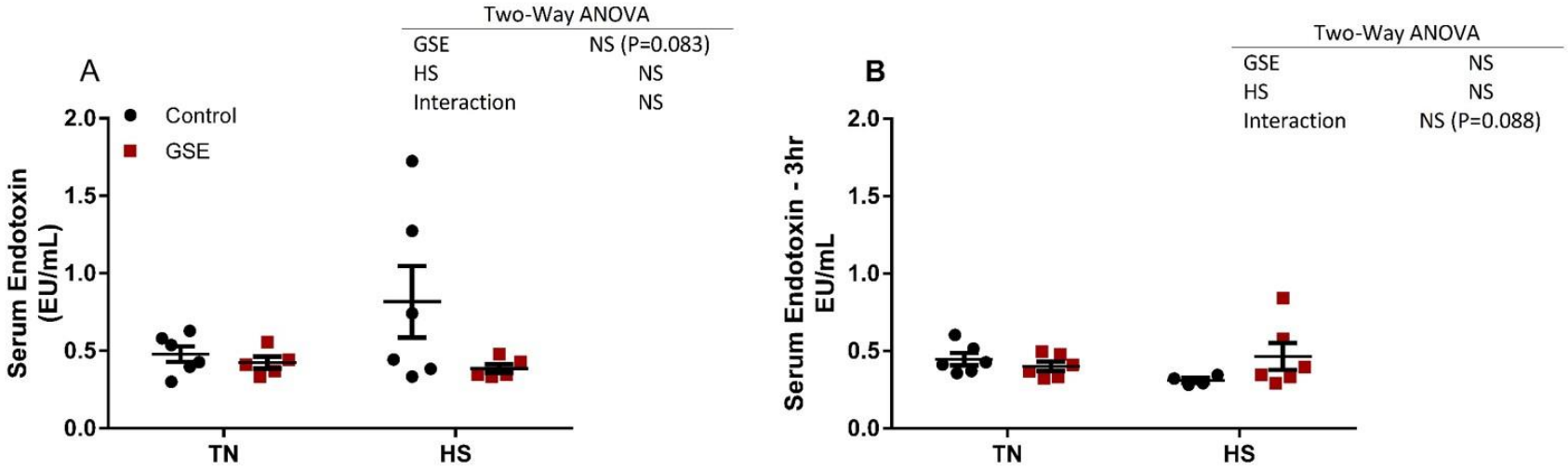


Figure 10. Endotoxin concentrations measured A) following an overnight (>12hr) fast and B) 3hr following the ingestion of a high carbohydrate load. Results were for the TN and HS-controls (black; circle) and TN and HS-GSE (red; square) supplemented (12mg/kg BW GSE) groups. Serum endotoxin concentrations are reported based on a 0.01 to 100 EU/mL E. coli 055:B5 endotoxin standard obtained from the kinetic turbidimetric Limulus Amebocyte Lysate (LAL) Pyrogent™ - 5000 assay (Lonza; Walkersville, MD, USA). EU/mL=Endotoxin unit per milliliter. Data analyzed via two-way ANOVA with Tukey's HSD test.

Fecal Calprotectin (**Figure 11**)

Fecal calprotectin levels (contents from the distal lumen) were measured following the HS period at sacrifice. Mean calprotectin levels were 9.52±0.75, 10.59±0.89, 12.42±0.55, and 10.97±1.22 ng/ml for the HS-Control, HS-GSE, TN-GSE, and TN-Control groups, respectively. HS-GSE had an 11.25% higher fecal calprotectin compared to the HS-control (10.59±0.89 vs. 9.52±0.75 ng/ml). TN-GSE group had a 13.18% higher fecal calprotectin vs. the control (12.42±0.55 vs. 10.97±1.22 ng/ml). There were no significant differences between treatments or groups ($P>0.05$). All outliers were statistically eliminated by Dixon's Q test. Measurements for fecal calprotectin were based off of N=4 (HS-Control), N=3 (HS-GSE), N=5 (TN-GSE), and N=6 (TN-Control) samples. Three samples, one HS-Control and two HS-GSE, were unaccounted for as there were no distal contents upon collection to analyze.

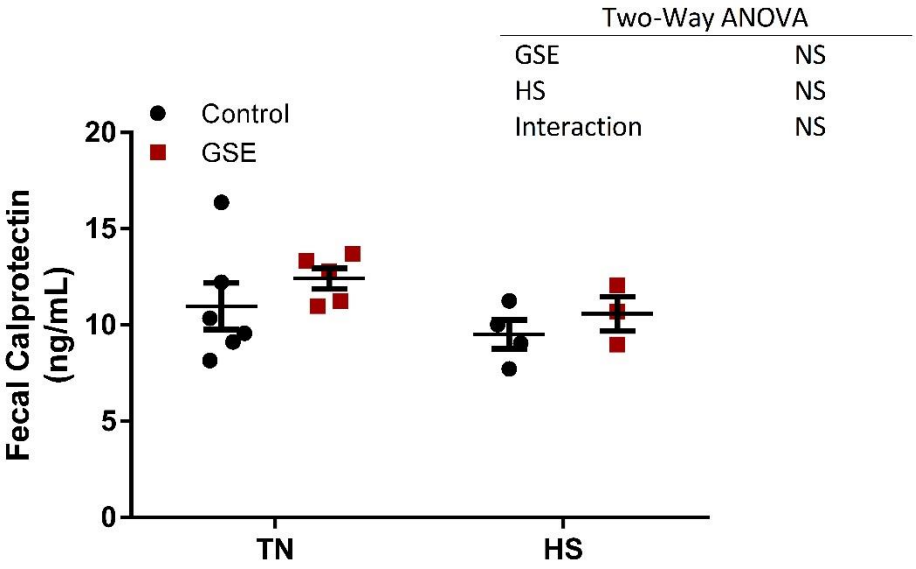


Figure 11. Calprotectin concentrations in the feces (collected from the luminal contents of the distal colon) were assessed for the TN and HS-controls (black; circle) and TN and HS-GSE (red; square) supplemented (12mg/kg BW GSE) groups (n=6 per group; n=3 did not contain contents). Calprotectin levels were quantified using a porcine calprotectin enzyme-linked immunosorbent assay (Bluegene Biotech CO., LTD; Shanghai, China). All values are expressed as mean±SEM. Data was analyzed via two-way ANOVA with Tukey's HSD test.

Discussion

To date, this is the first study evaluating the effects of grape seed extract used in a larger swine model to determine if GSE can provide the multifaceted health benefits as it has been shown in smaller animal models such as the rat or mouse. That said, this is also the first time GSE has been evaluated to alleviate the deleterious effects of heat stress in the animal. It is important at this time to note that GSE consumption had no adverse health effects on the animal which is similar to Mittal et al [146] and all other studies reviewed earlier who reported no adverse effects.

Initially in this study, pigs were weighed and placed into treatment groups based on weight in order to have similar mean weights among treatment groups as it minimizes the variation from group to group [147]. This was immediately confirmed by the baseline DXA scan upon arrival into the Litton Reaves facility as group means for BMD, BMC, Tissue (%fat), fat, and lean tissue were all similar and showed no significant differences among groups ($P>0.05$).

Before the initiation of HS, there were no significant differences between treatment groups for T_b and feed intake as expected; however, there was a significant difference between groups in RR in which TN groups had a significantly higher baseline RR than the HS groups. There is no explainable reason for this other than an increase in excitement or activity level, which led to more rapid breathing. Upon initiation of the HS period, environmental temperature was increased to 33-34°C which has been shown to induce stress in the animal as it is well above the animal's thermal neutral zone limit of 25.5°C for this weight range of pig as reported by Huynh et al [28]. Larger pigs have also been shown to be more effected at this temperature than

smaller, light-weight, and younger pigs [30]. This temperature caused distress in the animal as it not only visibly reduced level of activity causing the animal to lay down in order to reduce heat production and maximize heat displacement, but also physiologically was characterized by a 3.09 and 3.35-fold increase in respiration rates, a 1.51°C and 1.08°C increase in T_B, and an immediate 27.7% and 13.7% one day reduction in VFI for the HS-control and HS-GSE groups, respectively. Observations were similar to Ames et al [23]. Physiological data of increased RR and T_b was in agreement with [3, 4, 28, 78] as all authors showed significant increases in RR and T_b in HS environments. Most notably, RR and RT trends were congruent with Pearce et al [3] in that RR and T_b were at the maximum very early on in the HS period and began to decrease respectively thereafter with variable fluctuation. Reduction in VFI was also in agreement with one review [29] and multiple individual studies [3, 5, 28, 32, 33] in which authors showed a significant decrease in VFI under HS conditions compared to the TN-control. However, in those studies reduced FI appeared to remain constant for the entire HS period, whereas in this study, FI began to rebound in both HS groups following day 3.

At no time during the HS period did consumption of the GSE dose play a role on differing the RR or T_B which is consistent with the literature. Only one instance in the literature was it found that GSE lowered T_B in neonatal rats [148]. On the other hand, while there were no significant differences in FI between control and GSE supplemented groups in HS and TN conditions, the HS-GSE group did have approximately 0.4kg higher FI on 50% of the recorded FI days during the HS period providing a one day (day 3) in which GSE had a significantly different effect on the HS condition than the TN condition. Regarding FI, reported changes in feed intake have been variable. Balu et al [149] and Gonthier [150] reported no differences in

feed intake at GSE levels of approximately 100mg/kg BW; however, Wren et al [151] reported significant increases of approximately 300 to 2000 mg/kg BW in rats with more consistent significances coming at the highest dose.

In this study, the effects of environmental HS on body composition were shown as the changes from arrival DXA scan to the DXA scan immediately following the HS period can be seen. There were no significant changes in BMD or BMC. Bone mineral content is 15% lower in the HS-control compared to the HS-GSE. This is expected as HS conditions have shown, in birds, to decrease mineral content in bones as a resulted increase in excretion of minerals such as calcium, potassium, and sodium into the urine that could've played a role in bone formation [152]. Change in BMD and BMC was expected to be more pronounced in the GSE supplemented groups compared to the control groups as studies have shown a correlation of increased BMD in the femoral neck [153] and lumbar spine [153, 154] in addition to increased osteoblast activity in cell cultures [155] from the consumption of flavan-3-ols, primarily catechins (from tea) and procyanidins.

Continuing with the body composition analysis, percent fat and fat accumulation showed similar trends with a variety of results despite no significant differences in percent fat and only the environmental effect showing the TN control having significantly higher fat accumulation compared to the HS-control in fat accumulation. In fat accumulation, the significant difference between fat accumulation between the TN and HS controls could be attributed to the fact that these animals were not pair-fed and given the reduced VFI during HS, TN animals had higher nutrient consumption, which could lead to higher fat accumulation. On the other hand, the visible

differences between percent fat and fat accumulation were as expected under TN conditions, but actually the opposite of expected results during HS. During TN temperatures, studies have shown that GSE can reduce backfat mass [8], circulating triglycerides [8, 119, 120] as well as total phospholipid levels [120] through the proposed mechanisms of increased β-oxidation [8, 118] and the inhibition of lipases, specifically pancreatic lipase and lipoprotein lipase, therefore maximizing the amount of triglycerides oxidized, but also slowing down the rate at which they are metabolized [121]. It was expected that during the HS period these mechanisms would carry over and reduce lipid carcass traits; however, this was not the case as changes in percent fat and fat accumulation were higher in the HS-GSE groups compared to the control. A proposed mechanism for why this occurred is found in the multifaceted hormone, insulin. It has been shown, during periods of HS, that circulating insulin levels are higher than would be found under TN conditions [52]. In addition to increased levels of circulating insulin, GSE also has shown to improve insulin sensitivity [6, 7, 116]. Being that insulin is an anabolic hormone that prevents deregulates lipid oxidation in favor for lipid synthesis, it is proposed that increased insulin levels in combination with GSE during HS caused the promotion lipid synthesis and therefore further lipid storage [38, 49].

It can be seen that environmental factor played a significant role in lean tissue accretion in that the TN groups had approximately 45% higher lean tissue accretion than their HS counterparts. This was expected as during HS periods as the animal is expected to be in a catabolic state. Protein synthesis in the animal is reduced in addition to increased biomarkers for skeletal muscle catabolism, specifically glucocorticoids, catecholamines, among others [51, 156, 157]. However, this change in skeletal muscle metabolism is not completely understood due to

the HS related rise in circulating insulin concentrations. As stated previously, insulin is an anabolic hormone, but it does not appear to directly affect muscle insulin response (in HS cows) to therefore synthesize new protein [3, 158]. The literature does not show any significant results regarding GSE's ability to stimulate lean tissue accretion.

The final growth performance parameter measured during this study, percent increase in weight during the HS period. As expected, the environmental factor was extremely significant in which body weight gains for the HS groups were significantly less than their TN counterpart. Again, the difference in the percent increase in weight gained could be attributed to dissimilar feed intakes between TN and HS groups due to reduced VFI in the HS groups. While the diet played no significant role in differentiation from its control diet-pair, the percent increase in weight was visibly less than the control in both TN and HS conditions. As in the case with reduced lipid accumulation, GSE has also shown to reduce bodyweight through the same proposed mechanism of increased β-oxidation as found in the reduced lipid accumulation [8].

Fasting blood glucose was measured on day 6 and immediately following the HS period in order to evaluate the effects of GSE on carbohydrate metabolism under both TN and HS conditions. On day 6, there were no statistical differences of interest between the diet treatments, but the environmental effect tended toward significance (P=0.067) which showed fasting blood glucose levels in both the TN-GSE and HS-GSE were approximately 1.5-fold lower than their control counterpart. It is also to note at this time, two graphs were displayed for the fasting blood glucose due to the extreme variability in fasting blood glucose levels on day 6 compared to the minimal variation immediately following the conclusion of the HS period. On day 6, pigs were

fasted for >12 hours before baseline blood was drawn. Mean fasting blood glucose levels for the TN-GSE pigs were approximately 40mg/dl, three pigs of which recorded as ≤20mg/dl on the standard glucometer. During this time, pigs were removed from their pen and/or restrained via the snout snare in order to sample blood from the jugular vein. This physical restraint invoked stress in the animal causing an increase cortisol and epinephrine levels [159, 160]. However, the increase in blood glucose levels also raised insulin levels to regulate the rise in blood glucose concentrations [161]. Interestingly, it appears that the combination of acute insulin release and GSE improved insulin sensitivity in the GSE supplemented groups compared to the TN groups.

When blood samples were taken at the conclusion of the HS period, the animal was under isoflurane anesthesia for the DXA scan analysis. Fasting blood glucose under anesthesia showed less variability in samples and no significant differences between treatments. Anesthesia does diminish insulin action, therefore, reducing glucose uptake by the tissue and higher circulating blood glucose levels [162]. It is important to note that the improved insulin sensitivity seen under stress was absent during measurement under anesthesia as there was no acute insulin release. The level or extent to which GSE affects carbohydrate metabolism is hard to determine with this data and could have been better understood without the shortcomings of the failed glucose tolerance test.

A hallmark symptom of HS in the pig is reduction in intestinal integrity allowing endotoxin of Gram negative bacteria to leak from the inside of the intestine into circulating blood which increases for the risk of sepsis and subsequent death in the animal [66]. On day 6, serum endotoxin concentrations were measured following an overnight (>12hr) fast. As expected, there

were no significant differences between treatments in the TN group as there should have been no alteration in intestinal permeability at this temperature. In the HS environment on this day, serum endotoxin concentrations were much more variable and the mean serum concentration was approximately 2-fold higher than the GSE supplemented counterpart. HS-GSE serum concentration variation was extremely tight and were the lowest of all four treatment groups. Diet effect was borderline significant. Both the increase in HS-control serum endotoxin concentrations and reduced endotoxin concentrations in the HS-GSE supplemented group were as expected and in agreement with showing that GSE or other flavan-3-ol rich substances can reduce circulating endotoxin concentrations through promoting tight junction protein formation [82, 122, 123].

Following the consumption of a high carbohydrate load, endotoxin concentrations were again measured three hours later. Endotoxin concentrations for the TN groups remained similar as baseline values; however, HS-control differed in that endotoxin levels actually decreased following the consumption of this high carbohydrate load with minimal variation while the HS-GSE group had a slightly higher mean and more variation than at the baseline. This was not completely expected as it has shown in a chronic human study that the consumption of a high carbohydrate diet leads to increased metabolic endotoxemia; however, with the addition of the GSE supplement, this increase in endotoxin concentration should have been expected in the control group due to GSE's ability to form tight junction proteins and secure intestinal integrity [82, 163].

When interpreting and comparing results, a discussion regarding the endotoxin analysis is warranted due to the different types of assays, inaccuracies, and wide variety of results. When analyzing endotoxin concentration there are two commonly used methods. Those methods are the limulus amebocyte lysate (LAL) assay that has been around for over 30 years and the more recent discovery of the recombinant Factor C (rFC) fluorometric assay in order to eliminate the need of the horseshoe crab blood. The LAL assay involves using blood from the horseshoe crab which contains a primer, called Factor C, in which when the endotoxin is bound to this cascade it stimulates a coagulation cascade that can be measured over time via optical density. The rate at which the sample coagulates is inversely proportional to the amount of endotoxin present in the sample. The more recent test, rFC was made for a single step reaction that can measure endotoxin, therefore, potentially eliminating confounding factors that could occur in the later steps of the multistep cascade. In the sample itself, there are a variety of factors in blood or plasma such as bile salts, proteins, co-factors, and lipoproteins that could inhibit the assay and render it useless [164]. In order to account for this, serum was used in order to minimize potential inhibitors. In addition, when comparing and contrasting results, there are a wide variety of reported endotoxin concentrations as well as the levels that accurately constitute septic conditions. In a review by Boutagy et al [165], the author notes this discrepancy in an overview of two studies, one containing 116 healthy individuals and the other containing 345 healthy individuals, that report endotoxin concentration values nearly 600x apart (~0.1 EU/mL vs. 60 EU/mL), respectively, let alone noting that septic conditions have been seen in humans at approximately 1 EU/mL. With that said and even being seen in pig studies (Pearce et al [4] have reported 49 and 148 EU/mL for TN and HS, respectively, vs. 0.48 and 0.82 EU/mL for TN-

control and HS-control, respectfully, reported in this current study) it is incredibly difficult to effectively evaluate the true meaning of this data.

A fluorescently labeled polysaccharide (FITC-D_{10}) was given to the animal at a dose of 3.5mg/kg BW in order to measure intestinal permeability to a large macromolecule and would have been another indicator of GSE's ability to reduce intestinal permeability along with measuring endotoxin. Unfortunately, upon analyzing the samples, fluorescence was not detectable indicating that a larger dose is needed in pigs.

Finally, the last measurement of the effect of HS on the GI system was with the measurement of fecal calprotectin and how it would respond to chronic administration of GSE. Calprotectin, a protein produced by neutrophils in conjunction with the increased inflammation, provides a way to efficiently measure the extent of intestinal inflammation and if GSE can effectively reduce it [166]. Raised calprotectin levels have been shown in individuals with chronic inflammatory bowel diseases such as Crohn's disease and Ulcerative Colitis [167] as well as in response to increased levels of exercise in HS conditions compared to those at TN conditions [168]. In this study, fecal calprotectin levels were all very similar, showing no significant differences between groups, and did not appear to be altered in response to GSE administration. The trends of these results were also not in congruence with the literature in that the mean fecal calprotectin levels were higher in TN conditions compared to HS conditions and GSE fecal calprotectin levels were higher than the control diet. This is a bit surprising given the the results by Wang et al [99] which showed reduced neutrophil levels following GSE administration in a Crohn's (IL-10 deficient) rat model, and results by Goodrich et al [9] showed

drastic 10-fold reduction in fecal calprotectin levels following chronic GSE administration in a non-HS model.

While there was not many significant results provided by GSE in this study, there were some visible benefits in some results (endotoxin and blood glucose) that leads one to believe that the GSE dose was ineffective in order to adequately differentiate the treatments from the control. In the literature there has been a wide variety of GSE doses that have resulted in health benefits, but have been only tested in rats, mice, and humans. There is a possibility that in pigs, GSE's health benefits could be a factor of a U/J-shaped dose response curve or hormesis in that the benefits provided may be shown at very low concentrations (hormesis) or varying concentrations (U/J-shape dose response). In order to determine the dose which provides the maximum health benefits to the animal, it would be of interest to perform a dose-response experiment.

Due to some palatability complications with the GSE substance, a better delivery method is also needed. Dried molasses, a sweet substance that is given to pigs in industry, was considered; however, was not used due to the extremely fine consistency of the GSE. The GSE would be believed to separate from the molasses due to low miscibility and the entire dose might not have been consumed, therefore, cookie dough was used. Being that cookie dough was the chosen method of delivery, it is important to mention that bioavailability of polyphenols differs in a standalone situation compared to one in carbohydrate matrix. Bioavailability is lowered as a result of polyphenols binding to carbohydrates and making their release more difficult [169]. Bioavailability of GSE was not only potentially altered due to the method of delivery, but could also be altered due to the impact the environment had on the animal. GSE bioavailability could have been altered for better or worse in the HS groups due to increased intestinal permeability or

an alteration in gut microorganisms, but to what extent that bioavailability was altered is currently unknown; however, a look into native flavan-3-ols or flavan-3-ol metabolite with remaining serum samples left over from the current study concentrations is available. This would be an effective look into ways to improve bioavailability.

Conclusions and Future Work

This is the first time that supplementary GSE has been evaluated to alleviate the deleterious effects of HS in the pig. Based on results from this study, a supplemental dose of 12mg/kg BW GSE is not effective in enhancing growth performance or body composition in the animal given there were no significant differences in which GSE benefited growth parameters. These growth parameters such as BMD, BMC, fat, lean tissue, and percent BW gain, and body composition parameters such as RR, T_B, and fecal calprotectin were not significantly altered levels under HS conditions. However, GSE supplementation at this dose did tend toward having a significant reduction in circulating endotoxin values and may have been achieved with a larger sample size or different sampling day. The reduced circulating endotoxin values in the GSE group may indicate the formation of TJP's and improved intestinal integrity. In addition, GSE supplementation appeared to reduce the onset of reduced feed intake by 1 day during heat stress and implicated towards higher feed intake in the HS-GSE group compared to the HS-control. This result might have been accomplished given a longer measurement period or the elimination of the fasting period. Finally, GSE also appeared to have an influence on increased insulin sensitivity during periods of additional stress.

To continue on with samples from this research, it would be interesting to investigate the histology of intestinal samples that were preserved in formalin. Looking at the microvilli height and crypt depth could better tell the story of intestinal wall damage and GSE's effect on it.

Moving into the future, it would be more beneficial to not only find a the most effective GSE dose, but also find an improvement in delivery method over the one currently used among other various nuances. Investigating varying doses may have shown the results necessary to see some significant differences between groups. In addition, a completed glucose tolerance test using an intravenous catheter would be more beneficial to completely understand if GSE has any effect on carbohydrate metabolism than only the fasting blood glucose data provided in this study. Samples for endotoxin and fecal calprotectin taken earlier in the HS period may have also shown more vast differences between the treatment groups. This would be taken before the body has a chance to begin to repair itself from the initial damage [170]. As well, an improved delivery method would have been in a hard capsule filled with GSE that could be swallowed whole and not tasted by the animal. However, loading various doses into individual capsules on a daily basis is not practical for farmers or the industry, therefore, a new route in polyphenol research could be in the use of peanut skins. Peanut skins have very similar polyphenol content (A-type procyanidins) to that of the GSE, potentially less astringent taste, could be more easily mixed into the feed and consumed by the animal, and is completely discarded as a waste product [171].

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Appendix A - Procedure Protocols

DXA Scan Procedure

1. Fast pigs overnight for a minimum 8 hours
2. Anesthetize pig using conical anesthesia mask (for pigs ≤ 50kg)
    a. Maximum 5% isoflurane with 800ml/min of oxygen (dose will vary during procedure)
        i. If the pig cannot be safely restrained for gaseous induction, induction of anesthesia will be intramuscular
3. Perform DXA Scan – body composition analysis
4. Immediately following DXA Scan, obtain a blood sample via venipuncture method
    a. Venipuncture
        i. Restrain pig by placing them on their back with forelimbs pointed caudally
            1. One assistant hold head and forelimbs; second assistant performs venipuncture
        ii. Clean puncture area with 5% iodine solution (betadine), 70% isopropyl alcohol or other disinfecting solution
        iii. Using a sterile needle (18-22Ga by 1½ inches), puncture deepest point of the caudal part of the jugular groove, formed between the medial sternocephalic and lateral brachiocephalic muscles, and inserted in a dorsal-medial direction until blood flow is observed
            1. 10 mL of Blood is collected in 3 (4mL) BD Vacutainer SST containing a label (Animal ID [100-123], DXA Scan, Baseline [or Pre-HS, Post-HS], Blood)
                a. Invert BD Vacutainer SST 5 times to ensure complete mixing with clotting activator
                i. Leave clot for 30 minutes
        iv. Once blood sample has been collected, apply physical pressure (with gauze) to the puncture site until bleeding has stopped
5. Return Pig to its cage
6. Repeat on day 13 and 20 for pre and post-heat stress body composition analysis
7. After clotting, centrifuge BD Vacutainer SSTs at 1000-1300 RCF for 10 minutes at room temperature
    a. Pull off serum and place into cryogenic vial
        i. In a separate tube, place 300µL serum with 75µL 2% acetic acid/1% ascorbic acid solution (polyphenol preservation)
    b. Snap freeze in liquid N_2
    c. Store at -80°C until further analysis

Endotoxin

1. Fast pigs overnight for a minimum 8 hours
2. Anesthetize pig using conical anesthesia mask (for pigs ≤ 50kg)
    a. Maximum 5% isoflurane with 800ml/min of oxygen (dose will vary during procedure)
        i. If the pig cannot be safely restrained gaseous induction, induction of anesthesia will be intramuscular
3. Catheterize pig with a butterfly needle – ear vein catheter (21Ga x ¾”x7”)
4. Two hours following catheterization, obtain a baseline, 2mL blood sample in BD Vacutainer SST with a label for specific current pig (Animal ID [100-123], Endotoxin, Baseline, Blood)
    a. Invert BD Vacutainer SST 5 times to ensure complete mixing with clotting activator
        i. Leave clot for 30 minutes
5. Following baseline blood draw, feed pig its diet for the morning period
6. 3 hours following morning feeding session, obtain another 2mL blood sample (via ear vein catheter) into a BD Vacutainer SST with a label specific for current pig (Animal ID [100-123], Endotoxin, Post Feeding, Blood)
    a. Invert BD Vacutainer SST 5 times to ensure complete mixing with clotting activator
        i. Leave clot for 30 minutes
7. After clotting, centrifuge BD Vacutainer SSTs at 1000-1300 RCF for 10 minutes at room temperature
    a. Pull off serum and place into cryogenic vial
        i. In a separate tube, place 300µL serum with 75µL 2% acetic acid/1% ascorbic acid solution (polyphenol preservation)
    b. Snap freeze in liquid N_2
    c. Store at -80°C until further analysis

Glucose Tolerance Test/Intestinal Permeability

1. Fast pigs overnight for a minimum 8 hours
2. Anesthetize pig using conical anesthesia mask (for pigs ≤ 50kg)
    a. Maximum 5% isoflurane with 800ml/min of oxygen (dose will vary during procedure)
        i. If the pig cannot be safely restrained gaseous induction, induction of anesthesia will be intramuscular
3. Catheterize pig with butterfly needle – ear vein catheter (21Ga x ¾”x7”)
4. Two hours following catheterization, orally drench pig with a 50% (w/v) glucose/saline solution to provide 2g glucose/kg BW.
    a. At the same time, orally drench pig with 3.5mg/kg BW FITC-D_{10}
5. Collect 2mL blood samples in BD Vacutainer SST (Animal ID [100-123], GTT/IP, Time point, Blood) at the following time points**: 0, 15, 30, 45, 60, 90, 120, and 180** minutes post-gavage
    a. Invert BD Vacutainer SST 5 times to ensure complete mixing with clotting activator
        i. Leave clot for 30 minutes
6. Obtain a drop of blood to measure blood glucose levels using One Touch Ultra Blue test strips and standard glucometer
    a. Record results
7. After clotting, centrifuge BD Vacutainer SSTs at 1000-1300 RCF for 10 minutes at room temperature
    a. Pull off serum and place into cryogenic vial
        i. In a separate tube, place 300µL serum with 75µL 2% acetic acid/1% ascorbic acid solution (polyphenol preservation)
    b. Snap freeze in liquid N_2
    c. Store at -80°C until further analysis

Appendix B – Necropsy Procedure

Personnel required

1. Weighing/Euthanasia
2. Dissection/blood collection
3. Tissue/muscle collection (2 samples per person)
    a. Duodenum
    b. Jejunum
    c. Ileum
    d. Cecum
    e. Proximal colon
    f. Distal colon
    g. Longissimus dorsi
    h. Liver

Supplies

1. Gloves (Few boxes of each size)
2. Paper towels
3. Pencil
4. Pens (3; take 2 extra)
5. Markers (3; take 2 extra)
6. Test tube racks
7. 50 mL beakers for PBS (4)
8. 500 mL beakers for flushing waste (3)
9. Glass solvent bottle for disposal of flushing waste (2)
10. Scotch tape/Lab tape (1 of each)
11. Surgical tape
12. Lab mat (roll)
13. PBS (2L; may need more)
14. Squirt Bottle (for PBS)
15. Liquid N_2 dewar (4)
16. 2% acetic acid w/ 1% (w/v) solution (2L)
17. Histology sample bag (1/animal – double bagged)
    a. Tissue cassettes (8/animal)
18. Buckets for histology samples
19. 10% neutral buffered formalin (3L)
20. Spray bottle with 70% EtOH (1)
21. Ice Bucket (3)
    a. Ice

22. Plates
    a. Labeled: (1 for flushing contents/1 for tissue)
        i. Duodenum (2/animal)
        ii. Jejunum (2/animal)
        iii. Ileum (2/animal)
        iv. Cecum (2/animal)
        v. Proximal (2/animal)
        vi. Distal (2/animal)
        vii. Liver
        viii. L. Dorsi
23. Surgery tools:
    a. Scalpel
    b. Forceps
    c. Scissors
    d. hemostats
24. Blood collection supplies
    a. Blood Collection tubes (2/animal at sac)
    b. Sharp tipped needles for blood collection post-sacrifice?
25. Biohazard sharps container (2)
26. Biohazard bags
    a. For disposal of solid waste
27. 5mL cryo-tbues
    a. For plasma (at sacrifice)
        i. 4 serum cryo-tubes for serum
        ii. Each contain 0.25µL (2% acetic acid w/ 1% ascorbic (w/v) solution) per 100µL serum
    b. For tissue protein analysis (animal ID, segment ID, protein) – (8/animal)
        i. Segments:
            1. Duodenum
            2. Jejunum
            3. Ileum
            4. Cecum
            5. Proximal colon
            6. Distal colon
            7. Liver
            8. Longissimus Dorsi
        ii. 1mL Lysis buffer/vial
        iii. 1mL HALT protease inhibitor/vial
            1. Defined mass of tissue
    c. For tissue polyphenol analysis (animal ID, segment ID, polyphenol) – (8/animal)

        i. Segments: See 29b. i.
            1. 1mL 2% acetic acid/1%(w/v) ascorbic acid solution
    d. For tissue microbial analysis (animal ID, segment ID, microbial) – (8/animal)
        i. Segments: See 29b. i.
            1. 1 mL RNA later
    e. For content protein analysis (animal ID, contents, protein) – (8/animal)
        i. 1mL Lysis buffer
        ii. 1mLHALT protease inhibitor
    f. For content polyphenol analysis (animal ID, contents, polyphenol) – (8/animal)
        i. 1mL 2% acetic acid/1%(w/v) ascorbic acid solution
    g. For content microbial analysis (animal ID, contents, RNA) – (8/animal)
        i. 1mL RNA later
28. Vial labels
    a. For Tissues/contents
        i. Animal ID
        ii. Treatment
        iii. Segment ID
        iv. Contents or Tissue
        v. Designated Analysis
    b. For blood/plasma
        i. Animal ID
        ii. Treatment
        iii. Blood or plasma
29. Labeled tissue cassettes (Label with PENCIL ONLY!!)
    a. Labeled with:
        i. Animal ID
        ii. Treatment
        iii. Segment ID
    b. Segments
        i. Duodenum (1/animal)
        ii. Jejunum (1/animal)
        iii. Ileum (1/animal)
        iv. Cecum (1/animal)
        v. Proximal colon (1/animal)
        vi. Distal colon (1/animal)
        vii. Liver (1/animal)
        viii. Longissimus Dorsi (1/animal)
30. Freezer boxes (can fit approx. 100 samples per box)
    a. 2 for contents/polyphenol analysis
    b. 2 for contents/microbial analysis

c. 2 for contents/protein analysis
d. 2 for tissue/polyphenol analysis
e. 2 for tissue/microbial analysis
f. 2 for tissue/protein analysis

<u>Preparation</u>

1. Reserve room for euthanasia in advance (if needed)
2. Make sure all the tools are needed for sacs
3. Label histology cassettes with pencil
4. Label all sample vials
5. Prepare solutions
    a. PBS
    b. 2% acetic acid w/ 1% ascorbic acid (w/v)
6. Fill vials with required storage solutions
    a. Pre-weigh tube/cap and storage solution.
        i. Record weight
7. Stage vials in test tube racks
8. Prep sac room
9. Chill PBS in VWR bottle for >1hr
10. Prep plasma separation supplies in the lab:
    a. Labeled 1.5 or 2mL cryo tubes containing acetic acid/ascorbic acid solution
11. Prep stations in sac room with needed supplies:
    a. Euthanasia
        i. Balance
        ii. Pig snare
        iii. Paper Towels
    b. Dissection
        i. Unlabeled weigh boats for resting sterile tools (2)
        ii. Tools
            1. Scalpel
            2.Forceps
            3. Scissors
            4.Hemostats
        iii. Plates labeled with animal ET and “duodenum”, “jejunum”, “ileum”, etc. (1/animal)
        iv. Ice bucket
            1. Ice
        v. Biohazard sharps container

vi. Biohazard bags (for solid waste)

c. Duodenum prep:
    i. Unlabeled plates for resting sterile tools (2)
    ii. Plates labeled with animal ID and “duodenum” (1/animal)
    iii. Disposable spatulas (1/section)
    iv. Forceps
    v. Blunt tip 21 gauge needles (1/animal)
    vi. 10 mL syringes (1/animal)
    vii. 50 mL beaker for PBS (1)
    viii. 500 mL beaker for flushing waste (1)
    ix. Ice cold PBS (refill ~20-30mL from bottle for each animal immediately prior to flushing
    x. Scalpel handle (1)
    xi. Scalpel blades (1/animal)
    xii. Labeled 5mL cryo tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for tissue polyphenol analysis)
    xiii. Labeled 5mL cyro-tube with 1mL Lysis buffer/0.5mL HALT protease inhibitor (for tissue protein analysis)
    xiv. Labeled 5mL cryo-tube with 1mL RNAlater (for tissue microbial analysis)
    xv. Labeled 5mL cryo-tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for contents polyphenol analysis)
    xvi. Labeled 5mL cryo-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for contents protein analysiss)
    xvii. Labeled 5mL cryo-tube with 1mL RNAlater (for contents microbial analysis)
    xviii. Labeled histology cassettes (1/animal)
    xix. Bucket for cassettes
    xx. Double-bagged histology bags for cassettes, labeled “duodenum” (1)
    xxi. 350mL formalin (in bags)
    xxii. Liquid N_2 in dewar (1)

d. Jejunum
    i. Unlabeled plates for resting sterile tools (2)
    ii. Plates labeled with animal ID and “Jejunum” (1/animal)
    iii. Disposable spatulas
    iv. Forceps
    v. Blunt tip 21 gauge needles (1/animal)
    vi. 10 mL syringes (1/animal)
    vii. 50 mL beaker for PBS (1)
    viii. 500 mL beaker for flushing waste (1)
    ix. Ice cold PBS (refill ~20-30mL from bottle for each animal immediately prior to flushing

x. Scalpel handle (1)
xi. Scalpel blades (1/animal)
xii. Labeled 5mL cryo tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for tissue polyphenol analysis)
xiii. Labeled 5mL cyro-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for tissue protein analysis)
xiv. Labeled 5mL cryo-tube with 1mL RNAlater (for tissue microbial analysis)
xv. Labeled 5mL cryo-tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for contents polyphenol analysis)
xvi. Labeled 5mL cryo-tube with 1mL Lysis buffer/0.5mL HALT protease inhibitor (for contents protein analysiss)
xvii. Labeled 5mL cryo-tube with 1mL RNAlater (for contents microbial analysis)
xviii. Labeled histology cassettes (1/animal)
xix. Bucket for cassettes
xx. Double-bagged histology bags for cassettes, labeled “Jejunum” (1)
xxi. 350mL formalin (in bags)
xxii. Liquid N_2 in dewar (1)

e. Ileum
i. Unlabeled Plates for resting sterile tools (2)
ii. Plates labeled with animal ID and “Ileum” (1/animal)
iii. Disposable spatulas
iv. Forceps
v. Blunt tip 21 gauge needles (1/animal)
vi. 10 mL syringes (1/animal)
vii. 50 mL beaker for PBS (1)
viii. 500 mL beaker for flushing waste (1)
ix. Ice cold PBS (refill ~20-30mL from bottle for each animal immediately prior to flushing
x. Scalpel handle (1)
xi. Scalpel blades (1/animal)
xii. Labeled 5mL cryo tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for tissue polyphenol analysis)
xiii. Labeled 5mL cyro-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for tissue protein analysis)
xiv. Labeled 5mL cryo-tube with 1mL RNAlater (for tissue microbial analysis)
xv. Labeled 5mL cryo-tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for contents polyphenol analysis)
xvi. Labeled 5mL cryo-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for contents protein analysiss)
xvii. Labeled 5mL cryo-tube with 1mL RNAlater (for contents microbial analysis)

xviii. Labeled histology cassettes (1/animal)
xix. Bucket for cassettes
xx. Double-bagged histology bags for cassettes, labeled “Ileum” (1)
xxi. 350mL formalin (in bags)
xxii. Liquid N_2 in dewar (1)

f. Cecum

i. Unlabeled Plates for resting sterile tools (2)
ii. Plates labeled with animal ID and “Cecum” (1/animal)
iii. Disposable spatulas
iv. Forceps
v. Blunt tip 21 gauge needles (1/animal)
vi. 10 mL syringes (1/animal)
vii. 50 mL beaker for PBS (1)
viii. 500 mL beaker for flushing waste (1)
ix. Ice cold PBS (refill ~20-30mL from bottle for each animal immediately prior to flushing
x. Scalpel handle (1)
xi. Scalpel blades (1/animal)
xii. Labeled 5mL cryo tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for tissue polyphenol analysis)
xiii. Labeled 5mL cyro-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for tissue protein analysis)
xiv. Labeled 5mL cryo-tube with 1mL RNAlater (for tissue microbial analysis)
xv. Labeled 5mL cryo-tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for contents polyphenol analysis)
xvi. Labeled 5mL cryo-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for contents protein analysiss)
xvii. Labeled 5mL cryo-tube with 1mL RNAlater (for contents microbial analysis)
xviii. Labeled histology cassettes (1/animal)
xix. Bucket for cassettes
xx. Double-bagged histology bags for cassettes, labeled “Cecum” (1)
xxi. 350mL formalin (in bags)
xxii. Liquid N_2 in dewar (1)

g. Spiral Colon

i. Unlabeled Plates for resting sterile tools (2)
ii. Plates labeled with animal ID and “Spiral Colon” (1/animal)
iii. Disposable spatulas
iv. Forceps
v. Blunt tip 21 gauge needles (1/animal)
vi. 10 mL syringes (1/animal)

vii. 50 mL beaker for PBS (1)
viii. 500 mL beaker for flushing waste (1)
ix. Ice cold PBS (refill ~20-30mL from bottle for each animal immediately prior to flushing
x. Scalpel handle (1)
xi. Scalpel blades (1/animal)
xii. Labeled 5mL cryo tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for tissue polyphenol analysis)
xiii. Labeled 5mL cyro-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for tissue protein analysis)
xiv. Labeled 5mL cryo-tube with 1mL RNAlater (for tissue microbial analysis)
xv. Labeled 5mL cryo-tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for contents polyphenol analysis)
xvi. Labeled 5mL cryo-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for contents protein analysiss)
xvii. Labeled 5mL cryo-tube with 1mL RNAlater (for contents microbial analysis)
xviii. Labeled histology cassettes (1/animal)
xix. Bucket for cassettes
xx. Double-bagged histology bags for cassettes, labeled “Proximal Colon” (1)
xxi. 350mL formalin (in bags)
xxii. Liquid N_2 in dewar (1)

h. Distal Colon

i. Unlabeled Plates for resting sterile tools (2)
ii. Weigh Plates with animal ID and “Distal colon” (1/animal)
iii. Disposable spatulas
iv. Forceps
v. Blunt tip 21 gauge needles (1/animal)
vi. 10 mL syringes (1/animal)
vii. 50 mL beaker for PBS (1)
viii. 500 mL beaker for flushing waste (1)
ix. Ice cold PBS (refill ~20mL from bottle for each animal immediately prior to flushing
x. Scalpel handle (1)
xi. Scalpel blades (1/animal)
xii. Labeled 5mL cryo tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for tissue polyphenol analysis)
xiii. Labeled 5mL cyro-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for tissue protein analysis)
xiv. Labeled 5mL cryo-tube with 1mL RNAlater (for tissue microbial analysis)

xv. Labeled 5mL cryo-tube with 1mL; 2% acetic acid/1%(w/v) ascorbic acid solution (for contents polyphenol analysis)
xvi. Labeled 5mL cryo-tube with 1mL Lysis buffer/1mL HALT protease inhibitor (for contents protein analysiss)
xvii. Labeled 5mL cryo-tube with 1mL RNAlater (for contents microbial analysis)
xviii. Labeled histology cassettes (1/animal)
xix. Bucket for cassettes
xx. Double-bagged histology bags for cassettes, labeled "Distal colon" (1)
xxi. 350mL formalin (in bags)
xxii. Liquid N_2 in dewar (1)

12. Immediately prior to necropsy:
    a. Fill dewars with liquid N_2

Procedure

Euthanasia
1. Penetrative captive bolt
2. Exsanguination

Necropsy
1. Separation of intestinal tissue is as follows
    a. Distal Colon:
        i. Excise 4-6" above the rectum
        ii. Place distal colon onto labeled plate
        iii. Hand off to Distal colon tissue prep
    b. Spiral colon
        i. Grasp spiral colon just below the cecum, and cut the colon just below the cecum
        ii. Excise 4-6" of spiral colon and place onto plate
        iii. Hand off tospiral colon tissue prep
    c. Cecum
        i. Grasp the small intestine just above the cecum and cut the small intestine just above the cecum with the scissors
        ii. Place cecum onto plate
        iii. Hand off to cecum tissue prep
    d. Ileum
        i. Directly above the cecum, make a cut with the scissors. Make another cut approximately 1/3 the length of the small intestines
            1. The ileum is the terminal portion of the small intestines

ii. Cut out 4-6” of this (end) segment of small intestines
iii. Place ileum onto plate
iv. Hand off to ileum tissue prep

e. Jejunum
    i. Following the previous cut, make another cut, with scissors, approximately 1/3 the length further of the small intestines
        1. The jejunum is the middle portion of the small intestines
    ii. Cut out 4-6” of this (middle) segment of the small intestines
    iii. Place jejunum onto plate
    iv. Hand off to jejunum tissue prep
f. Duodenum
    i. Make the final cut for the small intestines immediately following the pyloric valve of the stomach
        1. The Duodenum is the first portion of the small intestines
    ii. Cut out 4-6” of the first segment of small intestines
    iii. Place Duodenum onto plate
    iv. Hand off to Duodenum prep
g. Liver
    i. Cut off portions of liver and place into labeled test tubes
        1. Take the same lobe of liver from each animal
    ii. Orient section in labeled histology cassette, snap closed, immerse in formalin
h. Longissimus Dorsi

Tissue Collection and Preparation (perform on each section)

2. Remove and prepare intestinal contents:
    a. Using 2 gloved fingers (1 on each side), manually squeeze intestinal contents out into a plate (may use forceps to manually flatten cecum too squeeze out contents due to its watery consistency at this point)
    b. Gently mix with disposable spatula to homogenize the contents
    c. Prep contents for:
        i. Polyphenol analysis
            1. Add contents to 1mL 2% acetic acid/1% (w/v) ascorbic acid in 5mL cryo-tube with disposable spatula or transfer pipette
            2. Weigh tube and cap with tissue/contents and record weight
            3. Vortex to disperse
        ii. Microbial analysis
            1. Add contents to 1mL RNALater reagent in 5mL cryo-tube
            2. Weigh tube and cap with tissue/contents and record weight
            3. Vortex to disperse

iii. Protein analysis
    1. Add contents to 1mL Lysis buffer/1mL HALT protease inhibitorreagent in 5mL cryo-tube
    2. Weigh tube and cap with tissue/contents and record weight
    3. Vortex to disperse
iv. Snap freeze samples in liquid N_2

3. Prepare the intestinal tissue:
    a. Flush the section to remove residual intestinal contents:
        i. Gently slide the intestines around the nozzle of a squirt bottle containing chilled PBS Gently grip intestines with fingers to ensure it doesn't slide off the nozzle
        ii. Gently flush with ice-cold PBS until intestines are free of contents (flush PBS into a plate , and discard in waste beaker)
            1. Clean nozzle of squirt bottle with 70% ethanol before next intestinal sample or animal
        iii. Place flushed section onto clean plate
    b. Divide the section for further processing:
        i. For intestinal tissue
            1. Cut off first 1" ring of intestines from top portion of intestines
                a. This ring will be used for histology
            2. Cut off second 1" ring (immediately following first cut)
                a. Divide section into roughly 3 equal segments
                b. Segment 1: polyphenol analysis
                c. Segment 2: Protein analysis
                d. Segment 3: Microbial analysis
4. Prepare each segment for analysis
    a. Polyphenol analysis
        i. Place tissue in 5mL cryo-tube containing 1mL of 2% acetic acid/1% ascorbic (w/v) solution
        ii. Weigh tube and cap with tissue/contents and record weight
        iii. Snap freeze in liquid N_2
    b. Protein
        i. Immerse samples in 1mL lysis buffer in 5mL cryo-tubes
        ii. Weigh tube and cap with tissue/contents and record weight
        iii. Snap freeze samples in liquid N_2
    c. Microbiota
        i. Immerse samples in 1mL RNALater in 5mL cryo-tubes
        ii. Weigh tube and cap with tissue/contents and record weight
        iii. Snap freeze samples in liquid N_2
    d. Histology

i. Orient the segment flat on the tissue cassette surface
ii. Snap cassette closed and immerge completely in 10% neutral buffered formalin

Between pigs

1. Change scalpel blades (discard used blades in biohazard sharps container)
2. Clean off scissors/PBS squirt bottle nozzle with 70% ethanol
3. Discard all waste, dirty weigh boats, etc (in biohazard bags)
4. Organize vials and cassettes for next animal to be euthanized
5. Get additional liquid N_2 if needed to keep samples frozen

Post-Necropsy

1. Clean up surgery room
2. Carcass disposal
3. Plasma prep and storage
    a. Centrifuge blood samples
    b. Aliquot serum into cryogenic vials
4. In a separate tube, place 300µL serum with 75µL 2% acetic acid/1% ascorbic acid solutionc. Snap freeze serum samples in liquid N_2Freeze all snap frozen samples at -80°C in labeled vial boxes
5. Processing and transfer of formalin-fixed tissue for staining
    a. TBD
6. Surgical Tools:
    a. Thoroughly scrub surgical tools with Alconox
    b. Place in autoclave packs (max 2 tools/pack)
    c. Autoclave tool packs
    d. Leave packs sealed, place in surgery box
7. Order any supplies needed for next necropsy or to refill for the lab